A COMPARISON OF SURFACE AND BIOLOGICAL PROPERTIES OF CHITOSAN/TANNIC ACID AND CHITOSAN/POLY(ACRYLIC ACID) MULTILAYERS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES OF MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

SEPTEMBER 2019

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ABSTRACT

A COMPARISON OF SURFACE AND BIOLOGICAL PROPERTIES OF CHITOSAN/TANNIC ACID AND CHITOSAN/POLY(ACRYLIC ACID) MULTILAYERS

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September 2019, 68 pages

Chitosan is a natural polysaccharide which finds use in various biomedical applications due to its important biological properties. Layer-by-layer (LbL) technique is a practical and economical method to functionalize surfaces using interacting polymer pairs. LbL technique can also be used to construct polymer platforms for controlled release of functional molecules from a surface. In this thesis, ultra-thin polymer coatings were prepared using Chitosan and two different weak polyacids, i.e. Tannic Acid (TA) and poly(acrylic acid) (PAA) through LbL technique at moderately acidic conditions. The driving force for multilayer assembly was electrostatic interactions between the protonated amino groups of Chitosan and phenolate groups of TA and protonated amino groups of Chitosan and carboxylate groups of PAA for Chitosan/TA and Chitosan/PAA films, respectively. In addition, hydrogen bonding interactions among the hydroxyl groups of Chitosan and phenolic hydroxyl groups of TA or carboxyl groups of PAA contributed to the association of the polymers at the surface. The surface properties of the films such as LbL growth profile, stability, wettability and surface morphology were examined. The remarkably different surface properties are explained by the chemical nature and acidity of the polyacids. The differences in biological properties of the films such as drug loading capacity, stimuliresponsive release and protein adsorption behaviours are explained by the differences in surface properties. This thesis study generated fundamental information about the structure-property relationship in Chitosan based LbL films and showed tuning both surface and biological properties of the multilayers by simply changing the nature of the polyacid.

Keywords: Chitosan, Layer-by-Layer films, Tannic acid, Poly(acrylic acid)

KİTOSAN/TANNİK ASİT VE KİTOSAN/POLİ(AKRİLİK ASİT) ÇOK KATMANLI FİLMLERİN YÜZEY VE BİYOLOJİK ÖZELLİKLERİNİN KARŞILAŞTIRILMASI

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Eylül 2019, 68 sayfa

Kitosan önemli biyolojik özellikleri sebebiyle birçok biyomedikal uygulamada kullanımı olan doğal ve doğrusal yapıda bir polisakkarittir. Katman-katman (LbL) kendiliğinden yapılanma yöntemi birbirleriyle etkileşen polimerler kullanarak yüzeylerin işlevselleştirilmesi için kolay uygulanabilir ve ekonomik bir yöntemdir. LbL yöntemiyle işlevsel moleküllerin yüzeyden denetimli salımı için polimer platformlar oluşturmak da mümkündür. Bu tez çalışmasında, LbL tekniği kullanarak, Kitosan ve iki farklı poliasit, Tannik Asit (TA) ve poli(akrilik asit) (PAA), kullanarak hafif asidik koşullarda ultra-ince polimer kaplamalar hazırlanmıştır. Kitosan/TA and Kitosan/PAA çok katmanlı filmlerinin oluşmasındaki itici kuvvet, Kitosanın protonlanmış amino grupları ile TA'nın fenolat grupları ve Kitosanın protonlanmış amino grupları ile PAA'nın karboksilat grupları arasındaki elektrostatik etkileşimidir. Buna ek olarak, Kitosanın hidroksil grupları ile TA'nın fenolik hidroksil grupları veya PAA'nın karboksil grupları arasındaki hidrojen bağları da polimerlerin yüzeyde birikmesine katkı sağlamıştır. Bu iki filmin LbL büyüme, kararlık, ıslanabilirlik ve yüzey morfolojisi gibi yüzey özellikleri incelenmiş ve karşılaştırılmıştır. Birbirinden oldukça farklı film özellikleri poliasitlerin kimyasal yapısı ve asit özellikleri ile açıklanmıştır. Bu iki filmin ilaç yükleme kapasitesi, uyarana duyarlı salım ve protein tutunması gibi biyolojik özellikleri arasındaki farklar da yüzey özellikleri ile ilişkilendirilmiştir. Bu tez çalışması, Kitosan esaslı LbL filmlerin yapı-özellik ilişkileri hakkında temel bilgi oluşturulmasına katkı sağlamış ve kullanılan poliasite bağlı olarak çok katmanlı filmlerin yüzey ve biyolojik özelliklerinin kontrol edilebileceğini göstermiştir.

Anahtar Kelimeler: Kitosan, Katman-katman, Tannik asit, Poli(akrilik asit)

To Feriha Bekcan

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deep gratitude to my supervisor, Assoc. Prof. Dr. İrem Erel Göktepe for her infinite patience and guidance. She encouraged me to do research and therewith solve the problems during my thesis study. Without her, this thesis would not be possible. I would also specially thank to my co-advisor, Prof. Dr. Sreeparna Banerjee for her valuable knowledge and guidance on biological importance of this thesis.

I am thankful to Assoc. Prof. Dr. Can Özen and Assoc. Prof. Dr. Çağdaş Devrim Son for their support and advices through my education.

I would like to thank my former and current lab members; Dilara Gündoğdu, Meltem Haktanıyan, Sinem Ulusan, Majid Akbar, Eda Çağlı, Esma Uğur and Gökçe Tidim. They spared time to answer my questions and have always been in a positive attitude. Also, special thank to Gencay Çelik for the helpful experimental information.

I am grateful to Ece, Iraz, Selin for being in my life.

Finally, I would like to thank my mother, my father and my sister for the motivation that they gave me to complete this thesis and supporting me no matter what happens.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AFM	Atomic Force Microscopy
ACh	Anionic derivative of Chitosan
B. cereus	Bacillus cereus
BCM	PS-b-PAA amphiphilic block copolymer micelles
BPEI	Branched Poly(ethyleneimine)
BSA	Bovine Serum Albumin
CCh	Cationic derivative of Chitosan
CIP	Ciprofloxacin
СМС	Carboxymetyl cellulose
CMCS	Carboxymetyl Chitosan
DI	Deionized
DDA	Degree of deacetylation
Dex-CHO	Dextran aldehyde
DOX	Doxorubicin
DHPA	3,4 Dihydroxyhydrocinnamic acid
E. coli	Escherichia coli
GO	2D amine functionalized graphane
HMS	Mesoporous silica
IBU	Ibuprofen

Interpenetrating polymer network
Luria Bertani
Layer by Layer
Lower critical solubility temperature
Lysozyme
Mueller Hinton
Molecular Weight
Polystyrene sulfate
Poly(acrylic acid)
Poly(allylamine hydrochloride)
Polyacrylamide
Phosphate Buffer Saline
β-cyclodextrin-based polymer
Poly(diallyldimetylammonium)
Poly(diallyldimetylammoniumchloride)
Poly(2-(dimethylamino)ethyl methacrylate)
Poly[2(dimethylamino)ethylmethacrylate-bpoly(2-
(diethylamino)ethyl methacrylate)]
Polydimetylsiloxane
Polyelectrolyte Multilayers
Poly(2-ethyl-2-oxazoline)
Poly(2-isopropyl-2-oxazoline)

PLL	Poly(L-lysine)
PMAA	Poly(methacrylic acid)
PMAs	Poly(2-methacryloyloxy-ethylphosphorylcholine-co- methacrylicacid)
PNIPAM	Poly(N-isopropylacrylamide)
PSS	Poly(styrenesulfonate)
PVCL	Poly(N-vinylcaprolactam)
PVPON	Poly(N-vinylpyrrolidone)
pHEMA	Poly(2-hydroxyethylmethacrylate)
pH _{crit}	The critical pH
S. aureus	Staphylococcus Aureus
TA-Cipro	Tannic acid and Ciprofloxacin
2D	Two-dimensional
3D	Three-dimensional

CHAPTER 1

INTRODUCTION

1.1. Chitosan

Polysaccharides, an important class of biopolymers are potential materials for human based biomedical applications, e.g. as pharmaceutical excipients in drug delivery or scaffolds for regenerative medicine [1,2].

Chitin, a polysaccharide composed of N-acetyl glucosamin, was first obtained from mushrooms and was identified by the French chemist, Henry Braconnot in the 19th century. Mushrooms are not the only living species to extract chitin. Cuticules of crustaoceans especially crab and shrimp shells also contain chitin [3].

Chitosan is mainly produced by enzymatic or chemical deacetylation of chitin (Figure 1.1). Chemical deacetylation is mostly preferred for commercial production due to its economic process and feasibility for mass production [4,5]. Among several characteristics of Chitosan, degree of deacetylation (DDA) and molecular weight (MW) are the most critical parameters determining characteristics of Chitosan including physical, mechanical as well as biological properties [4–6]. DDA gives information about the content of free amino groups in the polysaccharides and also it is used to distinguish Chitosan from chitin. For example, 75 % or above deacetylation of chitin corresponds to Chitosan [7]. Reaction conditions and method of purification specifies the degree of deacetylation [7,8].

Molecular weight of Chitosan varies with the initial source material (shrimps, fungi, insects etc.) [6,9]. It influences features of Chitosan such as water holding capacity, antimicrobial activity and blood coagulation activity [10]. Zheng et al. showed that for Chitosan with MW below 300 kDa, antimicrobial activity against gram positive bacteria, *S. Aureus* enhanced as the MW increased at constant concentration. On the

contrary, antimicrobial effect against gram negative bacteria, *E. coli* decreased [11]. In another study, Chitosan with varying MW and DDA was characterized for the blood aggregation property by Hattori et al. They showed that aggregation of whole blood which resulted from the electrostatic interaction between positively charged Chitosan and negatively charged erythrocytes, platelets and plasma protein was the highest for Chitosan with MW in the range of 8.6-247 kDa and DDA of 75 % to 88 % [12].

Chitosan has a highly crystalline structure and it is insoluble in aqueous solution at pH above 6. Chitosan has a pK_a of 6.5 [13]. It can be solved in dilute acidic solutions (pH<6) due to protonation of the amino groups at pH below its pK_a . It is challenging to study with Chitosan because of this solubility problem. Chitosan can be chemically modified through its amino groups to increase its solubility above neutral pH conditions and stability, making it a more adaptable biopolymer for various purposes. Chitosan based composites or derivatives can be obtained from its reactions with other small molecules or polymers. Chitosan hydrogel is one example of Chitosan based composites [14]. Chitosan hydrogel consists of a three-dimensional network composed of crosslinked polymer chains. It absorbs water and expands to a larger volume due to highly hydrophilic groups in its structure [15]. It is highly resistant to dissolve in water due to crosslinks between polymer chains [14,16]. In addition to lots of important properties of Chitosan hydrogels, e.g. biodegradability, fluid uptake and holding capacity, the compatibility of chitosan hydrogels to the tissue makes them a good candidate for biomedical applications. Recently, Chitosan hydrogels are used in the production of contact lenses, drug delivery systems, as tissue engineering scaffolds and wound dressings [17]. For example, interpenetrating polymer network (IPN) composed of poly(hydroxyethyl methacrylate) p(HEMA) hydrogels and Chitosan were found to avoid inflammatory complications and extend lifespan of contact lenses. This behaviour was attributed to the antifouling behaviour of Chitosan against proteins and other components in tear [18].

Chitosan is also used as one of the components for improving biocompatibility and immune-stimulatory activities of three-dimensional hydrogel cell scaffolds. Zheng

and co-workers reported that Chitosan promotes cell adhesion to the scaffold, giving a hope to create microstructures for tissue engineering [19].

Chitosan microparticles or nanoparticles which are embedded into hydrogels are also of specific interest for the development of novel drug delivery systems with controlled release properties. In these hydrogels, Chitosan particles are used as drug carriers. In a study of Seyfoddin and co-workers, levonorgestrel hormone was encapsulated in glutaraldehyde-crosslinked Chitosan microspheres and these microspheres were embedded in poly(vinyl alcohol) hydrogel matrix. Microspheres were reported to extend the levonorgestrel release duration compared to bare hydrogels [20]. In another study, amoxicillin loaded Chitosan/poly- γ -glutamic acid nanoparticles were incorporated into pH-sensitive alginate-calcium hydrogel. It was found that the hydrogel protected nanoparticles from destruction by the stomach acid and released them at higher pH, e.g. in case of gastric ulcer [21].

