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Engineering and Study of Biocompatible Nanoceramics

Kathrin Bogusz

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Engineering and Study of Biocompatible Nanoceramics

This thesis is presented as part of the requirement for the award of the Degree of

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Kathrin Bogusz, MSc., BSc.
DECLARATION

The technical content of this thesis is original work carried out in the laboratories at the Institute for Superconducting and Electronic Materials, the Electron Microscopy Centre, the Illawarra Health and Medical Research Institute, and the Centre for Medical Radiation Physics at the University of Wollongong, New South Wales, Australia. The content of this thesis does not contain any material that has been submitted for the award of any degree in this or any other University and, to the best of my knowledge, contains solely original work, unpublished or written by any other person, except where referenced or acknowledged.

Kathrin Bogusz

September, 2018
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ABSTRACT

Over the past decade, there has been increasing interest in the use of multifunctional nanoparticles for applications in the fields of chemistry, physics, biology, and medicine, as they offer unique electronic, optical, mechanical, magnetic, and thermal properties, compared to their bulk counterparts. Of particular interest is the use of nanoparticles in cancer treatment and cancer prevention in the form of an ultraviolet blocker in sunscreen products. An emerging field of research is the development of multifunctional nanoparticle systems, also called ‘theranostic systems’, which are able to combine multiple features, such as therapeutic, diagnostic, radiation dose enhancing, and targeting functions on one single platform. Inorganic bismuth- and tantalum-based nanoparticles are easily synthesized, have a high effective atomic number, are known to be biocompatible, and have not yet been well studied for cancer treatment or as potential ultraviolet blocker. Therefore, the aim of this project was to synthesize and characterize various bismuth- and tantalum-based nanomaterials regarding their physicochemical properties and their biological activity in cancer and normal cells in vitro.

Part of this doctoral work is focused on the materials and biological characterization of Bi(OH)\(_3\) and α-Bi\(_2\)O\(_3\) nanoparticles. Both types of nanoparticles, which have a particle size of 6 – 10 nm, display extreme toxicity towards gliosarcoma 9L and MCF-7 human breast cancer cells. Clonogenic assays reveal a mortality of over 90% in 9L and MCF-7 cells at a concentration of 50 µg/mL after incubation for 24 h. Moreover, the nanoparticles cause significant mortality of up to 60% in the cancer cells at the very low concentration of 6.25 µg/mL. In contrast, at the same concentration, the nanoparticles exhibit no noticeable mortality towards normal Madin-Darby canine kidney cells. The internalization of the nanoparticles was demonstrated using flow cytometry, and confocal microscopy was used to investigate when the loss of cell viability starts. The nanoparticles cause faster cell death in 9L cells compared with MCF-7 cells, demonstrated via the
Abstract

Identification of apoptosis through increased sub G₁ levels after 24 h of nanoparticle incubation. Cleavage is identified as the main apoptotic nuclear morphology in 9L, which suggests the presence of reactive oxygen species.

Given the ability of Bi(OH)₃ nanoparticles to kill cancer cells, while showing biocompatibility in normal cells, these nanoparticles were further investigated as a multifunctional ultraviolet filter for sunscreens. It was found that the absorbance of Bi(OH)₃ nanoparticles in the ultraviolet region is comparable to those of both the ZnO and TiO₂ nanoparticles used in commercial sunscreens. The in vitro photoprotection results show that the sunscreen fabricated in this study from a combination of TiO₂/Bi(OH)₃ was more efficient than TiO₂/ZnO over the whole ultraviolet range, with an increase in the sun protection factor of 28%. In addition, the homemade sunscreens show rheological properties comparable to those of commercial sunscreens. The combination of TiO₂/Bi(OH)₃ led to insignificant damage on pre-painted steel panels after exterior exposure for twelve weeks. Furthermore, the addition of Bi(OH)₃ nanoparticles reduced the degradation of crystal violet by photocatalytically active TiO₂ or ZnO nanoparticles under ultraviolet exposure.

To further examine new possibilities regarding the development of a novel inorganic ultraviolet filter, nanocomposite materials were synthesized, consisting of TiO₂ nanoparticles with homogeneously attached (BiO)₂CO₃ clusters on their surfaces. The TiO₂/(BiO)₂CO₃ nanocomposites exhibited an absorbance in the ultraviolet-visible range that was similar to that of TiO₂ nanoparticles and photocatalytic activity that is reduced by up to 60% compared to the TiO₂ nanoparticles when exposed to ultraviolet and visible light. In addition, the nanocomposites show high biocompatibility in normal HaCaT human skin cells and Madin-Darby canine kidney cells in vitro, and more importantly, they are capable of reducing the photo-generated toxicity of TiO₂ in HaCaT cells upon irradiation with simulated sunlight.
Finally, a nanotheranostic system was designed based on δ-Ta₂O₅ nanoparticles with a particle size of 27 nm, which were coated with poly(acrylic acid) with different layer thicknesses of 2 – 8 nm. The capability of the δ-Ta₂O₅-poly(acrylic acid) nanocomposites to provide anatomical contrast-enhancing features is demonstrated via computed tomography. The δ-Ta₂O₅-poly(acrylic acid) nanocomposite was further loaded with methotrexate, and the drug release was observed for a total of 72 h at pH values of 3.6, 5.4, 7.4, and 9.4. While the different layer thicknesses did not influence the drug release kinetics, a decrease in pH of the release medium results in a slower release of methotrexate. In most cases, the drug release mechanism was anomalous (non-Fickian), and under more alkaline pH conditions, near Case II transport was observed, which suggests a drug release mechanism highly influenced by macromolecular chain relaxation.

The work presented in this doctoral thesis highlights the multiple features of bismuth- and tantalum-based nanoparticles, which show selective toxicity towards cancer cells and are of particular interest, as they can deliver drugs at a sustained rate to target cancer cells, which can result in higher therapeutic efficiency and reduced systemic toxicity. Moreover, the examined bismuth-based compounds could potentially be applied as alternative ultraviolet filter components in sunscreen formulations, reducing the potential adverse effects associated with TiO₂ nanoparticles. Future work establishing the physiological relevance and mechanism of action – both on a molecular level and in the cellular environment – will be crucial in pursuing avenues for therapeutic intervention and prevention in cancer treatment.
# TABLE OF CONTENTS

DECLARATION ......................................................................................................................... II

ACKNOWLEDGEMENTS ........................................................................................................... III

ABSTRACT ............................................................................................................................. IV

TABLE OF CONTENTS ........................................................................................................... VII

LIST OF ABBREVIATIONS ................................................................................................... XVII

LIST OF SYMBOLS ............................................................................................................... XXII

LIST OF FIGURES ................................................................................................................ XXV

LIST OF TABLES .................................................................................................................. XXXIX

LIST OF PUBLICATIONS AND CONFERENCES .............................................................. XLII

SCHOLARSHIPS AND AWARDS .......................................................................................... XLIV

CHAPTER 1 – INTRODUCTION ............................................................................................. 1

1.1 Background and Motivation .......................................................................................... 2

1.2 Thesis Structure ............................................................................................................ 5

1.3 References ..................................................................................................................... 7
# Table of Contents

**CHAPTER 2 - LITERATURE REVIEW**

2.1 Cancer Characteristics

2.1.3 Cancer Cells and Normal Cells

2.1.4 Causes of Cancer

2.1.5 Types of Cancer

2.1.6 Current Cancer Prevention Strategies and Anti-Cancer Treatments

2.2 Ultraviolet Radiation and Melanoma

2.2.1 Ultraviolet Light Irradiation

2.2.2 The Human Skin Barrier

2.2.3 Effects of Solar Ultraviolet Radiation on the Human Skin

2.2.3.1 Solar Ultraviolet B Irradiation

2.2.3.2 Solar Ultraviolet A Irradiation

2.2.4 Impact of Ionizing Radiation on Deoxyribonucleic Acid

2.2.4.1 Direct Impact of Ionizing Radiation on Deoxyribonucleic Acid

2.2.4.2 Indirect Impact of Ionizing Radiation on Deoxyribonucleic Acid

2.2.4.3 Repairing Mechanisms of Deoxyribonucleic Acid after Exposure to Ionizing Radiation

2.2.5 Melanoma Risk Factors and Special Situation in Australia

2.2.6 Current Methods of Protection from Solar Ultraviolet Radiation

2.3 Theranostic Nanomaterials for Cancer Treatment

2.3.1 Definition of Nanomaterials

2.3.2 Definition of a Theranostic System

2.3.3 Nanomaterials for Theranostic Systems

2.3.3.1 Organic Nanoparticles

2.3.3.2 Inorganic Nanoparticles

2.3.3.3 Core-Shell Structures

2.3.4 Therapeutic Effects of Nanoparticles

2.3.4.1 Definition of Reactive Oxygen Species
2.3.4.2 Significance of Reactive Oxygen Species ........................................32
2.3.4.3 Nanoparticles as Reactive Oxygen Species Generators ..................34
2.3.4.4 Nanoparticles as Scavenger of Reactive Oxygen Species .................34
2.3.5 Chemotherapeutic Drugs ..................................................................35
  2.3.5.1 Structural Properties of Methotrexate ........................................36
  2.3.5.2 Application of Methotrexate in Cancer Therapy ...........................37
2.3.6 Radiation Therapy .............................................................................38
  2.3.6.1 Radioprotectors and Radiosensitizers ........................................38
  2.3.6.2 Mechanism of Radio-Dose Enhancing Therapy Based on Nanoparticles 39
  2.3.6.3 Radiosensitizers Based on Nanoparticles .....................................40
2.3.7 Diagnostics and Imaging ....................................................................41
  2.3.7.1 Computed Tomography .................................................................42
  2.3.7.2 Classical Contrast-Enhancing Agents ..........................................44
  2.3.7.3 Contrast-Enhancement Agents Based on Nanoparticles ................44
2.3.8 Examples of Theranostic Systems .......................................................46
2.4 Nanomaterials for Sun-Blocking Applications .......................................47
  2.4.1 Sunscreen Products ........................................................................47
    2.4.1.1 Organic Ultraviolet Filters .......................................................49
    2.4.1.2 Inorganic Ultraviolet Filters ....................................................49
  2.4.2 Sun Protection Factor .......................................................................51
    2.4.2.1 In Vivo Determination of Sun Protection Factor ...........................52
    2.4.2.2 In Vitro Determination of Sun Protection Factor .........................53
  2.4.3 Photochemistry and Photobiology ....................................................54
    2.4.3.1 TiO₂ .....................................................................................55
    2.4.3.2 ZnO .....................................................................................57
    2.4.3.3 Photocatalytic Activity of Semiconducting Nanoparticles ............58
    2.4.3.4 Determination of Photocatalytic Activity ....................................59
    2.4.3.5 Determination of Reactive Oxygen Species .................................61
2.5 Limitations of Current Sunscreens and Suggested Solutions ........................................ 63
  2.5.1 Skin Penetration of Inorganic Ultraviolet Filters..................................................63
  2.5.2 Inconclusive Toxicity of Inorganic Ultraviolet Filters..........................................65
    2.5.2.1 Reported Lack of Toxicity.............................................................................66
    2.5.2.2 Reported Toxicity.......................................................................................66
  2.5.3 Photostability and Efficiency................................................................................69
  2.5.4 Improvement of Existing Inorganic Ultraviolet Filters.........................................71
    2.5.4.1 Stability of Emulsions................................................................................71
    2.5.4.2 Broad Ultraviolet A/Ultraviolet B Protection..............................................72
    2.5.4.3 Reduction of Photoactivity.........................................................................72
  2.5.5 New Class of Nanoparticles for Sunscreen Formulations.......................................75
    2.5.5.1 Inorganic Nanoparticles.............................................................................76
    2.5.5.2 Organic Nanoparticles..............................................................................77

2.6 References ..................................................................................................................77

CHAPTER 3 – MATERIALS AND METHODOLOGY........................................... 100

3.1 Synthesis of Nanomaterials .........................................................................................101
  3.1.1 Materials............................................................................................................101
  3.1.2 Synthesis of Bi(OH)₃ Nanoparticles................................................................101
  3.1.3 Synthesis of α-Bi₂O₃ Nanoparticles..................................................................101
  3.1.4 Preparation of Nanocomposites of TiO₂ and (BiO)₂CO₃....................................102
  3.1.5 Synthesis of δ-Ta₂O₅ Nanoparticles................................................................102
  3.1.6 Coating of δ-Ta₂O₅ Nanoparticles with Poly(acrylic acid)...............................102

3.2 Physical and Chemical Characterization of Nanomaterials........................................104
  3.2.1 Materials............................................................................................................104
  3.2.2 Specific Surface Area (Brunauer-Emmett-Teller)..............................................105
  3.2.3 X-Ray Diffraction (XRD)................................................................................106
  3.2.4 X-Ray Photoelectron Spectroscopy (XPS).......................................................107
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.5</td>
<td>Fourier-Transform Infrared Spectroscopy (FTIR)</td>
<td>108</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Raman Spectroscopy</td>
<td>110</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Ultraviolet-Visible (UV-Vis) Spectroscopy</td>
<td>110</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Dynamic Light Scattering (DLS)</td>
<td>112</td>
</tr>
<tr>
<td>3.2.9</td>
<td>Thermogravimetric Analysis (TGA)</td>
<td>113</td>
</tr>
<tr>
<td>3.2.10</td>
<td>Computed Tomography</td>
<td>114</td>
</tr>
<tr>
<td>3.2.11</td>
<td>Polymer Swelling Studies</td>
<td>115</td>
</tr>
<tr>
<td>3.3</td>
<td>Morphological Characterization of Nanomaterials</td>
<td>116</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Scanning Electron Microscopy</td>
<td>117</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Transmission Electron Microscopy</td>
<td>118</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Energy-Dispersive X-Ray Spectroscopy</td>
<td>120</td>
</tr>
<tr>
<td>3.4</td>
<td>Photocatalytic Activity and Ultraviolet-Blocking Properties of Sunscreen Formulations</td>
<td>121</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Materials</td>
<td>121</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Preparation of Sunscreen Formulations</td>
<td>121</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Separation of Inorganic Sunscreen Components</td>
<td>121</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Photocatalytic Activity</td>
<td>122</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Sun Exposure of Sunscreen Formulations</td>
<td>124</td>
</tr>
<tr>
<td>3.4.5.1</td>
<td>Gloss Readings of the Test Panels</td>
<td>125</td>
</tr>
<tr>
<td>3.4.5.2</td>
<td>Scanning Electron Microscopy Imaging of the Test Panels</td>
<td>126</td>
</tr>
<tr>
<td>3.4.6</td>
<td>Rheological Properties</td>
<td>126</td>
</tr>
<tr>
<td>3.4.7</td>
<td>Photoprotection Efficiency</td>
<td>126</td>
</tr>
<tr>
<td>3.5</td>
<td>In Vitro Cell Culture Methods</td>
<td>128</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Materials</td>
<td>128</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Cell Lines</td>
<td>128</td>
</tr>
<tr>
<td>3.5.2.1</td>
<td>Human HaCaT Skin Cells</td>
<td>128</td>
</tr>
<tr>
<td>3.5.2.2</td>
<td>Madin-Darby Canine Kidney (MDCK) Cells</td>
<td>129</td>
</tr>
<tr>
<td>3.5.2.3</td>
<td>9L Rat Brain Tumour Cells</td>
<td>129</td>
</tr>
</tbody>
</table>
Table of Contents

3.5.2.4 Human MCF-7 Breast Cancer Cells ................................................. 130
3.5.3 Subculture of Cells .............................................................................. 131
3.5.4 Cell Counting ....................................................................................... 131
3.5.5 Cryopreservation of Cells .................................................................... 132
   3.5.5.1 Freezing of Cells ......................................................................... 132
   3.5.5.2 Thawing of Cells ......................................................................... 133

3.6 *In Vitro* Biological Characterization ..................................................... 133
   3.6.1 Materials ............................................................................................. 133
   3.6.2 Statistical Analysis ............................................................................. 134
   3.6.3 Preparation of Nanoparticle Suspensions ........................................ 134
   3.6.4 Live Cell Imaging ................................................................................ 135
      3.6.4.1 Confocal Microscopy ................................................................ 135
      3.6.4.2 Staining with Propidium Iodide .................................................. 136
      3.6.4.3 IncuCyte ZOOM System ............................................................... 137
   3.6.5 Flow Cytometry .................................................................................. 138
      3.6.5.1 Detection of Forward and Side Scatter ....................................... 139
      3.6.5.2 Cell Cycle Population ................................................................. 140
   3.6.6 Cell Survival and Proliferation ............................................................. 142
      3.6.6.1 Clonogenic Survival Assay .......................................................... 142
      3.6.6.2 MTT Proliferation Assay in Absence of Simulated Sunlight ........ 145
      3.6.6.3 MTT Proliferation Assay under Exposure to Simulated Sunlight .. 147
   3.6.7 Detection of Intracellular Reactive Oxygen Species .......................... 149
      3.6.7.1 Fluorescence Microscopy ............................................................... 149
      3.6.7.2 Staining with Hoechst 33342 ......................................................... 150
      3.6.7.3 Staining with 2',7'-Dichlorodihydrofluorescein Diacetate ........... 151
   3.6.8 *In Vitro* Drug Loading and Release Studies ....................................... 152
      3.6.8.1 *In Vitro* Drug Loading ................................................................. 152
      3.6.8.2 *In Vitro* Drug Release ................................................................. 153

3.7 References ............................................................................................... 154
CHAPTER 4 – BISMUTH-BASED COMPOUNDS FOR BIOMEDICAL APPLICATIONS ............................................................... 161

4.1 Introduction ........................................................................................................................................................................... 163
  4.1.1 Properties of Bismuth-Based Compounds ......................................................................................................................... 164
    4.1.1.1 Properties of Bi(OH)₃ ...................................................................................................................................................... 164
    4.1.1.2 Properties of Bi₂O₃ .................................................................................................................................................... 166
    4.1.1.3 Properties of (BiO)₂CO₃ .............................................................................................................................................. 169
  4.1.2 Diagnostic Properties .......................................................................................................................................................... 171

4.2 High Toxicity of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles Towards Malignant 9L and MCF-7 Cells .................................................. 171
  4.2.1 Identification, Size, Morphology, and Surface Composition of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles .................................................... 172
  4.2.2 Cellular Uptake of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles .................................................................................................................. 176
  4.2.3 Cytotoxicity of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles .................................................................................................................. 178
  4.2.4 Staining with Propidium Iodide ......................................................................................................................................... 182
  4.2.5 Cell Cycle Population ......................................................................................................................................................... 184
  4.2.6 Evaluation of Apoptotic Nuclear Morphologies and Intracellular Reactive Oxygen Species .................................................................. 186
  4.2.7 In Vitro Computed Tomography Imaging of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles ................................................................. 191
  4.2.8 Conclusions ...................................................................................................................................................................... 194

4.3 Biocompatible Bi(OH)₃ Nanoparticles with Reduced Photocatalytic Activity as Possible Ultraviolet Filter in Sunscreens ................................................. 195
  4.3.1 Overview of Tested Materials, Homemade Sunscreens, and Commercial Sunscreens .......................................................... 195
  4.3.2 Identification, Size, Morphology, and Surface Composition of Bi(OH)₃, TiO₂, and ZnO Nanoparticles .............................................. 197
  4.3.3 Ultraviolet-Visible Study of Bi(OH)₃, TiO₂, and ZnO Nanoparticles ......................................................................................... 201
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.4</td>
<td>Photocatalytic Activity of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles under Ultraviolet Irradiation</td>
<td>204</td>
</tr>
<tr>
<td>4.3.4.1</td>
<td>Under Ultraviolet Exposure (300 nm and 350 nm)</td>
<td>204</td>
</tr>
<tr>
<td>4.3.4.2</td>
<td>Under Ultraviolet Exposure (350 nm)</td>
<td>208</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Photocatalytic Activity of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles under Simulated Solar Irradiation</td>
<td>210</td>
</tr>
<tr>
<td>4.3.6</td>
<td>Sun Exposure of Homemade Sunscreens and Commercial Sunscreens</td>
<td>212</td>
</tr>
<tr>
<td>4.3.6.1</td>
<td>Gloss Readings of the Test Panels</td>
<td>212</td>
</tr>
<tr>
<td>4.3.6.2</td>
<td>Scanning Electron Microscopy Imaging of the Test Panels</td>
<td>215</td>
</tr>
<tr>
<td>4.3.6.3</td>
<td>Separation of Inorganic Sunscreen Components</td>
<td>216</td>
</tr>
<tr>
<td>4.3.7</td>
<td>Rheological Properties of Homemade Sunscreens and Commercial Sunscreens</td>
<td>217</td>
</tr>
<tr>
<td>4.3.8</td>
<td>Photoprotection of Homemade Sunscreens</td>
<td>221</td>
</tr>
<tr>
<td>4.3.9</td>
<td>Cytotoxicity of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles in HaCaT and MDCK Cells</td>
<td>222</td>
</tr>
<tr>
<td>4.3.10</td>
<td>Conclusions</td>
<td>224</td>
</tr>
<tr>
<td>4.4</td>
<td>TiO$_2$/(BiO)$_2$CO$_3$ Nanocomposites for Ultraviolet Filtration with Reduced Photocatalytic Activity</td>
<td>225</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Identification, Size, Morphology, and Surface Composition of TiO$_2$/(BiO)$_2$CO$_3$ Nanocomposites</td>
<td>225</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Optical Properties and Band Gap of the TiO$_2$/(BiO)$_2$CO$_3$ Nanocomposites</td>
<td>232</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Photocatalytic Activity of the TiO$_2$/(BiO)$_2$CO$_3$ Nanocomposites under Ultraviolet Exposure (300 nm and 350 nm) and under Simulated Solar Irradiation</td>
<td>233</td>
</tr>
<tr>
<td>4.4.4</td>
<td>In Vitro Cell Viability in HaCaT and MDCK Cells</td>
<td>237</td>
</tr>
<tr>
<td>4.4.4.1</td>
<td>In the Absence of Simulated Sunlight</td>
<td>237</td>
</tr>
<tr>
<td>4.4.4.2</td>
<td>In the Presence of Simulated Sunlight</td>
<td>238</td>
</tr>
<tr>
<td>4.4.5</td>
<td>Conclusions</td>
<td>241</td>
</tr>
<tr>
<td>4.5</td>
<td>References</td>
<td>242</td>
</tr>
</tbody>
</table>
# CHAPTER 5 – TANTALUM-BASED COMPOUNDS FOR BIOMEDICAL APPLICATIONS

5.1 Introduction ..........................................................................................................................255

5.1.1 Properties of Ta₂O₅ .......................................................................................................255
5.1.2 Polymers and Hydrogels ............................................................................................257
5.1.2.1 Properties of Poly(acrylic acid) ..............................................................................258
5.1.2.2 Polymerization of Acrylic Acid Monomers .............................................................259
5.1.3 Drug Delivery Systems Based on Hydrogels ...............................................................261
5.1.3.1 Principle of Drug Loading and Drug Release ..........................................................261
5.1.3.2 Existing Systems Based on Poly(acrylic acid) .........................................................262

5.2 Development of pH-Sensitive Methotrexate-Loaded δ-Ta₂O₅-Poly(acrylic acid) Nanocomposite with Controlled Drug Release ................................................................................263

5.2.1 Identification, Size, Morphology, and Surface Composition ........................................264
5.2.2 Fourier-Transform Infrared Spectroscopy .....................................................................266
5.2.3 Thermal Stability of δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites .................................268
5.2.4 Surface Composition Before and After Polymerization ...............................................269
5.2.5 Swelling Properties of δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites ............................272
5.2.6 Methotrexate Loading Capacity of δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites .........273
5.2.7 Effect of Poly(acrylic acid) Layer Thickness on the Release of Methotrexate from δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites ........................................................275
5.2.8 Effect of pH on the Drug Release Kinetics .................................................................278
5.2.9 In Vitro Computed Tomography Imaging of δ-Ta₂O₅ Nanoparticles and δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites ........................................................................281
5.2.10 Conclusions ...............................................................................................................284

5.3 References ..........................................................................................................................285
# Table of Contents

## CHAPTER 6 - CONCLUSIONS AND FUTURE PROSPECTS

6.1 Thesis Conclusions ........................................................................................................294
  6.1.1 Theranostic Nanoparticles ..................................................................................294
  6.1.2 Nanoparticles as Ultraviolet Filters in Sunscreens ..........................................296

6.2 Future Prospects ...........................................................................................................297
  6.2.1 Theranostic Nanoparticles ..................................................................................297
  6.2.2 Nanoparticles as Ultraviolet Filters in Sunscreens ..........................................300

6.3 References ....................................................................................................................301

APPENDIX I ......................................................................................................................304

APPENDIX II .....................................................................................................................316
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fu</td>
<td>5-Fluorouracil</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>Attenuated Total Reflectance</td>
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<td>Description</td>
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<td>DLS</td>
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<td>European Collection of Cell Cultures</td>
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<td>EPR</td>
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<td>Fluorescence-Activated Cell Sorting</td>
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<td>FBS</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FEGSEM</td>
<td>Field Emission Gun Scanning Electron Microscope</td>
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<td>FSC</td>
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<td>FWHM</td>
<td>Full Width at Half Maximum</td>
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<td>G</td>
<td>Guanine</td>
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<td>GU</td>
<td>Gloss Unit</td>
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<td>High-Angle Angular Dark-Field</td>
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<td>International Agency for Research on Cancer</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half Maximum Inhibitory Concentration</td>
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<td>Joint Committee on Powder Diffraction Standards</td>
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<td>L</td>
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<td>Minimum Erythematous Dose</td>
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<td>Mismatch Repair</td>
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<td>MOVPE</td>
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<tr>
<td>PAA</td>
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<td>PLA</td>
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<td>RNA</td>
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<td>X-Ray Diffraction</td>
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LIST OF SYMBOLS

A    Absorbance
A_t  Absorbance of treatment
A_c  Absorbance of control
a_{adsorbent}  Mass of adsorbent
A_m  Atomic mass
at. %  Atomic percentage
B    Constant
c    Concentration
c_0  Initial concentration
C_{BET}  BET constant
d    Spacing between diffraction planes
d_{crystallite}  Average crystallite size
D_{diffusion}  Diffusion coefficient
E    Energy
E_1  Heat of adsorption for the first layer
E_g  Band gap
E_L  Heat of adsorption for the second and higher layers
e^-  Electron
E_{\lambda}  Erythemal spectral effectiveness
G    Global
h    Planck's constant \(6.626 \times 10^{-34} \text{ (m}^2 \cdot \text{kg} \cdot \text{s}^{-1})\)
h^+  Hole
I    Intensity
I_0  Incident intensity
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<th>Symbol</th>
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<td>$K$</td>
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<td>$k$</td>
<td>Apparent rate constant</td>
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<td>$k_B$</td>
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<td>$K_f$</td>
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<td>$k_{kin}$</td>
<td>Kinetic constant</td>
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<td>$l$</td>
<td>Path length</td>
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<td>$m$</td>
<td>$m = 2$ for direct transition; $m = 0.5$ for indirect transition</td>
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<tr>
<td>$m_{electron}$</td>
<td>Mass of electron</td>
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<tr>
<td>$M_t$</td>
<td>Amount of drug released at time $t$</td>
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<tr>
<td>$M_\infty$</td>
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<tr>
<td>$n$</td>
<td>Diffusion exponent</td>
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<td>Adsorption cross-section of the adsorbing gas</td>
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<td>$t$</td>
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<td>$\tau$</td>
<td>Shear stress</td>
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</tbody>
</table>
LIST OF FIGURES

Figure 1.1. Estimated number of new cases and deaths for the most common cancer types in the United States in 2018. The data were obtained from the American Cancer Society [4] and the United States National Cancer Institute [2]. The breast cancer data include both female and male; the liver cancer data include intrahepatic bile duct cancer; and the lung cancer data include the bronchus. 2

Figure 2.1. Development of cancer from normal cells, adapted from [1]. Before cancer cells form in tissues of the body, the cells go through abnormal changes (hyperplasia and dysplasia). 12

Figure 2.2. Schematic illustration showing how cancer cells spread all over the body. Adapted from [4]. 12

Figure 2.3. Cancer is caused by certain changes to genes, which are the basic physical units of inheritance and are found in each cell in the nucleus. Adapted from [1]. 13

Figure 2.4. Types of cancer, categorized by the cellular origin of the tumour. The information was collected from the U.S. National Cancer Institute [1]. 14

Figure 2.5. Schematic representation of a skin cross-section, adapted from [13]. 17

Figure 2.6. Electromagnetic spectrum of visible and ultraviolet solar radiation and the effects on the human skin. Adapted from [6]. 18

Figure 2.7. Schematic representation of direct damage to deoxyribonucleic acid (DNA). 20

Figure 2.8. Schematic representation of indirect damage to deoxyribonucleic acid (DNA). 21

Figure 2.9. Variation of ultraviolet dosage with geographical location. Adapted from [6]. 22

Figure 2.10. Examples of different-sized materials, including the classification of nanoparticles (red arrow). Adapted from [36]. 24

Figure 2.11. Examples of compositions of theranostic systems. Abbreviations: deoxyribonucleic acid (DNA), short interfering ribonucleic acid (siRNA). 26
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12</td>
<td>Different types of organic nanomaterials that are applied in cancer therapy. Adapted from [38].</td>
</tr>
<tr>
<td>2.13</td>
<td>Different types of inorganic nanomaterials that are applied in cancer therapy. Adapted from [38].</td>
</tr>
<tr>
<td>2.14</td>
<td>Exemplary core-shell structure.</td>
</tr>
<tr>
<td>2.15</td>
<td>Overview of the role of reactive oxygen species in cancer cells and the induction of apoptosis. Adapted from [99].</td>
</tr>
<tr>
<td>2.16</td>
<td>Chemical structure of methotrexate.</td>
</tr>
<tr>
<td>2.17</td>
<td>Possible interactions of intracellular radiosensitizing nanoparticles with high effective atomic number Z, adapted from [154]. * Abbreviation: deoxyribonucleic acid (DNA).</td>
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<tr>
<td>2.18</td>
<td>Overview of imaging modalities for biomedical applications. Adapted from [161].</td>
</tr>
<tr>
<td>2.19</td>
<td>Working principle of computed tomography scans. Adapted from [164].</td>
</tr>
<tr>
<td>2.20</td>
<td>Axial computed tomography (CT) scans of dogs without administration of a contrast agent (left images) and after administration of 660 mg /kg Iohexol (right images) [171]. In the top row, a small (0.5 cm) hepatocellular carcinoma is identified via the white arrows. In the bottom row, a large hepatocellular carcinoma (12.5 cm) is visible.</td>
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<td>2.21</td>
<td>Working principles of chemical and physical sunscreens.</td>
</tr>
<tr>
<td>2.22</td>
<td>Absorbance of bulk TiO$_2$ and ZnO at room temperature. Adapted from [11].</td>
</tr>
<tr>
<td>2.23</td>
<td>Increase in ultraviolet protection with increasing sun protection factor (SPF). Adapted from [195].</td>
</tr>
<tr>
<td>2.24</td>
<td>Absorbance spectra of sunscreen products with a critical wavelength (a) smaller and (b) greater than 370 nm. Adapted from [198].</td>
</tr>
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<td>2.25</td>
<td>Mechanism of formation of electron-hole pairs, their recombination, and their transport to the particle surface of a semiconductor. Adapted from [201].</td>
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</tbody>
</table>
Figure 2.26. Crystal structures of anatase, rutile, and brookite TiO$_2$; obtained via Materials Studio software using Crystallography Open Database files 1010942, 1532819, and 8104269. ........................................................................................................................................... 56

Figure 2.27. Crystal structures of rocksalt, wurtzite and zinc blende ZnO; obtained via Materials Studio software using Crystallography Open Database files 1534836, 1537875, and 9008877. ........................................................................................................................................... 57

Figure 2.28. Fluorescence microscopy images of U937 human tumour monocytes double-labelled with Hoechst dye (nuclear morphology, top row) and 2',7'-dichlorodihydrofluorescein diacetate, showing the 2',7'-dichlorofluorescein (DCF) signal (reactive oxygen species content, bottom row). The left column represents viable cells, while the centre and right columns show apoptotic cells in budding and cleavage, respectively. Adapted from [121]. ........................................................................................................................................... 62

Figure 2.29. Histopathological evaluation of the skin, liver, spleen, and lung tissue of hairless mice after dermal exposure to TiO$_2$ with particle sizes of 10, 25, and 60 nm for 60 days. The black arrows indicate pathological changes in the tissue. Adapted from [233]. ........................................................................................................................................... 64

Figure 2.30. Dose-dependent cell viability curves of (a) ZnO nanoparticles and (b) TiO$_2$ nanoparticles following exposures of 4 h and 24 h on human skin fibroblasts [249]. ........................................................................................................................................... 67

Figure 2.31. (a) Effect of TiO$_2$ at a concentration of 100 μg/mL on the metabolic activity of HaCaT cells as a function of irradiation dosage. (b) Generation of hydroxyl radicals from TiO$_2$ under irradiation with ultraviolet A light (340 nm). The electron spin resonance spectra were recorded at room temperature in the presence of 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide and TiO$_2$ at a concentration of 0.1 mg/mL. Adapted from [252]. ........................................................................................................................................... 69

Figure 2.32. Survival of Saccharomyces Cerevisiae yeast cells after ultraviolet irradiation for 0 – 40 min. The yeast cells were treated without an ultraviolet filter (left image), with modified TiO$_2$ (centre image), and with azobenzone (right image). The number of yeast cells on the left was twofold greater than the number of cells on the right [268]. ........................................................................................................................................... 73
List of Figures

Figure 2.33. Absorbance curves of pure (black curve), 20 mol.% (red curve), and 40 mol.% (blue curve) CeO$_2$ nanoparticles. Adapted from [275].

Figure 3.1. Schematic illustration of the coating procedure involving the reaction of δ-Ta$_2$O$_5$ nanoparticles and acrylic acid (AA).

Figure 3.2. Schematic principle of X-ray diffraction by a crystalline sample with the angle $\theta$ of the incident beam on lattice planes with spacing $d$.

Figure 3.3. Working principle of X-ray photoelectron spectroscopy.

Figure 3.4. Working principle of a Fourier transform infrared spectrometer, adapted from [17].

Figure 3.5. Rayleigh and Raman scattering (Stokes and Anti-Stokes).

Figure 3.6. Working principle of an ultraviolet-visible spectrometer.

Figure 3.7. Scheme of Brownian motion of (a) small and (b) large particles [26].

Figure 3.8. Schematic diagram of a thermogravimetric analyser.

Figure 3.9. (a) Toshiba Asteion TSX-021A computed tomography scanner. (b) 1.5 mL vials with $\alpha$-Bi$_2$O$_3$ (top) and δ-Ta$_2$O$_5$ (bottom) nanoparticles suspended in phosphate buffered saline.

Figure 3.10. (a) Signals generated during the interaction of an electron beam with a specimen. (b) Interaction volumes in the depth range of an electron beam and a specimen.

Figure 3.11. Adapted schematic illustration of a scanning electron microscope [33].

Figure 3.12. Adapted schematic illustration of a transmission electron microscope [33].

Figure 3.13. Principle of energy-dispersive X-ray spectroscopy.

Figure 3.14. Separation of inorganic sunscreen components of (a) sunscreen emulsion containing TiO$_2$ and Bi(OH)$_3$ NPs (left) and commercial sunscreen formulations of (b) Nivea® anti-age (SPF 30), and (c) Nivea® (SPF 50). On each watch glass, the left side shows the sunscreen and the right side shows the obtained inorganic components after extraction.
Figure 3.15. Schematic procedure for assessment of photocatalytic activity of nanomaterials using crystal violet.................................................................123

Figure 3.16. Sun exposure of steel panels painted with different sunscreen formulations. (a) Front view of the exposure rack. (b) View of the exposure rack facing north at 45°...125

Figure 3.17. Microscopic image of normal HaCaT human skin cells.................................................................129

Figure 3.18. Microscopic image of normal Madin-Darby canine kidney cells.................................129

Figure 3.19. Microscopic image of 9L rat brain tumour cells .................................................................130

Figure 3.20. Microscopic image of MCF-7 human breast cancer cells.................................130

Figure 3.21. Cell counting using a Neubauer haemocytometer. Adapted from [44]........132

Figure 3.22. Working principle of a confocal microscope, adapted from [49]. .......................136

Figure 3.23. Working principle of cell staining with propidium iodide. .........................................137

Figure 3.24. Schematic illustration of a flow cytometer. Adapted from [58]..........................139

Figure 3.25. Forward and side scatter of radiation hitting a single cell. ........................................140

Figure 3.26. Schematic illustration of the cell cycle, showing the flow cytometric components of each phase. Adapted from [60].................................................................141

Figure 3.27. Schematic procedure of the clonogenic survival assay.................................143

Figure 3.28. Working principle of the MTT proliferation assay........................................145

Figure 3.29. Schematic procedure of the MTT proliferation assay........................................146

Figure 3.30. Light emission profile of the OSRAM Ultra-Vitalux® 300 W Sunlamp [71]. ...148

Figure 3.31. Working principle of a fluorescence microscope, adapted from [74]........150

Figure 3.32. Working principle of nuclear staining with Hoechst 33342 [76]..........................151

Figure 3.33. Working principle of cell staining with 2',7'-dichlorodihydrofluorescein diacetate.................................................................152

Figure 4.1. Commercially available (a) Pepto-Bismol® and (b) De-Nol® [5, 6].......................163

Figure 4.2. Molecular structure of PhBi(MeOx)2. An X-ray crystallographic study has shown that the molecule is a five coordinate monomer with distorted square pyramidal stereochemistry. Adapted from [8].................................................................164
Figure 4.3. Ball model of BiOCl; obtained via Materials Studio software using Crystallography Open Database file 1011175.

Figure 4.4. Crystal structure of α-Bi₂O₃; obtained via Materials Studio software with data from [32].

Figure 4.5. Ball model of (BiO)₂CO₃; obtained via Materials Studio software using Crystallography Open Database file 9004677.

Figure 4.6. XRD patterns of Bi(OH)₃ and α-Bi₂O₃ nanoparticles.

Figure 4.7. TEM (left) and HAADF STEM (right) images of (a) Bi(OH)₃ NPs and (b) α-Bi₂O₃ NPs. The HAADF image of Bi(OH)₃ NPs exposes lattice fringes spaces of 0.263 and 0.275 nm. The HAADF image of α-Bi₂O₃ NPs exposes lattice fringes spaces of 0.332 nm that correspond to (111) planes.

Figure 4.8. Hydrodynamic diameter in PBS determined via dynamic light scattering of (a) Bi(OH)₃ and (b) α-Bi₂O₃ nanoparticles. The concentration of both nanomaterials is 50 µg/mL.

Figure 4.9. High-resolution XPS spectra of (a) Bi 4f and (b) O 1s of Bi(OH)₃ (top) and α-Bi₂O₃ (bottom) nanoparticles.

Figure 4.10. Relative internalization of Bi(OH)₃ and α-Bi₂O₃ nanoparticles in 9L, MCF-7, and MDCK cells after 24 h of exposure, with concentrations of nanoparticles of 0, 6.25, 12.5, 25, and 50 µg/mL, obtained through flow cytometry.

Figure 4.11. Confocal images of 9L (top row), MCF-7 (centre row), and MDCK (bottom row) cells without nanoparticles (control, left column), with Bi(OH)₃ nanoparticles (centre column), and with α-Bi₂O₃ nanoparticles (right column). The concentration of both nanomaterials was 50 µg/mL, and the incubation time was 4 – 8 h. The black arrows indicate non-internalized nanoparticles, while the red arrow show internalized nanoparticles. The insets in each image show a close-up of a single cell.

Figure 4.12. Clonogenic assay of (a) 9L, (b) MCF-7, and (c) MDCK cells after 24 h exposure with Bi(OH)₃ and α-Bi₂O₃ nanoparticles at concentrations of 0, 6.25, 12.5, 25, and 50
µg/mL. The cells were trypsinized, plated at low density into 100 mm Petri dishes, and incubated for 15 doubling times at 37°C and 5% (v/v) CO₂. The surviving fraction was obtained by comparing the plating efficiencies of the control and the treatment samples. The * indicates P < 0.05, ** indicates P < 0.01, *** indicates P < 0.001, and **** indicates P < 0.0001 for the comparison with the ‘control’ (0 µg/mL). The b indicates P < 0.05, bb indicates P < 0.01, bbb indicates P < 0.001, and bbbb indicates P < 0.0001 for the comparison between the treatments (ns = not significant).

Figure 4.13. Confocal images of (a) 9L, (b) MCF-7, and (c) MDCK cells stained with propidium iodide without additives (control, left column) and after 24 h of incubation with Bi(OH)₃ nanoparticles (centre column) and α-Bi₂O₃ nanoparticles (right column). The concentration of both nanomaterials was 50 µg/mL.

Figure 4.14. Cell cycle distributions of (a) 9L, (b) MCF-7, and (c) MDCK cells without nanoparticle treatment (left panels) and after exposure to Bi(OH)₃ (centre panels) and α-Bi₂O₃ nanoparticles (right panels), with both at a concentration of 50 µg/mL for 24 h. The cells were trypsinized, fixed with ethanol, stained with propidium iodide, and analyzed using a flow cytometer. The percentages of cells in different phases in the populations are indicated in each panel.

Figure 4.15. 9L cells double-labelled (left columns), labelled with Hoechst stain alone (centre columns; nuclear morphology) and with 2',7'-dichlorodihydrofluorescein diacetate alone (right columns; ROS content; 2',7'-dichlorofluorescein (DCF) signal). The rows show the control cells (top rows); cells after treatment with Bi(OH)₃ nanoparticles for 21 h (centre rows), and cells after treatment with α-Bi₂O₃ nanoparticles for 15 h (bottom rows).

Figure 4.16. MDCK cells double-labelled (left columns), labelled with Hoechst stain alone (centre columns; nuclear morphology) and with 2',7'-dichlorodihydrofluorescein diacetate alone (right columns; ROS content; 2',7'-dichlorofluorescein (DCF) signal). The rows show the control cells (top rows); cells after treatment with Bi(OH)₃ nanoparticles for 21 h.
(centre rows), and cells after treatment with α-Bi₂O₃ nanoparticles for 15 h (bottom rows).

Figure 4.17. Additional number of apoptotic nuclei with cleavage morphology for 9L and MDCK cells after treatment with 50 μg/mL of Bi(OH)₃ and α-Bi₂O₃ nanoparticles for 0, 2, 4, 6, 9, 12, 15, 18, 21, and 24h, compared to non-treated cells. Cleavage frequencies among the total cells were evaluated by Hoechst and 2',7'-dichlorodihydrofluorescein diacetate staining.

Figure 4.18. Additional number of apoptotic nuclei with budding morphology for 9L and MDCK cells after treatment with 50 μg/mL of Bi(OH)₃ and α-Bi₂O₃ nanoparticles for 0, 2, 4, 6, 9, 12, 15, 18, 21, and 24h, compared to non-treated cells. Budding frequencies among the total cells were evaluated by Hoechst and 2',7'-dichlorodihydrofluorescein diacetate staining.

Figure 4.19. (a) Linear fitting of the CT number of Bi(OH)₃ nanoparticles as a function of the mass concentration of Bi in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of linear regression and the coefficient of determination (R²) value are indicated. (b) CT images of Bi(OH)₃ nanoparticles with Bi mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area that was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.

Figure 4.20. (a) Linear fitting of the CT number of α-Bi₂O₃ nanoparticles as a function of the mass concentration of Bi in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of the linear regression and the R² value are indicated. (b) CT images of α-Bi₂O₃ nanoparticles with Bi mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area which was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.

Figure 4.21. XRD patterns of Bi(OH)₃ (M1, top), ZnO (M3, centre), and TiO₂ (M2, bottom) nanoparticles.

Figure 4.22. TEM images of Bi(OH)₃ (M1), TiO₂ (M2), and ZnO (M3) nanoparticles.
Figure 4.23. Particle size distribution of (a) Bi(OH)₃ (M1), (b) TiO₂ (M2), and (c) ZnO (M3) nanoparticles.

Figure 4.24. High-resolution XPS spectra of the O 1s region of (a) Bi(OH)₃ (M1), (b) TiO₂ (M2), and (c) ZnO (M3) nanoparticles.

Figure 4.25. UV-visible absorption spectra of Bi(OH)₃ (M1), TiO₂ (M2), and ZnO (M3) nanoparticles at 25 µg/mL in deionized water. The concentration of the combined materials (ZnO and Bi(OH)₃; TiO₂ and Bi(OH)₃) is 25 µg/mL each, implying a total concentration of 50 µg/mL.

Figure 4.26. Tauc plots of Bi(OH)₃ (M1), TiO₂ (M2), and ZnO (M3) nanoparticles. The red lines represent extrapolated band gaps corresponding to Bi(OH)₃, TiO₂, and ZnO nanoparticles.

Figure 4.27. (a) Relative decrease in absorbance of crystal violet solutions containing only the dye, ZnO (M3, 5 mg/L), TiO₂ (M2, 5 mg/L), and Bi(OH)₃ (M1) nanoparticles with concentrations ranging from 1 to 5 mg/L, and combinations of M2 (5 mg/L) + M1 (1–5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (300 nm and 350 nm). The data represent the mean of three independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).

Figure 4.28. (a) Relative decrease in absorbance of crystal violet solutions containing only the dye, Bi(OH)₃ (M1, 5 mg/L), TiO₂ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles, and combinations of M2 (5 mg/L) + M1 (5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (350 nm). The data represent the mean of three independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).

Figure 4.29. (a) Relative decrease in absorbance of crystal violet solutions containing Bi(OH)₃ (M1, 5 mg/L), TiO₂ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles under exposure of AM 1.5 G one sun (100 mW/cm²). The data represent the mean of three
independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).

Figure 4.30. Gloss readings of pre-painted steel panels without, and panels treated with commercial sunscreen formulations (CS1: Nivea® (SPF 50); CS2: Nivea® anti-age (SPF 30)), the homemade base emulsion, and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2) after exposure to the Australian sun at a latitude of ~34.5°S (Wollongong, NSW) for six and twelve weeks.

Figure 4.31. SEM images of pre-painted steel panels without, and panels treated with, commercial sunscreen formulations (CS1: Nivea® (SPF 50); CS2: Nivea® anti-age (SPF 30)), the homemade base emulsion, and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2) after exposure to the Australian sun at a latitude of ~34.5°S (Wollongong, NSW) for twelve weeks.

Figure 4.32. XRD patterns of the extracted inorganic components of commercial sunscreens (CS1: Nivea® (SPF 50), bottom; CS2: Nivea® anti-age (SPF 30), centre), and the TiO$_2$-based homemade sunscreen (HS, top).

Figure 4.33. Viscosity measurements of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2).

Figure 4.34. Rheograms of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2).

Figure 4.35. Effects of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) nanoparticles on the mitochondrial function in non-cancerous human skin (HaCaT) cells and dog kidney (MDCK) cells. The cells were treated with the nanoparticles at concentrations of 0 (control), 5, 10, 25, 50, 100, 250, and 500 µg/mL for 24 h. At the end of exposure, the mitochondrial function was determined using the MTT reduction assay. The data are presented as the mean of three independently prepared experiments.
Figure 4.36. XRD patterns of commercial TiO$_2$ NPs, and the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites Bi/Ti 2, 4, and 8 at.%.................................................................................................................................226
Figure 4.37. SEM images of TiO$_2$ NPs (left), and the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios..................................................................................................................................................228
Figure 4.38. HAADF STEM images of TiO$_2$ NPs (left), the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios. The HAADF image of the nanocomposite with Bi/Ti 8 at.% (right) exposes lattice fringes spaces of 0.30 and 0.33 nm, corresponding to (001) and (110) planes of rutile TiO$_2$, respectively...................................................................................................................228
Figure 4.39. TEM images with high resolution EDS mapping of (a) TiO$_2$ NPs, (b) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 2 at.%, (c) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 4 at.%, and (d) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 8 at.%..................................................................................................................................................229
Figure 4.40. High resolution XPS spectra of the Bi 4f (left column) and C 1s (right column) regions of TiO$_2$ NPs, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios..................................................................................................................................................231
Figure 4.41. (a) UV-visible absorption spectra and (b) Tauc plots of TiO$_2$ NPs, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios. The absorption spectra were recorded at a concentration of NPs of 25 µg/mL in DI water. ..................................................................................232
Figure 4.42. (a) Relative decrease in absorbance of crystal violet solutions containing TiO$_2$, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios under UV light exposure (300 nm and 350 nm). The concentration of all materials is 5 mg/L. The data represent the mean of three independently prepared samples, which were measured separately. (b) Apparent rate constant curves for the UV-visible dye degradation of crystal violet, as shown in (a). .................................................................................................234
Figure 4.43. (a) Relative decrease in absorbance of crystal violet solutions containing TiO$_2$, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios under exposure of AM 1.5 G one sun (100 mW/cm$^2$). The concentration of all materials is 5 mg/L. The data represent the mean of three independently prepared samples, which were measured
separately. (b) Apparent rate constant curves for the dye degradation of crystal violet under exposure to AM 1.5 G one sun (100 mW/cm²), as shown in (a) ........................................236

Figure 4.44. Effects of the commercial TiO₂ NPs, and TiO₂/(BiO)₂CO₃ nanocomposites with different Bi/Ti atomic ratios on the mitochondrial function in non-cancerous a) HaCaT human skin cells and b) dog kidney (MDCK) cells. The cells were treated with the NPs at concentrations of 0 (control), 5, 10, 25, 50, 100, 250, and 500 µg/mL for 24 h. At the end of exposure, the mitochondrial function was determined using the MTT reduction assay. The data are represented as the mean of three independently prepared experiments....................238

Figure 4.45. Degradation of crystal violet solutions containing TiO₂ NPs at concentrations of 25, 50, and 100 µg/mL upon irradiation with simulated sunlight (300 W Sunlamp, Ultra-Vitalux®, OSRAM) for 15 min........................................................................................................239

Figure 4.46. Viability of HaCaT cells following exposure to TiO₂ or TiO₂/(BiO)₂CO₃ (Bi/Ti 8 a.%) NPs with and without additional simulated sunlight irradiation (300 W Sunlamp, Ultra-Vitalux®, OSRAM) for 15 min. The viability was assessed using the MTT assay and the tested concentrations were 25, 50, and 100 µg/mL. The data are expressed as percentage of viable cells compared to control cells that have not been exposed to nanomaterials. ........................240

Figure 5.1. Crystal structure of δ-Ta₂O₅; obtained via Materials Studio software with data from [9]................................................................................................................................................256

Figure 5.2. Principle of pH-dependence on the swelling behaviour of hydrogels.............259

Figure 5.3. Acrylic acid monomers can either react via addition polymerization or esterification to form poly(acrylic acid) or acrylic esters, respectively. Adapted from [51]. .................................................................................................................................................260

Figure 5.4. Principle of drug release of a drug-loaded nanoparticle-poly(acrylic acid) (PAA) conjugate................................................................................................................................................261

Figure 5.5. Release of DOX from CaF₂:Ce³⁺/Tb³⁺-PAA composites in PBS buffer with pH = 7.4, 4.0, and 2.0. Adapted from [58].................................................................................................................................................263
Figure 5.6. XRD patterns of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h.

Figure 5.7. (a) SEM and (b) TEM images of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The green arrows indicate the PAA layers. (c) Particle size distribution of uncoated $\delta$-Ta$_2$O$_5$ NPs. A total of 100 particles were measured, and the relative frequency was plotted as a function of particle size. (d) Plot of the layer thickness as a function of the polymerization time.

Figure 5.8. Expected reaction for the as-prepared $\delta$-Ta$_2$O$_5$ nanoparticles when dispersed in methanol (left) and etched with hydrochloric acid for 25 min (= surface activation). After centrifugation (centre), the hydroxyl-terminated nanoparticles were transferred into an acrylic acid (AA) solution (= physical adsorption of monomer) and heated up to 83 °C (right) for different polymerization times (= thermal induced chain polymerization).

Figure 5.9. FTIR spectra of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The dotted line indicates the wavenumber below which the absorption bands of $\delta$-Ta$_2$O$_5$ outweigh the absorption bands related to PAA.

Figure 5.10. (a) TGA curves of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. (b) First derivative of the TGA curve of $\delta$-Ta$_2$O$_5$-PAA with 5 h polymerization time.

Figure 5.11. Plot of the organics content as a function of the polymerization time.

Figure 5.12. (a) XPS survey spectra of $\delta$-Ta$_2$O$_5$ NPs, $\delta$-Ta$_2$O$_5$ NPs after HCl treatment, $\delta$-Ta$_2$O$_5$-PAA 1 h, and $\delta$-Ta$_2$O$_5$-PAA 2.5 h. High-resolution spectra of $\delta$-Ta$_2$O$_5$ NPs, $\delta$-Ta$_2$O$_5$ NPs after HCl treatment, and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1 and 2.5 h for b) the C 1s region and c) the O 1s region.

Figure 5.13. (a) Swelling ratio (SR) of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The swelling properties were measured at 37°C at pH 7.4 for 48 h. (b) The influence of the pH value of the buffer solution on the swelling ratio of the $\delta$-Ta$_2$O$_5$-PAA nanocomposite that was synthesized with a polymerization time.
of 5 h. The swelling properties were measured at 37°C at pH 3.6, 5.4, 7.4, and 9.4 for 48 h.

