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Polyelectrolyte complex materials from chitosan and gellan gum

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POLYELECTROLYTE COMPLEX MATERIALS FROM CHITOSAN AND GELLAN GUM

A thesis submitted in fulfillment of the
requirements for the award of the

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

KHAIRUL ANUAR MAT AMIN, B.Sc, M.Sc

SCHOOL OF CHEMISTRY

August, 2011

To my wife Majidah Hassan, my daughter Iman Huwaida and my mother Ramlah Khatib Mamat for their understanding and love.

CERTIFICATION

I, Khairul Anuar Mat Amin, declare that this thesis submitted in fulfilment of the requirement for the award of Doctor of Philosophy, in the School of Chemistry at the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for any qualifications at any other academic institution.

Khairul Anuar Mat Amin

August 2011

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Thank you.

ABSTRACT

The demand for wound management treatment especially advanced and active wound care products are huge. Polyelectrolyte complex (PEC) materials have been reported as a promising and novel way to develop wound dressings. The research presented in this thesis aimed to utilise the biopolymers chitosan (CH) and gellan gum (GG) in the preparation of PEC films. These films were prepared by dipping free-standing CH and GG films into oppositely charged solutions. Studies on PEC films showed that the mechanical characteristics depend on the solution and film pH, as well as the order of addition of the biopolymers. GG films immersed into CH solutions were found to consist of an inner GG layer and two outer CH layers, whereas in the reverse addition process, GG diffuses into the CH layer. The different in film composition was related to differences in pH and persistence length of GG and CH. Similar mechanical characteristics were observed for PEC coated wool fibres. The observation of the inability of CH solutions to diffuse into GG materials was used to develop dual layer films for future wound dressing applications. The upper layer was designed to act as a bacterial resistance layer, while the bottom layer was designed to promote cell viability and proliferation. Titanium dioxide (TiO₂), silver and zinc oxide nanoparticles as well as the antibiotic levofloxacin (Lev) were incorporated into CH and GG films to assess their ability to improve the biopolymers' antibacterial and cell viability properties. CH-Lev films displayed strong bacterial resistance against *Escherichia coli*, which was attributed to fast release (within 30 min) of levofloxacin. GG-TiO₂ composites exhibited the best cell viability and cell proliferation of all materials considered. Dual layer films consisting of a CH-Lev upper layer and GG-TiO₂ composite as bottom layer were found to be mechanically robust and flexible. The water vapour transmission rates (WVTR) of dual layer films is comparable to WVTR values reported for commercial wound dressing such as Cutifilm, Tegaderm, Bioclusive, Duoderm and Intrasite. As conclusion, PEC materials from CH and GG designed with future wound dressing applications were successfully prepared.

ABBREVIATIONS

γ	strain-at-break
Ag	silver
ALP	alkaline phosphatase
CD	circular dichroism
CFU	colony-forming unit
CH	chitosan
cm	centimetre
cP	centipoise
d	day
d	diameter
Da	Dalton
DD	degree of deacetylation
DMEM	Dulbecco's Modified Eagle's Medium
E	Young's modulus
EDTA	ethylenediaminetetraacetic acid
<i>Escherichia coli</i>	<i>E. coli</i>
FBS	fetal bovine serum
g	gram
GG	gellan gum
GlCNAc	N-acetyl-glucosamine
Gly	glycerine
h	hour

HMW	high molecular weight
kHz	kiloHertz
kN	kiloNewton
L929	mouse fibroblast cell
Lev	levofloxacin
m	metre
M	molar
mg	milligram
min	minute
mJ	milli-Joules
mL	millilitre
mm	millimetre
MMW	medium molecular weight
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H tetrazolium
Mw	molecular weight
mΩ	milliOhm
NH ₃ ⁺	amine group
nm	nanometre
η	viscosity
P	permeance
PEC	polyelectrolyte complex
PEMs	polyelectrolyte multilayers
ppm	part per million

RH	relative humidity
rpm	rotation per minute
s	second
SD	standard deviation
SEM	scanning electron microscopy
t	time
T	toughness
TCPP	tissue culture polystyrene plate
TiO ₂	titanium dioxide
TS	tensile strength
UV-Vis	ultraviolet visible
WVP	water vapour permeability
WVTR	water vapour transmission rates
ZnO	zinc oxide

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of wound management

1.1.1 What is a wound?

A wound is a type of injury in which the skin or living tissue is torn, cut or punctured as the results of surgical incisions, diseases, or burns ¹. Every year, there are approximately 165 million cases worldwide requiring wound treatment across different types of wounds as shown in Table 1.1. Surgical wounds are the vast majority of injuries (103 million) followed by lacerations (20 million), diabetic ulcers (11 million) and burn wounds (10 million) (Table 1.1) ². Surgical wounds are classified to three types: (i) skin grafts or muscle flaps, (ii) wound dehiscence, and (iii) surgically debrided wounds and left to close by secondary intention ¹.

Laceration is the common type of trauma that occurs as a result of a graze due to the dressing sticking to the wound. Chronic wounds such as diabetic and venous ulcers are caused by an autoimmune disorder of pancreatic β -cells and arterial problems, respectively ³. Burn injuries occur when energy is transferred from a heat source (electrical, thermal, chemical) to the body which may result in cell death

depending on burn size and depth ⁴. Burn depth is described in terms of degrees: (i) first-degree burns (superficial burns), involves only the epidermis ⁵, (ii) second-degree burns (superficial-thickness burns), involves the epidermis and dermis, (iii) third-degree burns (full-thickness burns), involves the epidermis and dermis in subcutaneous tissues ⁶, and (iv) fourth-degree burns (subdermal burns), involves epidermis, dermis, fat, muscle, tendon and/or bone ⁶.

Table 1.1: Global cases of wound management worldwide (Source: MedMarket Diligence report #S247).

Wound Type	Worldwide Prevalence (millions)
Surgical wounds	102.8
Traumatic wounds	1.5
Lacerations	19.9
Burn wounds (out-patient)	3.3
Burn wounds (medically treated)	6.3
Burn wounds (hospitalized)	0.2
Pressure ulcers	7.4
Venous ulcers	11.0
Diabetic ulcers	11.3
Amputations	0.2
Carcinomas	0.6
Melanoma	0.1
Complicated skin cancer	0.1
Total	164.7

There are more than 3000 wound products available on the market to fulfil the specific functions of healing ⁷. They cover a wide range of treatments such as passive dressings (gauze, bandages), advanced and active wound care products (semipermeable dressing, multilayer absorbent dressing, calcium alginate dressing, hydrogel, hydrocolloid), physical therapies for wound care (electrical stimulation, ultrasound), closure products (sutures, staples, tapes), internal wound products for surgery (locking plate, nails, screws), and last, but not least tissue engineering (scaffolds) ⁸.

Approximately 80% of the surgical wounds are treated with wound closure products such as sutures, staples and tapes, however in the case of surgically debrided wounds, a wound dressing is used to cover the open wound, which is then filled by granulation and closed by epithelialisation ⁹. A significant problem of this type of wound healing is the likelihood of pathological infection. The average level of infections in surgical wounds is 7 to 10%, dependent on the procedure, surgical discipline and skills. Infections can be prevented by wound care products with antibacterial properties ¹⁰.

It is crucial that laceration and chronic wounds are treated using advanced and active wound care products which provide a moist wound environment and absorb exudates, and an antibacterial barrier ^{11, 12}. Burn wounds (minor burns, medically treated, and hospitalized) have been healed by using hydrogel and advanced wound care products to increase tissue proliferation and to improve the healing process ¹³⁻¹⁷.

1.1.2 What is wound healing?

Wound healing is an organized process consisting of three overlapping phases, i.e. inflammation, proliferation, and maturation/ remodelling^{8, 18, 19}. Inflammation occurs within hours of wound formation. It allows the body to control blood loss and fight bacterial invasion. Polymorphs and macrophages combine in a defence against bacteria and begin the process of repairing by clearing debris, damaged tissue, as well as recruiting fibroblast cells²⁰. These cells are responsible for synthesizing collagen which reforms the structure of protein around the wound area. The cellular and enzymatic activities in the wound are responsible for increasing the osmolality (exudates) and fracturing of the skin edges around the wound site^{21, 22}. This process results in patients experiencing pain due to swelling, and leads to a slow healing process. Therefore, it is important to provide a wound product which is capable of absorbing the exudates.

The proliferation process begins within 48h²³ after injury and highly depends on the number of fibroblasts that produce the collagen¹⁸. The proliferation phase consists of three crucial parts: granulation tissue, wound contraction, and epithelialization²⁴. The cellular and chemical activity of the wound site results in the formation of granulation tissue¹⁸. Wound contraction occurs during this phase. It is the drawing together of the wound margins, and is affected by the shape, depth, and size of the wound.²⁵ As the wound area is filled with granulation tissue, epithelial cells (keratinocytes) begin to multiply and migrate across the wound bed²⁶. This process accelerates fast because of the moist environment around the wound. The proliferation phase is completed when the wound is resurfaced with epithelial tissue. During this process, the tensile strength of the wound is improved depending on the

area of the wound. The speed of the healing process is dependent on many factors such as, age of the patient and levels of red blood cells, oxygen and zinc in the body⁸.

The maturation phase is the last stage in the healing process, in which the vascularity of scar progressively decreases, the numbers of fibroblast are reduced, and the enlargement and reorientation of the collagen fibres strengthens the wound tissue. The strength of the wound site increases rapidly within 6 weeks and the amount of collagen formation in the scar increases for several months, or in some cases even up to 2 years^{23, 27}. Even after the wound area is fully remodelled, scar tissue is at most only 80% of the original tissue's strength and elasticity^{23, 28}.

There are three categories of wound healing: (i) primary healing, (ii) secondary healing and (iii) delayed primary healing⁸ (Figure 1.1). Primary healing relates to a wound that can be treated with simple sutures, tape or passive dressing (Figure 1 A). When a wound is left open and allowed to heal by proliferation, the term secondary healing (Figure 1 B) is used. Delayed primary healing relates to larger wounds that are infected or fail to successfully close by secondary healing (Figure 1.1 C).

Figure 1.1: (A) Primary healing, (B) secondary healing, and (C) delayed primary healing (reproduced from reference 8).

1.1.3 What is an ideal wound dressing?

An ideal wound dressing should be capable of maintaining a moist environment at the wound interface, be permeable to water, air and gas, able to remove an excess of exudates, offer an adequate toughness, be non-toxic, and be resistant to bacteria or micro-organism^{8, 29-33}. The human body is constantly losing water to the environment by evaporations through the skin to control the body's temperature. This process requires energy; approximately 2.43 mJ of energy is needed to evaporate one litre of water and an excessive water loss associated with enormous heat loss can affect the metabolism of the body³⁴. Normal healthy skin evaporates about $204 \text{ g m}^{-2} \text{ d}^{-1}$ at 35.8°C which increases depending on the activity³⁵. First degree burns evaporate about 36% higher than normal skin, and second degree burns evaporate almost 2000% higher compared to normal skin (Table 1.2).

When a wound is covered with non-evaporative materials, the evaporative water loss from the wound results in the build-up of exudates and can lead to back pressure on the wound which causes considerable pain to the patient³⁶. It is also possible for dressings to move around the wound in accommodating exudates build-up which can result in dehydration and bacterial infection. It also results in leakage from the edge of the dressing and forms a pocket of fluids, in a process to relieve the pressure associated with exudates build-up, thus causing pain and discomfort to the patient³⁷. Therefore, it is essential for wound dressing to have similar water vapour transmission rates (WVTR) as the skin, and provide a satisfactory moisture balance within the repairing wound. WVTR values which are too low might slow down the healing process, whereas WVTR values which are too high may result in faster wound drying, leading to formation of scars³⁸. The recommended WVTR is 500-

2000 g m⁻² d⁻¹, which would provide adequate moisture and prevent wound dehydration³⁷.

Table 1.2: The evaporative water loss of damaged skin (reproduced from reference 35).

Studied area	Evaporative water loss (g m ⁻² d ⁻¹)	Skin temperature (° C)
Normal skin	204.0 ± 12.0	35.8 ± 2.0
First-degree burn	278.4 ± 26.4	35.3 ± 0.1
Second-degree burn	4274.4 ± 132.0	35.3 ± 0.4
Third-degree burn	3436.8 ± 108.0	34.5 ± 0.4
Granulating wound	5138.4 ± 201.6	34.7 ± 0.2
Donor site	3590.4 ± 180.0	35.3 ± 0.2

Queen *et al.*³⁷ was reported the WVTR of a few commercial wound dressings that were measured at 35°C and 35% of relative humidity (RH), see Table 1.3. Wound dressings with the thickness below 0.2 mm exhibit WVTR values ranging from 139 g m⁻² d⁻¹ to 491 g m⁻² d⁻¹. WVTR values increased with decreasing thickness up to 3052 ± 684 g m⁻² d⁻¹ (Table 1.3). For example, in a recent study, membranes of chitosan and alginate polyelectrolyte complex with thickness about 0.015 mm resulted in WVTR values between 442 g m⁻² d⁻¹ - 618 g m⁻² d⁻¹ depending on amount of alginate³⁹. The WVTR values of poly(vinyl) alcohol/Dextran hydrogel are about 1900 g m⁻² d⁻¹ - 2000 g m⁻² d⁻¹⁴⁰. In addition, membrane with thickness < 0.1 mm will likely to record the WVTR values below than 1000 g m⁻² d⁻¹^{39, 41}, whereas values for thicker dressing (> 0.1 mm) such as hydrogel, hydrocolloid and foams are excess of 1000 g m⁻² d⁻¹^{40, 42-44}. Regardless, all these WVTR values are

within the recommended range ($500\text{-}2000\text{ g m}^{-2}\text{ d}^{-1}$) for providing a moist environment around the wound. Wu and co-workers⁴⁵ also reported the comparison of WVTR without and with dressing on different types of burns and ulcer and reported that the dressing reduced the water transmission in of all burn types.

Table 1.3: Water vapour transmission rates (WVTR) of commercial wound dressings (reproduced from reference 37).

Materials	Thickness (mm)	WVTR ($\text{g m}^{-2}\text{ d}^{-1}$)
Stretch 'n' seal	0.01	362 ± 44
Vigilon cover film	0.03	139 ± 23
Vigilon + cover film	1.16	168 ± 32
Tegaderm	0.03	491 ± 44
Bioclusive	0.10	382 ± 26
Lyofoam	10.3	3052 ± 684
Synthaderm	0.73	2005 ± 203
Coraderm	0.76	2859 ± 296
Geliperm	1.13	10973 ± 998
Corethium	0.36	1822 ± 385
Free water surface	-	10419 ± 689

The capability of a film, hydrogel, scaffold membrane or porous material to hold water is an important parameter in evaluating a wound dressing. Optimum water absorption might prevent the wound from accumulating of unwanted body fluid. This accumulation of wound exudates commonly causes maceration and

facilitates infection^{46, 47}. It also facilitates a moist wound environment, which is helpful for the healing process⁴⁸.

A wound dressing should provide an adequate toughness and flexibility to allow conformation to an uneven body surface. It should have a high tensile strength, and optimum strain-at-break to allow application on different body regions. As shown in Figure 1.2, no wound dressing is completely applicable to all body regions⁴⁹. Some wound dressings can cover multiple regions (e.g. Coraderm, Bioclusive, Tegaderm and Synthaderm). Highly flexible materials and strong adhesives are needed to apply on elbow and finger joints⁴⁹.

Figure 1.2: Radii of curvature of various body regions and suitable wound dressings (reproduced from reference 49).

Biocompatibility of wound materials or implants involves low or no toxicity or carcinogenic effects, either locally or systemically in local tissue⁵⁰⁻⁵³.

Biocompatibility evaluation consists of tissue compatibility (*in-vitro* and *in-vivo*) studies and blood compatibility^{54, 55}. Numerous studies have demonstrated successful *in-vitro* and *in-vivo* biocompatibility characteristics of biopolymers materials as dressings⁵⁶⁻⁶³, fixation devices, or replacement implants in musculoskeletal tissues⁶⁴⁻⁶⁶.

Bacterial contamination of a wound seriously threatens and affects the healing process. As an example, infection in burn wounds is the major complication reported, and it is estimated that about 75% of mortality cases following burn injuries is related to infection rather than to excessive exudate or hypervolemia⁶⁷. Infection can lead to the formation of a biofilm in the wound within approximately 10 hours after infection⁶⁸. This biofilm protects the bacteria, which then become immune to antibiotics^{69, 70}. Therefore, there is a need for the development of improved wound dressings that prevent, slow down or avoid the proliferation of bacteria and biofilms. An antimicrobial effect can be achieved by eluting germicidal compounds such as silver ions, titanium dioxide and iodine from the wound dressing⁷¹⁻⁷³.

1.1.4 Types of wound dressings available on the market

A great variety of dressing types fulfil a specific requirement such as type of healing, location and age of wound, size and stage, level of exudates, wound infection, tissue loss or maceration to improve the speed and effectiveness of wound healing^{3, 7, 8}. Gauze dressings (natural or synthetic fibres) are the most commonly used today because they are inexpensive and can be applied to most of body parts. However, the granulation tissue and keratinocytes get trapped within the dressing and these fragile

tissues are often dislodged together with the dressing thereby delaying wound healing⁷.

To improve on this problem, advance and active wound care products such as, semipermeable films, multilayer absorbent dressings, calcium alginate dressings, hydrogels, and hydrocolloids have been developed. Newer developments are the evaporation technique, gelation process, hydrogels, scaffold, dual layer film, dry/ wet phase separation method, and hybrid peptides⁷⁴⁻⁸³. Table 1.4 gives an overview of different types of commercially available wound dressings and describe the advantages and disadvantages of each of them.

Table 1.4: Different types of wound dressings available on the market (reproduced from reference 8).

Type	Example and manufacturer	Advantage	Disadvantage
Natural fibre dry dressing	Cotton wool, gauze, linen product	Provides dry wound healing environment	Granulation tissue can grow into the mesh of product and gives trauma to the patient
Synthetic fibre gauze	Topper 8, Johnson & Johnson	More absorbent than cotton	Creates a dry wound
Non-adherent, non-absorbent dressing	Tricotex, Smith & Nephew	Non-adherent dry dressing	Not absorbent
Non-adherent dry or film coated dressing	Melolin, Smith & Nephew Telfa, Kendall (Tyco)	Non-adherent contact layer, highly absorbent core and	Dressing may stick and dry out, causing trauma to

	Healthcare)	air-permeable, fluid	the patient
	Zetuvit, Hartmann	repellent	
Island dressing	Primapore, Smith & Nephew	Non-adherence	Not suitable for
		contact layer	highly exudating
		prevents wound	wounds
	Tegaderm Island, 3M	trauma	
	Cutifilm Plus, BSN/ Smith & Nephew		
Antimicrobial non-adherent film coated dressing	Telfa AMD, Kendal (Tyco healthcare)	Effective against <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Proteus mirabilis</i> , <i>Serratia marcescens</i> , <i>Enterobacter cloacae</i> , <i>Staphylococcus coagulase</i>	Requires adhesive tape
Non-adherent transparent polyamide net dressing	Tegapore, 3M	Non-adherent to moist wound bed Porous nature of dressing allows exudate to pass into secondary dressing	Haemostasis should be attained prior to application of Tegapore
Tulle Gras dressing	Jelonet, Smith & Nephew Adaptic, Johnson & Johnson Cuticern, BSN/ Smith &	Provides a moist wound environment	Cotton products can shed fibres into wound

	Nephew		
Semipermeable film dressing	Opsite Flexigrid, Flexifix, IV 3000, Smith & Nephew Tegaderm, 3M Bioclusive, Johnson & Johnson	Permeable to moisture vapour and gases Allows inspection of wound	Non-absorbent
Foam dressing	Lyof foam, Medlock Allevyn, Smith & Nephew Tielle, Johnson & Johnson Biatain, Coloplast Suprasorb P, Lohmann & Rausher Trufoam NA, Unomedical	Highly absorbent Facilitates a moist wound environment	Autolysis problem
Calcium alginate dressing	Kaltostat, ConvaTec Sorbsan, Unomedical Algoderma, Johnson & Johnson Curasorb, Kendall Tegagen, 3M Algisite M, Smith & Nephew	Absorbent Biodegradable in wounds	Gel may be confused for slough in wound
Hydrocolloids	Comfeel Plus, Coloplast DuoDERM, ConvaTec	Water repellent Gel formation	Not recommended on wounds

	Tegasorb, 3M	provides moist	clinically infected
	Hydrocoll, Hartmann	environment	with anaerobic
		Hydroactive particles	bacteria
		absorb wound	
		exudate	
Hydrogels	Intrasite gel, Smith & Nephew	Provides a moist environment for cell migration	Maceration of the surrounding skin
	DuoDERM gel, ConvaTec	Absorbs exudate	may occur with liberal use of gel
	Cutinova gel, BSN/Smith & Nephew		
	Aquform, Unomedical		
Hydrofibre dressing	Aquacel, ConvaTec	Non-traumatic dressing removal	Requires a secondary dressing
		Maintains a moist wound healing environment	
Multilayer Absorbent dressing	CombiDERM ACD, ConvaTec	Promotes moist wound healing	Require a cavity filling dressing
	Transorbent, Unomedical	Absorbs exudate	
	Alione, Coloplast		
Silicone dressing	Mepitel, Tendra	Minimizes wound trauma on removal	Need a secondary

1.2 Overview of polyelectrolyte complex

1.2.1 What is polyelectrolyte complex?

A polyelectrolyte complex (PEC) or electrostatic self-assembly (ESA) is formed by alternate absorption of a charged substrate into dilute solutions of a polycation or polyanion⁸⁴. This process has attracted the interest of many scientists not only to solve the mechanism of polyelectrolyte complexation⁸⁵⁻⁸⁷, but also to develop a wide range of applications, such as nanofiltration⁸⁸⁻⁹⁰, pervaporation^{91, 92}, coating^{93, 94}, wound healing⁹⁵, tissue engineering⁹⁶⁻⁹⁸ and encapsulation (capsules)^{99, 100}.

The PEC method was introduced by Decher's group in the early 1990s¹⁰¹⁻¹⁰³ and has greatly increased in popularity due to its simplicity to create nanolayers¹⁰⁴. A number of interacting macromolecules have been successfully observed for polyelectrolyte multilayer build-up, ranging from synthetic polyelectrolyte to charged biopolymers such as, polysaccharides, proteins and nucleic acids^{93, 105-107}. Figure 1.3 is a reproduction of the image Decher used in his Science publication to describe this process¹⁰³.

In this schematic, the films are alternately dipped into polycations and polyanion solutions. The adsorption of molecules allows for charge repulsion on the surface and charged molecules will be repulsed allowing for the formation of a single layer film. In the second step, an oppositely charged molecule can be adsorbed on top of the first one. This process can be repeated cyclically to form multilayer structures on the surface of a substrate¹⁰³.

Figure 1.3: (A) Schematic of the film deposition process, and (B) molecular picture of the first two steps of deposition (reproduced from reference 103).

The resulting multilayer films are made up of a number of layers each with its own individual structure and properties. They can then be tailored as biological compounds, conducting polymers, light emitting polymers, drug release agents by manipulating these self-assembled nanolayers onto a suitable substrate^{108, 109}. The strong electrostatic attraction between charged surfaces and oppositely charged molecules in solution is understood to be the dominant factor in the adsorption of polyelectrolytes as shown in Figure 1.4^{101, 110-112}.

The substrates are normally deposited into absorbate concentrations of polyelectrolyte solution. Adsorption duration per layer can be varied from minutes for polyelectrolyte to hours for certain colloids/gels depending on factors such as the molar mass, pH and concentrations of the polyelectrolyte solution⁸⁵.

Figure 1.4: The formation of a polyelectrolyte complex of two oppositely charged polyelectrolytes in aqueous solution of different pH (reproduced from reference 112).

The composition of the polyelectrolyte complex and the characteristics of the individual layers are affected by several aspects such as thickness. Polyelectrolyte systems can exhibit a linear growth in both the thickness and mass of the films depending on the number of deposition step, however growth is also observed to be of an exponential mechanism which gives an unpredictable thickness increment¹¹³.

Poly(styrene sulfonate)/poly(allylamine hydrochloride)^{114, 115}, poly(L-lysine)/alginate¹¹⁶, poly(L-lysine)/hyaluronan¹¹⁷ are the most prominent examples of

linearly growing systems or thickness, which usually form a stratified structure. Films of poly(L-glutamic acid)/poly(L-lysine)¹¹⁸, poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate)¹¹⁹ show a growing thickness that increases exponentially depending on alternate layers applied. The proposed explanation for this exponential growth mechanism is related to the diffusion of polyelectrolyte “in”, and “out” of the film^{118, 120}. Another hypothesis is that the exponential growth of polyelectrolyte complex is a result of an increase in surface roughness as the film build up^{115, 121}, but no observation of such a rough surface has been found on exponentially growing film made of polypeptides^{118, 120}. The linear and exponent thickness increases of polyelectrolyte complex are dependent on deposition conditions such as concentration of the polyelectrolyte, adsorption duration, ionic strength, temperature, dipping speed, drying duration^{113, 122} and the underlying surface. The charge density, local mobility, and salt concentration also appear to have an influence on the thickness of the layer^{113, 121-123}.