Hemostatic, mucoadhesive, bactericidal and pH-responsive characteristics of chitosan make it suitable for the treatment of chronic wounds. Chitosan hydrogels prepared using genipin, a crosslinker with lower toxicity than glutaraldehyde, were reported to neutralize the wound site environment. To mimic the alkaline environment of chronic wounds, hydrogels were immersed into PBS at pH 8.2/37 °C. It was found that hydrogels prepared with low MW Chitosan had 230 % fluid uptake and lowered the pH by 0.80 ± 0.24 units through swelling upon exposure to pH 8.2. When a similar experiment was conducted at pH 7.4, the pH drop was recorded as 0.30 ± 0.11 . The same study also reported that Chitosan hydrogels prevented *E. coli* growth by % 70 while enhanced cellular proliferation which are important properties for a wound healing material [22].

Recently, Onat et al. prepared Chitosan hydrogels whose surface was modified with water-soluble tannic acid and ciprofloxacin (TA-Cipro) complexes and poly(N-vinyl caprolactam) (PVCL) *via* layer-by-layer (LbL) technique. These hydrogels were found to have antibacterial effect against *E. coli* and *B. cereus* because of the

temperature-induced release of ciprofloxacin (CIP) from hydrogel surface as well as inherent antibacterial properties of Chitosan. The surface-modified Chitosan hydrogels were reported to promote human fibroblast cell proliferation and spreading significantly compared to bare hydrogels.



Figure 1.1. Chemical production of chitosan.

1.1. Layer-by-Layer Self-Assembly Technique

Production of layer-by-layer (LbL) polymer films comprise consecutive adsorption of oppositely charged polymers on a material surface [23] resulting in ultra-thin multilayers. The first study on LbL self-assembly was reported by Iler and co-workers in which oppositely charged colloidal particles were deposited at a surface in a LbL fashion [24]. This technique is later investigated for oppositely charged polymers by

Decher and Hong [25]. Briefly, the substrate is immersed in a polycation solution for about 10-20 minutes, followed by rinsing for about 1-2 minutes to remove weakly bounded polyelectrolytes. Then, the polycation coated substrate is immersed into polyanion solution for 10-20 minutes which was again followed by the rinsing step. This coating cycle continuous until desired number of layers are reached on the substrate surface [26–28]. Electrostatic interactions are the driving force for multilayer assembly. However, LbL technique can also be applied to species interacting *via* hydrogen bonding, coordination bonding, charge- transfer, metal-ligand and biospecific interactions [29–33]. Figure 1.2 shows LbL film assembly through dipcoating process. Note that, in addition to dip-coating, LbL assemblies can be also constructed using different deposition methods such as spin coating and spraying [34].



Figure 1.2. Schematic representation of layer-by-layer self-assembly technique.

LbL films can be fabricated onto two- dimensional (2D) substrates, e.g. glass, quartz, silicon wafer, mica [35] or three-dimensional (3D) substrates, e.g. silica

microparticles [36], porous calcium carbonate microparticles [37], calcium phosphate nanoparticles [38]. LbL method allows use of aqueous polymer solutions. Additionally, it is simple, efficient and reproducible, thus it is very convenient to create biologically relevant surfaces [39]. By adjusting the pH, temperature, ionic strength of assembly solutions, properties of polyelectrolyte multilayers can be altered [40]. Multilayer properties can also be tuned at the post-assembly step. The following subsections will provide information about controlling film properties at the assembly and post-assembly steps.

1.1.1. Effect of Layer-by-Layer Self-Assembly Conditions on Film Properties

1.1.1.1. Effect of Chemical Nature of Polyelectrolytes on LbL Growth

The functional groups in each repeating unit of polymers plays a crucial role on LbL film properties. Thus, the chemical nature of polyanions and/or polycations is the most critical factor affecting the film properties [41]. The thickness of LbL films and the composition of the multilayers can be controlled by proper selection of the polyelectrolytes and deposition ambient [42,43]. For example, Taketa et al. showed that under the same pH and ionic strength conditions, self-assembly of Chitosan and alginate provided multilayers with low thickness values, whereas Chitosan and hyaluronic acid formed thicker LbL films same [44].

The chemical nature of the polyelectrolytes also affects the multilayer growth regime. The growth pattern of LbL films is basically divided into two categories: linear growth and exponential growth [14]. The thickness of each layer increases constantly in linear growth. In exponential growth, the thickness of each layer increases exponentially as the layer number increases. Multilayer films of poly(L-lysine) (PLL)/alginate [45], and PLL/hyaluronan [46,47] were the first reports of LbL films in which exponential growth was detected by the mass and thickness measurements. There are two different explanations in the literature for the mechanism of exponential growth. The first

explanation suggests that exponential growth is correlated with the surface roughness which provides additional site for the deposition of polymers which eventually leads to exponential growth [46]. The second explanation suggests that at least one of the film components diffuse in and out throughout the self-assembly process which leads to greater amount of complexation at the interface and eventually exponential growth of multilayers [46,47].

In addition to nature of polyelectrolytes, polyelectrolyte chain length also plays a role on the LbL film growth. The short chain polymers have tendency to form unstable films which also lack uniform growth. In contrast, uniform film growth and stability were both found to be greater for long chain polymers [48]. Combination of polymer chains with different molar masses also did not provide robust film growth and stability. For example, Schlenoff et al. reported that when the surface coated with the short chain polymers was exposed to a solution of polymer with molar mass ~ 10^4 Da, the short chain polymers are partially stripped off from the surface by the oppositely charged long chain polymers resulting in a decrease in film thickness and eventually inhibition of multilayer growth [49].

1.1.1.2. Effect of Ionic Strength on LbL Growth

The total thickness which is determined in Angstrom precision changes with ionic strength of the solvents. The screening of charges through polyelectrolyte chains by adding salt ions makes polymer molecules more coiled, so thickness of the polyanion and polycation pairs become higher and rougher multilayers are obtained [50]. For example, Schmitt et al. prepared polystyrene sulfonate (NaPSS) solutions with varying sodium chloride concentrations and deposited NaPSS with poly(allylamine hydrochloride (PAH) at the surface using LbL technique. They found that NaPSS solution with the highest salt concentration provided higher multilayer thickness compared to NaPSS/PAH films deposited in the absence of additional electrolyte. The

higher film thickness in the presence of additional salt was explained by the loopy conformation of adsorbed polymer chains [51].

In another study, Blomberg and co-workers reported that for Chitosan and heparin multilayer films, the mass of the adsorbed multilayer film which was monitored by dual-polarization interferometry and quartz crystal microbalance increased when the ionic strength of Chitosan solution was increased from 0.1 mM to 150 mM at constant pH. The same effect was obtained when they increased the pH 4 to 5.8 at fixed ionic strength [52].

1.1.1.3. Effect of pH on LbL Growth

Controlling pH of LbL polymer deposition solutions is one way to obtain multilayer films of weak polyelectrolytes with remarkably different surface properties. Charge density of weak polyelectrolytes can be controlled by the pH of the environment. For example, Rubner et al. showed that slight changes in pH of polymer deposition solutions results in formation of very thick or very thin multilayers [53]. They investigated the effect of deposition pH on the layer thickness and organization of PAH/PAA multilayer thin films by changing pH of dipping solution systematically. Ionization degree of an adsorbed PAA chain varies from 20-30 % to 100 % between pH 2.5-6.5 range whereas PAH chains are almost fully ionized between this pH interval as determined by FTIR. In 2.5-4.5 pH region, when pH increases PAH layer thickness increases while PAA layer thickness decreases. The reason why PAA thickness decreased as the pH increased was the reduced number of loops and tails due to highly charged PAA chains. Conversely, PAH layer thickness increases because higher number of oppositely charged PAH chains are required to neutralize the more ionized chains of previously adsorbed PAA layer due to increase in pH. They also showed that surface roughness of PAH/PAA multilayer films (20-layers) increases with increasing pH at 2.5-4.5 pH region due to more loopy conformational state of adsorbed chains. On the other hand, when the multilayers constructed at pH range of 6-7.5, surface roughness is very low (<10 Å) since flat conformation of chains are adopted during assembly as polyions are fully charged.

In another study of Rubner et al., it was reported that the structure and composition of carboxymethyl cellulose (CMC)/Chitosan multilayer films were affected by the deposition pH [54]. When multilayers assembled at pH 5, thinner films were produced because both Chitosan ($pK_a \approx 6.5$) and CMC ($pK_a \approx 4$) were highly charged. In contrast, the multilayers constructed at pH 3 provided higher thickness although CMC chains were poorly charged at this pH. The reason for the thicker films at pH 3 films was explained by the hydrogen bonding interactions among the COOH groups of CMC and OH groups of Chitosan in addition to the electrostatic association between the carboxylate groups of CMC and protonated amino groups of Chitosan.

Recently, Gundogdu et al. showed that film deposition pH of poly[2-(dimethylamino)ethyl methacrylate]-*b*-poly[(2-(diethylamino)ethyl methacrylate)] (PDMA-*b*-PDEA) micelles and poly(styrenesulfonate) (PSS) multilayers importantly affected the thickness and pH-stability of the multilayer films [55]. It was reported that PDMA-*b*-PDEA micelles/PSS multilayers assembled at pH 8 were thinner than pH 8.5 films but intense due to higher charge density of PDMA coronal chains providing more binding points between the polymer pairs. When multilayers of pH 8 is exposed to mildly acidic conditions, they were stable however multilayers assembled at pH 8.5 were disassembled when exposed to neutral pH due to relatively weakly associating chains as micelle corona being less ionized compared to pH 8 films.

1.1.1.4. Effect of Temperature on LbL growth

Temperature is another critical parameter to tune the LbL film properties when at least one of the film components is a temperature-responsive polymer. For example, Quinn and Caruso constructed LbL thin films of PAA and thermosresponsive poly(N-isopropylacrylamide) (PNIPAAm). The effect of the temperature on film properties was investigated. The mass proportion of PNIPAAm in the film got higher as the deposition solution temperature was gradually increased from 10 °C to 21 °C and to 30 °C. Also, smoother surfaces were obtained when the film was prepared at the temperatures closer to the lower critical solubility temperature (LCST) of PNIPAAm (32 °C). The higher amount of PNIPAAm within the multilayers at higher temperatures was correlated with the solution state property of PNIPAAm which is related to coil to globular transition of polymer chains near to LCST. The smoother multilayer surfaces at high temperatures was explained by the tighter coil form of PNIPAAm chains which led to tighter packing during adsorption.

In another study, Tan et al. examined the thickness of polyelectrolyte multilayers (PEMs) composed of two strong polyelectrolytes, i.e. poly(diallyldimethylammonium chloride) (PDDA) and PSS at low and high temperatures (10 °C-70 °C) and proposed a new mechanism for temperature sensitive deposition. They observed that roughness of the films increased by high salt and temperature conditions and explained this observation by the temperature induced swelling and melting of the films. By rinsing after each step for 10 seconds in hot DI, the film roughness was found to be reduced. In addition to this, heat was suggested to increase the fluidity of the films. In a related study, the rms roughness of PDDA/PSS films was also reported to be reduced by annealing PEMs in salt solutions [56]. These researchers proposed that temperature could have provided acceleration for this annealing process [57].

Kankare and co-workers studied the effect of temperature on the building process of poly(diallyldimethylammonium) (PDADMA)/PSS and PSS/PAH multilayers. The increment in deposition temperature induced change on the rate of the layer-by-layer build-up process. They found that increasing temperature induced the exponential build-up regime in both systems. Linear growth regime was found when they deposited multilayers at 15 °C and 25 °C. The linear growth regime shifted to exponential growth regime in case deposition temperature was increased to 45 °C and

55 °C. They correlated the shift in the growth profile from linear to exponential with increasing temperature with the diffusion rate of the polymers [58].

1.1.2. Effect of Layer-by-layer Post-Assembly Conditions on Controlled Release of Functional Molecules

The improvements in fabrication of polymer containing platforms or drug loaded platforms has been achieved for therapeutic drug loading and release area. Various polymer platforms such as gels, liposomes, micelles and multilayers are developed to find the optimal drug release properties. LbL polymer films can be constructed through various polymer-polymer interactions. Similarly, the drug molecules can be loaded into the multilayers through varying polymer-drug interactions. This provides easy tuning of drug release properties from multilayers through disruption of polymer-drug interactions. LbL multilayer thin films constructed using stimuli responsive polymers give response to various conditions such as pH, ionic strength, temperature etc. By taking advantage of the stimuli-responsive behaviour of the films, it is possible to trigger the release of drug molecules from LbL multilayer films.

1.1.2.1. pH Responsive LbL Films

Multilayers composed of pH-responsive polymers can be used to trigger the release of functional molecules from the surface in a pH-responsive manner. For example, Hong et al. co-assembled PS-*b*-PAA amphiphilic block copolymer micelles (BCM) with 2D amine functionalized graphene oxide (GO), or branched polyethylenimine (BPEI) through electrostatic interactions between the carboxyl group of BCM and amine group of functionalized GO or BPEI. Drug incorporated PS-*b*-PAA micelles released the drug molecules more easily at pH 2 than that at pH 7.4 due to protonation of the carboxylate groups on the micellar corona and disruption of interactions among the LbL films. In comparison to GO/BCM film, BPEI/BCM film was found to release the drug more slowly at both pH 7.4 and 2 in PBS due to different disintegration profiles

of the two films. They explained that GO sheet and BCM multilayers disintegrated at once, whereas BPEI and BCM multilayers gradually dissociated [60].