Figure 5.14. (a) Absorbance of MTX solution at concentrations ranging from 2.5 to 45 µM. (b) Calibration curve and equation for the calculation of MTX concentration. ......................................................... 275

Figure 5.15. In vitro release profiles of methotrexate-soaked δ-Ta₂O₅ NPs and δ-Ta₂O₅-PAA nanocomposites with different polymerization times of 1, 2.5, and 5 h in buffer solution with pH 7.4. The drug release was monitored at 37°C for 72 h. ................................................................. 276

Figure 5.16. In vitro release profiles of methotrexate-soaked δ-Ta₂O₅-PAA with longest polymerization time of 5 h in buffer solutions with pH values of 3.6, 5.4, 7.4, and 9.4. The drug release was monitored at 37°C for 72 h. ................................................................. 278

Figure 5.17. (a) Structure of methotrexate. (b) Schematic route of the methotrexate (= drug) loading and release from the nanocomposites containing poly(acrylic acid) (PAA) at different pH values (3.6, 5.4, 7.4, and 9.4). ................................................................. 280

Figure 5.18. (a) Linear fitting of the CT number of δ-Ta₂O₅ nanoparticles as a function of the mass concentration of Ta in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of linear regression and the coefficient of determination (R²) value are indicated. (b) CT images of δ-Ta₂O₅ nanoparticles with Ta mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area that was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp. ................................................................. 282

Figure 5.19. (a) Linear fitting of the CT number of δ-Ta₂O₅-PAA nanoparticles (5 h polymerization) as a function of the mass concentration of Ta in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of the linear regression and the R² value are indicated. (b) CT images of δ-Ta₂O₅-PAA nanoparticles (5 h polymerization) with Ta mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area which was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp. .................................................................................................................................................. 282
LIST OF TABLES

Table 2.1. Subdivision of ultraviolet (UV) light according to the International Organization for Standardization (ISO) standard ISO-21348 [9] ................................................................. 16
Table 2.2. Examples of different types of reactive oxygen species.a) ........................................... 32
Table 2.3. Composition of exemplary theranostic systems for biomedical applications.a) ..... 46
Table 2.4. Selection of sunscreen ingredients and their amount of ultraviolet (UV) radiation protection, as approved by the US Food and Drug Administration (FDA) as of 7 December 2009 [185] ......................................................................................................................... 48
Table 2.5. Selection of dyes that are commonly used to assess the photocatalytic activity of TiO₂ in combination with solar/UV irradiation [209] ......................................................................................... 60
Table 2.6. Physico-chemical properties of selected commercially available coated TiO₂ nanoparticles [228] .......................................................................................................................... 74
Table 3.1. Information on HaCaT human skin cells [42] .............................................................. 129
Table 3.2. Information on Madin-Darby canine kidney cells [42] .................................................. 129
Table 3.3. Information on 9L rat brain tumour cells [42] .............................................................. 130
Table 3.4. Information on MCF-7 human breast cancer cells [42] .............................................. 130
Table 4.1. Polymorphs of Bi₂O₃ and the corresponding crystal systems and space groups. ............................................................................................................................... 166
Table 4.2. Hydrodynamic diameters and intensities (Int) of Bi(OH)₃ and α-Bi₂O₃ nanoparticles suspended in PBS after 2 h of sonication, determined via dynamic light scattering. The concentration of both materials is 50 μg/mL ........................................ 174
Table 4.3. IC₅₀ values for the inhibition of colony number of 9L, MCF-7, and MDCK cell lines following exposure to Bi(OH)₃ and α-Bi₂O₃ nanoparticles for 24 h. ......................................................... 180
Table 4.4. Overview of the materials (M) used. ........................................................................... 196
Table 4.5. Overview of the homemade sunscreens (HS) used...................................................... 196
Table 4.6. Overview of the commercial sunscreens (CS) used.................................................... 197
Table 4.7. Apparent rate constant $k$ for the degradation of crystal violet solutions containing TiO$_2$ (M2, 5 mg/L), ZnO (M3, 5 mg/L), and Bi(OH)$_3$ (M1) nanoparticles, in concentrations ranging from 1 to 5 mg/L, and combinations of M2 (5 mg/L) + M1 (1 – 5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (300 nm and 350 nm), as shown in Figure 4.27. The errors indicated are the standard deviation obtained through the linear regression.

Table 4.8. Apparent rate constant $k$ for the degradation of crystal violet solutions containing Bi(OH)$_3$ (M1, 5 mg/L), TiO$_2$ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles, and combinations of M2 (5 mg/L) + M1 (5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (350 nm), as shown in Figure 4.28. The errors indicated are the standard deviation obtained through the linear regression.

Table 4.9. Apparent rate constant $k$ for the degradation of crystal violet solutions containing Bi(OH)$_3$ (M1, 5 mg/mL), TiO$_2$ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles under exposure to AM 1.5 G one sun (100 mW/cm$^2$). The errors indicated are the standard deviation obtained through the linear regression.

Table 4.10. Flow index ($n_{flow}$) and consistency index ($K$) of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and the homemade sunscreens of TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2). The errors indicated are the standard deviation obtained through the linear regression.

Table 4.11. BET surface areas of TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites.

Table 4.12. High resolution XPS data of Bi 4f and C 1s of TiO$_2$ NPs and the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.

Table 4.13. Band gaps of TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites.

Table 4.14. Apparent rate constant $k$ for the degradation of crystal violet solutions containing TiO$_2$, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites under irradiation by UV light (300 nm and 350 nm) and AM 1.5 G one sun (100 mW/cm$^2$). The concentration of all
materials is 5 mg/L. The errors indicated are the standard deviation obtained through the linear regression.

Table 5.1. Polymorphs of Ta₂O₅ and the corresponding crystal systems and space groups.

Table 5.2. Selection of synthetic polymers that are used for drug delivery systems.

Table 5.3. pH values of different intracellular and extracellular compartments [58].

Table 5.4. XPS analysis including the C 1s region of δ-Ta₂O₅ NPs (before and after treatment with HCl) and δ-Ta₂O₅-PAA nanocomposites with polymerization times of 1 and 2.5 h.

Table 5.5. XPS analysis including the O 1 s region of δ-Ta₂O₅ NPs (before and after treatment with HCl) and the δ-Ta₂O₅-PAA nanocomposites with polymerization times of 1 and 2.5 h.

Table 5.6. Loading capacity (LC) and entrapment efficiency (EE) of δ-Ta₂O₅ NPs and δ-Ta₂O₅-PAA nanocomposites with different polymerization times at a weight ratio of MTX/composite of 1/1 (10 mg/mL).

Table 5.7. Drug release kinetic data for δ-Ta₂O₅-PAA nanocomposites with different polymerization times. The kinetic constant $k_{kin}$ and the diffusion exponent $n$ were obtained from the fit of the drug release experimental data to the Ritger-Peppas equation.

Table 5.8. Drug release kinetic data for the δ-Ta₂O₅-PAA nanocomposite with 5 h polymerization time, exposed to different pH environments. The kinetic constant $k_{kin}$ and the diffusion exponent $n$ were obtained from the fit of the drug release experimental data to the Ritger-Peppas equation.
LIST OF PUBLICATIONS AND CONFERENCES

Publications


Conferences


SCHOLARSHIPS AND AWARDS

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- Higher Degree by Research Scholarship, Institute for Superconducting and Electronic Materials, University of Wollongong, Australia, 2014.

- International Postgraduate Tuition Award, University of Wollongong, Australia, 2014.

- PROMOS Scholarship, German Academic Exchange Service, Germany, 2013.

- ERASMUS Scholarship, Leibniz Universität Hannover, Germany, 2012.

- Niedersachsen Scholarship, Leibniz Universität Hannover, Germany, 2009.

- Award for Best Performance in Chemistry, Gesellschaft Deutscher Chemiker, Germany, 2006
CHAPTER 1 – Introduction
1.1 Background and Motivation

Nanoparticles (NPs) are emerging as a promising class of therapeutics for the treatment of malignant neoplasms, which are commonly known as cancer and are one of the leading causes of death worldwide [1]. In 2012, over 14 million new cases of cancer were registered, and the number is expected to rise by about 70% over the next two decades [2]. In 2015, 8.8 million deaths were related to cancer, according to the World Health Organization [3]. Figure 1.1 displays the estimated numbers of new cases and deaths for the most common cancer types in the United States in 2018.

![Figure 1.1](image_url)

**Figure 1.1.** Estimated number of new cases and deaths for the most common cancer types in the United States in 2018. The data were obtained from the American Cancer Society [4] and the United States National Cancer Institute [2]. The breast cancer data include both female and male; the liver cancer data include intrahepatic bile duct cancer; and the lung cancer data include the bronchus.

Although chemotherapeutic drugs are widely applied in cancer therapy, a report in 2004 stated that “the overall contribution of curative and adjuvant cytotoxic chemotherapy to 5-year survival in adults was estimated to be 2.3% in Australia and 2.1% in the USA.” [5]. These numbers are very low, especially since the overall 5-year survival rate of cancer is over 60%. The reasons for the low impact of chemotherapeutic agents alone are the
increase in patient drug tolerance, the lack of cancer cell targeting, and altered drug-receptor sensitivity. Combinations of therapies and the design of theranostic systems seem therefore to be a reasonable approach to improve the efficiency of cancer therapy.

Early clinical trials suggested that therapeutics based on NPs showed higher therapeutic efficiency than commonly used anticancer drugs, while simultaneously minimizing side effects, as they are capable of delivering drugs in the optimum dosage and at the optimum frequency to cancerous tissue [6, 7]. Biodegradable NPs based on polymers are of particular interest, as they provide controlled release of pharmacologically active drugs to the action site [8]. Moreover, it is well known that NPs navigate easily across various biological barriers to preferentially accumulate in tumour tissues due to their enhanced permeability and retention effects [9]. This realization guided some of the work performed for this doctoral thesis.

By designing multifunctional NP systems, additional features can be included in these so-called theranostic systems, which also provide targeting features and enable active cellular uptake [7]. The use of compounds with a high atomic number can enhance the radiation dose, which ultimately maximizes the efficiency of radiation therapy. It has been shown that the energy of the radiation beam could be reduced to orthovoltage radiotherapy (< 500 keV), while still providing enough damage to some human tumours, which further minimizes the destruction of normal cells [10]. It is therefore desirable to combine as many functions as possible on one single platform, while keeping the overall complexity, chemical instability, and fabrication costs of the theranostic system low.

Besides the treatment of cancer, the prevention of melanoma – one form of skin cancer – is of recent interest. According to the United States National Cancer Institute, melanoma can affect persons of any age, although it is mainly diagnosed in adulthood [11]. An alarming development is the increase in lifetime risk of being diagnosed with melanoma over the past century: while in 1935, the lifetime risk of developing melanoma
was 1 in 1,500, this number increased significantly to 1 in 68 individuals in 2002 [12]. The formation of melanoma is typically linked to excessive exposure to ultraviolet (UV) radiation from the sun, which can result in photo-aging and genetic damage through the generation of reactive oxygen species (ROS) [13, 14].

Commonly used sunscreen formulations contain inorganic UV filters such as TiO$_2$ or ZnO NPs, which function through the absorption of UV light. It has been shown, however, that these NPs are biologically more reactive than the normal microscale bulk material, with the smaller size allowing them to penetrate the skin more readily and reach deeper tissue layers [15]. This penetration is critical, as recent findings reveal that both TiO$_2$ and ZnO NPs exhibit intrinsic cytotoxic and genotoxicity. All these processes can be significantly enhanced through their photocatalytic activity when exposed to UV radiation [16, 17]. The photogenerated ROS can cause the degradation of the organic additives that are employed in sunscreens, yielding the formation of by-products with unknown toxicity [17-19]. In addition, after topical application, the degradation of organic UV filters results in a continual reduction of the sun protection factor (SPF) under exposure [20, 21]. Approaches to minimizing the photocatalytic activity of the active inorganic sunscreen ingredients include the coating of TiO$_2$ NPs with silica (SiO$_2$) or aluminium oxide (Al$_2$O$_3$), but complete protection has not been achieved as yet [16]. It has also been shown, however, that the composite materials TiO$_2$/SiO$_2$ and TiO$_2$/Al$_2$O$_3$ can display enhanced photocatalytic activity, which emphasizes the importance of the search for alternative and less reactive inorganic UV filter [22].

The purpose of this work was to engineer and study different inorganic NPs, including nanoceramics, based on Bi and Ta for biomedical applications, with the focus on the prevention and treatment of cancer. Inorganic Bi- and Ta-based NPs are easily synthesized, are known to be chemically stable, are biocompatible, and have not yet been well studied for biomedical applications [23-25]. Due to their high effective atomic
number, these materials are excellent candidates for the development of theranostic systems that can provide, besides their therapeutic and targeting functions, diagnostic features and radiation dose enhancement [26-29]. Moreover, the easily modifiable surfaces of ceramic materials allow loading with anti-cancer drugs, which is typically achieved with conjugation or encapsulation by a polymer layer [30]. On the nanoscale, polymers can improve the half-life, solubility, and stability of drug delivery systems, while reducing potential side effects [31-33]. Another component of this doctoral thesis was the optimization of existing TiO₂-based UV filters, with an emphasis on the reduction of the photocatalytic activity associated with TiO₂, and the study of a completely novel Bi-based inorganic UV filter for sunscreen application. Therefore, the aim of this project was to synthesize and characterize various Bi- and Ta-based nanomaterials regarding their physicochemical properties and their biological activity in cancer and normal cells in vitro.

1.2 Thesis Structure

This doctoral work is composed of six chapters. A brief description of each chapter’s role is outlined below:

Chapter 1 introduces the theme and motivation for this doctoral thesis, and details its structure.

Chapter 2 provides an overview of the fundamentals of cancer, UV radiation, and melanoma, and provides an in-depth look at the fundamental idea behind theranostic systems. A detailed outline is given regarding different nanomaterials for the development of theranostic systems and their mechanism of action, with the focus on therapeutics, diagnostics, radiation dose enhancement, and targeting. A comprehensive review of the use of NPs as UV filters in sunscreens is presented, focusing on the limitations of current sunscreens and current strategies to improve their performance, and concluding with likely future directions for the development of inorganic UV filters.
Chapter 3 presents the synthesis methods used in the fabrication of the Bi- and Ta-based nanomaterials studied in this work, and the various physical, chemical, morphological, photocatalytic, and biological *in-vitro* characterization techniques that have been utilized. Relevant details on the materials used, and sample preparation and analysis are specified in the subsequent results chapters.

Chapter 4 summarizes the studies of all Bi-based compounds: the first section discusses the study of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs regarding their physicochemical properties and their bioactivity in 9L gliosarcoma cells, MCF-7 human breast cancer cells, and normal Madin-Darby canine kidney (MDCK) cells *in vitro*. There is a specific focus on the analysis of the oxidative features of apoptotic cells. The second section focuses on the study of Bi(OH)$_3$ NPs as a novel, multifunctional UV filter for sunscreens with reduced photocatalytic activity compared to TiO$_2$ and ZnO NPs, which are typically used as inorganic UV filters. The photoprotection, photostability, and rheological properties of in-house fabricated sunscreens containing Bi(OH)$_3$ NPs are compared to sunscreen products commercially available in Australia. The modification of TiO$_2$ NPs for UV blocking applications has been the target of great research interest. Hence, the third section presents the development of TiO$_2$/Bi$_2$CO$_3$ nanocomposites and the study of their UV properties and photocatalytic activity, especially when exposed to HaCaT human keratinocytes and simulated sunlight *in vitro*. This chapter compiles information published in the following three journal articles: K. Bogusz et al., *Materials Science and Engineering C, 2018*, 93, 958-967; K. Bogusz et al., *Materials Research Bulletin, 2018*, 108, 130-141; K. Bogusz, et al., *Journal of Materials Chemistry C, 2018*, 6, 22, 5639-5650.

Chapter 5 focuses on the development of a theranostic system based on a composite of δ-Ta$_2$O$_5$ and poly(acrylic acid) (PAA), which was loaded with the anticancer drug methotrexate (MTX). The drug release of the MTX-loaded δ-Ta$_2$O$_5$-PAA composites was studied at different pH, and the contrast-enhancing properties of the composites were
studied in vitro. This chapter is based on the following manuscript, which is currently under revision: K. Bogusz et al., submitted to *Journal of Colloid and Interface Science*, 2018.

Chapter 6 concludes this doctoral thesis by summarizing the conclusions and knowledge acquired from this work, and discusses future prospects for the development of multifunctional nanomaterials in general, and Bi- and Ta-based compounds in particular.

1.3 References


CHAPTER 2 – Literature Review
2.1 Cancer Characteristics

2.1.3 Cancer Cells and Normal Cells

Cancer is a group of diseases that are characterized by the uncontrollable, abnormal, rapid, and indefinite proliferation of cells with the potential to invade other body parts [1]. In contrast to normal cells, cancer cells are less specialized and are able to avoid programmed cell death or apoptosis by ignoring signals, which ultimately results in the continuous growth and division of cells [2]. In addition, cancer cells promote the construction of blood vessel and often invade the immune system, inhibiting immune responses and consequently, removing another control mechanism for the detection and removal of damaged and abnormal cells [1, 2].

Not every change in the body's tissue, however, is directly linked to cancer. Hyperplasia, for instance, is defined as the process by which cells within a tissue divide faster than normal cells [1]. As a consequence, extra cells build up that exhibit the properties of normal cells, when examined via microscopy. Dysplasia, in contrast, is characterized by a build-up of cells that show abnormal cell properties [1]. If not treated, the abnormal cells are likely to develop into cancerous structures. The different forms of normal cell growth and the transition to malignant cell growth are shown in Figure 2.1.

Almost every type of cell in the human body can develop into cancer cells, typically after they are genetically altered, which increases the complexity of treatment. Many cancers form solid neoplasms or tumours, which are masses or lumps of tissue [3]. Once a cancerous tumour is formed, it can spread within the body via both the lymphatic system and the bloodstream, forming metastatic tumours (Figure 2.2). Unlike malignant tumours, benign tumours typically do not spread into nearby tissues, and they do not tend to grow back once they have been removed [1].
2.1.4 Causes of Cancer

Cancer is a genetic disease and can be inherited or arises as a result of external influences that cause changes to the genes, which is schematically shown in Figure 2.3 [1]. Typical environmental exposures that can trigger genetic mutations include [5, 6]:

- **Physical carcinogens**: ultraviolet (UV) and ionizing radiation.
- **Chemical carcinogens**: components of tobacco smoke, aflatoxin, heavy metals, or polycyclic aromatic hydrocarbons.
- **Biological carcinogens**: bacteria and parasites.

![DNA structure](image)

**Figure 2.3.** Cancer is caused by certain changes to genes, which are the basic physical units of inheritance and are found in each cell in the nucleus. Adapted from [1].

The genetic changes that contribute to the formation of cancer typically affect three main types of genes: proto-oncogenes, tumour suppressor genes, and deoxyribonucleic acid (DNA) repair genes [1]. Proto-oncogenes are involved in the growth and division of normal cells, but may become oncogenes once these genes are altered [1]. An alteration of tumour suppressor genes, which are involved in controlled cell growth, can lead to uncontrolled cell division [1]. Mutations in DNA repair genes typically result in the development of mutations in other genes [1].
2.1.5 Types of Cancer

In total, there exist over 100 different types of cancer, typically named after the organs of origin. Figure 2.4 displays a selection of cancer types, categorized by the original cells where they arise [1].

Figure 2.4. Types of cancer, categorized by the cellular origin of the tumour. The information was collected from the U.S. National Cancer Institute [1].
2.1.6 Current Cancer Prevention Strategies and Anti-Cancer Treatments

The risk of cancer can be controlled and reduced by implementing prevention strategies, such as evasion of risk factors, including the reduction of exposure to UV radiation (see section 2.2.4 and 2.2.6) [7]. Furthermore, early detection of cancer can significantly increase the chances of surviving. Typical screening methods are mammography screening for breast cancer or visual inspection for cervical cancer [7].

Depending on the location, type, and size of the tumour, different types of cancer treatments are currently applied in hospitals. These treatments include:

- Surgery,
- Chemotherapy, and
- Radiation therapy.

Surgical removal of the tumour is usually preferred if no metastases are formed and the tumour is large in size and easy to reach. There are typical risks associated with the surgical treatment, however, such as bleeding and infection [8].

In chemotherapy, highly-effective drugs are given to the patient, which can spread to various parts of the body, where they destroy cancer cells and stop or slow-down their growth [8]. Such treatment is recommended if metastatic tumours are formed. This treatment is associated with many side effects, however, such as anaemia, infections, and nerve changes [8]. Furthermore, chemotherapy cannot completely kill all cancer cells in vivo within a certain time, so that the cancer can relapse or metastases are formed [8].

Radiation therapy uses ionizing radiation to control or kill cancer cells and causes less severe side effects in comparison to chemotherapy. The success rate of radiation therapy, however, depends strongly on the type and progress of the tumour: while the chances are high that single and very localized tumours will be destroyed, a synergistic therapy with chemotherapeutic drugs is used for less manageable tumours [8].
Besides these classical therapies, a number of individually applied methods exists, such as biological therapy, hormone therapy, and photodynamic therapy [8].

### 2.2 Ultraviolet Radiation and Melanoma

#### 2.2.1 Ultraviolet Light Irradiation

UV light has wavelengths of 10 – 400 nm and can be subdivided into a number of ranges as per the recommendation from the International Organization for Standardization (ISO) standard ISO-21348 [9]. Their selection of UV ranges is shown in Table 2.1.

<table>
<thead>
<tr>
<th>Ultraviolet range</th>
<th>Abbreviation</th>
<th>Wavelength (nm)</th>
</tr>
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<tbody>
<tr>
<td>Near ultraviolet</td>
<td>NUV</td>
<td>300 – 400</td>
</tr>
<tr>
<td>Middle ultraviolet</td>
<td>MUV</td>
<td>200 – 300</td>
</tr>
<tr>
<td>Far ultraviolet</td>
<td>FUV</td>
<td>122 – 200</td>
</tr>
<tr>
<td>Extreme ultraviolet</td>
<td>EUV</td>
<td>10 – 121</td>
</tr>
</tbody>
</table>

The ability to see UV rays strongly depends on the eye’s development of colour receptors for UV rays. Typically, UV rays are invisible to humans, although, under certain conditions, children are able to see UV down to a wavelength of 310 nm [10]. In addition, NUV radiation is visible to insects, some mammals, and birds [10].

#### 2.2.2 The Human Skin Barrier

Human skin is composed of the epidermis and dermis. The upper epidermal layer, the stratum corneum, is the skin’s primary protection against percutaneous penetration of exterior substances [11] and is densely packed with approximately 15 layers of dead cells (corneocytes), which are embedded in a lipid matrix and are mainly filled with keratin, water, and enzymes [12] (Figure 2.5). The unique lipid organization in the intercellular stratum corneum is essential for the functioning of the skin barrier. The viable epidermis
consists of three layers: the stratum basale, the stratum spinosum, and the stratum granulosum. Cells proliferate in the stratum basale and start to differentiate and migrate into the stratum spinosum and stratum granulosum [13]. At the interface between stratum granulosum and stratum corneum, viable cells are transformed into dead corneocytes [13].

![Skin Cross-Section Diagram](image)

**Figure 2.5.** Schematic representation of a skin cross-section, adapted from [13].

The skin barrier regulates the production of lipids, stops excessive water loss, and prevents stressors from entering deeper skin layers. These stressors can be [14]:

- **Physical assaults:** mechanical injury, UV radiation.
- **Microbial assaults:** pathogens such as bacteria, fungus, viruses.
- **Chemical assaults:** allergens, oxidative substances, irritants, toxins.

The corneocytes are oriented parallel to the surface and allow for substances to permeate along indirect pathways through the intercellular areas [13]. Besides the transcellular and paracellular routes, the transappendageal route includes the penetration of substances along hair follicles, sebaceous glands, and sweat pores [11]. Compromised skin often shows a reduced skin barrier efficiency, which can be linked to changes in the lipid composition or altered tight junctions [15, 16]. Once the skin barrier is altered, substances can pass into deeper skin layers and potentially cause harm, locally or systematically.
2.2.3 Effects of Solar Ultraviolet Radiation on the Human Skin

With regards to the penetration of solar UV radiation into the skin, a further subdivision of UV radiation is made into: ultraviolet A (UVA), ultraviolet B (UVB), and ultraviolet C (UVC) radiation (Figure 2.6). In contrast to solar UVA and UVB, solar UVC radiation has a wavelength of typically 100 – 280 nm, is completely absorbed by stratospheric oxygen, and hence does not reach the human skin.

**Figure 2.6.** Electromagnetic spectrum of visible and ultraviolet solar radiation and the effects on the human skin. Adapted from [6].

2.2.3.1 Solar Ultraviolet B Irradiation

More than 90% of the solar UVB rays with a wavelength of 280 – 320 nm are absorbed by ozone and thus, do not reach the human skin [6, 9]. The small percentage that penetrates the skin, however, is typically absorbed by the epidermis and does not reach deep skin layers [6].
The energy is absorbed by DNA and causes genetic damages and mutations via molecular rearrangements and the formation of cyclobutane-pyrimidine dimers (CPDs) and the more mutagenic 6,4 pyrimidine-pyrimidones [17]. UVB rays lead, besides their potential to cause cancer, to the formation of redness, sunburn, freckles, and eye damage. The occurrence of sunburn is particular high amongst children, which ultimately increases the risk of developing melanoma in adulthood [18, 19].

2.2.3.2 Solar Ultraviolet A Irradiation

Higher wavelengths of 320 – 400 nm are classified as UVA, with further distinction of UVA1 (340 – 400 nm) and UVA2 (320 – 340 nm). Solar UVA rays can deeply penetrate into the dermis, up to the dermis stratum papillare [9, 20]. Visible light penetrates even deeper into the tissue, which leads – together with the penetration of UVA rays – to genetic mutations and base pair breakage of the DNA, base modifications, and the generation of ROS [6, 20].

An excessive exposure to solar UVA radiation can therefore result in photo-aging by shrinking and depletion of collagen and elastin, immune-suppression, and sunburns, which can cause the formation of skin cancer, especially melanoma [20, 21].

The damage to the skin through solar UV radiation can further damage the skin barrier and lead to increased epidermal permeability, which ultimately increases the chances for particles to reach viable cells in the skin [22].

2.2.4 Impact of Ionizing Radiation on Deoxyribonucleic Acid

High-energy UV radiation is considered to be ionizing radiation and can cause, together with many other substances such as some chemicals or heavy metals, direct and indirect damage to DNA [23, 24].
2.2.4.1 Direct Impact of Ionizing Radiation on Deoxyribonucleic Acid

Alpha particles, beta particles or X-rays, due to their high energy, easily produce ions if in contact with living tissue, causing direct damage to DNA by physically breaking its sugar phosphate backbones [23, 25].

As a result, single and double strands can break, which can lead to the loss of DNA fragments and the formation of tumour cells, if for instance the deleted chromosomal area encoded a tumour suppressor or a protein with oncogenic potential (Figure 2.7) [25]. Furthermore, DNA base pairs can be damaged, as they are held together by weak hydrogen bonds. UV radiation can induce the formation of covalent linkages between consecutive bases along the nucleotide chain, which then causes the formation of CPDs and 6-4 photoproducts [23, 26-28].

![Direct DNA damage](image)

**Figure 2.7.** Schematic representation of direct damage to deoxyribonucleic acid (DNA).

2.2.4.2 Indirect Impact of Ionizing Radiation on Deoxyribonucleic Acid

Indirect damage to DNA occurs typically through free and highly reactive radicals, as shown in Figure 2.8 (see section 2.3.4). The high-energy radiolysis of H₂O molecules into H⁺ and OH⁻ radicals can result in the formation of superoxide (HO₂) and peroxide (H₂O₂) molecules, which can produce oxidative damage to DNA, lipids, proteins, and many metabolites [23, 24]. For instance, the DNA lesion 7,8-dihydro-8-oxoguanine can be caused through oxidative alterations and results in a mismatched pairing with adenine[29].
2.2.4.3 Repairing Mechanisms of Deoxyribonucleic Acid after Exposure to Ionizing Radiation

When DNA strains break, DNA typically repairs itself through a number of different processes [23, 25]:

- Photoreactivation,
- Base excision repair (BER),
- Nucleotide excision repair (NER),
- Mismatch repair (MMR).

Typically, damaged DNA is firstly removed by endonuclease, followed by a DNA synthesis by DNA polymerases. Besides these repair mechanisms, the cell has also cell-cycle checkpoints and programmed cell death [23, 27].

Although these DNA repair mechanisms are highly efficient, they are not always flawless. As a consequence, insertions of incorrect nucleotides are often observed, which can lead to cell death or mutation such as substitutions, transitions, transversions, frameshift mutations, insertions, or deletions [23, 27, 28]. During the repairing of double-strand breaks, for instance, the joining of non-homologous chromosomes can occur, which in the long term, can lead to tumorigenesis.

Figure 2.8. Schematic representation of indirect damage to deoxyribonucleic acid (DNA).
2.2.5 Melanoma Risk Factors and Special Situation in Australia

The amount and strength of solar UV radiation depends on the altitude and proximity to the equator. Before the sunlight reaches the surface of the Earth, it can be absorbed, reflected back into space, or scattered by atmospheric particles. The greater the atmospheric interference on the way to the Earth’s surface, the lower the corresponding UV content of the sunlight is (Figure 2.9). The equator is most closely located to the sun, resulting in low atmospheric interference and high UV content. Towards the North Pole and South Pole, the sunlight has to travel longer, which results in greater scattering and lower UV doses from sunlight.

![Figure 2.9. Variation of ultraviolet dosage with geographical location. Adapted from [6].](image)

The increased UV doses in areas close to the equator such as Australia are directly linked to the highest melanoma rates. As such, the lifetime risk of developing melanoma in Australia in 2002 was 1 in 50 individuals [30]. Due to the hot weather, people typically wear less clothes and spend more time outside, which ultimately increases the UV exposure and risk of developing melanoma [31]. In addition, the majority of the Australian population is composed of fair skinned people, who are known to have a higher risk of developing melanoma, as opposed to dark-skinned people [32].
Besides the exposure to solar UV radiation, a large number of risk factors exist that influence the formation of melanoma. These risk factors are: indoor tanning and ultraviolet A (PUVA) therapy, skin pigmentation and pre-existing moles, sunburns, old age, immunodeficiency, chemical carcinogens, and genetic factors [6].

### 2.2.6 Current Methods of Protection from Solar Ultraviolet Radiation

While not all risk factors, such as genetic predisposition, can be controlled, the awareness of other risk factors is important for significantly reducing possible exposure to them. Being one of the highest risk factors, reduction of exposure to harmful solar UV radiation can be achieved by various methods, including: seeking of shade [33], application of broad-spectrum sunscreens with a SPF of 50+, and wearing of clothes, a broad-trimmed hat, and sunglasses [6, 33, 34].

The most effective method of protection is the complete avoidance of sun exposure. When sun exposure cannot be avoided, it is preferable to pursue sun contact when the UV index is low. The UV index is used to categorize the strength of solar UV radiation at a particular time and place, and is an international standard measurement that aims primarily to inform the general public about the need for protection [35]. Typically, the UV index is highest between 10 am and 4 pm when the sun is the most intense [33].

These control mechanisms are of special importance to fair skinned people, who have to be more careful with solar UVA and UVB radiation due to their lack of epidermal melanin [6]. Moreover, indoor tanning should be avoided, and pre-existing moles should be monitored from an early age. Especially in older age groups, screening and monitoring of skin changes is important. With regard to chemical and biological carcinogens, the careful handling of all chemicals and attentive reading of ingredient labels and their associated side effects are strongly advised.
2.3 Theranostic Nanomaterials for Cancer Treatment

2.3.1 Definition of Nanomaterials

A new direction of anti-cancer treatment involves the development of drugs that are based on NPs. Nanosized materials are defined as materials with a size ranging from 1 to 100 nm (Figure 2.10), and they exhibit unique and versatile physicochemical properties that are attributable to their elemental composition, crystallinity, size, shape, and surface features [36-38].

![Figure 2.10. Examples of different-sized materials, including the classification of nanoparticles (red arrow). Adapted from [36].](image)

The unique properties of nanocrystalline materials led to their use in microelectronics and as components in sensors and for the aerospace industry [39]. NPs are also applied in catalysis, photocatalysis, and in the mechanical industry, particularly as coatings, lubricants, and adhesives [40-43]. An emerging field of research is the energy sector, including the generation of energy through renewable sources and energy storage [40]. Other applications include pollution prevention, and the removal of hazardous substances [40, 44]. Applications of NPs in biology and medicine include labelling, biosensors, drug and gene delivery, detection of pathogens and proteins, and tissue engineering [45-51]. Moreover, as the growing use of engineered NPs increases their...
release into the environment, NPs are thoroughly investigated for their geno-, eco-, and cytotoxicity, and their persistency and reactivity [52].

Various NPs have been studied for their toxicity in numerous different cancer cell types, both in vitro and in vivo [37]. Typically, NPs are characterized by their ability to easily perfuse out of the bloodstream and their high tissue permeability. More importantly, NPs typically exhibit the Enhanced Permeability and Retention (EPR) property, in which molecules with a certain size tend to accumulate in tumour tissue to a larger degree as compared to normal tissue [53]. The increased accumulation of NPs is typically linked to the increased need for stimulation to produce blood vessels for fast cell growth [53, 54].

2.3.2 Definition of a Theranostic System

The term ‘theranostics’ is defined as the combination of a therapeutic agent and an imaging agent in a single system, which can be, for instance, achieved by combining materials with different properties in form of a composite material. Besides these two fundamental properties, even more functionalities can be added to the system, such as a targeting agent, as displayed in Figure 2.11.

The design of a theranostic system typically includes a drug carrier on which biologically active materials, such as antibodies or anti-cancer drugs, are attached. In particular, the use of nanomaterials is of interest, since they provide, besides their small size and the associated ability to easily pass through cell membranes, a high surface area that allows functionalization [38, 55].
Moreover, by tailoring the size of the nanomaterials, their tissue accumulation can be controlled. NPs with a diameter of $\leq 5$ nm either accumulate in tissue through pinocytosis or extravasation, or are cleared from the blood through the kidney. The fast clearance can have the advantage of reducing the total exposure of the human body to the NPs, as in the case of drug delivery systems that have released their active components [38]. If the NPs reach diameters $\geq 200$ nm, however, their potential to accumulate in tissue significantly decreases, making them less favourable as drug carriers [56].

### 2.3.3 Nanomaterials for Theranostic Systems

The nanomaterials that are applied for cancer therapy can have various different natures, ranging from simple inorganic gold (Au) NPs to more complex nanographene. More recently, organic structures, such as dendrimers and polymer-drug conjugates have emerged, with the aim of combining multiple features of nanomaterials. As a matter of fact, the use of single therapeutic agents has been shown to not be as effective as desired [57]. Therefore, modern approaches try to combine the use of multiple drugs and the use of inorganic and organic nanomaterials on one single platform [58].

**Figure 2.11.** Examples of compositions of theranostic systems. *Abbreviations: deoxyribonucleic acid (DNA), short interfering ribonucleic acid (siRNA)*.
2.3.3.1 Organic Nanoparticles

In general, the use of polymer-based materials can offer various advantages, such as a high biodegradability, biocompatibility, high functionality, and prolonged circulation of the nanomaterials. Moreover, the use of pH-sensitive polymers is promising due to the different pH values present in the tumour environment, endosome, and lysosome [59, 60].

Polymeric micelles are spherical structures with a hydrophobic core and a hydrophilic shell, which enables them to load and deliver anti-cancer drugs to the appropriate site (Figure 2.12) [38]. Similar to polymeric micelles, polymeric NPs provide superior pharmacokinetic properties, such as drug loading and drug stability. Typically, the NPs are surrounded by a polymer matrix that can adsorb, dissolve, or entrap drugs, or incorporate diagnostic agents, such as gadolinium complexes and magnetic NPs [61-63]. By combining drugs and water-soluble polymers on a single platform through a direct linkage or a spacer, polymer-drug conjugates can be obtained, which are the most successful nanotherapeutics that have been studied in regard to cancer therapy [64].

![Organic nanoparticles](image)

**Figure 2.12.** Different types of organic nanomaterials that are applied in cancer therapy. Adapted from [38].

Dendrimers typically have a highly branched three-dimensional structure, which provides empty holes, the surfaces of which have a high density of functional groups. This high functionality allows drugs, proteins, genes, or imaging agents to be loaded onto dendrimers through electrostatic interactions, encapsulation, or covalent bonding [65, 66].

Liposomes consist of closed three-dimensional membrane structures, which are made through self-assembly of materials with hydrophobic and hydrophilic ends, such as
phospholipids. The core is generally an aqueous phase, in which water-soluble drugs, enzymes, and nucleic acids can be incorporated [67]. The membrane layer itself is hydrophobic and allows the entrapment of water-insoluble bioactive components [68].

### 2.3.3.2 Inorganic Nanoparticles

There are a great number of inorganic-based nanomaterials that have been explored as active components of theranostic systems, including quantum dots, metal NPs, metal oxides, carbon-based nanomaterials, and magnetic NPs (Figure 2.13).

Quantum dots are normally semiconductor nanocrystals with a tunable particle size ranging from 2 to 100 nm, which enables them to display narrow emission spectra with emission maxima at 450 – 850 nm [69]. They are excellent imaging agents, as they provide bright signals and are photostable over long-time imaging. Quantum dots can further act as either photosensitizers or activators for other photosensitizers by energy transfer [70].

![Inorganic nanoparticles](image)

**Figure 2.13.** Different types of inorganic nanomaterials that are applied in cancer therapy. Adapted from [38].

Au NPs are biocompatible, tailorable in size and shape, and can easily be functionalized by surface coatings. Moreover, they have been explored in cancer therapy due to their labelling and sensing properties, and can act as a radiosensitizer due to their high X-ray absorption [71, 72]. Ceramic materials, such as silicon dioxide (SiO$_2$) can be synthesized in various nanostructures, including rigid porous structures with high surface areas and high density of functional groups, which enables the entrapment of anti-cancer
drugs or imaging agents [73, 74]. In addition, these ceramic materials are typically photostable and show high biocompatibility [38].

Carbon-based nanomaterials include carbon nanotubes (CNTs), fullerenes, and graphene, and offer biocompatibility, thermal conductivity, and unique electrical, optical, and mechanical properties [75]. The modifiable surface of these structures allows π-π stacking, hydrophobic interactions, covalent conjugation, hydrogen bonding, and electrostatic interaction with various drug molecules [76]. Due to their strong absorbance in the near-infrared region, these materials can be used as a contrast agent, and more importantly, they can be used for as photothermal agents that can cause ablation of cancer cells via the induction of hyperthermia [38, 77, 78].

Various types of magnetic nanomaterials with tuneable particle size exist, such as iron oxides, pure metals, spinel-type ferromagnets, and alloys [79]. Bioactive components are typically introduced into the system through coating with surfactants or polymers [38]. Magnetic nanomaterials are of particular interest, as they provide magnetic targeting, magnetic resonance imaging (MRI), and can induce cell death in hyperthermia therapy [80].

### 2.3.3 Core-Shell Structures

Similar to polymer-drug conjugates, liposomes, and magnetic nanomaterials, inorganic nanomaterials, such as quantum dots, metal NPs, and ceramic NPs, can be functionalized by a polymer to form a core-shell structure. Typically, the core provides imaging capabilities, while the polymer shell enables further attachment of bioactive materials, such as anti-cancer drugs or antibodies [58].
Figure 2.14. Exemplary core-shell structure.

The biocompatibility and stability of core-shell structures is significantly improved with polymer coatings such as poly(ethylene glycol) (PEG) or dextran [38]. Moreover, due to the great variety of polymers, tailored attachment, encapsulation, or covalent bonding of particular bioactive components can be achieved [58]. Moreover, the coating itself can provide features, such pH-dependent swelling properties or reversible drug loading and release [81-84]. These controllable drug delivery systems are essential to reduce the dosage frequency and systemic toxicity [85]

Despite these advantages, core-shell nanomaterials suffer from characteristic limitations, arising from attempts to combine different features. On the one hand, diagnostic nanomaterials are designed to provide quick disease monitoring, which favours short circulation times to minimize exposure to the human body and the associated possible side effects. On the other hand, therapeutic nanomaterials require good biodistribution, good biodegradation and long-circulation times to ensure an effective therapeutic response and avoid multiple dosage [58].

**Physically tethered surface coatings**

Generally, there are two different approaches that are used to apply surface coatings onto substrates such as nanoceramic materials. In one case, the substrate does not provide any linking functional groups, so that the coating is physically adsorbed onto the nanomaterial’s surfaces, where it can further react to form a complete shell. The disadvantage of that technique lies in the possible formation of lumps or cross-linking, increasing the overall size of the composite [86, 87].
Chemically tethered surface coatings

In the other case, the substrate has functional groups on its surfaces, allowing the surface coating to be covalently attached to the substrate, which yields much stronger stability and a stronger structure that can withstand aggressive processing conditions. In addition, the coating is usually formed more evenly and homogeneously with a reduced risk of clump formation [88-90].

2.3.4 Therapeutic Effects of Nanoparticles

Chemotherapeutic drugs provide therapeutic effects, making them desirable for theranostic systems. The use of anti-cancer drugs is associated with multiple side effects, however, such as hair loss, nausea, and fatigue, and more importantly, these drugs show low stability [91]. Therefore, current research is focused on the development of alternative therapeutic resources that are capable of overcoming the above-mentioned drawbacks.

Anticancer drugs can affect cancer cells by interfering with processes involved in the cell cycle or through apoptosis, the latter one being of particular interest for novel strategies that include the selective activation of apoptosis in malignant cells or its suppression in normal cells [92]. It is commonly known that NPs mediate their toxicity through the generation of ROS and that the level of oxidative stress depends on the structure and chemistry of the NPs [93].

2.3.4.1 Definition of Reactive Oxygen Species

ROS are radicals, ions, or molecules that have a single unpaired electron in their outermost electron shell, making them highly reactive. Generally, ROS can be categorized into two groups: radical ROS and non-radical ROS, as displayed in Table 2.2.
Table 2.2. Examples of different types of reactive oxygen species.\textsuperscript{a})

<table>
<thead>
<tr>
<th>Free oxygen radicals</th>
<th>Non-radical reactive oxygen species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>Chemical name</td>
</tr>
<tr>
<td>(O_2^*)</td>
<td>Superoxide</td>
</tr>
<tr>
<td>(\cdot\text{OH})</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>RSSR</td>
<td>Disulfides</td>
</tr>
<tr>
<td>(\text{NO}^*)</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ROO(^*)</td>
<td>Peroxyl</td>
</tr>
<tr>
<td>RO(^*)</td>
<td>Alkoxyl</td>
</tr>
<tr>
<td>ROS(^*)</td>
<td>Sulfonyl</td>
</tr>
<tr>
<td>RS(^*)</td>
<td>Thiyl</td>
</tr>
<tr>
<td>R(^*)</td>
<td>Organic radicals</td>
</tr>
<tr>
<td>RSOO(^*)</td>
<td>Thiyl peroxyl</td>
</tr>
</tbody>
</table>

\textsuperscript{a}) R stands for R group, an abbreviation for any group in which a carbon or hydrogen atom is attached to the rest of the molecule.

2.3.4.2 Significance of Reactive Oxygen Species

Low levels of ROS are important for cellular function and survival signalling [94, 95], but excessive ROS can lead to damage to DNA, lipid peroxidation, and protein oxidation [96, 97], underlining that, for optimal cell function, a well-balanced level of ROS is essential. In addition, the type of generated ROS, the location where it is generated, and its local concentration are of importance for cellular functions. Normal cells have typically lower ROS contents, while the ROS level increases in cancer cells (Figure 2.15), where ROS promote many aspects of tumour development and progression [98].
Figure 2.15. Overview of the role of reactive oxygen species in cancer cells and the induction of apoptosis. Adapted from [99].

The major endogenous source of cellular ROS is the mitochondrial electron transport chain [100], whereas exogenous sources can be UV and ionizing radiation [101, 102], chemical irritants [103], and environmental toxins [104].

Novel therapeutic strategies aim to increase the ROS production in cancer cells, which consequently changes the ROS balance and can lead to apoptosis, which is typically mediated through the mitochondrial death pathway including the release of cytochrome c and the activation of kinases [105]. Prolonged treatment with the same drug reduces ROS levels and increases the drug resistance [99]. Exogenous ROS are applied in combination with drugs to sensitize resistant cancer cells and induce apoptosis [99].

Alternatively, the use of antioxidants in early stages of tumour development, where high levels of ROS are essential, might prevent further growth of cancer cells. It still remains a challenge, however, to target specifically cancer cells and further research is needed to fully understand the diverse stress-regulated cellular functions [98].
2.3.4.3 Nanoparticles as Reactive Oxygen Species Generators

The various physicochemical properties of engineered nanomaterials allow for the design of tailored nanomaterials that can be used to induce elevated levels of ROS in vitro.

Silver (Ag) NPs with particular particle sizes, for instance, have caused an increase of more than 10-fold in ROS levels in alveolar macrophages [106]. The induction of apoptosis in a human monocytic cell line (THP-1) by Ag NPs and Ag⁺ ions was associated with a drastic increase in ROS after an incubation period of 6 – 24 h [107].

Another widely investigated material is nanosized titanium dioxide (TiO₂), which has been shown to cause inflammation, cytotoxicity, and genomic instability in mammalian cells [108]. A study by [109] showed that TiO₂ NPs induce the generation of ROS in immortalized mouse brain microglia (BV2). The toxicity of TiO₂ NPs is further elaborated in section 2.5.2.2.

ZnO NPs have shown a selective toxicity towards human liver cancer (HepG2) and human lung adenocarcinoma (A549), while no impact on normal rat astrocytes and hepatocytes was observed. The toxicity was found to originate from the generation of ROS via the p53 pathway [110]. It was also shown that ZnO NPs intrinsically produce only a small amount of ROS, and the cytotoxicity in mouse macrophage Ana-1 cells is attributed to internalized ZnO NPs and dissolved Zn²⁺ ions [111].

2.3.4.4 Nanoparticles as Scavenger of Reactive Oxygen Species

Attempts have been made to not only design NPs that can be used as generators of ROS, but to also focus on the exploration of their use as scavengers of ROS, providing cell repair mechanisms, for instance, to normal cells that are exposed to harmful chemotherapeutic agents or radiation. The neutralization of intracellular excess ROS is typically realized via the addition of antioxidants such as vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), or extracts from various plant species [112].
A typical class of NPs that exhibit free radical scavenging properties is based on cerium oxide (CeO$_2$) [113-115]. Cerium (Ce) has the ability to exist in both +3 and +4 oxidation states. This property is important in the case of CeO$_2$ NPs, which are known to boost cell viability due to their ability to repair cellular damage [116-119]. It was shown that Ce$^{3+}$ states can be oxidized to Ce$^{4+}$ due to oxygen deficiencies, which can be found on the surfaces of CeO$_2$ NPs for particular particle sizes [120, 121].

For instance, the anti-oxidant process involving the decomposition of hydrogen peroxide in the presence of CeO$_2$ takes place as follows: first, H$_2$O$_2$ is adsorbed onto the surfaces of CeO$_2$ (Equation (2.1)). Then, the adsorbed H$_2$O$_2$ reacts with Ce$^{4+}$ to yield Ce$^{3+}$ states and oxygen vacancies ($V_o$) (Equation (2.2)), which can react with molecular oxygen or H$_2$O$_2$ to form Ce$^{4+}$ states again (Equation (2.3) and Equation (2.4)):

$$\text{H}_2\text{O}_2(\text{aq}) + \text{CeO}_2(\text{s}) \rightarrow \text{H}_2\text{O}_2(\text{ads}) + \text{CeO}_2(\text{s}) \quad (2.1)$$

$$\text{H}_2\text{O}_2(\text{ads}) + 2\text{Ce}^{4+}(\text{aq}) \rightarrow \text{O}_2(\text{g}) + 2\text{H}^+(\text{aq}) + 2\text{Ce}^{3+}(\text{aq}) + V_o \quad (2.2)$$

$$\text{O}_2(\text{g}) + 2\text{Ce}^{3+}(\text{aq}) + 2V_o \rightarrow 2\text{Ce}^{4+}(\text{aq}) + 2\text{O}^{2-}(\text{aq}) \quad (2.3)$$

$$\text{H}_2\text{O}_2(\text{ads}) + 2\text{Ce}^{3+}(\text{aq}) + 2\text{H}^+(\text{aq}) + V_o \rightarrow 2\text{H}_2\text{O}(\text{l}) + 2\text{Ce}^{4+}(\text{aq}) \quad (2.4)$$

Besides CeO$_2$, it is widely known that carbonate ions can act as scavengers of ROS, hydroxyl radicals in particular. Once the carbonate is in contact with hydroxyl radicals, the formation of carbonate radicals is observed, as shown in Equation (2.5) [122, 123]:

$$\bullet\text{OH}(\text{aq}) + \text{CO}_3^{2-}(\text{aq}) \rightarrow \text{OH}^-(\text{aq}) + \text{CO}_3^-(\text{aq}) \quad (2.5)$$

In contrast to hydroxyl radicals, which react rapidly with organic compounds, the carbonate radical is very selective and prolongs degradation processes, as typically observed in the purification of waste water [124-126].

2.3.5 Chemotherapeutic Drugs

Many chemotherapeutic drugs exist, such as MTX, doxorubicin (DOX), paclitaxel (PTX), and 5-fluorouracil (5-FU). Besides their individual use, there are many approaches
to combining the use of multiple drugs via either their simultaneous or sequential administration [127, 128]. It was shown that sequential treatment with MTX and 5-FU was the most effective in L1210 leukaemia and the mouse osteosarcoma C22LR, but also resulted in side effects, such as weight loss and early death [128].

Although chemotherapeutic drugs are widely applied in chemotherapy, a report in 2004 stated that “the overall contribution of curative and adjuvant cytotoxic chemotherapy to 5-year survival in adults was estimated to be 2.3% in Australia and 2.1% in the USA.” [129]. These numbers are very low, especially since the overall 5-year survival rate of cancer is over 60%. Reasons for the low impact of chemotherapeutic agents alone are the increase in patient drug tolerance, the lack of cancer cell targeting, and altered drug-receptor sensitivity. Combinations of therapies and the design of theranostic systems seem therefore to be a reasonable approach.

### 2.3.5.1 Structural Properties of Methotrexate

MTX was originally developed and continues to be used for chemotherapy to treat cancers, including breast cancer, leukaemia, lymphoma, lung cancer, osteosarcoma, and bladder neoplasms [130]. It typically enters cells through endocytosis, via an energy-dependent carrier protein, or through passive diffusion when the extracellular concentration of MTX is increased [131-134].

Structurally, MTX is an analogue of folic acid (FA, Figure 2.16), which is known to irreversibly bind to dihydrofolate reductase (DHFR) [135]. By actively competing with FA, MTX can bind to DHFR and inhibit the production of reduced folates, which results in a decrease in synthesis of purines, thymidylic acid, methionine, and serine [136-139]. The lack of protein synthesis consequently causes damage to DNA and RNA, and inhibits the further proliferation of cells. More importantly, DHFR receptors are typically overexpressed on the membranes of cancer cells in comparison to normal cells, making MTX not only a chemotherapeutic agent, but also a targeting agent [140, 141].
MTX has two different types of functional groups: two carboxylic groups on one end, and two amine groups at the other end of the molecule. These functionalities enable the MTX molecule to be conjugated with other bioactive compounds via covalent bonding. In addition, it can also be loaded onto drug carriers via physical adsorption, ionic bonding, or encapsulation in polymer shells [142].

2.3.5.2 Application of Methotrexate in Cancer Therapy

MTX is an antineoplastic agent that is used not only in cancer treatment, but also for autoimmune disorders such as arthritis and other rheumatic conditions [143]. In the clinical setting, the applied doses of MTX vary from patient to patient and have to be evaluated depending on the patient’s state of health, individual needs, and the location, size, and type of tumour. Patients with leukaemia or high-risk lymphoma are typically intravenously administered high doses of MTX (≥ 500 mg/m²) in several cycles within a single day [144]. Gestational trophoblastic disease requires usually medium doses of MTX ranging between 50 – 500 mg/m², while bladder, breast cancer, and T-cell large granular lymphocyte leukaemia are treated with low doses of MTX (< 50 mg/m²) [144].

Depending on the administered dose, MTX can cause side effects, such as the loss of hair, nausea, fatigue, liver damage, low blood cell counts, acute pneumonitis, or kidney failure. In addition, MTX is known to be prone to drug resistance, which, together with its rapid distribution within the body and its relatively short half-life, leads to multiple administrations of the drug, which are extremely painful for the patient [145, 146].
Hence, the development of controllable drug delivery systems is required, to not only reduce the dosage frequency, but also the systemic toxicity [85]. Compared to free MTX, the circulation half-life of MTX in blood has been increased over 180-fold via an attachment to human albumin [147]. In addition, the conjugation of MTX with polymers has been shown to increase the cellular toxicity [148, 149].

### 2.3.6 Radiation Therapy

Radiation therapy can either be applied externally through beam radiation, or can be applied from sources within the body, which is then called brachytherapy. The external beam radiation typically includes radiation with kilovoltage X-rays or megavoltage X-rays, depending on the type, stage, and location of the tumour. For superficial tumours that are easily reached, such as skin cancers, the kilovoltage X-ray irradiation is generally sufficient. For deeper-located tumours, such as in lung, brain, or prostate cancer, however, megavoltage X-rays are required to induce irreparable cell damage.

The cell ablation occurs either directly through the high-energy beam, or via the generation of reactive particles that can reduce the tumour size [150]. In general, the probability that tumours can be removed via radiation therapy is high, but due to the variation of different cancer types, their different natures, locations, and more importantly, their potential resistance, some cancers cannot fully be destroyed by radiation alone [91, 151]. More importantly, radiation therapy targets not only cancerous tissue, but also the normal cells that are usually exposed to radiation, which can, in turn, lead to side effects, such as skin changes, hair loss, nausea, vomiting, and fatigue [91, 151].

#### 2.3.6.1 Radioprotectors and Radiosensitizers

There are approaches to maximize the efficiency of radiation therapy in cancerous tissue and, at the same time, to minimize the destruction of normal cells due to harmful radiation. The attempts typically involve the application of radioprotectors and
radiosensitizers prior to radiation therapy with the aim of better controlling the cell responses to the delivered radiation.

**Radioprotectors**

Radioprotectors are drugs that aim to protect normal cells from radiation damage. In particular, these materials can promote cell repair mechanisms, and they can neutralize harmful ROS that are generated during the exposure to high-energy beams. Recently, CeO$_2$-based NPs have been investigated as potential radioprotectors, which were able to promote a boost in cell growth in normal tissue [116-119].

**Radiosensitizers**

Radiosensitizers, in contrast, are drugs designed to target cancer cells and to enhance their susceptibility to damage by radiation. Typically, radiosensitizers contain elements with high $Z$, which are able to locally increase the effect of the applied radiation dose by interfering with the incident energy beam. The number of photoelectron interactions increases with the atomic number for a given incident photon energy $E$ with $(Z/E)^3$ [152]. Moreover, by functionalizing these materials with bioactive ligands, such as DNA, proteins, or antibodies, the targeting function can be improved even further.

It has also been shown that the energy of the radiation beam could be reduced to orthovoltage radiotherapy (< 500 keV), while still providing enough damage to some human tumours, which further minimizes the destruction of normal cells [153].

