Coatings from Polyelectrolytes: Fundamentals of Buildup and Control over Mechanical Properties for Bioapplications

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COATINGS FROM POLYELECTROLYTES: FUNDAMENTALS OF BUILD-UP AND
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To my father Mohammad, my mother Zeinab,
to Hasan, Dina, Hanaa and to the love of my life Haifa
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ABSTRACT

Controlling cell fate via mechanical properties of the culture substrates provides an important tool for biomedical applications. Thin films of polyelectrolyte multilayers (PEMUs) have been used as substrates for cell culture due to their biocompatibility. Photocrosslinkable polyelectrolyte multilayers were prepared from poly(acrylic acid) grafted with photosensitive benzophenone (PAABp) as the polyanion and poly(allylamine hydrochloride) (PAH) as the polycation. Young’s modulus measured by force spectroscopy using nano-indentation showed smooth controlled increase after irradiation with UV light. The permeability of PEMUs for iodide ions, measured with a rotating disc electrode, decreased significantly. The surface wettability and charge density were not affected by irradiation, suggesting that surface chemistry and charge remained essentially unaltered. This provides substrate material for cell culture applications where the only variable is the mechanical stiffness. Since photocrosslinking provided control over the region of crosslinking, photomasks were used to prepare substrates with gradient elasticity. The behavior of rat aortic smooth muscle cells (A7r5) and osteosarcoma (U2OS) was followed on uniform substrates of increasing stiffness and on substrates with gradient elasticity. A7r5 cells detected the elasticity gradient and those that were on the soft side could polarize and orient towards the stiff side, where they showed better adhesion. U2OS cells also showed preference to the stiff side; however U2OS cells that were on the soft side did not adhere and underwent apoptosis.

Nano-indentation was used to study some important fundamental properties of polyelectrolyte multilayer from poly(diallyldimethylammounium chloride) PDADMAC as the polycation and sodium poly(styrene sulfonate) (NaPSS) as the polyanion, which allowed proposing mechanism for PEMU build-up. The degree of swelling varied depending on the type of polyelectrolyte on the surface, with PDADMA-ending films more swollen than PSS ending films. This observation correlated well with the change in modulus. This suggested that PDADMA-ending films are extrinsically compensated while PSS-ending films are more intrinsically compensated. Also using nano-indentation, we demonstrated that the surface features and roughness of polyelectrolyte multilayers were not due to phase separation.

Using nano-indentation, we demonstrated the role of water in controlling the mechanical properties of PEMUs. Previously, it was shown that salt plasticizes PEMUs and they were termed “saloplastics”. A complimentary study was done to investigate the plasticizing effect of
water. We used osmotic stress to control the amount of water in the films without affecting the degree of ionic crosslinks. Poly(ethylene glycol) (PEG) was used as the osmotic stressor. We showed that water increases free volume between the polyelectrolyte chains and acted as lubricant and the elastic modulus increases with decreasing water content. Water was also found to contribute to the viscoelastic properties of the polyelectrolyte multilayer, suggesting that water acts in plasticizing polyelectrolyte multilayers.
CHAPTER ONE

INTRODUCTION

1.1 Mechanism of cell interaction with the mechanical properties of their substrate

Surface properties of bio-implants play an important role in their success. Extensive research has been conducted in the field of biomaterials and tissue engineering to improve the properties of implanted devices such as mechanical strength, biocompatibility, among other surface properties, in order to control cellular adhesion and repulsion, and inflammatory responses. Cellular interactions are induced by chemical and physical signals from the substrate such as its chemical nature and physical properties like stiffness, and topography. Cells can analyze these signals and respond accordingly, which directs the final cell fate including cell morphology and differentiation. In this dissertation, we focused on the effect of substrate mechanical properties, i.e. elasticity, on the behavior and phenotypic expressions of cells.

The behavior of cells can be controlled by the mechanical properties of their substrate, for example, they can be directed to adhere to or repel from a substrate based on stiffness. Cardiomyocytes, smooth muscle cells, and fibroblasts, adhere better on stiffer substrates, which was demonstrated by the increase in projected cell area on stiffer substrates. On the other hand, neurons were found to behave normally on soft substrates, where they extended neurites with multiple branch points. The fact that substrate elasticity affect the phenotypic expression of cells is important for the improvement of biomedical devices such as coronary stents. Cell migration, which is important for a number of fundamental biological processes such as wound healing and angiogenesis, can be controlled and directed in a variety of ways such as “chemotaxis”, which involves the use of soluble chemicals, “haptotaxis” achieved by variation in the density of adhesive ligands, and recently “durotaxis” which is controlled by changes in substrate compliance where most cells are known to adhere better on stiffer substrates.

Cells interact with their environment for survival by adhering to other cells or to their extracellular matrix (ECM). Cell binding to ECM proteins, shown in Figure 1.1, creates cell-matrix adhesion sites. These sites are involved in the physical attachment of the cell to the underlying substrate through ligand proteins in the extracellular matrix such as collagen, fibronectin and vitronectin. The binding of cells to ECM protein on substrate creates
intracellular associations with cytoskeletal proteins and triggers the organization of molecular complexes that can activate intracellular signaling pathways, which in turn can regulate the morphology of cells such as adhesion, migration, and gene expression and also affects cellular differentiation.\textsuperscript{13,17-20} The main type of protein involved in cell-ECM binding is the transmembrane protein “integrin”. Integrins are transmembrane glycoproteins receptors for ECM constituents. They are heterodimeric in nature composed of 18α- and 8β-integrin subunits. The extracellular domain of integrins bind to the ECM protein ligands and the cytoplasmic domain binds to the components of actin cytoskeleton.\textsuperscript{13,20} These bindings result in reorganization of the actin cytoskeleton and associated proteins such as vinculin and talin that activate intracellular signaling pathways which in turn control cell fate.\textsuperscript{13,16}

![Diagram of integrin molecular connections](image)

**Figure 1.1** Representation of integrin molecular connections. Integrins involved in the formation of focal adhesions are presented. After integrins bind to ECM protein ligands on the surface of the substrate, the cytoplasmic domain of integrin binds to cytoplasmic proteins such as talin and vinculin and this activate intracellular signaling pathways that can regulate cell morphology, adhesion, migration, gene expression and differentiation.

### 1.1.1 Focal adhesion or focal contacts

Adhesion of cells to the underlying substrate via integrin-ligand binding induces the formation of focal adhesions, also known as focal contacts. Focal adhesions are known to induce many cellular events, such as gene expression, embryonic development and cellular motility. In
the region of integrin-matrix linkage, the cell is separated from the substrate by only 10-15 nm. Many cells including fibroblasts, and smooth muscle cells are found to form focal adhesions when plated on substrates. These focal adhesions are associated with stress fibers in the cells and are found to have important role in cell migration. Focal adhesions are usually highly concentrated in stationary cells and can be absent in highly motile cells. Focal adhesions are constituted of (1) the extracellular components, which include the ECM proteins to which cells attach, and those are rich in fibronectin and vitronectin, (2) the transmembrane components which are mainly the integrins that bind to the ECM protein and (3) the cytoplasmic components, which are rich in paxillin, viculin, and α-actinin proteins. Focal adhesions can be considered as chain of attachments connecting actin to integrin on the cell membrane and made through α-actinin, vinculin and talin as shown in Figure 1.1. Focal adhesions are usually localized at the terminal of actin stress fibers, at or behind the leading edge of the cell. Vinculin and paxillin can be used as markers for focal adhesions. Focal adhesions can be used as an indication of cellular motility. The presence of focal adhesions is accompanied by reduction in cellular motility.

1.1.2 Effect of substrate mechanical properties on cell locomotion “Mechanotransduction”

Cells like fibroblasts, epithelial, and smooth muscle cells have the ability to detect variations in the mechanical properties of their substrate and respond accordingly. Myosin motors play an important role in detecting the stiffness of the substrate via pushing/pulling of integrin receptors on the substrate with the aid of the cytoskeletal proteins. When integrins bind to ECM proteins the actin-myosin cytoskeleton starts exerting tension forces. On soft ECM, these forces are weak and the association with the cytoskeleton is unstable. On the other hand, when integrin anchors to rigid substrates, tension forces in the cytoplasmic domain increase. Response to substrate rigidity is associated with intracellular signals through the cytoskeleton. For example, increase in tyrosine phosphorylation is induced on stiff surfaces, which stimulates the formation of stable focal adhesions.
1.2 Polyelectrolyte multilayer (PEMU)

1.2.1 Overview

In 1966, the deposition of thin films of polyelectrolyte complexes from solution onto a glass substrate was described by Iler. This concept gained much attention in the early nineties, when Decher presented the layer-by-layer build-up of polyelectrolyte multilayer thin films through the sequential adsorption of oppositely charged polyelectrolytes from solution into a solid substrate. Since then, this method was applied by many research groups, who used both synthetic polyelectrolytes and biopolymers to develop thin films for a wide range of applications, from electronics to bioapplications. Polyelectrolyte multilayer films can be made by the direct, alternate deposition of a polycation and a polyanion on an oppositely charged surface. Initially, a negatively charged substrate is dipped into the polycation solution, followed by

Figure 1.2 Modeling the mechanism of “mechanotransduction” through which a cell can sense the stiffness of their underlying substrate. (A) In the absence of ECM protein ligands, integrin molecules are inactivated. (B) In presence of ECM protein and when cells are plated on a relatively soft substrate, integrin binds to the ECM proteins and this causes the actin-myosin cytoskeleton to associate with integrin and start to exert tension forces indicated as the black arrows, these tension forces increases on a more rigid substrate shown in (C) which stimulates more tyrosine phosphorylation and leads to more stabilized association with the cytoskeleton which stimulates the formation of more focal adhesions.
dipping into the polyanion solution. Between the two deposition steps, a rinsing step is required to remove excess polymers and salts and to avoid polymer solution contamination. The washing step is also needed to stabilize weakly attached polyelectrolytes.\(^{30}\)

The use of polyelectrolytes has several advantages over using small charged molecules in the layer-by-layer technique, because it allows good adhesion to the substrate which requires a certain number of ionic bonds. Polyelectrolyte adsorption is irreversible, which provides stability for their growth by limiting spontaneous desorption from the surface during build up.\(^{31}\) Salt concentration is the most important variable for controlling the film thickness. It was shown that multilayer thickness has linear dependence on salt concentration.\(^{32-33}\) Surface charge reversal and overcompensation are other important phenomena in multilayer build-up. In each cycle during a multilayer preparation, a reproducible amount of material is adsorbed to the surface reversing the charge and making it possible for other polyelectrolyte of opposite charge to adsorb.\(^{31}\) Low molecular weight polyelectrolyte can form quasi-soluble complexes, which leads to desorption from the surface and this affects film thickness. Factors that affect the thickness of the film also include the type of polymers, salt, temperature, solvent, deposition and rinse time, polymer concentration, drying between the deposition steps, and pH for weak acids and bases; however, when working at room temperature with high molecular weight polymers and in the presence of sodium chloride, the most important factors affecting film thickness are the type of polyelectrolytes used and salt concentration.\(^{34}\)

The mechanism of multilayer build-up is summarized in the following reaction and illustrated in Figure 1.3:

\[ Pol^+ A^- + Pol^- C^+ \leftrightarrow Pol^+ Pol_m^- + A^- C_{aq}^+ \quad (1.1) \]

\(Pol^+\) and \(Pol^-\) represent the positive and negative polyelectrolyte repeat units respectively. \(A^-\) and \(C^+\) are the counterions. \(m\) refers to components of the multilayer. In PEMUs, monomer units compensated by other monomer units within the film are termed intrinsic sites, and monomer units compensated by counter ions are termed extrinsic or doping sites.\(^{35}\) A representation of intrinsic and extrinsic sites is shown in Figure 1.4.
Figure 1.3 Mechanism of multilayer build-up. The blue and red polymers represent the polycation and polyanion respectively. Initially a negatively charge substrate is dipped into the polycation solution for 5 min., then followed by 3 rinsing steps in water for 1 minute each. The film is then dipped into the polyanion solution for 5 min, followed by rinsing. This cycle is repeated a number of times depending on the desired thickness for the film.

Figure 1.4 The blue and red polymers are the negative and positive polyelectrolytes respectively. Intrinsic sites are defined as monomer units compensated by monomer units. Extrinsic sites are monomer units compensated by counter ions. (a) is an example of ladder type of interaction between the polyelectrolytes and (b) is an example of network type of interaction.
1.2.2 Build-up mechanism: Charge overcompensation and internal structure

The key factor to a growing multilayer is charge overcompensation. When a polyelectrolyte is added it compensates the charge of the previously adsorbed polymer and reverses the charge on the surface. It was predicted that a constant surface charge develops at each step during multilayer build-up and the surface charge is overcompensated at each step.\(^{31,36-37}\) Schlenoff et al. used radiolabelled sulfate counter-ion in polyelectrolyte multilayers prepared from poly(styrene sulfonate) (PSS), as the polyanion, and poly(diallyldimethylammonium chloride) PDADMAC, as the polycation, to show that surface charge represents the charge identity of the last added layer.\(^{31,35,38}\) Two films were prepared one composed of 14 layers and ending in PSS (negative surface) and a 15 layer film ending in PDADMA (positive surface). When dipping the PSS-ending multilayer in radiolabelled sulfate solution, no sulfate ions were adsorbed. On contrary, sulfate ions adsorbed in the case of PDADMA-ending film.\(^{35}\) From this experiment, it was concluded that the polymer that is not in excess is completely ion-paired with the other polyelectrolyte, i.e. intrinsically compensated.\(^{31}\) Overcompensation lead to excess charge density of the last-added polyelectrolyte, this occurs only at the few top layers at the surface, and it was assumed that overcompensation is similar for both the negatively and positively charged polyelectrolytes.\(^{39-40}\) Overcompensation and surface charge density were predicted to decay exponentially as we move farther from the surface and into the bulk of the film. The excess polymer charge on the surface is balanced by counter ions (extrinsically compensated).\(^{31}\)

Polyelectrolyte multilayers are known to be interpenetrated rather than stratified meaning that both polyelectrolyte are present at each point within the film.\(^{30,25,32}\) Jomaa et al. used neutron reflectometry experiment to study the bulk structure of polyelectrolyte multilayers. The fuzzy stratification of freshly prepared film disappeared after dipping the PSS/PDADMA film in high salt solution as a result of polyelectrolyte interdiffusion, a process known as “annealing” and will be discussed in the next section. In addition, Jomaa et al. found that the diffusion of polyelectrolytes at the surface of the film is much faster than in the bulk, which supports the finding that the first few surface layers are extrinsically compensated providing more free volume for the polyelectrolytes to interdiffuse compared to the intrinsically compensated “compact” bulk.\(^{40}\) These films also showed evidence of some excess positive extrinsic charges in the bulk (residual extrinsic sites), detected using infrared active negative
counterions and estimated by approximately 6%. This percentage decreased to 2% after treating, i.e. annealing, the film at high salt concentration.41-43

1.2.3 Effect of Salt on the properties of PEMUs

1.2.3.1 Swelling and permeability

Salt plays a major role in controlling the properties of polyelectrolyte multilayers. Salt was found to control the thickness of PEMU by controlling the thickness growth of a single deposited later. Also salt concentration affected the permeability, swelling and the mechanical properties of PEMUs.33,42,44,47 The electrostatic interaction, between the monomers of oppositely charged polyelectrolytes result in ionically crosslinked structure with reversible physical junctions. The formation of PEMU is entropically driven by the release of water and counter ions into the solution and this is illustrated in Figure 1.5.42,48 These counter-ions may be introduced by exposing the film to salt solution. The effect of salt on the structure of PEMUs is represented in the following equilibrium which is the reverse of Equation 1.1:

\[
Pol^+ Pol^- + Na_{aq}^+Cl^{-}_{aq} \Leftrightarrow Pol^+Cl^{-}_{m} + Pol^-Na^+_{m} \quad (1.2)
\]

Where \( Pol^+ \) and \( Pol^- \) refer to positive and negative polyelectrolyte respectively. “m” refers to components in the multilayers phase.

Figure 1.5 The pairing of oppositely charged polyelectrolytes, the association is driven by the release of water and counter-ions into the solution in an entropy driven assembly.
Equation 1.2 indicates that increasing the salt concentration of the surrounding medium will break, or dope the ionic crosslinks between the monomers of the oppositely charged polyelectrolytes. On the other hand, decreasing the salt concentration will lead to the formation of more ionic crosslinks between the polyelectrolytes. Thus intrinsic sites can be converted into extrinsic sites by adding salt and vice versa. This phenomenon impacted the properties of PEMUs in many ways, for example, Dubas et al. found that the films swell on increasing salt concentration as a result of increasing counter-ion content in the film which are hydrated and thus bring more water to the film. This was termed as the “antipolyelectrolyte effect”. The efficiency of doping was also found to depend on the type of salt and polyelectrolyte used. Farhat et al. studied the permeability of ferrocyanide ions, which are electroactive species, through PDADMA/PSS multilayers using rotating disc electrode (RDE). When the electrode was coated with PDADMA/PSS multilayer the current decreased as a result of increased resistance to mass transfer through the film. Exposing the PEMU-coated RDE to increasing salt concentration solutions increased the current, which means that more ferrocyanide was able to diffuse through the multilayer towards the electrode. This was explained by the increased electroactive ion hopping rate through the film with increasing salt concentration. In other words, the film acted a “reluctant exchanger” on increasing salt content. Each site within the film that is compensated by counter-ion (Cl\(^{-}\)) can act as a charge carrying site; electroactive ions can then be transported through the film by hopping from one site to another. Increasing the salt concentration will increase the amount of extrinsic sites in the film thus increasing the amount of charge carrying sites. This resulted in increasing ionic diffusion through the film. Polyelectrolyte diffusion through the multilayer was also found to increase with increasing salt concentration.

