SERICIN-POLYMER CONJUGATES: PREPARATION AND PHYSICOCHEMICAL CHARACTERIZATION

A Thesis Submitted to
The Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
Abdulkadir GÜL

July, 2017
İZMİR

We approve the thesis of **Abdulkadir GÜL**

Examining Committee Members:

_____

Prof. Dr. Volga BULMUŞ
Department of Bioengineering, İzmir Institute of Technology

_____

Assoc. Prof. Dr. Murat BARSBAY
Department of Chemistry, Hacettepe University

_____

Assist. Prof. Dr. Ayben TOP
Department of Chemical Engineering, İzmir Institute of Technology

27 July 2017

_____

Prof. Dr. Volga BULMUŞ
Supervisor, Department of
Bioengineering
İzmir Institute of Technology

_____

Assoc. Prof. Dr. Engin ÖZÇİVİCİ
Head of the Department of Biotechnology and Bioengineering

_____

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGMENTS

Firstly, I would like to express my endless thanks to my supervisor Prof. Dr. Volga Bulmuş for her support, direction and the most importantly the chance she has given me to work with her laboratory team. Throughout my thesis studies, I have learned so much from her and also it has been an honor and happiness to me to experiment with her.

I also would like to thank to Assist. Prof. Dr. Hadi Zareie for his academic support and giving the opportunity to work with his lab team and for experimental aids.

I would like to acknowledge Izmir Institute of Technology for financial support through BAP #2016IYTE56.

I am most grateful to my labmates; Damla Taykoz, Aykut Zelçak, Esra Aydınlıoğlu, Vildan Güven, Gürbüz Dursun and Fazilet Akay. They deserve the biggest thanks for their help and support throughout my M.Sc. study. I will always love and never forget them.

Also, I would like to thank the staff of Biotechnology and Bioengineering Research and Application Center; especially Dane Rusçuklu who helped me for SDS-PAGE studies and Evrim Balcı for her help and support throughout the experiments in BİOMER.

I am warmly thankful to my close friends Aytaç Gül, Mehmet Salih Yıldız, Mehmet Ziya Okur, Cansu Önercan and Suna Sop for sustained encouragements to be social, help and guide.

Lastly and most importantly, I thank my family for supporting me during all my education and social life. I am grateful of my father Ramazan Gül, my sister Kübra Gül, my brother Süleyman Gül and of course my dear mother Bahire Gül for their endless love, support and patience during my thesis and all life.

ABSTRACT

SERICIN-POLYMER CONJUGATES: PREPARATION AND PHYSICOCHEMICAL CHARACTERIZATION

Sericin is a protein derived from silkworm, Bombyx mori, and has several useful properties as a natural biomaterial such as antioxidant character, moisturizing ability, hydrogel forming property and most importantly immunogenic inertness. The aim of this thesis is to prepare and physicochemically characterize sericin-polymer conjugates as potential natural-synthetic hybrid biomaterials with enhanced properties for drug delivery and tissue engineering applications.

For this purpose, three polymers having the same degree of polymerization (n~42) and varying chemical nature, i.e. poly(oligoethylene glycol methacrylate), P(OEGMA) hydrophilic and neutral, poly(hydroxyethylmethacrylate) P(HEMA) less hydrophilic and neutral, and poly(dimethylaminoethyl methacrylate) P(DMAEMA) hydrophilic and cationic after quaternization, were first synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization. Each polymer was characterized via nuclear magnetic resonance (^{1}H-NMR) and gel permeation chromatography (GPC). Separately, molecular weight and isoelectric point of sericin were characterized using various techniques including Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and High-Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). Polymers were then covalently conjugated to sericin using NHS/EDC chemistry. The conjugates were characterized using SDS-PAGE, GPC and DLS (Dynamic Light Scattering). The SDS-PAGE and GPC results showed the successful preparation of the conjugates. DLS revealed that the hydrodynamic size of P(OEGMA) and P(DMAEMA) polymers and their conjugates were between 1 and 10 nm as they are soluble in PBS and do not form aggregates. Unlike the other two polymers, although the size of P(HEMA) polymer was observed to be 3.24 ± 0.62 nm, the DLS measurements of P(HEMA) conjugates indicated the presence of self-organization and aggregation of Sericin-P(HEMA) conjugates in aqeuous solution. Consequently, the size of sericin-P(HEMA) conjugates were found to be 530 ± 60.83 and 223.3 ± 25.2, respectively.

.

ÖZET

SERİSİN-POLİMER KONJUGATLARI: HAZIRLANMASI VE FİZİKOKİMYASAL KARAKTERİZASYONU

Serisin, ipekböceği olan Bombyx mori'den türetilen bir proteindir ve doğal bir biyomalzeme olarak antioksidan karakter, nemlendirme kabiliyeti, hidrojel oluşturma özelliği ve en önemlisi immünojenik inertlik gibi birçok yararlı özelliğe sahiptir. Bu tezin amacı, ilaç taşınımı ve doku mühendisliği uygulamaları için geliştirilmiş özelliklere sahip potansiyel doğal-sentetik hibrid biyomalzemeler olarak serisin-polimer konjugatlarını hazırlamak ve fizikokimyasal olarak karakterize etmektir.

Bu amaçla, aynı polimerizasyon derecesine (n ~ 42) ve değişen kimyasal yapıya sahip, yani poli (oligoetilen glikol metakrilat), P(OEGMA) hidrofilik ve nötr, poli (hidroksietilmetakrilat) P(HEMA) daha az hidrofilik ve nötr ve (dimetilaminoetil metakrilat) P(DMAEMA) kuaternize olduktan sonra hidrofilik ve katyonik olan üç polimer, ilk olarak tersinir katılma ayrışma zincir transfer (RAFT) polimerizayonu ile sentezlendi. Her polimer nükleer manyetik rezonans (^{1}H-NMR) ve jel geçirgenlik kromatografisi (GPC) ile karakterize edildi. Ayrı ayrı serisinin molekül ağırlığı ve izoelektrik noktası, Sodyum Dodesil Sülfat-Poliakrilamid Jel Elektroforez (SDS-PAGE) ve Yüksek-Çözünürlüklü İki-Boyutlu Poliakrilamid Jel Elektroforezi (2D-PAGE) olmak üzere çeşitli teknikler kullanılarak karakterize edildi. Polimerler, NHS/EDC kimyası kullanılarak serisine kovalent bağlı olarak konjuge edildi. Konjugatlar, SDS-PAGE, GPC ve DLS (Dinamik Işık Saçılması) kullanılarak karakterize edildi. SDS-PAGE ve GPC sonuçları, konjügatların başarılı bir şekilde hazırlandığını gösterdi. DLS, P(OEGMA) ve P(DMAEMA) polimerlerinin ve bunların konjügatlarının hidrodinamik boyutunun 1-10 nm arasında olduğunu ve bunların PBS'de çözünerek agregatlar oluşturmadığını göstermiştir. Diğer iki polimerden farklı olarak, P(HEMA) polimerinin boyutu 3.24 ± 0.62 nm olarak gözlemlenmesine rağmen, P(HEMA) konjügatlarının DLS ölçümleri, Sericin-P(HEMA) konjügatlarının sulu çözeltide kendiliğinden organizasyonunu ve serisin konjügatlarının çözeltide bir araya toplandığını gösterdi. Sonuç olarak serisin-P(HEMA) konjugatlarının boyutları sırasıyla 530 ± 60.83 ve 223.3 ± 25.2 olarak bulundu.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The cocoons of mulberry silk derived from Bombyx mori composes of mainly two proteins. These are fibroin and sericin. Fibroin which is insoluble in water constitutes 70-80% whole silk and sericin which is water-soluble globular protein constitutes about 20-30% whole silk (Gulrajanı, 1988). Sericin can be generated from many silkworm sources such as silk fabric, cocoons and silk waste from the silk industry (Wu et al., 2007). Sericin is a type of silk protein containing 18 kinds of amino acids including essential amino acids and 32 percent of these amino acids are formed by the serine amino acid (Vogeli et al., 1993; Shaw and Smith, 1951). In addition, 18% and 16% of sericin are composed of aspartic acid and glycine, respectively. Sericin has high content of hydroxyl amino acids (45.8%), polar amino acids (42.3%) and non-polar amino acids residues (12.2%) (Padamwar and Pawar, 2004).

Sericin has attracted great attention and some important potential application areas like cosmetics, moisturizers, antioxidants and pharmaceuticals (Sara et al., 2003; Padamwar et al., 2005). Several properties of sericin have been investigated in various fields (Das et al., 2012). These investigations have shown that sericin has important properties as a biomaterial such as hydrogel forming and excellent moisture absorbing and desorbing properties. Furthermore, sericin can play important role in wound healing because of its high serine content (Yoshii et al., 2000). Sericin contains amino acids with strong polar side groups such as hydroxyl, carboxyl, and amino group. Because of this property, sericin can be used in various coating or blending processes with natural and artificial fibers, polymers and fabrics. Through the bioconjugation of sericin with polymers, new drug delivery systems have been developed to reduce immunogenicity and enhance stability of therapeutics in the circulatory system (Mandal and Kundu, 2009) as recent studies have shown that sericin has immunogenic inertness (Aramwit et al., 2012). All these features of sericin reported in the literature have been an inspiration for the investigation of sericin-polymer conjugates.

Polymers having well-defined molecular weight and end-group have great promise in forming sericin-polymer bioconjugates. Reversible addition-fragmentation

chain transfer (RAFT) polymerization appears to be one of the most amenable techniques for synthesis of well-defined polymers with end-group fidelity. With RAFT polymerization, copolymers and homopolymers with controlled molecular weights and narrow polydispersity index (PDI) can be synthesized. Besides, another important feature of RAFT polymerization is its ability to yield polymers with designed end-group functionality, which is required for bioconjugations.

The aim of this thesis is to prepare sericin-polymer conjugates and perform preliminary physicochemical characterizations to provide a preliminary basis for future explorations of the potential use of conjugates in drug delivery, tissue engineering, cosmetics, antioxidant and moisturizers. In accordance with this purpose, well-defined hydrophilic, relatively hydrophobic and charged polymer after quaternized poly(oligo(ethylene glycol) methyl ether methacrylate) (P(OEGMA)), poly(2-hydroxyethyl methacrylate) (P(HEMA)) and poly(2-dimethylamino ethyl methacrylate) (P(DMAEMA)) with controlled molecular weight and desired end-group functionality were synthesized using RAFT polymerization.

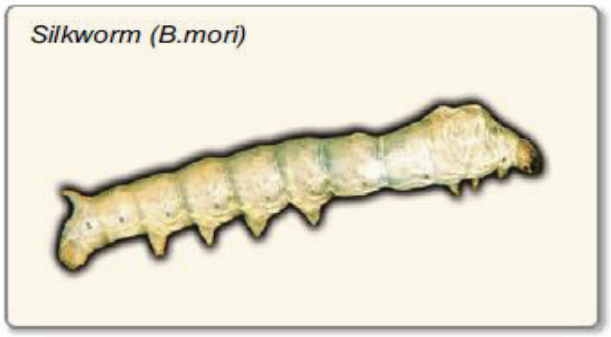
Characterizations of polymers were performed by NMR and GPC. Separately, sericin was characterized by SDS-PAGE and 2D electrophoresis to determine molecular weight, structure and isoelectric point. Sericin and polymers were conjugated via well-known NHS/EDC chemistry. Obtained bioconjugates were analysed by SDS-PAGE, GPC and DLS to verify the formation of conjugates. Literature review, experimental methods and the results and discussion are presented in Chapter 2, Chapter 3 and Chapter 4, respectively.

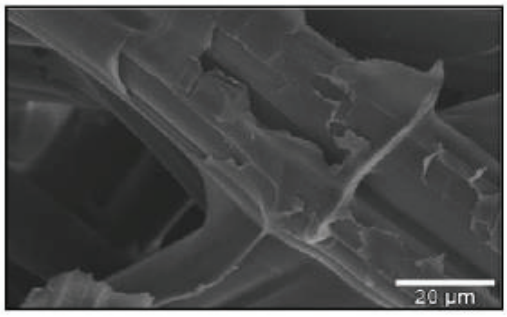
CHAPTER 2

LITERATURE REVIEW

2.1. General Properties of Sericin

In recent years, the natural silk-derived proteins have gained great importance especially for biomedical applications. In this context, silk derived from Bombyx mori (Figure 2.1) is one of the most remarkable. It consists of substantially two proteins. These are fibroin and sericin.





Electron micrograph of silkworm

Figure 2.1. Examples of silk fibers produced by silkworms and a schematic illustration (Source: John et al., 2008)

While 25-30% of silk protein is composed of sericin, fibroin and other materials form the remaining 70-75% (Table 2.1) (Rui, 1998). Sericin is on the outer layer of cocoons and it encloses the fibroin fibers (Zhang, 2002).

Table 2.1. Composition of Silk

(Source: Rui, 1998)

Component	%
Fibroin	70-80
Sericin	20-30
Carbohydrates	1.2-1.6
Inorganic matter	0.7
Wax matter	0.4-0.8
Pigment	0.2
Total	100

Today, the cocoon production in the world is about 1 million tons, most of it (400,000) is dry cocoon. 50,000 tons of sericin can be obtained from this amount. As a result, if this big amount that goes to waste every year is recycled, it can be used in many areas such as medical biomaterials, cosmetics and medical textiles etc. (Zhang, 2002) which would be very useful and beneficial in terms of the economy and the environment.

Because of the fact that sericin is a large protein, its molecular weight after purification from cocoons varies from about 10 to 300 kDa. The reason for this situation ows to the fact that the structure, hence the molecular weight of sericin is affected by factors like processing pH, temperature and time. Sericin mainly consists of 18 kinds of amino acids and most of these amino acids have strong polar side groups such as hydroxyl, carboxyl and amino groups. Amino acids that constitute large proportion of sericin are serine (about 33%), aspartic acid (15%) and glycine (about 14%) (Zhang, 2002). Table 2.2 shows the amino acid percentages in sericin and fibroin (Aramwit et al., 2010b; Genc et al., 2009).

Sericin is a water-soluble globular protein. While small sericin peptides can be soluble in cold water, the larger sericin peptides are soluble in hot water. The solubility profile of sericin peptides determines the usage areas of sericin (Zhang, 2002). Nevertheless, sericin is a hydrophilic protein and because of the fact that it has high content of serine and aspartic acid residues, it has high hydrophilicity (Garel et al., 1997).

Table 2.2. Amino acid composition of Sericin and Fibroin
(Sources: Aramwit et al., 2010b; Genc et al., 2009)

Symbol	Amino acid	Fibroin	Sericin
G	Glycine	45	14
A	Alanine	29	5
S	Serine	12	33
Y	Tyrosine	5	3
V	Valine	2	3
D	Aspartic acid	1	15
R	Arginine	1	3
E	Glutamic acid	1	8
I	Isoleucine	1	1
L	Leucine	1	1
F	Phenylalanine	1	1
T	Threonine	1	8
C	Cystine	0	0
H	Histidine	0	1
K	Lysine	0	4
M	Methionine	0	0
P	Proline	0	1
C	Tryptophan	0	0

2.1.1. Degumming Process of Sericin

Sericin can be recovered with several methods through the so-called degumming process of cocoons. Although there are a number of methods for degumming process, all of them have some advantages and disadvantages. For example, processes using heat or heat under pressure have an advantage because of the fact that they lead to no impurity.

However, since the treatment is too long and it induces significant fibroin damage, these methods are not preferred (Freddi et al., 2003).

Many degumming techniques have environmental issues. For example, ethanol can successfully precipitate sericin from silk wastewater and increase of ethanol concentration can augment product yield (Wu et al., 2007). However, when applied on an industrial scale, this technique was found to be harmful to the environment. In addition to this technique, various other techniques have been used for degumming process of sericin such as enzymatic hydrolysis (Vaithanomsat and Kitpreechavanich, 2008), freeze- and tray-drying (Vaithanomsat and Kitpreechavanich, 2008) and membrane filtration (Capar et al., 2008; Fabiani et al., 1996; Vaithanomsat and Kitpreechavanich, 2008).

Figure 2.2 shows the schematic of a degumming process to isolate fibroin and sericin from silk cocoons. Degumming process of silk cocoons is important in terms of properties of the final product. Each method results in products with different amino acid composition and molecular weight (Aramwit et al., 2010a). For example, while heat and acid extraction give sericin product having a molecular weight of 35-150 kDa, extraction with an alkali solution yields products with molecular weight of 15-75 kDa. As a result, the degumming method influences the properties of the final product such as molecular weight, isoelectric point, and amino acid sequence.

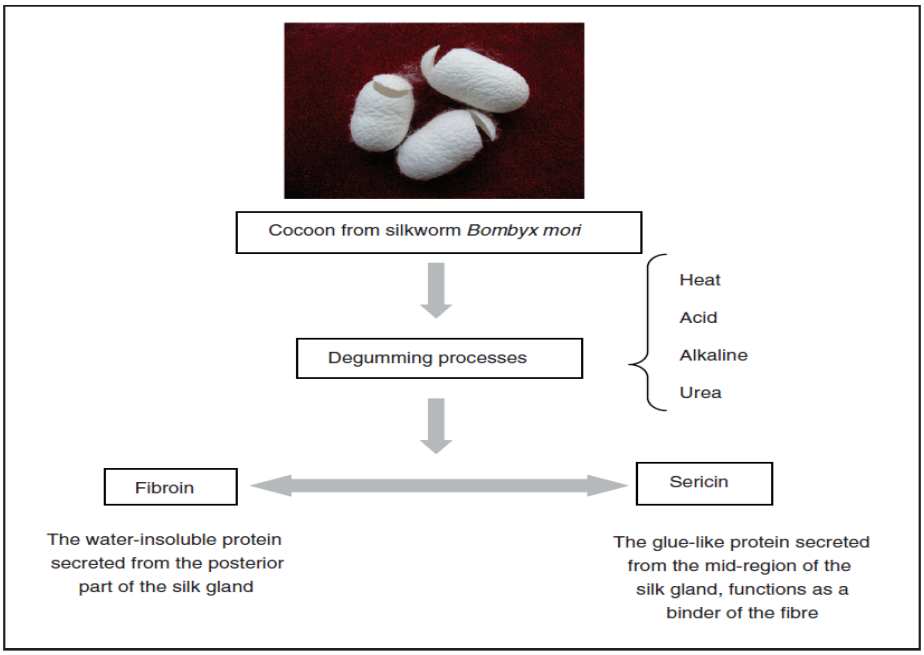


Figure 2.2. The schematic of a degumming process to isolate fibroin and sericin from silk worm cocoons (Source: Aramwit et al., 2012)

2.1.2. Applications and Properties of Sericin

Sericin has attracted increasing attention due to some properties such as moisture absorption and dispensation properties (Patel and Modasiya, 2011), UV resistance (Tamada et al., 2004), anticoagulant (Sarovart et al., 2003), antioxidant and anti-bacterial activity (Aramwit et al., 2010a) and inhibitory action of tyrosinase (Zhang, 2002). These properties of sericin are summarized in Figure 2.3. All these properties have made sericin an important potential biomaterial.

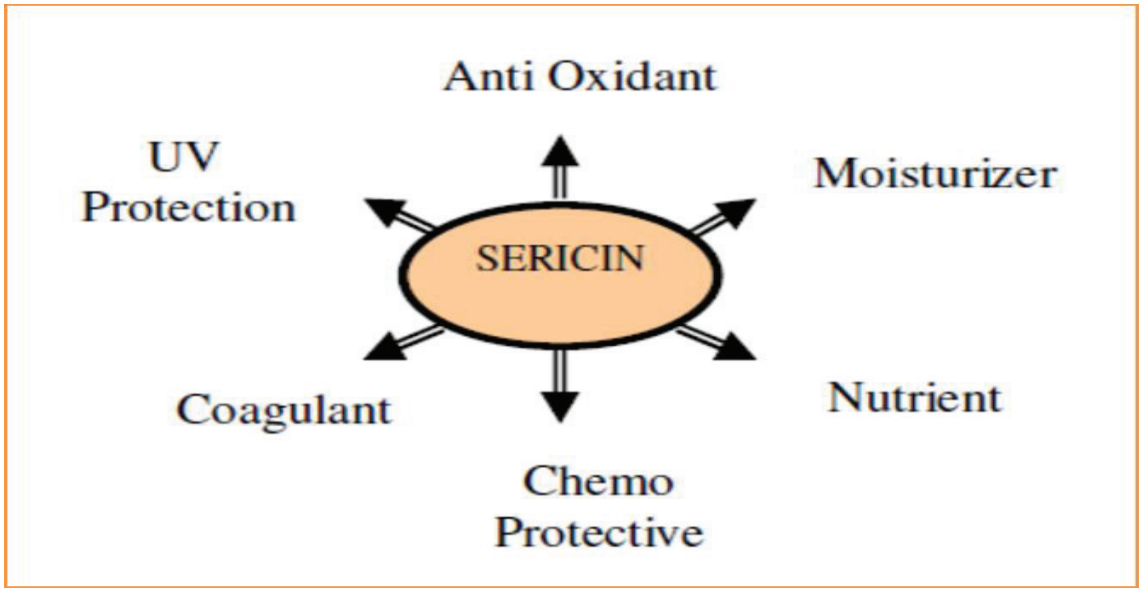


Figure 2.3. Diagrammatic representation of properties of sericin
(Source: Mondal et al., 2007)

Recent studies have shown that sericin can be used in many fields such as biomaterials (Padamwar et al., 2005), coating (Yamada et al., 2001), cosmetics (Hoppe et al., 1984; Yamada et al., 1993), food industry, films (Minoura et al., 1995; Zhaorigetu et al., 2001), moisture absorbents (Yamada and Fuwa, 1994), biomedical (Terada et al., 2005), bioconjugates (Madal and Kundu, 2009) and wound healing activity (Wu at al., 1996). In addition to these applications, one of the most important features of sericin is to reduce immunogenicity (Panilaitis et al., 2003) and increase drug stability in the circulatory system (Kundu et al., 2008).

2.1.3. Immunological Responses to Silk Sericin

One of the most important criteria for the construction of any biomaterial is its interaction with the immune system (Panilaitis et al., 2003), i.e. a critical factor determining biocompatibility of a material. This is especially important in drug delivery, tissue engineering and regenerative medicine applications (Brown and Badylak, 2013). Immune response is important because inborn immune system is the first block to any foreign material in the body and determines the reaction of the body to foreign matter (Franz et al., 2011).

Panilaitis et al. demonstrated that water-soluble sericin obtained from degumming process of natural silk has no immunological response and considerable macrophage activation (Panilaitis et al., 2003). Later it was determined that sericin promoted inflammatory mediators and proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). This increase caused the modulation of skin growth, repair and scarring (Aramwit et al., 2009). However, the maximum levels of TNF-α and IL-1b, after induction of the sericin, that released from monocyte and macrophage cells were 500 and 350 pg mL^{-1}, respectively. These cytokines levels did not cause an inflammatory response nor would it prevent cellular proliferation (Cosgrove et al., 2008; Khan et al., 2006; Wilusz et al., 2008), and these levels decreased considerably during wound healing.

As a result, researches have showed that sericin has no immunological response in the body for the drug delivery systems. This feature makes sericin a suitable material for drug delivery systems and biomedical applications.

2.1.4. Biomaterial Applications

Since sericin is inexpensive, readily available, biodegradable and biocompatible, with high serine content, water-soluble and highly hydrophilic, bioactive, antioxidant, anti-elastase, anti-tyrosinase, moisturizing and mitogenic, it has been investigated in various biomedical applications as a biomaterial (Figure 2.4). Also, an important feature of sericin is that it improves cell proliferation in serum-free media, suggesting its use in cell culturing (Table 2.3).

