Developing hybrid fibres for local protein delivery

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Recommended Citation
Abstract

Proteins have attracted considerable attention in the treatment of various chronic diseases due to their high potency and specificity. However, physiochemical properties of proteins and complex physiology of the non-invasive routes pose significant challenges for site specific delivery of these macromolecules. Various polymeric delivery systems have been developed to include bioactive cues by encapsulating proteins into the structure to form a protein-polymer composite system. Developmental work in the field of protein delivery structures has revealed a number of characteristics that promise great benefits to their possible use in a broad range of devices and applications including tissue scaffolds. There has, therefore, been much interest and many attempts to produce various structures with the ability to release the proteins in a sustained approach. The main aim of this thesis is to establish fabrication processes to develop three-dimensional structures encapsulating protein with a sustained release profile.

In systemic delivery of proteins, biodegradable microspheres as parenteral depot formulation occupy an important place because of several aspects like protection of sensitive proteins from degradation, prolonged or modified release, pulsatile release patterns. In this study, two types of protein-loaded microspheres composed of alginate (Alg) and poly(lactic-co-glycolic acid) (PLGA) were prepared (Chapter 2). Emulsion and double-emulsion fabrication methods were applied to produce Alg and PLGA microspheres, respectively and have been systematically characterized to show their ability to control the release profiles of a small protein Fluorescein isothiocyanate-bovine serum albumin (FITC-BSA). The mean sizes of the FITC-BSA loaded Alg and PLGA microspheres, loaded with low (type α) and high (type β) levels of protein, were 11, 26 and 12 micrometres, respectively. FITC-BSA releases from all types of microspheres showed the classic biphasic profile, which were governed by resolving or degradation (diffusion and polymer erosion) reasons for Alg and PLGA microspheres, respectively. In short, PLGA microspheres loaded with higher levels of protein provided an efficient system to achieve almost linearly controlled protein release at a rate suitable for further applications.

In addition, wet-spun microfibres have gained considerable interest as scaffolding substrates over the past decade. From a protein delivery perspective, wet-spinning is most similar to conventional microspheres-based protein encapsulation techniques and avoids the potential for thermal denaturation.
of therapeutics, unlike melt spinning and dry spinning. In addition, the high surface area-to-volume ratios of fibres help them to provide local and sustained delivery of proteins to the site of injury. With the aim of developing structures for long-term local delivery of proteins, a two-phase hybrid structure was devised by incorporation of protein-loaded microspheres into polymeric wet-spun fibrous structures. These hybrid fibres not only possess a long-term protein release profile, but also act as a promising candidate choice for longitudinal protein release applications. The structure was optimized to achieve a uniform fibre with sustained release profile (Chapter 3). The fibres were further morphologically, chemically, mechanically and thermally studied. The two-phase delivery matrices display retarded FITC-BSA release significantly in both initial and late stages compared to release from the PLGA microspheres or alginate fibre alone. It was found that fibres fabricated from Alg material (low viscosity type) with higher concentration, compared with Alg (medium viscosity type) with lower concentrations, can provide a lower level of burst release as well as a slower release profile over the observation time.

In recent years, there have been some attempts to develop concentration gradient structures with the ability of distributing proteins in a spatially graded manner. Despite all the efforts, there is still a challenge to develop a gradient structure which can be easily fabricated and programmed in terms of protein concentration and also be able to present a long-term release profile. As a result of this research, the production of protein-loaded microspheres concentration gradient fibre has been successfully achieved. This research presents a simple technique to develop programmable structures that have good potential for promotion and direction of guided tissue regeneration in “linear” systems such as muscle and nerve. Since in this study, Fluorescein isothiocyanate- bovine serum albumin was applied as a model protein which can be detected easily, the further studies can be done by optimizing the presented fabrication techniques by loading the growth factors.
Summary of Content

Delivery of bioactive molecules particularly proteins into the site of action is challenging, mainly because of their inherent instability as well as physiological barriers. This thesis aims to develop novel protein-friendly structured protein delivery systems for local treatments in tissue systems. Chapter 1 presents the general introduction and related literature review of this research field.

As a first step towards this aim, (Chapter 2), protein-loaded microspheres composed of alginate (Alg) and poly(lactic-co-glycolic acid) (PLGA) were considered. Emulsion and double-emulsion fabrication methods were applied to produce Alg and PLGA microspheres, respectively and have been systematically characterized to show their ability to control the release profiles of a model protein Fluorescein isothiocyanate-bovine serum albumin (FITC-BSA). The resulting FITC-BSA loaded Alg and PLGA microspheres displayed uniform morphology with size ranging from zero to 100 micrometres, and high efficiency protein encapsulation. The FITC-BSA loaded PLGA microspheres displayed a long lasting and linear release profile compared with protein loaded Alg microspheres. Therefore, FITC-BSA loaded PLGA microspheres were selected to be applied for further studies.

Subsequently, a composite delivery system was designed and implemented by incorporating the protein-loaded PLGA microspheres incorporated into Alg wet-spun microfibres to achieve a two-component sustained release system (Chapter 3). Through chapter 3, the effects of the spinning dopes properties and spinning operational parameters on final properties of the structures were investigated. Importantly, it was shown how the preparation parameters (i.e. the concentrations of spheres and/or alginate material) can affect the controllability of the protein release profile of the fibres. As a result, optimized conditions to prepare the uniform fibres with extended controlled release profile were introduced. The fibres were characterized chemically, mechanically and morphologically.

In Chapter 4, we introduce an adjustable gradient fabrication method to develop a novel PLGA microsphere concentration gradient Alg fibre for local delivery of proteins. The fabrication methodology applies some of the concepts of both wet-spinning and 3D-printing methods. The distribution of FITC-BSA loaded PLGA microspheres, through the fibrous matrix, was studied and
the produced fibre was characterized and the protein release profile from the concentration gradient fibre was investigated.

Chapter 5 provides a summary of the thesis and highlight directions for future research.
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Fahimeh Mehrpouya

University of Wollongong

March 2018
Declaration

I declare that due to the funding issues with my Ph.D., the title and focus of my Ph.D. project was changed (on 10/08/2014) to align with the milestones of the ARC Centre Excellence for Electromaterials Science (ACES). The fabrication skills I learnt during the first year of my PhD (under the supervision of Dr. Javad Foroughi and Prof. Gordon G. Wallace) were highly utilized during my PhD. I was trained to use many of the facilities, equipment and methodologies such as scanning electron microscopy, optical microscopy, FTIR, and various spinning methods. Based on my research work during my first year of Ph.D., a manuscript was published:

Certificate

I, Fahimeh Mehrpouya, declare that this thesis submitted in fulfilment of the requirements for awarding the degree of Doctor of Philosophy, from the University of Wollongong, is wholly my own work unless otherwise referenced. This document has not been submitted for qualifications at any other academic institution.

Fahimeh Mehrpouya

25th March 2018
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<th>Description</th>
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<tbody>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PDLA</td>
<td>Poly(D-lactic acid) poly(L-lactic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PAN</td>
<td>Poly(acrylonitrile)</td>
</tr>
<tr>
<td>Alg</td>
<td>Alginate</td>
</tr>
<tr>
<td>LVG</td>
<td>Guluronic acid rich alginate</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol) (2-isopropyl alcohol)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>LC-GO</td>
<td>Liquid crystal graphene oxide</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene dianimetetraacetic acid</td>
</tr>
<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>FITC-BSA</td>
<td>Fluorescein isothiocyanate-bovine serum albumin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factors</td>
</tr>
<tr>
<td>NTs</td>
<td>Neurotrophin factors</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
</tbody>
</table>
NT-4/5: Neurotrophin-4/5
Trk: Tyrosine receptor kinase
ECM: Extracellular matrix
AD: Alzheimer disease
K: Kilo ($10^3$)
T: Tera ($10^{12}$)
Da: Dalton
M: Molar
N: Newton
$m^2$: Square metres
L: Litre
p: Pico ($10^{-12}$)
Pa: Pascal
s: Second
cm: Centimetre
ml: Millilitre
m: Milli
μ: Micro
g: Gram
LE: Loading efficiency
PL: Practical loading
W/O: Water in Oil
W/O/W: Water/Oil/Water
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Chapter 1  Introduction
This study presents approaches for the biofabrication of novel delivery systems, to address current challenges in the controlled delivery of proteins. The main goal of this thesis is to establish simple, scalable and programmable manufacturing techniques to develop next generation of delivery systems towards promoting and directing tissue regeneration in “linear” systems.

This chapter introduces and provides a historical account of the various types of polymers, with biomedical applicability, with specific focus on their use(s) for delivery of bioactive molecules, in particular, proteins. Advantages and disadvantages of each method are discussed and compared between the polymers. Secondly, different structures for local delivery of proteins were compared and some of the previously established fabrication methods for delivery systems are briefly discussed. To conclude, the significance of developing novel structures for local delivery systems with favourable properties is highlighted.

1.1 Introduction to polymers for biomedical applications

Polymers are an exciting class of structural materials that can be used in various biomedical applications such as drug delivery (1). Polymers, for bio-applications, have a long history where some of them such as alginate and chitosan are made from natural sources and are categorized as natural-based polymers (2). The three types of natural-based polymers are: polysaccharides (i.e. alginate, chitin/chitosan, hyaluronic acid derivatives), or proteins (i.e. collagen, fibrin, silk), and polynucleotides (i.e. DNA, RNA) (3). But apprehensions over the complex structural composition of natural polymers, along with concerns associated with the use of animal-derived polymers including immunogenicity and pathogen transmission, have driven the development of synthetic polymers (4) as alternatives. This category of polymers includes: synthetic biodegradable polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) copolymers and poly(caprolactone) (PCL) (5, 6). Biocompatibility is a critical factor for polymer therapeutic applicability (7). These polymers based on the nature of their chemical bonds can be classified as non-degradable (i.e. poly(ethylene glycol) (PEG)), biodegradable (i.e. PLGA) or bioresolvable (i.e. alginate) (8, 9). Table 1-1 shows some of the basic limitations and benefits of natural-based and
synthetic polymers with biomedical applicability. It is obvious that the stability, thermal and mechanical properties of each type of polymers are highly dependent to its chemical structure and the shape (i.e. spheres, fibres and films) of the polymeric product.

Table 1-1. Limitations and benefits of natural-based biopolymers and synthetic polymers with biomedical applicability (7, 10, 11).

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Limitations</th>
<th>Benefits</th>
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| Natural-based polymers | 1. Weak thermal and mechanical properties. Their native structures are easily destroyed during high temperature processing  
2. Weak control of their degradation and release behaviour | 1. Biodegradable or bioresolvable  
2. Biocompatible |
| Synthetic polymers  | 1. Containing impurities or other compounds that can affect their biocompatibility | 1. Flexible in structural make up  
2. Worthy thermal stability (for most of them, thermal degradation happens higher than 300 °C) (10)  
3. Worthy mechanical properties compared with most natural-based polymers (i.e. up to 9.8 MPa for electrospun PCL nanofibrous mat) (11) |

1.1.1 Natural-based polymers

The first polymers (i.e. cellulose and starch) to be used in biomedical applications had natural-based sources (5, 12, 13). Naturally derived polymers can be fabricated in the form of thin films, fibres, sponges or scaffolds for potential biomedical applications. They can also be chemically and/or physically modified to improve their efficiency as biomaterials (1). Natural-based polymers have been used as carrier systems for the delivery of bioactive molecules and some of these systems have shown
to enhance biological interaction of the product with the surrounding host tissue (6, 14, 15). For example, Kolambkar et al. (16) developed a biocompatible alginate-based hybrid system for growth factor delivery in the functional repair of large bone defects. Their *in vivo* studies showed promising results in the regeneration of bones with large defects with no side effects on surrounding soft tissues. Amongst the natural-based polymeric systems developed for delivery of bioactive molecules, hydrogel forms of natural-based polymers are receiving increasing attention by reason of their capability to retain a great quantity of water, good biocompatibility, low interfacial tension and the minimal mechanical and frictional irritation that they cause (6).

### 1.1.2 Hydrogel forms of natural-based polymers

Hydrogels are defined as water-swollen polymeric networks that are typically stabilized by physical and/or chemical cross-linking (17, 18). Hydrogels are three-dimensional (3D) networks of hydrophilic polymers that have structural similarity to the macromolecular-based components in the body and are considered biocompatible. The most common hydrogel substrates are the natural-based polymers including alginate, collagen, gelatin, fibrin, chitosan and hyaluronate. A wide range of cross-linking strategies can be used, including bonding between chains through chemical reactions and/or UV photo-polymerization or physical methods such as melt polycondensation and cross-linking by crystallization (19, 20).

The properties of hydrogels have made them attractive in the different medical fields. For instance, due to their excellent biocompatibility, hydrogels are promising candidates for bioactive molecules delivery applications (6, 21). Recent studies have mostly focused on design and synthesis of novel hydrogel structures and their application in tissue engineering (22, 23) and bioactive molecules delivery (24-27). For instance, hydrogels-based on natural-based polymers have continued to be considered for encapsulation bioactive molecules as matrices for repairing and regenerating a wide variety of tissues and organs (28). The release of bioactive molecules from hydrogels is normally diffusion-controlled. Since the bioactive molecules are generally physically entrapped in the gels, the hydrogels usually present fast release with a huge amount of burst. Hence, enhancing the interactions between bioactive molecules and gels (i.e., electrostatic interaction), and/or using precursors which can form highly cross-linked structures, can prolong the release of entrapped bioactive molecules (29).
1.1.2.1 Alginate hydrogels

Alginate (Alg), the most frequently used polymer in cell and/or bioactive molecule encapsulation research, is a natural anionic polymer which has been isolated from Azotobacter vinelandii, several Pseudomonas species and algae. Alg is a linear polysaccharide composed of 1,4′-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (blocks) in different sequences (30) (Figure 1-1 A). These two variables have been associated with gel mechanical stability, viability of encapsulated cells, in vivo biocompatibility and diffusion properties of the Alg gel structure. Alg structures with higher G-content are more mechanically stable. Alg beads composed of high G-content are also known to be more porous than high M-content Alg, thus increasing diffusion of molecules into and out of the matrix (31).

Figure 1-1. (A) Chemical structure of Alg (28) and (B) mechanism of ionic interaction between Alg and divalent cations (specified as M) (31).

Alg can form a physical gel by cooperative binding with divalent cations, for instance Ba²⁺, Sr²⁺ and the most common one Ca²⁺ (Figure 1-1 B) (32-34). The ability to assemble Alg gels at neutral pH and mild temperatures makes Alg a promising choice for the encapsulation and delivery of cells, drugs and proteins (i.e. growth factors) (35). Attributable to the diffusion of cations from the gels under physiological conditions, ionically cross-linked gels reveal poor mechanical properties and uncontrolled degradation behaviour. Consequently, Alg structures usually show a relatively short-term release profile for encapsulated bioactive molecules with a high level of burst release. Consequently, a number of modification approaches have been applied to improve the Alg gels (36, 37). Table 1-2 shows some of the bioapplications of the Alg structures over the last five years (published from 2013...
There is a huge interest in applying Alg to develop different biostructures and biocomposites (with other polymers) for different bioapplications.

### Table 1-2. Some of the recent bioapplications of the Alg and Alg biocomposite structures.

<table>
<thead>
<tr>
<th>Physical form</th>
<th>Composition</th>
<th>Bioapplication</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Wet-spun fibres</td>
<td>Alg</td>
<td>Drug delivery</td>
<td>(38)</td>
</tr>
<tr>
<td>Wet-spun fibres</td>
<td>Alg</td>
<td>Nerve tissue engineering</td>
<td>(39-41)</td>
</tr>
<tr>
<td>Core-shell fibres</td>
<td>Alg, PLGA,</td>
<td>Drug delivery</td>
<td>(42, 43)</td>
</tr>
<tr>
<td></td>
<td>Chitosan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nano-gels</td>
<td>Alg</td>
<td>Protein delivery</td>
<td>(44)</td>
</tr>
<tr>
<td>Nano-carriers</td>
<td>Alg, Hydroxyapatite</td>
<td>Protein delivery</td>
<td>(45)</td>
</tr>
<tr>
<td>Microspheres</td>
<td>Alg</td>
<td>Protein delivery</td>
<td>(46-48)</td>
</tr>
<tr>
<td>Microspheres</td>
<td>Alg, PLGA, Fibroin</td>
<td>Protein delivery</td>
<td>(50, 51)</td>
</tr>
<tr>
<td>Microspheres</td>
<td>Alg</td>
<td>Tissue engineering</td>
<td>(48, 49, 52, 53)</td>
</tr>
<tr>
<td>Conduits</td>
<td>Alg, Gelatin</td>
<td>Nerve conduits</td>
<td>(54)</td>
</tr>
<tr>
<td>Films</td>
<td>Alg, Silk fibroin</td>
<td>Wound healing</td>
<td>(55)</td>
</tr>
<tr>
<td>Hydrogels</td>
<td>Alg, Gelatin</td>
<td>Tissue engineering</td>
<td>(56, 57)</td>
</tr>
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</table>

#### 1.1.3 Synthetic polymers

The concept of synthetic biodegradable polymers was introduced in the 1980s. Since then the field has experienced a steady and stable growth in application. Synthetic polymers are produced under controlled conditions and consequently the conditions can be adjusted, based on the specific application, to achieve required mechanical, physical, chemical, porosity, degradation and surface properties, and maintenance of their properties for a given time and degradation with minimal harmful
effects (58, 59). Hence, synthetic polymers typically have readily controlled structures with higher flexibility (60, 61). They experienced growing applications in bioactive molecule delivery and/or tissue engineering fields since their properties (e.g., porosity, degradation time and mechanical characteristics) can be readily tailored to specific applications and many synthetic polymers can be fabricated in the physical forms, such as hydrogels, to show physicochemical and mechanical properties compatible with biological tissues (62).

Many of the biodegradable synthetic polymers (i.e. PLGA and PCL) contain hydrolytic cleavage of site, such as ester bonds. In addition to ester derivatives, hydrolysis also acts on poly(anhydrides), poly(orthoesters), poly(phosphoesters), poly(phosphazenes) and poly(cyanoacrylate) derivatives (63). These biodegradable synthetic polymers meet the requirements for tissue engineering and sustained bioactive molecule release applications, attributed to their steady biodegradability (64). PLGA is the most commonly applied synthetic polymer in medical applications due to its biodegradability, biocompatibility and ease of processing (65).

1.1.3.1 PLGA

Poly lactic-co-glycolic acid (PLGA) is a copolymer of lactic acid and glycolic acid. Poly lactic acid (PLA) includes an asymmetric α-carbon which is typically described as the D or L forms in stereochemical terms. It means that the enantiomeric forms of the polymer PLA are poly D-lactic acid (PDLA) and poly L-lactic acid (PLLA). Table 1-3 shows some of the recent bioapplications of PLGA structures. PLGA is biocompatible and biodegradable, exhibits a wide range of erosion times, has tunable mechanical properties and most importantly, is a FDA approved polymer (66). PLGA biomaterials have been used for many bioapplications such as delivery of bioactive molecules. PLGA particulate systems, such as microspheres/ nanospheres and implants, have been shown to be capable of carrying and delivering a variety of biomolecules classes vaccines, peptides, and proteins as well as hydrophilic and hydrophobic drugs. Some advantages of using PLGA delivery systems include a reduction of injection frequency and formulations that can be tailored for any number of desired release profiles (67). In addition, they can be applied to develop composite structures with other biopolymers specifically hydrogels (i.e. chitosan and Alg).
Table 1-3. Some of the recent bioapplications of the PLGA and PLGA biocomposite structures.

<table>
<thead>
<tr>
<th>Physical form</th>
<th>Composition</th>
<th>Bioapplication</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microspheres</td>
<td>PLGA</td>
<td>Drug delivery</td>
<td>(67-70)</td>
</tr>
<tr>
<td>Microspheres</td>
<td>PLGA</td>
<td>Tissue engineering</td>
<td>(68, 71, 72)</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>PLGA</td>
<td>Drug delivery</td>
<td>(73)</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>PLGA, Chitosan, Alg</td>
<td>Protein delivery</td>
<td>(74-75)</td>
</tr>
<tr>
<td>Microspheres</td>
<td>PLGA</td>
<td>Protein (i.e. growth factor) delivery</td>
<td>(77, 78)</td>
</tr>
<tr>
<td>Wet-spun fibres</td>
<td>PLGA, Alg</td>
<td>Dual-drug delivery</td>
<td>(42)</td>
</tr>
<tr>
<td>Nanofibres</td>
<td>PLGA</td>
<td>Bone regeneration</td>
<td>(79)</td>
</tr>
<tr>
<td>Nanofibres</td>
<td>PLGA</td>
<td>Drug delivery</td>
<td>(80)</td>
</tr>
<tr>
<td>Microfibres</td>
<td>PLGA</td>
<td>Bone regeneration</td>
<td>(81, 82)</td>
</tr>
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The molecular weight (Mw) of PLGA is a determining parameter of the physicochemical properties of PLGA copolymers. Swelling behavior, mechanical strength, degradability (ability to undergo either hydrolysis and biodegradation) are directly affected by the relative composition (the proportion of PLA and PGA in the copolymer structure) of the PLGA (Figure 1-2) (83). Higher content of PGA causes a quicker rate of degradation with the exception of 50:50 ratio of PLA/PGA, which displays the fastest degradation, with higher PGA content leading to increased degradation interval below 50% (82). When PLGA structures are exposed to an aqueous media (i.e. phosphate buffered saline (PBS) and simulated body fluid (SBF)), the two mentioned polymeric units (PLA and PGA) slowly degrade by hydrolysis. The most commonly applied PLGA formulations are PLGA (85:15) polymer containing 85% L-lactic acid (LA) and 15% glycolic acid (GA) which degrades in approximately 5–6 months, PLGA (75:25) and PLGA (50:50) polymers take 4 to 5 months and 1 to 2 months,
respectively for degradation (65). The mentioned times could slightly differ based on the materials’ structures.

![Poly (lactic-co-glycolic acid) to Lactic acid and Glycolic acid](image)

**Figure 1-2. Schematic illustration of the degradation mechanism of PLGA copolymers by hydrolysis (84).**

### 1.2 Introduction to bioactive molecules

Any molecule that is produced by a living organism or can be used for the accelerated regeneration of tissues of a living organism is known as a bioactive molecule (85, 86). This category of materials includes large macromolecules such as proteins, polysaccharides, lipids, and nucleic acids, as well as small molecules such as low molecular weight drugs (aspirin), primary metabolites (i.e. ethylene) and secondary metabolites (i.e. antibiotics) (87, 88). Many peptides and proteins possess bioactivity properties and are considered as potential therapeutics. Peptides comprise up to 50 amino acids in length, but proteins are much larger than this (89, 90). Small molecule (peptides) treatment or delivery are not suitable approaches for a broad variety of biological applications and processes (89). In addition, proteins are the most important units of the living organism as they carry out all important physiological and biological processes like ligands for signaling, enzymes for biotransformation
reactions, receptors for pharmacological response elucidation and antibodies in immune system interactions (15). Hence, during the last decade, therapeutic proteins (such as protein drugs and growth factors) have gained significant applications in the field of bioactive molecule delivery and tissue engineering (14).

1.2.1 Bovine serum albumin

Albumins are synthesized by the parenchymal cells of the liver and exported as a non-glycosylated protein (91). Serum albumins are the major protein component of blood plasma and interstitial fluid of body tissues (92). They are the main soluble protein constituents of the circulatory system which contain several physiological functions (93). Serum albumins bind to many different types of amphiphilic biological molecules which play a significant role in determining their physiological function. The most important property of these proteins is that they serve as a depot and transport protein for many drugs, small bioactive molecules or growth factors (94). Many researchers use a model protein antigen such as bovine serum albumin (BSA) for protein release studies of structures. BSA is one of the most widely studied proteins due to its medical importance, availability, unusual ligand-binding properties, low cost compared to growth factors and being a homologous protein (95-99). BSA is comprised of a single chain of 583 amino acid residues involving 35 cysteines (forming 17 disulfide bridges) which confer a high stability to the protein (33) and its molecular weight (kDa), dimensions (Å), diffusion constant ($D_{20,w} \times 10^{-7}$ cm$^2$/s) and isoelectric point are 66.3, 140 × 40 × 40, 5.9 and 4.7, respectively (100).

1.2.2 Growth factors

Growth factors (GFs) are considered as soluble naturally occurring proteins or steroid hormones that bind to transmembrane receptors on the surface of target cells. GFs promote the growth, organization, and maintenance of cells and tissues. GFs play crucial roles in a variety of physiological processes, such as phenotypic activities of cells and information transfer between cells and their microenvironment (101-104). The signaling of GFs is mediated by interactions between the GFs and their receptors and can be further modulation by cellular interactions with the ECM proteins in the environment. It has been shown that the development of molecular complexes between GFs and ECM biofactors (mostly proteins (i.e. fibronectin (105), laminin (106-108) and agrin (109, 110)) and
carbohydrates (i.e. heparin (111)) can improve GFs signaling. In addition to binding to receptors, a large class of GFs has been observed to also bind to heparan sulfate proteoglycans (HSPGs) on cell surfaces and within the ECM. The interaction of GFs with HSPGs generally involves the binding to heparin chains, and in many instances these interactions have been demonstrated to modulate the cellular response to GFs (112). Moreover, it is reported that the binding of GFs (i.e. fibroblast growth factors) to heparan sulfate chains of proteoglycans protect the GFs from degradation (113). Due to the crucial role of GFs in controlling basic cellular functions, a wide range of GFs has been tested for therapeutic applications such as bone regeneration (114, 115), and nerve tissue engineering (116, 117). However, efficient GF delivery is an ongoing challenge for tissue regeneration therapies. Due to the short half-life in biological conditions, GFs need delivery systems to reach the target tissue with sufficient amount as required for specific biological function. Also, the accurate quantification of complex molecules such as GFs encapsulated in polymeric delivery devices, is equally critical and just as complex as achieving efficient delivery of active GFs (118).

1.2.2.1 Neurotrophic factors

The interest in GFs for bioapplications commenced in 1954 with the discovery of nerve growth factor (NGF). Since then, due to their continuous capacity to provide new insights into neural function, the motivation to study neurotrophics (NTs) has never subsided (119). They are able to prevent or reduce neuronal degeneration through their neurotrophic action on the specific neuronal population (120). NTs are frequently applied to enhance peripheral nerve regeneration by invoking their specific role(s) in regulation of development, maintenance and function of vertebrate nervous systems (121, 122).

