Studies of protein release mechanisms from polymer blends

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**NANYANG
TECHNOLOGICAL
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**STUDIES OF PROTEIN RELEASE MECHANISMS FROM
POLYMER BLENDS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2012

STUDIES OF PROTEIN RELEASE MECHANISMS FROM POLYMER BLENDS

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Abstract

The main objective of this work is to understand protein release mechanisms from polymer blends through microenvironment evaluation. Such evaluation would provide deeper insights on the importance of microenvironment properties especially polymer miscibility, protein partitioning and protein distribution, in affecting protein release mechanisms.

Quantification of protein partitioning in a phase-separated polymer blend gives important insights into the protein release mechanism. Here, the author reports on the first visualization of protein-PEG colocalization in PCL/PEG blends using a combined application of confocal Raman mapping and confocal Laser Scanning Microscopy (CLSM) imaging. The degree of protein-PEG colocalization was further quantified via a novel image processing technique. This technique also allowed the characterization of the 3-D protein distribution within the films. The results showed homogeneous protein distribution within the film matrix, independent of PEG content and film thickness. However, the degree of protein-PEG colocalization was inversely proportional to PEG content, ranging from 65 to 94%. This quantitative data on protein-PEG colocalization was used along with in vitro PEG leaching profile to construct a predictive model for overall protein release. The predictive model matched well with the experimental protein release profile, which is characterized by an initial burst release and a subsequent slower diffusional release. More importantly, the success of this predictive model has highlighted the influence of protein-PEG colocalization and PEG leaching on protein release mechanism. This study suggests that a formulation strategy of enhancing polymer-polymer interaction would be desired for the successful protein sustained formulation using polymer blend. This has led to the use of PCL-PEG diblock copolymer in optimizing such interaction.

The microenvironment evaluation for PCL-PEG copolymer blends validated its correlation with protein mechanisms. Protein release profiles of PCL/copolymer blends can be characterized by an initial burst release followed by a diffusional release. Interestingly, the magnitude of protein burst release is highly influenced by PCL-PEG block fractions. In spite of a homogeneous protein distribution found in both copolymer types, the massive burst release from copolymer blends of lower PCL-block fraction indicated a non-surface-segregation-dependent burst release. To sort out the cause of burst release, polymer miscibility of the copolymer blends was investigated. The results showed that the degree of polymer miscibility was proportional with the increasing PCL-block fraction, as an outcome of PCL-block partitioning into the homo PCL matrix. The better miscibility between PCL and copolymer of higher PCL-block fraction provides a non-leaching behaviour, which resulted in a low-burst-high-subsequent protein release profiles. The importance of microenvironment properties in governing protein release mechanisms has once again been highlighted.

Chapter 1. Introduction

1.1 Background

Since the approval of recombinant insulin by the US Food and Drug Administration (FDA), a remarkable expansion in the number of protein therapeutics has seen in the last 25 years [1]. To date, around 200 biopharmaceutical products are currently approved by the FDA for clinical use, of which 58 of them are approved in the recent 4 years [2], and many more are still in the pipeline. The global market size for protein therapeutics is projected grow robustly by 7 – 15% annually [2] for the next few years. According to a Market Watch published in Nature Reviews Drug Discovery, protein therapeutics are likely to represent four of the five top selling therapeutics (including synthetic drugs) by 2013 [3].

The earliest reported applications of proteins as therapeutic agents involved intravenous injection. This delivery method requires higher dosage that may generate undesired effects as it is not localized to the target tissue and is also not sustained due to the short half-lives of most proteins. This issue has motivated the development of controlled delivery systems that allow the localized, sustained and controlled delivery of therapeutic proteins. Among the different approaches to achieve protein delivery, the use of polymers as its vectors, specifically biodegradable polymers, holds great promise. In developing a polymeric protein controlled release system, the active compound should be delivered at a specific rate, while maintaining its therapeutical effect, at the same time, without exceeding its toxic limit

throughout the entire therapy regimen. Thus, controlling the release rates of proteins through various polymer combinations is crucial.

Blending of different polymers is an attractive way to control and tailor protein release rate. The advantages of polymer blends for controlled release applications include the possibility of controlling the release of both hydrophobic and hydrophilic proteins, and manipulation of carrier properties such as hydration rate, degradation rate and drug loading. These advantages have propelled the use of polymer blends into the spotlight of controlled delivery systems.

1.2 Problem Statement

A simple approach to modify release rates of a drug/protein from a biodegradable polymer is through blending. The intrinsic immiscibility of most polymers, however, causes phase separation during solvent casting in film preparation [4, 5]. The use of a hydrophilic polymer, especially, in the phase separated polymer blends may lead to fast polymer leaching [6-11] that complicates the understanding of release mechanisms. *To date, very few, if any, works have investigated the correlation of the fast leaching polymer with drug/protein release mechanism.* Furthermore, when the drug/protein is loaded in a phase separated system, its partitioning between the polymer blend components (or phases) determines the release characteristic from the blends. *However, it has been almost impossible to quantify the preferential partitioning of drug/protein into each of the phases, and this has limited the understanding of the release mechanisms and their manipulation.*

Currently available quantitative methods for drug distribution studies are limited to surface analysis, with a detection depth limited to nano-scale [12, 13]. Although valuable, the low penetration depths for these instruments limit their application for a wide variety of drug delivery systems, such as polymeric degradable system where the carrier thickness is usually in micron-scale. To overcome the detection depth limit, a more commonly used method for drug distribution studies is imaging, which involved mapping the distribution of components, providing a 3-D information of drug distribution within a polymer system. *However, very little, if any, quantitative work has been done to fully enhance the 3-D information of CLSM images in drug delivery formulation research.*

1.3 Objectives

The primary goal of this research is *to understand protein release mechanisms from polymer blends*. The focus of this thesis can be subdivided into two parts; that is to *quantitatively evaluate the microenvironment of the protein loaded polymer blends* and to *correlate the insights gained from this evaluation with protein release mechanisms to achieve a controllable protein release system*.

To understand the release mechanisms, it is important to quantitatively evaluate the protein distribution and its partitioning in the polymer blends; for this we plan to use what was formerly only a qualitative imaging method. Due to the possibility of polymer phase separation in the blends, further studies on polymer miscibility will also be done to elucidate its effect in affecting protein distribution and protein partitioning. This is especially

important given the fact that in an immiscible system, protein preferential partitioning in one polymer phase and polymer leaching of one polymer phase may occur.

The second part is to understand the protein release mechanisms by correlating the information gained from the quantitative evaluation with the protein release profiles. This would then allow a deeper appreciation of how the physicochemical properties may affect the behavior of protein release from the blend system. At the end of the thesis, tailoring of protein release profiles will also be done through the deeper understanding of the microenvironment-protein release correlation.

1.4 Scope

In order to achieve the objective mentioned, a blend of two commonly used polymers in the medical device industry, PCL and PEG, will be studied. A model protein, lysozyme, will be loaded into the blends of (1) PCL/PEG and (2) PCL/PCL-PEG diblock copolymer. Quantitative characterization of the protein distribution and protein partitioning will be carried out using confocal methods, and used to explain the observed release patterns of protein from different blends.

A preliminary study with various polymer systems and protein loading techniques will be conducted for screening purposes. The formulations and fabrications screening process will be based on the potential use of the system as a controlled delivery carrier, especially in terms of the controllability of the protein release profile. Although protein stability is not of

the focus of this research work, protein stability issue will also be considered in the screening process.

Microenvironment evaluation of the protein loaded blends will be focused on (1) protein distribution, (2) protein partitioning, and (3) polymer miscibility of the blends. Qualitative analysis of protein distribution and protein partitioning in the polymer blends will be studied using both confocal Raman mapping and confocal microscopy depth-profiling techniques. In addition to the qualitative analysis, quantitative analysis will also be done via a novel image processing technique of the CLSM images. This novel technique is meant to extract quantitative microenvironment information from the 3-D CLSM images of the polymer blends. As PCL, PEG and PCL-PEG diblock copolymers are semicrystalline, their crystallinity and thermal properties will be investigated to indicate the miscibility of the blends. In addition, the possibility of PEG or copolymer leaching from the blends will also be confirmed.

To better understand the protein release mechanisms, in vitro protein release from the two polymer blends will be explored. In the attempt to adjust protein release rate (as well as component leaching rate), the following approaches will be tested: (1) by varying the ratio of PEG or copolymer in the blends, and (2) by using PCL-PEG diblock copolymers of different PCL to PEG block fractions.

1.5 Overview

This thesis has been organized into seven chapters. The opening chapter highlighted the background and motivation that drives this research topic, approaches to achieve the objectives, and the novelty of this research. Chapter 2 reviewed previously reported works in relation to this research topic. These past achievements served as the basis and direction of this work. Chapter 3 outlined the experimental methodology to fulfill the objectives of this research work. Chapter 4, 5 and 6 formed the core of this thesis. In brief, Chapter 4 showed the progress of fabrication screening from the perspective of release controllability and protein stability, which includes polymer selection, protein loading methodology, and the use of excipient/surfactant. This screening process has become the foundation of this research that leads to an in-depth study of two PEG-based blend systems, reported in Chapter 5 and 6. Chapter 5 served as the climax of this thesis. This chapter outlined a new paradigm that permits the prediction of protein release from PCL/PEG blend system by the combined use of confocal Raman and CLSM imaging. These imaging methods extract qualitative and quantitative information on protein partitioning in the blends, and its distribution within the matrix. The quantitative information provides a new platform and a breakthrough for understanding the release mechanism in polymer blends from the aspect of protein partitioning. Chapter 6 reported protein release from PCL and PCL-PEG diblock copolymer blends. The microenvironment evaluation of the blend system gives deeper understanding on factors that play important role in protein release mechanism. Through the understanding, a low-burst-high-subsequent protein release profile was achieved via careful adjustment of the PCL-PEG block fraction and the amount of copolymer added. Finally, this thesis ends with Chapter 7 describing the final conclusions drawn from all research works, accompanied with some recommendations for future research.

Chapter 2. Literature Review

2.1 Protein drugs

As early as 1900s, the discovery of biomolecules with therapeutic potential that are purified from their native source (such as insulin from bovine/ porcine pancreas, and α -1-proteinase inhibitor from pooled human plasma) has been identified [1]. Despite their therapeutic potential, these “natural” biomolecules, however, are produced in low quantities; the extraction of therapeutically useful quantities is impractical. There has been little success in the production of biomolecules until two major discoveries in the 1970s: (1) the first production of rDNA by Paul Berg (1980 Nobel Prize winner in chemistry), and (2) the transformation of *Escherichia coli* cells with recombinant plasmid by Herbert Boyer, which enabled the large scale production of virtually any protein [14, 15]. The two biological breakthroughs have enabled the production of various protein therapeutics and have prompted a robust growth in biotechnology companies. The use of proteins and peptides as human therapeutics has expanded since then due to several advantages in comparison to small molecule drugs [1]:

1. Less interference with normal biological processes due to their high specificity.
2. They have complex functions that cannot be mimicked by small molecule drugs.
3. They are more compatible with human bodies.
4. They provide effective replacement treatment for most generic disorder diseases without the need of gene therapy, a field that has yet mature.

Despite their notable advantages, protein drugs are not suitable to be delivered in the form of tablets and capsules (these are the most common dosage forms in the current drug delivery market) due to the degradation by proteolytic enzymes in the gastrointestinal tract. Besides, the high molecular weight has resulted in poor permeability at intestinal mucosa. Consequently, almost every protein drug is administered by injections [16, 17]. This has become the bottleneck of the treatment effectiveness due to the chronic nature of some diseases (such as diabetics) and short biological half-life of proteins dictates periodic (such as insulin) and prolonged treatment by multiple injections. Due to fear of needles in some diabetics, the delay insulin injection has put them at the risk of blindness, even death. To overcome the limitations, much attention has been paid to prolong the biological activity in vivo. One interesting approach to prolong protein activity retention in body is by using a **controlled delivery system** to protect proteins from proteolytic degradation and antibody neutralization, which results in better efficacy and patient compliance.

2.2 Origin and evolution of controlled delivery system

This section depicts the earliest days of controlled delivery field, which includes the key person who discovered it, the pioneers who launched this field, and a few important devices with clinical success.

2.2.1 “Macro era”

Drug delivery system was born at Harvard in the mid 1960s when Judah Folkman was circulating rabbit blood with silicone tubing. By accident, he discovered that the rabbit would fall asleep if he exposed the tubing to anesthetic gasses on the outside. He then

proposed that a short, sealed segment of such tubing containing a drug, which later named by Silastic®, could be implanted as a constant rate drug delivery device [18, 19]. This has prompted the establishment of Alza, a company founded by Zaffaroni to design and develop drug delivery system, together with Folkman and Higuchi [20]. The first controlled delivery devices were designed in macroscopic scale using poly (ethylene-co-vinyl acetate) (PEVA), include Ocusert® and Progestesert® that released anti-glaucoma drug (pilocarpine) in the eye and contraceptive steroid (progesterone) in the uterine cavity, respectively. The PEVA served as the rate-controlling membrane to release drug in a zero-order profile. Devices that used silicone rubber tubing, as proposed by Folkman, are Norpant® and Vaginal Ring® that deliver contraceptive steroid at a constant rate. Other important zero-order drug release devices include scopolamine-containing skin patch product in the 1970s [21], osmotic pump capsules Oros® and Durons® in the 1980s and 1990s[22], and a hydrogel system Geomatrix® tablet by Peppas and Colombo in the 1990s [23].

In 1976, Langer and Folkman published a paper in Nature, to report on the incorporation and release of large molecular compounds, protein drugs, from PEVA matrix [24]. This is the first successful protein delivery in which the protein drugs were released in its active form from the hydrophobic polymer matrix. Although this device never reached clinical status, according to an interview with Langer, out of many of his published works and patents, this has been his favorite discovery [25].

2.2.2 “Micro era”

Schmitt and Polestina [26], in the 1960s, developed a biodegradable sutures of poly (glycolic acid) (PGA). Adopting this technology, the field of controlled drug delivery evolved from the zero-order macroscopic devices to microscopic biodegradable depot systems in the late 1960s. Inspired by the work of Langer on the incorporation and release of protein drugs from the non-degradable PEVA matrix as previously mentioned, most of the drugs tested in this era were protein drugs. To mention a few, in the late 1970s, Syntex, a drug delivery company developed a long-acting poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles containing leuprolide acetate, Lupron Depot® [27], followed by goserelin acetate loaded cylinder Zoladex™. Both are administered as an intramuscular injection containing a suspension of protein called gonadotropin releasing hormone super-agonist (GnRH agonist), a synthetic form of luteinising hormone-releasing hormone (LHRH), encapsulated in PLGA. They were initially designed for the treatment of prostate cancer but have since been used to treat hormone dependent tumors such as breast cancer, and some benign gynaecological disorders without any serious side effects or need for surgical castration [28-30]. These were the first clinically-approved, injectable, degradable drug delivery system and are still on the market today.

Another important device, Nutropin Depot™, a controlled-release system loaded with recombinant human growth hormone (rhGH) was developed to treat children with a growth deficiency [31, 32]. This device was produced by using ProLease™ technology, invented by Gombotz et. al. in the early 1990s [33] for fabricating uniform-sized, drug-loaded PLGA microparticles [20]. With Nutropin Depot™, the frequency of injection has reduced from daily injection to only one or two doses a month.

In the late 1980s, the well-known “Re-Gel®” of MacroMed Inc. was developed by Kim and Byun. It is a degradable system of PLGA-PEG di-block and tri-block copolymers and is one of the first thermal-responsive devices that have made it to clinical trials in Europe. Other degradable delivery systems such as a family of polyanhydrides were developed by the key pioneer in the controlled drug delivery field, Bob Langer and co-workers, have seen clinical success in the treatment of brain tumors [20].

2.2.3 “Nano era”

The field of controlled drug delivery evolved from the microscopic biodegradable depot systems to nanoscopic era in the late 1970s when the idea of polymer-drug conjugate arose. 3 key technologies that have stimulated nano-therapeutics in the late 1980s till present are (1) PEGylation, (2) active targeting and (3) Enhanced permeation and retention effect (EPR).

The term “PEGylation” is referred to poly(ethylene glycol) conjugated drugs or drug carriers. It is used to shield the drug, especially protein from recognition by the body’s immune system, which enhance circulation time in body and enhanced efficacy of the drug. This concept was first developed by Frank Davis in the late 1960s [34]. At that time, only a few non-human origin proteins were available for parenteral administration, whereby most of these foreign proteins produced adverse immunological responses in human. Prof. Davis then educated himself with a few months of library work at Rutgers University to search for an answer to the problem, which then he ended up with PEG-protein conjugation using monomethoxy PEGs. His findings have ignited an explosion in the research field of

polymer-drug conjugation and nano-therapeutics in academia, laboratories and industries. The widespread application in different carrier systems in the 1990s has resulted in a new class of protein drugs especially anticancer agents was developed (Table 2.1).

Table 2.1. Examples of PEG-protein conjugate in clinical use or development. Adapt from ref. [35].

Compound	Name	Status	Indication
PEG-L-asparaginase	Oncaspar	Market	Acute lymphoblastic leukaemia
PEG-granulocyte colony stimulating factor (GCSF)	Neulasta	Market	Prevention of neutropaemia associated with cancer chemotherapy
PEG-interferon- α (IFN α)	PEG-asys	Market	Hepatitis B and C
	PEG-Intron	Phase I/II	Melanoma, chronic myeloid leukaemia and renal-cell carcinoma
PEG-arginine deiminase	ADI-PEG20	Phase I	Hepatocellular carcinoma
PEG-glutaminase combined with a glutamine anti-metabolite 6-diazo-5-oxo-L-norleucine (DON)	PEG-PGA and DON	Phase I/II	Various cancers

2.2.4 Future of drug delivery system

Two biological breakthroughs, both reported in 1990s, are believed to be the driving force for the drug delivery field to move one step further: (1) Dolly the sheep born in February 1997 by cloning technology [36], and (2) successful cultures of human embryonic stem cells in 1998 [37]. Although the two biological miracles seem to have more impact on tissue engineering field, the increase understanding on cell biology, especially in DNA sequence

decoding is undeniable. As the understanding on cell biology increases, so as the ability to design a serum stable- high uptake-cell targeting nano-scale devices, the field of drug delivery system is believed to evolve to “Bio-era”. The understanding in decoding DNA sequences for a specific disease and the increased ability to use DNA sequences to optimize treatment in each individual, a more “personalized” and biologically precise medicine will be generated. Much excitement to come in the field of drug delivery, as what Prof Allan Hoffman stated in one of his review, “it will be even more exciting as the controlled drug delivery field goes from the Macro-Micro-Nano polymeric drug delivery system eras to the Bio-drug delivery system era of bio-controlled delivery systems for biomolecular drugs” [20].