Polyelectrolyte complexes have been successfully developed on a number of different substrates including glass, quartz, mica, silicone, gold and silver^{104, 118, 121, 124-126}. The choice of substrate depends on the application, as well as the convenience of analysis^{104, 127}. Each of the different types of substrate differs with regards to surface charge of the structure (regardless of roughness), smoothness and surface topology¹²⁸. Fou and Rubner proved this by modifying the surface charge of microscopic glass slides to hydrophilic, hydrophobic, negatively charged, and positively charged and found that the surface charge influenced the deposition time, thickness and layer uniformity of the substrates¹²⁸. Because of that, a polyelectrolyte complex can be applied on various substrates of different sizes and shapes based on

their surface charge. While glass, silicon wafers, gold coated particles, quartz and mica have been the dominating choice of substrates for polyelectrolyte complexes, the use of bio-polymer film and textile (wool fibre) as a substrate have rarely been considered.

1.2.2 Biopolymers as a potential substrate

At present, biopolymers are receiving greater attention than synthetic petrochemical-based polymers due to environmental concerns¹²⁹. The trend of using synthetic polymers started more than half a century ago to substitute natural materials in almost every daily used product because of their properties. Today, the trend has turned back to using natural abundant materials that are more environmentally sound in terms of biodegradability and pollution in general. A variety of renewable biopolymers such as polysaccharides, proteins, lipid, and their composites, derived from plant and animal resources have been studied for the development of edible/biodegradable packaging materials^{130, 131}, scaffold films¹³²⁻¹³⁴, wound dressing with antibacterial properties^{135-137, 138-142} and drug delivery^{141, 143, 144}.

1.2.3 Polysaccharides as substrate for a polyelectrolyte complex

Recently, polyelectrolyte complexes based on polysaccharides have been explored using combinations of chitosan-alginate^{145, 146}, chitosan-carrageenan¹⁴⁷⁻¹⁴⁹, chitosan-guar gum¹⁵⁰, chitosan-heparin¹²⁴, chitosan-xanthan¹⁵¹, chitosan-kondagogu gum¹⁵² and chitosan-gelatin¹⁵³. Chitosan-alginate is among the most popular polyelectrolyte complex studied. Research into this PEC has mainly focused on hydrogels for drug release^{154-156, 142, 143}, wet-spun nanocomposite-fibre^{157, 158}, wound healing⁹⁵, and scaffold in cartilage tissue engineering¹⁵⁹. Iwasaki *et al.*¹⁵⁹ reported the advantage of

this technique for cell adhesion (rabbit chondrocytes) on alginate-based chitosan hybrid polymer fibers that proved superior compared to alginate polymer fibers¹⁵⁹. In another study, Boddohi *et al.*¹⁶⁰ reported that the polyelectrolyte multilayers (PEMs) of chitosan-heparin induced greater cell density and higher proliferation rate of mesenchymal stem cells (MSC), which is suitable to apply on surface coatings to stabilize and potentiate growth factors in therapeutic applications¹⁶⁰.

Up to date, a limited number of CH-GG polyelectrolyte complexes have been reported as the focus has been on the development of capsules and fibers¹⁶¹⁻¹⁶⁷. For example, Yamamoto and co-workers studied the characteristic structures of the interface between CH-GG due to charge overcompensation on the formation of fibers and capsule¹⁶². This interface reaction could form a hemisphere, honeycomb shape or spaced droplets of polyelectrolyte complex. While the interface can be pulled out to form fibres, as reported by Amaike and co-workers¹⁶⁷. Another study by Fujii *et al.*¹⁶⁶ reported the encapsulation of alkaline phosphatase (ALP) by utilizing the polyelectrolyte complex technique of CH-GG as shown in Figure 1.5.

Figure 1.5: Encapsulation process of alkaline phosphatase by polyelectrolyte complex technique between CH-GG (reproduced from 166).

1.2.4 Textile as substrate for a polyelectrolyte complex

Not many studies have been published on coating and dyeing of textile fabrics by the polyelectrolyte complex technique^{168, 169}. Polowinski's team has popularized the use of the polyelectrolyte complex for modifying the surface of textile fabrics¹⁷⁰⁻¹⁷². The fabrics were treated by a grafting technique using acrylic or methacrylic or by chemical modification (irradiation). This changed the surface charge of textile fabrics, as well as their hydrophilicity and electro kinetic potential, while also improving dye ability. A basic group was then applied (poly (allylamine hydrochloride)) onto acidic treated fabric to form a polymer complex. Dubas *et al.*¹⁶⁹ also successfully coated nylon and silk fibers containing silver nanoparticles using a polyelectrolyte complex. The nylon and silk coated with silver nanoparticles exhibited antimicrobial activity against *Staphylococcus aureus* at 50% and 80%, respectively.

1.3 Biopolymers

1.3.1 Chitosan

Chitosan is a product of deacetylation of chitin either by chemical or microbiological treatments and it composed of randomly distributed β -(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Figure 1.6)^{173 174, 175}.

Figure 1.6: Structure of chitosan (*l*=acetylated unit, *m*=deacetylated unit).

CH is widely used in the field of biomedicine in wound dressings^{57, 176 141, 177, 178}, pharmaceutical delivery and medicine control¹⁷⁹, scaffolds¹⁸⁰, by promoting bone cell division as well as bone growth¹⁸¹ due to its biocompatibility¹⁸², biodegradability⁵⁴ and strong anti-bacterial properties¹³⁶. It is also used as a component in surgical appliances, contact lenses and in anticoagulant, and anticancer substances^{173, 183, 184}. The versatility of CH fabrication to form products such as membranes, three-dimensional structures (scaffold), fibers or by modifying the CH solution to form a polyelectrolyte promotes the usability of this material (Figure 1.7).

Figure 1.7: Schematic illustrating the versatility of fabrication of chitosan. At low pH (< 6), chitosan's amine is protonated and active as polyelectrolyte solution to be used in polyelectrolyte complex. At higher pH (> 6.5), chitosan's amine is deprotonated and the molecular structure can be altered by grafting or crosslinking to form film, fiber or gel network (reproduced from reference 185).

The properties of chitosan in solution depend on its molecular weight, degree of deacetylation, pH and ionic strength. CH is insoluble in water due to the pK_a of glucosamine which is around 6.2-7.8¹⁸⁶. CH is soluble in diluted acids on account of

a protonation of free amine groups. The dissociation constant K_b of an amine group is obtained from the equilibrium of:

$$K_b = \frac{[\text{NH}_3^+][\text{OH}^-]}{[\text{NH}_2]} \text{ and } \text{p}K_b = -\log K_b$$

while the dissociation constant of the conjugated acid, K_a is obtained from the equilibrium of:

$$K_a = \frac{[\text{NH}_2][\text{H}_3\text{O}^+]}{[\text{NH}_3^+]} \text{ and } \text{p}K_a = -\log K_a$$

The dissociation constant of CH depends on the degree of dissociation and the method of deacetylation used. The dissolved CH flocculates at a pH range of 5.5 - 6.0¹⁸⁶. At higher pH values, charge density of the chitosan solution is decreased due to less amine group being protonated, and vice versa, at lower pH (<6.5) the charge density of CH is increased due to a higher amount of protonated amino groups. This factor is therefore crucial for optimizing the formation of a polyelectrolyte complex.

Many factors such as molecular weight¹⁸⁷⁻¹⁸⁹, type of solvent^{190, 191}, different drying condition¹⁹², and humidity¹⁹³ contribute to the formation of CH films. Different sources and whether CH is prepared chemically or microbiologically contribute to varying degrees of deacetylation (DD), acetyl group arrangement, and chain length, which all affect the characteristics of CH^{136, 194}. Acetic acid has been

reported as the optimum acid to dissolve CH giving optimum tensile strength and resistance against water, compared to acids such as citric acid, formic acid and lactic acid¹⁹⁰. Such CH films (2% CH, 1% acetic acid) have high tensile strength and Young's modulus, because of strong electrostatic repulsion between chitosan chain segments¹⁹⁰.

Glycerine is reported to be the optimum plasticizer for CH films compared to ethylene glycol (EG), polyethylene glycol (PEG), and propylene glycol (PG)^{193, 195}. However, glycerine reduced the tensile strength and Young's modulus, and improved the strain-at-break. It also affected the hydrophobicity and hydrophilicity of the materials. Suyatma and co-workers found that chitosan films with glycerine improved surface hydrophobicity after 20 weeks¹⁹⁵ and a number of studies reported the influence of hydrophobicity and hydrophilicity of the materials on biological response¹⁹⁶⁻¹⁹⁸.

Cell viability on CH film of different cell lines such as on mouse connective tissue (L929), rat primary schwann cell, nerve cell (PC12), rat osteoblast (UMR-106) and baby hamster kidney cells (BHK21(C13)) is well known^{134, 199-203}. The biocompatibility is affected by the chemical composition or degree of deacetylation of CH²⁰³. A high degree of deacetylation of CH carries more active free amino groups, which protonate in acidic solution to form a cationic amine group (NH_3^+). The opposite charges between cell surface and NH_3^+ groups are responsible for ionic interaction. Prasitsilp *et al.*¹⁹⁹ studied the effect of different sources of CH (shrimp and cuttle fish) on the different degree of deacetylation and tested on mouse connective tissue (L929) and baby hamster kidney cells (BHK21(C13))¹⁹⁹. The

results show that the different sources of CH did not affect cell growth significantly, providing they had a high degree of deacetylation (> 85%). The same observation was found by Chatelet *et al.*²⁰⁴, CH films with the degree of deacetylation (53%-97.5%) are cytocompatible.

CH exhibits antibacterial activity depending on its concentration, molecular weight and degree of deacetylation^{135, 136, 188}. Liu *et al.*¹⁸⁸ reported that the effective concentration of CH to kill *Escheria. coli* is 200 ppm. CH with molecular weight range from 5.5×10^4 to 15.5×10^4 Da also showed antibacterial activity at the optimum concentration of 200 ppm. No *et al.*²⁰⁵ reported that Gram-positive (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaricus*) and Gram negative (*Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*) bacteria respond differently to varying concentrations and molecular weights of CH. Although many studies have been conducted showing that CH has antibacterial activity²⁰⁵⁻²⁰⁷, no clear evidence has been provided to demonstrate the relationship between the antibacterial activity of CH and surface characteristics of the bacterial cell wall. El-Ghaouth *et al.*²⁰⁸ have proposed possible antibacterial actions of CH. Firstly, CH is believed to react with the cell surface which alters cell permeability and further prevents the entry of material or causes leakage of material.

CH has also been reported to offer resistance to viral infection especially in plants, but also in some animal cells²⁰⁹⁻²¹¹. CH is able to imitate phytopathogens, and induces a wide spectrum of protective reactions and limits the spread of the viruses

through the plant^{212, 213}. For example, by spraying or inoculating leaves with CH, various plant species were protected against viruses such as, tobacco necrosis virus (TNV), peanut stunt virus (PSV), cucumber mosaic virus (CMC), potato virus X (PVX), and alfalfa mosaic virus (ALMV)^{210, 214, 215}. The effectiveness of CH to inhibit viral infection depends on the concentration, deacetylation degree, and molecular weight of CH besides charge value and the character of the chemical modifications of the molecule^{187, 211, 213}. In mammals, CH can stimulate the inductive phase of the immune response to virus antigens and nerves of the immune system.

1.3.2 Gellan gum

Gellan gum (GG) was discovered in 1977²¹⁶. It is produced by *Pseudomonas elodea* and consists of a repeating unit of tetrasaccharide: 1,3-linked β -D-glucose, 1,4-linked β -D-glucuronic acid, 1,4-linked β -D-glucose, and 1,4-linked α -L-rhamnose (Figure 1.7)²¹⁷. Glucose is the highest percentage of the main constituents (at 60 %) followed by rhamnose and glucuronic (20% each)²¹⁷. Gellan gum exists in two types, i.e. high acyl and low acyl and is differentiated by two acyl substituents of acetate and glycerate in the high acyl type (Figure 1.8)²¹⁸.

Figure 1.8: The structure of gellan gum; (A) high acyl gellan gum, and (B) low acyl gellan gum (Ac: acetate group, Gly: glycerate group) (reproduced from reference 219).

In low acyl gellan gum, both acyl groups are completely removed by alkaline treatment²²⁰. As a result, the high acyl gellan gum produces soft, elastic, non-brittle gels, whereas the low acyl form produces firm, non-elastic and brittle gels. In this study, the focus is on low acyl gellan gum due to its firmness which is important in the development of wound dressing.

Low acyl gellan gum is sensitive to an ionic environment and divalent ions during the evaporation process. Because of that, the properties of low acyl gellan gum such as gel strength, setting temperature, and melting temperature can be controlled by altering the cations and pH²²¹. Furthermore, heat (75°C) can further help to complete the evaporation process.

The conformation and structure of gellan gum depends on the concentration of polymer used, setting temperature, ionic environment and the presence of any cations in the solution²²²⁻²²⁴. In the absence of cations, low acyl gellan gum gels around 30 °C^{224, 225}. The gelation temperature increases with addition of cations. At high temperature (> 50 °C), gellan forms a coil structure or single-stranded and at low temperature (< 30 °C), gellan forms an ordered double helix^{223, 226}. The transition temperature from single-stranded to double helix is around 30°C. After transition, the double helix of gellan gum can be associated with cations (Ca^{2+} , Na^+ , K^+) to form junction zones, which will aggregate leading to the formation of an interconnected three-dimensional gel network.

Gellan gum is approved by the United States Food and Drug Administration (US FDA) and the European Union (it is labelled as E 415 in EU regulation) for use

in the food industry and is commonly used in gelled based products, beverages, dairy foods, emulsions, edible film to improve food experiences²²⁷⁻²²⁹. Gellan gum is also used as a culture medium for bacteria and plant tissue culture^{230,231}. Studies on the medical and pharmaceutical applications of gellan gum have also been reported. For example, it has been used as a scaffold material for the application of tissue engineering^{232, 233}, as a vehicle for ophthalmic drugs^{234, 235}, and in eye drops²³⁶. Gellan gum has shown to have a good compatibility in hydrogel form for live cells such as mouse fibroblast (L929 cell line)²³⁷, human dermal fibroblasts (HDFs), human fetal osteoblasts (hFOB 1.19)²³⁸, human nasal cartilage²³⁹, and rat bone marrow cells (rBMC)²³³.

1.4 The use of nanoparticles

Most bio-polymers have low tensile strength and Young's modulus compared to ceramics and metals. To overcome this problem, composites can be reinforced with micron-sized inclusions such as fibres, platelets, or particles to improve the polymer properties^{240, 241}. The processing techniques have been hugely improved in the last few years as the size of inclusions are now on a nanoscale (1-100 nm). This has improved the material properties dramatically especially on the mechanical level due to the extremely high surface to volume ratios^{242, 243}. Furthermore, inclusion of nanoscale into biopolymers also improved cell biocompatibility²⁴⁴, antibacterial and antiviral properties²⁴⁵⁻²⁴⁹.

In this study, three nanoparticles were chosen to improve the biopolymer composites: (i) titanium dioxide, (ii) silver and (iii) zinc oxide. Most of the reviews discussed in this chapter are based on incorporation of nanoparticles into chitosan. An

extremely limited number of researches have been done on gellan gum incorporated with nanoparticles.

1.4.1 Titanium dioxide nanoparticles

Titanium dioxide (TiO_2) is widely used in the cosmetic, coating and paint industry²⁵⁰. It also has been used as a filter to absorb heavy metal ions, organic pollutants²⁵¹ and in the preparation of a photodegradable insecticide²⁵².

Inclusion of TiO_2 nanoparticles into biopolymers has shown promising effects on mechanical properties, as well as on cell viability and antimicrobial activity^{253, 254}. As an example, chitosan and TiO_2 nanoparticles (diameter range 20-60 nm) have been used to prepare films by crosslinking a chitosan- TiO_2 -gelatin dispersion followed by evaporative casting and ultraviolet-sterilisation²⁵⁵. These composites showed 17% biodegradability after 6 days (against lysozyme), antibacterial activity against the Gram-positive bacterium *Staphylococcus aureus* (SA) and wound healing characteristics when applied to wounded Sprague-Dawley rats. Chitosan- TiO_2 scaffolds prepared through a freeze-drying technique resulted in improved mechanical properties and cell attachment (hepatic immortal cell lines- HL-7702) compared to pure chitosan scaffolds²⁵⁴. While hybrid multilayer film prepared via layer-by-layer assembly of chitosan-heparin films containing TiO_2 and Ag were found to act as contact-active and release-active antibacterial agents¹⁷⁷.

Incorporating TiO_2 nanoparticles (diameter ~100 nm) into electrospun chitosan-poly(vinyl alcohol) fibers enhanced the fiber's antibacterial activity against *Escherichia Coli* from 72% to 85%²⁵⁶. TiO_2 films also exhibited an antiviral effect

by causing significant changes in the virus structure and resulting in the loss of the ability by the virus to attack host cells ⁷². Other reports showed the ability of TiO₂ to destroy Hepatitis B virus surface antigen (HBsAg) ²⁵⁷.

TiO₂ antibacterial and antiviral activity are expected originates from its photocatalytic properties ^{72, 258-260}. Upon exposure to a light source that contains ultraviolet (UV) light, TiO₂ generates superoxide ions and hydroxyl radicals. It has been proposed that these ions and radicals operate in concert to attack phospholipids in bacteria. The resulting lipid peroxidation reaction causes disruption of the bacterial cell membrane, leading to loss of viability ⁷².

1.4.2 Silver nanoparticles

Antibiotic-resistance of bacterial strains has become a major problem in public health and the focus has shifted to silver (Ag), known for its ability to control infections ^{261, 262}. Continuous improvements are sought on products containing silver nanoparticles ranging from ointment to bandages for wound dressing that need to be effective in retarding and preventing bacterial infections ^{245, 248}.

The use of Ag cations in wound care and hygiene products implies that Ag is not considered to have adverse effects to humans ²⁶². It has been shown that combining chitosan or modified chitosan with Ag into composites resulted in films and hydrogel materials with enhanced cell antimicrobial activity, increased tensile strength, but decreased water vapour permeability ^{79, 244, 263}. The chitosan-Ag films yielded a significant reduction in the number of viable bacterial colonies compared to the control sample for both Gram-positive and Gram-negative bacteria ²⁴⁴.

Ag nanoparticles (diameter 15 nm) have also been incorporated into genipin-crosslinked chitosan films ²⁴⁴. This study showed that chitosan films with Ag nanoparticles contents between 50 and 200 ppm increased the number of viable L929 cells attached to the film surface, compared to genipin-crosslinked chitosan. The genipin-crosslinked chitosan film did not exhibit any antibacterial activity against the Gram-negative bacterium (*Escherichia Coli*). The composite films with Ag contents larger than 100 ppm were found to be highly effective, which was attributed to the leaching of Ag ions from the composite materials. Ag nanoparticles (diameter 34 nm) were also incorporated into hydrogels based on lactose-substitute chitosan ²⁶³. These hydrogels did not show any cytotoxic effect against three different eukaryotic cell lines, while being very effective against both Gram-negative and Gram-positive bacteria. This was attributed to the hydrogel's ability to impede the uptake of nanoparticles by the cells, while allowing these particles to exert their antibacterial effect through contact with the bacterial membrane ²⁶³. In other work, composite materials were prepared by immobilizing Ag on sterilized chitosan films which were found to promote wound healing and combat infection in Sprague-Dawley rats ²⁶⁴.

Chitosan-Ag composite materials with low Ag content (2.15% by weight) have also been prepared by converting AgNO₃ into Ag nanoparticles ²⁶⁵. The antibacterial activity of chitosan was significantly enhanced by the inclusion of Ag nanoparticles, while fluorescence spectroscopy measurements revealed that bacterial growth stopped immediately after exposure of *Escherichia Coli* to the composite. It has been proposed that Ag nanoparticles can act in three ways against Gram-negative bacteria (summarised in Figure 1.9): (i) they attach to the surface of the bacterial cell membrane, significantly inhibiting permeability and respiration; (ii) they penetrate

inside the bacteria and cause further damage; and (iii) they release silver ions resulting in bacterial deoxyribonucleic acid (DNA) losing its capacity for replication as well as inactivation of bacterial proteins^{245, 247}.

Figure 1.9: Schematic of mechanism of antibacterial activities exerted by Ag (reproduced from reference 247).

1.4.3 Zinc oxide nanoparticles

Similar to TiO_2 , zinc oxide (ZnO) is photocatalytic and has been used in cosmetics, sunscreens, coatings, and in the paint industry²⁶⁶. ZnO combined with eugenol, also known as zinc oxide eugenol, has been used as a filling or cement material in dentistry where it avoids the formation of bio-film.²⁶⁷

Up to date, no study has been reported on the biocompatibility of a bio-polymer incorporated with ZnO nanoparticles with a focus on antimicrobial activities²⁶⁸. Direct studies of ZnO nanoparticle in the media cultured with live cells such as L929, HeLa cell, and peripheral blood mononuclear cells show that this nanoparticle inhibits cell growth^{269, 270}. Furthermore, Dechsakulthorn and co-workers assessed the

cytotoxicity of ZnO nanoparticles (diameter range of 50-70 nm) in human skin fibroblasts using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS assay) and reported that higher toxicity was observed at both 4 and 24h ²⁷¹. Lin and co-workers also demonstrated that ZnO particles with diameters of 70 nm and 420 nm significantly reduced cell viability and caused oxidative deoxyribonucleic acid (DNA) damage in a dose and time dependant manner on adenocarcinomic human alveolar basal epithelial cells (A549 cells) ²⁷².

ZnO nanoparticles exhibit strong antibacterial activities on a broad spectrum of bacteria ²⁷³⁻²⁷⁵. The antibacterial property is considered to be due to the generation of hydrogen peroxide (H₂O₂) from ZnO ²⁷⁶ which leads to penetration and disorganization of the cell membrane ^{275, 277}. However, it is not yet clear what the role of Zn²⁺ is on antibacterial activity ^{266, 274}.

1.5 Fluoroquinolones as antimicrobial agents

The increasing concern about penicillin resistance of *Streptococcus pneumonia* has led to more intensive research into the use of new antibacterial agents. Fluoroquinolones are among the rapid growing class of antimicrobial agents that has proved effective in the treatment of bacterial infection ²⁷⁸. The general structure of fluoroquinolone, shown in Figure 1.10, has a fluorine at position 6 of a naphthyridine ring ²⁷⁹. Levofloxacin, gatifloxacin, lemovloxacin and pefloxacin are fluoroquinolone that have different functional groups (R₁, R₂, R₃, and R₄) ²⁷⁹.

Figure 1.10: General structure of fluoroquinolone (reproduced from reference 279).

1.5.1 Levofloxacin

Levofloxacin is (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate (Figure 1.11) ²⁸⁰.

Figure 1.11: The chemical structure of levofloxacin (reproduced from reference 281).

It is an antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria especially aerobes and with a limited activity against anaerobes ²⁸². It is also effective in the treatment of a wide range of clinical infections including respiratory pathogens, skin infections, and in the urinary tract ²⁸³.

It was discovered in 1962 and approved by the Food Drug Administration (FDA) in December 1996 and since then has been essentially used in broad pharmaceutical applications such as in oral administration ²⁸⁴.

Levofloxacin has been used actively in ophthalmic solutions ²⁸⁵⁻²⁸⁷ for the treatment of ocular bacterial infections ²⁸⁸. Bezwada *et al.* ²⁸⁹ reported the intrinsic cytotoxicity of ophthalmic solutions containing levofloxacin in on human corneal keratocytes (HCK) and human corneal endothelial cells (HCE). The results show that levofloxacin least affected on human corneal keratocytes (HCK) and human corneal endothelial cells (HCE). Levofloxacin also shows no effect on osteoblast-cell (MC3T3-E1) growth at 24h and relatively mild effect at 72h compared to ciprofloxacin and trovafloxacin ²⁹⁰.

The strong antibacterial activity of levofloxacin against broad spectrums of bacteria has been reported in many studied such as on *Escheria coli*, *Staphylococcus aureus*, *Xanthomonas malthophuia*, *Bacteroides fragilis* and *methicillin-resistant Staphylococcus aureus* ²⁹¹⁻²⁹³. Furthermore, *in-vivo* mouse protection studied indicated that levofloxacin more active than ciprofloxacin against selected Gram-negative organisms due to greater tissue penetration ²⁹¹.

