MODIFICATION OF BIOPOLYELECTROLYTES FOR BIOADHESIVE APPLICATIONS

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B.S., Chemistry, Abant İzzet Baysal University, 2009

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Chemistry
Boğaziçi University

2012

MODIFICATION OF BIOPOLYELECTROLYTES FOR BIOADHESIVE APPLICATIONS

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DATE OF APPROVAL: 02.07.2012

To my family

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my thesis advisor Assist. Prof. A. Başak Kayıtmazer for her support, endless patience and assistance throughout my studies. Everything I have learned about scientific research is thanks to her. There is no words to thank her enough for giving me the opportunity to work in this project and being always available whenever I need guidance.

I would like to thank the members of my committee: Prof. Duygu Avcı Semiz and Assist. Prof. Bora Garipcan for giving their valuable time in reviewing this thesis and for all the advices.

I would also like to thank Dr. Ayla Türkekul and Burcu Selen Çağlayan for NMR analysis and Dr. Bilge Gedik Uluocak for zeta potential analysis.

I thank to all members of Kayıtmazer Research Group, Alaaddin Faruk Köksal, Melike Belenli, Pınar Ansen for their help in my research and especially to Nihan Ataalp for her additional support and friendship. I must express my greatfulness to Ahmet Erdem, Murat Burak Türk and Hasan H. İnce for their endless friendship and valuable discussions. I would also like to thank all my friends, colleagues and all the members of the Chemistry Department.

Finally, my deepest thanks are for my dear family. They have always been there with their loving hearts and endless support whenever I needed.

This research has been supported in part by grant by Bogazici University Research Fund (BAP grant no: 5072) and European Union 7th Framework Marie Curie International Reintegration Grant (Grant no: 256498)

ABSTRACT

MODIFICATION OF BIOPOLYELECTROLYTES FOR BIOADHESIVE APPLICATIONS

Biomimetic research on the development of bioadhesives mimicing the adhesive proteins secreted by blue mussels have become very popular in the recent years. The most important feature of the adhesive proteins is that they can stick to any type of surfaces in dry or wet environment. Furthermore, these proteins can preserve their bulk adhesive properties over time in the presence of water in which most adhesives fail to manage. 3,4-dihydroxy-L-phenylalanine (L-DOPA) is an aminoacid found in the adhesive proteins of marine mussels. L-DOPA is the most abundant molecule at the interface of adhesion and the catechol group of DOPA is believed to be the key funtional group in mussel attachment. There have been numerous studies on catechol functionalization of polymers to mimic the adhesive feature of mussel adhesive proteins. However, the advantage of our method is that the polymers were modified by forming strong amide bond, which is more stable against hydrolysis, using catechol containing molecules. In this study, the catechol modification of two biopolyelectrolytes; hyaluronic acid (HA) and chitosan (CHI) with L-DOPA-Methyl ester and 3,4-dihydroxyhydrocinnamic acid (DOHA), respectively, was achieved via carbodiimization of active esters. The modification was confirmed with ^{1}H-NMR analysis, UV spectroscopy and gelation with periodate. The degrees of modification (% by mole) of polymers were calculated by UV-Vis spectroscopy. Potentiometric titrations were also carried out to investigate the effect of modification on pK_a of biopolymers. Higher pK_a values of modified polymers than that of unmodified ones also confirmed the modification.

ÖZET

BİYOYAPIŞTIRICI UYGULAMALARI İÇİN BİYOPOLİELEKTROLİTLERİN MODİFİKASYONU

Midyelerin salgılamış olduğu yapışkan proteinleri taklit eden biyoyapıştırıcıların geliştirilmesi üzerine yapılan biyomimetik araştırmalar son yıllar da çok popüler bir hale gelmiştir. Bu yapışkan proteinlerin en önemli özelliği ıslak veya kuru ortamda hemen her türlü malzemeye rahatlıkla yapışabilmeleridir. Bunun da ötesinde, bu proteinler yapışkanlık özelliklerini, birçok yapışkanın aksine, sulu ortam içerisinde de uzun zaman muhafaza edebilmektedir. 3,4-dihidroksi-L-fenilalanin (L-DOPA) bu proteinlerin yapısında bulunan bir aminoasittir. L-DOPA yapışma arayüzeyinde en yoğun oranda bulunan maddedir ve sahip olduğu katekol fonksiyonel grubunun midye yapışmasında anahtar rol oynadığı düşünülmektedir. Midye yapışkan proteinlerinin özelliklerini taklit etmek amacıyla polimerlerin katekol ile fonksiyonellenmesi üzerine bir çok çalışma yapılmıştır. Fakat bizim yöntemimizin avantajı, polimerlerin katekol içeren moleküllerle hidrolize karşı daha dayanıklı amid bağı oluşturularak modifiye edilmesidir. Bu çalışmada, hyalüronik asit ve kitosanın modifikasyonu, sırasıyla, katekol grubuna sahip L-DOPA ve 3,4-dihidroksihidrosinamik asit (DOHA) ile karbodimid kimyası kullanılarak gerçekleştirilmiştir. Modifikasyon ^{1}H-NMR analizi, UV spektrometri ve periyodat jelleşmesi yöntemleri ile teyit edilmiştir. Polimerlerin modifikasyon oranları (% mol olarak) UV-Görünür spektroskopisi yöntemiyle hesaplanmıştır. Ayrıca, potansiyometrik titrasyon ile modifkasyonun polimerlerin denge sabitine (pK_a) olan etkisi incelenmiştir. Modifiye edilen polimerlerin modifiye edilmemişlerden yüksek pK_a değerine sahip olması da modifikasyonu teyit etmiştir.