2.3 Biodegradable polymers

Biodegradable polymers, as introduced earlier, are particularly attractive to be applied in controlled delivery field. The major advantage in using a biodegradable polymer as drug carrier is once administrated into the body, no retrieval or further manipulation is required. Furthermore, these polymers are degraded into soluble, non-toxic by-products. Nair and Laurencin [38] summarized some of the important properties of biodegradable polymers as biomaterials (Text box 2.1).

Text box 2.1. Properties of biodegradable materials as biomaterials. Adapt from ref. [38].

- ❖ The material should not evoke a sustained inflammatory or toxic response upon implantation in the body.
- ❖ The material should have acceptable shelf life.
- ❖ The degradation time of the material should match the healing or regeneration process.
- ❖ The material should have appropriate mechanical properties for the indicated application and the variation in mechanical properties with degradation should be compatible with the healing or regeneration process.
- ❖ The degradation products should be non-toxic, and able to get metabolized and cleared from the body.
- ❖ The material should have appropriate permeability and processibility for the intended application.

Biodegradable polymers can be categorized into 2 groups based on their degradation mechanisms (Figure 2.1): surface erosion and bulk degradation (with or without autocatalysis):

(1) Surface erosion

When water is in contact with the polymer, hydrolytic chain scission of the polymer backbone occurred, producing oligomers and monomers. These oligomers and monomers will then diffuse out from the surface into the release media when they reached a certain chain length. Surface erosion happens only when the diffusion rate of oligomers into the surrounding is faster than the rate of water penetration into the bulk. Hence, the degradation only happens at polymer surface which resulted in the thinning of the polymer, while the molecular weight of the internal bulk generally remains unchanged.

(2) Bulk degradation (with and without autocatalysis)

When water is in contact with the polymer, random hydrolytic chain scission would take place throughout the entire polymer matrix, forming oligomers and monomers which then diffuse out into the release media. Bulk degradation happens only when the diffusion rate of oligomers into the surrounding is slower than the rate of water penetration into the bulk. Hence, this degradation mode happens within the entire matrix, which includes the internal bulk. On the other hand, internal autocatalysis happens when the surface oligomers diffuse freely into the surroundings, while the carboxyl and hydroxyl end group within the bulk produce an acidic gradient, which in turn accelerates the internal degradation. Once the internal oligomers or monomers reached a certain chain length, they will diffuse out through the outer layer rapidly, leaving a hollow inner structure.

Figure 2.1. Degradation modes. Graphic modified from ref. [39].

The most widely used and well characterized polymers is aliphatic poly(esters), such as poly(D,L-lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymer poly(D,L-lactic-co-glycolic acid) (PLGA). These polymers are particularly attractive as a drug delivery carrier due to their easy manipulation in drug release rate. For example, drug release rate in PLA carrier can be tailored by the variation in stereochemistry of the methyl groups, where the amorphous poly(D,L-lactic acid) releases drugs faster than the semi-crystalline poly(L-lactic acid). PLGA have also been utilized to achieve the desired release profile by altering the LA to GA ratio. Therefore, the duration of drug/protein release can be varied from a few weeks to more than a year. There are a number of examples of the successful commercial use of these poly(esters) for drug delivery. However, there are many significant challenges remaining for the delivery of protein. The major problems include protein instability especially during the protein release regime. For detail descriptions of this problem, readers are referred to Section 2.6.

Another poly(ester), Poly (ϵ -caprolactone) (PCL), a least common poly(ester) until the advent of tissue engineering in recent years (Figure 2.2). PCL was studied as early as the 1930s [40]. It is usually synthesized through the ring-opening polymerization of ϵ -caprolactone. PCL is a hydrophobic polymer with high crystallinity, which resulted in a slow degradation rate of 2-3 years via hydrolytic bulk degradation. It was initially investigated as a long-term drug delivery device. One successful device available in the market is Capronor®, a long-term contraceptive device with zero order release of levonorgestrel [38]. Due to its slow degradation rate and its ability to blend with other polymers to modify its degradation kinetics, which facilitates tailoring of desired drug release profile, extensive research into its potential application in controlled drug delivery system is stimulated.

Figure 2.2. Number of PCL related publications in the field of biomaterials and tissue engineering in the last 20 years. Graph taken from ref. [39].

2.4 Protein/drug release mechanism from PEG-based blends

Blending of different polymers to conserve their individual properties is an attractive way of obtaining new properties in the final mixture. The advantages of polymer blends for controlled release applications include easy fabrication of devices and manipulation of device properties such as hydration rate, degradation rate and drug loading. In this section, the author will focus on drug/protein release from PEG-based blends.

Drug/protein release mechanisms from PEG blend systems has been investigated as early as 1991. The earlier works involved the measurement of water uptake in PCL/PEG tablet [41] and PLA/PEG microspheres [42]. In these studies, the hydrophilic PEG was dissolved in the release medium, resulting in the formation of swollen structure with high water content, which subsequently increased the drug/protein release rate. On the other hand, others used

scanning electron microscopy (SEM) to observe the change in matrix porosity in PLGA/PEG microspheres [43], PLA/PEG microspheres [42], PCL/PEG tablet [8], and fibers [44]. In these studies, PEG acted as a pore former, where the matrix porosity increases with PEG content at the end of the release studies. It was proposed that the PEG aggregates presented on the surfaces were dissolved and leached out from the matrix when contacted with an aqueous medium; pores were consequently formed to allow drug/protein release.

More recent studies involved the measurement of PEG leaching rate together with drug/protein release rate from a variety of PEG blend systems such as lipid/PEG [7, 9, 45, 46] and ethylcellulose/PEG [10] blends. Koennings et al. [45] proposed a parallel release of PEG and protein, as the PEG release profile corresponds closely to protein release profile. Another group of researchers [7, 9, 46] fitted a mathematical model based on Fick's second law of diffusion to calculate the diffusion coefficient of protein and PEG. As a result, they discovered a non-constant protein diffusion coefficient. Their proposed protein release mechanism is as follows: the simultaneous dissolution and diffusion of PEG increase the porosity of the matrices, thus the diffusivity of protein (the mobility of protein molecules increases with increasing porosity) which subsequently increases the overall protein release.

Despite the extensive studies of PEG blend systems and the development of mathematical models, the release mechanisms remain uncertain. Nevertheless, it is well accepted that the addition of PEG increases the drug release rate by its fast leaching rate followed by the formation of an interconnected pore network alleviating drug/protein transport rate. Table 2.2 summarized the observations of various researchers in relation to drug/protein release mechanisms from PEG blends.

Table 2.2. Summary of blend systems using PEG and PEG-based surfactant for drug and protein delivery.

Reference	Blend	Materials properties	Drug/protein	Geometry	Observations
[47]	PLA/ Pluronic	Pluronic: L-101 (hydrophobic), P-104 (moderately hydrophilic), F-108 (hydrophilic)	Bovine serum albumin (BSA)	Film	• Water content increases with Pluronic content • Weight loss increases with Pluronic content • Similar degradation rate for formulations with or without Pluronic • Hydrophobic Pluronic reduces burst • Protein release rate decreases with the addition of hydrophobic Pluronic
[41]	PCL/PEG and PCL-PEG copolymer	PEG (Mw 6000)	5-fluorouracil	Tablet	• Water content increases with PEG content • Surface hydrophilicity of PCL-PEG copolymer is higher than that of PCL/PEG blend • Crystallinity of PCL-PEG copolymer is lower than that of PCL/PEG blend • Drug release rate increases with the PEG content • Drug release rate is higher for PCL-PEG copolymer than that of PCL/PEG blend
[43]	PLGA/PEG	PLGA 50:50 PEG (Mw 4600)	FITC-dextran, FITC-rabbit gamma immunoglobulin (FITC-IgG)	Microsphere	• Similar morphology, before and after release for all formulations, with and without PEG • PEG has no significant effect on PLGA degradation • Protein release rate increases with PEG content
[42]	PLA/PEG	PEG (Mw 10000, 35000)	Bovine serum albumin (BSA)	Microsphere	• Thermal properties indicates partial miscibility between PLA and PEG

					• Microspheres porosity increases with PEG content • Water uptake increases with PEG content • Protein release rate increases with PEG content
[8]	PCL/PEG	PCL (M _w 65000), PEG (M _w 10000)	Theophylline	Tablet	• No interaction between PCL and PEG from their thermal properties and FTIR spectra • Matrix porosity increases with PEG content after leaching • Drug release rate increases with PEG content
[44]	PCL/PEG	PCL (M _w 80000), PEG (M _w 35000)	FITC-BSA	Normal fiber, core- shell fiber	• Homogeneous fiber diameter for core-shell fiber. Beaded fiber morphology for blend-type fiber • Protein distributed evenly in the core of the core-shell fiber. More protein located at the beaded part of the blend-type fiber • After release, core-shell fiber collapsed from the previously cylindrical shape. Rough, porous and eroded-like surface were observed for blend-type fiber • Higher burst for blend-type fiber
[45]	Lipid/PEG	Glyceryl trimyristate, PEG (M _w 6000)	Lysozyme (14kDa), Bovine serum albumin (66kDa), alcohol dehydrogenase	Cylinder implant	• FITC-protein homogeneously distributed within the cylindrical lipidic implant • Semi-quantitative measurement of CLSM images visualized the mode of protein transport, i.e. water penetration followed by protein release

			(150kDa), Thyroglobulin (690kDa)		• Protein release rate increases with protein loading • Protein release rate increases with the addition of PEG • Slower release rate for higher molecular weight protein
[46]	Lipid/PEG	Tristearin, PEG (Mw 6000)	Rh-interferon α -2a (IFN- α)	Pellet	• Burst effect reduces with the addition of PEG • Protein release rate increases with PEG content • Protein solubility decreases with PEG content due to protein precipitation • Protein precipitation does not affect protein stability
[7]	Lipid/PEG	Tristearin, PEG (Mw 6000)	Rh-interferon α -2a (IFN- α)	Pellet	• Protein release rate increases with PEG content • Porosity of the implant increases with PEG content after release • Protein diffusivity increases with PEG where protein diffusion coefficient follows an exponential relationship with initial PEG content
[48]	PLGA/PEG	PLGA 50:50, PEG (Mw 2000)	Paclitaxel	Film	• Phase separation between PLGA and PEG was observed using CARS • Mapped images show colocalization of hydrophobic paclitaxel and hydrophilic PEG before release • Mapped images show the appearance of pores was delayed as a function of depth, as an

					• indication of PEG leaching from film surface • Addition of PEG increases burst but reduces the overall release rate
[49]	PLA/PEG	PEG (Mw 400, 750, 2000)	Bovine serum albumin (BSA)	Scaffold	• No difference in the mean pore size of PLA scaffold at different PEG loading • Water uptake increases as a function of PEG content and PEG Mw • Protein release rate increases with the addition of PEG
[11]	Lipid/PEG	Tripalmitin (Dynasan 116®), PEG (Mw 10000)	Theophylline	Cylindrical implant	• Drug release rate increases with PEG content • Matrix porosity increases with PEG content after release
[6]	PCL/PEG	PCL (Mw 50000), PEG (Mw 1500)	Praziquantel	Cylindrical implant	• Thermal properties show immiscibility between drug, PCL and PEG in their crystalline phase • Drug release rate increases with PEG content • PEG release rate increases with PEG content and the release rate is significantly faster than drug release rate • Porosity of the implant increases with PEG content

2.5 Drug/protein distribution

Blending of two polymers usually results in a heterogeneous mixture, due to the intrinsic immiscibility of most polymers, as a result of thermodynamics. Although blends have been studied, the effect of phase separation on drug/protein release, in particular, the preferential partitioning of drug/protein into each of the phases and how the drug/protein partitioning affects its distribution in a delivery system remain elusive. Thus it is important to probe the details of drug/protein distribution in the blend. This section highlighted a variety of drug/protein distribution characterization methods that can be categorized into two analyses groups.

2.5.1 Quantitative analysis

A traditional yet straight-forward way to quantify drug/protein distribution is by microtomy of a sample [50]. In this method, the sample was cut into a few layers, followed by separation of drug/protein from the carrier in solvent. The amount of the separated drug/protein in each layer was then determined. The drawback of this method, however, is the difficulty in microtoming a thin sample. Other quantification methods for drug distribution studies are limited to surface analysis. Belu and co-workers [12] utilized electron spectroscopy for chemical analysis (ESCA) to quantify the surface compositions of rapamycin on a coated stent with a detection depth limited to ~10 nm. X-ray photoelectron spectroscopy (XPS) depth profiling was used by Rafati *et al.* to ascertain quantitative weight concentration of drug loaded PLA films at depth of 96 nm [13]. Although valuable, the low penetration depths for these instruments limit their application for a wide variety of drug

delivery systems; such as polymeric degradable films [51, 52] and polymeric-coated drug-eluting stents [53] where the carrier thickness ranged from 10 μm – 160 μm .

2.5.2 Qualitative analysis

Confocal Raman microscopy, which has very high spatial resolution and is non-invasive, may be used to advantage for this quantification. Recent Raman imaging work employed coherent anti-Stokes Raman scattering (CARS) to monitor the release of paclitaxel from polymer films [48] (Figure 2.3a), while Windbergs *et. al.* performed a real time theophylline anhydrate dissolution to visualize the solid-state properties of lipid-based oral dosage forms [54].

Confocal laser scanning microscopy (CLSM), also non-destructive, has been extensively used and well accepted for research in cell biology [55]. Its application to characterize pharmaceutical systems, in contrast, has been far less documented. Recently, Pygall *et al.* [56] highlighted the imaging capabilities of CLSM to study a range of pharmaceutical systems, one of which involved mapping the distribution of component within a polymer blend [57]. Other groups used CLSM to qualitatively investigate the distribution of fluorescently labeled protein in microspheres [58, 59]. Recently, CLSM was used to visualize mass transport of protein as a function of time [45, 60] in a delivery system (Figure 2.3b). Their work has demonstrated a strong correlation between protein release and buffer penetration.

Transmission electron microscopy (TEM) is a powerful imaging technique with high resolution. Zhao and Rodgers [61] did an excellent job to visualize ovalbumin (OVA) distribution within a PLGA 75:25 microparticle (Figure 2.3c). The preparation work includes protein fixation, staining and microtomy. It should be noted that staining work can give rise to artifacts. In addition, these methods are destructive as the material of interest has to be cut and stained before it can be examined. Furthermore, sample preparation is time consuming, especially to obtain a 3-dimensional picture of the drug distribution of the blends, many samples are required.

Figure 2.3. Qualitative analysis of drug/protein distribution. (a) Distribution of paclitaxel (PTX) in PLGA/PEG blends [48]. (b) CLSM images of FITC-BSA distribution and buffer penetration in a microsphere as a function of time. Green = protein, red = buffer, orange = protein and buffer [60]. (c) Montage of ovalbumin (OVA) distribution in a microparticle [61].

2.6 Protein stability in controlled delivery system

Despite impressive successes in some diseases, protein delivery has presented several challenges that have yet to be broadly resolved. Protein stability, of many other challenges, can be considered as the most important hurdles in the application of protein sustained release formulations from a biodegradable polymeric system. According to US Food and Drug administration (FDA), a stable pharmaceutical product is defined as “no more than 10% deteriorates in 2 years” [62]. Protein stability can also be defined more accurately by considering its stability in the levels of protein structure, and it can be categorized into two groups:

Chemical stability: Chemical stability can be defined as protein integrity in terms of (1) its stability in the amino acid sequence (primary structure), (2) the folding of amino acids into geometrically ordered units (secondary structure), and (3) the assembly of secondary structure into a 3-D functional unit and the reactivity side chains (tertiary structure). Protein integrity can be damaged via proteolysis, which resulted in two or more degraded products of smaller molecular weights.

Physical stability: Physical stability can be defined as the ability to maintain its integrity by retaining its tertiary structure. The tertiary structure of a protein can be destroyed by two degradation pathways: chemical degradation pathway (deamidation, hydrolysis, acylation and disulfide formation) [17, 63] (Figure 2.4) and physical degradation pathway (reversible or irreversible denaturation via unfolding of protein backbone, adsorption, aggregation and precipitation).

Figure 2.4. Schematic depicting mechanisms of chemical degradation of proteins. Deamidation and acylation are primarily caused by lowering of pH within the PLGA matrix and formation of acidic degradation products. Image modified from ref. [17]

Protein instability in polymeric delivery systems has been extensively reviewed by the past few years [63, 64]. Strategies to improve protein stability can be divided into two groups, protein stabilization and prevention of protein denaturation mechanisms. Examples of such approaches are briefly mentioned as follow:

Protein stabilization strategies:

- (1) Chemical modification such as PEGylation [65, 66] and carboxy-methylation [67]
- (2) Neutralization of the acidic product using basic salt [68]
- (3) Promote protein refolding via metal-induced precipitation [69] and co-lyophilization with PEG [70, 71]
- (4) Using a viscous microenvironment such as starch [72] and poloxamer [73]

Prevention of protein destabilization mechanisms strategies:

- (1) Delay of polymer degradation using more crystalline polymer [74]
- (2) Preparation of porous carriers by blending with hydrophilic/amphiphilic additives [42]
- (3) Minimization of protein-polymer surface area such as reservoir-type microcapsules [75]

Van der Weert et al. [63] made an excellent summary on protein destabilization mechanisms and stabilization approaches in PLGA systems during fabrication, storage and release. Although their work focused on PLGA systems, many of the stress factors that affect protein stability as well as stabilization mechanisms (Table 2.3) can be utilized for other polymeric delivery systems. Herein, the used of PEG in protein stabilization will be highlighted and discussed.