1.6 Aims of this PhD project

As reviewed in this chapter, the development of wound care products are growing to cater different need of varied damage skins. Biopolymers such as chitosan (CH) and gellan gum (GG) have been receiving great attention particularly in the field of biomedicine due to their biocompatibility and biodegradability properties. Various

methods have been used in the development of wound dressing based on either CH or GG such as evaporation, gelation process and dry/wet phase separation method^{74, 75, 79, 82}, whereas polyelectrolyte complex (PEC) technique is rarely considered. The main aim of this study is to explore the formation of wound dressing through a simple polyelectrolyte complex using chitosan (CH) and gellan gum (GG). The mechanical properties, biocompatibility and antibacterial activity of free-standing CH and GG films are improved by incorporating titanium dioxide (TiO₂), silver (Ag) and zinc oxide (ZnO) nanoparticles²⁹³⁻²⁹⁷. Levofloxacin, an antibiotic is also included into CH films to improve the biopolymer's antibacterial properties. The polyelectrolyte complex films of CH and GG are attached to wound area which designed to act as a bacterial resistance on the upper layer and promote cell viability and proliferation at the bottom layer. The mechanical characteristics, water vapour transmission rates and swelling of the polyelectrolyte complex films are assessed and compared to commercial wound dressings.

The challenge faced in the formation of a CH-GG PEC-film is to prevent the formation of a precipitate in the form of fibers or a capsule^{162, 163, 167, 294}. In comparison, polyelectrolyte complexes from polysaccharides such as chitosan-alginate^{95, 295}, chitosan-carrageenan¹⁴⁹, chitosan-heparin¹⁶⁰ are mixable by stirring and the resulting solution can be easily cast into films. This problem is overcome in the development of a PEC-film of CH-GG by immersing a single biopolymer CH or GG substrate into an oppositely charge polyelectrolyte solution. The following steps were taken towards the aim of developing wound dressings based on PECs from CH and GG:

Aim 1: Improvement of mechanical characteristics of CH and GG films through addition of glycerine and nanoparticles (TiO_2 , Ag and ZnO) without comprising their ability to support cell growth and antibacterial activity (chapter 3 and 4);

Aim 2: To prepare polyelectrolyte complex-films (PEC-film) (chapter 5) and polyelectrolyte complex-wool fibres (PEC-wool fibres) (chapter 6) from CH and GG by optimised the parameters, i.e. duration of immersion, order of addition and pH to improve the mechanical characteristics;

Aim 3: To prepare a dual layer film consists of a bacterial resistance on upper layer and promote cell viability and proliferation at bottom layer for future wound dressing applications (chapter 7).

CHAPTER 2

EXPERIMENTAL TECHNIQUES

2.1 Materials

Chitosan (CH: medium molecular weight, 75% degree of deacetylation (DD), viscosity ≈ 453 cP, product number 448877 - lot number 07918TE; CHM: medium molecular weight, 79% DD, viscosity ≈ 915 cP, product number 448877 - lot number 04609LD; and CHH: high molecular weight, 75.6% degree of deacetylation, viscosity $\approx 1,406$ cP, product number 419419 - lot number 10305DD), glycerine (Gly), levofloxacin (Lev) ($\geq 98.0\%$, catalogue number: 28266), titanium dioxide nanoparticles (99.9% TiO_2 , diameter, $d < 100$ nm, lot number 12908CH), silver nanoparticles (99.5% Ag, $d < 100$ nm, lot number 07916BH), and zinc oxide nanoparticles (99.9% Zn, $d < 100$ nm, lot number 04011AH) were obtained from Sigma Aldrich, Australia. Viscosity (1% chitosan in 1% acetic acid, Brookfield method) and DD are as specified in Sigma-Aldrich's Certificate of Analysis. Gellan gum (GG; low acyl, $M_w \sim 2-3 \times 10^5$ Da, lot number 7K1383A) was a gift from CP Kelco, USA. According to the manufacturer's specification, all acyl groups have been removed from the glucose residue for this form of gellan gum. Raw threads (100% wool, dreamtime 4 ply, colour 0051 (light colour), lot number 760840) were

purchased from Patons, Australia. Food colours (blue and yellow) were purchased from Queen Essence Food Colours, Australia. All materials were used as received.

2.2 Sample preparation

2.2.1 Preparation of chitosan-nanoparticle composites

CH solutions were prepared by dissolving 2 g chitosan in an acetic acid solution (0.08 M) under continuous stirring (500 rpm) for 2h at 70 °C. CH-Gly solutions were prepared by additions of 15%, 30% and 50% of glycerine (by weight relative to CH) and hereafter referred to as CH15, CH30, and CH50, respectively. CH-TiO₂ and CH-Ag dispersions were prepared by bath sonication at 40 kHz (Unisonics FXP 12D, Australia) of 200 mg, 400 mg, and 600 mg TiO₂ or Ag in 90 mL Milli-Q water (18.2 MΩ) for 30 min. This was followed by additions of CH (2 g), glycerine (30% by weight relative to CH) and 10 mL acetic acid (5% v/v) under continuous stirring (500 rpm) for 2h at 70 °C. The dispersions were sonicated in bath sonicator for an additional 10 min prior casting.

Films were prepared by evaporative casting, i.e. solutions or dispersions were deposited onto an acrylic plate and allowed to dry for 2 days under controlled conditions (21 °C, 60 ± 10% relative humidity, RH) (Figure 2.1). The films were peeled off and cut into rectangular strips depending on the characterisation. The films were pre-conditioning in desiccators (21 °C, 50 ± 5% RH) for another 2 days prior to testing. The resulting CH-TiO₂ and CH-Ag composites containing 10%, 20% and 30% nanoparticles loadings (by weight relative to CH) are hereafter referred to CHTi10, CHTi20, CHTi30 and CHAg10, CHAg20, CHAg30, respectively.

Figure 2.1: Schematic of the preparation of free standing CH and CH films by evaporative casting technique.

2.2.2 Preparation of gellan gum-nanoparticle composites

GG solutions were prepared by dissolving 1 g gellan gum in 100 mL Milli-Q water (18.2 MΩ cm) under continuous stirring (500 rpm) for 2h at 70 °C. GG-Gly solutions were prepared by additions of 15%, 30%, 50%, 60% and 70% of glycerine (by weight relative to GG) and hereafter referred to as GG15, GG30, GG50, GG60 and GG70, respectively. GG-TiO₂, GG-Ag and GG-ZnO dispersions were prepared by bath sonication at 40 kHz (Unisonics FXP 12D, Australia) of 200 mg, 400 mg, and 600 mg TiO₂, Ag or ZnO in 100 mL Milli-Q water for 30 min. This was followed by an addition of GG (1 g), and glycerine (50% by weight relative to GG) under continuous stirring (500 rpm) for 2h at 70 °C. The dispersions were sonicated in bath sonicator for an additional 10 min prior casting.

Films were prepared by evaporative casting, i.e. solution or dispersions were deposited onto an acrylic plate and allowed to dry for 2 days in a temperature humidity chamber (Thermoline scientific, TRH-460) (21 °C, 50 ± 5% RH). The resulting films were cut to rectangular strips and pre-conditioned for another 2 days in

the humidity chamber at 21 °C, $50 \pm 5\%$ RH prior to testing. The resulting films of GG-TiO₂, GG-Ag and GG-ZnO containing 10%, 20% and 30% of nanoparticles loadings (by weight relative to GG) are hereafter referred to GGTi10, GGTi20, GGTi30, GGAgi10, GGAgi20, GGAgi30, GGZn10, GGZn20 and GGZn30, respectively.

2.2.3 Polyelectrolyte complex film of chitosan and gellan gum

CH solution (30% glycerine) and GG solution (50% glycerine) were prepared as described in section 2.2.1 and 2.2.2, respectively. pH modified solutions were prepared by adjusting the pH of CH to pH ~ 1.8 and GG to pH ~ 12 through additions of concentrated acetic acid (glacial, 17 M) and sodium hydroxide (1 M), respectively. These solutions with adjusted pH are hereafter referred to as CH (pH) and GG (pH). Solutions were deposited onto an acrylic plate and allowed to dry for 2 days in a temperature humidity chamber at 21 °C and $50 \pm 5\%$ RH before peeling off.

Polyelectrolyte complex (PEC) films were prepared by immersing the CH or GG film into the solution of oppositely charge for 30 s on each side, i.e. they were laid flat for 30 s, before turning over, and laid flat for another 30 s (Figure 2.2).

Figure 2.2: Schematic of the preparation of polyelectrolyte complex film. CH and GG films were laid flat into the solution of oppositely charge for 30 s on each side.

The resulting films were dried on a custom build rack under controlled ambient conditions (21 °C, 60 ± 10% RH) for 2 days. The films were then pre-conditioned for another 2 days in a temperature humidity chamber at 21 °C and 50 ± 5% RH prior to testing. The CH film immersed into the GG solution (70 °C) is hereafter referred to as CH^{into}GG, and the GG film immersed into the CH solution (25 °C) is hereafter referred to as GG^{into}CH. Meanwhile, a pH adjusted film or solution is named with pH after the film or solution. For example, a pH adjusted CH film into GG solution is hereafter referred to as CH(pH)^{into}GG, and a pH adjusted CH film into a pH adjusted GG solution is hereafter referred to as CH(pH)^{into}GG(pH).

2.2.4 Polyelectrolyte complex coated wool fibres using chitosan and gellan gum

CH solutions (30% glycerine) and GG solutions (50% glycerine) were prepared as described in section 2.2.1 and 2.2.2, respectively. CH and GG-dyed solutions were prepared by additions of 0.25% (v/v) of blue and yellow food dyes, respectively. The pH adjusted CH and GG-dyed solutions were prepared by adjusting the pH of CH to pH ~ 1.8 and GG to pH ~ 12 through additions of concentrated acetic acid (glacial, 17 M) and sodium hydroxide (1 M).

CH coated fibre (CH-fibre) and GG coated fibre (GG-fibre) were prepared by immersing pristine wool fibres (30 cm) into CH solution (25 °C) and GG solution (70 °C) in a beaker for 2, 5, 10, 20 and 30 min. CH-dyed and GG-dyed fibres were prepared by immersing wool fibres into CH-dyed and GG-dyed solutions for 20 min and 5 min, respectively. Meanwhile, pH adjusted CH and GG-dyed fibres were prepared by immersing wool fibres into pH adjusted CH and GG-dyed solutions for 20 min and 5 min, respectively. The coated wool fibres were dried by hanging on the

retort stand under controlled temperature (21 ± 5 °C, $60 \pm 10\%$ RH) for 2 days (Figure 2.3), and pre-conditioned for another 2 days in the temperature humidity chamber (21 ± 5 °C, $50 \pm 5\%$ RH) prior to testing.

PEC-coated wool fibres were prepared by immersing CH-dyed and GG-dyed fibres into dyed solutions of oppositely charge at 20 min and 5 min, respectively. CH-dyed fibre immersed into the GG-dyed solution is hereafter referred to as CH^{into}GG, meanwhile GG-dyed fibre immersed into the CH-dyed solution is hereafter referred to as GG^{into}CH. The pH adjusted of dyed-fibres or dyed solutions are named after pH. For example, a pH adjusted CH-dyed fibre into GG-dyed solution is hereafter referred to as CH(pH)^{into}GG, and a pH adjusted CH dyed fibre into a pH adjusted GG-dyed solution is hereafter referred to as CH(pH)^{into}GG(pH). The PEC-coated fibres were dried by hanging on the retort stand under controlled temperature (21 ± 5 °C, $60 \pm 10\%$ RH) for 2 days, and pre-conditioned for another 2 days in the temperature humidity chamber at 21 °C and $50 \pm 5\%$ RH prior to testing.

Figure 2.3: Schematic of the formation of polyelectrolyte complex (PEC) on wool fibre; (A) pristine wool fibre immersed into chitosan or gellan gum solutions at different immersion of durations, (B) wool fibres were dried on the retort stand, (C) coated wool fibres were immersed into solutions of oppositely charge at different immersion of durations and (D) the PEC-coated wool fibres were dried on the retort stand.

2.2.5 Polyelectrolyte complex film of chitosan–levofloxacin and gellan gum– TiO₂ nanoparticles

GGTi20 composites were prepared as described in section 2.2.2. CH-Lev01, CH-Lev1 and CH-Lev5 solutions were prepared by additions of levofloxacin at 0.4 w/w%, 4 w/w%, and 20 w/w% (percentage weight relative to CH) into 100 mL of 0.08 M acetic acid. Chitosan (2 g) and glycerine (30%, by weight relative to CH) were added into the levofloxacin solutions with stirring (500 rpm) for 2 hours at 70 °C. CH-Lev films were prepared by evaporative casting, i.e. solutions were deposited onto an acrylic plate and allowed to dry for 2 days in a temperature humidity chamber at 21 °C and 50 ± 5% RH. The films were peel off and cut into the rectangular strips depending on the characterisations. The films were pre-conditioning for another 2 days in the temperature humidity chamber at 21 °C and 50 ± 5% RH prior to testing.

PEC-films were prepared by pouring a CH-Lev01 solution onto the surface of a dry GGTi20 composite in an acrylic plate (Figure 2.4). This PEC-film hereafter referred to as dual layer films. The dual layer films were dried for 2 days in the temperature humidity chamber at 21 °C and 50 ± 5% RH. The dual layer films were then peeled off and cut into rectangular strips depending on the characterisation, and pre-conditioned for another 2 days in the humidity chamber at 21 °C and 50 ± 5% RH prior to testing.

Figure 2.4: Schematic of the preparation of dual layer films. CH-Lev01 solution is pouring onto the surface of GGTi20 composite in the acrylic plate and dried.

2.3 Characterisation of solutions, dispersions and films

2.3.1 Ultraviolet visible spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy of solutions, dispersions and films were performed using a spectrophotometer (Varian, Cary 500 UV-Vis NIR) with data interval=0.5 nm, scan speed=300 nm/min and wavelength range 200-800 nm. Quartz cells with path length 0.01 mm and 5 mm were used and detailed on caption to each figures. The UV absorption and transmittance of films were measured by attached the film on the cuvette's surface.

2.3.2 Circular dichroism

Circular dichroism (CD) spectra were recorded using a spectropolarimeter (Jasco J-810) equipped with a temperature controller (Jasco CDF-426S), at a scan rate 100 nm/min, temperature=20 °C, and a CD-matched cuvette with 0.01 mm and 5 mm path

lengths for solution and film, respectively (unless stated otherwise). CD intensity was converted into mean molecular ellipticity $[\Theta]$ ($\text{deg cm}^2 \text{decimol}^{-1}$) using:

$$[\Theta] = \Theta_p / (10 * n_r * C_p * l), \quad (\text{Eqn. 2.1})$$

where Θ_p is the ellipticity (CD intensity, mdeg) of the polysaccharide (i.e., minus the solvent), l is the path length (cm), n_r and C_p are the number and molar (moles/L) concentration of repeating tetrasaccharide units within a chain of CH and GG. The average number of repeating units of GG chain reported at 800²⁹⁶.

2.3.3 pH measurement

The pH of solutions, dispersions, films and coated-wool fibres were measured using a pH meter (Metrohm 826 pH mobile) (Figure 2.5). The pH of solutions and dispersions were determined by electrode plus glass electrodes (Metrohm), while the pH of films and coated-wool fibres were measured by a flat-membrane glass electrode (Metrohm) (Figure 2.5 C).

Figure 2.5: (A) Metrohm 826 pH mobile, (B) electrode plus glass electrode dips into the solution or dispersion and (C) flat-membrane glass electrode is attached on the film surface or coated-wool fibres.

2.3.4 Tensile testing

Stress-strain measurements were obtained using an Instron Universal Testing Machine (model 5566) with ± 10 kN grips and cross-head speed set at 20 mm/min. All films were cut to 25 mm x 100 mm, whereas the coated-wool fibres were cut to 100 mm. The films and coated-wool fibres were clamped in between of the grips at distance of 50 mm (Figure 2.6). The thickness of each film was measured using a hand-held micrometer (Mitutoyo) at 10 different positions along the gauge length of each specimen and the mean values were calculated. The diameters of coated-wool fibre were measured with an optical microscope (Leica Z16 APO) and analysed with software kit (Leica DFC290). At least 10 diameter values were measured on each coated-wool fibre and the mean values were calculated.

Figure 2.6: (A) Film and (B) coated-wool fibre are clamped between the grips for tensile testing, respectively.

Tensile strength (TS), Young's modulus (E) and toughness (T) were calculated from the slope of the linear part of the stress-strain curve, the maximum stress, and by integrating the area under the stress-strain curve, respectively.

Meanwhile, strain-at-break (γ) is the strain recorded when it breaks. A minimum of five independent stress-strain measurements were obtained per sample.

2.3.5 Optical and Electron Microscopy

The cross-section morphology of CH-composite, GG-composite and dual layer films were observed using a field emission scanning electron microscope (FE-SEM) (JEOL JSM-7500 FA). Samples were prepared for observation using the freeze-fracture technique; i.e. the samples were dipped in liquid nitrogen (-196 °C) and fractured at -150 °C.

The cross-section of the polyelectrolyte complex (film and coated-wool fibre) of CH and GG were observed using an optical microscope (Leica, Z16 APO). The film (2 cm x 2 cm) and coated-wool fibre (1 cm) were vertically aligned under the optical axis of the microscope, and photographs of the cross section were captured.

2.3.6 Swelling

Swelling was measured by using four types of solutions, i.e. Milli-Q water, and three buffer solutions, (pH 1, pH 7 and pH 12) (Sigma Aldrich) at 21 °C. The films were removed after 24h in solutions, gently wiped with a tissue to expel surface water and weighed. Water swelling (WS) was determined from the equilibrium-swelling ratio according to:

$$WS = (L_{\text{wet}} - L_{\text{dry}})/L_{\text{dry}}, \quad (\text{Eqn. 2.2})$$

where L_{dry} and L_{wet} are the weight of the dry and wet films, respectively. A minimum of five independent measurements were obtained per sample.

2.3.7 Contact angle

Contact angle (θ) is the angle at which a liquid interface meets the solid surface as shown in Figure 2.7.

Figure 2.7: Contact angle (θ) measurement of water droplet on the sample.

A goniometer (Dataphysics OCA40) video-based optical system was used for all contact angle measurements. 2 μL of distilled water was placed on the sample's surface. The images of the water spreading on the polymer surface were recorded by a video camera and analysed with the instrument's software kit (SCA20). The contact angle is recorded immediately after a droplet placed on the surface without waiting distilled water to reach equilibrium due to hydrophilic behaviour of the samples. A minimum of 5 measurements were made across the sample and the mean value calculated.

2.3.8 Water vapour transmission rates

The water vapour transmission rates (WVTR), water vapour permeability (WVP) and permeance (P) were measured following a modified ASTM International standard method²⁹⁷. Each film is fixed on the circular opening of a permeation bottle

(diameter = 1.5 cm, height = 5.0 cm) with the effective transfer area (A) of 1.33 cm² (Figure 2.8).

Figure 2.8: (A) Film is fixed on the circular opening of a permeation bottle and stored in a temperature humidity chamber at 21 °C and 50 ± 5% RH.

The permeation bottle is placed in the temperature humidity chamber at 21 °C and 50 ± 5% RH unless stated otherwise. The equilibrium moisture penetration was determined by weighing the bottles at 0h, 24h, 48h, and 96h, respectively. The WVTR, WVP and P were obtained using Eqn. 2.3, 2.4 and 2.5, respectively as below:

$$\text{WVTR} = (m/\Delta t)/A, \quad (\text{Eqn. 2.3})$$

$$\text{WVP} = [(m \cdot X)/(\Delta t \cdot A \cdot \Delta P)], \quad (\text{Eqn. 2.4})$$

$$P = [(m/(\Delta t \cdot A \cdot \Delta P))], \quad (\text{Eqn. 2.5})$$

where $m/\Delta t$ is the amount of water gain per unit time of transfer, X is the film thickness (m), A is the area exposed to water transfer (m²), and ΔP is the water vapour pressure difference between inside and outside of permeation bottle (Pa).

2.3.9 Kubelka-Munk analysis

The images of dyed-wool fibre were captured by microscope (Leica Z16 APO) which saved to 8-bit colour data in the red, green and blue channels. These images were analysed using the Kubelka-munk (K-M) equation for each of the three channels implemented in MATLAB software tools (Eqn. 2.6) ²⁹⁸;

$$K/S = (1-R)^2 / 2R, \quad (\text{Eqn. 2.6})$$

where K is the coefficient of absorption, S is scattering spectrum, and R is the fraction of light reflected, which the ratio (K/S) obtained are proportional to the concentration of the colourant ²⁹⁹. A minimum of 5 measurements were made across the dyed-wool fibre and the mean value calculated.

2.3.10 Levofloxacin release studies

Levofloxacin release studies from CH-Lev films was determined as follows, CH-Lev films (2 cm x 2 cm) were immersed in a 100 mL PBS buffer solution (pH = 7) at 37 °C. Thief samples were taken at 1, 2, 5, 10, 20, and 30 min to determine absorbance spectra (Varian Cary 500 Scan UV-VIS-NIR spectrophotometer, between 200 and 800nm). A standard curve was constructed from diluting a 1 mg/mL levofloxacin solution and measuring the absorbance (A) at 287 nm. The extinction coefficient was obtained using Beer-Lambert law:

$$A = \mathcal{E} l c, \quad (\text{Eqn. 2.7})$$

where $\mathcal{E}$ is the molar absorptivity, l is pathlength and c is the concentration of the solution. The resulting extinction coefficient was used to determine the levofloxacin concentration in PBS, which was then converted into fraction remaining in the film.

2.3.11 Elemental analysis

Elemental analysis (C, H, N) of raw powders (CH and GG) and films were carried out by the University of Otago, New Zealand. This information is converted into one matrix (M_s) for the raw powders, and several arrays, one for each of the films. The film's composition in terms of percentage of each of the biopolymers is then obtained from the matrix product of the films array and the inverse of the matrix M_s .

2.3.12 In-Vitro Cell Studies

Routine cell-culture

L929 mouse fibroblast cells (American Type Tissue Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% (v/v) foetal calf serum (FCS, Sigma Aldrich) and 1% antibiotic (Penicillin/Streptomycin, Sigma Aldrich) at 37 °C in a humidified atmosphere of 5% CO₂. Cells were sub-cultured every 3 days using established protocols. The cells were harvested at 60-80% of confluence, washed twice with DMEM, and then trypsinized for 5 min with 0.2% trypsin (Sigma Aldrich) containing 0.02% ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich). The resulting cell suspension was centrifuged and re-suspended in DMEM-FCS followed by cell counting with a hemocytometer (Hausser Scientific, USA).

Cell viability

Specimens were placed into wells of 96-well culture plates and sterilized in a laminar airflow chamber using UV radiation for 20 min. Prior to cell seeding, specimens in 96-well culture plates were soaked in media (DMEM only) for 24h and supernatant removed. Suspensions of L929 cells (5000 cell/well) were seeded into wells containing specimens and cultured at 37 °C in humidified atmosphere of 5% CO₂ for

24h, 72h and 144h. Media were replaced after 96h. Tissue culture polystyrene plates (TCPP) were used as control contained the same number of cells. Cell viability of specimens was imaged after 24h, 72h and 144h incubations by light microscope (Zeiss Axiovert 40 CFL).

CH-composite and GG-composite films were imaged by staining with calcein-AM (Fluka Biochemika). 1 μ L of calcein-AM added to each well containing specimens and incubated for 15 min prior to imaging. Calcein-AM is membrane-permeable and is cleaved in the cytosol of viable cells to release calcein, which remains trapped inside cells and endows them with bright green fluorescence when excited with blue light. Imaging of cells growing on specimens was achieved utilizing an inverted light microscope (Zeiss Axiovert 40 CFL) with fluorescent light source (HBO 50).

Indirect contact assay

Specimens were placed into wells of 96-well culture plates and sterilized in a laminar airflow chamber using UV radiation for 20 min. The media (DMEM, 10% FCS) were added into wells containing specimens and incubated at 37 °C in humidified atmosphere of 5% CO₂ for 24h. The specimens were removed and the media containing L929 cell in wells were then observed by light microscope (Leica DC 500).

Cell proliferation

Cell proliferations were quantified by MTS assay (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium)). Prior to addition of MTS reagent (1 mg/mL), the specimens-cell culture (L929) and specimens-free cell

culture (positive control) were transferred to new wells with fresh media and incubated for 4h at 37 °C in an atmosphere containing 5% CO₂. The absorbance of specimens measured at 490 nm with a microplate reader (FLUOstar Omega, BMG Labtech, Germany). The absorbance was then related to cell numbers using calibration curves of L929 cells in 96-well plates by triplicate serial dilution of known cell number in DMEM-FCS solution.