### 2.3.6.2 Mechanism of Radio-Dose Enhancing Therapy Based on Nanoparticles

The NPs that are located inside the cytoplasm of cancer cells represent an active interaction component when the cells are exposed to an X-ray beam, as displayed in Figure 2.17. Typically, the energy of the X-ray beam can be absorbed by the NPs, which can lead to the ejection of an outer shell electron from its orbital with a kinetic energy equivalent to the absorbed energy minus the binding energy of the electron. This kinetic energy can be
released to the surrounding tissue, which effectively increases the absorbed radiation dose in the cell.

![Possible interactions of intracellular radiosensitizing nanoparticles with high effective atomic number Z, adapted from [154]. Abbreviation: deoxyribonucleic acid (DNA).](image)

The energy beam can also lead to the ejection of an inner-shell electron, leaving a vacancy that can be filled by electrons from the outer shell, causing distinctive secondary radiation [152]. The emissions include fluorescent photons, Rayleigh scattering, the production of charged particles, Compton scattering, or the scattering of X-rays and photons. All these emissions lead to direct damage to the DNA [152]. In addition, ROS can be generated, which causes indirect DNA damage through a cascade of processes.

### 2.3.6.3 Radiosensitizers Based on Nanoparticles

Besides a high effective Z, to be suitable as a radiosensitizer, the NPs need to be biocompatible and tailorable. In addition, their clearance pathways need to be thoroughly studied.
One of the first types of NPs that were studied for their radio-dose enhancing properties was Au NPs ($Z = 79$), which have been widely investigated for biological applications, such as for contrast agents (see section 2.3.7.3) [152]. In fact, it has been shown that Au NPs can successfully enhance the effects of radiation therapy, in particular, for radio-resistant cells, such as SCCVII squamous cell carcinoma [155, 156]. In addition, Au NPs are biocompatible, show low systematic clearance out of the body, and can be easily attached with surfactants [156].

Besides Au, platinum (Pt, $Z = 78$) NPs have been widely investigated as radiosensitizing materials. Pt finds applications in biomedical areas due to its antibacterial properties, biocompatibility, and its facilely modifiable surface properties that allow coating with polymers or the attachment of further bioactive materials. In particular, coatings with PAA have been widely reported, enabling easy diffusion through cell membranes and binding to DNA fragments, which were damaged via the generation of secondary particles during radiation [157, 158].

A big drawback of the use of both Au and Pt NPs is their associated high cost, giving an impetus to research to find cheaper materials that can be applied in a wide range of applications. As mentioned in CHAPTER 4 and CHAPTER 5, metal oxides based on Ta and Bi offer highly effective atomic numbers and are therefore promising potential radiosensitizing materials. These compounds are usually biocompatible in the cellular environment and $\text{Bi}_2\text{O}_3$ in particular is cheap to produce [159].

2.3.7 Diagnostics and Imaging

Various non-invasive imaging modalities are currently used to not only detect cancer at early stages, but to also monitor drug responses and migration in real time. These medical imaging techniques include magnetic resonance imaging (MRI), X-ray computed tomography (CT), ultrasound (US), positron-emission tomography (PET), single-photon emission CT (SPECT), and optical fluorescence imaging, as shown in Figure 2.18 [160, 161].
2.3.7.1 Computed Tomography

CT is the one of the most prevalent and important diagnostic tools in hospitals with regards to availability and frequency of usage [162]. As estimated, over 45,000 CT scanners are currently operated worldwide, and their usage is still on an upward trend [163].

CT consists of computer-based programs able to process X-ray measurements taken from several angles to yield a tomographic image (‘slice’) of a particular area of a scanned object. These slices are collected and stacked together to generate a three-dimensional image of the object. The patient lies on a bed that slowly moves through a gantry, while simultaneously, the X-ray tube rotates around the patient (Figure 2.19) [164].

Figure 2.18. Overview of imaging modalities for biomedical applications. Adapted from [161].
CT scans can provide images of different body structures, such as bones and soft tissue, and can be crucial to identify abnormalities and tumours. The anatomical contrast strongly depends on the ability to absorb the incoming X-rays, which have energies ranging from 80 to 140 keV. A high tube potential is usually applied in larger patients to ensure quality images, while children are imaged with smaller energies, which increases the contrast [165].

While bone structures typically have a high electron density and provide sufficient contrast, CT imaging of internal soft tissue requires the addition of contrast-enhancing agents [166]. The difference in contrast is based on the degree of X-ray attenuation of the different body structures, as shown in Equation (2.6) [167]:

\[ I = I_0 \cdot e^{-\mu x} \] (2.6)

where \( I \) is the transmitted x-ray intensity, \( I_0 \) is the incident x-ray intensity, \( \mu \) is the linear attenuation coefficient (m\(^{-1}\)), and \( x \) is the thickness of the absorber medium (cm). The linear attenuation coefficient is characteristic of each material and depends on the X-ray energy \( E \) (eV), the atomic number \( Z \), the density \( \rho \) (g/cm\(^3\)), and the atomic mass \( A_m \) (Da), as shown in Equation (2.7):

\[ \mu \approx \frac{\rho \cdot Z^4}{A_m \cdot E^{3/2}} \] (2.7)
2.3.7.2 Classical Contrast-Enhancing Agents

To obtain images of soft tissues, materials based on iodine (atomic number, $Z = 53$) and barium sulphate ($Z = 56$) are typically used [168]. The iodine-based compounds are either small molecules with short imaging time or more complex systems based on liposomes, polymers, and micelles that can provide longer blood circulation times [169, 170].

Figure 2.20 shows axial CT images taken from dogs which were either not treated with a contrast agent (left images) or treated with Iohexol (right images), which is a conventional contrast agent [171]. After the administration of the contrast agent, the visibility of the hepatocellular carcinoma is significantly improved.

![Without contrast agent vs With contrast agent](image)

**Figure 2.20.** Axial computed tomography (CT) scans of dogs without administration of a contrast agent (left images) and after administration of 660 mg /kg Iohexol (right images) [171]. In the top row, a small (0.5 cm) hepatocellular carcinoma is identified via the white arrows. In the bottom row, a large hepatocellular carcinoma (12.5 cm) is visible.

2.3.7.3 Contrast-Enhancement Agents Based on Nanoparticles

The design of NPs for the use as contrast agent is focused on three main areas, depending on their target function to reach the diseases sites:
- Blood pool CT contrast agent,
- Passive targeting, and
- Active targeting.

Passive targeting utilizes non-specific pathways, such as the reticulo-endothelial system or the EPR effect. Active targeting, in contrast, can be achieved by conjugation of NPs with peptides, antibodies, or other bioactive materials, which can improve the accumulation of the NPs in specifically targeted tissue.

Besides their tailorable size, biocompatibility, and bioactivity, NPs offer tuneable blood circulation times and controllable clearance pathways, which is of interest for patient-specific diagnostic imaging.

Some metal NPs such as Au (Z = 79) have a much higher atomic number than iodine-based agents and provide greater chemical stability and electron density, which ultimately results in stronger X-ray attenuation and greater contrast [172]. In addition, these NPs can be functionalized introducing active targeting capabilities: the conjugation of Au NPs with antibodies resulted not only in a 1.6 times increase in the visibility of millimeter-sized breast tumours in comparison to passive targeting, but also in a 22 times greater accumulation in the tumour periphery than in the surrounding tissue [95].

Generally, the use of Au NPs is considered to be safe, although recent in vivo studies indicated that Au NPs might exhibit toxicity in certain particle sizes [173]. In addition, the use of Au NPs is associated with high costs, which is driving research on cheaper alternative materials with high Z[168].

Metal oxide NPs are usually cheap, widely available, can be easily synthesized, are biocompatible, and their size and surface properties are tailorable. High Z metal oxides include tantalum (Ta)-based (Z = 73) and bismuth (Bi)-based (Z = 83) materials, such as tantalum (V) oxide (Ta₂O₅) and bismuth (III) oxide (Bi₂O₃), respectively [174, 175].
Particular examples of contrast-enhancement agents based on Ta and Bi are presented in CHAPTER 4 and CHAPTER 5.

### 2.3.8 Examples of Theranostic Systems

Currently, the design of theranostic systems is focused on the implementation of multiple features on one single platform. While the original systems mostly contained only diagnostic and chemotherapeutic features, new approaches aim to combine even more features, such as targeting, sensing, photodynamic therapy, or enhancement of radiation therapy. The strategies include the use of both organic nanomaterials and inorganic nanomaterials. Compositions of some typical theranostic systems are shown in Table 2.3.

**Table 2.3. Composition of exemplary theranostic systems for biomedical applications.**

<table>
<thead>
<tr>
<th>Imaging agent</th>
<th>Therapeutic agent</th>
<th>Additional components</th>
<th>Further features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold DOX, cisplatin, FA</td>
<td>–</td>
<td>Targeting, radiation therapy,</td>
<td></td>
<td>[176, 177]</td>
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<tr>
<td>Iron oxide siRNA, DOX, MTX</td>
<td>PEG, APTES</td>
<td>Targeting, drug delivery, photodynamic therapy, hyperthermia therapy</td>
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<td>[140, 178, 179]</td>
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<tr>
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<td>PEI-PEG</td>
<td>Targeting</td>
<td></td>
<td>[180]</td>
</tr>
<tr>
<td>Carbon nanotubes PTX, antibody, salinomycin</td>
<td>PEG, MnO</td>
<td>photothermal therapy</td>
<td></td>
<td>[181, 182]</td>
</tr>
<tr>
<td>Quantum dots DOX, mitoxantrone</td>
<td>DNA aptamer</td>
<td>Targeting, sensing</td>
<td></td>
<td>[183, 184]</td>
</tr>
</tbody>
</table>

*a) Abbreviations: short interfering ribonucleic acid (siRNA), doxorubicin (DOX), methotrexate (MTX), paclitaxel (PTX), polyethyleneimine (PEI), polyethylene glycol (PEG), (3-aminopropyl) triethoxysilane (APTES)*
2.4 Nanomaterials for Sun-Blocking Applications

2.4.1 Sunscreen Products

Conventional sunscreen products are available as sticks, gels, ointments, or aerosols, and are based on an oil-in-water or water-in-oil emulsion. Emulsions are preferred as they offer cost efficiency, good rheological properties, and can provide skin smoothness in contrast to oil-based sunscreens, which leave an unpleasant residue on the skin [115, 185].

Sunscreen formulations contain either physical or chemical UV filters that can protect against both UVA and UVB radiation (Figure 2.21). The active ingredient is normally dissolved or dispersed in the hydrophobic oil-phase of the sunscreen formulation.

![Figure 2.21. Working principles of chemical and physical sunscreens.](image)

Physical sunscreens, which are often referred to as 'mineral' sunscreens, contain inorganic UV filters, such as TiO₂ or ZnO particles, that reflect, scatter, or absorb the incoming UV rays, depending on their particle size. Once applied, physical sunscreens form a protective layer on the skin's surface, so that harmful UV rays cannot penetrate into the skin [186, 187]. Chemical sunscreens, in contrast, have solely UV absorbing organic ingredients and are absorbed into the skin [11, 186-188]. Modern sunscreens often have a
combination of organic and inorganic UV filters to fully maximize the UV protection. A small overview of active sunscreen ingredients is presented in Table 2.4.

**Table 2.4.** Selection of sunscreen ingredients and their amount of ultraviolet (UV) radiation protection, as approved by the US Food and Drug Administration (FDA) as of 7 December 2009 [185].

<table>
<thead>
<tr>
<th>Active sunscreen ingredient</th>
<th>Maximum FDA-approved concentration (%)</th>
<th>Range of protection (nm)</th>
<th>Protection (UVA, UVB)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic UVB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Para-aminobenzoic acid</td>
<td>15</td>
<td>260 – 313</td>
<td>UVB</td>
</tr>
<tr>
<td>Octinoxate</td>
<td>7.5</td>
<td>280 – 310</td>
<td>UVB</td>
</tr>
<tr>
<td>Homosalate</td>
<td>15</td>
<td>290 – 315</td>
<td>UVB</td>
</tr>
<tr>
<td>Octylcrylene</td>
<td>10</td>
<td>287 – 323</td>
<td>UVB</td>
</tr>
<tr>
<td><strong>Organic UVA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxybenzone</td>
<td>6</td>
<td>270 – 350</td>
<td>UVB, UVA2</td>
</tr>
<tr>
<td>Sulisobenzone</td>
<td>10</td>
<td>250 – 380</td>
<td>UVB, UVA2</td>
</tr>
<tr>
<td>Dioxybenzone</td>
<td>3</td>
<td>206 – 380</td>
<td>UVB, UVA2</td>
</tr>
<tr>
<td>Avobenzone</td>
<td>3</td>
<td>310 – 400</td>
<td>UVA1, UVA2</td>
</tr>
<tr>
<td><strong>Inorganic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>25</td>
<td>290 – 350</td>
<td>UVB, UVA2</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>25</td>
<td>290 – 400</td>
<td>UVB, UVA1</td>
</tr>
</tbody>
</table>

Besides the base emulsion and the addition of the active UV filter, sunscreen formulations contain preservatives and additives, such as emulsifiers or thickeners, to combat microbial attacks and increase the stability of the emulsion, which prevents the separation of phases [115]. Emulsifiers are amphiphilic molecules, such as fatty acid soaps,
that reduce the interfacial tension between the water and oil phase. Thickeners, such as alginates, natural gums, cellulose derivatives, bentonite, and fumed silica, increase the viscosity, which reduces the coalescence and segregation rate [115].

2.4.1.1 Organic Ultraviolet Filters

The active ingredients of chemical sunscreens are based on organic structures, which are typically less photostable than physical sunscreens, since organic compounds can lose their stability over time [17, 186]. Organic-based sunscreens are usually colourless with a thin consistency, which enables easy application onto the skin. Typically, 20 min is required before organic-based sunscreens provide full protection, but they are less likely to be washed away, since they are absorbed into the skin. More importantly, however, is the fact that individual organic UV filters do not provide full broad-band UVA and UVB protection, resulting in the use of combinations of different organic UV filters [186].

2.4.1.2 Inorganic Ultraviolet Filters

While there are many organic UV absorbers approved by regulatory bodies, only two inorganic UV filters can be used, namely TiO$_2$ and ZnO [186, 189]. Their UV blocking properties are determined by their particle size, dispersion in the base emulsion, refractive characteristics, and film thickness [187].

Traditional sunscreens employing either of these two materials appear white on the skin because the particle size is greater than 100 nm. TiO$_2$ NPs, for instance, have been shown to reflect visible light the best when present in particle sizes of 200 – 500 nm [11]. This white coating acts as a ‘physical blocker’, functioning by scattering and reflecting the incident light.

Modern sunscreens employ nanosized materials, typically, NPs of ZnO or TiO$_2$, since, at suitable concentrations when well dispersed, their emulsions are transparent in the visible range and are thus invisible after topical application, a factor highly desirable
from a cosmetic standpoint [186, 190]. When they are in nano-particulate form (e.g. 10 – 40 nm), however, they are too small to reflect and scatter efficiently and thus, they function as inorganic UV absorbers, relying on their UV absorption spectrum for effectiveness. They only become physical blockers when poorly dispersed and agglomerated, and consequentially appear visibly white.

In fact, TiO$_2$ absorbs UV light much more strongly than it scatters UVA and UVB radiation [187]. Figure 2.22 displays the absorbance spectra of bulk TiO$_2$ and ZnO at room temperature. With regard to the recommended broad UVA/UVB protection, TiO$_2$ NPs provide good UVB protection, while ZnO NPs cover the UVA range.

![Absorbance of bulk TiO$_2$ and ZnO at room temperature. Adapted from [11].](image)

Since the TiO$_2$ and ZnO NPs are typically dispersed in the hydrophobic oil-phase of the sunscreen formulation, they are usually coated with organic-based dispersants to stabilize the dispersion, which ultimately enhances the stability of the sunscreen formulation [115]. The coatings include very thin layers of stearic acid in the case of TiO$_2$ (Eusolex® T-S TiO$_2$) or triethoxycaprylysilane in the case of ZnO (Z-COTE® HP1). To improve the visual appearance of the sunscreen products, iron (III) oxide (Fe$_2$O$_3$) pigments are occasionally added [187].
The great advantage of inorganic UV filters is their immediate protection once they are applied on the skin, their photostability, and their limited skin penetration [191]. As such, it has been shown that most particles are found in the first 1 µm of the upper layer of the stratum corneum [11, 190]. Furthermore, inorganic UV filters tend to cause less allergic reactions than organic UV filters, making them particularly suitable for sensitive skin [186, 191]. The thick consistency of TiO$_2$ and ZnO based sunscreens, however, can complicate correct application onto the skin. In addition, the layer of sunscreen may rub off easily.

### 2.4.2 Sun Protection Factor

The SPF is a universally accepted measure to express the efficiency of sunscreen products and is based on the definition of the minimum erythematous dose (MED), which is the smallest dose of energy necessary to result in the formation of erythema within 1 – 6 h that disappears within 24 h [192, 193]. By using the MED, the SPF can be determined by the following Equation (2.8) [194]:

$$SPF = \frac{\text{MED of with sunscreen protected skin}}{\text{MED of unprotected skin}}$$  \hspace{1cm} (2.8)

The efficiency of UV protection of sunscreen products typically increases with increasing SPF, as shown in Figure 2.23. Once correctly applied onto the skin, the period of protection is the product of the period of natural protection multiplied by the SPF. Considering the different sensitivity of human skin tones to UV radiation, it is of great importance to first determine the period of natural protection, before obtaining sunscreen products with different SPF.
2.4.2.1 *In Vivo* Determination of Sun Protection Factor

The SPF can be determined *in vivo* or *in vitro*, but is ideally evaluated in human volunteers to consider the real conditions of use in practice [192]. The *in vivo* tests usually involve human volunteer subjects onto whose skin 2 mg/cm² sunscreen formula is applied to an area on the mid-back [193]. The sunscreen is then dried for 15 min and five increasing UV radiation doses are applied to the treated area. The same series of UV doses is administered to untreated skin [193]. After 16 – 24 h, the irradiated skin sites are analyzed and the SPF is determined following Equation (2.8) [193].

![Figure 2.23. Increase in ultraviolet protection with increasing sun protection factor (SPF). Adapted from [195].](image)
According to the FDA, the SPF protection must be clinically tested on at least ten human volunteers, which is associated with high costs, long waiting periods, and more importantly, health risks due to the direct application of UV radiation to the skin [193, 196]. Thus, *in vitro* methods are emerging as non-invasive way to determine the SPF.

### 2.4.2.2 *In Vitro* Determination of Sun Protection Factor

The SPF describes the protection solely from UVB radiation and does not consider UVA radiation. Based on regulations that have been approved by the FDA in 2011, *in vivo* UVA testing is no longer required [197]. Instead, a simple *in vitro* critical wavelength reading based on spectrophotometry has been accepted as a replacement [194, 196, 198]:

1. First, sunscreen products are placed onto substrates such as quartz, and the UV absorbance is measured over the entire UV spectrum [194, 198].
2. The area beneath the obtained spectral absorbance curve is then calculated via mathematical integration [198].
3. The critical wavelength ($\lambda_c$) is defined as the wavelength below which 90% of the area under the absorbance curve resides [198].

**Critical wavelength and broad-spectrum UV protection**

The critical wavelength is crucial to evaluate the UV absorbing properties of active UV filters and provides information about broad-spectrum protection, which includes larger wavelengths in the UVA region. UVB filters, for instance, have critical wavelengths that are smaller than those of UVA filters (see Figure 2.24). The higher the critical wavelength, the better is the broad-spectrum photoprotection. In 2012, the FDA has introduced new regulations that allow the claim of ‘broad-spectrum’ protection only when the critical wavelength is $> 370$ nm [197, 199].

The determination of SPF and critical wavelength is considered to provide a sufficient description of the photoprotective properties of a sunscreen product: while the SPF describes the amplitude of protection at a given application thickness, the critical
wavelength provides information about the absorption capability over the entire UV spectrum, which is independent of application thickness [198].

![Absorbance spectra of sunscreen products](image)

**Figure 2.24.** Absorbance spectra of sunscreen products with a critical wavelength (a) smaller and (b) greater than 370 nm. Adapted from [198].

A study published in 2000 showed that only 10% of the studied 59 commercially available sunscreen products provide a critical wavelength ≥ 370 nm [200]. These products all contained UVA filters, such as ZnO, TiO₂, or avobenzone, but not all of the tested sunscreens with these ingredients displayed a sufficient critical wavelength. These findings underline the point that the simple addition of UVA filters does not necessarily ensure true broad-spectrum protection, but instead, a thorough study of the entire formulation is essential [200].

### 2.4.3 Photochemistry and Photobiology

TiO₂ and ZnO are both semiconductors, meaning that an energy band gap separates the orbitals that form the valence band (VB) and the conduction band (CB). If light is shone on TiO₂ with energy that equals or is larger than the band gap, an electron (e⁻) can be excited from the VB to the CB, leaving a hole (h⁺) in the VB [11], as shown in Equation (2.9), where $h = \text{Planck's constant} \times 10^{-34} \text{ (m}^2 \cdot \text{kg} \cdot \text{s}^{-1})$ and $\nu = \text{frequency (s}^{-1})$:

$$\text{TiO}_2 + h\nu \rightarrow e^- + h^+ \quad \text{(2.9)}$$
The e\textsuperscript{−} and h\textsuperscript{+} either recombine, or they can be transported to the surface of the particle, where they can participate in redox reactions, mostly with pre-adsorbed substrates such as molecular oxygen or water, as shown in Figure 2.25 [11].

![Diagram of electron-hole pairs, their recombination, and their transport to the particle surface of a semiconductor.](image)

**Figure 2.25.** Mechanism of formation of electron-hole pairs, their recombination, and their transport to the particle surface of a semiconductor. Adapted from [201].

The photoactivity of semiconducting NPs is linked to a number of factors, the most essential of which is the surface of the particles [187]. When reduced to nanometer scale, the band gap of semiconducting NPs increases since the electronic energy levels are interpreted as discrete energy levels. This ultimately leads to a blue shift of the absorption threshold [202]. Due to the high interfacial energy, however, NPs tend to agglomerate and aggregate, which ultimately influences their size and the band gap width [11].

### 2.4.3.1 TiO\textsubscript{2}

TiO\textsubscript{2} is a naturally occurring mineral that is typically nontoxic, inexpensive, and crystallizes in three common polymorphs: anatase (tetragonal, space group: \(I\text{\textsubscript{4}}/\text{amd}\)), rutile (tetragonal, space group: \(P4\text{\textsubscript{2}}/\text{mnm}\)), and brookite (orthorhombic, space group: \(P\text{\textsubscript{bca}}\)) [203, 204]. Ball-models of their crystal structures are shown in Figure 2.26. The rutile
phase is the most stable among all the structures, while the anatase and brookite structures are metastable at room temperature [204].

![Diagram of crystal structures of TiO₂](image)

**Figure 2.26.** Crystal structures of anatase, rutile, and brookite TiO₂; obtained via Materials Studio software using Crystallography Open Database files 1010942, 1532819, and 8104269.

Under particular conditions, there are also less common structures possible, such as TiO₂ II (columbite), TiO₂ III (baddeleyite), TiO₂ (H) (hollandite), TiO₂ (R) (ramsdellite), and TiO₂ (B) (monoclinic) [204].

TiO₂ is a known photocatalyst, with the band gap for bulk TiO₂ ranging from approximately 3.0 eV (rutile), to 3.13 eV (brookite) and 3.2 eV (anatase) [205]. Only the rutile and anatase polymorphs are relevant, however, in the preparation of sunscreens or in heterogeneous catalysis [187]. More importantly, anatase is photocatalytically more active than rutile TiO₂ [206, 207].

TiO₂ particles have been used as a white pigment, partly due to their refractive properties [11]. Polycrystalline and epitaxial films of rutile have an average refractive index of 4.0, while anatase has a refractive index of 3.6 [208]. TiO₂ was first reported as an
active sunscreen agent in 1952 [209]. Currently, TiO$_2$ NPs are still widely used as an active UV filter ingredient in sunscreens that protect from UVB and UVA2 (see section 2.4.1).

### 2.4.3.2 ZnO

ZnO is a naturally occurring mineral that can be found in the Earth's crust, although most ZnO is manufactured synthetically [11]. Three crystalline structures of ZnO exist: rocksalt (cubic, space group: $Fm\bar{3}m$), zinc blende (cubic, space group: $F\bar{4}3m$), and wurtzite (hexagonal, space group: $P6_3mc$), with wurtzite being the most stable and common form at room temperature [210]. The crystal structures of ZnO are displayed in Figure 2.27.

In contrast to TiO$_2$, ZnO exhibits significant mammalian toxicity, which can be related to the compound's solubility (see section 2.5.2) [211]. Furthermore, ZnO is associated with induction of oxidative stress and mitochondrial dysfunction [110, 111, 211].

The band gap of ZnO ranges from 3.2 eV (wurtzite) to 3.3 eV (zinc blende) [212] and the refractive index of ZnO was found to range between 2.3 and 2.0, which reduces the whitening effect of ZnO in comparison to TiO$_2$ [213]. In modern sunscreen formulations,
ZnO NPs are typically used together with TiO$_2$ NPs, as they offer the UVA1 protection that is essential for broad-spectrum protection (see section 2.4.1).

### 2.4.3.3 Photocatalytic Activity of Semiconducting Nanoparticles

The photocatalytic activity of TiO$_2$ has been used to explore possible applications in the purification of waste water, the removal of organic pollutants such as hydrocarbons, chlorinated hydrocarbons, and heavy metals, including Pt$^{4+}$, Pd$^{2+}$, Au$^{3+}$, Rh$^{3+}$, and Cr$^{3+}$, and the destruction of biological species such as bacteria, viruses, and moulds [214, 215]. The highly reactive radicals that are formed during the process, however, can pose health concerns when applied to the human body.

The photocatalytic reactions of TiO$_2$ are shown in Equation (2.10), Equation (2.11), Equation (2.12), and Equation (2.13), and can also be found for ZnO NPs [215]:

$$e^- + O_2(ads) \rightarrow O_2^\bullet (ads) \quad (2.10)$$

$$e^- + H^+ (ads) \rightarrow H^\bullet (ads) \quad (2.11)$$

$$h^+ + HO^- (ads) \rightarrow \cdot OH(ads) \text{ (in alkaline solution)} \quad (2.12)$$

$$h^+ + H_2O(ads) \rightarrow H^+ (ads) + \cdot OH(ads) \text{ (in neutral solution)} \quad (2.13)$$

Upon photoexcitation of TiO$_2$ an electron transfer from TiO$_2$ to molecular O$_2$ results in the formation of the superoxide anion radical O$_2^\bullet$. In contact with water, TiO$_2$ catalyses the release of an electron from H$_2$O, which ultimately leads to the formation of the hydroxyl radical $\cdot$OH. In acidic medium, the superoxide anion radical is protonated, which ultimately leads to the formation of the hydroperoxyl radical HO$_2^\bullet$ and hydrogen peroxide H$_2$O$_2$ [187]. Hydrogen peroxide can also be generated following radical chain steps involving hydroxyl radicals [187].

The photocatalytic activity depends on various factors, such as the crystalline state, textural properties, and surface properties, such as the surface area. For instance, amorphous TiO$_2$ is rarely photocatalytically active, which is related to the presence of non-
bridging oxygen atoms. The Ti-O atomic arrangement defects could cause the recombination of photogenerated electron-hole pairs [215, 216].

In addition, it has been shown that the calcination atmosphere can have an impact on the photocatalytic activity of TiO₂: nitrogen atmosphere or vacuum can lead to defects and low surface hydroxyl density, which reduces the photocatalytic activity [217]. In contrast, calcination in argon atmosphere promotes high surface coverage with hydroxyl groups, elevating the photoactivity [217].

2.4.3.4 Determination of Photocatalytic Activity

The hydroxyl radical is a very powerful oxidizing agent amongst the ROS and can easily attack organic compounds that are close to the surface of the photocatalyst [215]. The reactions that take place during the oxidation and decomposition of organic matter (OM) are shown in Equation (2.14) and Equation (2.15):

\[ \text{h}^+ + \text{OM}_{(aq)} \rightarrow \text{OM}^{\cdot-}_{(aq)} \text{ (oxidation)} \]  
\[ \cdot\text{OH}_{(ads)} + \text{OM}_{(aq)} \rightarrow \text{degradation products (CO}_2, \text{H}_2\text{O, etc.)} \]  

The degradation products are typically less toxic than the initial organic material, although there is the associated risk that by-products can also be formed, the toxicity or biological effects of which are unknown [215].

To assess the photocatalytic activity of materials, organic dyes are used, whose colour fades upon degradation, which is directly linked to the photogenerated ROS. The dyes can be categorized into five groups: azo compounds (monoazo, diazo, and polyazo dyes), indigoid dyes, anthraquinone dyes, triarylmethane dyes, and xanthene dyes. In the presence of solar/UV light and a photocatalyst such as TiO₂, the photogenerated reactive species usually attack sites near the chromophore, which results in fading of the dye [108, 215]. Table 2.5 displays a selection of dyes that are used to assess the photocatalytic degradation of TiO₂.
### Table 2.5. Selection of dyes that are commonly used to assess the photocatalytic activity of TiO₂ in combination with solar/UV irradiation [209].

<table>
<thead>
<tr>
<th>Colour Index name</th>
<th>Chemical structure</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Azo dyes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid Red 2 (Methyl Red, MeRed)</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>430</td>
<td>[218-220]</td>
</tr>
<tr>
<td><strong>Indigoid dyes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indigotin (Indigo)</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>610</td>
<td>[221]</td>
</tr>
<tr>
<td><strong>Anthraquinone dyes</strong></td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>596</td>
<td>[222, 223]</td>
</tr>
<tr>
<td>Reactive Blue 4 (Procion blue, MX-R, RB4)</td>
<td><img src="image4" alt="Chemical structure" /></td>
<td>596</td>
<td>[222, 223]</td>
</tr>
<tr>
<td><strong>Triarylmethane dyes</strong></td>
<td><img src="image5" alt="Chemical structure" /></td>
<td>550</td>
<td>[218, 224]</td>
</tr>
<tr>
<td>Basic Violet 3 (Crystal Violet)</td>
<td><img src="image6" alt="Chemical structure" /></td>
<td>550</td>
<td>[218, 224]</td>
</tr>
<tr>
<td><strong>Xanthene dyes</strong></td>
<td><img src="image7" alt="Chemical structure" /></td>
<td>550</td>
<td>[225, 226]</td>
</tr>
<tr>
<td>Rhodamine-B (Basic Violet 10, Brilliant Pink B)</td>
<td><img src="image8" alt="Chemical structure" /></td>
<td>550</td>
<td>[225, 226]</td>
</tr>
</tbody>
</table>
The chemical structure of the dye can have an impact on its reactivity in a TiO$_2$-based system. The use of monoazo dyes, for instance, results in increased photocatalytic degradation rates compared to anthraquinone dyes [215]. In addition, the degradation rate is increased when methyl and chloro groups are present in the dye molecule. Hydroxyl groups increase the electron resonance in the dye molecule. Nitrile groups and alkyl side chains lead to a decrease in the photocatalytic degradation rate, as, for instance, the latter ones decrease the solubility of the dye in water [215].

2.4.3.5 Determination of Reactive Oxygen Species

There are multiple ways of detecting intracellular and extracellular ROS, such as electron spin resonance (ESR) spectroscopy, chemiluminescent and fluorescent probes, and immunoassays [227].

**ESR spectroscopy**

ESR spectroscopy is used to investigate materials with unpaired electrons and is of particular interest for studies involving metal complexes or radicals [108, 228]. The spin-trapping technique includes spin traps that covalently bind free radicals, resulting in the formation of products that are detectable by ESR [229]. Spin probes, in contrast, are oxidized by ROS without binding [227]. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), for instance, is used as a spin-trap to detect superoxide anion radicals, although the associated reactions are very slow, which has led to the development of better methods [227].

**Chemiluminescent and fluorescent probes**

Superoxide anion radicals can also be detected by chemiluminescent and fluorescent probes. Various chemiluminescent probes exist, such as lucigenin, luminol, and coelenterazine. It has been reported, however, that the generation of radicals might be overestimated for particular probes, due to the observed redox-cycling, in which the probe radicals react with oxygen to form superoxide anion radicals [227].
Fluorescent probes such as dihydroethidium and red mitochondrial superoxide indicator (mitoSOX) have been used to evaluate mitochondrial and intracellular ROS. Other fluorescent probes are the popular 2',7'-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) for the detection of intracellular hydrogen peroxide, dihydrorhodamine for the detection of peroxynitrite, and N-acetyl-3,7-dihydroxyphenoxazine for the detection of extracellular hydrogen peroxide [227].

A double-labelling method has been developed that detects intracellular ROS using DCFH$_2$-DA and visualizes nuclear fragmentation, in particular budding and cleavage types, using Hoechst dye [121]. While the budding type is associated with an ROS-independent apoptotic pathway, the cleavage type indicates a ROS-dependent apoptotic pathway [121]. Figure 2.28 displays the fluorescence microscopy analysis of human tumour monocytes U937 double-labelled with DCFH$_2$-DA and Hoechst dye [121]. The 2',7'-dichlorofluorescein (DCF) signal in budding cells is comparable to that in viable cells, while cells in cleavage exhibit stronger staining [121].

**Figure 2.28.** Fluorescence microscopy images of U937 human tumour monocytes double-labelled with Hoechst dye (nuclear morphology, top row) and 2',7'-dichlorodihydrofluorescein diacetate, showing the 2',7'-dichlorofluorescein (DCF) signal (reactive oxygen species content, bottom row). The left column represents viable cells, while the centre and right columns show apoptotic cells in budding and cleavage, respectively. Adapted from [121].
2.5 Limitations of Current Sunscreens and Suggested Solutions

2.5.1 Skin Penetration of Inorganic Ultraviolet Filters

Commonly used sunscreens protect against UV radiation, but have also raised concerns regarding both organic and inorganic UV filtering additives. When evaluating the possible drawbacks of sunscreen products, many factors have to be considered, besides their cosmetic properties, such as the spreadability and the transparency of the products.

The skin penetration of a particle typically depends on its ability to penetrate the alternate hydrophobic and hydrophilic layers of the stratum corneum. The penetration of the stratum corneum is determined by many factors, such as the polarity, size, molecular weight, solubility, and lipophilicity of the particles [230]. Furthermore, the capacity of the particles to form hydrogen bonds and, in the case of acidic or alkaline particles, their acid dissociation constant (pKa) values are of importance when evaluating percutaneous absorption [230].

It is well known that organic UV filters have some of the highest penetration rates into the skin and can be found in viable skin layers [186]. There are a great number of studies involving the skin penetration of both TiO$_2$ and ZnO NPs. Although some past studies failed to specifically identify the presence of TiO$_2$ and ZnO NPs in deep skin layers [231, 232], more recent studies confirm their skin penetration, especially in the case of TiO$_2$ NPs [11]. Furthermore, some surfactants that are added to sunscreen formulations may alter the skin barrier, which can cause an increase in the skin penetration of NPs [11].

Typically, TiO$_2$ and ZnO NPs are found within the stratum corneum and/or hair follicles [11]. Some studies have shown that these NPs can also be detected in viable skin layers, much deeper than the stratum corneum [11, 233-235]. For instance, after the skin of Yucatan mini-pigs was repeatedly treated with sunscreen emulsions with 5 wt.% TiO$_2$ in
particle sizes of 20 – 500 nm, measureable levels of NPs could be located in the dermis [235].

When the skin barrier is damaged, however, NPs have been detected in much deeper skin layers. More interestingly, in vivo experiments with hairless mice revealed not only the transport of TiO$_2$ NPs with particle sizes of 10, 25, and 60 nm to viable skin layers, but long-term exposure of 60 days also showed that detectable levels of NPs were found in lung, liver, and spleen tissues, as shown in Figure 2.29 [233].

![Figure 2.29](image_url)

**Figure 2.29.** Histopathological evaluation of the skin, liver, spleen, and lung tissue of hairless mice after dermal exposure to TiO$_2$ with particle sizes of 10, 25, and 60 nm for 60 days. The black arrows indicate pathological changes in the tissue. Adapted from [233].
Another study included patients with atopic dermatitis, onto whose skin commercial ZnO emulsions were applied for a total of two weeks [233]. ZnO NPs with a size of 80 nm were found mostly in the stratum corneum and the stratum spinosum, which can further facilitate the transport to deeper viable skin layers [233].

### 2.5.2 Inconclusive Toxicity of Inorganic Ultraviolet Filters

Organic UV filters are known to interfere with hormones and cause allergic reactions [186]. Considering that they penetrate easily into the skin and are less photostable than physical UV filters, organic UV filters can lose their stability over time resulting in decomposition and release of heat [11, 186]. Since, in order to achieve broad UVA and UVB protection, multiple organic UV filters have to be added to the sunscreen base emulsion; these side effects have an even wider remit.

It was proved that, during exposure to UV-visible (UV-vis) irradiation, ZnO and TiO$_2$ NPs produce ROS, which can react with the organic filters in sunscreens, resulting in their degradation [236, 237]. This ultimately not only leads to compromised filtering activity, but also to the formation of side products with unknown toxicity [236, 238]. Furthermore, these radicals can induce DNA damage and cell death [187, 196].

The fundamental requirement that must be met to cause DNA damage and further toxicological effects is the transport of NPs through the skin barrier to deeper, viable skin layers [11, 187]. More importantly, the NPs must penetrate the cell nucleus [187]. As a matter of fact, to fully establish the connection between NPs and their genotoxicity, their size, shape, and surface properties are undeniable factors that need to be considered.

There are many reports on the toxicity of TiO$_2$ and ZnO NPs that highlight both their lack of toxicity and their associated geno- and cytotoxicity in vitro and in vivo. This inconclusiveness can be related to missing guidelines for skin toxicity, sensitization, and corrosion, as well as to the use of different testing protocols, cell lines, and unrealistic dose exposure [239-242].
2.5.2.1 Reported Lack of Toxicity

*In vitro* and *in vivo* studies have shown that neither TiO\(_2\) nor ZnO is toxic, both on the micro- and on the nanoscale [243, 244]. TiO\(_2\) NPs (20 nm) did not exhibit any cytotoxicity, genotoxicity, photocytotoxicity, or photogenotoxicity in rat liver epithelial cells when exposed to UVA irradiation [245]. Therefore, the Therapeutic Goods Administration (TGA) concluded in 2016 that no risk is posed by these inorganic UV filters [196]. ZnO NPs have been shown to display a higher cytotoxicity against cancer cells than normal cells, making these particles potentially interesting for cancer treatment [246].

2.5.2.2 Reported Toxicity

TiO\(_2\) has been classified by the International Agency for Research on Cancer (IARC) as a group 2B carcinogen [247]. According to IARC, these carcinogens are 'possibly carcinogenic to humans', since pigment-grade ultrafine TiO\(_2\) dust has been linked to the formation of respiratory cancer in rats [11, 247]. On the other hand, the FDA classified ZnO as generally safe, when used as directed following cosmetics directives [248].

*In the absence of UV irradiation*

The toxicity of TiO\(_2\) and ZnO NPs has been assessed in human fibroblasts in the absence of UV light [249-253]. ZnO (50 – 70 nm) and TiO\(_2\) (< 150 nm) NPs have been shown to cause reduced viability after exposure for 4 h and 24 h on skin fibroblasts, leading to a half maximum inhibitory concentration (IC\(_{50}\)) of 49.56 ± 12.89 ppm (Figure 2.30(a)) and 2696 ± 667 ppm (Figure 2.30(b)) for ZnO and TiO\(_2\), respectively [249]. The increased toxicity of ZnO NPs has been linked to the formation of Zn\(^{2+}\) ions and the generation of ROS [110, 111, 211].
Figure 2.30. Dose-dependent cell viability curves of (a) ZnO nanoparticles and (b) TiO$_2$ nanoparticles following exposures of 4 h and 24 h on human skin fibroblasts [249].

In the presence of UV irradiation

TiO$_2$ and ZnO NPs have been widely associated with potential DNA damage, which can also be linked to their photocatalytic activity [11]. ZnO NPs (< 200 nm) with concentrations of 105 – 320 µg/mL displayed a noticeable increase in genotoxicity during in vitro investigations using Chinese hamster ovary (CHO) cells under exposure to UV radiation at > 290 nm and doses of 0.35 and 0.7 J/cm$^2$ [254]. TiO$_2$ specimens, which were extracted from sunscreen products, after UV irradiation at 300 – 400 nm and 12 W/cm$^2$, caused single-strand and double-strand breaks on DNA plasmids and nuclei of human skin cells [255]. It was also shown that ultrafine TiO$_2$ NPs (< 30 nm) caused lipid peroxidation and production of H$_2$O$_2$ in rat lung alveolar macrophages [255]. Besides the formation of H$_2$O$_2$, superoxide anion radicals, hydroxyl radicals, and singlet oxygen, which are well-known to be cytotoxic and/or genotoxic, are often formed [108, 187, 255]. Interestingly, TiO$_2$ NPs do not show any significant DNA damage under dark conditions. Only after irradiation with UV rays, does TiO$_2$ cause genotoxicity [256].

Of particular importance is the study of the toxic effects of TiO$_2$ and ZnO when exposed to repeated UV radiation over long periods of time. ZnO NPs showed the formation of ROS inside keratinocytes and a decrease in their viability at a concentration of 15 µg/mL, while TiO$_2$ did not show any noticeable generation of ROS or toxicity [250].
More interestingly, it was discovered that after 3 months, both types of NPs accumulated in the form of aggregates within the cytoplasm, which resulted in reductions in mitochondrial function and changes in cellular morphology [250].

**Importance of crystal structure of TiO₂**

Although anatase TiO₂ is considered to be photocatalytically active, X-ray diffraction (XRD) analysis of the inorganic components that were separated from commercially available sunscreens identified either rutile TiO₂ or a combination of rutile and anatase TiO₂ as the active ingredient [206, 236]. In particular, the composition of commercial Aeroxide® (P25, Degussa) has been identified, which contains approximately 80% anatase and 20% rutile TiO₂ [228, 257]. Upon UV irradiation, the production of reactive hydroxyl radicals was shown to be significantly increased for anatase TiO₂ compared to rutile TiO₂ [258]. More importantly, the ROS production of P25 is considerably increased compared to anatase TiO₂ alone [259].

TiO₂ NPs have been studied *in vitro* in their pure phases (anatase, rutile) and their mixed form, in particular in form of P25 [249, 252, 253]. One study reported that no toxicity could be observed in HaCaT cells following exposure to TiO₂ in the form of rutile with a particle size of 100 nm (R100), anatase with an average particle size of 25 and 325 nm (A25, A325), and P25 (35 nm, 86:14 anatase:rutile) alone [252]. When irradiated with UVA at 340 nm, however, subsequent dose- and concentration-dependent toxicity was caused by the anatase and P25 samples, which was related to the production of ROS, while the rutile sample showed very low toxicity (Figure 2.31) [252]. These results have been confirmed by the authors of [253], who observed an 1.8 fold increase in the accumulation of intracellular ROS levels in HaCaT cells after exposure to 200 µg/mL of P25 (21 nm; 75:25 anatase:rutile) for 24 h and subsequent irradiation with UVA at 365 nm.
Figure 2.31. (a) Effect of TiO$_2$ at a concentration of 100 μg/mL on the metabolic activity of HaCaT cells as a function of irradiation dosage. (b) Generation of hydroxyl radicals from TiO$_2$ under irradiation with ultraviolet A light (340 nm). The electron spin resonance spectra were recorded at room temperature in the presence of 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide and TiO$_2$ at a concentration of 0.1 mg/mL. Adapted from [252].

2.5.3 Photostability and Efficiency

Besides the penetration of NPs into deep skin layers, their photoactivity and associated toxicity, the photostability of the formulation has a substantial impact on the overall performance of the sunscreen product. Three pathways exist for a molecule that absorbs UV radiation to convert or scatter the absorbed energy [187]:

- Radiative decay (emission),
- Non-radiative decay (heat), and
- Photochemical reactions.

**Chemical UV filters**

The photoinstability of chemical UV filters is commonly known, causing not only the degradation of organic compounds into by-products with unknown toxicity, but also the reduction of UV attenuation, which lowers the actual UVA/UVB protection [187, 236, 237]. Sunscreens based on avobenzone, for instance, were shown to have lost almost 50% of their advertised protection after an UV irradiation at 295 – 400 nm and 27 mW/cm$^2$ for
only 2 h [260]. This organic UV filter can even exhibit photooxidizing properties, and in combination with other chemical organic UV filters, the photoinstability can be enhanced [187]. The generated free radicals even persisted after the exposure to UV radiation had ended [261].

**Inorganic UV filters**

Another study investigated the photostability of commercially available sunscreen products containing inorganic UV filters [237]. The study showed that after a 2 h exposure of the sunscreens to simulated sunlight at wavelengths > 290 nm and 48 W/m², significant changes in attenuation occurred. Photodegradation of a sunscreen lotion with SPF 25 containing the UVA absorbers avobenzone and terephthalycamphor sulfonic acid, a camphor derivative as UVB filter, and the inorganic UV filter TiO₂ led to a 10% decrease in attenuation at 300 and 360 nm, and the formation of new photoproduct(s) at wavelengths < 290 nm [237]. A sunscreen lotion with SPF 15 containing octyl methoxycinnamate, octyl salicylate, and TiO₂ showed a 40% drop in attenuation at 300 nm. More interestingly, a sunscreen lotion based on inorganic TiO₂ and ZnO and unknown organic ingredients with SPF 25 exhibited a decrease in attenuation of almost 95% at a wavelength of 315 nm within the 2 h exposure to simulated sunlight [237].

It was further shown that the organic UV filter oxybenzone showed significantly greater photoinstability in combination with TiO₂ compared to a combination of oxybenzone and octyl methoxycinnamate, which further underlines the photoactivity and associated problems of TiO₂ [237]. The photocatalytic activity of inorganic NPs can cause the generation of ROS and ultimately the degradation of sunscreen ingredients and a decrease in SPF (see section 2.4.3) [262].

**Consequences for the end-user**

Since the SPF only accounts for UVB protection, its decrease is not the only concern. More importantly, a study published in 2000 showed that only 10 % of the
studied 59 commercially available sunscreen products provide broad spectrum protection with a critical wavelength ≥ 370 nm (see section 2.4.2) [200].

The majority of end-users are unaware of the lack of broad spectrum protection and the loss of UV attenuation of commercial sunscreens over time. As such, the general rate of re-application of sunscreen is low, together with a lack of generous coverage of all body parts with sunscreen lotion [187]. In a long-term study running over 5 years, over 1600 residents of Nambour, Queensland, Australia, were given either sunscreens or betacarotene as a placebo. The study showed that there was no detectable difference in basal-cell and squamous-cell carcinoma incident rates [263].

Of particular concern is also the fact that the exact sunscreen formulations are mostly unknown, although they are commonly available and used by a large number of people. This significantly complicates the determination of sunscreen safety and possible side effects that might arise with the use of photocatalytically active ingredients.

2.5.4 Improvement of Existing Inorganic Ultraviolet Filters

The concerns that have been raised regarding both organic and inorganic UV filtering additives have led to increasing research on sunscreen stability, safety, including that of inorganic NPs and organic UV filters, photoactivity, and efficiency. The main goal is to improve commercially available sunscreen formulations by improving the properties of their organic and inorganic ingredients.

2.5.4.1 Stability of Emulsions

Sunscreen formulations are based on emulsions that might break down once applied onto the skin, which can potentially increase the skin permeability for NPs [11]. It has been shown that the penetration levels of TiO₂ were higher for an oily dispersion in comparison to an aqueous dispersion [264]. It is therefore advantageous to stabilize the emulsion with the focus on an aqueous dispersion.
The addition of microsponge delivery systems to a modern sunscreen formulation, for instance, has improved its stability and cost-efficiency [265]. Once incorporated with active UV filtering materials, the microsponge systems release these only when the sunscreen is applied on the skin [265].

### 2.5.4.2 Broad Ultraviolet A/Ultraviolet B Protection

One problem with the use of nanosized TiO$_2$ and ZnO is their shift of the absorption threshold to shorter wavelengths, leading to more UVB absorption and an imbalance in the recommended UVA/UVB protection. The absorption of TiO$_2$ NPs in particular shifts to UVB only, so that broad spectrum protection is not achieved anymore. A possible solution to this problem is the addition of both nanosized and microsized ZnO to maintain UVA protection and transparency at the same time [11]. A study suggested that ZnO with a particle size of 130 nm had a more severe effect on UVA protection than ZnO particles with a size of only 20 nm [266].

### 2.5.4.3 Reduction of Photoactivity

The Scientific Committee on Consumer Safety (SCCS) has declared that sunscreens that contain TiO$_2$ as active ingredient should be composed of the less photocatalytically active rutile TiO$_2$ with a maximum of 5% anatase TiO$_2$ [267].

**Surface modifications**

In addition, there have been a number of attempts to reduce the photocatalytic activity of inorganic UV filters. For instance, the surface of TiO$_2$ was modified using a thermally assisted method, which resulted in a deactivation of the photocatalytic activity and further prevented DNA damage to human keratinocyte skin cells [268]. Saccharomyces Cerevisiae yeast cells showed greater survival rates with the inactivated TiO$_2$ NPs under UV radiation in comparison to azabenzene (Figure 2.32), and the inactivated NPs still exhibited their characteristic UVA/UVB attenuation [268].
Figure 2.32. Survival of Saccharomyces Cerevisiae yeast cells after ultraviolet irradiation for 0 – 40 min. The yeast cells were treated without an ultraviolet filter (left image), with modified TiO$_2$ (centre image), and with azobenzone (right image). The number of yeast cells on the left was twofold greater than the number of cells on the right [268].

**Surface coatings**

Typically, the formation of hydroxyl radicals, or other relevant ROS, takes place on the surface of the NPs, after the electron-hole pair has been formed [11]. By modifying the surface properties of these NPs, such as by applying coatings or ROS scavenging materials on their surfaces, their photocatalytic activity can be altered. The coated NP is entirely isolated from the surrounding medium (skin, water, and oxygen), preventing the generation of ROS [11]. Alternatively, the coatings can also actively scavenge the photogenerated ROS [11].

For a surface coating to be suitable, it needs to be non-toxic, highly biocompatible, photostable, and more importantly, it cannot influence the UV attenuation of the NPs. Such coatings are typically based on silica (SiO$_2$), aluminium oxide (Al$_2$O$_3$), aluminium hydroxide (Al(OH)$_3$), methicone, and poly(methacrylic acid) [11, 269].

The composition of the nanocomposites is of particular importance, since materials such as TiO$_2$/SiO$_2$ and TiO$_2$/Al$_2$O$_3$ can also display enhanced photocatalytic activity [270]. A comprehensive study investigating the generation of ROS by uncoated and coated TiO$_2$ NPs, either in the form of pure rutile or in a mixture of 80% anatase and 20% rutile (P25) was conducted by the authors of [228]. Table 2.6 gives an overview of the tested materials.
### Table 2.6. Physico-chemical properties of selected commercially available coated TiO₂ nanoparticles [228].

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Composition (%)</th>
<th>Crystal phase (%)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maxlight™ F-TS20</td>
<td>TiO₂, 75</td>
<td>Rutile, 100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SiO₂, 22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eusolex® T-AVO</td>
<td>TiO₂</td>
<td>Rutile, 100</td>
<td>&lt; 200</td>
</tr>
<tr>
<td></td>
<td>SiO₂</td>
<td></td>
<td>(needle-like)</td>
</tr>
<tr>
<td></td>
<td>TiO₂, 84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-Lite® SF</td>
<td>Al(OH)₃, 7</td>
<td>Rutile, 100</td>
<td>30 – 60 x 10</td>
</tr>
<tr>
<td></td>
<td>Dimethicone, 4.5</td>
<td></td>
<td>(needle-like)</td>
</tr>
<tr>
<td></td>
<td>TiO₂, 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SiO₂, 7.5</td>
<td></td>
<td>30 – 60 x 10</td>
</tr>
<tr>
<td>T-Lite® SF-S</td>
<td>Dimethicone, 5.5</td>
<td>Rutile, 100</td>
<td>(needle-like)</td>
</tr>
<tr>
<td></td>
<td>Al(OH)₃, 3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TiO₂, &gt; 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEGO® Sun TS Plus</td>
<td>SiO₂, 10 – 25</td>
<td>Anatase, 80</td>
<td>28 – 32</td>
</tr>
<tr>
<td></td>
<td>Trimethoxycaprylsilane, 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TiO₂, &gt; 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PW Covasil S</td>
<td>Trimethoxycaprylsilane, &lt; 5</td>
<td>Anatase, 80</td>
<td>28 – 32</td>
</tr>
<tr>
<td></td>
<td>Polymethyl methacrylate, 5</td>
<td>Rutile, 20</td>
<td></td>
</tr>
<tr>
<td>PW Covasil S-1</td>
<td>TiO₂, &gt; 95</td>
<td>Anatase, 80</td>
<td>28 – 32</td>
</tr>
<tr>
<td></td>
<td>Trimethoxycaprylsilane, &lt; 5</td>
<td>Rutile, 20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TiO₂, 100</td>
<td>Anatase, 80</td>
<td>28 – 32</td>
</tr>
<tr>
<td></td>
<td>Rutile, 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeroxide®</td>
<td>TiO₂, 100</td>
<td>Anatase, 80</td>
<td>28 – 32</td>
</tr>
<tr>
<td></td>
<td>Rutile, 20</td>
<td></td>
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</tr>
</tbody>
</table>

The lipid peroxidation was determined in porcine skin under UV irradiation at a wavelength of 250 – 800 nm and compared to control samples without TiO₂ [228].
Covasil S, PW Covasil S-1, T-Lite® SF, and standard Aeroxide® induced substantial lipid peroxidation, while the use of Maxlight™ F-TS20, TEGO® Sun TS Plus, and T-Lite® SF-S resulted in reduced lipid peroxidation [228]. The obtained results suggest that, besides the phase of TiO₂, the type of surface material has an impact on the overall capacity to reduce photoactivity, with silica coatings being the most promising.

Similar tests with the focus on cytotoxicity and genotoxicity were performed with uncoated and coated ZnO NPs (30 nm) [271]. The results indicate that the NPs that were coated with poly(methacrylic acid) or oleic acid exhibited reduced cytotoxicity in comparison with uncoated ZnO NPs. The ZnO NPs coated with poly(methacrylic acid), however, showed the highest genotoxicity [271].

**Addition of photostabilizers and antioxidants**

Besides the use of surface coatings, the degradation of active organic ingredients can be reduced by the addition of photostabilizers such as octocrylene, 4-methylbenzylidene camphor, butyloctyl salicylate, hexadecyl benzoate, butyloctyl benzoate, and polycrylene to sunscreen formulations [272].