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LIST OF SYMBOLS

% (w/v)	Mass-volume Percentage
g	Gram
hr	Hour
kcal	Kilocalorie
kDa	Kilo Daltons
kPa	Kilo Pascal
mg	Milligram
min	Minute
ml	Millilitre
MPa	Mega Pascal
Mw	Weight Average Molecular Weight
Pa	Pascal
pK_a	Acid Dissociation Constant
v/v	Volume-volume ratio
μm	Micrometer

LIST OF ACRONYMS/ABBREVIATIONS

Aam	Acrylamide
AFM	Atomic Force Microscopy
Ala	Alanine
CA	Cyanoacrylate
Ca	Calcium
CHI	Chitosan
DCC	N,N'-Dicyclohexylcarbodiimide
DD	Degrees of Deacetylation
DOHA	3,4-dihydroxy hydrocinnamic acid
DOPA	3,4-dihydroxy-L-phenylalanine
DOPA-OMe	3,4-dihydroxy-L-phenylalanine-methyl ester
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide Hydrochloride
HA	Hyaluronic Acid
HCl	Hydrochloric Acid
Hyp	Hydroxyproline
L-DOPA	3,4-dihydroxy-L-phenylalanine
Lys	Lysine
MAEP	Monoacryloxyethyl Phosphate
MAP	Mussel Adhesive Proteins
Mefp	Mytilus Edulis Foot Protein
Mg	Magnesium
MOPS	3-(N-Morpholino)propanesulfonic acid
MWCO	Molecular Weight Cut-off
NaCl	Sodium Chloride
$NaIO_4$	Sodium Metaperiodate
NaOH	Sodium Hydroxide
NHS	N-hydroxysuccinamide
NMR	Nuclear Magnetic Resonance Spectroscopy
PBS	Phosphate Buffer Saline

Pc	Phragmatopoma Californica
PEG	Poly(ethylene glycol)
PEG-dA	Poly(ethylene glycol)-diacrylate
Plu-SH	Pluronic Acid –Thiol
Pro	Proline
Ser	Serine
$SOCl_2$	Thionyl Chloride
Thr	Threonine
Tyr	Tyrosine
UV-Vis	Ultraviolet-Visible

1. INTRODUCTION

1.1. Biological Adhesives

An adhesive is a material connecting two interfaces to each other by surface bonding. Glue, paste, adhesive cement and gum are some of the examples used as synonyms of the term “adhesive”. A classification could be made among the adhesives on the basis of their setting mechanisms such as;

(i) curing in the absence of a chemical reaction (i.e. by applying heat or pressure)

(ii) curing via a chemical reaction (i.e. polymerization or crosslinking reaction)

The raw materials of the adhesives might originate from synthetic or natural sources. Polymer-based glues (i.e. epoxy resins) and casein (a phosphoprotein in milk) are examples for synthetic and natural adhesives, respectively. Having a variety of different raw materials, adhesives are being used in the daily life; for instance, in sticking two papers together as well as for industrial purposes.

In addition to the adhesive usage in many different areas from automotive to textile industries, adhesives have been used in medical applications for more than 4000 years. The term “bioadhesive” is used when the raw material is either a natural product or a synthetic material mimicking natural adhesives. Today, there is a huge interest in developing biological adhesives because of their potential applications inside or outside of the body such as tissue sealing [1]. Other motivations for this interest are the advantages of bioadhesives over sutures such as (i) avoiding the necessity of pinching a foreign substance such as a needle through the skin, and (ii) probability of scar to leave a permanent mark after the removal of the suture. Since bioadhesives are developed to be applied on the human tissue, it is required to have certain characteristics such as below [2-4]:

- Strong adhesive characteristics in the presence of other tissue elements such as proteins and fats.
- Ability to replace water molecules at the boundary layer betwwen the surface and the adhesive to have a maximum strength of adhesion.
- Adequate flow ability in its fluid state so that the bioadhesive can be applied easily.
- Quick and easy preparation in the surgery conditions.
- Rapid curing time under various conditions (i.e. physical conditions of the operating room) to minimize the duration of surgery.
- Following its curing, stability of the adhesive in the presence of mechanical force (tension, shear, compression)
- No release of heat upon curing (no exothermic reaction)
- Good adhesion to medical and dental implants.
- Biodegradable, biocompatible, nontoxic, and easily sterilizable without causing any damage to its properties.

The list above, all the preferred qualities of an ideal bioadhesive. There are already commercially available medical adhesives which meet some of the properties listed above. The ongoing research interest on bioadhesives focuses on developing bioadhesives that meet almost all of the listed properties above.

1.2. Commercial Bioadhesives and Their Disadvantages

Cyanoacrylates (CA) are the most widely used commercially available adhesives for medical applications such as hemostasis, blepharoplasty and wound closure. The adhesion strength of cyanoacrylates has a range of 10^4 to 10^6 Pa at the early stages of the treatment. However, the CA adhesion strength decreases over time in wet environments, especially if shorter acrylic esters are used [5]. Considering the low stability of shorter acrylates like methyl and ethyl, longer chain (such as butyl) acrylates are preferred. Longer acrylates degrade slower than the shorter ones and cause less inflammation of tissues [6]. The problem, however, with the longer CAs is that the curing time increases due to the slower rate of polymerization of the long alkyl chains. Another disadvantage of CA-based glues is the formaldehyde release, upon degradation [7]. Although the release is low in

concentration and could be lessened by choosing the optimum length of alkyl chains, even small amounts of formaldehyde is toxic which would lead to tissue inflammation.

Fibrin glue is another example for commercially available medical adhesives. Our body produces this bioadhesive in cases of injury or bleeding. Fibrinogen is cleaved with the help of thrombin to form fibrin which, then, precipitates and forms a mesh-like hydrogel; i.e. blood coagulation. Fibrin adhesives imitate the coagulation process and have been used as tissue glues[8], hemostatic agents [9], and tissue sealents. On the one hand, fibrin-type adhesives are biocompatible, non-toxic and rapidly curable, on the other hand, they have low adhesion strength (10^3 Pa) to tissues and bones [10]. High risk of transmission of pathogens such as viruses is a major concern in fibrin glues since fibrin is primarily obtained from blood.