2.3.13 Antibacterial studies

Culture conditions

A model microbe, i.e. a laboratory adapted version of the Gram-negative bacterium *E. coli* (JM 109) was used for antibacterial assays³⁰⁰. Luria-Bertani (LB) standard growth medium consisting (perliter) of 5 g yeast extract (Difco), 10 g tryptone (Difco) and 10 g NaCl are sterilized by autoclave for 20 min at 120 °C. *E. coli* was grown in LB broth and incubated aerobically at 41 °C in a rotary action incubator shaker (Bioline 472) at 200 rpm. *E. coli* suspensions were grown to an optical density of 1.0 (wavelength 600 nm) in LB broth which measured using a spectrophotometer (Ultrospec 10, Amersham Biosciences).

Qualitative study – solution/dispersion

100 uL of *E. coli* suspensions were evenly pipette-loaded on the solid LB agar and dried in a laminar airflow chamber. The solutions/dispersion were filled into holes on solid LB agar (diameter 6 mm) (Figure 2.9) and incubated at 37 °C for 24h. The presence of any clear zone around the hole on the plate was recorded as an indication of inhibition against the *E. coli*.

Figure 2.9: *Escherichia coli* suspension evenly spread on the surface of LB agar and solutions/dispersions were filled up into the holes.

Qualitative study – film

100 μL of *E. coli* suspensions were evenly spread on the solid LB agar and dried in a laminar airflow chamber. The sample discs (diameter ~ 6 mm) were then placed on the LB agar and incubate at 37 $^{\circ}\text{C}$ for 24h. The presence of any clear zone around the discs on the LB agar was recorded as an indication of inhibition against the *E. coli*.

Quantitative study – solution/dispersion

Solutions/dispersions (50 μL) were added into a 15 mL Falcon tube containing 4 mL LB broth and 1 mL of *E. coli* suspension. The mixing of LB broth and *E. coli* suspension is hereafter referred as inoculum. The solutions/dispersions were incubated at 37 $^{\circ}\text{C}$ with shaking at 200 rpm for 3h. The viable population of *E. coli* is enumerated by sequentially diluted 200 μL solution containing 20 μL inoculum and 180 μL phosphate buffered saline (PBS) in 96-well plates to obtain serial dilutions (10^1 - 10^8 CFU/mL). Then, 20 μL of each of the dilutions was aseptically plated in triplicate on LB agar plates and incubated at 37 $^{\circ}\text{C}$ for 24h. LB broth with inoculum was used as a positive control. Growth inhibition was also compared to the ampicillin solution (5 mg/ L) (Amresco). Colonies appearing on the plates after 24h of

incubation at 37 °C were counted and reported as the *E. coli* viable population in CFU per mL.

Quantitative study - film

A film (1 cm x 2 cm) was added into a 15 mL Falcon tube containing 4 mL LB broth and 1 mL of *E. coli* suspension. This falcon tubes with contents were incubated at 37 °C with shaking at 200 rpm. 10 µL inoculums were taken at intervals of 0h and 1h. The viable population of *E. coli* is enumerated by sequentially diluted 100 µL solution containing 10 µL inoculum and 90 µL phosphate buffered saline (PBS) in 96-well plates to obtain a serial dilution (10^1 - 10^8 CFU/mL). Then, 20 µL of each of the dilutions was aseptically plated in triplicate on LB agar plates and incubated at 37 °C for 24h. Colonies appearing on the plates after 24h of incubation at 37 °C were counted and reported as the *E. coli* viable population in CFU per mL.

2.3.14 Antiviral study

Antiviral studies were carried out in collaboration at the Centre for Biomedical, Molecular and Chemical Sciences (CBMCS), University of Canberra, Australia under supervision of Prof. Suresh Mahalingam.

Cell culture

Vero cells, a line of African green monkey kidney cells (T48) (American Type Culture Collection) were cultured in optiMEM culture media (Gibco, Invitrogen) containing 3% newborn bovine serum (NBS) (Gibco, Invitrogen) at 37 °C under an atmosphere of 5% CO₂. Cells were sub-cultured every 3 days using established protocols. The cells were harvested at 60-80% of confluence, washed with optiMEM,

and then trypsinized for 5 min with 0.2% trypsin (Sigma-Aldrich). The resulting cell suspension was centrifuged and re-suspended in optiMEM followed by cell counting with a hemocytometer (Hausser Scientific, USA).

Virus stock

The green fluorescent protein restrain of Ross-river virus (GFP-RRV) was kept in freezer at -80°C . The virus is thawed inside ice for 30 min prior use.

Virus survival

The CH films were cut to the rounded shape which fix the size of 24-well culture plates, then soaked into optiMEM media in 50 mL falcon tube for 3h. The films were then rinsed with 70% ethanol, and placed into 24-well culture plates and sterilized in a laminar airflow chamber under UV-light for 20 min. Suspension of Vero cells (1.5×10^5 cells/well) were seeded into well containing film and cultured at 37°C in atmosphere of 5% CO_2 for 20h. GFP-RRV virus (3×10^4 PFU/ well) were seeded into the well containing adherent Vero cell on CH film and incubated at 37°C in atmosphere of 5% CO_2 for 6h, 12h and 24h. Virus activity was then imaged using fluorescent microscope (Nikon ECLIPSE Ti) equipped with a light source (Nikon Intensilight, C-HGFI).

CHAPTER 3

CHARACTERISATION OF COMPOSITE MATERIALS FROM CHITOSAN WITH TITANIUM DIOXIDE AND SILVER NANOPARTICLES

Chitosan (CH) films containing glycerine (Gly) were prepared and characterised to find optimum mechanical properties (tensile strength, Young's modulus, toughness and strain-at-break) as well as swelling and water vapour transmission rates (WVTR). Titanium dioxide (TiO_2) and silver (Ag) nanoparticles were incorporated in CH film to improve the mechanical properties. The swelling, contact angle (CA) and WVTR values of CH-composites were measured. In addition, the cell viability and cell proliferation of all samples were investigated *in vitro* using mouse fibroblast cells (L929). Antibacterial and antiviral activities of the CH-composites were evaluated on Gram-negative bacteria (*Escherichia coli*) and green fluorescent protein restraint of Ross-river virus (GFP-RRV), respectively.

3.1 Spectroscopy

3.1.1 UV-Visible

The UV-visible absorbance spectra of the CH (without glycerine) and CH30 (containing 30% of glycerine by weight relative to CH) films as well as CH-Ag and CH- TiO_2 dispersions are shown in Figure 3.1. Addition of 30% glycerine (by weight

relative to CH) to the CH solutions increased the absorbance intensity in the spectral region (300-800 nm) which could be due to the formation of hydrogen bonding between CH and Gly (Figure 3.1 A).

Figure 3.1: (A) UV-visible absorption spectra of CH and CH30 films (pathlength=5 mm), (B) UV-visible absorbance spectra of CH-Ag dispersions with 10%, 20% and 30% of nanoparticle loadings (by weight relative to CH) (pathlength=0.01 mm); inset: characteristic absorption band of Ag at 420 nm (C) UV-visible absorbance spectra of CH-TiO₂ dispersions with 10%, 20% and 30% of nanoparticles loadings (by weight relative to CH) (pathlength=0.01 mm).

The addition of Ag and TiO₂ to CH solutions resulted in characteristic absorption bands at 420 nm and 330 nm, respectively (Figure 3.1 B and C)^{244, 263,301, 302}. The narrow absorption band observed for GG-Ag dispersions at 420 nm could be due to the electrostatic attraction between Ag ions and amine groups of chitosan (Figure 3.1 B)³⁰³. As expected, the intensity of the characteristic absorption bands (420 nm and 330 nm) increased with increasing Ag and TiO₂ concentration (Figure 3.1 B and C).

The transmittance of CH films in the visible wavelength range (500-750 nm) is 70% (Figure 3.2 A), see also Figure 3.3 A.

Figure 3.2: (A) Correlation of the transmittance (wavelength at 600 nm) against thickness of the CH films with 10% to 50% of glycerine (by weight relative to CH); inset: transmittance of the CH film and (B) transmittance of CH-Ag and CH-TiO₂ composite films at 10% of nanoparticles loading (by weight relative to CH).

Increasing the glycerine concentrations from 10% to 50% leads to an increase in CH film thickness from 70 ± 15 to 116 ± 7 μm, which resulted in a 10% reduction in transmittance (Figure 3.2 A). The CH-Ag and CH-TiO₂ composite films were not optically transparent as evident from the transmittance spectra (Figure 3.2 B) and the

photographs (Figure 3.3 B and C). CH solution and CH-composite dispersions were stable up to 6 months (Figure 3.3 D).

Figure 3.3: (A) CH30 film, (B) CHTi20 composite, (C) CHAg20 composite and (D) CH30, CHTi20 and CHAg20 (left to right) dispersions after 6 months. All measurements of UV absorbance were carried out at 21 °C. Films dimension are 2 cm x 2 cm.

3.1.2 Circular dichroism

Circular dichroism (CD) spectra of CH solutions show a band at 210 nm due to the presence of N-acetyl-glucosamine (GlcNAc) (Figure 3.4 A) ³⁰⁴⁻³⁰⁶. Addition of 30% glycerine to the CH solution resulted in enhanced molar ellipticity (at 210 nm) due to an increased number of hydrogen bonds between CH-Gly (Figure 3.4 A) ³⁰⁷. A comparison between CH30 solution and film shows that the CD band shifted from 210 nm to 226 nm (Figure 3.4 A and C). This shift corresponds to the CH chain adopting a helical conformation ³⁰⁸.

The temperature dependence of CH30 solutions showed that the molar ellipticity of the absorption band at 210 nm increased with decreasing temperature (Figure 3.4 B). This has been previously been attributed to changes in orientational order and the electronic dipole of the chromophoric groups of CH^{308, 309}.

Figure 3.4: (A) Comparison between CH and CH30 solutions (pathlength=0.01 mm), (B) typical temperature dependence of CH30 solution at 10 °C, 21 °C, 37 °C and 50 °C (pathlength=0.01 mm) and (C) CD spectra of CH30, CHAg10, CHAg20, and CHAg30 composite films (pathlength=5 mm). All measurements were carried out at 21 °C unless stated otherwise.

The CD spectra of the CH-Ag composites exhibited observable changes compared to CH30 film (Figure 3.4 C). The increase in molar ellipticity of the CH-Ag composites at 215-226 nm indicated that the helical conformational of CH30 film was changed, which could be a result of binding between amine groups and silver ions. The absorption bands of CH-Ag composites shifted to higher wavelength for higher nanoparticle loading (Figure 3.4 C). For example, the CD bands of CH-Ag composite with 10% nanoparticle loading (by weight relative to CH) is at 215 nm; and shifted to 226 nm with 30% nanoparticle loading (by weight relative to CH). It is not clear what the reason of this shifting.

3.2 Mechanical properties and microscopy

3.2.1 Effect of glycerine

CH films are brittle and not easy to handle. The tensile strength (TS), Young's modulus (E), toughness (T), and strain-at-break (γ) values of CH film are 39 ± 5 MPa, 1223 ± 173 MPa, $2.45 \pm 0.08 \text{ J g}^{-1}$ and $10 \pm 2\%$, respectively, (Figure 3.5 A and Table 3.1). Inclusion of glycerine significantly reduces the mechanical properties, but increases the strain-at-break (Table 3.1). This is a result of a decreased in hydrogen bonding interactions between CH-CH due formation of hydrogen bonding between CH-Gly. This decreases the intermolecular forces along CH chains³¹⁰, leading to an increase in the strain-at-break values. For example, addition of 30% glycerine (by weight relative to CH) to chitosan resulted in a decreases in E (from 1223 ± 173 MPa to 25 ± 7 MPa), TS (from 39 ± 5 MPa to 6 ± 1 MPa) and T (from $2.45 \pm 0.08 \text{ J g}^{-1}$ to $1.27 \pm 0.01 \text{ J g}^{-1}$), while the γ increased from $10 \pm 1\%$ to $32 \pm 2\%$ (Table 3.1).

Figure 3.5: Typical stress-strain curves of (A) CH film containing 0%, 15%, 30% and 50% glycerine (by weight relative to CH), (B) CH-TiO₂ composites containing 10%, 20% and 30% nanoparticle loadings (by weight relative to CH), (C) CH-Ag composites containing 10%, 20% and 30% nanoparticle loadings (by weight relative to CH) and (D) comparison between the CH30 film, CH-TiO₂ and CH-Ag composite films with 30% nanoparticle loadings (by weight relative to CH).

Table 3.1: Properties of chitosan films with different percentages of glycerine (Gly). Thickness (thick), tensile strength (TS), Young's modulus (E), toughness (T), strain-at-break (γ), swelling and contact angle (CA) are given for each film.

Gly	Thick	TS	E	T	γ	Swelling	CA
(%)	(μm)	(MPa)	(MPa)	(J g ⁻¹)	(%)	(%)	($^{\circ}$)
0	70 $\pm$ 15	39 $\pm$ 5	1223 $\pm$ 173	2.45 $\pm$ 0.08	10 $\pm$ 2	N/A	102 $\pm$ 3
15	72 $\pm$ 6	19 $\pm$ 8	559 $\pm$ 156	4.46 $\pm$ 0.32	27 $\pm$ 3	>>5,000	108 $\pm$ 2
30	100 $\pm$ 15	6 $\pm$ 1	25 $\pm$ 7	1.27 $\pm$ 0.01	32 $\pm$ 2	823 $\pm$ 31	111 $\pm$ 1
50	116 $\pm$ 7	2 $\pm$ 1	6 $\pm$ 1	0.75 $\pm$ 0.05	45 $\pm$ 5	331 $\pm$ 28	110 $\pm$ 1

3.2.2 Effect of different batch and molecular weight of CH

The effect of different batches of CH and molecular weight on mechanical characteristics was investigated by preparing CH30 films using: (i) medium molecular weight (MMW) with different batches (CH1 and CH2), and (ii) a high molecular weight (HMW) chitosan (CH3), see Table 3.2. The deacetylation degree (DD) values of the batches were similar for MMW, but there was a large difference in viscosity (η) as supplied by the manufacturer (Sigma-Aldrich), i.e. $\eta = 453$ cP for CH1 and $\eta = 915$ cP for CH2.

Table 3.2: Properties of films prepared using chitosan from various sources (source), degree of deacetylation (DD), viscosity (η), glycerin (Gly) (by weight relative to chitosan). “CH1” and “CH2” indicate two different batches of medium molecular weight chitosan, while “CH3” indicates high molecular weight chitosan, respectively. Tensile strength (TS), Young’s modulus (E), and strain-at-break (γ) of those CH films.

Source	DD	η	Gly	TS	E	γ
	(%)	(cP)	(%)	(MPa)	(MPa)	(%)
CH1, this work	75	453	30	6 ± 1.0	25 ± 7	32 ± 2
CH2, this work	79	915	30	8.0 ± 0.4	100 ± 30	34 ± 2
CH3, this work	76	1,406	30	22 ± 4.0	500 ± 134	44 ± 4
Ref. ¹⁹⁰	> 85	-	25	41.6 ± 5.9	-	42.4 ± 4
Ref. ⁷⁹	90	110	25	32.9 ± 0.7	-	54.6 ± 3
Ref. ¹⁹³	90	-	28	17.3 ± 2.8	230 ± 5.6	44.2 ± 8
Ref. ¹⁹⁵	98	-	20	31.8 ± 2.0	-	45.7 ± 3

It is well known that the viscosity of a polymer solution can be related to the molecular weight according to the Mark-Houwink-Sakurada (MHS) equation, which for CH has been determined as $\eta = 1.49 \cdot 10^{-4} M_w^{0.79}$ ³¹¹. Hence, the MHS equation suggests that the molecular weights of CH2 and CH3 are 1.7 and 2.4 times that of CH1, respectively. These higher molecular weight materials exhibited higher TS and E values, i.e. TS = 8.0 ± 0.4 MPa and E = 100 ± 30 MPa for CH2 compared to TS = 22 ± 4 MPa and E = 500 ± 134 MPa for CH3 (Table 3.2).

3.2.3 Effect of addition of nanoparticles

Inclusion of TiO₂ and Ag resulted in mechanical reinforcement of CH30 materials (Figure 3.5, 3.6 and Table 3.3). The mechanical properties of these films increased with increasing TiO₂ and Ag concentration. For example, addition of 30% TiO₂ (by weight relative to CH) resulted in an 11.8 fold increase in Young's modulus (from 25 ± 7 MPa to 294 ± 11 MPa). Tensile strength and toughness values are both increased approximately 3 and 6 fold, respectively, i.e. from 6 ± 1 MPa to 20 ± 6 MPa and from 1.27 ± 0.01 J g⁻¹ to 7.2 ± 1.5 J g⁻¹, respectively, while the strain-at-break was not affected. Meanwhile, an addition of 30% of Ag (by weight relative to CH) resulted in a 12.9 fold increase in Young's modulus, a 4.5 fold increase in tensile strength and a 6.3 fold increase in toughness, while the strain-at-break increased from $32 \pm 2\%$ to $38 \pm 4\%$. Our results demonstrate that CH-Ag materials have significantly higher tensile strength compared to CH-TiO₂ materials (Table 3.3).

It is suggested the improvement in the mechanical properties of CH-Ag and CH-Ti composites could result from electrostatic interactions between the amine group of CH with Ag ions and Ti ions³¹²⁻³¹⁴. In addition, it is likely that hydrogen bonding between CH with TiO₂ nanoparticles is also a contributing factor for the

observed increase in mechanical properties. For example, it has been reported that the increased of tensile strength in polymer-silicate clay composites can be attributed to the formation of hydrogen bonding between the polymer and silicate clay³¹⁵. While, it has also been suggested that mechanical reinforcement in composite materials depends on the possibility that the polymer optimizes its conformation with nanoscale components³¹⁶.

Table 3.3: Properties of composite films prepared using chitosan with titanium dioxide (TiO₂) and silver (Ag) nanoparticles. Nanoparticles content (NP), thickness (Thick), tensile strength (TS), Young's modulus (E), toughness (T), strain-at-break (γ), water vapour transmission rates (WVTR), swelling and contact angle (CA) are listed for the different composite materials. All samples were prepared with 30 % glycerine content by weight relative to chitosan.

Film	NP	Thick	TS	E	T	γ	WVTR	Swelling	CA
	(%)	(μm)	(MPa)	(MPa)	(J g ⁻¹)	(%)	(g m ⁻² d ⁻¹)	(%)	(°)
CH-TiO ₂	10	76 ± 3	13 ± 4	99 ± 38	2.45 ± 0.1	24 ± 2	413 ± 10	73 ± 11	97 ± 0.5
	20	79 ± 6	18 ± 1	276 ± 83	4.15 ± 0.4	18 ± 2	410 ± 11	100 ± 17	93 ± 2
	30	73 ± 8	20 ± 6	294 ± 11	7.20 ± 1.5	26 ± 4	408 ± 13	105 ± 15	85 ± 2
CH-Ag	10	75 ± 8	12 ± 2	103 ± 29	3.24 ± 0.1	33 ± 2	383 ± 25	>>1,000	101 ± 1
	20	88 ± 20	14 ± 2	310 ± 187	4.10 ± 0.4	35 ± 7	384 ± 26	1047 ± 90	103 ± 1
	30	71 ± 11	27 ± 4	322 ± 145	8.00 ± 1.5	38 ± 4	368 ± 26	1002 ± 51	106 ± 1

Figure 3.6: Scanning electron microscopy images of cross-sectional areas of (A) CH-Ag composite film with 10% nanoparticle loading (by weight relative to CH) (CHAg10), (B and C) enlarged view of CHAg10 composite film, (D) CH-Ti composite film with 10% nanoparticle loading (by weight relative to CH) (CHTi10), and (E-F) enlarged view of CHTi10 composite film.

3.3 Swelling

The swelling results show that addition of glycerine to the CH film results in a large reduction in water uptake (Table 3.1). For example, increasing the glycerine content

from 15% to 30% reduced the swelling from >>5000% to 823% (Table 3.1).

Photographs of the swollen CH15 and CH30 films are shown in Figure 3.7.

Figure 3.7: Swelling of films in Milli-Q water for 24h; (A) CH15 film, (B) CH50 film, (C) CHAg20 composite and (D) CH-Ti nacomposite. Scale bars represent 1 cm.

It is suggested that the swelling of CH materials is a result of electrostatic repulsion between CH chains³¹⁷. Previously, it has been hypothesised that swelling of CH composite films can be reduced by either prevention of chitosan chain movement or separation of the chitosan chains³¹⁸. Therefore, it is likely that the observed reduction in swelling is due to hydrogen bonding between glycerine and chitosan which limits chitosan chain movement.

In comparison to CH30, addition of Ag (10%, by weight relative to CH) resulted in a large increase in swelling (from $823 \pm 31\%$ to $4223 \pm 20\%$), which is an almost 5 fold increase (Table 3.1 and 3.3). This is due to the interaction of the amine group (NH_2) in CH with Ag ions ($\text{R-NH}_2 \text{Ag}^+$)³¹⁹. The structure of $\text{R-NH}_2 \text{Ag}^+$

breaks up in water to AgOH and $R-NH_3^+$ and the AgOH molecules could interact with Ag^+ and form bio-conjugates with CH. These bio-conjugates are responsible for water absorption. Similar results have been observed in water swelling for other CH-Ag composite films^{79, 244}.

Addition of TiO_2 nanoparticle decreased the swelling of CH-Ti composites. For instance, an addition of 10% (by weight relative to CH) of TiO_2 resulted in a significant reduction in swelling (from $823 \pm 31\%$ to $73 \pm 11\%$), while increasing the TiO_2 concentration to 30% increased the swelling to $\sim 105 \pm 15\%$ (Table 3.1 and 3.3). It is suggested that this can be attributed to the strong hydrogen bonding interactions between CH and TiO_2 , which contributed to further interconnecting the CH chains (in addition to interconnections via CH-Gly hydrogen bonding), thereby reducing swelling.

3.4 Contact angle

The hydrophobicity of the CH-composites was determined through contact angle (CA) measurements. The hydrophobicity of the CH films was found to increase with increasing glycerine content (Table 3.1). Additions of 10%, 20% and 30% (by weight relative to CH) of Ag-nanoparticles resulted in CA values of $101 \pm 1^\circ$, $103 \pm 1^\circ$, and $106 \pm 1^\circ$, respectively. In contrast, the CA of CH30 was reduced from $111 \pm 1^\circ$ to $85 \pm 2^\circ$ through addition of TiO_2 nanoparticles (Table 3.3).

It is not clear at present why our values are higher than reported by Rhim and co-workers⁷⁹. They had reported that the CA value of their CH film is at 45.6 ± 0.2 . It is likely that, factors such as differences in molecular weight, deacetylation degree

(DD), and the source of chitosan (as described in Table 3.2), could be responsible for the observed difference.

3.5 Water vapour transmission rates (WVTR)

Water vapour transmission rates (WVTR) of CH-composites are summarised in Table 3.3. The WVTR values of the CH-Ti and CH-Ag composites films were in the range of 408 - 413 g m⁻² d⁻¹ and 368 - 384 g m⁻² d⁻¹, respectively. These values are within the range of WVTR values (90 – 2,893 g m⁻² d⁻¹) reported for eight commercially available synthetic wound dressings²⁹⁷. In particular, the obtained values are directly comparable to those reported for the hydrocolloid based dressings known as IntraSite® (354 ± 42 g m⁻² d⁻¹) and Restore Cx® (482 ± 69 g m⁻² d⁻¹).

3.6 Cell study

3.6.1 Cell viability

CH film and CH-composites were neutralised with NaOH to remove any remaining acetic acid (necessary for solubility of CH in water). As prepared, CH30 films exhibited a low L929 cell viability (Figure 3.8 A). Several studies had reported that the potential cytotoxicity of chemically modified biomaterials could be from acetic acid residues^{320, 321}.

CH30 film treated with NaOH exhibit higher cell viability compared to as-prepared CH30 films without treatment. The L929 cells adhering on CH30 film changed their morphology from spherical to elongated/spindle-like, which is typical of L929 fibroblast cells (Figure 3.8 B). It has been suggested that the adherence of L929 cells on CH film is a result of electrostatic interaction between positively charged CH chains and negatively charged L929 cell membrane^{199, 322}. This suggests

that treated CH30 films support the attachment and growth of L929 cell. Our results are in agreement with previous studies of L929 on CH films^{200, 203, 323}.

Figure 3.8: (A) As prepared CH30 films (without 1 M NaOH treatment), (B) CH30 film with 1 M NaOH treatment, (C) fluorescent image of adherent L929 cells on CHTi10 composite, (D) fluorescent image of dying cells on CHAg10 composite, and (E) cell proliferation of CH30 film and CH-Ti composites cultured in L929 cell for 72h. Errors represent one standard deviation from the measured values (n=3). Scale bars represent 50 μ m.