Furthermore antioxidants, such as vitamins, are added to sunscreens to provide protection from DNA inactivation or cell damage [273, 274]. Although these organic additives may offer benefits, they are potential allergens and typically have lower photostability than inorganic components (see section 2.5.3) [237].

**2.5.5 New Class of Nanoparticles for Sunscreen Formulations**

Besides the improvement of existing UV filters, research is also focused on the development of new UV filters. Particular interest is paid to their photocatalytic activity, which cannot be completely prevented with surface coatings in the case of inorganic UV filters [11]. Besides having high physical and chemical stability, once added into the base emulsion, the optimum UV filter should ideally fulfil the following requirements [187]:
• Transparency,
• High photostability and low photocatalytic activity,
• High biocompatibility, low cytotoxicity, and low genotoxicity,
• Non-permeability into skin,
• Broad spectrum UVA/UVB protection, high UV attenuation, and high SPF.

2.5.5.1 Inorganic Nanoparticles

Since it is assumed that the generation of ROS is also related to NP toxicity in the absence of UV radiation, an optimal NP-based UV filtering component does not show any generation of ROS – neither in presence nor the absence of UV radiation [108-111]. In contrast, by introducing ROS scavenging properties into the NP system, the biocompatibility of the inorganic UV filter can be improved, and at the same time, the decomposition of organic sunscreen components can be reduced.

Materials of interest include pure CeO$_2$ [113-115] and rare earth- [113], alkaline earth- [113, 275], and transition metal [276] doped CeO$_2$. The absorbance curves of pure and Ca-doped CeO$_2$ NPs are shown in Figure 2.33. CeO$_2$ NPs provide selective absorbance in the UV range and it has been shown that sunscreen emulsions prepared with TiO$_2$/CeO$_2$ yielded in an increase of 27% in their SPF compared to a formulation containing the classical combination of TiO$_2$/ZnO [119]. More importantly, CeO$_2$ NPs show high biocompatibility and can scavenge free radicals (see section 2.3.4.4) [120, 121].

Nevertheless, these nanomaterials have a relatively high cost in comparison to TiO$_2$ NPs, making latter ones still preferable. Therefore, cheaper materials have been investigated, such as $\alpha$-Fe$_2$O$_3$ [277], Ce-doped $\alpha$-Fe$_2$O$_3$[278], and Fe$_2$O$_3$/CeO$_2$ nanocomposites [238], which showed scavenging of photogenerated hydroxyl radicals as well as improved UV absorbance. More recently, Ti-doped SnO$_2$ NPs have been synthesized that exhibit reduced photocatalytic activity compared with TiO$_2$ and ZnO NPs [279].
Figure 2.33. Absorbance curves of pure (black curve), 20 mol.% (red curve), and 40 mol.% (blue curve) CeO$_2$ nanoparticles. Adapted from [275].

2.5.5.2 Organic Nanoparticles

Besides the development of new inorganic NPs as UV filters, the investigation of organic NPs as potential sunscreen ingredients has aroused research interest. For instance, organic NPs were isolated from English ivy (*Hedera helix*) and exhibited greater UV blocking properties than TiO$_2$ NPs [280]. The biodegradable ivy NPs also showed greater biocompatibility and decreased cell toxicity in human epithelial HeLa cells compared with TiO$_2$ NPs. Mathematical models suggested that they have limited potential to penetrate the skin [280]. It is, however, necessary to establish the photostability of those types of NPs.

2.6 References


Chapter 2 – Literature Review


Chapter 2 – Literature Review


Chapter 2 – Literature Review


CHAPTER 3 – Materials and Methodology
3.1 Synthesis of Nanomaterials

3.1.1 Materials

Bismuth(III) nitrate pentahydrate (Bi(NO$_3$)$_3$·5H$_2$O, 98%), ammonium hydroxide (NH$_4$OH, 28–30%), tantalum ethoxide ((CH$_3$CH$_2$O)$_5$Ta, ≥ 99.98%), acrylic acid (AA, 99%), methanol (≥ 99.8%), hydrochloric acid (HCl, 37%), 2-propanol (99.5%), and denatured ethanol were purchased from Sigma-Aldrich. Nitric acid (HNO$_3$, 69%) was purchased from Merck and chloroform (1% ethanol) was purchased from Chem Supply.

TiO$_2$ (Aeroxide®, P25) and ZnO nanoparticles (NPs, < 100 nm) were obtained from Sigma-Aldrich. Commercial poly(acrylic acid) (PAA) with a molecular weight (MW) of 450,000 was purchased from Polysciences Inc. All commercial materials were used without further purification.

3.1.2 Synthesis of Bi(OH)$_3$ Nanoparticles

Bi(OH)$_3$ NPs were synthesized via a classical precipitation route. First, 4 g of Bi(NO$_3$)$_3$·5H$_2$O (8.25 mmol) was dissolved in 40 mL of 69% HNO$_3$. Then, 30% NH$_4$OH was added dropwise until a white precipitate was formed. The precipitate was filtered via centrifugation (Eppendorf, 5 min, 3920 g), washed eight times with deionized (DI) water, resuspended in 150 mL of DI water, and heated under stirring at 80–100°C for 5.5 h. The precipitate was then filtered (5 min, 3920 g), washed eight times with DI water (10 min, 3920 g), and dried in vacuum at 60°C for 48 h.

3.1.3 Synthesis of α-Bi$_2$O$_3$ Nanoparticles

To obtain α-Bi$_2$O$_3$ NPs, Bi(OH)$_3$ NPs, which were obtained through the classical precipitation method described above [1], were annealed in a horizontal tube furnace (LABEC) at 525°C and a heating rate of 10°C/min for 4 h under air atmosphere. After annealing, a yellow powder was obtained.
3.1.4 Preparation of Nanocomposites of TiO$_2$ and (BiO)$_2$CO$_3$

TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites were synthesized via a precipitation method. First, 600 mg (7.51 mmol) of TiO$_2$ NPs was suspended in 110 mL of DI water and sonicated for 1 h using a sonication bath (Branson 3800, Ultrasonics Corp). Then, according to the atomic ratio of Bi/Ti, Bi(NO$_3$)$_3$·5H$_2$O was dissolved in 0.75 – 2.95 mL (17.98 – 70.70 mmol) of 69% HNO$_3$. The acid was 50% neutralized with the dropwise addition of 30% NH$_4$OH. Then, the reaction mixture was added to the suspension of TiO$_2$ and stirred for 2 min. Further NH$_4$OH was added dropwise until the pH reached a value of 8. The precipitate was filtered via centrifugation (Eppendorf, 5 min, 157 g), washed eight times with DI water (5 min, 157 g), suspended in 35 – 50 mL DI water, and heated under stirring at 80 – 100°C for 5.5 h. The composites were filtered (5 min, 157 g) and washed eight times with DI water, and the white powder was dried in vacuum at 60°C for 48 h. The samples were denoted as Bi/Ti 2 at.%, Bi/Ti 4 at.%, and Bi/Ti 8 at.% for the composites with Bi:Ti atomic ratios of 0.02, 0.04, and 0.08, respectively.

3.1.5 Synthesis of δ-Ta$_2$O$_5$ Nanoparticles

δ-Ta$_2$O$_5$ NPs were synthesized using a classical precipitation route. First, 10 g (24.62 mmol) of (CH$_3$CH$_2$O)$_5$Ta was transferred carefully with exclusion of water into 200 mL of ethanol. The solution was stirred at room temperature, and then 60 mL of DI water was added quickly to the reaction mixture, which resulted in the formation of a white precipitate. The precipitate was filtered via centrifugation (Eppendorf, 10 min, 1656 g, room temperature), washed twice with DI water (10 min, 1656 g, room temperature), and dried at 90°C overnight. Then, the precipitate was annealed in a tube furnace (LABEC) at 730°C at a heating rate of 5°C/min for 30 min under air atmosphere.

3.1.6 Coating of δ-Ta$_2$O$_5$ Nanoparticles with Poly(acrylic acid)

To obtain structurally stable NP-polymer composite materials, the polymer is usually grafted onto the NP surfaces. If the surface of the substrate is activated via prior
etching, the number of hydroxyl groups increases, which also results in an increase in physical adsorption of AA monomers [2].

For instance, gold-coated silicon structures were formed following plasma etching treatment [2]. In another study, silicon NPs were first treated with a mixture of hydrofluoric acid and nitric acid, and then were grafted with PAA [3]. The etching of the NPs has been shown to increase the binding affinity of proteins, which can interact with a wider range of orientations [4]. Other approaches include ultraviolet-ozone soft etching of ceramic NPs before the polymer is grafted onto their surfaces [5]. Following this approach, the polymer chains grow around each individual ceramic particle, avoiding the wrapping of clusters of substrate NPs [6, 7].

The coating of δ-Ta₂O₅ NPs with PAA was performed in three steps:

- **First step:** surface activation using methanol to increase the number of hydroxyl groups on the NP surface.
- **Second step:** acidic etching using concentrated HCl to increase the physical adsorption of AA monomers.
- **Third step:** thermal induced organic polymerization in emulsion of acrylate chains of AA leading to PAA-coated NPs.

First, 330 mg (0.75 mmol) of δ-Ta₂O₅ NPs was added into a centrifuge tube containing 25 mL of methanol. The centrifuge tube was placed in a sonication bath (Branson 3800, Ultrasonics Corp) and sonicated for 25 min. The suspension was then centrifuged at room temperature (Eppendorf, 7 min, 980 g), the NPs were resuspended in 20 mL of concentrated HCl, and the suspension was stirred gently under nitrogen (N₂) flow for 25 min. The suspension was centrifuged at room temperature (5 min, 794 g), and the NPs were resuspended in 20 mL of AA (291.42 mmol) and placed back under N₂-flow. The suspension was stirred for a total of 1, 2.5, and 5 h, respectively, at a temperature of 83 ± 1 °C. After the desired amount of time, 10 mL of chloroform was added to the reaction
mixture to stop the polymerization process. The suspension was then centrifuged at room temperature (3 min, 157 g), washed three times with chloroform (3 min, 157 g, room temperature), and the PAA-coated NPs were dried at room temperature for 48 h. The coating procedure is schematically shown in Figure 3.1.

![Figure 3.1. Schematic illustration of the coating procedure involving the reaction of δ-Ta$_2$O$_5$ nanoparticles and acrylic acid (AA).](image)

To determine the surface properties of δ-Ta$_2$O$_5$ after treatment with HCl via X-ray photoelectron spectroscopy (see section 3.2.3), an aliquot was taken before resuspension in AA, followed by washing three times with methanol (3 min, 157 g, room temperature), and drying at room temperature for 48 h.

### 3.2 Physical and Chemical Characterization of Nanomaterials

#### 3.2.1 Materials

Phosphate buffered saline (PBS), denaturated ethanol, sodium acetate (≥ 99.0%), sodium carbonate decahydrate (≥ 99.99%), acetic acid (≥ 99.7%), and sodium bicarbonate (≥ 99.7%) were purchased from Sigma-Aldrich.
3.2.2 Specific Surface Area (Brunauer-Emmett-Teller)

The Brunauer-Emmett-Teller (BET) method for the determination of the specific surface area of nanopowders is based on the adsorption of gas molecules onto the surface of a material. It assumes that the adsorbate forms a weakly bonded monolayer, and the volume of gas is correlated with the surface area of the tested material [11].

This method makes it possible to experimentally determine the adsorbed and desorbed volume of an inert gas, usually nitrogen, at various relative pressures and at a constant temperature, which is then used to calculate the specific surface area [11, 12]. The BET model includes multilayer adsorption, where the first layer describes the heat of adsorption. Equation (3.1) describes the BET adsorption isotherm [11, 12]:

\[ V = \frac{V_{\text{mono}} \cdot C \cdot p}{p_0 \cdot (1 - \frac{p}{p_0}) \cdot (1 - \frac{p}{p_0} + C \cdot \frac{p}{p_0})} \]  

(3.1)

where \( V_{\text{mono}} \) is the adsorbed gas volume of a monolayer (m\(^3\)), \( p \) is the equilibrium pressure (Pa), \( p_0 \) is the saturation vapour pressure (Pa), and \( C_{\text{BET}} \) is the BET constant. The BET constant can be described by Equation (3.2) [11]:

\[ C_{\text{BET}} = \frac{E_1 - E_L}{R_{\text{gas}} \cdot T} \]  

(3.2)

where \( E_1 \) is the heat of adsorption for the first layer (J), \( E_L \) is the heat of adsorption for the second and higher layers (J), \( R_{\text{gas}} \) is the ideal gas constant (8.314 J \cdot mol\(^{-1}\) \cdot K\(^{-1}\)), and \( T \) is the temperature (K). The BET surface area \( S_{\text{BET}} \) (m\(^2\) \cdot g\(^{-1}\)) is defined by Equation (3.3) [11]:

\[ S_{\text{BET}} = \frac{V_{\text{mono}} \cdot N_{\text{Avogadro}} \cdot s}{V_{\text{a}} \cdot a_{\text{adsorbent}}} \]  

(3.3)

where \( N_{\text{Avogadro}} \) is the Avogadro’s number (6.022 \times 10\(^{23}\) mol\(^{-1}\)), \( s \) is the adsorption cross-sectional area of the adsorbing gas (m\(^2\)), \( V_{\text{a}} \) is the molar volume of adsorbed gas (m\(^3\) \cdot mol\(^{-1}\)), and \( a_{\text{adsorbent}} \) is the mass of adsorbent (g).

The BET surface area of all materials was measured using a Nova 1000 high speed gas sorption analyser from Quantachrome. The adsorption of N\(_2\) at the temperature of
liquid nitrogen was determined, and prior to measuring, the samples were degassed at 60°C for 15 h in vacuum.

### 3.2.3 X-Ray Diffraction (XRD)

XRD is widely used to identify crystal structures, which provide a diffraction grating for electromagnetic radiation such as X-rays with a wavelength in the range of the lattice plane distances, as shown in Figure 3.2.

![Figure 3.2. Schematic principle of X-ray diffraction by a crystalline sample with the angle $\theta$ of the incident beam on lattice planes with spacing $d$.](image)

X-rays that are directed through the atoms in the crystalline structure are scattered through the electron shell of the radiated atom. The radiation caused by constructive interference is recorded by a detector with a deflection angle of $2\theta$ and results in visible reflections in the XRD pattern. In contrast, destructive interference does not yield visible reflections. The conditions that need to be present for constructive interference are summarized in the Bragg equation (Equation (3.4) [8, 9]):

$$2 \cdot d \cdot \sin(\theta) = n_{\text{diffraction}} \cdot \lambda$$  

(3.4)

where $d$ is the spacing between diffraction planes (nm), $\theta$ is the incident angle (rad), $n_{\text{diffraction}}$ is the diffraction order, any integer, and $\lambda$ is the wavelength of the beam (nm).

In general, Cu Kα radiation is used with a wavelength of 0.1542 nm.
By assigning the Miller indices $hkl$ to the particular diffraction angles, the lattice plane difference can be calculated. If the crystal lattice structure is known, the lattice parameters of the unit cell ($a$, $b$, and $c$) can be determined, according to Equation (3.5) [9]:

$$\frac{1}{d^2} = \left(\frac{h}{a}\right)^2 \cdot \left(\frac{k}{b}\right)^2 \cdot \left(\frac{l}{c}\right)^2$$  \hspace{1cm} (3.5)

If the crystals are very small in size (100 – 200 nm), a broadening of the reflections occurs, which can be described by the Scherrer equation. The mean crystallite size can be calculated by the following Equation (3.6)) [10]:

$$d_{\text{crystallite}} = \frac{K_f \cdot \lambda}{\beta \cdot \cos(\theta)}$$  \hspace{1cm} (3.6)

where $d_{\text{crystallite}}$ is the average crystallite size (nm), $K_f$ is a form constant (assuming that the particles are spherical, $K_f = 0.89$), $\lambda$ is the wavelength of the X-ray radiation (nm), $\beta$ is the full width at half maximum (rad), and $\theta$ is the Bragg angle of diffraction (rad).

The phases of all produced and as-received materials were characterized via an Enhanced Mini-Materials Analyzer X-Ray Diffractometer (GBC Scientific) and a Mac Science M03XHF22 diffractometer with Cu Kα radiation at 40 kV and 25 mA. Lattice parameters were obtained via Rietveld refinement using the Marquardt least square method.

### 3.2.4 X-Ray Photoelectron Spectroscopy (XPS)

XPS is a surface-sensitive quantitative spectroscopic technique that gives information about the elemental composition of a sample and the chemical and electronic states of elements on the surface [13].

A sample is irradiated using a beam of X-rays, which penetrate the first 0 – 10 nm of the material [13]. The kinetic energy and emitted electrons are collected by a detector, as displayed in Figure 3.3. To minimize the loss of photoemitted electrons due to inelastic collisions or recombination, ultra-high vacuum (< 10⁻⁹ mbar) is needed. By counting the ejected electrons over the range of kinetic energies, the photoelectron spectrum is
recorded. The energies and intensities of the photoelectron peaks are characteristic of each element and allow their identification, except for hydrogen [13].

Figure 3.3. Working principle of X-ray photoelectron spectroscopy.

XPS was conducted using a SPECS PHOIBOS 100 Analyzer installed in a high vacuum chamber with base pressure below $10^{-8}$ mbar. X-ray excitation was provided by Al Kα radiation with a photon energy of 1486.6 eV at a voltage of 12 kV and power of 120 W. The XPS binding energy spectra were recorded with pass energy of 20 eV in fixed analyser transmission mode. Analysis of the XPS data was carried out using CasaXPS 2.3.15 software.

3.2.5 Fourier-Transform Infrared Spectroscopy (FTIR)

Infrared (IR) spectroscopy is used to identify organic compounds [14, 15]. Typically, a FTIR spectrometer consists of an IR source and an interferometer, which splits one beam of light into two (Figure 3.4). After the beams are recombined at the beamsplitter, constructive and destructive interference is generated, yielding an interferogram [16]. The combined beam passes through the sample, which absorbs all wavelengths characteristic of its spectrum, ultimately changing the interferogram. The detector measures these changes, and a computer transfers the digitized signal using the Fourier transform [16].
Chapter 3 – Materials and Methodology

**Figure 3.4.** Working principle of a Fourier transform infrared spectrometer, adapted from [17].

When IR radiation hits the sample, energy is absorbed by the individual molecules, which causes a change in the dipole moment and ultimately, the formation of different stretching and bending vibrations. The number of degrees of vibrational freedom of the molecule is directly linked to the number of absorption peaks in the FTIR spectrum, while the intensity of the peaks corresponds to the change of dipole moment [15, 16]. Not all fundamental vibrations, however, are visible in the FTIR spectrum, since certain modes are IR inactive if there is no change in the dipole moment, or they might have the same frequency. Furthermore, more bands can be generated due to overtones or combinations of fundamental frequencies [14].

FTIR spectra were recorded on an IR Prestige 21 Spectrometer from Shimadzu Corporation over the range of 4000 – 500 cm<sup>-1</sup> (64 scans, resolution of 4 cm<sup>-1</sup>) via attenuated total reflectance (ATR) using the MIRacle attachment. The ATR method is based on a crystal with high refractive index, such as diamond, through which a beam of IR light is passed at a certain angle. An evanescent wave is generated, which, due to total internal reflection, extends beyond the surface of the crystal into the sample [18].
3.2.6 Raman Spectroscopy

Similar to IR spectroscopy, Raman spectroscopy provides information about vibrational, rotational, and other low-frequency modes in a molecule. A laser is used as the source of monochromatic light in the visible, near infrared, or near ultraviolet range, which hits the sample and can be reflected, absorbed, or scattered [19].

When analysing samples that have a change in polarizability, the frequency of the scattered radiation shows, besides the incident radiation (Rayleigh scattering), also radiation that is scattered at different wavelengths (Stokes and Anti-Stokes Raman scattering), as pictured in Figure 3.5 [19]. This change in wavelength of the scattered photons can be used to identify chemical structures.

![Figure 3.5. Rayleigh and Raman scattering (Stokes and Anti-Stokes).](image)

Raman spectra were recorded on a Raman Jobin Yvon HR800 Spectrometer using ×50 LWD and ×100 LWD objectives over the range of 50 – 3500 cm⁻¹.

3.2.7 Ultraviolet-Visible (UV-Vis) Spectroscopy

UV-vis spectroscopy is used to determine the UV-vis absorption properties of samples, or, in the case of semiconducting materials, their band gap [20].

Typically, an UV-vis spectrometer consists of a UV-vis source and a beam splitter that separates the light beam into two equal intensity beams. One beam passes through a cuvette containing the sample and a solvent/dispersant, while the other beam passes
through a cuvette filled with only the solvent/dispersant (= reference) (Figure 3.6) [21]. Detectors measure the intensity of both light beams, where the intensity of the sample beam is defined as $I$ and the intensity of the reference is defined as $I_0$ [21].

![Figure 3.6. Working principle of an ultraviolet-visible spectrometer.](image)

The concentration $c$ of the sample (mol $\cdot$ L$^{-1}$) can be calculated via the Lambert-Beer law as shown in Equation (3.7) [22]:

$$A = \log \left( \frac{I_0}{I} \right) = \varepsilon \cdot c \cdot d = \alpha \cdot l$$

(3.7)

where $A$ is the absorbance, $\varepsilon$ is the extinction coefficient (m$^2$ $\cdot$ g$^{-1}$), $l$ is the path length through the sample (m), and $\alpha$ is the absorption coefficient (m$^{-1}$).

UV-vis light can either be reflected or absorbed by a molecule [21, 23]. If the energy is high enough, the molecules can undergo electronic transitions from the ground state to the excited state or from the valence band to the conduction band. For a superconductor, the optical absorption behaviour near the band edge follows Equation (3.8) [20]:

$$(\alpha \cdot h \cdot \nu)^m = B \cdot (h \cdot \nu - E_g)$$

(3.8)

where $\alpha$ is the absorption coefficient, $h$ is the Planck constant ($6.626 \times 10^{-34}$ m$^2$ $\cdot$ kg $\cdot$ s$^{-1}$), $\nu$ is the light frequency (s$^{-1}$), $B$ is a constant, and $E_g$ is the band gap (eV). The value of $m$ depends on the type of optical transition (i.e. $m = 2$ for a direct transition and $m = 0.5$ for an indirect transition) [20].
UV-vis measurements of suspended NPs

Suspensions were prepared in DI water at a concentration of 1 mg/mL and were sonicated for 2 h using a sonication bath (Branson 3800, Ultrasonics Corp). Dilutions were prepared at concentrations of 25 – 50 µg/mL to yield an absorbance close to 1.0. The absorbance was recorded on an UV-3600 spectrophotometer from Shimadzu Corporation over the range of 800 – 200 nm by using 1.4 mL quartz cuvettes with a path length of 1 cm.

UV-vis measurements of solutions

The UV-vis absorbance of solutions was measured directly, as described above, without sonication. For the calibration curve of methotrexate (MTX) and the drug release experiments, the supernatant of each sample was directly measured, and the quantity of MTX was calculated from the absorbance maximum at 303 nm (see section 3.6.8.2).

3.2.8 Dynamic Light Scattering (DLS)

DLS is used to determine the geometric size distribution profile of small particles (down to 1 nm in diameter) in suspension [24]. These particles include NPs, colloids, proteins, polymers, or emulsions. When in suspension with an aqueous dispersant, a hydration layer is typically formed surrounding the particle or molecule. The measured particle size is therefore given as a hydrodynamic diameter [24].

During the DLS measurement, a laser beam is focused on the sample and the fluctuations of scattered light are detected at a known scattering angle by a fast photon detector [25]. The fluctuations are caused by Brownian motion, which differs for small and large particles: while smaller particles show faster dynamics, larger particles tend to diffuse more slowly in suspension (Figure 3.7). The Stokes-Einstein equation shows the relation of the diffusion coefficient \( D_{\text{diffusion}} \) (m²/s) and hydrodynamic radius \( R_{\text{hydrodynamic}} \) (nm) of the particles (Equation (3.9)) [24]:

\[
D_{\text{diffusion}} = \frac{k_B \cdot T}{6 \cdot \pi \cdot \eta \cdot R_{\text{hydrodynamic}}} \tag{3.9}
\]
where $k_B$ is the Boltzmann constant $(1.38 \times 10^{-23} \text{ m}^2 \cdot \text{kg} \cdot \text{s}^{-2} \cdot \text{K}^{-1})$, $T$ is the temperature (K), and $\eta$ is the viscosity (Pa · s).

Figure 3.7. Scheme of Brownian motion of (a) small and (b) large particles [26].

The hydrodynamic diameter of the nanomaterials suspended in PBS at a concentration of 50 µg/mL was determined using a Malvern – Zetasizer APS2000. The light scattering experiments were conducted using an 830 nm laser in three independent measurements at room temperature. An autocorrelation function for the scattered light from the suspension was used to obtain the size distribution, utilizing the Malvern – Zetasizer 7.03 software.

3.2.9 Thermogravimetric Analysis (TGA)

TGA is typically used to investigate changes in the mass of a material during heating and provides information about the composition of a sample, and its stability and degradation. The sample is typically placed in a crucible that is made of an inert and high-temperature-resistant material such as platinum or aluminium oxide, which is then placed in a precisely controlled furnace, as shown in Figure 3.8. A heating program in different atmospheres, such as argon, nitrogen, air, or oxygen, with final temperatures of up to 1200°C is applied, and a highly sensitive balance registers any mass changes [27]. A loss of mass is typically related to certain processes, such as evaporation, sublimation, or the generation of volatile products, while a gain of mass can be caused by oxidation [27].
Figure 3.8. Schematic diagram of a thermogravimetric analyser.

The stability of the synthesized and as-received materials and the quantity of organics were investigated using a Metter Toledo TGA/DSC 1 STAR® system. Each sample was placed in an aluminium oxide cup and heated from 50 - 800°C at a heating rate of 5°C/min in air atmosphere.

3.2.10 Computed Tomography

The principle of CT, including the set-up of a CT scanner and the use of CT contrast enhancing agents, is discussed in section 2.3.7.1.

The anatomical contrast enhancement capability of α-Bi₂O₃, Bi(OH)₃, and δ-Ta₂O₅ NPs, and composites of δ-Ta₂O₅ and PAA were demonstrated using CT. Suspensions with concentrations of Bi and Ta ranging from 0 to 8 mg/mL in PBS were prepared in 1.5 mL vials and sonicated for 2 h. Then, the vials were imaged together using a Toshiba Asteion TSX-021A whole body X-ray CT scanner with a tube voltage of 100, 120, and 135 kV, as shown in Figure 3.9. The CT images were obtained using the standard patient image reconstruction algorithms included with the scanner.
Figure 3.9. (a) Toshiba Asteion TSX-021A computed tomography scanner. (b) 1.5 mL vials with α-Bi$_2$O$_3$ (top) and δ-Ta$_2$O$_5$ (bottom) nanoparticles suspended in phosphate buffered saline.

CT numbers (HU) were calculated using ImageJ by normalizing the obtained grey scale (tissue) against the control (water). The CT number is directly linked to the image pixel and the tissue voxel via the attenuation coefficient $\mu$ (cm$^2$ · g$^{-1}$), as shown in Equation (3.10) [28]:

$$\text{CT number} = \frac{\mu_{\text{tissue}} - \mu_{\text{water}}}{\mu_{\text{water}}}$$

3.2.11 Polymer Swelling Studies

PAA as a hydrogel is able to hold large amounts of water and to deswell reversibly [29]. The swelling studies of nanocomposites based on δ-Ta$_2$O$_5$ and PAA were conducted in buffer solutions with a pH of 3.6, 5.4, 7.4, and 9.4 at 37°C, as it was shown that the shrinking and expansion of PAA depends on the pH value (see section 5.1.2) [30].

Preparation of buffer solutions

Four different pH buffer solutions were prepared to yield a pH of 3.6, 5.4, 7.4, and 9.4, respectively, at 20°C. The acetate buffer with pH 3.6 was prepared by combining 92.5 vol. % of 0.1 M acetic acid with 7.5 vol. % of 0.1 M sodium acetate. To yield a pH of 5.4, 85.5 vol. % of 0.1 M sodium acetate was combined with 14.5 vol. % of 0.1 M acetic acid. For experiments that require a buffer with pH 7.4, PBS was used. The carbonate-bicarbonate buffer with a pH 9.4 was prepared by combining 70 vol. % of 0.1 M sodium bicarbonate with 30 vol. % of 0.1 M sodium carbonate.
Swelling of PAA

First, the desired weight of the NPs \( W_0 \) was added into the buffer solution with the desired pH value. At predetermined time intervals, the NPs were removed from the swelling medium, blotted with filter paper to remove excess buffer solution from the NP surfaces, and the weight of the swollen composite \( W_1 \) was determined. The swelling behaviour of the NPs was observed for a total of 48 h, and the swelling ratio (SR) was calculated, as shown in Equation (3.11) [31]:

\[
SR = \frac{W_1 - W_0}{W_0}
\] (3.11)

3.3 Morphological Characterization of Nanomaterials

Electron microscopy is mainly divided into scanning electron microscopy (SEM) and transmission electron microscopy (TEM), although there are also modes in between, such as scanning transmission electron microscopy (STEM). These techniques are based on an electron beam that is focussed on a specimen and interacts with its atoms, as shown in Figure 3.10. The generated signals can be used to obtain information about the particle morphology and size. The elemental composition of a specimen can be determined via electron energy loss spectroscopy (EELS) or energy dispersive X-ray spectroscopy (EDS).

In this thesis, SEM, STEM, and TEM images and EDS mappings were acquired using a JSM7500FA cold field emission gun scanning electron microscope (FEGSEM, JEOL), a JEOL JEM-2010 transmission electron microscope, and a JEOL JEM-ARM200F atomic resolution microscope.
3.3.1 Scanning Electron Microscopy

SEM is used to obtain indirect images of the specimen surface by scanning it with a very fine electron probe with energies ranging from 0.2 to 40 keV [32]. The position of the electron beam on the sample directly corresponds to a position in the image, so that information about the topography of the sample can be obtained.

The most common imaging modes are based on the emission of secondary electrons (SE) with energies < 50 eV and back scattered electrons (BSE) with energies > 50.
eV [32]. Depending on the angle between the surface and the beam, more or less SE are emitted and detected, resulting in an image showing the tilt of the surface [32]. To minimize the recombination and collision of electrons, ultra-high vacuum (10^{-3} Pa) is necessary. A schematic illustration of a SEM is shown in Figure 3.11.

Figure 3.11. Adapted schematic illustration of a scanning electron microscope [33].

SEM images were obtained using a JSM7500FA cold field emission gun scanning electron microscope with an accelerating voltage of 15 kV. STEM images were acquired at an accelerating voltage of 30 kV.

3.3.2 Transmission Electron Microscopy

In the TEM mode, a beam of electrons passes through a very thin specimen (≤ 100 nm thick), allowing the transmission of electrons, which ultimately results in the formation of an image [32]. A schematic illustration of a TEM is shown in Figure 3.12.

Due to the smaller de Broglie wavelength of electrons, the resolution of a transmission electron microscope is significantly increased in comparison to light microscopes (Equation (3.12)) [34]:

\[ \frac{\lambda}{\Delta x} = \frac{\hbar}{mE} \]

\[ \frac{\lambda}{\Delta x} = \frac{\hbar}{2mE} \]
\[ \lambda = \frac{h}{m_{\text{electron}} \cdot v_{\text{electron}}} \]  

(3.12)

where \( \lambda \) is the wavelength (m), \( h \) is the Planck constant \((6.626 \times 10^{-34} \text{ m}^2 \cdot \text{kg} \cdot \text{s}^{-1})\), \( m_{\text{electron}} \) is the mass of the electron (kg), and \( v_{\text{electron}} \) the electron velocity (m \cdot s^{-1}). According to de Broglie, an increase in the accelerating voltage results in an increase in the electron velocity and ultimately an increase in resolution. Thus, high-end transmission electron microscopes are capable of imaging single columns of atoms.

**Figure 3.12.** Adapted schematic illustration of a transmission electron microscope [33].

TEM images were obtained using a JEOL JEM-2010 transmission electron microscope with an accelerating voltage of 200 kV. High-resolution TEM images were obtained using a JEM-ARM200F atomic resolution microscope. The image processing was performed using Gatan Digital Micrograph, and particle size distributions were determined using ImageJ.
3.3.3 Energy-Dispersive X-Ray Spectroscopy

EDS units are typically connected to SEM and TEM microscopes and can be used to determine the elemental composition of a specimen. Upon exposure of a specimen to a high-energy electron beam, an electron in an inner shell can be excited and ejected, which results in the formation of an electron hole, as shown in Figure 3.13 [32]. Electrons from outer shells with higher energy can fill this hole, releasing energy in the form of X-rays, which are collected by a detector. The energy levels of the electron shells are characteristic of each atom, so that distinctive spectra are obtained [32].

![Diagram of energy-dispersive X-ray spectroscopy](image.png)

**Figure 3.13.** Principle of energy-dispersive X-ray spectroscopy.

EDS bulk analysis and EDS mappings were performed with a JSM7500FA cold field emission gun scanning electron microscope equipped with an X-Flash 4010 10 mm², 127 eV SDD energy dispersive X-ray detector (Bruker, Massachusetts, USA). An accelerating voltage of 20 kV was used to obtain X-ray spectra of photons between 0 – 20 keV with a real time acquisition period of at least 120 s.

High-resolution EDS mappings were acquired using a JEM-ARM200F atomic resolution microscope fitted with a Centrino SDD 100 mm² detector (JEOL, Akishima, Tokyo, Japan). The EDS spectral imaging acquisition was performed using Thermo Scientific COMPASS software for the NORAN System 7 X-ray Microanalysis System.

120
3.4 Photocatalytic Activity and Ultraviolet-Blocking Properties of Sunscreen Formulations

3.4.1 Materials

Crystal violet (≥ 90%), hexane (≥ 95%), and denatured ethanol were obtained from Sigma-Aldrich. TiO$_2$ (Aeroxide®, P25) and ZnO NPs (≤ 100 nm) were purchased from Sigma-Aldrich. ZnO NPs coated with triethoxycaprylylsilane (Z-COTE® HP1) were obtained from BASF. Eusolex® T-S TiO$_2$ NPs are coated with stearic acid and were obtained from Merck KGaA. All commercial nanopowders were used without further purification.

Four commercially available sunscreen products were purchased, including Nivea® (SPF 50) and Nivea® anti-age (SPF 30), which have inorganic ingredients (i.e. TiO$_2$ NPs). These sunscreens were tested for their photostability and photocatalytic activity. OMBRA® (SPF 50) and Auscreen® (SPF 50) are organic-based sunscreens and were used for rheological measurements.

3.4.2 Preparation of Sunscreen Formulations

A water-in-oil emulsion was prepared containing 25 wt.% organic filters and 5 wt.% commercially available TiO$_2$ (Eusolex® T-S) NPs. In order to test the performance of commercial ZnO and synthesized Bi(OH)$_3$ NPs, the emulsions were supplemented with either type of NP for a final concentration of 3 wt.% (Figure 3.14(a)).

3.4.3 Separation of Inorganic Sunscreen Components

The photostability of sunscreen formulations is related to the phase of the TiO$_2$ NPs present in the formulations, with anatase TiO$_2$ being photocatalytically more active than rutile TiO$_2$ [35, 36]. The separation of commercial sunscreen ingredients and the XRD analysis of the inorganic components identified either the rutile phase or a combination of rutile and anatase TiO$_2$ [35, 37].
To extract the inorganic components from the commercial sunscreens used for the sun exposure tests (see section 3.4.5) and the TiO$_2$-based emulsions (see section 3.4.2), 1 – 2 g of sunscreen formulation was placed in a centrifuge tube, as described in the literature [37]. After 30 mL of hexane was added, the mixture was shaken to remove the active organic components, and centrifuged at room temperature (Eppendorf, 5 min, 1411 $g$). The supernatant was discarded, and 30 mL of ethanol was added to the residue to remove the remaining organics. After centrifugation (5 min, 1411 $g$, room temperature), the supernatant was discarded, and 30 mL of DI water was added to the residue to remove the surfactants from the inorganic components. The mixture was shaken, centrifuged at room temperature (5 min, 1411 $g$), and the remaining inorganic components were washed several times with ethanol (5 min, 1411 $g$, room temperature). The inorganic components were dried overnight, and the crystalline phase was determined via XRD. The extracted inorganic components are shown in Figure 3.14.

![Separation of inorganic sunscreen components](image)

**Figure 3.14.** Separation of inorganic sunscreen components of (a) sunscreen emulsion containing TiO$_2$ and Bi(OH)$_3$ NPs (left) and commercial sunscreen formulations of (b) Nivea® anti-age (SPF 30), and (c) Nivea® (SPF 50). On each watch glass, the left side shows the sunscreen and the right side shows the obtained inorganic components after extraction.

### 3.4.4 Photocatalytic Activity

The degradation of crystal violet is used as an indicator to investigate the presence of hydroxyl radicals in NP suspensions upon exposure to UV-vis light. Hydroxyl radicals typically react with crystal violet, resulting in multiple by-products and a concomitant loss of colour, which can be monitored by UV-vis spectrometry [38].
Suspensions of Bi(OH)$_3$, TiO$_2$, and ZnO NPs, and combinations of these nanomaterials were prepared in DI water and sonicated for 2 h using a sonication bath (Branson 3800, Ultrasonics Corp). The concentration of TiO$_2$ and ZnO remained constant at 5 mg/L, while the concentration of Bi(OH)$_3$ was varied between 1 – 5 mg/L. Crystal violet dye at an initial concentration of 12 µmol/L was added to the NP suspensions, which were equilibrated by stirring in the dark for 60 min, as indicated in Figure 3.15. An aliquot was taken prior to irradiation and the absorbance was measured at 590 nm using an UV-3600 spectrophotometer from Shimadzu. In a typical run, the aliquot was firstly centrifuged to remove the photocatalyst (Eppendorf, 1 min, 157 g, room temperature), and after UV-vis measurement, the aliquot was resuspended and returned to the reaction mixture.

**Figure 3.15.** Schematic procedure for assessment of photocatalytic activity of nanomaterials using crystal violet.
**Exposure to UVA/UVB**

After determination of the initial absorbance, the reaction mixture was placed in a RPR-200 photochemical reactor (Rayonet, Branford, CT, USA), equipped with 300 nm and 350 nm lamps. Upon exposure to UV light, aliquots were withdrawn every 5 min for a total of 30 min, and the absorbance was measured.

**Exposure to simulated sunlight**

For the sun simulation experiments, a halogen lamp (50 W power) was used, and the degradation of crystal violet was investigated under illumination of global (G) air mass (AM) 1.5 G one sun (100 mW/cm²). The irradiation time ranged from 0 – 6 h, and the absorbance was measured in intervals of 30 min.

The kinetics of the dye degradation was investigated using a first-order reaction. The apparent rate constant \( k \) (min\(^{-1}\)) was calculated according to Equation (3.13) [32]:

\[
c = c_0 \cdot e^{-k \cdot t}
\]  

(3.13)

where \( c \) is the concentration (mol \cdot L\(^{-1}\)) as the reaction progresses, \( c_0 \) is the initial concentration (mol \cdot L\(^{-1}\)), and \( t \) is the time (min).

**3.4.5 Sun Exposure of Sunscreen Formulations**

Another cheap and reliable method for evaluating potential photocatalytic sunscreen activity has been described by [37] and a similar protocol was employed here.

Flat panels (250mm × 150mm) of pre-painted steel product were obtained from a production run of a coil-coating paintline in Port Kembla NSW. The steel panels were painted with five different sunscreen formulations: three prepared emulsions (see section 3.4.2) and two commercial sunscreens (Nivea® (SPF 50) and Nivea® anti-age (SPF 30)) were tested.

The sunscreen formulations were applied to the steel panels using a #10 drawdown bar, which gives a uniform applied wet-film thickness of ~13µm. Typically, 3 g
of the emulsion was placed halfway along the length of the test panel and was drawn down, thus covering half the panel. The top half of the panel remained uncovered and was used as a negative control for comparison. The panels were then mounted lengthwise on an open, fence-type, exterior exposure rack facing north at 45°, at a latitude of ~34.5°S (Wollongong, NSW) for a total of twelve weeks, as shown in Figure 3.16. The exposure time of twelve weeks was chosen since significant differentiation after this particular time can be expected if photocatalytic components are present in the formulation under test [37].

![Figure 3.16. Sun exposure of steel panels painted with different sunscreen formulations. (a) Front view of the exposure rack. (b) View of the exposure rack facing north at 45°.](image)

### 3.4.5.1 Gloss Readings of the Test Panels

A 30 mm wide test strip was cut of each panel after six and twelve weeks of sun exposure, and prepared for gloss readings. Firstly, the cut strips were cleaned, particularly to reduce any residues of the weathered emulsions. A soft brush was used to clean the lower parts of the strips under running water with a temperature of 40°C. Then, a solution of diluted detergent was placed on a soft paper towel, which was carefully used to rub the surface of the strips. After 1 min of rubbing, the strip was rinsed under running water (40°C), and then dried at room temperature. With the help of a 60° gloss meter (Byk-Gardner), the gloss of each sample was determined.
3.4.5.2 Scanning Electron Microscopy Imaging of the Test Panels

Changes in the surface roughness were monitored over time using SEM. A 4.5 cm × 2.0 cm area was cut from each panel after twelve weeks exposure and cleaned of debris under warm running water with a fine hair paint brush. The test strip was then coated with a thin layer of platinum, and the surface roughness was examined using a JEOL JSM-7001F scanning electron microscope. SEM images were acquired at an accelerating voltage of 5 kV.

3.4.6 Rheological Properties

Rheological properties were determined for TiO$_2$/ZnO and TiO$_2$/Bi(OH)$_3$ emulsions and two sunscreen formulations commercially available in Australia: OMBRA® (SPF 50) is a viscous cream dispensed from a bottle, while Auscreen® (SPF 50) is applied through a spray dispenser. These two commercial products were chosen in order to compare their viscosity and ease of topical application to those of the prepared emulsions.

For a typical measurement, a small amount of emulsion was placed on the base plate of a Physica MCR 301 (Anton Paar, Graz, Austria) rheometer, and the viscosity was measured at room temperature with a test gap of 0.3 mm over a range of rotational shear rates, which were increased from 0.01 to 1000 s$^{-1}$. The viscosity was recorded every 5 s for a total of 50 measurement points. The obtained rheograms were analyzed, and the flow index $n_{flow}$ was calculated using Equation (3.14) [39]:

$$\tau = K \cdot r^{n_{flow}}$$

(3.14)

where $\tau$ is the shear stress (dyne ⋅ cm$^{-2}$), $r$ is the shear rate (s$^{-1}$), and $K$ is the consistency index.

3.4.7 Photoprotection Efficiency

In-vitro SPF and UVA protection factor (UVA-PF) measurements of the prepared emulsions were performed using a Labsphere UV2000S in accordance with ISO
24,443:2012, in order to evaluate the photoprotection efficiency in the UVA and UVB ranges.

30 mg of emulsion was placed on the surface of a standard poly(methyl methacrylate) (PMMA) plate (Europlast, Aubervilliers, France) and spread homogeneously across its surface by finger. After spreading, 15 ± 0.5 mg of emulsion remained on the PMMA plate, which was left to dry under ambient conditions. Four plates were prepared for each emulsion and the transmission was measured between 290 and 400 nm at six different locations on the PMMA plate. The SPF was calculated using Equation (3.15) [40]:

$$\text{SPF} = \frac{\int_{290}^{400} E_{\lambda} S_{\lambda} \, d\lambda}{\int_{290}^{400} E_{\lambda} T_{\lambda} \, d\lambda}$$  \hspace{1cm} (3.15)

where $E_{\lambda}$ is the erythemal spectral effectiveness (W ∙ m$^{-2}$), $S_{\lambda}$ is the solar spectral irradiance (W ∙ m$^{-2}$ ∙ nm$^{-1}$), and $T_{\lambda}$ is the spectral transmittance of the emulsion.

The UVA-PF was also calculated from the obtained measurements, according to Equation (3.16) [40]:

$$\text{UVA-PF} = \frac{\int_{320}^{400} E_{\lambda} S_{\lambda} \, d\lambda}{\int_{320}^{400} E_{\lambda} S_{\lambda} T_{\lambda} \, d\lambda}$$  \hspace{1cm} (3.16)

The critical wavelength is typically used to evaluate the efficiency of protection in the UVA region and was determined by using the first value of wavelength for which the following ratio, $R$, in Equation (3.17) is ≥ 0.9 [41]:

$$R = \frac{\int_{290}^{\lambda} A_{\lambda} \, d\lambda}{\int_{290}^{400} A_{\lambda} \, d\lambda}$$  \hspace{1cm} (3.17)

where $A_{\lambda}$ is the absorbance.
3.5 In Vitro Cell Culture Methods

3.5.1 Materials

Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Dulbecco’s Phosphate Buffered Saline (DPBS, with/without Ca\(^{2+}\) and Mg\(^{2+}\)), PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)), and trypsin ethylenediaminetetraacetic acid (trypsin–EDTA, 0.05%) were purchased from Life Technologies. DMEM was supplemented with phenol red, glucose (4.5 g/L), and L-glutamine. Dimethyl sulfoxide (DMSO, ≥99.9%), Trypan blue solution (0.4%), and denaturated ethanol were purchased from Sigma-Aldrich.

All in vitro cell culture methods were conducted inside a Euroclone – Safemate 1.2 ABC Class II biological safety cabinet.

3.5.2 Cell Lines

Four different cell lines were available for the investigation of biological activity of selected nanomaterials: two normal cell lines, and two malignant cell lines. The cells were purchased from the European Collection of Cell Cultures (ECACC) and the American Type Culture Collection (ATCC).

The four cell lines allow the use of the same growth medium, which results in a better comparison of biological interactions of the nanomaterials with different cell types, reducing side effects that may arise due to the use of different medium formulations.

3.5.2.1 Human HaCaT Skin Cells

HaCaT human skin cells are transformed keratinocytes derived from histologically normal adult skin [42]. The skin cells were used to investigate the biocompatibility of the materials for sunscreen applications. Information on HaCaT cells is displayed in Table 3.1, and the cell morphology is shown in Figure 3.17.
### Table 3.1. Information on HaCaT human skin cells [42].

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Skin</td>
</tr>
<tr>
<td>Disease</td>
<td>–</td>
</tr>
<tr>
<td>Growth mode</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>95% (v/v) growth medium + 5% (v/v) DMSO</td>
</tr>
</tbody>
</table>

*Abbreviation: dimethyl sulfoxide (DMSO)*

![Microscopic image of normal HaCaT human skin cells.](image)

3.5.2.2 Madin-Darby Canine Kidney (MDCK) Cells

As a second non-malignant cell line, MDCK cells were used, which were derived from an adult female cocker spaniel [42]. Information on MDCK cells is displayed in Table 3.2, and the cell morphology is shown in Figure 3.18.

### Table 3.2. Information on Madin-Darby canine kidney cells [42].

<table>
<thead>
<tr>
<th>Species</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Kidney</td>
</tr>
<tr>
<td>Disease</td>
<td>–</td>
</tr>
<tr>
<td>Growth mode</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Epithelial</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>90% (v/v) growth medium + 10% (v/v) DMSO</td>
</tr>
</tbody>
</table>

*Abbreviation: dimethyl sulfoxide (DMSO)*

![Microscopic image of normal Madin-Darby canine kidney cells.](image)

3.5.2.3 9L Rat Brain Tumour Cells

To study the potential selectivity of the tested bioactive NP systems, the nanomaterials were tested on 9L rat brain tumour cells, which were derived from an N-
nitrosomethylurea-induced tumour and are known to exhibit extremely high resistance to both chemotherapy and radiotherapy [42, 43]. Information on 9L cells is displayed in Table 3.3, and the cell morphology is shown in Figure 3.19.

**Table 3.3.** Information on 9L rat brain tumour cells [42].

<table>
<thead>
<tr>
<th>Species</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Brain</td>
</tr>
<tr>
<td>Disease</td>
<td>Glioma</td>
</tr>
<tr>
<td>Growth mode</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>90% (v/v) growth medium + 10% (v/v) DMSO</td>
</tr>
</tbody>
</table>

*Abbreviation: dimethyl sulfoxide (DMSO)*

**3.5.2.4 Human MCF-7 Breast Cancer Cells**

MCF-7 cells are radiosensitive breast adenocarcinoma cells, which were established from a 69-year old female [42]. Information on MCF-7 cells is displayed in Table 3.4, and the cell morphology is shown in Figure 3.20.

**Table 3.4.** Information on MCF-7 human breast cancer cells [42].

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Breast</td>
</tr>
<tr>
<td>Disease</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Growth mode</td>
<td>Adherent</td>
</tr>
<tr>
<td>Morphology</td>
<td>Epithelial</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>95% (v/v) growth medium + 5% (v/v) DMSO</td>
</tr>
</tbody>
</table>

*Abbreviation: dimethyl sulfoxide (DMSO)*

![200 μm](image1)

**Figure 3.19.** Microscopic image of 9L rat brain tumour cells.

![200 μm](image2)

**Figure 3.20.** Microscopic image of MCF-7 human breast cancer cells.
3.5.3 Subculture of Cells

The cells were routinely maintained in the exponential growth phase in T75 cm$^2$ cell culture flasks with a vented screw cap (Greiner Bio-one) containing DMEM with phenol red, L-glutamine, and glucose, supplemented with FBS (10% (v/v)) and penicillin/streptomycin (1% (v/v)). All cell cultures were maintained at 37°C and 5% (v/v) CO$_2$ in a cell culture incubator (Heracell 150i). The cell concentration ranged between 2 – 4 × 10$^4$ cells/cm$^2$, and the cells were passaged after confluence was reached for up to 30 passages.

During a subculture, the growth medium was discarded and the monolayer of cells was washed twice with 10 mL of pre-warmed DPBS (without Ca$^{2+}$ and Mg$^{2+}$). Then, 0.05% trypsin-EDTA was added and the cells were incubated for 5 – 10 min at 37°C and 5% (v/v) CO$_2$. After detachment, the trypsin-EDTA was neutralized with pre-warmed complete growth medium, and an aliquot was taken for a cell count. The desired volume of cell suspension was then transferred into a new T75 cm$^2$ flask containing 25 mL of fresh growth medium. All maintained cell cultures were routinely tested for mycoplasma contamination.

3.5.4 Cell Counting

The concentration of cells in suspension was determined using a Neubauer haemocytometer. Typically, an equal volume of cell suspension was mixed with a solution of 0.4% Trypan blue. Then, 10 µL of the mixture was placed inside the counting chamber and the amount of viable cells was determined (Figure 3.21). Trypan blue is a dye that can enter dead cells and stains them blue, while viable cells are impermeable and remain clear. The cell concentration was calculated by only counting the viable cells.
3.5.5 Cryopreservation of Cells

The cryopreservation of cells includes the freezing and thawing of cells, as further described below.

3.5.5.1 Freezing of Cells

Prior to freezing, 4 – 6 T75 cm² flasks were seeded for confluence on the day of freezing, and their growth medium was replaced 24 h before freezing.

On the day of freezing, the cells, which were in the exponential growth phase, were washed and trypsinized, as described for the standard subculture. After successful cell detachment, pre-warmed growth medium was added and the cell concentration was determined. Meanwhile, the cell suspension was placed on ice, and the cells were centrifuged at 4°C (Eppendorf, 5 min, 300 – 469 g) to obtain the cell pellet. The supernatant was discarded and the cell pellet was resuspended in ice-cold freezing medium (see section 3.5.2) to reach a concentration of $2 - 4 \times 10^6$ cells/mL. The freezing suspension was then transferred into 2 mL Nalgene® cryogenic vials and placed in a Nalgene® Mr. Frosty freezing container with 250 mL of isopropanol, allowing the cells to freeze at a cooling rate of 1 – 3°C/min once they were placed in a freezer (-80°C).
On the next day, the frozen vials were transferred to a permanent gas phase liquid nitrogen storage vessel. These vials were denoted as the 'master stock'. To confirm successful freezing of the cells, a frozen vial of the 'master stock' was taken after 24 h storage inside the liquid nitrogen vessel and thawed. Once viability was confirmed, the thawed cells represented the 'working stock' and were maintained for 30 passages.

3.5.5.2 Thawing of Cells

First, the frozen cryogenic vial was removed from the liquid nitrogen storage vessel and placed in a 50 mL tube containing pre-warmed 70% ethanol. The tube was then placed in a water bath at 37°C, where it was left just until the cell suspension was thawed. The cells were transferred into a new T75 cm² flask, and pre-warmed, fresh growth medium was added dropwise to equilibrate the cells to the medium. The cell suspension was gently mixed and an aliquot was taken for a cell count. The flask was maintained at 37°C and 5% (v/v) CO₂.

On the next day, after the cells had attached to the flask and expelled the DMSO, the old growth medium was replaced with fresh pre-warmed growth medium. The cell growth was monitored until a confluence of 80 – 90% was reached, and the cells were passaged.

3.6 In Vitro Biological Characterization

3.6.1 Materials

Hoechst 33342 nucleic acid stain (≥ 98%), propidium iodide (PI, ≥ 94%), methotrexate hydrate (≥98%), crystal violet solution (2.3% crystal violet, 0.1% ammonium oxalate, 20% ethyl alcohol), 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA, ≥ 97%), PBS sachets (pH 7.4), ribonuclease A (RNase A), Trypan blue solution (0.4%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 98%), sodium bicarbonate (≥ 99.7%), acetic acid (≥ 99.7%), sodium hydroxide (NaOH, 99.99%), methanol (≥ 99.8%), sodium acetate (≥ 99.0%), sodium carbonate decahydrate (≥ 99.99%), DMSO (≥99.9%),
and denatured ethanol were purchased from Sigma-Aldrich. DMEM, FBS, penicillin,
streptomycin, DPBS (with/without Ca\(^{2+}\) and Mg\(^{2+}\)), PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)), and
trypsin–EDTA, 0.05% were purchased from Life Technologies. DMEM was supplemented
with phenol red, glucose (4.5 g/L), and L-glutamine.

TiO\(_2\) (Aeroxide\(^{®}\), P25) and ZnO NPs (≤ 100 nm) were obtained from Sigma-Aldrich
and used without further purification. Bi(OH)\(_3\), α-Bi\(_2\)O\(_3\), and TiO\(_2\)/(BiO)\(_2\)CO\(_3\)
nanocomposites were synthesized (see section 3.1.2, 3.1.3, and 3.1.4).