Hammond et al. studied hydrolytically degradable LbL thin films which were constituted with polymer poly(β -amino ester) and a series of model therapeutic polysaccharides (heparin, low molecular weight heparin, and chondroitin sulfate). It was found that heparin loaded films degraded linearly at pH 7.4 and pH 6.2 because of decomposition of poly(β -amino ester). Poly(β -amino ester) degraded more faster in basic conditions than acidic conditions. Degradation was due to the cleavage of the main chain of poly(β -amino ester) which occurred through nucleophilic attacks of free hydroxyl ions or intramolecular amine groups of poly(β -amino ester) or the nearby molecules. The drug released from multilayers more faster at pH 7.4 than pH 6.2 due to faster degradation of poly(β -amino ester) at higher pH values [61].

Li et al. selected ibuprofen (IBU) as a model drug and integrated ibuprofen into the hollow mesoporous silica (HMS). They reported that when HMS spheres which have 300-400 nm average diameter coated with PAH and PSS, the system can give controlled release of IBU on specific pH and salt conditions. The electrostatic interactions between the oppositely charged PEM layers become weak at the higher salt concentration. As a results of incompact PAH/PSS multilayers, the drug diffuses into the release medium easily. On the contrary, at the increased pH value such as pH 8, compact PAH/PSS multilayers show sealing effect and decreases permeability of IBU [62].

1.1.2.2. Temperature Responsive LbL Films

Besides pH, temperature is also one of the most commonly used triggering mechanisms in controlled release applications.

Recently, Haktanıyan et al. reported pH and temperature-induced release of anticancer drug, doxorubicin (DOX) from poly(2-isopropyl-2-oxazoline) (PIPOX) and TA LbL films. It was found that the amount of DOX release increased when the temperature of the release medium was raised from ambient to physiological temperature. It was explained by LCST behaviour of PIPOX and the conformational changes within the multilayers resulting in formation of voids and facilitating the drug release [63]. In another study of the same research group, temperature-induced release of CIP was reported from PIPOX/TA multilayers, whereas insignificant temperature effect on CIP release was detected for poly(2-ethyl-2-oxazoline) (PEOX)/TA multilayers [64].

Sukhishvili and co-workers fabricated temperature and pH responsive multilayers. They found that the critical pH (pH_{crit}) at hydrogen-bonded LbL films disintegrate changed with temperature for multilayers composed of dual responsive, i.e. pH and temperature-responsive polymers. For example, the pH_{crit} increased in the temperature range of 10-37 °C for multilayers of poly(methacrylic acid) (PMAA) and LCST-type poly(N-isopropylacrylamide) (PNIPAM), while the pH_{crit} decreased in the same temperature range for PMAA and UCST-type polyacrylamide (PAAm) [65].

1.1.3. Chitosan Based LbL Self-Assembly Films

LbL deposition of Chitosan has also been of interest for various biomedical applications due to important biological properties of Chitosan and many advantages of LbL technique. In this section Chitosan based LbL films and their potential applications are explained.

Lu and co-workers used catechol to functionalize Chitosan and hyaluronic acid to increase the stability of Chitosan and hyaluronic acid LbL films under physiological conditions 3,4 Dihydroxyhydrocinnamic acid (DHPA) was grafted on Chitosan through chemical reaction between the amino groups on Chitosan and carboxyl groups on DHPA using carbodiimide chemistry. Hyaluronic acid was modified by dopamine which have also catechol groups in its structure. Modified hyaluronic acid and Chitosan were then used for the construction of LbL multifunctional films which were

then used as a platform for loading drug (ASC14). The films showed swelling in acidic environment and shrinking in alkaline environment. So, more amount of drug could be released at pH 6.5 than that at pH 7.4 in PBS. Authors suggested use of such a drug delivery system in tissue engineering applications [66].

In another study, Pérez-Anes et al. produced β -cyclodextrin-based polymer (PCD) and Chitosan multilayers using LbL method for local delivery of therapeutics from implants. They obtained PCD by the polycondensation reaction between citric acid and β -cyclodextrin. PCD/Chitosan multilayers strongly attached to the titanium biomaterial surface through polydopamine precursor film which acted as a bridge between PCD/Chitosan multilayers and the titanium substrate. They loaded gentamicin antibiotic into the coated titanium multilayers for the purpose of constructing a drug-eluting system to treat infections [67].

Yu and Pishko prepared paclitaxel nanoparticles and coated them with Chitosan and dextran-sulfate through LbL method. Surface of the Chitosan/dextran-sulfate coated paclitaxel nanoparticles were then conjugated with poly(ethylene glycol) and fluorescence labeled wheat germ agglutinin to create biocompatible, hydrophilic and targeted drug delivery system. The released amount of paclitaxel was regulated by the number of Chitosan and dextran-sulfate layers. The nanoparticle based drug delivery system was considered as an appropriate candidate for the treatment of colon cancer [68].

Mohanta et al. constructed nanocarriers which consisted of Chitosan/alginate LbL self-assembled films coated onto mesoporous silica nanoparticles (MSNs). Coated MSNs exhibited low hemolytic and cytotoxic activity against red blood cells compared to uncoated MSNs.

Therefore, they may be promising materials for intravascular drug delivery. In the same study, the anticancer drug DOX was loaded into LbL modified MSNs. Efficacy of DOX loaded Chitosan/alginate coated MSNs was assessed through *in vivo* and *in vitro* studies. The results of *in vitro* studies showed that DOX-loaded nanocarriers

exhibited a long-term retention in HeLa cell nucleus and prolonged intracellular DOX release. *In vivo* studies showed that DOX loaded nanocarriers were found to stay longer in rats and showed slower elimination from blood plasma of rats than free DOX [69].

Liu and co-workers coated the surface of fatty acid containing nanoliposomes (NLs) using Chitosan and sodium alginate through LbL technique. Chitosan/sodium alginate multilayer coating was reported to provide stability and prevent leakage of encapsulated food ingredients such as fatty acids from NLs. The enzymic digestion stability results showed that coated NLs have better resistance to lipolytic degradation and allow a lower level of encapsulated component release at gastrointestinal conditions. The coated NLs could be used for development of functional food ingredients delivery systems in gastrointestinal tract [70]. Similarly, in another study, liposomes containing Hepatitis B surface antigen were coated with Vitamin B₁₂ conjugated Chitosan and sodium alginate. Intestinal antigen presenting cells were chosen as a target for coated liposomes. Rough surface for oral delivery of the antigen was formed by the rearrangement of layers on liposomes at intestinal conditions because improved surface roughness increased biological interaction and facilitated uptake in the target cell. Also coating prevented the degradation of liposomes in gastric conditions [71].

When biological implants come into contact with the physiological fluids, proteins in biofluids covers immediately the surface of the implant. Protein adsorption on biological implants leads to failure of devices and accelerates the formation of biofilm. At this point, antifouling surfaces gain interest due to inhibiting properties of microfouling, macrofouling organisms and protein adhesion [72].

Liu et al. reported that crosslinked Chitosan and hyaluronan LbL films on polydimethylsiloxane (PDMS) exhibited stronger resistance to adsorption of proteins in human saliva [73]. Similarly, Zhu and co-workers demonstrated that paclitaxel/Chitosan nanofibers coated with poly(2-methacryloyloxy-

ethylphosphorylcholine-co-methacrylicacid) (PMAs)/Chitosan through LbL method were effective against platelet adhesion. Also, significant inhibition of fibrinogen and bovine serum albumin adhesion were recorded [74].

By achieving LbL assembly of dextran aldehyde (Dex-CHO)/carboxymethyl Chitosan (CMCS) films, self-polishing multilayer coatings were carried out for combating biofouling. The self-polishing property was accomplished through breakage of the imine linkage between Dex-CHO and CMCS at acidic conditions followed by the release of the topmost layer containing the attached bacteria and protein. The self-polishing capability of multilayers was needed for antifouling of high concentrations of bacteria [75].

Nowakowska and co-workers constructed LbL films which consisted of only ionic derivatives of Chitosan. The components of the multilayers were cationic derivative of Chitosan (CCh) and anionic derivative of Chitosan (ACh). LbL film growth achieved with the interaction between quaternary ammonium groups of CCh and sulfonate groups of ACh. They studied antifouling and antibacterial properties of films and revealed the relation between the physicochemical properties and biological activity of the films [76].

In another study, Chitosan/Alginate LbL films which were crosslinked by glutaraldehyde showed enhanced stability under physiological conditions. It was found that the deposition of human albumin protein onto crosslinked multilayers could be controlled by physicochemical properties of the multilayer system [77].

1.2. The aim of the thesis

The aim of this thesis was:

i) to construct two different Chitosan based LbL films using different types of polyacids, i.e. polycarboxylic acid and polyphenol with varying pK_a values;
ii) to examine the surface properties of the films such as LbL growth, stability, surface morphology and wettability;

iii) to investigate the biological properties such as protein adhesion and drug loading capacities of the films

iv) to understand the structure-property relationship in these two different Chitosan based LbL films and correlate the surface properties with the protein adhesion and drug release properties of the films.

CHAPTER 2

EXPERIMENTAL PART

2.1. Materials

Acetic acid (\geq 99 %), Bovine Serum Albumin (BSA) (lyophilized powder), Branched Poly(ethyleneimine) (M_w = 25000), Low molecular weight Chitosan (mol wt 50000 -190000 Da and % 75-85 deacetylated), Ciprofloxacin (\geq 98 %, HPLC), Lysozyme (LYS) from chicken egg white (lyophilized powder, \geq 40000 units/mg protein), Phosphate Buffer Saline (PBS) (tablet), Poly(acrylic acid) (M_w = 1800) were purchased from Sigma-Aldrich Chemical Co. Hydrochloric acid fuming (HCl) (37 %), Luria Bertani (LB) broth (MILLER), Mueller-Hinton (MH) broth, Sulfuric acid (H₂SO₄) (95-97 %), Sodium dihydrogen phosphate dihydrate (NaH₂PO₄.2H₂O), Sodium Hydroxide (NaOH) (Pellets), Tannic Acid (M_w = 1701.20) were purchased from Merck Chemicals. Dulbecco's Phosphate Buffered Saline solution were purchased from Biological Industries. The deionized water was purified by Milli-Q system (Millipore) at 18.2 MΩ.

2.1. Methods and Equipments

2.1.1. Preparation of Polymer Solutions

Chitosan was dissolved in 0.001 % (v/v) acetic acid solution with a final Chitosan concentration of 1 mg/mL. PAA was dissolved in 10 mM phosphate buffer with a final PAA concentration of 1 mg/mL. Both Chitosan and PAA solutions were left for overnight at 25 °C in a Lauda Alpha RA 8 chiller circulator for complete dissolution. TA was dissolved in 10 mM phosphate buffer solution with a final TA concentration of 1 mg/mL. In contrast to Chitosan and PAA solutions, TA solution was always

prepared fresh before film preparation to avoid degradation of TA during an overnight waiting period.

2.1.2. Preparation of Chitosan/TA and Chitosan/PAA Multilayers

Silicon wafers or glass slides were immersed into concentrated sulfuric acid (% 98) for 85 minutes. After acid treatment, the substrates were rinsed first with distilled and then DI water for several minutes and dried under a flow of nitrogen gas. Then, the substrates were put into 0.25 M NaOH solution for 10 minutes, followed by rinsing with first distilled and then DI water and dried under nitrogen flow. Prior to construction of the multilayers, the substrates were coated with a precursor layer which was BPEI. BPEI was dissolved in 10 mM phosphate buffer solution with a final BPEI concentration of 0.5 mg/mL. The precursor layer deposition was performed at pH 5.5 for 30 minutes followed by rinsing with 10 mM phosphate buffer at pH 5.5 for 1 minute.

Multilayer deposition was started was immersing the positively charged, BPEI coated substrates into the polyanion solution which was either TA solution (1 mg/mL, pH 5) or PAA solution (1 mg/mL, pH 4) for 15 minutes. This was followed by rinsing the substrates for two times with 10 mM phosphate buffer at pH 5 (for TA coating) or pH 4 (for PAA coating) each for 1 minute. Second layer deposition was performed by immersing the substrates into 1 mg/mL Chitosan solution at either pH 5 (for Chitosan/TA films) or pH 4 (for Chitosan/PAA films) for 15 minutes. Substrates were then rinsed twice using 10 mM phosphate buffer solution at pH 5 (for Chitosan/TA films) or pH 4 (for Chitosan/PAA films) each for 1 minute. This cycle was continued until desired number of layers were deposited at the surface.