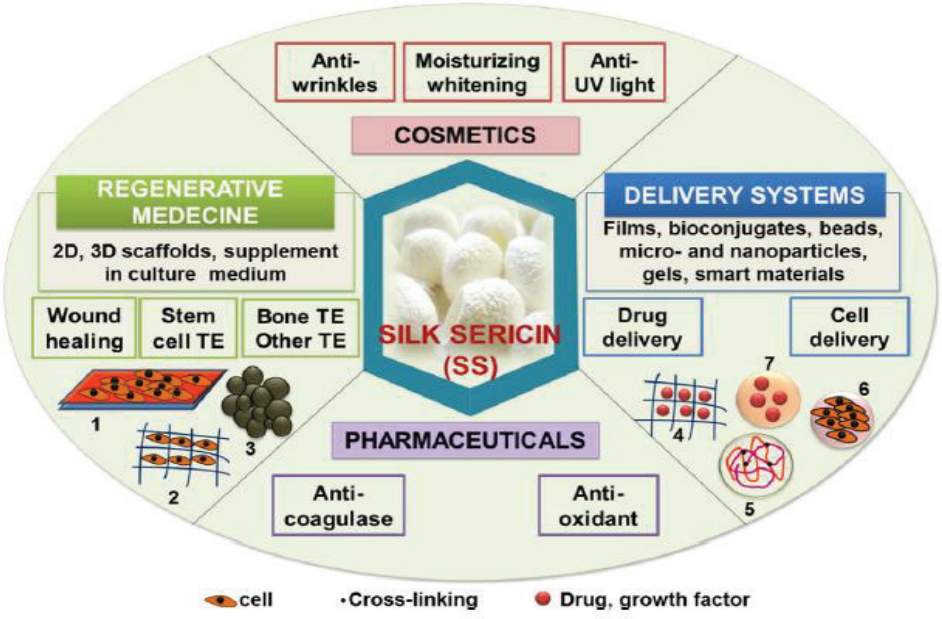


Figure 2.4. Biomedical applications of sericin (TE = tissue engineering Biomedical applications of silk sericin (SS). TE = tissue engineering; 1 = coating with SS; 2 = cell scaffolding; 3=SS-induced hydroxyapatite nucleation; 4 = drug loading and controlled release; 5 = stabilization of bioactive molecules by conjugation; 6 = SS particles for targeted cell delivery; 7 = SS particles or gels for targeted drug delivery) (Source: Lamboni et al., 2015)

Table 2.3. Sericin properties important for biomedical applications

Comments	Authors
Stable scaffolds; good cell attachment and proliferation; reduced expression of gelatinase A	Nayak and Kundu (2014)
Good cytocompatibility, high porosity and swellability	Mandal et al. (2009)
SS induced cell migration and proliferation in cultures; accelerated wound healing in second degree burns	Aramwit et al. (2013)
Injectable in situ forming hydrogel for cell scaffolding and tissue regeneration, pH-responsive	Kundu and Kundu (2012)
Improve serum-free mammalian cell culture	Terada et al. (2005)
Enhance wound healing by sericin cream	Aramwit and Sangcakul (2007)

Sericin can be used as antioxidant material because of suppressing in vitro lipid peroxidation. Besides, it inhibits tyrosinase activity, which is an important feature for food industry and cosmetics. It has also forming ability. During gel formation mechanism α-random coil structure of sericin is converted to β-sheet structure (Kato et al., 1998). If sericin gets solubilized in water with time, gelation speed increases at room temperature (Jun et al, 1997). In brief, Table 2.4 shows exploitation of sericin for variety of biomaterials applications.

Table 2.4. Exploitation of sericin properties as a biomaterial

Material	Advantage	Authors
Improving by the cross-linked of protein Dimethylolurea	High strength and water permeability cross-linked sericin membranes	Gimenes et al. (2007)
Genipin	Good mechanical properties and sericin can be released efficiently for promoting collagen production	Aramwit et al. (2010c)
SS-poly(vinyl alcohol) scaffolds, EtOH-treated	Enhanced scaffold stability, cell attachment and proliferation, collagen III production, and acceleration of wound healing in vivo	Siritienthong et al. (2012), Siritientong et al. (2014)
A blended hydrogel made of sericin and fibroin and PVA	Excellent moisture absorbing and desorbing properties and elasticity	Pushpa et al. (2013)
IPN hydrogel prepared from alginate and sericin	Enhancing mechanical strength improving degradation kinetics	Zhang et al. (2015)

2.1.5. Bioconjugates

Bioconjugation with natural or synthetic polymers facilitates the transport of drugs, such as peptides, enzymes and oligonucleotides (Veronese and Morpurgo, 1999). Because of the fact that it reduces immunological response and reduces stability, bioconjugation (such as polymer-protein conjugations) is highly advantageous. Polymer conjugation with drugs enhances tumor targeting through enhanced permeability and retention (Vicent and Duncan, 2006). Several researchers have used sericin as a natural

polymer for bioconjugation with therapeutic proteins, enzymes and polysaccharides (Zhang et al., 2004; Zhang et al., 2006a; Zhang et al., 2006b; Anghileri et al., 2007).

Sericin can be conjugated with polymers via its –OH, -COOH and $–NH_2$ active groups on its surface (Kundu et al., 2008). Polymers such as polyethylene glycol (PEG), polyethylene oxide (PEO), hydroxymethyl propyl cellulose (HPMC) and others may be used in this manner. The success achieved with the application of sericin is due to its hydrophilic nature with its low antigenicity and immunogenic properties (Panilaitis et al., 2003) and increasing the period of retention by the kidneys (Vicent and Duncan, 2006). Sericin-polymer conjugates lead to new potential drug delivery systems with increased drug stability and reduced immunogenicity (Mandal and Kundu, 2009) (Table 2.5).

Table 2.5. Sericin for conjugation of proteins and drug
(Source: Kundu et al., 2008)

Polymer-protein/drug conjugates	Properties	Comment	Authors
Sericin-L-asparaginase	Lower *K*m value, higher thermostability, prolonged half-life period, and reduced immunological response	Against acute lymphoblastic leukemia	Zhang et al. 2004; Zhang et al. 2006a
Sericin-β-glucosidase	Stable on re-use and storage, retains >86% activity after 30 days	Glucose sensor	Zhang 2002
Sericin–insulin	Bioavailability, increased half-life, and reduced immunogenicity	Control blood sugar level	Zhang et al. 2006b

Sericin's surface can be modified by grafting vinyl monomers. This strategy allows overcoming some obstacles of sericin such as instability in hot water, poor solubility in organic solvents and low resistance to microbial attacks (Nakamura and Hatakeyama, 1984; Wei et al., 1989; Yao et al., 2003).

2.1.6. Cosmetics

Sericin has high content of serine (about 33%) and glycine (about 14%) so it can be used as a moisturizer in the cosmetic industry (Kato et al., 1998). This is achieved by preventing transepidermal water loss and restoring natural moisturizing factors (Voegeli et al. 1993). Also, it has both anti-wrinkle and anti-aging effects because of its collagen promoting activity (Aramwit and Sangcakul, 2007; Ogawa and Yamada, 1999; Voegeli et al., 1993; Yamada et al., 1998). It reduces damage of hair surface by binding hair if 0.02-2% of sericin is contained in hair products along with other substances (Hoppe et al., 1984). Another feature of sericin is that it has wound-healing properties in cosmetic industry (Wu et al., 1996). This feature can be owing to the fact that sericin may give rise to target cells responsible for wound healing such as fibroblasts and myofibroblasts (Hino et al.,2006; Padol et al., 2012).

2.2. Protein-Polymer Conjugates

Protein-polymer conjugates have been widely used in various areas such as medicine, biotechnology and nanotechnology. New structures formed by combination of proteins and polymers have some advantages including high protein stability, solubility, and biocompatibility (Caliceti and Veronese, 2003; Vandermeulen and Klok, 2004).

In the mid-1970s, works in the field of polymer bioconjugates first appeared and in this sense, polymers were conjugated with enzymes to enhance their heat stability (Epton et al., 1977). Also, Davis and Abuchowski uncovered polyethylene glycol (PEG) constructs that do not cause immunological side effects and developed protein-PEG constructs called PEGylation that prevented the recognition of proteins by the immune system and allowed them to stay in the circulatory system for a long time (Abuchowski et al., 1977).

Subsequent studies investigated the conjugation of various enzymes and other proteins in more detail with stimulant-responsive polymers and other water-soluble polymers (Van-Dijk-Wolthuis et al., 1999; Veronese, 2001). The potential of enzyme / protein-polymer conjugates has begun to be explored in many areas such as bioseparations, biological reactions, diagnostics and drug delivery.

2.3. Other Conjugates of Polymers with Biofunctional Molecules

There are many biomolecules and biofunctional molecules, other than proteins and peptides, that can be conjugated to polymers such as sugars and polysaccharides, single and double-stranded oligonucleotides and DNA plasmids, simple lipids and phospholipids, and a wide spectrum of recognition ligands and synthetic drug molecules (Figure 2.5).

The conjugation of a synthetic polymer and a biomolecule produces a new, hybrid molecule species that can synergistically combine the individual properties of the two components to achieve novel and unusual properties. It can be said that such bioconjugates are "double smart". Polymer-drug (Fuertges and Abuchowski, 1990) conjugates and polymer-protein (Hoffman, 1998) conjugates are among the most important polymer-biomolecule or polymer-biofunctional molecule conjugates. Medicine and biotechnology have had a number of successful applications for such smart polymer-biomolecule systems, and therefore represent a significant extension of polymeric biomaterials beyond their known use in implants and medical devices.

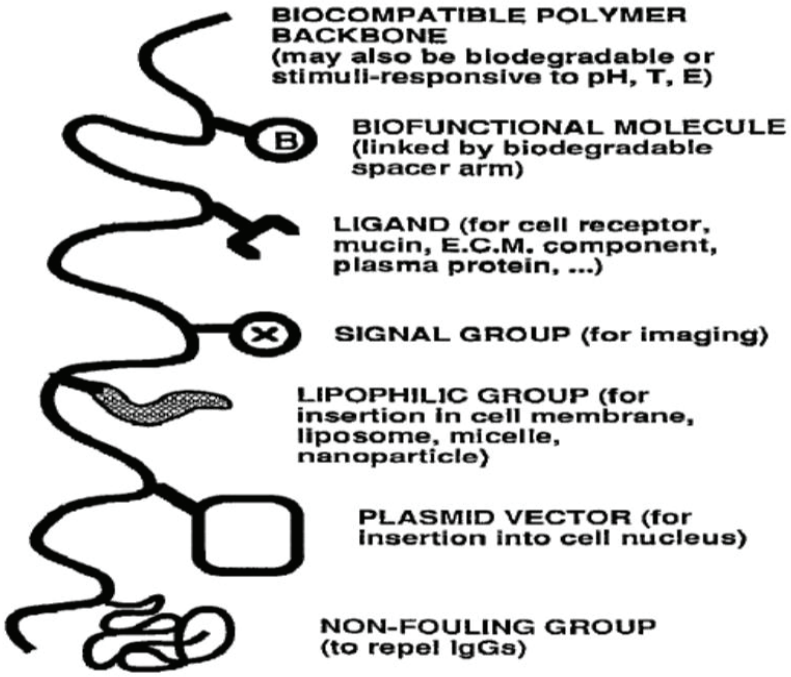


Figure 2.5. Schematic representation of the diversity of natural or synthetic biomolecules that can be conjugated with a polymer (Source: Hoffman, 1995)

In some cases, only one molecule such as a recognition protein capable of binding to a reactive terminal group of the polymer can be conjugated, or it may be linked at a reactive pendant group along the polymer backbone. In other cases, multiple molecules can be conjugated along the polymer backbone, such as a targeting ligand multiple drug molecules as well as multiple drug molecules (Source: Hoffman, 1995).

2.4. Synthesis of Biomolecule-Polymer Conjugates

There are three basic methods available for obtaining protein-polymer conjugates (Thordarson et al., 2006; Grover and Maynard, 2011) (Fig. 2.6). In the first of these methods, a preformed polymer is directly conjugated with a protein. The name of this method is the grafting-to approach and provided either by covalent attachment to the amino acid side chain of a reactive functional group of the polymer, or vice versa, or through a ligand-apoprotein interaction. In the latter case, a cofactor or ligand is covalently attached to a polymer chain. In grafting to method, usually an α, ω-telechelic polymer which permits conjugation with protein or polypeptide at one end is first synthesized (Tasdelen et al., 2011).

An alternative way to create the reactive group is the modification post-polymerization of the polymer end groups (Willcock and Reilly, 2010). This method is an indirect protein-polymer conjugation. In grafting-from approach, a moiety capable of mediating or initiating a polymerization process is introduced into an amino acid side chain of protein or peptide. As a result, a macro-initiator is obtained and the polymer chain is directly amplified from the protein.

The third path follows the grafting-through approach in which various protein reactive groups are incorporated by the use of monomers in a growing polymer chain. These monomers may react either directly or after polymerization, by means of reactive moieties introduced with peptides or proteins (Stukel et al., 2010). Remarkably, this third approach does not cause conjugation of a protein / peptide to a polymer chain, but results in the conjugation of many proteins / peptides to a polymer chain. For example, PEG conjugates have been made by the grafting to method since PEG has the lack of side chains and the absence of the possibility of propagation of the PEG chain from a macro protein initiator through an ionic polymerisation mechanism.

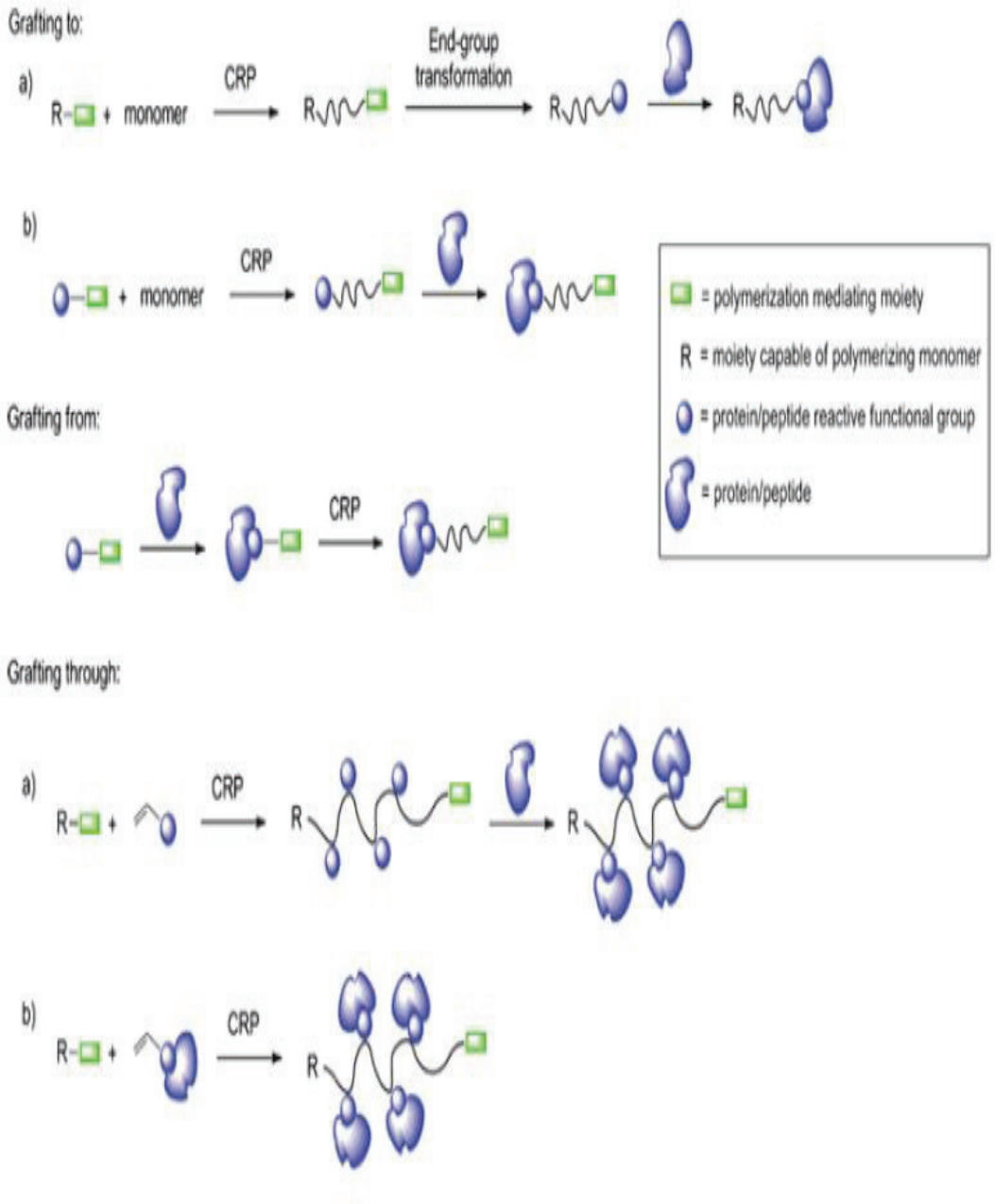


Figure 2.6. Combination strategies for protein-polymer conjugate
(Source: Broyer et al., 2011)

On the contrary, polymers obtained by radical polymerization are suitable for possible chemical modifications (Nicolas et al., 2007). Controlled radical polymerization processes used in this field is reversible addition-fragmentation chain transfer (RAFT) polymerization (Barner-Kowollik, 2008; Moad et al., 2005; Favier and Charreyre, 2006), atom transfer radical polymerization (ATRP) (Patten and Matyjaszewski, 1998) and

nitroxide mediated polymerization (NMP) (Sciannamea et al., 2008). All of these methods lead to polymers having low polydispersity and a predetermined molecular weight, and most importantly selective conjugation through the end groups allows obtaining well-defined protein-polymer conjugates.

In addition, RAFT and ATRP find out the possibility of forming a grafting-from approach by conjugating both chain transfer agents and initiator systems to proteins / peptides, respectively. Other advantages of RAFT and ATRP are the possibility of polymerisation in a wide range of solvents, reaction conditions and various suitable monomers, assuming that the conditions are compatible with the protein / peptide.

Proteins can either be attached to one end of a random polymer or pendant groups along the polymer backbone (Fig. 2.7). Normally, lysine amino groups are reactive sites for random polymer conjugation to proteins and N-succinimide bonding chemistry is most commonly used. Other possible attachment sites include serine or tyrosine-OH groups, -COOH groups of aspartic or glutamic acid, and -SH groups of cysteine residues.

The most probable binding site can be determined by the reactive group on the polymer and the reaction conditions, in particular by pH. Since these conjugations are usually made in a non-specific manner, they can interfere sterically with the protein's active site, or alter the microenvironment, and the biological activity of the protein generally decreases (Ding et al., 1998).

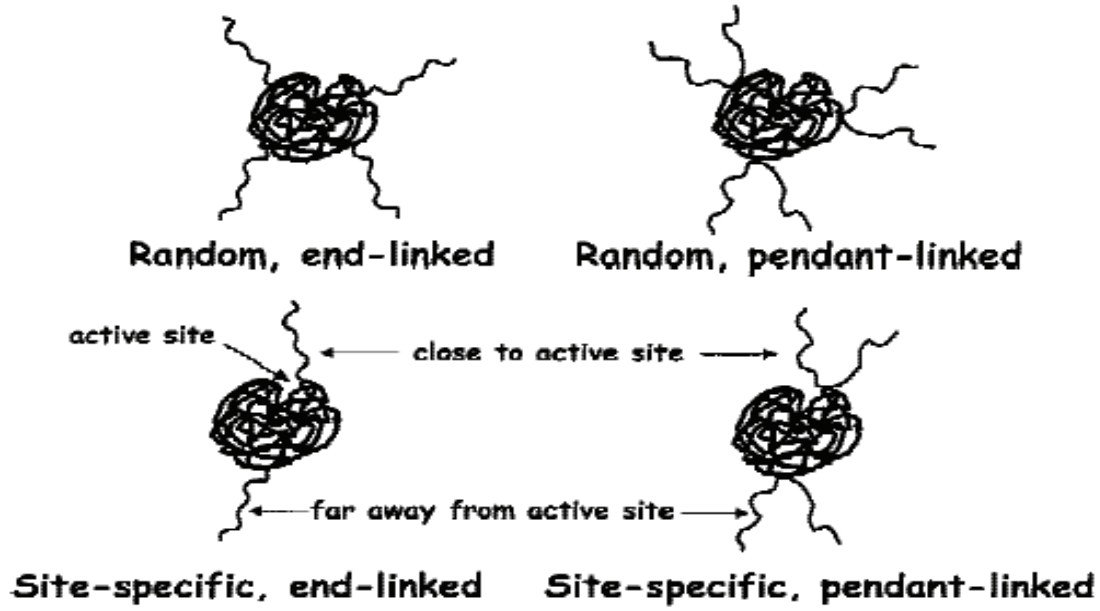


Figure 2.7. Various types of random and site-specific smart polymer-protein conjugates. In the latter case, it is desirable that binding near the active site of the protein results in stimulus control of the recognition process for the protein ligand, while conjugation far away from the active site should avoid any interference of the polymer with the protein's natural activity (Source: Chilkoti et al., 1994; Stayton et al., 1995)

It is however possible to conjugate polymers to a specific site on certain proteins by adding a specific reactive amino acid such as a cysteine with a reactive -SH thiol group (Fig. 2.8). This is genetically engineered into the DNA sequence of the protein of a region-specific mutation protein, and then cloning the mutant in cell culture. Hence, this method is applicable only to proteins whose complete peptide sequence is known. The preparation of the reactive polymer is similar to the method described above, but now the reactive terminal or side groups and reaction conditions are specially designed to favor conjugation to -SH groups instead of $-NH_2$ groups. Typical groups of mercaptyl reactive polymers include maleimide and vinyl sulphone groups.

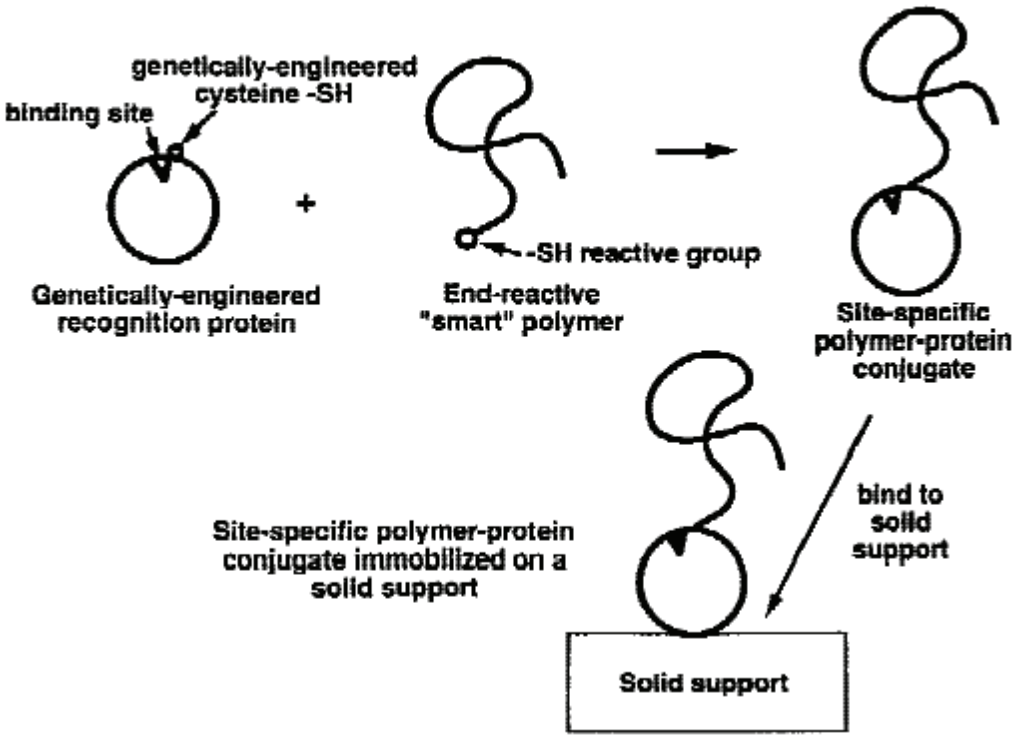


Figure 2.8. Schematic expression of the process for preparing a site-specific conjugate of a smart polymer with a genetically engineered mutant protein (Source: Chilkoti et al., 1994; Stayton et al., 1995)

2.4.1. EDC/sulfo-NHS-Mediated Bioconjugation

EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride], one of the most widely used carbodiimides in bioconjugation processes, is a water-soluble molecule and it catalyses the formation of amide bonds between the carboxy and amine groups. The reason for its widespread use is due to its high water solubility and the easy removal of by-products. In addition, sulfo-NHS (N-hydroxy sulphosuccinimide) is utilized to enhance the stability of active intermediates in coupling reactions by formation of active ester functional groups with carboxylates (Figure 2.9).