1.2.3.2 Mechanical properties

The effect of salt on hydration and permeability of PEMUs should impact the mechanical properties of the film. PEMUs are amorphous gels stabilized by reversible physical junctions (ionic crosslinks), and the density of these crosslinks controls their mechanical properties. Also it was demonstrated that PEMU’s structure has 70% ladder-type interactions and 30% network interactions. In a ladder structure, consecutive monomer charges on a polyelectrolyte chain pair-up with other two oppositely charged monomers forming a zip-like structure (Figure 1.4). Those percentages were assumed and used as fixed parameter when studying the mechanical properties of PEMUs made from PDADMA and PSS. Being of nanometer range thickness and restricted
to surfaces such as glass made it difficult to test their mechanical properties with the traditional techniques such as tensile testing and rheometry. Quartz crystal microbalance,\textsuperscript{52-53} strain induced buckling,\textsuperscript{54} and osmotic swelling\textsuperscript{55-57} were first used to assess the mechanical properties of PEMUs. Later, Jaber et al. were able to prepare thick PDADMA/PSS multilayer on fluorinated surfaces, which made it possible to peel the film from the surface giving free standing multilayer, suitable for tensile testing.\textsuperscript{46} Due to the small thickness of the films obtained, an apparatus that is used to test individual muscle fibers was employed to test the mechanical properties of PDADMA/PSS multilayers. They found that the elastic modulus of the film decreased on increasing the ionic strength of the surrounding medium.\textsuperscript{46} They mainly related the elastic modulus of the film to crosslinking density, where high salt concentration acts in breaking the ionic crosslinks between the polyelectrolyte chains leading to more extrinsic sites (lower crosslinking density) and thus lower elastic modulus. On contrary, at low salt concentrations, more ionic crosslinks (intrinsic sites) were formed between the polyelectrolytes which resulted in higher crosslinking density and thus higher modulus values. The damping efficiency of PDADMA/PSS films also increased with increased doping of the film with salt.\textsuperscript{58} The behavior of PEMU on increasing salt concentration could be explained by a behavior similar to glass-to-rubber transition observed in polymer networks on increasing the temperature of the medium.\textsuperscript{46,58} Thus, PEMUs can be softened by adding salt similar to the effect of temperature in other polymeric networks. This was termed as \textit{counter-ion induced plasticization} and PEMUs were later named “saloplastics”.\textsuperscript{46,59-60}

\textbf{1.2.3.3 Annealing}

Dubas et al. showed that exposing PEMU to high salt concentration smoothen the surface of PSS/PDADMA multilayer.\textsuperscript{45} They measured the surface roughness of films by AFM after immersing the film in 1M NaCl for different times, and they found that surface roughness decreased with time. They attributed this to a phenomenon known as “annealing”, where at high salt concentrations intrinsic sites in the PEMU are converted to extrinsic sites. This phenomenon resulted in swelling by driving more water into the film and thus providing more space polyelectrolyte chains to interdiffuse and enhancing chain mobility.\textsuperscript{45} Jomaa et al. found that annealing can also affect the bulk of the film and not only the surface. They used neutron reflectivity studies on as made PSS/PDADMA multilayer and on the multilayers dipped in increasing salt concentrations. They measured Bragg peaks that result from interference between
neutrons reflected from evenly separated deuterated PSS within the film. The as-made PEMU showed Bragg peaks, which indicated the presence of some stratification in the structure of the PEMU. Dipping the multilayer in increasing salt decreased the Bragg peak as result of the loss of stratification and increased interpenetration of polyelectrolyte chains within the film. This observation was also justified as due to increased mobility of the polyelectrolyte chains on increasing salt, which also acted in lubricating and freeing the polyelectrolyte segments allowing the chains to intermingle and relax to a lower energy state.\textsuperscript{40}

One might think that dipping as-made multilayers in water at the end of assembly should result in a film with no extrinsic charge (no counter ions). However, due to the kinetic control on the assembly of polyelectrolytes, some extrinsic sites can remain trapped within the bulk of the film and are known as residual extrinsic sites. 6\% residual extrinsic sites were detected in the as-made multilayer using ATR-FTIR by incorporating IR active negative counter ions.\textsuperscript{41-42} Annealing the multilayer allowed some of the trapped extrinsic sites to diffuse out as a result of the slow intermingling of the polyelectrolyte chains. The percentage of trapped extrinsic sites was found to decrease to 2\% after annealing.\textsuperscript{41-42} A schematic diagram illustrating this observation is shown Figure 1.6.
Figure 1.6 As-made multilayer was immersed in high salt concentration. Trapped extrinsic sites diffuse out of the film upon annealing since polyelectrolyte chains are more mobile at high salt concentration, this allowed them to interdiffuse and mix resulting in a lower energy structure. It was found that the percentage of trapped extrinsic sites decreased from 6% to 2% when annealing PSS/PDADMA multilayer in 1M NaCl.

1.3 Polyelectrolyte multilayer for bio-applications

1.3.1 General introduction

The use of polyelectrolyte multilayers in bioapplications gained more attention in 2001, and since then, they had been studied for drug release applications, cellular adhesion and repulsion, non-fouling films, coatings for bio-implants to enhance anti-bacterial properties and biocompatibility, and for localized gene therapy. Polyelectrolyte multilayers (PEMUs) are good candidates for bio-applications: they are characterized by their ease of preparation, controllable thickness, tunable mechanical and surface properties, and they can almost coat any surface which make them good for coating bioimplants. Also a wide variety of polyelectrolytes exists, some are synthetic and others are naturally available, and most of which are biocompatible. In the next few paragraph, a summary on the usage of PEMUs in bioapplications is presented.
1.3.2 Protein adsorption

Salloum et al. studied the mechanism of protein adsorption on PEMUs. Protein adsorption on surface coatings is important in blood-contacting devices and in enhancing cellular adhesion.\textsuperscript{67,73-74} It is known that proteins adsorb on the surface via electrostatic, hydrogen bonding and hydrophobic interactions.\textsuperscript{75} Surfaces of opposite charge to the protein were the most efficient in promoting protein adsorption, and thicker films were more efficient in adsorbing protein since they act as “sponge” in protein loading.\textsuperscript{73} Hydrophobic surfaces are also known to induce greater protein adsorption. The presence of hydrophilic neutral poly(ethylene oxide) on the surface decreased the efficiency of protein adsorption.\textsuperscript{76-77} Enhancing cellular adhesion and proliferation on PEMUs after pre-coating the surface with ECM proteins, mainly fibronectin, was extensively studied. In these studies, PEMUs prepared from synthetic or natural polyelectrolytes were employed. It was found that adsorption of extracellular matrix proteins on the surface of PEMU promotes cellular adhesion and proliferation, for example, Wittmer at al. found that coating PEMUs, prepared from natural polyelectrolytes poly(l-lysine) (PLL) as polycation and dextran sulfate (DEXS) as a polyanion, with a layer of fibronectin enhanced the adhesion and spreading of human umbilical vein endothelial cells.\textsuperscript{78} A similar study was conducted by McShane and coworker using synthetic polyelectrolytes, poly(allylamine hydrochloride) (PAH) as the positive polyelectrolyte and poly(styrene sulfonate) (PSS) as the negative one. The adhesion and proliferation of smooth muscle cells was enhanced after coating the PAH/PSS surface with fibronectin.\textsuperscript{79}

1.3.3 Cellular interaction

A major advantage of using PMEUs for bioapplications is the ease of tuning their surface properties to be cell repellent or cell-adhesive, depending on the target application. Surfaces with cell-repellent properties are important in biomedical devices, such as stents, where the main drawback involves the invasion of vascular smooth muscle cells (A7r5) and building layers of tissues on the surface which can block the blood flow.\textsuperscript{80} Salloum et al. were able to direct adhesion and repulsion of vascular smooth muscle cells on PEMUs depending on the surface charge and surface hydrophobicity of the terminating layer.\textsuperscript{67} They found that negatively charged surfaces were more cell adhesive than positively charged ones. This was explained by the fact that may positively charged surfaces act as surfactants and are capable of disrupting the cell
membrane. Cell adhesion was greatly limited on PEMU surfaces ending in zwitterionic groups made from poly(acrylic acid) grafted with zwitterionic, (3-[2-(acrylamido)ethyl]dimethylammonio) propane sulfonate (AEDAPS), which acted as a hydrophilic surface. Increasing the grafting degree of the zwitterion on PAA made the surface more cell-repellent; on the other hand, A7r5 cells were highly adhesive on PEMUs terminated with hydrophobic nafion. This could be a result of the high swelling of hydrophilic surfaces, which make them soft and spongy. Salloum et al. were able to show that a single layer on the surface can change to cell-adhesive or repulsive, regardless of the underlying bulk material. Other strategies that were used to selectively enhance cell-adhesion on the surface of PEMUs include grafting one of the polyelectrolytes with ECM peptide sequence that is known to bind to integrin receptors at the surface of cells. The most famous sequence is the arginine-glycine-aspartic acid (RGD) sequence. Using the well-known EDC coupling chemistry, Picart et al. grafted the polyanion, poly(L-glutamic acid) (PGA), with RGD and this was adsorbed onto a poly(l-lysine) (PLL) coated film. The adhesion and proliferation of primary human osteoblasts were significantly enhanced when cells were cultured on a PLL/PGA film with PGA-RGD as the top layer compared to the unmodified PLL/PGA films. Berg et al. found that coating cell-inert film, made from a hydrogen bonded multilayer of poly(acrylic acid) and polyacrylamide (PAAm), with poly(allylamine hydrochloride) grafted with RGD peptide, enhanced the adhesion of fibroblasts to the surface. Also increasing the ligand density on the surface was found to result in better adhesion. Another protein that can enhance cell adhesion in similar way to RGD is laminin-5. A film prepared from PLL and PGA grafted with laminin-5 enhanced the proliferation of epithelial cells. The ability to functionalize polyelectrolyte with groups that control cell interactions on the surface and the ability of PEMUs to coat any surface provide vital tool for improving the surface properties of bio-implants. For example, an important problem encountered in titanium implants for bone repair is the unpredictable implant integration with the host bone, and this can lead to the risk of implant loosening over time. Wang et al. coated titanium substrates with a polyelectrolyte multilayer prepared from hyaluronan (HA) and chitosan (CH) and terminated in RGD containing peptide. Without RGD, HA/CH inhibited the adhesion of osteoblasts, however, immobilization with RGD had a significant beneficial influence on the adhesion and proliferation of osteoblasts resulting in improved their orthopedic application of the material. The same strategy was followed in dental applications, where the
success of an implant (titanium) depends on its anchorage to the surrounding bone. It was demonstrated that coating the implant with poly(L-lysine) grafted with RGD and poly(ethylene glycol) (PEG) enhanced the attachment of epithelial cells, fibroblasts and osteoblasts.\textsuperscript{86}

1.3.4 Anti-bacterial

Another drawback in bio-implants is the adhesion of bacteria on the surface, which result in infection and the failure of the implant. This will require surgery to remove and replace the implant in the body. The potential of PEMUs in antibacterial coatings has been demonstrated.\textsuperscript{87-91} Certain polyelectrolytes, such as chitosan, are known to have antibacterial properties. Bratskaya et al. prepared a PEMU from chitosan/k-carrageenans and tested its antibacterial capability. This kind of PEMU was found to significantly decrease bacterial growth on the surface, providing a promising system to be used as antibacterial coatings.\textsuperscript{69} Another polyelectrolyte that prevented adhesion of bacteria is heparin. Multilayer prepared from chitosan and heparin was found to kill and prevent the adhesion of \textit{E. coli} bacteria on the surface.\textsuperscript{92} The same group prepared chitosan/heparin film containing silver nanoparticles that was found to have better antibacterial properties.\textsuperscript{70} Other PEMU systems that were tested for their antibacterial properties are prepared from poly(L-glutamic acid) (PGA) and lysozyme.\textsuperscript{93}

1.3.5 Non-fouling

Another potential use of PEMUs is in non-fouling coatings to prevent the adhesion of serum proteins on the surface, an application that is important in enhancing the efficiency of drug delivery vehicles. Several methods were reported for grafting polyelectrolytes with polyethylene glycol (PEG), a highly hydrated polymer that is known to render a surface protein resistant. Polyelectrolytes grafted with PEG were then deposited on the surface of PEMUs giving the latter non-fouling characteristics.\textsuperscript{77,94} Salloum et al. synthesized PAA-co-AEDAPS by grafting poly(acrylic acid) PAA with the zwitterion AEDAPS (3-[2-(acrylamido)-ethyldimethyl ammonio] propane sulfonate. Interestingly, coating the surface of PEMUs with PAA-co-AEDAPS also prevented the adhesions of cells and proteins on the surface similar to polyelectrolytes with PEG moieties.\textsuperscript{67}
1.3.6 Drug release

An interesting advantage of PEMUs is the ability to load them with molecules such as growth factors, antibacterial agents, and drugs, and the ability to control the release of these molecules. Rubner and co-workers reported the preparation of hydrogen bonded multilayer loaded with anti-bacterial silver (Ag) nanoparticle. Others reported the loading and release of different molecules such as enzymes, and low molecular cationic molecules from PEMU, which is important in drug delivery applications.

1.3.7 Tunable mechanical properties

In addition to chemical signals that affect the cell’s behavior, mechanical properties of substrates used for cell culture were found to direct the fate of cells. The ability to engineer materials for cell culture with decoupled mechanical and chemical stimuli is still a growing field. Tuning the mechanical properties of PEMUs can be easily achieved by adjusting the pH and ionic strength of the surrounding environment. However when working under physiological conditions, the pH and ionic strength of the medium have to remain within a narrow range close to the pH and ionic strength of the body, which are 7.4 and 0.15 M, respectively. It is possible to adjust the mechanical properties of PEMUs by chemically crosslinking the amine and carboxyl groups within the film using the carbodiimide chemistry. This resulted in converting the ionic crosslinks in the film into covalent bonds. The same type of transformation can be induced by heating the film, a process known as thermal crosslinking. A more recent approach that was introduced later is photocrosslinking. This can be achieved by grafting one of the polyelectrolytes with a photosensitive molecule, such as benzophenone or vinyl benzene. Photocrosslinking provided several advantages over chemical and thermal crosslinking, most importantly it provided the possibility of patterning and tuning the stiffness of PEMUs without affecting the chemistry and charge density of the film which is a requirement when testing the effect of substrate mechanical properties on the behavior of the cells.
1.4 Polyelectrolytes

![Polymeric structures](image)

**Figure 1.7** Structure of the polyelectrolytes that will be used in experiments done in this dissertation. PAA, PAH, PEI, PSS, PDADMA are all commercially available, PAABp was synthesized and its synthesis procedure will be discussed in the coming chapters.

1.5 Dissertation outline

The experimental details and the equipments used in this dissertation are illustrated in Chapter 2. More details about the experiments are listed in each of the chapters.

In Chapter 3, an approach based on photocrosslinking was used to tune the stiffness of PEMU prepared from poly(acrylic acid) (PAA) as polyanion and poly(allylamine hydrochloride) (PAH) as polycation. PAA was grafted with a photosensitive molecule with benzophenone group. The product, PAABp, was characterized by NMR. A multilayer was then prepared from PAH and PAABp. PAH/PAABp showed a controlled crosslinking degree after irradiation with UV light. Photocrosslinking impacted the mechanical properties of the film, measured by nano-
indentation, and the permeability of film to iodide ions. On the other hand, the wettability and surface chemistry of the film remained unaffected.

In Chapter 4, PAH/PAABp was applied to prepare a film with gradient in elasticity and to test the effect of substrate mechanical properties on the behavior of cells. Two different gradients were prepared, one using a photomask (neutral density filter). PAH/PAABp film was prepared, covered with neutral density, and then irradiated with UV, which resulted in a continuous variation in elasticity along the film. In another approach, half of the PAH/PAABp film was covered with a black mask and the other half was irradiated with UV, and therefore, an edge gradient was prepared. Smooth muscle cells and osteosarcoma cells were plated on the films with gradient elasticity. Both cell lines showed higher preference to the stiffest end of the gradient. On the edge gradient smooth muscle cells were able to sense the gradient, and cells on the soft side migrated to the stiff side.

In Chapter 5, nano-indentation by atomic force microscope was used to characterize the swelling, modulus, viscoelasticity, and topography of PEMU prepared from poly(diallyldimethylammonium chloride) (PDADMAC) as polycation and sodium poly(styrene sulfonate) (NaPSS) as polyanion. It was shown that a multilayer ending in PDADMA swelled more than a multilayer ending in PSS, and this correlated well with change in modulus where PDADMA ending films were softer than PSS-ending films. The modulus of the film was shown to decrease with increasing salt concentration as a result of increased extrinsic compensation. Measuring the modulus of PDADMA/PSS multilayer at increasing indentation rates, which are equivalent to different strain rates, showed evidence of viscous-like behavior of PDADMA/PSS films. AFM images of the surface of PDADMA/PSS showed surface roughness that increased on swelling. Measuring the modulus of the features on the surface of the multilayer showed no difference between the hills and valleys. This showed that surface roughness of PDADMA/PSS multilayer was not due to phase separation.

In Chapter 6, the effect of water on the mechanical properties of PDADMA/PSS multilayer was illustrated. A strategy to control the water content in the film, without affecting the degree of extrinsic and intrinsic compensation, was followed based on osmotic stress technique. Poly(ethylene glycol) (PEG) was used as an osmotic stressor. Immersing PDADMA/PSS films in PEG solutions with increasing weight percentages decreased the water content of the film, and this affected the elasticity and viscoelasticity of the film significantly.
The presence of water in the film provided free volume for chain mobility and relaxation, which proved that water acts in plasticizing PDADMA/PSS multilayers.

In Chapter 7, some important conclusions regarding this dissertation are provided with future remarks that can be important to proceed further with the work and take it to the next step.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Chemicals

Poly(acrylic acid) (PAA Mw = 100,000 g mol$^{-1}$), poly(allylamine hydrochloride) (PAH Mw = 56,000 g mol$^{-1}$), Poly(4-styrenesulfonic acid) (PSS Mw = 7.5x10$^4$ g mol) and poly(diallyldimethylammonium chloride) (PDADMAC Mw = 40x10$^4$ – 50x10$^4$ g mol$^{-1}$) were used as received from Aldrich. Poly(ethyleneimine) (PEI Mw = 70,000 g mol$^{-1}$) obtained from Polysciences. NaCl (ACS grade) was used to adjust the ionic strength of the polyelectrolyte solutions. Potassium iodide (ACS reagent) from Aldrich was used for the permeability studies. 4-hydroxybenzophenone from AlfaAesar, potassium carbonate anhydrous from Fisher Scientific and 2-bromoethanol from Acros Organic were used in the synthesis of the photocrosslinker. N,N’-dicyclohexylcarbodiimide (DCC) from Fluka was used for coupling PAA to benzophenone. Polyethylene glycol (PEG Mw = 8000 g mol$^{-1}$) was purchased from Alfa Aesar. All solutions were prepared using deionized water (Barnstead, 18 MΩ E-pure).

2.2 Instrumentation

2.2.1 Ellipsometry\textsuperscript{114-115}

Ellipsometry is an optical technique used to measure the thickness of thin films. It is based on analyzing the change in polarization of laser light upon its reflection after hitting the surface. The fact that the ellipsometry measure changes in polarization makes ellipsometer able to measure thickness as low as a monolayer. A typical ellipsometric measurement involves striking the sample (PEMU film or bare silicon wafer) with monochromatic light that is linearly polarized and then the reflected light from the sample changes polarization to become elliptically polarized. The incident light on the sample is characterized by a fixed incident angle. The reflected elliptical light is characterized by amplitude, $\Psi$, which is the relative ratio between the two components of the electric field of the light beam, and relative phase shift, $\Delta$, which is the phase difference between the two components of the electric field of the beam. Using computer software the difference in $\Psi$ and $\Delta$ between the incident light and the reflected light from the sample is calculates and then used to measure the thickness of the film.\textsuperscript{114}
In our lab we use Gaertner Scientific L116S autogain variable angle stokes ellipsometer that has a He-Ne laser that has a wavelength equal to 632.8 nm and strike the sample at an incident angle equal to 70°. The refractive index of the sample was then estimated by the computer software with a typical sample that has a thickness of about 100 nm. This estimated refractive index was then employed and the thickness measurements were performed at a fixed refractive index. Usually 10 different thickness measurements were made on each sample and the thickness was taken as the average.