Addition of TiO₂ to CH film promotes L929 cell growth, whereas inclusion of Ag nanoparticles inhibits growth. Fluorescence imaging showed that cells on CH-Ti composite are viable (Figure 3.8 C). However, the degree of fluorescence observed for cells on Ag containing films indicates that most cells are dying (Figure 3.8 D). Previously, it has been shown that chitosan-Ag films crosslinked with genipin were biocompatible with L-929 cells ²⁴⁴, while lactose-substitute chitosan-alginate-Ag hydrogels were found to be non-cytotoxic towards mouse-fibroblast-like (NIH-3T3), human hepatocarcinoma (HepG2) and human osteosarcoma (MG63) cells ²⁶³. Our results may suggest that the observed cell death was due to nanoparticle contact with the cells ³²⁴. As such, it is possible that the cell viability of the CH-Ti composites can be explained by the prevention of nanoparticle diffusion. Hence, it is likely that a nanoparticle containing material will be non-cytotoxic as long as contact between nanoparticles and cells is prevented, as is the case for the aforementioned hydrogel and cross-linked chitosan ^{244, 263}.

This observation was further investigated by a so-called indirect contact assay on the supernatant of CH-Ti and CH-Ag composites. Figure 3.9 shows that the L929 cells in the supernatant containing CH-Ti composite are viable, i.e. the cells elongated and adhered to the tissue culture polystyrene plate (TCPP). In contrast, L929 cells in supernatant containing CH-Ag composite were not viable (Figure 3.9). It is suggested that the high swelling ratio of CH-Ag composite compared to CH-Ti composite (Table 3.3) may result in diffusion of Ag nanoparticle into the medium, and kill the cells.

Figure 3.9: In-direct contact assay of the CH-composites after incubated with L929 cell for 72h; (A) TCPP, (B) CHTi10, and (C) CHAg10. Scale bars represent 100 μm .

3.6.2 Cell proliferation

Glycerine in CH film exhibits a significant effect on the L929 cell proliferation (Figure 3.8 E). For instance, the cell proliferation of CH30 multiples to 3200 cells/well after incubated for 72h, whereas CH film is only at 1000 cell/well (Figure 3.8 E). Cell proliferation on the CH-Ti composites was improved compared to CH30 film (Figure 3.8 E). For instance, CHTi10 composite and CH30 film recorded 4900 cells/well and 3200 cells/well, respectively after incubated for 72h. However, cell proliferations were decreased upon additions 20% and 30% (by weight relative to CH) of nanoparticle loadings (Figure 3.8 E). In contrast, the CH-Ag composites show none of the cell proliferates on substrate which expected due to diffusion of Ag nanoparticles into the media and killed the cells.

A number of studies reported the influence of physic-chemistry of the surface toward biological response of viable cells such as hydrophobicity and hydrophilicity of materials^{196, 198, 325-330}. An addition of glycerine was reported to improve the hydrophobicity and lead to a positive biological response of viable cells on the substrate^{60, 331, 332}. This behaviour is in agreement with the cell proliferation recorded on CH30 and CH films. Incorporation of nanoparticles has been reported to increase surface area to volume ratio of nanocomposite for cells to adhere thus increases cell proliferation³³³. As consequent, this factor explained the higher cell proliferation obtained on the CH-Ti composites compared to CH30 film (Figure 3.8 E). Not limited to that, inclusions of nanoparticles also had been reported to change surface topology of the nanocomposite which could affected cell proliferation³³⁴⁻³³⁶.

3.7 Antibacterial study

The antibacterial activity of CH30 solution and CH-composites dispersion was quantified by a viable cell count method (quantitative study). Table 3.4, shows that CH30 solution against *Escherichia coli* (*E. coli*) results in a 1 log cycle reduction compared to control (Table 3.4) which is in agreement with previously reported values^{136, 206, 212, 337, 338}. As a comparison, the commercial antibiotic ampicillin (5 mg/L) exhibited a 5 log cycle reduction compared with the control (Table 3.4).

Table 3.4: The number of viable bacterial colonies after 24 hours (n_{viable}) of control, commercial antibiotic (ampicillin), CH30, and CH-composites. Errors represent one standard deviation from the measured mean values (n=3).

Sample	n_{viable} (CFU/ml)
control	$8.0 \pm 0.5 \times 10^8$
ampicillin	$1.3 \pm 0.1 \times 10^4$
CH30	$7.0 \pm 0.7 \times 10^7$
CHAg10	$8.0 \pm 1.4 \times 10^6$
CHTi10	$9.0 \pm 1.6 \times 10^6$

Chitosan is well-known for its antimicrobial effect, which has been attributed to its ability to enter the bacterial cell wall through pervasion and to form a polymer membrane on the surface of the cell wall^{135, 337}. The former prevents nutrients from entering the bacterial cell, while the latter disturbs the physiological activity of the bacterium¹³⁵. Chitosan has been reported to be effective against both Gram-negative and Gram-positive bacteria, although its effectiveness depends on its molecular weight, degree of deacetylation (DD) and concentration^{135, 212, 337}.

CH-TiO₂ and CH-Ag composite films were characterised by two methods; i.e. quantitative (disk method) and qualitative (viable count method). The inclusion of Ag and TiO₂ nanoparticles in chitosan solution increases the effectiveness against *E. coli* to a 2 log cycle reduction compared to the control (Table 3.4). Similar results were reported for addition of Ag⁺ ions to chitosan and Ag nanoparticles to genipin-crosslinked chitosan materials^{79, 244}. The effectiveness of the CH-composite dispersions is similar to that reported for crosslinked chitosan-TiO₂-gelatin films, albeit that this was tested against the Gram-positive bacteria *S. aureus*²⁵⁵. The antibacterial activity of TiO₂ materials originates from its photocatalytic properties²⁵⁸⁻²⁶⁰. Upon exposure to a UV light containing light source, TiO₂ generates superoxide ions and hydroxyl radicals²⁵⁸⁻²⁶⁰. It has been proposed that these ions and radicals operate in concert to attack phospholipids in *E. coli*²⁵⁹. The resulting lipid peroxidation reaction causes disruption of the bacterial cell membrane, leading to loss of viability by *E. coli*²⁵⁹.

The qualitative antibacterial studies of CH-Ag and CH-TiO₂ dispersions and films showed that distinctive inhibition zones were only visible for CH-Ag dispersions and films (Figure 3.10). The antibacterial activity increases with Ag nanoparticle contents, which is an agreement with previous study reported⁷⁹. The CH-Ag dispersions were found to be more effective towards *E. coli* compared to film. This may be due to the ability of the dispersions to diffuse into the Lysogeny broth agar (Figure 3.10).

Figure 3.10: Antibacterial activity of samples quantified by a qualitative method against *Escherichia coli* incubated at 37 °C for 24h. (A) CHAg20 dispersion, (B) CHTi20 dispersion, (C) CHAg20 composite, (D) CHTi20 composite, and (E) inhibition zone on the CH-Ag dispersions and films. Errors represent one standard deviation from the measured mean values (n=3).

The antibacterial activity of Ag nanoparticles and Ag⁺ ions is well known^{245, 248, 339}, and it has been suggested that silver nanoparticles act in three ways against

Gram-negative bacteria: (i) they attach to the surface of the bacterial cell membrane, significantly inhibiting permeability and respiration; (ii) they penetrate inside the bacteria and cause further damage; (iii) they release silver ions resulting in bacterial DNA losing its capacity for replication as well as inactivation of bacterial proteins²⁴⁵. Our materials yield promising antibacterial activities, which is further addressed in chapter 7.

3.8 Antiviral activity in vitro

The antiviral activity of CH30 films were investigated against green fluorescent protein restraint of Ross-river virus (GFP-RRV). The CH30 films were seeded with Vero cell (1.5×10^5 cells/well), and incubated for 20h. Then, the GFP-RRV was added and further incubated for 6h, 12h and 24h. The Vero cells infected with GFP-RRV on CH30 film were comparable to a control after incubation for 6h and 12h (Figure 3.11 A - D). A few Vero cells were clearly infected by GFP-RRV, as evident from the debris around the cells (Figure 3.11 A – D).

It was assumed that the GFP-RRV were killed in the same way as the bacteria, as described previously²⁴⁵. The GFP-RRV penetrates into the Vero cells and replicates. The replication process resulted in death of Vero cells, leading debris around the cells. After 24h, the numbers of infected Vero cells were increased on the TCPP, but decreased on the CH30 film (Figure 3.11 E – F). This result indicates that CH30 films exhibit antiviral activity by protecting the Vero cells from infection by GFP-RRV. No clear explanation on the mechanism of this observation has been reported in the literature to date. Further studies using a plaque assay (quantitative) are needed to ascertain the number of cells infected. It was not possible to test CH-Ag

and CH-Ti composite films, because of their opaque property, which prevents observation of the cell growth and infected cells.

Figure 3.11: Vero cells infected by green fluorescent protein restrain of Ross-river virus (in boxes) on tissue culture polystyrene plate (TCP) (as control) and CH30 film after incubated for 6h, 12h and 24h. Scale bars represent 50 μm .

3.9 Conclusion

The physical properties, cell proliferation and antibacterial activities of TiO₂ and Ag containing chitosan composite materials were investigated. Inclusion of glycerine in chitosan made the films mechanically weaker, but more flexible. This was attributed to a reduction in the attractive energy between CH-CH, due to formation of hydrogen bonding between CH-Gly.

TiO₂ and Ag containing composite materials exhibited a significant mechanical reinforcement compared to chitosan films. For example, addition of 30% (by weight relative to CH) TiO₂ resulted in an 11.8-fold increase in Young's modulus, a 3-fold increase in tensile strength, and a 6-fold increase in toughness. In comparison, addition of 30% (by weight relative to CH) Ag resulted in 12.9- fold and 6-fold increases in Young's modulus and toughness, respectively, but only a 3-fold increase in tensile strength. The extensibility of Ag containing materials was higher than TiO₂ containing materials. The water vapour transmission rates (368 - 413 g m⁻² d⁻¹) were similar for both materials, and comparable to commercially available wound dressings. However, inclusion of Ag lowered the water resistance (increased swelling) of chitosan films from 823% up to 4,000%, while incorporation of TiO₂ resulted in a significant improvement in water resistance (to 100%). It is hypothesised that these observations are related to the effect of the nanoparticles on the interconnection of chitosan chains, i.e. the strong hydrogen bonding between CH and TiO₂ increases the interconnection, while Ag disrupts these interconnections, thereby increasing swelling.

In vitro cell studies found that CH-TiO₂ films supported the growth of cell, whereas CH-Ag films did not. Proliferations of L929 cells on all TiO₂ containing

chitosan films were almost comparable to CH film. Fluorescence imaging showed that most cells on Ag containing films were dead. The antibacterial effectiveness by a quantitative method against *E. coli*, was similar for both Ag and TiO₂ containing materials. In contrast, a qualitative method revealed that CH-Ag composite displayed superior antibacterial activity compared to CH-TiO₂ composite. Preliminary results showed that chitosan is also effective in inhibiting virus (GFP-RRV) growth.

CHAPTER 4

CHARACTERISATION OF COMPOSITE MATERIALS FROM GELLAN GUM WITH TITANIUM DIOXIDE, SILVER AND ZINC OXIDE NANOPARTICLES

Low acyl gelan gum (GG) produces firm, non-flexible and brittle films ²¹⁹. Glycerine was incorporated into GG to improve the flexibility and thereby the handle-ability of the films ³⁴⁰. GG-composite films containing titanium dioxide (TiO₂), silver (Ag) and zinc oxide (ZnO) nanoparticles were characterised to investigate the effect of nanoparticles on the mechanical properties, swelling and water vapour transmission rates (WVTR). Biological activities (cell viability and cell proliferation) on the GG and GG-composite films were studied *in vitro* using mouse fibroblast (L929) cells. Furthermore, the antibacterial activities of GG film and GG-composites were evaluated using a Gram-negative bacterium, i.e. *Escherichia coli* (*E. coli*).

4.1 Spectroscopy

4.1.1 UV-visible

GG film and GG-composites were prepared by evaporative casting technique. The addition of glycerine does not change the absorption peak of the GG film (Figure 4.1

A). The absorption peak observed for both GG and GG50 films at 250 nm corresponds to glucuronic acid of gellan gum³⁴¹.

Figure 4.1: (A) UV-visible absorbance of the GG and GG50 films (pathlength=5 mm), (B) UV-visible absorbance of the GG-composite dispersions (pathlength=0.01 mm), (C) UV-visible transmittance of the GG-composites films (pathlength=5 mm); inset: transmittance of the GG50 film. All measurements of UV absorbance were carried out at 21 °C.

The addition of anatase TiO_2 and ZnO nanoparticles to GG solutions resulted in an absorption of specific characteristic bands in the near-UV spectral region at 320 nm^{246, 301, 302} and 360 nm^{342, 343}, respectively (Figure 4.1 B). It has been suggested that the blue shifted (transition to shorter wavelengths) of ZnO absorption from 385 nm (literature)³⁴⁴ to 360 nm (this study) is due to a smaller particle size³⁴⁴. The characteristic absorption band of Ag atoms in GG-Ag dispersions at 420 nm is barely observed as the Ag ions are expected to attach to the carboxylic groups of gellan gum³⁰³ (Figure 4.1 B).

The transmittance of GG50 film is around 70% in the visible wavelength range (500-750 nm) (inset in Figure 4.1 C) which is evident from the images shown in Figure 4.2 A. The films of the GG-composites were not optically transparent as evident from the transmittance spectra (Figure 4.1 C) and the images (Figure 4.2 B, C and D).

Figure 4.2: Photographs of the films; (A) GG50, (B) GGTi20 composite, (C) GGAg20 composite and (D) GGZn20 composite. Films dimension are 2 cm x 2 cm.

4.1.2 Circular dichroism

The effect of glycerine on GG spectra is shown in Figure 4.3 A. GG films show a single band at 212 nm, while GG50 films exhibit dual bands at 191 nm and 212 nm. The absorption bands at 191 nm and 212 nm correspond to D-uronic and L-uronic acid residues of glycosidic acid in the gellan gum repeating unit³⁴⁵. The band observed at 191 nm in GG50 film is a result of an increases in the number of carboxyl groups from glycerine surrounding the gellan backbone (Figure 4.3 A)³⁴⁵.

Figure 4.3: Circular dichroism (CD) spectra: (A) GG and GG50 films (pathlength=5 mm), (B) GG50 solution (pathlength=0.01 mm) and film (pathlength=5 mm), and (C) GG50 and GG-composite dispersions (pathlength=0.01 mm). All measurements were carried out at 21 °C.

The comparison of GG50 solution and film shows that these materials exhibit two bands, i.e. at 197 nm and 212 nm for solutions, and at 191 nm and 212 nm for films (Figure 4.3 B). These bands originate from D-uronic and L-uronic acid residues of glycosidic acid, respectively³⁴⁵. The band of GG50 solutions (197 nm) shifted to 191 nm in film which expected due to the transformation of GG from a random coil formation (solution) to a helical structure (film)^{345, 346, 347}.

The addition of 20% (by weight relative to GG) TiO₂, Ag, and ZnO nanoparticles to GG50 solution decreased the band intensity at 197 nm and 212 nm (Figure 4.3 C). This is possibly due to the attraction of those nanoparticles to the carboxyl groups of D-uronic and L-uronic acid residues of glycosidic acid and reduced the signal for both bands.

4.2 Mechanical properties and microscopy

4.2.1 Effect of glycerine

GG films are brittle, and not easy to handle, which makes it very difficult to measure their mechanical properties. The addition of glycerine significantly reduces their Young's modulus, but increases the toughness and strain-at-break values (Figure 4.4 A and Table 4.1). For example, the tensile strength (TS) and Young's modulus (E) of GG15 films decreased by 20% (from 36 ± 14 MPa to 30 ± 1 MPa) and 280% (from 2620 ± 82 MPa to 690 ± 40 MPa), respectively; and toughness (T) and strain-at-break (γ) by 385% (from 0.33 ± 0.07 J g⁻¹ to 1.6 ± 0.2 J g⁻¹) and 300% (from $1.8 \pm 0.4\%$ to $7.8 \pm 0.4\%$), respectively, compared to GG70 films (Table 4.1).

Figure 4.4: Typical stress-strain curves; (A) gellan gum (GG) with 15% (GG15), 30% (GG30), 50% (GG50), 60% (GG60) and 70% (GG70) (by weight relative to GG) glycerine contents and (B) comparison of GG50 film and GG-composites films containing 20% (by weight relative to GG) of TiO₂ (GGTi20), Ag (GGA20) and ZnO (GGZn20) nanoparticles loading.

The mechanism of formation of the GG films is closely related to the conformational transition from coil-to-helix structure. In aqueous solution at high temperatures (~ 60 °C), gellan gum chains are in disordered single coil state. Upon cooling from 60 to 30 °C³⁴⁸, the gellan solution promotes the formation of double helices stabilised by internal hydrogen bonding²¹⁶. This facilitates the tight packing GG chains, resulting in brittle films. The addition of glycerine, which contains three hydroxyl groups per repeating unit promotes the formation of hydrogen bonds between GG-Gly and replaces the hydrogen bonds between GG chains. The GG-Gly bonds decrease the intermolecular forces along polymer chains, which decreased Young's modulus and improves the strain-at-break values³¹⁰.

Table 4.1: Mechanical properties of gellan gum film containing different percentages of glycerine. Thickness (Thick), tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) for the different GG materials are given.

Glycerine (%)	Thick (μm)	TS (MPa)	E (MPa)	T (J g^{-1})	γ (%)
0	30 ± 2	-	-	-	-
15	36 ± 2	36 ± 14	2620 ± 82	0.33 ± 0.07	1.8 ± 0.4
30	33 ± 4	40 ± 2	1657 ± 333	0.34 ± 0.04	3.6 ± 0.3
50	44 ± 3	41 ± 2	1097 ± 162	0.68 ± 0.1	5.4 ± 0.3
60	52 ± 4	33 ± 1	810 ± 172	1.9 ± 0.02	9.4 ± 0.5
70	54 ± 2	30 ± 1	690 ± 40	1.6 ± 0.2	7.8 ± 0.4

4.2.2 Effect of addition of nanoparticles

Addition of TiO_2 , ZnO and Ag nanoparticles improved the T and γ of GG50 films (Figure 4.4 B, Table 4.2). In contrast, the TS and E values were decreased. The T values increase for GG- TiO_2 composites with increasing nanoparticles, but decreases for GG-ZnO composites, while T has maximum at 20% (by weight relative to GG) for GG-Ag composites (Table 4.2). In contrast, the E values decreased for GG- TiO_2 and GG-Ag composites with increasing nanoparticles, and optimum at 20% (by weight relative to GG) for GG-ZnO composite.

Table 4.2: Properties of GG50 and GG-composite films. Thickness (Thick), tensile strength (TS), Young's modulus (E), toughness (T), strain-at-break (γ) and swelling for the different composite materials. All samples were prepared with 50% glycerine content by weight relative to gellan gum.

Samples	Thick	TS	E	T	γ	Swelling (%)	
	(μm)	(MPa)	(MPa)	(J g^{-1})	(%)	pH 1	pH 7
GG50	44 ± 2	42 ± 5	1097 ± 163	0.68 ± 0.1	5 ± 1	51 ± 1	311 ± 2
GGTi10	43 ± 6	27 ± 7	407 ± 90	1.22 ± 0.1	13 ± 1	77 ± 1	590 ± 37
GGTi20	63 ± 9	28 ± 3	237 ± 50	1.29 ± 0.4	18 ± 1	78 ± 1	490 ± 24
GGTi30	65 ± 9	27 ± 5	256 ± 54	1.76 ± 0.2	16 ± 1	70 ± 3	456 ± 6
GGZn10	59 ± 5	28 ± 5	256 ± 19	4.33 ± 0.6	20 ± 3	98 ± 5	574 ± 17
GGZn20	63 ± 4	31 ± 3	360 ± 43	3.34 ± 0.2	16 ± 2	95 ± 2	579 ± 48
GGZn30	57 ± 2	26 ± 4	264 ± 34	1.48 ± 0.5	13 ± 2	91 ± 5	571 ± 22
GGAg10	45 ± 2	26 ± 2	425 ± 50	1.63 ± 0.1	12 ± 2	80 ± 5	462 ± 10
GGAg20	62 ± 7	30 ± 4	315 ± 40	2.93 ± 0.4	16 ± 1	79 ± 2	451 ± 7
GGAg30	63 ± 1	24 ± 2	253 ± 25	1.06 ± 0.1	12 ± 1	64 ± 3	408 ± 5

The conformational transition of GG from aqueous solution at high temperature ($\sim 60\text{ }^{\circ}\text{C}$) to solid state ($\sim 30\text{ }^{\circ}\text{C}$), i.e. from disordered single coil state to double helix leads to the intercalation of TiO_2 , ZnO and Ag nanoparticles into the GG matrix (Figure 4.5).

Figure 4.5: (A) Illustration of the intercalation of nanoparticles into GG matrix, (B-D) SEM images of cross-section of GG-composite films; (B) GGTi20 composite, (C) GGAg20 composite, (D) GGZn20 composites, (E-G) SEM images of nanoparticles agglomeration; (E) GGTi20 composite, (F) GGAg20 composite, and (G) GGZn20 composite.

This phenomenon has been previously observed and is common in polymer/clay nanocomposites^{349, 350}. The intercalation process allows the GG-composite to restrain the strain in tensile testing and thus improved the T and γ of the GG-TiO₂, GG-ZnO and GG-Ag composite films compared to GG50 films³⁵¹ (Table 4.2). In contrast, this leads to less interaction between GG chains and therefore decreases in TS and E. The aggregation of nanoparticles within the GG matrix (Figure 4.5 E-G) could also contribute to the observed decrease in TS and improved γ ³⁵².

4.3 Water vapour transmission rates (WVTR)

The water vapour transmission rates (WVTR) of GG50 films were found to be $\sim 977 \text{ g m}^{-2} \text{ d}^{-1}$, which is within the range of an ideal wound dressing ($500\text{-}2000 \text{ g m}^{-2} \text{ d}^{-1}$) as suggested by Queen and co-workers³⁷ (Table 4.3). The WVTR values of GG50 films increased upon addition of 10% (by weight relative to GG) TiO₂, ZnO and Ag nanoparticles. For example, the WVTR values of GGTi10, GGZn10 and GGAg10 composites were $1094 \text{ g m}^{-2} \text{ d}^{-1}$, $1094 \text{ g m}^{-2} \text{ d}^{-1}$ and $1108 \text{ g m}^{-2} \text{ d}^{-1}$ compared to $977 \text{ g m}^{-2} \text{ d}^{-1}$ for GG 50 film. These WVTR values increased which can be expected based on the intercalation of nanoparticles in the GG matrix, lead to a less tight packing of GG chains (see Figure 4.5), which promotes water vapour transport rates. The WVTR values of GG-TiO₂, GG-ZnO and GG-Ag composites at 20% and 30% (by weight relative to GG) of nanoparticles loadings were between 1042 and $1168 \text{ g m}^{-2} \text{ d}^{-1}$ (Table 4.3). The WVTR values of these GG-composites were expected to be related to the distribution of nanoparticles in the GG matrix. Permeance (P) values of the GG50 and GG-composites films show the same observations as WVTR values (Table 4.3).

Table 4.3: Water vapour properties of GG50 and GG-composites films. Water vapour transmission rates (WVTR), water vapour permeability (WVP), and permeance (P) for the different composite materials are given.

Samples	Thickness	WVTR	WVP	P
	(μm)	($\text{g m}^{-2} \text{d}^{-1}$)	$10^{-14} \times (\text{kg Pa}^{-1} \text{m}^{-1} \text{s}^{-1})$	$10^{-9} \times (\text{kg Pa}^{-1} \text{m}^{-2} \text{s}^{-1})$
Blank	-	3685 ± 10	-	-
GG50	44 ± 3	977 ± 5	4.3 ± 3	0.96 ± 5
GGTi10	50 ± 5	1094 ± 6	5.4 ± 3	1.08 ± 6
GGTi20	68 ± 8	1042 ± 8	7.0 ± 7	1.03 ± 8
GGTi30	67 ± 8	1048 ± 1	6.9 ± 3	1.04 ± 5
GGZn10	55 ± 4	1094 ± 2	5.9 ± 1	1.08 ± 2
GGZn20	47 ± 3	1058 ± 8	5.6 ± 5	1.05 ± 8
GGZn30	60 ± 1	1168 ± 13	7.0 ± 6	1.16 ± 1
GGAg10	53 ± 2	1108 ± 10	5.8 ± 7	1.09 ± 1
GGAg20	64 ± 6	1117 ± 6	6.9 ± 5	1.10 ± 5
GGAg30	61 ± 1	1079 ± 4	6.5 ± 1	1.07 ± 4

4.4 Swelling

The swelling behaviour of the GG-composite films in buffer solutions at pH 1 and pH 7 is summarised in Table 4.2. GG50 films in a buffer of pH 1 exhibit low swelling at $51 \pm 1\%$ due to anionic property, and those in a buffer of pH 7 swell $311 \pm 2\%$. Swelling increased dramatically upon addition of nanoparticles. In a buffer of pH 1, GGTi10, GGAg10 and GGZn10 composite films swelled to about $77 \pm 1\%$, $98 \pm 5\%$ and $80 \pm 5\%$ respectively; and in a buffer of pH 7 at $590 \pm 37\%$, $574 \pm 17\%$ and $462 \pm 10\%$, respectively (Table 4.2). It is postulated that the increase of swelling upon inclusion of nanoparticles is due to the intercalation of nanoparticles in GG-matrix.