**Stock solutions**

Hoechst 33342 (10 mg/mL) and RNAse A (1 mg/mL) were dissolved in DI water;
PI (1 mg/mL) was dissolved in PBS; DCFH\(_2\)-DA (125 µg/mL) was dissolved in denatured
ethanol. MTT (5 mg/mL) was dissolved in PBS. MTX was dissolved in bicarbonate-
carbonate buffer.

**3.6.2 Statistical Analysis**

Data are presented as means ± standard deviation (SD). Significant differences
between the treatments and the control were evaluated using the one-way analysis of
variances (ANOVA) and Tukey's multiple comparison test. With P being the probability, a
value of P < 0.05 was considered statistically significant.

**3.6.3 Preparation of Nanoparticle Suspensions**

All nanomaterials that were used for *in vitro* biological characterization, were
firstly autoclaved (Getinge, H5 5510 EC1) at 121°C and 15 psi, and then suspended in PBS
(without Ca\(^{2+}\) and Mg\(^{2+}\)) at a concentration of 1 mg/mL. The suspensions were sonicated
for 2 h using a sonication bath (Branson 3800, Ultrasonics Corp) and added to the growth
medium to yield concentrations of 6.25 – 500 µg/mL.
3.6.4 Live Cell Imaging

Live cells were imaged using confocal microscopy and the InuCyte ZOOM system. While the former one allows high resolution imaging and can give information about the cellular uptake of NPs, the latter method is used to observe cell growth over time. Furthermore, both methods allow the use of staining agents such as PI to identify apoptotic cells [45].

3.6.4.1 Confocal Microscopy

Confocal microscopy is widely used for biological specimens and materials science, as it offers control of the depth of field, reduced background noise, and the ability to obtain three-dimensional images. The specimen preparation is similar to that for conventional fluorescence microscopes and allows imaging of both fixed and living cells and tissues with multiple thicknesses.

In a traditional wide-field fluorescence microscope, an incoherent mercury or xenon lamp illuminates the entire specimen, which leads to the excitation of different optical paths at the same time. The photodetector then detects a large part of the unfocused background noise, reducing the overall optical resolution, especially in thicker specimens (> 2 µm). In contrast, the confocal microscope (Figure 3.22) uses point illumination from a laser, which is reflected by a dichroic mirror, and a spatial pinhole that blocks out-of-focus light in the image formation process [46, 47]. By using this setup, the specimen is excited by the light source at one depth at a time, and only photons that are close to the focal plane are detected, which ultimately results in increased optical resolution [48].
Figures 3.22. Working principle of a confocal microscope, adapted from [49].

Live cell images of 9L, MCF-7, and MDCK cells were collected with the confocal microscope after exposure to the NPs using a Leica – TCS SP5 Advanced System with Live Cell Imaging (CO₂ chamber).

3.6.4.2 Staining with Propidium Iodide

PI is a fluorescent dye that is widely used in fluorescence microscopy, confocal microscopy, flow cytometry, and fluorometry [45].

PI is membrane-impermeant and reaches the nucleus either by permeabilization of the cells, or by passing through disordered areas of cell membranes, as shown in Figure 3.23 [50]. Once PI gains access to nucleic acids and intercalates between the base pairs of the double stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), its fluorescence is enhanced 20- to 30-fold, which is used to identify dead cells in a cell population [45, 51].
Figure 3.23. Working principle of cell staining with propidium iodide.

9L, MCF-7, and MDCK cells were seeded on Nunc\textsuperscript{TM} Lab-Tek\textsuperscript{TM} Chambered Coverglass, and Bi(OH)\textsubscript{3} and α-Bi\textsubscript{2}O\textsubscript{3} NPs were added at a concentration of 50 μg/mL. The cells were imaged every 2 – 4 h using confocal microscopy (see section 3.6.4.1) to investigate when cellular uptake of the NPs begins. The cell viability was determined after a total exposure time of 24 h was reached by the addition of 10 μM PI 5 min prior to imaging. The emission images were recorded using Leica LAS X software.

3.6.4.3 IncuCyte ZOOM System

The IncuCyte Zoom system offers real-time and non-invasive imaging of live cells and can be used to monitor cell growth and workflow, and perform live cell assays [52]. Typically, the cells are seeded and maintained either in microplates, or T25 cm\textsuperscript{2} or T75 cm\textsuperscript{2} flasks and placed in the IncuCyte system inside a cell culture incubator at 37°C and 5% (v/v) CO\textsubscript{2}. Different objectives are installed underneath the cells, allowing the capture of bright-field and fluorescence images in a chosen time interval.
Chapter 3 – Materials and Methodology

The cell growth was observed for all cell lines using an Essen Bioscience – IncuCyte ZOOM system with three different approaches: imaging of the separate cultures, the exchange of growth medium, and co-culturing.

**Separate cell cultures**

9L, MCF-7, MDCK, and HaCaT cells were seeded in T25 cm² flasks (Falcon) allowing for attachment, and suspensions of Bi(OH)₃, α-Bi₂O₃, TiO₂, and TiO₂/(BiO)₂CO₃ were added at a concentration of 50 µg/mL. Upon NP addition, the flasks were instantly placed inside the IncuCyte system, and live cell images were obtained every 4 h for a total of up to 48 h.

**Growth medium exchange**

9L cells were first seeded on T25 cm² flasks and treated with the different NPs for 24 h. Then, the growth medium was removed from the cell culture, centrifuged at 469 g at room temperature for 5 min, and added to healthy cultures of 9L and MDCK cells, whose growth medium had been removed in advance. The cell viability was imaged for 24 h.

**Co-culture**

For the co-culture of 9L and MDCK cells, 9L cells were seeded on T25 cm² flasks 24 h before the MDCK cells were seeded in the same flask. After another 24 h, the NPs were added to the culture flask, and the cell viability was imaged for 24 h.

**3.6.5 Flow Cytometry**

Flow cytometry measures the physical and biochemical characteristics of cells or particles in suspension and is used for cell counting and cell sorting [53, 54]. Furthermore, the technique allows the detection of proteins or other biomolecules that bind to specific cell compartments, such as DNA. With the obtained results, information can be gained regarding cell cycle kinetics and DNA damage (cell cycle population) [55].

Typically, a flow cytometer consists of three systems: fluidics, optics, and electronic systems. The sheath fluid (= fluidics system) separates the cells, so that only one cell at a
time reaches the laser (= optics system), where two observations can be made: first, if the cells or particles were stained prior to their introduction into the flow cytometer, the emission of fluorescence can be detected [56, 57] (Figure 3.24). The second detection is the forward angle scatter (FSC) and side angle scatter (SSC) of light when it hits the cell [54]. The electronic system converts the detected signals into digital data.

![Schematic illustration of a flow cytometer. Adapted from [58].](image)

**Figure 3.24.** Schematic illustration of a flow cytometer. Adapted from [58].

The internalization of NPs and the cell cycle population were investigated using a Becton Dickinson fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR II; BD Biosciences, USA).

### 3.6.5.1 Detection of Forward and Side Scatter

When a cell or particle is hit by a focused laser beam, the laser light is scattered in all directions, as shown in Figure 3.25. While the intensity of the FSC light is proportional to the size of the cell and does not change with the degree of internalisation, the SSC light is proportional to the cell granularity and can be used to examine the cellular uptake [59].
Figure 3.25. Forward and side scatter of radiation hitting a single cell.

9L and MDCK cells were seeded on T12.5 cm² flasks (Falcon), and suspensions of Bi(OH)₃ and α-Bi₂O₃ NPs were added at concentrations of 0, 6.25, 12.5, 25, and 50 µg/mL. After 24 h of NP exposure, the cells were trypsinized, and the cell concentration was determined. Around $2 \times 10^6$ cells were centrifuged at 300 – 469 g at room temperature for 5 min, and the obtained cell pellet was washed twice with PBS (without Ca²⁺ and Mg²⁺). The cell pellet was resuspended in 500 µL PBS, transferred into 5 mL polystyrene flow tubes, and analyzed with a flow rate of 60 mL/min until 10,000 events were recorded. The degree of internalization of the NPs was determined with FACSDiva software, where cell doublets and aggregates were gated out.

### 3.6.5.2 Cell Cycle Population

The cell cycle population was determined via an assay utilizing cell staining with the fluorescent dye PI, as further described in section 3.6.4.2. The assay generates a cell population histogram in respect to the fluorescent intensity of PI, which is correlated with the amount of DNA within each cell: cells that are in the $G_0/G_1$ phase will have half the DNA of $G_2$ cells or cells in the mitosis stage [60].

In Figure 3.26, the $G_0/G_1$ phase is indicated as diploid (2N, with N being the haploid complement of chromosomes), while the $G_2/M$ phase has a tetraploid (4N) DNA content [60]. Since the cells in the S-phase are replicating, they will have events that are in between
the G₁ and the G₂/M phase [60]. The sub G₁ level indicates apoptotic cells, since DNA exits cells after the membrane is made permeable, leading to a decrease in the intracellular content [61].

![Cell Cycle Diagram](image)

**Figure 3.26.** Schematic illustration of the cell cycle, showing the flow cytometric components of each phase. Adapted from [60].

The cell cycle population was investigated using a method described in [62]. 9L and MDCK cells were seeded on T12.5 cm² flasks and suspensions of Bi(OH)₃ and α-Bi₂O₃ NPs were added at a concentration of 50 µg/mL. After 24 h of NP exposure, the cells were trypsinized, and the cell concentration was determined. Around $2 \times 10^6$ cells were centrifuged at 300 – 469 g and 4°C for 5 min and washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺). The cells were fixed with dropwise addition of 1 mL ice-cold 70% ethanol and stored at -20°C. After 48 h, the cell suspension was centrifuged and washed twice with ice-cold PBS. The cell pellet was resuspended in 200 µL of ice-cold PI solution (8 µL of PI stock solution, 20 µL of RNAse stock solution, and 172 µL of PBS) and transferred into 5 mL polystyrene flow tubes. The flow tubes were incubated for 60 min at 37°C and 5% (v/v) CO₂ and the stained cells were analyzed using a flow rate of 60 mL/min until 10,000 events were recorded. The stained nuclei were analyzed for DNA-PI fluorescence, and the
population of cells in all phases of the cell cycle (G₀/G₁, S, and G₂/M) was determined with FACSDiva software.

### 3.6.6 Cell Survival and Proliferation

Cell survival and proliferation was investigated using the clonogenic assay and the MTT assay. The clonogenic assay is widely used to evaluate cell survival and proliferation, since the results have a certain degree of correlation with the clinical response [63]. This assay is associated with a slow turn-around time, however, and low efficiency, leading to investigations to find faster and simpler methods to assess drug sensitivity.

The MTT assay is an alternative, which is fast, easy to handle, and widely used in cell biology [64]. The drug sensitivity was evaluated in five human lung cancer cell lines using both methods, and it was shown that the correlation coefficient between the results obtained through the clonogenic assay and MTT assay depend on the tested treatment [63]. While the correlation coefficient \( r_{\text{correlation}} \) was high for platinum analogues (\( r_{\text{correlation}} = 0.939 \)), the correlation decreased for anthracyclines/anthracenedione (\( r_{\text{correlation}} = 0.611 \)) [63].

In this doctoral thesis, clonogenic assays were used to evaluate cell survival of 9L, MCF-7, and MDCK cells when exposed to Bi(OH)₃ and α-Bi₂O₃ NPs. The MTT assay was used to investigate the biocompatibility of TiO₂, TiO₂/(BiO)₂CO₃, ZnO, and Bi(OH)₃ NPs for potential sunscreen application in normal HaCaT and MDCK cells. Furthermore, the MTT assay was utilized in combination with irradiation by simulated sunlight.

#### 3.6.6.1 Clonogenic Survival Assay

The clonogenic survival assay is an *in vitro* method and based on the ability of a single cell to form a colony, which is made up of at least 50 cells. Also referred to as the colony formation assay, this assay is regarded as the gold standard sensitivity assay, which evaluates every single cell and its capability to theoretically go through unlimited cell
division [65, 66]. It is the method of choice for investigation of the effects of drugs, cytotoxic agents, or ionizing radiation on the survival and proliferation of cells [65, 67].

Typically, the clonogenic assay consists of four steps, including the seeding of cells, their exposure to drugs, cytotoxic agents, or ionizing radiation (for the control = no exposure), and the incubation of plated cells for fifteen doubling times to allow the growth of colonies, which, in the last step, are fixed, stained, and counted under a microscope (Figure 3.27).

The number of cell colonies is used to determine the surviving fraction (SF in %) of the cells, which is the ratio of the plating efficiency of the treatment ($PE_T$) and the plating efficiency of the control ($PE_C$), as shown in Equation (3.18) [68]:

$$SF = \frac{PE_T}{PE_C}$$
Chapter 3 – Materials and Methodology

\[ SF = \frac{PE_t}{PE_c} \times 100 \]  

(3.18)

The plating efficiency (PE) is the ratio of counted cell colonies \( (N_{\text{colonies}}) \) to the initial seeding number \( (S_{\text{seeding}}) \), as displayed in Equation (3.19):

\[ PE = \frac{N_{\text{colonies}}}{S_{\text{seeding}}} \]  

(3.19)

**Determination of optimum seeding density**

To determine the optimum seeding density of cells that yields approximately 100 cell colonies after the incubation for fifteen doubling times, a cloning efficiency test was performed. Firstly, 9L, MCF-7, and MDCK cells were seeded into T12.5 cm\(^2\) flasks (Falcon). At a confluence of 70 – 90\%, the growth medium was discarded, and the cells were gently washed with DPBS (without Ca\(^{2+}\) and Mg\(^{2+}\)). The cells were detached using trypsin-EDTA, the cell concentration was determined, and the cells were plated in triplicate into 100-mm tissue culture dishes (BD Falcon), which were filled with 10 mL complete growth medium. In total, eight densities of cells were chosen for each cell line ranging from 200 to 10,000 cells/plate. The cells were incubated for fifteen doubling times at 37\( ^\circ \)C and 5% (v/v) CO\(_2\) (Heracell 150i). Then, the tissue culture dishes were washed with DPBS (with Ca\(^{2+}\) and Mg\(^{2+}\)) and stained with a solution of 25% crystal violet and 75% ethanol. Cell colonies were counted by optical microscopy, provided that they contained at least fifty healthy cells.

**Treatment with NPs**

As described above, 9L and MDCK cells were seeded on T12.5 cm\(^2\) flasks, and suspensions of Bi(OH)\(_3\) and \( \alpha \)-Bi\(_2\)O\(_3\) NPs were added at concentrations of 0, 6.25, 12.5, 25, and 50 µg/mL. After 24 h of NP exposure, the growth medium was discarded, and the cells were washed and trypsinized. The cells were then seeded at low densities (as determined above) into tissue culture dishes and incubated for fifteen doubling times. For each treatment, at least three different cell densities were seeded in triplicate. Then, the cells were fixed and stained, the cell colonies were counted, and the SF was determined.
3.6.6.2 MTT Proliferation Assay in Absence of Simulated Sunlight

The MTT proliferation assay is a quantitative calorimetric method based on the reduction of water-soluble yellow tetrazolium salt to water-insoluble purple formazan, which takes place in mitochondria of viable cells, as shown in Figure 3.28 [69].

![Diagram of MTT Proliferation Assay](image)

**Figure 3.28.** Working principle of the MTT proliferation assay.

The reduction of MTT to formazan depends on mitochondrial oxidoreductase enzymes. Since the metabolic activity of dying cells decreases, the amount of produced formazan is directly proportional to the number of viable cells and can therefore be used to determine cell viability [69]. The water-insoluble formazan crystals are typically dissolved using DMSO, an acidified ethanol solution, or a solution of sodium dodecyl sulfate in diluted hydrochloric acid [69, 70]. The absorbance of the light-sensitive purple solution can then be determined at a wavelength between 500 – 600 nm, as schematically shown in Figure 3.29 [70].
Determination of optimum seeding density

To determine the optimum seeding density of cells that yields an absorbance of approximately 1.0 after the incubation with MTT dye, a calibration curve was constructed based on different seeding densities of HaCaT and MDCK cells. Firstly, the cells were seeded in a 96-well plate and incubated at 37°C and 5% (v/v) CO₂. Before the addition of MTT, the culture medium was removed from each well and replaced with 100 µL of new growth medium and 10 µL of MTT stock solution. The plate was incubated for 4 h at 37°C and 5% (v/v) CO₂ until a purple-coloured formazan product was formed. All but 25 µL of the test solution was removed, and the formazan crystals were dissolved in 100 µL of DMSO. After incubation at 37°C for 10 min, the well plate was centrifuged at 300 g at room temperature for 5 min. Then, 100 µL of supernatant was transferred to a new well in a 96-
well plate, and the absorbance was measured at a wavelength of 540 nm on a SpectraMax 384 Plaus (Molecular Devices) microplate.

**Treatment with NPs**

HaCaT and MDCK cells were seeded with an optimized seeding density (as determined above) in 96-well plates and incubated at 37°C and 5% (v/v) CO₂. Suspensions of TiO₂, TiO₂/(BiO)₂CO₃, ZnO, and Bi(OH)₃ NPs were added to the cells to yield concentrations of 0, 5, 10, 25, 50, 100, 250, and 500 µg/mL and the cells were incubated for 24 h. As described above, at the end of the exposure, the culture medium was removed, replaced with fresh medium, and supplemented with MTT. After 4 h of incubation, the formazan was dissolved in DMSO. The well plate was incubated for 10 min, centrifuged (10 min, 1875 g, room temperature), and the absorbance of the supernatant was measured at 540 nm. The cell viability (in %) is the ratio of absorbance of the treatment (Aₜ) to the absorbance of the control (Aᵦ), as shown in Equation (3.20):

\[
\text{Cell viability} = \frac{Aₜ}{Aᵦ} \times 100
\]

(3.20)

**3.6.6.3 MTT Proliferation Assay under Exposure to Simulated Sunlight**

MTT tests were performed with HaCaT cells, which, besides the treatment with NPs, were irradiated with simulated sunlight. This experimental setup allows investigation of the effects of photocatalytically active nanomaterials on cell viability.

**Degradation of crystal violet**

To ensure that the nanomaterials developed sufficient photocatalytic activity, TiO₂ NPs were suspended in DPBS (with Ca²⁺ and Mg²⁺, 1 mg/mL), sonicated for 2 h, and added to a solution of crystal violet in DPBS in a 96-well plate at concentrations of 25, 50, and 100 µg/mL. After a dark adsorption of 60 min, the absorbance was measured at 590 nm after removal of the photocatalyst. The TiO₂ NPs were then resuspended in the crystal violet solution, and the 96-well plate was then placed (without the lid) on an ice block.
underneath the light source (300 W Sunlamp, Ultra-Vitalux®, OSRAM), which was pre-run for 30 min to ensure stabilization of the output. The light emission profile of the lamp is shown in Figure 3.30. The light intensity was measured using a calibrated UVA/B light meter (model 850009, Sper Scientific) and was found to be 6 mW/cm² at the surface of the wells. The 96-well plate was irradiated for 15 min, which equals a dose of 5.4 J/cm². Afterwards, the photocatalyst was removed via centrifugation (5 min, 1875 g, room temperature), the absorbance of the crystal violet solution was measured, and the amount of degraded crystal violet was determined.

Figure 3.30. Light emission profile of the OSRAM Ultra-Vitalux® 300 W Sunlamp [71].

Exposure of HaCaT cells to simulated sunlight

HaCaT cells were seeded in 96-well plates and the growth medium was replaced with 100 µL of DPBS prior to the addition of the NP suspensions. Suspensions of TiO₂ and TiO₂/(BiO)₂CO₃ NPs were added to the cells at concentrations of 25, 50, and 100 µg/mL. This concentration range was chosen to ensure the observation of detectable photocatalytic effects while keeping the toxicity related to the nanomaterials to a minimum. The control cells were treated with the equivalent amount of PBS. The cells were incubated with the NPs for 45 min at 37°C and 5% (v/v) CO₂. The culture plate lid was then removed, and the 96-well plate was placed on an ice block underneath the light source. The cells were irradiated under the same conditions as described above (for 15 min with simulated sunlight at a light intensity of 6 mW/cm², which equals a dose of
5.4 J/cm²). After irradiation, DPBS was replaced with 100 µL of supplemented growth medium, and the 96-well plate was incubated for 24 h before fresh medium and MTT dye were added and the absorbance was measured, as described in section 3.6.6.2.

### 3.6.7 Detection of Intracellular Reactive Oxygen Species

Apoptotic cells are characterized by a change in nuclear morphology, which is identifiable by fluorescence microscopy after DNA staining. While the budding type is associated with an ROS-independent pathway of apoptosis, the cleavage type indicates a ROS-dependent apoptotic pathway [72]. In addition, intracellular ROS can be detected directly using dyes that show fluorescence changes in presence of ROS [72].

In this thesis, 9L and MDCK cells were double-stained using Hoechst 33342 and DCFH₂-DA. The staining procedure is described in section 3.6.7.1, while further information on the dyes is provided in sections 3.6.7.2 and 3.6.7.3.

#### 3.6.7.1 Fluorescence Microscopy

Fluorescence microscopy is widely used to not only observe fluorescence in biological specimens, but also to image live cells and obtain morphological and structural information. Although some biological structures, such as chlorophyll, have primary fluorescence, the majority of biological specimens need to be linked with fluorescent molecules (fluorochromes) to generate fluorescence [73].

Typically, the fluorescence microscope has emission and excitation filters, which are able to separate the excitation and emission wavelengths, as shown in Figure 3.31 [73]. Since the objective acts as both a condenser lens (excitation light) and an objective lens (emission light), a dichroic mirror is used to reflect shorter wavelengths of light and allow longer wavelengths to pass [73]. This epi-illumination type of light pathway provides a dark background, which in turn increases the visualization of fluorescence. The light source is typically a mercury or xenon arc lamp [73].
First, 9L and MDCK cells were seeded on a Nunc™ Lab-Tek™ Chambered Coverglass. Before the addition of Bi(OH)$_3$ or α-Bi$_2$O$_3$ NPs at a concentration of 50 μg/mL and after 2, 4, 6, 9, 12, 15, 18, 21, and 24 h of NP exposure, the cells were washed with DPBS (with Ca$^{2+}$ and Mg$^{2+}$), stained with Hoechst stain (16 μM), and treated with DCFH$_2$-DA (10 μM). Emission images were obtained using a Leica – DMi8 fluorescence microscope with attached environmental chamber (37°C, 5% (v/v) CO$_2$) and Leica LAS X software, as shown in Figure 3.31. A minimum of 300 cells was counted in at least four randomly selected microscopic fields, and the fraction of apoptotic nuclei with increased levels of green fluorescence was determined [72, 75].

3.6.7.2 Staining with Hoechst 33342

Hoechst 33342 can be used for either fixed or live cell fluorescent staining of DNA. Before it is applied to the biological specimen, the dye is yellow in colour, with excitation at a wavelength of 350 nm [51]. Once in contact with cells, the cell-membrane-permeable Hoechst 33342 binds to double-stranded DNA, preferably into the minor groove of adenine-thymine regions, and emits blue fluorescence at a wavelength of 460 – 490 nm, as shown in Figure 3.32 [51].
3.6.7.3 Staining with 2',7'-Dichlorodihydrofluorescein Diacetate

DCFH$_2$-DA is typically used to identify intermediates of ROS in neutrophils and macrophages. The dye is initially non-fluorescent and can pass through the plasma membrane, which results in a de-esterification (Figure 3.33, gray marquees, top) and the formation of 2',7'-dichlorodihydrofluorescein (DCFH$_2$) by cytosolic esterases [75]. The non-fluorescent DCFH$_2$ undergoes a two-electron oxidation (grey marquees, centre) in the presence of intracellular ROS to yield 2',7'-dichlorofluorescein (DCF), which is highly fluorescent [75]. In aqueous solution, DCF has an absorption maximum at 503 nm and an emission maximum at 523 nm [51]. The green fluorescence is typically monitored by confocal or fluorescence microscopy, or by flow cytometry [75].

Figure 3.32. Working principle of nuclear staining with Hoechst 33342 [76].
3.6.8 In Vitro Drug Loading and Release Studies

The in vitro drug loading was performed in a solution of MTX with a pH of 7.4, while the drug release was studied over the period of 72 h in release media (buffer solutions) with pH of 3.6, 5.4, 7.4, and 9.4. The same buffer solutions were used for the polymer swelling studies; their preparation is described in section 3.2.11.

3.6.8.1 In Vitro Drug Loading

Anti-cancer drugs such as MTX can be used to develop controllable drug release delivery systems, which not only increases their efficiency, but could also lower systemic
toxicity [77]. In this doctoral thesis, a nanocomposite based on $\delta$-Ta$_2$O$_5$ and PAA hydrogel was placed in a solution of MTX and allowed to swell until equilibrium was reached. This method allows the preservation of the drug, as opposed to the addition of MTX during the hydrogel synthesis, which requires temperatures $> 80^\circ$C [31, 78-80].

For each polymerization time, 200 mg of $\delta$-Ta$_2$O$_5$-PAA NPs was added to 20 mL of a solution of 10 mg/mL MTX in PBS. For the stock solution, 200 mg of MTX was dissolved in 6.67 mL of carbonate-bicarbonate buffer (pH = 9.4) and diluted with 13.33 mL of PBS. The suspension was placed on an orbital shaker (Bioline) at a speed of 30 rpm and mixed for a total of 48 h at 24°C. Then, the suspension was allowed to sediment, and the supernatant was decanted. The soaked NPs were rinsed with PBS and dried under vacuum until the weight remained constant. The loading capacity (LC) was calculated using Equation (3.21), and the entrapment efficiency (EE) was determined via Equation (3.22):

$$\text{LC} (%) = \frac{\text{Weight of entrapped drug (g)}}{\text{Weight of dry composite (g)}} \cdot 100$$ \hspace{1cm} (3.21)

$$\text{EE} (%) = \frac{\text{Weight of entrapped drug (g)}}{\text{Weight of drug used for entrapment (g)}} \cdot 100$$ \hspace{1cm} (3.22)

3.6.8.2 In Vitro Drug Release

To study the release profiles for the MTX-loaded $\delta$-Ta$_2$O$_5$-PAA NPs, 10 mg of the dry MTX-loaded NPs was added into glass vials, to which 10 mL of release medium was added. The release medium varied in pH value (pH: 3.6, 5.4, 7.4, and 9.4). The reaction vials were placed on an orbital shaker at a rotation rate of 50 rpm and a constant temperature of 37 ± 1°C. After predetermined time intervals (0, 15, 30, 45 min, and 1, 2, 6, 12, 24, and 72 h), the suspension was allowed to sediment for 2 min, and 5 mL aliquots were taken. UV-vis spectroscopy was used to determine the concentration of MTX present in the supernatant at a wavelength of 303 nm [81]. The absorbance was recorded on an UV-3600 spectrophotometer from Shimadzu over the range of 800 to 200 nm.
To determine the concentration of MTX, a calibration curve was established with MTX solutions of known concentration. The total amount of soaked MTX was estimated by centrifugation of the 72 h sample at 5208 g for 10 min (room temperature). The remaining pellet was suspended in 0.1 M NaOH and sonicated for 1 h. Then, methanol was added to the solution, which was sonicated for an additional 1 h before the absorbance was measured at 303 nm [81].

### 3.7 References


[57] Becton Dickinson Biosciences. BD LSR II user’s guide. 2007, Becton, Dickinson and Company, San Jose, United States.


CHAPTER 4 – Bismuth-Based Compounds for Biomedical Applications

This chapter is based on three published/accepted articles:


Author contributions: KB synthesized the materials, designed and performed all experiments and characterization, and collected, analysed, and interpreted all data, unless otherwise specified. DC assisted with acquiring the TEM images. KB wrote the manuscript and generated figures. MT, DC, ML, AR, SXD, HKL, and KK provided intellectual support, guidance, and helped editing the manuscript.

Author contributions: KB synthesized the materials, designed and performed all experiments and characterization, and collected, analysed, and interpreted all data, unless otherwise specified. DC helped with the preparation of sunscreen formulations and the measurements of the SPF and rheological properties. KB wrote the manuscript and generated figures. DC, MT, TB, TD, AR, SXD, HKL, and KK provided intellectual support, guidance, and helped editing the manuscript.


Author contributions: KB synthesized the materials, designed and performed all experiments and characterization, and collected, analysed, and interpreted all data. KB wrote the manuscript and generated figures. MT, ML, SXD, HKL, and KK provided intellectual support, guidance, and helped editing the manuscript.
Chapter 4 – Bismuth-Based Compounds for Biomedical Applications

4.1 Introduction

One of the major issues for the implementation and use of nanomaterials in nanomedicine is nanotoxicity. Bismuth and its compounds are considered to be the least toxic of the heavy metals. The study of toxicity of Bi (III) nitrate in human proximal tubular cells showed the biocompatibility of Bi, even at the highest tested dose of 100 mM [1].

Bismuth is reasonably cheap, available in great amounts and many of its compounds have already been used in medicines or cosmetics. Inorganic Bi salts, such as bismuth subsalicylate (Pepto-Bismol®), tripotassium dicitrato bismuthate (De-Nol®), and ranitidine bismuth citrate (Tritec®) are used for the treatment and prevention of gastric and duodenal ulcers (Figure 4.1) [2]. Bismuth oxychloride (BiOCl) is used in mineral powder in makeup, and bismuth (III) oxide (Bi₂O₃) has been used as an astringent [3, 4].

![Figure 4.1. Commercially available (a) Pepto-Bismol® and (b) De-Nol® [5, 6].](image)

Organobismuth compounds have antifungal and antimicrobial activity [7], and have shown anti-tumour effects in various human cancer cell lines [7, 8]. Heterocyclic organobismuth (III) can induce increased levels of intracellular ROS and the activation of cascades and mitochondrial perturbations [7]. Bismuth (III) dithiocarbamate complexes have been shown to be more effective than cisplatin, DOX, 5-FU, and etoposide, and in vivo studies indicate significant anticancer activity against ovarian cancer OVCAR-3 and colon carcinoma HT-29 cells [9].

Although organobismuth compounds are promising candidates for cancer therapies, they are usually structurally complex, and their synthesis involves multiple
steps [7, 8, 10]. For example, the molecular structure of PhBi(MeOx)₂ is shown in Figure 4.2. Moreover, these compounds are chemically and physically less stable compared to inorganic Bi compounds, which makes the latter ones preferable for further study [11].

![Molecular structure of PhBi(MeOx)₂](image)

**Figure 4.2.** Molecular structure of PhBi(MeOx)₂. An X-ray crystallographic study has shown that the molecule is a five coordinate monomer with distorted square pyramidal stereochemistry. Adapted from [8].

### 4.1.1 Properties of Bismuth-Based Compounds

In this doctoral thesis, Bi(OH)₃ and α-Bi₂O₃ NPs and nanocomposites based on TiO₂ and (BiO)₂CO₃ have been synthesized and characterized. The properties of these Bi-based compounds are described below.

#### 4.1.1.1 Properties of Bi(OH)₃

Bismuth (III) hydroxide (Bi(OH)₃) is also found under the name of bismuth hydrate, bismuth oxide hydrate, and bismuth oxyhydrate, and is typically a colourless powder [12-14]. Little is known regarding its structure with early claims of the compound being amorphous [13-16]. Later studies, however, suggested that crystalline forms of Bi(OH)₃ exist [13, 14, 17], the formula of which can also be written as [Bi₂O₂]²⁺(OH)₂⁻ × 2H₂O, underlining its structural relationship to the layered bismuth oxyhalides. In particular, its structure is comprised of (OH)⁻ groups that are adjusted between layers of (Bi₂O₂)²⁺ [13, 14]. Bi(OH)₃ crystallizes in the tetragonal space group P4/mmm, as observed for bismuth
oxychloride (BiOCl) [14]. Since no reference data exists for Bi(OH)_3, Figure 4.3 displays a projected ball model of BiOCl with (Bi_2O_2)_2^2- layers interleaved by slabs comprising Cl^- ions.

![Ball model of BiOCl](image)

**Figure 4.3.** Ball model of BiOCl; obtained via Materials Studio software using Crystallography Open Database file 1011175.

It has been shown that the reflections in its X-ray diffraction pattern are broadened when it is milled in a high energy ball miller for 15 min, reducing the particle size from 100 μm initially to sub-micrometre and nanosize [17].

Bi(OH)_3 NPs can be synthesized using classical precipitation, where a base such as ammonium hydroxide (Equation (4.1)) or sodium hydroxide (Equation (4.2)) is added to a solution of bismuth (III) nitrate [18-20]:

\[
\text{Bi}^{3+}_{(aq)} + 3\text{NH}_3_{(aq)} + 3\text{H}_2\text{O}_{(aq)} \rightarrow \text{Bi(OH)}_3_{(s)} + 3\text{NH}_4^+_{(aq)} \quad (4.1)
\]

\[
\text{Bi}^{3+}_{(aq)} + 3\text{OH}^-_{(aq)} \rightarrow \text{Bi(OH)}_3_{(s)} \quad (4.2)
\]

Bi(OH)_3 has also been synthesized via a hydrothermal method using bismuth (III) nitrate, calcium nitrate, and ammonium hydroxide, yielding plate-like particles with a width of approximately 10 nm and lengths on the microscale [14].

**Applications**

Bi(OH)_3 is an inorganic intermediate usually that is used to obtain α-Bi_2O_3 after annealing at 300°C [18, 19], or in compounds, such as bismuth subcarbonate ((BiO)_2CO_3)
[21] and bismuth titanate (Bi₄Ti₃O₁₂) [22]. It is also used on its own as a pigment, or in various applications such as glasses and ceramics [12, 23]. A recent study shows an attempt to synthesize nano-energetic systems based on aluminium and Bi(OH)₃ (Al-Bi(OH)₃) [17].

Furthermore, Bi(OH)₃ has been used in combination with BiOCl to form heterojunctions, which have been studied for their photocatalytic activity towards the degradation of rhodamine B and acetophenone [13, 14]. While Bi(OH)₃ and BiOCl showed negligible efficiency, their heterojunction exhibited significant photoactivity [13, 14]. The band gap of Bi(OH)₃ has been determined theoretically with a band gap of 2.81 eV [13, 14].

Bi(OH)₃ has already been used for biomedical applications, underlining its great potential. A suspension of (BiO)₂CO₃ and Bi(OH)₃ in water is typically used in milk of bismuth, a protective agent used in the treatment of gastrointestinal diseases [24].

### 4.1.1.2 Properties of Bi₂O₃

Bismuth (III) oxide can exist in six crystalline polymorphic forms, most of which are not stable at room temperature (Table 4.1).

<table>
<thead>
<tr>
<th>Polymorph</th>
<th>Stability</th>
<th>Crystal system</th>
<th>Space group</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Bi₂O₃</td>
<td>Stable</td>
<td>Monoclinic</td>
<td>P₂₁/c</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>β- Bi₂O₃</td>
<td>Metastable</td>
<td>Tetragonal</td>
<td>P̅42₁c</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>δ- Bi₂O₃</td>
<td>Stable</td>
<td>Face-centered cubic</td>
<td>Fm̅3m</td>
<td>[27]</td>
</tr>
<tr>
<td>γ- Bi₂O₃</td>
<td>Metastable</td>
<td>Body-centered cubic</td>
<td>I23</td>
<td>[28]</td>
</tr>
<tr>
<td>ε- Bi₂O₃</td>
<td>Metastable</td>
<td>Orthorhombic</td>
<td>Pbnb</td>
<td>[27]</td>
</tr>
<tr>
<td>ω- Bi₂O₃</td>
<td>Metastable</td>
<td>Triclinic</td>
<td>P1</td>
<td>[29]</td>
</tr>
</tbody>
</table>
At room temperature, only the α-Bi$_2$O$_3$ is stable, which when heated up to 727 °C phase transitions into δ-Bi$_2$O$_3$, followed by a melting of Bi$_2$O$_3$ at 825°C [30, 31]. The crystal structure of α-Bi$_2$O$_3$ is displayed in Figure 4.4.

![Crystal structure of α-Bi$_2$O$_3$](image)

**Figure 4.4.** Crystal structure of α-Bi$_2$O$_3$ obtained via Materials Studio software with data from [32].

If δ-Bi$_2$O$_3$ is cooled to 639°C, a phase transition to γ-Bi$_2$O$_3$ occurs, after which – if not cooled slowly enough – it transforms to the α-Bi$_2$O$_3$ at 500°C [26]. If δ-Bi$_2$O$_3$ is cooled to 639°C, a phase transition to β-Bi$_2$O$_3$ occurs, which is transformed to α-Bi$_2$O$_3$ at 303°C [26]. ε-Bi$_2$O$_3$ and ω-Bi$_2$O$_3$ have been obtained through hydrothermal treatment and the heating of α-Bi$_2$O$_3$ on a beryllium oxide substrate to 800°C, respectively [27, 29].

Depending on the synthesis method, Bi$_2$O$_3$ has been obtained in several sizes and shapes. The different synthesis strategies are mainly divided into four methods:

- Classical precipitation [15],
- Flame spray pyrolysis [33],
- Solution combustion [34], and
- Electrodeposition [35].

Stable α-Bi$_2$O$_3$ NPs are typically synthesized using classical precipitation and further annealing of the obtained Bi(OH)$_3$ NPs at temperatures above 300°C [18, 19]. This synthesis method yields plate- or needle-like particles with a mean particle size of 50 nm [36]. Although the use of sodium hydroxide as precipitating agent is associated with
impurities such as sodium ions, it has been shown that, by using an additional ageing process, crystalline \(\alpha\)-Bi\(_2\)O\(_3\) NPs can be obtained without annealing [37, 38].

By adding additional surfactants and dispersants to the precipitation, the shape and size of \(\alpha\)-Bi\(_2\)O\(_3\) NPs can be controlled. For instance, the addition of PEG yields spherical \(\alpha\)-Bi\(_2\)O\(_3\) NPs with a mean particle size of 60 nm [39]. Granular \(\alpha\)-Bi\(_2\)O\(_3\) NPs with a mean particle size of 40 – 100 nm have been synthesized using polyvinylpyrrolidone (PVP) as an additional dispersant, and the use of oleic acid resulted in \(\alpha\)-Bi\(_2\)O\(_3\) nanowires [38, 40].

**Applications**

Bi\(_2\)O\(_3\) has unique optical, electrical, and ion-conducting properties and exhibits a high refractive index, as well as high dielectric permittivity, photoconductivity, and photoluminescence [41]. Its band gap depends on the particular polymorph and varies between 2.00 – 3.96 eV [30] with \(\alpha\)-Bi\(_2\)O\(_3\) being a semiconductor with a direct band gap of 2.80 – 2.85 eV [40, 42]. All these unique properties make Bi\(_2\)O\(_3\) a versatile material that has been investigated for several applications, including superconductors [43], solid-state electrolytes [41], electrical ceramics [44], gas sensors [38, 45], and photocatalysis [40, 46].

A recent study suggests dependence of the toxicity of \(\alpha\)-Bi\(_2\)O\(_3\) NPs on the processing conditions, particularly the annealing atmosphere. It has been shown that annealing in argon results in oxygen deficiency, which can in turn alter the intracellular interactions of \(\alpha\)-Bi\(_2\)O\(_3\) NPs [47]. Compared to air-annealed \(\alpha\)-Bi\(_2\)O\(_3\) NPs, which showed biocompatibility of 70% and 80% in 9L glioma malignant cells and Madin-Darby Canine Kidney (MDCK) cells, respectively, the argon-annealed \(\alpha\)-Bi\(_2\)O\(_3\) demonstrated biocompatibility of over 100% in both cell lines [47]. It is assumed that the oxygen vacancies that are located on the surface of the argon-annealed \(\alpha\)-Bi\(_2\)O\(_3\) are able to scavenge free ROS, which are typically associated with NP toxicity [47].
4.1.1.3 Properties of \((\text{BiO})_2\text{CO}_3\)

Bismuth carbonate, also known as bismuth subcarbonate, with the molecular formula \((\text{BiO})_2\text{CO}_3\) or \(\text{Bi}_2\text{O}_2\text{CO}_3\), crystallizes in the orthorhombic space group \(\text{Imm}2\) [48]. Figure 4.5 shows a projected ball model of \((\text{BiO})_2\text{CO}_3\) with \((\text{Bi}_2\text{O}_2)^{2+}\) layers interleaved by slabs comprising \(\text{CO}_3^{2-}\) groups [49].

![Ball model of \((\text{BiO})_2\text{CO}_3\)](image)

**Figure 4.5.** Ball model of \((\text{BiO})_2\text{CO}_3\); obtained via Materials Studio software using Crystallography Open Database file 9004677.

Several different synthesis methods have been reported to obtain \((\text{BiO})_2\text{CO}_3\) NPs with different particle sizes and structures, including nanotubes, nanoplates, nanosheets, and hollow microspheres [50]. In the field of photocatalysis, more complex structures of \((\text{BiO})_2\text{CO}_3\) have been developed with flower- and persimmon-like morphologies. The synthesis methods include:

- Hydrothermal and solvothermal synthesis [21, 51],
- Reflux method [52], and
- One-pot template-free synthesis methods [53].
In general, a precursor like bismuth citrate or bismuth nitrate is mixed with reagents such as sodium carbonate, ammonium carbonate, and urea, which are further accompanied by the addition of surfactants such as cetyltrimethylammonium bromide (CTAB) to improve the uniformity of (BiO)₂CO₃ in size and shape [50]. A very simple approach involves the refluxing of bismuth citrate and urea in ethylene glycol, yielding (BiO)₂CO₃ nanotubes [52]. Nanostructures are also obtained via hydrothermal and solvolothermal synthesis, whereas the one-pot template-free synthesis method typically yields microstructures of (BiO)₂CO₃ [50].

**Applications**

(BiO)₂CO₃ has been widely investigated as an ingredient in protective agents for gastrointestinal diseases [24], as filler in radiopaque catheters [54], and in pollution prevention, as it shows photocatalytic and antibacterial activity [21, 52]. Moreover, it is well known that the carbonate ion acts as a hydroxyl radical scavenger, reducing the degradation of organic molecules in waste water (see section 2.3.4.4) [55, 56].

(BiO)₂CO₃ has a large band gap (2.9 – 3.6 eV) that is tuneable, depending on its morphological properties [50]. Diverse (BiO)₂CO₃ nanostructures have been synthesized and studied regarding their photocatalytic activity, using dyes such as rhodamine B, methyl orange, and methylene blue [50, 51, 57]. The photocatalytic activity of (BiO)₂CO₃ has typically been reported for a large concentration of the active material, 1 g/L [51, 58].

(BiO)₂CO₃ has been further investigated in combination with other materials to form BiVO₄/(BiO)₂CO₃ nanocomposites [59], (BiO)₂CO₃/BiOI heterojunctions [60], and α-Bi₂O₃/(BiO)₂CO₃ heterojunctions [61] as efficient photocatalysts for the removal of toxic pollutants. Recently, a graphene and TiO₂ co-modified (BiO)₂CO₃ heterojunction composite was developed that displays a higher photocatalytic activity than the individual compounds [62].
4.1.2 Diagnostic Properties

Bi-based compounds have a high effective atomic number of $Z = 83$, making them potential candidates for radiation-dose enhancement in radiation therapy. Furthermore, Bi-based diagnostic agents are promising candidates to replace iodine-based CT contrast agents, as well as gold and platinum NPs [63]. Bismuth NPs and bismuth(III) ions have shown a significantly greater X-ray attenuation than iodine, particularly for large tube potentials, which are used in clinical CT scanners [64]. Similarly, biocompatible dendrimer- and polymer-stabilized bismuth sulphide NPs exhibit greater X-ray attenuation than iodine-based CT contrast agents at the same molar concentration of the active element [65, 66]. For instance, PVP-coated $\text{Bi}_2\text{S}_3$ NPs displayed a fivefold better X-ray absorption than iodine and experiments in mice indicate a good performance of the PVP-coated $\text{Bi}_2\text{S}_3$ NPs as a blood pool agent [66]. In a different study, human-serum-albumin-coated $\text{Bi}_2\text{O}_3$ NPs were explored as a contrast agent for X-ray imaging [67]. Moreover, Bi-based compounds are known to exhibit contrast enhancement that varies little with the X-ray tube potential [63, 64, 68].

Besides the use of single Bi-based compounds, the combination of $\text{BiOCl}/\text{Bi}_2\text{O}_3$ encapsulated in single-walled carbon nanotubes (~50 nm) has been investigated for improved X-ray CT imaging in pig-bone-marrow-derived mesenchymal stem cells [69]. Moreover, these multifunctional materials showed high biocompatibility in vitro. Other multifunctional nanohybrids are based on bismuth and iron and can provide, besides CT contrast enhancement, also MRI contrast enhancement [68].

4.2 High Toxicity of $\text{Bi(OH)}_3$ and $\alpha$-$\text{Bi}_2\text{O}_3$ Nanoparticles Towards Malignant 9L and MCF-7 Cells

This section discussed the materials characterization of $\text{Bi(OH)}_3$ and $\alpha$-$\text{Bi}_2\text{O}_3$ NPs, which were obtained through a facile synthesis with an average single particle size of 6 – 10 nm. The synthesis procedure and all characterization methods are described in
CHAPTER 3. The antitumor activity of the Bi(OH)$_3$ and $\alpha$-Bi$_2$O$_3$ NPs was determined *in vitro* using two different tumour cell lines: 9L gliosarcoma cells and MCF-7 human breast cancer cells, exposed to the NPs with different concentrations. Biological interactions were also studied in normal MDCK cells. The 9L cells have a fibroblast-like morphology and were chosen due to their high radio-resistance and their use for both *in vivo* and *in vitro* models [70]. MCF-7 cells were chosen as a human cancer cell line with epithelial-like morphology. Apoptotic nuclear morphologies were determined in 9L cells using a double labelling with Hoechst dye and DCFH$_2$-DA.

### 4.2.1 Identification, Size, Morphology, and Surface Composition of Bi(OH)$_3$ and $\alpha$-Bi$_2$O$_3$ Nanoparticles

The XRD patterns of the obtained nanomaterials are shown in Figure 4.6. The diffraction pattern of the non-annealed Bi-based compound matches the XRD pattern obtained elsewhere [17], which identifies the material as bismuth hydroxide. The annealed material is identified as single phase $\alpha$-Bi$_2$O$_3$ (JCPDS 01-076-1730), which is the most stable polymorphic form at room temperature [71]. Since Bi(OH)$_3$ is structurally not yet well defined, no JCPDS file was found in literature, and Miller indices could not be assigned to the reflections.

![Figure 4.6](image)

*Figure 4.6.* XRD patterns of Bi(OH)$_3$ and $\alpha$-Bi$_2$O$_3$ nanoparticles.

172
Figure 4.7 presents TEM and high-angle angular dark-field (HAADF) images of both nanomaterials. The average single particle size is $6.04 \pm 0.89$ nm for Bi(OH)$_3$ and $10.13 \pm 0.95$ nm for $\alpha$-Bi$_2$O$_3$, with round and ellipsoidal shape (left images). The HAADF images (right images) show $\alpha$-Bi$_2$O$_3$ NPs with interplanar spacing of 0.332 nm, which is in accordance with the $d$-spacing of the (111) plane.

![Figure 4.7. TEM (left) and HAADF STEM (right) images of (a) Bi(OH)$_3$ NPs and (b) $\alpha$-Bi$_2$O$_3$ NPs. The HAADF image of Bi(OH)$_3$ NPs exposes lattice fringes spaces of 0.263 and 0.275 nm. The HAADF image of $\alpha$-Bi$_2$O$_3$ NPs exposes lattice fringes spaces of 0.332 nm that correspond to (111) planes.](image)

Dynamic light scattering analysis was performed directly after sonication, in order to examine the hydrodynamic diameters of both nanomaterials suspended in PBS; the intensity-based size distributions of the particles are shown in Table 4.2. The results show that both nanomaterials are present in form agglomerates: some of them are still in nanoscale and some have sizes larger than 100 nm. The original dynamic light scattering data is displayed in Figure 4.8. Although the stability of the NPs could potentially be improved by stabilizing agents [72], the goal of this doctoral thesis was to avoid possible effects of stabilizing agents on the cellular experiments, and to only study the interactions of pristine Bi(OH)$_3$ and Bi$_2$O$_3$ NPs with the cells.
Table 4.2. Hydrodynamic diameters and intensities (Int) of Bi(OH)\(_3\) and α-Bi\(_2\)O\(_3\) nanoparticles suspended in PBS after 2 h of sonication, determined via dynamic light scattering. The concentration of both materials is 50 μg/mL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1 (nm)</th>
<th>Int 1</th>
<th>Peak 2 (nm)</th>
<th>Int 2</th>
<th>Peak 3 (nm)</th>
<th>Int 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi(OH)(_3)</td>
<td>26.8 ± 2.0</td>
<td>6.3%</td>
<td>200.4 ± 23.8</td>
<td>24.3%</td>
<td>679.8 ± 121.1</td>
<td>69.4%</td>
</tr>
<tr>
<td>α-Bi(_2)O(_3)</td>
<td>86.4 ± 8.9</td>
<td>10.2%</td>
<td>322.5 ± 57.9</td>
<td>89.8%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.8. Hydrodynamic diameter in PBS determined via dynamic light scattering of (a) Bi(OH)\(_3\) and (b) α-Bi\(_2\)O\(_3\) nanoparticles. The concentration of both nanomaterials is 50 μg/mL.

XPS was performed in order to investigate both the surface composition and the properties within the nanomaterials. The high-resolution XPS spectra of the Bi 4f peak are displayed in Figure 4.9(a). The peaks located at 158.10 ± 0.28 eV and 164.36 ± 0.75 eV can be assigned to the binding energies of Bi 4f\(_{7/2}\) and Bi 4f\(_{5/2}\), respectively, with a separation of 6.26 ± 0.03 eV and full-width at half-maximum (FWHM) of 2.14 (58%) and 2.06 (42%) eV.
for the Bi(OH)$_3$ NPs, and 2.26 (59%) and 2.07 (41%) eV for the α-Bi$_2$O$_3$ NPs [73, 74]. These symmetric peaks are ascribed to Bi$^{3+}$ ions in the Bi–O bonds, whereas no peaks with an asymmetric shape could be identified as corresponding to metallic Bi [74].

Two Gaussian peaks were used to fit the experimental data in the high-resolution XPS spectra of O 1s (Figure 4.9(b)). The peak at the lower binding energy of 530.23 ± 0.04 eV with a Full Width at Half Maximum (FWHM) of 2.77 eV (Bi(OH)$_3$) and 2.9 eV (α-Bi$_2$O$_3$) can be assigned to O$^{2-}$ ions in the Bi–O bonds [74]. A major O 1s peak at the binding energy of 532.61 ± 0.13 eV (FWHM = 2.73 eV (Bi(OH)$_3$) and 3.3 eV (α-Bi$_2$O$_3$)) can be identified as onto the NP surface absorbed hydroxide species, as described for other metal oxides [74, 75]. The spectral area of the higher binding energy peak is significantly larger for the Bi(OH)$_3$ NPs, with 52% of the total O 1s area as compared to α-Bi$_2$O$_3$ NPs with 41%, which can be explained by the presence of more hydroxyl groups on the surface of Bi(OH)$_3$. The XPS survey spectra and high-resolution XPS spectra of N 1s and C 1s are displayed in Figure A1 in the Appendix I.

**Figure 4.9.** High-resolution XPS spectra of (a) Bi 4f and (b) O 1s of Bi(OH)$_3$ (top) and α-Bi$_2$O$_3$ (bottom) nanoparticles.

175
4.2.2 Cellular Uptake of Bi(OH)₃ and α-Bi₂O₃ Nanoparticles

The relative cellular uptake was examined by comparing the mean SSC of the untreated 9L, MCF-7, and MDCK cells with the mean SSC of the cells treated with either Bi(OH)₃ or α-Bi₂O₃ NPs at concentrations of 6.25, 12.5, 25, and 50 µg/mL after 24 h exposure (Figure 4.10).

![Figure 4.10](image)

**Figure 4.10.** Relative internalization of Bi(OH)₃ and α-Bi₂O₃ nanoparticles in 9L, MCF-7, and MDCK cells after 24 h of exposure, with concentrations of nanoparticles of 0, 6.25, 12.5, 25, and 50 µg/mL, obtained through flow cytometry.

In general, the internalization of both nanomaterials increased relative to the control and was dose-dependent. Furthermore, both NPs show a very similar relative internalization in 9L and MCF-7 cells with the Bi(OH)₃ NPs displaying a slightly greater uptake compared to α-Bi₂O₃ NPs. In contrast, the nanomaterials show only a moderate cellular uptake in MDCK cells.

Figure 4.11 displays live cell confocal images of 9L, MCF-7, and MDCK cells, non-treated and treated with Bi(OH)₃ and α-Bi₂O₃ NPs at a concentration of 50 µg/mL. The images are representative for a large number of cells and have been chosen to visually show the uptake of the NPs into the cells. An exposure time of 4 - 8 h was chosen, since significant changes in cellular uptake of both NPs were already observable. For both
materials, all NPs initially within a certain distance of the cell were internalized into 9L cells (top row, centre image), as indicated by the particle-free space around the cells. Similar observations have been made with 1,2-dilauroyl-sn-glycero-3-phosphocholine-modified (DLPC-modified) bismuth NPs (denoted as Bi@DLPC NPs) towards MDA-MB-231 cells (breast cancer cells) [76].

**Figure 4.11.** Confocal images of 9L (top row), MCF-7 (centre row), and MDCK (bottom row) cells without nanoparticles (control, left column), with Bi(OH)$_3$ nanoparticles (centre column), and with α-Bi$_2$O$_3$ nanoparticles (right column). The concentration of both nanomaterials was 50 μg/mL, and the incubation time was 4 – 8 h. The black arrows indicate non-internalized nanoparticles, while the red arrow show internalized nanoparticles. The insets in each image show a close-up of a single cell.

Compared with 9L cells, it appears that the NPs do not internalize as fast into MCF-7 cells, as indicated by the visibly larger amount of NPs located ultimately next to the cells.
for this early stage of NP incubation time (centre row, centre image). A similar trend is observed for MDCK cells, in which the NPs do not internalize easily. These observations are in agreement with the relative internalization obtained via flow cytometry.