Each GF protein from NTs class is a homodimer formed by identical peptide chains of about 120 amino acids. The classic members of the class of NTs are NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are derivatives from a common ancestral gene and are almost similar in sequence and structure (120). Since different mature neurons express different types of neurotrophic receptors, each of the NTs activates a special neuronal population, with limited overlap. NGF, which was discovered by a Nobel Prize winner (Rita Levi-Montalcini), was the first characterized GF and during the past decades, it remained as the most often studied member of the NTs family (123).
NGF is an important protein for the survival, regulation of growth and differentiation of sympathetic and spinal cord primary sensory neurons in the peripheral nervous system. It also plays a crucial role for the differentiation and survival of cholinergic neurons of the basal forebrain during the development of the nervous system. Moreover, NGF is responsible for the maintenance of the neuronal phenotype after maturity. It has other broader effects over endocrine and inflammatory mechanisms (124, 125). The three chains of the NGF structure are designates α, β and γ. Among NGF subunits, only the β-moiety (which has a higher molecular weight (26,518 Da)) holds nerve growth promoting activity and is responsible for the maintenance of adrenergic neurons in vitro. In 1991, the structure of β-NGF was discovered by Neil McDonald (126).

In recent years, NGF has been frequently used for clinical treatments. For example, in 1992, Olson et al. in a case report showed that the NGF infusion in Alzheimer’s disease (AD) is effective and NGF can stimulate central cholinergic neurons which are the known losses of cholinergic innervation of the cortices in AD (127). After that many studies have been done to optimize the dosage and delivery structure of NGF (128, 129). Moreover, it is reported that NGF can promote axon growth. For instance, Kong-Min et al. reported that NGF, released from a PLGA nerve conduit, could promote the axon outgrowth in chick dorsal root ganglia (DRG) cells up to 100 µm while the maximum released concentration of NGF in the culture media was equal to 5 ng/ml. It was shown that by increasing the concentration of NGF in the media from 10 to 50 ng/ml, the sensory neurons and superior cervical ganglion axon outgrowth were significantly improved. Further, NGF application stimulation regulated axonal growth of PC-12 cells in vitro (130, 131). This study focuses on biofabrication perspectives of structures for mostly linear protein delivery applications. These structures might have the potential to be applied for nerve tissue engineering and/or improving neuromuscular junction functionality. Hence, BSA and NGF have been applied as proteins for encapsulation purposes. The properties and functions of these two proteins have been previously discussed in here and also in section 1.21.

1.3 Introduction to protein delivery

Proteins can be administered either systemically or locally. Many proteins are poorly bioavailable and not metabolically susceptible via traditional oral or subcutaneous injections administration routes (132). Proteins bioavailability via the oral route is poor for molecules with molecular mass of greater
than several hundred daltons (8). So that, a strong challenge in the application of these therapeutic proteins is their delivery to the target site without any premature degradation or loss of activity. The development of appropriate formulations that modify pharmacodynamics and pharmacokinetics may not be possible for many proteins, and also the period of activity and half-life of proteins cannot readily be preserved over injections of the naked protein. Thus, protein pharmaceuticals must be repeatedly injected over extended periods (i.e. months or years) for clinical applications. In addition, proteins are susceptible to hydrolysis and modification at gastric pH levels and can be further degraded by proteolytic enzymes in the small intestine and not be absorbed across the endothelial barrier (8). Some types of proteins (such as GFs) need to be delivered to the site of action for tissue engineering applications. Consequently, due to the relatively large size of proteins, their short biological half-lives resulting in rapid loss of bioactivity, tissue specificity and the potential toxicity at high levels, these conventional routes are often limited in utility. Thus, a sustained protein delivery system offers a number of potentially important clinical advantages (103, 133, 134).

To address these issues and improve the bioavailability of proteins, many studies have been devoted to the control and maintenance of long-term release. Among the administration methods that have been developed, encapsulation of proteins (into a delivery system) has been shown to increase the stability and provides the selective entrapment of proteins in biopolymers matrix.

Polymers have shown promise as delivery system which are able to protect proteins from extreme conditions. Ideally, the controlled release delivery system should be easily administered and does not require surgical removal (133, 135). Overall, protein therapy involves the targeted transport and sustained release of therapeutic proteins (i.e. GFs) using biodegradable polymeric delivery systems which can provide protection for proteins against the loss of their bioactivity in physiological conditions as well as long-term (sustained) release of protein at the target site (101).

The earliest controlled polymeric delivery systems were “reservoir” types. In such systems, a polymer coating would surround the proteins which resulted in the regulation of the rate of the protein release and protection of the protein from the bio-environment (shown in Figure 1-3). The typical polymers applied for this purpose (silicon elastomers or poly (ethylene-co-vinyl acetate) were biocompatible.
and non-degradable in physiological conditions. Too slow diffusion of large molecules (such as GFs) was the main weakness associated with these systems.

Figure 1-3. Release from a reservoir delivery system. The protein (dark blue) is contained within a central core and a polymeric membrane is surrounding it. As the protein diffuses through the polymeric membrane and into the surrounding tissue (light blue cloud), the concentration of protein within the central core reduces (136).

A growing interest in developing next generation protein-polymer matrices has now ensued. These protein-polymer matrices are fabricated by dispersing the protein within a solid matrix (in any form) of polymers. In such delivery systems, the protein is incorporated in the form of solid particles which leads to the formation of a heterogeneous structure within the matrix (136). Figure 1-4 shows the schematic of protein release from protein-biodegradable polymer delivery systems in different forms.
Figure 1-4. Examples of biodegradable polymer delivery systems. Solid particles (dark blue) of a protein are shown suspended within a solid polymer matrix in different forms. Schematic of protein release from polymer devices (i.e. Alg and PLGA) in different forms such as disks or (A) particles or a piece of a fibre and (B) spheres, and from (C i) degradable and (C ii) swelling hydrogels (136).

1.3.1 Local protein delivery treatments

Although some growth factors (such as platelet derived growth factor (PDGF) or epidermal growth factor (EGF)) are highly effective and selective in vitro, they might have significant systemic side effects. Hence, a protein should be delivered directly to the site of action to avoid systemic side effects. Afterwards it needs to remain at the target location sufficiently long to play its role in treatment. Local delivery is the only feasible strategy if locally high concentrations of a protein are
desired without incurring any side effects to non-target sites, or local concentration gradients need to be established (137). Moreover, local delivery is an effective route to reduce waste of expensive proteins. For instance, the increasing number of grafting procedures and the disadvantages associated with graft harvesting (e.g. limited graft quantity and donor site morbidity) drive the quest for local delivery of bioactive molecules that are instrumental in the initiation of auto-induction by bone. In this regard, Kempen et al. (138) studied the effect of local sequential vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs) delivery on ectopic and orthotopic bone regeneration. VEGF is one of the key regulators of angiogenesis during bone formation and BMPs are multi-functional GFs that belong to the transforming growth factor beta superfamily which play a central role in most bone regeneration strategies. BMP activities are regulated at different molecular levels which play a central role in most bone regeneration strategies. It is reported that members of the BMP family can initiate the complete cascade of bone formation, including the migration of mesenchymal stem cells and their differentiation into osteoblasts. As a result, these researchers showed that the advantage of local release of combination of angiogenic and osteogenic GFs in enhancing bone regeneration.

Protein delivery systems and structures for local delivery applications need to combine an easy fabrication, easy application, good tissue compliance, efficient and appropriate protein administration during the treatment, as well as protein stability. In the following sections, some of the most efficient and common delivery systems are introduced.

1.4 Proteins delivery structures

Protein delivery systems should preserve and improve the conformational stability of proteins. Protein stability might be compromised at different stages: during fabrication of polymer delivery system, throughout the storage time before application and after implantation (88). The complexity of the structure of proteins is one of the main reasons behind loss of stability and biological activity through fabrication and use of biodelivery systems. Throughout the fabrication process, any possible structural changes (for instance denaturation) of the proteins should be avoided. These changes result in the loss of native structure and function, and interaction with surroundings by adsorbing to surfaces or aggregating with other protein molecules (15). Hence, the design and fabrication of delivery structures
plays a critical role for controlled delivery of functional proteins. The chemical composition (i.e. polymer and/or composites of polymers) and geometry (such as spheres and fibres) of delivery systems affect the protein release profile \((101, 139, 140)\). As such, the final application will dictate the required mechanical and degradation characteristics, and the mode of delivery (prefabricated or injectable). Degradable biopolymer systems offer advantages for full tissue integration and reduced invasiveness. The optimal carrier should metabolically dissolve or degrade at a rate appropriate to the tissue restoration requirements while sustaining release of the proper concentration of protein. Appropriate ratio of surface area to volume is also desired for adequate mass transport to the biological environment \((101)\). Considering that no delivery system is ideal for all the applications, the delivery systems and structures that have been extensively used for protein delivery applications are discussed in the following sections.

### 1.4.1 Microspheres

Recently, polymeric microspheres have been widely used as controlled delivery systems because they can release proteins in a controlled rate and targeted manner, and can also be applied as implantable or injectable delivery systems. Microspheres are defined as monolithic spheres or therapeutic agent dispersed within the polymer matrix or as structure made up of continuous phase of one or more miscible polymers in which protein molecules are distributed at the molecular or macroscopic level \((141, 142)\). The inherently small size and large surface area, high protein loading efficiency as well as high diffusibility and mobility of protein-loaded microspheres, place microspheres as effective delivery systems for many proteins \((143-145)\). The average optimal diameter of the microspheres ranges from few micrometres to ideally less than 200 micrometres. This means that the larger microspheres may be comparable to cells or bigger \((146)\). The microspheres vary in materials composition, sphericity, porosity, uniformity of particle and particle size distribution. The microspheres’ properties determine the kinetics of the protein release from microspheres. Microspheres fabrication parameters (i.e. polymer concentration, solvent volatility, voltage and feeding rate for electrospraying) affect the final size distribution of produced microspheres. Hence, the release kinetics and profiles from microspheres can be controlled by optimization of the fabrication variables \((147)\).
The approach of using microspheres for protein delivery is to encapsulate the proteins into a polymeric spherical matrix. Protein loading into microspheres can be achieved via post-loading or pre-loading. In the post-loading method, the protein is adsorbed onto the spheres’ surface and, depending on the spheres’ properties (e.g. porosity and materials composition), might penetrate the inner structure. In terms of the pre-loading method, the protein is encapsulated into the polymeric matrix through the fabrication process of the microspheres. The protein release, depending on the chemical composition and geometry of the microspheres, can happen through desorption, diffusion, or degradation of microspheres. Release profiles of pre-loaded proteins usually present sustained release kinetics favourable for long-term delivery in comparison with the post-loaded proteins (84). Since the protein is not subjected to harsh fabrication conditions, the post-loading method looks more promising in keeping the bioactivity of the proteins with very short half-lives. Post-loading of protein into microspheres is highly dependent on the loading capacity of the spheres’ material, porosity and hydrophilicity of the microspheres and therefore, it cannot be applied for many types of polymeric microspheres such as PLGA microspheres.

Furthermore, the choice of appropriate biocompatible polymers can significantly affect the proteins release profile (148). Various synthetic and natural materials are used for the preparation of microspheres, and each of these biopolymers present different release kinetics. For the controlled delivery of proteins, biodegradability of polymers is an important factor. Degradable polymers do not demand surgical removal but, however, the degradation products need to be permissive to tissue repair and function. Prominently, the release of the protein can be regulated by the degradation and diffusion behaviour of the polymers (29). Polymers are classified based on the rate of hydrolysis of the functional groups which determines the final degradation behaviour of the structures. For instance, bulk-eroding polymers, such as PLGA which has received a great deal of attention in protein delivery applications, allow the infusion of water (present in release media) into the polymer matrix. Hence, PLGA microspheres degrade throughout the microsphere matrix and the resulting monomers (lactic acid and glycolic acid), oligomers and protein diffuse out of the structure and into the surrounding medium (Figure 1-5). The first protein release phase is often a “burst” released during the first few hours of incubation. It is assumed that the burst is a result of the protein sited on or close to the surface of the sphere or in pores connected to the surface. In the following incubation in aqueous
medium, water penetrates the microspheres, protein hydrated and diffused. This process is further facilitated by PLGA degradation which produce voids facilitating diffusion of proteins. Also, PLGA molecules separate from the microspheres surface resulting partially-degraded PLGA chains diffusing out and so that the protein release continues slowly from the microspheres through diffusion. The diffusion of the monomers and protein into the surrounding medium leads to formation of a network of pores through the structure. This phase is continued through the increasing of the size of water-filled pores till the sphere falls apart. If some proteins still remains, there may be a third phase, in which the remaining protein is released as a result of the collapse of the remaining structure (149, 150).

Figure 1-5. (A) Schematic of polymer erosion in and protein release from protein-loaded PLGA microspheres. Individual protein-loaded PLGA microspheres might consist of surface and internal pores (white). (B) Proteins distribution through the PLGA microsphere structure is shown by black dots. PLGA microspheres degradation is due to surface erosion and release of proteins. (C) Release of protein by diffusion is caused by internal PLGA hydrolytic degradation and diffusion of degradation products (monomers and oligomers) through the pores to the external medium. (D) A degraded skeleton (151).
Alg as a naturally occurring anionic polysaccharide, has been frequently applied to form microspheres for delivery of proteins (14). Alg is a bioresorbable material in physiological conditions, whereby biodegradation occurs due to the breakdown of the polymer network architecture as a result of cation exchange. The protein might be released either from the surface and/or during the dissolution of the structure which happens through a swelling-erosion mechanism. This process makes the gel structure weaker and therefore, the remaining protein would be released over time (152).

1.4.1.1 Microspheres fabrication methods

The fabrication methods differ based on the initial polymers and the type of desired protein structure that can be efficiently encapsulated. Several main points should be considered in evaluating the method. First, the method should produce microspheres with a reasonable yield and with no contamination. In addition, reproducible control over the size of the microspheres is necessary. Ultimately, the fabrication method must be compatible with the protein to be encapsulated (loading through either pre-loading or post-loading processes), avoiding extreme conditions such as high temperatures, presence of organic solvents, toxic surfactants or physical forces that might lead to loss of bioactivity of the protein (149, 153). Several fabrication approaches are commonly used for the fabrication of polymeric microspheres involving polymerization, spray drying methods (i.e. electrospray) and emulsion/solvent evaporation (154). Among them, polymerization methods are quite different since the polymer precursors (starting monomers) are needed as the starting materials. Moreover, the polymerization methods usually provide a very poor control of the size of the produced microspheres (a wide range between 40 to 1000 µm) and the resulting microspheres usually display a relatively high polydispersity. Although there are some reports on improving the polymerization chemistry, these methods are not very favourable for protein encapsulation applications because they need various harsh preparation conditions (in terms of pH, temperature and pressure, etc) resulting in loss of bioactivity of the proteins (155-157). Also, there is a possibility of undesirably affecting the bioactivity of the proteins through the required conditions for completion of the polymerization (158). In contrast, spray drying and emulsion/solvent evaporation methods, employ preformed polymers. Here, the general features of each of these methods are discussed.
I. Spray drying

The spray drying approach is an important method in the pharmaceutical and biochemical fields which can be applied for encapsulation of some types of bioactive molecules. This class of fabrication methods can be applied for preparation of microspheres loaded by heat resistant or heat sensitive bioactive molecules and whether the bioactive molecules are both water soluble or water insoluble. Spray drying involves spraying a complex liquid mixture, containing a bioactive molecule dissolved, dispersed, or emulsified with a biopolymer in an aqueous or an organic solvent, into an environment for solidification followed by rapid evaporation of solvent (159) (Figure 1-6). This approach is based on the isolation of microspheres, and the final microspheres characteristics depend on the initial formulation whether in the form of a solution, a suspension or an emulsion (160, 161).

![Figure 1-6. Formation of protein-loaded polymer microspheres using the general spray drying method (162).](Image)

The bioactive molecule loaded microspheres produced by this method have a potential to provide new types of administering routes, like oral dosage forms and targeting systems to organs and tissues (163). As an example, Giunchedi et al. applied this method for fabrication of small BSA-loaded PLGA microspheres (size range: 2-5 µm) with high protein encapsulation efficiency (~70 – 80 %)
Although some of the spray drying methods are adaptable to an industrial scale, rather than some of the other fabrication procedures which are limited to laboratory-scale operation (165), but there are a few principal drawbacks with these methods: i) not all types of biopolymers can be used; ii) the rapid drying process might cause a loss of crystallinity of polymeric structure and loss of bioactivity of bioactive molecules. Each of the spray drying methods based on applied techniques for making droplets as well as drying them can affect the properties of the final microspheres. One of the most well-known methods, using the spray drying technique, is electrospraying. Here, some of the advantages and disadvantages of this method are reported.

**Electrospraying**

Electrospraying (electrohydrodynamic spraying) is defined as a method of liquid atomization using the electrical forces (Figure 1-7). In this process, a high voltage is applied to a liquid flowing out of a capillary nozzle. The electric field forces the liquid to be dispersed into droplets. The electrical charge of the formed droplets facilitates their motion. The size of electrosprayed droplets can range from few nanometres to hundreds of micrometres, and their size distribution can be nearly monodispersed. Adjusting the fabrication conditions, like feed rate, voltage, the distance from needle to collector, needle diameter and polyelectrolyte concentrations, the droplet size can be controlled.

![Figure 1-7. Schematic representation of the electrospray process (147).](image)
Applying this method, protein-loaded micro or nanospheres can be fabricated (142). Since the droplets cannot be cross-linked before solidification (drying step), electrospraying is not able to fabricate microspheres from some of the natural polymers (i.e. Alg) and so the method needs some improvements such as combining the method with ionotropic gelation (165). Moreover, there are some limits in the choice of solvents and the solvents with lower boiling temperatures are preferred for this method for ease of evaporation (166).

Some other modified methods, based on the same technique of making droplets from polymeric solution or emulsion and recovery of the produced microspheres have been developed. One of them is the nozzle-based ink-jet printing which can be applied to produce microspheres from both an aqueous or an organic polymer solution.

Ink-jet printing

Inkjet printing is a non-contact approach that enables processing of droplets of liquid, with a volume of 1–100 pL, into two-dimensional and three-dimensional structures. Like spray drying methods, this method also involves dissolving or dispersing the bioactive molecules and polymers in a liquid to prepare the initial ink. Then the liquid is ejected from a micro-nozzle to form the drops. Inkjet printing is associated with several advantages including low processing costs and generation of minimal waste (167). Figure 1-8 shows the schematic of the experimental process for the synthesis of microspheres using the inkjet printing method. Nozzle-based inkjet methods have been long favoured in the fabrication of microspheres due to its good size distribution and controllability. For example, Chung et al. fabricated dextran-loaded Alg microspheres (size range: 12.2 - 37.9 µm) by printing aqueous Alg solution into a cross-linking bath containing calcium chloride and Lutrol F127 (168). But the same drawbacks of the spray drying methods limit the use of this approach for different polymers. Furthermore, the molecular weight of the polymer, rheology (viscosity) behaviour of the initial liquid and surface tension of the solvent play key roles throughout this process since they can easily cause blockage of the small nozzles which can negatively affect the amount of produced microspheres per hour (yield of the method) (169). Moreover, the two phases of the liquids (i.e. hydrophilic bioactive molecule with a hydrophobic biopolymer dissolved in mixture of the solvents) are not optimal for this method. Hence, in regards of protein delivery applications, this method can be mostly applied for
encapsulation of proteins (as hydrophilic biomolecules) into water-soluble natural-based polymers (i.e. Alg) rather than synthetic polymers (i.e. PLGA).

![Initial biopolymer liquid](Image)

**Figure 1-8.** Experimental process for the synthesis of microspheres using an inkjet printing method (I68). The initial polymeric liquid is transferred to a cartridge which will be sited in a nozzle-based ink-jet printing set-up. Then the liquid is ejected from a micro-nozzle to form the drops into a cross-linking solution or directly on a solid collector plate.

II. Emulsion/solvent evaporation methods

One of the most common methodologies for fabrication of microspheres is founded on emulsification/solvent evaporation. The initial concept of these methods is based on emulsification of the polymer solution (it also can be in combination with the protein) into a continuous phase, solvent evaporation through the emulsion/air interface resulting in polymer precipitation and finally spheres hardening. At the end of the process, the microspheres are separated from the continuous phase by filtration and then they are washed and recovered (I70). Based on the nature of the polymer and protein and the desired loading process, either the conventional (single) emulsion or multiple (double) emulsion method can be applied for preparation of the microspheres.

**Single emulsion technique**

There are many studies focusing on fabrication of natural based polymers such as Alg microspheres using this technique (I71). Here, natural-based polymers are first dissolved in aqueous medium (water phase- W) and then dispersed in non-aqueous medium (oil phase) (water in oil- W/O) continued by cross-linking of dispersed globule. The cross-linking usually was achieved through chemical or
physical cross-linking processes (ionic gelation process) (172). For example, in 2014, Baimark et al. (173) prepared Alg microsphere (size > 100 µm) using a W/O emulsion method. They reported that by increasing the concentration of cross-linking agent (Ca$^{2+}$), they could improve the release profile of a model drug (Blue dextran 2000) up to a few days. It is worth mentioning that the size of the initial forming droplets, that determine the final spheres diameter, can be controlled by the mixing speed. Using a high speed mechanical stirrer or homogenizer to mix the suspension (particularly for surfactant-free suspensions) before cross-linking the spheres, can help to make smaller microspheres (diameter < 50 µm) (163). Lemoine et al. (174) produced BSA-loaded Alg microspheres through a single emulsion method using a homogenizer at 8,000 rpm. They reported that the final protein-loaded spheres formed from low viscosity Alg type had a mean diameter of 11.9 ± 0.1 µm (Figure 1-9). The main drawback of the encapsulation of protein through this system is that the stirrer high speed and the mechanical tension can affect the bioactivity of proteins. To address this issue and achieve higher encapsulation efficiency, the protein post-loading method into the produced Alg microspheres, and protein function preservation, might be helpful.

![Figure 1-9. Alg microspheres prepared with low viscosity Alg (volume mean diameter = 11.9±0.1 µm). Scale bar 62.5 µm (174).](image)

**Double emulsion technique**

Synthetic biopolymer microspheres (e.g. PLGA) are manufactured using various techniques and among them water-oil-water (W/O/W) double emulsion is considered as the most common one for
encapsulation of water-soluble bioactive molecules (i.e. proteins). Due to the difficulty in trapping hydrophilic proteins into a hydrophobic polymer matrix, a high-energy mixing process is needed. To improve the loading efficiency into microspheres and minimize the loss of bioactivity of proteins because of exposure to organic solvents, the fabrication process is completed in two steps. At first, a primary W/O emulsion is formed by dispersing the aqueous protein solution in an organic polymer solution. Then the second emulsion (W/O/W) is formed by dispersion of the first emulsion in an aqueous continuous phase (i.e. poly(vinyl alcohol) (PVA) as a stabilizer) (175). There are numerous studies on encapsulation of proteins into PLGA microspheres via this technique and the optimization of the effects of operational factors on the mean size, protein encapsulation efficiency, protein release profile and protein’s bioactivity. For example, Yang et al. (176) created BSA-loaded PLGA microspheres via a double emulsion method and they studied the effect of BSA loading on microspheres’ mean size and release profile. They reported that higher loading of BSA encapsulated into the spheres caused formation of bigger microspheres (size > 189 µm) and the release profile was faster with higher level burst at first day. Blanco et al. (177), using two model proteins (BSA and lysozyme) and two different molecular weights of PLGA, studied the effect of protein loading and polymer composition on dual protein release from PLGA microspheres. They showed the effect of the polymer-protein interactions on the inner structure, encapsulation efficiency and protein release from PLGA microspheres. Ravi et al. (178) reported that by optimizing the manufacturing conditions, they could produce very small BSA-loaded microspheres with a narrow size range (3.98 to 8.74 µm). The main drawbacks of their work were: i) very high speed homogenization (unsuitable for proteins with low stability), as well as ii) low loading efficiency (~ 40%). Moreover, there have been some efforts expended to make the surface morphology of PLGA microspheres more porous with the aim of improving the release profile of model proteins (179, 180). The challenges of having highest possible protein loading through preparing microspheres with appropriate sizes in order to achieve desired release profiles are not quite resolved. Hence, based on the final application and protein type, researchers optimize their emulsion fabrication system. Also, there are more reports on encapsulation of proteins with less stability (such as GFs) into the PLGA microspheres via double emulsion method. Table 1-4 shows some of the studies on encapsulation of NGF (and other proteins and/or polymers as the protector and stabilizer for NGF) into PLGA microspheres via double emulsion fabrication methods.
Table 1-4. Examples of encapsulation of NGF in PLGA microspheres via double-emulsion method.

<table>
<thead>
<tr>
<th>Microspheres mean size (µm)</th>
<th>NGF stabilizer</th>
<th>Details of release</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>PEG</td>
<td>In <em>vitro</em>- Continues long term release (seven weeks)</td>
<td>(181)</td>
</tr>
<tr>
<td>20</td>
<td>Human serum albumin (HSA)</td>
<td>In <em>vitro</em></td>
<td>(182)</td>
</tr>
<tr>
<td>---</td>
<td>BSA</td>
<td>In <em>vivo</em>- significantly improve the ability of spatial learning and memory of the rats with fimbria-fornix lesion</td>
<td>(183)</td>
</tr>
<tr>
<td>&lt;59</td>
<td>N/A</td>
<td>In <em>vitro</em> and <em>in vivo</em>- long term delivery (23 days) with the rate of 0.9%-2.2% per day with linear kinetics</td>
<td>(184, 185)</td>
</tr>
</tbody>
</table>

1.4.2 Fibrous structures

In recent years, due to the high mechanical properties, ease of fabrication and desirable protein release profile, there has been growing interest in applying micro- and nanofibres for the purpose of protein delivery (186). The morphology of the fibres makes them suitable to be applied as delivery system for proteins (186, 187). The cylindrical shape of the fibres provides a relatively high surface area for them. The fibres’ surface area to volume ratio can be controlled by changing length as well as the cross-sectional radius. Additionally, fibres are the only candidate for a two-dimensional delivery structure that also performs a structural role. The protein-loaded fibres can be applied to develop novel implantable delivery platforms that can provide healing with more precision and efficacy than other methods in use (188).

1.4.2.1 Fibres fabrication methods

There are many techniques to produce biopolymer fibres, but only a few of them are suitable for protein encapsulation (189). Dependent on the final application, fibres with different shapes and
structures, such as hollow, flat, and ribbon shaped, can be produced. Electrospinning, microfluidic fibre fabrication, self-assembly and wet-spinning are the most common methods. Among them, electrospinning and wet-spinning fibre fabrication approaches are the most favoured to develop structures for bioactive molecule delivery. Each of them provides some advantages that make them suitable for specific delivery applications.