TA solution was refreshed every hour to avoid degradation of TA during film construction. Rinsing solutions were refreshed after deposition of every 4 layers to avoid contamination in the rinsing solutions. LbL growth was followed by measuring the dry film thickness after every layer deposition using a spectroscopic ellipsometer

of Optosense, USA (OPT-S6000). The ellipsometry angles were arranged to 65° and fitted in the wavelength range of 400-1100 nm.

2.1.3. Crosslinking of Chitosan/PAA Multilayers

Chitosan/PAA multilayers were crosslinked through thermal treatment. Films were placed in a glass petri dish and put into Nüve EV 018 vacuum oven for 45 min at 150 °C or 110 °C.

2.1.4. AFM Imaging of Multilayer Films

AFM imaging of the films was performed using an NT-MDT Solver P47 AFM in tapping mode with Si cantilevers. Roughness values were taken from images with 2 x 2 μ m scan size. Multilayers were deposited into 2 cm x 1.5 cm glass slide as described in Section 2.2.2.

2.1.5. Determination of Wettability

Static contact angle measurements were carried out by Attension Theta Lite optical tensiometer. Approximately 1.5 μ L droplet of deionized water was formed and dropped onto multilayers constructed onto glass slides. Nine water droplets were deposited onto each sample. For each droplet, 10 independent measurements were recorded with 464 millisecond frame intervals.

2.1.6. Stability of Multilayers

Multilayer stability was followed at two different conditions: 1) in PBS at pH 7.4 and 37°C and 2) in PBS at pH 5.5 and 37 °C. First PBS was prepared by dissolving 1 tablet in 200 mL DI water. Multilayers were then immersed into PBS at pH 7.4 and 37 °C

or PBS at pH 5.5 and 37 °C. Films were rinsed with monobasic buffer at 37 °C before ellipsometric thickness measurements. Temperature was kept constant throughout the stability experiments using a water bath. Stability was followed by measuring the thickness of the remaining film at the surface as a function of time. Thickness values were normalized to the initial film thickness to calculate the remaining fraction at the surface.

2.1.7. Protein Adsorption onto Multilayers

BSA solution was prepared by dissolving BSA in PBS at a final BSA concentration of 1 mg/mL. Similarly, Lysozyme solution was prepared by dissolving LYS in PBS at a final LYS concentration of 1 mg/mL. Silicon wafers coated with Chitosan/TA or Chitosan/PAA multilayers and blank samples (uncoated silicon wafers) were immersed into either1 mg/mL BSA or LYS solution and incubated for 1 hour at 37 °C on an orbital shaker table. After protein deposition, substrates were rinsed two times using 10 mM phosphate buffer at pH 7.4 followed by drying under nitrogen flow. Ellipsometric thickness was measured before and after protein adsorption and an increment in thickness was attributed to protein adsorption onto multilayers.

2.1.8. CIP Loading into Multilayers

CIP was dissolved in 1 mM phosphate buffer at pH 2.5 to form 0.1 mg/mL solution and stirred magnetically overnight for complete dissolution. pH of CIP solution was raised to 7.4 prior to loading into multilayers. Glass substrates (2 cm x 2 cm) coated with 20-bilayer Chitosan/TA or crosslinked Chitosan/PAA films on both sides (total 40-bilayers) were put into 20 mL of 0.1 mg/mL CIP solution at pH 7.4 for 12 hours at room temperature. Multilayer coated substrates were then rinsed with 1 mM phosphate buffer at pH 7.4 for 1 minute.

2.1.9. CIP Release from Multilayers

40-bilayer (20-bilayer on each side of the glass slide) CIP loaded Chitosan/TA or Chitosan/PAA films were immersed into 20 mL PBS at pH 5.5 and pH 7.5. The temperature of solutions was fixed at 37 °C with a water-bath. CIP release was followed by taking aliquot from the release solution at certain time intervals and monitoring the evolution of the fluorescence intensity of CIP at 414 nm (for release at pH 7.4) or 445 nm (for release at pH 5.5) as a function of time using a Hitachi F-2500 fluorescence spectrophotometer. Excitation wavelength was 280 nm. The excitation and emission slits were taken 5.0 nm and 5.0 nm. Calibration curves were prepared separately under both release conditions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Characterization of Multilayers

3.1.1. Layer-by-Layer Growth of Chitosan Based Multilayers

Chitosan has a pK_a of 6.5 [13] and the primary amino groups of chitosan give it a cationic nature especially at pH below 6.5 (Figure 3.1). Chitosan was self-assembled with either TA or PAA at the surface using LbL technique. TA is a natural polyphenol (Figure 3.1). It has 25 hydroxyl groups per molecule. The pK_a of TA depends on the source that it was extracted. Two pK_a values have been recently reported for the one that was used in this study in another work of Erel Research Group [63]. PAA is a synthetic polycarboxylic acid with a pK_a of 4.5 [78] (Figure 3.1). Multilayer growth was followed using ellipsometry technique. Both films were constructed onto a BPEI precursor layer for a more robust film growth. The first layers were either TA or PAA.



Figure 3.1. Molecular structures of Tannic acid, Poly(acrylic acid) and Chitosan.

The driving force for multilayer growth was both electrostatic and hydrogen bonding interactions between Chitosan and the polyacids (TA or PAA). Chitosan/TA and Chitosan/PAA films were constructed at pH 5 and pH 4, respectively. At pH 4 and pH 5, chitosan had protonated amino groups. Although multilayers were constructed at pH values below the pK_a values of TA and PAA, the association between Chitosan/TA and Chitosan/PAA was expected to occur through both electrostatic and hydrogen bonding interactions. The ionization of TA and PAA possibly enhanced in the presence of positively charged Chitosan leading to electrostatic association among primary amino groups of Chitosan and phenolate groups of TA or carboxylate groups of PAA. On the other hand, Chitosan has hydroxyl groups which also possibly

associated with phenolic hydroxyl groups of TA and protonated carboxylic acid groups of PAA through hydrogen bonding interactions.

The two films demonstrated remarkably different growth profiles. For Chitosan/TA multilayers, the amount of Chitosan that could be deposited at the surface was significantly low compared to that of TA (Figure 3.2A). Alternatively, Chitosan could have dissolved some of the TA that was already deposited at the surface to form water-soluble Chitosan/TA complexes. The comparison of the thickness values for every increasing bilayer showed a linear growth profile with 0.5-3.1 nm increment per bilayer. Similar observations were recorded by Lvov and co-workers for TA/PAH LbL films [79]. They found that the film thickness of TA/PAH rised between 1.2-5.7 nm for a single TA layer and then decreased after deposition of the PAH layer especially at low pH. Also, the growth profile of TA/PAH is comparable to TA/CHI multilayers reported in this study.

For Chitosan/PAA films, an exponential growth profile was recorded (Figure 3.2B). The thickness of multilayer films reached 48.3 nm even after 8 layers of deposition. The thickness of multilayers could not be measured via ellipsometry beyond layer number 8 due to whitish color of the film surface at higher layer numbers resulting in scattering of the light and unreliable thickness measurements. Exponential growth regime is explained by two different mechanism: i) the increase in surface roughness with increasing layer number provides progressively increased surface area for polymer deposition, leading to an exponential growth of multilayers [80–82] and ii) in and out diffusion of at least one of the polymer through the multilayers resulting in deposition of higher amount of material with increasing layer number [83–85]. In and out diffusion mechanism was suggested by Schaaf and co-workers for PLL/hyaluronan multilayers. When HA terminated multilayers were exposed to PLL solution, most of the PLL chains diffused into the entire film besides being deposited on the top. During the deposition of the next hyaluronan layer, PLL chains diffused outward, formed PLL/hyaluronan complexes and deposited at the interface which led to additional increment in thickness [86]. Similarly, in addition to roughness

increment, the significant increase upon deposition of the 8th, PAA layer in case of Chitosan/PAA films might be also due to in and out diffusion of PAA through the multilayers.



Figure 3.2. (A) Evolution of film thickness of TA/CHI film as a function of bilayer number at pH 5 and 25 °C. Inset shows the LbL growth of the multilayers. (B) LbL growth of Chitosan/PAA films at pH 4 and 25 °C. For both films, multilayers were constructed onto BPEI precursor layer with a thickness of ~ 2.5 nm. The precursor thickness was added into the thickness values in the graph.

3.1.2. Surface Morphology of Chitosan Based Multilayers

AFM imaging was performed to contrast the surface morphology of Chitosan/TA and Chitosan/PAA multilayers. Figure 3.3 shows AFM height images and the corresponding roughness values of 13-layers of Chitosan/TA and Chitosan/PAA films. The difference in surface morphology of the two multilayers can be attributed to the difference in the binding strength among the layers. Chitosan/PAA films. The number of intermolecular contacts between TA and Chitosan was possibly higher than that between Chitosan and PAA due to greater extent of hydrogen bonding interactions between TA and Chitosan. In addition to hydrogen bonding, the dendrimer-like structure of TA could have provided enhanced association between TA and Chitosan multilayers. The higher number of intermolecular contacts among the Chitosan and TA layers provided intense multilayers with smooth surfaces (roughness ~ 1 nm), whereas a roughness of 7.6 nm was recorded for relatively weakly interacting PAA and Chitosan layers. It must be borne in mind that PAA has a pK_a of 4.5 and at the multilayer deposition pH of 4, PAA has lower number of hydrogen donor groups compared to TA. Therefore, the contribution of hydrogen bonding to Chitosan/PAA multilayers was lower than Chitosan/TA films. The loose multilayer structure in Chitosan/PAA films might have resulted in more loopy conformation of polymer chains in the films, leading to higher surface roughness. Kharlampieva and Sukhishvili correlated the more loopy conformation of polymer chains within the multilayers with the lower number of interlayer adhesion points among weakly interacting hydrogen bonding polymer pairs [87]. Shiratori and Rubner found that surface roughness increased significantly due to more loopy conformational arrangement of polymers in electrostatic multilayer films [53]. On the other hand, in and out diffusion mechanism suggests deposition of Chitosan and PAA complexes at the interface. In such a case, the next polymer layer preferably deposits onto higher parts of the surface which significantly increases the surface roughness with increasing layer number.



Figure 3.3. AFM height images (5x5 µm scan size) and roughness values of 13-layer Chitosan/PAA and Chitosan/TA films

3.1.3. Wettability of Chitosan Based Multilayers

Chitosan/TA and Chitosan/PAA films were compared with respect to their wettability. Static contact angle measurements were taken for 6-, 7-, 12-, 13-, 18- and 19-layer films which were deposited onto glass substrates. For Chitosan/TA films, wettability was always higher when the topmost layer was TA except 6- and 7-layer films which behaved similarly possibly due to very low amount of material at the surface and low surface coverage. The surface wettability is exceedingly responsive to the outmost layer composition [88]. When the amount of TA at the film surface is higher, the surface had a greater tendency to spread water leading to lower contact angle values. Because of the phenolic groups of TA, substrate surface coated with TA had greater wettability [89]. It must be borne in mind that, as it is seen in Figure 3.4A, thickness values of 6- and 7-layer films are much lower than 12-, 13-, 18-,19-layer films and they are closer to first layer. In case of Chitosan/PAA system, films with Chitosan topmost layer had lower contact angle values compared to Chitosan/TA films with

similar layer number. This could be due to lower association among the layers and higher extent of interpenetration between Chitosan and PAA films providing the contribution of both polymers' functional groups to the topmost layer. Note that for both types of the Chitosan films, the lower contact angle value for 6-layer films with Chitosan topmost layer might be correlated with the lower surface coverage at low layer numbers and the contribution of the protonated amino groups of the anchoring layer (BPEI) to the surface hydrophilicity [90].





Figure 3.4. Evolution of static contact angle of Chitosan/TA (A) and Chitosan/PAA (B) films as a function of increasing layer number. Films with odd layer number refer to multilayers with TA topmost layer, whereas those with even layer number refer to multilayers with Chitosan topmost layer. Uncoated glass substrate was used as control.

3.1.4. Stability of Multilayers

To mimic the biological environment, the stability of Chitosan/TA and Chitosan/PAA multilayers were assessed in PBS at pH 7.4 and 37 °C. In addition, the stability of multilayers was also examined at moderately acidic conditions to due local pH drops at an infectious site in the body. Stability was followed by measuring the film thickness as a function of time after immersing the multilayers into PBS at 37 °C followed by drying under N₂ flow. At each time point, the thickness was normalized to the initial film thickness.