The intercalation leads to a less packing of GG chains which promotes the water absorption, and thus increased the swelling percentage.

4.5 Cell studies

4.5.1 Cell viability

4.5.1.1 Effect of glycerine

The effect of glycerine on L929 cell behaviour was investigated by morphological evaluation at time intervals of 24h, 72h, and 144h after seeding, as shown in Figure 4.6. The L929 cells were viable on the GG and GG50 films which indicate that both films support cell growth and therefore are unlikely to be cytotoxic.

4.5.1.2 Effect of nanoparticles

The effects of TiO₂, Ag and ZnO nanoparticles incorporated in GG50 films on the behaviour of L929 cells were investigated. Within 24h after cell seeding, the bright green fluorescent L929 cells were visible on the GGTi20 composite film (Figure 4.7 A) whereas lower numbers of viable cells were observed on GGAg20 and GGZn20 composites films (Figure 4.7 B and C). The lower number of L929 cells at 72h on the GGAg20 and GGZn20 composite films indicate that cells on these films had died (Figure 4.7 B and C). Within 72h, the viable cells number increased on the GGTi20 composite film (Figure 4.7 D).

The additions of these nanoparticles have been reported to have both beneficial^{244, 353} and deleterious^{270, 354} effects on cell behaviours. Most studies have reported that TiO₂, Ag and ZnO nanoparticles kill cells if they are in direct contact to the nanoparticle which happens, for example when the nanoparticles are added to a

medium containing cells^{269, 270, 354-356}. In contrast, the likelihood of nanoparticles killing cells is reduced by incorporating nanoparticles into a polymer matrix^{244, 263, 357}. Liu *et al.*²⁴⁴ reported that chitosan-Ag films crosslinked with genipin were compatible to L929 cells. Lactose-substituted chitosan-alginate-Ag hydrogels were found to be non-cytotoxic towards mouse-fibroblast-like (NIH-3T3), human hepatocarcinoma (HepG2), and human osteosarcoma (MG63) cells^{244, 263}.

Figure 4.6: L929 cells grown on GG (A-C) and GG50 (D-F) films at different time intervals; (A and D) 24h, (B and E) 72h, and (C and F) 144h. The cells were viable on both films which indicate the films support cell growth. Scale bars represent 50 μm .

Figure 4.7: Fluorescence microscope images of the cytotoxic effect of incorporated TiO₂, Ag and ZnO nanoparticles into the GG films after culture in the medium containing L929 cell lines for (A-C) 24h and (D-F) 72h; (A and D) GGTi20 composite, (B and E) GGAg20 composite, and (C and F) GGZn20 composite. Scale bars represent 50 μm.

An indirect contact assay was carried out to confirm the effect of TiO₂, Ag and ZnO nanoparticles incorporated GG matrix on the L929 cells. The GG-TiO₂, GG-Ag

and GG-ZnO composites films were incubated into wells containing L929 cell suspension for 24h. The films were removed and the remaining cells in the media were imaged using light microscopy.

Figure 4.8: Cytotoxicity of the media containing L929 cells incubated with GG-TiO₂, GG-Ag and GG-ZnO composites for 72h; (A) TCP (negative control), (B) GGTi20 (C) GAg20 and (D) GGZn20. Scale bars represent 50 μm.

The results show that the L929 cell morphology in the media after incubating with GG-Ag and GG-ZnO composites indicates that they are dying. This was expected due to leaking of Ag and ZnO nanoparticles into the media, which reduces the viability of L929 cells (Figure 4.8 C and D). In contrast, L929 cells incubated in the presence of CH-TiO₂ composite were adhering and elongating on the tissue culture polystyrene

plate (TCPP) (Figure 4.8 B). This suggests that the TiO_2 nanoparticles in the GG matrix are prevented from diffusion into the media.

The L929 cell viability for GGTi20 composites was investigated further after culturing for 48h and 144h. Figure 4.9 shows that viable L929 cells are distributed throughout the GGTi20 composite film, and some of them are clustered (Figure 4.9 D).

Figure 4.9: Representative fluorescent micrographs of live (green) surface of L929 cells in GGTi20 composite film after (A, B and C) 48h and (D, E, F) 144h of culture.

4.5.2 Cell proliferation

The L929 cell proliferation on GG, GG50, and GGTi20 composite films was evaluated using the MTS assay as shown in Figure 4.10 D. The L929 cells were seeded at 5000 cells/well into a 96 well-plate that contained films and incubated for 24h, 72h and 144h in culture media. Prior to addition of the MTS reagent, the supernatant in wells was removed to remove cells that were not attached on the films, and fresh culture media added.

Figure 4.10: (A-C) Schematic of the attachment and proliferation of L929 cells in response to the gellan gum surface after 24h, 72h and 144h following cell seeding and (D) the increase in attached L929 cell numbers after 24h, 72h and 144h of culture on GG, GG50 and GGTi20 composite films. Error bars represent standard errors of triplicate assays.

Within 24h period following cell seeding, the viable number of cells/well on GG, GG50 and GGTi20 composite films was approximately 300, 600 and 1600,

respectively. In comparison, the number of L929 cells on TCP was 9000 cells/well. Within 72h period following cell seeding on GG film, the number of L929 cells attached to the film increased to 1800 cells/well or a 3 fold increase compared to that after 24h. The same increase (3 fold) was observed on GGTi20 composite film which increased to 5000 cells/well by 72h. For GG50 films the attached cell number increased to 2700 cells/well, which is a 9 fold increase compared to 24h of culture. The number of attached cells climbed sharply after 144h of culture (Figure 4.10 D).

As illustrated in Figure 4.10 A, the L929 cell started to respond and adhere on the GG films' surface after incubation for 24h. The cell response to biomaterials depends on the physical property (hardness), hydrophilicity or hydrophobicity, and surface roughness of the materials^{196, 198, 328-330, 358-362}. For example, Hatano *et al.*³⁵⁹ found that surface roughness of modified polystyrene promotes the proliferation and gene expression levels of alkaline phosphatase (ALP) and osteocalcin of the calvarial cells which decreased at a higher roughness of polystyrene. A low viable cell number recorded on the GG50 film after 24h of culture at 300 cells/well could be due to the removal of unattached cells from the surface of the film while pipetting the supernatant to replace with fresh medium prior addition of MTS reagent. Once the cells were attached to the surface of the film after 24h of culture, the attached cells proliferated at approximately equal rates every 24h until 72h on the GG and GGTi20 composite films. Within 144h of culture, the cell numbers increased at faster rates compared to 72h of culture.

4.6 Antibacterial study

The antibacterial activity of GG50 film and GG-composite films was measured by qualitative (disk method) using the Gram-negative bacterium *E. coli*. The result shows

that GG50 film does not show any inhibition around the disk (Figure 4.11 A). Similar results were reported on konjac glucomannan/gellan gum film³⁶³. Interestingly on GG-composite films containing 10%, 20% and 30% (by weight relative to GG) of TiO₂, Ag and ZnO nanoparticles, no inhibition was found which could be due to the limited contact of nanoparticles have with the LB agar to kill the bacteria (Figure 4.11 B-D). This is supported by SEM imaging which showing a little of nanoparticles exposed on the surfaces' of GG-Ag composite, GG-TiO₂ composite and GG-ZnO composite films, thus reduce the effectiveness of nanoparticles to kill the *E. coli* (Figure 4.12).

Figure 4.11: (A-D) Antibacterial activity of the GG50 film and GG-composites films quantified by qualitative method after incubated at 37 °C for 24h; (A) GG50 film, (B) GGAg30 composite, (C) GGTi30 composite and (D) GGZn30 composite.

Figure 4.12: (A-C) SEM images of surfaces' of GG-composites; (A) GGAg20 composite, (B) GGTi20 composite and (C) GGZn20 composite. Scale bars represent 1 μm .

4.7 Conclusion

This study prepared gellan gum films and characterised their physical and biological properties. Circular dichroism spectroscopy confirmed the transition of GG from random coil (solution) to helix (film). The evaluation of the mechanical properties showed that inclusion of glycerine into the GG film improved the flexibility at a cost of reduced tensile strength and Young's modulus, which was attributed to changes in hydrogen bonding. Incorporation of TiO₂, Ag, and ZnO nanoparticles enhanced the toughness and strain-at-break of the GG50 films.

The water vapour transmission rates (WVTR) values were in the recommended range of an ideal wound dressing (977 – 1168 g m⁻² d⁻¹). The swelling of GG-composites decreased upon addition of higher nanoparticles loadings. *In-vitro* tests performed with mouse fibroblast (L929 cells) on GG-composite films revealed that the cells remain viable on GG-TiO₂ composite and most of the cells on Ag and ZnO containing films died due to leakage of nanoparticles into the cultured media. Increases in cell proliferation were reported on longer incubation of the GG, GG50 and GG-TiO₂ composite films (up to 144h). The antibacterial activity showed that none of the GG50 or GG-composite films was resistant against *E. coli*. In summary, it can be concluded that gellan gum containing TiO₂ nanoparticle films have generated promising results that can be applied in the development of wound dressing which is further investigate in chapter 7.

CHAPTER 5

CHARACTERISATION OF POLYELECTROLYTE COMPLEX FILMS FROM CHITOSAN AND GELLAN GUM

Many studies have been devoted to the preparation of wound dressing from polysaccharides based polyelectrolyte complex (PEC) techniques^{95, 310, 364-366}. Chitosan-alginate^{95, 295}, chitosan-carrageenan¹⁴⁹ and chitosan-heparin¹⁶⁰ are mixable by stirring and the resulting solution can be cast into films. In contrast, chitosan (CH) and gellan gum (GG) solutions are unable to be mixed together due to insoluble gellan gum in acidic medium. The mixing between CH and GG solutions results in the formation of a precipitate due to charge overcompensation at the interface in the form of fibres, capsules or coacervate structures depending on the complexation conditions^{162, 163, 167, 294}. There are no literature reports on the formation of CH-GG PEC-films. In this chapter, free standing CH and GG films are prepared by evaporative casting technique. PEC-films are formed by dipping free-standing films of either CH or GG into solutions of opposite charge. The effects of the order of addition and adjustment of pH of films and solutions on the mechanical properties, swelling and water vapour transmission rates (WVTR) are discussed.

5.1 Spectroscopy

5.1.1 UV-Visible

UV-visible spectra of CH, GG and PEC-films are shown in Figure 5.1. The CH and GG films show a peak at 200 nm and 250 nm corresponding to N-acetylglucosamine and the carboxylic group, respectively^{341, 367} (Figure 5.1 A). pH adjusted films using CH (pH=1.80) and GG (pH=12.00) solutions showed no changes were observed in peaks and absorbance intensities compared to unadjusted CH and GG films (Figure 5.1 B).

Figure 5.1: UV-visible absorbance spectra; (A) CH and GG films, (B) pH adjusted CH and GG films using CH (pH=1.80) and GG (pH=12.00) solutions and (C-D) polyelectrolyte complex (PEC)-films of CH and GG.

PEC-films reveal that the order of addition of $\text{GG}^{\text{into}}\text{CH}$ and $\text{CH}^{\text{into}}\text{GG}$ greatly affected the absorbance spectra regardless of pH modifications (Figure 5.1 C and D). For example, UV-Vis of $\text{GG}^{\text{into}}\text{CH}$, $\text{GG}^{\text{into}}\text{CH}(\text{pH})$, and $\text{GG}(\text{pH})^{\text{into}}\text{CH}$ PEC-films show two bands at 200 nm and 250 nm corresponding to CH and GG, respectively (Figure 5.1 C). In contrast, UV-Vis spectra of $\text{CH}^{\text{into}}\text{GG}$ and $\text{CH}(\text{pH})^{\text{into}}\text{GG}$ PEC-films revealed that the CH peak was dominant, but the GG peak had disappeared. The spectra for $\text{CH}^{\text{into}}\text{GG}(\text{pH})$ and $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ PEC-films showed that the CH peak was dominant with a low absorbance intensity of GG (Figure 5.1 D). All CH, GG and PEC-films either with the order of addition of $\text{CH}^{\text{into}}\text{GG}$ or $\text{GG}^{\text{into}}\text{CH}$ were transparent as evident from Figure 5.2 and 5.3.

Figure 5.2: (A-B) Free-standing films; (A) CH, (B) CH(pH), (C-F) PEC-films with the order of addition of $\text{CH}^{\text{into}}\text{GG}$; (C) $\text{CH}^{\text{into}}\text{GG}$, (D) $\text{CH}(\text{pH})^{\text{into}}\text{GG}$, (E) $\text{CH}^{\text{into}}\text{GG}(\text{pH})$ and (F) $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$. The naming of PEC-films as followed. For example, free-standing CH(pH) films dipped into GG solution is hereafter named as $\text{CH}(\text{pH})^{\text{into}}\text{GG}$.

Figure 5.3: (A-B) Free-standing films; (A) GG, (B) GG(pH), (C-F) PEC-films with the order of addition of GG^{into}CH; (C) GG^{into}CH, (D) GG(pH)^{into}CH, (E) GG^{into}CH(pH) and (F) GG(pH)^{into}CH(pH). The naming of PEC-films as followed. For example, free-standing GG(pH) films dipped into CH solution is hereafter named as GG(pH)^{into}CH.

5.2 Mechanical properties

5.2.1 Chitosan and gellan gum films

A comparison between CH and GG films revealed that CH films exhibit lower tensile strength (TS) and Young's modulus (E), but higher toughness (T) and strain-at-break (γ) compared to GG films (Figure 5.4 and Table 5.1). In other words, CH films are flexible, while GG films are brittle.

Figure 5.4: Stress-strain curves of films; (A) CH and CH(pH) and (B) GG and GG(pH).

5.2.2 pH adjusted chitosan and gellan gum films

It is assumed that changes in the pH of the solutions lead to changes in the ionic nature of the solutions. For example, most of the CH's amine groups will be protonated in a CH(pH) solution at pH ~ 1.8 , and most of the GG's carboxy groups will be deprotonated in a GG(pH) solution at pH ~ 12 . The effect of solution pH on the films' mechanical properties is profound. For example, films prepared using a CH(pH) solution at pH ~ 1.8 exhibit a 4.8-fold increase in E compared to films prepared using CH solutions with pH ~ 5.6 (Table 5.1). Smaller increases were observed for T (1.9 fold), TS (1.7 fold) and γ (1.4 fold). In contrast, films prepared using GG solutions at pH ~ 12 resulted in large increases in T (6.5 fold) and γ (2.8 fold), but at the cost of a reduced E (1.9 fold decrease) and TS (1.3 fold) compared to films prepared using GG solutions at pH ~ 5.4 . The increase in TS values for the CH materials are likely related to the observed increase in density, from $655 \pm 71 \text{ kg m}^{-3}$ to $1,372 \pm 40 \text{ kg m}^{-3}$ (CH(pH) film). The increased density would result in a closer packing of the biopolymers, which may inhibit sliding of the chains under stress in the

load direction³⁶⁸. The opposite occurs for the chains in GG films, with a decrease in both density and TS.

5.2.3 Polyelectrolyte complex-films

The mechanical characteristics of CH and GG films change as a result of the PEC process. Immersing CH films into GG solution ($\text{CH}^{\text{into}}\text{GG}$) resulted in an improvement of T, E and TS values coupled with a decreased in γ values compared to CH films (Figure 5.5). PEC-films formed through immersing of GG films into CH solution ($\text{GG}^{\text{into}}\text{CH}$) resulted in the opposite trends: γ improves, but E and TS values decreased compared to GG films.

Figure 5.5: Stress-strain curves of films; (A) CH and $\text{CH}^{\text{into}}\text{GG}$ and (B) GG and $\text{GG}^{\text{into}}\text{CH}$.

Changing the order of addition alters the biopolymer composition of the PEC-films (Table 5.1). For example, $\text{CH}^{\text{into}}\text{GG}$ exhibits higher CH:GG ratios compared to $\text{GG}^{\text{into}}\text{CH}$. The difference in the composition of PEC-films is reflected in the mechanical properties (Table 5.1).

Table 5.1: Summary of composition of the films determined using elemental analysis, CH:GG ratios, tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) values.

Film	C (at.-%)	H (at.-%)	N (at.-%)	CH (%)	GG (%)	CH:GG	TS (MPa)	E (MPa)	T (J g ⁻¹)	γ (%)
CH	0.75	0.12	0.13	100	0	-	15 ± 6	25 ± 7	1.27 ± 0.01	32 ± 2
CH ^{into} GG	0.78	0.13	0.09	60	40	1.5	12 ± 2	103 ± 4	2.72 ± 0.28	19 ± 1
CH ^{into} GG(pH)	0.80	0.14	0.06	32	68	0.47	26 ± 6	130 ± 34	7.20 ± 2.58	45 ± 5
CH(pH)	0.75	0.12	0.13	100	0	-	10 ± 1	120 ± 42	2.44 ± 0.21	45 ± 2
CH(pH) ^{into} GG	0.80	0.14	0.06	32	68	1.0	16 ± 1	322 ± 177	3.47 ± 1.07	20 ± 1
CH(pH) ^{into} GG(pH)	0.79	0.13	0.08	51	49	0.56	23 ± 4	202 ± 38	4.11 ± 1.36	26 ± 3
GG	0.87	0.13	0	0	100	-	42 ± 5	1097 ± 163	0.68 ± 0.03	5 ± 1
GG ^{into} CH	0.83	0.14	0.03	18	82	0.22	20 ± 8	282 ± 123	0.76 ± 0.31	12 ± 1
GG ^{into} CH(pH)	0.85	0.13	0.01	7	93	0.08	27 ± 2	1345 ± 120	0.81 ± 0.81	3 ± 1
GG(pH)	0.87	0.13	0	0	100	-	32 ± 8	576 ± 136	4.45 ± 1.19	15 ± 4
GG(pH) ^{into} CH	0.82	0.14	0.04	21	79	0.27	30 ± 5	329 ± 81	5.12 ± 0.12	22 ± 3
GG(pH) ^{into} CH(pH)	0.85	0.14	0.01	5	95	0.05	21 ± 3	170 ± 37	2.05 ± 1.37	19 ± 2

For example, changing the order of addition from CH^{into}GG to GG^{into}CH results in a 3.6-fold decrease in γ , i.e., from 2.72 J g⁻¹ to 0.76 J g⁻¹. A smaller 1.6 fold reduction was observed for γ , while E and TS values improved 1.7 and 2.7 times, respectively.

5.2.4 pH adjusted polyelectrolyte complex (PEC) films

Using CH(pH) and GG(pH) solution for either the formation of CH and GG films, or the immersion solutions, or both, resulted in improvements in the mechanical characteristics. TS and density values of PEC-films prepared by immersing CH films into GG solutions increase linearly (Figure 5.6 A and D) with increasing CH:GG ratio (Figure 5.6 D).

Figure 5.6: (A) Tensile strength, (B) toughness, (C) strain-at-break and (D) density as a function of CH:GG ratio for polyelectrolyte complex-films prepared by dipping CH into GG (spheres) and GG into CH (squares).

Although there is no linear relationship, it is clear that T and γ values increased (Figure 5.6 B and C) with increasing CH:GG ratio (Table 5.1). In contrast, it is not possible to observe any trends for PEC-films prepared by dipping GG films into CH solutions ($\text{GG}^{\text{into}}\text{CH}$). Overall, it appears that the highest mechanical values are observed for PEC-films with a CH:GG ratio of 0.47, i.e. for films containing twice as much GG as CH (Table 5.1).

5.3 Optical microscopy

A cross-sectional analysis of these films shows that GG appears to diffuse into CH films (Figure 5.7 B), but CH does not diffuse into GG film (Figure 5.7 D). Optical microscopy suggests that the $\text{GG}^{\text{into}}\text{CH}$ PEC-film consists of an inner GG layer and two outer CH layers.

Figure 5.7: Optical microscope images of cross-section of films; (A) CH, (B) $\text{CH}^{\text{into}}\text{GG}$, (C) GG and (D) $\text{GG}^{\text{into}}\text{CH}$.

It is well-known that the multilayer structure of PEC-films is affected by polymer charge density, polymer ionic strength, additive salt and type of polyion³⁶⁹. There are number of differences between the CH and GG films and the solutions used:

(i) CH has an intrinsic $pK = 6.5$ and requires acidic conditions to be soluble in water¹. GG has an intrinsic $pK = 3.5$ and is soluble in water³⁷⁰, (ii) The pH of CH films is always near neutral, regardless of the pH of the solutions. For example, CH solutions of $pH = 1.8$, $pH = 2.8$ and $pH = 5.4$ resulted in films with pH values of 6.9, 6.7 and 7.4, respectively. GG solutions of $pH = 5.6$ and 12.0 resulted in films with pH values of 4.7 and 9.0, respectively; (iii) Chain stiffness or the persistence length of GG is reported to be 9.4 nm at 40 °C³⁷¹. The temperature of GG solution during the formation is even higher (70 °C). Unlike CH, gellan gum chains converts from a double-helix structure to random coils upon heating, and will be in random coil conformation at 70 °C. The reported persistence length for CH (DD = 93%) is 81 nm at 25 °C³⁷². In other words, it is suggested that CH chains are less flexible compared to GG chains. Therefore, it is likely that the difference in PEC composition shown in Table 5.1 may be attributed to the difference in persistence length, as well as being driven by alkalinity and acidity of films and solutions. For example, immersing an acidic GG film ($pH = 4.7$) into an acidic CH solution ($pH = 1.8$, temperature = 25 °C) will impede diffusion of CH into GG due to electrostatic repulsion of the chains. This may result in the build-up of a separate CH layers on the GG film as shown in Figure 5.7 D. It is also possible that the small persistence length of GG in the 70 °C solution may aid the diffusion leading to the composition shown in Figure 5.7 B.

5.4 Water vapour transmission rates (WVTR)

The water vapour transmission rates (WVTR), water vapour permeability (WVP) and permeance (P) of the CH, GG and PEC-films are summarized in Table 5.2. GG films show higher values of WVTR compared to those observed for CH films. The PEC-films exhibited a narrow range of WVTR values, between 910-1056 $g\ m^{-2}\ d^{-1}$ that fall into the suggested range (500-2000 $g\ m^{-2}\ d^{-1}$) of providing adequate moisture that

prevents wound dehydration^{37, 373}. The ability of GG to diffuse into CH films ($\text{CH}^{\text{into}}\text{GG}$), but not vice versa, is reflected in the WVTR values. For example, $\text{CH}^{\text{into}}\text{GG}$ films exhibit higher WVTR ($925 \text{ g m}^{-2} \text{ d}^{-1}$) compared to $\text{GG}^{\text{into}}\text{CH}$ films ($910 \text{ g m}^{-2} \text{ d}^{-1}$). Higher WVTR values were also observed for $\text{CH}^{\text{into}}\text{GG}(\text{pH})$ ($974 \text{ g m}^{-2} \text{ d}^{-1}$) compared to $\text{GG}(\text{pH})^{\text{into}}\text{CH}$ ($932 \text{ g m}^{-2} \text{ d}^{-1}$). The lower WVTR values of the $\text{GG}^{\text{into}}\text{CH}$ films could be due to the layered structure, which may and slows the transport of water vapours through the GG layer and two outer CH layers (Figure 5.7 D). Compared to $\text{CH}^{\text{into}}\text{GG}$, no separation layer formed (Figure 5.7 B), thus the water vapours could transfer easily and recorded higher WVTR (Table 5.2).

Table 5.2: Water vapour transmission rates (WVTR), water vapour permeability (WVP), permeance (P) values, and percentage swelling upon immersion into buffer of pH 1 and pH 7 for 24 hours.