Table 2.3. Approaches to maintain protein integrity during preparation, storage and release. Table modified from ref. [63].

Stage	Stress factor	Methods involved/ descriptions	Stabilization approach	Stabilization mechanism
Preparation	Water/organic solvent interfaces	- Water-in-oil emulsion (protein loading)	Add sugars, polyols, PEG	Increase of Gibbs free energy for unfolding, shielding from interfaces by preferential hydration
		- Solid-in-oil emulsion (protein loading)		
		- Protein dissolution in oil phase (protein loading)	Increase protein loading Add other proteins	Reduction of interface Competition for interfaces
		- Oil-in-water emulsion and solvent evaporation (protein encapsulation)	Avoid aqueous process	Absence of water/organic solvent interfaces
		- Oil-in-oil emulsion and solvent evaporation (protein encapsulation)	Pre-encapsulate protein in hydrophilic core	Shielding from interfaces
		- Spray-drying (protein encapsulation)		
	Protein-polymer contacts	- Water-in-oil emulsion (protein loading)	Add other proteins Pre-encapsulate protein in hydrophilic core	Competition for polymer Shielding from polymer
		- Protein dissolution in oil phase (protein loading)	Hydrophobic ion pairing	Shielding from polymer
	Shear	- Water-in-oil emulsion (protein loading)	Add surfactants	Competition for interfaces
			Reduce homogenization time	Minimized exposure to shear

		- Solid-in-oil emulsion (protein loading)		
		- Spray-drying (protein encapsulation)		
	Drying	- Air-drying	Add lyoprotectants	Increase Gibbs free energy of unfolding, water substitution
		- Vacuum-drying		
		- Lyophilization	Avoid lyophilization, use other drying method	Absence of freezing step
Storage	Moisture dehydration		Reduce residual solvent level	Minimized mobility and water-induced degradation
			Add lyoprotectants	Increase Gibbs free energy of unfolding, water substitution
Release	Acidification	- Fast degrading polymer such as PLGA	Add basic compounds	Buffering
	Protein-polymer contacts	- Protein adsorb onto polymer interface via hydrophobic interaction and ionic interaction	Add other proteins Add surfactants Add sugars	Competition for polymer Shielding from polymer Increase of Gibbs free energy of unfolding, shielding from polymer by preferential hydration

2.6.1 *Role of PEG during protein encapsulation*

Protein can be encapsulated in polymeric system via emulsion, suspension and dissolution. In these methods, protein is exposed to water/organic solvent interface, organic solvent and in contact with polymer solution. These stress factors are known to cause major loss of protein activity. Pérez-Rodriguez and co-workers investigated the effect of α -chymotrypsin exposure to water/DCM interface by homogenization of protein aqueous solution in DCM, with and without excipients. It was found that the exposure of protein to water/DCM interface caused a drop in the specific activity to 70% of its initial value (Figure 2.5). Compared to the addition of PEG and other surface active excipients, PEG improved the protein specific activity to 96%, which is very encouraging. Other research groups investigated the mechanisms involved in protein stabilization with PEG 400 [76] and PEG 3,350 [77]. It was proposed that the addition of PEG (1) decreased protein adsorption at the interface by competitive adsorption or (2) formed a hydration layer at protein surface and shielded the protein from the interface.

Figure 2.5. Effect of excipients/additives on specific activity of α -chymotrypsin in buffer solution after homogenization with DCM. Graph plotted from data given in ref. [78].

2.6.2 Role of PEG during protein release

Jiang and Schwendeman [42] utilized PEG 10,000 and PEG 35,000 to stabilize BSA in PLA/PEG blend microspheres. After 29 days of incubation, only 15% of the remaining BSA was still in water-soluble form, while 25% had become water-insoluble non-covalent aggregates (control group). In contrast, no insoluble protein was observed in formulations with <20% PEG loading. The authors further investigated the mechanisms involved in protein stabilization by examining the pH change and water uptake after 4 weeks of incubation. It was found that the release medium for PLA/PEG formulations was slightly more acidic than the control group; while the water uptake rate in the blend was overwhelmed by the strong water absorption of PEG. Hence, protein stabilization mechanisms by PEG were proposed to be (1) the increase in water content in the blends diluted the acidic species, and (2) the dissolution of PEG in the release medium created a

more porous matrix, and thereby increased the diffusion rate of the acidic species, leading to a less acidic environment within the matrix. Similar observations were found by Lavelle et al. [79] where the addition of PEG significantly improved ovalbumin (OVA) stability in PEG blend formulations. The above mentioned hypotheses on protein stabilization mechanisms were recently visualized by Ding and Schwendeman [80] using CLSM, which further confirmed the applicability of PEG in protein stabilization.

Chapter 3. Experimental Techniques

3.1 Materials

The materials used in this study are listed in Table 3.1.

Table 3.1. List of materials used in this study.

Materials	Abbreviation	Company
<i>Polymer</i>		
Poly (DL-lactide-co-glycolide) 53/47, IV 1.05 dl/g	PLGA 53/47	Purac Far East Pte
Poly (ϵ -caprolactone), Mn 80,000 g/mol	PCL	Aldrich
Polyethylene glycol, Mw 2,000 g/mol	PEG	Merck-Schuchardt
Fluorescein isothiocyanate tagged PEG, Mw 2,000 g/mol	FITC-PEG	Nanocs Inc.
Poly (ϵ -caprolactone)-Polyethylene glycol di-block copolymer, Mw:		Advanced Polymer Materials Inc.
PCL(1.5k)-PEG(5k)-CH ₃	PCL-PEG	
PCL(10k)-PEG(5k)- CH ₃		
PCL(1.5k)-PEG(5k)-NH ₂	PCL-PEG-	
PCL(10k)-PEG(5k)- NH ₂	NH ₂	
<i>Protein</i>		
Lysozyme from chicken egg white	Lys	Sigma
Rhodamine B labelled lysozyme	Rh-B Lys	Nanocs Inc.

Solvent

Dichloromethane (HPLC grade)	DCM	Tedia Chemical Company
Acetonitrile (HPLC grade)	ACN	Tedia Chemical Company
Chloroform (HPLC grade)	-	Tedia Chemical Company
Dimethylformamide (ACS grade)	DMF	Tedia Chemical Company

Acid

Trifluoroacetic acid (HPLC grade)	TFA	Tedia Chemical Company

Reagent

micro-Bicinchoninic acid protein assay kit	BCA	Thermo Scientific
Kaiser test kit	-	Fluka

Others

Buffer solution, pH 7.4	-	OHME Scientific
Fluorescein isothiocyanate monomer	FITC	Sigma-Aldrich
Dialysis tubing, Por 7 (3.5k MWCO)	-	Spectra

3.2 Methods

3.2.1 Film preparation

Polymer pellets (PLGA 53/47 or PCL) were dissolved in DCM and stirred overnight to obtain homogenous polymer solutions while lysozyme was dissolved in water at 0.2 mg/ μ L. For polymer blends, PEG or copolymer flakes at various weight fractions were co-dissolved with the homo polymer in DCM. Later, lysozyme aqueous solution was added into the PCL or PCL/PEG or PCL/copolymer solutions, and was stirred for 1 hour to form a single emulsion. The solutions were casted on a glass plate and dried under ambient conditions overnight before drying in a vacuum oven at 37 °C for 1 week. Solvent residue analysis was

conducted with Thermogravimetric Analyzer, TGA (TA Instruments Q500). The following steps were taken during the process: samples (10-20 mg) were heated from room temperature to 135 °C at 10 °C/min at nitrogen environment, after which it was held isothermally for 30 mins. The resulting weight loss revealed < 0.5 wt% residual DCM in all samples. Films that were used for CLSM imaging and PEG leaching experiments were prepared in the same way using rhodamine B-tagged lysozyme and FITC-tagged PEG.

3.2.2 *Surface morphology*

The surface morphology of polymer blend films was studied using scanning electron microscopy (SEM) (JEOL, model 6360). The as-prepared films were gold coated at the following conditions: gas pressure of 20 mbar, current of 18 mA, and coating time of 50 s. Finally, the SEM micrographs were observed at 3 kV.

3.2.3 *Polymer miscibility*

The miscibility of polymer blends was investigated based on their thermal properties and crystallinity:

3.2.3.1 Thermal properties

The thermal properties of polymer blends were investigated using differential scanning calorimetry (DSC). A TA Instruments DSC Q-10 was used to measure the melting temperature (T_m) of polymer blends. The DSC data were obtained from both the first and second heating profile of the films, in order to observe the thermal history imparted by the

film preparation process and thermal behavior in the molten stage. The following steps were performed for thermal properties measurement: samples of 5-10 mg were heated from -90 °C to 160 °C at 10 °C/min and were held isothermally for 5 mins. The samples were then cooled slowly back to -90 °C at the cooling rate of 5 °C/min and were subsequently heated again to 160 °C at 10 °C/min.

3.2.3.2 Crystallinity

The crystallinity of the as-prepared films was investigated using X-ray diffraction (XRD). The X-ray diffraction patterns were recorded using a Shimadzu XD-5 diffractometer (40 kV $\times$ 30 mA) equipped with Cu K α radiation. Divergence, scatter and receiving slits were set at 1°, 1° and 0.3 mm, while scans were collected from 5° – 65° 2 θ with a step size of 0.02° and 1 seconds count time at each point.

3.2.4 FITC-copolymer conjugation

FITC-copolymer conjugate was prepared with an NH₂-terminated PCL-PEG copolymer as shown in Figure 3.1. First, 1 g of PCL(1.5k)-PEG(5k)-NH₂ and PCL(10k)-PEG(5k)-NH₂ were weight separately, followed by the addition of 2x excess of FITC isomer. Next, the mixture was dissolved in 50 mL DMF and was left to reaction in the dark for 24 hours. Later, the reaction mixture was placed in a dialysis bag (MWCO = 3.5k) and dialyzed against DMF for one week to remove unconjugated FITC. During this time frame, the external solvent was changed several times to enhance the dialysis efficiency. Then the FITC-conjugated block copolymers were obtained by solvent evaporation of DMF at 37 °C for one week.

Figure 3.1. FITC conjugation with PCL-PEG-NH₂ copolymer.

3.2.5 ¹H NMR of copolymer

A 400-MHz NMR spectrometer (Mercury) was used to obtain ¹H NMR spectra of the amphiphilic copolymers. The copolymer solutions in CDCl₃ were prepared at a concentration of 1% (w/v), with tetramethylsilane 0.03% (v/v) as an internal standard.

3.2.6 Qualitative analysis of protein distribution using confocal Raman microscopy

Qualitative analysis of protein distribution in PCL/PEG blends were characterized using confocal Raman microscopy. Raman point-by-point mapping of both film surfaces were performed at scanned area of 100 μm × 100 μm with a step size of 5 μm. Renishaw InVia

Reflex confocal Raman microscope equipped with a deep-depleted thermoelectrically (Peltier) cooled CCD array detector (576×384 pixels) was used. The setup was equipped with a 785 nm near infrared diode laser, where by the samples were irradiated at ~ 50 mW, and the backscattered light was collected with a 50× objective lens (NA 0.5) of a high grade Leica microscope. The samples were scanned for 30 s per spectrum that ranges from 700 to 1800 cm^{-1} using a static 1800 groove per mm dispersive grating. Spectral preprocessing such as spectral baseline correction and spike removal was preformed prior to the collected data which was further analyzed using the band-target entropy minimization (BTEM) algorithm [81], a novel technique developed by Dr. Effendi Widjaja from Institute of Chemical and Engineering Sciences (ICES).

3.2.7 BTEM Raman spectral construction

BTEM algorithm is a powerful technique to reconstruct the pure component spectra from a set of mixed spectra without the need of priori known spectral libraries. It has been proven that this algorithm is able to reconstruct the pure component spectra even for the minor components [82]. The pre-processed mapped image of protein loaded PCL/PEG was analyzed using the BTEM algorithm. After spectrum reconstruction, the raw spectra of each mapped points was projected back onto the baseline-corrected normalized data set to calculate the contributions of each components. Subsequently, the spatial distribution of protein, PEG and PCL at the film surface can thus be generated. For a more detailed description of this algorithm, readers are referred to the previous work [81].

3.2.8 *Quantitative analysis of protein distribution using CLSM*

Protein distribution was acquired by scanning across dual channel images of FITC-PEG and rhodamine B-lysozyme loaded film in a 2-D fashion at scanned area of $246 \times 246 \mu\text{m}$. By repeating the same process at depth interval of $0.1 \mu\text{m}$ for the entire film thickness, a 3-D distribution profile could be constructed. Leica TCS SP5 confocal microscope equipped with a Leica DMI 6000 CS inverted microscope was used. The setup was equipped with a 488 nm line and a 543 nm line Ar ion laser, and the laser was split with a TD 488/543/633 excitation beam splitter to excite FITC and rhodamine B, respectively. The resulting emissions were filtered at 495 to 521 nm for FITC and 550 to 584 nm for rhodamine B, and were collected with a HCX PL APO lambda blue 63 $\times$ oil objective lens (NA 1.4). Pinholes were set to one Airy unit ($95.6 \mu\text{m}$) and the pixel sizes were $0.48 \times 0.48 \times 0.77 \mu\text{m}^3$. Laser power and detection gain were adjusted so that the level of autofluorescence was insignificant. Sequential scanning mode was used to remedy the problem of emission cross talk, and this was checked by acquiring dual channel images of film with single fluorophore (either FITC-PEG or rhodamine B lysozyme). No detectable cross talk was observed for these experiments. Due to the limit of penetration depth ($\sim 25 \mu\text{m}$ within the bulk) and possible signal losses along the z -direction, protein and PEG distribution were characterized by scanning the film from both surfaces.

3.2.9 *Quantification of protein-PEG colocalization*

Colocalization of a system can be visualized using three images consisting of the red-green dual channel images, and a third overlay image generated from the combined channels where overlapping pixels turn to yellow. These yellow pixels are subjected to the relative intensity

of the R-G dual channels. In other words, these yellow pixels that represent the overlap of red and green pixels are reliable only when the signal strengths of the two imaging fluorochromes are equal [83]. Thus, image processing is required to evaluate the colour components of the selected pair of channels. Raw images acquired by CLSM were exported to Image Pro Plus Version 4.5 in TIFF format. The dual colour images first underwent background subtraction and were then subjected to RGB channel assignment before quantitative analysis. The degree of overlap was described by a few coefficients with different sensitivity and applicability:

Pearson's correlation coefficient (R_r) [84] describes the degree of overlap between two patterns. This correlation is indicated by coefficient values ranging between -1 and 1. A “-1” coefficient value means complete exclusion, “0” indicates no correlation, and “1” reflects perfect overlap.

Overlap coefficient according to Manders (R) [85] indicates the true degree of overlap as it measures the overlap of the signals. This correlation is not sensitive to intensity variations in the image analysis and the limitations of fluorescence-related imaging issues, such as photobleaching of a sample [55]. The measurement of Manders coefficient is indicated by coefficient value ranging from 0 to 1. A “0” coefficient value corresponds to non-overlapping signal, while “1” reflecting complete overlapping of two images.

Overlap coefficients (k_1 and k_2) [85] illustrates the differences in intensities of two channels. No fixed value is applicable for k_1 and k_2 .

3.2.10 Quantification of protein distribution

For morphometric analysis, the images in TIFF format underwent image enhancement and were then filtered by a Flatten filter. All images were processed in the same manner before the selection of protein-rich domains, using a pseudo-automated adaptive thresholding process based on the image intensity histogram. The non-segmented domains were separated manually followed by a smoothing process. Once all the desired domains were selected, “area” and “size” descriptor were calculated to allow the quantification of protein-rich domain at selected optical layers. The amount of protein coverage (in terms of area) at these optical layers can be estimated by the following equation:

$$\text{Protein coverage per layer (\%)} = \frac{\text{Total area of protein rich domain } (\mu\text{m}^2)}{\text{Known image scanned area } (246 \mu\text{m} \times 246 \mu\text{m})} \times 100$$

3.2.11 In vitro protein release

Protein release studies were conducted by incubating the films of 15×15 mm at 37 °C in glass bottles, each containing 3 mL of buffer solution (pH 7.4). At each sample recovery time point, the release medium was completely removed and replaced with fresh buffer. Protein concentration in the release medium was analyzed using micro- bicinchoninic acid (BCA) protein assay. All samples were prepared and tested in triplicates while the data is presented as mean ± standard deviation of the mean.

Details on micro-BCA protein assay are as follow:

This protein assay works on the reduction of Cu^{2+} by protein to Cu^{1+} in an alkaline medium (STEP 1) and form interaction with two molecules of BCA to give a purple-coloured Cu^{1+} -BCA complex absorbing at 562 nm (STEP 2). The amount of reduction is proportional to the protein concentration.

The measurement was performed according to PIERCE micro-BCA protein assay protocol. In short, working reagent of MA, MB and MC were mixed in the ratio of 50:48:2. These reagents were then mixed with protein samples in 1:1 volume ratio, and were incubated at a 60 °C water bath for an hour. Later, the absorbance at 562 nm was recorded by UV-Vis spectrophotometer (UV-2501, Shimadzu) and the concentration is calculated based on a calibration curve (Figure 3.2). The calibration curve was created using freshly prepared protein standards of known concentration.

Figure 3.2. Calibration curve of lysozyme in buffer solution (pH 7.4) using micro-BCA protein assay kit.

3.2.12 *In vitro* PEG leaching

PEG leaching from PCL/PEG blends was conducted in a similar way as protein *in vitro* release stated in Section 3.2.11. The amount of FITC-PEG release was measured using a microplate reader (Tecan infinite M200) at the following settings: excitation wavelength = 495 nm, emission wavelength = 521 nm, and gain = 50. All samples were prepared and tested in triplicates while the data are presented as mean $\pm$ standard deviation of the mean. The conversion of fluorescence readings to FITC-PEG concentration is calculated based on the calibration curve shown in Figure 3.3.