1. Electrospinning

Electrospinning (Electrohydrodynamic spinning) is a well-known method to form small diameter fibres in the range of several nanometres to micrometres. It is a broadly used technology for electrostatic fibre formation which applies electrical forces to produce polymer fibres (Figure 1-10.) (190). This idea of using electrostatic forces to produce fibres was developed and applied for the first time in the 1930s. In the electrospinning process, different processing and solution variables play key roles on fibres’ diameter, architecture and mechanical properties.

Figure 1-10. A typical horizontal electrospinning setup (189).

Among the biomedical applications of electrospun mats, bioactive molecule delivery is one of the most promising areas. Electrospinning provides the possibilities to have high bioactive molecule loading (up to 60%), encapsulation efficiency (up to 100%), and ability to modulate release (191). Also, this method, like wet-spinning, allows the co-processing of polymers merely by applying
ternary solutions of the components for electrospinning or by co-electrospinning resulting in core-shell fibres (192). There are some reports on applying this method to develop interconnected porous fibrous mats for protein delivery. For example, Chew et al. (193) fabricated electrospun copolymer of ε-caprolactone and ethyl ethylene phosphate (PCLEEP) nanofibrous mat loaded with human β-NGF and they used BSA as a carrier and stabilizer protein. They reported a first-order sustained release of NGF over three months from the structure and as indicated by PC-12 neurite outgrowth assay, the bioactivity of NGF was preserved during the release period.

Although electrospinning is able to provide structure with some specific advantages there are challenges limiting the use of this method for many applications. Polymer solutions with insufficient entanglements (such as low concentrations, low viscoelasticity or molecular weight) may produce beaded fibres or droplets. Hence, the usage of low molecular weight polymers for electrospinning is not common (194). Moreover, some polymers such as Alg (as a natural-based polyelectrolytic polymer) cannot be individually applied to form fibres through electrospinning and should be blended with a flexible and uncharged synthetic polymer (i.e. polyvinyl alcohol or polyethylene glycol) (195). The other restricting factor for the application of electrospun fibrous mats is that they are not suitable for two-dimensional applications such as providing platforms to guide the cell growth in a specific direction.

II. Wet-spinning

Wet-spinning was developed in the 1930s to allow extrusion of a polymer solution (spinning dope). Generally, after fully dissolving the polymers in a suitable solvent, solutions are extruded through a spinneret into a miscible nonsolvent coagulation bath. The direct contact between the polymer solution and nonsolvent coagulant cause a phase separation. Nonsolvent-induced phase separation transforms liquid polymer streams into solid continuous fibre (196). The produced microfibres might be drawn through one or a series of rollers to achieve a higher mechanical strength, elasticity and elongation. Because of complex interactions of different elements (i.e. solvent/nonsolvent miscibility parameters, precipitation strength, residence time and extrusion rate) that might affect the phase
separation of the polymer solution, the step of gelation or cross-linking of the polymer solution determines the final structure and dimensions of microfibres. The diameters of microfibres fabricated via wet-spinning are usually between four to a few hundred micrometres (197). Figure 1-11 presents the schematic of a uniaxial lab-scale wet-spinning setup.

![Figure 1-11. Schematic of a uniaxial lab-scale wet-spinning setup (198).](image)

Wet-spinning offers many benefits to protein delivery technologies since it can be done at ambient temperatures and is most similar to microsphere-based encapsulation techniques. Moreover, the wet-spun microfibres have a diameter of suture-like scale and they allow easy implantation/removal, anchor-ability in tissue and mechanism stability (42). This method might prevent the potential denaturation of proteins since they do not face any extreme conditions such as high temperatures and/or strong and high voltage field, unlike melt spinning, dry spinning and electrospinning methods. Hence, wet-spinning can be applied successfully for many bioactive molecules delivery and/or cell encapsulation. Scaffolds and structures involving microfibre-based orientation are also able to provide unidirectional alignment of growing cells which makes them an interesting choice for some of the studies in the field of tissue engineering (199). Wet-spun fibres can be woven, knitted, braided or embroidered into macro-level scaffolds and afford superstructures for the clinical reconstruction of damaged tissues/organs (190).
Synthetic polymers can be applied to form fibres via most spinning fabrication methods due to their ease of manipulation and reproducibility under various processing conditions. Although natural-based polymers, such as some of hydrogels like chitosan or Alg, are often desired because of their ability to closely mimic the physiochemical properties of the extracellular matrix, usually most of the fibre spinning methods (i.e. electrospinning) are not appropriate for processing them. Wet-spinning can produce uniform continuous fibres from hydrogels. For instance, chitosan/Alg microfibres (with diameter range of 63-140 µm) were successfully developed for encapsulation and delivery of human hepatocellular carcinoma cells (199). Lin et al. (200) also developed BSA-loaded Alg fibrous dressings via wet-spinning and studied the operating parameters on the release profile of the protein. Obviously, based on the type of polymers and the final application, the spinning process must be optimized. For example, some researchers developed core-shell fibres via wet-spinning to extend the release profile of the bioactive molecules, to have a dual bioactive molecule release or to protect bioactive molecules from the fabrication process (42, 201).

### 1.4.3 Customization of structural format

Many researchers have been trying to develop new delivery systems via new techniques to achieve their final desired applications. For instance, many multi-layer structures (spheres or fibres) have been fabricated to modulate the release profile of proteins. Some approaches such as surface polymerization, layer-by-layer deposition, emulsion-solvent evaporation, coaxial or triaxial spinning or spraying methods and vapour deposition polymerization have been applied to fabricate of core-shell delivery systems. These systems can be specifically applied for encapsulations of hydrophilic bioactive molecules (such as proteins) in hydrophilic/hydrophobic biocomposite structures, for dual-bioactive molecules delivery or achieving an extended or improved release profile. For example, Zhai et al. (50) in 2010 reported encapsulation of BSA in PLGA/Alg microspheres through a double-emulsion method. They reported a modulated release profile of BSA from PLGA/Alg rather than PLGA microspheres. One of the advantages of core-shell delivery systems is that the shell can protect the protein from degradation while allowing sustained release over time (202). Wanawananon et al. (42) fabricated a core-shell PLGA/Alg fibre via a wet-spinning method as a dual-drug delivery system.
Furthermore, three-dimensional printing (3D-printing) has received a great deal of attention in recent years. 3D-printing technology is a more complicated method able to fabricate customized structures using a computer design technology. The inner and outer architectures, including the pore shape, porosity, and the interconnectivity of the scaffolds, can be controlled by accurate computer designing. Despite numerous advantages of this method, there are still some limitations (i.e. choice of polymers) for application of this method. The significant issue for achieving a successful structure by printing is the choice of polymers with a good printability which is directly dependent on the rheological properties (such as viscosity) of precursors (203). Hydrogels (such as Alg) are one the most common category of polymers applied for developing structures via 3D-printing. But each range of the hydrogels’ molecular weight is compatible with only just one type of bio-printers (inkjet, laser assisted and extrusion-based). Each of these methods differ in both fabrication and gelation steps, and so their ability to develop structures with proper stability differs (204, 205).

Despite some significant advantages, the success of many of these delivery systems is limited due to their short residence at the site of absorption because of their fast degradation, insufficient protein release profile and/or incompatible mechanical properties (206). Therefore, with the aim of overcoming the problems associated with each of the delivery systems, some studies have been done on developing delivery systems that incorporate two or more delivery structures to provide regulated long-term release profiles with desirable final properties (i.e. proper mechanical properties). This study develops a new strategy that incorporate bioactive molecule (i.e. protein)-loaded microspheres into a continuous matrix (microfibres) of polymers, and hence gain optimal retention of bioactive molecules, and simultaneously provide bulk materials with enhanced features for sustained release of bioactive molecules.

1.5 References

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Chapter 2  Fabrication of Protein-loaded Microspheres for Controlled Release Applications

The aim of this chapter is to investigate the properties of microspheres as potential biomolecule delivery systems with specific applications in controlled release systems. Despite the recognized role of polymers in the encapsulation and release of proteins, the final properties of the protein-loaded microspheres have not been sufficiently characterized. In this research work, the fluorescently-labelled bovine serum albumin (FITC-BSA) was chosen as the model protein to be encapsulated. For this study, two different types of polymers have been applied for preparing the microspheres. Alginate (Alg) and D, L-lactide and glycolide copolymer (PLGA) microspheres were prepared by a W/O emulsion and W/O/W double emulsion method, respectively. Different properties of the protein-loaded microspheres, such as the mean diameter, loading efficiency, release profiles, chemical and thermal properties were investigated. Both types of microspheres showed a smooth surface morphology. The release profile of FITC-BSA from microspheres presented two phases, an initial burst release phase due to the protein adsorbed on the microsphere surface, followed by a slower release phase corresponding to the protein entrapped within the polymer matrix. FITC-BSA showed a slower release rate from PLGA microspheres compared to Alg microspheres. Moreover, the release rate was different for different protein loading levels into the PLGA microspheres. Consequently, these microspheres provide a good option for use in future applications in controlled release devices for protein delivery. In addition, some attempts have been made to encapsulate the nerve growth factors (β-NGF) into PLGA microspheres. The results demonstrate that some further studies can be done to optimize the affecting fabrication parameters to achieve microspheres with ability of releasing growth factors in a sustained approach.

2.1  Introduction to Microspheres for protein delivery

Polymeric microspheres offer significant advantages as bioactive molecule delivery structures, most notably the possibility to control the release rate of the incorporated bioactive molecule. There are many reports of encapsulation of various categories of bioactive molecules, for instance drugs,
proteins (i.e. GFs) within microspheres. An ideal fabrication approach, for developing efficient microspheres, should be able to provide consistent microsphere diameter, high bioactive molecule encapsulation (loading) efficiency and controlled sustained release of the loaded bioactive molecules (1, 2). Increasing or controlling the encapsulation efficiency is desirable, as it can help to extend the duration and dosage of treatment (3, 4). The fabrication technique and the process applied for loading of bioactive molecules into the microspheres are the main parameters affecting encapsulation efficiency and release of bioactive molecules.

There are some restrictions in terms of bioactive molecule release from biopolymer microspheres, such as:

1. The burst release effect and very fast release profile of bioactive molecules particularly from hydrogel-based microspheres.
2. Low release rate of bioactive molecules and, therefore, insufficient dosage at later times, and incomplete release due to bioactive molecule entrapment within the microspheres usually in the case of synthetic biopolymer microspheres (5).

Emulsion evaporation has been used for a long time to form polymeric spheres from as-prepared polymer solutions (6-8). In this chapter, we introduce optimized, reproducible methods for fabrication of two types of biopolymer-based microspheres. Alg (hydrogel) and PLGA (synthetic polymer) microspheres were successfully fabricated by applying emulsion and double-emulsion methods, respectively. Fabrication parameters and polymer and bioactive molecule concentrations, as well as other ingredients (such as calcium chloride as the cross-linking agent for Alg), have been optimized. Fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) was used as the model protein and it was encapsulated into Alg and PLGA microspheres for release studies. Protein loading methods into Alg and PLGA microspheres were based on post-loading and pre-loading, respectively. The microsphere morphology, size distribution and encapsulation efficiency were assessed. The release tests from Alg and PLGA microspheres were performed in a simulated body fluid (SBF). The results have shown promising controlled release profiles for the as-prepared Alg and PLGA microspheres.
2.2 Materials and Methods

2.2.1 Materials

Ultrapure sodium alginites (trade name PRONOVA) were purchased from Novamatrix, now part of FMC Bio-Polymer, as guluronic acid rich alginate (LVG, vis of 1%w/w: 20-200 mPa.s). The D, L-lactide and glycolide copolymer (PLGA, lactide : glycolide (50:50) – mol wt 30,000-60,000, inherent viscosity 0.4 dl/g) was purchased from Corbion. Poly(ethylene glycol) (PEG, typical M_n 2,000) was obtained from Sigma Aldrich, USA. Calcium chloride dehydrated, ethylenediaminetetraacetic acid (EDTA), sodium citrate, 2-isopropyl alcohol and fluorescein isothiocyanate-conjugated albumin, bovine serum (FITC-BSA) were all purchased from Sigma Aldrich, USA. Recombinant Human β-NGF is obtained from PeproTech, USA. Albumin, Bovine serum (BSA, Fraction V, RIA and ELISA Grade) was purchased from Merck, USA. The EASYstrainer™ Cell Sieves filters (size of pores = 70 µm) were purchased from Greiner Bio-One. Analytical grade dichloromethane (DCM) was obtained from Chem. Supply Pty Ltd. Milli-Q water (18 MΩ cm⁻¹) was used in the preparation of aqueous solutions. A simulated body fluid (SBF) solution, with ion concentrations close to those of human blood plasma and primed with the following ion concentrations of 142 mM Na⁺, 5 mM K⁺, 1.5 mM Mg²⁺, 2.5 mM Ca²⁺, 103 mM Cl⁻, 27 mM HCO₃, 1.0 mM HPO₄²⁻ and 0.5 mM SO₄²⁻ with a final adjusted pH of 7.4±0.05 (9), was used as the medium for the release studies.

2.2.2 Methods

2.2.2.1 Preparation of the FITC-BSA loaded Alg microspheres

The fabrication method for FITC-BSA-loaded Alg microspheres involved two steps. The first step involved preparation of blank calcium alginate (Ca-Alg) microspheres, and the second involved post-loading of FITC-BSA protein into the Alg microspheres. The schematic of the whole procedure is shown in Figure 2-1.

Fabrication of the blank calcium alginate (Ca-Alg) microspheres

The fabrication of Ca-Alg microspheres followed a surfactant-free water in oil (W/O) emulsification method (Figure 2-1). A typical example of the procedure is given here. An aqueous sodium Alg solution with a concentration of 2% (w/v) was prepared. 5ml of Alg solution was added drop by drop into 50 ml of olive oil which was being homogenized at 11000 rpm. The high stirring rate and proper
stirring time (5 min) helped to form small Alg droplets before cross-linking; therefore, the stirring conditions can extremely affect the size distribution of the Alg microspheres. The temperature of the olive oil was controlled using an ice bath prior to (for 5 min) and during the homogenization. The mixture was then homogenized for 5 min under the same conditions and transferred onto a magnetic stirrer, and stirred at 1500 rpm. 3ml of aqueous CaCl$_2$ solution (concentration of 1M) was added drop by drop (at an average rate of 1ml/min) to induce cross-linking of the Alg droplets. The stirring was continued for 1.5 h, after which 20 ml of isopropanol (as a strong dehydration agent which can harden and dehydrate the produced alginate microspheres) (10, 11) was added to the suspension with stirring and was maintained for a further 10 min. The spheres were collected by use of centrifuge and washed 3 times with isopropanol. After the last wash, the microspheres were dispersed into an aqueous solution of 2mM CaCl$_2$. The microspheres were then recovered by freeze-drying and stored at -20 °C.

**Post-loading of fluorescent-labelled bovine serum albumin (FITC-BSA) into Alg microspheres**

5 mg of freeze-dried blank Ca-Alg microspheres was placed in a 1.5 ml eppendorf tube and 20 μl of aqueous solution of 2 mg/ml FITC-BSA solution was added. This concentration was chosen after considering a report by Li et al. (12) who studied the effect of the BSA solution concentration on loading efficiency and capacity of hydrogel-based microspheres. They showed that a solution of 1 to 2 mg/ml of BSA contains the most efficient concentration. The loading procedure was accomplished by incubating the suspension at 4 °C overnight. After this time, the sample was washed with Milli-Q water to remove the free unloaded FITC-BSA. In a further step, the FITC-BSA loaded Alg microspheres were freeze-dried for further studies.

![Figure 2-1. Schematic of the fabrication process of FITC-BSA loaded Alg microspheres.](image-url)
2.2.2.2 Preparation of FITC-BSA loaded PLGA microspheres

The FITC-BSA loaded PLGA microspheres were prepared via a modified water/oil/water (W/O/W) double-emulsion method. The schematic of the whole process is shown in Figure 2-2. Briefly, 1 g of PLGA was dissolved in 5 ml of DCM to form the oily phase (O). FITC-BSA was dissolved in Milli-Q water in 10 and 50 mg/ml concentrations (to study the effect of FITC-BSA concentration on loading efficiency and capacity) to obtain an inner aqueous phase (W1). 250 µl of 10 mg/ml solution (final product designated as PLGA microspheres type α) or 500 µl of 50 mg/ml solution (final product designated as PLGA microspheres type β) was dispersed into the O phase by sonication (112 W, 5 min). The resulting primary emulsion (W1/O) was poured into 10 ml of 4% w/v aqueous PVA solution (as the outer aqueous phase (W2)) and sonicated at 112 W for 2 min. The mixture was then added into 250 ml of aqueous 0.4% w/v PVA solution and the resultant double-emulsion was stirred at 1500 rpm for 2 h to evaporate the organic solvent at room temperature. The produced particles were filtered using an EASYstrainer™ Cell Sieves filters (size of pores = 70 µm). The residual PLGA microspheres were then collected by centrifuging and washed three times with Milli-Q water. The microspheres were freeze-dried, and stored at -20 °C.

![Figure 2-2. Schematic of the double emulsion fabrication method applied for fabrication of FITC-BSA loaded PLGA microspheres.](image)

2.2.2.3 Preparation of β-NGF loaded PLGA microspheres

β-NGF loaded PLGA microspheres were prepared using a similar procedure as mentioned above. Some modifications have been applied to the fabrication process of β-NGF loaded PLGA
microspheres to prevent the loss of bioactivity of NGF as much as possible (discussed further in section 2.4.5). In this regard, a mixture of DCM with acetone (with the ratio of 2:1) was used as solvent for PLGA. Moreover, an aqueous solution of three biomaterials with the ratio of NGF: BSA: PEG of 1:50:100 was used as protein solution. A further discussion of the effect of applied materials is provided in section 2.4.5. All the conditions and ratios of materials were maintained as previously described in the W/O/W double emulsion method. At the end of the fabrication process, the fabricated NGF loaded PLGA microspheres were freeze-dried, and stored at -80 °C.

2.3  Characterization

2.3.1  Rheology of the solutions

The rheological behaviour of initial polymer solutions (Alg or PLGA solutions) was analyzed using an AR-G2 rheometer (TA Instruments, New Castle, DE) equipped with a Peltier plate thermal controller. A2°/36mm cone and plate geometry was used in all measurements. The solutions were set at the equilibrium temperature for 1 min prior to performing the experiments. Viscosity and shear stress were measured as a function of shear rate at 25 °C.

2.3.2  Optical microscopy

Images of as-prepared, dried and wet microspheres were recorded via an optical microscope (Leica M205A, Australia). Average diameters of the microspheres were estimated with ImageJ image visualization software (n=150).

2.3.3  Scanning Electron Microscopy

The dried microspheres were mounted on metal stubs using conductive double-sided tape. Sample’s morphologies (after immersing for 45 seconds in liquid nitrogen) were observed by use of a scanning electron microscope (JEOL JSM-6490 LV, Japan) at an accelerating voltage of 15 kV.

2.3.4  Laser Confocal Scanning Microscopy

The morphology of FITC-BSA loaded PLGA microspheres type α was also examined on a laser confocal scanning microscope (LCSM, Leica TCS SP2, Australia). The excitation and emission wavelengths were 488 and 520 nm, respectively.
2.3.5 Fourier Transform Infrared Spectroscopy (FT-IR)
The FITC-BSA loaded Alg and PLGA microspheres were characterized by FT-IR, using a FT-IR spectrometer (Shimadzu IRPrestige-21 infrared spectrometer, Japan). The samples were air-dried at 20°C. Fourier transform infrared (FTIR) spectra were measured between 750 and 4000 cm\(^{-1}\).

2.3.6 Differential Scanning Calorimetry (DSC) thermograms
DSC analysis was performed on a DSC Q100 from TA Instruments at a rate of 10 °C min\(^{-1}\) between 25 and 200-300 °C on 3.5 mg samples. The reported data are the results of the first heating run.

2.3.7 Thermogravimetric analysis (TGA) Profiles
Thermogravimetric analysis (TGA) was performed using TA Instruments TGA Q500 on 5 mg of samples with a heating rate of 10 °C min\(^{-1}\) under a nitrogen atmosphere.

2.3.8 Quantification of protein loading efficiency
**FITC-BSA loaded Alg microspheres.** To assess the loading efficiency of FITC-BSA protein in the Alg microspheres, 5 mg of dry FITC-BSA loaded Alg microspheres were suspended in 1 ml of aqueous solution of 10 mM EDTA and 10 mM citric acid for 4 hours at room temperature until the Alg microspheres were dissolved. After vigorous agitation, the suspension was centrifuged (3000 rpm, 5 min) and a 200 µl sample was extracted for further analysis using a microplate reader (Ex\(_{\text{max}} = 485\) nm and Em\(_{\text{max}} = 520\) nm). The protein loading (µg/mg microspheres) was quantified using a standard curve (R\(^2 = 0.998\)) of fluorescence intensity vs FITC-BSA concentration. The results were expressed as mean ± standard deviation (n = 3).

**FITC-BSA loaded PLGA microspheres.** For assessment of the PLGA microspheres, the microspheres (5 mg) were dissolved in 0.5 ml DCM and then extracted with 0.5 ml Milli-Q water overnight. The mixture was centrifuged (3000 rpm, 5 min) and the aqueous phase was collected for quantification of protein loading, using the method described above.
2.3.9 Protein Release Study

Release studies from FITC-BSA loaded Alg and PLGA microspheres (type α and β) were carried out in SBF at 37 °C, respectively. A typical example is given here. 20 mg of microspheres was placed in a 1.5 ml Eppendorf tube with 300 µl of SBF. The entire setup was placed in an incubator shaker in slow shaking mode. At each time point, the mixture was centrifuged with the supernatant being collected for further analysis and replenished with fresh SBF and re-incubated until the next time point. The amounts of FITC-BSA released from the Alg and PLGA microspheres were quantified using a fluorescence spectrometer at Ex$_{\text{max}}$ = 485 nm, Em$_{\text{max}}$ = 520 nm.

Based on the release studies, the PLGA microspheres were chosen for further studies. To study the β-NGF release from PLGA microspheres, a similar procedure was adopted. Briefly, 20 mg of β-NGF loaded PLGA microspheres was immersed in a release media containing phosphate-buffer saline (PBS), with 0.1% (w/v) BSA (as a carrier and protector protein for NGF). The supernatants were collected at specific time points and stored in -80 °C for further study. The amounts of β-NGF released from the PLGA microspheres were determined by an immune-enzymatic assay (ELISA) following the protocol provided by the supplier.

2.4 Results and discussion

2.4.1 Rheological behaviour of initial polymer solutions

The rheological behavior of solutions is the main affecting parameter on the final size distribution of the droplets and microspheres. It is reported that solutions with too high viscosities usually form bigger droplets while the solutions with too low viscosities are not able to form stable droplets or beads (13, 14). The proper viscosity of each solution differs based on polymers, solvents and final application. The rheological behaviour of the initial blank Alg and PLGA solutions used for the fabrication of microspheres was studied based on their viscosity and shear stress versus shear rate curves. The viscosity and shear stress of the aqueous Alg solution (2% w/v) are reported at various shear rates (between 1 to 1000 1/s) in Figure 2-3. As can be seen, the Alg solution experiments show an almost constant value of viscosity in lower shear rates (from 1 to 20 1/s) and a slightly downward trend with increasing shear rates. Moreover, in terms of shear stress, we observe a constant increasing
trend according to the expected Newtonian behaviour. It can be seen that a decrease in the viscosity occurred at higher shear rates which is attributed to the swelling behavior of Alg (15).

It is reported that the viscosity of Alg solution must be above 60 mPa.s to make spheres with good mechanical properties (16, 17). Too high viscosity (>500 mPa.s) of the initial solution can lead to deformation in the final Alg beads. The analysis shows that the viscosity of the solution is suitable for further fabrication applications.

Figure 2-3. (A) Viscosity and (B) shear stress vs. shear rate curves of initial aqueous Alg solution used to make microspheres.
Rheological behaviour and specifically viscosity is one of the major controlling parameters of PLGA microspheres development (18). Figure 2-4 illustrates the variations in viscosity and shear stress of the PLGA solution (20% w/v in DCM) as a function of shear rate. In low shear rate range with high viscosity, many forces present in the oil phase (PLGA solution) which need to be overcome to form fine particles (19). Moreover, molecular weight distribution, concentration of polymer solution and interaction of polymer molecules with the solvent are the most important variables affecting practical processing and performance of polymeric materials as a result of affecting their rheological behaviour.

A high molecular weight or a high concentration increase how far the material can stretch before rupturing (20). It is reported that highly viscous PLGA solutions produce microspheres with a dense core, which are supposed to show less initial protein burst release (19). Microencapsulation processes are based on the principle of so-called "solvent extraction/evaporation". The viscosity of a polymer solution can affect the rate of solvent extraction and evaporation, which in turn affects the microsphere properties like the ability to encapsulate proteins, specific surface area and porosity (21).

In both solvent extraction and evaporation, the solvent of the disperse phase, i.e., protein/matrix dispersion must be slightly soluble in the continuous phase so that partitioning into the continuous phase can occur leading to precipitation (solidification) of the matrix material (22). As can be seen, there is a continuous decrease in viscosity (from 109 to 68 mPa.s) and consistent increase in shear stress with increasing shear rate. It is reported that the viscosity range of 32 to 440 mPa.s is required for producing microspheres encapsulating bioactive molecules for controlled and sustained delivery purposes (23). It is worth mentioning that this range might slightly differ for various bioactive molecules and fabrication procedure conditions. As can be seen in Figure 2-4 (A), the viscosity of the PLGA solution, with applied concentration in this study, perfectly matches with the mentioned range and it means that the applied molecular weight and concentration, as the main parameters determining the rheological behaviour of solutions, are suitable for fabrication of PLGA microspheres encapsulating proteins.

As discussed above, polymer chemical structure, hydrophilicity, molecular weight distribution, concentration in a solution, solvent type and its molar mass and density are some of the determining parameters that affect the rheological properties of solutions. Comparing the viscosity curves of Alg and PLGA materials reveals that .
Figure 2-4. (A) Viscosity and (B) shear stress vs. shear rate curves of initial PLGA solution in DCM used to make microspheres.

Figure 2-5 illustrates the variations in viscosity and shear stress of the PLGA solution (20% w/v in DCM and acetone) as a function of shear rate. Although that DCM is miscible in acetone, but using the mixture of them as solvent leads to some changes in rheological behaviour of PLGA solution.