2.2.2 Atomic force microscope

Atomic force microscopy is an important tool to study the topography of materials with high resolution. Briefly, AFM studies involve the use of a cantilever with a sharp tip at its end to scan the sample. The movement of the cantilever is controlled by nanometer precise piezoelectric device (in x, y, z directions). When the tip interacts with the sample, changes in the z-direction measured from the deflection of a laser beam off the metalized backside of the cantilever into a position sensitive photodiode detector can give information on the surface morphology and thickness (step height) of the material (film). Measurements can be performed both in wet and dry conditions. A schematic diagram of the basic component of an AFM is shown in Figure 2.1.

![Figure 2.1 A schematic diagram of the basic components of an AFM instrument.](image-url)
The two most common used methods used in atomic force microscope are the contact mode and the AC mode. A typical contact mode experiments can take place in dry and wet condition and can be accomplished by scanning the tip over the sample while measuring the cantilever deflection. The tip touches the sample through the adsorbed fluid layer. A constant cantilever deflection that is equal to the set point is maintained by the feedback loop that allows the movement of the piezoelectric scanner in the z-direction at each x,y data point, this lead to a constant force between the sample and the tip measured by Hooke’s law shown in Equation 2.1.

\[ F_{\text{applied}} = Kd \quad (2.1) \]

Where \( F_{\text{applied}} \) is the applied force on the sample, \( K \) is the force constant and \( d \) is the deflection of the cantilever. The movement of the scanner in the z direction to maintain a constant force reflects the topographical image of the sample.

For the AC mode or tapping mode, which can also be performed in wet and dry conditions, the cantilever is oscillating while the tip scans the surface of the sample. The cantilever is oscillated at or at either side of the resonance frequency with constant setpoint amplitude that ranges usually from 20nm to 100nm. The piezoelectric scanner maintains constant amplitude by vertical movement at each x,y data point, thus a constant tip-sample interaction is maintained. The vertical movement of the scanner is stored by the computer as the topographical image of the surface.

For our experiments we use MFP-3D asylum research AFM with Igor software. AFM images were obtained in AC mode for both the wet and dry images. To obtain the thickness of the films, the film was scratched and the tip was scanned over the scratch to obtain the step height as shown in Figure 2.2.
The mechanical properties using AFM can be obtained by the use of force spectroscopy which is based on force-indentation measurements and is considered the most reliable technique to study the mechanical properties of materials of nanometer scale thickness. The movement of the cantilever is controlled by a piezo device; then a force curve is based on measuring the deflection of the cantilever indenting the surface of a sample as a function of the movement of the piezo in the z-direction. Usually a force curve is conducted initially on a hard sample.

**Figure 2.2** A scratch made on a polyelectrolyte multilayer film, the AFM tip was scanned over the scratch to obtain the thickness (step height). The thickness can be obtained from the difference in height between the scratch and the film (shown as the two blue marks). The thickness of this film is 1200 nm.
typically a glass slide as a reference then on the sample of interest. The measurement on the reference and the sample should be made using the same tip and under the same environmental conditions and without changing the position of the laser on the cantilever. Figure 2.3 is a schematic representation of the deflection of the cantilever on a hard (glass) and soft sample. When the cantilever is in air the deflection is constant as a function of the z-movement of the piezo; when the cantilever poke the hard sample the deflection is directly to the movement of the piezo, on the other hand if the cantilever poked the soft sample, the deflection is smaller as a result of tip indentation in the sample. The difference in z-distance of the cantilever taken at the same deflection on glass and on the sample of interest is used to calculate the tip indentation in the sample according to the Equation 2.2 and the force applied on the sample can then be obtained according to Equation 2.3:

\[
\delta = (z - d) \quad (2.2)
\]

\[
F_{\text{applied}} = Kd = K(z - \delta) \quad (2.3)
\]

Where \(\delta\) is the indentation distance of the tip in the material, \(z\) is the distance of the tip relative to the material in the z-direction, \(d\) is the deflection of the tip, \(k\) is the spring constant of the cantilever.

From Equations 2.2 and 2.3 we can obtain a plot of force vs. indentation known as force curves. Force curves are analyzed based on tip geometry, by using the three mathematical models of Hertzian contact mechanics that provide solutions to the indentation of a semifinite substrate with a hard sphere. The Sneddon’s model when a conical tip is used give the F-ind relation represented by Equation (2.4):

\[
F_{\text{cone}} = \frac{2E_{\text{surface}}}{\pi(1 - \nu^2_{\text{surface}})} \tan(\alpha)(z - d)^2 \quad (2.4)
\]

Sphere and punch tips are represent in Equations (2.5) and (2.6) respectively:

\[
F_{\text{sphere}} = \frac{4}{3} \frac{E_{\text{surface}}}{(1 - \nu^2_{\text{surface}})} \sqrt{R(z - d)^{\frac{3}{2}}} \quad (2.5)
\]
\[ F_{\text{punch}} = 2 \frac{E}{(1 - \nu^2)} R(z - d) \] (2.6)

\( E \) is the modulus of the material, \( R \) is the radius of the indenter, \( \nu \) is the poisson ratio of the material, and \( \alpha \) is the half angle of the tip.\textsuperscript{15,121}

The difference between the three profiles is related to the difference in power dependences on the indentation,\textsuperscript{15,122} where for the cone tip, a quadratic relation is obtained between the force and the indentation,\textsuperscript{1,33} in the sphere model the force is proportional to the indentation to the power 1.5, and finally in the punch fit a linear relation between the force and the indentation is obtained.\textsuperscript{15,122} It is always important to keep the contact area between the tip and the sample smaller than the sample thickness, otherwise, the substrate stiffness will contribute to the measurements.\textsuperscript{118} Figure 2.4 shows the three indentation profiles of different tip geometries.

**Figure 2.3** Schematic representation of the deflection of the cantilever as a function of z-movement of the piezo. (a) The cantilever is in air and the deflection is constant. (b) The cantilever poking the hard sample and the deflection is directly proportional to the z-movement of the piezo. (c) The cantilever indenting the soft sample.
Contact angle is the measurement of the wetting of a solid by the liquid. Cahn Instruments, DCA 300 (Figure 2.5) was used to measure the dynamic contact angle, both advancing $\Theta_a$ and receding $\Theta_r$, through the Wilhelmy plate technique. The instrument is formed of a moving stage and a highly sensitive balance and thus the forces exerted on a solid sample are recorded when immersed in the test liquid. For PEMUs the dynamic contact angle was obtained by immersing the film built on a double sided silicon wafer, in water and then pulled out at a rate of 100 $\mu$m sec$^{-1}$. First the film is hung on the balance and then tared. The film is then brought in contact with water at a speed of 100 $\mu$m sec$^{-1}$. When the film touches the water the forces exerted on the film are recorded by the tensiometer at zero depth of immersion and then it continue to record the forces as the sample is immersed in the liquid. Advancing angle is the angle obtained when the film is dipped in the solution; however the receding angle is the angle when the film is pulled out of the water. With the aid of the instrument software, the linear region of the plot of force versus stage displacement corresponding to the advancing and receding meniscus was fit to the least square which was extrapolated to zero depth of immersion to take out the effect of buoyant force as shown in Figure 2.6, and then these values were used to determine the advancing and receding contact angle according to Equations 2.7 and 2.8.

$$F_{\text{total}} = F_{\text{wet}} + \text{weight of probe} - \text{buoyancy} \quad (2.7)$$
The buoyancy and the weight of probe are zeroed by the instrument and thus the wetting force can be deduced. The wetting force can be related to the contact angle according to Equation 2.8.

\[ F_{\text{wet}} = \gamma P \cos \Theta \quad (2.8) \]

Where \( \gamma \) is the surface tension of water, in which the experiments are conducted and is equal to 72.3 mN m\(^{-1}\), \( P \) is the wetted perimeter of the sample obtained by measuring the width and thickness of the sample and \( \Theta \) can be the advancing and the receding angle.

**Figure 2.5** Cahn Instruments, DCA 300 used to measure dynamic contact angle both advancing and receding of PEMUs
The static contact angle was performed using KSV instruments LTD model Cam 200. A drop of water that has a 10 µl was dropped on the sample and pictured with the camera, a technique called sessile drop and is shown in Figure 2.7. The software was used to obtain the static contact angle which is the angle formed between the baseline of the drop and the line that is tangent to it. Measurements were done at 3 different locations on the sample and the static contact angle was reported as the average of the three locations.

**Figure 2.6** Schematic representation of the stages describing the wetting of the solid by liquid on immersion in liquid to obtain the advancing angle and after emersion from liquid to obtain the receding angle. $F_a$ is the advancing force built up as the sample is immersed in water. $F_b$ is the receding force obtained as the sample is being pulled out of the liquid (water). (a) Represents the point where the sample touches the liquid (zero depth of immersion). (b) Represents the advancing interface. (c) The receding interface. (d) The point when the sample is pulled out from water.
2.2.4 Fourier Transform Infrared spectroscopy (FTIR)\textsuperscript{124}

FTIR is a spectroscopic technique used to obtain information on the chemical functional groups and bonds of molecules. The energy of the IR photon needs to match the spacing of the vibrational levels of the molecule for the photon to be absorbed. This technique is based on the Michelson interferometer which can be described as follows a beam of light from the source is split into two beams which are then reflected into a stationary and a moving mirror. The beam of light coming from the stationary and moving mirrors are now travelling with different path length and recombine and gets reflected towards the sample. The different in path length between the beams of light result in constructive and destructive interferences called interferogram. Fourier transform is then used to convert time domain data into frequency domain. FTIR measures absorptions at all wavelength simultaneously which is an advantage over other absorption techniques. A chemical bond can be described as an oscillating spring that is capable of absorbing infrared energy. The absorbed energy depends on the mass (size) and the strength of bond according to the following Equation:

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Static contact angle of a film with PDADMA ending layer and another one with PSS ending layer. Software was used to obtain the static contact angle which is the angle between the baseline and the line tangent to the drop. The angle at both sides (right and left) of the drop was obtained. The static contact angle for PDADMA ending films was 70° and for PSS ending films was 20°}
\end{figure}
\[ \bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{k}{\mu}} \]  

(2.9)

Where \( \bar{\nu} \) is the wavenumber, \( k \) is the force constant, and \( \mu \) is the reduced mass for the atoms forming the bond and is shown in Equation 2.10.

\[ \mu = \frac{m_1 m_2}{m_1 + m_2} \]  

(2.10)

Where \( m_1 \) and \( m_2 \) are the masses of the atoms forming the bond. From equation 2.9 and 2.10 we can deduce that lighter atoms and stronger bonds absorb at higher energy.

The FTIR data was obtained using a nitrogen-purged FTIR (Nicolet Nexus 470 with a deuterated triglycerine sulfate (DTGS) detector) spectrometer. The resolution was 4 cm\(^{-1}\), and 256 scans were taken of the sample. The PEMUs were prepared on double sided silicon wafer, and before build-up a spectrum for the bare silicon wafer was taken and used as background. Powder samples were prepared as KBr pellets using Carver Laboratory Press (model C). Omni software which was provided by manufacture was used to analyze the FTIR data.

**2.2.5 UV-Visible spectroscopy**

UV-Vis spectroscopy measures the light transmitted after it passes through the sample. When a light is shined on a sample, it undergoes electronic transition from the ground state to the excited state. Only light with energy that matches the electronic transition will be absorbed and the rest will be transmitted. Transmittance can then be defined as the ratio of intensity of transmitted light to the initial incident light as shown on Equation 2.11. The absorbance can be related to the concentration of the sample according to Lambert-Beer law shown in Equation 2.13.

UV-Vis measurement was carried out on a Varian Cary UV/VIS/NIR spectrophotometer. All measurements were done in double beam mode. PEMUs were prepared on a UV fused silica substrate which is transparent to UV-Vis region (200-800 nm) and this was used as the reference during measurement.
\[
T = \frac{I}{I_0} \quad (2.11)
\]
\[
A = -\log_{10}\left(\frac{I}{I_0}\right) \quad (2.12)
\]
\[
A = \varepsilon \ell C \quad (2.13)
\]

Where \( T \) is the transmittance, \( I \) and \( I_0 \) are the intensity of the transmitted and incident light respectively, \( A \) is the absorbance, \( \varepsilon \) is the molar absorptivity or extinction coefficient, \( \ell \) is the path length of the light in the sample, and \( C \) is the concentration of the sample.

### 2.2.6 Nuclear magnetic resonance spectroscopy (nmr)\(^{125-126}\)

NMR is a spectroscopic technique used to determine the number of magnetically distinct atoms of the type being studied such as hydrogen and carbon. Atomic nuclei have a property called spin, any atom that has an odd atomic or mass number is characterized by a quantized spin angular momentum and thus a magnetic moment. In the absence of applied magnetic the spin states of a given nucleus have the same energy and are said to be degenerate. When a magnetic field is applied the nucleus will have its magnetic moment either aligned with the field or in opposite direction to the field. For example, a hydrogen atom can have a clockwise (+1/2) spin state or counter clockwise spin state (-1/2). In the presence of magnetic field the spin state (+1/2) is of lower energy since is aligned with the field and the spin state (-1/2) is of higher energy because it is opposed to the applied field. The greater the applied magnetic, the higher the energy difference between the spin states. When a magnetic field is applied, the nucleus start to precess around its own axis with a frequency called Larmor frequency proportional to the applied magnetic field. When a radiofrequency wave is supplied to the precess proton and if the radiofrequency matches the frequency of the generated electric field, the energy can be absorbed by the nucleus and lead to spin change, a process called resonance. The generated electric field depends on the environment of the nucleus under study and thus based on the environment protons within a molecule can have different chemical shift. Using NMR the structure of the synthesized molecule can be predicted.

The synthesized organic products were characterized by AC300 Brucker spectrometer operating at 300 MHz to acquire \(^1\)H NMR.
2.2.7 Hydrodynamic rotating disc electrode (RDE) voltammetry

RDE is used to study the permeability through PEMUs by using electrochemically active ions such as Iodide or ferricyanide. The system consists of three electrodes, the working electrode which is a 5 mm platinum rotating disc electrode, the reference electrode which is a saturated KCl-Calomel electrode, and the counter electrode which is a platinum foil. The voltage is applied between the working and the reference electrode and the current flows between the working and counter electrodes.

When using a reduced electrochemical species, scanning the voltage in the positive direction lead to oxidation, and then as the scan direction is reversed the molecule is reduced. In cyclic voltammetry the flow of current is limited by rate of mass transfer to the electrode surface and the kinetic of electrochemical reactions. Prior to using the working electrode, the electrode is polished with 0.05 micron alumina and then sonicated in distilled water. For the bare electrode the mass transfer effect is taken out by rotating the electrode enough to overcome the diffusion barrier. When the electrode is coated with PEMU, the redox species will experience mass transfer to reach the electrode surface and the current will decrease. This is an important method to study the permeability through PEMUs as will be seen in chapter 3.

2.2.8 Polyelectrolyte multilayer (PEMU) build-up

PEMU are prepared via the layer-by-layer technique with the aid of a robot (StratoSequence V, nanoStrata Inc.) on a 1 inch diameter single side polished silicon wafer for AFM measurements, on double side polished silicon wafer for FTIR measurements, on quartz slides for UV-Vis measurements and on glass coverslips (cover glass No. 1, 22 mm sq., Corning) for cell culture studies. The substrates were cleaned in piranha (70% H$_2$SO$_4$ and 30% H$_2$O$_2$) for 20 minutes (caution: piranha is a strong oxidizer and should not be stored in closed containers) or by using air plasma, and then rinsed vigorously with distilled water (18 MΩ) and dried with N$_2$. All polyelectrolyte solutions were prepared at 10 mM concentrations (with respect to the repeat unit) in water or tris buffer depending on the experiment and the pH of the solutions was adjusted using HCl or NaOH. The ionic strength of the polymer solutions was adjusted using NaCl. The substrate was mounted on a shaft that rotated at 300 rpm. The dipping time in each of the polymer solutions was 5 min followed by three 1 min rinsing steps in water.
CHAPTER THREE
PHOTOCROSSLINKING: A CONTROLLABLE METHOD TO TUNE THE MECHANICAL PROPERTIES OF POLYELECTROLYTE MULTILAYERS

3.1 Introduction

The bulk modulus of PEMU is controlled by the ion-pair crosslinking density between the oppositely charged polyelectrolytes which in turn controlled by the ionic strength of the surrounding medium for example increasing the salt concentration of the medium will rupture the ionic crosslinks between the polyelectrolytes and this will cause the multilayer to be softer on the other hand decreasing the salt concentration will lead to the formation of more ionic crosslinks between the polyelectrolyte chains and thus the multilayer will become stiffer.\textsuperscript{38,46,58-60}

We are interested in the potential use of PEMUs in biological applications and in investigating the behavior of cells on polymer surfaces of variable mechanical properties. When working under physiological conditions the ionic strength of the medium has to remain close to the body conditions. So, different approached were used to vary the stiffness of polyelectrolyte multilayers usually by incorporating a crosslinking moiety into the bulk of the film that enables the polyelectrolyte chains to crosslink by the formation of covalent bonds between the polyelectrolytes. A potential PEMU system for such kind of applications consist of poly(allylamine hydrochloride) (PAH) as the polycation and poly(acrylic acid) (PAA) as the polyanion due to the presence of the amine and carboxyl functional groups that can serve as the crosslinking moieties within the film. These functional groups were crosslinked either thermally or chemically. Thermal crosslinking was induced by extensively heating the film at elevated temperatures resulting in formation of amide bonds.\textsuperscript{99,103,108,128-130} For chemical crosslinking, mainly carbodiimide (EDC) chemistry was for coupling.\textsuperscript{65,105-106,131-133}

Photocrosslinking is another method used to modify the stiffness of polymeric films, it allow systematic control over the crosslinking density without using any chemical reagents that might be toxic to cells and thus require further purification.\textsuperscript{11,66,134} Photocrosslinking which can be performed in vitro or in vivo,\textsuperscript{135} also provide control over regions of crosslinking making it
possible for photo-patterning which can not be achieved by thermal and chemical crosslinking.\textsuperscript{11,134} Photocrosslinking can be performed by incorporating a photosensitive molecule into the bulk of the PEMU by grafting it to one of the polyelectrolytes.\textsuperscript{62,66,111-112,136-141}

Thermal and chemical crosslinking were first used to control the permeability through PEMUs where both decreased the ion transport through these films.\textsuperscript{103,142} It was shown that permeability was affected by the water content of the film where high water content makes the film softer and thus more permeable in addition to the effect of the ionic content of the film.\textsuperscript{41} Both thermal and chemical crosslinking consumes the carboxylic acid and amine functional group and thus significantly affecting the ion content of the film.\textsuperscript{15} So when using these approaches to study the effect of substrate mechanical properties on the behavior of cells, it appeared that mechanical properties were not the only variable. So, it is important to have control over the crosslinking density without affecting the properties of the film such as its ionic content. Photocrosslinking accomplished through the benzophenone chemistry and described in this chapter kept the charge content within the film unaffected which made it possible to study the permeability and mechanical properties of PEMUs as a function of the degree of crosslinking.