Figure 3.3. FITC-PEG calibration curve.

3.2.13 Protein stability

The amount of stable protein in the release medium was measured using Reversed-phase high performance liquid chromatography (RP-HPLC). An Agilent ZORBAX poroshell 300SB –C18 (2.1×75 mm, 5 µm) column is connected with Agilent 1100 Series instrument equipped with a VW detector. Two mobile phases of water/ACN/TFA at different ratios were used, and a gradient mode was selected according to Table 3.2. Other operating conditions were as follow: flow rate = 1mL/min, sample volume = 10 µL, column temperature = 40 °C, detector = 215 nm and stop time = 14 min.

Table 3.2. Gradient mode setting.

Time (min)	95:5 water/ACN + 0.1% TFA	5:95 water/ACN + 0.07% TFA
0	90	10
2.5	90	10
10	60	40
11	60	40
12	90	10

Quantitative analysis was carried out using Agilent ChemStation 2008. Integration settings of protein peak were as follow: New exponential tangent skim mode, area reject = 0.04, height reject = 0.04, and integration time from 8.98 to 9.5 min. The protein concentration is calculated based on a calibration curve (Figure 3.4), created using freshly prepared protein standards of known concentration.

Figure 3.4. Calibration curve of lysozyme in buffer solution (pH 7.4) using RP-HPLC.

3.2.14 Polymer degradation

The degradation of polymers was characterized in terms of change in mass and molecular weight. Films of 10 mm × 10 mm dimensions were cut from each polymer combination. All films were weighed, completely immersed in buffer solution (pH 7.4) and incubated at 37 °C. At predetermined time point, the release medium was completely removed and replaced with fresh buffer. At these time point, some films were removed and rinsed with distilled water and vacuum dried at 37 °C for a week before characterization test.

3.2.14.1 Mass loss

In this study, the dried polymer films were weighed and the % mass loss was calculated according to the following equation:

$$\% \text{ Mass Loss} = \frac{W_o - W_t}{W_o} \times 100$$

where W_o = initial weight; W_t = dry weight at time, t .

3.2.14.2 Molecular weight analysis

In this study, the dried polymer samples were dissolved in chloroform at a concentration higher than 1 mg/mL, and filtered before molecular weight measurement using a gel permeation chromatography (GPC, Agilent series 1100) through a PLGel MIXED-C column (300×7.5 mm, Agilent). Other conditions used in this characterization test were as follow: chloroform flow rate = 1 mL/min, analysis temperature = 35 °C, RID signal = 254 nm and

injection volume = 50 μL . The molecular weight of the samples was obtained relative to a calibration curve from polystyrene standards (M_w between 165 and 5000 g/mol).

Chapter 4. Formulations and Fabrications Screening

4.1 Introduction

Since the first application of biodegradable polymer poly(glycolic acid) (PGA) in the field of pharmaceuticals in the 1960s [26], controlled release system has expanded considerably. The most commonly used biodegradable polymers is poly(esters) such as PLA, PGA, PLGA and PCL. These polymers are commercially available in different molecular weights and compositions which allow control of degradation of the polymer. Therefore, the duration of drug/protein release can be varied from a few weeks to more than a year. Readers are referred to Section 2.3 for more details on properties and degradation mode of these polymers.

At present, a wide variety of biodegradable polymers and encapsulation techniques, have been employed to deliver agents ranging from small therapeutic drugs to huge protein molecules. This chapter focuses on preliminary work to help the author to select a suitable polymer system and protein loading technique before moving on to in-depth studies of protein release mechanisms described in the next chapter. The basis of formulations and fabrications selection is by the tailor-ability of the protein release profile to achieve a more complete protein release, as well as protein stabilization by the additive.

4.2 Results and discussion

4.2.1 PLGA system

Release kinetics profiles of lysozyme at different protein loading are shown in Figure 4.1. The release profiles can be divided into four phases: (1) an initial burst release in the first 18 hours, (2) a plateau over 20 days, (3) a second burst due to mass erosion and (4) an additional spurt and incomplete release. In the earlier stage of the release study, higher burst effect is observed for 6wt% protein loading formulation. Nearly 80% of the protein loaded remains within the polymer films after 75 days incubation. This indicates that the protein could not be completely released from the film over the release durations.

Figure 4.1. Protein release from PLGA 53/47 films.

The PLGA 53/47 hydrolytic degradation such as the molecular weight change and mass loss is monitored to examine the influence of polymer degradation on protein release. Figure 4.2 shows the change of molecular weight as a function of degradation time. It can be seen that the molecular weight decreases linearly without a lag time for all formulations. During this period, protein is released slowly by diffusion through the PLGA matrix. Degradation of the PLGA 53/47 polymer appears to be slightly affected by the incorporated protein. The molecular weight of the protein loaded films is lower than that of the blank PLGA film. This is probably due to the loading procedure, where homogenization can partially degrade the polymer [86].

Figure 4.2. Weight average molecular weight change for PLGA 53/47 films.

The mass loss data shown in Figure 4.3 depicts a biphasic profile, with an induction period of 22 days followed by a rapid mass loss, as a result of oligomers leaching. This is in agreement with the previously reported papers from our group [87, 88]. Comparing the mass loss data with molecular weight change data, less than 1% of the PLGA films eroded within the first 20 days of incubation period, however, nearly 80% molecular weight change during the corresponding intervals. The disproportionate in both data can be explained by the polymer degradation mechanism as mentioned in Section 2.3. When the PLGA films are in contact with water, hydrolytic chain scission of the PLGA occurs, producing oligomers. These oligomers remain in the matrix which explains the induction period in the mass loss data. Once the oligomers achieve a critical chain length, they leach out rapidly, as reflected in the abrupt mass loss after day 22. The mass erosion of the PLGA matrix is thus identified as the cause of the spurt in protein release profile that occurs at around day 22.

Figure 4.3. Mass loss for PLGA 53/47 films.

Polymer degradation can also be observed via the changes in its surface morphology. Immediately after preparation, the protein loaded films showed an intact surface with clear bubble-like droplets in polymer matrix Figure 4.4. The films degraded very rapidly, and after 32 days of incubation they appeared highly eroded and porous. It is worth mentioning that the blank PLGA 53/47 films appeared to visually degrade faster than the protein loaded formulations. Despite the resemblance in the molecular weight change and mass loss data for both blank and protein loaded PLGA films, the blank PLGA films had collapsed into a rubbery mass at ~ 25 days of incubation while this is not observed in protein loaded films. Similar observation has been reported by Park et.al. [89, 90] that the porosity and specific surface area of the blank PLGA microspheres is significantly higher than that of a carboxymethylated-BSA loaded microspheres. It is possible that some form of interaction between protein and PLGA may have occurred, either by protein adsorption on the polymer surface, or carboxylic end group interaction with protein, that have hindered the collapse of the PLGA matrix.

Figure 4.4. Morphology of protein loaded PLGA53/47 films, before and after release.

Although significant polymer degradation occurred, a reduction in the rate of release of protein can be seen in the later stage. For instance, for 3 wt% protein loaded film, 65% of the 53/47 film degraded over 11-49 days, yet less than 20% of the loaded protein was released overall. This incomplete protein release (in spite of substantial polymer erosion) suggests a protein-polymer interaction that favors retention of protein in the matrix. In the initial stages of polymer erosion, some pore formation occurs that facilitates protein release via diffusion through water-filled pores. However, as degradation proceeds, a critical stage is reached when the number of carboxylic end-groups is high enough to interact significantly with the amide groups on the protein which then retards protein release. As mass erosion occurs, the oligomers leached out, thus the number of chain-end is expected to decrease to a

point where there is no enough chain-end to interact with protein (Figure 4.5), consequently, an acceleration in release occurred after day 50 for PLGA 53/47 at the end of the release study. It should be noted that the carboxylic-amide interaction formed during polymer degradation could have also affect the protein concentration measurement using micro BCA protein assay. Since the number of peptide bonds (amide bonds) is responsible for the colour formation with BCA, such protein-polymer interactions may have shield the peptide bonds from reacting with the BCA reagents, resulting in a lower protein detection, and an underestimated protein release.

For 6 wt% protein loaded film, the protein-carboxylic interaction is more pronounced, manifested by the retardation of release after the initial burst. This observation can be explained based on the isoelectric point (pI) of lysozyme, which is around 11.3, implying that lysozyme is positively charged throughout the release study. Hence, in higher protein loading, an increased amount of positively charged lysozyme at the pH of the receptor fluid and in the bulk of the polymer is suspected to interact more strongly with the carboxylic end group, leading to overall decreased release.

Figure 4.5. Amount of remaining protein (mol) and number of polymer chain-end (mol) as a function of time. Degradation studies were done only until day 40 due to difficulties in mass measurement.

Figure 4.6. Effect of PEG on protein release from PLGA 53/47 films.

The addition of surfactant such as PEG is known to facilitate the drug/protein release from a delivery system. The rate of drug/protein release is very much affected by the PEG molecular weight, i.e. the rate of drug/protein release is higher when using a low molecular weight PEG than that of the higher ones. In this work, PEG 2000 is selected to facilitate protein release rate from PLGA 53/47. Since it is uncertain on the effect of PEG composition to protein release rate, the author started with a low blend ratio of 5 wt%. The release profiles of lysozyme from PLGA 53/47 with and without PEG blending are shown in Figure 4.6. It is obvious that the addition of PEG increases protein release rate from PLGA matrix. In the first day of protein release, the initial burst from PEG blended film increases from 5% to 15%. The effect of PEG addition appears to be more dominant at the end of the release study manifested by the overall increment of 60% protein release at day 75. As mentioned earlier, protein release from PLGA matrix can be divided into two stages: (1)

diffusional controlled and (2) degradation controlled. When the protein loaded matrix is exposed to release medium, the protein molecules will diffuse through the pre-existing pores across the film thickness. These pores, which become more interconnected during polymer degradation and erosion increases the overall matrix porosity that subsequently increase protein release rate at the later stage. The addition of hydrophilic PEG is expected to increase water uptake of the PLGA matrix, which subsequently increases protein diffusion in the first stage of release study, followed by the increase in polymer degradation rate. Hence, the polymer degradation of PLGA/PEG blends is monitored.

Figure 4.7. Molecular weight change for PLGA 53/47 based matrix, with and without the addition of PEG and protein.

Figure 4.7 shows the effect of PEG on molecular weight change of PLGA matrix. The addition of PEG led to remarkable increase in polymer degradation rate. Instead of a linear molecular weight drop for pure PLGA 53/47 film, an exponential decay of molecular weight

is observed for 5% PEG blend film, independent of protein addition. When water is in contact with the PLGA/PEG blends, random hydrolytic chain scission would take place throughout the entire polymer matrix, forming oligomers and monomers. The addition of hydrophilic PEG is expected to increase water penetration into the hydrophobic PLGA. This can also explain the accelerated hydrolysis of PLGA chains, as evidenced by the rapid decrease in molecular weight, leading to earlier onset of the polymer mass erosion (Figure 4.8). As reported earlier, pure PLGA film shows a biphasic mode with an induction period prior to a rapid rate of erosion at day 22. For PLGA/PEG blends, this induction period is shortened to 12 days, followed by the onset of rapid mass erosion. As a result, protein release from the PEG blend was greatly accelerated.

Figure 4.8. Mass loss for PLGA 53/47 based matrix, with and without the addition of PEG and protein.

Despite of the enhanced protein release via the addition of PEG, the problem of incomplete release remains. In addition, the protein-carboxylic interaction that is known to cause protein denaturation has certainly become one of the important hurdles for successful protein delivery from PLGA matrix. Strategies to improve protein stability have been investigated intensely in literature. Readers are referred to Section 2.6 for more details. In brief, the approach to maintain protein stability can be divided into two groups, protein stabilization and prevention of protein destabilization mechanisms. In this work, rather than stabilizing the protein released from PLGA matrix, the author has focused on the prevention of protein destabilization by delaying polymer degradation, using PCL as protein carrier. These experiments were based on the reasoning that protein release profiles can be optimized if the release is controlled purely by diffusion mechanism.

4.2.2 PCL system

As from the previous section, protein stability has been recognized as a major problem in protein delivery. Herein, the identification of protein denaturation factors and possible approaches to minimize protein denaturation in the film fabrication stage is investigated. The experiment is designed to mimic 2 conditions that have the most impact on protein denaturation during film fabrication: (1) solvent exposure during protein loading, and (2) heat exposure in the film drying process.

Figure 4.9 shows protein stability after exposure to denaturation factors. Stability ratio at the y-axis of the graph can be defined as the fraction of stable protein in the released solution. This can be calculated by dividing the HPLC measurements (amount of stable protein) with micro BCA measurements (amount of total protein). Protein in solution-form appears to be very sensitive to denaturation factors. The heat exposure at 37 °C for a week has caused 13% protein denaturation, while the addition of solvent further destabilized the protein. Upon drying, proteins are subjected to the loss of hydrating water shell, which subsequently resulted in protein unfolding. The presence of aqueous/organic solvent interface has been identified as a major cause of protein denaturation [63]. The formation of interfaces is a common protein destabilizing factor that results in interfacial adsorption followed by unfolding and aggregation [63, 91]. PEG addition in the protein aqueous phase has slightly reduced protein denaturation by increasing of Gibbs free energy of unfolding, and shielding protein from the water/ organic solvent interface by preferential hydration [63]. In contrast to protein in its solid-form, protein denaturation does not occur when it is exposed to DCM as evidenced by the same stability ratio between single denaturation factor and both DCM+heat denaturation factors, accompanied without any further improvement on protein

stability with the addition of PEG. This is due to the lack of protein conformational mobility in its solid state, in comparison to the large structural mobility in its solution state, hence, drastically maintain its bioactivity.

Figure 4.9. Effect of organic solvent and heat exposure on protein stability. Blue bars represent protein in its solid form, while red bars represent protein solution

It should be noted that the addition of PEG is not solely to improve protein stability. As mentioned in Section 2.3, PCL undergoes hydrolytic bulk degradation with a slow degradation rate of 2-3 years due to its hydrophobic and high crystallinity nature. Hence, protein release from PCL matrix is known to be very slow and often incomplete. This has motivated the use of PEG, a pore-former, to increase the overall protein release rate.

Protein release from PCL system is assessed based on the release profile tailor-ability using 2 protein loading techniques:

- (1) Solid dispersion: protein is added into the polymer solution in its solid form. Solid dispersion is formed by mechanical stirring.
- (2) Emulsion: protein is dissolved in aqueous solution prior adding into the polymer solution. Emulsion is formed by mechanical stirring.

Figure 4.10. Protein release profiles from PCL based matrix by different protein loading methods.

Figure 4.10 shows protein release profiles from PCL/PEG blend films fabricated via the above mentioned techniques. In general, the release is biphasic; consisting an initial burst within the first day, and a first-order release thereafter. The addition of PEG in the “solid dispersion” formulation shows minimal effect on the protein release profile. A statistical *t*-test was conducted for all data points, indicating insignificant differences ($p > 0.05$) between pure PCL and PCL/PEG formulations, as also indicated by the overlapping error bars in the release profile. SEM micrographs for “solid dispersion” formulations show clear protein crystal presence on the film surface, independent on PEG loading (Figure 4.11). This explains the similarity in their corresponding release profile. On the other hand, protein loading via emulsion method shows much tailor-ability in their release profiles. The presence of PEG exerted a significant influence on the release profiles, particularly on the magnitude of the initial burst release.

From protein stability point of view, protein loading using solid dispersion method is more favorable. Due to the ability of maintaining bioactivity in the solid state, the proteins are least prone to harsh environment during film fabrication. However, its comparable release profiles in formulations with and without PEG indicated that this protein loading method is indeed ineffective in fabricating a protein controlled-release system. Conversely, the emulsion method that provides much tailor-ability in protein release profile is thus chosen as the film fabrication technique for the subsequent release study. Protein stability can be improved with the addition of PEG, however, ironically, the protein burst effect was more pronounced with the increasing excipient system that stabilizes the protein. Such an influence of excipients, particularly for low molecular weight PEG, on the release profiles was expected because the presence of PEG increases the porosity of the polymer matrix by

its fast leaching rate. Protein release mechanism from PCL/PEG blends will be discussed in greater depth in the next chapter.

Figure 4.11. Surface morphology of protein loaded PCL and PCL/PEG films, fabricated via solid dispersion and emulsion protein loading method.

Chapter 5. PCL/PEG Blends

5.1 Introduction

PCL, a biodegradable polymer, has been extensively studied for application in protein delivery. Due to its high degree of hydrophobicity and crystallinity, PCL degrades very slowly and results in a slow release rate of slowly-diffusing species, such as proteins. Several approaches have been used to modify protein release rate. These include blending the hydrophobic matrix with polymers of greater hydrophilicity, such as PEG. A wide range of PEGs (400 g/mol – 50,000 g/mol [6, 49]) have been used to modify drug/protein release rate. These PEGs were blended with hydrophobic matrix such as PCL [6, 8, 42, 44], PLGA [43, 48], PLA [42, 49] and more recently with lipid [7, 11, 45, 46]. Despite the vast drug/protein delivery systems investigated, all drug/protein release profiles from these blends showed similar trend, that their release rate was significantly increased. For such blends, it has been proposed that the drug/protein release is purely diffusion-controlled, either in a Fickian or a non-Fickian diffusion mode, through pores formed by the leached-out PEG. The PEG aggregates presented on the surfaces were dissolved and leached out from the matrix when contacted with an aqueous medium; pores were consequently formed and filled with water to allow drug/protein release [7-10]. The author refers to this release mechanism as the “pore-diffusion model”. Readers are referred to Section 2.4 for a more detailed review on drug/protein release mechanisms from PEG based blends.