3.1.4.1. Stability of Chitosan/TA Multilayers

Figure 3.5 shows the fraction retained at the surface as a function of time for 12-layer Chitosan/TA films. The difference between Panel A and Panel B figures is the selfassembly procedure. Figure 3.5A belongs to multilayers in which films were dried after every layer deposition. Figure 3.5B belongs to multilayers in which films were dried only at the end of film construction. 12-layer Chitosan/TA film which was dried after each layer deposition was completely stable for a 12-hour period. No significant change in film thickness was recorded for the first 5 hours. Multilayers swelled when left in PBS at 37 °C for an additional 7-hour period. The reason for swelling can be explained by penetration of salt ions in PBS into the multilayers. When salt ions pair with polyelectrolytes, it improves hydrophilicity of polymer complexes. Thus it is expected that additional water molecules might have been entrapped within the multilayers [63,91]. Distinctly, 35 % of 12-layer Chitosan/TA film which was dried only at the end of 12th layer deposition was lost in the first 2 hours in PBS at 37 °C. No further decrease in film thickness was recorded between 2-5 hours. Similar to multilayers which were dried after every layer deposition, multilayers swelled when long-term exposed to PBS at 37 °C. This result clearly demonstrates the effect of drying on the film stability. Effect of drying on LbL film growth and stability has been reported earlier. The chemically heterogeneous domains, defects on the PEMs surface could be eliminated by drying process. Defects are formed in the beginning of assembly process and decreases progressively with increasing layer number. Adsorbed polyelectrolyte chains in liquid/solid interface could possess free by form of tails and loops into liquid. Drying decreases water content and increases hydrophobic interactions. As a result of it, loops and tails are compensated with the oppositely charged polymer. A uniform and less defected surfaces stay more stable [92].



Figure 3.5. Fraction retained at the surface as a function of time for 12-layer Chitosan/TA films which was dried after every layer (A) or dried only after 12th layer (B)

3.1.4.2. Effect of Layer Number on the Stability of Chitosan/TA Multilayers

To examine the effect of layer number on the stability of multilayers, stability of 19layer Chitosan/TA films was followed in PBS at 37 °C as a function of time and the results were contrasted with 12-layer films (Figure 3.6). Unlike to 12-layer Chitosan/TA film, 19-layer Chitosan/TA film which was dried after every layer deposition lost 20 % of its initial thickness in the first 1 hour. Similarly, 19-layer film swelled when long-term exposed to PBS at 37 °C. The greater stability of 12-layer films can be correlated with the greater mechanical support provided by the substrate. Sukhishvili and coworkers reported that despite the layers in poly(Nvinylpyrrolidone) (PVPON)/PMAA, PVCL/PMAA and PNIPAM/PMAA films strongly integrated and showed linear growth regime, the films dispersed as the distance increased from the substrate. This was explained by the stronger chain coiling at the distance which was far from the substrate [87].



Figure 3.6. Fraction retained at the surface as a function of time for 19-layer Chitosan/TA film which was dried after every layer. 12-layer Chitosan/TA films prepared under identical conditions were plotted for comparison.

3.1.4.3. Stability of Chitosan/PAA Multilayers

In contrast to Chitosan/TA films, Chitosan/PAA films were not stable in PBS at 37 °C. The thickness of 8-layer Chitosan/PAA film decreased by approximately 35 % in the first 1 hour (Figure 3.7). Almost ~ 50 % of the film released from the surface at the end of five hours. Unlike to Chitosan/TA films, no swelling was recorded after long-term exposure to PBS at 37 °C. The difference between the stability of the multilayers can be correlated with the binding strength among the film layers. TA has a dendrimer-like structure which might have provided additional stability to the multilayers. In addition, TA has higher pK_a values than that of PAA which possibly lead to greater extent of hydrogen bonding interactions between Chitosan/TA films resulting in greater film stability.



Figure 3.7. Fraction retained at the surface of 8-layer Chitosan/PAA films as a function of time upon exposure to PBS at 37 °C.

3.1.4.4. Crosslinking of Chitosan/PAA multilayers

To enhance the stability of Chitosan/PAA multilayers at physiologically related conditions, thermal crosslinking was applied among the layers. Thermal crosslinking between polybases bearing amine groups and polycarboxylic acids has been reported in the literature [93]. 8-layer Chitosan/PAA films were prepared and exposed to first 110 °C for 45 minutes in an oven. However, no improvement in the stability was recorded (Figure 3.8). Enhanced stability of the multilayers can be explained by the crosslinking of the layers through amide bond formation between the primary amino groups of Chitosan and carboxylic acid groups of PAA. Crosslinking temperature was critical for efficient crosslinking of Chitosan/PAA multilayers. Increasing the crosslinking temperature to 150 °C provided stability and resulted in swelling by ~ 15 % of Chitosan/PAA films.



Figure 3.8. Fraction retained at the surface of crosslinked 8-layer Chitosan/PAA films at 150 °C for 45 min (square), at 110 °C for 45 min (circle) as a function of time. Non-crosslinked (triangle) film was plotted for comparison.

Finally, the effect of crosslinking on the surface morphology of multilayers was examined. Figure 3.9 contrasts AFM height images and roughness values of 13-layer non-crosslinked and crosslinked Chitosan/PAA films. The surface roughness slightly decreased upon crosslinking possibly due to formation of more intense films with slightly smoother surfaces.



Figure 3.9. AFM height images (5x5 µm scan size) and respectively roughness values of noncrosslinked and crosslinked 13-layer Chitosan/PAA multilayers.

3.2. Protein Adsorption onto Multilayers

3.2.1. Protein Adsorption onto Chitosan/TA multilayers

Two model proteins were used for protein adsorption experiments, i.e. BSA and LYS. Chitosan/TA multilayers were prepared with varying number of layers and BSA or LYS adsorption onto surface was evaluated. In addition, the effect of topmost layer on protein adsorption was examined. Multilayers were prepared and exposed to protein solution (1 mg/mL, prepared in PBS) at pH 7.4 and 37 °C for 1 hour. Films

were shaked using orbital shaker during protein adsorption experiments. Figure 3.10 shows evolution of film thickness upon protein adsorption for bare silicon wafer, 6-, 7-, 12-, and 13-layer Chitosan/TA films. The lowest amount of BSA adsorption was observed for 13-layer film with TA topmost layer (Figure 3.10A). Factors like hydrophilicity and hydrophobicity of protein or surface, electrostatic or other interactions (van der Waals and Hydrogen) among surface and protein, chemical characteristic of surface influence adsorption process of protein onto surface. Hydrophilic surface has large energy barrier for the replacement of water molecules from the surface [94]. Therefore, charge interactions between hydrophilic surface and protein and surface-induced conformational changes in protein provide energy change which usually leads to protein adsorption [95]. Although it is difficult to explain the reason behind the lowest amount of BSA deposition onto 13-layer Chitosan/TA film due to complicated surface-protein interactions, it is suggested that BSA with pI of 4.9 [96] carries net negative charge at neutral pH conditions, thus the deposition was lower onto 13-layer film with TA topmost layer due to electrostatic repulsion between TA and BSA. BSA adsorption onto 12-layer film with Chitosan topmost layer was slightly higher than 13-layer film with TA topmost layer which might be explained by the electrostatic association between negatively charged BSA and slightly positively charged Chitosan. The difference between 6- and 7-layer films was not significant. Interestingly, the amount of BSA deposited onto 6- and 7- layer films was greater than that deposited onto bare silicon wafer. This might be due very small thickness increments during LbL assembly of Chitosan/TA pair and relatively low surface at the layer number 6 or 7 resulting in higher BSA adsorption promoted by the positively charged BPEI precursor layer. In case of LYS adsorption (Figure 3.10B), the highest amount of adsorption was recorded for films with TA topmost layer, supporting the effect of charge interactions on protein adsorption. pI of LYS is 11.4 [96], thus LYS is positively charged at neutral pH conditions and the surfaces with negatively charged TA topmost layer were more promoting for Lysozyme adsorption. The films with Chitosan topmost layer did not also behave anti-adhesive against LYS adsorption. This can be explained with low amount of Chitosan deposition at the surface as well as low amount of positively charge on Chitosan chains which might not provide enough repulsion against LYS adsorption.



Figure 3.10. Evolution of film thickness before and after BSA adsorption (A) and LYS adsorption (B) onto Chitosan/TA multilayers with varying layer number. Dark gray parts represent the initial thickness of the films. Light gray parts correspond to the increment in film thickness upon BSA adsorption (A) and Lysozyme adsorption (B). Uncoated silicon wafer was used as control.

3.2.2. Protein Adsorption onto Chitosan/PAA multilayers

Protein adsorption experiments onto Chitosan/PAA multilayers could not be conducted using ellipsometry technique for 12- and 13-layer films due to high thickness values and whitish color of the surface leading to scattering of light and unreliable thickness measurements. Micro BCA technique which relies on releasing the adsorbed protein from the surface and quantification through spectroscopic techniques was also used to analyze the behavior of the surfaces against protein adsorption. However, microBCA technique also did not provide reliable results due to very low amount of protein extracted from the surface. For this reason, BSA and LYS adsorption could only be examined for 6-layer films with Chitosan topmost layer and 7-layer films with PAA topmost layer. Chitosan/PAA films did not show any antiadhesive behaviour. The amount of BSA or LYS adsorbed onto 6- and 7-layer films were greater than that deposited onto the control substrate which was uncoated silicon wafer. A comparison between Chitosan/TA and Chitosan/PAA multilayers showed that the amount of BSA or LYS adsorbed onto Chitosan/PAA was higher than that deposited onto Chitosan/TA multilayers. This can be due to higher surface roughness of Chitosan/PAA films which might have provided additional site for BSA or LYS adsorption. Importantly, the amount of LYS adsorbed onto 7-layer film with PAA topmost layer was remarkably high when compared to the BSA amount deposited onto 7-layer Chitosan/TA film with TA topmost layer. This might be due to the difference on the charge density of TA and PAA at pH 7.4. PAA with a pK_a of 4.5 is fully ionized at pH 7.4, whereas TA has a pK_a of 6.5 and 8 and it is partially ionized at pH 7.4. Therefore, the amount of LYS attracted towards the surface through electrostatic association might have been higher for 7-layer Chitosan/PAA films. In addition, the relatively loose Chitosan/PAA film matrix might have provided both incorporation of LYS into the multilayers and deposition onto the surface.



Figure 3.11. Evolution of film thickness before and after BSA adsorption (A) and LYS adsorption (B) onto Chitosan/PAA multilayers with 6- and 7- layer numbers. Dark gray parts represent the initial thickness of the films. Light gray parts correspond to the increment in film thickness upon BSA adsorption (A) and LYS adsorption (B). Uncoated silicon wafer was used as control.

3.3. Release of Ciprofloxacin from Multilayers

CIP is a second generation broad spectrum fluoroquinolone antibiotic which kills or slow down growth of both Gram-positive and Gram-negative bacteria [97]. CIP loaded 40-bilayer Chitosan/TA and Chitosan/PAA films were produced by immersing substrates into 0.1 mg/mL CIP solution (prepared in 1 mM phosphate buffer) at pH 7.4 for 12 hours at room conditions. For Chitosan/TA multilayers, electrostatic interactions among phenolate groups of TA ($pK_{a,1} = 6.5$ and $pK_{a,2} = 8$ [63]) and protonated piperazinyl amino groups of CIP (pK_a of piperazinyl amino groups = 8.7 [98]) mostly assured CIP loading into multilayers [64]. Similarly, carboxylate groups of PAA (pK_a of carboxyl groups = 4.5 [78,99]) electrostatically interacts with piperazinyl amino groups of CIP molecules resulting in CIP loaded Chitosan/PAA multilayers. In addition, the pK_a of carboxylic acid groups of CIP is 6.1 [98]. Therefore, free amino groups of Chitosan and carboxylic acid groups of CIP might have contributed to CIP loading into multilayers for both films. In addition to electrostatics, CIP loading into polymer multilayers might have occurred through hydrogen bonding interactions. CIP association with Chitosan/TA film matrix might have been also through hydrogen bonding interactions between carbonyl groups of CIP and phenolic hydroxyl groups of TA. Contribution of hydrogen bonding interactions into Chitosan/PAA multilayers is less likely because PAA is fully deprotonated at the CIP loading pH of 7.4. Figure 3.12 and Figure 3.13 shows chemical structure of CIP and schematic representation of association between CIP molecules and polymer multilayer components.





Chitosan

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44

PAA-CIP interaction



Figure 3.13. Schematic representation of hydrogen bonding and electrostatic interactions between CIP molecules and polymers.

CIP release studies were conducted at both pH 7.4 and pH 5.5. The reason for conducting the release experiment also at moderately acidic conditions was to mimic the acidic environment at an infectious site in the body. Prior to conducting release experiments at pH 7.4 and pH 5.5, stability of multilayers was also assessed at pH 5.5. Figure 3.14 shows fraction retained at the surface of 12-layer Chitosan/TA (Panel A) and 8-layer Chitosan/PAA (Panel B) films as a function of time upon exposure to PBS at pH 5.5 and 37 °C. As seen in the figure, both films were stable at pH 5.5. The

stability was even enhanced possibly due to protonation of the phenolate and carboxylate groups of TA and PAA, respectively and enhanced hydrogen bonding interactions among Chitosan and TA or PAA.