Probing the mechanical properties of PEMUs is a challenging procedure since PEMUs are thin film usually in the nanometer to micrometer range thickness and constrained to a solid substrate, so the use of the more common ways to study the mechanical properties of materials such as tensile and rheology encounters many challenges and difficulties. Jaber et al. was able to study the mechanical properties of polyelectrolyte multilayers by using tensile tests and making use of the stress relaxation technique and this was done with the aid of special apparatus used to study the mechanical properties of a single muscle fiber. However, this procedure, although affective, posed many difficulties including the need of relatively thick films (500 layers were need for PSS/PDAD multilayer \( @ \ 1 \ M \) salt); also they had to prepare the multilayer on fluorinated substrates to peel them off for tensile testing.\textsuperscript{46,58} Other techniques have been used to mechanically characterize PEMUs such as quartz crystal microbalance (QCM),\textsuperscript{36,52-53} osmotic swelling,\textsuperscript{55-56} and strain-induced buckling.\textsuperscript{54} Recently nano-indentation measurements had arisen as a simple and relevant technique to measure the Young’ modulus of relatively thin samples\textsuperscript{15,65-66,102,121,132-133,143} which rely on indenting the sample by a nanometer-size tip and the elastic response can be deduced from the loading force applied as a function of indentation.\textsuperscript{119}
In this chapter we describe the synthesis of photocrosslinkable PEMU based on benzophenone moiety grafted on poly(acrylic acid). UV irradiation was used to tune the mechanical properties of the PEMU and the modulus was measured by force spectroscopy using nano-indentation. The elastic modulus of the film was affected by photocrosslinking however the charge density within the film remained unaffected. The crosskinking density and the stiffness of the film were correlated with the permeability of iodide ion through the film.

### 3.2 Materials and Methods

Poly(acrylic acid) (PAA $M_w = 100,000$ g mol$^{-1}$) and poly(allylamine hydrochloride) (PAH $M_w = 56,000$ g mol$^{-1}$) were used as received from Aldrich. NaCl (ACS grade) was used to adjust the ionic strength of the polyelectrolyte solutions. Poly(ethyleneimine) (PEI $M_w = 70,000$ g mol$^{-1}$) obtained from Polysciences. Potassium iodide (ACS reagent) from Aldrich was used for the permeability studies. 4-hydroxybenzophenone from AlfaAesar, potassium carbonate anhydrous from Fisher Scientific and 2-bromoethanol from Acros Organic were used in the synthesis of the photocrosslinker. N,N'-dicyclohexylcarbodiimide (DCC) from Fluka was used for coupling PAA to benzophenone.

#### 3.2.1 Synthesis of 4-(2-hydroxyethoxy) benzophenone (1)

3.96 g of 4-hydroxybenzophenone (16 mmol) was dissolved in 24 mL of acetone, to which 5.6 g of $K_2CO_3$ (40 mmol) was added. The mixture was stirred under reflux for 30 min. 5.46 g of 2-bromoethanol (44 mmol) was added drop by drop to the reaction mixture and the reaction was left to stir for 10 h. After the reaction mixture was cooled to room temperature, 16 mL of water was added and the product was extracted with diethylether. Diethylether was evaporated under reduced pressure, and the product was recrystallized from ethanol giving white crystals. The reaction is shown in Figure 3.1. Yield = 40%. $^1$H NMR (CDCl$_3$, 300 MHz, δ in ppm) (Figure 3.2): 7.82-7.84(d,2H), 7.75-7.77(d,3H), 7.46-7.60(m,2H), 6.98-7(d,2H), 4.16-4.19(t,2H), 3.99-4.04(m,2H), 2-2.04(t,1H).

With the help of the Prof. Alabugin research group, Dr. W. Yang helped to improve the yield of the reaction using 18-crown-6-ether as a catalyst and followed the following procedude: 13 mL of 2-Bromoethanol (100.9 mmol) was added to the mixture of 4-hydroxybenzophenone (5 g, 25.2 mmol), $K_2CO_3$ (7.7 g, 55.49 mmol) and 18-crown-6-ether (0.4 g, 1.3 mmol) in 50 mL of acetone. The reaction mixture was refluxed for 14 hours. The solvent was removed under
vacuum and the residue was dissolved in CH₂Cl₂. The organic solution was washed with H₂O and dried over Na₂SO₄. After the evaporation of the solvent, the desired product (4.1 g, 67 %) was recrystallized with benzene.

\[ \text{HOBr} \quad \text{Acetone} \rightarrow \text{K₂CO₃} \quad \text{OH} \quad \text{O} \quad \text{OH} \]

**Figure 3.1** Sn₂ Reaction of 2-bromoethanol with 4-hydroxybenzophenone to form a spacer linker.

\[ \text{ppm} \]

**Figure 3.2** \(^1\)H NMR spectrum for 4-(2-hydroxyethoxy)benzophenone. \(^1\)H NMR (CDCl₃, 300 MHz, \(\delta\) in ppm): 7.82-7.84(d, 2H), 7.75-7.77(d, 3H), 7.46-7.60(m, 2H), 6.98-7(d, 2H), 4.16-4.19(t, 2H), 3.99-4.04(m, 2H), 2.2.04(t, 1H).

**3.2.2 Synthesis of poly(acrylic acid) “PAA” grafted with 4-(2-hydroxyethoxy)benzophenone (Bp) “PAABp”**

3.42 g (47 mmol) of PAA (protonated/acid form) was dissolved in 90 mL of anhydrous dimethylformamide (DMF), then 2.87 g (11 mmol) of 4-(2-hydroxyethoxy)benzophenone was
added to the solution and the mixture stirred for 10 min at 65 °C. 1.95 g of dicyclohexylcarbodiimide (9.48 mmol) was added and the reaction mixture was heated for 12 h at 65 °C under stirring. The reaction was then cooled and the white crystals of dicyclohexyl urea were removed under centrifuge. The mixture was concentrated under reduced pressure and 5 mL of DMF was added to the residue which leads to the formation of more urea crystals which were also centrifuged out. The product was precipitated in ethyl acetate to give white crystals of PAA grafted with benzophenone “PAABp”. The grafting percent, determined from a UV-Vis calibration curve, was found to be 5 and 18 mol% from two different syntheses. The reaction is shown in figure 3.4. ¹H NMR (DMSO, 300 MHz, δ in ppm) (Figure 3.3): 7.694 (br m), 7.531 (br m), 7.091 (br m), 4.275 (br m), 2.204 (br s), 1.731 (br s), 1.469 (br, s).

Figure 3.3 ¹H NMR spectrum for PAABp ¹H NMR (DMSO, 300 MHz, δ in ppm) 7.694 (br, m), 7.531 (br, m), 7.091 (br, m), 4.275 (br, m), 2.204 (br, s), 1.731 (br, s), 1.469 (br, s).
3.2.3 Polyelectrolyte multilayer (PEMU) build-up

PEH/PAABp multilayers were built with the aid of a robot (StratoSequence V, nanoStrata Inc.) on a 1 inch diameter single side polished silicon wafer for AFM measurements and on quartz slides for UV-Vis measurements. The substrates were cleaned in piranha (70% H₂SO₄ and 30% H₂O₂) for 20 minutes (caution: piranha is a strong oxidizer and should not be stored in closed containers) and then rinsed vigorously with distilled water (18 MΩ) and dried with N₂. Each of the polymer solutions were 10 mM (with respect to the repeat unit) in 10 mM tris buffer at pH 7.4. The ionic strength of the polymer solutions was set to 150 mM using sodium chloride salt. The coating of the substrate was maintained by initial dipping in PEI solution for 30 minutes, followed by rinsing with distilled water, the substrate was then mounted on a shaft that rotates at 300 rpm to allow better diffusion of the polymers, thus yielding smoother films. The dipping time in each of the polymer solutions was set to 5 minutes followed by three 1 min rinsing steps in distilled water. The dipping time in each of the polymer solutions was 5 min followed by three 1 min rinsing steps in water. PEI(A/B)ₙ and PEI(A/B)ₙA indicate a multilayer ending in B and A respectively, where A represents the polyanion and B the polycation.

The dry thickness of the PEMU film was obtained by using a Gaertner Scientific L116S autogain variable angle Stokes ellipsometer unless stated otherwise. Ten thickness measurements on each PEMU were averaged.
Static contact angle was used to measure the effect of photocrosslinking on the wettability of PAH/PAABp multilayer at different irradiation times. The static contact angle was determined using a KSV Cam 200. The volume of water droplet was 10 µl for all measurements and each measurement point was an average of 3 points taken at different locations on the PEMU.

### 3.2.4 PAH/PAABp photocrosslinking

The PEMU was irradiated a dry state in a CL-1000 Model 254 nm UV box (Ultra-Violet Products Ltd., wavelength range 200 - 280 nm) equipped with a calibration photodiode. The UV crosslinker consisted of 5 UV lamps (8 W each) having dimensions of 12.70 x 30.5 x 25.4 cm. Samples were placed at the center of the box. The UV intensity at the sample was 4.9 mW cm$^{-2}$.

The FTIR data was obtained using a nitrogen purged FTIR (Nicolet Nexus 470 with a DTGS detector) spectrometer. The resolution was 4 cm$^{-1}$ and 256 scans were taken on the sample. The samples were held at 15° off perpendicular to avoid interference fringes.

The efficiency of crosslinking was studied using a Cary 100 Bio UV-Visible spectrophotometer. UV-Vis measurements were performed on a multilayer built on a quartz slide to monitor the absorption of benzophenone unit following different irradiation times.

### 3.2.5 Force spectroscopy

Force indentation measurements, or force curves, were obtained using an AFM$^{118}$ as described by Moussallem et al.$^{15}$ An MFP-3D AFM unit was equipped with an ARC2 controller (Asylum Research Inc., Santa Barbara, CA) and Igor Pro software. AC240-TS tips that have a spring constant of ~2 N m$^{-1}$ were used for indentation. After calibrating the optical lever sensitivity (OLP) of the tip, the spring constant was calibrated in air using the thermal fluctuation technique. The tip was immersed in the buffer solution and the OLP recalibrated. Force maps of 4 x 5 were performed on the bare silicon wafer where the tip was recalibrated and then on the PEMU with a scan size of 20 x 20 µm. All force maps were performed in situ with the sample immersed in Tris buffer at pH= 7.4 and in 0.15 M NaCl. The distance from the surface and the velocity of the tip was set to 500 nm and 1µm s$^{-1}$ respectively.

The force applied, which deflects the cantilever, and the resulting indentation is given by Equation (2.2) and (2.3).
In this Chapter, force curve analysis was based on Sneddon’s model, that describes a conical tip \(1^{20}\) where force vs. indentation is represented by Equation 2.4 and a punch model represented in Equation (2.6)\(^{15,121-122}\).

Force vs. indentation for uncrosslinked films were quadratic thus a cone fit was used to obtain the Young’s modulus. For crosslinked films a linear dependence of force on indentation was obtained and thus a punch model was used. The apparent modulus, \(E_a\), can be related to Young’s modulus of the indented material and the indenter using Equation 3.5.\(^{143}\) Force vs. indentation graphs were fitted up to 20% indentation in order to minimize effects of the substrate on measured sample stiffness. The half angle and the radius of the tip were 18° and 10 nm respectively (provided by the manufacturer).

\[
E_a = \left( \frac{1 - \nu_1^2}{E_1} + \frac{1 - \nu_2^2}{E_2} \right)^{-1} \tag{3.1}
\]

\(E_a\) is the apparent modulus, \(E_1\) and \(\nu_1\) are the Young’s modulus and Poisson ratio of the indented material respectively, \(E_2\) and \(\nu_2\) are the Young’s modulus and Poisson ratio of the silicon indenter. The value for \(E_2\) was set to 150 GPa and the of \(\nu_2\) was set to 0.27. The value for \(\nu_1\) was set to 0.5 as the PEMUs were considered as to be isotropic elastic materials in the range of loads applied.\(^{15}\)

Images of the surface topology were obtained using the same equipment in scanning mode. AC mode was used to obtain the morphology of the surface. The scan size was set to 20 x 20 \(\mu\)m and the scan rate was 0.5Hz. Thickness measurements were determined by scratching the film with a tweezers, and then scanning a 90 x 90 \(\mu\)m area over the scratch. The thickness was measured by drawing a line using Igor software, across the scratch and the height of the film was obtained, the height was obtained at different positions of the image and the average height was reported. The rms roughness of the image was obtained by using a 1 x 1 \(\mu\)m square patch and determined by the software, ten values were recorded at different positions of the image and the average value was reported.
3.2.6 Permeability of iodide ion at different irradiation times

A 100 mL electrochemical cell was equipped with a water jacket thermostat was filled with approximately 50 mL of water with 0.15 M NaCl and 1 mM KI solution. The potential were quoted against a platinum counter electrode, and KCl saturated calomel electrode. The Working electrode was a 5 mm diameter rotating platinum disk (RDE, from pine instruments) mounted on a pine AFMSRCE rotator and speed controller. Potential ramps were generated with the aid of pine AFCBP1 potentiostat and the resulting voltammograms were recorded using aftermath software. Before coating the electrode with PAH/PAABp multilayer, the electrode was polished with 0.05 µm alumina, sonicated and then rinsed with water. The electrode was then coated with 31 layers of alternating PAH and PAABp similar to the procedure described in the above.

3.3 Results and discussion

3.3.1 Effect of substrate stiffness on force spectroscopy measurements: Reliable thickness for nano-indentation

The modulus of polymer thin films when measured by nano-indentation is affected by the stiffness of the substrate, and thus depends on how the measurement is conducted and on the thickness of the film.\textsuperscript{144-146} To avoid reporting false modulus values for PEMUs when measured by AFM, we wanted to determine the thickness and consequently the number of layers needed to do force curve measurements on nanometer thin PAH/PAA film without being affected by the stiffness of the substrate. Two PAH/PAA multilayers were prepared, one composed of 31 layers and has a wet thickness of 262 nm and one formed of 23 layers with a wet thickness of 146 nm and both films had PAH as the terminating layer. Force curve measurements were performed on the films while immersed in tris buffer at pH= 7.4 and 0.15 M NaCl and the resulting force vs. indentation profiles were fitted to different indentation profiles from 0 to 10 nm, then 10 to 20 nm, then 20 to 30 nm, and up to the maximum indentation achieved as shown in Figure 3.5. A comparison of the modulus values obtain at each indentation distance for both films showed that for the thick film the modulus values remained constant irrespective of indentations, however for the thinner film the modulus values was constant up to about 1/3 of the thickness (49 nm), for indentation values higher than 49 nm the modulus values started to increase since the measurements started to be affected by the stiffness of the substrate. This observation showed that for a reliable force spectroscopy measurement and when using a tip diameter of around 20
nm, the minimum thickness needed is around 100 nm. This lower limit for thickness could be lowered if using sharper tips.

![Figure 3.5](image-url) Dependence on apparent modulus as a function of indentation distance for a multilayer of (□) 31 layers; and (◊) 23 layers of PAH/PAA. At higher indentations the tip “feels” the substrate and the apparent modulus increase significantly. All force curve measurements were performed at room temperature while the multilayer is immersed in Tris buffer (pH= 7.4, 0.15 M NaCl).

### 3.3.2 PAH/PAABp multilayer and photocrosslinking

Enhancing the stability and mechanical properties of polyelectrolyte multilayer against varying pH\textsuperscript{102} or ionic strength\textsuperscript{45} was mainly employed by converting the weak ion pairing that keep the film held together into covalent bonds. This could be achieved by chemical\textsuperscript{65} or thermally\textsuperscript{108} crosslinking functional groups such as amine and carboxylic acids to form amide bonds or by photocrosslinking which can be achieved by incorporating a photosensitive molecule into the bulk of the multilayer.\textsuperscript{66,112,137-138,147}

The use of Photocrosslinking goes back to the pre-biblical times. People used to dip linen cloth into a solution of lavender oil that contains Syrian asphalt containing the light sensitive unsaturated aromatic and heterocyclic compounds used to cause the cloth to harden when exposed to light. In the nineteenth century, people discovered that gelatin can be immobilized when exposed to light in the presence of potassium dichromate.\textsuperscript{148}
Photocrosslinking is a rapid and energy efficient technique. For a system to be a candidate for photocrosslinking it must have a chromophore that absorbs light from the source. In order for absorption to occur, the wavelength of light emitted from the source must match the energy for electronic transition from the ground state to the excited state according to Equation 3.6:

\[ h\gamma = E_a - E_g \quad (3.2) \]

Where \( h \) is Plank’s constant, \( \gamma \) is the frequency of the incident light, \( E_a \) and \( E_g \) are the energies of the ground and excited state respectively.

Benzophenone, serving as light absorbing molecule within the bulk of the polyelectrolyte multilayer was grafted on PAA using the procedure described by Park et al. Irradiating the film with UV causes the benzophenone molecule to absorb UV light and the excited benzophenone by \( n,\pi^* \) transition has a biradical character (ketyl radical) which is able to abstract hydrogen from the surrounding and leads to crosslinking by the formation of covalent bond or it can go back to ground state by phosphorescence. The shorter arm of the benzophenone side chain linker then what was prepared by Park et al. is anticipated to yield a stiffer material.

It was shown that the reactivity of benzophenone as hydrogen atom abstractor is comparable to alkoxy radical. The quantum yield of the photoreduction of benzophenone was found to decrease with decreasing the concentration of the hydrogen donor; hydrocarbons and alcohol were found to react with ketyl radical predominantly via a hydrogen abstraction mechanism, also the quantum yield decreases in the presence of oxygen which can react with the biradical and regenerate benzophenone. The photochemical reaction involving benzophenone molecule starts by absorbing light at the wavelength of its \( n-\pi^* \) transition and this usually takes around \( 10^{-12} \) sec. This step is then followed by intersystem crossing to the triplet state, a step that takes around \( 10^{-9} \) sec, and then hydrogen abstraction takes place in around \( 10^{-5} \) sec. Therefore, the reaction of hydrogen abstraction is the slowest step and thus is considered the rate determining step in the photoreduction of benzophenone. This reaction is also estimated to be exothermic by about 102 kcal mol\(^{-1}\). In addition, the rate of intersystem crosslinking from the singlet state to the triplet state is much faster then the rate of deactivation of excited singlet...
state so the excited triplet state of benzophenone is always responsible for the hydrogen abstraction.\textsuperscript{152} The photocrosslinking procedure is described in Figure 3.6.