The third commercially available bioadhesive is based on gelatin, which is a protein found in skin. Gelatin-based glues include formaldehyde and resorcinol [11]. Resorcinol is introduced into gelatin to increase adhesive strength while formaldehyde is used as a crosslinking agent between gelatin and resorcinol. Gelatin-based adhesives have better adhesion strength than fibrin (10^5 Pa) [10], but presence of formaldehyde in the main formula of these adhesives is still a disadvantage.

1.2. Biomimetics

Biomimetics studies imitation or adaption of functional biological particularities of nature with the help of biotechnology. Biomimesis, biomimicry, bionics, biognosis are all used as synonyms of the term “biomimetics”. Several disciplines ranging from chemistry to electronics have taken advantage of biologically inspired designs.

Inspiration from nature has a long history, i.e first artificial silk was produced in 1855 [12]. Numerous examples of bioinspired technology or materials have been made in the known history of humanity. Inspired by eyes [13] and wings of insects[14], and leaves of plants growing in tropical forests [15], anti-reflective surfaces, which enhanced the capture of light by 10% in solar panels, have been developed. Lotus flower has been an

inspiration for developing ultrahydrophobic surfaces; i.e.commercial self-cleaning dyes have been produced [16]. Dyes including no pigments at all have been made by mimicing surface structure of the wings butterflies such as Blue morpho.

One of the most important and desired targets of biomimetic research is to find environmentally harmless and naturally adaptive materials [17]. This target is a result of the growing population of world and diminishing natural resources and pollution. In the last decade, natural and synthetic proteins, and polysaccharides have been heavily studied as a biomaterial, because they are mostly biocompatible and biodegradable. Nature, which has its own and unique characteristics, does not always allow people to adapt all of its biological attributes without making substantial changes. When faced with such challanges, synthetic pathways are involved like in the case of development of mimetic adhesive materials.

1.3. Adhesion of Marine organisms

The adhesive proteins secreted by marine organisms such as blue mussels, sandcastle worms, caddisfly and barnacles have become an object of biomimetic research in the recent years [18-20]. These proteins have showed remarkable adhesive strength under aqueous environment [21]. Adhesive proteins not only gave inspiration for producing biomimetic adhesives but also boosted studies on developing antifouling surfaces for biomaterials [22].

The detected proteins involved in adhesion are found to be mostly phenolic protein [23, 24]. The advantage of phenolic proteins is that they are biodegradable and nontoxic which are two important properties for an ideal bioadhesive.

The main point in biomaterial studies is the adhesion mechanism at the interface, because these adhesive proteins can stick any type of substrate surfaces. Since it is difficult and time consuming to extract adhesive proteins directly from these marine creatures, developing synthetic systems is more convenient for obtaining higher supplies of biomaterials.

1.3.1. Blue Mussels

Blue mussel, *Mytilus Edulis,* is a bivalve living in marine environment. What makes mussels interesting for researchers is their capability to attach themselves to surfaces, either organic or inorganic, via their byssus [25, 26] (Figure 1.1).

Figure 1.1. Image of blue mussel on mica sheet [27]

The byssus, or byssal thread, is a bundle of threads, 3-4 cm long, coming out of the mussel shell and serves as an attachment structure. Each thread has an adhesive plaque at the adhesion interface [28]. There are five different adhesive proteins detected in a thread; i.e. *Mytilus Edulis* foot protein-1 (*mefp*-1), through mefp-5. Each protein is supposed to have a different function and has been found at different places in the byssus (Figure 1.2).

Mefp-1 has a molecular weight of ~110 kDa and abundant at the outer layer of the thread [23]. The aminoacid sequence of mefp-1 is N-Ala-Lys-Pro-Ser-Tyr-Hyp-diHyp-Thr-DOPA-Lys-C. This protein is believed to function as a protective coating for the thread and the adhesive plaque [29].

Figure 1.2. The byssal thread and the distrubution of adhesive proteins with mol % DOPA content [30]

Mefp-2 and mefp-4 have a molecular weight of ~ 40-42 kDa and~ 79 kDa, respectively. They are located inside of the plaque and might have a role of stabilizing the adhesive plaque, since they constitute 30% of total adhesive plaque weight [31-33].

Mefp-3 (~6 kDa) and mefp-5 (~9.5 kDa) are found at the interface where adhesive plaque is tethered to a substrate surface and are believed to be mainly responsible for mussel attachment. Aminoacid sequences of Mefp-3 and Mefp-5 proteins revealed that they have 48 and 74 aminoacids, respectively [31, 34, 35].

The remarkable feature of adhesive proteins is building of soft glue at the time of secretion, which hardens after a couple of minutes under water. Moreover, adhesive proteins can preserve their bulk adhesive properties over time in the presence of water which is something that most of the adhesives fail to manage [32].

There is a common molecule in the protein sequences of these proteins called 3,4-dihydroxyphenyl-L-alanine (L-DOPA). Mefp-1 has 10-15 mol% [36], mefp-2 has 2-3

mol%, and mefp-4 has 4 mol% of DOPA [32]. Two proteins having the highest concentrations of DOPA is mefp-3 (21 mol %) and mefp-5 (27 mol %) [35]. Since these proteins are abundant at the adhesion interface, researchers proposed that DOPA might be the key molecule of adhesion [25, 37].