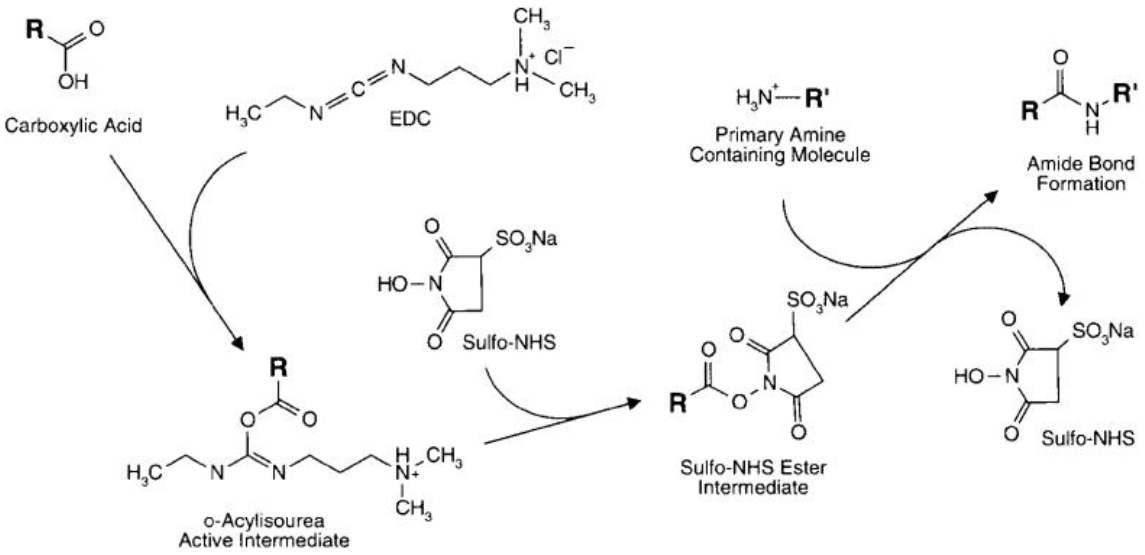


Figure 2.9. The EDC catalyzes the formation of amide bonds between the carboxy and amine groups. The sulfo-NHS (N-hydroxy sulphosuccinimide) is utilized to enhance the stability of active intermediates in coupling reactions by formation of active ester functional groups with carboxylates (Source: Nakajima and Ikada, 1995)

A few examples on this topic are as follows: Thilakarathne et al. showed that Hemoglobin (Hb) having lysine amino groups is covalently bound to carboxyl functional groups on the hydrophilic polymer, poly(acrylic acid) (PAA) and consequently polymer-Hb nanogels which are swollen with the lightly cross-linked solvent are obtained. Cross-linked hydrophilic polymers used PAA are carbopols and tested for protein delivery (MacLean-McDavitt et al., 2003), controlled release (Muramatsu et al., 2000) and other biomedical applications. For this purpose, a 450 kDa PAA known to have reduced cytotoxicity as compared to the lower molecular weight PAA was used (Arshady, 2003). The use of high molecular weight PAA provides easy machinability compared to conventional sensing materials such as metal oxides and solid electrolytes (Adhikari and Majumdar, 2004; Ding et al., 2005). The Hb-PAA system described herein showed the biological activity and sequestering, and one of its greatest advantages is its long shelf life compared to unmodified hemoglobin.

In another study, a zwitterionic block copolymer, poly(methyl acrylate-*b*-sulphobetaine methacrylate) (PMAA-*b*-PSBMA or PMS) was designed for altering the protein uricase to study the protection of bioactivity and pH sensitive separation. The

reaction of PMAA with the conventional 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC / HCl) and N-hydroxysuccinimide (NHS) chemistry was carried out in order to increase the activity of the -COOH groups in the polymer. This was provided by an increase in the local concentration of -COOH groups caused by charge-induced adsorption to the positively charged region on the uricase. Consequently, the resulting PMS-uricase conjugates can be resuspended in a physiological solution with pH adjustment from pH 5.35 to 7.4, as well as easily separable from the reaction solution. Moreover, in anti-trypsin digestion, PMS-uricase conjugates have a better property than unmodified uricase (Nishimura et al., 1979; Ganson et al., 2006; Bomalaski et al., 2002; Sundy et al., 2007).

2.5. Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization

Reversible addition−fragmentation chain transfer (RAFT) polymerization was firstly reported in 1998 by a group at CSIRO (Commonwealth Scientific and Industrial Research Organization, Australia). This method is a living radical polymerization (LRP) technique, which minimizes termination reactions (Bulmus, 2011).

Thiocarbonylthio moiety containing chain transfer agent (RAFT agent) must be used for the realization of RAFT polymerization (Figure 2.10). RAFT agent consists of R and Z group in addition to reactive thiocarbonylthio moiety. Whereas R group in charge of initiating the growth of polymeric chains, Z group actives the thiocarbonyl bond toward radical addition and then stabilizes the resultant adduct radical in the ω-end of the polymeric chain (Bulmus, 2011).

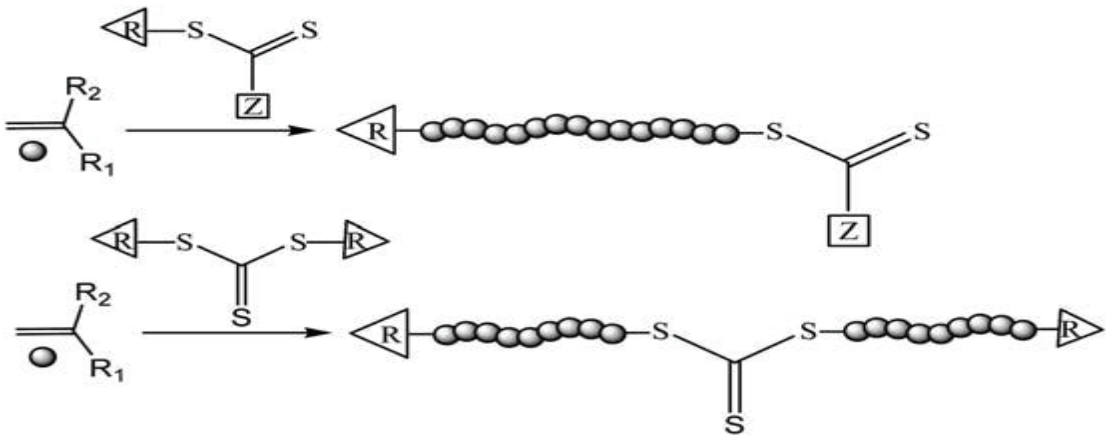


Figure 2.10. Schematic expression of polymer synthesis by RAFT process using a thiocarbonylthio or a symmetric trithiocarbonate RAFT agent (Source: Bulmus, 2011)

According to kinetics of RAFT polymerization, the theoretical molecular weight ($M_{n(theo)}$) of polymers is determined via Equation 2.1 .

$$M_{n(theo)}=\frac{[M]}{[RAFT]}xMw_{monomer}\ x\ Conv\% + Mw_{RAFT} \quad (2.1)$$

where;

[M] is the monomer concentration

[RAFT] is the RAFT agent concentration

$Mw_{Monomer}$ is the molecular weight of the monomer

Mw_{RAFT} is the molecular weight of the RAFT agent

Polymer synthesis by RAFT polymerization has many advantages. Generally, well-defined polymers can be synthesized with controlled molecular weight and low polydispersity index (PDI) using the RAFT technique. Also, high conversions and commercially acceptable polymerization rates can be obtained by RAFT polymerization. Polymers having various structures such as linear polymers, brush or comp-type, star polymers, block and graft copolymers can be obtained by RAFT polymerization (Gregory and Stenzel, 2012). Also, polymers synthesized by RAFT polymerization have defined

end-group functionality, which can be perfectly used for bioconjugations (Boyer et al., 2009).

The reason for the use of RAFT polymerization in this thesis is its suitability for the synthesis ofprotein-polymer conjugates. Due to the fact that it forms living polymers from a wide variety of functional groups and creates well-defined end-group functionality, RAFT is well suited for the synthesis of bioconjugates including protein-polymer conjugates (Falatach et al., 2015).

Generally, in order to increase the efficiency of conjugation, it is necessary to use carboxylic acid activating agents in bioconjugation reactions between carboxylic acid and amine or hydroxyl groups. Hence, N-hydroxysuccinimide (NHS) (Aqil et al., 2008; Aamer and Tew, 2007), pentafluorophenyl (PFP) (Golden, 2010) or 2-mercaptothiozalin is used to activate the carboxylic acid-terminated polymers synthesized by RAFT polymerization (Xu et al., 2009). For example, in order to conjugate to amine groups of anti-streptavidin and anti-plasmodium falciparum histidine-rich protein 2 (PfHRP2) antibodies, the carboxyl end group of semi-telechelic poly(N-isopropylacrylamide) (pNIPAAm) synthesized by RAFT polymerization using 2-ethylsulfanylthiocarbonylsulfanyl-1-2-methyl propionic acid as a carboxylic acid-bearing RAFT agent was modified with tetrafluorophenol to obtain amine reactive ester groups. These conjugates were used to capture and detect a model streptavidin antigen (Golden, 2010).

Another example of protein-polymer conjugates was reported by Falatach et al. in 2015. α-Chymotrypsin which is used as a fat protease was modified with oligomers synthesized by RAFT polymerization. N, N-dimethylacrylamide (DMAm) or oligo (ethylene oxide) methyl ether acrylate (OEOA) monomers were polymerized for this purpose. Using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide / N-hydroxysuccinimide (EDC / NHS) chemistry, the polymers were conjugated to free amine groups on chymotrypsin. The resulting conjugates retained the enzymatic activity and conjugates formed with high molecular weight DMAm and OEOA polymers significantly increased stability due to the inhibition of α-chymotrypsin autolysis of the high molecular weight polymer (Falatach et al., 2015).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Sericin Bombyx mori (silkworm) was purchased from Sigma-Aldrich. 2-Hydroxyethyl methacrylate (HEMA; Mw: 130.14 g/mol), oligo(ethylene glycol) methyl ether methacrylate (OEGMA; Mw: 467 g/mol) and 2-dimethylamino ethyl methacrylate (DMAEMA; Mw: 157.21 g/mol) monomers were purchased from Sigma-Aldrich. All monomers were de-inhibited by passing through a basic aluminum oxide column before use.

Chain transfer agent, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) was purchased from Sigma-Aldrich. The initiator, 2,2′-Azobis(2-methylpropionitrile) (AIBN) was used after recrystallization twice in methanol.

Dichloromethane (for organic trace analysis UniSolv®) was purchased from Merck while 1,4-dioxane (ACS Reagent 99% purity), hexane (anhydrous 95% purity), N,N-dimethylacetamide (DMAc) (CHROMASOLV® Plus, for HPLC, ≥99.9% purity), deuterium oxide (D_2O), deuterated chloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d6), N,N-dimethylformamide (DMF), diethylether (>99.7%), acetonitrile (>99.9) were purchased from Sigma-Aldrich.

PBS (phosphate buffer saline solution, pH 7.4, 0.1 mM) was prepared using relevant mono and dibasic salts and NaCI in order to use in conjugation procedures. These salts were purchased from Merck. Dialyses membrane (MWCO=3500 Da) was purchased from Spectrum ®Laboratories.

N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Merck and N-hydroxysulfosuccinimide (Sulfo-NHS) was purchased from Therm.

3.2. Instruments

3.2.1. Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR spectroscopy (Varian, VNMRJ 400 spectrometer) was used to determine conversion of the monomers to polymers throughout polymerizations and the chemical structure of the obtained compounds. Also, theoretical molecular weight and purity of polymers were determined by ^{1}H NMR. Deuterated dimethyl sulfoxide (d_6) or chloroform ($CDCl_3$) was used as NMR solvent. For NMR analysis, samples were dissolved at about 5-10 mg/mL concentration in NMR solvent.

3.2.2. Gel Permeation Chromatography

The molecular weight and molecular weight distribution (polydispersity index) of polymers synthesized during the study were determined using gel permeation chromatography (GPC). A Shimadzu modular system comprising an SIL-10AD auto injector, PSS Gram 30 Å and 100 Å (10 μM, 8x300 mm) columns, an RID-10A refractive-index detector and SPD- 20A prominence UV/vis detector calibrated with low polydispersity poly(methyl methacrylate) standards (410-67000g/mol) were used. The mobile phase was N, N dimethylacetamide (DMAc) containing 0,05 % w/v LiBr.

GPC samples were prepared by dissolving polymers in DMAc at 2 mg/mL concentration. Prepared samples were filtered by using 0,45 μm syringe type membrane filter before analysis.

3.2.3. DLS Analysis

DLS was used to measure the hydrodynamic radius (Rh) of samples. Malvern NanoZS Particle Analyzer was used for DLS experiments (measurement range 0.3 nm-10.0 microns; light source He-Ne laser 633 nm Max 5mW; Power 100 VA).

3.2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine molecular weight and purity of sericin and also to verify the formation of sericin-polymer conjugates. A BIO-RAD electrophoresis device was used. Proteins of known mass were also utilized to determine molecular weight of samples.

3.2.5. High-Resolution Two-Dimensional Polyacrylamide Gel Ectrophoresis (2D-PAGE)

High-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to determine sericin's iso-electric point (pI). For this purpose, aBio-Rad PROTEAN® IEF cell and ReadyStrip™ IPG strips (17 cm; pH 3-10) were utilized to run first dimension and load the sample, respectively. Also, rehydration/equilibration trays were used to load the strips with the protein samples. 12% gel was prepared to run protein sample in the second dimension. The power source for the SDS-PAGE system was provided by SDS-PAGE electrophoresis cell (PROTEAN II XL cell; 16 mA/gel for 30 min, then 24 mA/gel for 4 hr).

3.3. Methods

3.3.1. RAFT Polymerization of Oligo(ethylene glycol) Methyl Ether Methacrylate (OEGMA)

Oligo(ethylene glycol) methyl ether methacrylate (OEGMA, Mw: 467 g/mol) was polymerized via RAFT polymerization using AIBN as an initiator and 4-cyano-4 (phenylcarbonothioylthio) pentanoic acid as a RAFT agent (CPADB).

A degree of polymerization of approximately 42-43 yielding a molecular weight of 20,000 g/mol was targeted for all polymerization processes. Also low monomer conversions were targeted to ensure the end-group fidelity. In this context, [OEGMA]/[RAFT]/[AIBN] mole ratio was adjusted to be 150/1/0.2 in polymerizations. Corresponding amounts of OEGMA (1.13 M), RAFT agent (7.5×10^{-3} M) and AIBN

($1.5x10^{-3}$ M) were dissolved in acetonitrile (2.5 mL). Before polymerization, the polymerization mixture was deoxygenated by purging with nitrogen for about 15-30 minutes. The mixture was then sealed and put into an oil bath set at 65 ^{o}C for varying polymerization times between 45 minutes and 240 minutes to obtain polymer having a M_n of 20,000 g/mol. The polymer with the desired molecular weight was found to be synthesized at 90 minutes under the giving conditions. When the polymerization time is over, the reaction solution was cooled in an ice bath and exposed to air.

Polymers were purified to remove excess of monomer, CPADP and AIBN. In purification procedure, acetonitrile was first evaporated under vacuum and then polymers were precipitated in cold diethyl ether three times followed by centrifugation and then dried in vacuum oven at 40 ^{o}C overnight.

Each sample was analysed by GPC using dimethylacetamide (DMAc) as a mobile phase to determine the number average molecular weight (Mn) and molecular weight distribution (PDI) of the polymers. Chemical structure and monomer conversions were determined by ^{1}H-NMR spectroscopy using $CDCl_3$ as solvent. The polymerization scheme is given in Figure 3.1.

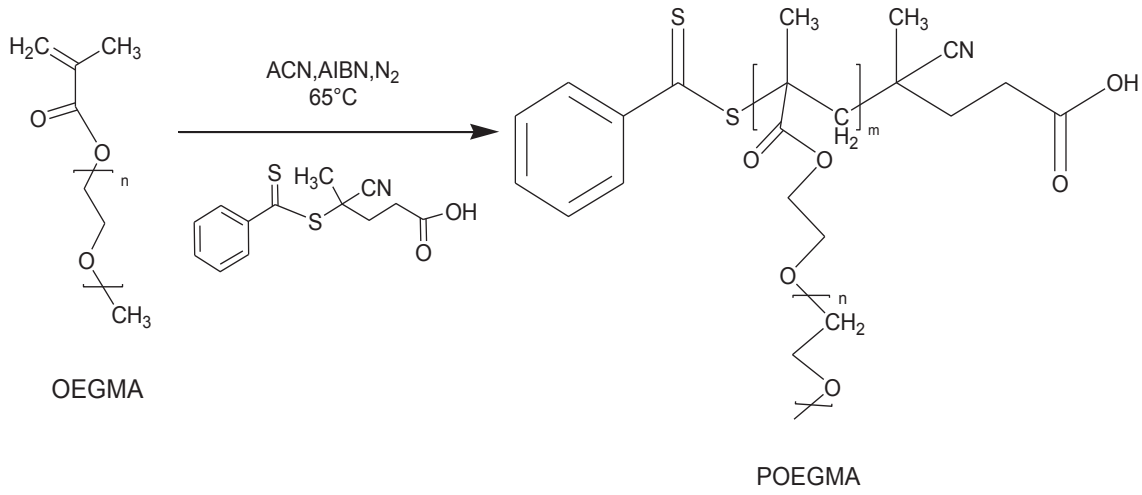


Figure 3.1. Synthesis of poly(oligoethylene glycol) methyl ether methacrylate P(OEGMA) via RAFT polymerization

3.3.2. RAFT Polymerization of 2-Hydroxyethyl Methacrylate (HEMA)

2-Hydroxyethyl methacrylate (HEMA, Mw: 130.1 g/mol) was polymerized via RAFT polymerization using AIBN as an initiator and CPADB as a RAFT agent.

The polymerization conditions were set to target a degree of polymerization of approximately 42-43 to obtain different polymers with comparable chain lengths. P(HEMA) having a degree of polymerization of 42-43 yields a polymer with a molecular weight of approx. 5500 g/mol. Accordingly, in polymerization experiments, [HEMA]/[RAFT]/[AIBN] mole ratio was adjusted as 100/1/0.2. HEMA (0.5 M, 0.33 g, 2.5 mmol), RAFT Agent (Mw: 279 g/mol, 13.8 mg, 0.025 mmol) and AIBN (Mw: 164 g/mol, 1.64 mg, 0.82 mmol) were dissolved in 1,4-dioxane (4,4 mL). Before polymerization, the mixture was purged with nitrogen for about 15-30 minutes to remove oxygen from the medium. The mixture was sealed and then put into an oil bath set at 65 ^{o}C for varying polymerization times between 60 and 210 minutes to obtain polymer having a M_n of about 5,500 g/mol. The polymer with the desired molecular weight was found to be synthesized at 150 minutes. The polymerization was terminated by cooling the reaction solution in an ice bath and exposing to air.

Polymers were purified to remove unreacted monomer, CPADP and AIBN. In purification procedure, synthesized polymers were precipitated in cold diethyl ether at least three times followed by centrifugation and then dried in vacuum oven at 40 ^{o}C overnight.

The Mn and PDI of the polymers were determined by GPC using DMAc as a mobile phase. Chemical structure and monomer conversions were determined by ^{1}H-NMR spectroscopy using DMSO-d_6 as a solvent. The polymerization scheme is given Figure 3.2.

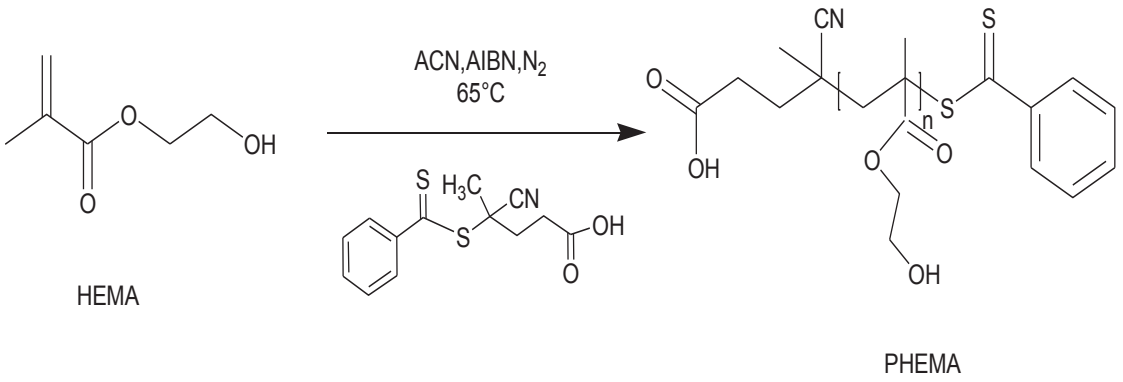


Figure 3.2. Synthesis of poly(2-hydroxyethyl methacrylate) P(HEMA) via RAFT polymerization

3.3.3. RAFT Polymerization of 2-Dimethylamino Ethyl Methacrylate (DMAEMA)

2-Dimethylamino ethyl methacrylate (DMAEMA, Mw: 157.21 g/mol) was polymerized according to the literature with some modifications (Vuoriluoto et al., 2015).

Similar to previous polymerizations, the polymerization conditions were set to target a degree of polymerization of approximately 42-43. Hence P(DMAEMA) with a molecular weight of approx. 6,600 g/mol was expected to be synthesized. Accordingly [DMAEMA]/[RAFT]/[AIBN] mole ratio was adjusted as 130/0.6/0.2. DMAEMA (1.3 M, 1.65 g, 10.5 mmol) as a monomer, CPADB ($6x10^{-3}$ M, 14.1 mg,0.51 mmol) as a RAFT agent and AIBN ($2x10^{-3}$ M, 2.5 mg, 0.015 mmol) as an initiator were dissolved in 1,4-dioxane (8 mL). Before polymerization, the mixture was sealed with a rubber septum and bubbled with nitrogen for about 15-30 minutes to remove oxygen from the medium. The mixture was put into an oil bath set at 65 ^{o}C for varying times between 165 minutes and 260 minutes. The polymer with the desired molecular weight was found to be synthesized at 260 minutes. Polymerization was stopped by immersing the solution into an ice bath and exposing it to the air.

Polymers were purified to remove unreacted monomer, CPADP and AIBN. In purification step, synthesized polymers were precipitated in hexane at least three times followed by centrifugation and then dried at 40 ^{o}C in vacuum oven overnight to yield a pure polymer.

Each sample was analysed by GPC using DMAc as a mobile phase to determine the Mn and PDI of the polymers. Chemical structure and monomer conversions were determined by ^{1}H-NMR spectroscopy using $CDCl_3$ as a solvent. The polymerization scheme is given in Figure 3.3.

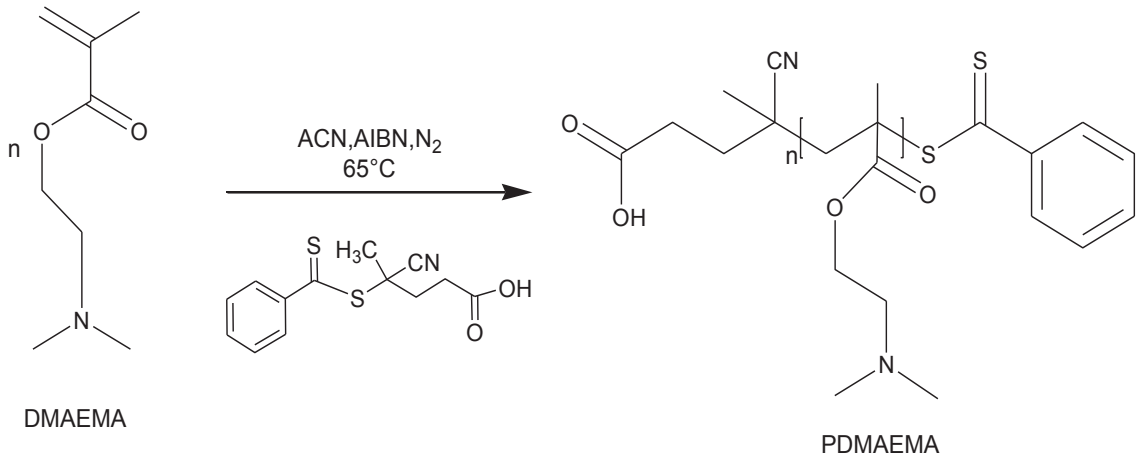


Figure 3.3. Synthesis of poly(2-dimethylamino ethyl methacrylate) P(DMAEMA) via RAFT polymerization

3.3.4. Characterization of Sericin

The characteristics of sericin purchased have not been obtained from the manufacturer. Sericin was therefore needed to be characterized before conjugation experiments. In this content, SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) and high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were performed to determine the molecular weight and isoelectric point (pI) of sericin.

3.3.4.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Sericin

SDS-PAGE was used to determine molecular weight and purity of sericin. Firstly, a separating gel (12%) was prepared by mixing 3.35 mL of distilled water, 2.5 mL of 1.5 Tris-HCI (pH 8.8), 4 mL of acrylamide/bisacrylamide (30% T, 2.67% C stock), 100 µl of 10% (w/v) SDS, 50 µl of 10% ammonium persulfate (fresh) and 5 µl of TEMED. The prepared solution was poured into the thin space between two glasses and waited for 45 min to let it gelation in the incubator. Then, the 4% stacking gel was prepared by mixing 6.1 mL of distilled water, 2.5 mL of 0.5 Tris-HCI (pH 6.6), 1.3 mL of acrylamide/bisacrylamide (30% T, 2.67% C stock), 100 µl of 10% (w/v) SDS, 50 µl of 10% ammonium persulfate (fresh) and 10 µl of TEMED and the stacking gel was cast on the top of separating gel. The mixture was poured on top of the separation gel and waited for about 30 minutes for the gel to polymerize completely. The 40 µl of protein samples (sericin 1 (0.8 mg/100µl) and sericin 2 (0.6 mg/100µl)) prepared for SDS-PAGE analysis were mixed well with 60 µl of SDS sample buffer and denatured by heating in the presence of a sample buffer and boiled for 10 minutes then cooled to room temperature. Then, 5 µl of standard protein markers (10 kDa-170 kDa), 15 µl of sericin 1-SDS mixture and 20 µl of sericin 2-SDS mixture were loaded into three different wells. The electrophoresis was carried out at 120 V for about 1 hour. The gels were then stained with silver staining.