Benzophenone offers several advantages over other moieties used as photophores, first they are chemically more stable then diazirine and aryl azides. Second they can be operated in ambient light. Third they have the ability to react with unreactive C-H bond.\textsuperscript{154} Several factors affect the reactivity of benzophenone. Benzophenone can react with geometrically accessible C-H. This issue can be solved and thus the efficiency can be enhanced by using flexible linkers on the benzophenone molecule.\textsuperscript{154} In addition to geometrical factors the efficiency of covalent attachment by benzophenone depends on the electronic and chemical environment of the latter and the H-donor molecule.\textsuperscript{153} The strength of the bond being broken and the stability of the resulting alkyl radical are major factors that affect efficiency with C-H bond being favored over O-H and N-H bonds. In terms of stability the reactivity of C-H bond goes in the following order NCH\textsubscript{x} > SCH\textsubscript{x} > C=CCH\textsubscript{2} > CH\textsubscript{2} > CH\textsubscript{3}.\textsuperscript{154} The type of substituent on benzophenone can also affect its reactivity significantly. Large substituent can decrease the reactivity due to steric hindrance and decreased accessibility to the C-H bond especially of they are in the ortho and meta positions.\textsuperscript{154} Electron withdrawing groups can enhance the efficiency of hydrogen abstraction. On the other hand electron donating groups decrease the efficiency of hydrogen abstraction by shifting the electron transition from n-\pi* to \pi-\pi* , the latter triplet state is less reactive towards hydrogen abstraction.\textsuperscript{154}
The grafting degree of PAA with benzophenone was determined from a calibration curve obtained by dissolving variable concentrations of benzophenone in dimethyl formamide (DMF) and then doing UV-Vis measurements to acquire the absorbance of benzophenone as a function of concentration. The concentration of the solutions ranged from 1.146µM to 110.2µM. (Figure 3.7). The grafting degree was determined to be 5 and 18 mol% from two different synthesis reactions that we performed. The PAA grafted with benzophenone named PAABp was used as the polyanion to prepare a multilayer with PAH as the polycation.

Figure 3.6 Mechanism of benzophenone photo-crosslinking. The first step is biradical formation after UV irradiation, this step is followed by hydrogen abstraction from the surrounding, the last step is coupling (crosslinking)
To determine the percentage of benzophenone on PAA the following calculation steps was used:

0.014 g of the synthesized PAABp was dissolved in 10 mL dimethylformamide (DMF) and then diluted 10 times in DMF. UV-Vis spectrum of the diluted solution was taken and the absorbance of benzophenone was determined to be 1.51.

The linear fit line equation from the calibration curves is \( y = 0.0189x - 0.0127 \), where \( y \) is equal to 1.51 and thus \( x \) which is the concentration of benzophenone (species capable of absorbing) can be calculated to be 80 µM, hence the concentration before dilution can be deduced to be 800 µM.

In 10 mL DMF the number of moles of benzophenone is 0.000008 moles, thus the mass is 0.0019g (Mw. of benzophenone is 242.27 g mol\(^{-1}\)).

The percentage by mass of benzophenone on PAA is

\[
\% \text{ by mass} = \frac{\text{mass of benzophenone}}{\text{mass of PAA}} = \frac{0.0019g}{0.014g} \times 100 = 13\% \quad (3.3)
\]

At this point the molecular weight of PAABp can be calculated as follows
\[ M_{wPAABp} = \frac{13 \times 296 + 72.06 \times 87}{100} = 101 \text{g mol}^{-1} \] (3.4)

Where 296 and 72.06 are the molecular weight of the acrylic acid grafted with benzophenone and acrylic acid monomer units respectively. The number of moles of PAA is:

\[ \frac{0.014 g}{101 \text{g mol}^{-1}} = 0.00014 \text{moles} \] (3.5)

Therefore the percentage by moles on PAA is

\[ \%_{bymoles} = \frac{0.000008 \text{moles}}{0.00014 \text{moles}} \times 100 = 5\% \] (3.6)

Layer by layer build up of PAH/PAABp (5%) (PAA grafted with 5% benzophenone) was investigated by building the film on a silicon wafer. The polyelectrolytes were prepared at 10 mM concentration in tris buffer at pH = 7.4 and 0.15 M NaCl. The multilayer was prepared with the aid of a robot, the dipping time in each of the polymer solution was set to be 5 min followed with 3 rinsing step after each polymer solution with water each for 1 min. Thickness was measured by ellipsometry after each layer. The thickness vs. layer number was shown in Figure 3.8. The film showed a smooth increase in thickness similar to what was usually obtained using commercially available polyelectrolytes.\textsuperscript{33,155}
Figure 3.8 Layer by layer build up of PAH/PAABp. The polyelectrolytes were prepared in Tris buffer, pH= 7.4 with 0.15 M NaCl

PAH/PAA and PAH/PAABp (18%) (PAA grafted with 18% benzophenone) films under the same buildup conditions were built on a double sided silicon wafer for FTIR experiments. Comparison of the FTIR spectra of PAH/PAA multilayer (blue), and unirradiated PAH/PAABp (18%) (red) was shown in Figure 3.9. FTIR for PAH/PAABp (18%) revealed the presence of the ester bond at 1729 cm$^{-1}$, benzophenone carbonyl peak at 1600 cm$^{-1}$ and other peaks as a result of the attached benzophenone. All these peaks are absent in the spectrum for PAH/PAA.
To determine the crosslinking density or the percentage of crosslinked benzophenone by UV irradiation, a multilayer was prepared on UV fused silica substrate and then irradiated with UV at different irradiation times. The absorption of benzophenone decreased with increasing irradiation as a result of the loss of the carbonyl as shown in Figure 3.7 and the crosslinking density defined as the percentage of crosslinked benzophenone was obtained from equation 3.7.

\[
\% Bp_{\text{crosslinked}} = \left( \frac{A_0 - A_t}{A_0} \right) \times 100 \quad (3.7)
\]

Where \( \% Bp_{\text{crosslinked}} \) is the percentage of crosslinked Bp within the multilayer, \( A_0 \) is the initial absorption of Bp before irradiation, and \( A_t \) is the absorption of Bp after irradiation for a given time \( t \).

The first 30 seconds of irradiation showed a big jump in the percentage of crosslinked benzophenone since benzophenone molecules were more accessible and the polyelectrolyte chains were more mobile. As the irradiation time was increased the crosslinking rate started to decrease as a result of the multilayer becoming more compact thus made it more difficult to crosslink the remaining uncrosslinked benzophenone molecules. So after 100 mins of irradiation only 80% of the benzophenone molecules within the film were crosslinked.
Figure 3.10 shows the efficiency of crosslinking of PEI(PAH/PAABp)₁₅ built on quartz slide at different irradiation times. (A) UV-Visible spectra of Bp, the arrow indicates the decrease in Bp peak before and after 0.5, 1, 1.5, 2, 3, 5, 7.5, 10, 15, 20, 25, 35, 45, 70, and 100 minutes of irradiation. (B) The increase in percentage of crosslinked benzphenone at the different irradiation times obtained from Equation 7.

The crosslinking mechanism proposed in Figure 3.6 was proved by IR spectroscopy. In this experiment PAA grated with 18% benzophenone was used to prepare the PAH/PAABp multilayer, and was built on a double sided polished silicon wafer which is a requirement to do
IR spectroscopy on PEMU films. The PAA with the 18% benzophenone was used because the stretching mode of the PAA with 5% benzophenone was too weak to be seen by IR and as a consequence very high number of layers was required to distinguish the peaks for the benzophenone molecule. Figure 3.11 showed the FTIR spectra of PAH/PAABp (18%) film before and after irradiation with UV. Only the benzophenone carbonyl band at 1600 cm\(^{-1}\) and the benzophenone peaks between 1256-1318 cm\(^{-1}\) decreased with irradiation however the ester bond at 1729 cm\(^{-1}\) remained intact. This showed that UV irradiation affected only the benzophenone molecules within the film and had no effect on the rest of the polymer chain.

![FTIR spectra of PEI(PAABp/PAH) before (red) and after (blue) 100 min irradiation, and of 4-(2-hydroxyethoxy)benzophenone (grey). The peak at 1729 cm\(^{-1}\), indicated by an arrow, is from the ester bond between PAA and 4-(2-hydroxyethoxy)benzophenone. The peak corresponding to the diarylketone at 1600 cm\(^{-1}\), indicated by an asterisk, decreased significantly after irradiation. In this PEMU the PAABp was 18% substituted by Bp.]

3.3.3 Effect of photocrosslinking on the mechanical properties of PAH/PAABp multilayer

The mechanical properties of PEI(PAH/PAABp)\(_{15}\) (317 nm wet thickness) was studied by force spectroscopy using nano-indentation. The apparent modulus of the film varied over a wide range from about 16 MPa before irradiation up to about 350 MPa after irradiating the film for 100 min (see Figure 3.12-A). For the uncrosslinked (soft films) cone fit was utilized to obtain the apparent modulus because a quadratic relation was obtained between the force and indentation,
for the irradiated (stiff) films the force varied linearly with indentation so a punch fit was used. Figure 3.13 showed sample force vs. indentation graphs obtained experimentally for uncrosslinked film and fitted to cone model (A) and a photocrosslinked film fitted to punch model (B).

The biggest jump in apparent modulus was obtained the first 10 sec of irradiation where the benzophenone molecules were more available. As irradiation time increased the crosslinking density also increased making the film more compact and thus it was different to crosslink the other benzophenone molecules, thus the rate of modulus change decreased. The crosslinking density increased to 80% after irradiation for 100 min. Measuring the modulus of materials, $E_a$, by nanoindentation is model dependent thus it is more precise rather than accurate and best used to compare relative values, so comparing our values to prior work done with the same apparatus was considered a valid approach. The initial (uncrosslinked) modulus of PAH/PAABp was 16 MPa lower than the initial modulus of PAH/PAA obtained by Moussallem et al. which was 6 MPa.\textsuperscript{15} This might be due to the presence of the hydrophobic benzophenone molecule within the film which can decrease the hydration of the films and this can make it stiffer. Using thermal crosslinking in our lab to crosslink PAH/PAA multilayer lead to modulus values of around 8GPa which is much higher than what was obtained here by photocrosslinking, this was due to the fact that all the amine and carboxylic acid monomer units are involved in crosslinking leading to high crosslinking degree which can be close to 100%. The system used in this experiment had 5% Benzophenone only on one of the polyelectrolytes adding that there is a possibility that not all radical generated by UV were able to crosslink to other chains. Also for comparison, the chemical crosslinking of PLL and HA increased the modulus from 12 KPa to 450 KPa,\textsuperscript{132} similarly in this case all amines and carboxylic acids are involved in crosslinking and the formation of amide bonds. Comparing our system to other polyelectrolyte multilayer system that also employed photocrosslinkable moieties, Vazquez et al., grafted hyaluronan (HA) polyanion with vinyl benzene (VB), HA with different densities of VB was prepared and the modulus of the film increased from 35 KPa at 14% grafting percentage to 140 KPa at 37% grafting degree. Beyond 37% HA-VB was insoluble.\textsuperscript{66} Going back to our system, it showed a 22-fold increased in modulus with PAA grafted with 5% benzophenone, however when using the PAA with 18% benzophenone modulus values of around 5 GPa could be obtained see Figure
3.12-B which was comparable to what was obtained by thermal crosslinking. These results showed that a wide range of modulus could be obtained by photocrosslinking.

**Figure 3.12** A and B Variation of apparent modulus of PEI(PAH/PAABp)$_{15}$ with PAABp (5%) and (18%) respectively as a function of crosslink density. Measurements were obtained from force curves using atomic force microscope. The measurements were conducted under wet conditions where the multilayer was immersed in tris buffer at pH= 7.4 with 0.15 M NaCl

**Figure 3.13** (A) Fitting example of force vs. indentation for a (PAH/PAA)$_{17}$PAH multilayer, The measurements were conducted in 10 mM Tris-HCl with 0.15 M NaCl at pH =7.4. Quadratic behavior describes the plot and thus a cone fit was used. The half angle of the cone was set to 18°. (B) Fitting example for a force vs. indentation plot for a PEI(PAABp/PAH)$_{15}$ multilayer after 100 min irradiation. Measurements were performed in 10 mM Tris-HCl with 0.15 M NaCl at pH =7.4. Linear behavior was obtained between the force and indentation and a punch fit was used, the radius of the punch was set to 10 nm.
3.3.4 Effect of photocrosslinking on the morphology and swelling of PAH/PAABp

Ideally to explore the effect of substrate mechanical properties on the behavior of cells its important to keep other parameters that affect the cell’s behavior such as surface chemistry, wettability and charge density unaffected. So, we attempted to check for the effect of photocrosslinking on the surface topography of PAH/PAABp (5%) films that will be used later in cell culture studies (see chapter 4). The morphology of PAH/PAABp multilayer before and after crosslinking was studied using AFM. PEI(PAH/PAABp) was imaged in AC mode before irradiation and then after irradiating the film in UV-light for 100 min (see Figure 3.14). Roughness values, defined as the average deviation, before and after crosslinking was obtained by using a 5 x 5 μm square patch and estimated by Igor software. There was no effect of photocrosslinking on surface roughness which appeared to be 7nm before and after irradiation. On the contrary Schneider et al. showed that chemically crosslinking PLL and HA to form amide bond with the aid of EDC as a coupling agent had a big effect on the surface roughness of the film.

To verify the effect of crosslinking on the swelling of the film, dry and wet thickness (in tris buffer @ pH=7.4 and 0.15 M NaCL) for PEI(PAH/PAABp) was also determined by AFM. Thickness measurements were determined by gently scratching the film with a tweezers, and then scanning a 90 x 90 μm area over the scratch. Swelling percentage was evaluated as the difference in wet and dry thickness divided by the dry thickness. The swelling percentage was not affected by crosslinking and it appeared to be 29% for uncrosslinked and crosslinkined multilayer corresponding to hydrophobicity index of 1.29. The fact that the swelling was not affected by photocrosslinking showed that the ion content of the film remained unchanged after photocrosslinking, and thus mechanical properties were the only variable, unlike other crosslinking strategies (ionic, thermal and chemical) that significantly change the ion content.
Another parameter that affects the cell’s behavior is the surface wettability. Surface hydrophobicity can be obtained by measuring the contact angle of water at the surface by the standard sessile drop technique. We saw in literature that chemical crosslinking significantly changed the surface wettability of PLL/HA multilayer\textsuperscript{132} we wanted to check for the change in contact angle with photocrosslinking.

The static contact angle was obtained at different irradiation times for PAH and PAABp terminated films. Only little changes in contact angle was observed upon crosslinking, however overall the film appeared to maintain its surface wettability over a wide range of crosslinking as shown in Figure 3.15. Contact angles for PAH terminated PEMUs were about 35°, similar to those obtained by Yoo et al. for PAH/PAA films\textsuperscript{155} whereas the PAABp terminated film had contact angles of about 50°, much higher than observed for PAH/PAA, probably due to the presence of the hydrophobic Bp molecule.

\textbf{Figure 3.14} A and B are AFM images of PEI(PAH/PAABp)$_{15}$ before and after 100 mins crosslinking respectively. Both images had a roughness value of 7 nm.

\textbf{3.3.5 Variation of surface wettability with photocrosslinking}

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3.3.6 Permeability of KI as a function of crosslinking

The permeability of PAH/PAABp to iodide ion was studied as a function of crosslinking density (irradiation time). Harris et al. studied the effect of thermal crosslinking on the permeability of PAH/PAA films to various ions. In this experiment the permeability was studied as the rate of electrochemical oxidation for PAH/PAABp film that was built on a rotating disc electrode (RDE). All other parameters that affect the permeability were kept constant: the salt concentration of the film was set to 0.15 M NaCl, the potassium iodide concentration was 1mM, and the speed of rotation of the electrode was 1000 rpm. The final layer was kept positive (PAH) to take out the effect of negative-negative ion charge repulsion a description of the set-up is shown in Figure 3.16 and 3.17. Before coating the electrode with PAH/PAABp multilayer, the electrode was polished with 0.05 µm alumina, sonicated, and then rinsed with water. The electrode was then coated with PEI(PAABp/PAH)$_{15}$ as described above.

The ion flux is estimated directly by the electrochemical current. For bare electrode the limiting current is represented by the Levich equation 3.8.

$$i_{lev} = 0.620nFAD_{aq}^{2/3} \omega^{1/2} \nu^{-1/6} C_{aq}$$  \hspace{1cm} (3.7)
where \( n \) is the number of electrons transferred, \( F \) is Faraday’s constant (96 490 C mol\(^{-1}\)). \( D_{aq} \) (cm\(^2\) s\(^{-1}\)) is the solution diffusion coefficient of the electroactive species, \( \nu \) is the kinematic viscosity, \( C_{aq} \) is the solution concentration, \( \omega \) is the angular velocity (rad s\(^{-1}\)), and \( A \) is the electrode area.

The theory of current-voltage responses for membranes on RDEs was developed by Gough and Leypoldt,\(^{156}\) and applied by Ikeda et al.\(^{157}\) to study the permeation of electroactive solutes through ultrathin films on RDEs.\(^{157}\) After coating the RDE with PAH/PAABp the limiting current, \( i_l \), decreased as a result of increased resistance to mass transfer as indicated in Equation 3.9.\(^{157}\)

\[
\frac{1}{i_l} = \frac{1}{i_m} + \frac{1}{i_{iev}} \tag{3.8}
\]

where \( i_m \) is the current limited by permeation through the membrane (membrane current). As seen in Figure 3.18, the current decreased as the irradiation time increased. The decrease in current upon crosslinking can be explained by the physical barrier created after photocrosslinking which makes the film more compact and less permeable to iodide ions. The control of permeability is also indicated by the linear decrease of membrane current as a function of \( \% \) of also shown in Figure 3.18. A decrease in ion permeability for a photocrosslinked multilayer was also seen by Kang et al. using cyclic voltammograms.\(^{140}\) Others controlled the permeability of polyelectrolyte multilayer by changing the salt concentration where at higher salt concentration the multilayer is swelled and is less resistant to ion transport.\(^{38}\) Here we provide a system with controllable permeability by grafting the PAA with 5 \% benzophenone. Here we provide a system with controllable permeability without changing its charge content, charge content is a major factor that controls permeability.
Figure 3.16 PAH/PAABp film that was built on rotating disc electrode. This set-up is used to measure the ion-flux through polyelectrolyte multilayers.