1.3.2. Sandcastle Worm

The sandcastle worm (*Phragmatopoma Californica*) is a marine polychaete and lives in sandcastle-like tubular dwellings. Similar to mussels, these worms secrete adhesive proteins to build their tubes by gluing sand grains to each other under water. The glue is a creamy white fluid when secreted, sets approximately in 30s and turns to reddish brown colored solid after couple of hours [38]. The glue is composed of acidic and basic proteins along with Ca^{+2} and Mg^{+2}.

a)

b)

Figure 1.3. Glass beads glued by sandcastle worm. a) The color of glue is white after secreted. b) The color of glue turned to brown after several hours due to oxidation of proteins [39]

Five proteins, referred to as *Phragmatopoma californica*-1 (Pc1), Pc2, Pc3x, Pc4 and Pc5, are found in sandcastle worm glue [24, 40]. The studies on these proteins illustrate that glycine or serine are the dominant aminoacids (more than 50% of the total aminoacids) and Pc1, -2, and -5 are the dopa-rich proteins, but the exact place of abundances of these proteins whether at the adhesion interface or not is unknown. Tyrosine is another aminoacid that exists in proteins and might be transformed (post-translational hydroxylation) into 3,4-dihydroxy-L-phenylalanine (L-DOPA) [24]. The hardening of adhesive fluid and the color change is believed to be the result of the oxidative crosslinking of o-quinones (formed by oxidation of dopa) [39].

1.3.3. Properties and structure of L-DOPA

3,4-dihydroxy-L-phenylalanine (L-DOPA) (Figure 1.4) is a chemical that is common in the aminoacid sequences of adhesive proteins of mussels and sandcastle worms. DOPA is actually very well-known molecule, since it is the precursor to the neurotransmitter dopamine and the 2001 Nobel prize in chemistry is given to the asymmetric synthesis of L-DOPA. This compound can go into reactions to form coordination bonds with metals [41], and to form didopa via aryl coupling or Michael-type addition [42].

Figure 1.4. Chemical structure of L-DOPA

The catechol group of DOPA is believed to have a major responsibility in mussel attachment [23]. Catechol unit has an absorption wavelength of 280 nm. When oxidized, catechol gave an absorption peak at 320 nm [42]. Catechol is highly sensitive to light, air, and pH (at alkaline pH values, since pKa of hydroxyl group is 9.2).

Messersmith and his co-workers [43] have performed an atomic force microscopy (AFM) study to examine the effect of oxidation to adhesive properties and functionality of

catechol group. Lee *et al*. have modified the tip of AFM with PEG molecule end-functionalized with single DOPA residue and analyzed the adhesion strength when the tip is in contact with a titanium surface in water. As mentioned above, catechol can be oxidized by air, light or pH. The strength of adhesion was measured for both oxidized and unoxidized catechol units. The results were remarkable: The unoxidized dopa had the highest inorganic surface adhesion strength ever measured 22.2 kcal/mol. When oxidized, however, calculated adhesion strength was 5.3 kcal/mol.

1.3.4. Proposed adhesion mechanism

Different adhesion mechanisms were proposed for mussels, however, the exact nature of attachment at the interface is still unclear (Figure 1.5). Suggested reaction pathways for the curing of adhesive plaque (or catechol group of L-DOPA) includes Michael type-additon and aryloxy free radical coupling [44].

Figure 1.5. Proposed adhesion mechanism at the interface through catechol coordination bond with surface molecules [45]

Dopaquinone is the oxidation product of dopa and can undergo reactions via addition, redox exchange, and tautomerizm [46]. o-Dopaquinone has α,β unsaturated bonds which are available for attacks by nucleophiles. The well known example is the reaction of side-chain amine with dopaquinone via intramolecular Michael-type-addition. These addition reactions depend on the pK_a of both primary and secondary amines; i.e. the lower the pK_a the faster the adduct form [47].

Although quinones can react by means of Michael-addition (Figure 1.6), the existance of this reaction in mussel adhesive proteins still needs further investigation. Significantly diminished lysine levels were found in some mefp-1 decapeptides, yet no crosslinkining formation was estimated [42].

Figure 1.6. Michael addition crosslinking reaction pathway of o-dopaquinone [44, 47]

Free radical coupling leading to the formation of diDOPA is another suggested mechanism for the setting of adhesive plaque. McDowell *et al*.[48] have showed the formation of 5,5-di(3,4-dihydroxy-L-phenylalanine) cross-links by using ^{13}C rotational echo double resonance NMR analysis of byssal attachment plaques secreted by mussels that have been allowed to grow in the presence of ^{13}C and ^{2}H containing analogs of

tyrosine. Another study carried out with model decapeptides copying the aminoacid sequence of mefp-1 have also showed such cross-linkinking formation following oxidation to quinopeptides [42].

Figure 1.7. Proposed reaction pathway for cross-linking in oxidized mefp-1 derived decapeptides [42]

The curing of adhesive cement of sandcastle worm is proposed to be similar to mussel adhesive proteins. Initial secreted proteins were set, then tyrosine residues are hydroxylated to form dopa following the formation of covalent bond between two dopa molecules which act as a crosslinking agent [39].

1.3.4. Adhesives inspired by mussels and sandworms

Having remarkable adhesive features, marine creatures have recived substantial interest to find desired bioadhesives. The extraction of adhesive proteins from these organisms is difficult and cost-ineffective due to the requirement of considerably high number of such organisms. Researchers have focused to make synthetic biomimetic systems copying the characteristics of those proteins. The main functional group that all systems have in common is catechol.

Shao *et al*. [49] have synthesized copolymers having catechol functional units imitating the sandcastle worm glue poteins Pc3 and Pc1. Synthesis of dopa containing monomer has been done by reacting methacryloyl chloride with a borate dopamine complex at a pH higher than 9 followed by breakage of the bond between borate and hydroxyl groups of catechol. Synthesized monomer was then copolymerized with monoacryloxyethyl phosphate (MAEP), and acrylamide (Aam) via free radical polymerization (Pc3 monolog). The adhesive produced by mimetic polymers have the strength 3-fold less than the natural sandworm adhesive which has a strength of 350 kPa. Moreover, it is lower than the adhesive strength of mussel adhesive proteins which is ranging from 320 to 750 kPa (depending on season).