Despite the extensive studies of PEG blend systems and the development of mathematical models, the release mechanisms remain incompletely understood. This is probably due to the intrinsic immiscibility of most polymers, which causes phase separation in polymer blends [4, 5]. Furthermore, the use of a hydrophilic polymer may lead to fast polymer leaching [7-10] that complicates the understanding of release mechanisms. In particular, it has been almost impossible to quantify the preferential partitioning of drug/protein into each of the phases and thus understand how this partitioning affects the drug/protein release. To achieve this quantification, high lateral and depth resolution techniques were employed.

This chapter outlines a new paradigm that permits the prediction of protein release from a polymer blend system by the combined use of CLSM and confocal Raman imaging. The major objectives of this chapter are: (1) characterization of protein loaded PCL/PEG blends, (2) quantification of protein distribution and protein-PEG colocalization via a novel image processing technique, and (3) prediction and measurement of the protein release profile. The CLSM image processing approach was used to extract important quantitative information in the microenvironment of the blend and to better understand the protein release mechanisms. Table 5.1 summarizes the films prepared for quantitative and qualitative protein distribution studies, and in vitro protein and PEG release studies.

Table 5.1. Composition of the prepared formulations and their corresponding usage.

Sample Name	Polymer ratio (%)			Protein loading (wt% of total film)	
	PCL	PEG	FITC- PEG	Lysozyme	Rhodamine B-Lysozyme
<i>In vitro protein release and Raman mapping for qualitative analysis</i>					
PCL 80 μm	100	-	-	3	-
10% PEG 80 μm	90	10	-	3	-
30% PEG 80 μm	70	30	-	3	-
PCL 40 μm	100	-	-	3	-
10% PEG 40 μm	90	10	-	3	-
30% PEG 40 μm	70	30	-	3	-
<i>PEG leaching and CLSM imaging for quantitative analysis</i>					
10% FITC-PEG 80 μm	90	-	10	-	3
30% FITC-PEG 80 μm	70	-	30	-	3
10% FITC-PEG 40 μm	90	-	10	-	3
30% FITC-PEG 40 μm	70	-	30	-	3

5.2 Results

5.2.1 Surface morphology

Figure 5.1. Surface morphology of protein loaded PCL/PEG films (80 µm) before release studies.

Figure 5.2. Surface morphology of protein loaded PCL/PEG films (40 μm) before release studies.

Figure 5.1 shows surface morphology of protein loaded PCL/PEG blends. Clear “spherulitic” crystalline domains are observed in pure PCL film surface. In contrast, rougher spherulites (pin-pores) can be seen for PEG blends. The pore size increases with PEG content, forming uneven sizes of “land-lake” morphology at the film surface. This is especially pronounced in 20, 30 and 40% PEG loading formulations for 80 μ m films. At high PEG loading (40%), clear PEG “precipitates” are observed on the surface. These precipitates can also be observed in most of the 40 μ m formulations (Figure 5.2). This phase-separated morphology is due to polymer immiscibility in the binary system. Hence, the miscibility between PCL and PEG is investigated in more detail in the next section.

5.2.2 Polymer miscibility

Figure 5.3 shows DSC traces of pure PEG crystal, protein loaded PCL, and their blends with various constituent compositions. Thermal properties for the as-prepared film (first heating scan) shows sharp endothermic peak at 54 °C, and a broad endothermic peak at 62 °C, representing the melting temperature of pure PEG crystal and pure PCL film, respectively. Their blends, however, show more complicated behavior. Phase separation between PCL and PEG can be clearly observed with a distinguishable PCL melting peak, and a multi-modal PEG melting peak. These multiple PEG endothermic peaks could be due to crystal perfection in the heating scan.

In the second heating scan, melting peaks of PEG and PCL are observed at 52 and 56 °C, respectively. These endothermic peaks could also be seen in their blends with different areas corresponding to the blend compositions without significant peak shifts, thus confirming that

PCL and PEG crystalline phases are separate. No significant differences could be observed in thermal properties for blank polymer films (results not shown) probably due to the low amount of protein added.

Figure 5.3. DSC thermograms of PEG crystal, protein loaded PCL, and their blends.

Polymer miscibility can also be determined using XRD. Figure 5.4 shows XRD profiles of PCL, PEG and their blends. PCL film revealed 3 distinct peaks at 21.4° , 22° and 23.8° , while PEG revealed 2 peaks at 19.1° and 23.3° , indicating their crystalline nature. High PEG loading blends (30% and 40%) show two sets of peaks, each represents PCL or PEG. The PEG peaks in these formulations, however, are quite weak. Nevertheless, the XRD profiles confirmed a phase separated phenomenon, similar to that observed in the DSC thermograms.

Figure 5.4. XRD profiles of PCL, PEG and their blends. The black dotted lines represent the diffractions from PCL and the red dotted lines from PEG.

5.2.3 Qualitative analysis of protein-PEG colocalization

Figure 5.5. Raman spectra of protein, PEG and PCL in the 1800 – 700 cm⁻¹ region, generated using BTEM analysis.

Figure 5.5 shows the reconstructed Raman spectral estimate of protein, PEG and PCL via BTEM analysis. The reconstructed Raman spectral estimate of protein concurs well with other literature [92-97]. All peak positions and their corresponding band assignment are summarized in Table 5.2. The bending mode $\delta(\text{CH}_2)$ is observed at 1459 and 1448 cm^{-1} ; the stretching of C-N is observed at 1128, 1109 and 1078 cm^{-1} ; while the stretching of C-C is observed at 936 and 900 cm^{-1} . The ring stretching modes of Phenylalanine (Phe), a side-chain residue group of lysozyme, give rise to strong Raman bands at 1585 cm^{-1} , ring “breathing” modes are associated with Raman bands at 1006 cm^{-1} , and CH in-plane deformation at 1207 cm^{-1} . The ring stretching and breathing modes of Tyrosine (Tyr) are similar to those of Phe. Its doublet at 858 and 836 cm^{-1} has been assigned to a Fermi resonance between a ring breathing and the overtone of an out-of-plane ring-bending vibration. The indole ring of Tryptophan (Trp) gives rise to many ring modes, of which Raman band at 1363, 1338, 1014, 879 and 761 cm^{-1} . The polypeptide chain mode of lysozyme can be characterized by its three Amide bands. The Amide I region is characterized by a strong broad peak of C=O stretching between 1600 and 1700 cm^{-1} while the sharp Amide II band is observed at 1552 cm^{-1} . The weaker Amide III band is characterized by a broad band composed of three peaks at 1274, 1262 and 1240 cm^{-1} .

The BTEM estimates of PEG and PCL (Figure 5.5) both show a semi-crystalline characteristic, as denoted by their sharp Raman peaks and the peak splitting. The frequencies of the Raman bands of both PEG and PCL are listed in Table 5.3, together with their probable band assignments. For PEG, the bending mode of CH_2 is characterized by a strong band composed of three peaks at 1487, 1473 and 1448 cm^{-1} . The combination of C-C stretching and CH_2 wagging is observed as two weak Raman peaks positioned at 1398 and

1364 cm^{-1} . The twisting mode of CH_2 is characterized by the two strong peaks at 1283 and 1236 cm^{-1} . The bands at region between 1139 and 846 cm^{-1} arise from combinations of chain backbone modes, in particular the C-O-C stretching vibration, and the CH_2 rocking modes [98, 99].

In the case of PCL, a sharp peak was observed at 1724 cm^{-1} , which was assigned to carbonyl stretching mode $\nu(\text{C=O})$ of PCL. Raman bands that reflect the semi-crystalline domains of PCL include the CH_2 bending mode $\delta(\text{CH}_2)$ between 1470 – 1418 cm^{-1} , the CH_2 wagging and twisting mode between 1307 – 1285 cm^{-1} , and skeletal stretching between 1110 – 1040 cm^{-1} and 915 cm^{-1} [100-103].

The three BTEM spectral estimates of protein, PEG and PCL shown in Figure 5.5 were then fitted back onto the original Raman mapping data set in order to determine the associated spatial distribution. Figure 5.6 shows the spatial distributions of the three components at the top and bottom surfaces of 80 μm films at various PEG loading. For protein-loaded PCL, protein-rich domains can be observed at both surfaces of the film. These protein islands are surrounded by a continuous matrix of PCL. At 10% PEG loading, the spatial distribution of these three components clearly show that there is phase segregation between PCL and PEG, and between PCL and protein. Interestingly, the spatial distributions of both the protein and PEG are seen to be similar. This confirms the extensive colocalization of protein and PEG in the same domain. The colocalization of protein-PEG is also observed in the 30% PEG film; however, the overlapping between the two components is visually lesser especially in the 40 μm film (Figure 5.7). This visualization of protein-PEG colocalization is only qualitative using this method.

Table 5.2. Band assignment for lysozyme [94-97].

Frequency (cm ⁻¹)	Band assignment
761	Tryptophan (Trp): indole ring
836	Tyrosine (Tyr): Fermi resonance between ring fundamental and overtone
858	Tyrosine (Tyr): Fermi resonance between ring fundamental and overtone
879	Tryptophan (Trp): indole ring
900	$\nu(\text{C-C})$
936	$\nu(\text{C-C})$
1006	Phenylalanine (Phe): ring breathe
1014	Tryptophan (Trp): ring breathe
1078	$\nu(\text{C-N})$
1109	$\nu(\text{C-N})$
1128	$\nu(\text{C-N})$
1207	Phenylalanine (Phe): CH in-plane deformation
1240	Amide III: in-plane $\delta(\text{N-H}) + \nu(\text{C-N})$
1262	Amide III: in-plane $\delta(\text{N-H}) + \nu(\text{C-N})$
1274	Amide III: in-plane $\delta(\text{N-H}) + \nu(\text{C-N})$
1338	Tryptophan (Trp): indole ring
1363	Tryptophan (Trp): indole ring
1432	$\delta(\text{N-H})$ of the indole ring
1448	C-H deformation vibration, $\delta(\text{CH}_2)$
1459	C-H deformation vibration, $\delta(\text{CH}_2)$
1552	Amide II: in-plane $\delta(\text{N-H}) + \nu(\text{C-N})$
1585	Phenylalanine (Phe): ring stretch
1660	Amide I: $\nu(\text{C=O})$

ν = stretching; δ = bending

Table 5.3. Band assignment for PEG [98, 99] and PCL [100-103].

PEG		PCL	
Frequency (cm ⁻¹)	Band assignment	Frequency (cm ⁻¹)	Band assignment
846	r(CH ₂)	915	v(C-COO)
862	r(CH ₂) _s + v(COC) _s	960	v(C-C)
1065	r(CH ₂) _a + v(COC) _s	1040	v(C-CH ₃)
1075	r(CH ₂) _s + v(COC) _s	1066	v(C-C) + v(COC) _s
1128	v(COC) _s	1110	v(C-C) + v(COC) _{as}
1139	v(COC) _a	1285	t(CH ₂)
1236	t(CH ₂) _a	1307	w(CH ₂) + t(CH ₂)
1283	t(CH ₂) _s + t(CH ₂) _a	1418	δ(CH ₂)
1364	w(CH ₂) _s + v(C-C)	1443	δ(CH ₂)
1398	w(CH ₂) _s + v(C-C)	1470	δ(CH ₂)
1448	δ(CH ₂) _s	1724	v(C=O)
1473	δ(CH ₂) _a		
1487	δ(CH ₂) _s		

v = stretching; δ = bending; t = twisting; r = rocking; w = wagging;
s = symmetric; as = asymmetric

Figure 5.6. Spatial distribution of protein, PEG and PCL in 80 μm films.

Figure 5.7. Spatial distribution of protein, PEG and PCL in 40 μm films.

5.2.4 Quantitative assessment of protein-PEG colocalization

Figure 5.8 shows dual channels images acquired by CLSM, where the red pixels represent protein distribution while the green pixels represent PEG distribution. The degree of overlap between protein and PEG is seen in the overlay image as yellow regions when the green and red pixels overlap. This observation of colocalization is in agreement with the Raman mapping images and is supported by their corresponding scatterplots. In the scatterplot, pure red and pure green pixels are cluster toward the axes of the plot, while colocalized pixels that appear as orange and yellow hotspots scatter diagonally toward the upper right hand corner. The degree of protein-PEG colocalization is quantified and is listed in Table 5.4. Coefficients calculated for 10% PEG blends at 40 μm and 80 μm were: $R_r = 0.83$ and 0.64 , $R = 0.94$ and 0.83 (represents as 94% and 83% colocalization). On the other hand, the calculated coefficients for 30% PEG blends were: $R_r = -0.15$ and -0.11 , $R = 0.65$ and 0.65 for 40 μm and 80 μm , respectively. Overlap coefficients k_1 and k_2 showed the contribution of protein and PEG to colocalization, i.e. equal contribution from both red and green channel for all the formulations except for 30% PEG (80 μm) where $k_1 \ll k_2$. Readers are referred to Section 3.2.9 for the description and applicability of the above mentioned coefficients.

Figure 5.8. Dual channels (red and green) and overlay CLSM images along with their corresponding scatterplots for PCL/PEG blend films.

Table 5.4. Comparison of colocalization parameters calculated from dual channels images in Figure 5.8 (mean $\pm$ standard deviation, $n = 3$).

	10% PEG 40 μm	10% PEG 80 μm	30% PEG 40 μm	30% PEG 80 μm
Pearson's coefficient, R_r	0.83 ± 0.03	0.64 ± 0.12	-0.15 ± 0.20	-0.11 ± 0.16
Manders overlap coefficient, R	0.94 ± 0.02	0.83 ± 0.09	0.65 ± 0.03	0.65 ± 0.01
Overlap coefficient, k_1	0.95 ± 0.11	0.85 ± 0.22	0.67 ± 0.37	0.20 ± 0.12
Overlap coefficient, k_2	0.93 ± 0.15	0.81 ± 0.10	0.63 ± 0.19	2.10 ± 0.46

5.2.5 Protein distribution

Protein distribution in a delivery system has often been ignored in most protein release studies, although this is one of the important factors that will affect the protein release mechanisms, especially the burst effect. Only until recently when CLSM imaging has been used intensively in pharmaceutical products, visualization of protein distribution has become possible.

Protein distribution of PCL/PEG blends is investigated via CLSM imaging, to assess the possibility of phase segregation at the film surface during film preparation (solution casting), and the effect of PEG on protein distribution. Due to the consideration of signal losses while performing in-depth scanning, protein-PEG distribution within 25 μm from both top and bottom film is analyzed.

Figure 5.9 shows a 3-dimensional projection of protein and PEG distribution in PCL/PEG blends. As explained in the previous section, the red pixels represent protein distribution; the green pixels represent PEG distribution, and orange-yellow correspond to the overlapping of protein and PEG. It can be seen that the protein and PEG distribution of the top layer and bottom layer are similar, i.e. no protein or PEG segregation in either of the layers, indicating homogeneous distribution of protein and PEG in all formulations.

The protein-PEG colocalization can also be visualized in the projection images. In 10%PEG 40 μm formulation, protein-PEG colocalization is indicated by the strong yellow signal, homogeneously distributed throughout the film. This signal appears to be dimmer in 10%PEG 80 μm formulation, as represented by an orange signal, also evenly distributed within the film. At 30% PEG loading, strong green signal (PEG) can be seen in the entire film. Protein-PEG colocalization in this formulation, in contrast, is visually much lesser, represented by a few faint orange spots. This observation is consistent with the results presented in Section 5.2.3 and 5.2.4, that protein-PEG colocalization at high PEG loading (30%) is lower than that in low PEG loading (10%) formulations.

Figure 5.9. 3-D projection of protein (red) and PEG (green) distribution in PCL/PEG blends from the top and bottom film surface.

On top of the qualitative investigation of protein distribution, quantitative assessment of protein distribution throughout the film thickness is done to provide better insight. For each formulation, protein coverage at a few selected optical layers are quantified according to the procedure describe in Section 3.2.10. Figure 5.10 shows the area coverage of protein-rich domains for PCL/PEG blend films at 40 and 80 μm . A homogeneous protein distribution can be seen for all formulations, where the area coverage of protein-rich domains ranged from 10% to 35% of the total scanned area. No sign of surface segregation is observed.

Figure 5.10. Quantification of protein distribution in terms of the total area coverage of protein-rich domains at specific optical layer.

Figure 5.11. Size distribution of protein-rich domain throughout the film thickness.

The size of protein-rich domain at a few selected optical layers in PCL/PEG blends is shown in Figure 5.11. The protein-rich domain sizes are divided into 3 groups: small droplets which area is smaller than $60 \mu\text{m}^2$, medium droplets where the area is between 60 and $120 \mu\text{m}^2$, and large droplets with area larger than $120 \mu\text{m}^2$. For 10%PEG 40μm formulation, majority of the protein droplets are small. In a thicker film (80 μm), these “small-size” droplets are lesser. Protein droplets in 30% PEG formulations, on the other hand, are larger. Approximately 40% and 60% of the protein droplets are in the size of $< 120 \mu\text{m}^2$. It is

postulated that in the solvent casting process, protein droplets coalesce forming larger droplets. The coalescent of protein droplets is more apparent in a thicker film as the solvent evaporation process is longer, providing more time for the droplets to migrate and coalesce. At high PEG content, viscosity of the polymer solution is lower. This eases the coalescent of protein droplets, manifested by its larger droplet size. Nevertheless, the author does not rule out other possibilities that might have affected protein droplet size, such as protein crystallization in high PEG content formulations. Readers are referred to Section 5.3 for more discussion.

5.2.6 *In vitro* release

Protein released from PCL and PCL/PEG blend films were compared on the basis of same protein loading (Figure 5.12a), for two different film thicknesses. The release profiles for all cases consist of two stages: an initial burst effect followed by a diffusional-controlled slow release. In the first stage, an initial burst of up to 35% was observed for the 30% PEG blends at both thicknesses while lower bursts are seen for 10% PEG blends. After this initial burst, a slower diffusion-controlled release contributed to a gradual increase of 7 – 10% in the protein cumulative release for all formulations. Protein-loaded PCL films exhibit similar release profile, but with much lower burst release that ranged from 5% to 10% for 40 μm and 80 μm respectively.