3.3.4.2. High-Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) of Sericin

High-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) method was performed to determine the isoelectric point (pI) of sericin. The experimental procedure was as follow; 0.7 mg of sericin sample was diluted with 400 µl of rehydration solution (5 g urea, 200 mg 1-4% CHAOS, 75 mg DTT, 10 µl Bio-Lytes and 10 µl bromophenol blue) corresponding to 17 cm pH 3-10 ReadyStrip IPG Strips and placed on a strip holder, and covered with DryStrip and cover fluid for 11-16 hours at room temperature. IEF was started with a gradient procedure: 100 V, 2 h; 500 V, 2 h; 1000 V,

2 h; 2000 V, 1 h; 60000 Vh. The strips were then carefully equilibrated in equilibration buffer I (6 M, 7.2g urea, 0.4 g 2% SDS, 5 mL 0.375M Tris-HCI (pH 8.8), 4 mL 20% glycerol and 200 mg DTT) and equilibration buffer II (containing 250 mg of idoacetamide instead of DTT). Then, the SDS-PAGE gel was prepared with 12% separating gel (20.1 mL of distilled water, 15 mL of 1.5 Tris-HCI (pH 8.8), 24 mL of acrylamide/bisacrylamide (30% T, 2.67% C stock), 600 µl of 10% (w/v) SDS, 300 µl of 10% ammonium persulfate (fresh) and 30 µl of TEMED). After loading on a %12 separating gel for the second-dimension separation, the SDS-PAGE gel was then stained with a high-resolution image scanner.

3.3.5. Conjugation of Sericin and Polymers

PBS (phosphate buffer saline solution, pH 7.4, 0.1 mM and PH 7.0, 0.1 mM) was first prepared using relevant mono and dibasic salts and NaCl for conjugation. Polymer (P(OEGMA), P(HEMA) or P(DMAEMA)) (10 mmol) was weighted and dissolved in 100 µl of PBS (pH 7.0, 0.1 mM salt concentration). EDC (1.15 mg 600 µl, 10 mmol) was first added to the polymer solution and then Sulfo-NHS (1.1 mg, 200 µl, 25 mmol) was added and the mixture was left to react for 30 minutes. 40 mg sericin (20 mmol) was then weighted and dissolved in 200 µl of PBS (pH 7.4, 0.1 mM salt concentration). Polymer mixture was added to the sericin mixture and allowed to react overnight. Then, the whole mixture (V_T=1100 µl) was dialyzed against ultrapure water for 4 days using a dialysis membrane with a MWCO of 3500 and finally freeze-dried.

In a separate experiment, polymer:sericin mole ratio was changed. In this second set of experiment, all the procedure and conditions were the same except the polymer: sericin mole ratio. In this case, excess of polymer was used when compared with sericin. The polymer:sericin mole ratio was 10. In sericin-P(OEGMA) conjugates, P(OEGMA) (40mg, 2µmol) was weighted and dissolved in 260 µl of PBS (pH 7.0, 0.1 mM salt concentration). EDC (20 µl, 5 µmol) was first added to the polymer solution and then Sulfo-NHS (20 µl, 4.5 mM) was added and the mixture was left to react for 30 minutes. 2 mg sericin (0.2 µmol) was then weighted and dissolved in 100 µl of PBS (pH 7.4, 0.1 mM salt concentration). Polymer mixture was added to the sericin mixture and allowed to react overnight. Then, the whole mixture (V_T=400 µl) was dialyzed against ultrapure

water for 4 days using a dialysis membrane with a MWCO of 3500 Da and finally freeze-dried.

For sericin-P(HEMA) and sericin-P(DMAEMA) conjugates, P(HEMA) (6.1mg, 1μmol) and P(DMAEMA) (6.4mg, 1μmol) was separately weighted and dissolved in 130 μl of PBS (pH 7.0, 0.1 mM salt concentration). EDC (10 μl, 5 μmol) was first added to the polymer solutions and then Sulfo-NHS (10 μl, 4.5 mM) was added and both mixtures were left to react for 30 minutes. For both mixtures, 1 mg sericin (0.1 μmol) was then weighted and dissolved in 50 μl of PBS (pH 7.4, 0.1 mM salt concentration). Both polymer mixtures was added to the sericin mixtures and allowed to react overnight. Then, the whole mixture (V_T=200 μl) was separately dialyzed against ultrapure water for 4 days using a dialysis membrane with a MWCO of 3500 Da and finally freeze-dried for both mixtures.

In a control experiment, sericin was subjected to the dialysis procedure without any conjugation reactions. The same dialysis procedure applied after polymer conjugations was performed for pure sericin only to investigate whether purification procedure after conjugations alter the molecular weight characteristics of sericin. The sample is coded as purified sericin In addition, to investigate if sericin characteristics are affected by reaction conditions, only sericin without polymer was exposed to EDC and Sulfo-NHS reagents using the exact same conditions applied for polymer conjugations reactions and then purified via the same method used after polymer conjugations. The sample is coded as polymer-free sericin

3.3.6. Characterization of Sericin-Polymer Conjugates

Sericin-polymer conjugates obtained after dialysis and freeze-drying along with sericin samples obtained from control experiments were analyzed via SDS-PAGE, dynamic light scattering (DLS) and gel permeation chromatography (GPC).

3.3.6.1. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) Method

SDS-PAGE was performed to determine the presence of sericin-polymer conjugates. The positions of the conjugates on the gel were examined with the aid of marker and original sericin (with no modification). The same gels (as in the above molecular weight determination of sericin) were prepared to determine the conjugation of sericin and polymers. 5 µl of standard proteins (both small marker (3.5kDa-30kDa) and large marker (10kDa-170kDa)), different amounts of sericin, conjugates, purified sericin and polymer-free sericin were loaded into different wells in different gels. The electrophoresis of the gels was carried out at 120 V for about 1 hour and stained with silver staining.

3.3.6.2. Dynamic Light Scattering (DLS)

For DLS analysis, polymers, sericin-polymer conjugates, sericin, polymer-free sericin and purified sericin solutions were dissolved in PBS (phosphate buffer saline solution, pH 7.4, 0.1 mM) at certain concentrations and filtered using 0,45 µm filter. DLS analyses were performed for each sample solution. For measurements, the prepared solutions were taken into ZEN0040 Microcuvettes and placed into a Zetasizer Nano ZS (Malvern, UK) (measurement range 0.3nm – 10.0 microns; light source He-Ne laser 633nm Max 5mW; Power 100 VA) in Malvern disposable polystyrene cuvettes at room temperature. The measurements were replicated 3 times with 3 repeats for each sample solution.

3.3.6.3. Gel Permeation Chromatography (GPC)

Each conjugate sample along with corresponding free polymer was analysed by GPC using DMAc as a mobile phase to determine the conjugation. Sericin-polymer conjugates and free polymers were prepared by dissolving conjugates in DMAc at 2

mg/mL concentration. Then, prepared samples were filtered by using 0,45 μm syringe type membrane filter before analysis.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this thesis, three different polymers having varying chemical properties were intended to be synthesized to conjugate with sericin protein. For this purpose, a highly hydrophilic and neutral polymer, poly(oligo (ethylene glycol) methyl ether methacrylate) P(OEGMA), a less hydrophilic and neutral polymer poly(hydroxyethyl methacrylate) P(HEMA) and a hydrophilic and charged polymer after quaternized, poly(dimethylamino ethyl methacrylate) P(DMAEMA) were first synthesized. These polymers were selected since they all are commonly used as components of biomaterials in varying fields of biomedical applications including tissue engineering and drug delivery. All three polymers were intended to be synthesized at a predetermined degree of polymerization and a defined end-group to be able to compare the conjugation results and final conjugates of different polymers with sericin. The intended degree of polymerization by NMR was about 26, considering the molecular weight range of non-degradable polymers, such as vinyl polymers including P(OEGMA), P(HEMA) and P(DMAEMA), used in drug delivery and tissue engineering applications. A defined end-group was needed to be able to covalently conjugate polymers to sericin. Carboxylic acid moiety was chosen as a functional end-group as it allows easy conjugation of polymers with protein's amino groups via commonly applied chemistries such as NHS/EDC chemistry. Accordingly, reversible addition fragmentation chain transfer (RAFT) polymerization was chosen as a polymerization method since RAFT polymerization provides control over the molecular weight and offers end-group fidelity of polymers. It is also suitable for polymerization of varying functional monomers under mild conditions such as at low temperatures and in aqueous solutions.

4.1. Synthesis and Characterization of P(OEGMA)

Poly(oligo (ethylene glycol) methyl ether methacrylate) P(OEGMA) is a hydrophilic, neutral polymer which have been increasingly used in biomedical applications as an alternative to well-known biocompatible polymer, poly(ethylene

glycol) (PEG). P(OEGMA) is known to be non-toxic, non-immunogenic, water-soluble polymer. It has been increasingly used in drug delivery systems including protein conjugates (Magnusson et al., 2010).

OEGMA (Mw: 467 g/mol) was polymerized via RAFT polymerization by using AIBN as a free radical initiator and 4-cyano-4 (phenylcarbonothioylthio) pentanoic acid as a RAFT agent (CPADB).

Molecular weight and molecular weight distribution (polydispersity index (PDI)) of purified polymers were determined via Gel Permeation Chromatography (GPC). The chemical structure of synthesized polymers was determined via Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. The obtained results by NMR and GPC analyses are given Figures 4.1 and 4.2, respectively.

The formation of P(OEGMA) was confirmed via ^{1}H-NMR spectroscopy as shown in Figure 4.1. In the spectrum, the proton peaks at 4.75 ppm and 3.66 ppm are assigned to the methylene groups of oligo(ethylene glycol) side chains. The signals at 7.88-7.31 ppm belong to 5 protons of the RAFT Z-group. Other characteristic peaks that belong to protons in the polymer are presented in Figure 4.1. The analysis above indicates that P(OEGMA) have been successfully synthesized.

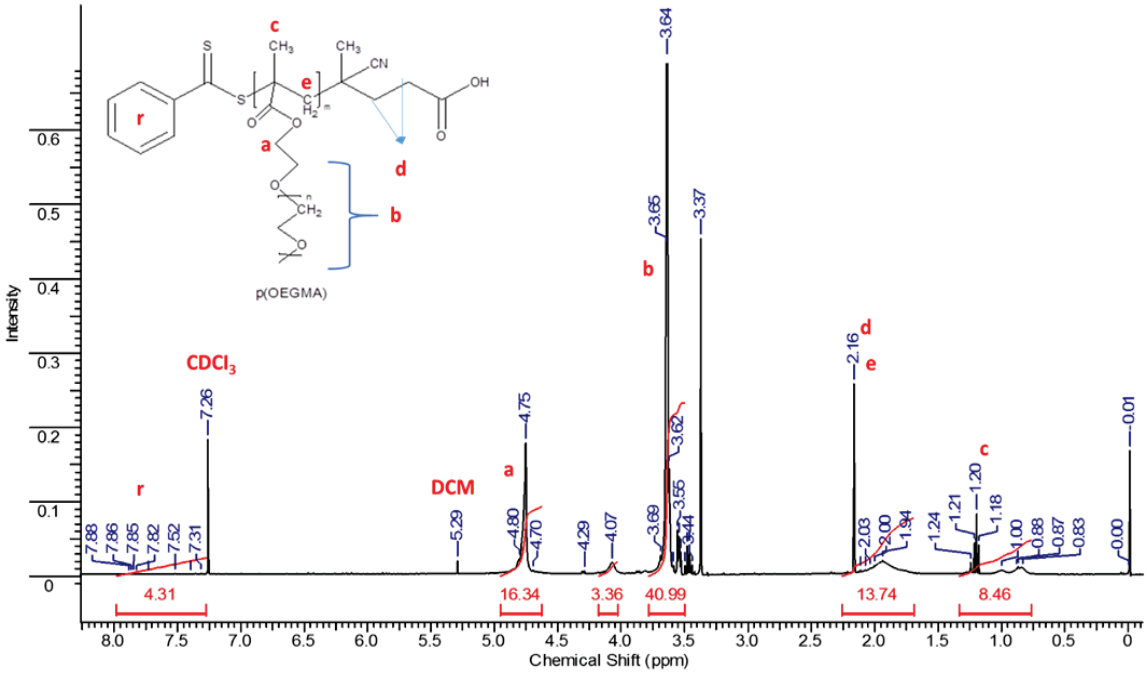


Figure 4.1. ^{1}H-NMR spectrum of purified P(OEGMA) (in $CDCl_3$) [(Mn:20,000 g/mol) synthesized using a [OEGMA]/[CPADB]/[AIBN] mol ratio of 150/1/0.2]

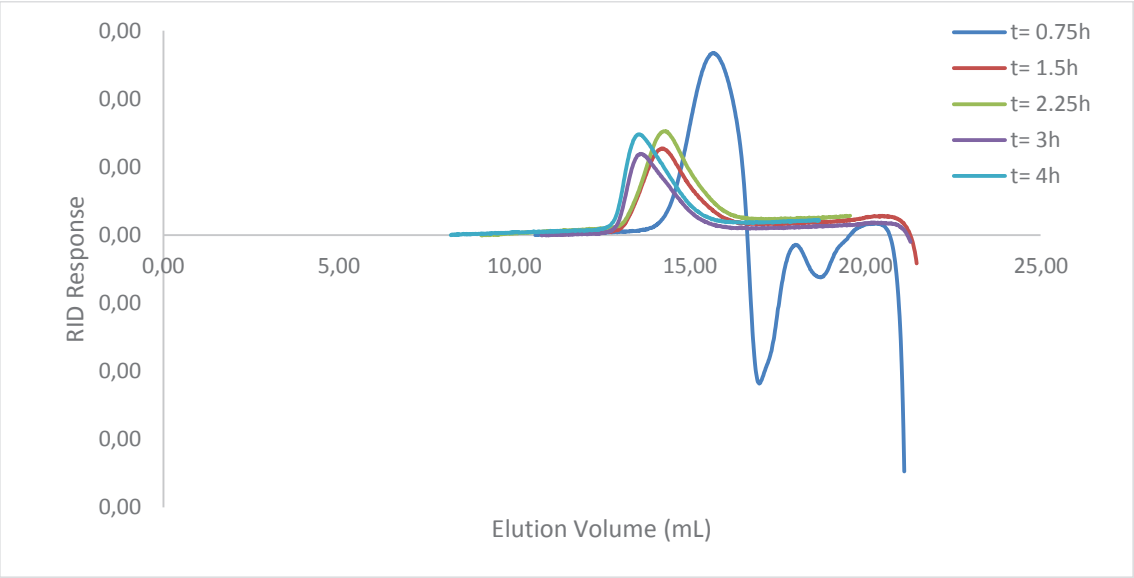


Figure 4.2. GPC chromatograms of P(OEGMA) synthesized using a [OEGMA]/[CPADB]/[AIBN] mol ratio of 150/1/0.2]

The number average molecular weight (M_n) and molecular weight distribution (PDI) of polymers obtained by GPC are given in Table 4.1. As seen in Table 4.1, polydispersity index (PDI<1.55) values were slightly high, compared to polymers synthesized by RAFT-controlled polymerization (usually PDI<1.2). The PDI values could be improved by decreasing [M]/[RAFT] ratio in polymerizations. Nevertheless, the PDI values obtained were much lower than the PDI values commonly obtained for polymers synthesized via free radical polymerizations with no chain transfer agents, indicating that the polymerization was mostly RAFT mechanism controlled.

Table 4.1. Polymerization conditions and the properties of P(OEGMA) polymers obtained in this study

Sample Code	**[M]/[R]/[I]**	**Time(min)**	**[a]$Mn_{NMR(g/mol)}$**	**[b]$\overline{DP}$**	**[c]$Mn_{GPC(g/mol)}$**	**[d]PDI**
K1	150/1/0.2	45	3,604	7	4,640	1.21
K2	150/1/0.2	90	12,154	25	20,000	1.45
K3	150/1/0.2	135	14,054	29	21,000	1.40
K4	150/1/0.2	180	20,229	42	30,000	1.51
K5	150/1/0.2	240	21,654	45	32,000	1.53

[a-b] Calculated by using Equation 4.1 and 4.2 respectively from ^{1}H-NMR spectrum of purified sample, [c](Mn_{GPC}) and [d](PDI) determined by gel permeation chromatography (GPC).

$$Mn_{NMR} = \frac{\int Polymer}{\int RAFT} \; x \; Mw \; of \; monomer + Mw \; of \; RAFT \tag{4.1}$$

$$\text{Degree of Polymerization } (\overline{DP}) = \frac{\int_{3,62}^{Polymer}/2}{\int_{7,5}^{RAFT}/5} = \frac{a/2}{r/5} \tag{4.2}$$

The Mn (Mn_{NMR}) and degree of polymerization ($\overline{DP}$) were determined via ^{1}H-NMR spectroscopy. Degree of polymerization ($\overline{DP}$) and Mn_{NMR} of polymers were calculated according to Equations 4.1 and 4.2 respectively, using the integration of the characteristic signals belonging to RAFT agent (r signal, Figure 4.1) and oligoethylene glycol side chains (a signal, Figure 4.1) in ^{1}H-NMR spectrum. For calculation of Mn_{NMR} and $\overline{DP}$, it was assumed that there were negligible termination reactions in polymerizations.

4.2. Synthesis and Characterization of P(HEMA)

Poly(2-hydroxyethyl methacrylate) (P(HEMA)) was among the polymers synthesized in this study. P(HEMA) has good biological and blood compatibility. P(HEMA) is perhaps the most commonly used polymer for making hydrogels because of its water content similar to living tissues (Rayment et al., 2008; Schiraldi et al., 2004; Bhawal et al., 2004). Furthermore, because of its high oxygen permeability, good mechanical properties and appropriate refractive index value, soft contact lenses and intraocular lenses are the most important applications of this polymer (Lee et al., 1996; Karlgard et al., 2004). While HEMA monomer and P(HEMA) having molecular weight less than 5000 g/mol are water-soluble, P(HEMA) with larger molecular weights are water insoluble.

HEMA (Mw: 130.1 g/mol) was polymerized via RAFT polymerization using AIBN as an initiator and CPADB as a RAFT agent. The polymers were characterized by NMR and GPC analysis (Figure 4.3 and 4.4, respectively).

The formation of P(HEMA) was confirmed by ^{1}H-NMR spectroscopy as shown in Figure 4.3. In the spectrum, the broad signal at 0.78–1.94 ppm is associated with the methylene groups in the backbone of the P(HEMA) chain. The peaks around 0.76-1.22 ppm confirmed the presence of protons of C-CH_3 methyl groups. The peaks at 3.57–3.88 ppm are assigned to methylene protons adjacent to the oxygen moiety in HEMA unit. A broad peak at 4.11 ppm is ascribed for the hydroxyl proton of HEMA. Peaks at 7.82-7.46 ppm belong to 5 protons of the RAFT agent. The analysis above indicates that P(HEMA) has been successfully synthesized.

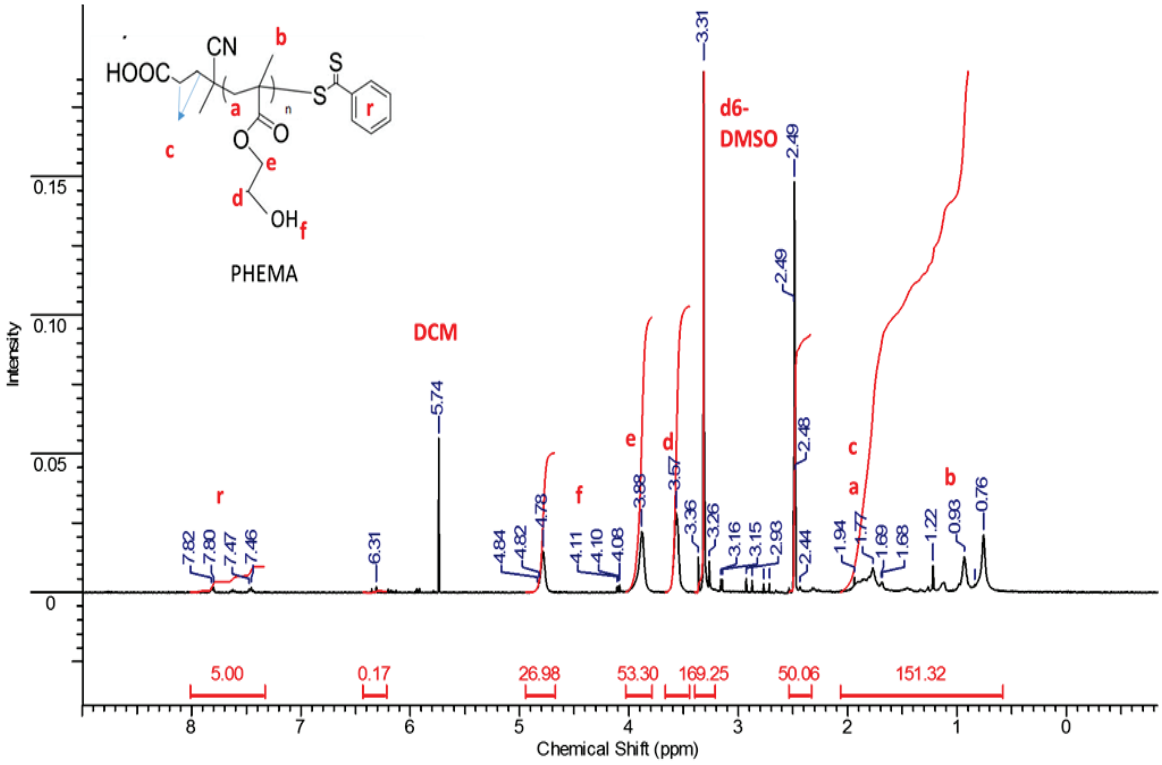


Figure 4.3. ^{1}H-NMR spectrum of purified P(HEMA) (in DMSO-d6) [(Mn: 6,129g/mol) synthesized using a [HEMA]/[CPADB]/[AIBN] mol ratio of 100/1/0.2]

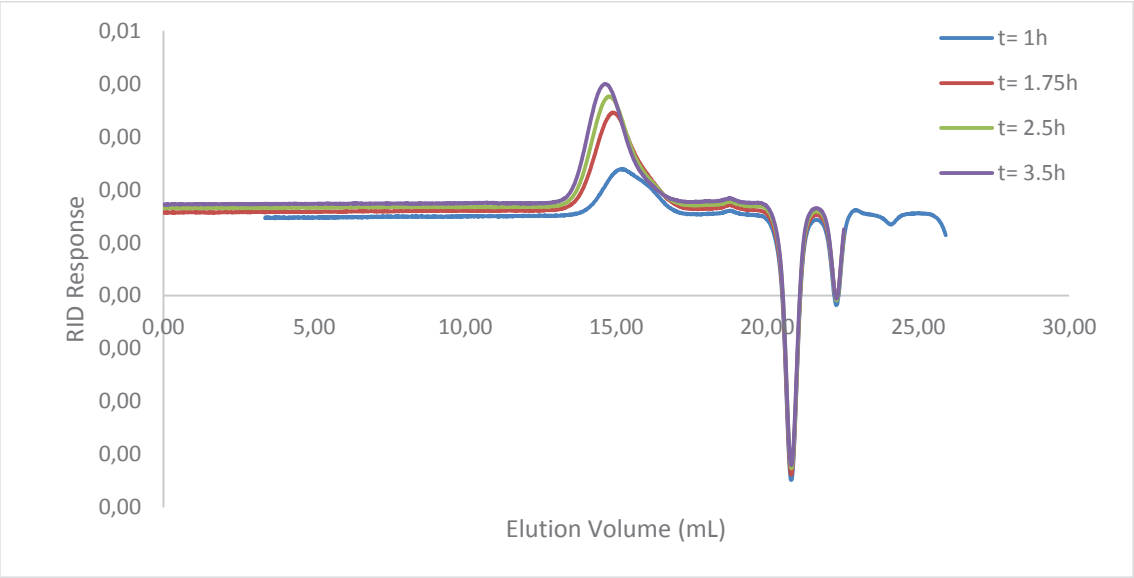


Figure 4.4. GPC chromatograms of P(HEMA) synthesized using a [HEMA]/[CPADB]/[AIBN] mol ratio of 100/1/0.2]

The M_n and PDI values obtained during this study are given in Table 4.2. As seen in Table 4.2, the low PDI (<1.25) values implied narrow molecular weight distribution of polymers and thus RAFT controlled polymerization mechanism.

Table 4.2. Polymerization conditions and the properties of P(HEMA) polymers obtained in this study

Sample Code	**[M]/[R]/[I]**	**Time(min)**	**[a]$Mn_{NMR(g/mol)}$**	**[b]$\overline{DP}$**	**[c]$Mn_{GPC(g/mol)}$**	**[d]PDI**
D2-1	100/1/0.2	60	1,320	8	4,719	1.19
D2-145	100/1/0.2	105	1,840	12	5,604	1.22
D2-230	100/1/0.2	150	3,662	26	6,129	1.21
D2-330	100/1/0.2	210	4,313	31	6,800	1.22

[a-b] Calculated by using Equation 4.1 and 4.2 respectively from ^{1}H-NMR spectrum of purified sample, [c](Mn_{GPC}) and [d](PDI) determined by gel permeation chromatography (GPC).