Film	WVTR	WVP	P	pH 1	pH 7	pH 12
	$(\text{g m}^{-2} \text{ d}^{-1})$	$10^{-14} \times (\text{kg pa}^{-1} \text{ m}^{-1} \text{ s}^{-1})$	$10^{-10} \times (\text{kg pa}^{-1} \text{ m}^{-2} \text{ s}^{-1})$	(%)	(%)	(%)
Blank	3685	-	-			
CH	918	9.09	9.09	101 ± 2	311 ± 2	96 ± 0.03
GG	977	4.25	9.67	51 ± 0.3	509 ± 13	^{a)}
$\text{CH}^{\text{into}}\text{GG}$	925	8.97	9.15	132 ± 7	224 ± 10	87 ± 4
$\text{GG}^{\text{into}}\text{CH}$	910	7.20	9.01	95 ± 1	493 ± 12	^{b)}
$\text{CH}^{\text{into}}\text{GG}(\text{pH})$	974	10.60	9.64	52 ± 19	317 ± 11	104 ± 37
$\text{GG}(\text{pH})^{\text{into}}\text{CH}$	932	9.86	9.23	115 ± 5	553 ± 2	66 ± 38

CH(pH) ^{into}	1041	10.70	10.30	83 ± 15	392 ± 17	41 ± 6
GG(pH)						
GG(pH) ^{into}	1056	8.78	10.50	145 ± 84	674 ± 22	53 ± 2
CH(pH)						

a) film dissolved, b) film split into separate layers

5.5 Swelling

The swelling of CH, GG and PEC-films in buffer solutions at pH 1 and pH 7 are summarised in Table 5.2. GG films absorb more water than CH films in buffer solution at pH 7, i.e. $509 \pm 13\%$ compared to $311 \pm 2\%$. It has been suggested that GG absorbs more water due to the higher number of hydroxyl groups compared to CH³⁷⁴. GG films were found to completely dissolve in buffer solutions of pH=12.

For PEC-films, the order of addition played a significant role in the swelling behaviour. For example, in buffer at pH 7, CH^{into}GG, CH^{into}GG(pH) and CH(pH)^{into}GG(pH) PEC-films were less susceptible to swelling compared to GG^{into}CH, GG(pH)^{into}CH and GG(pH)^{into}CH(pH), see Table 5.2. The larger amount of swelling observed for GG^{into}CH, GG(pH)^{into}CH and GG(pH)^{into}CH(pH) PEC-films may be attributed to the layered structure of these films. These films consist of a GG layer sandwiched between two outer layers of CH (Figure 5.7 D).

In buffer solution at pH 12, the GG^{into}CH PEC-film split into two layers. The GG layer in the middle of the film was dissolved and two layers remaining were CH layer which covered the GG film (Figure 5.7 D). Interestingly, the GG(pH)^{into}CH and

GG(pH)^{into}CH(pH) PEC-films did not exhibit separation of the layers. It is likely that this can be attributed an increase in the charge density of GG(pH) film, and thus improved electrostatic interaction with CH.

5.6 Conclusion

PEC-films were prepared by dipping free-standing films of GG into CH solution (GG^{into}CH) and CH into GG solution (CH^{into}GG). It was shown that the composition and mechanical characteristics depended on the solution and film pH, as well as the order of addition. The results indicate that toughness, tensile strength, strain-at-break and density values of PEC-films prepared by immersing CH films into GG solutions increased with an increasing CH:GG ratio. For example, tensile strength increased from 12 MPa to 26 MPa for an increase in CH:GG ratio from 0.47 to 1.50. In contrast, it was not possible to observe similar trends for PEC-films prepared by the reverse addition. It is suggested that the observed behaviour can be attributed to differences in their film compositions, i.e. GG^{into}CH films consist of an inner GG layer and two outer CH layers, whereas for CH^{into}GG films GG has diffused into the CH layer. The film composition; for example GG^{into}CH PEC-film also affected the WVTR and swelling. The GG^{into}CH film shows low amount of water vapours were able to diffuse into layered structure of the film, whereas absorbed higher swelling compared to CH^{into}GG PEC-film.

CHAPTER 6

MECHANICAL PROPERTIES OF POLYELECTROLYTE COMPLEX COATED WOOL FIBRES USING CHITOSAN AND GELLAN GUM

A polyelectrolyte complex (PEC) between chitosan (CH) and gellan gum (GG) on wool fibres was investigated. Pristine wool fibres were first coated in either CH or GG to modify surface charges. The single-coated wool fibres were then immersed in solution of opposite charge to form PEC-coated wool fibres. The effects of immersion duration, dyed, pH adjusted and order of addition of single-coated and PEC-coated wool fibres on the mechanical properties are discussed. Kubelka-Munk analysis was used to detect colorant changes in the PEC-coated wool fibres.

6.1 Mechanical properties and microscopy

6.1.1 Single-coated wool fibres

The mechanical properties of pristine wool fibres immersed in the CH solution (CH-fibre) and GG solution (GG-fibre) for 2, 5, 10, 20 and 30 min are shown in Figure 6.1 and Table 6.1. CH-fibre and GG-fibre results showed significant improvements in Young's modulus (E) values after 2 min of immersion; i.e. E values have improved more than doubled compared to pristine wool fibres (Figure 6.1 B, Table 6.1). Smaller

increases were observed for tensile strength (TS) and toughness (T) values, while strain-at-break (γ) values decreased.

Figure 6.1: (A) Tensile strength, (B) Young's modulus, (C) toughness (D) strain-at-break and (E) diameter of CH-fibre (triangles) and GG-fibre (squares) as function of immersion duration time (min).

The E values of CH-fibre increased up to 20 min of immersion. In contrast, the E values of GG-fibre only needed 5 min of immersion to reach their maximum value. The γ of CH-fibre and GG-fibre shows small changes after 5, 10, 20 and 30

min of immersions (Figure 6.1 D). The diameter for both CH-fibre and GG-fibre decreased (shrinkage) after 2 min of immersion and reached plateau values after 20 min and 5 min of immersions, respectively (Figure 6.1 E). The shrinkage observed for CH-fibre (20 min) and GG-fibre (5 min) is in agreement with the optimum E values for both fibres (Table 6.1).

Table 6.1: Mechanical properties of CH-fibre and GG-fibre at different immersion duration time. Tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) for different single-coated fibre are given.

Sample	Immersion (min)	Diameter (μm)	TS (MPa)	E (MPa)	T (J g^{-1})	γ (%)
Pristine wool fibre	-	850 ± 30	41 ± 1	270 ± 24	19 ± 11	64 ± 2
CH-fibre	2	930 ± 10	49 ± 4	662 ± 52	31 ± 8	47 ± 2
	10	880 ± 40	55 ± 5	1103 ± 117	30 ± 3	48 ± 2
	20	800 ± 30	65 ± 4	1447 ± 191	33 ± 1	50 ± 3
	30	760 ± 40	73 ± 7	1400 ± 250	27 ± 8	49 ± 6
GG-fibre	2	870 ± 70	48 ± 8	567 ± 181	32 ± 2	50 ± 1
	5	800 ± 70	61 ± 9	1001 ± 155	38 ± 1	54 ± 1
	10	780 ± 50	61 ± 7	932 ± 143	34 ± 4	54 ± 1
	20	770 ± 20	69 ± 7	1004 ± 56	36 ± 7	56 ± 1

Wool fibre has various functional groups such as hydroxyl, amino, imino and sulphhydryl^{375, 376}. These functional groups are expected to bind with the amine (NH_3^+) and hydroxyl groups (OH) of chitosan as well as with the hydroxyl (OH) and carboxyl groups (COOH) of gellan gum³⁷⁷. The E of CH-fibre were higher than that

of GG-fibre which may be attributed to stronger hydrogen bonding between functional groups of CH and the wool fibre^{378, 379}.

Figure 6.2: (A-C) Cross sectional area of single-coated fibres; (A) pristine wool fibre, (B) CH-fibre after 20 min of immersion, (C) GG-fibre after 5 min of immersion, (D-F) fractured topography (side view) of coated fibres from tensile testing; (D) pristine wool fibre, (E) CH-fibre after 20 min of immersion and (F) GG-fibre after 5 min of immersion.

A strong inter-fibre bonding is evident from the cross-section of the CH-fibre after 20 min of immersion (Figure 6.2 B) as well as the fracture topography after tensile testing (Figure 6.2 E). This is in support of the proposed argument for stronger hydrogen bonding in CH-fibres compared to GG-fibres. In contrast, GG-fibre shows the fibres are evenly bonded in the matrix after 5 min of immersion (Figure 6.2 C) but the number of fibres pulled out from the GG matrix (Figure 6.2 F) which resulted in higher strain-at-break values compared to CH-fibre (Table 6.1).

CH-fibre required a longer immersion (20 min) to reach optimum TS, E and T values, whereas GG-fibre reached optimum TS, E and T values after 5 min of immersion. The longer immersion time for CH-fibre is expected due to difference in persistence length between CH and GG. The persistence length of CH (degree of deacetylation, DD=93%) has been reported at 81 nm, meanwhile GG at 9.4 nm. In other words, it is suggested that GG chains are more flexible compared to CH chains and thus diffuse easily into wool fibre. In what follows, CH-fibre (20 min) and GG-fibre (5 min) are used in the preparation of dyed-fibre, pH adjusted fibre and PEC-coated fibres.

6.1.2 Dyed single-coated fibre

Adding a dye to CH solution significantly improved the TS and E values compared to pristine wool fibre and CH-fibre (Table 6.2). For example, both the TS and E values of CH-dye fibres improved to 2-folds of CH-fibre. GG-dyed fibre shows 1-fold improvement in TS compared to GG-fibre. The T of CH-dyed and GG-dyed fibres decreased, whereas γ does not significantly changes compared to fibres without dye modification (Table 6.2).

Table 6.2: Mechanical properties of dyed single-coated fibre (unless stated otherwise), pH adjusted single-coated fibre and dyed with pH adjusted polyelectrolyte complex (PEC)-coated fibres. CH-fibres and CH solutions were dyed with food colour (blue); and GG-fibres and GG solutions were dyed with yellow colour. Single-coated fibres immersed in CH and GG solution for 20 min and 5 min, respectively. For PEC-coated fibres, CH single-coated fibre immersed in GG solution for 20 min ($\text{CH}^{\text{into}}\text{GG}$) and GG single-coated fibre immersed in CH solution for 5 min ($\text{GG}^{\text{into}}\text{CH}$). Tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) of coated fibres are given.

Sample	Diameter (μm)	TS (MPa)	E (MPa)	T (J g^{-1})	γ (%)
Pristine wool fibre	850 ± 30	41 ± 1	270 ± 24	19 ± 11	64 ± 2
CH (without dye)	800 ± 30	65 ± 4	1447 ± 191	33 ± 1	50 ± 3
CH	620 ± 40	105 ± 9	2380 ± 275	28 ± 1.5	48 ± 2
GG (without dye)	800 ± 70	61 ± 9	1001 ± 155	38 ± 1	54 ± 1
GG	680 ± 50	72 ± 9	959 ± 103	31 ± 0.3	58 ± 2
CH(pH)	740 ± 60	82 ± 9	1577 ± 257	30 ± 1.4	52 ± 1
GG(pH)	660 ± 40	42 ± 4	814 ± 150	23 ± 2.0	63 ± 3
$\text{CH}^{\text{into}}\text{GG}$	620 ± 60	111 ± 14	2356 ± 108	26 ± 0.4	48 ± 2
$\text{GG}^{\text{into}}\text{CH}$	690 ± 50	92 ± 7	1290 ± 109	32 ± 0.6	58 ± 1
$\text{CH}^{\text{into}}\text{GG}(\text{pH})$	730 ± 40	43 ± 3	1065 ± 111	17 ± 0.6	57 ± 2
$\text{GG}^{\text{into}}\text{CH}(\text{pH})$	700 ± 60	69 ± 16	798 ± 140	36 ± 1.5	59 ± 2
$\text{CH}(\text{pH})^{\text{into}}\text{GG}$	790 ± 70	64 ± 3	949 ± 83	34 ± 0.9	58 ± 2
$\text{GG}(\text{pH})^{\text{into}}\text{CH}$	690 ± 30	63 ± 1	918 ± 66	28 ± 0.1	70 ± 5
$\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$	750 ± 60	36 ± 2	769 ± 60	20 ± 0.8	60 ± 2
$\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$	650 ± 30	60 ± 3	945 ± 155	26 ± 0.6	70 ± 1

Food colours (blue and yellow) have active ingredients such as concentrated dye (anthraquinone and triphenylmethane), citric acid, a synthetic colorant (E124,

Ponceau for blue and E102, Tartrazine for yellow) and preservative (E211, sodium benzoate). Citric acid may form H-bonds with hydroxyl groups and amino groups of CH as well as enabling ester crosslinking^{380, 381}. Anthraquinone and triphenylmethane compounds also expected to interact with hydroxyl groups and amino groups in CH³⁸² which reflected in improved TS and E values of CH-dyed fibre. On the other hand, GG-dyed fibre results showed that the TS improved which could be due to hydrogen bonding between carboxyl groups of GG with citric acid³⁸³.

6.1.3 pH adjusted single-coated fibre

Pristine wool fibres immersed in pH adjusted dyed solutions of CH (pH=1.8) and GG (pH=12), respectively showed that TS and E values decreased, while γ values increased compared to those samples prepared using dyed solutions without pH modification (Table 6.2). For example, the TS and E of CH-dyed (pH) fibres decreased to 82 ± 9 MPa and 1577 ± 257 MPa, respectively compared to CH-dyed fibre (TS= 105 ± 9 MPa and E= 2380 ± 275 MPa) (Table 6.2). In contrast, the toughness improved for CH-dyed (pH) fibre, but decreased for GG-dyed (pH) fibre compared to those prepared without pH modification.

6.1.4 Dyed and pH adjusted polyelectrolyte complex-coated fibre

The mechanical properties of dyed and pH adjusted polyelectrolyte complex (PEC)-coated fibres are summarised in Table 6.2. CH^{into}GG PEC-coated fibres exhibited an increase in TS values, but no significant change in E, T and γ values compared to single-coated CH-dyed fibres. Modification of the pH of CH^{into}GG PEC-coated fibre; i.e. fibre and/or solution (CH^{into}GG(pH), CH(pH)^{into}GG, and CH(pH)^{into}GG(pH)) resulted no mechanical reinforcement compared to single-coated CH-dyed and CH-dyed (pH) fibres. Those PEC-coated fibres (pH-modified) showed that the mechanical

characteristics are comparable to single-coated GG-dyed or GG-dyed (pH) fibres (Table 6.2). For example, the TS, E, T and γ values of CH^{into}GG(pH) PEC-coated fibre were 43 ± 3 MPa, 1065 ± 111 MPa, 17 ± 0.6 J g⁻¹ and $57 \pm 2\%$, respectively. In comparison, the TS, E, T and γ values of single-coated GG-dyed (pH) fibre were 42 ± 4 MPa, 814 ± 150 MPa, 23 ± 2 J g⁻¹ and $63 \pm 3\%$. The same observations were made for the CH(pH)^{into}GG and CH(pH)^{into}GG(pH) PEC-coated fibres (Table 6.2). These fibres exhibit mechanical characteristics comparable to those of single-coated GG-dyed and GG-dyed (pH) fibres, respectively.

These observations are supported by changes in color of the CH^{into}GG, CH^{into}GG(pH), CH(pH)^{into}GG, and CH(pH)^{into}GG(pH). For example, Figure 6.3 showed the colour changed in CH(pH)^{into}GG(pH). Initially, CH-dyed (pH) and GG-dyed (pH) (both fibres and solutions) are blue and yellow colors, respectively. By dipping CH-dyed (pH) fibres into GG-dyed (pH) solution, the color of this PEC-coated fibre altered to green after 0.5, 1 and 5 min of immersion (Figure 6.3 A). The green colour partially changed to yellow after 10 min of immersion and completely changed to yellow after 20 min immersion (Figure 6.3 A). These colour changes were also followed using Kubelka-munk analysis as detailed in experimental (chapter 2)³⁸⁴. The results showed that the K/S values of red are smaller than the green values after 0.5, 1 and 5 min of immersion, which is reflected in the change to green colour of pH-modified PEC-coated fibre (Figure 6.3 B).

Figure 6.3: (A) Colour transformation of $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ PEC-coated fibre at 0.5, 1, 5, 10, 15 and 20 min of immersions and (B) K/S values of red (◇) and green (□) channels as a function of duration of immersions.

In contrast, $\text{GG}^{\text{into}}\text{CH}$ PEC-coated fibre showed significant improvements in TS and E values compared to GG-dyed fibre (Table 6.2). pH modification of $\text{GG}^{\text{into}}\text{CH}$ (fibres and solutions) resulted in mechanical reinforcement of $\text{GG}^{\text{into}}\text{CH}(\text{pH})$, $\text{GG}(\text{pH})^{\text{into}}\text{CH}$ and $\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$ PEC-coated fibres compared to single-coated GG-dyed or GG-dyed (pH) fibres (Table 6.2). For example, the $\text{GG}(\text{pH})^{\text{into}}\text{CH}$ PEC-coated fibre exhibited the following characteristics, $\text{TS}=63 \pm 1$ MPa, $\text{E}=918 \pm \text{MPa}$, $\text{T}=28 \pm 0.1 \text{ J g}^{-1}$ and $\gamma=70 \pm 5\%$. For comparison, the TS, E, T and γ values of single-coated GG-dyed (pH) fibres were 42 ± 4 MPa, 814 ± 150 MPa, $23 \pm 2 \text{ J g}^{-1}$ and $63 \pm 1\%$, respectively. The same mechanical trends were observed for $\text{GG}^{\text{into}}\text{CH}(\text{pH})$ and $\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$ PEC-coated fibres compared to GG-dyed and GG-dyed (pH) fibres (Table 6.2). For colour changes, this order of addition resulted in PEC-coated fibres with a cross-section showing a green colour covering the outer layer and a yellow colour in the core of these fibres (Figure 6.4).

Figure 6.4: (A) Single-coated fibres GG-dyed fibres before and after pH modification, (B-D) PEC-coated fibres; (B) $\text{GG}^{\text{into}}\text{CH}$, (C) $\text{GG}^{\text{into}}\text{CH}(\text{pH})$ and (D) $\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$. Those PEC-coated fibres are covered with a green colour on the outer layer and yellow colour at core of each fibre.

Why the mechanical characteristics as well as colour of PEC-coated wool changed by reversing the order of addition, for example in $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ and $\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$. As explained in chapter 5 (polyelectrolyte complex-films), there are a number of differences between CH and GG materials which influenced those properties as followed. (1) CH has an intrinsic $\text{pK}=6.5$ ³⁸⁵ and GG has an intrinsic $\text{pK}=3.5$ ³⁸⁶ over a wide range of solution pH; (2) The pH of CH-dyed fibre are always near neutral, regardless of the pH of CH-dyed solution used to immerse the fibres. For example, CH-dyed solutions of $\text{pH}=1.76 \pm 0.04$ and $\text{pH}=5.54 \pm 0.04$ resulted in CH-dyed fibres with the pH values of 6.8 ± 0.05 and 7.3 ± 0.05 , respectively. GG-dyed solutions of $\text{pH}=5.57 \pm 0.04$ and 12.15 ± 0.12 resulted in GG-dyed fibres with pH values of 5.91 ± 0.09 and 8.95 ± 0.13 , respectively; and (3) Chain stiffness or the persistence length of CH (DD=93 %) is 81 nm at 25 °C³⁷², whereas GG is 9.4 nm at 40 °C³⁷¹. From the difference in pH of CH-dyed fibre and GG-dyed solution as well

as persistence length of CH and GG, it is likely that the mechanical characteristics of $\text{CH}^{\text{into}}\text{GG}(\text{pH})$, $\text{CH}(\text{pH})^{\text{into}}\text{GG}$, and $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ PEC-coated fibres are governed by diffusion of GG-dyed solution into CH-dyed fibre. This argument is in agreement with colour changes observed, for example in $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ PEC-coated fibres as shown in Figure 6.3 A. For this PEC-coated fibre, GG-dyed (pH) solutions are expected to have high degree of dissociation of COO^- ²²¹ ($\text{pH}=12.15 \pm 0.12$). Unlike GG-dyed (pH) solution, CH-dyed (pH) fibres are expected to have low degree of dissociation of NH_3^+ ³⁸⁷ ($\text{pH}= 6.8 \pm 0.05$). During short immersion times (0.5, 1 and 5 min), the COO^- groups of the GG-dyed (pH) solutions are expected to interact with NH_3^+ from CH-dyed (pH) fibre, and change to green colour (Figure 6.3 A). But, after a long immersion time (20 min), the charge density of CH-dyed (pH) fibre in GG-dyed (pH) solution is expected to reduce, which leaves the COO^- from GG-dyed (pH) solution is only in CH-dyed (pH) fibre. As a result, this interaction altered the colour of $\text{CH}(\text{pH})^{\text{into}}\text{GG}(\text{pH})$ PEC-coated fibre from green to yellow colour (Figure 6.3 A).

In contrast, CH is unable to penetrate into GG in $\text{GG}^{\text{into}}\text{CH}$, $\text{GG}^{\text{into}}\text{CH}(\text{pH})$ and $\text{GG}(\text{pH})^{\text{into}}\text{CH}(\text{pH})$ PEC-coated fibres as shown in Figure 6.4. This could be due to higher persistence length of CH (81 nm at 25 °C) ³⁷² compared GG (9.4 nm at 40 °C) ³⁷¹. However, the electrostatic interaction is expected to occur between CH-dyed solution (cationic) and GG-dyed fibre (anionic). This interaction reinforced those PEC-coated fibres which resulting in improved mechanical characteristics (Table 6.2). The electrostatic interaction is also in agreement with the observed colour change of those PEC-coated fibres to green colour in the outer layer of fibre, but maintained the yellow colour in the core of PEC-coated fibres (Figure 6.4).

6.2 Conclusion

In this chapter, single-coated and PEC-coated fibres using CH and GG were successfully developed. Pristine wool fibre immersed in CH and GG solutions showed optimum TS, E, T and γ values after immersion for 20 min and 5 min, respectively. CH-dyed and GG-dyed fibres showed that the mechanical characteristics were improved compared to without dye modification. In contrast, pH modified of CH-dyed and GG-dyed fibres decreased the mechanical characteristics. PEC-coated fibres were prepared by dipping CH-dyed fibres into GG-dyed solution (CH^{into}GG) and GG-dyed fibre into CH-dyed solution (GG^{into}CH). The order of addition was found to affect the mechanical characteristics of PEC-coated fibres; i.e. CH^{into}GG showed no mechanical reinforcement. In contrast GG^{into}CH showed mechanical reinforcements compared to single-coated CH-dyed and GG-dyed fibres, respectively. pH modification of these PEC-coated fibres showed similar mechanical behaviour. It is suggested that the difference in observed mechanical characteristics is due to the ability of GG-dyed solution to diffuse into CH-dyed fibre. This behaviour governed the mechanical characteristics of CH-dyed fibre, which is also evident from colour changes of this PEC-coated fibre. In contrast, CH-dyed solution was unable to diffuse into GG-dyed fibre. Electrostatic interaction was expected to occur on the outer layer of GG-dyed fibre (anionic) with CH-dyed solution (cationic) which resulted in improvement of mechanical characteristics. This is in agreement with a green colour observed on the outer layer of this PEC-coated fibre and a yellow colour in the core of the fibre.

CHAPTER 7

POLYELECTROLYTE COMPLEX FILMS CONSISTING OF ANTI-BACTERIAL AND CELL SUPPORTING LAYERS

This chapter discusses polyelectrolyte complex films (hereafter referred to as dual layer films) for wound dressing applications. The upper layer was designed to act as a bacterial resistance layer and the bottom layer to promote cell viability and proliferation. In chapters 3 and 4, the quantitative and qualitative of antibacterial properties of CH and GG-composites against *Escherichia coli* (*E. Coli*) showed that, both of those composites exhibit low antibacterial resistance. Therefore, in this chapter, levofloxacin, an antibiotic was incorporated into CH films (CH-Lev) to improve the biopolymer's antibacterial properties. GG-composites incorporated with titanium dioxide (GG-TiO₂) exhibit optimum cell viability and proliferation on mouse fibroblast cells (L929) (chapter 4), and thus these were used as bottom layer. The dual layer films were prepared by pouring a CH-Lev solution onto the surface of a GG-TiO₂ composite film in a mould. The mechanical characteristics, water vapour transmission rates (WVTR) and swelling of dual layer films and a commercial wound dressing (Cutifilm) were assessed. In addition, the cell viability and cell proliferation

of dual layer films tested using L929 cells and antibacterial activity of CH-Lev against *E. coli* are discussed.

7.1 Spectroscopy

7.1.1 UV-visible and circular dichroism

CH-Lev films exhibited specific characteristic absorption bands of levofloxacin in the ultraviolet region at 227 nm and 298 nm (Figure 7.1 A)^{279, 388-390}. The intensity of the CH-Lev films (at 298 nm) increased with increasing amounts of levofloxacin (Figure 7.1 A). Dual layer films consisting of CH-Lev01 (0.4% w/w of levofloxacin in CH film) and GGTi20 layers exhibit none of the characteristic absorption bands of levofloxacin, i.e. at 227 nm and 298 nm. It is likely that these bands are masked by the strong absorbance of GGTi20 composite in this wavelength region.