Figure 2-5 shows that with increasing shear rate, the viscosity and shear stress of the solution have been decreased and increased, respectively. It can be seen in Figure 2-5 A that despite the similar trends (Comparing Figure 2-4 and Figure 2-5), the viscosity range of polymer solution in a mixture of DCM and acetone (in shear rates less than 100 1/s) is much higher than solution made from dissolving PLGA in DCM. It could be explained considering the interactions between two mixed liquids. Viscosity is determined by interactions between the randomly coiled and swollen macromolecules and
the surrounding solvent molecules (in this case: mixture of DCM and acetone). If two liquid (i.e. DCM and acetone) with similar intermolecular interactions are combined, they are said to be miscible with each other and so that they will mix to form a homogenous solution. This phenomenon can lead to formation of some inter-forces between the molecules of two liquids (24). In addition, dissolving the PLGA 50:50 (applied in this study) in either DCM or acetone cause formation of weak interactions between macromolecules of PLGA and solvent molecules. The strength of these interactions depends on molecular weight and polarity of solvents. Both of these solvents have polar and non-polar side. It suggests that smaller molecules of acetone (with average molecular weight of 58.08 g/mol) can cause slightly stronger interactions with PLGA macromolecules in compare to DCM molecules with 84.93 g/mol.

Figure 2-5. (A) Viscosity and (B) shear stress vs. shear rate curves of initial PLGA solution in DCM and acetone used to make microspheres.
2.4.2 Morphological study of the microspheres

Ca-Alg microspheres were produced by applying a W/O emulsion method. The as-prepared (dispersed in water and freeze-dried Ca-Alg) microsphere morphologies were studied by optical and LV-SEM microscopy, respectively. Figure 2-6 A shows that the as-prepared Ca-Alg microspheres were well disperse in water with rounded shape and smooth surface. In order to study the morphology of the hydrated spheres after protein loading, a small drop of the sample was placed on a sample holder and then it was immersed in liquid nitrogen for 45 seconds. Figure 2-6 B shows the LV-SEM images of FITC-BSA loaded Alg microspheres. As expected, the rehydrated spheres are almost reformed and they present an almost spherical, smooth morphology. It appears that the re-swelling of spheres in aqueous media caused an agglomeration of the spheres (Figure 2-6 B).

![Figure 2-6](image)

Figure 2-6. (A) Optical microscopy of as-prepared blank Ca-Alg microspheres dispersed in water, and (B) LV-SEM images of hydrated FITC-BSA loaded Alg microspheres in SBF, (B-i) LV-SEM image of a single hydrated FITC-BSA loaded Alg microsphere in SBF specifying the sphere dimensions.

PLGA copolymers have been used for fabrication of microspheres loaded with FITC-BSA protein by applying a double emulsion method. Basically, the final size of the generated spheres is smaller than that of the formed droplets in the first and second emulsions. That is due to the evaporation of the solvent (DCM) in the final step of the process which causes a slight shrinkage in the microspheres (25). Figure 2-7 shows the morphology of the FITC-BSA loaded PLGA microspheres. The generated microspheres (containing different levels of protein) were spherical in shape and there is no evidence of agglomeration. The optical microscopy images of the as-prepared FITC-BSA loaded PLGA
microspheres type α and type ß, while they are dispersed in water, are present in Figure 2-7 A and B. These images show no evidence of agglomeration in microspheres.

Figure 2-7 C and D illustrate the confocal laser microscopy images of the FITC-BSA loaded PLGA microspheres. The emission of green fluorescence suggests that the preloaded fluorescent-labelled protein was encased within the PLGA microspheres. Moreover, Figure 2-7 A and B suggest the presence of pores inside the microspheres structure with a thin outer layer. The size range of the internal pores (Figure 2-7 A and B) was 0.2 to 13.1 µm (measured using ImageJ software). Moreover, Figure 2-6 F-i represents the LV-SEM image of the internal structure of a single microsphere representing the spatial arrangement of the pores inside a single microsphere. This phenomenon can be explained by considering the fabrication progress. As already mentioned, in the first emulsion (W1/O), aqueous FITC-BSA solution droplets were dispersed into PLGA solution to form initial microcapsule droplets. Through this step, water (aqueous solution) is used to produce water droplets (internal aqueous phase) inside the PLGA phase. This is a result of using an ultrasonic probe machine, which plays a key role to form porous structures of the PLGA microspheres (26). In the next step (W1/O/W2), the formed droplets were effused internally into the internal PVA solution. The second PVA solution (named as external PVA solution with lower concentration) can cause the formation of the internal pores during the solvent evaporation and the subsequent polymer solidification. The kinetics of the possible internal reaction which creates these pores and the parameters affecting the size of them are imperceptible (26). There are a few studies on the development of different methods to fabricate spheres with interior pores such as adding hydrogen peroxide in first emulsion (27) or ammonium bicarbonate as a sparkling salt in the primary droplets (28) with the aim of improving the bioactive molecules release profiles. It is reported that the higher level of water present in first emulsion step, which can be provided by applying an appropriate amount of aqueous solution of protein, results in formation of PLGA microspheres with an internal porous structure (27). Furthermore, it is reported that the molecular weight of PLGA has a critical effect on the creation of pores through the microsphere structure (27). In this study, a PLGA material with the molar ratio of 50:50 was applied. Bae et al. (27) studied the effect of the molar ratio of PLGA copolymer on formation of internal pores through the microspheres structure. They reported that using PLGA with the molar ratio of 50:50 results in fabrication of well-organized, covered porous (high number of
internal pores) microstructure PLGA microspheres while other molar ratios (such as 75:25, 85:15, and 95:5) present a poor morphology with enlarged internal pores under similar fabrication circumstances (27).

Figure 2-7 E and F display the LV-SEM images of the FITC-BSA loaded PLGA microspheres. As can be seen, PLGA microspheres show a smooth surface with a few displaying surface pores (shown with orange arrows (Figure 2-6 E)). The surface pores develop as a result of the evaporation of DCM. Larger microspheres have a comparatively smaller ratio of surface to volume and thus the evaporation rate is slower. Hence, larger microspheres need a longer time to solidify and therefore undergo more severe deformation, which results in the formation of surface pores and even collapsing for some of them (shown with green arrows (Figure 2-7 E)).
Figure 2-7. (A and B) Optical microscopy images of as-prepared PLGA microspheres type $\alpha$ and $\beta$, respectively. (C and D) Confocal laser microscopy and (E and F) LV-SEM microscopy images of the microspheres type $\alpha$. *Orange arrows: surface pores. **Green arrows: collapsed structures. (F-i) LV-SEM microscopy image of internal structure of a single microsphere type $\alpha$ representing the spatial arrangement of internal pores.

Based on a study by Pean et al. (29), a mixture of DCM with acetone (with the ratio of 2:1) as solvent for PLGA has a less detrimental effect on NGF stability than DCM alone. They reported that using acetone can increase the permeability of final microspheres (30). So that, in this study, a mixture of
solvents (DCM and acetone) was used for dissolving the PLGA, which might affect the formation of spheres due to the faster evaporation during the fabrication process. Figure 2-8 presents the surface morphology of β-NGF loaded PLGA microspheres. Figure 2-8 does not display any significant defect on surface morphology of microspheres which can be attributed to the fast evaporation of acetone.

Figure 2-8. LV-SEM image of β-NGF loaded PLGA microspheres.

2.4.3 Study of the size distribution of produced microspheres

The mean diameters and the size distributions of as-prepared Alg and FITC-BSA loaded PLGA microspheres and β-NGF loaded PLGA microspheres were determined by measuring 100-150 beads from optical images (Figure 2-9 and Figure 2-10 A, B and C). Different processing parameters i.e. the polymer concentration, viscosity of the solutions, composition of the dispersed and continuous phases, temperature, homogenizer speed and the stirring rate in the emulsion steps (first or second) may affect the droplet formation and, consequently, the mean microsphere diameter. For instance, it is reported that the higher the concentration of polymer, the larger the microspheres that are produced (3, 28, 31).

Based on the numerous studies, smaller spheres of hydrogel with narrow size distribution afford enhanced properties such as the release profile and lower possibility of the rupture of the beads (32-34). Traditionally, the fabrication of Alg microspheres has been done by extruding an Alg solution from a needle into a divalent cationic solution to activate the gelation process. Although this method enables the retention of full biological activity of biomolecules, there is no control over the bead size and size distribution of the product. Therefore, the final beads might be too large or polydispersed to
be suitable for some bioapplications and also they cannot be incorporated into continuous structures such as fibres.

Figure 2-9. Size distribution of blank Ca-Alg microspheres.

To investigate the particle sizes under conditions that mimic the environment of a potential application (i.e. dispersed in aqueous media), the size distribution of hydrated Alg microspheres was studied. The results are shown in Figure 2-9. It can be seen that the produced Alg spheres have a relatively narrow size distribution with a mean diameter of 11 (±3.1) µm and approximately 94% of them are smaller than 20 µm, which could be due to the rather low viscosity of the initial polymer solution (35).
Figure 2-10. Size distribution of (A) FITC-BSA loaded PLGA microspheres type α, (B) FITC-BSA loaded PLGA microspheres type β, and (C) β-NGF loaded PLGA microspheres.

The mean sizes of PLGA microsphere types α and β were 26 (± 14.7) and 12 (± 10.1) μm, respectively, determined by using the optical microscopy images of as-prepared microspheres (Figure 2-10 A and B). These numbers were 13 (± 6.3) and 9.55 (± 18.1) for NGF loaded PLGA microspheres. The size of both types of FITC-BSA loaded PLGA microspheres changed from several to tens of microns but it is noticed that the type β spheres, which were loaded with almost ten times
more protein had a smaller mean size. It suggests that the presence of higher volumetric ratio of W/O in first emulsion (the presence of more water (with lower relative volatility rather than DCM) as the solvent of protein in first emulsion) can postpone the formation of spheres as a result of fast extraction/evaporation of solvent (36). It provides the chance for them to form with a more uniform size distribution (less number of large spheres and lower STD deviation (comparing Figure 2-10 A and B)). In addition, the frequency-based size distribution results of NGF loaded PLGA microspheres (Figure 2-10 C) reveals that the average diameter of ß-NGF loaded PLGA microspheres are almost similar to FITC-BSA loaded PLGA microspheres type ß. Figure 2-10 C also shows that the number of large microspheres are less in compare to (Figure 2-10 A and B) and hence most of the microspheres have a diameter less than 20 µm. The NGF loaded PLGA microspheres are produced using a similar procedure of FITC-BSA loaded PLGA microspheres type ß. Polymer concentration, solvent evaporation rate, the volume ratio of primary aqueous protein solution as well as volume ratio and concentration of the PVA solution are introduced as the main affecting parameters on size of forming PLGA microspheres (37). Among them, the only parameter that has been changed for NGF loaded PLGA microspheres is the solvent mixture (using a mixture of DCM : acetone with the ratio of 2:1). The combination of acetone with DCM has affected the elimination of solvent as a result of changing the boiling point and density of the solvent (the two determining parameters of relative volatility). Also, it seems that acetone (with higher boiling point rather than DCM) plays the similar role of having extra amount of water in primary emulsion. In addition, miscibility of water and acetone leads to formation of some inter-forces between these two liquid which need to be overcome during the solvent extraction step to form fine particles. This phenomenon also might postpone the formation of final spheres and help in formation of spheres with more uniform size range. Hence, its presence leaded to retarding the fast extraction/evaporation of solvent and slowing the formation of spheres.

2.4.4 Assessing the yield and loading efficiency of fabrication methods and loading capacity of microspheres

The yield (productivity) of the fabrication method, loading efficiency (LE) and practical loading (PL) values of microspheres were calculated according to the Equations 2-1, 2-2 and 2-3, respectively (12, 38-42). All the experiments to assess the yield, loading efficiency and practical loading of microspheres fabrication method have been repeated three times and an average of the results are reported.
Table 2-1 shows the effect of volume and concentration of cross-linking solution on final yield of fabrication method of Alg microspheres. Due to the results present in Table 2-1, less amounts of CaCl₂ solution led to less yield whereas higher amounts did not significantly affect the productivity of the method. Also, while the amount of CaCl₂ is similar, the higher volume of solution caused a higher yield. Hence, the volume of 3 ml of a CaCl₂ solution with the concentration of 1 M is considered as the optimized amount of cross-linking solution. As can be observed in Table 2-1, the yield of fabrication for Alg microspheres (produced with the optimized amount of cross-linking solution) was 55% (±13).
Table 2-1. Investigation of the effect of CaCl₂ solution on final yield of fabrication method of Alg microspheres.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of moles</th>
<th>Concentration of CaCl₂ solution (M)</th>
<th>Volume of CaCl₂ solution (ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>17 ±10</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>0.5</td>
<td>3</td>
<td>25 ±3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.5</td>
<td>4</td>
<td>38 ±14</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>15 ±8</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>36 ±19</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>55 ±13</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>53 ±16</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>59 ±6</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>32 ±8</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>2</td>
<td>1.5</td>
<td>47 ±19</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>57 ±7</td>
</tr>
</tbody>
</table>

The yield of fabrication was 64% (±5) for PLGA microspheres type α and 72% (±2) for PLGA microspheres type β. Considering the larger mean size of PLGA microspheres type α, it could be argued that the initial batch of the product possibly contained more spheres larger than 70 µm as compared to type β; therefore, they were removed after filtration and, consequently, type α has a lower yield. Figure 2-11 shows the efficiency of fabrication methods in encapsulation of FITC-BSA. It is not a surprise that some of the added protein and resultant microspheres were lost during the fabrication processes, specifically, the washing and filtration stages.
Figure 2-11. Loading efficiency of FITC-BSA into the Ca-Alg and PLGA microspheres and practical loading of microspheres. *PLGA microspheres type α. **PLGA microspheres type β.

2.4.5 FITC-BSA release profile from Alg and PLGA microspheres

The encapsulation of FITC-BSA into Alg microspheres, via the presented preparation method, resulted in an almost long-term release of the protein into the surrounding media. The cumulative release of FITC-BSA from Alg microspheres is shown in Figure 2-12. The studies ran over a period of 21 days and, at specific time intervals, the whole release medium (SBF) was collected and replaced with an equal volume of fresh medium. In the first 24 h, the initial burst release of the model protein (FITC-BSA) rose to more than 20% and, over 21 days, up to ~78%, of completion which is much slower than those reported from Alg microspheres of similar sizes (43). This can be due to the relatively higher degree of cross-linked structure of the Alg spheres as a result of its high level of G-block content (44, 45). In this study, the ultrapure low viscosity sodium alginates (trade name PRONOVA) was applied to fabricate the alginate microspheres. The G to M blocks ratio of this type of alginate is ≥1.5. This ratio is equal to 1 for the common commercial alginate materials which are applied in other studies (44, 45).
Calcium chloride (CaCl₂), acts as the cross-linker agent for Alg hydrogels by forming electrostatic bridges between the negatively charged polysaccharides. These divalent cations make junction zones in that they contribute to the intermolecular binding between the moderately stiff G-blocks on different Alg molecules. This phenomenon results in the formation of a three-dimensional spherical network gel. This binding zone between G-blocks is referred to as the “egg-box model”. The cross-linking procedure is schematically shown in Figure 2-13. In the first step of cross-linking, the CaCl₂ reacts with sodium alginate on the surface of the beads, to form a thin outer layer of calcium alginate. When enough Ca²⁺ ions are available, the gradual diffusion of CaCl₂ into the core of the particles would harden the liquid core into rigid microspheres (31, 46). This phenomenon explains how the Alg composition with higher G-block ratio can form beads with stiffer structure. In 2010, Khanna et al. (47) studied the effect of Alg composition on the release rate of the encapsulated model protein (BSA) and growth factors. They reported that Alg type LVG which has a ratio of G to M-blocks (equal to 1.5, similar to the Alg material used in this thesis), has the lowest initial burst release. Moreover, Figure 2-12 reveals that the FITC-BSA release profile from Alg microspheres continued (after the first day) at lower levels compared to the reported studies (48-51) over 3 weeks which confirms the efficiency of the fabrication method and cross-linking procedure and time for the Alg microspheres.
Figure 2-13. (A) The Ca\(^{2+}\) (released from CaCl\(_2\)) dependent cross-linking process and the formation of the “egg-box model”, (B) forming hard gel (51).

On the other hand, bioactive molecules release from Alg structures is typically modulated by a swelling–dissolution–erosion process (52). Theoretically, swelling in hydrogels regulates the speed and ease of a liquid to penetrate in both the processes of protein loading into hydrogel beads and/or protein release from them. Due to the stiff structure of the produced Alg microspheres, they cannot be easily dissolved in release fluid such as SBF. SBF contains a low amount of various positively charged ions such as Ca\(^{2+}\). The di-axial configuration of G-blocks permit a more favorable interaction with the cation, which results in a greater degree of gelation, so that the breaking of cross-linking bonds and, consequently, the dissolving and degradation of the Alg structure may occur in days or even months. This was confirmed by measuring the residual mass of microspheres following a 21 days’ release experiment, which was 57% (±11%) of the initial mass.

Figure 2-14 A and B show the FITC-BSA release profile of PLGA microspheres type α and β, respectively. As can be observed, for both types of spheres, less than 60% of loaded protein was released during the observation period. It is known that the diffusion of the protein through a
polymeric matrix and the actual polymer-erosion rate itself cause release of the encapsulated protein from the PLGA microspheres in an aqueous media (53-56).

Figure 2-14. The cumulative release profiles of FITC-BSA loaded PLGA microspheres (A) type α and (B) type β in SBF.

The degradation of PLGA generates a build-up of acidic monomers and oligomers, which causes a decrease in the pH level of the polymeric matrix where proteins tended to aggregate and denature. Conspicuously, the bioactivity of encapsulated protein can be partly lost due to the release of acidic degradation products of PLGA which lead to the denaturation of proteins (57). This phenomenon can
lead to unexpected modifications of the expected protein release profile from PLGA microspheres (even after a long observation time) due to protein denaturation (57-60).

The protein release profiles from synthetic polymer structures are entirely consistent with the microspheres’ degradation/erosion (58). As can be observed in Figure 2-14, the protein-loaded PLGA microspheres, type α and β show a biphasic release profile, with an initial stage of burst release (up to 33% of the loaded mass of protein) and a second phase of sustained release related to the erosion of PLGA, which confirms the previous studies (57, 59, 60).

The initial burst release is mostly attributed to the proteins on the surface of the spheres. The next steps of release happen due to diffusion of the proteins through the swelling matrix. Swelling micro-cavities can hasten the release of FITC-BSA from PLGA microspheres during the burst and diffusion phases. The remaining release corresponds to PLGA erosion (61). Comparing the profiles of type α and β spheres indicates that the spheres with smaller mean size (type β) have a continuous sustained release profile with an almost linear trend over the test period. This can be explained by considering the size distribution of the spheres. As was expected, the wide size range of spheres type β (Figure 2-10 B) causes the different release speeds from the spheres. The larger microspheres lead to a slower release profile because of their lower surface area to volume ratio for protein diffusion (61). As can be seen in Figure 2-14, the overall amounts of protein released and also the initial burst release level from type β microspheres were lower than type α microspheres. Therefore, the encapsulation of proteins into PLGA microspheres through the established method for type microspheres avoids the initial local overdose and also enables the sustained longer-term protein release profile. A sustained release of proteins may overcome many of the overdose drawbacks such as causing the side effects and wasting the expensive proteins. Moreover, the proteins should remain at the target location sufficiently long to play their roles in treatment. Overall, considering the relatively large size of proteins, their short biological half-lives resulting in rapid loss of bioactivity, tissue specificity and the potential toxicity at high levels, the microspheres providing a sustained extended release profile are preferred. In addition, since the fabricated microspheres would be further incorporated into alginate solution to form fibres through a wet-spinning process, the microspheres with slower release profile which are able to provide less amount of burst release are of interest. This can help to avoid release of huge amount of
protein prior to final spinning process. Consequently, the FITC-BSA loaded PLGA microspheres (type β) with almost linear release profile were chosen for further studies.

Serum albumins play different role during the encapsulation procedure of NGF into PLGA microspheres (26, 62). They are known as carrier and protector protein for NGF in aqueous solutions and a stabilizer for the primary emulsion. Hence, many researchers, focusing on developing structures for protein delivery, use a model protein antigen such as bovine serum albumin (BSA) for protein release studies of structures to avoid the waste of expensive NGF. BSA is one of the most widely studied proteins due to its medical importance, availability, unusual ligand-binding properties, low cost compared to NGF and being a homologous protein (26, 62-64). Moreover, Stroh et al (65) showed an almost similar tortuosity and diffusion coefficient in solution for BSA and NGF. Tortuosity (as one of the release profiles’ determining parameters) is defined as the square root of the ratio of the free diffusion coefficient in solution (Df) to the apparent diffusion coefficient in tissue (Db). They reported that both proteins assigned to the high-tortuosity category and they reported the tortuosity amounts of ~2.24 and 2.14 and Df values (and hence hydrodynamic radius) of ~11.9 and 12.1 cm²s⁻¹ for albumins and NGF, respectively. Since BSA has a negatively charged structure, it won’t behave as a component or competitor for multi-charged structured NGF in attaching to the slightly negatively charged cells’ surface. It makes BSA as a good candidate to be applied as a carrier for NGF during the encapsulation processes. BSA is considered as a small protein (Mw ~ 66500 Da) and a potential carrier which can help in preservation of NGF activity for further studies on NGF. Hence from biofabrication aspects, the encapsulation properties of BSA (as the major component for NGF in protein solution) must be investigated. Moreover, in this study, fluorescently-labelled BSA has been applied to be able to study of the protein distribution into structures easier. Figure 2-15 shows the release profile of NGF for PLGA microspheres in media containing phosphate-buffer saline (PBS), with 0.1% (w/v) BSA. Based on a study by Pean et al. (26), polyethylene glycol (PEG) was used as stabilizer and carrier for NGF. These researchers have shown that PEG could significantly improve the stability of NGF (29). On the other hand, De Boer et al. (62) studied the effect of using BSA in NGF aqueous solution as the carrier protein and they reported that BSA can successfully help in stabilization of NGF. Consequently, an aqueous solution of these three biomaterials as protein solution to be encapsulated was chosen. From the results presented in Figure 2-15, less than 4% of
NGF was released in the first day and the release continued at a slow rate during the following days of observation. Notably, these results are much slower than the reported rates in literature (62-64). For example, Pean et al. (29, 30) reported an average of 20% burst followed by a continuous release, and after 21 days more than 50% of NGF was released. Menei et al. (66) reported a high level of burst, more than 30% at the first day. Sun et al. (67) reported an almost low level of burst release (~10%) of NGF from PLGA microspheres, produced via a double-emulsion method. They stated that the GFs were fully released (more than 90%) after four weeks while they were encapsulated with different protein carriers such as PEG, BSA, human serum albumin and ovalbumin in the PLGA microspheres. It seems that future optimization of the encapsulation procedure, including polymer concentration and protein carriers, is needed to improve the release profile of NGF.

![Graph](image)

**Figure 2-15.** Release profile of β-NGF loaded PLGA microspheres in a media containing phosphate-buffer saline (PBS), with 0.1% (w/v) BSA.

On the other hand, the preservation of the bioactivity of NGF, during the encapsulation process, has a significant effect on their functionality *in vitro* and *in vivo*. Since De Boer et al. (62, 68) fabricated NGF loaded PLGA microspheres and they assessed the bioactivity of released NGF from PLGA microspheres using a rat DRG bioassay. The released NGF promoted extension of neurites after day 8,
11, 14, 17, 20 and 23. Pean et al. (26) investigated how having PEG in protein solution and acetone as cooperative solvent (with DCM) can lead to improved NGF stability and retained NGF bioactivity during fabrication of NGF loaded PLGA microspheres. They assessed the NGF stability by comparing the amount of released NGF as measured by radioactivity counting and by ELISA. Their results showed PEG (in corporation with albumins) is a good candidate to protect NGF against physical denaturation by contact with an organic phase during emulsion. In addition, DCM/acetone mixture seemed less detrimental to NGF stability that DCM alone. As a result, they reported that applying a mixture of DCM/acetone as organic phase and a solution of NGF as aqueous phase, albumins and PEG can increase the number of activated NGF delivered by up to more than 8 times compared to not having these two materials (26). In a different study by this group, they reported an optimised volume ratio of 2:1 for DCM/acetone as the organic phase (29). Moreover, Johnson et al. (69) studied the effect of PEG:NGF mass ratios on maintaining the activity of NGF. Their results demonstrated the usefulness of using such optimised concentration of PEG in both acting as a progen to modulate release and aiding in the preservation of activity of NGF (6). In this study, the De Boer et al. (62, 67) approach for fabrication of NGF loaded PLGA microspheres were followed and some modifications (applying a mixture of DCM/acetone as organic phase and a solution of NGF as aqueous phase) have been applied to increase the preservation of NGF bioactivity. The NGF release profile of microspheres prepared by this methodology in this study (analysed by ELISA assay) has shown the presence of released amounts of NGF in release media, but the functionality of the released NGF protein is still unclear. However, adsorption of the NGF epitope to its antibody (which was generated using functional NGF) in the ELISA assay may suggest that at least at the structural level, the post-release NGF was structurally sound, but in vitro analysis of specific activity for the released NGF fell outside the scope of this study. The body of work presented in this thesis is mostly focused on engineering aspects of establishing adjustable fabrication techniques and biofabrication of two-phase longitudinal delivery systems and characterizations of them (protein-loaded spheres incorporated into microfibrous structures, as discussed in Chapter 3 and 4). Furthermore, comparing the NGF release profile from PLGA microspheres presented in this chapter with literature suggests that future optimization of the encapsulation procedure, including polymer concentration and protein carriers, is needed to improve the release profile of NGF. Hence, the prepared NGF-loaded PLGA microspheres are still too pre-matured for further biological assessments. Further cell culture studies and optimizing the fabrication
steps which ascertain the functional status of the released NGF (after optimizing the methodology) are highly recommended as a subject for future studies.

2.4.6 Physicochemical characterizations of FITC-BSA loaded Alg and PLGA microspheres

Understanding how the chemical composition and thermal behaviour of microspheres are affected by protein encapsulation processes can provide significant insight into the fabrication processes and how they might be modified specifically for applications in further studies. It may also contribute to an understanding of the involvement of microspheres in protein release (70). The objective of this section is to investigate the effects of the encapsulation of protein on expected physicochemical behaviour (based on literature review) of Alg or PLGA microspheres.

2.4.6.1 Study of the chemical structure of FITC-BSA loaded Alg and PLGA microspheres via their FTIR spectra

The FITC-BSA loaded Alg and PLGA microspheres were characterized by FTIR (Shimadzu IRPrestige-21 infrared spectrometer). The samples were air-dried spheres at 20°C. Fourier transform infrared (FTIR) spectra were measured between 750 and 4000 cm\(^{-1}\).