The Mn (Mn_{NMR}) and degree of polymerization ($\overline{DP}$) were determined via ^{1}H-NMR spectroscopy. Degree of polymerization ($\overline{DP}$) and Mn_{NMR} of polymers were calculated according to Equations 4.1 and 4.2 respectively, using the integration of the characteristic signals belonging to RAFT agent (r, Figure 4.3) and the methylene groups in the backbone of the P(HEMA) chains (a, Figure 4.3) in ^{1}H-NMR spectrum. For calculation of Mn_{NMR} and $\overline{DP}$, it was assumed that there were negligible termination reactions in polymerizations.

4.3. Synthesis and Characterization of P(DMAEMA)

Poly(2-dimethylamino ethyl methacrylate) (P(DMAEMA)) is a well known, cationic upon quaternization, water-soluble, hydrophilic polymer. It is also a stimuli-responsive polymer having one or more properties that can be changed to a large extent in a controlled manner by temperature and pH (Gil and Hudson, 2004). P(DMAEMA) is widely used in wastewater treatment, filtration techniques, therapy, gene delivery, self-assembly of block copolymers. Since P(DMAEMA) is a cationic polymer after quaternization, it is used especially as gene delivery vectors (Cherng et al., 1997; Du and Zhao, 2004; Gohy et al., 2001; Mahltig et al., 2001; van de Wetering et al., 1997).

DMAEMA (Mw: 157.21 g/mol) was polymerized via RAFT polymerization according to the literature with some modifications (Vuoriluoto et al., 2015). AIBN and CPABD were used as an initiator and a RAFT agent. The polymers were characterized by NMR and GPC analyses (Figure 4.5 and 4.6, respectively).

The formation of P(DMAEMA) was confirmed by the ^{1}H-NMR spectroscopy as shown in Figure 4.5. In the spectrum, the proton peaks at 4.07 ppm and 2.58 ppm are assigned to the methylene groups on the side chain. The signal at 2.29 ppm is the characteristic peak of methyl group adjacent to nitrogen (N-CH_3). The peaks at 1.83-2.18 ppm and 0.87-1.28 ppm can be attributed to the methylene and methyl groups on the backbone, respectively. Finally, the signals at 7.88-7.53 ppm belong to 5 protons of the RAFT Z-group. The analysis above indicates that P(DMAEMA) was successfully synthesized.

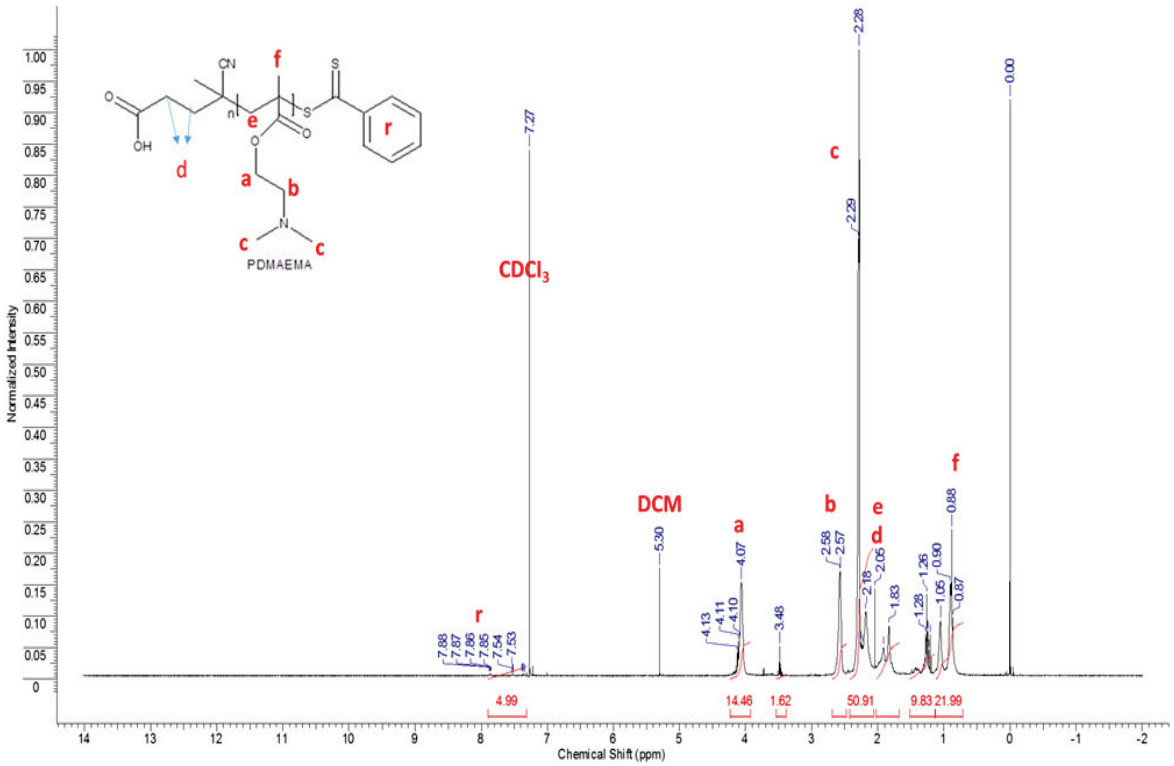


Figure 4.5. ^{1}H-NMR spectrum of purified P(DMAEMA) (in $CDCl_3$) [(Mn: 6,400g/mol) synthesized using a [DMAEMA]/[CPADB]/[AIBN] mol ratio of 130/0.6/0.2]

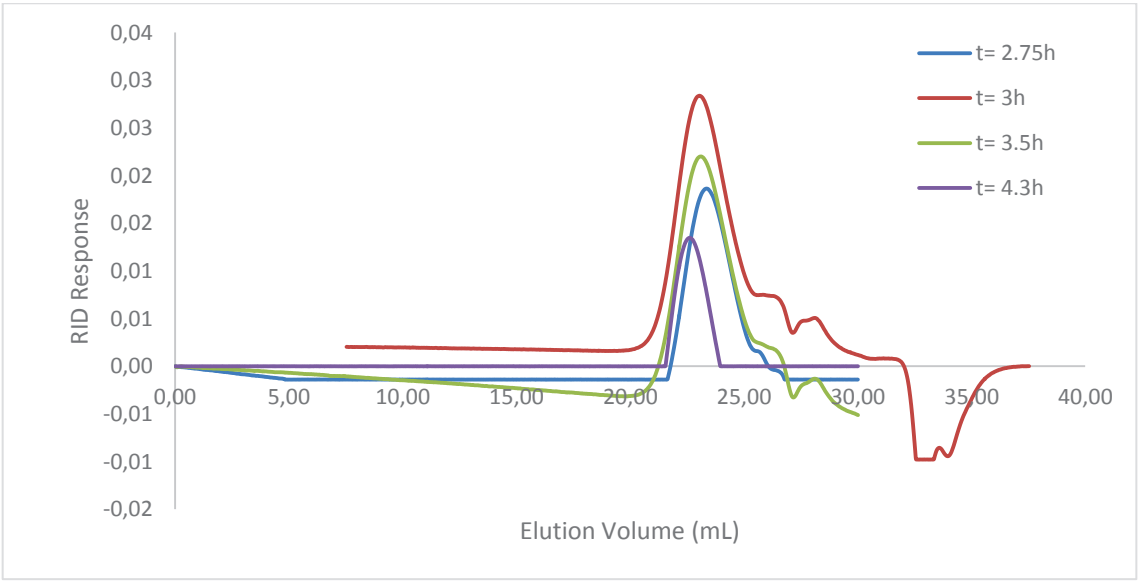


Figure 4.6. GPC chromatograms of P(DMAEMA) synthesized using a [HEMA]/[CPADB]/[AIBN] mol ratio of 130/0.6/0.2]

The M_n and PDI values obtained for P(DMAEMA) are given in Table 4.3. As seen in the Table 4.3, the low the polydispersity index (PDI<1.2) values implied narrow molecular weight distribution of polymers and thus, RAFT controlled polymerization mechanism.

Table 4.3. Polymerization conditions and the properties of P(DMAEMA) polymers obtained in this study

Sample Code	**[M]/[R]/[I]**	**Time(min)**	**[a]$Mn_{NMR(g/mol)}$**	**[b]$\overline{DP}$**	**[c]$Mn_{GPC(g/mol)}$**	**[d]PDI**
N-2.45	130/0.6/0.2	165	1,693	9	3,800	1.17
N-3	130/0.6/0.2	180	2,165	12	4,400	1.20
N-3.3	130/0.6/0.2	210	2,637	15	4,500	1.20
N-4.15	130/0.6/0.2	260	4,052	24	6,400	1.1

[a-b] Calculated by using Equation 4.1 and 4.2 respectively from ^{1}H-NMR spectrum of purified sample, [c](Mn_{GPC}) and [d](PDI) determined by gel permeation chromatography (GPC).

The Mn (Mn_{NMR}) and degree of polymerization ($\overline{DP}$) were determined via ^{1}H-NMR spectroscopy. Degree of polymerization ($\overline{DP}$) and Mn_{NMR} of polymers were calculated according to Equations 4.1 and 4.2 respectively, using the integration of the characteristic signals belonging to RAFT agent (r, Figure 4.5) and the methylene groups in the backbone of the P(DMAEMA) chains (e, Figure 4.5) in ^{1}H-NMR spectrum. For calculation of Mn_{NMR} and $\overline{DP}$, it was assumed that there were negligible termination reactions in polymerizations.

4.4. Characterization of Sericin

It has been demonstrated by various research groups that the physical and chemical properties of sericin are affected by its molecular weight and structure (Freddi et al., 2003; Vaithanomsat and Kitpreechavanich, 2008; Zhang, 2002). Sericin is usually extracted via different methods involving varying pH, temperature and processing time

and varying extraction conditions yield different amino acid compositions (Aramwit et al., 2010a).

Degumming methods for obtaining sericin affect molecular weight of sericin. For example, heat and acid extraction yields sericin with a molecular weight of 35-150 kDa, while sericin obtained via an alkaline solution has a molecular weight of 15-75 kDa. When sericin is extracted via heat, acid and alkaline solutions it normally displays large molecular weight distribution visible by a smear of bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, when it is extracted using urea, it provides significant bands on SDS-PAGE between 10 and 225 kDa. .

The characteristics of sericin used in this thesis were not known as the manufacturer did not provide such information. SDS-PAGE and high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) experiments were therefore performed to determine the molecular weight and isoelectric point of sericin. The UV absorption spectrum of sericin was also determined.

4.4.1. Molecular Weight Distribution of Sericin

Sericin is a family of proteins having a molecular weight ranging from 20 kDa to 400 kDa. While Gamo et al. estimated that the molecular mass of sericin could be 309, 177, 145, 134 and 80 kDa, Sprague has reported at least 15 different polypeptides with a molecular weight ranging from about 20 kDa to about 200 kDa at the anterior portion of the middle silk gland (Gamo et al., 1977; Sprague, 1975). Also, while Wu et al. showed that the sericin had a continuous distribution between 97 kDa and 14 kDa, some bands were observed above and below 97 kDa and 14 kDa, respectively (Wu et al., 2007).

In this study, SDS-PAGE method was used to determine the molecular weight distribution of sericin using molecular weight markers of 10-170 kDa. The results are shown in Figure 4.7. The SDS-PAGE results showed diffused bands with molecular weights ranging between 10 and 72 kDa. However, a region where sericin proteins are most dense was observed at around 10-15 kDa. The presence of a large smear on SDS-PAGE gel revealed that sericin sample was composed of a large number of polypeptide fragments, possibly due to the degumming process of the manufacturer. Such a large distribution in molecular weight can have significant undesirable effects on the properties and applications of sericin.

It has been reported in literature that sericin with lower molecular mass (< 50kDa) is readily soluble in cold water (Gupta et al., 2014). Sericin used in this study was also soluble in cold water, supporting the data obtained by SDS-PAGE. In addition, in summary, the SDS-PAGE electrophoresis results showed that while sericin used in this study was composed of polypeptide fragments between 10 kDa and 72 kDa, fragments between 10 kDa and 15 kDa predominates in the sample.

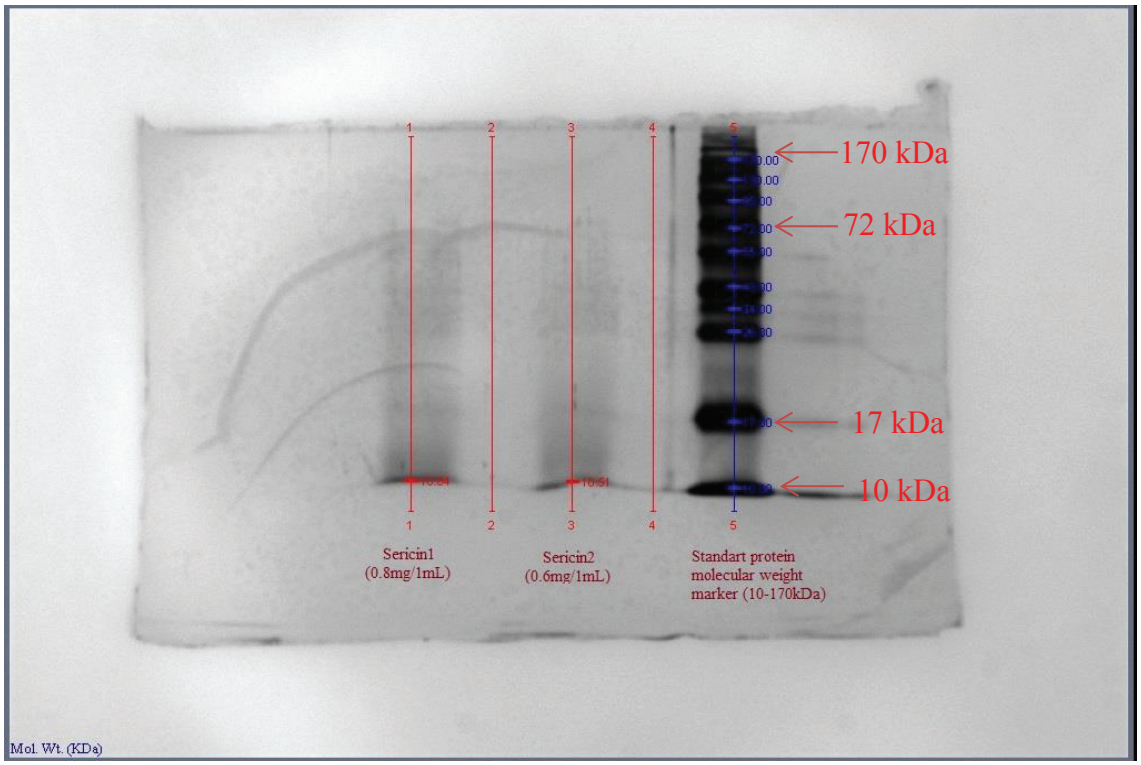


Figure 4.7. SDS-PAGE analyses of sericin (Line 1: Sericin (0.8 mg/ml), Line 3: Sericin (0.6 mg/ml) and Line 5: standart protein molecular weight marker)

4.4.2. Identification of Sericin by High-Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

High-resolution two-dimensional polyacrylamide gel electrophoresis (2D PAGE) method was performed to determine the isoelectric point (pI) of sericin. In 2D-PAGE gel, sericin runs in the gel according to its net charge (pI) via isoelectric focusing (IEF) in the first dimension and according to its size (Mr) via SDS-PAGE in the second dimension.

As seen in Figure 4.8, protein smears were observed at molecular weights ranging between 3.5 and 35 kDa (which was the range observable under the experimental conditions) and a pI of about 4-4.8. The results were similar to those of Kurioka and Yamazaki (2002). These authors analysed sericin with a low molecular mass of 6027 extracted from the cocoon shell of silkworm, Bombyx mori. 2D-PAGE resolved this protein into a single spot with a pI of 4.3 and a Mr of 6000.

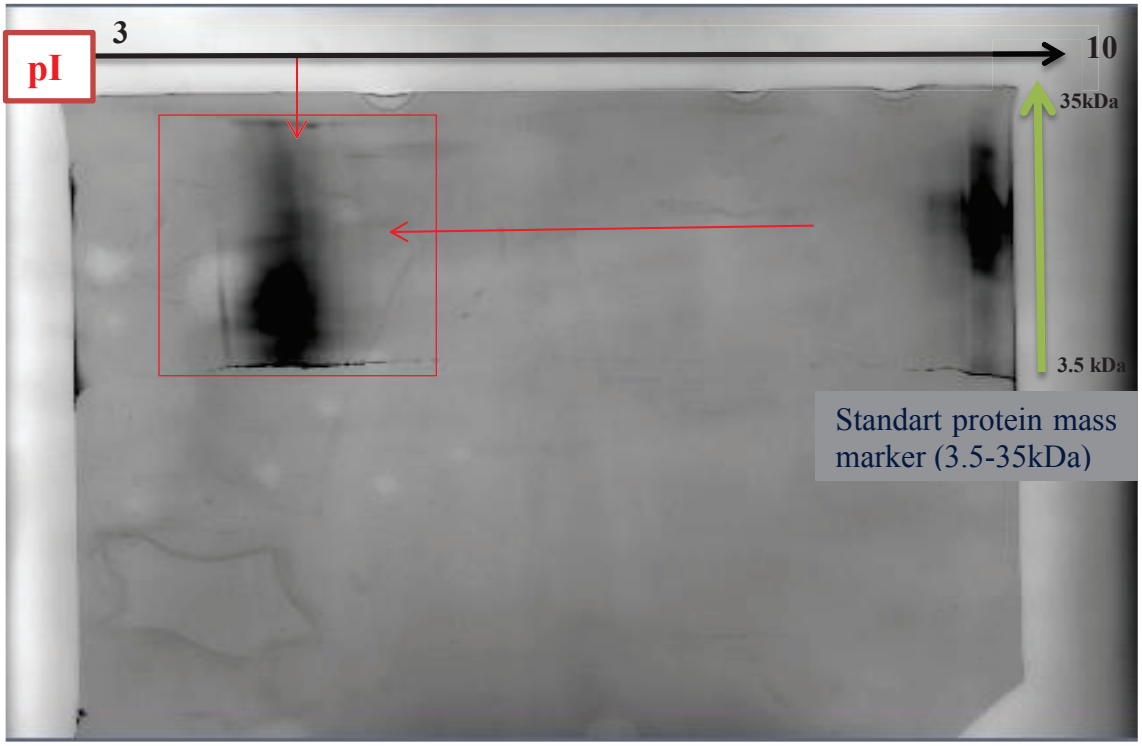


Figure 4.8. 2D-map of sericin protein (Sericin sample was placed on pH 3-10, 17 cm ReadyStrip IPG strips for electrophoresis (7 h) followed by SDS-PAGE (12% gel, 4 h) and silver staining)

4.5. Synthesis and Characterization of Sericin-Polymer Conjugates

Sericin-polymer conjugates (sericin-P(HEMA), sericin-P(OEGMA) and sericin-P(DMAEMA)) were synthesized via Sulfo-NHS/EDC chemistry using polymer's carboxylic acid (-COOH) end-group and amine groups of protein. Sulfo-NHS/EDC chemistry is well-known for preparation of bioconjugates of polymers. The water-soluble carbodiimide EDC can be used together with NHS (sulfo-NHS), a water-soluble

compound, to form active ester functionality with carboxylate groups. The resulting sulfo-NHS esters are hydrophilic reactive groups which react rapidly with amines in the target molecules (Staros, 1982; Denney and Blobel, 1984; Kotite et al., 1984; Beth et al., 1986). Conjugations were made using polymers having the same degree of polymerization (DP) of 42 determined by GPC P(OEGMA) Mn= 20,000 g/mol and PDI= 1.45 ; P(HEMA) Mn= 6,129 g/mol and PDI= 1.21; P(DMAEMA) Mn= 6,400g/mol and PDI= 1.1). Two different conjugation reactions were performed using two different polymer:protein ratios (10:1 and 0.5:1 molar ratio, polymer concentration= 5 mM and 0.9 mM, respectively) in reaction solutions.

In order to determine whether the covalent conjugations took place between polymers and sericin, the conjugates were characterized after dialysis (MWCO= 3500 Da) via various techniques including SDS-PAGE, dynamic light scattering (DLS) and GPC. Also, commercial sericin, commercial sericin after dialysis (MWCO= 3500 Da) (purified sericin) commercial sericin exposed to conjugation conditions without polymers (polymer-free sericin) and free polymers were used as control samples to confirm the presence of conjugates in these characterization procedures.

4.5.1. Characterization of Conjugates via SDS-PAGE

SDS-PAGE method was performed to verify the presence of sericin-polymer conjugates. Standard protein markers (both small marker (3.5 kDa - 35 kDa) and large marker (10 kDa - 170k Da)), sericin, sericin-P(OEGMA) conjugates, sericin-P(HEMA) conjugates, sericin-P(DMAEMA) conjugates, purified sericin and polymer-free sericin were loaded into different wells.

Figures 4.9 A and B separately show gels for each conjugate (A: Sericin-P(OEGMA), B: Sericin-P(HEMA) and Sericin-P(DMAEMA)) prepared at low polymer concentration (polymer:protein mol ratio= 0.5:1) while Figure 4.9 C shows all conjugates together (prepared at 0.5:1 ratio) along with control samples (i.e. purified sericin and polymer-free sericin) on the same gel. As seen in Figures 4.9A, B and C, the conjugate samples of P(OEGMA) and P(DMAEMA) showed significantly different bands (at around 40 kDa and 15 kDa, respectively) when compared with original sericin and control sericin samples, indicating polymer conjugation to sericin took place. In sericin-P(HEMA) sample, there was a slight change in the position of the dense region in the

protein smear, also suggesting P(HEMA) conjugation to sericin. It should be noted that the control sericin samples indicated slight decrease in the intensity of low molecular weight peptide fragments in the smear profile of original, untreated sericin sample. This is expected since dialysis step removes smaller peptide fragments. From the band of polymer-free sericin sample, it was concluded that there was no significant reaction between sericin peptides under the conjugation reaction conditions.