Figure 3.14. Fraction retained at the surface of (A) 12-layer Chitosan/TA and (B) 8-layer crosslinked Chitosan/PAA films as a function of time upon exposure to PBS at pH 5.5 and 37 °C.

After assuring the film stability at neutral and moderately acidic conditions, emission spectra of CIP in PBS at pH 7.4 and pH 5.5 were recorded to clarify the peak positions of CIP and specify the expectations during release studies. Emission spectra of CIP showed peaks centered at 445 nm and 414 nm at pH 5.5 and pH 7.4, respectively (Figure 3.15).



Figure 3.15. Emission spectra of CIP at pH 5.5 (A) and pH 7.4 (B).

After these preliminary studies, CIP release from both multilayers was followed at 37 °C and at pH 7.4 and pH 5.5. Multilayers were first exposed to PBS at pH 7.4 at 37 °C. A sample from release solution was taken at certain time intervals. The fluorescence intensity of the peaks at 414 nm and 445 nm was followed as a function of time. As seen in Figure 3.16, at pH 7.4 and 37 °C, significant amount of CIP released from both types of films in the first half an hour. Importantly, the amount of CIP released from Chitosan/PAA films was greater than that released from Chitosan/TA films. This was due to lower loading capacity of thinner Chitosan/TA films. It must be borne in mind that Chitosan/PAA multilayers showed exponential growth profile and the amount of CIP loaded into relatively loose Chitosan/PAA multilayers was expected to be much higher compared to Chitosan/TA films. The amount of CIP loaded into Chitosan/TA and Chitosan/PAA films was calculated by measuring the fluorescence intensity of CIP before and after immersing the multilayers into CIP solution (prepared with monobasic buffer at pH 7.4). Concentration of CIP was too high to get a reliable emission spectrum. For this reason, CIP solution was diluted by adding 1 mM monobasic buffer at pH 7.4 and 25 °C into $14 \,\mu\text{L}$ of CIP solution to form a final volume of 2 mL prior to fluorescence intensity measurements. The CIP amount in the solution before and after CIP loading into multilayers was quantified using calibration curves of CIP prepared under identical conditions (see appendix Figure A1). The dilution factor was considered during calculations. The loading amount of CIP into multilayers were found 72 µg and 295 µg for 40-bilayer Chitosan/TA and Chitosan/PAA films, respectively. In addition to the high loading capacity, in contrast to thin and intense Chitosan/TA films, Chitosan/PAA films might have facilitated the release of CIP molecules from the surface due to their loose film structure. The release at pH 7.4 can be explained by self-diffusion as well as salt-induced disruption of CIP/polymer interactions. It is worth mentioning that CIP loading was performed using CIP solution prepared in 1 mM phosphate buffer solution. However, CIP release was performed in PBS which had significantly higher amount of salt content (0.002 mol L^{-1} NaH₂PO₄, 0.008 mol L^{-1} Na₂HPO₄, 0.0027 mol L^{-1} KCl and 0.137 mol L^{-1} NaCl). As the salt ions diffused into the multilayer matrix, the electrostatic association among CIP/TA, CIP/PAA and CIP/Chitosan possibly disrupted resulting in CIP release from multilayers. Release of CIP from the surface was quantified using calibration curves prepared under identical conditions with release studies. Unfortunately, the amount of CIP released from Chitosan/PAA films was too high to quantify through calibration curves. Therefore, a sample of solution was taken at the very end of the release, diluted 1:8 using PBS at pH 7.4 or pH 5.5, quantified through calibration curves (see appendix Figure A2, Figure A3) and the CIP amount after 24 hours was recalculated by taking into account the dilution factor. Then % CIP release was calculated through dividing the amount of CIP released from multilayers by the amount of CIP loaded into the multilayers. As it was also clear from Figure 3.17, % CIP released from Chitosan/PAA multilayers was significantly higher than that released from Chitosan/TA films due to loose structure of exponentially grown Chitosan/PAA films which provided high loading capacity to the multilayers. However, different from Chitosan/TA films, Chitosan/PAA multilayers did not show a significant pH responsive release behaviour. The difference between these two film systems can be correlated with the difference in the pK_a values of TA and PAA. As mentioned earlier, TA has two pK_a values, i.e. 6.5 and 8. Therefore, changing pH from 7.4 to 5.5 creates a significant change in the number of phenolate groups being converted to phenolic hydroxyl groups which induced a loss in association between TA and CIP. However, PAA has a pK_a of 4.5 and PAA is almost fully ionized at both pH 7.4 and pH 5.5. Therefore, unlike TA, decreasing pH from 7.4 to 5.5 does not result in a significant change in the charge density of PAA and affect the association among PAA and CIP molecules.



Figure 3.16. Release of CIP from Chitosan/TA and Chitosan/PAA multilayers at (A) pH 5.5 and (B) pH 7.4 at 37 °C.



Figure 3.17. % release of CIP from 40-bilayer Chitosan/TA and Chitosan/PAA films at pH 5.5 and pH 7.4 after 24 h.
CHAPTER 4

CONCLUSION

In this study, Chitosan based multilayer films were prepared through LbL technique using TA and PAA as the counterparts. The driving force for Chitosan and TA selfassembly at pH 5 was the electrostatic interactions between the protonated amino groups of Chitosan and phenolate goups of TA together with hydrogen bonding interactions between hydroxyl groups of Chitosan and phenolic hydroxyl groups of TA. For Chitosan/PAA system, multilayers could be constructed in a LbL fashion at pH 4 through the electrostatic association among the protonated amino groups of Chitosan and carboxylate groups of PAA as well as hydrogen bonding interactions between the hydroxyl groups of Chitosan and carboxyl groups of PAA. Chitosan/TA films showed linear growth profile, whereas Chitosan/PAA films demonstrated exponential growth profile. The difference in the stability of the films was remarkable in physiologically related conditions. Chitosan/PAA films easily disintegrated in PBS at pH 7.4 and 37 °C, while Chitosan/TA multilayers were quite stable under similar conditions. The difference was correlated with the greater extent of hydrogen bonding interactions in case of Chitosan/TA due to higher pK_a of TA and greater amount of protonated phenolic hydroxyl groups on TA and the dendrimer-like structure of TA providing additional stability to the multilayers. The instability of Chitosan/PAA films was attributed to the relatively weak association between Chitosan and PAA films. Chitosan/PAA films found to be rendered stable in PBS at pH 7.4 and 37 °C only when the multilayers were thermally crosslinked. The weak association between Chitosan and PAA provided high surface roughness due to more loopy conformation of the polymers in the multilayers. In contrast, Chitosan and TA multilayers were relatively smooth with lower surface roughness than that of Chitosan/PAA films with the same layer number. Chitosan/TA multilayers exhibited some anti-adhesive

behaviour against BSA and LYS adsorption. Chitosan/PAA films showed higher amount of protein adsorption, if compared with Chitosan/TA.

The two multilayers were also contrasted with respect to their stimuli-responsive release behaviour. CIP, a broad spectrum antibiotic was loaded into multilayers at pH 7.4 and the release was examined in PBS at pH 7.4/37 °C and pH 5.5/37 °C. The relatively loosely bound Chitosan/PAA multilayers released approximately x7 times greater amount of CIP compared to Chitosan/TA films with the same layer number after 24 hours. This significant difference in the amount of CIP released from two multilayers was correlated with the relatively loosely bound Chitosan/PAA multilayers which had greater amount of room for CIP loading. Importantly, Chitosan and TA multilayers showed pH-responsive CIP release, whereas the difference in the amount of CIP released from Chitosan/PAA multilayers at pH 5.5 and 37 °C was insignificant. This was due to the difference in the acid strength of TA and PAA. TA has two pK_a values, i.e. 6.5 and 8 and decreasing pH from 7.4 to 5.5 had a more significant effect on the number of phenolate groups being converted into phenolic hydroxyl groups resulting in loss of electrostatic interaction between CIP and TA and release of CIP from the surface in a pH-responsive manner. However, in case of Chitosan and PAA films, PAA has a pK_a of 4.5, thus decreasing pH from 7.4 to 5.5 did not result in a significant change in the number of carboxylate groups on PAA and a further loss in association between PAA and CIP.

This thesis study contributed to the generation of fundamental information on structure-property relationship in Chitosan multilayer films. Considering the important biological properties of Chitosan, these multilayers may find use in surface functionalization or controlled release studies from surfaces.

The future work on this study will consist of the following part:

1. Bacterial anti-adhesive properties of Chitosan/TA and Chitosan/PAA films and antibacterial activity of CIP loaded Chitosan/TA and Chitosan/PAA films will be

performed using model gram-negative and gram-positive bacteria, i.e. *E.coli* and *S.aureus*.

REFERENCES

[1] D. Pasqui, M. De Cagna, R. Barbucci, Polysaccharide-Based Hydrogels: The Key Role of Water in Affecting Mechanical Properties, Polymers (Basel). 4 (2012) 1517–1534. doi:10.3390/polym4031517.

[2] A. Aravamudhan, D.M. Ramos, A.A. Nada, S.G. Kumbar, Natural Polymers: Polysaccharides and Their Derivatives for Biomedical Applications, Elsevier Inc., 2014. doi:10.1016/B978-0-12-396983-5.00004-1.

[3] A. Domard, M. Domard, Chitosan: Structure-Properties Relationship and Biomedical Applications, Polym. Biomater. (2nd Ed.). (2002) 187–212.

[4] N.N. Inamdar, V. Mourya, Chitosan and Low Molecular Weight Chitosan: Biological and Biomedical Applications, Adv. Biomater. Biodevices. 9781118773 (2014) 183–242. doi:10.1002/9781118774052.ch6.

[5] I. Tsigos, A. Martinou, D. Kafetzopoulos, V. Bouriotis, Chitin deacetylases: new, versatile tools in biotechnology, TibTech. 18 (2000) 305–312. doi:10856926.

[6] Y. Yuan, B.M. Chesnutt, W.O. Haggard, J.D. Bumgardner, Deacetylation of chitosan: Material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures, Materials (Basel). 4 (2011) 1399–1416. doi:10.3390/ma4081399.

[7] T.A. Khan, K.K. Peh, H.S. Ch'ng, Reporting degree of deacetylation values of chitosan: The influence of analytical methods, J. Pharm. Pharm. Sci. 5 (2002) 205–212. doi:10.1080/00131726609339665.

8] I. Younes, M. Rinaudo, Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications, Mar. Drugs. 13 (2015) 1133–1174. doi:10.3390/md13031133.

[9] C.M. de Moura, J.M. de Moura, N.M. Soares, L.A. de A. Pinto, Evaluation of molar weight and deacetylation degree of chitosan during chitin deacetylation reaction: Used to produce biofilm, Chem. Eng. Process. Process Intensif. 50 (2011) 351–355. doi:10.1016/j.cep.2011.03.003.

[10] M.L. Tsaih, R.H. Chen, The Effect of Reaction Time and Temperature During Heterogenous Alkali Deacetylation on Degree of Deacetylation and Molecular Weight of Resulting Chitosan, J. Appl. Polym. Sci. 88 (2003) 2917–2923. doi:10.1002/app.11986.

[11] L.Y. Zheng, J.F. Zhu, Study on antimicrobial activity of chitosan with different molecular weights, Carbohydr. Polym. 54 (2003) 527–530. doi:10.1016/j.carbpol.2003.07.009.

[12] H. Hattori, M. Ishihara, Changes in blood aggregation with differences in molecular weight and degree of deacetylation of chitosan, Biomed. Mater. 015014 (2015) 15014. doi:10.1088/1748-6041/10/1/015014.

[13] Q.Z. Wang, X.G. Chen, N. Liu, S.X. Wang, C.S. Liu, X.H. Meng, C.G. Liu, Protonation constants of chitosan with different molecular weight and degree of deacetylation, Carbohydr. Polym. 65 (2006) 194–201. doi:10.1016/j.carbpol.2006.01.001.

[14] R.C.F. Cheung, T.B. Ng, J.H. Wong, W.Y. Chan, Chitosan: An update on potential biomedical and pharmaceutical applications, Mar. Drugs. 13 (2015). doi:10.3390/md13085156.

[15] N. Bhattarai, J. Gunn, M. Zhang, Chitosan-based hydrogels for controlled, localized drug delivery, Adv. Drug Deliv. Rev. 62 (2010) 83–99. doi:10.1016/j.addr.2009.07.019.

[16] E.M. Ahmed, Hydrogel: Preparation, characterization, and applications: A review, J. Adv. Res. 6 (2015) 105–121. doi:10.1016/j.jare.2013.07.006.