Figure 3.17 An illustration of ion-flux variation after photocrosslinking. A is am non-crosslinked PAH/PAABp film and B is a film that was photocrosslinked. More iodide ions are expected to penetrate the non-crosslinked and result in higher current then the photocrosslinked one.
3.4 Conclusions

Thermal and chemical crosslinking had been widely used to tune the stiffness of PEMU. As these two techniques are extremely efficient in increasing the stiffness of the films however those two methods lack control over sites of crosslinking and they were found to affect the
chemistry, charge density, and hydrophobicity of the films. So when using thermal and chemical crosslinking to study the effect of substrate mechanical properties on the behavior of cells, there will be a strong confusion if cells are responding to the change in stiffness of the substrate or responding to the change in other properties of the films. Photocrosslinking is introduced as an alternative. Here a benzophenone containing group was synthesized and coupled with PAA that was used in preparing photocrosslinkable PAABp/PAH multilayers. Photocrosslinking made it possible to systematically tune the Young’s modulus and permeability of the multilayer while preserving its hydrophillicity and surface topography. This system is a good candidate to be used in a wide variety of applications mainly as a replacement for tissue culture dishes and biocoatings for implants as will be seen in chapter 4.
CHAPTER FOUR
DUROTACTIC RESPONSE OF A7R5 AND U2OS CELLS ON
PHOTOCROSSLINKABLE POLYELECTROLYTE
MULTILAYERS

4.1 Introduction

Motility and adhesion are two important features regarding the physiological function of cells. The mechanical properties of cell’s microenvironment affect the behavior of cells such as their motility, adhesion, protein and gene expression, and differentiation of stem cells (mesenchymal). Cells are generally known to adhere more on stiff substrates, and they show a directed migration from the soft part of the substrate to the stiff part when plated on surfaces with gradient elasticity, a process known as “durotaxis”. Cells adhere to the substrate through the binding of integrins, transmembrane proteins, to the extracellular matrix (ECM) protein ligands, this binding induces a process known as mechanotransduction that transfers mechanical signals to the cytoskeleton that mediate actin polymerization and actin-myosin contractility. The stimulation of these signaling pathways controls the cell’s behavior on the surface such as their spreading, motility, proliferation and differentiation.

Polylelectrolyte multilayer thin films (PEMUs) have been widely used as friendly coatings for biomedical devices and for cell culture applications, mainly to study the effect of substrate stiffness on the behavior of cells. This is due to their ease of preparation, controllable thickness, biocompatibility, and the ability to coat any material. Also another important advantage is the ability to easily tune their mechanical properties, either by changing the polymer chemistry or salt concentration. Changing the salt concentration is not a valid approach for bioapplications, where the salt concentration has to remain within a narrow value close to the body conditions. So, others ways were put through by introducing a crosslinking moiety into the bulk polyelectrolyte multilayer film. Three major routes were used and were discussed in details in Chapter 3. Briefly, crosslinking can be achieved either by thermal crosslinking, chemical crosslinking, or photocrosslinking. Photocrosslinking offers several advantages over chemical and thermal crosslinking. It does not involve the use of chemical reagents that can be toxic and require
further purification, it can be performed both in vitro and in vivo, can be easily monitored with UV-Vis spectroscopy, and most importantly it provides control over the regions of crosslinking and preserve the chemistry and charge density within the film. On the other hand, thermal and chemical crosslinking are time consuming they lack the control over regions of crosslinking and it was shown that thermal and chemical crosslinking altered the topography, surface wettability, and the chemistry of PEMUs.

Vascular smooth muscle cells and osteosarcoma were used and were tested for their motility and adhesion on PEMUs. These cells have highly-adhesion dependent characteristics and are capable of establishing stress fibers and vinculin containing focal adhesions when plated on surfaces, and thus, were used to understand their interaction with surfaces of variable mechanical properties, which is important for the improvement of biomedical devices such as endovascular stents and for bone fracture repair.

In this chapter PAH/PAABp was used to prepare films that showed gradient in elasticity. Two types of gradients were used; one that showed a continuous change of stiffness along the film and this was prepared using a neutral density filter and one with an edge (steep) gradient. The behavior of A7r5, rat aortic smooth muscle, cells and U2OS, osteosarcoma, cells was tested on the films with uniform elasticity or on the gradients. Culturing cells on surfaces with gradient in stiffness enables us to check if cells can sense variations in mechanical properties of their substrates and if they can respond and migrate to a preferred substrate.

### 4.2 Materials and methods

#### 4.2.1 Reagents

Poly(acrylic acid) (PAA $M_w = 100,000$ g mol$^{-1}$) and poly(allylamine hydrochloride) (PAH $M_w = 56,000$ g mol$^{-1}$) were used as received from Aldrich. NaCl (ACS grade) was used to adjust the ionic strength of the polyelectrolyte solutions. Poly(ethyleneimine) (PEI $M_w = 70,000$ g mol$^{-1}$) obtained from Polysciences. PAA grafted with 5% photosensitive benzophenone was synthesized according to Lehaf et al.
4.2.2 Polyelectrolyte multilayer preparation

PAH/PAABp multilayers were built with the aid of a robot (StratoSequence V, nanoStrata Inc.) on a 1 inch diameter single side polished silicon wafer for AFM measurements and on 22x22 glass coverslips for AFM and cell culture experiments. The substrates were cleaned with air plasma for 1 min or with piranha solution and then rinsed vigorously with distilled water (18 MΩ) and dried with N₂. Each of the polymer solutions were 10 mM (with respect to the repeat unit) in Tris buffer at pH 7.4. The ionic strength of the polymer solutions was adjusted to 150 mM using NaCl which is equivalent to the physiological salt concentration. Surfaces were coated with a layer of PEI first then they were placed on a shaft that rotated at 300 rpm. The dipping time in each of the polymer solutions was 5 min followed by three 1 min rinsing steps in water.

PEI(A/B)_x and PEI(A/B)_y A indicate a multilayer ending in B and A respectively, where A represents the polyanion and B the polycation. After coating the polyelectrolyte multilayer on coverslips and before usage in cell culture, these coverslip were submerged in tris buffer at pH = 7.4 that have 0.15 M NaCl.

4.2.3 Photocrosslinking

The PEI(PAABp/PAH)_15 films were irradiated in dry state in a CL-1000 Model 254 nm UV box (Ultra-Violet Products Ltd., wavelength range 200 - 280 nm) equipped with a calibration photodiode. The UV crosslinker consisted of 5 UV lamps (8 W each) having dimensions of 12.70 x 30.5 x 25.4 cm. Samples were placed at the center of the box. The UV intensity at the sample was 4.9 mW cm⁻².

4.2.4 Force spectroscopy

Force curve were conducted as described by Moussallem et al.¹⁵ and Lehaf et al.¹¹³ to obtain the apparent modulus of PEMUs. An MFP-3D AFM unit was equipped with an ARC2 controller (Asylum Research Inc., Santa Barbara, CA) and Igor Pro software. Multi75AI tips with a spring constant of ~2 N m⁻¹ were used for indentation. All force curves were performed under wet conditions where the sample was immersed in Tris buffer set at pH = 7.4 and 0.15 M NaCl. Force maps were performed on the uniform multilayers to obtain its apparent modulus at different irradiation time. Also, for the multilayer with gradient elasticity, force maps were
performed at different distances along the film. The applied force and the resulting indentation are given by Equations 2.2 and 2.3.

The force vs. indentation profiles obtained were fitted using the punch model according to Equation 4.1.

\[ F_{punch} = 2E_c R(z - d) \]  

Where \( E_c \) is the convoluted modulus of the material and \( R \) is the radius of the indenter. After fitting the force vs. indentation graph, \( E_c \) was obtained and can be plotted in Equation 4.2 to obtain \( E_1 \) which is the apparent modulus of the material.

\[ E_c = \left( \frac{1 - \nu_1^2}{E_1} + \frac{1 - \nu_2^2}{E_2} \right)^{-1} \]  

\( E_2 \) is the modulus of the indenter material which was set to 150 GPa, \( \nu_1 \) and \( \nu_2 \) are the poisson ratios of the multilayer and the silicon indenter respectively. \( \nu_2 \) was set at 0.27, \( \nu_1 \) was assumed to be 0.5 as the PEMUs were considered to be isotropic elastic materials in the range of loads applied.

4.2.5 Cell culture

A7r5 cells (rat aortic smooth muscle cells) and U2OS osteosarcoma cells were purchased from American type culture collection, and then propagated as cell line in the lab for several years. The A7r5 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100 units mL\(^{-1}\) penicillin G, 100 µg mL\(^{-1}\) streptomycin, and 10µg mL\(^{-1}\)1 gentamicin. U2-O5 cells were cultured the same way but using DMEM F-12 as culture medium.

4.2.6 Imaging

Nikon Ti-E inverted microscope and Nikon TS100 tissue culture were used to obtain differential interface contrast (DIC) images of cells and for phase contrast microscopy.
Immunofluorescent imaging of vinculin and actin were conducted according to the following procedure: A7r5 and U2OS at 5 x 10^4 cells per well were seeded on PEMUs that were built on glass coverslips and placed in a six well plate. The cells were cultured for 48 hour then were washed with PBS buffer at 37°C three times for 5 minutes each and then fixed with 3.7% formaldehyde for 15 minutes. Following the treatment with formaldehyde the cells were washed with PBS buffer at room temperature three times for 5 minutes each and then treated with 0.2% Triton x-100 in PBS for 10 minutes, next the cells were treated with 0.05% Triton x-100 in PBS and then incubated in 1% bovine serum albumin to block nonspecific binding sites. After blocking, the cells were stained following the protocol of Moussallem et al.\textsuperscript{15} The primary antibodies IgG (mouse) (1:200 dilution) (vinculin staining) were added to the coverslips and incubated at 37°C for one hour. The coverslips were then washed with 1% goat serum goat serum/0.05% Triton X-100/PBS followed adding the goat anti-mouse secondary antibodies which had the Alex Fluor 488 (green) or 546 (red) (1:2000 dilutions). Also cells were stained with phalloidin Alexa 488 (F-actin staining from Molecular Probes, Inc.). When all the antibodies were applied, the coverslips were washed with 1% goat serum/0.05% Triton X-100/PBS for five minutes, followed by two five minutes washes with 1x-PBSA. After the washes, 10x DAPI was prepared in 1x-PBS at 1: 20000 µl dilutions and were overlaid on the coverslips for five minutes. The coverslips was then washed with 1x-PBSA twice followed by three quick washes with sterile distilled water.

4.3 Results and discussion

4.3.1 A7r5 cells on uniform Substrates

Five uniformly crosslinked PEI(PAABp/PAH)\textsubscript{15} irradiated for 0 (native), 2, 10, 35, and 100 mins, these irradiation times corresponded to 0, 40, 58, 67, and 78 % of crosslinked benzophenone which was measured by UV-Vis spectroscopy on a multilayer prepared on quartz slides. The resulting apparent modulus varied from about 21 MPa for the non-irradiated films up to 100 MPa for the ones irradiated for 100 mins as shown in Figure 4.1
A7r5 cells were plated on the films with increasing percent of crosslinking and thus increasing stiffness. The adhesion of cells to the underlying substrates can be achieved by the binding of integrin to extracellular matrix protein ligands. This binding induces the formation of focal adhesions. Focal adhesions provide attachment points to the substrate and are rich in vinculin and are usually localized at the terminal of actin stress fibers. A7r5 cells responded to the increasing stiffness by increasing their surface area and increasing the number of vinculin adhesion (green color) shown in Figure 4.2. These morphological changes was also seen by Moussallem et al. where A7r5 cells phenotype varied from synthetic “motile” phenotype to sessile “contractile” phenotype when cultured on PAH/PAA films on increasing stiffness induced by thermally crosslinking the amine groups and the carboxyl groups to form amide linkages. Other research groups also studied polyelectrolyte multilayers as prospective coatings for biomaterials. Richert et al. used another polyelectrolyte combination of poly (L-lysine) (PLL) as the polycation and Hyaluronan “a biopolymer” as the polyanion to prepare their PEMUs. The amine group of PLL and the carboxyl group of HA were chemically crosslinked, in the presence of water soluble EDC/NHS as the coupling agent forming the amide bond which resulted in a significant increase in stiffness. They used Chondrosarcoma cells (HCS2/8) to

Figure 4.1 Variation of apparent modulus of PEI(PAH/PAABp)15 multilayer obtained by force curve measurements using atomic force microscope as a function of crosslink density. The measurements were conducted under wet conditions where the multilayer was immersed in tris buffer at pH= 7.4 with 0.15 M NaCl.
study the effect of stiffness on cellular adhesion; it was shown that the adhesion and spreading of HCS2/8 cells were enhanced on the crosslinked multilayer.\textsuperscript{65} Ren et al. studied the effect of substrate elasticity on the differentiation of myoblasts stem cells. They also used PLL/HA films as substrates of controllable stiffness. They showed that stiffness not only affected the proliferation and adhesion of myoblasts, but it also has a significant impact on their differentiation, and that elongated and thin striated myotubes formed on the more rigid substrates.\textsuperscript{167} Recently Pozos Vázquez et al. controlled cell adhesion by photocrosslinking where they used Hyaluronan (HA) biopolymer grafted with vinylbenzyl group (VB) that acted as a photocrosslinkable molecule within the multilayer. The stiffness of the multilayer varied depending on the percentage of VB on HA.\textsuperscript{66}

![Increasing percent crosslinking](image)

**Figure 4.2** Morphological changes of A7r5 cells when plated on PEI(PAABp/PAH)\textsubscript{15} of increasing stiffness. The percentage of crosslinking varied from 0, 20, 40, 60, and 80 percent and on glass coverslip (CS). The cells were plated and then cultured for 24 hours then fixed for imaging. The green stain is the vinculin focal adhesions. The blue stain is for the nucleus. Scale bar 20 μm

**4.3.2 Cellular response on PEMU with gradient elasticity**

PEI(PAH/PAABp)\textsubscript{15} was prepared on glass coverslips for cell culture and on silicon wafer for AFM measurements as described before. A gradient in stiffness on the PEMU was formed by using a rectangular metallic neutral density filter purchased from Thorlabs which was fabricated from UV fused silica substrate with an Inconel metal coating on the surface. The optical density of the filter varied from 0.04 to 2. A high optical density resulted in less light transmitted through the filter and thus less photocrosslinking and this is illustrated in Figure 4.3.
The sample was placed in UV crosslinked box covered with the filter and then irradiated with UV. Irradiation time was set to 200 minutes. Force curve measurements were performed along the film. The AFM had a scanner able to scan and move the substrate in the X and Y directions and thus force curves were obtained all along the multilayer at different positions. The scan size of each measurement was set to 10 \( \mu m \). The apparent modulus versus the distance (mm) along the multilayer parallel to the gradient direction was represented in Figure 4.4. The apparent modulus along the film varied from about 55 MPa (soft edge) to about 144 MPa (stiffest edge).

\[ \text{Figure 4.3 Continuous gradient in stiffness prepared on PEMU with the use of neutral density filter. The optical density along the filter varied from 0.04 to 2. At low optical density more UV will transmitted through the filter which will result in higher modulus values. The film was placed covered with the neutral filter and irradiated with UV in the UV crosslinker box} \]
To test if cells can sense variations in mechanical properties of their substrates and if they can respond and migrate to a preferred substrate, we plated A7r5 cells on the films with gradient elasticity. A7r5 cells showed a higher preference to the stiffer end of the gradient where they were more spread with higher surface, however they kept a non-adhesive rounded morphology on the softer side.

**Figure 4.4** Variation of apparent modulus along the PEI(PAH/PAABp)$_{15}$ multilayer obtained by force curve measurements using atomic force microscope as a function of the distance along the film. The measurements were conducted under wet conditions where the multilayer was immersed in tris buffer at pH= 7.4 with 0.15 M NaCl.
From Figure 4.5 we can clearly see that A7r5 cells showed the highest response to compliance and thus the biggest difference in morphology at the two extreme edges of the continuous gradient (the stiffest and the softest side). So, we decided to prepare PEI(PAH/PAABp)\textsubscript{15} with an edge gradient. In this case half of the multilayer was covered with a black shield and the other half was exposed to UV for 100 min as described in Figure 4.6. This resulted in the steepest change in compliance possible as shown in Figure 4.7. The apparent modulus varied from 38 MPa on the unexposed part of the film to 120 MPa on the side that was exposed to 100 min of UV irradiation.

**Figure 4.5** A7r5 cells plated on the continuous gradient. The modulus along the gradient varied from about 60 MPa up to about 160 MPa. A7r5 cells were more spread and thus adhered better on the stiffest side of the gradient and they kept a non-adhesive rounded morphology on the softer side. The red stain is for actin. Scale bar 100 μm
Figure 4.6 Edge gradient prepared on PEI(PAH/PAABp)$_{15}$. In this case half of the film was covered with black mask and the other half was irradiated with UV.

Figure 4.7 Variation of apparent modulus of PEI(PAH/PAABp)$_{15}$ multilayer with edge gradient. Force curve measurements were conducted in tris buffer at pH = 7.4 and 0.15 M NaCl.
A7r5 cells and U2OS osteosarcoma cells that could interact with bioimplants coated with polyelectrolyte multilayers thin films such as endovascular stents and titanium, titanium is used to repair bone fracture, were then tested on the edge gradient. These cells were seeded at $5 \times 10^4$ cells per well and then their behavior was monitored after 24, 48, and 72 hours on the edge gradient, native uniform (native correspond to uncrosslinked film), and photo-crosslinked uniform films (see Figure 4.9). All coverslips with the edge gradient were marked with a black marker at the position of the black mask during irradiation. During imaging the black marker worked as the identification of the position of the edge gradient. A7r5 cell that were on the stiffer side of the edge gradient had an adhesive morphology (widely spread and higher surface area) and they kept a non-adhesive rounded morphology on the softer side, also on the edge gradient after 48 hours the cells were highly oriented towards the stiffer side, and some cells were able to cross the edge towards the stiffer side where they undergone a transition in morphology from being non-adhesive to highly adhesive (see Figure 4.10). Same behavior was observed for the U2OS cells however these cells demonstrated a bigger non-adhesive morphology on the softer side of the edge where a lot of these cells came out of the surface and went into apoptosis due to their adhesive dependent survival regulatory pathway. To better see the behavior of A7r5 cells of the edge gradient Figure 4.10 showed a zoom-in of the image of A7r5 cells on the edge at different times.
**Figure 4.8** A7r5 cells (A1-A12) and U2OS (U1-U12) plated on edge, uniform native, uniform crosslinked, and glass followed after 24, 48, and 72 hours. Scale bar 100 μm
To further study the effect of stiffness on the behavior of A7r5 cells, these cells were stained for actin stress fibers (red) see Figure 4.12 and the reorganization of these microfilaments network was followed. The cells that were on the native PEI(PAH/PAABp)15 showed a reduction in the amount of stress fibers and they then established a higher concentration of actin stress fibers when plated on the uniform crosslinked films (stiffer). The cells that were on the edge gradient reflected the same trend, the cells on the softer side of the edge had a lower concentration of actin stress fibers then those that were on the stiffest edge. The increasing amount of actin stress fibers on the stiffest substrates reflected higher adhesion.