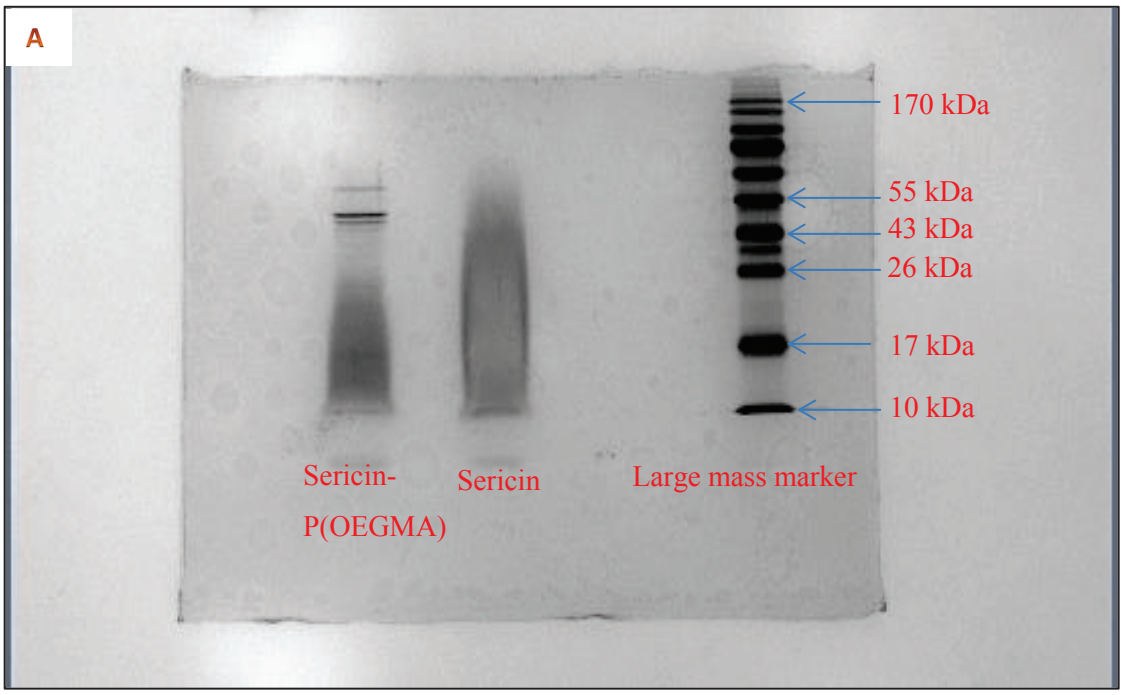
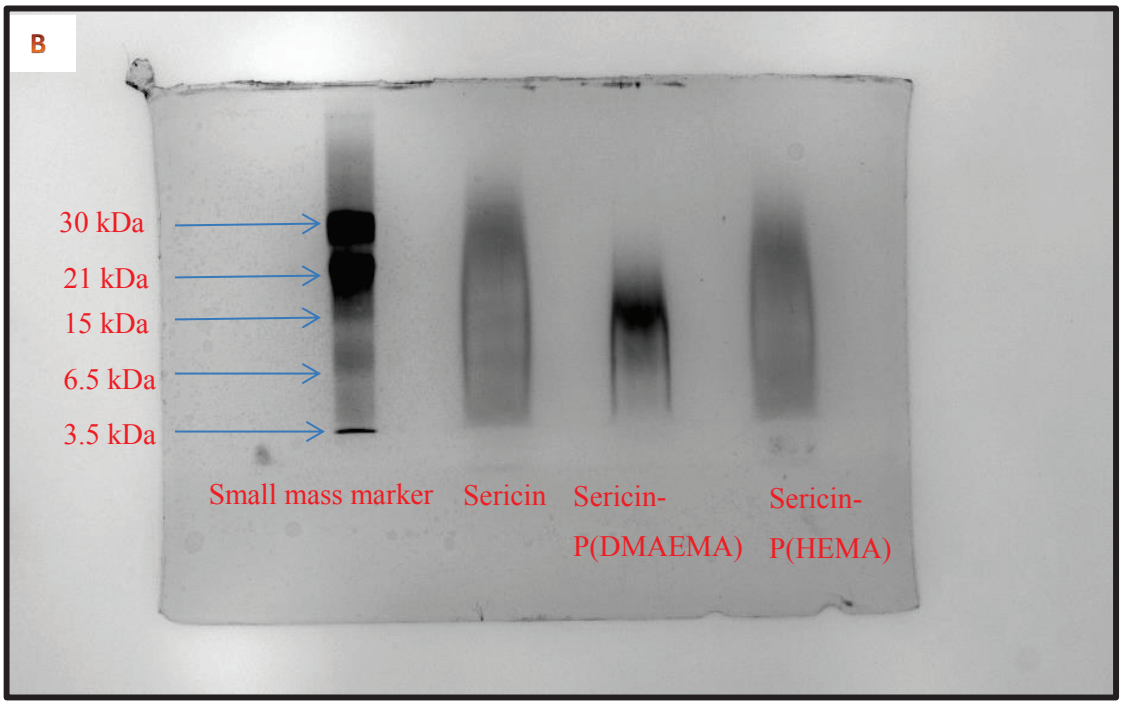


Figure 4.9. SDS-PAGE analyses of (A) sericin-P(OEGMA) and (B) sericin-P(DMAEMA) and sericin-P(HEMA) conjugates prepared at a polymer: protein ratio of 0.5:1 along with original sericin sample. (C) SDS-PAGE analysis of the same conjugate samples together with control samples (i.e. purified sericin and polymer-free sericin) (Line 1: Sericin, Line 2: Sericin-P(DMAEMA) conjugate Line 3: Sericin-P(HEMA) conjugate, Line 4: Sericin-P(OEGMA) conjugate, Line 5: Polymer-free sericin, Line 6: Purified sericin and Line 7: Large standart protein marker)

(Cont. on next page)



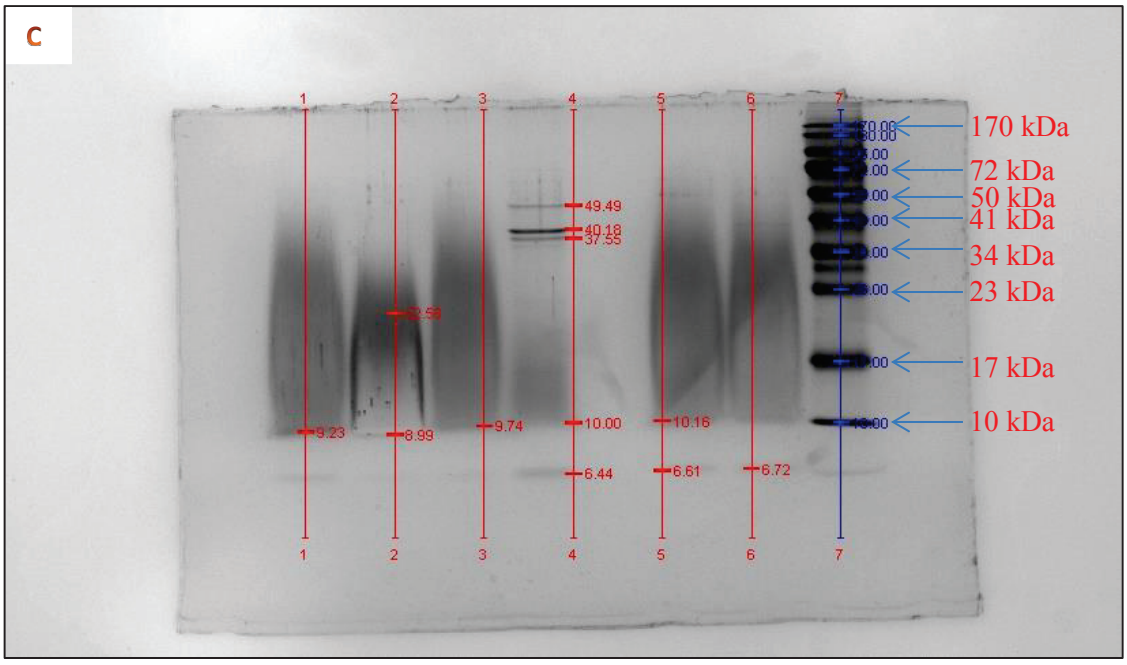


Figure 4.9. (Cont.)

Figures 4.10 shows the SDS-PAGE gel for conjugates prepared at high polymer: protein ratio (polymer: protein mol ratio= 10:1) along with control samples (i.e. purified sericin and polymer-free sericin). In this case, small protein markers (3.5-35 kDa) were

used. As it can be seen, at high polymer:protein ratio, while the bands of sericin-P(OEGMA) and sericin-P(HEMA) conjugates were distinctly different than purified sericin and original sericin samples, there was no observable change in the band of sericin-P(DMAEMA) conjugate.

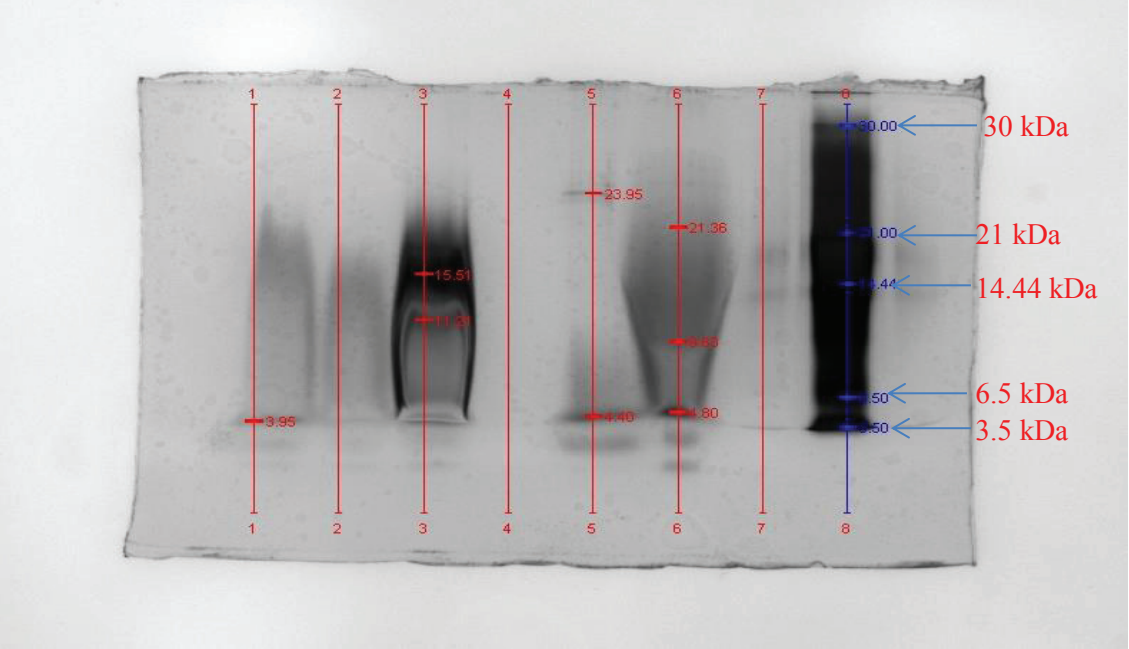


Figure 4.10. SDS-PAGE analysis of conjugates prepared at a polymer:protein ratio of 10:1 (Line 1: Sericin, Line 2: Purified sericin, Line 3: Sericin-P(DMAEMA) conjugate, Line 5: Sericin-P(OEGMA) conjugate, Line 6: Sericin-P(HEMA) conjugate and Line 7: Small standard protein marker)

Overall the SDS-PAGE results indicated the formation of sericin-polymer conjugates for all three different polymers upon the conjugation reactions performed.

4.5.2. Characterization of Conjugates via GPC

Sericin-polymer conjugates prepared at low polymer: protein mol ratio (0.5:1) were examined via GPC. Each conjugate sample was analyzed by GPC using dimethylacetamide (DMAc) as a mobile phase. The GPC results of each conjugate were compared with the GPC results of polymers only since sericin only could not be dissolved in organic solvent. All conjugates were analyzed via GPC after dissolving in DMAc and

filtering through 0.45 µm filter. GPC results of conjugates and polymers are given in Figures 4.11 A, B and C. Sericin-P(OEGMA) conjugate had significantly longer retention time when compared with that of free P(OEGMA) polymer, clearly indicating the formation of the protein-polymer conjugate. On the other hand, the other two conjugates had slightly different retention time than free polymers, also suggesting the formation of conjugates of polymers possibly with smaller peptide fragments at a lower extent reaction. It is also possible that the insoluble component of the conjugate sericin might interact with GPC colon.

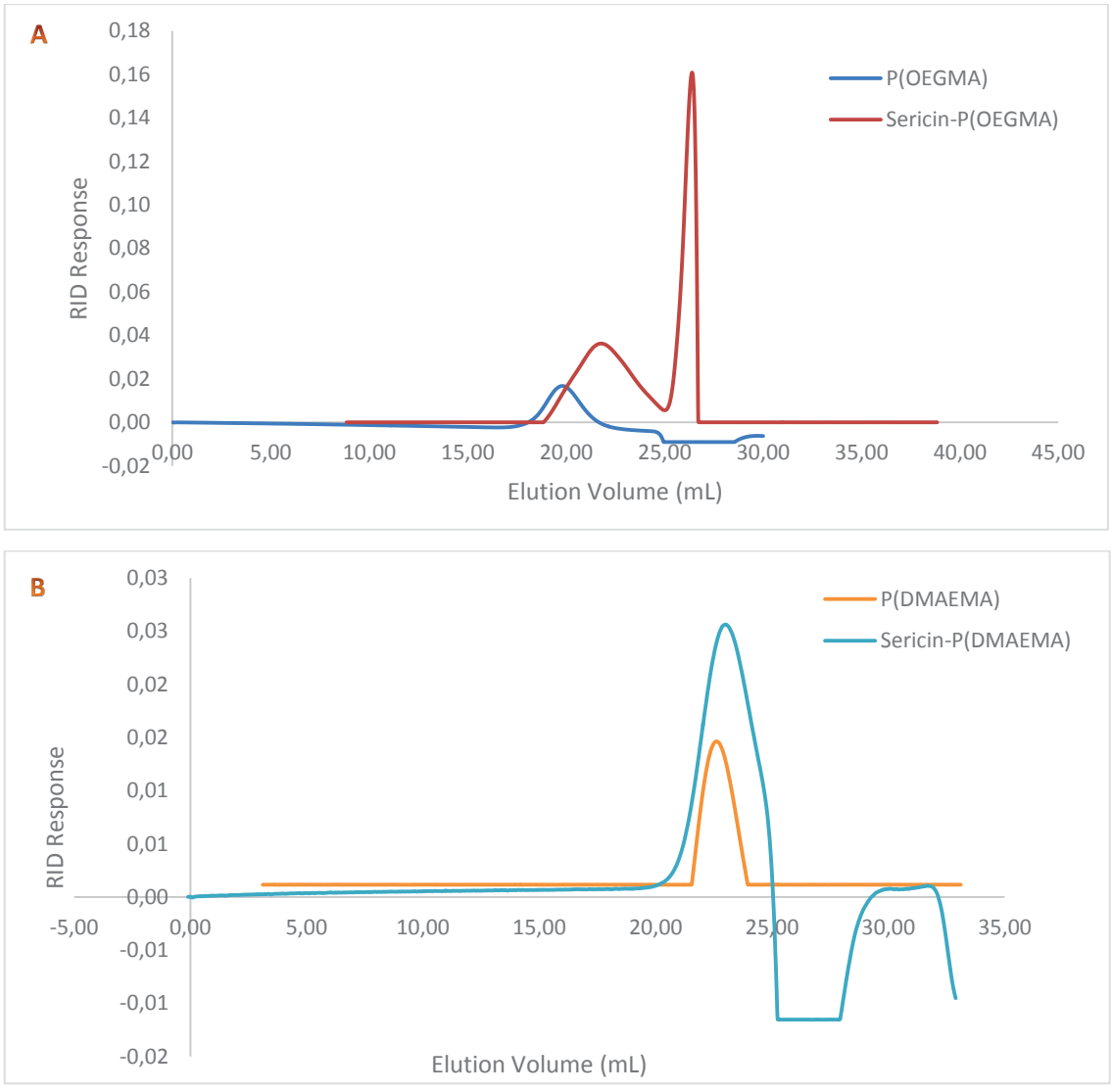


Figure 4.11.GPC chromatograms of (A) P(OEGMA) and Sericin-P(OEGMA) conjugate; (B) P(DMAEMA) and Sericin-P(DMAEMA) conjugate; (C) P(HEMA) and Sericin-P(HEMA) conjugate

(Cont. on next page)

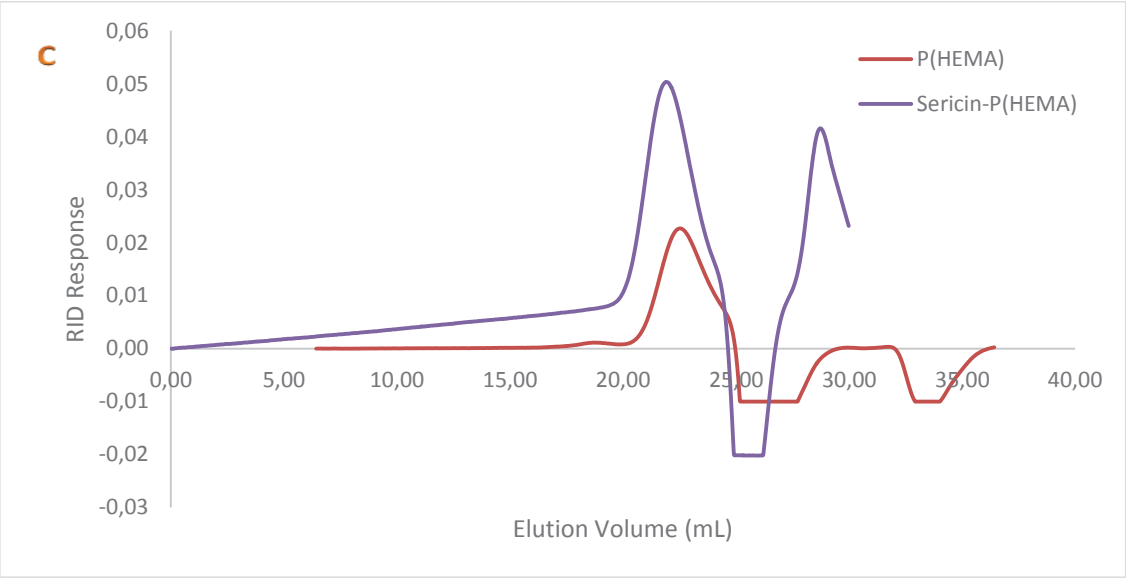


Figure 4.11. (Cont.)

4.5.3. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) method is used to determine the hydrodynamic size of molecules such as polymers and proteins, and it is also commonly used to characterize larger structures such as spherical nano- and micro-particles (Kersey et al., 2012). Sericin-polymer conjugates were characterized via DLS to determine the hydrodynamic size differences between sericin and its polymer conjugates. For DLS analysis, polymers, sericin-polymer conjugates, sericin and purified sericin samples were dissolved in PBS (phosphate buffer saline solution, pH 7.4, 0.1 mM) at a fixed certain concentration (0.3 mg polymer/100 µl PBS). The measurements were replicated 3 times with 3 repeats for each sample solution. The results of each measurement are given Figure 4.12. The summaries of hydrodynamic sizes are also presented in Table 4.4.

Table 4.4. The average number-based hydrodynamic sizes (nm) determined by DLS experiments (Conjugate1: polymer:protein molar ratio 0.5:1, Conjugate2: polymer:protein molar ratio 10:1)

Samples	Average number-based hydrodynamic size (nm) (std dev.)
Sericin	1,91 ± 0,96
P(DMAEMA)	4,2 ± 0,15
Sericin-P(DMAEMA) Conjugate1	4,3 ± 0,58
Sericin-P(DMAEMA) Conjugate2	4,7 ± 0,56
P(HEMA)	3,24 ± 0,62
Sericin-P(HEMA) Conjugate1	530 ± 60,83
Sericin-P(HEMA) Conjugate2	223,3 ± 25,2
P(OEGMA)	6,1 ± 0,1
Sericin-P(OEGMA) Conjugate1	5,8 ± 0,63
Sericin-P(OEGMA) Conjugate2	5,7 ± 1,1

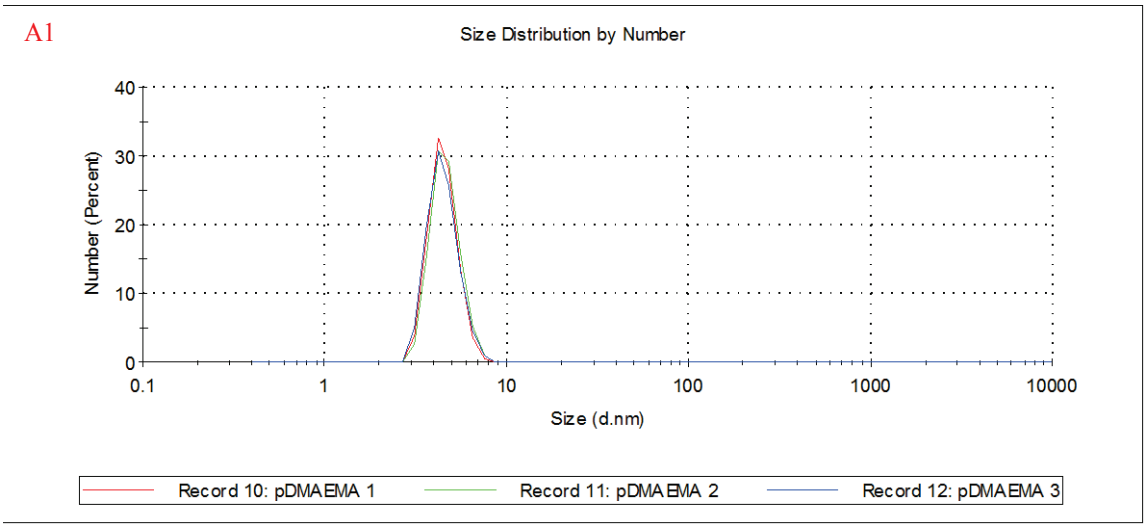
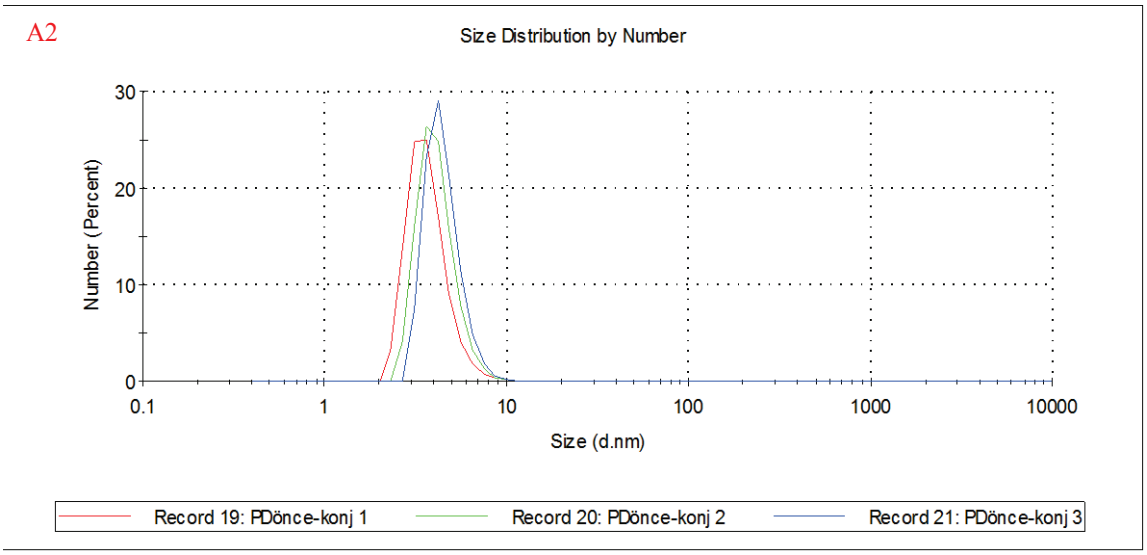
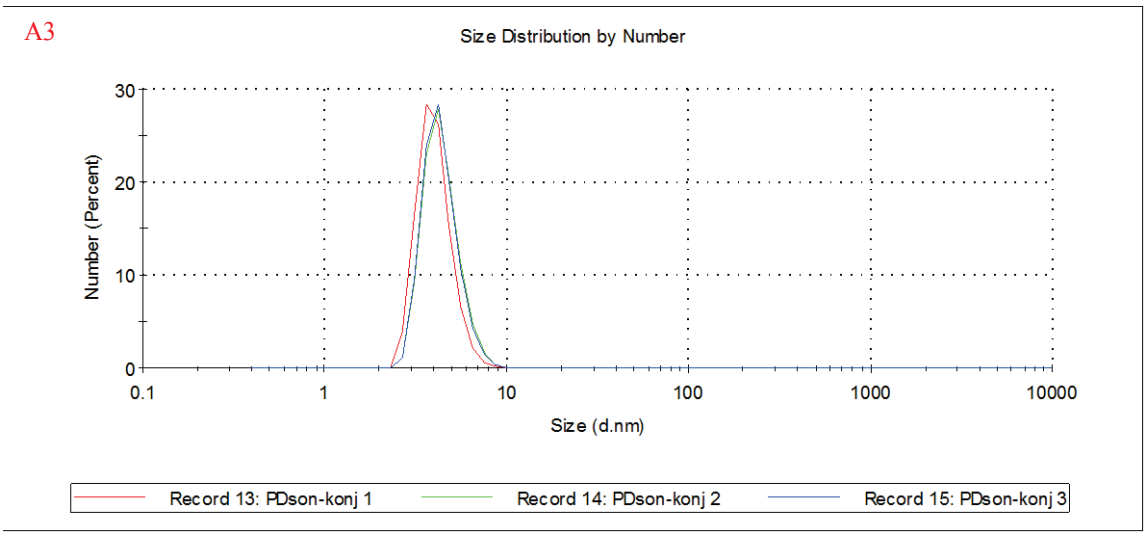


Figure 4.12. Hydrodynamic size distribution of conjugates determined by DLS (A1:P(DMAEMA),A2:Sericin-P(DMAEMA) conjugate1 (polymer:protein molar ratio 0.5:1), A3: Sericin-P(DMAEMA) conjugate2 (polymer:protein molar ratio 10:1); B1:P(OEGMA), B2:Sericin-P(OEGMA) conjugate1 (polymer:protein molar ratio 0.5:1), B3: Sericin-P(OEGMA) conjugate2 (polymer:protein molar ratio 10:1); C1:P(HEMA), C2: Sericin-P(HEMA) conjugate1 (polymer:protein molar ratio 0.5:1), C3: Sericin-P(HEMA) conjugate2 (polymer:protein molar ratio 10:1); D: purchased sericin)

(Cont. on next page)