FTIR spectrum analysis of FITC-BSA loaded Alg is shown in Figure 2-16. The FTIR spectrum of FITC-BSA loaded Alg microspheres shows the Alg characteristic peaks at 3606 cm\(^{-1}\) because of stretching of O-H, 1594 cm\(^{-1}\) due to the stretching of -COO (asymmetric), 1421 cm\(^{-1}\) due to the stretching of –COO and the typical C-O-C stretching vibration which is located at 1080 cm\(^{-1}\), respectively (71-77). Also, a characteristic peak of BSA at 2924 cm\(^{-1}\) due to C-H (alkyl) stretching can be observed in FITC-BSA loaded Alg microspheres spectrum (73). Due to these results, no indication of a strong chemical interaction, changing the nature of the functional groups of polymers, can be detected.
Figure 2-16. FTIR spectrum of FITC-BSA loaded Alg microspheres. The characteristic peaks of Alg and BSA materials are specified.

The FTIR spectrum of FITC-BSA loaded PLGA microspheres (Figure 2-17) shows a vibrational peak at ~1745 wavenumber which is attributed to carbonyl (C=O) stretching of PLGA. Two more characteristic peaks between 1050-1250 cm\(^{-1}\) due to stretching of C-OH groups and the stretching of (CO)-OC groups are detected in the protein-loaded PLGA microspheres spectrum (23, 26, 78-85). The main peak of BSA is also detected in the FTIR spectrum of FITC-BSA loaded PLGA microspheres.
2.4.6.2 Study of the thermal behaviour of FITC-BSA loaded Alg and PLGA microspheres (type β)

Thermogravimetric analysis (TGA) was performed using TA Instruments TGA Q500 on 5 mg of microspheres samples with a heating rate of 10 °C.min\(^{-1}\) under a nitrogen atmosphere. Differential scanning calorimetry (DSC) analysis was performed on a DSC Q100 from TA Instruments at a rate of 10 °C.min\(^{-1}\) between 25 and 200-300 °C on 3.5 mg samples.

The thermal weight loss of the freeze-dried protein-loaded microspheres was studied to investigate the thermogravimetric change of the material components. As can be seen in Figure 2-18, for the FITC-BSA loaded Alg microspheres, the first mass loss, which was approximately 18% at <100 °C, is due to the evaporation of water (86). A significant weight loss occurs from 200 °C to 340 °C as a result of the thermal decomposition process of the protein and Alg chains. Moreover, the weight loss from 560 °C to 620 °C corresponds to the decomposition of Alg monomers (87-90). The remaining 23% includes elements that decompose above 630 °C.

![FTIR spectrum of FITC-BSA loaded PLGA microspheres (type β). The characteristic peaks of PLGA and BSA materials are specified.](image-url)
The TGA profile for FITC-BSA loaded PLGA microspheres (type β) can be seen in Figure 2-19. The first slight weight loss from 30 °C to 250 °C is attributed to the evaporation of the moisture absorbed by the protein and the beginning of polymer degradation. The second phase of weight loss, between 280 °C and 400 °C, is associated with the decomposition of protein and PLGA (88). The thermal decomposition of PLGA chains is reported to occur between 180 °C and 320 °C (92-94).
Furthermore, DSC thermograms of protein-loaded Alg (between 30 °C to 300 °C) and PLGA (between 30 °C to 200 °C) microspheres were studied (Figure 2-20). DSC is considered as an essential tool to investigate the possible interactions between protein and polymer and physical state of protein and microsphere structure. These features could affect the release profiles of the protein from the systems. This information could be acquired by considering the appearance, shift, or disappearance of endothermic or exothermic peaks in the thermograms (95, 96).
Under the experimental conditions, the DSC thermogram of FITC-BSA loaded Alg microspheres (Figure 2-20) presents a broad exothermic peak between 25 °C and 200 °C attributed to the dehydration process (97-101). Moreover, a strong endothermic peak at 260°C corresponding to the decomposition of the –COO\(^-\) is observed (102). It was expected to be a decomposition peak at 226 °C, as reported in the literature for blank Ca-Alg microspheres (102). It seems that loading FITC-BSA into the spheres caused the mentioned characteristic peak of blank Ca-Alg microspheres (at 226 °C) to shift to higher temperatures (102-104).

Furthermore, Figure 2-21 shows the DSC thermogram of FITC-BSA loaded PLGA microspheres. It can be observed that the specific \(T_g\) peak at 46 °C overlapped with that of PLGA (26, 105, 106). On the other hand, based on the results from the TGA profile of the spheres, it seems that the downward trend starting from about 220 °C corresponds to the gradual degradation of the polymer.

The reported melting point of BSA is between 68 °C to 70 °C. The DSC thermograms of the protein-loaded microspheres (Figure 2-20 and 2-21) indicates that there is no change in the physical state of
the protein. The absence of an endothermic peak for the BSA at the mentioned temperature suggests that the protein existed in an amorphous or disordered crystalline phase as a molecular dispersion in the polymeric matrix \((75, 102, 107)\), which is in agreement with the results from the thermogravimetric study of the structure. Moreover, based on the results from Figures 2-17, 2-18, 2-19 and 2-20, both types of produced microspheres are relatively stable within the practical and physiological temperature ranges.

![DSC thermogram of FITC-BSA loaded PLGA microspheres (type β).](image)

**2.5 Conclusions**

In this chapter, the fabrication of Alg and PLGA microspheres through emulsion and double-emulsion methods were presented. The surface morphology, fabrication yield, practical loading and encapsulation efficiency, protein release from the produced microspheres, chemical and thermal properties of the protein-loaded microspheres were studied. The mean sizes of the FITC-BSA loaded Alg and PLGA microspheres, loaded with low (type α) and high (type β) levels of protein, were 11, 26 and 12 micrometres, respectively. FITC-BSA releases from all types of microspheres showed the classic biphasic profile, which were governed by resolving or degradation (diffusion and polymer erosion) reasons for Alg and PLGA microspheres, respectively. In short, PLGA microspheres loaded
with higher levels of protein provided an efficient system to achieve almost linearly controlled protein release at a rate suitable for further applications. Furthermore, due to the results from thermal analysis of the FITC-BSA loaded microspheres, they are thermally stable in further practical (room temperature) and physiological (~ 37 °C) temperature ranges.

2.6 References


Chapter 3  Development of Alginate Fibres Containing Protein-loaded PLGA Microspheres for Long-term Controlled Release Applications

Wet spun alginate microfibers containing (FITC-BSA) loaded PLGA microspheres were fabricated to achieve an extended protein release profile. The cumulative protein release profile revealed significant dependence on alginate relative viscosity range (low or medium viscosity alginate materials) and concentration as well as concentration of protein-loaded PLGA micropsheres. It was shown that the alginate fibrous matrix retarded protein release significantly in both early and late stages. The final morphology, chemical structure and mechanical properties of prepared fibres were investigated.

3.1  Introduction to fibre structures for controlled delivery of bioactive molecules

Developing fibre structures, in recent years, has offered exciting opportunities in several diverse fields i.e. energy (1-3), environment (4-6) and designing novel biomaterials for bioapplications (7-9). Microfibres have received a great deal of attention in the field of biomedical engineering such as wound healing (10-12), cell or bioactive molecule encapsulation (13-15) and tissue engineering (16-18). One of the main reasons for this is their high ratio of surface area to volume which is essential for efficient bioactive molecule release applications. The fibres’ surface area to volume ratio can be controlled by changing length as well as the cross-sectional radius. In addition, the high mechanical properties, ease of fabrication and desirable protein release profile of fibres leaded to a growing interest in applying micro- and nanofibres for the purpose of protein delivery (19). Additionally, in comparison other structures such as films and nanofibrous mats, microfibres are the only candidate for a two-dimensional delivery structure that also performs a structural role (20). Since this study focuses on developing next generation of delivery structures for linear protein release, microfibers might be the best applicant for this aim because of their ease of fabrication and high surface area in compare to other possible two-dimentional structures (i.e films or twisted electrospun mat yarns).The application
of wet-spun microfibres as vehicles for bioactive molecule delivery for regenerating targeted tissues has been greatly studied (21-24). It has been mostly done by encapsulating bioactive molecules inside the polymer matrices of wet-spun fibres. This system can also be applied for controlled delivery of bioactive molecules to the surrounding cells (25-27).

Wet-spun Alg microfibres present great potential for the design of multifunctional polymeric systems (25, 28, 29) with their properties and functionality being improved by incorporating diverse organic and/or inorganic additives into the fibre matrix (26). Fibre structures used for bioapplications need to provide structural support and controlled delivery of bioactive molecules with a proper release rate. Understanding aspects of bioactive molecule delivery and other properties of fibres may assist in the optimization and development of wet-spun fibre platforms (30).

Based on the results from Chapter 2, it was found that FITC-BSA loaded PLGA microspheres (type β) can provide a long lasting and almost linear release profile. Therefore, these types of microspheres were chosen to incorporate into the hydrogel-based platform to improve the efficiency of the platform system in the controlled release of model protein. In Chapters 2 and 3, FITC-BSA was applied as an analog to the GFs that can be utilized in further studies. BSA is frequently used as both a surfactant and a model protein for bioactive molecule release studies. Moreover, BSA is often incorporated as a protective protein into microspheres loaded with GFs and, hence, applying it as a surfactant in such applications is helpful or even necessary (31).

Various attempts have been made, in terms of incorporation of spheres into fibre structures, to combine the advantages of both structures for different applications. For instance, in 2013, Tang et al. (32) used a technique combining electrospinning and electrospraying to prepare PLGA fibre-microsphere scaffolds for loading bioactive substances. This simultaneous electrospinning and electrospraying technique was also used by other researchers to make nanofibrous membranes and characterizations of such structures has subsequently been performed (33, 34). Poor mechanical properties and rapid bioactive molecule release were found to be two major drawbacks of these structures.
In this chapter, wet-spun fibres containing protein-loaded PLGA microspheres have been developed to minimalize the burst release of protein and deliver the protein with an extended controlled release profile. The effect of incorporation of microspheres on the final properties of the fibres have been studied. The surface morphology of the prepared fibres were studied using optical, LV-SEM and laser confocal microscopy. Moreover, other properties of the fibres, such as the chemical structure (via FTIR spectra), mechanical strength (via stress-strain curves) and cumulative release profiles have also been characterized.

3.2 Materials and Methods

3.2.1 Materials

Alginic acid sodium salt from brown algae (low viscosity, vis of 1% w/v: 4-12 cP, 1%w/v in H₂O, 25 °C) (Medium viscosity, vis of 2% w/v in H₂O: ≥ 2,000 cP, 25 °C), CaCl₂ dehydrated and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA), poly(vinyl alcohol) (Mw 50,000-85,000 Da 96% hydrolyzed) were purchased from Sigma Aldrich, USA. For ease of understanding, two labels of MV and LV are defined to represent the alginate material type. Alg MV stands for low viscosity, vis of 1% w/v: 4-12 cP, 1%w/v in H₂O, 25 °C and Alg LV stands for (low viscosity, vis of 1% w/v: 4-12 cP, 1%w/v in H₂O, 25 °C) alginate materials.

The D, L-lactide and glycolide copolymer (PLGA, 50:50– inherent viscosity 0.4 dl/g, 25 °C, 0.5 g/dL in CHCl₃) was purchased from Corbion. Analytical grade dichloromethane (DCM) was obtained from Chem. Supply Pty Ltd. Milli-Q water (18 MΩ cm⁻¹) was used in the preparation of aqueous solutions. A simulated body fluid (SBF) solution was prepared with the following ion concentrations of 142 mM Na⁺, 5 mM K⁺, 1.5 mM Mg²⁺, 2.5 mM Ca²⁺, 103 mM Cl⁻, 27 mM HCO₃, 1.0 mM HPO₄²⁻ and 0.5 mM SO₄²⁻ with the final adjusted pH of 7.4±0.05 (35) was used as the medium for the protein release study. EASY strainer sterile filter (pore size = 70 µm) and Millex® Syringe sterile filters (pore size = 0.22 µm) were obtained from Greiner Bio-One (Austria) and MERCK (Germany), respectively.
3.2.2 Methods

3.2.2.1 Fabrication of FITC-BSA loaded PLGA microspheres (type β)

Based on the results from protein release studies from different microspheres (present in Section 2.4.5), the PLGA microspheres type β, with an almost linear release profile, were chosen for incorporation into Alg fibrous host structures. The fabrication process for FITC-BSA loaded PLGA microspheres type β has been described in Chapter 2 (Section 2.2.2.2). The final freeze-dried protein loaded spheres were stored at -20 °C.

3.2.2.2 Wet-spinning set-up and fabrication of Alg fibres containing FITC-BSA loaded PLGA microspheres (Alg/PLGA fibre)

Alg solutions of different materials (labelled as medium and low viscosity – Alg MV and Alg LV, respectively) and concentrations with different loadings of PLGA microspheres were obtained. The ingredients of the spinning dopes are listed in Table 3-1.
Table 3-1. Ingredients of spinning dopes and average diameter of the as-spun fibres. Alg/PLGA A and Alg/PLGA B stand for fibres made from solutions containing 1% and 2% of Alg MV, respectively and the mass ratio of PLGA spheres to Alg was 0.25 for both. Alg/PLGA C1, Alg/PLGA C2 and Alg/PLGA C3 stand for fibres made from Alg LV 4% solution and the mass ratio of PLGA spheres to Alg were 0.053, 0.11 and 0.25 for each, respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alg MV 1%</th>
<th>Alg MV 2%</th>
<th>Alg LV 4%</th>
<th>Alg/PLGA A</th>
<th>Alg/PLGA B</th>
<th>Alg/PLGA C1</th>
<th>Alg/PLGA C2</th>
<th>Alg/PLGA C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg MV (%wt)</td>
<td>1.00</td>
<td>2.00</td>
<td>---</td>
<td>1.00</td>
<td>2.00</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Alg LV (%wt)</td>
<td>---</td>
<td>---</td>
<td>4.00</td>
<td>---</td>
<td>---</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Mass ratio of PLGA spheres to Alg</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.25</td>
<td>0.25</td>
<td>~0.053</td>
<td>~0.11</td>
<td>0.25</td>
</tr>
<tr>
<td>PLGA concentration in dried fibre (%wt)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>20</td>
<td>20</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Average diameter of as-spun fibre (µm)</td>
<td>540±12</td>
<td>593±5</td>
<td>581±16</td>
<td>583±23</td>
<td>642±46</td>
<td>614±11</td>
<td>616±35</td>
<td>630±43</td>
</tr>
</tbody>
</table>
To prepare the spinning dopes, three Alg spinning solutions with fixed concentrations of 1 and 2% (w/v) of medium viscosity Alg and 4%w/v of low viscosity Alg were prepared. The Alg/PLGA spinning suspensions were prepared by mixing the Alg spinning dopes with the different ratios of PLGA microspheres using the ratios listed in Table 3-1. The resulting Alg/PLGA spinning suspensions were stirred (1500 rpm) for approximately 5 min and treated ultrasonically (112 W, 2 min). The Alg fibres containing BSA-FITC loaded PLGA microspheres (Alg/PLGA fibres) were prepared using a wet-spinning method obtained from Speakman (36). Wet-spinning is based on coagulation, where a prepared polymer spinning dope is extruded into the non-solvent and coagulation occurs (36-38). The wet-spinning conditions, such as feeding rate and collecting rate, were optimized to achieve continuous fibers with almost consistent diameter along the structure. The solutions were extruded through a needle (gauge = 19) at a flow-rate of 40 mL.h⁻¹ and drawn into the coagulation bath of 2% (w/v) CaCl₂ at room temperature. Due to the small size of the microspheres (size ≤ 55 µm, mean diameter = 12 µm), they could easily pass through the needle during the wet-spinning process along with the rest of the spinning dope. The spun fibres were collected on a rotating drum (rotational speed = 70 rpm) and then soaked in Milli-Q water for 20 s. They were then rinsed and dried in a dark room (to avoid any loss of activity of Fluorescent-labeled proteins) to achieve a constant weight.

3.2.2.3 Wet-spinning set-up and fabrication of FITC-BSA loaded Alg fibres

For comparison, FITC-BSA loaded Alg fibre (loading protein directly into Alg Fibrous structure without encapsulating it into PLGA microspheres) was also prepared using the wet-spinning procedure explained above. To prepare the spinning dopes, first the three Alg spinning solutions with fixed concentrations of 1 and 2% (w/v) of medium viscosity Alg (Alg MV) and 4%w/v of low viscosity Alg (Alg LV) were prepared. In the next step, 2, 4 and 8 mg of FITC-BSA were added to Alg MV 1%, Alg MV 2% and Alg LV 4% solutions, respectively. This ensures that the mass ratio of Alg to FITC-BSA is constant at 500 in all solutions. The protein was dissolved in Alg solution by stirring for 10 min at 1500 rpm at room temperature.
3.3 Characterization

3.3.1 Rheology of the solutions

The rheological behaviour of the wet-spinning solutions was analyzed by using an AR-G2 rheometer (TA Instruments, New Castle, DE) equipped with a Peltier plate thermal controller. A2º/36mm cone and plate geometry was used in all measurements. The solutions were set at the equilibrium temperature for 1 min prior to performing the experiments. Viscosity and shear stress were measured as functions of shear rate at 25 °C.

3.3.2 Optical microscopy

Images of as-spun and dried fibres were recorded via an optical microscope (Leica M205A, Austria).

3.3.3 Scanning Electron Microscopy

The as-spun fibres were mounted on metal stubs using conductive double-sided tape. Sample morphology was observed using a scanning electron microscope (JEOL JSM-6490 LV, Japan) at an accelerating voltage of 15 kV. The cross-sectional imaging was also performed by inserting the fibres into a hole (1 or 1.5 mm diameter) which had been pre-drilled into a small brass block (~25 mm diameter ~10 mm). The holes allow the fibres to be inserted upright and protrude from the brass block. The block containing the mounted fibres was then plunged into liquid nitrogen for about 45 s and a liquid nitrogen cooled razor blade was run across the surface of the block to fracture the fibres. The block was then quickly transferred to the LVSEM for examination. Average diameter of the fibres were estimated with ImageJ image visualization software (n=3).

3.3.4 Laser Confocal Scanning Microscopy

To confirm the presence and distribution of protein-loaded microspheres throughout the wet-spun fibres, observations of hydrated fibres deposited on microscope glass slides were performed on a laser confocal scanning microscope (LCSM, Leica TCS SP2, Australia). The excitation and emission wavelengths were 488 and 520 nm, respectively. The Alg LV 4% fibre was used as a negative control: initially to adjust all parameters, including the laser intensity and gain, until fluorescent signals could
not be seen; then, without changing any settings, the same parameters were used to observe the fibres containing FITC-BSA loaded PLGA microspheres.

3.3.5 Fourier Transform Infrared Spectroscopy (FT-IR)

The FITC-BSA loaded PLGA microspheres, Alg LV 4% and Alg/PLGA fibres C1, C2 and C3 (Table 3-1) were characterized by FT-IR, using a FT-IR spectrometer (Shimadzu, Japan). The samples were air-dried fibres at 20°C.

3.3.6 Protein Release Study

Protein release studies from FITC-BSA loaded PLGA microspheres or Alg and Alg/PLGA fibres were undertaken using a similar condition. A brief description is given here. The release evaluation test of FITC-BSA loaded PLGA microspheres has been explained in Chapter 2 (Section 2.3.5).

Release studies were carried out in SBF at 37 °C, with empty Alg fibres as controls. The fibres (3 cm lengths) were placed in 1.5 ml Eppendorf tubes with 300 µl of SBF. The release was performed in an incubator shaker under slow shaking conditions. At each time point (0, 1, 3, 5, 7, 10, 14 and 21 days), the SBF supernatant was removed and replaced with fresh SBF and re-incubated until the next designated sampling time point. Collected samples were stored at -20 °C and the protein content of the samples were quantified using a fluorescence microplate reader at Ex$_{\text{max}}$ = 485 nm and Em$_{\text{max}}$ = 520 nm (the excitation and emission wavelengths, respectively) (n=3).

3.3.7 Quantification of protein loading

Alg fibres. The fibres (length = 3 cm, n = 7) were weighed and dissolved in an aqueous solution containing EDTA (50 mM) and sodium citrate (100 mM). After vigorous agitation, this suspension was centrifuged (3000 rpm, 5 min) and a 200 µL sample was extracted for further analysis using a microplate reader (Ex$_{\text{max}}$ = 485 nm and Em$_{\text{max}}$ = 520 nm). The protein loading was quantified using a standard curve of fluorescence intensity vs. FITC-BSA concentration.
**Alg/PLGA fibres.** A similar process was followed to dissolve the Alg matrices. Then the suspensions were filtered using Millex® Syringe sterile filters (pore size = 0.22 µm). After filtering the suspension, the remaining microspheres were washed with water to remove residual salt, dried at 37 °C and weighed. Based on the protein loaded into the PLGA microspheres (discussed in Section 2.4.4) and mass of PLGA microspheres present in each section of fibre, the amount of protein loading in the fibres was calculated (Equation 3-1).

\[
M_{\text{FITC-BSA}} = \text{LE} \times M_{\text{PLGA}}
\]  
**Equation 3-1**

Where:

- \(M_{\text{FITC-BSA}}\) is the mass of FITC-BSA present in each piece of fibre,
- LE is the loading Efficiency of FITC-BSA loaded PLGA microspheres (type \(\beta\)) (as per Section 2.4.4) and \(M_{\text{PLGA}}\) is the mass of FITC-BSA loaded PLGA microspheres (type \(\beta\)).

### 3.3.8 Mechanical Properties

Tensile tests of the prepared fibres were carried out on a dynamic mechanical tester (EZ-L Tester from Shimadzu, Japan), at a rate of 1 mm.min\(^{-1}\) and a gauge length of 30 mm. Average values of tensile strength and maximum strain were determined from testing 3 fibres of each type.

### 3.4 Results and Discussion

#### 3.4.1 Study of rheological behaviour of spinning dopes

With the aim of studying the rheological properties of spinning dopes, the viscosity vs. shear rate graphs of spinning dopes (introduced in Table 3-1) were considered (Figures 3-1 to 3-3). During the spinning process, the rheological behavior of the spinning dopes have significant effects on flow-rate distribution through the spinneret needle and the fibre formation step (39). Hence, the structural fibre properties (such as the diameter along the fibre) could be affected by rheological properties. The concentration of spinning dope constituents and their relative ratios must be appropriate to prepare suspensions with proper viscosities for consistent solution processing into fibres. Low concentrations of spinning dopes may compromise the stability of the spinning solution and too high apparent
dynamic viscosity may disturb the fibre formation process. Both of these issues result in formation of discontinuous or ununiformed pieces of fibres (40).

To extrude the Alg spinning dopes through a needle (spinneret) to form fibres, the viscosity of the dope typically should be in the range of 0.01 to 0.2 Pa.s (41-43), which can be produced either from high concentration solutions made of Alg with a low molecular weight (Alg LV), or lower concentrations with a medium molecular weight (Alg MV) (41). The variation and influence of Alg concentrations on the rheological behaviors of the solutions are illustrated in Figure 3-1.

![Figure 3-1](image)

**Figure 3-1.** (A) Viscosity and (B) shear stress of initial blank Alg spinning solutions. The labels in the figure are detailed in Table 3-1.
The literature reveals that Alg solutions are shear-thinning non-Newtonian fluids without a flow limit which is in agreement with our results (Figure 3-1) (40, 41). As can be seen in Figure 3-1 A, for the Alg solutions at different concentrations and made from both types (Alg MV and Alg LV), viscosity slightly decreases with increasing shear rate, which is typical behaviour of polymer solutions (38, 44, 45). Moreover, shear stress (Figure 3-1 B) increases less than proportionally with the shear rate. All solutions exhibited typical shear thinning behaviour which is attributed to alignment of their molecules in the flow (46, 47). It also can be observed that the viscosity of the Alg solutions, made from Alg MV, increases strongly as the concentration is doubled. Theoretically, higher molecular weight causes a higher level of inter-chain bonding and greater fibre strength (48-50). Based on practical experiences, using any of these three Alg aqueous solutions as spinning dopes for the wet-spinning process, we were able to produce continuous uniform Alg fibres.

Figure 3-2 shows the rheological properties of Alg/PLGA spinning dopes containing similar concentrations of ingredients. Comparing the rheological behaviour of Alg solutions (Figure 3-1) with Alg/PLGA spinning dopes (Figure 3-2), shows that the presence of the PLGA spheres within the dopes does not lead to any significant changes in the overall trends of viscosity (Figure 3-2 A) and shear stress (Figure 3-2 B) vs. shear rate. It also can be seen in Figure 3-2 A that the dispersion of PLGA microspheres caused higher levels of variations in viscosity trend of Alg/PLGA fibre A in lower shear rates. It suggests that in solutions with lower concentrations of Alg (1%), the polymer concentration might not be sufficient for well-dispersity of microspheres and, therefore, the rheological behaviour of the spinning dope will be affected.
Figure 3-2. (A) Viscosity and (B) shear stress of Alg/PLGA spinning dopes applied to fabricate the Alg/PLGA fibres A, B, and C-3. The labels in the figure are detailed in Table 3-1.

Figure 3-3 shows the effect of concentration of PLGA microspheres on the rheological behaviour of spinning dopes. As can be seen, the viscosity range of the dope has not been significantly affected by increasing this concentration. Considering Figure 3-1, 3-2 and 3-3 clarifies that introducing PLGA microspheres to the initial Alg solutions causes some slight variations in the viscosity of the spinning
dopes at lower shear rates. As it does not have significant effects on the general viscosity of the spinning dopes, the spinability of the dopes will not be significantly affected.

Figure 3-3. (A) Viscosity and (B) shear stress of Alg/PLGA spinning dopes applied to fabricate Alg/PLGA fibres C-1, C-2, and C-3. The labels in the figure are detailed in Table 3-1.
3.4.2 Protein (FITC-BSA) release study of FITC-BSA loaded Alg and Alg/PLGA fibres

It is reported that the protein release from all wet-spun fibres depends heavily on protein molecular weight (25). To study the effect of the Alg precursor type, the release of FITC-BSA from pure Alg fibres was studied by encapsulating the protein directly into the fibrous matrix. The released protein from each fibre was calculated based on a calibration curve which had $r^2$ values greater than 0.98. The results are shown in Figure 3-4. As can be seen, all the pure Alg fibres showed very fast release profiles, although the Alg LV type showed a relatively longer release with almost less burst.