Figure 7.1: (A) UV-visible absorption of CH, CH-Lev01, CH-Lev1 and CH-Lev5 films, (B) UV-visible absorption of CH-Lev01 film and dual layer films (C) transmittance of the CH, CH-Lev01, CH-Lev1, and CH-Lev5 films and (D) circular dichroism spectra of CH film with different amounts of levofloxacin. All measurements were carried out at 21 °C using a 5 mm cuvette.

The transmittance of CH and CH-Lev01 films in the visible wavelength range (500-750 nm) is 80% (Figure 7.1 C and Figure 7.2 A). The transmittance of films with higher levofloxacin concentrations decreases sharply, i.e. the values for CH-Lev1 (4.0% w/w) and CH-Lev5 (16.0% w/w) films are 20% and 5%, respectively (Figure 7.1 C).

Figure 7.2: Photographs of films; (A) CH-Lev01, (B) CH-Lev1, (C) CH-Lev5, (D) Cutifilm™ and (E-G) dual layer films at different angles. The dual layer films were robust and flexible which could be easily folded and rolled-up without breaking. Scale bars represent 1 cm.

The effect of levofloxacin in CH films was measured by circular dichroism (CD) (Figure 7.1 D). Addition of levofloxacin to CH resulted in a shift of the CD band to a longer wavelength, i.e. from $\lambda=210$ nm to $\lambda=218$ nm, as well as an increase in the molar ellipticity. For example, the molar ellipticity of CH films is at ~5 mdeg and increased to ~40 mdeg for CH-Lev5 film. The observed changes CD band shift and intensity increase could be due to ionic interactions (NH_3^+ - COO^-) and hydrogen bonding (H-OH) between CH and levofloxacin^{391,345, 389}.

7.2 Mechanical properties and microscopy

7.2.1 Chitosan-levofloxacin films

The mechanical properties of CH film; i.e. tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) values are 21 ± 3 MPa, 657 ± 236 MPa, 5.32 ± 0.11 J g⁻¹, and $32 \pm 2\%$, respectively. The addition of levofloxacin (0.4% w/w in CH-Lev01 film) increased the TS and γ compared to those observed for CH film (Figure 7.3 A, 7.4, see also Table 7.1). However, addition of higher amounts of levofloxacin resulted in significant decreases in film toughness and ductility. For example, the T and γ values of CH-Lev5 films reduced by 67% and 60% compared to that of CH film, respectively. The decrease in mechanical characteristics (TS, E, T and γ) of the CH-Lev1 (4% w/w) and CH-Lev5 (16% w/w) films compared to the CH film may be attributed to disruption of CH intra-chain interactions as a result of intermolecular interactions between CH and levofloxacin (Table 7.1).

Figure 7.3: (A) Typical stress-strain curves of the CH-Lev01, CH-Lev1 and CH-Lev5 films, and (B) comparison stress-strain curves of the CH-Lev01 film, GGTi20 composite and dual layer films.

Figure 7.4: Representative of (A) tensile strength, (B) Young's modulus, (C) toughness and (D) strain-at-break of chitosan films as a function of levofloxacin contents (by weight relative to CH).

Table 7.1: Mechanical properties of CH film, CH-Lev01 film, CH-Lev1 film, CH-Lev5 film, GGTi20 composite, dual layer films and CutifilmTM. Thickness (Thick), tensile strength (TS), Young's modulus (E), toughness (T) and strain-at-break (γ) for the different materials are given.

Film	Levofloxacin (w/w,%)	Thick (μm)	TS (MPa)	E (MPa)	T (J g ⁻¹)	γ (%)
CH	-	96 $\pm$ 15	21 $\pm$ 3	657 $\pm$ 236	5.32 $\pm$ 0.11	32 $\pm$ 2
CH-Lev01	0.4	115 $\pm$ 7	25 $\pm$ 2	646 $\pm$ 143	4.84 $\pm$ 0.19	38 $\pm$ 2
CH-Lev1	4.0	99 $\pm$ 10	18 $\pm$ 3	534 $\pm$ 252	1.90 $\pm$ 0.11	16 $\pm$ 4
CH-Lev5	16.0	103 $\pm$ 10	12 $\pm$ 2	491 $\pm$ 92	1.71 $\pm$ 0.19	14 $\pm$ 3
GGTi20	-	63 $\pm$ 9	28 $\pm$ 3	237 $\pm$ 14	1.29 $\pm$ 0.4	18 $\pm$ 1
Dual layer	-	144 $\pm$ 2	35 $\pm$ 1	228 $\pm$ 25	3.61 $\pm$ 0.3	24 $\pm$ 1
Cutifilm TM	-	60 $\pm$ 4	6.5 $\pm$ 2	132 $\pm$ 19	40.0 $\pm$ 1.0	400 $\pm$ 30

7.2.2 Dual layer films

The mechanical robustness and flexibility of films is crucial in wound dressing applications. CH-Lev01 and GGTi20 composites were selected to form dual layer films as they offer the best combination of mechanical characteristics (Figure 7.3 B and Table 7.1). The dual layer films were mechanically robust and could be easily folded and rolled-up without breaking (Figure 7.2).

Table 7.1 shows that there is a synergistic effect between the layers as the mechanical characteristics are different from the values of the individual layers. For example, the TS value of the dual layer films is more than 20% higher compared to that of the individual CH-Lev01 and GGTi20 composite. In addition, the T and γ values of the dual layer films are in between the values of the individual layers. In

comparison, a commercial dressing, Cutifilm[™] showed low tensile strength (6.5 ± 2 MPa) and Young's modulus (132 ± 19 %), but extreme strain-at-break at $400 \pm 30\%$, which may be a result of the reinforcement effect of adhesive (use to stick the dressing to the wound) in the film (Table 7.1).

The cross sections of the dual layer films are shown in Figure 7.5. No layer separation between the CH-Lev01 and GGTi20 composite could be observed indicating perfect formation of the dual layer films. Aggregates of TiO₂ nanoparticles (~100 nm) throughout the GG matrix were clearly observed (Figure 7.5 C and D).

Figure 7.5: (A) Optical imaging of cross-section of dual layer films (upper layer is CH-Lev01, bottom layer is GGTi20 composite), (B) field emission-SEM (FE-SEM) imaging of a freeze-fractured dual layer films, (C) enlargement of finely dispersed titanium dioxide (TiO₂) nanoparticles throughout the GG matrix and (D) aggregation of TiO₂ nanoparticles in the GG matrix.

7.3 Swelling

The swelling of a wound dressing reflects its capability to absorb body fluid (exudates)³⁹². GGTi20 composites are able to absorb more water than CH-Lev01 films in buffer solution at pH 7, i.e. $490 \pm 24\%$ compared to $73 \pm 1\%$, respectively (Table 7.2). It has been suggested that gellan gum can absorb more water compared to chitosan due to a higher number of the hydroxyl groups³⁷⁴. Furthermore, the presence of 20% (by weight relative to GG) TiO₂ nanoparticles leads to a less tight packing of GG chains, which promotes swelling. Combining these films into a dual layer films results a similar swelling behaviour in buffer solution at pH 7 ($430 \pm 89\%$). As a comparison to dual layer films, CutifilmTM, which is designed as waterproof dressing showed a small amount of swelling in buffer solution at pH 7 ($10 \pm 4\%$).

Table 7.2: Properties of films. Thickness (Thick), water vapours transmission rates (WVTR), water vapour permeability (WVP), permeance (P) and swelling for the different materials are given.

Samples	Thick (μm)	WVTR ($\text{g m}^{-2} \text{d}^{-1}$)	WVP $10^{-14} \times (\text{kg Pa}^{-1} \text{m}^{-1} \text{s}^{-1})$	P $10^{-10} \times (\text{kg Pa}^{-1} \text{m}^{-2} \text{s}^{-1})$	Swelling (%)		
					pH 1	pH 7	pH 12
Blank	-	3685	-	-	-	-	-
CH-Lev01	105 ± 7	866	9.0	8.6	108 ± 1	73 ± 1	96 ± 2
GGTi20	63 ± 9	1042	7.0	10.3	78 ± 0.1	490 ± 24	a)
Double layer films	148 ± 8	853	12.4	8.4	91 ± 2	430 ± 89	b)
Cutifilm TM	60 ± 4	197	1.17	1.95	5 ± 2	10 ± 4	3 ± 3

a) film dissolved; b) film split to separate layers

7.4 Water vapour transmission rates (WVTR)

The water vapour transmission rates (WVTR) of GGTi20 composites are higher compared to that of CH-Lev01 films, i.e. $1042 \text{ g m}^{-2} \text{ d}^{-1}$ compared to $866 \text{ g m}^{-2} \text{ d}^{-1}$, respectively (Table 7.2). Combining these films into a dual layer films results in a WVTR value of $853 \text{ g m}^{-2} \text{ d}^{-1}$ which is within the recommend range ($500\text{-}2000 \text{ g m}^{-2} \text{ d}^{-1}$) for wound dressings³⁷. The WVTR value of the dual layer films is comparable to reported WVTR values for commercial wound dressing such as, for example CutifilmTM ($197 \text{ g m}^{-2} \text{ d}^{-1}$), Tegaderm ($491 \pm 44 \text{ g m}^{-2} \text{ d}^{-1}$)³⁷, Bioclusive ($382 \pm 26 \text{ g m}^{-2} \text{ d}^{-1}$)³⁷, Duoderm ($886 \pm 60 \text{ g m}^{-2} \text{ d}^{-1}$)³⁷³ and Instrasite ($354 \pm 42 \text{ g m}^{-2} \text{ d}^{-1}$)³⁷³.

7.5 Cell studies

7.5.1 Cell viability and proliferation

The effect of levofloxacin in CH films (0.4% w/w relative to CH) on L929 cells behaviour was investigated by morphological evaluation at time intervals of 24h, 72h and 144h, as shown in Figure 7.6. The L929 cells were viable on the surface of CH-Lev01 films. The increase in cell density between 24h and 144h indicates that CH-Lev01 films supported cell growth, and are therefore unlikely to be cytotoxic. These results are in agreement with previous studies showing that levofloxacin had a relatively mild detrimental effect on cells^{289,290}.

The cell viability of cells on dual layer films was also investigated. Due to the non-transparency of GGTi20 composite (chapter 4), the films were stained with calcein-AM to confirm cell viability. Fluorescent microscopy images showed that 72h after cell seeding bright green fluorescent L929 cells were clearly visible on both sides of dual layer films, i.e. on the surface of the CH-Lev01 and GGTi20 layers (Figure 7.7).

Figure 7.6: Fluorescence microscope images of L929 cells on the CH-Lev01 film after (A) 24h, (B) 72h and (C) 144h. Scale bars indicate 20 μm .

Figure 7.7: Fluorescence microscope images of L929 cells on the (A) CH-Lev01 and (B) GGTi20 surfaces of a dual layer film after incubating for 72h. Scale bar represents 20 μm .

Cell proliferation on dual layer films was evaluated using the MTS assay. The proliferation studies revealed that cell numbers on the GGTi20 surface of dual layer films after 72 and 144h were 5300 and 23000 cells/well, respectively. This suggests that the release of levofloxacin does not adversely affect cell viability and proliferation on dual layer films.

Several studies have investigated the cell behaviour on commercial wound dressings^{56, 393-395}. Phan *et al.*³⁹⁵ reported that keratinocyte cells in TegadermTM grew at a slow rate even after being cultured for 120h. In another report, Draye *et al.*⁵⁶ studied the cytotoxicities with MTT assay of semi-occlusive polyurethane (PU) dressings (Tegaderm and Opsite) and a hydrocolloid dressing (DuoDERM) on epidermal keratinocytes. The results showed that after incubation for 144h, the keratinocyte cell survival increased on Opsite, decreased on Duoderm, while cell survival rates were retained on Tegaderm.

7.6 Antibacterial activities

Antibacterial activities of CH-Lev films were quantified by qualitative and *in vitro* quantitative methods against Gram-negative bacteria (*E. coli*). The qualitative studies showed that the inhibition zones around the CH-Lev disks increased with increasing amount of levofloxacin (Figure 7.8 and 7.9 A). For example, the inhibition zone of a CH-Lev5 film was 1.7 times larger than that of a CH-Lev01 film.

Figure 7.8: Photograph of qualitative antimicrobial test of (A) CH-Lev01, (B) CH-Lev1 and (C) CH-Lev5 films against *E. coli*. The inhibition zone is indicated by the arrow. Scale bars indicate 6 mm.

Figure 7.9 : (A) Inhibition zones for CH-Lev films against *E. coli* after 24h of incubation and (B) Effectiveness expressed in a viable colony counts (CFU/mL) for CH-Lev01, CH and control (innoculum without film) against *E. coli* after 1h. “0 h” indicates the number of colonies in the inoculums.

In the quantitative tests, the antibacterial effectiveness of CH and CH-Lev01 films was evaluated using a viable cell count method of the Gram-negative bacterium *E. coli*. Release studies showed that virtually all the levofloxacin is released from the CH-Lev film within 30 min in PBS at 37 °C (Figure 7.10 A and B). The diffusion constant for levofloxacin release from the film was obtained by fitting the loss data to the expected profile for Fickian diffusion from a thin film³⁰⁰. The molar absorption coefficient obtained from Figure 7.10 C was used to convert the data from Figure 7.10 B into Figure 7.10 D curve. Figure 7.10 D shows that this loss profile fits a diffusion coefficient $1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. This rapid release of levofloxacin is responsible for the observed effectiveness in reducing bacterial growth. For example, after 1h incubation only a small amount of bacterial colonies were surviving on the CH-Lev01 films, indicating a 7-8 log cycle reduction compared with the control (Figure 7.9 B). In contrast, CH films demonstrated a bacteriostatic effect against *E. coli*, which is consistent with previous observations⁷⁹.

Figure 7.10: (A) UV-vis spectra of PBS solution at 37 °C at increasing CH-Lev01 film immersion time, (B) absorbance of levofloxacin from a CH-Lev01 film at 287 nm versus immersion time, (C) absorbance of levofloxacin from a CH-Lev01 film at 287 nm versus levofloxacin concentration, and (D) loss of levofloxacin from a CH-Lev01 film (squares) compared with theoretical prediction of calculated loss assuming diffusion coefficient of $1.0 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ (solid line).

The strong antibacterial activity of levofloxacin against *E. coli* has been reported in many studies through inhibition replication and transcription of bacteria DNA^{283,291-293,396}. For example, Fu *et al.*²⁹¹ measured the antibacterial activity of levofloxacin, ofloxacin, ciprofloxacin and norfloxacin against number of organisms such as *Staphylococcus aureus* (*S. aureus*), *Xanthomonas malthophuia* (*X. malthophuia*), *Escherichia coli* (*E. coli*), *Bacteroides fragilis* (*B. fragilis*) and methicillin-resistant *Staphylococcus aureus* (MRSA). Results showed that levofloxacin is more active than ciprofloxacin and other fluoro-quinolones against *S. aureus* including MRSA, *X. malthophuia* and *B. fragilis*. Furthermore, *in-vivo* results

indicated that levofloxacin was more active than ciprofloxacin against selected Gram-negative organisms due to greater tissue penetration.

Holland and co-workers³⁹³ reported the antibacterial activities of commercial wound dressings (Opsite and Tegaderm) against *Escherichia coli* (*E. coli*), *staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*) and *Pseudomonas aeruginos* (*P. aeruginos*). After 2h of incubation against *E. coli*, Opsite and Tegaderm recorded significant reduction in viable bacterial colonies. It is suggested that the anti-bacterial activity of the CH-lev01 films is comparable to that of commercially available materials.

7.7 Conclusion

The dual layer films were prepared by pouring a CH-Lev01 solution onto the surface of a GGTi20 composite. The choice of materials to form the dual layer films was justified as follows. The addition of small amounts of levofloxacin (0.4% w/w) did not significantly affect the mechanical characteristics of CH films. Release studies show that the antibacterial effectiveness of the CH-Lev01 results from the fast release of levofloxacin. Addition of titanium dioxide (TiO₂) nanoparticles enhances the ductility of GG resulting in tougher materials, coupled with a loss in Young's modulus and tensile strength. Furthermore, GG films incorporate TiO₂ supported the growth of viable L929 cells. It was evident that CH-Lev01 and GGTi20 films offered the best combination of mechanical and bio-characteristics and these were combined to form dual-layered films. These dual layer films exhibit anti-bacterial activity on one side, while supporting the growth of L929 cells on the other side. This chapter contributes towards the development of PEC materials for future wound dressing applications.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 General conclusion

This work successfully explored the formation of wound dressing through a polyelectrolyte complex using chitosan (CH) and gellan gum (GG), i.e. polyelectrolyte complex (PEC) films (PEC-film) and PEC-coated wool fibres. PEC-films and PEC-coated wool fibres were prepared by dipping free-standing CH and GG films (chapters 3-4, aim 1), and CH and GG-coated fibres into oppositely charged biopolymer solutions (chapters 5-6, aim 2), respectively. The results showed that GG solutions were able to diffuse into CH materials. In contrast, CH solutions were unable to diffuse into GG materials resulting in a layered structure, i.e. an inner GG layer and two outer CH layers. The formation of this layered structure was applied in the development of PEC-films (hereafter referred as dual layer films) with an eye towards future wound dressing applications (chapter 7, aim 3). The dual layer films consisted of two layers. The upper layer was designed to act as a bacterial resistance layer to minimise the infection in the wound, while the bottom layer was to promote cell viability and proliferation.

CH films were incorporated with TiO₂ and Ag nanoparticles (chapter 3, aim 1). *In-vitro* tests performed using mouse fibroblasts cells (L929) found that the CH-TiO₂ composite were not cytotoxic, while CH-Ag composite were toxic to these cells. Proliferation of L929 cells on CH-TiO₂ composites did not show improvement compared to CH films. Qualitative antibacterial studies showed that the inhibition zones around CH-Ag (composite and dispersion) against *Escherichia coli* (*E. coli*) were superior compared to CH-Ti. Quantitative antibacterial tests revealed similar numbers of viable cell colonies of *E. coli* on both composites. Due to the low antibacterial resistance of these composites (CH-Ag and CH-TiO₂), an antibiotic, levofloxacin was incorporated in CH films (chapter 7). Qualitative results indicated that even the lowest levofloxacin concentration (0.4% w/w in CH-Lev01 film) exhibited film inhibition zones which were 4-fold larger compared to those observed for the highest nanoparticle loading (30% by weight relative to CH) in CH-Ag composites. In addition, the viable cell count method for CH-Lev01 films showed that only a small amount of bacterial colonies survived after 1h incubation, i.e. a 7-8 log cycle reduction compared to the inoculum without films. The rapid antibacterial response to the gram-negative bacterium, *E. coli* colonies was attributed to fast release (within 30 min) of nearly all of the antibiotic from the film into the solution.

GG films were incorporated with TiO₂, Ag and ZnO nanoparticles (chapter 4, aim 1). *In-vitro* tests, GG-TiO₂, GG-Ag and GG-ZnO composites revealed that the L929 cells remain viable on GG-TiO₂ composite. Most of the cells on Ag and ZnO containing composites died due to the leakage of nanoparticles into the culture media. CH-Ti composites showed significant increases in cell proliferation, i.e. about 50% compared to GG film after incubation for 144 h. The antibacterial activity of the GG-

TiO₂, GG-Ag and GG-ZnO composites quantified by inhibition method showed that none of the films was resistant against *E. coli*.

The mechanical characteristics and composition of PEC-films depended on the solution and film pH, as well as the order of addition (chapter 5, aim 2). The results indicated that toughness, tensile strength and strain-at-break of PEC-films of biopolymers prepared by immersing CH films into GG solutions (CH^{into}GG) increased with increasing CH:GG ratio. In contrast, it was not possible to observe similar trends in the mechanical characteristics for PEC-films prepared by the reverse addition (GG^{into}CH). It is suggested that the observed behaviour (mechanical characteristics) can be attributed to differences in their film composition. GG^{into}CH films were found to consist of an inner GG layer and two outer CH layers, whereas for CH^{into}GG films, GG diffuses into the CH layer. The diffusion of GG solution into CH layer was related to differences in pH and persistence length of GG and CH.

Similar mechanical characteristics were observed for PEC-coated wool fibres (chapter 6, aim 2). PEC-coated wool fibres prepared by dipping CH-dyed fibres into GG-dyed solutions (CH^{into}GG), and GG-dyed fibres into CH-dyed solutions (GG^{into}CH) showed that the order of addition influences the mechanical behaviour. For example, fibres prepared using CH^{into}GG showed no mechanical reinforcement compared to single-coated (CH-dyed) fibres. In contrast, GG^{into}CH fibre exhibited improved mechanical characteristics compared to single-coated GG-dyed fibres. pH modification of these PEC-coated wool fibres resulted in similar observations. It is suggested that the different mechanical characteristics observed for CH^{into}GG fibres were due to the ability of the GG-dyed solution to diffuse into CH-dyed fibre. In

contrast, CH-dyed solution was unable to diffuse into GG-dyed fibre (GG^{into}CH). These hypotheses are supported by colour changes in PEC-coated wool fibres.

An ideal wound dressing should be capable of being resistance to bacteria and non-toxic to cells. In addition, it should offer an adequate mechanical toughness and be permeable to water vapour. In the development of dual layer films (chapter 7, aim 3), the choice of materials was justified as follows. CH-Lev01 films were used as the upper layer as it displayed promising bacterial resistance against *E. coli*, compared to CH-Ag composites. GG-TiO₂ composite films were chosen as the bottom layer as it exhibited the best cell viability and cell proliferation, compared to CH and GG films. The dual layer films, (prepared by pouring a CH-Lev01 solution onto the surface of a GG-TiO₂ composite) were mechanically robust and could be easily folded and rolled up without breaking (chapter 7). Combining these two layers resulted in a synergistic effect on the mechanical characteristics, i.e. the tensile strength values of the dual layer films are > 20% higher compared to either the CH-Lev01 or the GG-TiO₂ layers. The toughness and strain-at-break values of the dual layer films are in between those of the values observed for the individual layers. The water vapour transmission rates (WVTR) of the dual layer films are 853 g m⁻² d⁻¹, which is within the recommended range (500-2000 g m⁻² d⁻¹) for wound dressings⁵². Furthermore, the WVTR value of dual layer films is comparable to WVTR values reported for commercial wound dressing such as, Cutifilm, Tegaderm, Bioclusive, Duoderm and Intrasite^{37, 373}. It was evident that combining CH-Lev01 and GG-TiO₂ composite into dual layer films offered the best combination of mechanical and bio-characteristics (chapter 7). In particular, the dual layer films exhibit antibacterial activity on one side, while supporting the growth of L929 cells on the other side. As

such, it should be clear that this thesis contributes to the development of PEC materials for future wound dressing applications.

8.2 Future recommendations

The release of levofloxacin in CH-Lev films discussed in this thesis is a good indicator that the dual layer films (consisting of CH-Lev01 and GG-TiO₂ composite) could be suitable as drug release materials. Drug release studies involving polyelectrolyte complexes from chitosan, poly (dimethyldiallyl ammonium chloride), carboxymethyl cellulose sodium, dextran sulphate, alginate, and poly(styrenesulfonate) have been reported^{397, 398}. These studies investigated the release of ibuprofen^{397, 399}, ketoprofen and cytochalasin D⁴⁰⁰, which could be incorporated into CH film and provide pain relief^{401, 402}.

The use of polyelectrolyte films from CH and GG as implanted materials is another interesting prospect. For example, levofloxacin could be used to provide an antibacterial barrier²⁷⁸ without compromising the ability to support cell growth. It has already been shown that multilayer polyelectrolyte films incorporated with defensin can be used to combat bacterial colonisation on implanted materials⁴⁰³. Bacterial-resistance against penicillin such as *Staphylococcus aureus* (*S. aureus*) and methicillin (known as methicillin-resistant *Staphylococcus aureus*, MRSA) is causing major problems in hospitals⁴⁰⁴. *S. aureus* can spread easily, especially in hospitals, through contact with pus from an infected wound, skin-to-skin contact with infected person, or contact with objects such as towels, sheets or clothing used by an infected person^{405, 406}. The incorporation of antibiotics such as levofloxacin into PEC-coated wool fibres should be investigated to fight those anti-biotic resistant bacteria and help to stop the spreading of MRSA in hospitals.

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Khairul Anuar Mat Amin and Marc in het Panhuis. Polyelectrolyte complex materials from chitosan and gellan gum. *Carbohydrate Polymers* 2011; **86**: 352-358.

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