Figure 4.9 A7r5 cells plated on the edge gradient and monitored after 24, 48, and 72 hours. After 24 hours A7r5 cells on the soft side of the gradient were rounded indication of non-adhesive morphology, after 48 hours the cells on the soft side were clearly oriented and polarized towards the stiffer side and after 72 hours some of the cells on the soft side were able to migrate towards the stiff side and their morphology changed from being rounded and non-adhesive to highly spread and adhesive. Scale bar 100 μm
The behavior of different kind of cells on substrates with gradient elasticity was mainly studied on substrates formed of polyacrylamide gels. The mechanical properties of polycrylamide gel were varied by keeping the acrylamide percentage constant while varying the percentage of bis-acrylamide.\textsuperscript{3,9,134,173-174} Pelham et al. showed that 3T3 fibroblast and NRK (Normal Rat Kidney) epithelial cells were able to detect the flexibility of their surrounding environment. On soft substrates, 3T3 fibroblasts and NRK cells of normal rat kidney migrated faster and exhibited elevated lamellipodium activity however, on rigid substrates these cells adhered and expressed stable focal adhesions.\textsuperscript{2} Lo et al. tested the directionality of 3T3 fibroblasts on substrates with gradient rigidity. These cells migrated from the soft part of the gradient crossing the boundary towards the stiffest side.\textsuperscript{162} Flanagan et al. also described the branching of neuron on substrates of variable stiffness. It was found that neurons adhere and spread on both soft and stiff substrates, however, on soft substrates they showed higher degree of branching correlating well with their biological microenvironment ($E_{\text{brain}}$=0.1-1 KPa).\textsuperscript{12} Wong et al. studied the directionality of vascular smooth muscle cells on a polyacrylamide gel with gradient compliance. They prepared their films via photopolymerization of 8% acrylamide and 0.3% bis-acrylamide. They used mask patterns to control the intensity of UV light that reaches

\textbf{Figure 4.10} Microfilament rearrangement of A7r5 cells in response to increasing stiffness on uniform native (A), uniform crosslinked (B), glass (C), and edge gradient (D). These showed higher concentration of actin stress fibers on stiffer (crosslinked) substrates. Scale bar 20 $\mu$m for A, C, D and 100 $\mu$m for D.
the film; a gradient with a continuous variation of elasticity was obtained, it was shown that vascular smooth muscle cells were able to migrate from the soft part of the gradient to the stiff part.$^{134}$ Recently, Cheung et al. prepared a gel with gradient mechanical compliance from polyethylene glycol diacrylate (PEGDA) supplemented with a constant amount of PEG monoacrylate linked bovine fibrinogen. They prepared a step-wise gradient using microfluidics-based lithography; with a step size as small as 27µm. Directed migration of human foreskin fibroblasts towards the rigid end of the gradient was observed.$^{163}$ Differentiation of stem cells on hydrogels with tissue-like elasticity was also studied. Mesenchymal stem cells (MSCs) were able to differentiate into different cell types.$^{174}$ Engler et al.,$^{3,161}$ Even Ram et al.,$^{166}$ and Zajac et al.,$^{175}$ showed that MSCs differentiated into neuronal cells on the soft part of the gradient, muscle cells on the intermediate stiffness and bone cells on rigid surfaces.

4.4 Conclusion

The use of photocrosslinkable PAH/PAABp polyelectrolyte multilayer provided control over the regions to be crosslinked. The use of neutral density filter allowed preparing a PEMU film with continuous gradient in elasticity. A7r5 cells were able to sense variations in mechanical properties of their underlined substrate and responded by orienting and polarizing towards the stiffer end. Since A7r5 cells showed the biggest difference in morphology at the extreme edges of the gradient (the softer and the stiffer side), a film with edge gradient was prepared by covering half of the film with a black mask and exposing the other half to UV. This resulted in the steepest change in modulus possible. A7r5 and U2OS cells were plated on the edge gradient and both cell types adhered on the stiffer side. U2OS cells were only able to detect stiffness where they adhered on the stiff side of the edge gradient and went into apoptosis on the soft side. On the other hand, A7r5 cells were highly oriented and polarized towards the stiffer end and were able to undergo morphological transition from being rounded and nonadhesive to highly adhesive when durotaxing towards the stiffer side. In the future more analysis and quantification had to done regarding the amount of actin stress fibers and vinculin expressed. Also analysis on the degree of orientation and polarization should be done. Orientation can be measured by calculating the angle between the axis of the cell and the gradient. Polarization can be obtained by measuring the long and short axis of the cell. More analysis involve using windrose plots to
track the migration of cells on the uniform and gradient substrates. We would like also to use mesenchymal stem cells and study them on the gradient and see if mesenchymal stem cells will be able to differentiate into different cells based on the underlying elasticity given that mesenchymal stem cells are known to differentiate into neuron on soft substrate, muscle cells on intermediate stiffness, and bone cells on highly rigid substrates.
CHAPTER FIVE

STRUCTURE AND BUILD-UP MECHANISM OF POLYELECTROLYTE MULTILAYERS STUDIED BY NANO-INDENTATION

5.1 Introduction

Polyelectrolyte multilayer films (PEMUs) are composed of hydrated mixture of positively and negatively charged polymers that are kept together by the ionic interaction interactions of there oppositely charged monomer units. Using the traditional theory of rubber elasticity, it was found that their stiffness in managed by the ion-pair crosslink density which is in turn controlled by the properties of the external environment such as ionic strength, pH or redox potential. We have seen in literature and in chapter 4 that the mechanical properties primarily the elastic modulus of substrates used for cell culture were found to affect the behavior and phenotypic expressions of cells adding that cells were able to sense variations in mechanical properties of their substrates. PEMUs acted in regulating the adsorption of proteins on the surface that are important for cell adhesion. Engler et al. found that in order for the cells to sense the elasticity of their substrate they have to pull against the matrix. As most of the matrices surrounding the cells in vivo and synthetic substrates used for cell culture are viscoelastic and thus have separable elastic or storage modulus (G’) and viscous or loss modulus (G’’). Studies concerning the behavior of cells on substrates were mainly focused on substrate elasticity or rigidity. It is now believed that the viscous component have an important role in tailoring the behavior of cells on their substrates. Previously in our lab we were able to use tensile stress relaxation experiments to study the elasticity and viscoelasticity of sufficiently thick PEMUs. Due to the challenges arising from the need to peel off the film from the surface and the obligation to have high number of layers (>500), nano-indentation is a more reliable technique to study the mechanical properties of nanometer thin films. The viscous component is not well addressed in nano-indentation measurements and is often ignored. In this chapter we will present an easy way to address the viscoelastic properties of PEMUs by nano-indentation.
Swelling of gels under external stimuli results in the formation of periodic structures and
buckles on the surface.\textsuperscript{188-189} Folding is an important process in many biological and natural
processes such as the folding of proteins, wrinkling of the skin and leaves and brain folding.\textsuperscript{190-191} As the explanation for these natural phenomena are still elusive. The use of the synthetic
responsive material might provide an insight on how to interpret such complicated natural
phenomena. The swelling of polymer gels that are constrained at one end and free at the other
end resulted in periodic features or buckles on the surface as described by Tanaka et al.\textsuperscript{188-189}
Tanaka reported the loss of surface stability upon swelling of gels lead to the formation of
intersecting line of cusps. Nano-indentation will be used to describe the features (roughness) that
appear on the surface of PEMUs.

The alternating deposition of a polycation and polyanion result in switching of surface
charge between a positive and negative potential respectively, which was termed as the “odd vs.
even” effect.\textsuperscript{192-193} Also the contact angle that is surface hydrophobicity which reflects the
surface energy of the terminating layer reversibly changed when changing the type of
polyelectrolytes on the surface.\textsuperscript{96,155,194} Odd vs. even effect was also observed in the mobility of
the hydration water in multilayer measured by NMR relaxation rates.\textsuperscript{195-196} The adsorption of
PAH on the surface decreased the water mobility for PAH/PSS systems.\textsuperscript{195-197} This provided a
complex view on the internal and surface properties of PEMUs.\textsuperscript{196,198} We attempt to use
nanoindentation to get better insight into build-up mechanism of PEMU.

In the present work we use nanoindentation to reveal some important fundamental
properties of PEMUs made from poly(diallyldimethylammonium chloride), PDAMDAC, and
poly(styrene sulfonate), PSS. The results in addition to the extensive knowledge of PEMUs
prepared from PDADMA and PSS enabled conclusions on their surface morphology and build-
up mechanism.

5.2 Materials and methods

Poly(4-styrenesulfonic acid) ($M_w = 7.5 \times 10^4$ g mol$^{-1}$), poly(diallyldimethylammonium
chloride) ($M_w = 40 \times 10^4 - 50 \times 10^4$ g mol$^{-1}$), poly(acrylic acid) (PAA $M_w = 100,000$ g mol$^{-1}$) and
poly(allylamine hydrochloride) (PAH $M_w = 56,000$ g mol$^{-1}$) were used as received from Aldrich.
NaCl (ACS grade) from Aldrich was used to adjust the ionic strength of the polyelectrolyte
solutions and for the doping solutions. All solutions were prepared using deionized water (Barnstead, 18 MΩ E-pure).

5.2.1 Polyelectrolyte multilayer preparation

Multilayers made from PDADMA and PSS were built with the aid of a robot (StratoSequence V, nanoStrata Inc.) on silicon wafer substrates that were cleaned with “piranha” reagent and then rinsed vigorously with distilled water followed by drying with a flow of N₂. Polyelectrolyte solutions were 10 mM based on the monomer repeat unit, and the ionic strength was fixed using NaCl at 1.0 M. The substrates were mounted on a shaft that rotated at 300 rpm. The dipping time in the polymer solutions was 5 min followed by three 1 min rinsing steps in water after each layer. (A/B)ₓ and (A/B)ₓA indicate a multilayer ending in B and A respectively, where x is the number of layer pairs, A represents the polycation and B the polyanion.

A Gaertner Scientific L116S autogain variable angle Stokes ellipsometer was used to obtain the dry thickness of PEMU films unless stated otherwise. Ten thickness measurements on each PEMU were averaged.

A KSV Cam 200 was used to determine the static contact angle of the PEMU films by the sessile drop technique. The volume of water droplet was 10 µl for all measurements and each data point was an average of 3 measurements taken at different spots on the PEMU. Dynamic contact angles, DCAs, (both advancing Θₐ and receding Θᵣ) were recorded using the Wilhelmy plate technique (Cahn Instruments, DCA 300). For DCA, the immersion/emersion rate of the PEMU, built on a double-side-polished Si wafer, in water was 100 µm sec⁻¹.

5.2.2 Force spectroscopy

The stiffness of the films, represented by the apparent modulus, was determined by force-indentation measurements, or force curves, using an atomic force microscope (AFM). An MFP-3D AFM unit was equipped with an ARC2 controller (Asylum Research Inc., Santa Barbara, CA) and Igor Pro software. Multi75AI tips were used, with spring constant ~ 2 N m⁻¹. The optical lever sensitivity (OLP) of the tip was first calibrated in air and then its spring constant was determined by the thermal fluctuation technique. The sample was then immersed in salt solution at a given concentration and the OLP of the tip was recalibrated. Force maps of 4 x 5 points were performed on the PEMU in situ with a scan size of 20 x 20 µm. The distance from the surface and the velocity of the tip were set to 500 nm and 1µm s⁻¹ respectively unless stated
otherwise. The term “apparent” modulus is used to describe the modulus recorded at a specific tip velocity. For viscoelasticity studies, force curves were performed at indentation velocities ranging from 50 nm sec\(^{-1}\) to 3.17 µm sec\(^{-1}\) on the PDADMA/PSS multilayer and this is equivalent to different strain rates. The applied force and the resulting indentation are given by Equations 2.2 and 2.3

All force curves were analyzed using Hertzian contact mechanics for a punch model as in Equation 4.1. Equation 4.1 predicts force is linearly proportional to indentation, which was the case observed here, as shown in the sample force curve in Figure 5.1.

\[ \text{Force} = k \times \text{Indentation} \]

\[ k = \frac{2 \sqrt{E_1 E_2} R}{3 \sqrt{1 - v_1^2}} \]

By fitting the force vs. indentation curves shown in Figure 5.1, \( E_c \) can be obtained and related to the apparent modulus \( E_1 \) of the indented material and \( E_2 \) (of the indenter) using Equation 4.2. The radius of the AC240-TS tip was 10 nm as provided by the manufacturer.

**5.2.3 Surface imaging**

Wet and dry images of the surface of PEMUs were conducted under AC and contact mode to obtain the morphology, surface roughness and thickness of the films. Multi75AI tips were used for dry and wet imaging of PSS ending multilayers and a softer tip, TR400PSA (spring constant ~ 0.02N m\(^{-1}\) and 20 nm radius) for PDADMA-ending films in liquid. The cantilever was tuned to resonate below its resonance frequency by 10%. The scan size was set to
5 x 5 µm or 10 x 10 µm and scan rate was 0.5 Hz. Thickness measurements were determined by scratching the film with a tweezers, and then scanning a 90 x 90 µm area over the scratch. The thickness was measured by drawing a line across the scratch and the edge (step) height of the film was obtained, the thickness was obtained at different positions of the image and the average height was reported. The roughness, defined as the rms roughness of the image was obtained on a 1 x 1 µm square patch, ten values were recorded at different positions of the image and the average value was reported.

5.3 Results and discussion

5.3.1 Swelling and mechanical properties

We were interested in investigating the effect of final layer identity on the stiffness of PEMUs as people had seen these alternations on other properties such as ion permeability, surface charge, and water content. A multilayer composed from PAH/PAA was prepared in such a way that to monitor the change in modulus as a function of the identity of the terminating layer (odd vs. even) and to correlate it to the thickness measured at each layer. 14 layers were first built in a row to remove the effect of substrate stiffness on the measurements and to get thick enough films and then odd/even were measured every 3 layers i.e. 14, 15, 18, 19, 22, 23, 26, 27, 30, 31, 34, 35, 38, 39. Dry thickness was measured by ellipsometry at each layer and force curve measurements were measured while the film was immersed tris buffer at pH = 7.4 with 0.15 M NaCl (see Figure 5.2). The wet thickness of the film was deduced from the index of hydrophobicity which is measured as the ratio of the dry to wet thickness of the PEMU. To measure the index of hydrophobicity of PAH/PAA multilayers, the film was built on a silicon wafer and then scratched gently with a tweezer, next the thickness before (dry) and after immersing the films in tris buffer at pH = 7.4 and 0.15 M NaCl was measured by scanning the AFM over a scratch. The ratio of the wet to the dry thickness was 1.22 and this corresponded to the index of hydrophobicity. We observed that at low number of layers < 25 at a thickness below 180 nm, the modulus was affected by the rigidity substrate which resulted in higher modulus values than the real values. Surprisingly and on contrary to the nano-indentation measurements obtained by Thompson et al. who saw that the modulus was independent on the terminating layer, we found a strong dependence of apparent modulus on the identity of the final layer. The modulus of PAH ending films was one third that
of PAA ending films (see Figure 5.2). We were surprised to find that the film terminated in the hydrophilic PAA was more glassy (stiffer) than those terminated in PAH, PAA is known to make swellable, weakly bound multilayer which can be decomposed by increasing the salt concentration or changing the pH of the medium. On the other hand PAH makes less hydrated films that are harder to swell. So the properties of the individual dry polyelectrolyte is different then when they are part of the multilayer. We found that with enough thickness the modulus of the film oscillated between 70 MPa for PAA ending films and 20 MPa for PAH ending films as shown in Figure 5.2. The modulus of polyelectrolyte complexes is strongly controlled by stoichiometry, meaning that a multilayer that has a lot of extrinsic site that is counter ions (non-stoichiometric) is expected to be softer then the one with less extrinsic sites (more intrinsic). So it might be that the PEMUs ending in PAA are more intrinsically compensated and thus stiffer then the ones terminated in PAH. The effect of final layer identity of PEMU on the modulus was not seen for the exponentially growing highly hydrated PLL/HA films.

![Figure 5.2](image)

**Figure 5.2** Dry film thickness as a function of the number of layers for PAH/PAA multilayer buildup. ◊; variation of apparent Young’s modulus as a function of film thickness and the final layer. The modulus was obtained by in situ AFM force curves measured in Tris buffer at pH= 7.4 and 0.15 M NaCl. The last point in the thickness series has a greater error.

The stiffness as a function of the identity of the terminating layer was also investigated for a different set of polyelectrolytes, both having pH-independent charges. PDADMA/PSS multilayers that were prepared from 1.0M NaCl were studied by nanoindentation, we started by
14 layers that were built in a row which resulted in a film that is thick enough for nanoindentation measurements independent of the substrate (wet thickness was about 500 nm). Following each layer the multilayer was rinsed in water then dried gently with a stream of nitrogen and then the film was dipped in 10 mM NaCl for 12 hours, 10 mM NaCl was used to prevent doping of the film and annealing of the surface features which usually occur over time at high NaCl concentration. Buildup continued from 1.0 M NaCl and thickness (both dry and wet) and force curves were measured by AFM after each layer; 14, 15, 18, 19, 22, 23, 26, 27, 30, 31, 40, 41, 50 and 51. Thickness was measured by scanning the AFM tip over the scratch, and force curve measurements were performed in situ in 10 mM NaCl at each layer indicated above. The modulus fluctuated between 80 MPa for PSS ending films and 7 MPa for PDADMA ending films, and this correlated with the wet thickness measurements: when PDADMA was absorbed on the surface the multilayer swelled more, and thus was more hydrated, once PSS was added on the surface, it either kept or to some extent decreased the wet thickness of the film as shown in Figure 5.3.