The amount of PEG leached from PCL/PEG films is shown in Figure 5.12b. It can be seen that a very high amount of PEG was leached from the polymer films, and the amount of PEG leaching increases with increasing PEG content, and with decreasing thickness. Within the

first 6 hrs, approximately 20 – 30% of PEG was released from 10% PEG blends, while 55 – 65% PEG leached out from 30% PEG blends. This was followed by a slower, gradual release.

Figure 5.12. In vitro release studies for actual protein release (a) and PEG leaching (b).

Figure 5.13. Comparison between actual (black diamond) and estimated (grey line) protein release profile. Statistical analysis was performed using t-test, and was shown beside the release profile. The probability value of $p > 0.05$ was considered to indicate statistical insignificance.

With the above data, it is clear that the addition of PEG increases the burst release as well as the subsequent release rate. To estimate the significance of protein-PEG colocalization and the contribution of the PEG leaching rate to the observed release profile, each PEG leaching profile was multiplied with its corresponding Manders overlap coefficient (refer to Table 5.4:

this coefficient is the coefficient of choice for reasons to be discussed in Section 5.3) to estimate the amount of protein release from PCL/PEG blends. These results were compared with the actual protein release data measured in an independent *in vitro* release experiment using micro-BCA assay (Figure 5.13). Interestingly, both sets of protein release profile were in agreement with each other ($R^2 > 0.92$), with only ~5 – 10% systematic deviations observed. It was noted that the calculated protein release in all formulations was slightly higher than the actual one. The slight discrepancy could be due to an over-estimation of colocalization using our method. Nevertheless, statistical analysis of the differences between the actual and estimated protein release showed $p > 0.05$ in all formulations; this was considered to indicate statistical insignificance in the discrepancy.

5.2.7 Protein Stability

The stability of protein released during the release regime is investigated using HPLC method as described in Section 3.2.13. Instead of showing protein release profiles quantified by HPLC, fractions of stable protein in the release medium are shown. The ratio of stable protein to total protein is calculated by dividing the HPLC measurements with micro BCA measurements at the same release time point, as shown in Figure 5.14. The fraction of stable protein released for PCL film drops drastically (~50%) over the release duration. At the end of the release (day 70), only 20% stable protein is found in the released solution. In contrast to PCL film, the addition of PEG greatly improved the fraction of stable protein throughout the entire release regime. A constant drop of 20% is observed at the end of release study for both PEG compositions.

Figure 5.14. Ratio of stable protein to total protein in the release studies.

5.3 Discussion

Confocal Raman mapping and CLSM imaging were used in conjunction to investigate protein distribution in PCL/PEG blends. Qualitative analysis of Raman mapping showed phase separation of polymers, where PEG was separated into individual domains from the PCL continuous matrix, forming PEG-rich islands. This is in agreement with the DSC and XRD results that indicate the immiscibility between the crystalline phase of PCL and PEG. Interestingly, the mapped image for protein showed a similar phase separated pattern that replicated the mapped image of PEG, indicating colocalization of protein-PEG in the same domain. To date, colocalization of protein and PEG has not been reported qualitatively or quantitatively; hence, in addition to Raman mapping, CLSM imaging was also carried out using fluorescence-tagged protein and PEG, to quantify the colocalization. Both sets of imaging confirmed extensive protein-PEG colocalization.

Image processing of dual channel fluorescence microscopic images further quantify the amount of colocalized domain, as described by a few coefficients. The resulting Pearson's coefficients for 10% PEG blends indicate a positive correlation between protein-PEG overlapping domain and a high degree of colocalization. However, the negative value of Pearson's coefficients for 30% PEG blends need not imply an inverse correlation. According to a review by Zinchuk *et al.* [104], negative values of Pearson's correlation should be interpreted very cautiously. Manders overlap coefficient should be employed if the intensity of one component is stronger than the others, as it allows quantification of colocalization coefficients in such images more reliably. In addition, Bolte *et al.* [83] assessed the use of Pearson's correlation in four different colocalization situations (complete colocalization, complete colocalization with different intensities, partial colocalization and exclusion) and concluded that Pearson's correlation coefficient values point to colocalization (value close to 1) when the images of two colour channels are completely colocalized; however, this correlation rarely discriminates the differences between partial colocalization and exclusion. The authors state that "value other than those close to 1 and especially mid-range coefficients (-0.5 to 0.5) do not allow conclusions to be drawn". The evaluation of colocalization using Pearson's correlation may be ambiguous in this work, probably due to the variations in fluorescence intensities, noise, and heterogeneous colocalization relationship between protein and PEG. Hence, only Manders coefficients were used to estimate protein release from PCL/PEG blends in the subsequent analysis.

From Manders coefficients, the degree of protein-PEG colocalization was found to be higher for 10% PEG blends (83% – 94%) compared to 30% PEG blend systems (65%). At present,

the author does not have an explanation for this observation. It may be related to the solubility limit of protein in PEG-containing solutions [9, 46, 105, 106]. Herrmann et al. [46] performed a solubility test of protein (IFN- α) in concentrated aqueous PEG 6000 solutions. In this study, IFN- α spontaneously crystallized in PEG 6000 concentration above 3%, and almost negligible protein solubility at PEG concentration above 10%. The crystallization of proteins occurs when the linear PEG chain occupied the solvent space around protein side chain, causing an exclusion of protein side chain, or sterical hindrance protein from the solvent space. A similar phenomenon may account for our observations here with respect to lysozyme solubility in PEG 2000 at 10% PEG in PCL and at 30% PEG in PCL, although the two systems are not strictly comparable. Protein crystallization may have also been responsible in causing the enlargement in the size of protein-rich domains for high PEG content formulations. As reported in Section 5.2.5, the protein-droplet-size-quantification test via image processing shows that nearly 50% of the protein-rich domains in 30% PEG blends are composed of large protein droplets, while only around 10-20% of these large droplets can be found in the 10% PEG blend. However, this hypothesis is not conclusive at the moment. More thorough investigation is needed as proposed in Section 7.2.1.

In order to examine the effect of film thickness on protein release rate, we fabricated protein loaded films of 40 μm and 80 μm at the same protein loading. As expected, almost all of the 40 μm films exhibited a higher protein release rate, likely due to a faster water penetration rate in a thinner film as compared to the 80 μm formulations. Blends with 30% PEG loading, however, exhibited similar protein release rate for both thicknesses. To explain these observations, protein distribution of all formulations was investigated via our novel image processing method. Quantitative analysis of protein distribution showed homogeneous

distribution throughout the entire film, independent of PEG contents. Therefore, the presence of different PEG loading did not substantially alter the distribution of protein within the thickness of the film. However, film thickness was found to have a slight effect on protein distribution where-by the protein coverage for the 40 μm films was slightly higher than the 80 μm formulations for PCL and 10% PEG loading. This could account for the increase in protein release rate, in addition to the higher water penetration rate mentioned earlier. On the other hand, similar protein coverage was observed for 30% PEG loading for both film thicknesses, thus accounting for the lack of dependence of release on thickness in this case.

From the *in vitro* release studies, the addition of PEG significantly amplified the magnitude of burst effect and total amount of released protein. Similar findings were observed by other research groups [7-10]. In most cases, the effect of protein burst release was attributed to surface segregation or loosely bound protein at the surface layer [59]. However, in this system, the results show that the protein does not accumulate on the surface; thus another cause must be sought for the increased burst and subsequent release of protein. To answer this question, PEG leaching was also quantified as a function of time. Combining the overlapping coefficient according to Manders together with the PEG leaching data, a comparison was made between the estimated protein release, to the actual protein release profile measured by BCA analysis. Clearly, good agreement was obtained between calculated and experimental release. What this implies is that the release of protein is dominated by the release of PEG with associated protein. The other contributions to the burst and subsequent release, such as surface segregation, or diffusion through the PCL matrix, are not significant in this system. Thus, the picture that emerges is shown in Figure

5.15: when PCL/PEG films are exposed to aqueous media, there is an initial burst release of PEG together with the colocalized proteins. Voids, which were formed by the leaching of PEG, later facilitated the diffusion of PEG entrapped deeper in the film matrix (but also associated with protein) and hence increased the overall rate and amount of protein released. Non-colocalized protein, on the other hand, permeated much more slowly through the highly crystalline matrix. Incomplete protein release in all formulations is understandable as the PEG/proteins embedded deeper within the bulk were not expected to be released until complete biodegradation of PCL occurs or until all pores became inter-connected.

Figure 5.15. Cartoon of protein release mechanism in PCL/PEG blend films.

So does the proposed mechanism vary appreciably from the “pore-diffusion model” mentioned in Section 5.1? Does the colocalization data distinguish sufficiently between the two? In the “pore-diffusion model”, PEG leaches out first, creating pores which then allow easier diffusion of protein (the pores could be water-filled). In this “pore-diffusion model”, the initial burst is due mostly to undissolved protein at the surface. In comparison to the proposed colocalization model, the initial burst is due to a burst of PEG release, which carries with its associated protein. This is responsible not only for the initial burst, but also

for subsequent release. The CLSM data clearly shows that there is no surface segregation, hence ruling out this as a cause of the burst. Following burst, the PEG release rate is similar regardless of PEG content or film thickness. This similarity is also reflected in the protein release rates, which affirms that the entire release profile reflects the release of PEG from the film, carrying with it the colocalized protein. The rather close fitting of the experimental release curves using the Manders coefficients clearly supports this model for both phases of release. Hence, it can be concluded that protein release from PCL/PEG blend system is mainly governed by the amount of colocalized protein in the PEG phase, and PEG leaching rate.

On a practical level, the ability to visualize and quantify protein-PEG colocalization via this novel image processing technique has provided a new platform for understanding the release mechanism from the aspect of protein partitioning. It should be noted that the protein-PEG colocalization coefficients calculated from this work may not be generalized to all protein loaded PEG-based blends delivery systems, i.e. for a different protein or a different PEG in a PCL/PEG blend system. However, the candidate maintain that colocalization of protein into the PEG in a phase separated PEG blend, is the driving factor in protein release. As such the concept should be applicable to different geometries, such as fibers or microspheres, provided the PEG is phase separated, and has a higher solubility for protein than the dispersing phase. If it is possible to quantify the extent of colocalization by a suitable technique, and if the PEG leaching can be measured or predicted, then the protein release mechanism can be predicted. It is hoped that this predictive capability allows for manipulation of formulations to attain a desired protein release profile. In the past, it has been almost impossible to quantify protein/drug partitioning into each phase of a blended

system, without destroying the samples. Using this approach, the distribution of protein throughout a given sample can be quantified, although this method does not yield quantitative compositional data (as given by ESCA and XPS) for the mass of protein distributed as a function of depth. The sample depth over which our method works depends on the materials used; in this work, protein distribution up to a depth of $\sim 25\ \mu\text{m}$ can be analyzed. As compared to the currently available quantification instruments that are limited to $< 100\ \text{nm}$ [12, 13], this larger analysis volume resulted in higher accuracy and may lead to wider applications in drug/protein delivery systems.

For a successful protein delivery system, besides maintaining the therapeutic concentration of the protein for a proper duration, the released protein should also be in its stable/active form to provide its therapeutic effect. Hence, a protein release study is never complete without protein stability measurement. From this study, the fraction of stable protein released for PCL formulation is low. In contrast to the huge loss in the protein stability for PCL film, it is obvious that the addition of PEG greatly improves the fractions of stable protein release throughout the entire release regime. Readers are referred to the next chapter Section 6.3 for discussion and hypothesis of such improvement. Despite the huge improvement in protein stability for PCL/PEG blends, its high burst and low subsequent release of protein from is undesirable. For a protein therapeutic, the high dosage of protein released at the beginning of treatment may have exceeded the toxic plasma concentrations leading to adverse reactions, while the low release of protein at the later stage might be too low for a desirable therapeutic effect. This has motivated the study on a non-leachable PEG-based blend, which will be discussed in greater length in the next chapter.

Chapter 6. PCL/Copolymer Blends

6.1 Introduction

Since the first protein incorporation into biodegradable polymer in the 1970s [107], intensive research on protein drug delivery has been carried out. Among the plethora of protein release publications, numerous studies have addressed the burst phenomena [108-113]. High initial release rate is desirable for the applications such as wound treatment, targeted delivery and pulsatile release [110]. However, the burst effect is often considered as a negative outcome for a long-term controlled release device, as it leads to both drug wastage as well as potentially severe side effects in the case of a potent drug. A summary of findings have been the focus of two reviews [110, 114] on the hypothetical mechanisms of “burst” release and potential strategies to minimize it.

In order to minimize the burst effect in PCL/PEG blends as discussed in Chapter 5, the use of a diblock PCL-PEG copolymer to replace PEG is evaluated. PCL-PEG diblock copolymer consists of a non-water soluble hydrophobic PCL block, covalently bound with a water soluble hydrophilic PEG block. Combining the two distinctive properties, it is reasonable to propose that in a PCL/copolymer blend system, the hydrophobic PCL block of the copolymer which is compatible with the PCL continuous matrix will form PCL chain-entanglement, preventing the leaching of the copolymer from the blends, while the hydrophilic PEG block will increase protein diffusivity through the crystalline matrix. The author hypothesize that a non-leaching PCL/copolymer blends will provide better

controllability on protein burst effect, while at the same time increasing protein subsequent release.

This chapter focuses on the role of the PCL-PEG copolymer in controlling protein release from PCL/copolymer blend system by (1) characterization of protein loaded PCL/copolymer blends, (2) quantification of protein distribution via a novel image processing technique, and (3) correlation of the microenvironment information to protein release mechanism. Table 6.1 summarizes the films prepared for qualitative protein distribution studies, and in vitro protein release studies.

Table 6.1. Composition of the prepared formulations and their corresponding usage.

Sample Name	Polymer ratio (%)			Protein loading (wt% of total film)	
	PCL	PCL(1.5k)- PEG(5k)	PCL(10k)- PEG(5k)	Lysozyme	Rhodamine B- Lysozyme
<i>In vitro protein release and mass loss analysis</i>					
PCL 80 μm	100	-	-	3	-
10% co1.5k 80 μm ^a	90	10	-	3	-
30% co1.5k 80 μm ^a	70	30	-	3	-
10% co 10k 80 μm ^b	90	-	10	3	-
30% co 10k 80 μm ^b	70	-	30	3	-
PCL 40 μm	100	-	-	3	-
10% co1.5k 40 μm ^a	90	10	-	3	-
30% co1.5k 40 μm ^a	70	30	-	3	-
10% co 10k 40 μm ^b	90	-	10	3	-
30% co 10k 40 μm ^b	70	-	30	3	-
<i>CLSM imaging for quantitative analysis (colocalization)</i>					
10% co1.5k-FITC 80 μm ^c	90	10	-	-	3
30% co1.5k-FITC 80 μm ^c	70	30	-	-	3
10% co 10k-FITC 80 μm ^d	90	-	10	-	3
30% co 10k-FITC 80 μm ^d	70	-	30	-	3
10% co1.5k-FITC 40 μm ^c	90	10	-	-	3
30% co1.5k-FITC 40 μm ^c	70	30	-	-	3
10% co 10k-FITC 40 μm ^d	90	-	10	-	3
30% co 10k-FITC 40 μm ^d	70	-	30	-	3

CLSM imaging for qualitative analysis (protein morphology)

10% co1.5k 80 μm ^a	90	10	-	-	3
30% co1.5k 80 μm ^a	70	30	-	-	3
10% co 10k 80 μm ^b	90	-	10	-	3
30% co 10k 80 μm ^b	70	-	30	-	3
10% co1.5k 40 μm ^a	90	10	-	-	3
30% co1.5k 40 μm ^a	70	30	-	-	3
10% co 10k 40 μm ^b	90	-	10	-	3
30% co 10k 40 μm ^b	70	-	30	-	3

^a co1.5k = PCL(1.5k)-PEG(5k)-CH₃

^b co 10k = PCL(10k)-PEG(5k)-CH₃

^c co1.5k-FITC = PCL(1.5k)-PEG(5k)-FITC

^d co 10k-FITC = PCL(10k)-PEG(5k)-FITC

6.2 Results

6.2.1 Surface morphology

Figure 6.1. Surface morphology of protein loaded PCL/copolymer PCL(1.5k)-PEG(5k) blends before release studies.

Figure 6.2. Surface morphology of protein loaded PCL/copolymer PCL(10k)-PEG(5k) blends before release studies.

Surface morphology of protein loaded PCL/copolymer blends is shown in Figure 6.1 and Figure 6.2. Clear spherulites crystalline domains were observed in protein loaded PCL film

surface. These spherulitic crystalline domains can also be observed in the PCL/copolymer blend systems, with a smoother “creamy” or gel-like surface, independent of the molecular weight difference of the copolymers and film thickness. In addition, the spherulites boundary in all the copolymer blends is somewhat blurred compared to that of PCL system.

6.2.2 Polymer miscibility

Figure 6.3 shows DSC traces of pure PCL(1.5k)-PEG(5k) diblock copolymer, protein loaded PCL, and their blends with various constituent compositions. The thermal properties for protein loaded PCL shows a broad endothermic peak at 62 °C, while a monomodal melting peak at 57 °C is seen for pure PCL(1.5k)-PEG(5k) diblock copolymer. Nojima et al. [115] established that the crystallinity of the PCL and PEG chains decreases with decreasing individual block fraction and falls to zero when the corresponding block fraction is less than 25% in the system. Their work is in agreement with other research groups that only the longer segment of PCL or PEG is crystallizable when the weight fraction of one of the segment is below 20% [116, 117]. In current work, the single melting peak observed for pure PCL(1.5k)-PEG(5k) copolymer is hence due to PEG, while that of the PCL is not observed due to a low block fraction of PCL.