### 4.2.3 Cytotoxicity of Bi(OH)$_3$ and α-Bi$_2$O$_3$ Nanoparticles

In addition to the lactate dehydrogenase assay, the clonogenic assay is also used to assess biocompatibility of NPs [76-81]. The surviving fractions of 9L, MCF-7, and MDCK cells after 24 h treatment with Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs at concentrations of 0 (control), 6.25, 12.5, 25, and 50 µg/mL using the clonogenic assay method are illustrated in Figure 4.12.

For both materials, the surviving fractions of the malignant cell lines significantly decrease at higher concentrations of NPs, with a value of < 1% and < 10% in 9L and MCF-7 cells, respectively, at 50 µg/mL. This value was therefore chosen as a standard concentration for further biological characterization. The IC$_{50}$ values are shown in Table 4.3.
Figure 4.12. Clonogenic assay of (a) 9L, (b) MCF-7, and (c) MDCK cells after 24 h exposure with Bi(OH)$_3$ and α-Bi$_2$O$_3$ nanoparticles at concentrations of 0, 6.25, 12.5, 25, and 50 µg/mL. The cells were trypsinized, plated at low density into 100 mm Petri dishes, and incubated for 15 doubling times at 37°C and 5% (v/v) CO$_2$. The surviving fraction was obtained by comparing the plating efficiencies of the control and the treatment samples. The * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and **** indicates $P < 0.0001$ for the comparison with the ‘control’ (0 µg/mL). The b indicates $P < 0.05$, bb indicates $P < 0.01$, bbb indicates $P < 0.001$, and bbbb indicates $P < 0.0001$ for the comparison between the treatments (ns = not significant).
**Table 4.3.** IC<sub>50</sub> values for the inhibition of colony number of 9L, MCF-7, and MDCK cell lines following exposure to Bi(OH)<sub>3</sub> and α-Bi<sub>2</sub>O<sub>3</sub> nanoparticles for 24 h.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>Bi(OH)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>α-Bi&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9L</td>
<td>6.35 ± 1.53</td>
<td>7.18 ± 0.94</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>16.80 ± 0.87</td>
<td>16.53 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>MDCK</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td></td>
</tr>
</tbody>
</table>

Organic bismuth compounds have been studied in breast (MDA-MB-435S, MCF-7, EVSA-T), colon (Colo201, DLS-1, WIDR), renal (A498), pancreatic (MIA paca), lung (H226, A549, H596), gastric (KATO3), cervical (Hela), and ovarian (IGROV) cancer, and neuroblastoma (SK-N-SH), osteosarcoma (MG-63), fibrosarcoma (HT1080), and melanoma (M19) [7, 82]. The greatest potency was observed against IGROV and MCF-7 cells, with IC<sub>50</sub> values of < 0.005 µM and < 0.004 µM, respectively.

It was shown that Bi@DLPC NPs displayed negligible cytotoxicity towards MDA-MB-231 and MCF-10A cells (breast epithelial cells) at concentrations of 0 – 500 µg/mL, with a cell viability of 87% at the highest concentration [76]. Only when in combination with near-infrared laser irradiation did the number of dead cells in MDA-MB-231 increase [76]. Similarly, a theranostic system based on Bi NPs, which have been capped with thiol ligands and surface-modified with poly(ethylene-glycol)-modified phospholipids (denoted as Bi-SR-PEG NPs) showed no significant toxicity (< 10%) in murine breast cancer 4T1 cells, even at the highest concentration of 100 µg/mL [83], which emphasizes the use of Bi NPs for biological application. Only after additional exposure of the cells and NPs to X-ray irradiation with different radiation doses (0, 2, 4, and 6 Gy), did the surviving fraction decrease to ~10% at the highest dose [83].

α-Bi<sub>2</sub>O<sub>3</sub> NPs with a particle size of 50 – 80 nm have been studied in 9L cells and exhibited a cytotoxicity of ≤ 30% at a concentration of 50 µg/mL. The difference in
cytotoxicity, compared to our study, is possibly related to differences in the single particle size of the NPs and their suspension preparation [78]. It has been shown that the optimization of dispersion method is crucial for biological in vivo and in vitro studies [84]. Bi(OH)$_3$ has been studied in L1210 mouse leukaemia cells and showed an IC$_{50}$ > 5 μM. The study, however, does not mention any structural, morphological or physical properties of the Bi(OH)$_3$ particles [8].

In this thesis, Bi(OH)$_3$ NPs are very potent towards 9L and MCF-7 cells, even at the lowest tested concentration. In contrast, CeO$_2$ NPs for instance, showed a surviving fraction of 9L cells of > 90% and > 60% after NP exposure for 24 h at a concentration of 50 and 500 μg/mL, respectively [85]. Even the additional irradiation of 9L cells exposed to CeO$_2$ NPs with 10 MV or 150 kVp X-ray beams with an absorbed dose up to 8 Gy did not yield in the same mortality as observed for the Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs.

Similarly, gold and silver NPs show a significantly reduced toxicity in MCF-7 cells [86, 87]. For instance, the use of gold NPs at a concentration of 25 and 50 μg/mL yielded in cell viability greater than 80 and 70%, respectively. Even the highest tested dose of 200 μg/mL did not result in cell viability lower than 50% [86]. In another study, the cell viability of gold NPs was tested at concentrations of 0 – 200 μM/mL, and no significant toxicity was observed, even at the highest concentration [87]. Similarly, the 24 h exposure of MCF-7 cells to silver NPs at a concentration of 50 μg/mL resulted in cell viability greater than 50% [88].

The use of ZnO NPs was shown to cause a toxicity similar to the one observed for Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs at a concentration of 25 and 50 μg/mL [89]. However, at lower concentrations (< 12.5 μg/mL) the Bi(OH)$_3$ NPs show a significantly increased efficiency towards MCF-7 cells, in comparison to ZnO NPs, which highlights the overall high potency of both Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs towards 9L and MCF-7 cells. More importantly, this high
potency is achieved without further treatment such as X-ray or near-infrared irradiation, as for Bi@DLPC and Bi-SR-PEG NPs [76, 83].

In addition, both nanomaterials show a very low-to-moderate toxicity in MDCK cells for the highest concentration of 50 µg/mL, with a surviving fraction of up to 95% (Bi(OH)₃) and 58% (α-Bi₂O₃), which could be related to the low internalization of the nanomaterials. The biocompatibility of Bi-based NPs has been reported widely [76, 83].

4.2.4 Staining with Propidium Iodide

Figure 4.13 shows confocal images of 9L, MCF-7, and MDCK cells that were untreated and treated with each NP at a concentration of 50 µg/mL and with the DNA stain PI. After 24 h exposure of 9L cells to Bi(OH)₃ NPs (Figure 4.13(a), centre column) and α-Bi₂O₃ NPs (right column), a significant number of cells with disordered areas of dysfunctional cell membranes can be identified, whereas control 9L cells do not exhibit any fluorescence (left column). Interestingly, the incubation with α-Bi₂O₃ NPs leads to a greater number of cells with dysfunctional membranes than the exposure to Bi(OH)₃ NPs, which can be explained by a faster killing mechanism, since the clonogenic assays show a similar high toxicity for both materials, with even higher mortality rates for the Bi(OH)₃ NPs.

In contrast to 9L cells, the DNA stain with PI shows a reduced number of dysfunctional cell membranes in MCF-7 cells (Figure 4.13(b)), indicating that the mechanism of killing is slower than for 9L cells. The DNA stain with PI shows no noticeable or only a very small number of dysfunctional cell membranes in MDCK cells for the Bi(OH)₃ and α-Bi₂O₃ NPs, respectively (Figure 4.13(c)).
Figure 4.13. Confocal images of (a) 9L, (b) MCF-7, and (c) MDCK cells stained with propidium iodide without additives (control, left column) and after 24 h of incubation with Bi(OH)$_3$ nanoparticles (centre column) and α-Bi$_2$O$_3$ nanoparticles (right column). The concentration of both nanomaterials was 50 μg/mL.
To examine when cell death starts, 9L, MCF-7, and MDCK cells were imaged repeatedly every 4 h for a total of 48 h after addition of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs at a concentration of 50 μg/mL. After addition of the NPs, the 9L cells maintain their capacity to grow and proliferate until an incubation time of 12 h (Bi(OH)$_3$) and 8 h (α-Bi$_2$O$_3$) is reached (Figures A2 – A4 in the Appendix I) [90-92]. Most of the cells start to lose their viability, after approximately 20 h (Bi(OH)$_3$) and 16 h (α-Bi$_2$O$_3$). Moreover, the cells do not recover over the total incubation time of 48 h. In general, the α-Bi$_2$O$_3$ NPs show faster cell death, which is in agreement with the results from the PI stain.

In contrast to 9L, MCF-7 and MDCK cells show a high viability over the whole exposure of 48 h (Figures A5 – A10 in the Appendix I); however, the capacity of MCF-7 cells to grow and proliferate seems to be reduced compared with control cells that were not exposed to the nanomaterials [90-92].

4.2.5 Cell Cycle Population

Flow cytometry analysis was used to determine the DNA content and cell cycle of 9L (Figure 4.14(a)), MCF-7 (Figure 4.14(b)), and MDCK (Figure 4.14(c)) cells, untreated (left panels) and treated with Bi(OH)$_3$ (centre panels) and α-Bi$_2$O$_3$ (right panels) NPs at a concentration of 50 μg/mL. The percentage of cells with apoptotic sub-G$_1$ DNA content is significantly increased for NP-treated 9L cells from 2.4 ± 0.9% for the control, to 22.0 ± 7.7% and 16.4 ± 5.8% for the treatment with Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs, respectively. In addition, the population of the G$_1$ phase is significantly reduced, which indicates that the cells have lost their metabolic activity, and the noticeable reduction of the S-phase suggests the possible influence of both nanomaterials on the process of DNA replication.
Figure 4.14. Cell cycle distributions of (a) 9L, (b) MCF-7, and (c) MDCK cells without nanoparticle treatment (left panels) and after exposure to Bi(OH)$_3$ (centre panels) and α-Bi$_2$O$_3$ nanoparticles (right panels), with both at a concentration of 50 μg/mL for 24 h. The cells were trypsinized, fixed with ethanol, stained with propidium iodide, and analyzed using a flow cytometer. The percentages of cells in different phases in the populations are indicated in each panel.

Similarly to 9L, MCF-7 cells show a noticeable increase in apoptotic sub-G$_1$ DNA content and a reduction of G$_1$ phase after treatment with the NPs. However, the impact on
the cell cycle is not as pronounced as in 9L cells and the S-phase shows a similar population as in control cells. The findings support the observations from the confocal imaging, which indicate a faster mechanism of killing of the NPs in 9L cells. In contrast to 9L and MCF-7 cells, the flow cytometric analysis of the DNA content of MDCK cells after 24 h of treatment with Bi(OH)₃ and α-Bi₂O₃ NPs shows no significant difference compared to the control.

4.2.6 Evaluation of Apoptotic Nuclear Morphologies and Intracellular Reactive Oxygen Species

The two main apoptotic nuclear morphologies, namely budding and cleavage, are easily recognisable after nuclear staining and are the result of two independent morphological routes that are differently modulated by stress or physiological apoptogenic agents like intracellular glutathione [93, 94].

Similarly to nanoceria (CeO₂), which affects only the oxidation-dependent apoptosis, we hypothesized that Bi(OH)₃ and α-Bi₂O₃ NPs may also exhibit similar behaviour, which is characterized by the cleavage morphology [95]. To analyse the oxidative features of apoptotic cells, a double labelling was performed with Hoechst stain to identify the nuclear morphology and the ROS dye H₂DCFDA. It has been demonstrated that the DCF signal in budding cells is comparable to that in viable cells, whereas the cells in cleavage display stronger staining [95].

The relative intracellular levels of ROS in 9L and MDCK cells were quantified by evaluating the effect of Bi(OH)₃ and α-Bi₂O₃ NPs on the abundance of the cleavage cell morphology in combination with the intensity of the DCF signal. The 9L cells were chosen for the double labelling as the nanomaterials exhibited the greatest effect on these cells, in comparison to MCF-7. MDCK cells were chosen, as the nanomaterials showed only low toxicity in this cell line. Due to the very fast induction of apoptosis by both materials at a concentration of 50 µg/mL in 9L cells, different incubation times of Bi(OH)₃ and α-Bi₂O₃
NPs, ranging from 0 to 24 h, were chosen in order to investigate the link between intracellular ROS levels and cell death.

The emission images of 9L and MDCK cells without and with NP incubation for 21 h (Bi(OH)₃) and 15 h (α-Bi₂O₃) are shown in Figure 4.15 and Figure 4.16, respectively. These particular incubation times with the NPs were chosen because they resulted in mostly dead cells for 9L, as shown via live cell imaging.

![Overlay, Hoechst, DCF images](image)

**Figure 4.15.** 9L cells double-labelled (left columns), labelled with Hoechst stain alone (centre columns; nuclear morphology) and with 2',7'-dichlorodihydrofluorescein diacetate alone (right columns; ROS content; 2',7'-dichlorofluorescein (DCF) signal). The rows show the control cells (top rows); cells after treatment with Bi(OH)₃ nanoparticles for 21 h (centre rows), and cells after treatment with α-Bi₂O₃ nanoparticles for 15 h (bottom rows).
**Figure 4.16.** MDCK cells double-labelled (left columns), labelled with Hoechst stain alone (centre columns; nuclear morphology) and with 2',7'-dichlorodihydrofluorescein diacetate alone (right columns; ROS content; 2',7'-dichlorofluorescein (DCF) signal). The rows show the control cells (top rows); cells after treatment with Bi(OH)$_3$ nanoparticles for 21 h (centre rows), and cells after treatment with α-Bi$_2$O$_3$ nanoparticles for 15 h (bottom rows).

Whereas the 9L control cells only show a weak DCF signal (Figure 4.15, top right image), the incubation with Bi(OH)$_3$ or α-Bi$_2$O$_3$ NPs leads to a significant increase in green fluorescence (centre and bottom right images). Furthermore, the treatment of 9L cells with each nanomaterial leads to the cleavage morphology of apoptotic nuclei (middle and bottom row, centre column images, as indicated by the red arrows). The elevated levels of ROS with no noticeable increase in budding but significant increase in cleavage morphology of apoptotic nuclei suggest an apoptosis pathway dependent on ROS.

In contrast to 9L cells, the treatment of MDCK cells with either Bi(OH)$_3$ or α-Bi$_2$O$_3$ NPs does not cause a significant increase in the DCF signal (Figure 4.16, centre and bottom right images), compared to the untreated MDCK cells (top right image), which emphasizes the high biocompatibility of both materials. Furthermore, MDCK cells do not exhibit a
significantly increased number of apoptotic nuclei with either budding or cleavage morphology when treated with Bi(OH)$_3$ or α-$\text{Bi}_2\text{O}_3$ NPs (middle and bottom row, centre column images), but show nuclei with similar shapes and sizes compared to untreated cells (top centre image).

Figure 4.17 displays the additional number of 9L and MDCK cells (compared to the control) that show cleavage due to the treatment with Bi(OH)$_3$ or α-$\text{Bi}_2\text{O}_3$ NPs, which is linked with increased intracellular levels of ROS. The number of 9L cells generating ROS increases slowly in the early stages of NP incubation until a maximum is reached at approximately 21 h (Bi(OH)$_3$) and 15 h (α-$\text{Bi}_2\text{O}_3$), confirming a difference in the rapidity of apoptosis induced by the two materials. As shown via live cell imaging, where cells lost their capacity to grow and proliferate after approximately 20 h (Bi(OH)$_3$) and 16 h (α-$\text{Bi}_2\text{O}_3$) of NP exposure, the obtained ROS profile suggests a strong correlation between cell death and elevated intracellular levels of ROS. The decrease in the number of cells with high ROS levels at longer exposure times can be explained by, firstly, the reduced number of viable cells and, secondly, the washing off of loosely attached cells during the preparation process for imaging.

In contrast to 9L, MDCK cells do not generate significantly more ROS than control cells throughout the whole period of 24 h. The number of 9L and MDCK cells that show budding morphology of apoptotic nuclei remains comparable to that for the control during the whole period of 24 h (Figure 4.18).
Figure 4.17. Additional number of apoptotic nuclei with cleavage morphology for 9L and MDCK cells after treatment with 50 μg/mL of Bi(OH)$_3$ and α-Bi$_2$O$_3$ nanoparticles for 0, 2, 4, 6, 9, 12, 15, 18, 21, and 24h, compared to non-treated cells. Cleavage frequencies among the total cells were evaluated by Hoechst and 2',7'-dichlorodihydrofluorescein diacetate staining.

Figure 4.18. Additional number of apoptotic nuclei with budding morphology for 9L and MDCK cells after treatment with 50 μg/mL of Bi(OH)$_3$ and α-Bi$_2$O$_3$ nanoparticles for 0, 2, 4, 6, 9, 12, 15, 18, 21, and 24h, compared to non-treated cells. Budding frequencies among the total cells were evaluated by Hoechst and 2',7'-dichlorodihydrofluorescein diacetate staining.

The relative number of 9L cells showing oxidation-dependent apoptosis is very similar for both materials, although the relative internalization of Bi(OH)$_3$ NPs in 9L cells is significantly increased compared to α-Bi$_2$O$_3$ NPs. As mentioned in section 4.2.2, the relative
cellular uptake of both nanomaterials in 9L cells is higher in comparison with MDCK cells. In order to show that the generation of ROS occurs selectively in 9L cells and is not due to a higher uptake of NPs, the concentrations of both Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs in MDCK cells were increased to a point where their relative internalization matched that of 9L cells, which was obtained for 50 μg/mL and an incubation time of 24 h. A similar relative cellular uptake of 495 ± 23% (Bi(OH)$_3$) and 388 ± 11% (α-Bi$_2$O$_3$) in MDCK cells was achieved for a concentration of 700 and 100 μg/mL, respectively, while using the same incubation time as for 9L cells. This increased concentration of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs in MDCK cells did not result in a larger number of nuclei with cleavage morphology, with 0.3 ± 0.8% and 0.8 ± 0.3%, respectively, for the exposure time of 24 h. Treatment of 9L cells with increased concentrations of 700 μg/mL of Bi(OH)$_3$ NPs and 100 μg/mL of α-Bi$_2$O$_3$ NPs showed that the amount of nuclei with cleavage morphology remained constant with 22.5 ± 3.6% and 16.3 ± 1.8%, respectively.

The generation of ROS has been shown to be a mechanism of action of ZnO NPs [96] that typically need to be internalized to induce elevated levels of intracellular ROS in mouse macrophage Ana-1 cells [97]. The obtained results in this thesis suggest a similar mechanism of action that involves the generation of ROS, as shown for 9L cells. The observed toxicity can furthermore be affected by different internalization rates. As the present results indicate, the nanomaterials do not internalize easily in MDCK cells and cause only moderate to low toxicity, while the cellular uptake is significantly higher in malignant cells. Further tests, however, are required to study the exact mechanism.

4.2.7 In Vitro Computed Tomography Imaging of Bi(OH)$_3$ and α-Bi$_2$O$_3$ Nanoparticles

The anatomical contrast enhancement capability of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs was demonstrated using a clinical CT scanner, with the resulting CT number as a function of the Bi concentration, as shown in Figure 4.19 and Figure 4.20. The CT values in Hounsfield
Units (HU) for each concentration were determined for multiple regions along the central axis of the vial to account for possible sedimentation of the suspended composites (see red dashed lines).

![Figure 4.19](image)  

**Figure 4.19.** (a) Linear fitting of the CT number of Bi(OH)$_3$ nanoparticles as a function of the mass concentration of Bi in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of linear regression and the coefficient of determination ($R^2$) value are indicated. (b) CT images of Bi(OH)$_3$ nanoparticles with Bi mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area that was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.

The data was plotted and fitted to deduce the relation between the CT number and the Bi concentration. The average CT number was chosen across the suspension height. In general, the contrast increased with increasing concentration of bismuth. A noticeable difference in CT number was observed at the highest concentration of 8 mg/mL for the centre of the sediment and the centre of the suspension height. The CT number reached a value of up to 3500 HU and 5000 HU for the sediment at a Bi concentration of 8 mg/mL for Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs, respectively.
Figure 4.20. (a) Linear fitting of the CT number of α-Bi$_2$O$_3$ nanoparticles as a function of the mass concentration of Bi in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of the linear regression and the R$^2$ value are indicated. (b) CT images of α-Bi$_2$O$_3$ nanoparticles with Bi mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area which was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.

X-ray attenuation also depends on the X-ray photon energy. Typically, for most elements, including iodine, the X-ray attenuation decreases with increasing X-ray energy (peak tube potential) [64]. It was shown that Bi$_2$O$_3$ NPs, which were stabilized with human serum albumin, provided an enhanced contrast at a tube potential of 80 kVp and a concentration of 3 mg/mL and 5 mg/mL with CT numbers of 260 HU and 380 HU, respectively [67]. Dextran-coated bismuth iron oxide nanohybrids displayed similar CT values of 50 – 300 HU at a concentration of 9.4 mg/mL, depending on the formulation of the nanohybrids and the tube potential [68]. It was shown that iron oxide attenuates X-rays poorly, with a CT number of approximately 50 HU for tube potentials between 80 – 140 kVp [68]. The inclusion of bismuth yielded an increase in X-ray attenuation, which increased gradually with the added amount of bismuth in the formulations. Similarly to the results obtained in this doctoral thesis, the attenuation was strongest for small tube potentials. For higher tube potentials, however, the attenuation did not change significantly. This observation was also made for bismuth NPs and highlights the potential of Bi-based nanomaterials for CT imaging [64].
In contrast, iodine-based CT agents show a much greater decrease in CT number for large X-ray tube potentials [63, 64, 68]. The superior contrast enhancement of the Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs across the diagnostic CT X-ray spectrum, ranging from 100 to 130 kVp, is of particular interest in a clinical setup [98]. Although it is beneficial for pediatric patients to reduce the radiation dose, the ability to use high X-ray energies is important for the imaging of large patients, as a sufficient penetration of X-rays needs to be ensured to obtain images of the desired quality.

4.2.8 Conclusions

Although Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs have not been explored as chemotherapeutic agents for cancer treatment so far, they offer multiple attractive features such as: imaging, possible radio-dose enhancement, and, as has been demonstrated for the first time in this thesis, they exhibit high in vitro toxicity towards malignant cells, which makes them promising theranostic systems for cancer treatment. With this combination of properties, the use of anti-cancer drugs can potentially be refined and therefore, the spectrum of side effects reduced while maintaining similar therapeutic effects.

The high mortality (> 90 - 99%) of chemo-resistant 9L glioma and MCF-7 breast cancer cells after only 24 h of NP treatment indicates that both materials might exhibit high toxicity in other cancer cell lines, which needs further investigation. Furthermore, the successful transition into in vivo models needs to be demonstrated in order to fully examine the anti-cancer activity. In contrast, the nanomaterials showed only low to moderate toxicity in normal MDCK cells. Apoptosis of 9L cells was identified via cleavage morphology of the nuclei and increased sub G$_1$ levels, whereas the number of cells entering the G$_1$ and S phases was reduced.
4.3 Biocompatible Bi(OH)$_3$ Nanoparticles with Reduced Photocatalytic Activity as Possible Ultraviolet Filter in Sunscreens

In this section, readily synthesized Bi(OH)$_3$ nanoparticles with a particle size of approximately 6 nm are investigated as a novel, multifunctional ultraviolet filter for sunscreen with low photocatalytic activity. The synthesis procedure and all characterization methods are described in CHAPTER 3. The SPF was determined in accordance with ISO 24443:2012 for an in-house fabricated sunscreen containing TiO$_2$/Bi(OH)$_3$ and compared to a ‘classical’ sunscreen of TiO$_2$/ZnO. The physical properties of the colourless Bi(OH)$_3$ NPs alone, and in combination with photocatalytically active TiO$_2$ and ZnO NPs, were investigated through monitoring of crystal violet degradation under UVA/UVB irradiation and simulated solar irradiation. The cytotoxicity of the Bi(OH)$_3$ NPs was determined upon healthy HaCaT keratinocytes and MDCK cells using the MTT assay.

4.3.1 Overview of Tested Materials, Homemade Sunscreens, and Commercial Sunscreens

In this section, an overview is presented of the different materials (M, Table 4.4), homemade sunscreens (HS, Table 4.5), and commercial sunscreens (CS, Table 4.6) used. The Bi(OH)$_3$ (M1) NPs were the only material that was synthesized in this thesis. Details about the Bi(OH)$_3$ NP synthesis and the preparation of homemade sunscreens are presented in CHAPTER 3. For all characterization techniques that involved combinations of TiO$_2$ and Bi(OH)$_3$ NPs, or ZnO and Bi(OH)$_3$ NPs, different concentrations of separately suspended NPs were mixed mechanically.

The materials M1 – M3 were used for photocatalytic activity tests, which involve the degradation of crystal violet. Furthermore, the UV filtering properties and the cytotoxicity of these materials were investigated.
### Table 4.4. Overview of the materials (M) used.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sample</th>
<th>Trade name/Supplier</th>
<th>Particle size (nm) and morphology</th>
<th>Crystal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Bi(OH)$_3$</td>
<td>Synthesized in this thesis</td>
<td>6.04 ± 0.89 (spherical)</td>
<td>–</td>
</tr>
<tr>
<td>M2</td>
<td>TiO$_2$</td>
<td>Aeroxide®, P25</td>
<td>24.08 ± 1.60 (ellipsoidal, cubic)</td>
<td>80% anatase, 20% rutile</td>
</tr>
<tr>
<td>M3</td>
<td>ZnO</td>
<td>Sigma Aldrich</td>
<td>44.86 ± 6.38 (spherical, hexagonal, rectangular)</td>
<td>Zincite</td>
</tr>
</tbody>
</table>

The homemade sunscreens HS1 and HS2 were used for photocatalytic activity tests, which involve the exterior exposure of pre-painted steel panels. Furthermore, rheological properties were determined, as well as the photoprotection efficiency (SPF). The results were compared to those of the commercial sunscreen formulations.

### Table 4.5. Overview of the homemade sunscreens (HS) used.

<table>
<thead>
<tr>
<th>ID</th>
<th>UV filter</th>
<th>Trade name</th>
<th>Particle size (nm) and morphology</th>
<th>Crystal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS1</td>
<td>TiO$_2$</td>
<td>Eusolex® T-S</td>
<td>Rod-like: 20 (width) and 100 (length) [99]</td>
<td>Rutilte</td>
</tr>
<tr>
<td></td>
<td>ZnO</td>
<td>Z-COTE® HP1</td>
<td>30 – 200, (rod-like, isometric) [100]</td>
<td>Wurtzite</td>
</tr>
<tr>
<td>HS2</td>
<td>TiO$_2$</td>
<td>Eusolex® T-S</td>
<td>Rod-like: 20 (width) and 100 (length) [99]</td>
<td>Rutilte</td>
</tr>
<tr>
<td></td>
<td>Bi(OH)$_3$</td>
<td>Synthesized in this thesis (M1)</td>
<td>6.04 ± 0.89 (spherical)</td>
<td>–</td>
</tr>
</tbody>
</table>
The commercial sunscreens CS1 and CS2 were used for photocatalytic activity tests, which involve the exterior exposure of pre-painted steel panels. In addition, the inorganic sunscreen components were separated from these two sunscreens. Rheological properties were tested of the commercial sunscreens CS3 and CS4.

Table 4.6. Overview of the commercial sunscreens (CS) used.

<table>
<thead>
<tr>
<th>ID</th>
<th>UV filter</th>
<th>Sunscreen</th>
<th>Particle size (nm) and morphology</th>
<th>Crystal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS1</td>
<td>TiO$_2$</td>
<td>Nivea® (SPF 50)</td>
<td>~ 25 (ellipsoidal, cubic)</td>
<td>80% anatase,  20% rutile</td>
</tr>
<tr>
<td>CS2</td>
<td>TiO$_2$</td>
<td>Nivea® anti-age (SPF 30)</td>
<td>Rod-like: 20 (width) and 100 (length) [99]</td>
<td>Rutile</td>
</tr>
<tr>
<td>CS3</td>
<td>Organics</td>
<td>OMBRA® (SPF 50)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CS4</td>
<td>Organics</td>
<td>Auscreen® (SPF 50)</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

4.3.2 Identification, Size, Morphology, and Surface Composition of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles

Figure 4.21 displays the XRD patterns of the synthesized Bi(OH)$_3$ (M1) NPs and the as-received TiO$_2$ (M2) and ZnO (M3) nanopowders. The XRD pattern of the Bi(OH)$_3$ NPs shows reflections which are identified as single phase but are broadened due to the very small particle size [17]. Since no JCPDS file can be assigned to the Bi(OH)$_3$, the XRD pattern is not indexed. The XRD pattern of the as-received ZnO NPs shows the single zincite structure (JCPDS 00-036-1451), whereas the TiO$_2$ NPs are composed of mixed phases of anatase (JCPDS 01-075-2552) and rutile (JCPDS 01-075-1753).
Chapter 4 – Bismuth-Based Compounds for Biomedical Applications

**Figure 4.21.** XRD patterns of Bi(OH)$_3$ (M1, top), ZnO (M3, centre), and TiO$_2$ (M2, bottom) nanoparticles.

Figure 4.22 shows TEM images of all the nanomaterials. The Bi(OH)$_3$ (M1) NPs are mostly agglomerates ~100 nm in size, with an average single particle size of $6.04 \pm 0.89$ nm, and round and ellipsoidal shapes (left images). The chosen image does not represent the average particle size of 6 nm, but still falls into the particle size distribution that varies between 2 – 14 nm, as shown in Figure 4.23.

**Figure 4.22.** TEM images of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) nanoparticles.

The TiO$_2$ (M2) NPs are mostly spherical, ellipsoidal, and cubic in shape, with similar particle sizes of $24.08 \pm 1.60$ nm (centre right image). The ZnO (M3) NPs are composed of particles with various morphologies, such as ellipsoidal, cubic, spherical, and hexagonal, with an average particle size of $44.86 \pm 6.38$ nm (Figure 4.23(c)). There is also a significant amount of rectangular plate-like particles with an average length of $92.99 \pm 20.97$ nm and width of $44.52 \pm 5.50$ nm, making the ZnO NPs the largest in comparison to the Bi(OH)$_3$
and TiO$_2$ NPs. Moreover, since the particle sizes vary greatly, the associated error is consequently much higher. For the particle size distribution of the ZnO NPs, the cross-section of the rectangular-shaped particles was used to calculate an equivalent spherical diameter.

**Figure 4.23.** Particle size distribution of (a) Bi(OH)$_3$ (M1), (b) TiO$_2$ (M2), and (c) ZnO (M3) nanoparticles.
The BET specific surface area of all materials was determined to be $47.8 \pm 0.5 \text{ m}^2/\text{g}$, $21.8 \pm 0.2 \text{ m}^2/\text{g}$, and $6.4 \pm 0.1 \text{ m}^2/\text{g}$ for the TiO$_2$ (M2), Bi(OH)$_3$ (M1), and ZnO (M3) NPs, respectively.

To investigate if the surface composition deviates from the bulk composition, XPS analysis was performed. Figure 4.24 displays both the high-resolution XPS spectra of the O 1s peak and the deconvolution results for the Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) NPs. The survey spectra of all nanomaterials are shown in Figure A11 in the Appendix I. The O 1s peak for all tested nanomaterials is resolved into multiple peaks with different intensities relative to each other. The O 1s peaks for Bi(OH)$_3$ at binding energies of 530.43 ± 0.04 eV and 532.81 ± 0.13 eV can be assigned to O$^-$ ions in Bi–O bonds [74] and hydroxide species (Bi–OH) adsorbed on the NP surfaces [74, 75], respectively.

Similarly to Bi(OH)$_3$, the O 1s peak for ZnO at 530.36 ± 0.25 eV represents O$^-$ ions in Zn–O bonds, and the peak at 531.95 ± 0.10 eV can be assigned to hydroxide and chemisorbed oxygen species (Zn–OH) [101].

The O 1s spectrum of TiO$_2$ displays peaks at 528.96 ± 0.08 eV associated with lattice oxygen in Ti–O and at 530.90 ± 0.11 eV, which is ascribed to hydroxyl groups (Ti–OH) on the surface, while the peak at 531.95 ± 0.16 eV corresponds to chemisorbed water (Ti–OH$_2$) [102].
Figure 4.24. High-resolution XPS spectra of the O 1s region of (a) Bi(OH)$_3$ (M1), (b) TiO$_2$ (M2), and (c) ZnO (M3) nanoparticles.

4.3.3 Ultraviolet-Visible Study of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles

The UV-visible absorption spectra of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) NPs are shown in Figure 4.25. The absorption spectra were all obtained at the same concentration (25 µg/mL) and indicate a higher and more selective absorbance of TiO$_2$ NPs within the UV region compared to ZnO and Bi(OH)$_3$ NPs. A comparison of the latter two
shows a significant increase in the absorbance maximum for the Bi(OH)$_3$ NPs, which is similar to that of TiO$_2$ in lower wavelengths < 300 nm, while the ZnO NPs show a reduced and broader absorbance throughout the whole UV range. Therefore, TiO$_2$ NPs are considered UVB and UVA2 (320 – 340 nm) blocker, while ZnO NPs provide protection in the UVB and UVA1 (340 – 400 nm) area [36]. According to these categories, Bi(OH)$_3$ NPs would – similarly to TiO$_2$ – be a suitable blocker of UVB radiation, and thus, a potential candidate as active sunscreen ingredient.

![Absorption spectra of Bi(OH)$_3$, TiO$_2$, and ZnO nanoparticles](image)

**Figure 4.25.** UV-visible absorption spectra of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) nanoparticles at 25 µg/mL in deionized water. The concentration of the combined materials (ZnO and Bi(OH)$_3$; TiO$_2$ and Bi(OH)$_3$) is 25 µg/mL each, implying a total concentration of 50 µg/mL.

This observation is reflected in the increase of the optical direct band gap of the Bi(OH)$_3$ NPs with 4.06 ± 0.08 eV, compared to TiO$_2$ NPs with 3.39 ± 0.07 eV and ZnO NPs with 3.08 ± 0.05 eV. The band gaps were calculated using a Tauc plot (Figure 4.26). There is no literature available in regard to the measurement or calculation of the band gap of Bi(OH)$_3$, and the results, which were obtained in this thesis, suggest the presence of a direct band gap at 4.06 ± 0.08 eV.
Figure 4.26. Tauc plots of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) nanoparticles. The red lines represent extrapolated band gaps corresponding to Bi(OH)$_3$, TiO$_2$, and ZnO nanoparticles.

With its wide band gap, Bi(OH)$_3$ NPs have a high optical transparency in the visible light range, making it potentially viable as a cosmetic material. The band gaps are in agreement with the literature, where the band gap for ZnO ranges from 3.1 to 3.3 eV [103]. The band gap for TiO$_2$ ranges from approximately 3.0 eV (rutile), to 3.13 eV (brookite) and 3.2 eV (anatase) [104].
The combination of materials, in particular TiO\textsubscript{2} and Bi(OH)\textsubscript{3}, and ZnO and Bi(OH)\textsubscript{3} with equal concentrations (each material = 25 µg/mL) results in absorption spectra that show the characteristics of both materials. For instance, the combination of ZnO and Bi(OH)\textsubscript{3} NPs clearly shows the peak maximum at approximately 375 nm, which corresponds to ZnO, and a linear increase in absorbance starting at approximately 300 nm, which is ascribed to Bi(OH)\textsubscript{3}.

**4.3.4 Photocatalytic Activity of Bi(OH)\textsubscript{3}, TiO\textsubscript{2}, and ZnO Nanoparticles under Ultraviolet Irradiation**

Intuitively, the presence of photocatalytically active anatase TiO\textsubscript{2} would not be expected in sunscreen formulations for topical application since TiO\textsubscript{2}, in particular the anatase phase, is known to be a highly active photocatalyst, which generates •OH radicals upon exposure to UV light [105]. Several recent studies, however, have confirmed the presence of a highly photoactive P25 phase in commercial formulations [105-107].

**4.3.4.1 Under Ultraviolet Exposure (300 nm and 350 nm)**

The photoactivity of Bi(OH)\textsubscript{3} (M1), TiO\textsubscript{2} (M2), and ZnO (M3) NPs was evaluated under UV irradiation using lamps with emission at 300 nm (UVB) and 350 nm (UVA), and is shown in Figure 4.27(a). The exposure of a crystal violet solution with TiO\textsubscript{2} (M2, 5 mg/L) leads to significant degradation, with only 10% of the initial absorbance present after irradiation with UV light for 30 min. Similarly, the addition of ZnO NPs, which are also known for their photocatalytic activity, also resulted in degradation albeit at a reduced rate.
Figure 4.27. (a) Relative decrease in absorbance of crystal violet solutions containing only the dye, ZnO (M3, 5 mg/L), TiO\(_2\) (M2, 5 mg/L), and Bi(OH)\(_3\) (M1) nanoparticles with concentrations ranging from 1 to 5 mg/L, and combinations of M2 (5 mg/L) + M1 (1–5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (300 nm and 350 nm). The data represent the mean of three independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).
In contrast, the exposure to Bi(OH)$_3$ NPs at the same concentration leads to little dye degradation over a similar period, meaning that the NPs do not generate many ROS upon exposure to UV light. Moreover, the small photocatalytic activity of the Bi(OH)$_3$ NPs is most likely not related to the reduced number of active sites for the photo degradation reaction in comparison to TiO$_2$, since the surface area of the ZnO NPs is even smaller, with the NPs displaying greater photodegradation than the Bi(OH)$_3$ NPs. The large band gap of > 4 eV for Bi(OH)$_3$ makes the excitation of electrons from the valence band to the conduction band less likely.

When added to suspensions of TiO$_2$ or ZnO prior to irradiation, the Bi(OH)$_3$ NPs cause a significant decrease in crystal violet degradation and apparent rate constant $k$, as shown in Table 4.7. The plots used to find the apparent rate constant were calculated with help of the Langmuir–Hinshelwood model [108] and are shown in Figure 4.27(b). More importantly, the dye degradation is reduced close to that of M1 alone indicating almost complete reduction of the photocatalytic activity of TiO$_2$.

Since TiO$_2$ is the more active photocatalyst, this reduction in activity was studied further by varying the concentration of mechanically added Bi(OH)$_3$ NPs. The reduction in photocatalytic activity is greater for the addition of lower concentrations of Bi(OH)$_3$ NPs. This effect is most likely not only related to the effects of absorption and scattering from the additional particles, as a substantial decrease in the degradation of crystal violet can also be observed for the lowest concentration of Bi(OH)$_3$ NPs used (1 mg/L).
Table 4.7. Apparent rate constant \( k \) for the degradation of crystal violet solutions containing TiO\(_2\) (M2, 5 mg/L), ZnO (M3, 5 mg/L), and Bi(OH)\(_3\) (M1) nanoparticles, in concentrations ranging from 1 to 5 mg/L, and combinations of M2 (5 mg/L) + M1 (1–5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (300 nm and 350 nm), as shown in Figure 4.27. The errors indicated are the standard deviation obtained through the linear regression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( k \times 10^{-2} ) (min(^{-1}), UV irradiation (300 &amp; 350 nm))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye only</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>TiO(_2) (5 mg/L)</td>
<td>7.08 ± 0.22</td>
</tr>
<tr>
<td>ZnO (5 mg/L)</td>
<td>1.45 ± 0.07</td>
</tr>
<tr>
<td>Bi(OH)(_3) (5 mg/L)</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>ZnO (5 mg/L) + Bi(OH)(_3) (5 mg/L)</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>TiO(_2) (5 mg/L) + Bi(OH)(_3) (5 mg/L)</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>Bi(OH)(_3) (2.5 mg/L)</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>TiO(_2) (5 mg/L) + Bi(OH)(_3) (2.5 mg/L)</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>Bi(OH)(_3) (1 mg/L)</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>TiO(_2) (5 mg/L) + Bi(OH)(_3) (1 mg/L)</td>
<td>0.47 ± 0.02</td>
</tr>
</tbody>
</table>

CeO\(_2\), for instance, has the ability to ‘scavenge’ free radicals due to the presence of a large number of surface defects on the surfaces of CeO\(_2\) NPs for small particle sizes, which can lead to oxygen deficiencies and reversible oxidation state changes in the cation [95, 109]. This mechanism enables scavenging of excess free radical species, and consequently, the photocatalytic activity of photocatalysts is reduced [110].

It is therefore likely that the reduced degradation of crystal violet is related to the scavenging of •OH radicals generated from the photocatalytically active TiO\(_2\) and/or the reduction of active surface sites on the TiO\(_2\) surface through the adsorption of Bi(OH)\(_3\) NPs. Very low concentrations of Bi(OH)\(_3\) NPs are enough to reduce the photocatalytic activity of TiO\(_2\) significantly: by adding a suspension of Bi(OH)\(_3\) NPs that resulted in a concentration
of 1 mg/L to a suspension of TiO$_2$ at a concentration of 5 mg/L prior to irradiation, the degradation of crystal violet decreased by more than 90%. This ultimately means that in the final suspension the ratio Bi(OH)$_3$:TiO$_2$ is 1:5.

There are no reports on the scavenging effects of Bi(OH)$_3$ NPs, but the very small diameter of the NPs suggests that the effect might be related to the surface properties of the material. The Bi(OH)$_3$ NPs display a very small particle size with low crystallinity, resulting in the presence of more surface defects, which, in turn, can act as ROS scavengers.

### 4.3.4.2 Under Ultraviolet Exposure (350 nm)

In order to exclude the possibility that Bi(OH)$_3$ NPs absorb the radiation in the UV range and thus reduce the effective UV radiation for the TiO$_2$ and ZnO NPs, photocatalytic activity tests were performed with a modified UV range. As shown via UV-visible spectroscopy, the band gap of Bi(OH)$_3$ NPs lies at 305 nm (4.06 eV), and for TiO$_2$ and ZnO at 366 nm (3.39 eV) and 403 nm (3.08 eV), respectively. By limiting the UV radiation only to lamps with an emission at 350 nm (UVA), the absorbance of UV radiation by Bi(OH)$_3$ NPs can be minimized. At the same time, the energy is sufficient to induce photocatalytic effects in TiO$_2$ and ZnO NPs. The degradation of crystal violet, including the apparent rate constant, is shown in Figure 4.28, which gives results for ZnO, TiO$_2$, and Bi(OH)$_3$ NPs at a concentration of 5 mg/L.
Figure 4.28. (a) Relative decrease in absorbance of crystal violet solutions containing only the dye, Bi(OH)$_3$ (M1, 5 mg/L), TiO$_2$ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles, and combinations of M2 (5 mg/L) + M1 (5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (350 nm). The data represent the mean of three independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).

The photodegradation properties of crystal violet, for all nanomaterials under light irradiation at 350 nm are summarized in Table 4.8. The performance of the ZnO NPs remains comparable to a full irradiation with both 350 (UVA) and 300 (UVB) nm lamps, most probably due to the absorbance in the broader wavelength range, with the absorbance maximum reached at approximately 375 nm, while TiO$_2$ shows a clear absorbance peak at wavelengths < 300 nm.
Table 4.8. Apparent rate constant \( k \) for the degradation of crystal violet solutions containing \( \text{Bi(OH)}_3 \) (M1, 5 mg/L), \( \text{TiO}_2 \) (M2, 5 mg/L), and \( \text{ZnO} \) (M3, 5 mg/L) nanoparticles, and combinations of M2 (5 mg/L) + M1 (5 mg/L) and M3 (5 mg/L) + M1 (5 mg/L) under ultraviolet light exposure (350 nm), as shown in Figure 4.28. The errors indicated are the standard deviation obtained through the linear regression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( k \times 10^{-2} ) (min(^{-1}), UV irradiation (350 nm))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye only</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>( \text{TiO}_2 ) (5 mg/L)</td>
<td>1.25 ± 0.06</td>
</tr>
<tr>
<td>( \text{ZnO} ) (5 mg/L)</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>( \text{Bi(OH)}_3 ) (5 mg/L)</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>( \text{TiO}_2 ) (5 mg/L) + ( \text{Bi(OH)}_3 ) (5 mg/L)</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>( \text{ZnO} ) (5 mg/L) + ( \text{Bi(OH)}_3 ) (5 mg/L)</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

The addition of \( \text{Bi(OH)}_3 \) NPs to the crystal violet solution results in very low dye degradation with an apparent rate constant \( k = 0.08 \pm 0.01 \) min\(^{-1}\), which is very similar to the control sample of only crystal violet with \( k = 0.02 \pm 0.01 \) min\(^{-1}\). It is therefore likely that the irradiation at 350 nm does not lead to any significant absorbance by \( \text{Bi(OH)}_3 \) and that the decreased photocatalytic activity of \( \text{TiO}_2 \) NPs can be assigned to either a mechanism related to the active scavenging of photogenerated intermediates, or, more likely, the decrease in active surface area through the physical adsorption of \( \text{Bi(OH)}_3 \) NPs. Consequently, the addition of a suspension of \( \text{Bi(OH)}_3 \) (5 mg/L) to a suspension of \( \text{ZnO} \) (5 mg/L) appeared to completely inhibit crystal violet degradation, while addition of \( \text{Bi(OH)}_3 \) to \( \text{TiO}_2 \) (5 mg/L) reduced the photocatalytic activity by 70%.

4.3.5 Photocatalytic Activity of \( \text{Bi(OH)}_3 \), \( \text{TiO}_2 \), and \( \text{ZnO} \) Nanoparticles under Simulated Solar Irradiation

In order to test the photocatalytic activity under conditions that are more realistic for sunscreen applications, suspensions of \( \text{Bi(OH)}_3 \) (M1), \( \text{TiO}_2 \) (M2), and \( \text{ZnO} \) (M3) NPs in presence of crystal violet were exposed to AM 1.5 G one sun (100 mW/cm\(^2\)) for a total time
of 6 h. The degradation curves and apparent rate constant curves are displayed in Figure 4.29, and highlight the degradation of crystal violet when exposed to TiO$_2$ and ZnO NPs.

![Degradation curves](image)

**Figure 4.29.** (a) Relative decrease in absorbance of crystal violet solutions containing Bi(OH)$_3$ (M1, 5 mg/L), TiO$_2$ (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles under exposure of AM 1.5 G one sun (100 mW/cm$^2$). The data represent the mean of three independently prepared samples. (b) Apparent rate constant curves for the dye degradation shown in (a).

Although there is some visible decrease in dye degradation for the treatment with Bi(OH)$_3$ NPs, these NPs show a much lower photocatalytic activity compared to TiO$_2$ and ZnO NPs, especially in the early hours of irradiation (0 – 2 h) Table 4.9.
Table 4.9. Apparent rate constant \( k \) for the degradation of crystal violet solutions containing Bi(OH)\(_3\) (M1, 5 mg/mL), TiO\(_2\) (M2, 5 mg/L), and ZnO (M3, 5 mg/L) nanoparticles under exposure to AM 1.5 G one sun (100 mW/cm\(^2\)). The errors indicated are the standard deviation obtained through the linear regression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( k \times 10^{-2} ) (min(^{-1}), sunlight irradiation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye only</td>
<td>0.47 ± 0.10</td>
</tr>
<tr>
<td>TiO(_2) (5 mg/L)</td>
<td>46.67 ± 0.99</td>
</tr>
<tr>
<td>ZnO (5 mg/L)</td>
<td>18.91 ± 2.23</td>
</tr>
<tr>
<td>Bi(OH)(_3) (5 mg/L)</td>
<td>4.58 ± 0.12</td>
</tr>
</tbody>
</table>

4.3.6 Sun Exposure of Homemade Sunscreens and Commercial Sunscreens

One major drawback of modern sunscreen formulations is the aggressive degradation of third party applications, such as surface coatings on wood and steel, which is mediated by photocatalytic reactions [111, 112]. The photocatalytic mechanism includes the formation of radicals if oxygen, water, and light are present.

In order to test the reduced photocatalytic activity of the Bi(OH)\(_3\) NPs in comparison to the photocatalytically more active ZnO NPs, both homemade sunscreen emulsions (with additional TiO\(_2\) NPs: HS1, HS2) were applied on dark blue pre-painted steel sheets. The base emulsion was also tested to determine the photocatalytic activity of the homemade sunscreens without added NPs. In addition, two commercially available sunscreen formulations were tested: CS1 (Nivea® (SPF 50)) and CS2 (Nivea® anti-age (SPF 30)), which also have organic compounds in addition to TiO\(_2\) NPs as active ingredients.

The degradation of the test panels was assessed via determination of the gloss and SEM imaging of the panel’s surfaces, as discussed below.

4.3.6.1 Gloss Readings of the Test Panels

After an exterior exposure of six and twelve weeks, a 30 mm wide test strip was cut of each panel, and the gloss was measured (Figure 4.30). While the blank test panel...
maintained the same gloss after twelve weeks, the treatment with commercial sunscreen CS1 (Nivea® (SPF 50)) lead to a significant decrease in gloss. In addition, the surfaces were roughened and disfigured after only six weeks exposure, and the pigment started to disappear after twelve weeks exposure, which underscores the aggressive, photocatalytically initiated degradation [111].

![Graph showing gloss readings of pre-painted steel panels](image)

**Figure 4.30.** Gloss readings of pre-painted steel panels without, and panels treated with commercial sunscreen formulations (CS1: Nivea® (SPF 50); CS2: Nivea® anti-age (SPF 30)), the homemade base emulsion, and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2) after exposure to the Australian sun at a latitude of ~34.5°S (Wollongong, NSW) for six and twelve weeks.

In contrast, steel panels that were treated with the homemade base emulsion (‘all organic’) or the homemade sunscreens containing TiO$_2$/ZnO and TiO$_2$/Bi(OH)$_3$, showed only a small decrease in gloss and no visible signs of defects after the full exposure of twelve weeks.

Interestingly, after twelve weeks of sun exposure, the gloss readings of the panels treated with the base emulsion (26.1 GU), and the TiO$_2$/ZnO (27.8 GU) and TiO$_2$/Bi(OH)$_3$
(37.5 GU) emulsions are much higher than of the untreated control steel panels (22.7 GU). An increase in gloss has been observed earlier [111] and is associated with a very strong adhesion of the emulsion to the surface of the panels, which leads to a reduced removal due to rainfall over the whole tested exposure time.

While the ingredients of sunscreen formulations have particles with sizes of 200 – 300 nm (TiO$_2$), the matting agents, which were used to coat the steel panels before the sunscreen formulations were applied, are made of particles large in size (7 – 10 µm) and irregular in shape, which ultimately leads to a roughening of the panels [111]. The homemade sunscreens reduced the initial roughness of the pre-painted steel panels, which leads to a higher gloss reading than for the untreated panels.

In the period of twelve weeks, however, the homemade sunscreens can lose their adhesion to the surface of the panels, which ultimately results in a decrease in gloss reading that is not related to the photocatalytic activity of the sunscreen ingredients [111]. The coating with the base emulsion, for instance, yields reduced gloss of the panels, although it does not contain any photocatalytically active inorganic particles.

The overall higher gloss readings without any significant disfiguration of the sample’s surface of the homemade sunscreen containing TiO$_2$/Bi(OH)$_3$ indicates a reduced photocatalytic activity in comparison to the homemade sunscreen containing TiO$_2$/ZnO. This conclusion is also supported by the fact that the only difference of the emulsion is the replacement of ZnO NPs with Bi(OH)$_3$ NPs, while keeping all other ingredients the same. Furthermore, it has been shown that – similarly to TiO$_2$ – ZnO NPs can be photocatalytically active, reducing the gloss data in comparison to untreated steel panels [111, 113].

Since the reduction in gloss of the panels is attributable to both photocatalytically active sunscreen ingredients and the loss of adhesion of the applied sunscreen to the panel’s surface [111], the panels have also been characterized via SEM, as shown below.
4.3.6.2 Scanning Electron Microscopy Imaging of the Test Panels

After an exterior exposure of twelve weeks, SEM images of the test panels were collected to investigate the surface morphology (Figure 4.31). The untreated control test panel (top row, left image) maintained a smooth surface after the exposure, with the surface coating still intact. The panels treated with the commercial sunscreens, however, showed signs of photocatalytic degradation: while the treatment with CS2 (top row, centre image) results in minor damage to the panel’s surface, the treatment with CS1 (top row, right image) clearly leads to the degradation of the surface coating of the steel panels. It has been shown that the photocatalytically active particles will first destroy the sunscreen matrix, followed by a decomposition of the coating [111].

![SEM images of test panels](image)

**Figure 4.31.** SEM images of pre-painted steel panels without, and panels treated with, commercial sunscreen formulations (CS1: Nivea® (SPF 50); CS2: Nivea® anti-age (SPF 30)), the homemade base emulsion, and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2) after exposure to the Australian sun at a latitude of ~34.5°S (Wollongong, NSW) for twelve weeks.

Similarly, the steel panels treated with the homemade TiO$_2$/ZnO emulsion (HS1, bottom row, centre image) display a visible surface roughness, although it is lower in comparison to a treatment with CS1.
In contrast, panels coated with the base emulsion prepared in this work (‘all organics’; bottom row, left image) and the TiO$_2$/Bi(OH)$_3$ emulsion (HS2, bottom row, right image) showed no visible degradation of the panel surface, underlining the lack of photocatalytic activity of the TiO$_2$/Bi(OH)$_3$ emulsion in comparison to the TiO$_2$/ZnO emulsion. This conclusion is also supported by the fact that the only difference in the emulsions is the replacement of ZnO NPs with Bi(OH)$_3$ NPs, while keeping all other ingredients the same.

These results support the findings that were obtained from the photocatalytic degradation of crystal violet. Compared with TiO$_2$ and ZnO, Bi(OH)$_3$ NPs showed the lowest photocatalytic activity and more importantly, Bi(OH)$_3$ NPs were able to reduce the photocatalytic activity of TiO$_2$ NPs. It was previously shown that the formation of defects is associated with the photocatalytic activity of particular sunscreen components and that the photocatalytic degradation accelerates the weathering of the coating by 100 fold [111].

4.3.6.3 Separation of Inorganic Sunscreen Components

The difference in photostability of the tested commercial sunscreens is related to the phase of the TiO$_2$ NPs present in the formulations. The separation of the sunscreen ingredients and the XRD analysis of the inorganic components identified either the rutile phase or a combination of rutile and anatase TiO$_2$ [106, 111, 114]. In the present case, sunscreen CS1 contains an anatase and rutile composite phase similar to M2, while sunscreen CS2 contains a single rutile TiO$_2$ phase as shown in Figure 4.32.