Stewart and his co-workers [50] have tried another strategy to increase the adhesion strength of the above mimetic glue system. Polyethylene glycol-diacrylate (PEG-dA) was introduced into the complex coacervates prepared by mixing Pc3 and Pc1 analogs of copolymers and Ca^{+2}. When PEG-dA is entrapped into coacervate phase, its polymerization was done to form extra crosslinking between mimetic copolymers. They have succeded to increase adhesion strength from 300 kPa to 1.2 MPa.

S. Lim *et al*. [51] designed a biomimetic system with hybrid mussel adhesive proteins (MAP) (fp-151 and fp-131). Tyrosine residues of hybrid proteins were transformed into dopa by modifying them with ascorbic acid and mushroom tyrosinase in phosphate buffered saline (PBS) (pH: 7.0). Modified hybrid MAPs were then complexed with hyaluronic acid to produce mussel mimetic adhesive. The adhesive strength tests of produced bioadhesive revealed that the bulk adhesive strength of mfp-151/HA was 3.17±0.51 MPa and mfp-131/HA was 4.00± 0.53 MPa. Their strengths were higher than sole adhesive proteins (1.98±0.40 MPa for mfp-151 and 1.87±0.24 MPa for mfp-131)

Lee *et al*. [52] used modified chitosan and pluronic acid polymers to develop a new type of bioglue. Chitosan, in deionized water (pH:5.5), was modified with 3,4-dihydroxy hydrocinnamic acid (DOHA) (catechol containing molecule) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). Hydrogels were prepared with modified chitosan and thiol terminated pluronic acid (Plu-SH). They have tested the adhesion strength of mimetic glue by universal testing machine by placing a tissue sample to the probe of machine. The results showed that the CHI/Plu-SH hydrogel has a detachment stress of 15 kPa. The same hydrogel system without modification with catechol showed a detachment stress of 1.9 kPa.

1.4. Hyaluronic Acid

Hyaluronic acid (HA) (Figure 1.8) is a biopolymer and a member of glycosaminoglycans having disaccharide repeating units of N-acetyl glucosamine and glucuronic acid connected by β (1→4) and β (1→3) glycosidic bonds. The important feature of HA which distinguishes it from other glycosaminoglycans is its molecular structure with no sulfate groups. HA is found in cartilage, vitreous humuor, skin, extracellular matrix and synovial fluid [53]. Being biocompatible and biodegradable, HA has gained great interest for biomedical applications such as wound healing[54], drug delivery [55], and cell proliferation [56].

Figure 1.8**.** Chemical structure of hyaluronic acid

1.5. Chitosan

Chitosan (CHI) is one of the few natural cationic polysaccharide which is obtained by alkaline deacetylation of chitin (Figure 1.9). Chitosan contains β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) repeating units. Chitosan has been used in many different applications such as drug delivery [57], biosensors [58], tissue engineering [59], and antimicrobial coating [60].

Figure 1.9. Chemical structure of chitosan

What makes chitosan attractive for researchers is its biocompatibility, biodegradability, and non-toxicity [61]. The dissociation constant (pK_a) for the amine group of CHI ranges from 6.2 to 6.6 depending on the degrees of deacetylation of chitosan and measurement conditions such as ionic strengthof the medium, concentration of polymer or type of salt used as a solvent [62-64].

1.6. Carbodimide Chemistry

Carbodiimidization of active esters was used to modify hyaluronic acid and chitosan. Carbodiimides are unsaturated compounds with allene-type chemical structure. They have mostly been used in peptide synthesis and protein modification. Among other carbodiimides, especially water-soluble 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), has been widely used as the so-called zero-length crosslinker, because no spacer molecule is necessary to form amide bond when EDC is used.

Figure 1.10. Rearrangement from O-acylurea to N-acylurea [65]

Carboxylic acid activation with EDC was problematic because of the unstability of intermediate O-acylurea and the intramolecular rearrangement of reactive O-acyl urea to unreactive N-acylurea (Figure 1.10) [65, 66]. Anderson *et al*. [67], for the first time,

introduced NHS to form reactive yet stable activated ester in peptide synthesis. The produced active ester can then be reacted with an amine group to form an amide bond.

2. OBJECTIVES AND METHODOLOGY OF THE PROJECT

There is an on-going research on finding new bioadhesive materials having better adhesive strength yet meet the qualities of ideal bioadhesive properties mentioned previously. There are commercially available bioadhesives, but they all have some disadvantages; i.e cyanoacrylates have good adhesion strength but toxic due to formaldehyde release or fibrin is biocompatible, however, its adhesion strength is low. There are also bioadhesives based on mussel adhesive proteins, but some of them have low adhesion strength and some of them use hybrid proteins which are hard to produce. There is still a need for new materials and production methods to obtain a better biological adhesive.

The aim of this project is to modify biopolyelectrolytes with catechol containing molecules to mimic adhesive proteins of mussels. Hyaluronic acid and chitosan were chosen for this purpose because these two polysaccharides are naturally occuring biocompatible, biodegradible and non-toxic which made them popular in medical applications. Carbodiimide chemistry was used to achive this goal. EDC is a well-known reagent in peptide synthesis and protein modification due to its easier purification than that of other carbodiimides such as DCC and its water solubility.