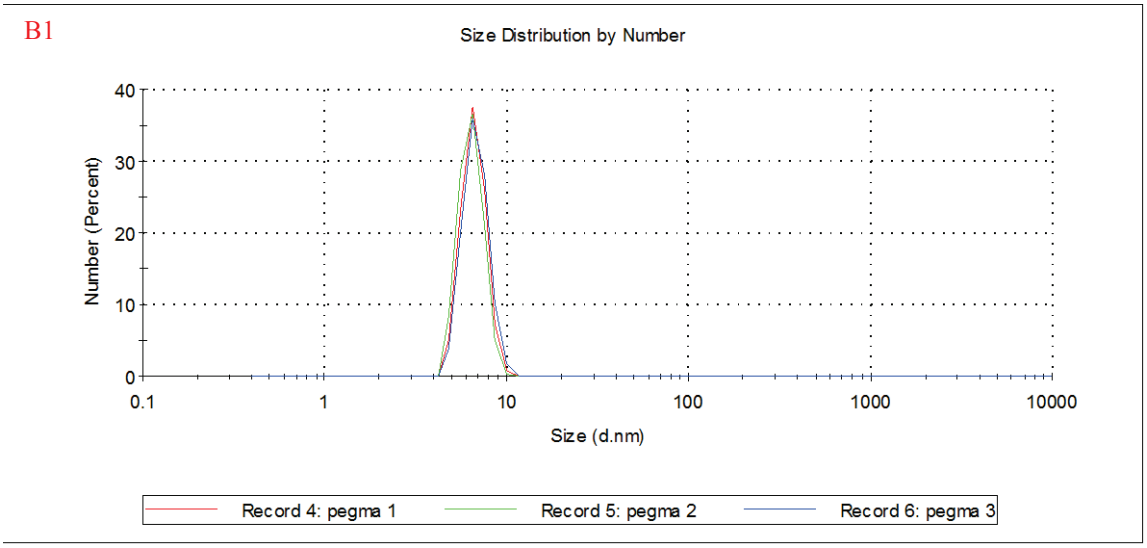
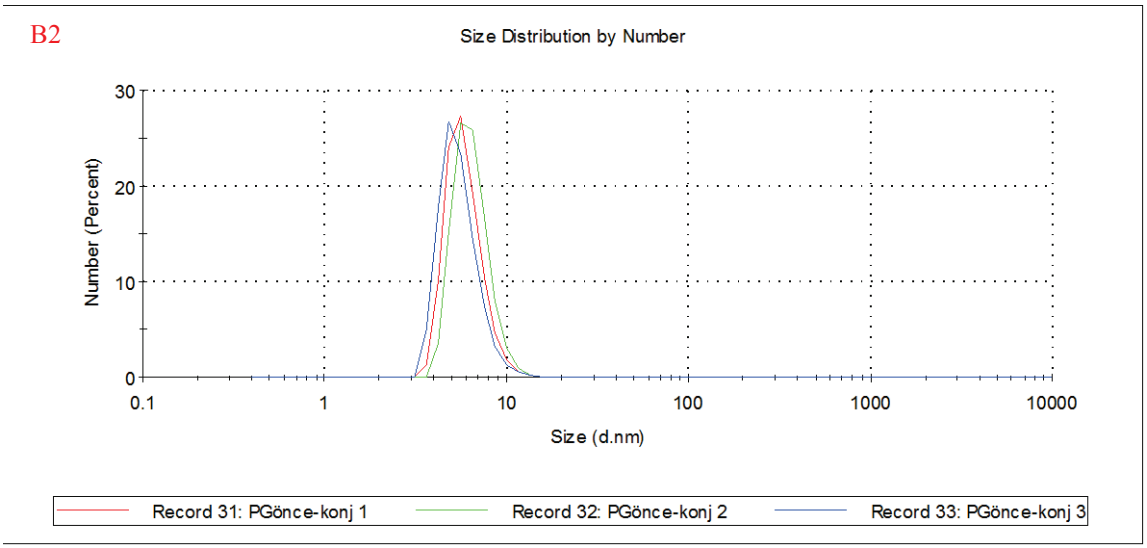
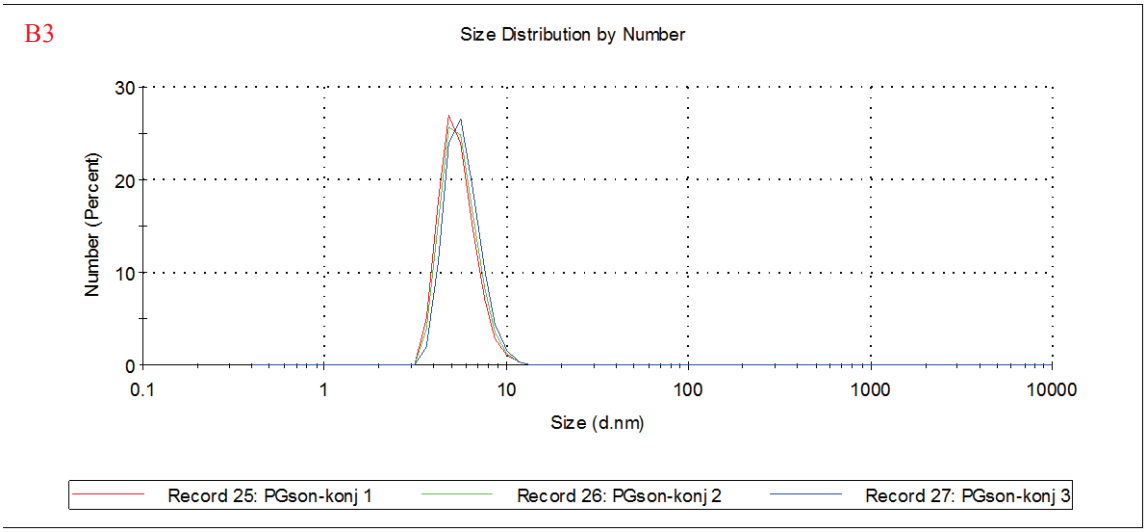


Figure 4.12. (Cont.)

(Cont. on next page)





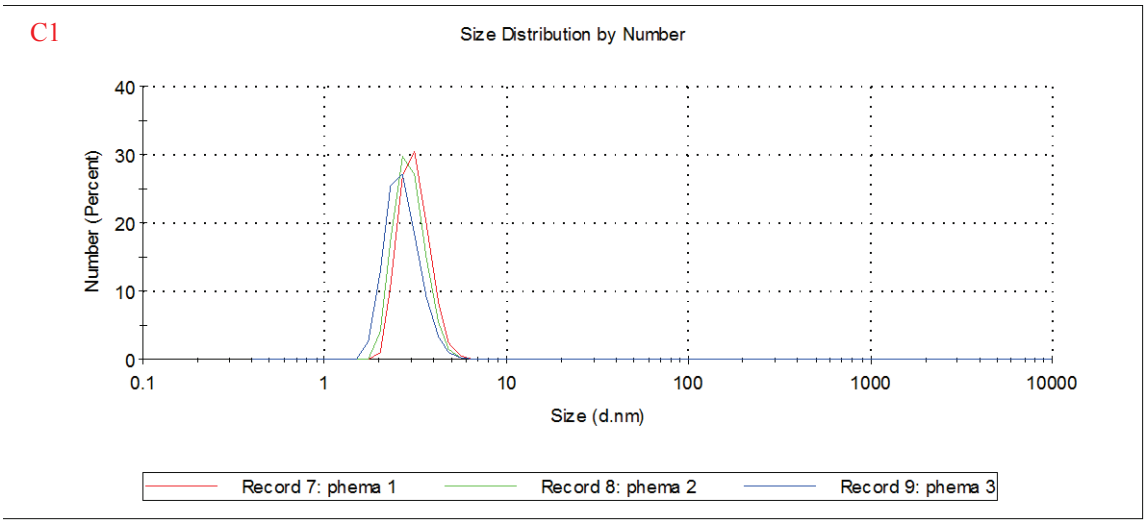
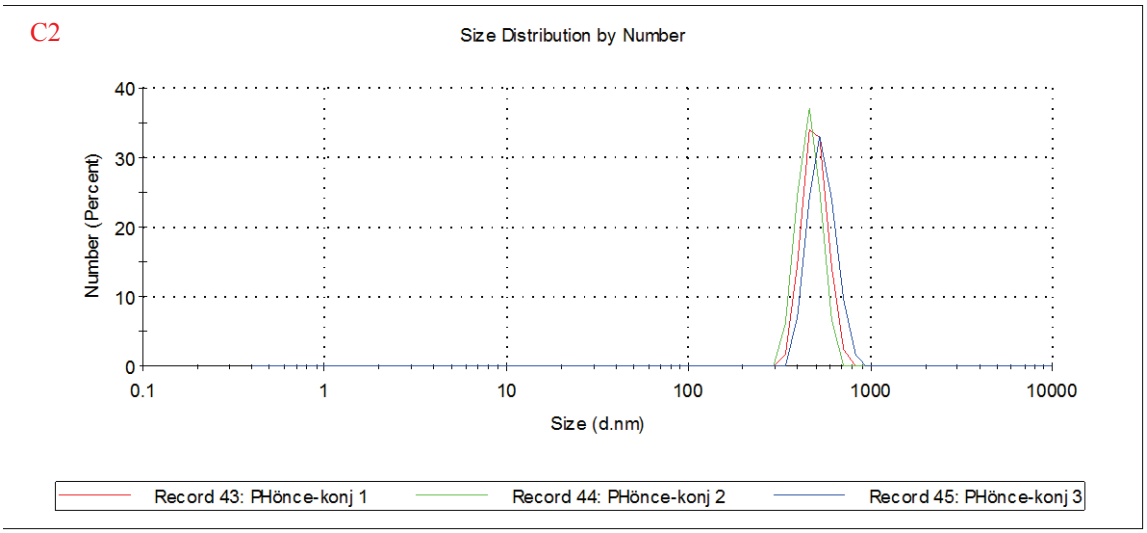
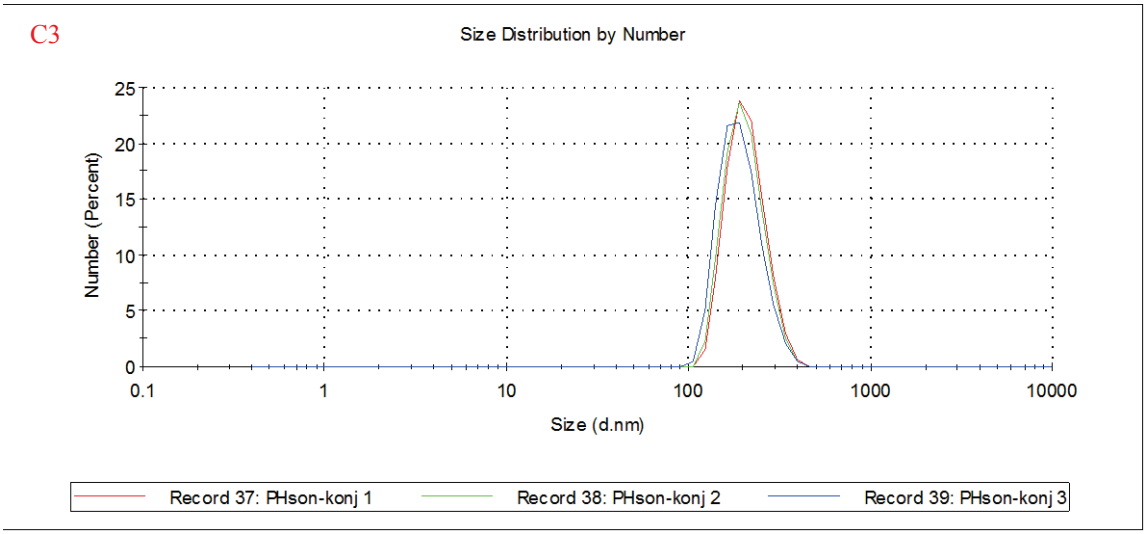


Figure 4.12. (Cont.)

(Cont. on next page)





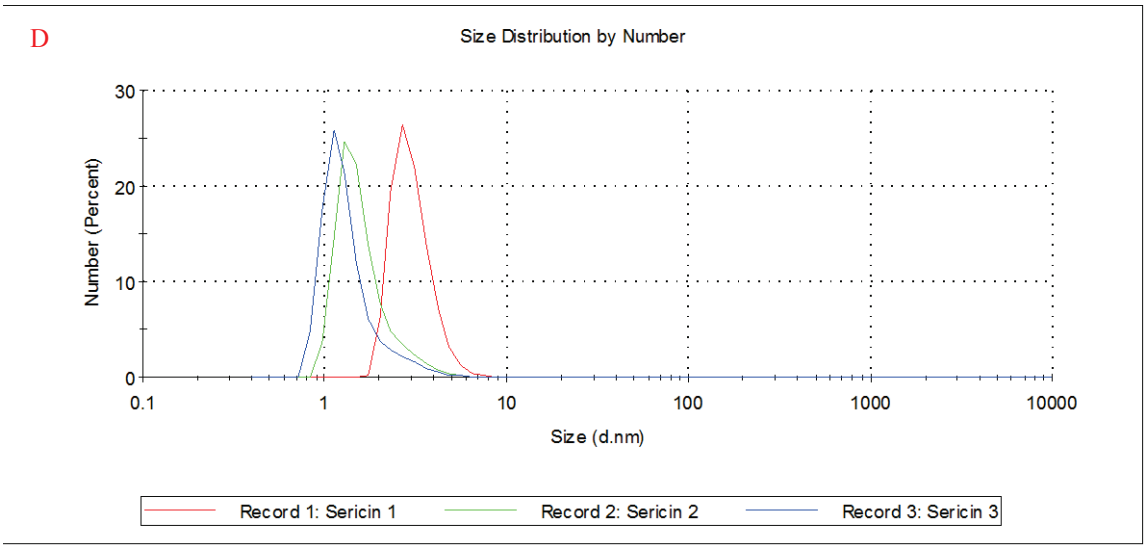


Figure 4.12. (Cont.)

As seen in Figure 4.12, the size of P(DMAEMA) and P(OEGMA) polymers was found to be between 1-10 nm. Since these polymers are highly hydrophilic, they dissolve in PBS and do not form aggregates. In the same way, sericin-P(DMAEMA) and sericin-P(OEGMA) conjugates present as unimers with no sign of self-organization in aqueous solution. Accordingly, the size of these conjugates were between 1-10 nm. Unlike the other two polymers, although the size of P(HEMA) polymer was observed to be 3.24 ± 0.62 nm, conjugate results observed by DLS indicated the presence of self-organization and aggregation of its sericin conjugates in aqeuous solution. Consequently, the sizes of sericin-P(HEMA) conjugates were found to be 530 ± 60.83 and 223.3 ± 25.2 nm. This result also showed sericin-P(HEMA) conjugates have been successfully obtained since the conjugates showed larger sizes when compared with corresponding free polymer, possibly due to the formation of amphihilic protein-polymer conjugate structure.

CHAPTER 5

CONCLUSION

The aim of this thesis is to prepare sericin-polymer conjugates and perform preliminary physicochemical characterizations to provide a preliminary basis for future explorations of the potential use of conjugates in drug delivery, tissue engineering, cosmetics, antioxidant and moisturizers. For this purpose, three polymers i.e. poly(oligoethylene glycol methacrylate), P(OEGMA), poly(hydroxyethylmethacrylate) P(HEMA) and poly(dimethylaminoethyl methacrylate) P(DMAEMA) having the same degree of polymerization (n~26) and different molecular weights of 12,154 g/mol, 3,662 g/mol and 4,052 g/mol, respectively, were first synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization. Via RAFT polymerization, copolymers and homopolymers with controlled molecular weights and narrow polydispersity index (PDI) could be synthesized. Besides, another important feature of RAFT polymerization is its ability to yield polymers with designed end-group functionality, which is required for bioconjugations. In this study, polymers having carboxylic acid functional end-group were synthesized via RAFT polymerization. It should be noted that polymers having the living group (reactive thiocarbonylthio group) synthesized by RAFT polymerization may be an important source of cellular toxicity. However, after the polymerization, it is possible to remove thiocarabonylthio groups by modification of the polymers. The aminolysis reaction is one of the most widely used methods, which iscarried out in the presence of amines to ω-end group (Pissuwan et al., 2010) and producing polymers with thiol end groups (Barner-Kowollik, 2008).

Each polymer was characterized via nuclear magnetic resonance (^{1}H-NMR) and gel permeation chromatography (GPC). The results obtained from GPC measurements and NMR spectroscopy confirmed the successful synthesis of polymers at defined molecular weight and narrow polydispersity under the synthesis conditions. Silk sericin was purchased for conjugation with polymers. Separately sericin was characterized by SDS-PAGE and 2D electrophoresis to determine molecular weight, structure and isoelectric point. The SDS-PAGE electrophoresis results showed that while sericin used in this study was composed of polypeptide fragments between 10 kDa and 72 kDa,

fragments between 10 kDa and 15 kDa predominates in the sample. Also, the 2D electrophoresis showed that protein smears were observed at molecular weights ranging between 3.5 and 35 kDa (which was the range observable under the experimental conditions) and a pI of about 4-4.8. The results were similar to those of Kurioka and Yamazaki (2002).

The final step of the study was to obtain sericin and polymer conjugates via well-known NHS/EDC chemistry using polymer's carboxylic acid (-COOH) end-group and protein's amine groups. Conjugations were made using polymers having the same degree of polymerization (DP) of 26 by NMR and 42 determined by GPC (P(OEGMA) Mn= 20,000 g/mol and PDI= 1.45 ; P(HEMA) Mn= 6,129 g/mol and PDI= 1.21; P(DMAEMA) Mn= 6,400g/mol and PDI= 1.1). Two different conjugation reactions were performed using two different polymer:protein ratios (10:1 and 0.5:1 molar ratio, polymer concentration= 5 mM and 0.9 mM, respectively) in reaction solutions. Also, commercial sericin, commercial sericin after dialysis (MWCO= 3500 Da) (purified sericin), commercial sericin exposed to conjugation conditions without polymers (polymer-free sericin) and free polymers were used as control samples to confirm the presence of conjugates in these characterization procedures. Obtained bioconjugates were analysed by SDS-PAGE, GPC and DLS to verify the formation of conjugates. Overall the SDS-PAGE results indicated the formation of sericin-polymer conjugates for all three different polymers.

Sericin-polymer conjugates prepared at low polymer: protein mol ratio (0.5:1) was examined via GPC. The GPC results of each conjugate were compared with the GPC results of polymers only since sericin only could not be dissolved in organic solvent. Sericin-P(OEGMA) conjugate had significantly longer retention time when compared with that of free P(OEGMA) polymer, clearly indicating the formation of the protein-polymer conjugate. On the other hand, the other two conjugates had slightly different retention time than free polymers, also suggesting the formation of conjugates of polymers possibly with smaller peptide fragments at a lower yield.

Finally, sericin-polymer conjugates were characterized via DLS to determine the hydrodynamic size differences between sericin and its polymer conjugates. DLS revealed that the hydrodynamic size of P(OEGMA) and P(DMAEMA) polymers and their conjugates were between1-10 nm and they dissolve in PBS and do not form aggregates. Unlike the other two polymers, although the size of P(HEMA) polymer was observed to be 3.24 ± 0.62 nm, the DLS result indicated the sizes of sericin-P(HEMA) conjugates to

be 530 ± 60.83 and 223.3 ± 25.2, revealing the presence of self-organization and aggregation of sericin-P(HEMA) conjugates in aqeuous solution. These results showed that the successful preparation of the conjugates.

As conclusion, sericin possesses some important potential application areas like cosmetics, moisturizers, antioxidants and pharmaceuticals. Some investigations have shown that sericin has important properties as a biomaterial such as hydrogel forming and excellent moisture absorbing and desorbing properties. Sericin contains amino acids with strong polar side groups such as hydroxyl, carboxyl, and amino group. Because of this property, sericin can be used in various coating or blending processes with natural and artificial fibers, polymers and fabrics. Through the bioconjugation of sericin with polymers, new drug delivery systems can be developed to reduce immunogenicity and enhance stability of therapeutics in the circulatory system as recent studies have shown that sericin has immunogenic inertness. Thus sericin has many advantages both singly and in conjugation with polymers. This thesis presents new polymer conjugates of sericin having potential as components of new drug delivery systems.

This study can be further developed with the following additional experiments. Amino acid sequence of sericin used in this study may be found by mass spectrometry techniques such as MALDI-TOF. Separately, sericin and sericin-polymer conjugates can be examined by a water GPC since they are not completely soluble in DMAc used as mobile phase in GPC experiments in this study. The properties and effects of sericin-polymer conjugates can be examined using in vitro cultured cells.

REFERENCES

Aamer, K. A., & Tew, G. N. (2007). RAFT polymerization of a novel activated ester monomer and conversion to a terpyridine-containing homopolymer. *Journal of Polymer Science Part A: Polymer Chemistry, 45*(23), 5618-5625.

Abuchowski, A., Van Es, T., Palczuk, N. C., & Davis, F. F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *Journal of Biological Chemistry, 252*(11), 3578-3581.

Adhikari, B., Majumdar, S. (2004). Polymers in sensor applications. *Prog. Polym. Sci., 29*(7), 699-766.

Aqil, A., Qiu, H. J., Greisch, J. F., Jerome, R., De Pauw, E. and Jerome, C. (2008). Coating of gold nanoparticles by thermosensitive poly(N-isopropylacrylamide) end-capped by biotin. *Polymer, 49*(5), 1145-1153.

Anghileri, A., Lantto, R., Kruus, K., Arosio, C., & Freddi, G. (2007). Tyrosinase-catalyzed grafting of sericin peptides onto chitosan and production of protein–polysaccharide bioconjugates. *Journal of Biotechnology, 127*(3), 508-519.

Aramwit, P., & Sangcakul, A. (2007). The effects of sericin cream on wound healing in rats. *Bioscience, Biotechnology, and Biochemistry, 71*(10), 2473-2477.

Aramwit, P., Kanokpanont S., De-Eknamkul W. and Srichana T. (2009). Monitoring of inflammatory mediators induced by silk sericin. *Journal of Bioscience and Bioengineering, 107*(5), 556–561.

Aramwit, P., Damrongsakkul, S., Kanokpanont, S., & Srichana, T. (2010a). Properties and antityrosinase activity of sericin from various extraction methods. *Biotechnology and Applied Biochemistry, 55*(2), 91-98.

Aramwit, P., Kanokpanont, S., Nakpheng, T., & Srichana, T. (2010b). The effect of sericin from various extraction methods on cell viability and collagen production. *International Journal of Molecular Sciences, 11*(5), 2200-2211.

Aramwit, P., Siritientong, T., Kanokpanont, S., & Srichana, T. (2010c). Formulation and characterization of silk sericin–PVA scaffold crosslinked with genipin. *International Journal of Biological Macromolecules, 47*(5), 668-675.

Aramwit, P., Siritientong, T., & Srichana, T. (2012). Potential applications of silk sericin, a natural protein from textile industry by-products. *Waste Management & Research, 30*(3), 217-224.

Aramwit, P., Palapinyo, S., Srichana, T., Chottanapund, S., & Muangman, P. (2013). Silk sericin ameliorates wound healing and its clinical efficacy in burn wounds. *Archives of Dermatological Research, 305*(7), 585-594.

Arshady, R. (2003). Introduction to Polymeric Biomaterials; *Citus Books: London*, p 46.

Barner-Kowollik, C. (2008). Handbook of RAFT polymerization. Darmstadt, Germany, WILEY-VCH.

Beth, A. H., Conturo, T. E., Venkataramu, S. D., & Staros, J. V. (1986). Dynamics and interactions of the anion channel in intact human erythrocytes: an electron paramagnetic resonance spectroscopic study employing a new membrane-impermeant bifunctional spin-label. *Biochemistry, 25*(13), 3824-3832.

Bhawal, S., Reddy, L. H., Murthy, R. S. R., & Devi, S. (2004). Effect of a polymerizable cosurfactant on the microstructure and drug-release properties of nanoparticles synthesized through emulsion polymerization. *Journal of applied polymer science, 92*(1), 402-409.

Bomalaski, J. S., Holtsberg, F. W., Ensor, C. M. and Clark, M. A. (2002). Uricase formulated with polyethylene glycol (uricase-PEG 20): Biochemical rationale and preclinical studies. *J. Rheumatol., 29*(9), 1942–1949.

Boyer, C., Bulmus, V., Davis, T. P., Ladmiral, V., Liu, J., & Perrier, S. b. (2009). Bioapplications of RAFT polymerization. *Chemical reviews, 109*(11), 5402-5436.

Brown, B. N., & Badylak, S. F. (2013). Expanded applications, shifting paradigms and an improved understanding of host–biomaterial interactions. *Acta biomaterialia, 9*(2), 4948-4955.

Broyer, R. M., Grover, G. N., & Maynard, H. D. (2011). Emerging synthetic approaches for protein–polymer conjugations. *Chemical Communications, 47*(8), 2212-2226.

Bulmus, V. (2011). RAFT polymerization mediated bioconjugation strategies. *Polymer Chemistry, 2*(7), 1463-1472.

Caliceti, P., & Veronese, F. M. (2003). Pharmacokinetic and biodistribution properties of poly (ethylene glycol)–protein conjugates. *Advanced drug delivery reviews, 55*(10), 1261-1277.

Capar, G., Aygun, S. S., & Gecit, M. R. (2008). Treatment of silk production wastewaters by membrane processes for sericin recovery. *Journal of membrane science, 325*(2), 920-931.

Cherng, J. Y., Van de Wetering, P., Talsma, H., Crommelin, D. J., & Hennink, W. E. (1997). Freeze-drying of poly ((2-dimethylamino) ethyl methacrylate)-based gene delivery systems. *Pharmaceutical research, 14*(12), 1838-1841.