[17] E. Caló, V. V. Khutoryanskiy, Biomedical applications of hydrogels: A review of patents and commercial products, Eur. Polym. J. 65 (2015) 252–267. doi:10.1016/j.eurpolymj.2014.11.024.

[18] H.L. Lim, H.J. Kim, J. Jun, Development of Hydrogel Lenses Functionalized with an Interpenetrating Chitosan Network for Reduction of Protein Adsorption, J. Nanosci. Nanotechnol. 16 (2016) 11952–11956. doi:10.1166/jnn.2016.13624.

[19] H. Yu, J. Liu, Y.-Y. Zhao, F. Jin, X.-Z. Dong, Z.-S. Zhao, X.-M. Duan, M.-L. Zheng, Biocompatible Three-Dimensional Hydrogel Cell Scaffold Fabricated by Sodium Hyaluronate and Chitosan Assisted Two-Photon Polymerization, ACS Appl. Bio Mater. 2 (2019) 3077–3083. doi:10.1021/acsabm.9b00384.

[20] J. Long, A.E. Etxeberria, C. Kornelsen, A. V. Nand, S. Ray, C.R. Bunt, A. Seyfoddin, Development of a Long-Term Drug Delivery System with Levonorgestrel-Loaded Chitosan Microspheres Embedded in Poly(vinyl alcohol) Hydrogel, ACS Appl. Bio Mater. 2 (2019) 2766–2779. doi:10.1021/acsabm.9b00190.

[21] C.H. Chang, Y.H. Lin, C.L. Yeh, Y.C. Chen, S.F. Chiou, Y.M. Hsu, Y.S. Chen, C.C. Wang, Nanoparticles Incorporated in pH-sensitive Hydrogels as Amoxicillin Delivery for Eradication of Helicobacter pylori, Biomacromolecules. 11 (2010) 133–142. doi:10.1021/bm900985h.

[22] A.M. Heimbuck, T.R. Priddy-Arrington, M.L. Padgett, C.B. Llamas, H.H. Barnett, B.A. Bunnell, M.E. Caldorera-Moore, Development of Responsive Chitosan-Genipin Hydrogels for the Treatment of Wounds, ACS Appl. Bio Mater. 2 (2019) 2879–2888. doi:10.1021/acsabm.9b00266.

[23] P.K.B. Palomaki, P.H. Dinolfo, A Versatile Molecular Layer-by-Layer Thin Film Fabrication Technique Utilizing Copper(I)-Catalyzed Azide-Alkyne Cycloaddition, Langmuir. 26 (2010) 9677–9685. doi:10.1021/la100308j.

[24] R. K. Iler, Multilayers of Colloidal Particles, J. Colloid Interface Sci. 21 (1966) 569–594.

[25] G. Decher, J.D. Hong, J. Schmitt, Buildup of ultrathin multilayer films by a self-assembly process: III. Consecutively alternating adsorption of anionic and cationic polyelectrolytes on charged surfaces, Thin Solid Films. 210–211 (1992) 831–835. doi:10.1016/0040-6090(92)90417-A.

[26] J. Alongi, F. Carosio, A. Frache, G. Malucelli, Layer by Layer coatings assembled through dipping, vertical or horizontal spray for cotton flame retardancy, Carbohydr. Polym. 92 (2013) 114–119. doi:10.1016/j.carbpol.2012.08.086.

[27] M. Michel, V. Toniazzo, D. Ruch, V. Ball, Deposition Mechanisms in Layerby-Layer or Step-by-Step Deposition Methods: From Elastic and Impermeable Films to Soft Membranes with Ion Exchange Properties, ISRN Mater. Sci. 2012 (2012) 1– 13. doi:10.5402/2012/701695.

[28] E. V. Skorb, D. V. Andreeva, Layer-by-Layer approaches for formation of smart self-healing materials, Polym. Chem. 4 (2013) 4834–4845. doi:10.1039/c3py00088e.

[29] L. Wang, Z. Wang, X. Zhang, J. Shen, L. Chi, H. Fuchs, A new approach for the fabrication of an alternating multilayer film of poly(4-vinylpyridine) and poly(acrylic acid) based on hydrogen bonding, Macromol. Rapid Commun. 18 (1997) 509–514. doi:10.1002/marc.1997.030180609.

[30] S. Leininger, B. Olenyuk, P.J. Stang, Self-Assembly of Discrete Cyclic Nanostructures Mediated by Transition Metals, Chem. Rev. 100 (2002) 853–908. doi:10.1021/cr9601324.

[31] Y. Shimazaki, M. Mitsuishi, S. Ito, M. Yamamoto, Preparation of the Layerby-Layer Deposited Ultrathin Film Based on the Charge-Transfer Interaction, Langmuir. 13 (2002) 1385–1387. doi:10.1021/la9609579.

[32] H. Xiong, Z. Zhou, Z. Wang, X. Zhang, J. Shen, New approach to fabrication of a self-organizing film of heterostructured polymer/CdS nanoparticles, Supramol. Sci. 5 (1998) 623–626. doi:10.1016/S0968-5677(98)00089-3.

[33] Y. Lvov, K. Ariga, I. Ichinose, T. Kunitake, Layer-by-layer Architectures of Concanavalin A by means of Electrostatic and Biospecific Interactions, J. Chem. Soc., Chem. Commun. (1995) 2313–2314.

[34] J. Borges, J. F. Mano, Molecular Interactions Driving the Layer-by-Layer Assembly of Multilayers, Chem. Rev. 114 (2014) 8883–8942. doi:10.1021/cr400531v.

[35] P. Bertrand, A. Jonas, A. Laschewsky, R. Legras, Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: suitable materials, structure and properties Scheme of the electrostatic layer-by-layer self-assembly (ESA), Macromol. Rapid Commun. 21 (2000) 319–348.

[36] M. Mihai, S. Schwarz, A. Janke, C.A. Ghiorghiță, E.S. Drăgan, Silica microparticles surface coating by layer-by-layer or polyelectrolyte complex adsorption, J. Polym. Res. 20 (2013). doi:10.1007/s10965-013-0089-5.

[37] A.I. Petrov, D. V. Volodkin, G.B. Sukhorukov, Protein-Calcium Carbonate Coprecipitation: A Tool for Protein Encapsulation, Biotechnol. Prog. 21 (2005) 918–925. doi:10.1021/bp0495825.

[38] M.S. Suh, J. Shen, L.T. Kuhn, D.J. Burgess, Layer-by-layer nanoparticle platform for cancer active targeting, Int. J. Pharm. 517 (2017) 58–66. doi:10.1016/j.ijpharm.2016.12.006.

[39] J. Almodóvar, L. W. Place, J. Gogolski, K. Erickson, M. J. Kipper, Layer-by-Layer Assembly of Polysaccharide-Based Polyelectrolyte Multilayers: A Spectroscopic Study of Hydrophilicity, Composition, and Ion Pairing, Biomacromolecules. 12 (2011) 2755–2765. doi:10.1021/bm200519y.

[40] K. Tang, N.A.M. Besseling, Formation of polyelectrolyte multilayers: ionic strengths and growth regimes, Soft Matter. 12 (2016) 1032–1040. doi:10.1039/c5sm02118a.

[41] M. Ferreira, M.F. Rubner, Molecular-Level Processing of Conjugated Polymers. 1. Layer-by-Layer Manipulation of Conjugated Polyions, Macromolecules. 28 (1995) 7107–7114. doi:10.1021/ma00125a012.

[42] J. Almodóvar, S. Bacon, J. Gogolski, J. D. Kisiday, M. J. Kipper, Polysaccharide-Based Polyelectrolyte Multilayer Surface Coatings can Enhance Mesenchymal Stem Cell Response to Adsorbed Growth Factors, Biomacromolecules. 11 (2010) 2629–2639. doi:10.1021/bm1005799.

[43] L. Richert, P. Lavalle, E. Payan, X.Z. Shu, G.D. Prestwich, J.F. Stoltz, P. Schaaf, J.C. Voegel, C. Picart, Layer by Layer Buildup of Polysaccharide Films: Physical Chemistry and Cellular Adhesion Aspects, Langmuir. 20 (2004) 448–458. doi:10.1021/la035415n.

[44] T.B. Taketa, M.M. Beppu, Layer-by-layer thin films of alginate/chitosan and hyaluronic acid/chitosan with tunable thickness and surface roughness, Mater. Sci. Forum. 783–786 (2014) 1226–1231. doi:10.4028/www.scientific.net/MSF.783-786.1226.

[45] D.L. Elbert, C.B. Herbert, J.A. Hubbell, Thin Polymer Layers Formed by Polyelectrolyte Multilayer Techniques on Biological Surfaces, Langmuir. 15 (1999) 5355–5362. doi:10.1021/la9815749.

[46] C. Picart, Ph. Lavalle, P. Hubert, F.J.G. Cuisinier, G. Decher, P. Schaaf, J.-C. Voegel, Buildup Mechanism for Poly(L-lysine)/Hyaluronic Acid Films onto a Solid Surface, Langmuir. 17 (2001) 7414–7424. doi:10.1021/la010848g.

[47] C. Picart, J. Mutterer, L. Richert, Y. Luo, G.D. Prestwich, P. Schaaf, J.-C. Voegel, P. Lavalle, Molecular basis for the explanation of the exponential growth of polyelectrolyte multilayers, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12531–12535. doi:10.1073/pnas.202486099.

[48] S. Micciulla, S. Dodoo, C. Chevigny, A. Laschewsky, R. Von Klitzing, Short versus long chain polyelectrolyte multilayers: A direct comparison of self-assembly and structural properties, Phys. Chem. Chem. Phys. 16 (2014) 21988–21998. doi:10.1039/c4cp03439b.

[49] Z. Sui, D. Salloum, J.B. Schlenoff, Effect of Molecular Weight on the Construction of Polyelectrolyte Multilayers: Stripping versus Sticking, Langmuir. 19 (2003) 2491–2495. doi:10.1021/la026531d.

[50] R. V. Klitzing, Internal structure of polyelectrolyte multilayer assemblies, Phys. Chem. Chem. Phys. 8 (2006) 5012–5033. doi:10.1039/b607760a.

[51] G. Decher, J. Schmitt, Fine-Tuning of the film thickness of ultrathin multilayer films composed of consecutively alternating layers of anionic and cationic polyelectrolytes, Trends Colloid Interface Sci. VI. 164 (2007) 160–164. doi:10.1007/bfb0116302.

[52] M. Lundin, F. Solaqa, E. Thormann, L. MacAkova, E. Blomberg, Layer-by-Layer Assemblies of Chitosan and Heparin: Effect of Solution Ionic strength and pH, Langmuir. 27 (2011) 7537–7548. doi:10.1021/la200441u.

[53] S.S. Shiratori, M.F. Rubner, pH-dependent Thickness Behavior of Sequentially Adsorbed Layers of Weak Polyelectrolytes, Macromolecules. 33 (2000) 4213–4219. doi:10.1021/ma991645q.

[54] T.B. Taketa, D.M. Dos Santos, A. Fiamingo, J.M. Vaz, M.M. Beppu, S.P. Campana-Filho, R.E. Cohen, M.F. Rubner, Investigation of the Internal Chemical Composition of Chitosan-Based LbL Films by Depth-Profiling X-ray Photoelectron Spectroscopy (XPS) Analysis, Langmuir. 34 (2018) 1429–1440. doi:10.1021/acs.langmuir.7b04104.

[55] D. Gundogdu, V. Bütün, I. Erel-Göktepe, Preparation of Layer-by-Layer Films with Remarkably Different pH-Stability and Release Properties Using Dual Responsive Block Copolymer Micelles, 1800128 (2018) 1–12. doi:10.1002/macp.201800128.

[56] S.T. Dubas, J.B. Schlenoff, Factors Controlling the Growth of Polyelectrolyte Multilayers, Macromolecules. 32 (1999) 8153–8160. doi:10.1021/ma981927a.

[57] H.L. Tan, M.J. McMurdo, G. Pan, P.G. Van Patten, Temperature Dependence of Polyelectrolyte Multilayer Assembly, Langmuir. 19 (2003) 9311–9314. doi:10.1021/la035094f.

[58] M. Salomäki, I.A. Vinokurov, J. Kankare, Effect of Temperature on the Buildup of Polyelectrolyte Multilayers, Langmuir. 21 (2005) 11232–11240. doi:10.1021/la051600k.

[59] D. Choi, J. Hong, Layer-by-layer assembly of multilayer films for controlled drug release, Arch. Pharm. Res. 37 (2014) 79–87. doi:10.1007/s12272-013-0289-x.

[60] U. Han, Y. Seo, J. Hong, Effect of pH on the structure and drug release profiles of layer-by-layer assembled films containing polyelectrolyte, micelles, and graphene oxide, Sci. Rep. 6 (2016) 1–10. doi:10.1038/srep24158.