![Figure 3-4. Cumulative release of FITC-BSA protein from FITC-BSA loaded Alg fibres. (Data indicate significance difference at p < 0.05). The labels in the figure are detailed in Table 3-1.](image)

The release profiles of the FITC-BSA from Alg/PLGA A, B and C-3 fibres, were assessed by immersing the fibres in SBF (pH 7.4, 37 °C) for up to 21 days and the results are shown in Figure 3-5. All these fibres contain the same ratio of FITC-BSA loaded PLGA microspheres to Alg material (Table 3-1). Overall,
the protein release from the Alg/PLGA fibers was delayed more than that from the PLGA microspheres or Alg matrix alone. Among these three fibres, Alg/PLGA fibre A showed a faster release profile for FITC-BSA over the course of the study and this result can be understood by considering the Alg cross-linking process. The Alg wet-spun fibres are formed by ionic cross-linking bonds between the COO$^-$ groups of Alg and Ca$^{2+}$ present in the coagulation bath. The cross-linking level can be increased by raising the concentration of Alg. This leads to the presence of more COO$^-$ in the initial spinning solution and, consequently, more ionic bonds will be formed during coagulation. Therefore, fibres formed from spinning dopes containing lower concentrations of Alg might swell and degrade sooner resulting in a faster protein release profile (51-54). In addition, Kong et al. (55) showed that under similar conditions, the higher molecular weight Alg experiences greater levels of chain breakage, which is one of the main reasons for degradation of the Alg material. This is attributed to the higher fraction of more flexible blocks (mannuronic acid (M) blocks) in these Alg chains.
Figure 3-5. Cumulative release profile of FITC-BSA from (A) Alg fibres, (B) PLGA microspheres, and (C) Alg/PLGA fibres. The labels in the figure are detailed in Table 3-1.
Based on the release data from Alg/PLGA fibres A, B and C-3, it was found that the spinning dopes made with Alg LV 4% as the initial polymer solution provided lower levels of burst release as well as a slower release profile (~ 35%). Hence, the Alg LV 4% was chosen as the initial polymeric solution for subsequent experiments. To investigate the effect of mass ratio of protein loaded PLGA microspheres to Alg on the release profile, the amount of released protein from the fibres with different amounts of FITC-BSA loaded PLGA microspheres was assessed. Figure 3-6 shows the release profile of FITC-BSA from Alg LV 4%, PLGA microspheres as well as Alg/PLGA fibre C-1, C-2 and C-3. As can be seen, incorporating the microspheres into the fibre structures led to a decrease in the initial burst release percentage and it extended the release time. The burst release is most likely happening because of protein present on the surface of the microspheres. Later in the time course, the encapsulated protein (in inner layers) finds the chance to escape to the Alg matrix (56). The data (Figure 3-6) suggests that the incorporation of the protein-loaded PLGA microspheres into the hydrogel Alg fibre matrix results in a decrease in the burst release amount and makes the release profile slower.
Figure 3-6. Cumulative release profiles of FITC-BSA from (A) Alg LV 4%, (B) PLGA microspheres, and (C) Alg/PLGA fibres C1, C2 and C3. The labels in the figure are detailed in Table 3-1.

The general release trend of all mass ratios (of PLGA microspheres to Alg) was almost the same; involving a lower level of burst release (compared to FITC-BSA loaded PLGA microspheres). The
release profiles continued with a reasonable fast release in the first week followed by a slower release profile during the rest of the experimental period. The higher the mass ratio, the faster the FITC-BSA is released. It is expected that the Alg matrix can act as a protection shell for protein encapsulated in spheres. Commonly, in first time points, only FITC-BSA loaded to spheres near the surface of fibre can be released. The protein loaded in microspheres located in the inner part of the fibre cannot be released until the spheres and Alg matrix swell or diffuse to a certain extent to provide pores large enough (57). This is a diffusion-based process and the lower the concentration of protein-loaded spheres, the slower the release profile will be.

3.4.3 Study of the chemical structure via FTIR Spectra

Figure 3-7 shows the FTIR spectra of Alg LV 4%, Alg/PLGA fibres C-1, C-2 and C-3 and protein-loaded PLGA microspheres. The characteristic peaks of Alg can be observed at 2924 cm$^{-1}$ and 1405 cm$^{-1}$ due to stretching $–$CH$_2$ and the carboxylic groups, respectively. Moreover, the peak at 1594 cm$^{-1}$ indicates the asymmetric stretch of C–O–H. Other main peaks attributed to the Alg are seen at 3311 cm$^{-1}$ and 1120 cm$^{-1}$, corresponding to the stretching vibration of O–H and stretching vibration of secondary alcohol (C–O), respectively (58, 59). For the FITC-BSA loaded PLGA microspheres, the strong characteristic absorption bands at approximately 1745 cm$^{-1}$ denotes the stretching vibration of C=O bond and the bands at 1161 cm$^{-1}$ can be allotted to the methyl group C–O bond of PLGA (60-63).
Figure 3-7. FTIR spectra of Alg LV 4% fibre, Alg/PLGA fibres C-1, C-2 and C-3, and protein-loaded PLGA microspheres. The labels in the figure are detailed in Table 3-1.

Considering the FTIR spectra of protein-loaded PLGA microspheres and Alg LV 4% fibre, it can be seen that the ratio of the intensities of the main peak of PLGA (at approximately 1745 cm$^{-1}$) to one of the characteristic peaks of Alg (at 1594 cm$^{-1}$) has increased as a result of the increase in the concentration of protein-loaded PLGA microspheres into Alg/PLGA fibres C-1, C-2, and C-3.
3.4.4 Mechanical properties of Alg LV 4% fibre and Alg/PLGA fibres C-1, C-2, and C-3

The mechanical properties of bioactive molecule loaded wet-spun microfibres are very critical to their functionality as therapeutic platforms in the clinical arena (25, 64, 65). The structures for protein release applications should possess a sufficient mechanical strength to avoid damage during manufacturing processes, storage, and transportation. Low elastic moduli were found to be advantageous to prevent the initiation and propagation of cracks and thus reduced the risk of dose dumping (66). To determine the effect of concentration of protein-loaded PLGA microspheres on the mechanical properties of wet-spun fibres, samples were loaded under uniaxial tension until break. The typical stress-strain curves of Alg LV 4% fibres and Alg/PLGA fibres are presented in Figure 3-8.

![Stress-strain curves of Alg LV 4% fibre and Alg/PLGA fibres C-1, C-2, and C-3.](image.png)

Figure 3-8. Stress-strain curves of Alg LV 4% fibre and Alg/PLGA fibres C-1, C-2, and C-3. The labels in the figure are detailed in Table 3-1.

The elastic modulus is defined as the initial slope of the stress–strain curves of the fibres (before the stress plateaus off or fracture occurs). The elastic modulus was calculated using the slope of the curve between tensile stress and tensile strain in the linear viscoelastic range (67). The highest stress that
fibres can bear is referred as tensile strength. In addition, tensile strain at break was calculated by dividing the elongated distance of the fibres at break by the initial length of the specimen (L = 30 mm). The mechanical properties of Alg LV 4% fibre and Alg/PLGA fibres C-1, C-2 and C-3 are shown in Table 3-2. The mechanical properties of Alg materials are mainly due to junctions created by the G-blocks with a strong auto-cooperative binding of Ca^{2+} between the chains. The M-blocks have much lower selectivity for Ca^{2+} and no autocooporative binding mechanism (65, 68).

Table 3-2 shows that the tensile strength, elastic modulus and tensile strain at break of Alg LV 4% fibres is greater than Alg/PLGA fibres. Incorporation of microspheres into the Alg matrix has significantly changed the properties of fibres and the tensile strength of fibres has decreased with the increasing concentration of PLGA microspheres. It suggests that as the PLGA microspheres are dispersed between the Alg polymeric chains, they will reduce the ability of G-blocks of the Alg material to cross-link (via Ca^{2+} ions) and form the “egg-box” which was described in Chapter 2. Hence, the PLGA microspheres act as structural weak points in the Alg host matrix and the increase in concentration caused a significant decrease in mechanical properties of the Alg/PLGA fibres. However, due to the different nature of Alg (hydrophilic) and PLGA (hydrophobic) materials, no interaction can be postulated between them that might lead to an increase in the strength of the fibre.

Table 3-2. Mechanical properties of Alg LV 4% fibre, and Alg/PLGA fibres C-1, C-2 and C-3. The labels in the figure are detailed in Table 3-1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average diameter of air-dried fibre (µm)</th>
<th>Elastic Modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Tensile strain at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg LV 4%</td>
<td>393±11</td>
<td>20.3</td>
<td>52.9±6.2</td>
<td>27.2±5.3</td>
</tr>
<tr>
<td>Alg/PLGA C-1</td>
<td>418±8</td>
<td>11.5</td>
<td>31.8±3.9</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>Alg/PLGA C-2</td>
<td>421±29</td>
<td>17.9</td>
<td>24.6±4.3</td>
<td>5.3±1.2</td>
</tr>
<tr>
<td>Alg/PLGA C-3</td>
<td>472±31</td>
<td>8.01</td>
<td>8.8±4.4</td>
<td>8.8±0.6</td>
</tr>
</tbody>
</table>
3.4.5 Morphological study of the Alg/PLGA fibres C-1, C-2, and C-3

Optical microscopy allowed for determination of average diameter of as-spun fibres and the results are reported in Table 3-1. As was expected, the average diameter of the fibres increased with the Alg concentration in spinning dopes (Comparing Alg/PLGA fibre A and Alg/PLGA fibre B). The average diameter of as-spun Alg/PLGA A, Alg/PLGA fibre B (Table 3-1) are 583±23 and 642±46, respectively. In addition, due to Table 3-1, adding protein-loaded PLGA microspheres also caused an increase in the average diameter of the fibres (Comparing Alg/PLGA fibres C-1, C-2, and C-3). The average diameter of as-spun Alg/PLGA fibres C-1, C-2, and C-3 (Table 3-1) are 614±11, 616±35, and 630±43 µm, respectively. Figure 3-9 shows the confocal laser microscopy image of as-spun Alg/PLGA fibres C-1, C-2, and C-3. Due to the Figure 3-9, there is no clue of aggregation of PLGA microspheres through the fibrous matrixes.

![Figure 3-9. Confocal laser microscopy images of as-prepared Alg/PLGA fibres C-1, C-2, and C-3.](image)

In addition, the surface morphology of Alg LV 4% and Alg/PLGA fibres, as well as the distribution of protein-loaded PLGA microspheres into the fibre Alg matrix, have been studied using optical, confocal laser microscopy and LV-SEM microscopy. Figure 3-10 A shows a schematic of the produced Alg/PLGA fibres. As an example, the longitudinal (Figure 3-10 B, D, E, and F) and cross-sectional images (Figure 3-10 C) of Alg/PLGA fibre C-3 are presented. Also Figure 3-11 provides a
closer look to distribution of FITC-BSA loaded PLGA microspheres along a piece of Alg/PLGA fibre C-3.

Figure 3-10. (A) Schematic of Alg/PLGA fibres. (B) Longitudinal and (C) Cross-sectional LV-SEM, (D) Longitudinal optical microscopy and (E) Confocal laser microscopy images of as-prepared Alg/PLGA fibre C-3 (Average diameter: 630±43 µm). (F) Longitudinal optical microscopy image of dried Alg/PLGA C-3 fibre.

It can be noted (Figure 3-10) that the Alg/PLGA fibre C-3 (with highest concentration of protein loaded PLGA microspheres) is straight, with some spheres being observed on its surface (Figure 3-10 B). With regards to the images from as-prepared fibres (Figure 3-10 B, C, D and E) and Figure 3-11, no evidence of aggregates of PLGA microspheres were observed on the surface or inside the fibre structure, which is an indication of the good dispersibility of the protein loaded PLGA microspheres with the Alg fibre. The surface morphology of the fibre contain striations that are attributed to roughness in the spinneret opening (69). Figure 3-10 F presents the morphology of the air-dried Alg/PLGA fibre C-3. The dehydration of the hydrophilic structure of Alg causes shrinkage which results in reduction of the average diameter of the fibre.
3.5 Conclusion

With the aim of developing structures for bioactive molecule delivery with a controlled extended release profile, a wet spinning process was used to fabricate Alg microfibres containing protein-loaded PLGA microspheres. The two-phase delivery matrices display retarded FITC-BSA release significantly in both initial and late stages compared to release from the PLGA microspheres or alginate fibre alone. The concentration of Alg material and protein-loaded PLGA microspheres were optimized to achieve uniform fibres with a long-lasting in-vitro release profile. It was found that fibres fabricated from Alg material (low viscosity type) with higher concentration, compared with Alg (medium viscosity type) with lower concentrations, can provide a lower level of burst release as well as a slower release profile over the observation time. Based on observations by optical and SEM microscopy, the microspheres were embedded well and homogeneously dispersed within the fibres. Incorporation of the microspheres into the Alg fibre matrix was verified by FTIR and their effect on the final mechanical properties of air-dried fibres was investigated. In next chapter, all the studies focus on properties of fibres with a short length of 3 cm; hence, to facilitate tracking the effects of presence of FITC-BSA loaded PLGA microspheres, the highest concentration of protein-loaded PLGA microspheres was chosen for further studies in the next chapter.


3.6 References


Chapter 4  A Simple Technique for Development of Fibres with Programmable Microsphere Concentration Gradients for Local Protein Delivery

Alginate has long been shown to be a biologically viable option for controlled local delivery of bioactive molecules (i.e. proteins and growth factors) \textit{in vitro} and \textit{in vivo}. However, the defined bioactive molecule-specific, zero-order release kinetics directly associated with its structure/composition, often preclude its use for complex delivery profiles: Specific bioactive molecule release profiles are achieved by controlling polymer composition/concentration, which also defines modulus of the alginate hydrogel. Thus, achievement of desired release profiles/kinetics often results in an alginate construct incompatible with implantation into target tissues. This largely limits alginate-mediated bioactive molecule delivery to single-factors of uniform concentration applications rather than applications that may involve multiple growth factors or bioactive molecules delivered at a concentration gradient for chemotactic purposes. In this study, a two-phase PLGA/alginate delivery system composed of poly D, L-lactic-co-glycolic acid (PLGA) microspheres containing protein (fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA)) wet-spun into alginate fibres to provide an additional element of release controllability was evaluated \textit{in vitro}. An extended controlled release profile was achieved and the fabrication system devised was shown to have the capability to produce wet-spun fibres with a protein concentration gradient (G-Alg/PLGA fibre). The morphology, diameters, mechanical properties and protein release profile of the fibres were characterized. The two-phase delivery matrices display retarded FITC-BSA release significantly in both initial and late stages compared to release from the PLGA microspheres or alginate fibre alone. In addition, it was observed that incorporation of higher concentrations of protein-loaded PLGA microspheres increased protein release compared to fibres with lower concentrations of BSA-loaded microspheres. The “programmable” microspheres concentration gradient fibre methodology presented here may enable development of novel alginate scaffolds with the ability to guide tissue regeneration through tightly-controlled release of one or more proteins in various spatio-temporal concentration configurations within the structure.
4.1 Introduction

Over the past two decades, localized delivery of therapeutic proteins (such as protein drugs and growth factors) to dysfunctional/damaged tissues has gained significant momentum in a number of biomedical areas, and in particular in tissue engineering applications (1). Local delivery systems overcome drawbacks associated with systematic approaches, whose distribution of therapeutic molecules via the circulatory system often results in diffuse efficacy and side-effects in non-target tissues (2, 3). Therefore, fabrication of effective and versatile protein delivery systems is important to most if not all bioactive molecules delivery, pharmaceutics, tissue engineering, and regenerative medicine applications (4, 5). From this viewpoint, the general biocompatibility and hydrophilic nature of alginate (Alg) based structures have made Alg one of the most common platforms being investigated for therapeutic protein delivery applications (6-8).

One of the ongoing challenges in the field of bioactive molecules delivery and tissue engineering is fabricating a structure that delivers the appropriate bioactive molecule(s) for an extended period in a manner that effectively directs the regeneration of target tissue (9). Microencapsulation of protein within the biodegradable polymers has been recognized as an attractive way to control the sustained delivery of bioactive molecules (10, 11). For instance, poly D, L-lactic-co-glycolic acid (PLGA) microspheres, because of their modestly-priced and reproducible fabrication processes, have been extensively studied for controlled sustained delivery of proteins (12-14). PLGA microspheres’ long term degradation facilitates bioactive molecule release up to several months (15, 16). Further incorporating the bioactive molecule-loaded PLGA microspheres into a hydrogel structure has shown to provide an added element of control to the release profile by mitigating the initial burst release that is often associated with the delivery directly from Alg and other polymers alone (2, 17-19). As an example, Zhai et al. (20) in 2015 reported that with addition of Alg (as the shell material) to cover the PLGA microspheres (the core), they could modulate the release profile (around 30% less release after the observation time) of a model protein (BSA) as well as decrease the level of burst release.

A number of structures have been fabricated and evaluated for their application in targeted local bioactive molecule delivery and cell guidance/chemotactaction (21-23). In these applications, biofactor gradients within the structures provide chemotactic cues for directionally-guided cell growth and/or
migration. However, all these structures required highly complex technologies for fabrication and very few were able to generate a non-burst release profile that: i) effectively controlled delivery concentrations to those required of the target cell/tissue system, and ii) did not deliver most of the bioactive molecules in a very short period.

Fibrous structures have gained a great deal of attention as bioactive molecules delivery systems. This is due to the simplicity of bioactive molecules loading in the spinning dope as well as their large surface areas across which bioactive molecules are transported to the target site (24). Electrospinning (25, 26) and wet spinning (27, 28) have been intensively explored to produce fibrous structures for a range of applications including bioactive molecules delivery, tissue engineering and regenerative medicine. Among them, wet-spinning has the potential to convert biomaterials into fibres for controlled release without the requirement for high voltages during the manufacturing process, which is less likely to cause protein denaturation (29). Wet-spinning is a non-solvent-induced phase inversion technique that is used to form continuous polymer microfibres by injecting the polymer solution into a coagulation bath composed of a poor solvent or solvent mixture with respect to the polymer. This method can produce filaments into complex geometries to address the needs for different bioactive molecule delivery applications (30, 31).

In this chapter, a simple and programmable approach for fabrication of Alg fibres containing protein-loaded microspheres is presented; whereby the protein loaded microspheres are incorporated into the fibre structure to achieve long term controlled release of protein in a gradient manner conducive to chemotactic applications. This novel gradient fibre is specifically programmable in terms of the presence of bioactive molecules along the fibre length while also potentially providing a platform for directing cell growth.

4.2 Materials and methods

4.2.1 Materials

D, L-lactide and glycolic acid (PLGA, 50:50, inherent viscosity 0.4 dL/g (25 °C, 0.5 g/dL in CHCl₃)) was purchased from Corbion. Alginic acid sodium salt from brown algae (Alg) (vis: 4-12 cP, 1 % in H₂O 25 °C), poly (vinyl alcohol) (PVA) (Mw 50,000–85,000 Da 96% hydrolyzed), calcium chloride
dihydrate, ethylenediaminetetraacetic acid (EDTA), sodium citrate and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) were all purchased from Sigma Aldrich, Australia. Analytical grade dichloromethane (DCM) was obtained from Chem. Supply Pty Ltd. Milli-Q water (18 MΩ cm\(^{-1}\)) was used in the preparation of aqueous solutions. A simulated body fluid (SBF) solution, with ion concentrations equal to those of human blood plasma and primed with the following ion concentrations of 142 mM Na\(^{+}\), 5 mM K\(^{+}\), 1.5 mM Mg\(^{2+}\), 2.5 mM Ca\(^{2+}\), 103 mM Cl\(^{-}\), 27 mM HCO\(_3\)^{-}, 1.0 mM HPO\(_4\)^{2-}\) and 0.5 mM SO\(_4\)^{2-}\) and with a pH adjusted to 7.4 (32), was used as the medium for the protein release study. EASYstrainer sterile filter (pore size = 70 µm) and Millex® Syringe sterile filters (pore size= 0.22 µm) were obtained from Greiner Bio-One (Austria) and MERCK (Germany), respectively.

4.2.2 Methods

4.2.2.1 Preparation of the protein-loaded PLGA microspheres (type β)

The fabrication process for FITC-BSA loaded PLGA microspheres type β has been described in Chapter 2. The final freeze-dried protein loaded spheres were stored at -20 °C for further use.

4.2.2.2 Wet spinning set-up and fabrication of Alg fibres with programmable loading of the protein-loaded PLGA microspheres

A spinning technology was developed employing two spinning dopes (Figure 4-1). Spinning dope A was composed of 4%w/v Alg in water and spinning dope B (Alg/PLGA spinning dope) was prepared by mixing 4% w/v Alg with the FITC-BSA loaded PLGA microspheres at a weight ratio of Alg to PLGA equal to 3:2. The mixing procedure involved magnetic stirring of the Alg/PLGA spinning suspension for 5 min followed by ultrasonication for 2 min. The two spinning dopes were extruded via syringe A and B respectively, with the extrusion rates being controlled using a CM Printing System-LinuxCNC via an in-home-designed coded software. The two spinning were dopes mixed together in a conjugation tube (with the length of 39 mm and interior diameter of 3.5 mm) where an in-home-designed interior blender (with a diameter of 3.2 mm) was installed to facilitate the mixing of the two spinning dopes to produce the final spinning dope. The final spinning dope was injected into a coagulation bath containing an aqueous CaCl\(_2\) solution (4% w/v). The coagulation bath was
programmed to move at a predetermined speed of 50 cm.min⁻¹ in parallel to the direction of spinning to avoid any stress to the as-spun fibre.

To minimize the dead volume and waste of protein-loaded PLGA microspheres, the conjugation tube was prefilled with Alg spinning dope prior to the spinning process. Thus the as-spun fibre contained an initial Alg-only part. The length of the Alg-only section can be calculated based on the extrusion rate of final spinning dope and the volume of the conjugation tube. At the completion of the spinning process, the initial Alg only part was removed (at cutting point shown in Figure 4-1) to produce the desired fibre.

In this study, two types of microsphere-loaded Alg fibres were fabricated and characterized: Alg fibre with uniform distribution of the protein-loaded PLGA microspheres (U-Alg/PLGA fibre), and Alg fibre with a concentration gradient of protein-loaded PLGA microspheres (G-Alg/PLGA fibre). U-Alg/PLGA fibres were prepared by control of the ratio of extrusion rate of the two spinning dopes, Alg spinning dope : Alg/PLGA spinning dope, at 1:1, using the procedure described above. Both Alg and Alg/PLGA spinning dopes were extruded at a fixed rate of 24 ml/h and mixed together via the conjugation tube to form the final spinning dope that was then extruded into the coagulation bath at a rate of 48 ml/h.

The G-Alg/PLGA fibres were produced using a similar procedure, but the ratio of extrusion rate of Alg spinning dope and Alg/PLGA spinning dope was programmed to vary linearly from 1:0 to 0:1 over a period of 15 seconds. It means that the spinning procedure begun with an extrusion rate of 48 ml/h for Alg spinning dope, and 0 ml/h for Alg/PLGA spinning dope, respectively, and ended with an extrusion rate of 0 ml/h for Alg spinning dope and 48 ml/h for Alg/PLGA spinning dope. During the spinning process, the extrusion rate of final spinning dope (from the needle through the coagulation bath) was fixed at 48 ml/h. By controlling the extrusion rate ratio of the two spinning dopes, programmable loading of the protein-loaded PLGA microspheres in Alg fibres can be readily achieved.
For comparison, FITC-BSA loaded Alg fibre (loading protein directly into Alg fibrous structure without encapsulating it into PLGA microspheres) was also prepared using the procedure for U-Alg/PLGA fibres. To do so, spinning dope A contained aqueous 4%w/v Alg solution, and spinning dope B contained FITC-BSA dissolved in Alg solution 4% w/v (FITC-BSA/Alg, w/w, 1/90).

Figure 4-1. (A) Schematic of the novel programmable wet-spinning technology. The extrusion rates of Alg spinning dope (in syringe A) and Alg/PLGA spinning dope (in syringe B) are
adjusted using a CM Printing System-LinuxCNC via an in-home-designed coded software. The two spinning dopes joined in a conjugation tube to form the final spinning dope. Then the final dope was extruded into a needle to form a fibrous structure. (B) The magnified schematic of the conjugation tube, where a small flexible blender was inserted to facilitate the mixing of the two spinning dopes. (C) Schematic illustration of U-Alg/PLGA and G-Alg/PLGA fibres produced using the above procedure.

4.3 Characterization

4.3.1 Rheology of the Alg spinning dopes

The rheological behaviour of the wet-spinning solutions was analysed using an AR-G2 rheometer (TA Instruments, New Castle, DE) equipped with a Peltier plate thermal controller. A2°/36mm cone and plate geometry was used in all measurements. The solutions were held at 25 °C for 1 min prior to performing the experiments. Viscosity and shear stress were measured as a function of shear rate at 25 °C.

4.3.2 Optical microscopy

Images of the as-spun and dried fibres were recorded via optical microscopy (Leica M205A, Austria).

4.3.3 Scanning Electron Microscopy

The protein-loaded microspheres were mounted on metal stubs using conductive double-sided carbon tape and sputter-coated with a thin layer of gold (thickness=15 nm). Microspheres diameters were analysed with image visualization software, Image J (n=150).

For longitudinal and cross-sectional imaging, five sections of hydrated G-Alg/PLGA fibre (at specific distances) were cut and inserted into pre-drilled holes (1 or 1.5 mm diameter) in a brass mounting block. The holes were of a depth which allowed fibres to be inserted upright and protrude from the surface. The block containing the mounted fibres was then plunged into liquid nitrogen for approximately 45 s and the frozen fibres were fractured using a liquid nitrogen cooled razor blade.
The block was then quickly transferred to the LVSEM (JEOL JSM-6490 LV, Japan) for examination. Sample morphologies were then observed in secondary electron imaging mode at an accelerating voltage of 15 kV.

4.3.4 Confocal Laser Scanning Microscopy

To confirm the presence and distribution of protein-loaded microspheres in the wet-spun fibres, the hydrated fibres were deposited on microscope glass slides and examined on a laser confocal scanning microscope (LCSM, Leica TCS SP2, Australia). The excitation and emission wavelengths used were 488 nm and 520 nm, respectively. Blank Alg fibre was used as a negative control: firstly, to adjust all the parameters including the laser intensity and gain, until fluorescent signals could not be seen from the Alg fibre sample; then, without changing any settings, the same parameters were used to observe the fibres containing fluorescently labelled protein-loaded PLGA microspheres.

4.3.5 Fourier Transform Infrared Spectroscopy (FT-IR)

The U-Alg/PLGA and G-Alg/PLGA fibres (air-dried at 20 °C) were characterized using an FT-IR spectrometer. The surface spectra were studied using an infrared microscope (AIM-8800, Shimadzu, Japan) in a reflectance mode equipped with a MCT (Mercury-Cadmium-Telluride) detector connected to the FT-IR instrument.

4.3.6 Mechanical Properties

Tensile tests were carried out on a dynamic mechanical tester (EZ-L Tester from Shimadzu, Japan), at 1 mm/min and a gauge length of 30 mm. Average values of tensile strength and maximum strain were determined after repeating each test three times.