Generally, anatase TiO$_2$ is photocatalytically more active than rutile TiO$_2$ [114, 115], but the mixed phase with 85% anatase and 15% rutile is particularly active [116]. The homemade TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2) sunscreens have Eusolex® T-S as ingredient, which is an inorganic UV filter composed of rutile type TiO$_2$ and thus, less photocatalytically active.
4.3.7 Rheological Properties of Homemade Sunscreens and Commercial Sunscreens

An important factor that needs consideration when formulating sunscreen products is their uniformity, which is related to rheology. The dependence of the viscosity on the shear rate is shown in Figure 4.33 and was measured for the homemade sunscreens of TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2), as well as for two commercially available sunscreen products. In order to classify the spreadability of the homemade emulsions, two sunscreen products (CS3, CS4) with different viscosity were chosen.

The commercial sunscreen, which is dispensed directly from the bottle (CS3: OMBRA® (SPF 50)), showed the highest viscosity, while the sunscreen applied through a spray dispenser (CS4: Auscreen® (SPF 50)) displayed the lowest viscosity. Both homemade sunscreen emulsions of TiO$_2$/ZnO and TiO$_2$/Bi(OH)$_3$ exhibit a viscosity in between the two commercially available sunscreen products, which emphasizes the adequate spreadability of the homemade emulsions and the associated eligibility for the use in sunscreen products.
formulations. More importantly, both homemade emulsions demonstrate comparable rheological properties, exhibiting greater viscosity for lower shear rates.

![Image](image.png)

**Figure 4.33.** Viscosity measurements of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and homemade sunscreens containing TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2).

While both commercial sunscreens show a linear relationship between the viscosity and the shear rate, the homemade sunscreens show non-linearity for higher shear rates. The increase in viscosity is associated with the presence of agglomerates of NPs, since the concentration of ceramic NPs is 8 wt.%. This effect is less pronounced for the homemade emulsion of TiO$_2$/Bi(OH)$_3$, which underscores the greater homogeneity in comparison to the ‘classical’ emulsion of TiO$_2$/ZnO.

The relationship between the shear rate and the shear stress is shown in Figure 4.34. All tested sunscreen formulations exhibited nonlinear behaviour and consequently can be referred to as non-Newtonian fluids [117]. The flow index $n_{flow}$ classifies the deviation of a system from Newtonian behaviour, which is found for $n_{flow} = 1$ [118]. Pseudoplasticity or shear thinning is typically indicated by $n < 1$, whereas shear thickening is present for values of $n_{flow} > 1$ [119].
Chapter 4 – Bismuth-Based Compounds for Biomedical Applications

Figure 4.34. Rheograms of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and homemade sunscreens containing TiO₂/ZnO (HS1) and TiO₂/Bi(OH)₃ (HS2).

As displayed in Table 4.10, the sunscreens showed a flow index ranging from 0.24 to 0.68, indicating pseudoplastic behaviour for all tested sunscreens. The more viscous OMBRA® (SPF 50) sunscreen displayed the lowest flow index, which agrees with the generally lower flow rate of thicker bases. The advantage of a pseudoplastic fluid lies in its shear thinning properties, allowing for it to be easily spread on the skin, while simultaneously preventing the applied film from running, as its viscosity instantly increases when no shear is applied [120]. In contrast, a Newtonian fluid runs quickly once applied to the skin, consequently reducing the protective effect of the applied film [121].

It is important to note that the flow index of the homemade TiO₂/Bi(OH)₃ (HS2) sunscreen is very similar to that of the commercially available Auscreen® (SPF 50) sunscreen, whereas the ‘classical’ homemade sunscreen of TiO₂/ZnO (HS1) exhibits a significantly greater flow index closer to those of Newtonian fluids.
Table 4.10. Flow index ($n_{flow}$) and consistency index ($K$) of two commercially available sunscreens (CS3: OMBRA® (SPF 50); CS4: Auscreen® (SPF 50)) and the homemade sunscreens of TiO$_2$/ZnO (HS1) and TiO$_2$/Bi(OH)$_3$ (HS2). The errors indicated are the standard deviation obtained through the linear regression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$n_{flow}$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMBRA® (SPF 50) (CS3)</td>
<td>0.238 ± 0.003</td>
<td>3175.411 ± 63.955</td>
</tr>
<tr>
<td>Auscreen® (SPF 50) (CS4)</td>
<td>0.455 ± 0.001</td>
<td>29.000 ± 1.682</td>
</tr>
<tr>
<td>TiO$_2$/ZnO emulsion (HS1)</td>
<td>0.679 ± 0.016</td>
<td>62.765 ± 6.475</td>
</tr>
<tr>
<td>TiO$_2$/Bi(OH)$_3$ emulsion (HS2)</td>
<td>0.481 ± 0.023</td>
<td>180.082 ± 27.271</td>
</tr>
</tbody>
</table>

The consistency index $K$ of the formulations was calculated to range between 29 for the Auscreen® (SPF 50) sunscreen and 3175 for the more viscous OMBRA® (SPF 50) sunscreen. These values are in agreement with commonly reported consistency indexes [119]. Generally, a high consistency index is associated with low spreadability, which is in agreement with the obtained results. More importantly, the homemade TiO$_2$/Bi(OH)$_3$ (HS2) sunscreen showed a consistency index significantly smaller than that of the OMBRA® (SPF 50) sunscreen, which underscores the improved spreadability properties of the TiO$_2$/Bi(OH)$_3$ sunscreen.

It was further shown that the viscosities of seven tested sunscreen formulations had a correlation with their SPF values: an increased SPF value always coincided with a higher viscosity [119]. These findings are in agreement with the obtained results for the two homemade sunscreens: the TiO$_2$/Bi(OH)$_3$ (HS2) sunscreen with a larger SPF value also shows greater viscosity in comparison to the ‘classical’ sunscreen with TiO$_2$/ZnO (HS1) (see section 4.3.8).
4.3.8 Photoprotection of Homemade Sunscreens

The UV absorbance of the 'classical' sunscreen made of TiO$_2$/ZnO (HS1), and of the sunscreen made of TiO$_2$/Bi(OH)$_3$ (HS2) was determined in vitro over the entire UV spectrum (290 – 400 nm) using substrate spectrophotometry.

According to the recommendation of the European Commission, the ‘classical’ sunscreen with a measured SPF of 39.02 ± 1.26 can be classified in the category of ‘high protection’ [100]. In addition, this sunscreen shows the characteristics of a broad spectrum sunscreen which can give photoprotection against longer UV wavelengths, since the critical wavelength of 372.88 ± 0.07 nm is higher than 370 nm. The UVA-PF is 14.99 ± 0.39.

The sunscreen made of TiO$_2$ and Bi(OH)$_3$ is significantly more efficient in the UVA and UVB range, with a SPF of 49.93 ± 1.66, which represents an increase of 28% compared to the ‘classical’ sunscreen. Furthermore, the TiO$_2$/Bi(OH)$_3$ sunscreen shows a UVA-PF of 16.57 ± 0.38, which is equivalent to an increase of 11% in comparison to the TiO$_2$/ZnO sunscreen. The UVA-PF is 33.2% of the total SPF for the TiO$_2$/Bi(OH)$_3$ sunscreen and therefore falls within the recommendations of the United States Food and Drug Administration (FDA) [122].

The obtained SPF is significantly larger than previously reported SPF values, such as for CeO$_2$ NPs. For instance, it was shown that a sunscreen of TiO$_2$ and calcium-doped CeO$_2$ NPs resulted in a maximum SPF of approximately 40 with a UVA-PF of approximately 11.5 [123].

The critical wavelength of the TiO$_2$/Bi(OH)$_3$ sunscreen was determined to be 372.01 ± 0.08 nm, resulting in broad-spectrum protection according to the FDA [122]. Since the critical wavelength only depends on the shape and width of the absorbance spectrum, and not on the amplitude or the thickness of sunscreen application, the protection in the high-wavelength UVA region of both tested sunscreens is underlined.
An *in-vitro* study of 59 commercially available sunscreen products with UVA-filters, such as TiO$_2$ and ZnO, showed that only 10% of the tested sunscreens had a critical wavelength ≥ 370 nm [117]. This study demonstrates that a simple addition of long wavelength UVA active ingredients does not ensure true broad-spectrum protection, and thus underscores the importance of the obtained results for the homemade sunscreen of TiO$_2$/Bi(OH)$_3$.

### 4.3.9 Cytotoxicity of Bi(OH)$_3$, TiO$_2$, and ZnO Nanoparticles in HaCaT and MDCK Cells

In order to be considered a potential material in sunscreen formulations, the Bi(OH)$_3$ NPs need to be biocompatible. The cytotoxicity of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) NPs was determined using the MTT assay, which is often employed to evaluate the effects of anticancer drugs [96].

Mitochondria are crucial for the maintenance of cellular function via aerobic adenosine triphosphate production and are therefore targets for toxic injury by numerous types of compounds [124]. Since the reduction of the tetrazolium salt occurs only in functional mitochondria, a decrease in MTT dye reduction indicates mitochondrial damage.

Non-cancerous human skin cells (HaCaT) and dog kidney cells (MDCK) were chosen, which were exposed to the NPs for 24 h at concentrations ranging from 5 to 500 µg/mL (Figure 4.35). While HaCaT keratinocytes are typically used to examine potential adverse effects of NPs in sunscreens [125, 126], MDCK cells are typically used to determine biocompatibility of nanomaterials since kidney cells represent a possible detoxification route [127, 128]. So in addition to providing the essential results on HaCaT cells, the MDCK results provide a point of reference for other workers in the NP research area.
Figure 4.35. Effects of Bi(OH)$_3$ (M1), TiO$_2$ (M2), and ZnO (M3) nanoparticles on the mitochondrial function in non-cancerous human skin (HaCaT) cells and dog kidney (MDCK) cells. The cells were treated with the nanoparticles at concentrations of 0 (control), 5, 10, 25, 50, 100, 250, and 500 µg/mL for 24 h. At the end of exposure, the mitochondrial function was determined using the MTT reduction assay. The data are presented as the mean of three independently prepared experiments.

In general, a reduction in cell viability was observed in a dose-dependent manner for all the tested nanomaterials, which was more distinct in MDCK cells. The results indicate a significant decrease in mitochondrial activity for the ZnO NPs, with a cell viability of 48.7% and 0.5% in HaCaT and MDCK cells, respectively, at the highest tested concentration of 500 µg/mL. The TiO$_2$ NPs demonstrate only moderate cytotoxicity with a cell viability ranging between 61.5 – 71.2% at the same concentration. Similar findings regarding the cytotoxicity of ZnO and TiO$_2$ NPs in HaCaT cells are described in the literature [125].

The Bi(OH)$_3$ NPs exhibited only a minor change in the reduction of MTT dye for all the tested concentrations in both cell lines, indicating that the mitochondrial function was not altered much at these doses. At the highest tested dose of 500 µg/mL, the NPs showed
a cell viability of 83.5% and 82.5% in HaCaT and MDCK cells, respectively. The high biocompatibility of Bi(OH)$_3$ NPs compared to the controls points to their suitability for use in sunscreens as opposed to ZnO NPs.

### 4.3.10 Conclusions

The free radical generating properties of the TiO$_2$ and ZnO NPs currently used in some commercial sunscreen products could have the potential to cause adverse health effects, either through the direct interaction of these radicals with human tissue, or through the accelerated decomposition of other organic compounds within the formulation. These reactions not only have the potential to decrease the SPF of the formulation more rapidly, but also yield products with unknown effects on the human body.

In this thesis, the potential of colourless Bi(OH)$_3$ NPs that were synthesized through a facile, low-temperature route as an active ingredient in sunscreen formulations was investigated. The Bi(OH)$_3$ NPs showed low photocatalytic activity throughout the whole UV-visible spectrum, and more importantly, they were able to reduce the photocatalytic activity of TiO$_2$ and ZnO NPs. It was also shown that sunscreen formulations containing TiO$_2$/Bi(OH)$_3$ have better photostability than commercially available sunscreen formulations, while displaying excellent rheological properties.

With its comparable absorbance to ZnO and TiO$_2$ in the UV region, and its significantly increased SPF and UVA-PF, Bi(OH)$_3$ NPs offer a novel and cost-effective approach to improve the UV blocking properties of commercial sunscreen formulations and act as an antioxidant stabilizer for the organic components of the product, reducing the potential harm of these products to the health of the consumer.

Moreover, the Bi(OH)$_3$ NPs exhibited high biocompatibility in healthy HaCaT and MDCK cells, while introduction of ZnO NPs resulted in a significant decrease in cell viability over the exposure time of 24 h at the same tested concentrations.
4.4 TiO$_2$/\((\text{BiO})_2\text{CO}_3\) Nanocomposites for Ultraviolet Filtration with Reduced Photocatalytic Activity

In this section, the UV blocking properties and photocatalytic activity of nanocomposite materials consisting of TiO$_2$ NPs and homogeneously attached \((\text{BiO})_2\text{CO}_3\) clusters with a size < 10 nm onto their surfaces is presented. The nanocomposites were prepared using a facile precipitation approach with different atomic ratios of Bi/Ti of 0.02, 0.04, and 0.08. The synthesis procedure and all characterization methods are described in CHAPTER 3. The photocatalytic activity of the TiO$_2$/\((\text{BiO})_2\text{CO}_3\) nanocomposites and pristine TiO$_2$ NPs were investigated through monitoring crystal violet degradation under UVA/UVB irradiation and simulated solar irradiation. The cytotoxicity was determined in normal HaCaT and MDCK cells using the MTT assay at a concentration ranging from 5 to 500 µg/mL and an exposure time of 24 h.

4.4.1 Identification, Size, Morphology, and Surface Composition of TiO$_2$/\((\text{BiO})_2\text{CO}_3\) Nanocomposites

Figure 4.36 displays the XRD patterns of the commercial TiO$_2$ NPs and the synthesized nanomaterials, which can be identified as nanocomposites of the type TiO$_2$/\((\text{BiO})_2\text{CO}_3\). The XRD pattern of the TiO$_2$ NPs shows reflections attributed to the anatase (JCPDS 01-075-2552) and rutile (JCPDS 01-075-1753) phases. The nanocomposites do not alter the diffraction patterns of the core materials, but lead to additional reflections in the XRD pattern which are assigned to \((\text{BiO})_2\text{CO}_3\) (JCPDS 00-041-1488). Moreover, the relative intensity of the reflections increases with increased bismuth present in the nanocomposite.
Figure 4.36. XRD patterns of commercial TiO$_2$ NPs, and the TiO$_2$/$(\text{BiO})_2\text{CO}_3$ nanocomposites Bi/Ti 2, 4, and 8 at.%.

Typically, the synthesis of $(\text{BiO})_2\text{CO}_3$ requires the use of templates or hydrothermal treatments. In this work, however, the TiO$_2$ NPs act as primary nucleation centres during the nanocomposite synthesis. It was shown that TiO$_2$ NPs can act as substrate seeds on which a single spherical domain of inverse spinel iron oxide can be epitaxially grown [129]. The seeds can act as efficient catalysts and provide a lower energy barrier for heterogeneous nucleation, in comparison to homogeneous nucleation [130].

The two-step precipitation method used in this work includes the addition of excess ammonium hydroxide, which was used as the precipitating agent, yielding $(\text{BiO})_2\text{CO}_3$. It has been shown that the concentration of precipitating agent has a significant influence on the physical properties of $(\text{BiO})_2\text{CO}_3$ NPs [131]. Since no carbon source was added to the reaction mixture, the carbonate formation occurred due to the presence of aqueous CO$_2$. In aqueous solution, carbonic acid, carbon dioxide, bicarbonate, and carbonate exist together in a dynamic equilibrium, as shown in Equation (4.3), Equation (4.4), and Equation (4.5) [132]:
In acidic environments, the equilibrium shifts to \( \text{CO}_2(\text{aq}) \), while the addition of a base can shift the equilibrium to the carbonate. In this work, \((\text{BiO})_2\text{CO}_3\) clusters were precipitated using a very slow and dropwise addition of 30% \(\text{NH}_4\text{OH}\), increasing the pH value gradually. The reaction was complete at a pH of 8 – 9. These principles of carbonate equilibrium chemistry were already used to monitor the rate of inorganic carbon uptake by a variety of algal species [133].

The BET specific surface area of all materials was measured and is displayed in Table 4.11. TiO\(_2\) NPs typically show a high specific surface area close to 50 m\(^2\)/g [134]. The nanocomposites display a small decrease in surface area, associated with the coverage of the TiO\(_2\) surface by the \((\text{BiO})_2\text{CO}_3\) clusters, due to the increase in the overall size of the NPs.

**Table 4.11.** BET surface areas of TiO\(_2\)/\((\text{BiO})_2\text{CO}_3\) nanocomposites.\(^a\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>BET surface area (m(^2)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO(_2)</td>
<td>47.8 ± 0.5</td>
</tr>
<tr>
<td>Bi/Ti 2 at.%</td>
<td>47.2 ± 0.5</td>
</tr>
<tr>
<td>Bi/Ti 4 at.%</td>
<td>45.5 ± 0.5</td>
</tr>
<tr>
<td>Bi/Ti 8 at.%</td>
<td>43.6 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\) Data provided for the pure TiO\(_2\) NPs, and the TiO\(_2\)/\((\text{BiO})_2\text{CO}_3\) nanocomposites with different Bi/Ti atomic ratios. The indicated errors are the standard deviation.

Figure 4.37 and Figure 4.38 show SEM and STEM images of the pure TiO\(_2\) NPs, and the TiO\(_2\)/\((\text{BiO})_2\text{CO}_3\) nanocomposites with Bi/Ti atomic ratios of 0.02, 0.04, and 0.08. The commercial TiO\(_2\) is made up of mostly rectangular, spherical, ellipsoidal, and prism-like particles with an average particle size of 23.39 ± 8.59 nm. Figure 4.37 shows the SEM
images of the nanocomposite with Bi/Ti atomic ratios of 0.02, 0.04, and 0.08. For all nanocomposites, the SEM analysis does not show any significant difference in morphology to uncoated TiO$_2$.

![SEM images of TiO$_2$ NPs (left), and the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.](image)

**Figure 4.37.** SEM images of TiO$_2$ NPs (left), and the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.

The STEM images of the TiO$_2$/(BiO)$_2$CO$_3$ composites clearly show that the surfaces of the TiO$_2$ NPs are covered with very small (< 10 nm) (BiO)$_2$CO$_3$ clusters (Figure 4.38). The coverage with the (BiO)$_2$CO$_3$ clusters is homogeneous and increases with added (BiO)$_2$CO$_3$. The HAADF image of the nanocomposite with Bi/Ti 8 at.% (right image) reveals the interface between TiO$_2$ and (BiO)$_2$CO$_3$ clusters and shows rutile TiO$_2$ with interplanar spacings of 0.30 and 0.33 nm, which is in accordance with the d-spacings of (001) and (110) planes, respectively.

![HAADF STEM images of TiO$_2$ NPs (left), the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.](image)

**Figure 4.38.** HAADF STEM images of TiO$_2$ NPs (left), the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios. The HAADF image of the nanocomposite with Bi/Ti 8 at.% (right) exposes lattice fringes spaces of 0.30 and 0.33 nm, corresponding to (001) and (110) planes of rutile TiO$_2$, respectively.

The high resolution EDS mapping indicates that the uncoated TiO$_2$ NPs feature no Bi (Figure 4.39) while a significant proportion of Bi can be found on the surfaces of the
particles for the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites. The EDS maps of the nanocomposites displayed a homogeneous distribution of the Bi on the surfaces of the TiO$_2$ particles. Uncovered TiO$_2$ NPs were not found anywhere in the imaged specimens, even for the composite with the smallest amount of (BiO)$_2$CO$_3$ (Bi/Ti 2 at.%). The Bi/Ti ratio of each of the composites was measured from EDS measurements. The obtained ratio for the 2, 4 and 8 at.% nanocomposites was 1.73 ± 0.1, 3.76 ± 0.1, and 8.11 ± 0.25 at.%, respectively.

![TEM images with high resolution EDS mapping](image)

**Figure 4.39.** TEM images with high resolution EDS mapping of (a) TiO$_2$ NPs, (b) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 2 at.%, (c) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 4 at.%, and (d) TiO$_2$/(BiO)$_2$CO$_3$ sample Bi/Ti 8 at.%.

The surface composition of the nanocomposites was characterized using high resolution XPS. Figure 4.40 displays the high resolution XPS spectra of the Bi 4f orbitals.
(left column) and the C 1s orbital (right column), and the peak deconvolution results for the pure TiO$_2$ and TiO$_2$/\((\text{BiO})_2\text{CO}_3\) nanocomposites. While the TiO$_2$ NPs do not display any Bi 4f orbitals, the presence of \((\text{BiO})_2\text{CO}_3\) clusters in Bi/Ti 2 at.% yields a significant signal of the two symmetric Bi 4f peaks with a binding energy of 159 eV (Bi 4f$_{7/2}$) and 164 eV (Bi 4f$_{5/2}$), which indicates the presence of Bi–O bonds with bismuth in the +3 oxidation state, as found in \((\text{BiO})_2\text{CO}_3\) [53, 74]. The intensity of the Bi 4f peak increases with increasing atomic ratio of Bi/Ti in the nanocomposite materials.

Two Gaussian peaks were used to fit the experimental data in the high resolution XPS spectra of C 1s (right column). The peak at the lower binding energy of 284.83 eV can be assigned to C–C bonds in adventitious carbon [53]. The second C 1s peak at 287 eV is ascribed to the C–O bonds that are usually present in CO$_3^{2-}$, as found in \((\text{BiO})_2\text{CO}_3\) [53]. Similarly to the Bi 4f peak, the carbonate-related peak increases in intensity with increasing amounts of \((\text{BiO})_2\text{CO}_3\) on the surface of TiO$_2$. The carbon element in pure TiO$_2$ corresponds mainly to adventitious hydrocarbon and is not related to the presence of \((\text{BiO})_2\text{CO}_3\).

All XPS data are summarized in Table 4.12. The XPS analysis supports the findings from the XRD and TEM analysis and confirms the presence of \((\text{BiO})_2\text{CO}_3\) in the nanocomposite materials. The XPS spectra for N 1s, O 1s and Ti 2p are shown in Figure A12 in the Appendix I. There were no peaks detected in the N 1s area, which excludes the presence of bismuth oxynitrates [135].
Figure 4.40. High resolution XPS spectra of the Bi 4f (left column) and C 1s (right column) regions of TiO$_2$ NPs, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.
Table 4.12. High resolution XPS data of Bi 4f and C 1s of TiO₂ NPs and the TiO₂/(BiO)₂CO₃ nanocomposites with different Bi/Ti atomic ratios.¹)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bi 4f₅/₂ (eV)</th>
<th>Bi 4f₇/₂ (eV)</th>
<th>C 1s C=O (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂</td>
<td>-</td>
<td>-</td>
<td>286.92 ± 0.28</td>
</tr>
<tr>
<td>Bi/Ti 2 at.%</td>
<td>164.68 ± 0.45</td>
<td>159.31 ± 0.28</td>
<td>287.57 ± 0.24</td>
</tr>
<tr>
<td>Bi/Ti 4 at.%</td>
<td>164.85 ± 0.40</td>
<td>159.34 ± 0.21</td>
<td>287.56 ± 0.24</td>
</tr>
<tr>
<td>Bi/Ti 8 at.%</td>
<td>165.85 ± 0.39</td>
<td>160.51 ± 0.43</td>
<td>287.25 ± 0.23</td>
</tr>
</tbody>
</table>

¹) The C 1s peak of adventitious carbon for all samples is found at 284.83 eV.

4.4.2 Optical Properties and Band Gap of the TiO₂/(BiO)₂CO₃ Nanocomposites

The UV-visible absorption spectrum of each investigated material was measured in order to determine its UV filtering properties and the effects of the (BiO)₂CO₃ formed onto the TiO₂ surface on the optical band gap (Figure 4.41).

![Figure 4.41.](image)

Figure 4.41. (a) UV-visible absorption spectra and (b) Tauc plots of TiO₂ NPs, and TiO₂/(BiO)₂CO₃ nanocomposites with different Bi/Ti atomic ratios. The absorption spectra were recorded at a concentration of NPs of 25 µg/mL in DI water.

TiO₂ NPs are a common additive to commercial sunscreen formulations with a high and selective absorbance within the UV region (Figure 4.41(a)). The TiO₂/(BiO)₂CO₃ nanocomposites exhibit a very similar absorption over the entire UV range as compared to uncoated TiO₂ NPs, with only slightly reduced intensities, depending on the amount of (BiO)₂CO₃ clusters present. The reduced absorbance in the visible range is related to the
presence of more transparent (BiO)_2CO_3 in comparison to TiO_2, which is of advantage in regard to potential application in sunscreen formulations [50]. Moreover, there is only a very small blue shift identified for all tested nanomaterials.

These findings suggest that the (BiO)_2CO_3 clusters have a negligible impact on the optical properties of the heterojunctions. This is most accurately reflected in a minor increase in the optical band gap of the nanocomposite materials, ranging from 2.69 to 2.83 eV compared to the TiO_2 NPs with an optical band gap of 2.68 ± 0.08 eV, as seen in Table 4.13. The band gaps of all the materials were calculated using Equation (3.8) and represent indirect band transitions. The Tauc plots are shown in Figure 4.41(b).

**Table 4.13. Band gaps of TiO_2/(BiO)_2CO_3 nanocomposites.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Band gap (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO_2</td>
<td>2.68 ± 0.08</td>
</tr>
<tr>
<td>Bi/Ti 2 at.%</td>
<td>2.83 ± 0.09</td>
</tr>
<tr>
<td>Bi/Ti 4 at.%</td>
<td>2.79 ± 0.07</td>
</tr>
<tr>
<td>Bi/Ti 8 at.%</td>
<td>2.69 ± 0.05</td>
</tr>
</tbody>
</table>

a) Data provided for the pure TiO_2 NPs, and the TiO_2/(BiO)_2CO_3 nanocomposites with different Bi/Ti atomic ratios. The indicated errors are the standard deviation.

### 4.4.3 Photocatalytic Activity of the TiO_2/(BiO)_2CO_3 Nanocomposites under Ultraviolet Exposure (300 nm and 350 nm) and under Simulated Solar Irradiation

Each of the nanocomposite materials and the pure TiO_2 NPs were tested for their photocatalytic activity within a broad UVA/UVB spectrum and under simulated solar light irradiation. Crystal violet was utilized as an indicator of the photocatalytic activity and the associated production of hydroxyl radicals (see section 3.4.4).

The discoloration of crystal violet in the absence of any photocatalyst was negligible, both under UV and under solar light irradiation. Upon addition to the crystal
violet solution of a suspension of TiO₂ at a final concentration of 5 mg/L, the reaction mixture degrades almost completely due to photocatalytic reactions when exposed to UVA and UVB light for 30 min, as shown in Figure 4.42(a).

![Figure 4.42](image)

**Figure 4.42.** (a) Relative decrease in absorbance of crystal violet solutions containing TiO₂ and TiO₂/(BiO)₂CO₃ nanocomposites with different Bi/Ti atomic ratios under UV light exposure (300 nm and 350 nm). The concentration of all materials is 5 mg/L. The data represent the mean of three independently prepared samples, which were measured separately. (b) Apparent rate constant curves for the UV-visible dye degradation of crystal violet, as shown in (a).

The photocatalytic activity of TiO₂ is generally linked to the particular phase: while anatase TiO₂ exhibits strong photocatalytic activity, the rutile phase is less active [112, 115]. Here, commercial Aerioxide® P25 was used, which is a combination of both anatase and rutile TiO₂. The addition of 5 mg/L of the nanocomposite materials resulted in a significant decrease in degradation of crystal violet in comparison to the pure TiO₂, suggesting a reduction in photocatalytic activity.

Furthermore, the reduction in the degradation of crystal violet increased with increasing amounts of (BiO)₂CO₃ clusters on the surface of TiO₂. More interestingly, there is only a small difference in degradation between the nanocomposites, which indicates that a coating with (BiO)₂CO₃ that results in a Bi/Ti atomic ratio of 0.02, leads to a significant reduction in the photocatalytic activity of the TiO₂ NPs, without affecting the UV blocking
properties of TiO$_2$. The apparent rate constant $k$ for each of the materials was calculated using the Langmuir-Hinshelwood model and is shown in Table 4.14, whereas the plots are displayed in Figure 4.42(b) [136].

**Table 4.14.** Apparent rate constant $k$ for the degradation of crystal violet solutions containing TiO$_2$, and TiO$_2$/B$_2$O$_3$ nanocomposites under irradiation by UV light (300 nm and 350 nm) and AM 1.5 G one sun (100 mW/cm$^2$). The concentration of all materials is 5 mg/L. The errors indicated are the standard deviation obtained through the linear regression.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k \times 10^{-2}$ (min$^{-1}$), UV irradiation</th>
<th>$k \times 10^{-2}$ (min$^{-1}$), sunlight irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye only</td>
<td>0.02 ± 0.01</td>
<td>0.47 ± 0.10</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>6.64 ± 0.22</td>
<td>44.81 ± 0.82</td>
</tr>
<tr>
<td>Bi/Ti 2 at.%</td>
<td>1.15 ± 0.04</td>
<td>11.43 ± 0.18</td>
</tr>
<tr>
<td>Bi/Ti 4 at.%</td>
<td>1.03 ± 0.04</td>
<td>10.57 ± 0.18</td>
</tr>
<tr>
<td>Bi/Ti 8 at.%</td>
<td>0.92 ± 0.03</td>
<td>9.93 ± 0.3</td>
</tr>
</tbody>
</table>

As displayed in Figure 4.43, the degradation of crystal violet under simulated solar light irradiation showed a similar trend to that under UV light in the same system, for an exposure time of 6 h. TiO$_2$ displays close to 100% degradation after 6 h of simulated solar light irradiation. The apparent rate constants for the nanocomposite materials are 3.9 – 4.5 times smaller than for the pure TiO$_2$, further confirming the photodegradation protection provided by (B$_2$O$_3$) at small concentrations.

The decrease in photocatalytic activity can be attributed to a number of factors, such as a decrease in the BET specific surface area, although the coating with (B$_2$O$_3$) in Bi/Ti 2 at.% leads to a decrease in the BET specific surface area of less than 1.5% in comparison to TiO$_2$. Yet, the photocatalytic activity of that nanocomposite is reduced by over 55% compared to TiO$_2$. Furthermore, the (B$_2$O$_3$) clusters, which are located on the surface of TiO$_2$, can absorb incoming UV light, reducing the overall radiation that reaches TiO$_2$. It is shown, however, that even the very small addition of (B$_2$O$_3$) to TiO$_2$ which
results in Bi/Ti 2 at.% is sufficient to cause a significant decrease in the degradation of crystal violet. Further addition beyond Bi/Ti 2 at.% has a negligible effect. Therefore, the reduction in photocatalytic activity does not appear to be related to a decrease in the surface area or increased absorption of UV radiation by the (BiO)$_2$CO$_3$ clusters.

**Figure 4.43.** (a) Relative decrease in absorbance of crystal violet solutions containing TiO$_2$, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios under exposure of AM 1.5 G one sun (100 mW/cm$^2$). The concentration of all materials is 5 mg/L. The data represent the mean of three independently prepared samples, which were measured separately. (b) Apparent rate constant curves for the dye degradation of crystal violet under exposure to AM 1.5 G one sun (100 mW/cm$^2$), as shown in (a).

Another explanation for the observed reduced dye degradation can be the very small size of the (BiO)$_2$CO$_3$ clusters (< 10 nm), which leads to their very high surface/volume ratio. Due to the low crystallinity, the surface is enriched with defects, which can act as ROS scavengers, as shown for CeO$_2$ NPs [95, 137]. More importantly, it is widely known that carbonate acts as a hydroxyl radical scavenger. Once the carbonate is in contact with hydroxyl radicals, the formation of carbonate radicals is observed, as shown in Equation (2.5) [138, 139]. The carbonate radical is very selective and typically prolongs degradation processes [55, 56].

Typically, nanomaterials are designed that increase the photocatalytic activity with the goal of improving the catalyst efficiency [140, 141]. Although the use of (BiO)$_2$CO$_3$ NPs
for photocatalytic purposes is widely reported, \((\text{BiO})_2\text{CO}_3\) can also be used as a photoprotector, reducing the photocatalytic activity of the strong photocatalyst \(\text{TiO}_2\). \((\text{BiO})_2\text{CO}_3\) is a weak photocatalyst on its own, especially at low concentrations such as those used in this work. Noticeable photocatalytic degradation of rhodamine B [58] and methyl orange [52] under UV-visible light irradiation was achieved at concentrations of 1.0 and 0.1 g/L, respectively. The application of \((\text{BiO})_2\text{CO}_3\) clusters as a photoprotector is of interest and importance, since TiO\(_2\) NPs are widely used as the active component in sunscreen formulations, although it is known that they generate free radicals [111, 142].

### 4.4.4 In Vitro Cell Viability in HaCaT and MDCK Cells

The *in vitro* cell viability was determined in non-malignant human skin cells and kidney (MDCK) cells in two different settings: under the absence of simulated sunlight (HaCaT and MDCK) and under presence of simulated sunlight (HaCaT). HaCaT keratinocytes are typically used to investigate possible adverse effects of NPs in sunscreens [125]. MDCK cells are used to examine the biocompatibility of NPs, since the kidney represents a possible detoxification route [127, 128].

As of now, bismuth oxychloride \((\text{BiOCl})\) is the only bismuth-based compound that has been studied in HaCaT cells, although without the application of UVA and UVB light and simulated sunlight [143]. Neither the toxicity of \((\text{BiO})_2\text{CO}_3\) in HaCaT cells, nor the inhibition of photo-induced toxicity of TiO\(_2\) due to the presence of \((\text{BiO})_2\text{CO}_3\) upon irradiation with simulated sunlight has yet been reported, which ultimately highlights the novelty of the presented work.

#### 4.4.4.1 In the Absence of Simulated Sunlight

The cytotoxicity of the commercial TiO\(_2\) NPs, and the TiO\(_2\)/\((\text{BiO})_2\text{CO}_3\) nanocomposite materials was determined using the MTT assay, which is based on the conversion of MTT into formazan crystals by viable cells [144]. HaCaT and MDCK cells
were exposed to the NPs for 24 h at concentrations ranging from 5 to 500 µg/mL (Figure 4.44).

In general, the reduction of cell viability was observed in a dose-dependent manner for all tested nanomaterials. In both cell lines, the pristine TiO\textsubscript{2} NPs displayed the highest cytotoxicity of all the tested materials. Nevertheless, at the highest tested concentration of 500 µg/mL, the decrease in mitochondrial activity is still moderate for the TiO\textsubscript{2} NPs, with cell viability of 71.2 ± 4.5% and 61.5 ± 6.6% in HaCaT and MDCK cells, respectively. The TiO\textsubscript{2}/(BiO)\textsubscript{2}CO\textsubscript{3} nanocomposites show cell viabilities that lie above that for the TiO\textsubscript{2} NPs. In addition, the biocompatibility of the nanocomposites increases with increasing atomic ratio of Bi/Ti.

![Figure 4.44](image_url)

**Figure 4.44.** Effects of the commercial TiO\textsubscript{2} NPs, and TiO\textsubscript{2}/(BiO)\textsubscript{2}CO\textsubscript{3} nanocomposites with different Bi/Ti atomic ratios on the mitochondrial function in non-cancerous a) HaCaT human skin cells and b) dog kidney (MDCK) cells. The cells were treated with the NPs at concentrations of 0 (control), 5, 10, 25, 50, 100, 250, and 500 µg/mL for 24 h. At the end of exposure, the mitochondrial function was determined using the MTT reduction assay. The data are represented as the mean of three independently prepared experiments.

### 4.4.4.2 In the Presence of Simulated Sunlight

To confirm that the toxicity of TiO\textsubscript{2} is reduced by the inhibition of photocatalytic activity due to (BiO)\textsubscript{2}CO\textsubscript{3}, cell viability assays with HaCaT have been performed under presence of TiO\textsubscript{2}, TiO\textsubscript{2}/(BiO)\textsubscript{2}CO\textsubscript{3} (Bi/Ti 8 at.%), and simulated sunlight.
To ensure that the chosen concentrations of tested nanomaterials are sufficient to induce detectable photocatalytic effects, the degradation of crystal violet was evaluated in presence of TiO$_2$ NPs at 25, 50, and 100 µg/mL and simulated sunlight for 15 min (Figure 4.45). A concentration of 25 µg/mL has been shown to be sufficient to cause a degradation of crystal violet of over 33%.

![Graph showing degradation of crystal violet](image)

**Figure 4.45.** Degradation of crystal violet solutions containing TiO$_2$ NPs at concentrations of 25, 50, and 100 µg/mL upon irradiation with simulated sunlight (300 W Sunlamp, Ultra-Vitalux®, OSRAM) for 15 min.

The HaCaT cell viability following exposure to TiO$_2$ or TiO$_2$/(BiO)$_2$CO$_3$ (Bi/Ti 8 at.%) NPs at concentrations of 25, 50, and 100 µg/mL with and without additional simulated sunlight irradiation is shown in Figure 4.46. When compared to the toxicity of the nanomaterials alone (non-irradiated), it is clearly visible that the additional exposure to simulated sunlight initiates processes that cause a higher toxicity in HaCaT cells. For instance, at a concentration of 100 µg/mL, the cell viability after treatment with only TiO$_2$ NPs is high with a survival of 86.4 ± 2.9%, while after additional irradiation with simulated sunlight the cell viability decreases to 64.1 ± 4.4%. This increase in cell mortality is most likely related to the photocatalytic activity of TiO$_2$ and the subsequent generation of ROS, while the composite is capable of inhibiting a significant amount of photocatalysis [145, 146]. In particular, for the same conditions, the treatment with TiO$_2$/(BiO)$_2$CO$_3$ results in a cell viability of 74.5 ± 4.7% after irradiation with simulated sunlight.
More interestingly, it is further shown that a significant effect of photocatalysis can only be observed at concentrations ≥ 50 µg/mL, which implies that the impact of photocatalytic activity of TiO₂ on the viability of HaCaT cells is negligible at concentrations ≤ 25 µg/mL.

**Figure 4.46.** Viability of HaCaT cells following exposure to TiO₂ or TiO₂/(BiO)₂CO₃ (Bi/Ti 8 a.%) NPs with and without additional simulated sunlight irradiation (300 W Sunlamp, Ultra-Vitalux®, OSRAM) for 15 min. The viability was assessed using the MTT assay and the tested concentrations were 25, 50, and 100 µg/mL. The data are expressed as percentage of viable cells compared to control cells that have not been exposed to nanomaterials.

The decrease of cell viability of human skin fibroblasts upon exposure to TiO₂ NPs and UV light has been reported widely [145-147]. For instance, the use of rutile TiO₂ and anatase TiO₂ at concentrations of 50 and 100 µg/mL was assessed in HaCaT cells under UVA irradiation with doses of 0 – 10 J/cm² [146]. No significant toxicity was found towards HaCaT cells exposed to TiO₂ at a concentration of 100 µg/mL without irradiation. Upon irradiation with UVA, however, the anatase TiO₂ treatment resulted in a decrease in cell viability of over 40% at the highest tested dose of 10 J/cm², while rutile TiO₂ displayed phototoxicity of less than 20% [145].

Interestingly, the use of a mixture of anatase and rutile (P25), similarly to this work, showed a decrease in cell viability of over 80 and 85% for a dose of 5.4 and 10 J/cm²,
respectively. Although the results in this work show higher cell viability at a dose of 5.4 J/cm², the observed trend highlights the need of more research into the reduction of photocatalytic activity of TiO₂, in particular P25, which is still widely applied in commercial sunscreen formulations [111, 142].

The phototoxicity is generally linked to the generation of ROS in presence of UVA [145, 146]. In particular, an increase in accumulation of intracellular ROS levels by 1.8-fold has been observed in HaCaT cells after 24 h exposure to 200 µg/mL of P25 and irradiation with UVA (365 nm) [146].

4.4.5 Conclusions

The commonly used TiO₂ NP additives in sunscreen formulations pose a potential health risk in regard to their ability to generate free radical species that, in turn, can cause cyto- and genotoxicity, either directly through penetration into deep skin tissue or indirectly through the degradation of organic sunscreen additives. The latter case could result in the formation of intermediates with unknown toxicity, which – besides the reduction of SPF – can cause adverse side effects.

By reducing the photocatalytic activity of TiO₂ NPs and simultaneously maintaining the UV filtering properties, the health risk of these sunscreen additives can be minimized. Using a simple two-step precipitation approach, TiO₂/(BiO)₂CO₃ nanocomposites were synthesized with different atomic ratios of Bi/Ti (0.02, 0.04 and 0.08). The (BiO)₂CO₃ clusters are smaller than 10 nm and are evenly distributed throughout the surfaces of the TiO₂ NPs.

It is demonstrated that, by introducing a small amount of (BiO)₂CO₃ clusters onto the surface of TiO₂, which results in an atomic ratio of Bi/Ti of 0.02, its photocatalytic activity was decreased and its biocompatibility in healthy cells increased, while maintaining its size, morphology, and UV blocking ability. Moreover, the nanocomposites displayed a reduced toxicity in HaCaT cells upon irradiation with simulated sunlight in
comparison to TiO$_2$ NPs, which indicates that the composites are capable of reducing the photo-induced generation of ROS.

### 4.5 References


[125] F. Rancan, B. Nazemi, S. Rautenberg, et al., *Ultraviolet Radiation and Nanoparticle Induced Intracellular Free Radicals Generation Measured in Human Keratinocytes by*


CHAPTER 5 – Tantalum-Based Compounds for Biomedical Applications

This chapter is based on one submitted manuscript:


Author contributions: KB designed and performed all experiments and characterization, and collected, analysed, and interpreted all data, unless otherwise specified. KB and MZ synthesized the materials. NT assisted with acquiring the CT images. KB wrote the manuscript and generated figures, and VS assisted with the reaction scheme. MZ, VS, MT, ML, NT, AR, SXD, HKL, and KK provided intellectual support, guidance, and helped editing the manuscript.
Chapter 5 – Tantalum-Based Compounds for Biomedical Applications

5.1 Introduction

Similar to bismuth, tantalum and its compounds have also shown great biocompatibility [1, 2]. Tantalum is a shiny, blue-grey transition metal that is chemically inert, and, when in contact with air, it usually forms a protective layer of Ta$_2$O$_5$ on its surface [3]. Despite its much higher cost in comparison to Bi-based materials, tantalum is highly corrosion- and temperature-resistant and thus, finds applications in aircraft engines, in the chemical industry, and as a component in laboratory equipment [3]. Due to its high biocompatibility, tantalum is used for surgical implants and coatings [1, 3].

5.1.1 Properties of Ta$_2$O$_5$

Ta$_2$O$_5$ is a transition metal oxide that can exist in multiple structural polymorphs, including low-temperature (L) and high-temperature (H) phases, as displayed in Table 5.1. The identification of Ta$_2$O$_5$ structures has been a subject of on-going research for over 50 years and has resulted in the description of multiple L-phases, in which multiple integers (5, 8, 11, 13, 14, 19, 22, and 25) of the parameter $b$ of the orthorhombic sub-cell have been found to result in slight variations of the overall L-Ta$_2$O$_5$ structure [4, 5].

<table>
<thead>
<tr>
<th>Polymorph</th>
<th>Other names</th>
<th>Crystal system</th>
<th>Space group</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ta$_2$O$_5$</td>
<td>High-temperature</td>
<td>Tetragonal</td>
<td>$I4_1$/amd</td>
<td>[6, 7]</td>
</tr>
<tr>
<td></td>
<td>(H-Ta$_2$O$_5$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Ta$_2$O$_5$</td>
<td>Low-temperature</td>
<td>Orthorhombic</td>
<td>$Pccm$ (11L-Ta$_2$O$_5$)</td>
<td>[4-6, 8, 9]</td>
</tr>
<tr>
<td></td>
<td>(L-Ta$_2$O$_5$)</td>
<td></td>
<td>$C112/m$ (19L-Ta$_2$O$_5$)</td>
<td></td>
</tr>
<tr>
<td>δ-Ta$_2$O$_5$</td>
<td>–</td>
<td>Hexagonal</td>
<td>$P6/mmm$</td>
<td>[8-10]</td>
</tr>
<tr>
<td>Z- Ta$_2$O$_5$</td>
<td>–</td>
<td>Monoclinic</td>
<td>$C2$</td>
<td>[11]</td>
</tr>
<tr>
<td>Trigonal Ta$_2$O$_5$</td>
<td>–</td>
<td>Trigonal</td>
<td>$R3$</td>
<td>[5]</td>
</tr>
</tbody>
</table>
On the nanoscale, the irreversible transition from $\delta$-Ta$_2$O$_5$, the crystal structure of which is shown in Figure 5.1, to $\beta$-Ta$_2$O$_5$ occurs at a temperature $\geq 750^\circ$C [8]. The reversible transition of $\beta$-Ta$_2$O$_5$ to $\alpha$-Ta$_2$O$_5$ has been observed at 1320$^\circ$C [6]. Trigonal Ta$_2$O$_5$ thin films were obtained upon thermal deposition of amorphous precursors on Si(001) wafers [5].

![Crystal structure of $\delta$-Ta$_2$O$_5$](image)

**Figure 5.1.** Crystal structure of $\delta$-Ta$_2$O$_5$; obtained via Materials Studio software with data from [9].

To synthesize Ta$_2$O$_5$, tantalum alkoxides such as tantalum ethoxide (Equation (5.1)) or halides, such as tantalum chloride (Equation (5.2)), are used, which, once dissolved, form a Ta$_2$O$_5$ precipitate after the addition of water. The subsequent annealing of the Ta$_2$O$_5$ precursor at 730$^\circ$C leads to crystalline $\delta$-Ta$_2$O$_5$ NPs [12]:

$$Ta_2(OEt)_{10(l)} + 5H_2O(l) \rightarrow Ta_2O_5(s) + 10EtOH(aq) \quad (5.1)$$

$$2TaCl_5(l) + 5H_2O(l) \rightarrow Ta_2O_5(s) + 10HCl(aq) \quad (5.2)$$

**Applications**

Ta$_2$O$_5$ is chemically inert [13], biocompatible [2], shows good radiopacity [14], and is nearly insoluble, except in very strong bases and hydrofluoric acid [15, 16]. Its extraordinary corrosion resistance makes Ta$_2$O$_5$ a valuable material for laboratory equipment [17], whereas its high refractive index enables the use of Ta$_2$O$_5$ for the preparation of photographic lenses and coatings [18].

256
In addition, $\text{Ta}_2\text{O}_5$ displays great dielectric properties [17, 19] and promising piezoelectric behaviour in form of thin films [5], which are typically obtained using metalorganic vapour phase epitaxy (MOVPE) [20]. Together with its wide band gap of 3.9 – 4.0 eV for the bulk material [21, 22], these properties have led to the use of $\text{Ta}_2\text{O}_5$ in electronics, particularly in tantalum capacitors [17, 20], and its investigation as a memory device [23].

**Diagnostic properties**

Ta-based compounds have a high effective atomic number of $Z = 73$ and density of 16.6 kg/m$^3$, and have been investigated for dose enhancement on radio-resistant 9L rat gliomasarcoma cancer cells in an megavolt photon field [12] and their ability to increase anatomical contrast [13, 24]. *In vitro* studies have shown that, for equal molar concentrations of tantalum and iodine, the image contrast was larger with tantalum across the diagnostic X-ray spectrum [13].

The easily modifiable surfaces of $\text{Ta}_2\text{O}_5$ allow loading with anti-cancer drugs, which is typically achieved with conjugation or encapsulation by a polymer layer [25]. These core-shell structures can further be of benefit for CT imaging, as they can be administered intravenously and display subsequent renal clearance [13, 26].

**5.1.2 Polymers and Hydrogels**

Studies of polymers for personalized and tailored medicine have been extensively reported. On the nanoscale, polymers can improve the half-life, solubility, and stability of drug delivery systems, while reducing potential side effects [27-29]. The polymers that are used for the design of drug delivery systems can be divided into two categories [30]:

- Natural polymers, and
- Synthetic polymers.

Protein based natural polymers include collagen, albumin, and gelatin, while natural polysaccharides are typically complexly branched agarose, hyaluronic acid,
dextran, chitosan, and cyclodextrins [30]. A selection of synthetic polymers used for the
design of drug delivery systems is displayed in Table 5.2.

Table 5.2. Selection of synthetic polymers that are used for drug delivery systems.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>IUPAC name</th>
<th>Chemical structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ethylene glycol) (PEG)</td>
<td>Poly(oxyethylene)</td>
<td>H[O_\text{O}]_\text{H}_n</td>
<td>[31, 32]</td>
</tr>
<tr>
<td>Poly(ethylene imine) (PEI)</td>
<td>Poly(iminoethylene)</td>
<td>[\text{N}_\text{H}]_n</td>
<td>[31]</td>
</tr>
<tr>
<td>Poly(lactic acid) (PLA)</td>
<td>Poly(2-hydroxypropionic acid)</td>
<td>[\text{O}_{\text{O}}]_\text{n}</td>
<td>[33]</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>1-Ethenylpyrrolidin-2-one</td>
<td>[\text{O}_{\text{N}}]_\text{n}</td>
<td>[34]</td>
</tr>
<tr>
<td>Poly(acrylic acid) (PAA)</td>
<td>Polyacrylic acid</td>
<td>[\text{O}_{\text{OH}}]_\text{n}</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (PVA)</td>
<td>1-Hydroxyethylene</td>
<td>[\text{OH}]_\text{n}</td>
<td>[37]</td>
</tr>
</tbody>
</table>

Besides their individual use, these polymers can be coupled with other polymers,
also in form of copolymers, to increase the hydrophilic properties of the nanocarriers [33].

5.1.2.1 Properties of Poly(acrylic acid)

PAA is a biodegradable, water-soluble, and bioadhesive polymer, which does not
require further treatment after insertion into the body [38, 39]. Due to the hydrophilic
carboxylic acid present in the polymeric material, PAA as a hydrogel is able to hold large
amounts of water in its three-dimensional network and to reversibly deswell [39]. Other
common water-soluble polymers, such as PVA, PVP, PEG, polyacrylamide, and some
polysaccharides, can also form hydrogels.
The great advantage of hydrogels is their tailorable design, enabling the control of responses to environmental changes, which can cause shrinking or swelling of the hydrogel. The stimuli include [35, 40]:

- **Physical stimuli**: temperature, electric and magnetic fields, light, pressure, sound.
- **Chemical stimuli**: solvent composition, ionic strength, pH value.

The shrinking and swelling of PAA, in particular, depends on the pH value: an alkaline environment leads to swelling of the PAA, while acidic conditions cause a shrinking of the polymer (Figure 5.2) [41]. This phenomenon is mostly linked to the presence of carboxylic groups that are able to associate with water molecules.

![Figure 5.2. Principle of pH-dependence on the swelling behaviour of hydrogels.](image)

Because of these unique properties, hydrogels have found applications in artificial muscles and tissue engineering [42, 43], food additives [44], pharmaceuticals [45], diagnostics [46], drug delivery [47], and in the separation of biomolecules or cells [48]. For instance, PAA-coated Pt NPs have been explored as a radiosensitizer, where the polymer coating improves the diffusivity through the cell membrane and the binding to DNA, which is damaged upon exposure to radiation via secondary radiation [49, 50].

### 5.1.2.2 Polymerization of Acrylic Acid Monomers

Acrylic acid (AA) is a monomer and intermediate that is used in acrylic esters for superabsorbent polymers, plastics, and synthetic rubber (Figure 5.3) [51]. Structurally, the AA monomers consist of an alkene chain and a carboxylic moiety, which remains intact after polymerization and can be used for further functionalization. After polymerization, a
three-dimensional network is typically generated via cross-linkage of polymeric chains through covalent bonds in particular, but also hydrogen bonds, van der Waals bonds, or physical adsorption.

Figure 5.3. Acrylic acid monomers can either react via addition polymerization or esterification to form poly(acrylic acid) or acrylic esters, respectively. Adapted from [51].

The polymerization of AA takes place via addition polymerization, also known as polyaddition, and does not produce any condensates [51]. Polyaddition is typically based on the formation of radicals, whereas condensation polymerization generates small low molecular weight molecules and can be achieved via radical polymerization, cationic addition polymerization, and anionic addition polymerization [52].

The radical polymerization can typically be initiated using organic peroxides [52] or via the absorption of visible, UV, or gamma irradiation [53]. The polymerization proceeds as follows [52]:

1. **Initiation**: generation of radicals that act as active centres.

2. **Chain growth**: the active centre of the growing polymer can attack a new monomer, resulting in a polymer that is one repeat unit longer. The active centre is transferred to the new polymer end.

3. **Chain termination**: disproportionation or combination of radicals lead to the loss of active centres.
Although an initiator is usually added for the conventional batch polymerization of AA, the monomer itself shows very high reactivity at elevated temperatures and is characterized by a high polymerization rate [54, 55]. The exothermic nature of the polymerization therefore requires precise temperature control to avoid the formation of clumps or a gel [55].

### 5.1.3 Drug Delivery Systems Based on Hydrogels

Due to their water absorption capability, hydrogels are excellent candidates for drug delivery systems, as they can incorporate large amounts of drugs, which can be released in response to physical or chemical stimuli. Moreover, due to their water-solubility and biocompatibility, the removal of hydrogels from biological systems is not required [38]. The principle of drug delivery systems based on hydrogels is displayed in Figure 5.4.

**Figure 5.4.** Principle of drug release of a drug-loaded nanoparticle-poly(acrylic acid) (PAA) conjugate.

#### 5.1.3.1 Principle of Drug Loading and Drug Release

In general, there are two methods to load hydrogels as drug carriers. In the first method, the drug is added to the monomer mixture during polymerization, which allows trapping of the drug within the polymer matrix. Since the conditions of the polymerization reaction may have deleterious effects on the drug properties, this method is not always favoured [56]. In the second approach, the hydrogel is placed in a solution containing the drug, and allowed to swell until equilibrium is reached [36, 56-58].
Once the drug is loaded into the system, its release can be studied in different release environments, i.e. different pH values. This is of importance, since the pH values of different cellular compartments differ, as displayed in Table 5.3.

### Table 5.3. pH values of different intracellular and extracellular compartments [58].

<table>
<thead>
<tr>
<th>Compartment</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood plasma</td>
<td>7.4</td>
</tr>
<tr>
<td>Extracellular tumour matrix</td>
<td>5.8 - 7.2</td>
</tr>
<tr>
<td>Endosome</td>
<td>5 – 6</td>
</tr>
<tr>
<td>Lysosome</td>
<td>4 – 5</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.5 – 3.5</td>
</tr>
</tbody>
</table>

#### 5.1.3.2 Existing Systems Based on Poly(acrylic acid)

Various systems have been developed that contain PAA as a hydrogel matrix [35, 36, 58-62]. For instance, PAA-coated magnetite (Fe₃O₄) NPs were synthesized, loaded with bleomycin, and the drug release was studied at a pH of 7.5 [59]. In another study, PAA-coated magnetite NPs were loaded with DOX, and the release was monitored at a pH of 4.2 and 7.4 [60]. More recently, systems have been developed that contain two or more polymers, with a view to controlling the drug loading and tailoring the pH-response.