[61] K. C. Wood, J. Q. Boedicker, D. M. Lynn, P. T. Hammond, Tunable Drug Release from Hydrolytically Degradable Layer-by-Layer Thin Films, Langmuir. 21 (2005) 1603–1609. doi:10.1021/la0476480.

[62] Y. Zhu, J. Shi, W. Shen, X. Dong, J. Feng, M. Ruan, Y. Li, Stimuli-Responsive Controlled Drug Release from a Hollow Mesoporous Silica Sphere/Polyelectrolyte Multilayer Core-Shell Structure, Angew. Chemie - Int. Ed. 44 (2005) 5083–5087. doi:10.1002/anie.200501500.

[63] M. Haktaniyan, S. Atilla, E. Cagli, I. Erel-Goktepe, pH- and temperatureinduced release of doxorubicin from multilayers of poly(2-isopropyl-2-oxazoline) and tannic acid, Polym. Int. 66 (2017) 1851–1863. doi:10.1002/pi.5458.

[64] E. Cagli, E. Ugur, S. Ulusan, S. Banerjee, I. Erel-Goktepe, Effect of side chain variation on surface and biological properties of poly(2-alkyl-2-oxazoline) multilayers, Eur. Polym. J. 114 (2019) 452–463. doi:10.1016/j.eurpolymj.2019.02.031.

[65] A. Zhuk, S. Pavlukhina, S.A. Sukhishvili, Hydrogen-Bonded Layer-by-Layer Temperature-Triggered Release Films, Langmuir. 25 (2009) 14025–14029. doi:10.1021/la901478v.

[66] B. Lu, D. Luo, A. Zhao, H. Wang, Y. Zhao, M.F. Maitz, P. Yang, N. Huang, pH responsive chitosan and hyaluronic acid layer by layer film for drug delivery applications, Prog. Org. Coatings. 135 (2019) 240–247. doi:10.1016/j.porgcoat.2019.06.012.

[67] A. Pérez-Anes, M. Gargouri, W. Laure, H. Van Den Berghe, E. Courcot, J. Sobocinski, N. Tabary, F. Chai, J.F. Blach, A. Addad, P. Woisel, D. Douroumis, B. Martel, N. Blanchemain, J. Lyskawa, Bioinspired Titanium Drug Eluting Platforms Based on a Poly-β-cyclodextrin-Chitosan Layer-by-Layer Self-Assembly Targeting Infections, ACS Appl. Mater. Interfaces. 7 (2015) 12882–12893. doi:10.1021/acsami.5b02402.

[68] X. Yu, M. V. Pishko, Nanoparticle-Based Biocompatible and Targeted Drug Delivery: Characterization and in Vitro Studies, Biomacromolecules. 12 (2011) 3205–3212. doi:10.1021/bm200681m.

[69] W. Feng, W. Nie, C. He, X. Zhou, L. Chen, K. Qiu, W. Wang, Z. Yin, Effect of pH-Responsive Alginate/Chitosan Multilayers Coating on Delivery Efficiency, Cellular Uptake and Biodistribution of Mesoporous Silica Nanoparticles Based Nanocarriers, ACS Appl. Mater. Interfaces. 6 (2014) 8447–8460. doi:10.1021/am501337s.

[70] W. Liu, J. Liu, T. Li, C. Liu, Improved Physical and in Vitro Digestion Stability of a Polyelectrolyte Delivery System Based on Layer-by-Layer Self-Assembly Alginate-Chitosan-Coated Nanoliposomes, J. Agric. Food Chem. 61 (2013) 4133–4144. doi:10.1021/jf305329n.

[71] A.K. Verma, S. Sharma, P. Gupta, D. Singodia, S. Kansal, V. Sharma, P.R. Mishra, Vitamin B 12 Grafted Layer-by-Layer Liposomes Bearing HBsAg Facilitate Oral Immunization: Effect of Modulated Biomechanical Properties, Mol. Pharm. 13 (2016) 2531–2542. doi:10.1021/acs.molpharmaceut.6b00274.

[72] F. Zhou, Antifouling Surfaces and Materials: From Land to Marine Environment, Antifouling Surfaces Mater. (2015) v–vi. doi:10.1007/978-3-662-45204-2.

[73] J.H.H. Bongaerts, J.J. Cooper-White, J.R. Stokes, Low Biofouling Chitosan-Hyaluronic Acid Multilayers with Ultra-Low Friction Coefficients, Biomacromolecules. 10 (2009) 1287–1294. doi:10.1021/bm801079a.

[74] Y. Liu, X. Ma, T. Zhou, R. Wang, J. Hou, J. Tang, B. Zhu, Y. Su, X. Zhu, Layer by layer assembled phosphorylcholine groups on paclitaxel/chitosan nanofibers coatings for hemocompatibility improvement, Surf. Coatings Technol. 357 (2019) 984–992. doi:10.1016/j.surfcoat.2018.10.074.

[75] G. Xu, P. Liu, D. Pranantyo, K.-G. Neoh, E.-T. Kang, Dextran- and Chitosan-Based Antifouling, Antimicrobial Adhesion, and Self-Polishing Multilayer Coatings from pH-Responsive Linkages-Enabled Layer-by-Layer Assembly, ACS Sustain. Chem. Eng. 6 (2018) 3916–3926. doi:10.1021/acssuschemeng.7b04286.

[76] M. Bulwan, K. Wójcik, S. Zapotoczny, M. Nowakowska, Chitosan-Based Ultrathin Films as Antifouling, Anticoagulant and Antibacterial Protective Coatings, J. Biomater. Sci. Polym. Ed. 23 (2012) 1963–1980. doi:10.1163/092050611X601711.

[77] G. V. Martins, E.G. Merino, J.F. Mano, N.M. Alves, Crosslink Effect and Albumin Adsorption onto Chitosan/Alginate Multilayered Systems: An in situ QCM-D Study, Macromol. Biosci. 10 (2010) 1444–1455. doi:10.1002/mabi.201000193.

[78] M. Wiśniewska, T. Urban, E. Grządka, V.I. Zarko, V.M. Gun'ko, Comparison of adsorption affinity of polyacrylic acid for surfaces of mixed silica-alumina, Colloid Polym. Sci. 292 (2014) 699–705. doi:10.1007/s00396-013-3103-x.

[79] T. Shutava, M. Prouty, D. Kommireddy, Y. Lvov, pH Responsive Decomposable Layer-by-Layer Nanofilms and Capsules on the Basis of Tannic Acid, Macromol. 38 (2005) 71272–71272.

[80] C. Jiang, X. Liu, C. Luo, Y. Zhang, L. Shao, F. Shi, Controlled exponential growth in layer-by-layer multilayers using high gravity fields, J. Mater. Chem. A. 2 (2014) 14048–14053. doi:10.1039/c4ta02437k.

[81] D.M. DeLongchamp, M. Kastantin, P.T. Hammond, High-Contrast Electrochromism from Layer-By-Layer Polymer Films, Chem. Mater. 15 (2003) 1575–1586. doi:10.1021/cm021045x.

[82] J. Ruths, F. Essler, G. Decher, H. Riegler, Polyelectrolytes I: Polyanion/Polycation Multilayers at the Air/Monolayer/Water Interface as Elements for Quantitative Polymer Adsorption Studies and Preparation of Hetero-superlattices on Solid Surfaces, Langmuir. 16 (2000) 8871–8878. doi:10.1021/la000257a.

[83] E. Guzmán, J.A. Cavallo, R. Chuliá-Jordán, C. Gómez, M.C. Strumia, F. Ortega, R.G. Rubio, pH-Induced Changes in the Fabrication of Multilayers of Poly(acrylic acid) and Chitosan: Fabrication, Properties, and Tests as a Drug Storage and Delivery System, Langmuir. 27 (2011) 6836–6845. doi:10.1021/la200522r.

[84] Ph. Lavalle, C. Gergely, F.J.G. Cuisinier, G. Decher, P. Schaaf, J.C. Voegel, C. Picart, Comparison of the Structure of Polyelectrolyte Multilayer Films Exhibiting a Linear and an Exponential Growth Regime: An in Situ Atomic Force Microscopy Study, Macromolecules. 35 (2002) 4458–4465. doi:10.1021/ma0119833.

[85] D.T. Haynie, E. Cho, P. Waduge, "In and Out Diffusion" Hypothesis of Exponential Multilayer Film Buildup Revisited, Langmuir. 27 (2011) 5700–5704. doi:10.1021/la104516a.

[86] P. Lavalle, C. Picart, J. Mutterer, C. Gergely, H. Reiss, J.-C. Voegel, B. Senger, P. Schaaf, Modeling the Buildup of Polyelectrolyte Multilayer Films Having Exponential Growth X, J. Phys. Chem. B. 108 (2004) 635–648. doi:10.1021/jp035740j.

[87] E. Kharlampieva, V. Kozlovskaya, J.F. Ankner, S.A. Sukhishvili, Hydrogen-Bonded Polymer Multilayers Probed by Neutron Reflectivity, Langmuir. 24 (2008) 11346–11349. doi:10.1021/la802502c.

[88] H. Luo, Q. Shen, F. Ye, Y.F. Cheng, M. Mezgebe, R.J. Qin, Structure and properties of layer-by-layer self-assembled chitosan/lignosulfonate multilayer film, Mater. Sci. Eng. C. 32 (2012) 2001–2006. doi:10.1016/j.msec.2012.05.023.

[89] Y.N. Jin, H.C. Yang, H. Huang, Z.K. Xu, Underwater superoleophobic coatings fabricated from tannic acid-decorated carbon nanotubes, RSC Adv. 5 (2015) 16112–16115. doi:10.1039/c4ra16074f.

[90] M. Kolasińska, R. Krastev, P. Warszyński, Characteristics of polyelectrolyte multilayers: Effect of PEI anchoring layer and posttreatment after deposition, J. Colloid Interface Sci. 305 (2007) 46–56. doi:10.1016/j.jcis.2006.09.035.

[91] S.T. Dubas, J.B. Schlenoff, Swelling and Smoothing of Polyelectrolyte Multilayers by Salt, Langmuir. 17 (2001) 7725–7727. doi:10.1021/la0112099.

[92] L. Wang, L. Weng, Z. Su, Surface defects in polyelectrolyte multilayers: Effects of drying and deposition cycle, Soft Matter. 7 (2011) 4851–4855. doi:10.1039/c1sm05158j.

[93] Y. Guo, W. Geng, J. Sun, Layer-by-Layer Deposition of Polyelectrolyte–Polyelectrolyte Complexes for Multilayer Film Fabrication, Langmuir. 25 (2009) 1004–1010. doi:10.1021/la803479a.

[94] T.R. Kyriakides, Molecular Events at Tissue-Biomaterial Interface, Elsevier Inc., 2015. doi:10.1016/B978-0-12-800196-7.00005-0.

[95] S. Ulusan, V. Bütün, S. Banerjee, I. Erel-Goktepe, Biologically Functional Ultrathin Films Made of Zwitterionic Block Copolymer Micelles, Langmuir. (2018). doi:10.1021/acs.langmuir.8b01735.

[96] D.S. Salloum, J.B. Schlenoff, Protein Adsorption Modalities of Polyelectrolyte Multilayers, Biomacromolecules. 5 (2004) 1089–1096. doi:10.1021/bm034522t.

[97] D. Sharma, R.P. Patel, S.T.R. Zaidi, M.M.R. Sarker, Q.Y. Lean, L.C. Ming, Interplay of the Quality of Ciprofloxacin and Antibiotic Resistance in Developing Countries, Front. Pharmacol. 8 (2017) 1–7. doi:10.3389/fphar.2017.00546.

[98] C. Gu, K.G. Karthikeyan, Sorption of the Antimicrobial Ciprofloxacin to Aluminum and Iron Hydrous Oxides, Environ. Sci. Technol. 39 (2005) 9166–9173. doi:10.1021/es051109f.

[99] T. Swift, L. Swanson, M. Geoghegan, S. Rimmer, The pH-responsive behaviour of poly(acrylic acid) in aqueous solution is dependent on molar mass, Soft Matter. 12 (2016) 2542–2549. doi:10.1039/c5sm02693h.

APPENDIX A

CALIBRATION CURVES



Figure A.1. Calibration curve of CIP solution in Monobasic Buffer at pH 7.4 and 37 °C. Fluorescence intensity at 414 nm is plotted as a function of CIP concentration (mg/mL).



Figure A.2. Calibration curve of CIP solution in PBS at pH 5.5 and 37 °C. Fluorescence intensity at 445 nm is plotted as a function of CIP concentration (mg/mL).



Figure A.3. Calibration curve of CIP solution in PBS at pH 7.4 and 37 °C. Fluorescence intensity at 414 nm is plotted as a function of CIP concentration (mg/mL).