Chilkoti, A., Chen, G., Stayton, P. S., & Hoffman, A. S. (1994). Site-specific conjugation of a temperature-sensitive polymer to a genetically engineered protein. *Bioconjugate chemistry, 5*(6), 504-507.

Cosgrove, B. D., Cheng, C., Pritchard, J. R., Stolz, D. B., Lauffenburger, D. A., & Griffith, L. G. (2008). An inducible autocrine cascade regulates rat hepatocyte proliferation and apoptosis responses to tumor necrosis factor-α. *Hepatology, 48*(1), 276-288.

Das Dipayam, Pradhan Arun Kumar, Chattopadhyay and Singh S. N. (2012). Mixed fibrous filter media for separation most penetrating particles, *J. Environ. Res. Develop., 7*(1), 146-152.

Denny, J. B., & Blobel, G. (1984). 125I-labeled crosslinking reagent that is hydrophilic, photoactivatable, and cleavable through an azo linkage. *Proceedings of the National Academy of Sciences, 81*(17), 5286-5290.

Ding, B., Yamazaki, M., Shiratori, S. (2005). Electrospun fibrous polyacrylic acid membrane-based gas sensors. *Sens. Actuators, B, 106*(1), 477-483.

Ding, Z., Chen, G., & Hoffman, A. S. (1998). Unusual properties of thermally sensitive oligomer–enzyme conjugates of poly (N-isopropylacrylamide)–trypsin. Journal of Biomedical Materials Research Part A, 39(3), 498-505.

Du, R., & Zhao, J. (2004). Properties of poly (N, N-dimethylaminoethyl methacrylate)/polysulfone positively charged composite nanofiltration membrane. *Journal of membrane science, 239*(2), 183-188.

Epton, R., Marr, G., & Morgan, G. J. (1977). Soluble polymer-protein conjugates: 1. Reactive N-(sym-trinitroaryl) polyacrylamide/acrylhydrazide copolymers and derived carbonic anhydrase conjugates. *Polymer, 18*(4), 319-323.

Fabiani, C., Pizzichini, M., Spadoni, M., & Zeddita, G. (1996). Treatment of waste water from silk degumming processes for protein recovery and water reuse. *Desalination, 105*(1), 1-9.

Falatach, R., Li, S., Sloane, S., Mcglone, C., Berberich, J. A., Page, R. C., Konkolewicz, D. (2015). Why synthesize protein e polymer conjugates ? The stability and activity of chymotrypsin-polymer bioconjugates synthesized by RAFT. *Polymer, 72*, 382–386.

Favier, A., & Charreyre, M. T. (2006). Experimental Requirements for an Efficient Control of Free-Radical Polymerizations via the Reversible Addition-Fragmentation Chain Transfer (RAFT) Process. *Macromolecular Rapid Communications, 27*(9), 653-692.

Franz, S., Rammelt, S., Scharnweber, D., & Simon, J. C. (2011). Immune responses to implants–a review of the implications for the design of immunomodulatory biomaterials. *Biomaterials, 32*(28), 6692-6709.

Freddi, G., Mossotti, R., & Innocenti, R. (2003). Degumming of silk fabric with several proteases. *Journal of Biotechnology, 106*(1), 101-112.

Fuertges, F., & Abuchowski, A. (1990). The clinical efficacy of poly (ethylene glycol)-modified proteins. *Journal of controlled release, 11*(1-3), 139-148.

Ganson, N. J., Kelly, S. J., Scarlett, E., Sundy, J. S., & Hershfield, M. S. (2005). Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly (ethylene glycol)(PEG), in a phase I trial of subcutaneous PEGylated urate oxidase. *Arthritis research & therapy, 8*(1), R12-R22.

Gamo, T., Inokuchi, T., & Laufer, H. (1977). Polypeptides of fibroin and sericin secreted from the different sections of the silk gland in Bombyx mori. *Insect Biochemistry, 7*(3), 285-295.

Garel, A., Deleage, G., & Prudhomme, J. C. (1997). Structure and organization of the Bombyx mori sericin 1 gene and of the sericins 1 deduced from the sequence of the Ser 1B cDNA. *Insect biochemistry and molecular biology, 27*(5), 469-477.

Genc, G., Bayraktar O., BaSal G. (2009). A research on the production of silk sericin powders by using spray drying method. *Tekstil ve Konfeksiyon. 19(4),* 273-279.

Gil, E. S., & Hudson, S. M. (2004). Stimuli-reponsive polymers and their bioconjugates. *Progress in polymer science, 29*(12), 1173-1222.

Gimenes, M. L., Liu, L., & Feng, X. (2007). Sericin/poly (vinyl alcohol) blend membranes for pervaporation separation of ethanol/water mixtures. *Journal of Membrane Science, 295*(1), 71-79.

Gohy, J. F., Antoun, S., & Jérôme, R. (2001). ph-dependent micellization of poly (2-vinylpyridine)-b lock-poly ((dimethylamino) ethyl methacrylate) diblock copolymers. *Macromolecules, 34*(21), 7435-7440.

Golden, A. L., Battrell, C. F., Pennell, S., Hoffman, A. S., J. Lai, J., & Stayton, P. S. (2010). Simple fluidic system for purifying and concentrating diagnostic biomarkers using stimuli-responsive antibody conjugates and membranes. *Bioconjugate chemistry, 21*(10), 1820-1826.

Gregory, A., & Stenzel, M. H. (2012). Complex polymer architectures via RAFT polymerization: From fundamental process to extending the scope using click 41 chemistry and nature's building blocks. *Progress in Polymer Science, 37*(1), 38-105.

Grover, G. N., & Maynard, H. D. (2010). Protein–polymer conjugates: synthetic approaches by controlled radical polymerizations and interesting applications. *Current Opinion in Chemical Biology, 14*(6), 818-827.

Gulrajani, M. L. (1988). Degumming of silk; Silk dyeing printing and finishing. Department of Textile Technology. *Indian Institute of Technology*, New Delhi.

Gupta, D., Agrawal, A., & Rangi, A. (2014). Extraction and characterization of silk sericin. *Indian Journal of Fibre & Textile Research (IJFTR), 39*(4), 364–372.

Hardy, J. G., Römer, L. M., & Scheibel, T. R. (2008). Polymeric materials based on silk proteins. *Polymer, 49*(20), 4309-4327.

Hino, R., Tomita, M., & Yoshizato, K. (2006). The generation of germline transgenic silkworms for the production of biologically active recombinant fusion proteins of fibroin and human basic fibroblast growth factor. *Biomaterials, 27*(33), 5715-5724.

Hoffman, A. S. (1995). "Intelligent" polymers in medicine and biotechnology. *In Macromolecular symposia* (Vol. 98, No. 1, pp. 645-664). Hüthig & Wepf Verlag.

Hoffman, A. S. (1998). A commentary on the advantages and limitations of synthetic polymer-biomolecule conjugates. In: Okano T, editor. Biorelated Functional Polymers: Controlled Release and Applications in Biomedical Engineering. New York: *Academic Press.* p 231–248.

Hoppe, U., Koerbaecher, K., & Roeckl, M. (1984). Hair and bath preparations containing sericin. Ger Offen DE 3233388 A1, 15.

Jun, L., Yaw, J., Li, Y., Hirabayashi, K., & Arai, M. (1997). Studies on physical properties of sericin gel. *Canye Kexe, 23*(1), 47-52.

Karlgard, C. C., Sarkar, D. K., Jones, L. W., Moresoli, C., & Leung, K. T. (2004). Drying methods for XPS analysis of PureVision™, Focus® Night&Day™ and conventional hydrogel contact lenses. *Applied surface science, 230*(1), 106-114.

Kato, N., Sato S., Yamanaka A., Yamada H., Fuwa N., & Nomura M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Bioscience, Biotechnology and Biochemistry 62*(1) 145–147.

Kersey, F. R., Merkel, T. J., Perry, J. L., Napier, M. E., & DeSimone, J. M. (2012). Effect of aspect ratio and deformability on nanoparticle extravasation through nanopores. *Langmuir, 28*(23), 8773-8781.

Khan, F. D., Roychowdhury, S., Nemes, R., Vyas, P. M., Woster, P. M., & Svensson, C. K. (2006). Effect of pro-inflammatory cytokines on the toxicity of the arylhydroxylamine metabolites of sulphamethoxazole and dapsone in normal human keratinocytes. *Toxicology, 218*(2), 90-99.

Kotite, N. J., Staros, J. V., & Cunningham, L. W. (1984). Interaction of specific platelet membrane proteins with collagen: evidence from chemical crosslinking. *Biochemistry, 23*(13), 3099-3104.

Kundu, B., & Kundu, S. C. (2012). Silk sericin/polyacrylamide in situ forming hydrogels for dermal reconstruction. *Biomaterials, 33*(30), 7456-7467.

Kundu, S. C., Dash, B. C., Dash, R., & Kaplan, D. L. (2008). Natural protective glue protein, sericin bioengineered by silkworms: potential for biomedical and biotechnological applications. *Progress in Polymer Science, 33*(10), 998-1012.

Kurioka, A., & Yamazaki, M. (2002). Purification and identification of flavonoids from the yellow green cocoon shell (Sasamayu) of the silkworm, Bombyx mori. *Bioscience, Biotechnology, and Biochemistry, 66*(6), 1396-1399.

Lamboni, L., Gauthier, M., Yang, G., & Wang, Q. (2015). Silk sericin: a versatile material for tissue engineering and drug delivery. *Biotechnology Advances, 33*(8), 1855-1867.

Lee, S.D., Hsiue, G.H., Kao, C.Y., & Chang, P.C.T. (1996). Artificial cornea: surface modification of silicone rubber membrane by graft polymerization of pHEMA via glow discharge. *Biomaterials, 17*(6), 587–595.

MacLean-McDavitt, D. S., Robertson, J. D., & Jay, M. (2003). Monitoring the in vivo delivery of proteins from carbomer hydrogels by X-ray fluorescence. *Pharmaceutical research, 20*(3), 435-441.

Magnusson, J. P., Bersani, S., Salmaso, S., Alexander, C., & Caliceti, P. (2010). In Situ Growth of Side-Chain PEG Polymers from Functionalized Human Growth Hormone's A New Technique for Preparation of Enhanced Protein− *Polymer Conjugates*. *Bioconjugate chemistry, 21*(4), 671-678.

Mahltig, B., Müller-Buschbaum, P., Wolkenhauer, M., Wunnicke, O., Wiegand, S., Gohy, J. F., ... & Stamm, M. (2001). Highly regular polyampholytic structures adsorbed directly from solution. *Journal of colloid and interface science, 242*(1), 36-43.

Mandal, B. B., & Kundu, S. C. (2009). Self-assembled silk sericin/poloxamer nanoparticles as nanocarriers of hydrophobic and hydrophilic drugs for targeted delivery. *Nanotechnology, 20*(35), 355101.

Mandal, B. B., Priya, A. S., & Kundu, S. C. (2009). Novel silk sericin/gelatin 3-D scaffolds and 2-D films: fabrication and characterization for potential tissue engineering applications. *Acta Biomaterialia, 5*(8), 3007-3020.

Minoura, N., Aiba, S. I., Gotoh, Y., Tsukada, M., & Imai, Y. (1995). Attachment and growth of cultured fibroblast cells on silk protein matrices. *Journal of Biomedical Materials Research Part A, 29*(10), 1215-1221.

Moad, G., Rizzardo E., Thang S. H. (2005). Living radical polymerization by the RAFT process. *Aust J Chem 58*, 379-410.

Mondal, M., Trivedy, K., & Kumar, S. N. (2007). The silk proteins, sericin and fibroin in silkworm. *Bombyx mori Linn., - a review, 5*(2), 63–76.

Muramatsu, M., Kanada, K., Nishida, A., Ouchi, K., Saito, N., Yoshida, M., Shimoaka, A., Ozeki, T., Yuasa, H., Kanaya, Y. (2000). Application of Carbopol to controlled release preparations I. Carbopol as a novel coating material. *Int. J. Pharm., 119*(1), 77-83.

Nakajima, N., & Ikada, Y. (1995). Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. *Bioconjugate Chemistry, 6*(1), 123-130.

Nakamura K., & Hatakeyama H. (1984). Graft copolymerization of vinyl monomers on to sericin. *Sen'I Gakkaishi 40*(9), T327–T331.

Nayak, S., & Kundu, S. C. (2014). Sericin–carboxymethyl cellulose porous matrices as cellular wound dressing material. *Journal of Biomedical Materials Research Part A, 102*(6), 1928-1940.

Nicolas, J., Mantovani, G., & Haddleton, D. M. (2007). Living Radical Polymerization as a Tool for the Synthesis of Polymer-Protein/Peptide Bioconjugates. *Macromolecular rapid communications, 28*(10), 1083-1111.

Nishimura, H., Ashihara, Y., Matsushima, A., & Inada, Y. (1979). Modification of yeast uricase with polyethylene-glycol-disappearance of binding ability towards anti-uricase serum. *Enzyme, 24*(4), 261–264.

Ogawa A., & Yamada H. (1999). Antiaging cosmetic containing sericin or hydrolysates and saccharomyces extracts. *Japan Patent Publication Number 11193210 A2 to Noevier Co Ltd. and Seiren Co Ltd*.

Padamwar, M. N., & Pawar, A. P. (2004). Silk sericin and its applications: A review. *J. Sci. Ind. Res., 63*(1), 323-329.

Padamwar, M. N., Pawar, A. P., Daithankar, A. V., & Mahadik, K. R. (2005). Silk sericin as a moisturizer: an in vivo study. *Journal of Cosmetic Dermatology, 4*(4), 250-257.

Padol, A. R., Jayakumar, K., Shridhar, N. B., Narayana Swamy, H. D., Mohan, K., & Manochaya, S. (2012). Efficacy of the silk protein based biofilms as a novel wound healing agent. *Int J Toxicol Appl Pharmacol, 2*(3), 31-6.

Panilaitis, B., Altman, G. H., Chen, J., Jin, H. J., Karageorgiou, V., & Kaplan, D. L. (2003). Macrophage responses to silk. *Biomaterials, 24*(18), 3079-3085.

Patel, R. J., Modasiya, M. K. (2011). Sericin: Pharmaceutical Applications. *Int J Res Pharm Biomed Sci 2*, 913-917.

Patten, T. E., & Matyjaszewski, K. (1998). Atom transfer radical polymerization and the synthesis of polymeric materials. *Advanced Materials, 10*(12), 901-915.

Pissuwan, D., Boyer, C., Gunasekaran, K., Davis, T. P., & Bulmus, V. (2010). In vitro cytotoxicity of RAFT polymers. *Biomacromolecules, 11*(2), 412-420.

Pushpa, A., Vishnu, B. G., & Reddy, T. K. (2013). Preparation of nano silk sericin based hydrogels from silk industry waste. *Journal of Environmental Research And Development, 8*(2), 243.

Rayment, E. A., Dargaville, T. R., Shooter, G. K., George, G. A., & Upton, Z. (2008). Attenuation of protease activity in chronic wound fluid with bisphosphonate-functionalised hydrogels. *Biomaterials, 29*(12), 1785-1795.

Rui, H. G. (1998). Quality of Cocoon Filament. *Oxford & IBH Publication Co. Pvt. Ltd., New Delhi,* 58-69.

Sarovart, S., Sudatis, B., Meesilpa, P., Grady, B. P., & Magaraphan, R. (2003). The use of sericin as an antioxidant and antimicrobial for polluted air treatment. *Rev Adv Mater Sci, 5*(3), 193-198.

Schiraldi, C., D'Agostino, A., Oliva, A., Flamma, F., De Rosa, A., Apicella, A. and De Rosa, R.M. (2004). Development of hybrid materials based on hydroxyethylmethacrylate as supports for improving cell adhesion and proliferation. *Biomaterials, 25*(17), 3645–3653.

Sciannamea, V., Jérôme, R., & Detrembleur, C. (2008). In-situ nitroxide-mediated radical polymerization (NMP) processes: their understanding and optimization. *Chemical reviews, 108*(3), 1104-1126.

Shaw, J. T. B., & Smith, S. G. (1951). Amino-acids of silk sericin. *Nature, 168*(4278), 745-745.

Siritienthong, T., Ratanavaraporn, J., & Aramwit, P. (2012). Development of ethyl alcohol-precipitated silk sericin/polyvinyl alcohol scaffolds for accelerated healing of full-thickness wounds. *International journal of pharmaceutics, 439*(1), 175-186.

Siritientong, T., Angspatt, A., Ratanavaraporn, J., & Aramwit, P. (2014). Clinical potential of a silk sericin-releasing bioactive wound dressing for the treatment of split-thickness skin graft donor sites. *Pharmaceutical research, 31*(1), 104-116.

Sperague, K. (1975). Bombyx mori silk proteins. Characterization of large polypeptides. *Biochemistry, 14*(5), 925-931.

Staros, J. V. (1982). N-hydroxysulfosuccinimide active esters: bis (N-hydroxysulfosuccinimide) esters of two dicarboxylic acids are hydrophilic, membrane-impermeant, protein cross-linkers. *Biochemistry, 21*(17), 3950-3955.

Stayton, P. S., Shimoboji, T., Long, C., Chilkoti, A., Chen, G., Harris, J. M., Hoffman, A. S. (1995). Control of protein-ligand recognition using a stimuli-responsive polymer. *Nature,378*(6556), 472–474.

Stukel, J. M., Li, R. C., Maynard, H. D., & Caplan, M. R. (2009). Two-step synthesis of multivalent cancer-targeting constructs. *Biomacromolecules, 11*(1), 160-167.

Sundy, J. S., Ganson, N. J., Kelly, S. J., Scarlett, E. L., Rehrig, C. D., Huang W., & Hershfield, M. S. (2007). Pharmacokinetics and pharmacodynamics of intravenous PEGylated recombinant mammalian urate oxidase in patients with refractory gout. *Arthritis Rheum., 56*(3), 1021–1028.

Tamada, Y., Sano, M., Niwa, K., Imai, T., & Yoshino, G. (2004). Sulfation of silk sericin and anticoagulant activity of sulfated sericin. *Journal of Biomaterials Science, Polymer Edition, 15*(8), 971-980.

Tasdelen, M. A., Kahveci, M. U., & Yagci, Y. (2011). Progress in Polymer Science Telechelic polymers by living and controlled / living polymerization methods. *Progress in Polymer Science, 36*(4), 455–567.

Terada, S., Sasaki, M., Yanagihara, K., & Yamada, H. (2005). Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture. *Journal of Bioscience and Bioengineering, 100*(6), 667-671.

Thordarson, P., Le Droumaguet, B., & Velonia, K. (2006). Well-defined protein–polymer conjugates—synthesis and potential applications. *Applied microbiology and biotechnology, 73*(2), 243-254.

Vaithanomsat, P., & Kitpreechavanich, V. (2008). Sericin separation from silk degumming wastewater. *Separation and Purification Technology, 59*(2), 129-133.

Vandermeulen, G. W., & Klok, H. A. (2004). Peptide/protein hybrid materials: enhanced control of structure and improved performance through conjugation of biological and synthetic polymers. *Macromolecular Bioscience, 4*(4), 383-398.

Van de Wetering, P., Schuurmans-Nieuwenbroek, N. M. E., Van Steenbergen, M. J., Crommelin, D. J. A., & Hennink, W. E. (2000). Copolymers of 2-(dimethylamino) ethyl methacrylate with ethoxytriethylene glycol methacrylate or N-vinyl-pyrrolidone as gene transfer agents. *Journal of controlled release, 64*(1), 193-203.

van Dijk-Wolthuis, W. N. E., Van de Wetering, P., Hinrichs, W. L. J., Hofmeyer, L. J. F., Liskamp, R. M. J., Crommelin, D. J. A., & Hennink, W. E. (1999). A versatile method for the conjugation of proteins and peptides to poly [2-(dimethylamino) ethyl methacrylate]. *Bioconjugate chemistry, 10*(4), 687-692.

Veronese, F. M., & Morpurgo, M. (1999). Bioconjugation in pharmaceutical chemistry. *Il Farmaco, 54*(8), 497-516.

Veronese, F. M. (2001). Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials, 22*(5), 405-417.

Vicent, M. J., & Duncan, R. (2006). Polymer conjugates: nanosized medicines for treating cancer. *Trends in biotechnology, 24*(1), 39-47.

Voegeli, R., Meier, J. & Blust, R. (1993). Sericin silk protein: unique structure and properties. *Cosmet Toilet, 108*; 101-108.

Vuoriluoto, M., Orelma, H., Johansson, L. S., Zhu, B., Poutanen, M., Walther, A., … Rojas, O. J. (2015). Effect of Molecular Architecture of PDMAEMA-POEGMA Random and Block Copolymers on Their Adsorption on Regenerated and Anionic Nanocelluloses and Evidence of Interfacial Water Expulsion. *Journal of Physical Chemistry B, 119*(49), 15275–15286.

Wei, D. Q., Li, G. J., Tao, J. L., Liu, Z. H., & Zhang, X. M. (1989). Investigation of the graft copolymerization of styrene onto silk sericin. *Acta Polymerica Sinica, 6*, 740-744.

Willcock, H., & O'Reilly, R. K. (2010). End group removal and modification of RAFT polymers. *Polymer Chemistry, 1*(2), 149-157.

Wilusz, R. E., Weinberg, J. B., Guilak, F., & McNulty, A. L. (2008). Inhibition of integrative repair of the meniscus following acute exposure to interleukin-1 in vitro. *Journal of Orthopaedic Research, 26*(4), 504-512.

Wu, C., Tian, B., Zhu, D., Yan, X., Cheng, W., Xu, G., Guo, Y., Wu, Y., Jia, S. (1996). Wound protection film and its preparation method. Faming Zhuanli Shenging Gongai Shuomingshu CN 1121836 (to Suzhou Silk Engineering Collage, China), 8 May 11996; *Chem Abstr. 130*:100662.

Wu, J. H., Wang, Z., & Xu, S. Y. (2007). Preparation and characterization of sericin powder extracted from silk industry wastewater. *Food Chemistry, 103*(4), 1255-1262.

Xu, J., Boyer, C., Bulmus, V., & Davis, T. P. (2009). Synthesis of dendritic carbohydrate end-functional polymers via RAFT: Versatile multi-functional precursors for bioconjugations. *Journal of Polymer Science Part A: Polymer Chemistry, 47*(17), 4302-4313.

Yamada, H., Fuwa, N., & Nomura, M. (1993). Synthetic fiber having improved hygroscopicity. *Japan patent.*

Yamada, H., & Fuwa, N. (1994). Protein-containing synthetic high molecular material and its preparation. *Japan patent*.

Yamada, H., Fuha, Y., Yuri, O., Obayashi, M., & Arashima, T. (1998). Collagen formation promoters containing sericin or its hydrolyzates and antiaging cosmetics. *Jpn Kokai Tokkyo Koho, JP* 10226653 A2, 8.

Yamada. H., Yamasaki, K., Zozaki, K. (2001). Nail cosmetics containing sericin. *Chem Abstra 134*(14), p 15.

Yao, Y. Q., Bian, P. F., Chen, W. X., Ling, R. G., & Shen, Z. Q. (2003). Synthesis and properties of degradable graft copolymer of vinyl acetate and sericin. *Chemical Journal of Chinese Universities-Chinese, 24*(12), 2327-2329.

Yoshii, F., Kume, N., Makuuchi, K. and Sato, F. (2000). Hydrogel composition containing silk protein. *Japan Patent 06*-017373A.

Zhang, Y. Q. (2002). Applications of natural silk protein sericin in biomaterials. *Biotechnology Advances, 20*(2), 91-100.

Zhang, Y. Q., Tao, M. L., Shen, W. D., Zhou, Y. Z., Ding, Y., Ma, Y., & Zhou, W. L. (2004). Immobilization of L-asparaginase on the microparticles of the natural silk sericin protein and its characters. *Biomaterials, 25*(17), 3751-3759.

Zhang, Y. Q., Tao, M. L., Shen, W. D., Mao, J. P., & Chen, Y. H. (2006a). Synthesis of silk sericin peptides–L-asparaginase bioconjugates and their characterization. *Journal of Chemical Technology and Biotechnology, 81*(2), 136-145.

Zhang, Y. Q., Ma, Y., Xia, Y. Y., Shen, W. D., Mao, J. P., & Xue, R. Y. (2006b). Silk sericin–insulin bioconjugates: Synthesis, characterization and biological activity. *Journal of Controlled Release, 115*(3), 307-315.

Zhang, Y., Liu, J., Huang, L., Wang, Z., & Wang, L. (2015). Design and performance of a sericin-alginate interpenetrating network hydrogel for cell and drug delivery. *Scientific reports, 5*, 12374.

Zhaorigetu, S., Sasaki, M., Watanabe, H., & Kato, N. (2001). Supplemental silk protein, sericin, suppresses colon tumorigenesis in 1, 2-dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. *Bioscience, Biotechnology, and Biochemistry, 65*(10), 2181-2186.