3. EXPERIMENTAL

3.1. Materials and Methods

Sodium hyaluronate (M_W : 51, 234, 150, and 752 kDa) was obtained from Lifecore Biomedical, LLC (Chaska, MN, USA) while chitosan was purchased from Novamatrix (Sandvika, Norway). N-hydroxysuccinamide (NHS), 3,4-dihydroxy-L-phenylalanine (L-DOPA), 3-(N-Morpholino)propanesulfonic acid (MOPS) and its sodium salt (MOPSNa), 3,4-dihydroxyhydrocinnamic acid (DOHA) and toluene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) was purchased from Fluka. Methanol (dried max. 0.003% H_2O) , thionyl chloride ($SOCl_2$), disodium hydrogen phosphate, potassium dihydrogen phosphate, and sodium metaperiodate ($NaIO_4$) were acquired from Merck KgaA (Darmstadt, Germany). All substances were used without further purification.

3.2. Synthesis

3.2.1. Synthesis of L-DOPA-Methyl ester

3,4-dihydroxy-L-phenylalanine (L-DOPA) was converted into L-DOPA methyl ester as described in the literature [68] . Briefly, L-DOPA (0.025 mol) in dry methanol (250 ml) was mixed with thionyl chloride ($SOCl_2$, 0.0505 mol) added in small quantities over 1 hr at 0 ^{o}C under Ar. After refluxing the mixture over 24 hr under Argon at 70 ° C, toluene was added to the sample, and was rotavaped. Coevaporation with toluene was performed three times to get rid of all the methanol.

The ^{1}H-NMR (400 MHz, D_2O), spectra displayed the following peaks: δ = 4,64 (^{1}H,CH_2CHN), δ = 3.69 (3H, OCH_3), 3.04 (^{1}H, CH_aH_bCHN), 2.97 (^{1}H, CH_aH_bCHN) ppm.

3.2.2. Modification of Hyaluronic Acid with L-DOPA Methyl Ester

Sodium hyaluronate (HA) (1 mg/ml, M_W : 130 kDa) was dissolved in MOPS buffer (0.1 M pH : 6.8) under Ar. 20-fold molar excess of N-hydroxysuccinamide (NHS) and 3,4-dihydroxy-L-phenylalanine-methyl ester (DOPA-OMe) were added to this solution. 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) was dissolved in Milli-Q water (Millipore, 18.2 MΩ), and added to the mixture (Fresh EDC solution was added every 2 hrs at the first 8 hours of the reaction to reach a final amount of 20-fold molar excess over the carboxylic acid groups of HA). The reaction was allowed to run overnight. The pH was checked to make sure it is below 7 since the catechol group of DOPA oxidizes at alkaline pH. The mixture was dialyzed (SnakesSkin, MW: 3.5 kDa) against Milli-Q water at pH: 3.5, and then freeze-dried. Modified HA was dissolved in 5 % (w/v) NaCl solution and 10 volume equivalent ethanol was added to precipitate HA. After decanting ethanol and redispersing the HA in water, the purified and precipitated polymer was again lyophilized.

The ^{1}H-NMR (400 MHz, D_2O) spectra peaks are as follows: δ = 4,49 (^{1}H,CH_2CHN), 2.89 (^{1}H, CH_aH_bCHN), 2.86 (^{1}H, CH_aH_bCHN) ppm.

3.2.3. Modification of Chitosan with DOHA

250 mg Chitosan (Novamatrix, 310 kDa, D.A : 95%) was dissolved in 220 ml 0.01 M MOPS buffer (pH:6.88) under Ar. To a 25 ml round bottom flask were added 837.7 mg DOHA and 30 ml 0.01 M MOPS buffer. After DOHA was dissolved, first batch of EDC (293.8 mg in 4 ml Milli-Q water for each batch) and 529.2 mg NHS were added, respectively (the solution became turbid because of the side product urea). Then, this solution was immediately mixed with chitosan solution. Second and third batches of EDC were added to the mixture at every 2 h in the first 4 hours of reaction. The reaction was stirred overnight under Ar. The next day, The solution was filtered through cellulose acetate filter against pH:3.5 Milli-Q water (Snakeskin dialysis tubing, MWCO :10 kDa). The dialyzed sample was filtered through 0.45 μm cellulose acetate filter paper to get rid of by product urea. The polymer were freezed and lyophilized. After that, they were

dissolved in 5 % by weight NaCl, precipitated with ethanol (ethanol:sample 10:1 v/v, sample was added drop by drop to ethanol which was being stirred vigirously), redisperse in water (pH : 3.0) and lyophilized.

3.3. Characterization

3.3.1. ^{1}H Analysis

^{1}H nuclear magnetic resonance (NMR) analysis were performed by using a Varian Gemini 400 MHz spectrometer.

3.3.2. UV-Visible Spectrometry

The degrees of modification of modified hyaluronic acid (HA-Cat) and chitosan (CHI-Cat) was determined by UV absorbance measurements using Shimadzu UV-1700 spectrophotometer. 3,4-dihydroxyhydrocinnamic acid (DOHA) was used as a standard. DOHA solutions containing different catechol concentrations were prepared in 0.015 M HCl. Absorbances at 280 nm and 320 nm (UV absorbance wavelengths of catechol and oxidized catechol, respectively) were recorded and a calibration curve was drawn. The modified polymer sample was dissolved in 0.015 M HCl solution and UV absorbances at the wavelengths mentioned above were recorded. Corresponding catechol concentrations were determined from the standard calibration curve.

Figure 3.1. The chemical structure of DOHA

3.3.3. Gelation Test

Modified polymers were dissolved in 1X phosphate buffer saline (PBS) (pH : 7.4) at a various concentrations depending on the mole % of catechol modification of the concerned polymer. Sodium metaperiodate ($NaIO_4$) powder were added to polymer solutions at an estimated mole ratio of 1:1 (catechol:$NaIO_4$).