4.3.7 Quantification of protein loading

FITC-BSA loaded PLGA microspheres. The PLGA microspheres (10 ~ 15 mg) were weighed and dissolved in 0.5 ml DCM and then 0.5 ml Milli-Q water was added. After vigorous agitation, the suspension was centrifuged (3000 rpm, 5 min) and a 200 µl sample from the aqueous phase was
extracted for further analysis using a microplate reader (Ex\textsubscript{max} = 485 nm and Em\textsubscript{max} = 520 nm). The protein loading (µg/mg microspheres) was quantified using a standard curve of fluorescence intensity vs FITC-BSA concentration. The results were expressed as mean ± standard deviation (n = 3).

**U-Alg/PLGA fibres.** The fibres (length = 3 cm, n = 7) were weighed and dissolved in an aqueous solution containing EDTA (50 mM) and sodium citrate (100 mM) (8, 33). After filtering the suspension, the remaining microspheres were washed with water to remove residual salt, dried at 37 °C and weighed. The result was expressed as mean ± standard deviation (n = 7). Based on the protein loading of the PLGA microspheres and mass of PLGA microspheres present in each section of fibre, the amount of protein loading in the fibres was calculated.

**G-Alg/PLGA fibres.** The total protein loading in the G-Alg/PLGA fibres (length = 3 cm, n = 7) was determined using the same procedure as for U-Alg/PLGA fibres. In addition, to characterise the protein concentration gradient structure, the G-Alg/PLGA fibres (n = 7) were cut into half. Both the low protein-loading end and high protein-loading end were characterised for protein loading using the method mentioned above.

### 4.3.8 Protein Release Studies

Protein release studies from FITC-BSA loaded PLGA microspheres and Alg fibres with or without FITC-BSA loaded PLGA microspheres were performed in SBF at 37 °C, with empty PLGA microspheres used as controls. Briefly, the microspheres were placed in 1.5 ml Eppendorf tubes with 300 µl of SBF and placed in an incubator shaker under slow shaking conditions. At each time point (0, 1, 3, 5, 7, 10, 14, 21, 28 and 35 days) the SBF supernatant was removed and replaced with fresh SBF and re-incubated until the next designated sampling time point. The SBF samples were stored at -20 °C and the protein content of the samples were quantified using a fluorescence microplate reader at Ex\textsubscript{max} = 485 nm and Em\textsubscript{max} = 520 nm (the excitation and emission wavelengths, respectively) (n=3).

The premature protein release (protein released from the FITC-BSA loaded microspheres during the mixing with the alginate prior to wet-spinning) was assessed. To do so, a mock wet-spinning process was followed (i.e., the extruded material did not interact with the coagulation bath). Briefly, 1 ml of
alginate dope containing the FITC-BSA microsphere was extruded from the needle and collected and diluted 10 times, centrifuged and syringe filtered (pore size 0.22 µm). The filtered solution was again diluted 10 times followed by evaluation of the protein content.

For G-Alg/PLGA fibres, in addition to monitoring the protein release as a function of time, the influence of protein concentration gradient structure (Figure 4-1 C) on protein release was also assessed. The G-Alg/PLGA fibres (n = 7) were cut into half, and both the low protein-loading end and high-protein loading end were monitored for protein release as a function of time, using the same protocol as described for FITC-BSA loaded PLGA microspheres.

4.4 Results and Discussion

4.4.1 Rheology of the spinning dopes

The study of rheological behaviour of spinning dopes is critical for production of a wet-spun product. For co-spinning of multiple-component (i.e. two) dopes, matching the rheology of the components is also important: any non-compliance in rheology of the two dopes will result in poor mixing of spinning dopes and subsequently formation of inhomogeneous final spinning dope that lead to poor quality wet-spun fibres.

Under shear, Alg polymer chains are in a less expanded conformation and become less entangled causing their viscosity to fall. Figures 4-2 A and B show the variations in viscosity and shear stress of Alg and Alg/PLGA spinning dopes as a function of shear rate, respectively. To extrude the Alg spinning dopes through a needle (spinneret) to form microfibres, the viscosity of the dope typically should be in the range of 0.01 to 2 Pa.s vs. the shear rate range between 1 to 100 1/s (34, 35). The presence of PLGA microspheres caused slight variations in rheological behaviour of Alg/PLGA spinning dope at shear rates lower than 10 (1/s), but both dopes follow a similar trend as the shear rate increased. Considering the relative similar rheological behaviour of these two spinning dopes (under the supposed shear rate range for wet-spinning), it is expected that they could be well-mixed.
4.4.2 Morphological study of the U-Alg/PLGA fibres

Figure 4-3 A and B show the typical optical (depth of focus = 240 µm) and LVSEM images of U-Alg/PLGA as-spun fibre.
It appears that the U-Alg/PLGA fibres have a relatively smooth surface morphology and the FITC-BSA loaded microspheres are uniformly distributed inside the fibres. This structure could be attributed to the difference between the interior diameter of the conjugation tube (≈ 3.5 mm) and the blender’s diameter (≈ 3.2 mm) (Figure 4-1). As aforementioned, prior to the spinning process, the conjugation tube (Figure 4-1) was initially filled with the Alg spinning dope and through the spinning process, the blender then mixed the two extruded spinning dopes in the middle of the conjugation tube. This produced a final spinning dope bearing an outer thin layer of Alg spinning dope, which then resulted in wet-spun fibres with a coating of Alg.

### 4.4.3 Investigation of the chemical structure via FTIR Spectra

This discussion is supported by our FT-IR spectroscopy study of the surfaces of U-Alg/PLGA and G-Alg/PLGA fibres (Figure 4-4). For G-Alg/PLGA fibres, FT-IR spectra were recorded at five distinct sections from the low protein concentration end to the high protein concentration end and the results are demonstrated in Figure 4-5. Our results showed no discernible difference in the spectra gathered at these positions.
Furthermore, both U-Alg/PLGA and G-Alg/PLGA fibres showed similar spectra as with pure alginate, indicating the presence of alginate structure at the fibre surface. The Major peaks of alginate can be observed at 2924 cm\(^{-1}\) and 1405 cm\(^{-1}\) due to stretching –CH\(_2\) and the carboxylic groups, respectively. Moreover, the peak at 1594 cm\(^{-1}\) indicates the asymmetric stretch of C–O–H. Other main peaks attributed to the Alg are seen at 3311 cm\(^{-1}\) and 1120 cm\(^{-1}\), corresponding to the stretching vibration of O–H and stretching vibration of secondary alcohol (C–O), respectively (36, 37). Minor peaks at 1754 cm\(^{-1}\) and 1161 cm\(^{-1}\) were noted in both spectra, which are associated with the carbonyl bond C=O stretching vibration and the C–O stretching respectively; both characteristic of PLGA (38, 39).
Figure 4-5. FT-IR spectra of G-Alg/PLGA fibre at five distinct sections. G-Alg/PLGA fibre (i-v) are specified in Figure 4-6 C.

4.4.4 Morphological study of the G-Alg/PLGA fibres

The morphology of G-Alg/PLGA fibres was studied to obtain information on the gradient structure. Figure 4-6 A shows the longitudinal LVSEM image of hydrated G-Alg/PLGA fibre at high protein-loading end and Figure 4-6 B and C show the schematic and longitudinal optical image of G-Alg/PLGA fibre, respectively. The G-Alg/PLGA fibre, like U-Alg/PLGA fibres, has a relatively smooth surface morphology even in the region with the highest concentration of microspheres (Figure 4-6 A inset). This microsphere concentration gradient structure of the G-Alg/PLGA fibre can be observed in Figure 4-6 C. Figure 4-6 (i to v) represent the cross-sectional LVSEM images of hydrated G-Alg/PLGA fibre at 5 insets (specified in Figure 4-6 C). These images present the distribution of microspheres inside the fibre structure. Figure 4-6 iv-a is a closer view of the central part of Figure 4-6 iv. It can be seen (Figure 4-6 (i to v) and particularly (iv-a)) that the fabrication process and incorporation of protein-loaded microspheres into the Alg fibrous matrix did not cause any deformation or collapse in PLGA microspheres structure and they retained their spherical
morphology. The laser confocal scanning image of G-Alg/PLGA fibre (containing a fluorescently labelled (FITC-BSA, green) (Figure 4-6 D) shows the gradient distribution of the microspheres, which is characterized by the intensity mapping of the green fluorescence that increase progressively from the right end (low concentration of microspheres) to the left end of the fibre (high concentration of microspheres). On closer observation (Figure 4-6 D-a to D-d and Figure 4-6 E-a to E-g), it is evident that the number of microspheres in the three insets increases from right to left.
Figure 4-6. (A) The longitudinal LVSEM image of high protein-loading end of G-Alg/PLGA fibre. (B) Schematic and (C) optical microscopy image
of G-Alg/PLGA fibre. (i-v) The cross-sectional LVSEM images at five insets (specified in section (C) from low protein-loading end (i) to high protein-loading inset (v)) of G-Alg/PLGA. (iv-a) The magnified LVSEM image of a FITC-BSA loaded PLGA microsphere incorporated into G-Alg/PLGA fibre. (D) The longitudinal laser confocal scanning microscopy image of G-Alg/PLGA fibre. (D-a to D-d) and (E-a to E-g) The laser confocal scanning microscopy images at different specific areas of G-Alg/PLGA fibre.

The quantification of the distribution of the FITC-BSA protein along the G-Alg/PLGA fibre has been done using an image-processing technique to measure the number of pixels detected as green colour (demonstrating the FITC-BSA protein). Also, the percentage of green colour area (FITC-BSA) to whole image (not just fibre) was achieved by calculation of green pixels’ ratio to the whole number of image pixels (black + green). Table 4-1 shows the results of quantification assessment of presence of FITC-BSA in images E-a to E-g (as specified in Figure 4-1).

Table 4-1. Quantification of presence of FITC-BSA in images E-a to E-g (specified in Figure 4-6). Each image has a total number of 86,846 pixels.

<table>
<thead>
<tr>
<th>Image</th>
<th>E-a</th>
<th>E-b</th>
<th>E-c</th>
<th>E-d</th>
<th>E-e</th>
<th>E-f</th>
<th>E-g</th>
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<tr>
<td>Number of pixels detected as FITC-BSA (green colour) (pixels)</td>
<td>14</td>
<td>122</td>
<td>496</td>
<td>987</td>
<td>1345</td>
<td>2826</td>
<td>6333</td>
</tr>
<tr>
<td>Percentage of green colour area to whole image (%)</td>
<td>0.02</td>
<td>0.14</td>
<td>0.57</td>
<td>1.14</td>
<td>1.55</td>
<td>3.25</td>
<td>7.3</td>
</tr>
</tbody>
</table>

4.4.5 Mechanical properties of U-Alg/PLGA and G-Alg/PLGA fibres

The mechanical properties of the U-Alg/PLGA and G-Alg/PLGA fibres were investigated to assess the effect of the distribution gradient of the FITC-BSA loaded PLGA spheres on the final properties of these fibres. All the fibres were cut into 3 cm long pieces for this testing. Figure 4-7 and Table 4-2 show the mechanical properties of air-dried fibres. As discussed in Chapter 3, the low compatibility of alginate structure with PLGA microspheres on the surface cause the spheres work as weak points of
the structure. Phase-separation of the hydrophilic alginate and hydrophobic PLGA may lead stress-concentration at the phase interface of two materials. This phenomenon would lead to faster break of fibres and consequently minor tensile strength. The modulus also could be reduced by low adhesion on the surface. Comparing the mechanical properties (Chapter 3 (section 3.4.4)) of Alg/PLGA fibre C-3 containing the similar concentration of ingredients, the U-Alg/PLGA and G-Alg/PLGA fibres showed relatively higher strengths which can be explained by considering their plasticity behavior undergoing stress. As can be seen in Figure 4-7, U-Alg/PLGA and G-Alg/PLGA fibres showed typical alginate fibre structure behaviour (while undergoing stress) by displaying an extra elongation before breaking completely (highlighted with the light grey dashed box in Figure 4-6) (40).

![Figure 4-7. Stress-strain curves of Alg/PLGA fibre C-3, U-Alg/PLGA and G-Alg/PLGA fibres. The dashed box represents the elongation part before the complete breakage of the fibre while undergoing stress.](image)

Tensile strength and strain at break of the fibres was measured by a dynamic mechanical tester (EZ-L Tester from Shimadzu, Japan) at a strain rate of 0.1 cm.min\(^{-1}\). The tensile strength was calculated according to the following formula:

\[ \sigma = \frac{F}{A} \]  

**Equation 4-1**
where $F$ is the maximum force (N) and $A$ is the cross-sectional area of the fibre (m$^2$) which is considered as the maximum stress in the Stress-strain curve of the fibres. Moreover, elastic modulus of U-Alg/PLGA and G-Alg/PLGA fibres is defined as the ratio of tensile stress to tensile strain in the elastic region of the stress-strain curve.

From the results presented in Figure 4-7 and Table 4-2, G-Alg/PLGA fibres have a lower mechanical strength compared with U-Alg/PLGA fibres which have a uniform distribution of PLGA microspheres inside and along the fibre. This observation is likely the result of the non-uniform gradient distribution of the microspheres inside and along the G-Alg/PLGA fibres.

### Table 4-2. Mechanical properties of Alg wet-spun fibres containing BSA-FITC loaded PLGA spheres.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean diameter of as-spun fibres (µm)</th>
<th>Mean diameter of dried fibres (µm)</th>
<th>Elastic Modulus (MPa)</th>
<th>Tensile Strength (MPa)</th>
<th>Tensile Strain at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-Alg/PLGA</td>
<td>567±14</td>
<td>368±11</td>
<td>9.48</td>
<td>12.4±3.2</td>
<td>6.8±0.6</td>
</tr>
<tr>
<td>G-Alg/PLGA</td>
<td>588±19</td>
<td>379±12</td>
<td>9.46</td>
<td>10.6±1.8</td>
<td>8.8±0.9</td>
</tr>
</tbody>
</table>

#### 4.4.6 Protein (FITC-BSA) release study

Table 4-3 shows the protein loading in each structure developed in this study. Firstly, the FITC-BSA release profiles of protein-loaded PLGA microspheres and Alg fibres were assessed to investigate the effect of both PLGA and alginate encapsulation on protein release. As shown in Figure 4-8 A, FITC-BSA loaded PLGA microspheres showed a continued release for more than 35 days, up to ~ 55% of the loaded protein was released within the observed period. By contrast, FITC-BSA directly encapsulated into Alg fibres shows a rapid release, with a burst release of more than ~44% in the first day and more than 70% released in the first week (Figure 4-8 B).
Table 4-3. The Protein-loaded PLGA microspheres and protein content of each structure.

<table>
<thead>
<tr>
<th>Samples</th>
<th>FITC-BSA loaded PLGA microspheres</th>
<th>U-Alg/PLGA fibre</th>
<th>G-Alg/PLGA fibre</th>
<th>Low protein-loading end</th>
<th>High protein-loading end</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-BSA loading</td>
<td>15.2</td>
<td>3.0</td>
<td>3.0</td>
<td>1.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>

The FITC-BSA release profiles of U-Alg/PLGA and G-Alg/PLGA fibres are presented in Figure 4-8 C. Despite the difference of distribution of spheres along the fibres, both U-Alg/PLGA and G-Alg/PLGA fibres were designed to contain an entire amount of 20% (mass ratio) of protein-loaded PLGA microspheres, and the protein contents of U-Alg/PLGA and G-Alg/PLGA fibres are reported in Table 4-3. Due to the lack of available experimental release studies to investigate the localized release profile, in this part the release profiles of whole fibres (with 3 cm length) consequently total amount released from fibres (not local release) have been studied. They showed relatively similar release profiles which confirms the efficiency of programming technique in loading ingredients. Since the concentrations of ingredients along the G-Alg/PLGA fibre are changing with a constant rate, it is expected that the protein local release profile (at each point) will be different similar to what have been showed previously in Chapter 3 for fibres with different concentrations of BSA-loaded PLGA microspheres as well as the different release rates from high protein-loading end and low protein-loading end of G-Alg/PLGA fibre. But the similarity in overall BSA release profile from U-Alg/PLGA fibre and G-Alg/PLGA fibre showed that the effect of ingredients concentration could be equilibrated. This suggests the efficiency of technique in increasing the Alg content and PLGA microspheres in opposite directions in a constant rate. This two-phase gradient fibre provides the possibility of guided chemotaxis by gradual presentation of cytokines or other biofactors (i.e. growth factors) along a concentration gradient in a spatially directional manner along a fibre. Both fibres
showed an initial slight burst release at day 1 (approximately 6% for both fibres), followed by continuous release trends until day 10. The protein release from U-Ag/PLGA and G-Ag/PLGA fibres continued slowly to up to approximately 32% at the end of the observation period (35 days). The assessment of the protein premature release from the microspheres into spinning dope revealed that approximately 6.1 (±4.2) % of loaded protein was released into the spinning dope during preparation and prior to the solidification of fibres which can cause a fast release profile from U-Ag/PLGA and G-Ag/PLGA fibres, during the first week. The cumulative release of U-Ag/PLGA and G-Ag/PLGA fibres showed that 3 days after the burst, they demonstrated a much slower release than either the FITC-BSA loaded PLGA microspheres or the FITC-BSA loaded Alg fibres alone. Consequently, the incorporation of FITC-BSA loaded microspheres into the Alg fibrous matrix helped to achieve a more extended release profile. Similar studies by others provide some insight into the protein release profiles observed with U-Ag/PLGA and G/Alg/PLGA fibres. Alsmadi et al. (21) developed a protein loaded gradient structure within hydrogel (agarose) microchannels. They applied BSA to study the release profile of the two halves of the structure (low protein-loading end and high protein-loading end). In first 24 hours ~20% and ~50% of the whole protein loaded into low protein-loading end and high protein-loading end was released, respectively. They also reported application of a mixture of BSA and nerve growth factor (NGF) (as biofactors) to investigate the success of the structure in cell growth directory. Despite their successful in vitro results in terms of directing sensory axonal growth along NGF gradients, growth factor release from the structure showed a large burst release (in the first day) with 100% release occurring over a 7-day period. Although their results confirm the efficiency of concentration gradient longitudinal structures in directing the cell re-growth but also it clarifies the need of developing novel concentration gradient structures that can deliver the proteins for an extended time and avoid the local overdose. Comparing the reported BSA release profile from the Alsmadi structure with what have achieved from the G-Ag/PLGA fibre, it is expected that the concentration gradient fibre established with the methodology developed in this study can help in achieving a longer-term protein release profile with less burst release. This can be explained by considering the encapsulation of proteins into microspheres and then incorporation of these into fibrous structure. This two-phase structure leads to the least contact between the protein and surrounding media which cause less burst release and slower release profile. As already discussed, the burst release from the developed structures in this study is mostly attributed to the pre-mature protein
release. It is expected that the developed technique through this chapter can help the future studies in developing NGF gradient fibres with a longer-term release profile and less burst release. This can be achieved because of NGF encapsulation into PLGA microspheres. The preliminary results of assessing the NGF release profile from PLGA microspheres showed a slow NGF release with very low burst amount; but as discussed in Chapter 2, the NGF encapsulation process and the fabrication parameters of making NGF gradient fibres can to be optimised in future studies to achieve a desirable release profile. Johnson et al. (41), developed 3D-printed nerve pathways to deliver two types of bioactive molecules (NGF and glial cell line-derived neurotrophic factor) in a gradient fashion. They loaded the bioactive molecules directly through the host matrix (gelatin methacrylate hydrogel) and reported that the release was completed in 21 days. Compared to the findings reported by Alsmadi and Johnson, in this thesis, incorporation of protein-loaded PLGA microspheres into an Alg fibrous matrix in a gradient manner, as reported here, results in much longer (controllable) release profiles suitable for longer term release applications.

This thesis also considers the release profile of the two half-sections (low protein-loading end and high protein-loading end, specified in Figure 4-6 C) of G-Alg/PLGA fibre with the results as shown in Figure 4-8 D. The low protein-loading end has a lower concentration of protein loaded microspheres (practical loading ~ 28.6% of the whole mass of microspheres loaded into the G-Alg/PLGA fibre) and the high protein-loading end contains the higher concentration of protein loaded microspheres (practical loading ~ 69.2% of the whole mass of microspheres loaded into the G-Alg/PLGA fibre). As shown in Figure 4-8 D the low protein-loading end and high protein-loading end, like G-Alg/PLGA fibre, showed an initial slight burst release followed by a continuous release until day 10. Then their release rate became much slower until the end of the observation period (35 days). The high protein-loading end demonstrated a faster release rate, in the first 10 days, than the low protein-loading end. It seems that with the increasing concentration of microspheres, the Alg concentration as the “shell” of the fibres is reduced; resulting in an increased release rate. This suggests that the concentration of protein along the fibres can be spatiotemporally regulated by modulating the gradient distribution of protein-loaded microspheres within the structure.
Figure 4-8. Cumulative release of FITC-BSA protein from PLGA microspheres (A), FITC-BSA loaded Alg fibre (B), U-Alg/PLGA and G-Alg/PLGA fibres (C) and G-Alg/PLGA fibre and the low protein-loading end and high protein-loading end (D).

There are some other delivery configurations, developed in recent years, which are capable of providing guiding (directing) platforms. For instance, Ostrovidov et al. (22) applied a microfluidic gradient generator to develop a microengineered hydrogel with a concentration gradient of a drug (okadaic acid) for high-throughput analysis of drug-cell interactions. The drug was released from the hydrogel in a gradient manner and resulted in a gradient of cell viability. Although most of the gradient platforms published could successfully release factors in a specific direction and direct axonal regrowth, they required complicated fabrication processes for achieving the gradient structure (21, 23). In addition, they displayed a rapid release profile up to a few days, with the majority of bioactive molecules being released in a phased manner during this time (21, 22, 42, 43). In contrast, the two-phase Alg fibre structure containing FITC-BSA loaded PLGA microspheres presented here
allows construction of a protein concentration gradient and delivery over a sustained (longer term) period that cannot be achieved with PLGA microspheres or Alg fibre alone.

### 4.5 Conclusion

In this study, a technique to fabricate a two-phase Alg/PLGA fibre protein delivery system has been presented. FITC-BSA (the model protein) was encapsulated in PLGA microspheres and then the microspheres were incorporated into an Alg fibre structure via a custom designed programmable wet-spinning method. The process demonstrated reliable production of loaded Alg fibres, which showed release of embedded proteins into the environment over a sustained period. These fibres showed longer release than from PLGA or Alg fibre alone. In addition, the system was demonstrated to facilitate the formation of fibres that contained a defined microsphere concentration gradient that could be readily varied/tailored according to specific release requirements of the protein species in question. The Alg matrix was shown to effectively retard protein release from PLGA microspheres and less than 35% of the whole loaded protein was released after 35 days. The concentration gradient aspect of the two-phase Alg/PLGA fibre system presented here provides the possibility of guided chemotaxis by gradual presentation of cytokines or other growth factors along a concentration gradient in a spatially directional manner along a fibre. This has good potential for promotion and direction of guided tissue regeneration in ‘linear’ systems such as muscle and nerve.

### 4.6 References


Chapter 5  Conclusions and Recommendations for Future Research

5.1  General Conclusion

The main aim of this study was to develop controlled protein delivery systems for long-term release applications. The microspheres were developed as carrier systems for proteins. Despite the recognized role of biopolymers in the encapsulation and release of proteins, the final properties of the protein-loaded microspheres have not been sufficiently characterized and studied in literature. In this study, protein-loaded Alginate (Alg) and PLGA microspheres were developed and their properties, as potential bioactive molecule delivery systems with specific applications in controlled release systems, were investigated. The hydrophilic fluorescently-labelled bovine serum albumin (FITC-BSA) was chosen as the model protein to be encapsulated. Alg and PLGA microspheres were prepared by a W/O emulsion and a W/O/W double emulsion method, respectively. Different properties of the protein-loaded microspheres, such as the mean diameter, loading efficiency, release profiles, chemical and thermal properties were investigated. Both types of microspheres showed a smooth, non-porous surface morphology. Moreover, the statistical analysis results showed almost narrow size distributions of the microspheres. The release profile of FITC-BSA from microspheres presented two phases, an initial burst release phase due to the protein adsorbed on the microsphere surface, followed by a slower release phase corresponding to the protein entrapped within the polymer matrix. FITC-BSA showed a slower release rate from PLGA microspheres compared to Alg microspheres. Moreover, the release rate was different for different protein loading levels into the PLGA microspheres. Based on these results, the PLGA microspheres with higher protein loading might provide a good option for use in future applications in controlled release devices for protein delivery.

After successful fabrication of the model-protein loaded microspheres, Alg wet-spun fibres containing protein (FITC-BSA)-loaded PLGA microspheres were fabricated to achieve an extended controlled protein release profile. Alg and PLGA have long been shown to be biologically well-tolerated options for controlled local delivery of proteins in vitro and in vivo. In this study, two-phase Alg/PLGA delivery systems composed of PLGA microspheres containing protein (FITC-BSA) wet-spun into Alg
fibres to provide an additional element of release controllability was evaluated in vitro. The fibres were fabricated from two different molecular weight Alg materials with different concentrations as well as different FITC-BSA loaded PLGA microspheres loadings. The two-phase delivery matrices display retarded FITC-BSA release significantly in both initial and late stages compared to release from the PLGA microspheres or Alg fibre alone. Moreover, the model protein release profiles revealed their dependence on Alg molecular weight and also concentration of ingredients.

On the other hand, the defined protein-specific, first-order release kinetics directly associated with its structure/composition, often preclude Alg’s use for complex delivery profiles: specific protein release profiles are achieved by controlling polymer composition/concentration, which also defines modulus of the Alg hydrogel. Thus, achievement of desired release profiles/kinetics often results in an Alg construct incompatible with implantation into target tissues. This largely limits Alg-mediated protein delivery to single-factors of uniform concentration applications rather than applications that may involve multiple proteins (i.e. GFs) delivered at a concentration gradient for chemotactic purposes. In Chapter 4, we introduced a fabrication technology to develop programmable microspheres concentration gradient fibres (G-Alg/PLGA fibre). The morphology, diameters, mechanical properties and protein release profile of the fibres were characterized. Extended controlled release profiles were achieved and the fabrication system devised was shown to have the capability to produce wet-spun fibres with a protein concentration gradient. In addition, it was observed that incorporation of higher concentrations of protein-loaded PLGA microspheres increased protein release compared to fibres with lower concentrations of BSA-loaded microspheres. This "programmable" microspheres concentration gradient fibre, presented in this study, may enable development of novel Alg scaffolds with the ability to guide tissue regeneration through tightly-controlled release of one or more proteins in various spatio-temporal concentration configurations within the structure.