Although the crystallization and melting behaviour of the diblock PCL-PEG copolymers has been investigated since the last 20 years, it is surprising that the thermal properties of PCL/copolymer blends are far less documented. In the PCL(1.5k)-PEG(5k) blends, phase separation between PCL and copolymer can be clearly observed with two distinguishable melting peaks. Since the PCL-block of the PCL(1.5k)-PEG(5k) copolymer is not

crystallizable, the higher temperature endothermic peak is attributed to the melting of the homopolymer PCL crystalline phase, while the lower temperature endotherm corresponds to the melting of the copolymers' PEG crystal phase. It is obvious that endothermic peak of copolymer shifted from 57 °C towards lower temperature (52 °C) with the decreasing copolymer loading in the blends. From literatures, it is known that PCL crystallized first followed by the crystallization of PEG [117]. Hence during solvent casting, the PCL continuous matrix is expected to crystallize faster, disturbing the crystallization of copolymer, leading to the shifting of copolymer melting peak. This effect is stronger in the blend system with higher PCL constituent.

Figure 6.3. DSC thermograms of protein loaded PCL, PCL(1.5k)-PEG(5k) diblock copolymer and their blends.

Figure 6.4. DSC thermograms of protein loaded PCL, PCL(10k)-PEG(5k) diblock copolymer and their blends.

Figure 6.4 shows DSC curves for PCL(10k)-PEG(5k) blends. For pure PCL(10k)-PEG(5k) copolymer, two melting peaks can be observed, where the higher temperature endothermic peak at 58 °C is ascribed to melting of the PCL crystal phase, while the bimodal lower temperature endotherms at 39 °C and 45 °C correspond to the melting of the PEG crystal phase. The two melting peaks of PEG segment might be due to the melting of PEG lamellae with a different fold number [117, 118]. In spite of the covalent bonded PCL and PEG blocks, both are able to crystallize and form two separate crystalline phase, however, with the melting temperature lower than their homopolymer of 62 °C and 58 °C for pure PCL and pure PEG, respectively. The depression in the melting temperature occurs when the crystallization of each block extremely influence each other to yield imperfect PCL and PEG crystals simultaneously [117].

In the PCL(10k)-PEG(5k) blends, co-crystallization of PCL-block with the homopolymer PCL is observed as evidence by the shifting of PCL-block melting peak from 58 °C to 62 °C, overlapping with the endothermic peak of PCL homopolymer. During the co-crystallization, the presence of the PCL-block crystal affects the crystal growth of the PCL homopolymer as reflected by the broadening of the endothermic peak. The PEG-block, conversely, also shows peak shifting towards higher temperature. However, it is not certain if the PEG-block is miscible with the system based on the broad shoulder formation in the DSC curves.

Polymer miscibility can also be determined using XRD. Figure 6.5 shows the XRD diffraction patterns of PCL, PCL-PEG diblock and their blends. The diffraction peaks of PCL(1.5k)-PEG(5k) and PCL(10k)-PEG(5k) can be successfully indexed, showing superimposition of the pure PCL (21.4°, 22° and 23.8°) and PEG (19.1° and 23.3°) crystals, with the intensity of the diffractions corresponding to the fraction of PCL and PEG-blocks. The blends of PCL(1.5k)-PEG(5k) with PCL shows phase separation behavior, indicated by the increase in the diffraction intensity at 19.1° and broadening of the diffraction peak at 23.3°, with the increasing copolymer compositions, in agreement with the DSC data. This trend, however, is not observed in the PCL(10k)-PEG(5k) blends. In this blend system, the diffraction pattern from the PEG-block of the copolymer is not visible even at 30% copolymer loading. This could be due to the retardation of PEG-block crystal formation by the PCL-block crystal, as also reflected in the PEG endothermic peak broadening in DSC.

Figure 6.5. XRD profiles of PCL, diblock copolymer and their blends. The black dotted lines represent the diffractions from PCL and the red dotted lines from PEG.

6.2.3 FITC-copolymer tagging

FITC-conjugated copolymer of two different molecular weight were synthesized as described in Section 3.2.4, and were characterized by ^1H NMR. ^1H NMR resonance signals

of OH-PCL-PEG-NH₂ were fully attributed, as shown in Figure 6.6a and Figure 6.7a. The methylene protons of the oxyethylene unit from the PEG block correspond to the singlet at $\delta = 3.65$ ppm (labeled *f+g*). Meanwhile, the methylene protons of the oxycarboxy-1, 5-pentamethylene unit of the PCL blocks appear at $\delta = 4.05$, 2.28, 1.61 and 1.36 ppm (labeled *a*, *e*, *b+d* and *c*, respectively). The small triplet at $\delta = 4.2$ ppm is attributed to the protein resonance of the methyleneoxyl group linking the PEG and PCL blocks.

(a) PCL(1.5k)-PEG(5k)-NH₂

(b) PCL(1.5k)-PEG(5k)-FITC

Figure 6.6. ¹H NMR spectra of PCL(1.5k)-PEG(5k) diblock copolymer before (a) and after (b) FITC conjugation.

(a) PCL(10k)-PEG(5k)-NH₂

(b) PCL(10k)-PEG(5k)-FITC

Figure 6.7. ¹H NMR spectra of PCL(10k)-PEG(5k) diblock copolymer before (a) and after (b) FITC conjugation.

From the chemical structure of copolymer and FITC (Figure 3.1), the functional group at the end of the PEG chain and the PCL chain is amine and hydroxyl, respectively. Since only the amine group can be reacted with isocyanate, FITC can therefore be attached to the end of the PEG chain. The success of the FITC-PEG conjugation is confirmed by ¹H NMR spectra (Figure 6.6b and Figure 6.7b). The signal of methylene protons of amine is found at 2.76

ppm for both copolymers before FITC labeling. After FITC conjugation, this resonance disappears completely (as shown in the inset), suggesting a high yield in the conjugation reaction. The ^1H NMR resonance signals of FITC, however, is hardly observed in both spectras probably due to its limited solubility in CDCl_3 .

6.2.4 Quantitative assessment of protein-copolymer colocalization

Figure 6.8 shows dual channels images acquired by CLSM, where the red pixels represent protein distribution while the green pixels represent PCL-PEG-FITC copolymer distribution. The degree of overlap between protein and copolymer is shown in the overlay image as yellow-orange regions when the green and red pixels overlap. Quantification of the overlapping pixels is done as described in Section 3.2.9 and is listed in Table 6.2. Coefficients calculated for 10% and 30% PCL(1.5k)-PEG(5k) blends were: $R_r = 0.90$ and 0.94 , $R = 0.92$ and 0.93 (represents as 92% and 93% colocalization). On the other hand, the calculated coefficients for 10% and 30% PCL(10k)-PEG(5k) blends were: $R_r = 0.70$ and 0.76 , $R = 0.79$ and 0.82 . Overlap coefficients k_1 and k_2 showed the contribution of protein and copolymer to colocalization. Higher contribution from protein to colocalization is observed as compared to copolymer ($k_1 \gg k_2$) for all formulations except for 30% PCL(1.5k)-PEG(5k) blend. Readers are referred to Section 3.2.9 for the description and applicability of the above mentioned coefficients.

Figure 6.8. Dual channels (red and green) and overlay CLSM images along with their corresponding scatterplots for PCL/copolymer blend films.

Table 6.2. Comparison of colocalization parameters calculated from dual channels images in Figure 6.8.

	10% co1.5k	30%co1.5k	10% co10k	30% co10k
	40 μm	40 μm	40 μm	40 μm
Pearson's coefficient, R_r	0.90	0.94	0.70	0.76
Manders overlap coefficient, R	0.92	0.93	0.79	0.82
Overlap coefficient, k_1	2.33	0.43	1.83	1.67
Overlap coefficient, k_2	0.36	0.76	0.34	0.40

6.2.5 Protein distribution

Protein distribution in the PCL/copolymer blends was investigated quantitatively via the novel image processing technique as described in Section 3.2.10. Due to the consideration of signal losses while performing in-depth scanning, protein distribution within 25 μm from both top and bottom surface was analyzed. Figure 6.9 shows the area coverage of protein-rich domains in PCL/copolymer blend films of two thicknesses. In general, copolymer blends of 10% gives a more homogeneous protein distribution, independent of film thickness. In contrast, protein coverage variation is larger ($\sim 20\%$) between each optical layer for 30% copolymer blends. More protein-rich domains can be found at 10 to 20 μm below the film surface. On the other hand, Figure 6.10 shows a comparison of the area coverage of protein in the blends, before and after FITC conjugation. No obvious different is observed between the two sets of formulations, though it should be noted that there are slightly lesser protein distributed at the top surface of 30% PCL(1.5k)-PEG(5k), and more protein distributed at the middle layer of 10% PCL(10k)-PEG(5k) after FITC conjugation. Nevertheless, no sign of surface segregation is observed for all blend formulations, with and without FITC labeling.

Figure 6.9. Protein distribution (area coverage) in PCL/copolymer blends.

Figure 6.10. Protein distribution (area coverage) in PCL/copolymer blends, before and after FITC conjugation.

Figure 6.11 shows protein droplets size distribution of copolymer blends. Similar to the analysis done for PCL/PEG system (refer to Figure 5.11), the protein-rich domain sizes in PCL/copolymer system are grouped according to the area coverage of $< 60 \mu\text{m}^2$, $60 - 120 \mu\text{m}^2$, and $> 120 \mu\text{m}^2$. In contrast to PEG blend formulations, it is surprising that majority of the protein droplets are huge. Furthermore, film thickness does not seem to affect protein droplet size in the copolymer blends, as it does for PEG blends. The amount of copolymer loading has slight effect on protein droplet size. Even though the size differences are small; it can be observed that the amount of large protein droplets increases with copolymer loading. Similar observation is found for PCL(1.5k)-PEG(5k) blends after FITC conjugation where there is an slight increment in the size of protein-rich domains with increasing copolymer compositions (Figure 6.12). The size of protein droplets in PCL(10k)-PEG(5k)-FITC blends, however, are in dependant of the blend ratio. Furthermore, the protein-rich domains size distribution for PCL(10k)-PEG(5k) appears to be very different after FITC conjugation, as more smaller protein droplets can be found at the top and bottom surface of the film.

Figure 6.11. Protein droplets size distribution in PCL/copolymer blends.

Figure 6.12. Protein droplets size distribution in PCL/copolymer blends, before and after FITC conjugation.

6.2.6 *Morphology of protein-rich domains*

The morphology of protein-rich domains in the copolymer blends is shown in Figure 6.13. Interestingly, a core-shell-like morphology can be observed for the protein-rich domains in the copolymer blends before FITC conjugation (Figure 6.13 left column). The core-shell protein droplet formation is highly dependent on copolymer loading, and copolymer chain length. For 10% PCL(1.5k)-PEG(5k) blends, the core-shell droplets can be observed clearly for most of the protein-rich domains. In contrast, only a few core-shell protein droplets can be found in the 10% PCL(10k)-PEG(5k) formulation. The core-shell morphology is more manifested in the 30% copolymer blends where the core is surrounded by a dense shell. This core-shell morphology, however, is not observed for those domains in the FITC-conjugated copolymer blends (Figure 6.13 middle column). After FITC conjugation, all protein droplets appear to be rounded without any visible shell even at 30% copolymer blend ratio. These rounded-filled protein-rich domains can also be found in PEG blends formulations at a smaller droplet size (Figure 6.13 right column). PCL film, on the other hand, shows ring-type protein droplets morphology.

Figure 6.13. Morphology of protein-rich domains in copolymer blends (left column), FITC-tagged copolymer blends (middle column), PEG blends and PCL (right column).

In order to provide a better insight into the core-shell morphology, CLSM depth profiling images for both 30% PCL(1.5k)-PEG(5k) and 30% PCL(10k)-PEG(5k) are shown in Figure 6.14 and Figure 6.15, respectively, with the protein droplet of interest being focused at the center of each images. The most remarkable difference between the two formulations is the morphology at the shell. For 30% PCL(1.5k)-PEG(5k) film, a fluffy-like boundary for the protein-core can be seen, while the protein-core in 30% PCL(10k)-PEG(5k) film is surrounded by a fractal-like boundary. Measurements of core-shell sizes are shown in Table 6.3. Despite the fluffy and fractal-like morphology differences at the shell, the core size and shell thickness for the two formulations are not significantly different. Both formulations have an average protein-core diameter of $\sim 20\text{ }\mu\text{m}$, and a shell thickness between $9 - 13\text{ }\mu\text{m}$, giving a shell:core ratio of ~ 0.6 . The observation of core-shell-like morphology certainly supports the findings in protein droplets size distribution (Section 6.2.5), that this morphology has contributed to the overall increment in protein droplets size as compared to that in pure PCL and PEG blends.

Figure 6.14. Depth-profiling of CLSM images for 30% PCL(1.5k)-PEG(5k)-CH₃ copolymer blends.

Figure 6.15. Depth-profiling of CLSM images for 30% PCL(10k)-PEG(5k)-CH₃ copolymer blends

Table 6.3. Core-shell size measurement for protein-rich domains in 30% copolymer blends. Each measurement is an average of 5 protein droplets.

	30% PCL(1.5k)-PEG(5k)	30% PCL(10k)-PEG(5k)
Core diameter (μm)	20.4 ± 5.4	19.2 ± 7.4
Shell thickness (μm)	13.5 ± 5.4	9.7 ± 2.3
Shell/Core	0.66 ± 0.13	0.51 ± 0.15

6.2.7 *In vitro* release

Protein released from PCL(1.5k)-PEG(5k) and PCL(10k)-PEG(5k) blends was investigated to understand the influence of copolymer chain length on protein release. For PCL(1.5k)-PEG(5k) blends, the release profiles consist of two stages: an initial burst effect followed by a diffusional-controlled release (Figure 6.16). An initial burst of ~ 5% is observed for PCL films. It is obvious that the addition of copolymer increases the burst release within the first 6 hours. Up to 60 – 70% protein burst effect is observed for the 30% copolymer blends while this amount is lower for 10% copolymer blends. In the second stage of release, a slow diffusion-controlled release is observed for pure PCL films. The addition of copolymer increases the amount of subsequent release, contributed to 40% of the overall protein released in the 30% copolymer blends, leading to a complete protein release at day 70.

Protein-loaded PCL(10k)-PEG(5k) blends exhibit similar release profile, but with a much lower burst release (Figure 6.17). In fact, the amount of burst is similar to that exhibited in the pure PCL system for 80 μm film thickness, while ~ 10% higher in the 40 μm film formulations. The amount of copolymer loading does not seem to affect the release much in the first 6 hours of the release study. However, the loading does affect the second stage of protein release where the overall protein release is significantly increased. In the 10% copolymer loading formulations, an additional 20 – 30% protein release is observed after initial burst, while in the 30% copolymer blends, 50 – 60% protein subsequent release is observed. Compared to merely 7% subsequent release in the pure PCL formulations, the increment of 50% protein release for copolymer blends in the second stage is indeed a vast improvement.

Figure 6.16. Protein release profiles from PCL(1.5k)-PEG(5k)-CH₃ blends.

Figure 6.17. Protein release profiles from PCL(10k)-PEG(5k)-CH₃ blends.

Figure 6.18. Mass loss data reflecting copolymer leaching from PCL/copolymer blends.

Mass loss study is conducted to investigate the possibility of copolymer leaching from the blends (Figure 6.18). It can be seen that the mass loss in PCL(1.5k)-PEG(5k) copolymer blends is high, and the magnitude of mass loss increases with increasing copolymer content. Within the first 6 hours, approximately 2% mass loss can be observed in the 10% copolymer blends, while ~11% mass loss for 30% copolymer blends. In contrast, mass loss profile for PCL(10k)-PEG(5k) blends is very similar to that of pure PCL formulations, independent of

copolymer loading and film thickness. The mass loss information can also be translated to indicate copolymer leaching from the blends. From the mass loss data, it is confirmed that PCL(1.5k)-PEG(5k) copolymer leaches out within the first 6 hours, while minimal PCL(10k)-PEG(5k) copolymer leaching is seen from the blends.

6.2.8 Protein Stability

Figure 6.19 shows the fraction of stable protein released from PCL and PCL(1.5k)-PEG(5k) blends. No significant different is observed for all 3 formulations at the beginning of the release study. From day 7 onwards, however, a huge different in the protein stability ratio is found between the PCL and copolymer blends. The fraction of stable protein released for PCL film drops drastically (~50%) over the release duration. At the end of the release (day 70), only 20% stable protein is found in the released solution. Copolymer blends, on the other hand give a more consistent stable protein release throughout the release study. The good performance is especially noticeable for 30% PCL(1.5k)-PEG(5k) blend, where only 10% drop in the fraction of stable protein release is found at the end of the release study. Protein stability data for PCL(10k)-PEG(5k) blends is not available for the time being.

Figure 6.19. Ratio of stable protein to total protein in the release studies.

6.3 Discussion

The potential use of PCL-PEG copolymer to tailor protein release from PCL blends was evaluated using different PCL-PEG chain lengths. From the *in vitro* release studies, polymer blends of PCL(1.5k)-PEG(5k) and PCL(10k)-PEG(5k) showed significant difference in the protein release profile, where the use of short PCL block significantly amplified the magnitude of burst effect. The amplification of burst effect can be due to two possibilities: (1) heterogeneous protein distribution i.e. protein surface segregation, and (2) copolymer leaching from the polymer blends. Heterogeneous drug/protein distribution is usually due to drug/protein migration during drying of the delivery system [110, 119]. During drying, as the solvent evaporates, drug/protein may diffuse together with solvent by convection towards film surface, causing higher drug/protein concentration at the surface, which subsequently leaves uneven drug/protein distribution within the film. When this happened, the

drug/protein is released immediately upon contacting with the release medium. Polymer leaching, on the other hand, is usually due to the immiscibility between two polymers, especially a blend system that involved the incorporation of hydrophilic polymer such as PEG. In Chapter 5, the author has showed the importance of PEG leaching measurement in relation to protein release mechanism. Hence, in this study, investigation on protein distribution and copolymer leaching has become necessary to understand the effect of copolymer chain length differences in relation to protein burst release.