3.3.4. Potentiometric Titration

pH titrations were performed with Orion-star pH meter equipped with Beckmann ATC temperature probe under N_2 at 25.0 ± 0.5 ^oC. Carbondioxide from the atmosphere may dissolve in the solution causing a decrease in its pH. N_2 gas was bubbled above the solution to prevent this decrease. Gas inlet was placed very close to the surface of the titrate solution to blanket the surface with nitrogen gas. Modified polymer solutions were prepared in 50 mM NaCl solutions at a concentration of 0.1 mg/ml. Initial pH's of solutions were adjusted to 2.00 ± 0.01 for HA-Cat and to 3.00 ± 0.01 for CHI-Cat with 1 N HCl solution. 2.0 ml and 0.2 ml microburettes (Gilmont Instrument Co., Barrington, IL, USA) were used to add 0.01 N NaOH solution to a 15 ml solution of the modified polymer. All measurements were carried out by increasing the pH from low to high by incremental addition of 0.01 N NaOH solution. Each titration was accompanied with a blank titration (containing only the NaCl solution without any polymer). The pH meter was calibrated with pH = 4.01, 7.00, and 10.01 buffer solutions.

3.3.5. Zeta Potential Measurements

Zeta potential of chitosan (DA:95 %, 310 kDa) was measured with Brookhaven ZetaPALS instrument. A chitosan stock solution was prepared by dissolving powdered chitosan in 10 mM NaCl solution at a concentration of 1 mg/ml. The polymer solution was stirred overnight at room temperature to ensure complete dissolution. Chitosan solutions with different pH values were prepared from stock solution without changing the concentration and ionic strength of the stock solution. pH was adjusted with 0.1 N HCl and 0.1 N NaOH solutions.

4. RESULTS AND DISCUSSIONS

4.1. Modification of Hyaluronic Acid with L-DOPA Methyl Ester

4.1.1. Synthesis of L-DOPA Methyl Ester (L-DOPA-OMe)

The carboxylic acid moiety of L-DOPA was protected with methanol in order to prevent possible side reaction of carboxylic acid group with modification reagents (Figure 4.2). ^{1}H-NMR spectrum (Figure 4.4) of L-DOPA-OMe showed a characteristic methoxy hydrogen peak at 3.69 ppm.

Figure 4.1. Synthesis of L-DOPA-OMe

4.1.2. Catechol functionalization of Hyaluronic Acid

Modification of HA has some challenges because of the sensitive nature of catechol group of L-DOPA methyl ester. Catechol unit has two hydroxyl groups which are sensitive to oxidation by air, light, and pH. A careful experimental set up should be prepared to prevent oxidation of catechol group.

An important reagent in the modification reaction is 1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide (EDC). EDC is responsible for reacting with carboxylic acid group in hyaluronic acid making carbonyl group of carboxylic acid moiety more electron deficient. Once the carbonyl group became more positive, NHS can easily attack and form activated ester. A new approach for the modification is the addition of fresh EDC solution at every 2 hours since activated ester loses its activity within 2 hours [69].

The success of modification reaction was analyzed by 1H-NMR, UV-Vis spectrometry and gelation test. The ^{1}H-NMR spectrum (Figure 4.5) of modified polymer illustrated two different peaks at around 7.0 ppm and 2.9 ppm. These peaks belong to the phenyl ring hydrogens and benzylic hydrogens of L-DOPA methyl ester, respectively. Since carboxylic acid group of HA will be needed in further studies, our aim is just to modify some of the present groups on the polymer backbone. Weak intensity of peaks is because of low mol % catechol modification.

The UV-Vis analysis of catechol modified HA was done to determine the mol % of catechols per chain. The absorbances (at 280 nm) of DOHA solutions with different known catechol concentrations were recorded. These obtained data was linearly fitted and catechol concentration of corresponding absorbance value of modified HA was calculated. The percent modification of HA ranged from 4.0 mol% to 7.0 mol % although all the reactions were carried out at pH 6.8 -7.0. However some of the amine groups of L-DOPA-OMe will be protonated depending on the pH of the experiment. Thus, small deviations of pH may lead to a change in the number of protonated amine groups during the reaction, so this might be the reason of different modification degrees as well as low degrees of modification.

The gelation test was also done to ensure the presence of L-DOPA which is not physically adsorbed to HA. Proposed adhesion mechanism of mussel adhesive proteins includes diDOPA formation via aryloxy free radical coupling which form a covalent crosslinking between proteins. $NaIO_4$ is known as an oxidizing agent for catechol unit, so in the presence of sodium meta periodate the modified HA should form a gel while native HA does not. After the addition of powder $NaIO_4$ (sample : $NaIO_4$ mole ratio 1:1) , yellow colored gel (Figure 4.2) formed within 23 minutes.

Figure 4.2. Picture of gel formed after the addtion of $NaIO_4$

Figure 4.3**.** Schematic illustration of modification of HA

Figure 4.4. ^{1}H-NMR (D_2O, 400 MHz) spectrum of L-DOPA Methyl Ester

Figure 4.5. ^{1}H-NMR Spectrum (D_2O, 400 MHz) of native and modified HA

4.2. Modification of Chitosan with DOHA

Carbodiimide catalyzed functionalization of chitosan was performed via carbodiimide chemistry using a method given in the literature [52] with small changes (Figure 4.6). We have made these changes because the procedure given in the literature yielded low degrees of modification and high % oxidation of catechol groups which are introduced to polymer chain. The changes we have made were that (i) chitosan was dissolved in MOPS buffer to keep the pH stable, (ii) NHS was added to the EDC-DOHA mixture to facilitate the reaction by making carbonyl group more reactive against nucleophilic attacks, and (iii) fresh EDC solution was added at every 2 hours in the first six hours of the reaction.