5.2 Recommendations for Future Research

- The morphology studies of uniform and microsphere concentration gradient fibres (U-Alg/PLGA and G-Alg/PLGA fibres) reveal the ability of the programmable fabrication method in producing fibres with modified morphology comparing to the wet-spinning method. It would be beneficial if
the wet-spinning approach could be exploited further to enable the formation of fibres with higher surface homogeneity, as this may positively impact on drug release and mechanical properties.

- Development of biopolymer microspheres and/or microfibres allowing a controlled and sustained release of them (neurotrophic factors, such as NGF) is an ongoing challenge. It is because of the instability of these proteins that they can lose their bioactivity during the preparation or the release phase (1). In Chapter 2, a report on a microencapsulation process to prepare NGF-loaded PLGA microspheres is presented. It would be beneficial to optimize the processes to achieve a desired continuous in vitro release of different neurotrophic factors considering their specific properties such as half-life time, molecular weight and isoelectric point. Moreover, the preservation of the bioactivity of NGF, during the encapsulation process, has a significant effect on their functionality in vitro and in vivo. Investigation of the preservation of growth factors bioactivity during the encapsulation and fabrication processes is suggested as a subject for further studies by biology researchers. It can ascertain the functional status of the released NGF from the structures prepared using the established methodologies for encapsulation of NGF into PLGA microspheres and incorporation of them into fibrous structures.

- The development of tissues is normally driven by the action of several GFs. One ongoing challenge in the field of protein delivery is development of structures that allow the local controlled release of appropriate combinations of multiple factors without affecting the bioactivity of each single-factor (2). Encapsulation of GFs into the microspheres and incorporation of them into a programmable Alg fibrous matrix can provide the opportunity of multiple GFs release in a required controlled manner.

- Long-term, localized delivery of neurotrophic factors (specifically NGF) in a gradient manner may help regenerate lost structure and function in diseased or traumatized tissues (3, 4). It would be desirable to study the ability of programmable NGF-loaded microspheres concentration gradient fibres to promote and direct nerve regeneration (along an increasing concentration of β-NGF) in the nervous system as a platform for molecularly guided tissue repair.
Moreover, luminal and multi-luminal nerve scaffolds are of particular interest as they provide increased surface area for nerve regeneration compared to fibres. It is recently reported that multi-luminal nerve scaffolds fabricated with hydrogel microchannels efficaciously bridged a nerve injury gap, by linearly restricting axonal regeneration and enticing the regeneration of fascicular-like tissue (3). The introduced fabrication technology (Chapter 4) can be easily applied to develop luminal and multi-luminal GF-loaded microspheres concentration gradient structures. The schematic of the suggested structures are presented in Figure 5-1.

Figure 5-1. Luminal (A) and multi-luminal (B) GF-loaded microspheres concentration gradient fibres.

5.3 References


Chapter 6 (First-year work)

Due to funding changes, I had to change the scope of my project at the completion of the first-year of my Ph.D. Herein; the work undertaken during my first-year is presented in the chapter 6. Aspects of the work detailed in appendix have been published (F. Mehrpouya, J. Foroughi, S. Naficy, J. M. Razal, M. Naebe, Nanostructured Electrospun Hybrid Graphene/Polyacrylonitrile Yarns. Nanomaterials 7, 293 (2017). doi: 10.3390/nano7100293)

Nanostructured electrospun hybrid graphene/polyacrylonitrile yarns

Graphene oxide/Polyacrylonitrile (PAN) nanofibrous yarns were prepared by electrospinning of solutions containing poly (acrylonitrile) (PAN) and liquid crystal graphene oxide (LCGO). In the course of electrospinning, the well-dispersed LCGO were oriented along the fibre axis in an electrified thin liquid jet. LCGO, a sort of novel two-dimensional (2D) macromolecule, provides a means by which to produce high performance graphene based fibres and yarns. During this process, the LCGO dispersed in N, N-Dimethylformamide (DMF) was incorporated into PAN dissolved in the same solvent. Nanofibrous mats were produced using conventional electrospinning. The GO sheets were well dispersed in the polar organic solvent, forming nematic and lamellar LCs upon increasing concentration. The nanofibers were spun and aligned in the same direction. Notably, the addition of LCGO at almost very low weights fraction improved the mechanical properties of the nanofibrous composite twisted yarns. This offers a new approach for the fabrication of continuous, strong and uniform composites yarns.

6.1 Introduction to hybrid nanofibres

Polyacrylonitrile is an important polymer commercially, mainly because it is the fibre precursor to about 90 % of the carbon fibre manufactured today (1). Carbon fibres exhibit high thermal stability, resistance to most solvents, high strength and high stiffness. Notably, the PAN-based carbon fibres are the preferred reinforcement material for structural composites where their superior strength and stiffness is combined with their light weight and low production cost compared to most metallic
components (2). Moreover, continuous advanced carbon nanofibrous yarns can be produced by carbonizing electrospun PAN-based yarns with improved mechanical properties (3,4). These carbon nanofibers (CNFs) have been used in a wide variety of nanotechnology applications, including development of materials and devices for energy storage, environmental, biomedical, electronics, and structural applications (3,5).

The degree of orientation of graphitic planes is the main parameter in determining the mechanical properties of CNFs. CNFs inherit their properties from the starting nanofibrous structure and it has been reported that reinforced CNFs can be made from precursors with higher mechanical properties (3,6-10). Hence, improving the properties of the PAN nanofibres (NFs) and their composites have been the subject of intensive investigations (1,11,12). For instance, nanofillers such as montmorillonite, carbon nanotubes and/or graphene oxide (GO) have been added to PAN to make nanofibrous composite with improved mechanical strength, electrical conductivity, or thermal stability (13-15). Although the previous studies presented that carbon-based fillers at a loading of a few percent could enhance the mechanical properties of PAN nanofibres, but developing a perfectly structured precursor with optimum concentration of ingredients has been remained as a challenge (16). In this study, a novel preparation method of PAN/LCGO composite nanofibrous twisted yarns is presented, which can be used as a precursor for carbon fiber production. The results indicate the improved mechanical properties of the product with optimized concentration of ingredients. On the other hand, the introduced fabrication method of hybrid nanofibrous twisted yarns can be applied to develop functional materials from other 2D components such as transition metal dichalcogenides or 2D boron sheets (instead of GO) to improve different properties (17-19).

Graphene, a free-standing 2D crystal with one-atom thickness, has become one of the most recent topics in the fields of materials science, physics, chemistry, and nanotechnology (20,21). It has a large theoretical specific surface area (2630 m$^2$·g$^{-1}$), high intrinsic mobility (200 000 cm$^2$·V$^{-1}$·s$^{-1}$), high Young’s modulus (~1.0 TPa), high thermal conductivity (~5000 Wm$^{-1}$·K$^{-1}$), and good electrical conductivity (22). It is worth mentioning that in recent years, graphene fiber has become as a new carbonaceous fiber with novel high mechanical and functional prospects (23).
Unlike pristine graphene that has limited processability, graphene oxide (GO) can be easily dispersed in many solvents due to the presence of various polar functional groups on its surfaces and edges (24-26). In view of their excellent mechanical and physical properties, graphene and GO based composites are expected to demonstrate enhanced properties compared to conventional composites (28). The use of liquid crystalline GO (LCGO) dispersions enables the development of unique 3D assembly with highly ordered, macroscopic structures (4,27-30).

As mentioned above, this paper aims to create PAN/LCGO nanofibrous twisted composite yarns that can be used as a precursor for the fabrication of reinforced carbon fibers. These fibres could also be used for different applications in the fields of energy storage (e.g. supercapacitors) (31,32), bioapplications (e.g. enzyme immobilization) (1), and ultra-fast microfiltration of oil-water emulsion (33). Highly oriented molecular structure is needed for high-performance carbon fibers. Since a minor quantity of graphene sheets restrain the disorientation of the chain segments, and this will cause an improvement in molecular orientation of precursor fibers during spinning and stabilization (34). For example, Chien et al. (35) fabricated composite carbon fibers with higher mechanical properties using continuous PAN/GO nanoribbon composite fibers as the precursor. Here, liquid crystal graphene oxide (LCGO) dispersed in an organic solvent was incorporated into PAN dissolved in the same solvent to prepare composite nanofibrous mats. These mats then were twisted to produce PAN/LCGO nanofibrous twisted composite yarns. The morphology, chemical and mechanical properties of the composites were studied by optical microscopy, field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), Raman spectroscopy, differential scanning calorimetry (DSC), and mechanical properties testing.

6.2 Materials and Methods

6.2.1 Materials

LCGO dispersion in N, N-Dimethyl formamide (DMF) was synthesized in home-laboratory, following a method presented elsewhere (36-40). The PAN (average molecular weight ~ 150,000 g.mol⁻¹) powder was obtained from Sigma-Aldrich and used without further purification. DMF was used as received (Sigma-Aldrich, Australia).
6.2.2 Methods

Known amounts of LCGO in DMF were added to PAN solutions (according to Table 1) and mixed by magnetic stirring for 48 h to prepare the electrospinning dope solutions. The mass ratio of the PAN was 10 wt% in all solutions. The electrospinning set-up consisted of a syringe which injected the electrospinning solution at the rate of 1 ml.hr$^{-1}$ onto a drum collector rotating at 2000 rpm. The distance and voltage between the syringe tip and the collector was maintained at 15 cm and 15 kV, respectively. Electrospinning was carried out at ambient temperature inside a humidity controlled chamber with humidity between 30 % and 40 %.

The nanofiber ribbons with average initial length of 57 cm were easily removed from the collector to be twisted into yarns. For this purpose, each ribbon was first immersed in ethanol and then connected to two motors’ shafts which rotated at 42 rpm for 17 minutes; so that, the ribbons were twisted into yarns. It is worth mentioning that further increase in twist resulted in breakage of the yarns (32). Table A-1 shows the percentage of LCGO in NFs mats which was calculated due to equation A-1.

\[
\text{GO (in NFs mats)} = \frac{m(\text{GO})}{m(\text{GO}) + m(\text{PAN})} \times 100
\]  

\text{Equation A – 1}

6.3 Characterization

6.3.1 Rheology of the solutions

The rheological behavior of initial dispersions were analyzed using an AR-G2 rheometer (TA Instruments, New Castle, DE) equipped with a Peltier plate thermal controller. A$^\circ$/36mm cone geometry was used in all measurements. The solutions were set at the equilibrium temperature for 1 min prior to performing the experiments. Viscosity and shear stress were measured as a function of shear rate at at 25 °C.

6.3.2 Optical microscopy

Images of as-prepared, dried and wet microspheres were recorded via an optical microscope (Leica M205A, Australia). Average diameters of the microspheres were estimated with ImageJ image visualization software (n=150).
6.3.3 Scanning Electron Microscopy

The nanofibres mats (coated with a 15 nm gold layer) were mounted on metal stubs using conductive double-sided tape. Sample’s morphologies were observed by use of a scanning electron microscope (JEOL JSM-6490 LV, Japan) at an accelerating voltage of 15 kV.

6.3.4 Raman Spectroscopies

Raman spectroscopy measurements in the region of 800-2200 cm⁻¹ were performed using a JY HR800 Raman spectrometer which was capable of performing Raman analysis with spatial resolution down to 1um and spectral resolution starting at 1.5 and down to 0.35 cm⁻¹.

6.3.5 Differential Scanning Calorimetry (DSC) thermograms

Differential scanning calorimetry (DSC) analysis was performed on a DSC Q100 from TA Instruments at a rate of 10 °C min⁻¹ between 25 and 400 °C on 10 mg of nanofibrous mats.

6.3.6 Mechanical Properties

Tensile tests of the prepared nanofibrous mats [size: 10mm × 5mm] were carried out on a dynamic mechanical tester (EZ-L Tester from Shimadzu, Japan), at rate of 1 mm.min⁻¹ and a gauge length of 10 mm. Average values of tensile strength and maximum strain were determined from testing 3 fibres of each type.

6.4 Results and Discussion

6.4.1 Study of rheological behavior of LCGO and PAN/LCGO composite suspensions

To evaluate properties of PAN and hybrid PAN/LCGO solutions, rheological behavior of the spinning solutions were carried out. The rheological behavior of LCGO and PAN/LCGO dispersions were
shown in Figure A-1. Shear stress and viscosity of PAN solution and PAN/LCGO dispersions were characterized as the function of shear rate at 25 °C using the cone-plate configuration.

Figure 6-1. (A) Viscosity and (B) shear stress vs. shear rate curves of LCGO dispersions in DMF and PAN and PAN/LCGO dispersions in DMF.

It can be seen in Figure 6-1 that shear stress increases with shear rate while viscosity decreases. Moreover, while PAN solution and dilute PAN/LCGO dispersions exhibit Newtonian behavior,
LCGO dispersions at higher concentration display a shear thinning trend. The highly-concentrated PAN/LCGO dispersions have considerably higher viscosity and shear stress at low shear rates compared to PAN solution and dilute PAN/LCGO dispersions (more than 100 times). At higher shear rates however, the viscosity continuously decreases with shear rate, converging to that of PAN and dilute PAN/LCGO. Similarly, the shear rate of PAN solution and dilute PAN/LCGO dispersions increases linearly with shear rate, while more concentrated PAN/LCGO dispersions behave differently. After a sharp increase in shear stress of concentrated PAN/LCGO dispersions with increasing shear rate, shear stress continues to increase linearly as a function of shear rate and again follows the dilute PAN/LCGO dispersions pattern at high shear rates. The observed shear thinning in rheological behavior of concentrated PAN/LCGO can be attributed to the alignment of LCGO plates in the dispersion at higher shear rates. Also, the change in the slope of shear stress as a function of shear rate at low shear rate values in concentrated PAN/LCGO dispersions agrees with this observation.

### 6.4.2 Morphological study of as-Prepared hybrid electrospun nanofibers

The fabrication of PAN and hybrid PAN/LCGO nanofiber were carried out using electrospinning machine. The 4 cm wide ribbons consisting of aligned polymer nanofibers were formed on the drum collector (Figure 6-2 A to E). Remarkably, with GO embedding, the color of electrospinning solutions and electrospun nanofibrous mats changes from white to dark brown, suggesting that GO nanosheets have been dispersed in the PAN substrate (Figure 6-2) (31,32). In addition the TEM image of PAN/LCGO spinning solutions were carried out to evaluate the dispersion of LCGO in PAN as the polymeric matrix. The TEM image of PAN/LCGO-D hybrid nanofiber is presented in Figure A-2 F.
Figure 6-2. Photography of the as-prepared electrospun mate (A) PAN, (B) PAN/LCGO-B, (C) PAN/LCGO-C, (D) PAN/LCGO-D and (E) PAN/LCGO-E. (F) TEM image of PAN/LCGO-D shows distribution of LCGO sheets through the nanofibrous structure.

6.4.3 Morphological study of PAN/LCGO nanofibrous mats and twisted yarns

Free-standing electrospun nanofibrous mats were successfully prepared from all PAN/LCGO concentrations presented in Table A-1. The overall morphology of as-spun nanofibres (NF) showed in Figure A-3. As can be seen, the electrospun nanofibers had variable fibre diameters and structure, which was significantly affected by the addition of LCGO. As can be seen from Table A-1, with the increase of LCGO loading, the average diameters of the composite nanofibres also increased.

Figure 6-3. FESEM images of as-spun nanofibres with different concentrations of LCGO, (A) PAN-A, (B) PAN/LCGO-B, (C) PAN/LCGO-C, (D) PAN/LCGO-D, (E) PAN/LCGO-E and (F)
the high magnification FESEM image of PAN/LCGO-E to study the morphology of an individual nanofibre with highest concentration of LCGO.

Figure 6-3 A to E also show that most of the nanofibres are oriented in one direction, which is on the direction of the rotation of the drum collector. As can be seen in Figure 6-3 the individual nanofibres with any concentrations of LCGO have an almost smooth structure.

Table 6-1. Electrospinning solutions and final percentage of GO in nanofibrous mats.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>PAN (wt%)</th>
<th>LCGO (wt%)</th>
<th>GO (in NFs mats) %</th>
<th>Average NFs diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN-A</td>
<td>DMF</td>
<td>10</td>
<td>----</td>
<td>---</td>
<td>252</td>
</tr>
<tr>
<td>PAN/LCGO-B</td>
<td>DMF</td>
<td>10</td>
<td>0.01</td>
<td>0.099</td>
<td>251</td>
</tr>
<tr>
<td>PAN/LCGO-C</td>
<td>DMF</td>
<td>10</td>
<td>0.031</td>
<td>0.309</td>
<td>311</td>
</tr>
<tr>
<td>PAN/LCGO-D</td>
<td>DMF</td>
<td>10</td>
<td>0.157</td>
<td>1.546</td>
<td>345</td>
</tr>
<tr>
<td>PAN/LCGO-E</td>
<td>DMF</td>
<td>10</td>
<td>0.314</td>
<td>3.044</td>
<td>490</td>
</tr>
</tbody>
</table>

On the other hand, the trend in the average diameter of nanofibers (Figure 6-3) can be explained by the fact that higher concentration of LCGO resulted in more viscous PAN/LCGO dispersions and hence, affected the drawing of filaments during the whipping motion of filaments during the electrospinning process resulting into thicker nanofibers (11). Apart from viscosity, other material variables such as electrical conductivity and surface tension of the electrospinning solutions are impacted by the LCGO loading. All these parameters are influential variable in defining the final morphology of the electrospun fibers (11).
The electrospinning parameters were optimized to obtain uniform nanofiber structures as is evident from Figure 6-3. But as can be observed in Figure 6-3, the sample PAN/LCGO-E with highest concentration of LCGO displayed rough and thicker regions which suggest it contain large graphene oxide sheets. It is known that GO sheets bend and fold easily into various shapes contingent of substrate or temperature (3). It is worth mentioning that a small amount of LCGO can remarkably change the solution properties of PAN. Because LCGO disperses well on DMF, the composite polymer solution, with higher concentrations of LCGO, can be divided into GO-rich domain and GO-scarce domain, which may lead to instability of the liquid jet during electrospinning process. So, the beaded structures were formed.

By twisting the electrospun mat, twisted yarns of electrospun nanofibres were obtained. Figure 6-4 A to C show the surface optical microscopy and longitudinal and cross-sectional SEM images of the PAN/LCGO-D nanofibrous twisted yarn. As can been seen from the surface morphology, the nanofibres were uniform, and predominantly oriented with a helix angle of ~35° to the yarn axis (Figure 6-4 A and B).

Figure 6-4. As-prepared electrospun nanofibres: (A) the surface optical microscopy, (B, C and D) longitudinal and cross-sectional SEM images of the PAN/LCGO-D nanofibrous twisted
yarn, respectively. (E and F) Longitudinal and cross-sectional FESEM images of individual PAN/LCGO-D nanofibre, respectively.

Similar to conventional textile yarns, twisting is a useful fibre processing technique to improve yarn strength. Clearly, untwisted yarn displayed poor mechanical properties and usually mechanical properties of twisted yarns are improved with the increase of twist level (37). As-prepared electrospun PAN and hybrid PAN/LCGO nanofibres were transformed into yarn by twisting. The as-spun PAN/LCGO-E nanofibres showed an enormously ununiformed structure and could not be twisted uniformly into the yarn.

6.4.4 Raman spectroscopies of PAN/LCGO nanofibrous twisted yarns

Raman spectroscopy plays an important role in the structural characterization of graphitic materials (25). A D-band in Raman spectra of pure PAN at 1320–1345 cm$^{-1}$ corresponds to sp$^3$ C–C bonds, indicating the disordered turbostratic structures, amorphous carbon or defects in the curved graphene nanosheets. The G-band at 1580–1597 cm$^{-1}$ corresponds to the in plane tangential stretching mode of sp$^2$ C–C bonds, expressing the ordered graphite crystallite structure and tangential shearing mode of the carbon atoms (18,26,27). The relative intensity ratio of the D-band to the G-band, ID/IG, which is called the “R-value”, indicates the amount of quantitative characterization of the structurally ordered graphite crystallites in the carbonaceous materials. The width of the G band is also often used as an indicator of the level of graphitization (narrower G band indicates better graphitic structure) (27).

Raman spectroscopy measurements in the region of 800-2200 cm$^{-1}$ were performed to study the internal structure of the PAN/ LCGO nanofibrous mats. The Raman spectra of pure PAN and LCGO/PAN nanofibrous samples (Figure 6-5 A) show significant differences between the two materials. The I_D/I_G ratio for the nanofibrous mats showed a peak at the 1.546 wt. % LCGO fraction (sample PAN/LCGO-D) and this difference indicates that this sample has more ordered graphite crystallites (Figure 6-5 B). Above 1.546 wt. % LCGO fraction, the R-value increased (i.e. the crystallinity decreased) (14,32,36). The samples with higher concentration of LCGO showed improved graphitic structure as indicated by smaller R (Figure 6-5 B). These results suggest that the
significant improvements in the graphitic structure of the resulting CNFs were a direct consequence of the addition of a small amount of LCGO into the electrospinning solutions.

Figure 6-5. (A) Raman spectra of PAN/LCGO nanofibrous mats, and (B) R-values for the same samples.
6.4.5 DSC thermograms of PAN/LCGO nanofibrous twisted yarns

The effect of LCGO addition on the thermal properties of electrospun PAN/LCGO composite nanofibrous twisted yarns were investigated using DSC. For comparison, the DSC exothermic curve of PAN nanofibrous mat is also illustrated in Figure 6-6. The decomposition temperature (Tm) and heat of fusion ($\Delta H$) for blends are demonstrated in Table 6-2. The exothermic peak for pure PAN is 324 °C. Addition of only 0.3 wt. % LCGO to the PAN solution led to an increase in Tm to 329 °C for the resultant mat. In general, the decomposition temperature increased because of LCGO addition. At the same time, a significant decrease in the heat of fusion (from 5036 to 4366 J/g) is observed with inclusion of LCGO in electrospun nanofibres.

![DSC thermograms of the PAN and PAN/LCGO nanofibrous twisted yarns.](image)

Considering the DSC thermograms of the twisted yarns, a clear increase in decomposition temperature and a corresponding decrease in $\Delta H$ can be realized with inclusion of LCGO (38,39,40). The increase in the Tm of PAN/LCGO fibres upon addition of LCGO content as well as the decrease in the $\Delta H$ could be due to the intermolecular interactions between PAN and LCGO particularly when being heat
treated during thermal analysis. This intermolecular interaction justifies the improvement in graphitic structure shown by Raman analysis as a result of LCGO inclusion. These findings are significant for the development of high performance carbon nanofiber structures using nano-enhanced precursor materials.

Table 6-2. The Tm and ΔH of PAN and PAN/LCGO nanofibrous twisted yarns.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DSC Max peak (ºC)</th>
<th>ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN-A</td>
<td>324</td>
<td>5036</td>
</tr>
<tr>
<td>PAN/LCGO-B</td>
<td>323</td>
<td>4827</td>
</tr>
<tr>
<td>PAN/LCGO-C</td>
<td>325</td>
<td>4660</td>
</tr>
<tr>
<td>PAN/LCGO-D</td>
<td>327</td>
<td>4818</td>
</tr>
<tr>
<td>PAN/LCGO-E</td>
<td>329</td>
<td>4366</td>
</tr>
</tbody>
</table>

6.3.1 Mechanical properties of PAN/LCGO nanofibrous twisted yarns

The mechanical properties of twisted electrospun PAN and hybrid PAN/LCGO yarns are shown in Figure A-7. Stress-strain curves obtained from each of the twisted PAN and hybrid PAN/LCGO yarns showed a significant difference in mechanical properties. Analysis of these curves indicates a stress at break of 41.1 MPa with 119.1% strain for the as-prepared twisted PAN/LCGO-D yarn, compared with 19.6 MPa stress with 75.53% strain for the as-prepared twisted PAN-A yarn. Young’s modulus of these yarns was 366.42 MPa and 145.35 MPa for the hybrid twisted PAN/LCGO-D and PAN-A yarns respectively. The average values of tensile modulus and ultimate tensile strength of the as-prepared PAN/LCGO nanofibrous twisted yarns are summarized in Table 6-3.
Figure 6-7. Stress vs. strain curves for as-prepared electrospun twisted PAN and PAN/LCGO yarns.

The sample with higher ratio of the graphene (PAN/LCGO-E) showed lower mechanical properties compared to other (Table 6-3). This phenomenon could be explained by aggregation of graphene in the polymer matrix due to higher mount of LCGO in spinning solution. Consequently, the addition of LCGO increased yarns Young’s modulus and their strength. Yield points (maximum in the peak (stress-strain curve)) associated with a deformation mechanism can be detected for the different composites. From the slope of the in elastic part of the stress–strain curve, increasing the concentration of LCGO in samples improved the mechanical properties of PAN/LCGO nanofibrous twisted yarns. As it was previously shown using fibers with improved mechanical properties as precursors for making carbon fibers can result in carbon fibers with improved properties (4). The low twisting saturation number for PAN/LCGO-E due to its high average diameter and diameter heterogeneity resulted in the deviation from the trend for mechanical properties. The comparison of the result of Raman spectroscopy and stress-strain plots show that increasing the concentration of LCGO in electrospinning solutions can improve mechanical properties by improving the graphitic structure.
Table A-3. Mechanical properties of PAN/LCGO nanofibrous twisted yarns at Yield points.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PAN-A</th>
<th>PAN/LCGO-B</th>
<th>PAN/LCGO-C</th>
<th>PAN/LCGO-D</th>
<th>PAN/LCGO-E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Young's Modulus</strong> (MPa)</td>
<td>145.35</td>
<td>312.5</td>
<td>332.45</td>
<td>366.42</td>
<td>233.56</td>
</tr>
<tr>
<td><strong>Stress</strong> (MPa)</td>
<td>19.60</td>
<td>22.90</td>
<td>31.20</td>
<td>41.10</td>
<td>16.5</td>
</tr>
<tr>
<td><strong>Strain</strong> (%)</td>
<td>75.53</td>
<td>62.32</td>
<td>93.40</td>
<td>119.10</td>
<td>13.15</td>
</tr>
</tbody>
</table>

6.5 Conclusions

The incorporation of very small amount of liquid crystal graphene oxide into PAN matrix have shown to have significant effects on the graphitic structure and preferred orientation of the composite nanofibrous twisted yarns formed from these dispersions. Consequently, the PAN/LCGO composite nanofibrous twisted yarns with improved mechanical properties compared to pure PAN yarns were prepared. The results revealed that the incorporation of LCGO effectively enhanced the mechanical properties of the composite nanofibres. It is expected that far better properties can be attained from the carbon yarns which will be made from these precursors, but still more studies are needed to further understand and manipulate the effect of using LCGO in electrospinning dispersions to produced advanced carbon yarns.

A.6 References