**Example 1**

Polyelectrolyte hydrogels based on cationic guar gum and AA, which provides anionic properties, have been shown to be highly sensitive to changes in the pH environment [35]. The loading and release of ketoprofen as a model drug indicated that the pH also influences the drug release kinetics. For instance, with alkaline pH of the release medium, Case II transport was observed, which suggest that the drug transport strongly depends on macromolecular chain relaxation. At lower pH values, the transport mechanism appeared to be both diffusion and relaxation controlled.
Example 2

CaF$_2$:Ce$^{3+}$/Tb$^{3+}$-PAA composite spheres were synthesized and loaded with DOX, and the electrostatic interactions between the drug and the polymer were studied [58]. The presence of PAA prevented the initial burst release of DOX, and the inorganic CaF$_2$:Ce$^{3+}$/Tb$^{3+}$ provided green fluorescence upon excitation with UV light. The release of DOX increased with decreasing pH from 7.4 to 4.0 and 2.0 (Figure 5.5).

![Figure 5.5. Release of DOX from CaF$_2$:Ce$^{3+}$/Tb$^{3+}$-PAA composites in PBS buffer with pH = 7.4, 4.0, and 2.0. Adapted from [58].](image)

5.2 Development of pH-Sensitive Methotrexate-Loaded δ-Ta$_2$O$_5$-Poly(acrylic acid) Nanocomposite with Controlled Drug Release

This work shows a route that uses the high surface area available in individual Ta$_2$O$_5$ nanoparticles with a particle size of 27 nm to polymerize a nanometric layer of PAA with a thickness of 2 – 8 nm, by applying different polymerization times ranging from 1 to 5 h. The synthesis procedure and all characterization methods are described in CHAPTER 3. The evolution of the polymer layer with the polymerization time was assessed and further correlated with the loading capacity of MTX that was measured by swelling of the hydrogel in the drug solution at pH 7.4. The drug release of MTX was studied at different
pH values, ranging from 3.6 up to 9.4 for a total of 72 h, and the released amount of MTX was determined via UV-vis spectroscopy.

### 5.2.1 Identification, Size, Morphology, and Surface Composition

XRD was used to determine the phase of the \( \text{Ta}_2\text{O}_5 \) NPs before and after PAA polymerization on the surface of the NPs. The obtained diffraction patterns (Figure 5.6) show the presence of a single \( \delta \) hexagonal phase with all reflections matching the JCPDS card no. 00-019-1299. Furthermore, the coating of \( \delta\text{-Ta}_2\text{O}_5 \) with PAA preserves the crystalline phase. The crystallite size was determined using the Scherrer equation and showed a mean size of 26.7 ± 5.4 nm for \( \delta\text{-Ta}_2\text{O}_5 \).

![Figure 5.6. XRD patterns of \( \delta\text{-Ta}_2\text{O}_5 \) NPs and \( \delta\text{-Ta}_2\text{O}_5\)-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h.](image)

SEM and TEM images of the \( \delta\text{-Ta}_2\text{O}_5 \) NPs and the \( \delta\text{-Ta}_2\text{O}_5\)-PAA nanocomposites are shown in Figure 5.7. The NPs are mostly spherical and ellipsoidal in shape and tend to form agglomerates > 100 nm in size, which is observed for both \( \delta\text{-Ta}_2\text{O}_5 \) and \( \delta\text{-Ta}_2\text{O}_5\)-PAA and is related to their high surface energy\(^\text{39}\). The single particle size of the uncoated \( \delta\text{-Ta}_2\text{O}_5 \) NPs varies between 13 – 57 nm with an average particle size of 26.6 ± 5.9 nm.
These findings indicate that the $\delta$-Ta$_2$O$_5$ NPs are an excellent candidate for the design of theranostic systems, since it has been shown that a particle size of up to 50 nm results in high cellular uptake [63].

**Figure 5.7.** (a) SEM and (b) TEM images of $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The green arrows indicate the PAA layers. (c) Particle size distribution of uncoated $\delta$-Ta$_2$O$_5$ NPs. A total of 100 particles were measured, and the relative frequency was plotted as a function of particle size. (d) Plot of the layer thickness as a function of the polymerization time.

The TEM images of the $\delta$-Ta$_2$O$_5$-PAA nanocomposites, which were obtained after different polymerization times, show homogeneous coverage of the surfaces of individual $\delta$-Ta$_2$O$_5$ NPs with layers of PAA. The layer thickness increases with increasing polymerization time: while polymerization for 1 h yields a polymer thickness of 2.1 ± 0.7
266 nm, a polymerization time of 2.5 and 5 h leads to a thickness of 3.9 ± 0.9 nm and 8.2 ± 1.4 nm, respectively.

The surfaces of the individual δ-Ta₂O₅ NPs were activated to induce preferential polymerization sites for the AA monomer to start to polymerize, as shown in Figure 5.8. This approach ensures that the polymer chains grow around each individual ceramic particle, avoiding the wrapping of clusters of δ-Ta₂O₅ [64, 65]. The thickness of the layer has a direct impact on the amount of drug which can be accumulated and, consequently, influences the drug release properties.

**Figure 5.8.** Expected reaction for the as-prepared δ-Ta₂O₅ nanoparticles when dispersed in methanol (left) and etched with hydrochloric acid for 25 min (= surface activation). After centrifugation (centre), the hydroxyl-terminated nanoparticles were transferred into an acrylic acid (AA) solution (= physical adsorption of monomer) and heated up to 83 °C (right) for different polymerization times (= thermal induced chain polymerization).

### 5.2.2 Fourier-Transform Infrared Spectroscopy

FTIR spectroscopy was used to confirm that PAA was successfully immobilized on δ-Ta₂O₅ NPs. Figure 5.9 shows the FTIR spectrum of uncoated δ-Ta₂O₅ NPs with characteristic bands at around 880 and 680 cm⁻¹, which are assigned to the Ta-O-Ta bridge and stretching vibrations of Ta-O bonds, respectively [66].
Figure 5.9. FTIR spectra of $\delta$-Ta$_{2}$O$_{5}$ NPs and $\delta$-Ta$_{2}$O$_{5}$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The dotted line indicates the wavenumber below which the absorption bands of $\delta$-Ta$_{2}$O$_{5}$ outweigh the absorption bands related to PAA.

The FTIR spectra of the $\delta$-Ta$_{2}$O$_{5}$-PAA nanocomposites show the presence of Ta-O and Ta-O-Ta vibrations in low wavenumbers $< 900$ cm$^{-1}$, which overlap with the stretching and bending vibrations related to PAA. Typically, O-H out-of-plane bending and CH$_2$ and C-COOH stretching is observed at 914 and 797 cm$^{-1}$, respectively [67]. Besides these bands, all other characteristic absorption bands that correspond to PAA are present in the $\delta$-Ta$_{2}$O$_{5}$-PAA nanocomposites: the absorption bands at 2946, 1451, and 1108 cm$^{-1}$ correspond to CH$_2$ or CH stretching, CH$_2$ stretching, and C-CH$_2$ stretching, respectively [67, 68]. In addition, the absorption bands at 1704 and 1240 cm$^{-1}$ are ascribed to the C=O stretching and C=O stretching, respectively [67]. It was interesting to observe that the vibration band with a maximum at 1704 cm$^{-1}$ increased with the polymerization time. Furthermore, the O-H stretching vibrations at 3085 and 2598 cm$^{-1}$ confirm the presence of PAA on the surfaces of the $\delta$-Ta$_{2}$O$_{5}$ NPs [67].

The monomer AA typically shows absorption bands at 1600 – 1680 cm$^{-1}$, which are assigned to C=C stretching [69]. The absence of these absorption bands in the FTIR spectra of the $\delta$-Ta$_{2}$O$_{5}$-PAA nanocomposites indicates a complete polymerization. The increase in
polymerization time generally leads to an increase in the intensity of the absorption bands of the characteristic functional groups, without changing their wavenumber.

### 5.2.3 Thermal Stability of δ-Ta$_2$O$_5$-Poly(acrylic acid) Nanocomposites

The stability of the nanomaterials was investigated by TGA, and the mass loss of organics was determined for all thermogravimetric curves (Figure 5.10). Uncoated δ-Ta$_2$O$_5$ NPs did not show any mass loss for the entire temperature scan range, while the δ-Ta$_2$O$_5$-PAA nanocomposite materials exhibited characteristic mass losses that were due to the presence of the polymer on their surfaces. To determine when the mass losses occurred, the derivative weight was plotted versus temperature [70].

![Graph showing TGA curves and derivative weight plots](image)

**Figure 5.10.** (a) TGA curves of δ-Ta$_2$O$_5$ NPs and δ-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. (b) First derivative of the TGA curve of δ-Ta$_2$O$_5$-PAA with 5 h polymerization time.

A mass loss starting at 126°C can be observed for all polymer nanocomposite materials and corresponds to the loss of unbound water. The amount of water was determined to be 0.1, 1.4, and 4.3 wt. % for the δ-Ta$_2$O$_5$-PAA nanocomposite with a polymerization time of 1, 2.5, and 5 h, respectively. For all the PAA-containing materials, three additional mass losses were identified at 253, 295, and 530°C, which are related to the release of organic compounds. The second mass loss can be attributed to the formation of PAA anhydride, and the third mass loss corresponds to the degradation of the PAA anhydride [71]. The fourth mass loss is ascribed to the thermal decomposition of PAA [72].
The amount of organics significantly increases with increasing polymerization time, with a total mass loss of organics of 1.4, 8.7, and 34.4 wt. % for the nanocomposites with a polymerization time of 1, 2.5, and 5 h, respectively (Figure 5.11).

![Graph showing the content of organics as a function of polymerization time.]

**Figure 5.11.** Plot of the organics content as a function of the polymerization time.

### 5.2.4 Surface Composition Before and After Polymerization

The surface composition of the δ-Ta₂O₅ NPs was evaluated via XPS and compared to δ-Ta₂O₅ after additional treatment with HCl to show that latter sample’s surfaces are functionalized with hydroxyl groups, which facilitate the layer-by-layer polymerization of AA onto the NPs. Figure 5.12 displays the survey spectra and the high-resolution XPS spectra of the O 1s and C 1s regions for the nanomaterials before and after polymerization.

The survey spectra show typical peaks that can be ascribed to the presence of carbon, oxygen, and tantalum. All XPS data are summarized in Table A1 and A2 in the Appendix II. The peaks at lower binding energy of ~285 and ~286 eV can be assigned to CH₂ and CH, respectively [71]. The peak at ~288 eV is ascribed to C–O bonds, which indicates the presence of adventitious carbon. The nanocomposites display an additional peak at ~290 eV, which corresponds to the O–C=O groups that are found in PAA [71].
Figure 5.12. (a) XPS survey spectra of δ-Ta₂O₅ NPs, δ-Ta₂O₅ NPs after HCl treatment, δ-Ta₂O₅-PAA 1 h, and δ-Ta₂O₅-PAA 2.5 h. High-resolution spectra of δ-Ta₂O₅ NPs, δ-Ta₂O₅ NPs after HCl treatment, and δ-Ta₂O₅-PAA nanocomposites with polymerization times of 1 and 2.5 h for b) the C 1s region and c) the O 1s region.
Table 5.4 displays the C 1s envelope ratios for all samples and shows – besides the increase in the ratio of C/O from ~143% (δ-Ta₂O₅) to ~217% (δ-Ta₂O₅-PAA) – an increase in the amount of CH bonds that are typically found in the polymer backbone chains, underlining the successful polymerization of PAA on the NP surfaces. The O 1s envelope ratios are shown in Table 5.5. These findings are further supported by the presence of an O 1s peak at ~534 eV for the O–C=O group, which is only found in the δ-Ta₂O₅-PAA nanoconposites [71].

Table 5.4. XPS analysis including the C 1s region of δ-Ta₂O₅ NPs (before and after treatment with HCl) and δ-Ta₂O₅-PAA nanocomposites with polymerization times of 1 and 2.5 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>XPS C 1s envelope ratios (%)</th>
<th>C/O ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH₂</td>
<td>CH</td>
</tr>
<tr>
<td>δ-Ta₂O₅</td>
<td>61.05</td>
<td>25.24</td>
</tr>
<tr>
<td>δ-Ta₂O₅ (HCl treated)</td>
<td>55.87</td>
<td>28.51</td>
</tr>
<tr>
<td>δ-Ta₂O₅-PAA 1 h</td>
<td>41.70</td>
<td>44.81</td>
</tr>
<tr>
<td>δ-Ta₂O₅-PAA 2.5 h</td>
<td>40.39</td>
<td>40.40</td>
</tr>
</tbody>
</table>

Table 5.5. XPS analysis including the O 1s region of δ-Ta₂O₅ NPs (before and after treatment with HCl) and the δ-Ta₂O₅-PAA nanocomposites with polymerization times of 1 and 2.5 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>XPS O 1s envelope ratios (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ta–O</td>
</tr>
<tr>
<td>δ-Ta₂O₅</td>
<td>65.71</td>
</tr>
<tr>
<td>δ-Ta₂O₅ (HCl treated)</td>
<td>77.31</td>
</tr>
<tr>
<td>δ-Ta₂O₅-PAA 1 h</td>
<td>86.84</td>
</tr>
<tr>
<td>δ-Ta₂O₅-PAA 2.5 h</td>
<td>81.87</td>
</tr>
</tbody>
</table>
The O 1s spectra also display peaks that correspond to Ta–O bonds and –OH groups at a binding energy of ~531 and 533 eV, respectively [73]. The presence of hydroxyl groups on the surface of δ-Ta$_2$O$_5$ after treatment with HCl is of particular importance, since the –OH groups act as activation sites that react with the AA monomers on the NP surfaces to form a layer of PAA, as shown via TEM (Figure 5.7).

### 5.2.5 Swelling Properties of δ-Ta$_2$O$_5$-Poly(acrylic acid) Nanocomposites

Investigation of the swelling behaviour of PAA-containing nanomaterials is of importance, since the swelling property influences the diffusion of small molecules through the polymer matrix. It has been shown that the more PAA that is present in the hydrogel, the larger is the affinity of the hydrogels for water, which ultimately results in higher swelling ratios (SR) [35]. The swelling kinetics of the δ-Ta$_2$O$_5$ NPs and the δ-Ta$_2$O$_5$-PAA nanocomposites for a time frame of 0 – 48 h at 37°C and pH 7.4 are displayed in Figure 5.13. As the thickness of the polymer layer increases, the volume of hydrogel increases, consequently increasing the swelling ratio. In general, the swelling ratio increases rapidly in the first 4 h and reaches equilibrium after around 24 h.

![Figure 5.13](image)

**Figure 5.13.** (a) Swelling ratio (SR) of δ-Ta$_2$O$_5$ NPs and δ-Ta$_2$O$_5$-PAA nanocomposites with polymerization times of 1, 2.5, and 5 h. The swelling properties were measured at 37°C at pH 7.4 for 48 h. (b) The influence of the pH value of the buffer solution on the swelling ratio of the δ-Ta$_2$O$_5$-PAA nanocomposite that was synthesized with a polymerization time of 5 h. The swelling properties were measured at 37°C at pH 3.6, 5.4, 7.4, and 9.4 for 48 h.
To study the effect of pH on the swelling ratio, the nanocomposite with highest amount of PAA (δ-Ta₂O₅-PAA 5 h) was chosen and exposed to buffer solutions with different pH (pH 3.6, 5.4, 7.4, and 9.4) at 37°C. The swelling kinetics are shown in Figure 5.13. The pH value has a significant impact on the swelling behaviour of the δ-Ta₂O₅-PAA nanocomposite. The difference in swelling ratio is ascribed to the accessibility of free volume for the expanded polymer matrix, the polymer chain relaxation, and the availability of ionizable functional groups [35]. For instance, the presence of carboxyl groups can lead to the formation of hydrogen bonds with water. In addition, the swelling behaviour depends on the network swelling pressure, the dissociation equilibrium, the presence of ions in the swelling medium, and impurities [74].

The carboxylic acid group in PAA tends to dissociate at pH values greater than 4.5, which ultimately leads to an increase in osmotic pressure inside the polymer matrix and explains the low swelling ratio in the buffer solution of pH 3.6 [35]. With increasing pH, the dissociation of carboxylic groups increases, which leads to negatively charged polymer chains that repel each other. The higher the pH of the buffer solution, the more the carboxylic groups will dissociate, and the more the polymer chains will repel each other, leading to more free space within the polymer network [35], ultimately leading to an increase in the swelling ratio.

5.2.6 Methotrexate Loading Capacity of δ-Ta₂O₅-Poly(acrylic acid) Nanocomposites

Different polymerization times during the δ-Ta₂O₅-PAA synthesis lead to composites with different polymer layer thicknesses (Figure 5.7), which affects the drug loading capacity. The MTX entrapment per 1 mg of nanocomposite, and the loading capacity and entrapment efficiency of all tested samples were determined and are listed in Table 5.6. Since a weight ratio of drug/composite of 1/1 was chosen, the entrapment efficiency is equivalent to the loading capacity. The loading capacity increased with
increasing layer thickness of PAA in the nanocomposites: while the drug loading capacity was 1.2% for a polymerization time of 1 h, it increased to 7.7% and 12.6% for a polymerization time of 2.5 and 5 h, respectively.

**Table 5.6.** Loading capacity (LC) and entrapment efficiency (EE) of δ-Ta$_2$O$_5$ NPs and δ-Ta$_2$O$_5$-PAA nanocomposites with different polymerization times at a weight ratio of MTX/composite of 1/1 (10 mg/mL).

<table>
<thead>
<tr>
<th>Sample</th>
<th>PAA/ 1 mg (TGA) [µg]</th>
<th>MTX/ 1 mg (weight) [µg]</th>
<th>MTX LC and EE [%]$^a$</th>
<th>MTX LC and EE [%]$^b$</th>
<th>MTX/ 1 mg (UV-vis) [µg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ-Ta$_2$O$_5$</td>
<td>-</td>
<td>11.5 ± 0.7</td>
<td>1.2 ± 0.1</td>
<td>-</td>
<td>8.3 ± 1.31</td>
</tr>
<tr>
<td>δ-Ta$_2$O$_5$-PAA 1 h</td>
<td>22.3 ± 0.5</td>
<td>21.3 ± 2.5</td>
<td>2.1 ± 0.3</td>
<td>95.5 ± 11.2</td>
<td>20.2 ± 2.9</td>
</tr>
<tr>
<td>δ-Ta$_2$O$_5$-PAA 2.5 h</td>
<td>101.2 ± 2.1</td>
<td>76.6 ± 9.3</td>
<td>7.7 ± 0.9</td>
<td>75.7 ± 9.2</td>
<td>67.5 ± 5.6</td>
</tr>
<tr>
<td>δ-Ta$_2$O$_5$-PAA 5 h</td>
<td>399.4 ± 8.2</td>
<td>126.4 ± 8.7</td>
<td>12.6 ± 0.9</td>
<td>31.6 ± 2.2</td>
<td>118.0 ± 4.1</td>
</tr>
</tbody>
</table>

$^a$ Normalized to mass of composite; $^b$ Normalized to mass of PAA.

When the hydrogel is placed into the aqueous solution containing MTX, the polymer network starts to swell, and the drug molecules fill the space between the polymer chains. The thicker the polymer layer, the larger is the swelling ratio (Figure 5.13). The MTX molecules and PAA chains experience electrostatic repulsion at a pH of 7.4, however, which could explain the low increase in loading capacity for the nanocomposite with thickest polymer layer [58, 75, 76]. The effects are described in more detail in section 5.2.8.

The MTX entrapment per 1 mg of nanocomposite was also determined via UV-vis spectroscopy after the *in vitro* drug release experiments and the treatment of the composites with 0.1 M NaOH. The calibration curve of different concentrations of MTX was determined and is shown in Figure 5.14. The MTX entrapment obtained via UV-vis spectroscopy is similar to the value calculated through the dry weight of the composite after the drug loading (Table 5.6).
The reported drug loading capacities for similar systems range from 12 – 80%, based on the delivery system components, the drug entrapment method, the incubation time, and the drug concentration used for the entrapment [58, 60, 76, 77]. For instance, it was shown for DOX as a model drug and a system consisting of mesoporous silica and PAA, that by increasing the weight ratio of drug/hydrogel from 0.1 to 0.25, 0.5, and 1.0, the loading capacity increased from 2.2 to 11.3, 24.5, and 40.2%, respectively [57]. The entrapment efficiency also increased from 25.1 to 51.2, 64.8, and 80.4%, respectively [57]. Magnetite NPs functionalized by PAA showed an entrapment efficiency towards Bleomycin of 52.44%; the drug loading concentration was 10 mg/mL [59]. Therefore, a weight ratio of MTX/composite of 1/1 and a concentration of MTX of 10 mg/mL were chosen. It should be noted, however, that δ-Ta$_2$O$_5$ contributes significantly to the overall mass of the composite and therefore, reduces the overall drug loading capacity. When normalized to the mass of polymer present in 1 mg of composite, the loading capacity and entrapment efficiency of MTX increased up to 95% for the composite with the thinnest PAA layer (Table 5.6).

5.2.7 Effect of Poly(acrylic acid) Layer Thickness on the Release of Methotrexate from δ-Ta$_2$O$_5$-Poly(acrylic acid) Nanocomposites

The calibration curve used to determine the MTX concentration and the release kinetics of MTX from δ-Ta$_2$O$_5$ and the δ-Ta$_2$O$_5$-PAA nanocomposites in phosphate buffer
solution with pH 7.4 at 37°C is shown in Figure 5.14. The corresponding drug release kinetic data is displayed in Figure 5.15 and Table 5.7 and was obtained by fitting the drug release experimental data to the Ritger-Peppas equation, as shown in Equation (5.3):

$$\frac{M_t}{M_\infty} = k_{\text{kin}} \cdot t^n$$  \hspace{1cm} (5.3)

where $M_\infty$ is the total amount of drug, $M_t$ is the amount of drug released at time $t$, $k_{\text{kin}}$ is a kinetic constant, and $n$ is the diffusion exponent. The fractional amount of drug released is expressed by $M_t/M_\infty$, and for the modelling, only fractions $< 0.6$ were considered [78].

Figure 5.15. In vitro release profiles of methotrexate-soaked $\delta$-Ta$_2$O$_5$ NPs and $\delta$-Ta$_2$O$_5$-PAA nanocomposites with different polymerization times of 1, 2.5, and 5 h in buffer solution with pH 7.4. The drug release was monitored at 37°C for 72 h.

Besides the diffusion of water into the hydrogel, the Ritger-Peppas model considers the swelling as water enters, the formation of the gel, the diffusion of the drug out of the hydrogel, and the dissolution of the polymer matrix. The diffusion exponent is used to characterize the release mechanism for a thin hydrogel: a value of $n = 0.5$ indicates Fickian diffusion, and a value of $n = 1$ points to Case II (relaxational) transport, which leads to a zero-order release [35, 78]. When $0.5 < n < 1$, anomalous transport is observed [35, 78].
Table 5.7. Drug release kinetic data for $\delta$-$\text{Ta}_2\text{O}_5$-PAA nanocomposites with different polymerization times. The kinetic constant $k_{\text{kin}}$ and the diffusion exponent $n$ were obtained from the fit of the drug release experimental data to the Ritger-Peppas equation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k_{\text{kin}}$ ± Error</th>
<th>$n$ ± Error</th>
<th>$R^2$</th>
<th>Transport mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta$-$\text{Ta}_2\text{O}_5$-PAA 1 h</td>
<td>1.54 ± 0.01</td>
<td>0.54 ± 0.03</td>
<td>0.990</td>
<td>Diffusion and relaxation</td>
</tr>
<tr>
<td>$\delta$-$\text{Ta}_2\text{O}_5$-PAA 2.5 h</td>
<td>1.60 ± 0.01</td>
<td>0.61 ± 0.03</td>
<td>0.992</td>
<td>Diffusion and relaxation</td>
</tr>
<tr>
<td>$\delta$-$\text{Ta}_2\text{O}_5$-PAA 5 h</td>
<td>1.78 ± 0.02</td>
<td>0.71 ± 0.05</td>
<td>0.987</td>
<td>Diffusion and relaxation</td>
</tr>
</tbody>
</table>

For all tested samples, a burst release of MTX occurred in the first hour, which is due to desorption of MTX from the gel surface. The following release of the drug was slower and yielded a total release of MTX of 100% for the $\delta$-$\text{Ta}_2\text{O}_5$ NPs, and 85.6, 88.8, and 91.8% for the $\delta$-$\text{Ta}_2\text{O}_5$-PAA nanocomposites with a polymerization time of 1, 2.5, and 5 h, respectively. The fast release of MTX from the uncoated $\delta$-$\text{Ta}_2\text{O}_5$ NPs is related to the absence of a hydrogel layer.

When the MTX-loaded hydrogel is placed in the release medium, the polymer starts to swell, followed by polymer chain relaxation and volume expansion. This enables the trapped MTX to diffuse into the external release medium [75]. The swelling ratio increased rapidly in the first hour and is therefore another explanation for the initial burst release of MTX. The observed release rate tends to increase with increasing polymer layer thickness, which is directly connected to the increase in drug loading [79].

The analysis of the release data showed that it essentially follows a non-Fickian process, since the diffusion exponent $n$ had a value > 0.5 for all the synthetized samples. The drug release kinetics of $\delta$-$\text{Ta}_2\text{O}_5$ NPs was impossible to determine due to the boost of drug release to the buffer solution that started immediately after immersion of the particles in the media. A value for $n$ between 0.54 and 0.71 (Table 5.7) was obtained for all the nanocomposites and indicates that the drug transport mechanism appears to be
anomalous: the drug release is controlled by a coupled effect of Fickian diffusion and chain relaxation of the hydrogels.

### 5.2.8 Effect of pH on the Drug Release Kinetics

To evaluate the effects of pH on the overall drug release and its mechanism, the drug release from $\delta$-Ta$_2$O$_5$-PAA (5 h polymerization time) was studied in release buffers with pH of 3.6, 5.4, 7.4, and 9.4. The drug release profiles are shown in Figure 5.16. The experimental data was fitted to the Ritger-Peppas model (Equation (5.3)) and is listed in Table 5.8. The drug release from the nanocomposites follows a similar profile for all tested pH values, starting with the initial burst release of MTX, which is followed by the slow release at longer incubation times.

**Figure 5.16.** *In vitro* release profiles of methotrexate-soaked $\delta$-Ta$_2$O$_5$-PAA with longest polymerization time of 5 h in buffer solutions with pH values of 3.6, 5.4, 7.4, and 9.4. The drug release was monitored at 37°C for 72 h.

Greater drug release was observed as the pH value of the buffer solution was increased. While the total release of MTX is only 79.1% at a pH of 3.6, the drug release increases to 84.4, 91.8, and 96.0% for pH of 5.4, 7.4, and 9.4, respectively. Moreover, the initial burst release of MTX was significantly reduced for the lowest pH of 3.6. It was shown that the swelling of the PAA nanolayer is the principal parameter ruling drug release [80]. Since the swelling ratio increases with increasing pH of the buffer solution, more
incorporated drug can be released from the hydrogel as the structure becomes more open.

The importance of the swelling behaviour of the hydrogel regarding drug release has been reported in the literature. For instance, hydrogels that were synthesized with cross-linking agents displayed a significantly slower drug release than unmodified hydrogels. The drug release was significantly hindered as the hydrogel cross-linking density was increased [75].

**Table 5.8.** Drug release kinetic data for the δ-Ta₂O₅-PAA nanocomposite with 5 h polymerization time, exposed to different pH environments. The kinetic constant \( k_{\text{kin}} \) and the diffusion exponent \( n \) were obtained from the fit of the drug release experimental data to the Ritger-Peppas equation.

<table>
<thead>
<tr>
<th>Release medium</th>
<th>( k_{\text{kin}} )</th>
<th>( n )</th>
<th>( R^2 )</th>
<th>Transport mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.6</td>
<td>1.39 ± 0.01</td>
<td>0.28 ± 0.03</td>
<td>0.966</td>
<td>Diffusion</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>1.61 ± 0.01</td>
<td>0.54 ± 0.03</td>
<td>0.992</td>
<td>Diffusion and relaxation</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>1.78 ± 0.02</td>
<td>0.71 ± 0.05</td>
<td>0.987</td>
<td>Diffusion and relaxation</td>
</tr>
<tr>
<td>pH 9.4</td>
<td>1.88 ± 0.01</td>
<td>0.84 ± 0.01</td>
<td>0.999</td>
<td>Relaxation</td>
</tr>
</tbody>
</table>

In addition to the effect of the swelling ratio on the drug release, the presence of electrostatic interactions between the drug and the hydrogel can also influence the drug release [58, 75, 76], as schematically shown in Figure 5.17. MTX has three \( pK_a \) values at 3.8, 4.8, and 5.6, while the dissociation of the carboxylic group of PAA takes place at \( pK_a \) of 4.25 [81]. At pH 3.6, the nitrogen atom in the pteridin ring should be mainly protonated, while the carboxylic groups on the hydrogel are hardly ionized. This leads to the presence of weak hydrogen bonding between the carboxylic groups of MTX and PAA. At pH 5.4, the carboxylic groups on the PAA chains should be partly deprotonated to form carboxylate anions, which leads to fewer hydrogen bonds. Moreover, the carboxylic groups of the MTX molecule start to dissociate, which causes further negative charge and consequently, increases the electrostatic repulsion between the MTX molecule and the PAA chains. This ultimately results in an increase in drug release rate.
At pH of 7.4 and 9.4, the nitrogen atom of the pteridine ring should be mostly deprotonated, which further increases the electrostatic repulsion between the MTX molecule and the PAA. The increase in pH shifts the equilibrium further to dissociated components and increases the electrostatic repulsion and the release of MTX. Moreover, the solubility of PAA increases with increasing pH, which can lead to the degradation and erosion of the polymer matrix and thus, to the increased release of entrapped MTX, especially for long incubation times [82, 83]. The principle of electrostatic repulsion and attraction has been shown to play an important role in controlled drug release. DOX, for instance, is a chemotherapeutic drug that has no carboxylic groups and its amino group is mostly protonated (pKₐ 8.2). When combined with PAA, different pH sensitivity can be obtained: the release of DOX is faster at pH 4.2 and slows down at a higher pH of 7.4 [60].

Figure 5.17. (a) Structure of methotrexate. (b) Schematic route of the methotrexate (= drug) loading and release from the nanocomposites containing poly(acrylic acid) (PAA) at different pH values (3.6, 5.4, 7.4, and 9.4).
The analysis of the drug release kinetic data indicates that the release mechanism strongly depends on the pH of the release medium, as shown in the literature [35, 56, 75]. The diffusion exponent $n$ increased as the pH of the release medium became more alkaline, which underlines the pH sensitivity of PAA-containing hydrogels. For a pH of 3.6, the release followed Fickian diffusion ($n < 0.5$). With regard to the release of MTX, Fick’s law includes the adsorption of buffer on the polymer matrix and the simultaneously desorption of MTX via diffusion [56]. For pH values of 5.4 and 7.4, the increase in the diffusion coefficient ($n = 0.54 \pm 0.03$ to $0.71 \pm 0.05$) indicates that the release mechanism is a result of the contributions of both Fickian diffusion and relaxation of polymer chains. At a pH of 9.4, the extensive ionization of the functional groups significantly influences the polymer chain relaxation and consequently, leads to a transport mechanism that is closer to Case II transport ($n = 0.84 \pm 0.01$). Such an increase in the diffusion exponent to values close to 1 at high pH has been reported [64, 78].

5.2.9 **In Vitro Computed Tomography Imaging of $\delta$-Ta$_2$O$_5$ Nanoparticles and $\delta$-Ta$_2$O$_5$-Poly(acrylic acid) Nanocomposites**

The anatomical contrast enhancement capability of uncoated $\delta$-Ta$_2$O$_5$ NPs and the $\delta$-Ta$_2$O$_5$-PAA nanocomposite with high polymerization time (5 h) was demonstrated using a clinical CT scanner, with the resulting CT number as a function of the tantalum concentration, as shown in Figure 5.18 and Figure 5.19. The CT values in HU for each concentration were determined for multiple regions along the central axis of the vial to account for possible sedimentation of the suspended composites (see red dashed lines).
Chapter 5 – Tantalum-Based Compounds for Biomedical Applications

**Figure 5.18.** (a) Linear fitting of the CT number of δ-Ta2O5 nanoparticles as a function of the mass concentration of Ta in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of linear regression and the coefficient of determination (R²) value are indicated. (b) CT images of δ-Ta2O5 nanoparticles with Ta mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area that was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.

**Figure 5.19.** (a) Linear fitting of the CT number of δ-Ta2O5-PAA nanoparticles (5 h polymerization) as a function of the mass concentration of Ta in mg/mL at different tube potentials of 100, 120, and 135 kVp. The equation of the linear regression and the R² value are indicated. (b) CT images of δ-Ta2O5-PAA nanoparticles (5 h polymerization) with Ta mass concentrations of 0 – 8 mg/mL; the red dashed line indicates the area which was used to determine the CT number. The tube potential varied between 100, 120, and 135 kVp.
In general, the contrast increased with increasing concentration of tantalum, and the suspension remained stable up to a concentration of 8 mg/mL for the δ-Ta$_2$O$_5$-PAA nanocomposite. For the uncoated δ-Ta$_2$O$_5$ NPs, however, a noticeable difference in CT number was observed at the highest concentration of 8 mg/mL for the centre of the composite sediment and the centre of the suspension height, which ultimately underlines the stabilization effect of PAA on the suspension.

The data was plotted and fitted to deduce the relation between the CT number and the Ta concentration. The average CT number was chosen across the suspension height. It should be noted that the CT number reached a value of 1000 – 2000 HU in the sediment at a Ta concentration of 8 mg/mL for uncoated δ-Ta$_2$O$_5$, which highlights the potential of Ta$_2$O$_5$ as an anatomical contrast enhancement agent. These results agree with reported CT numbers for Ta-containing compounds and underline the improved suspension stability and reduced beam hardening in comparison to previously reported Bi$_2$O$_3$ NPs [84, 85].

X-ray attenuation also depends on the X-ray photon energy. Typically, for most elements, including iodine, the X-ray attenuation decreases with increasing peak tube potential. In vitro studies have shown that for the same concentration of tantalum and iodine, the tantalum produced a significantly greater image contrast than iodine across the diagnostic CT X-ray spectrum, ranging from 80 to 140 kVp [13, 86].

In contrast to iodine, tantalum has a k-edge within the diagnostic X-ray spectrum, which ultimately results in an increase in attenuation at discrete energy levels near the k-edge [13]. Upon application of a polychromatic X-ray spectrum, the image contrast remains relatively constant for different tube potentials, as attenuation at discrete energy levels below and above the k-edge contribute to the net attenuation. The superior contrast enhancement and contrast-to-noise ratio of δ-Ta$_2$O$_5$-PAA for high X-ray energies are of particular interest in a clinical setup [86]. Although it is beneficial for pediatric patients to reduce the radiation dose, the ability to use high X-ray energies is important for the
imaging of large patients, as a sufficient penetration of X-rays needs to be ensured to obtain images of the desired quality.

5.2.10 Conclusions

In order to design a theranostic system with controlled drug release properties, δ-Ta₂O₅-PAA nanocomposites were prepared through the polymerization of AA on the surfaces of individual δ-Ta₂O₅ NPs. By varying the polymerization time, different layer thicknesses of PAA on the surfaces of the ceramic particles were obtained, which ultimately had an impact on the drug loading capacity.

MTX-loaded δ-Ta₂O₅-PAA matrices were prepared by soaking δ-Ta₂O₅-PAA in a solution of MTX, and the release mechanism was studied by the Ritger-Peppas model. It was demonstrated that the δ-Ta₂O₅-PAA nanocomposites were highly sensitive to different pH environments. Firstly, the swelling ratio increased with increasing pH of the swelling medium. Secondly, an acidic release medium slowed down the release of MTX, while more alkaline pH values led to a quick release of MTX from the MTX-loaded δ-Ta₂O₅-PAA matrices. The diffusion exponent $n$ increased from 0.28 to 0.84, which suggests that, with increasing pH, polymer chain relaxation contributes to the transport mechanism. These results indicate that such a system may, under certain conditions, provide release characteristics approaching zero-order release.

Furthermore, the δ-Ta₂O₅-PAA nanocomposites showed a contrast enhancement for tube potentials ranging from 100 to 135 kVp in vitro. The image consistency at high X-ray energies is of particular interest in a clinical setup, as it ensures sufficient penetration of X-rays in larger patients.

The results obtained in this research work lead to the conclusion that δ-Ta₂O₅-PAA nanocomposites can be successfully used as drug delivery systems, providing imaging, therapeutic, targeting, and potentially, radiation dose enhancement functionalities on one single platform.
5.3 References


Chapter 5 – Tantalum-Based Compounds for Biomedical Applications


Chapter 5 – Tantalum-Based Compounds for Biomedical Applications


CHAPTER 6 – Conclusions and Future

Prospects
6.1 Thesis Conclusions

6.1.1 Theranostic Nanoparticles

In this doctoral work, various aspects related to the fabrication and characterization of nanomaterials for biomedical applications have been explored and developed. Recently, work in the area of theranostic materials has been focused not only on the development of systems with therapeutic and diagnostic features, but also on the addition of more features such as selectivity and targeting functions. Various current state-of-the-art nanomaterials were thoroughly reviewed in regard to their advantages and disadvantages compared to other multifunctional materials used in the field of cancer research. Typically, organic NPs, inorganic NPs, or a combination of multiple materials are used to incorporate many features into one single platform and optimize their physiological interactions [1, 2].

A classical precipitation synthesis method was used to fabricate Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs. Only a small number of reports exist on the overall physicochemical characterization of Bi(OH)$_3$, particularly with regard to its cellular interactions. In contrast, α-Bi$_2$O$_3$ has been studied widely regarding its physical and chemical properties, although less consideration has been given to its bioactivity. This work sought to evaluate, for the first time, the use of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs as chemotherapeutic agents. It was demonstrated that both types of NPs exhibit high in vitro toxicity towards malignant 9L and MCF-7 cells, which makes them promising theranostic agents for cancer treatment. More importantly, these nanomaterials showed only low to moderate toxicity in normal, non-cancerous, MDCK cells, which also suggests high selectivity. It is commonly known that inorganic nanomaterials such as ZnO NPs mediate their toxicity through the generation of ROS [3, 4]. In this doctoral thesis, the first insights were gleaned regarding the apoptotic pathway of the tested malignant 9L cells, which was found to be oxidation-dependent. Cleavage was identified in 9L cells after exposure to the NPs, while no
significant increase in cleavage was observed in MDCK cells. The enhancement of anatomical contrast is also demonstrated in this work. Considering the potential radiation dose enhancement properties of Bi-based materials [5], these theranostic materials could potentially display four different functions: therapeutics, selectivity, imaging, and radiation dose enhancement. With this combination of properties, current cancer therapies can potentially be refined, and therefore, the spectrum of side effects can be reduced while maintaining similar therapeutic effects.

In this doctoral thesis, nanocomposites in the form of $\delta$-Ta$_2$O$_5$-PAA were synthesized for the first time and loaded with the chemotherapeutic drug MTX, which is known to also provide targeting features [6, 7]. The novelty of this work lies also in the synthesis method, which includes activation of the surfaces of the $\delta$-Ta$_2$O$_5$ NPs prior to the polymerization of acrylic acid on the surface of $\delta$-Ta$_2$O$_5$, resulting in controllable layer-by-layer-formation of PAA. It was demonstrated that the $\delta$-Ta$_2$O$_5$-PAA nanocomposites were highly sensitive to different pH environments, which is desirable, as it provides the ability to reduce the dosage frequency and systemic toxicity of the administered drugs. While an acidic release medium slowed down the release of MTX, alkaline pH values caused a quick release of MTX from the MTX-loaded $\delta$-Ta$_2$O$_5$-PAA matrices. Via the Ritger-Peppas model, it was shown that, with increasing pH, polymer chain relaxation contributes to the transport mechanism. In addition, the Ta$_2$O$_5$-PAA nanocomposites displayed anatomical contrast enhancement and provided image consistency at high X-ray energies, which is highly valuable in a clinical setup, as it ensures sufficient penetration of X-rays in larger patients. This theranostic system potentially displays four different features on one single platform: while the imaging and radiation dose enhancement properties are ascribed to $\delta$-Ta$_2$O$_5$ [8], the therapeutic and targeting features are associated with MTX [6, 7].
6.1.2 Nanoparticles as Ultraviolet Filters in Sunscreens

This doctoral work has also highlighted the main challenges related to the current state-of-the-art of commercial sunscreens that typically contain photosensitive organic UV filters, or worse, photoactive inorganic UV filters, such as TiO₂ and ZnO NPs [9]. The photocatalytic activity associated with the NPs reduces the overall photoprotection and lowers the SPF as a consequence of photogenerated ROS, which can cause further damage through the generation of unknown by-products with unknown toxicity [10-12]. ZnO NPs in particular have been shown to exhibit intrinsic geno- and cytotoxicity, which ultimately resulted in the search for alternative materials [13-15].

In this doctoral thesis, the UV protective and photocatalytic properties of Bi(OH)₃ NPs have been investigated for the first time. Bi(OH)₃ NPs are colourless, exhibit a comparable absorbance in the UV region to ZnO and TiO₂ NPs, and display greater biocompatibility than TiO₂ and ZnO NPs, which makes them highly attractive as a potential ingredient in sunscreens. The Bi(OH)₃ NPs showed low photocatalytic activity throughout the whole UV-visible spectrum and, more importantly, were able to reduce the photocatalytic activity of TiO₂ and ZnO NPs. In order to determine the suitability of Bi(OH)₃ NPs as an alternative to ZnO NPs as an active UV filter in sunscreens and, especially, to compare their performance to commercially available sunscreens, sunscreen emulsions were prepared in-house containing a mixture of either TiO₂/Bi(OH)₃ or TiO₂/ZnO. The homemade sunscreen made of TiO₂/Bi(OH)₃ displayed rheological properties comparable to those of commercial sunscreens and significantly increased photostability, SPF, and UVA-PF when compared to the classical combination of TiO₂/ZnO or other commercial sunscreens. Considering the excellent performance of Bi(OH)₃, this material offers a novel and cost-effective approach to improving the UV-blocking properties of commercial sunscreen formulations, with the added benefit of acting as an antioxidant stabilizer for the organic components of sunscreens, reducing their potential harm to the health of the consumer.
Examining the development of nanocomposites based on TiO$_2$ and (BiO)$_2$CO$_3$ as an alternative to the introduction of a completely novel inorganic UV filter was also a primary aim of this doctoral work. Due to the complex and especially long-lasting process required for approval of new compounds for biomedical applications, alternative approaches exist to reduce the photocatalytic activity of TiO$_2$ NPs while maintaining their UV filtering properties. Typically, thin coatings based on silica, aluminium oxide, aluminium hydroxide, methicone, and poly(methacrylic acid) are used [16, 17]. To further explore the possibilities of Bi-based compounds, (BiO)$_2$CO$_3$ clusters with a size < 10 nm were precipitated onto the surfaces of TiO$_2$ NPs for the first time. Reports have previously indicated that (BiO)$_2$CO$_3$ acts as a photocatalyst when used in large concentrations, although its activity at low concentrations had not been determined. The TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites were synthesized via precipitation without the application of templates or hydrothermal processes, which are typically used to obtain (BiO)$_2$CO$_3$ [18, 19]. By introducing only a small amount of (BiO)$_2$CO$_3$ clusters onto the surface of TiO$_2$, its photocatalytic activity was decreased and its biocompatibility in healthy cells increased, while maintaining its size, morphology, and UV blocking ability. Moreover, the nanocomposites displayed reduced toxicity in HaCaT cells upon irradiation with simulated sunlight, when compared to TiO$_2$ NPs, which indicates that the composites are capable of reducing the photo-induced generation of ROS. The TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites are therefore a promising novel material for further investigation as an active UV filtering additive in sunscreens.

6.2 Future Prospects

6.2.1 Theranostic Nanoparticles

Despite the many decades of research on therapeutic NPs, there are still uncertainties about their mechanism of action and their physiological interactions. Once inside the body, the NPs can interact with various substances in the blood and cells, while
being exposed to different pH environments, which affects the stability, aggregation, and agglomeration of the nanomaterials [20]. As a result, the materials can change in size, dissociate, or dissolve, forming unknown by-products whose bioactivity and interactions are yet largely unknown. This doctoral work aims to provide the first insights into the physicochemical properties of Bi- and Ta-based nanomaterials and their mechanism of action in the cellular environment in vitro. Due to the promising obtained results, further studies in an advanced setting would be beneficial, including the use of more experiments such as caspase activity assays in vitro, or in vivo studies.

As for the study of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs, a possible mechanism of action was proposed for the extreme toxicity observed in 9L cells. By using self-developed experiments, the first insights into the processes involved in the cell death were obtained. Although the toxicity of the NPs can be connected to increased ROS levels, the mechanism of action can also be related to other factors, such as the different natures of the investigated cell lines, differential gene expression and sensitivity responses to stimuli, different pH environments, or differences in cellular uptake. Since only initial experimental evidence is provided in this work, biological in vitro testing with cell lines of the same origin should be performed in order to ultimately answer these questions. Unfortunately, such cell lines were not available for this doctoral thesis. More importantly, more cell lines need to be tested to verify the early claims made in this work regarding the selective toxicity of Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs. While radiation dose enhancement by α-Bi$_2$O$_3$ NPs has recently been reported [5], this feature needs to be further evaluated using Bi(OH)$_3$ NPs. Since 9L cells are known to be highly radiation-resistant, this cell line could be a preferred starting point.

Future consideration can also be given to improvement of the stability of the Bi(OH)$_3$ and α-Bi$_2$O$_3$ NPs, which tend to agglomerate in suspension. To avoid possible effects of stabilizing agents on the cellular experiments that were carried out in this
doctoral thesis, the use of stabilizing agents was avoided. The goal was to study the interactions of pristine Bi(OH)$_3$ and Bi$_2$O$_3$ NPs with the cells. To increase cellular uptake and stabilize the particles in suspension, future work can involve suitable surface coatings that do not affect the NPs’ bioactivity [21]. This approach would also be suitable with regard to the long-term goal of intravenous delivery of the NPs, such as with currently used chemotherapeutic drugs.

The structural characterization of Bi(OH)$_3$ NPs also lacks detail: while the literature for all the other Bi- and Ta-based compounds that were investigated in this doctoral thesis provides sufficient data regarding their crystal structure, no information was available for Bi(OH)$_3$. Since Bi(OH)$_3$ NPs exhibit great potential for application in cancer treatment and as an inorganic UV filter in sunscreens, it could be beneficial to further research their crystal structure. Unfortunately, it wasn’t possible to further characterize the Bi(OH)$_3$ NPs during this doctoral thesis.

In this doctoral work, a thorough study was conducted on the loading and release of MTX in δ-Ta$_2$O$_5$-PAA nanocomposites, as well as their contrast-enhancing properties. Future work can include the optimization of synthesis time of the δ-Ta$_2$O$_5$-PAA nanocomposites. For instance, the influence of the organic polymerization rate on the kinetics of drug release can be studied using real time FTIR. In addition, delivery kinetics between physisorbed and chemisorbed hydrogels can be elaborated in further studies.

To fully investigate the efficiency of the nanometric theranostic system, the nanocomposites should also be characterized biologically. The therapeutic effects of δ-Ta$_2$O$_5$-PAA nanocomposites can be evaluated through in vitro experiments in cancer and normal cells, followed by the characterization of the targeting function currently attributed to MTX [6, 7]. In order to fully characterize the theranostic system, in vitro tests would be required to assess its capability for radiation dose enhancement.
6.2.2 Nanoparticles as Ultraviolet Filters in Sunscreens

Similarly to the use of NPs as therapeutic agents, their topological application in form of sunscreen emulsions also suffers from well-known difficulties. Reports regarding the skin penetration of NPs and, in particular, their cytotoxicity vary significantly, making it challenging to find full acceptance for their use in healthcare [17, 22-24]. This inconclusiveness can be related to missing guidelines for skin toxicity, sensitization, and corrosion, as well as to the use of different testing protocols, cell lines, and unrealistic dose exposure [25-28]. In order to provide a better comparison to other reports in the research on NPs for medical application, MDCK and HaCaT cells were chosen to investigate the cytotoxicity of Bi(OH)$_3$ and TiO$_2$/(BiO)$_2$CO$_3$ nanomaterials. The results obtained in this doctoral work justify further studies regarding the effects of the Bi-based nanomaterials in animal models in vivo, such as on pig skin, which is often used to evaluate the skin penetration of NPs. This setup can also be utilized to examine their photoprotection in vivo.

The sunscreen emulsions made of TiO$_2$ and Bi(OH)$_3$ NPs showed excellent photoprotection and rheological properties, and much lower photoactivity and photodegradation when compared to commercial sunscreens. Their excellent performance, especially in comparison to the classical combination of TiO$_2$/ZnO, justifies further in vitro testing. Considering the bioactivity of Bi(OH)$_3$, which was evaluated as part of this doctoral work, the Bi(OH)$_3$ NPs should be tested in skin cancer cells, where they could potentially induce apoptosis, as was shown in 9L and MCF-7 cells. As shown in this doctoral thesis, Bi(OH)$_3$ NPs exhibit high biocompatibility in normal skin cells, which justifies initiating in vivo tests to further investigate their properties.

This doctoral work also showed that the TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites exhibit significantly reduced photocatalytic activity when compared to pristine TiO$_2$ NPs, while still having comparable UV blocking properties. Future investigations should focus on the various mechanisms of action, which was not possible to do during this doctoral thesis.
work, as the resources were not available. For instance, the scavenging properties of the carbonate ion could be studied via ESR. Moreover, the band structure of (BiO)$_2$CO$_3$ should be investigated, since the reduced photocatalytic activity of TiO$_2$/(BiO)$_2$CO$_3$ may be associated with the band structures of TiO$_2$ and (BiO)$_2$CO$_3$. Similarly to the study of Bi(OH)$_3$ NPs as a potential UV filter, the nanocomposites can also be incorporated into sunscreen emulsions, and both their photoprotection and photodegradation could be investigated and compared to commercial sunscreen formulations.

6.3 References


Chapter 6 – Conclusions and Future Prospects


Figure A1. (a) XPS survey spectra and high-resolution XPS spectra of (b) C 1s and (c) N 1s regions of Bi(OH)$_3$ (top) and $\alpha$-Bi$_2$O$_3$ (bottom) nanoparticles.
Figure A2. BF images of untreated 9L cells, incubated for a total time of 48 h.
Figure A3. BF images of 9L cells incubated with Bi(OH)$_3$ nanoparticles at a concentration of 50 μg/mL for a total time of 48 h.
Figure A4. BF images of 9L cells incubated with α-Bi$_2$O$_3$ nanoparticles at a concentration of 50 μg/mL for a total time of 48 h.
Figure A5. BF images of untreated MCF-7 cells, incubated for a total time of 48 h.
Figure A6. BF images of MCF-7 cells incubated with Bi(OH)$_3$ nanoparticles at a concentration of 50 μg/mL for a total time of 48 h.
Figure A7. BF images of MCF-7 cells incubated with $\alpha$-Bi$_2$O$_3$ nanoparticles at a concentration of 50 $\mu$g/mL for a total time of 48 h.
Figure A8. BF images of untreated MDCK cells, incubated for a total time of 48 h.
Figure A9. BF images of MDCK cells incubated with Bi(OH)$_3$ nanoparticles at a concentration of 50 μg/mL for a total time of 48 h
Figure A10. BF images of MDCK cells incubated with α-Bi$_2$O$_3$ nanoparticles at a concentration of 50 μg/mL for a total time of 48 h.
Figure A11. XPS survey spectra of (a) Bi(OH)$_3$ (M1), (b) TiO$_2$ (M2), and (c) ZnO (M3) nanoparticles.
Figure A12. High-resolution XPS spectra of the N 1s (left column), O 1s (centre column), and Ti 2p (right column) regions for TiO$_2$ nanoparticles, and TiO$_2$/(BiO)$_2$CO$_3$ nanocomposites with different Bi/Ti atomic ratios.
APPENDIX II

**Table A1.** High resolution XPS data for the C 1s region of Ta\textsubscript{2}O\textsubscript{5} NPs (before and after treatment with HCl) and the Ta\textsubscript{2}O\textsubscript{5}-PAA nanocomposites with polymerization times of 1 and 2.5 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CH\textsubscript{2} (eV)</th>
<th>CH (eV)</th>
<th>C=O (eV)</th>
<th>O=C=O (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}</td>
<td>284.8</td>
<td>286.0</td>
<td>288.4</td>
<td>–</td>
</tr>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5} (HCl treated)</td>
<td>284.8</td>
<td>285.9</td>
<td>288.1</td>
<td>–</td>
</tr>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}-PAA 1 h</td>
<td>284.8</td>
<td>286.1</td>
<td>288.5</td>
<td>290.0</td>
</tr>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}-PAA 2.5 h</td>
<td>284.8</td>
<td>286.0</td>
<td>288.2</td>
<td>290.1</td>
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</table>

**Table A2.** High resolution XPS data for the O 1s region of Ta\textsubscript{2}O\textsubscript{5} NPs (before and after treatment with HCl) and the Ta\textsubscript{2}O\textsubscript{5}-PAA nanocomposites with polymerization times of 1 and 2.5 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ta–O (eV)</th>
<th>Ta–OH or C–OH (eV)</th>
<th>C=O (eV)</th>
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<tbody>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}</td>
<td>530.0</td>
<td>532.2</td>
<td>–</td>
</tr>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5} (HCl treated)</td>
<td>530.4</td>
<td>532.5</td>
<td>–</td>
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<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}-PAA 1 h</td>
<td>531.6</td>
<td>533.2</td>
<td>534.1</td>
</tr>
<tr>
<td>δ-Ta\textsubscript{2}O\textsubscript{5}-PAA 2.5 h</td>
<td>531.4</td>
<td>533.1</td>
<td>534.1</td>
</tr>
</tbody>
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