Quantitative analysis of protein distribution showed homogeneous distribution for most formulations. Although higher variation of area coverage was found in 30% copolymer loading; no sign of protein surface segregation was observed. In fact, the amount of protein distributed at surface was less than that within the bulk for all formulations. Comparing the protein coverage at both surfaces among each formulation, all films exhibited a relatively similar amount protein at the surface. Similar observation is found for FITC conjugated copolymer blends. Therefore, neither the block fraction of copolymer, copolymer loading and copolymer modification did not substantially alter the distribution of protein nor caused surface segregation across the films, hence ruling out this cause of the burst release.

Mass loss study was conducted to find out the possibility of copolymer leaching from the blends. Although mass loss study is not a direct indication of copolymer leaching, if the mass loss is greater than the measured protein release amounts, then it is indicative of copolymer leaching. From the mass loss data, it was confirmed that extensive copolymer leaching occurred in the PCL(1.5k)-PEG(5k) blends, where the amount of copolymer

leaching increased with the amount of copolymer added. In contrast, the flat mass loss profile of the PCL(10k)-PEG(5k) blends indicated no copolymer leaching from the system.

The occurrence of copolymer leaching is highly related to its miscibility with the continuous matrix, PCL. As referred to polymer miscibility data by DSC and XRD, clear phase separation was found in PCL(1.5k)-PEG(5k) blends. Since the short PCL-block of the copolymer is too short to interact with the PCL homopolymer, the copolymer behaved more like a PEG molecule in the blends. When the PCL(1.5k)-PEG(5k) blend films are exposed to the release medium, the copolymer leached out quickly into the aqueous media, which account for the protein burst effect. Compared to PEG leaching from PCL/PEG blends as presented in Section 5.2.6, the amount of copolymer leaching is much lower. Although it has been proven that the PCL-block chain length of 1.5k of the copolymer is neither crystallizable nor entangled to the carrier, its presence would reduce the overall copolymer affinity to water, that makes it more stable (less leachable) in the blends. The similar amount of copolymer leaching from different film thickness certainly supports a more stable polymer blend system, as compared to a film-thickness-dependant PEG leaching in the PCL/PEG blends. Miscibility between the PCL(10k)-PEG(5k) and PCL appears greater as suggested by the DSC and XRD data. The longer PCL block of the copolymer is believed to form chain entanglement with the PCL continuous matrix and/or co-crystallize with the PCL homopolymer. The PCL block of the copolymer functioned as an anchor, anchoring onto the matrix, thus preventing the leaching of copolymer from the blends. This explains the minimal protein burst effect in the PCL(10k)-PEG(5k) blends.

From Chapter 5, extensive protein-PEG colocalization in the PCL/PEG blends has been confirmed. Hence, it is reasonable to anticipate a certain degree of protein partitioning into the PEG-segment of copolymer blends. As expected, a high degree of protein-copolymer colocalization is quantified. In contrast to PEG blends, protein-copolymer colocalization for both PCL(1.5k)-PEG(5k) and PCL(10k)-PEG(5k) is independent of the copolymer composition. Interestingly, the degree of protein partitioning is higher than that of the PEG blends at 30% blend ratio. At present, the author does not have an explanation for this observation. It is suggested that this lesser exclusion effect of protein from copolymer domains may be affected by polymer miscibility with the PCL continuous matrix. In other words, the exclusion effect may be more manifested in a highly phase separated blends such as PCL/PEG blends compare to a less phase separated PCL/copolymer blends. This has resulted in a lower protein-PEG colocalization PEG blends and a higher protein-copolymer colocalization for copolymer blends.

From the CLSM images, it is found that the formation of core-shell-like protein droplets increased with the amount of copolymer added, while this morphology is not observed in pure PCL and PEG blend systems. Hence, some sort of protein-copolymer interaction must have caused the formation of core-shell-like morphology in the copolymer blends. From literatures, it is well-known that PCL-PEG diblock copolymer forms core-shell micellar structure in aqueous environment [120-126]. The core-shell structure of block copolymer can assemble spontaneously following placement of amphiphilic polymer structures into selective solvents [122]. In aqueous, the hydrophobic-hydrophilic diblock copolymer is especially effective, producing core-shell structures in which the hydrophobic block (PCL) collapses to generate the core, leaving the hydrophilic block (PEG) as the surrounding shell

[123, 124]. For current study, in reverse, PCL-PEG diblock copolymer was added in the PCL continuous matrix that was dissolved in organic solvent, while water was introduced into the system together with protein, forming minority aqueous-rich domains in the system. Thus, it is likely that the PCL-PEG diblock self-assembled in a way where the hydrophilic PEG-block partitioned into the protein-core, extending outward from the central core, forming a shell layer around the protein-rich domain, while the PCL-block co-crystallize and form chain entanglement with the PCL continuous matrix. The core-shell protein morphology, however, is not found in FITC conjugated copolymer blends. It is likely that the FITC conjugation has modified the properties of the PCL-PEG copolymers. Since FITC is a hydrophilic molecule, its conjugation with PCL-PEG copolymer may have also increased the overall hydrophilicity of the copolymer. When protein (in aqueous) is introduced into the FITC labelled copolymer blends, most of the hydrophilic copolymers may have partitioned into the protein-rich domains, while the effect of the PCL segment of copolymer diffusing out of the protein-rich domains has become lesser.

Protein droplets morphology in PCL film, on the other hand, shows interesting ring-type pattern. This is probably due to protein adsorption onto the PCL matrix via hydrophobic interaction. This protein-polymer contact may be responsible for protein degradation as evident in the decrease in stable protein release throughout the release time frame. As listed in the literature review Table 2.3, one of the approaches to minimize protein-polymer contact is by shielding the protein from polymer with surfactant, in this work, by adding hydrophilic PEG and PCL-PEG copolymer into the PCL continuous matrix. The effect of these blends has greatly improved protein stability during the release by ~20% and ~40% for PEG blends and copolymer blends, respectively. The success of protein shielding from PCL is also

visible by the different form of protein droplets morphology in the blends, i.e a solid filled protein droplets for PEG blends and core-shell pattern for copolymer blends. Although the protein stability data for PCL(10k)-PEG(5k) is not available, it is reasonable to assume an improved protein stability in comparison to pure PCL formulation by its similar core-shell protein droplets morphology as PCL(1.5k)-PEG(5k) blends.

It should be noted that the proposed PCL-PEG chain arrangement is not conclusive at the moment. In addition, it is not clear if a small proportion of the PEG chain close to the junctions of the PCL-block were brought into the PCL continuous matrix. Despite the uncertainty of copolymer chain arrangement, it is certain that the hydrophobic PCL-block partitions into the homo PCL matrix, especially for PCL(10k)-PEG(5k) blends. The higher block fraction of PCL enabled the blend system to be more stable by the chain entanglement formed with the homo PCL, preventing copolymer leaching, which subsequently minimized the protein burst effect. Since the copolymer is non-leachable from the blends, when water penetrates into the film during the release study, the hydrophilic PEG block swells upon contacting with the water, which then increased the diffusivity of protein molecule and resulted in a high subsequent protein release. Thus, a low-burst-high-subsequent protein release delivery system can be achieved by careful adjustment of the PCL-PEG block fraction and copolymer loading in the polymer blends.

Chapter 7. Conclusions and Recommendations

7.1 Conclusions

In this section, conclusions from each result chapters will be mentioned first, followed by final conclusions drawn from all research works.

7.1.1 Formulations and fabrications screening

Protein release from PLGA 53/47 films exhibited 3 phases in the release profile: an initial burst release followed by a plateau over 20 days, a second burst due to mass erosion and an additional spurt after day 50 with an overall incomplete protein release. The additional spurt after day 50 was governed by protein-carboxylic end group interaction (due to relatively rapid degradation of the PLGA matrix), which was not favorable from protein stability point of view. Hence, this polymer formulation was not considered for further studies.

Protein release from PCL matrix fabricated by solid dispersion and emulsion methods exhibited similar release profiles. Both showed an initial burst release, with a subsequent slower diffusional release (no second and third spurt was observed). The addition of PEG in the polymer system substantially increased the overall protein release in films fabricated by the emulsion method. However, the addition of PEG did not successfully modify the protein release profile for the solid dispersion fabrication method due to protein precipitation at the film surface. The lack of protein release profile controllability/tailor-ability in solid

dispersion formulations is thus pointed to an unfavorable protein loading technique for a protein delivery system, although this technique appeared to be more attractive to maintain protein stability. Therefore, only PCL/PEG formulations fabricated by emulsion method were considered for in-depth studies of protein release mechanisms.

7.1.2 PCL/PEG blends

Polymer miscibility of PCL/PEG blends was analyzed using DSC and XRD. The results showed distinctive melting endotherms and diffraction peaks of PCL and PEG, which confirmed phase separated crystalline domains between PCL and PEG. The distribution of protein and PEG in PCL/PEG blends was visualized by confocal Raman and CLSM. From both imaging methods, PEG segregation was observed in all of the PCL/PEG blends, in agreement with the DSC and XRD results. More importantly, protein was found to partition preferentially into the hydrophilic PEG domains. Quantification of protein-PEG colocalization by the novel image processing technique showed extensive protein-PEG colocalization, with the degree of protein-PEG colocalization inversely proportional to PEG content, ranging from 65 to 94%.

In vitro protein release and PEG release showed similar profiles: an initial burst effect followed by a slower diffusional release. In addition, the magnitude of burst release for both protein and PEG increased proportionally with the amount of PEG added. A predicted protein release profile was calculated using the quantitative measurement of protein-PEG colocalization coupled to PEG leaching. This novel approach was found to be in good

agreement with the actual protein release profile, highlighting the role of protein-PEG colocalization and PEG leaching in protein transport mechanisms.

7.1.3 *PCL/copolymer blends*

PCL-PEG copolymers were explored in order to reduce the substantial leaching (and re-altered initial burst) seen with the PCL/PEG blends. Protein release profiles from PCL(1.5k)-PEG(5k) and PCL(10k)-PEG(5k) blends are very different from one another. For PCL(1.5k)-PEG(5k) blends, significant protein burst release in the first 6 hours was found, where the burst increased proportionally with copolymer content in the blends, followed by a slower subsequent release that contributed to a complete protein release. In contrast, for PCL(10k)-PEG(5k) formulations, the addition of copolymer did not substantially affect the amount of protein burst release. In fact, the amount of protein burst in the first 6 hours for formulations with or without copolymer addition was marked at the same level, followed by an increased protein subsequent release for copolymer blends. As protein release profiles from film of different thicknesses are almost identical, it can be concluded that film thickness for copolymer blends has no impact on protein release.

The difference in protein release profiles for copolymer blends are related to their different microenvironment properties, as proven by polymer miscibility, copolymer leaching and protein distribution measurements. Polymer miscibility studies by DSC and XRD confirmed immiscible blend between PCL and PCL(1.5k)-PEG(5k), while a miscible blend between PCL and PCL(10k)-PEG(5k). Copolymer leaching measurement as translated from mass loss data showed massive copolymer leaching from the blends of PCL(1.5k)-PEG(5k)

copolymer, in contrast to the non-leaching behaviour of PCL(10k)-PEG(5k) copolymer blends. Nevertheless, the leaching of copolymer is much lower than PEG leaching of the same blend ratio. Quantitative protein distribution and protein-copolymer colocalization studies via CLSM image processing confirmed a homogeneous protein distribution in both blend systems and extensive protein-copolymer colocalization. The core-shell-like protein droplets morphology observed in CLSM images is believed to be responsible in the improved protein stability by shielding the protein droplets from PCL matrix. This morphology is believed to be constructed by PEG-blocks partitioning into the protein-rich domains while the hydrophobic PCL-blocks were brought into the PCL matrix, although this is not conclusive due to the different protein droplets morphology after FITC conjugation. In spite of this, the PCL-block partitioning in the homo PCL matrix was indeed conclusive. The better miscibility in PCL(10k)-PEG(5k) blends which has contributed to its non-leaching behaviour is therefore believed to be the key factor in the greater reduction in protein burst effect.

7.1.4 Overall conclusions

The first part of the objective is to *quantitatively evaluate the microenvironment of the protein loaded polymer blends* while the second part of the objective is to *correlate the insights gained from this evaluation with protein release mechanisms to achieve a controllable protein release system*. The results obtained show conclusive evidence of the correlation between microenvironment properties (protein distribution, protein partitioning and polymer miscibility) and protein release mechanisms. The key findings: that the protein colocalizes with the PEG phase, and that the PEG is readily leaches out upon contact with

water, help to explain the protein release profile over most of its duration. In spite of homogeneous protein distribution across the film thickness, massive protein burst release can be seen in a highly leachable blend system. The novel quantification technique of protein-PEG colocalization coupled with the PEG leaching profiles has enabled a prediction of protein release profiles. The good agreement between the predicted model and the actual protein release profiles has highlighted the importance of protein partitioning and polymer leaching in understanding protein release mechanisms. From this understanding, the use of a non-leaching PCL-PEG copolymer blend is indicated for protein release optimization. The resulting low-burst-high-subsequent protein release profiles further confirmed the correlation between microenvironment properties and protein release mechanisms. In addition to protein release mechanisms, an insight into protein droplets morphology in relation to its stability has bring to light the success of protein shielding using PEG and PCL-PEG copolymer.

From the above findings, it can be concluded that the work done in this thesis has clearly met the primary goal of this research, which is *to understand protein release mechanisms from polymer blends*.

The novel contributions from this work may be summed up as follows:

1. Visualization of protein-PEG and protein-copolymer colocalization for the first time using confocal Raman and CLSM
2. Quantification of protein partitioning into the PEG and copolymer phase using a novel image processing technique of CLSM images

3. Quantification of protein distribution across film thickness using the novel image processing technique
4. Extensive characterization of the phase separation between PCL and PEG, and PCL and PCL-PEG diblock copolymers.
5. Demonstration that protein release from PCL/PEG and PCL/copolymer blends is dominated by the leaching of the low-molar mass component
6. Providing insight into methods for obtaining various controlled-release profiles for proteins from biodegradable polymer blends.
7. Visualization of protein droplets morphology in relation to the improved protein stability for the first time.

7.2 Recommendations

Based on existing findings of the factors contributed in protein release mechanisms, it will help set directions for the future work that can be conducted. Some recommendations of studies that can be done in the near future are the following:

7.2.1 Protein solubility in PEG

As discussed in Chapter 5, protein-PEG colocalization in PCL/PEG blend system is highly dependence on PEG content in the system. It is postulated that the degree of protein-PEG colocalization is affected by protein solubility in PEG. When the amount of PEG in the system has exceeded protein solubility limit, the protein would crystallize as the linear PEG chain occupy the solvent space around protein side chain. It would thus be of interest to investigate the relationship between protein crystallization and PEG content, and how the

protein crystallization affects protein droplet size. In addition, it will be interesting to investigate the solubility of various proteins (of different hydrophobicity) in PEG of various molecular weights. The protein solubility test can be conducted by dissolving protein solution of fixed concentration, in concentrated aqueous PEG solutions of various concentrations. Protein crystallization can then be observed under optical microscope.

7.2.2 Release profile tailoring

From current study, the use of PCL(10k)-PEG(5k) blends appear to provide great potential in the tailoring of protein release profiles. While having a low protein burst effect and high subsequent release, it would be interesting to investigate the effect of higher copolymer loading (40% – 60% blend ratio) on protein release. Here, the author hypothesizes that a low-burst-cum-complete-release can be achieved within 70 days of release duration for high copolymer loading formulations.

On top of this, another approach is to use PCL-PEG copolymer of different chain length. As it has been proven that PCL-block of 10,000 g/mol is enough to co-crystallize and form chain entanglement with the PCL homopolymer, it is reasonable to fix the PCL-block chain length while varying PEG-block chain length. It is postulated that the increased amount of hydrophilic PEG in the blends would also resulted in a higher subsequent protein release while at the same time having a low protein burst effect from the non-leaching behavior of the copolymer in PCL blends.

7.2.3 Protein stability in relation to protein droplets morphology

The interesting core-shell, solid filled and ring-type protein droplets morphology presented in Section 6.2.6 is believed to be related to how protein droplets interacted with polymer matrix. This interaction greatly affects protein stability in the matrix. Initial findings of protein adsorption onto the polymer matrix may be visualized as a ring-type protein droplet that gives the lowest protein stability. Conversely, a solid-filled or a core-shell protein droplet may indicate the success in protein shielding from the polymer matrix that subsequently yield improved protein stability.

At present, it is unknown whether core-shell morphology is more preferable than a solid-filled morphology in relation to protein stability. In addition, it is uncertain if different copolymer block fraction or PEG molecular weight will change the protein droplet morphology in the matrix and how will the changes affect protein stability. It will also be interesting to find out the effect of PEG or copolymer leaching on protein stability where real-time protein droplets morphology monitoring can be done.

7.2.4 In vivo studies on protein therapeutics

In this research work, lysozyme, a model protein is used to study its release mechanisms from polymer blends. From the success in protein release profile tailoring and improvement in the protein stability for PCL/copolymer blend system, it would be interesting to use this system for the delivery of protein therapeutics.

Lisy et.al. [127] have engineered a novel chimeric peptide CD-NP that represents the fusion of the 22-amino acid peptide C-type natriuretic peptide (CNP) together with the 15-amino acid linear C-terminus of *Dendroaspis* natriuretic peptide (DNP). Their studies demonstrated that CN-NP *in vitro* activates cyclic guanosine monophosphate (cGMP) and inhibits cardiac fibroblast (CF) proliferation, induced by cardiotrophin-1, which is known to be activated in heart failure and myocardial infarction. The author proposed that a CDNP loaded PCL/copolymer film that act as a biodegradable heart stent to be implanted into the artery of a pig model, for the inhibition of cardiac smooth muscle cell growth as an effect of a heart stent implantation.

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Publications

Kerh Lin Liu, Effendi Widjaja, Yingying Huang, Xu Wen Ng, Say Chye Joachim Loo, Freddy Yin Chiang Boey, Subbu S. Venkatraman, A new insight for an old system: protein-PEG colocalization in relation to protein release from PCL/PEG blends, *Molecular Pharmaceutics*, 10.1021/mp200513b.

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