The peaks for hydrogens of the phenyl ring appeared between 6.4 and 6.8 ppm in the ^{1}H-NMR analysis (Figure 4.9). The degrees of modifications calculated was varied from 2.5 to 6.8 mol %. The difference is due to the different degrees of deacetylations (DD) of chitosan samples. Chitosan with higher DD has had higher % modification degree which may be due to the increasing number of available amine groups.

The gelation test results showed that modified polymers formed red colored gels within less than 30 sec. after the addition of $NaIO_4$ (Figures 4.7 and 4.8).

Figure 4.6. Reaction scheme for the modification of chitosan

Figure 4.7 Picture of gel. Chitosan D.A : 46% M_w:650 kDa, modification % (by mole) : 2.52

Figure 4.8 Picture of gel. Chitosan D.A : 95% M_w:310 kDa, modification % (by mole) : 6.83

Figure 4.9. ^{1}H-NMR Spectrum (D_2O, 400 MHz) of native and modified chitosan

4.3. Potentiometric Titration and ζ-Potential Measurement

The effect of modification on acid dissociation constant (pK_a) of polymers was investigated. pK_a's of both native and modified polymers were determined by potentiometric titration. pK_a of chitosan is dependent on the degrees of deacetylation (DD). As DD of chitosan decreases, pK_a increases due to the decreasing number of free amine groups [64]. Data presented in figures 4.11 to 4.16 was obtained by subtracting volume of NaOH added to the sample and volume of NaOH added to blank [$dV = V_{NaOH}$ (sample) - V_{NaOH} (blank)]. This subtraction eliminates the contribution from water and gives only the volume change due to the polymer. Figure 4.11 and 4.16 show the change in volume as a function of pH. At low pH's where volume change (dV) was constant, the degrees of protonation (β) is assumed to be 1.0; meaning all amine groups are protonated. At higher pH's where dV is constant β is assumed to be 0; meaning all amine groups are deprotonated.

Zeta potential measurement was also done to investigate the total charge of chitosan at different pH's and to confirm our assumption.

Figure 4.10. ζ-potential vs pH graph for unmodified chitosan (D.D : 95% M_W:310 kDa) in 10 mM NaCl.

The instrument calculates zeta potential by measuring electrophoretic mobility of polymer molecules moved in applied electrical field. Electrophoretic mobility is determined by electrophretic ligth scattering (ELS). Figure 4.10 shows the change of zeta potential of unmodified chitosan as a funtion of pH. Chitosan is a polycation due to its amine functional groups on its repeating units. ζ-potential of chitosan was positive in the adjusted pH range. As expected, the positive charge on chitosan decreased with increasing pH due to deprotonation of amine groups. At high pH's, the charge of chitosan is close to zero. Chitosan started to precipitate at pH 6.0. The interference of scattered light due to precipitated chitosan might be the reason for large deviations in measurements.

pK_a of chitosan samples was determined at $\beta = 0.5$ by fitting (Boltzmann non-linear curve fitting) all the recorded values. The midpoint of $\beta = 0$ and $\beta = 1$ where dV is constant was assumed to be $\beta = 0.5$. Corressponding pH at half point was then found. pK_a was 6.18 for unmodified chitosan and 6.37 for modified chitosan at degrees of deacetylation of 95 %.

pK_a increased as expected, because modification with catechol group caused a decrease in the number of free amine groups that makes the polymer slightly basic.

Figure 4.11. dV vs pH for modified chitosan (310 kDa, %modification 6.09). Polymer conc. 0.1 mg/ml, ionic strength 50 Mm

Figure 4.12. dV vs pH for native chitosan (310 kDa). Polymer conc.0.1 mg/ml I : 50 mM.

Hyaluronic acid has carboxlyic acid groups which gives HA its negative charge. pK_a of HA in 0.01 $NaNO_3$ reported in the literature was 2.9 [70]. Potentiometric titration was carried out for HA with different molecular weights with or without catechol functionalization (Figure 13 – 16). Data treatment was same as previously described for chitosan. Results are given in the Table 4.1.

Table 4.1. pKa values determined by potentiometric titration

Polymer	pKa (±0.02 pH units)
HA (Mw : 51 kDa)	2.39
HA (Mw : 150 kDa)	2.48
HA-Cat (Mw : 51 kDa)	2.59
HA-Cat (Mw :150 kDa)	2.70

The results showed that there is an increase in pKa as the molecular weigth of HA increases. The higher pKa of NaHA (M_W:150 kDa) may be due to less effective ion-exchange from protons to Na^+ ions due to its higher chain length. Modified polymers have

higher pKa as expected. Modification results in a decrease in the number of available ionizable groups (carboxylic acid groups) which makes polymer slightly basic.

Figure 4.13. dV vs pH graph for HA-unmodified (M_w:51 kDa)

Figure 4.14. dV vs pH graph for modified HA (M_w:51 kDa), % modification: 4.36

Figure 4.15. dV vs pH graph for HA-unmodified (M_w:150 kDa)

Figure 4.16. dV vs pH graph for modified HA (M_w:150 kDa), % modification: 7.02

5. CONCLUSIONS AND FUTURE WORK

Modification of hyaluronic acid and chitosan was successfully done with 3,4-dihydroxy-L-phenylalanine and 3,4-dihydroxyhydrocinnamic acid, respectively. Carbodimide chemistry with the use of activated esters was followed as a modification method. Modification of polyelectrolytes was characterized by ^{1}H-NMR analysis, UV-Vis spectrometry, gelation test and potentiometric titration. The degrees of modifications that were determined for two polymers range from 2.5 to 7.0 mol %. Formation of phenyl ring hydrogens and benzylic hydrogen in H-NMR spectrum confirmed the modification. pK_a values of modified polymers were higher than that of native ones because of the decrease in available ionizable groups due to modification.

As a future work, adhesive properties of modified polyelectrolytes will be determined and new synthesis methods to increase the modification degree will be investigated.

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