![Figure 5.3](image)

**Figure 5.3** (○) Wet thickness of PDADMA/PSS multilayer as a function of number of layers. (◊) apparent surface Young’s modulus obtained by force curve measurement taken at layer number 14, 15, 18, 19, 22, 23, 26, 27, 30, 31, 40, 41, 50 and 51. All measurements were performed in situ in 10 mM NaCl. An odd number of layers represents a PEMU capped with PDADMA and even corresponds to PSS-terminated film.
The data of Figure 5.3 is tabulated in Table 5

**Table 5.1** Wet thickness and modulus measurements at different number of layers. This data is plotted in Figure 5.1

<table>
<thead>
<tr>
<th>Number of layers</th>
<th>Wet thickness (nm)</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>184</td>
<td>375</td>
</tr>
<tr>
<td>15</td>
<td>527</td>
<td>1.23</td>
</tr>
<tr>
<td>18</td>
<td>483</td>
<td>119.6</td>
</tr>
<tr>
<td>19</td>
<td>756</td>
<td>1.35</td>
</tr>
<tr>
<td>22</td>
<td>836</td>
<td>98.6</td>
</tr>
<tr>
<td>23</td>
<td>1220</td>
<td>9.8</td>
</tr>
<tr>
<td>26</td>
<td>1170</td>
<td>87.4</td>
</tr>
<tr>
<td>27</td>
<td>1630</td>
<td>8.3</td>
</tr>
<tr>
<td>30</td>
<td>1740</td>
<td>74.6</td>
</tr>
<tr>
<td>31</td>
<td>1970</td>
<td>9.1</td>
</tr>
<tr>
<td>40</td>
<td>2448</td>
<td>52.6</td>
</tr>
<tr>
<td>41</td>
<td>2560</td>
<td>12</td>
</tr>
<tr>
<td>50</td>
<td>3000</td>
<td>76</td>
</tr>
<tr>
<td>51</td>
<td>3070</td>
<td>9.7</td>
</tr>
</tbody>
</table>

This remarkable difference in stiffness and hydration between PDADMA and PSS ending films was only observed when the experiments were conducted under wet condition and there was no oscillation in the DRY modulus and thickness as see in Figure 5.4, noting that the dried modulus in this case was measured by ellipsometry. Odd vs. even effect was also studied by other people using different techniques, for example Miller et al. used ellipsometry to study the swelling as a function of final layer, ionic strength, and the swelling solvent for different PEMUs. They were able to see that films terminating in PDADMA swelled more than those ended in PSS in agreement with our data. Also Ramos et al. used ellipsometry and QCM-D in a single device to monitor the water content of PDADMA/PSS multilayer, they found that PDADMA terminated multilayers had a higher water content then PSS terminated ones, and thus the film was softer. The effect of final layer identity on the swelling of PEMUs was also seen...
by Wong et al. where a PSS ending multilayer was more swollen than a PAH ending multilayer, it was interpreted that despite the presence of aromatic back bone, a multilayer ending in PSS was more hydrated than a multilayer ending in PAH and this was attributed to the higher charge density on the surface when PSS was deposited as the final layer then PAH since PSS is considered a stronger polyelectrolyte. Smith et al., and McCormick et al. used $^{13}$C nmr and $^1$H nmr to study the properties of polyelectrolyte multilayers, by $^{13}$C nmr they found that the mobility of PSS remains constant during the build up while that of PDADMA decreases when increasing the number of layers. Also, by $^1$H nmr they found that water showed a higher mobility and chemical shift when PDADMA was the final layer.

$\textbf{Figure 5.4 A}$ Dry and wet thickness of PDADMA/PSS multilayer starting from 14 layers. Wet thickness was obtained in 10 mM NaCl using AFM by scanning over a scratch, while dry thickness was measured by ellipsometry. Films with odd number of layers were PDADMA-ending while even number of layers corresponded to PSS-ending films. $\textbf{B}$ The dry modulus for PSS and PDADMA terminated measured by force spectroscopy using AFM

5.3.2 Effect of substrate stiffness on the apparent modulus of PDADMA/PSS during layer by layer build

As shown in Figure 5.2, indentation measurements started at the 14$^{th}$ layer because a minimum film thickness was required to avoid contributions from the substrate on the modulus measurement and therefore the PEMU would appear stiffer than expected and this was shown before for PAH/PAA films and elucidated in chapter 3. To estimate this minimum thickness
required for reliable force curve measurements, films with 14, 18, 30, and 50 layers were first compared. All films ended in PSS and force curve measurements were performed in 10 mM NaCl. The resulting force vs. indentation profile for all the films was fitted at different indentation distances from 0 to 2 nm, then 2 to 4 nm and up to 30 nm. \( \frac{E_{\text{app}}}{E_{\text{PEMU}}} \) was plotted as a function of indentation, where \( E_{\text{app}} \) was defined as the apparent modulus at each indentation distance and \( E_{\text{PEMU}} \) is the modulus of the PSS-terminated film at “infinite” thickness (i.e. where the tip no longer “feels” the substrate), and was taken here as 70 MPa. Figure 5.5 showed the deviation of the apparent modulus from \( E_{\text{PEMU}} \) as a function of indentation depth. It was found that as the indentation distance increased contributions from the substrate on the rigidity of the films started to contribute to modulus measurement. For thinner films \( E_{\text{app}} \) started to deviate at shorter indentation depths than for thicker films. For sufficiently thick films the apparent modulus remained independent of indentation depth.

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\[ \]

**Figure 5.5** Films with 14 layers (□), 18 layers (○), 30 layers (△), and 50 layers (×) (all ending in PSS). Force curves were performed for each film in 10 mM NaCl. Force vs. indentation curves were fitted from 0 to 2 nm, 2 to 4 nm, and so on, up to 30 nm indentation. For thinner films, \( E_{\text{app}} \) deviated from \( E_{\text{PEMU}} \) at shorter indentation as compared to thick films.
5.3.3 Modulus vs. doping by salt

It is well known that at high salt concentration doping of polyelectrolyte multilayers break the ionic bond between the monomers of the oppositely charged polyelectrolytes, thus converting the intrinsic sites into extrinsic. This phenomenon resulted in the swelling of the film by driving more water into the bulk of the multilayer as a result of increased ions content which are hydrated this illustrated in scheme 5.1.\cite{46,53} This increase in volume is an example of “antipolyelectrolyte” behavior, free polyelectrolytes are known to shrink on increasing salt. A decrease in modulus is expected as a result of the increased hydration of the film; however we saw a dramatic decrease in modulus as a result of breaking the ion-pair crosslinks that hold the film together.\cite{46}

Non-Stoichiometric polyelectrolye multilayers that are not 1:1 had a considerable counter ion population within the film (residual extrinsic charges), even if the film is dipped in water (it is obvious to expect that no counter ions remain in the film when immersed in water). Early AFM measurements on the swelling of multilayers revealed a minimum in the thickness versus [NaCl] data.\cite{45} At low salt concentration (i.e. close to zero), the residual extrinsic charges exerted osmotic pressure that drove water into the film and lead to “hyperswelling”. Adding more salt resulted in higher osmotic pressure on the outer medium, driving water out of the film and this caused the PEMU to dehydrate and therefore shrank; this observation was termed as the “polyelectrolyte effect”. Adding even more salt on the outer medium and when the external salt concentration was high enough, expansion due to doping became much stronger then the phenomena of osmotic dehydration and the former took dominance. This effect disappeared by exposing the multilayer to high NaCl (annealing) because the trapped extrinsic sites could be annealed out of the film as a result of interdiffusion of the polyelectrolyte chains (high NaCl promote increased polyelectrolyte chain mobility) and this process is schematically represented in Figure 1.6.\cite{45,201} It was demonstrated that the percentage of residual extrinsic sites decreased from 6% to 2% after annealing the PEMU in 1M NaCl.

As a complimentary study to the swelling data obtained before\cite{42} and although modulus is known to decrease with doping,\cite{46,53} we wanted to study the effect of salt concentration on the modulus of PDADMA/PSS films by nano-indentation which has not been reported before. Figure 5.6 showed that the modulus of the film had a small peak when increasing the salt concentration for the freshly prepared PEMU. This behavior correlated well with the thickness
vs. NaCl concentration obtained by Schlenoff et al.\textsuperscript{42} which showed a significant decrease in hydration (wet thickness) at about 0.25 M due to the osmotic swelling phenomenon explained above.\textsuperscript{45,201} After reaching 1.5M NaCl the multilayer was annealed and this feature was lost. In the latter case the film only showed “antipolyelectrolyte” behavior (meaning that increasing salt increases swelling and decreases the modulus). The apparent modulus values in Figure 5.6 are much higher than those reported by Jaber et al.\textsuperscript{46} at matching [NaCl] this is because the modulus values measured by Jaber et al. were collected by stress relaxation after the viscous components of the viscoelasticic PEMU were allowed to decay.

![Figure 5.6](image)

\textbf{Figure 5.6} Apparent Young’s modulus of a (PDADMA/PSS)\textsuperscript{15} multilayer prepared at 1 M NaCl with increasing [NaCl] (O); then decreasing [NaCl] (□). The tip velocity was 1\,\mu m\,sec\textsuperscript{-1}. The dotted line represents the doping level as a function of [NaCl].\textsuperscript{42}

\textbf{5.3.4 Nano-viscoelastic nature of PEMUs}

The viscous part of PEMU mechanical response is now believed to impact the behavior of cells grown on them. This was inspired by recent work that was done to study the effect of substrate viscoelasticity on the behavior of cells.\textsuperscript{187} They prepared a series of polyacrylamide gels that have the same storage modulus (elasticity) but different loss modulus (viscoelasticity). It was found that by increasing the loss modulus, the spread and proliferation of human mesenchymal stem cells increased, however the size and maturity of focal adhesions decreased.
Also the change in loss modulus had an impact on the differentiation of these cells with more differentiation on the substrates with higher loss modulus.\textsuperscript{187}

Tensile measurements were used to measure the viscoelasticity of thick PDADMA/PSS that were peeled from the surface.\textsuperscript{46,58} The results showed that the mechanical properties of these films had time/frequency dependence. At low frequency or long times, the material does not have enough time to relax and thus the viscous component vanish leaving only the “equilibrium” elastic modulus. Viscoelasticity is rarely measured by nano-indentation due to experimental challenges, we were able to adjust the indentation rate during force curve measurements and this will be equivalent to different strain rates used to measure viscoelasticity by tensile. This finding enabled us to study the viscoelasticity of our nanometer thin films by nano-indentation. Here, force curve measurements were performed on un-annealed PSS terminated (PDADMA/PSS)\textsubscript{15} multilayer, and on the same multilayer that was annealed in 1 M NaCl for 5 days, at different indentation velocities. All force curve measurements were made in 10 mM NaCl. A plot of apparent Young’s modulus vs. indentation velocity, shown in Figure 5.7 showed a large increase in apparent modulus with increasing indentation rate from 21 MPa to 83 MPa pointing the viscoelastic nature of PDADMA/PSS multilayer. The modulus at zero strain rates which corresponded to the “equilibrium” elastic modulus was 15 MPa, and was obtained by extrapolating the curve of Figure 5.7 to zero indentation velocity (zero strain rate). This value was close to what was obtained before using tensile testing on PDADMA/PSS microcoupons.\textsuperscript{46} The apparent modulus at different indentation velocities showed no difference between the annealed and the un-annealed multilayer. This result indicated that PSS terminating surfaces has mechanical properties and composition similar to bulk, in other words for PSS ending films there is a 1:1 stoichiometric compensation between the PDADMA and PSS at the surface.
The viscous response of PEMUs is not highly addressed in nanoindentation measurements. Mueller et al. prepared PDADMA/PSS multilayers hollow capsules. Their mechanical properties were measured by AFM, it was found that this material undergo a glass-melt transition at around 35°, beyond this temperature the stiffness of the multilayer showed a big decrease and the material behaved elastically. Beyond the transition temperature and by varying the deformation rate, the elastic constant above the transition temperature became rate dependent, indicating the viscoelasticity of the material. Using a home made AFM and a spherical indenter on a thick PEMU prepared from PLL/HA, Francius et al. the cantilever deflection was recorded at different approach velocity as a function of piezodrive position to extract the viscoelastic properties of PLL/HA multilayer upon the deposition of n layers PSS/PAH. Lu et al. characterized the linear viscoelastic behavior of single wall carbon nanotubes/polyelectrolyte multilayer using nanoindentation where the viscoelastic function of materials were measured using one of two methods: (1) the direct differentiation method from the load-displacement data and (2) by fitting the analytical load-displacement relation to the nanoindentation data known as the material parameter extraction method.

Figure 5.7 Apparent Young’s modulus of (PDADMA/PSS)15 multilayer surfaces, one un-annealed (□), the other annealed in 1 M NaCl (△), at different indentation velocities. The salt concentration of the polyelectrolyte solution was 1 M NaCl during build up and 10 mM for these in situ indentations, which probe the top 50 nm of the PEMU. The indentation velocity varied from 50 nm sec\(^{-1}\) to 3.15 µm sec\(^{-1}\).
5.3.5 Surface roughness is not due to phase separation

 Generally, it was shown that the surface roughness of PEMU increases with increasing the thickness of the film. Also, we found that the surface roughness of PEMUs decreased when they are present in enough salt concentration as a result polyelectrolyte chains intermingling promoting the smoothness of the surface. The mechanism responsible for surface roughness had always been a strong debate in the field. Recently, some people claimed that the appearance of surface features are due to induced phase separation (spinodal decomposition) between the different polyelectrolytes.

In terms of polyelectrolyte multilayers, there were several approaches to explain the appearance of characteristic features on the surface. Mendelsohn et al. described the formation of micropores in thin films formed of poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH). They described the phenomena by spinodal decomposition upon changing the pH that lead to the protonation of carboxylic acid allowing reorganization and phase separation from the acidic water solvent. Fery et al. described the formation of nanopores in polyelectrolyte multilayers formed from PAH/PAA; this was done by exposing the multilayer prepared from salt containing polyelectrolyte solutions to pure water. They showed that rinsing the multilayers with pure water during build up resulted in features with lateral periodicity on the surface. This lateral periodicity (brain structures) depended on the thickness of the film. However, when rinsing with salt concentration similar to the concentration of salt in the polyelectrolyte solutions, the resulting films were smoother with no vermiculate structures. But, when this film was dipped in pure water, nano-pores were created in the films as a result of structural changes. They illustrated the phenomena as a dewetting mechanism rather than spinodal decomposition as the latter mechanism was a bulk effect and should not be dependent on film thickness. The periodic vermiculate structures that appear on the surface of polyelectrolyte multilayers were also seen and discussed by McAloney et al. They described the formation of vermiculate worm like structures on the surface of PDADMA/PSS multilayers; Surface morphology was studied for a PSS ending layer at different polyelectrolyte salt concentration during deposition. It was found that at low salt concentration, a flat and smooth surface was obtained; however at high salt concentration the surface presented the vermiculate structures. This was not attributed to drying effects, however, it was related to the different configuration of polyelectrolytes in solution as follows: at low salt concentration the extended rod configuration of the polyelectrolyte resulted
in its flat adsorption and no vermiculate structure was seen, however at high salt concentration, the polyelectrolyte was adsorbed in the globular coil conformation, which resulted in the vermiculate morphology however no data was reported for PDADMA ending films.\textsuperscript{196,209} In this paper we described the morphology of the surface of PDADMA/PSS multilayers for films ending in PDADMA and others ending in PSS according to Tanaka where a polymer or a gel undergoes extensive swelling, this could lead to the formation of nice periodic features on the surface. Tanaka explained that when a surface layer was swollen, and this layer is under opposing mechanical constraints, where one end is fixed to a substrate and the other end is free to expand, the outer surface undergo buckling and the features appear.\textsuperscript{188-189}

Figure 5.3 showed that the surface modulus under wet conditions (film dipped in 10 mM NaCl) is a strong function of composition (PSS ending films were stiffer then PDADMA ending films), s any variation in composition at the surface (for example in phase separation) should result a difference in modulus. To illustrate this, a (PDADMA/PSS)\textsubscript{16} multilayer was prepared from 1 M NaCl and the surface modulus was mapped using Multi75AI, contact mode AFM image was taken on the multilayer \textit{in situ} while immersed in 10 mM NaCl, and this was preceded by calibrating the instrument for force curve measurements. The image shown in Figure 5.8 had a scan size of 10 x 10 µm and surface roughness of 70 nm, Using Igor software different points (positions) on the image that correspond to “hills” and “valleys” were chosen and force curve was performed on each while the film still in situ. The location of the various points was shown in Figure 5.8 indicated as red crosses. In summary 14 different points as hills and 14 different points as valleys were selected and corresponded to an average modulus of 47±4 MPa and 49±4 MPa respectively. The same experiment was done under dry conditions on a multilayer ending in PSS and then on the same multilayer after adding PDADMA layer (see Figure 5.9). Again in this case force curves were performed on various spots along the images corresponding to “hills” and “valleys” (see Figure 5.9). In this case the film was quite stiff so an AC160-TS tip with a spring constant of 40 N m\textsuperscript{-1} was used. For PSS ending film (Figure 5.9-A), the modulus of the hills was 1.8 ±0.2 GPa and that of the valleys was 1.9 ±0.3 GPa. For PDADMA ending films (Figure 5.9-B), the modulus of the hills was 2.6 ±0.1 GPa, and that of the valleys was 2.4 ±0.1 GPa. This also showed that even with the surface roughness, the composition was homogeneous even for the dry films. So we can conclude that despite the rough surface, the surface composition was homogeneous and thus there was no indication of phase separation.
To show that Multi75AI tip is capable of contouring the surface, Figure 5.8 showed a demonstration in real scale of the surface features of PEMU and the Multi75AI that has a cone angle of 35° as provided in the tip specifications sheet and height of 14 µm (here only 1 µm was shown). In these experiments only 50 nm of the total indentation was fit to obtain the Young’s modulus of hills and the valleys. Figure 5.8 showed clearly that the tip that had a diameter of 20 nm was small enough to interrogate the surface features of the film. Also as schemed in the graph, the shape and size of the tip was suitable to carry out force spectroscopy on the hills and valleys.

Looking at AFM images of PEMUs is somehow misleading, the surface usually looks rougher then it really is. To further understand the presence of features on the surface of PEMUs, the surface and z-axis had been replotted with an equal scale (see Figure 5.10). Generally, AFM images are zoomed in the z-axis by default, thus the surface appear much rougher then how it really looks as shown in Figure 5.10.
Figure 5.8 AFM image of a (PDADMA/PSS)$_{16}$ multilayer, was taken in 10 mM NaCl. The red crosses represent positions where force curves were taken. 14 force curves on the hills (bright grey spots) gave an average modulus of 47 MPa, and 14 force curves in the valleys (dark grey area) resulted in an average modulus of 49 MPa. The 4 µm-long solid line was drawn through point 20 (valley) and 9 (hill) and the contour shown in the height vs. distance graph. The thickness of the PEMU was measured at a scratch edge.
Figure 5.9 A and B are AFM images of (PDADMA/PSS)$_{15}$ and (PDADMA/PSS)$_{15}$PDADMA respectively. The images were recorded under dry conditions. Force curves were taken on various spots on the images that corresponded to peaks and valleys.
5.3.6 Implications for the mechanism of multilayer buildup: “Asymmetric Growth”

The results obtained above provided key information into the way a polyelectrolyte adds to a growing multilayer. This led us to put forward a conclusion on the build-up mechanism of PSS/PDADMA multilayers. When a PDADMA “layer” adsorbs on the surface, it bring a lot of extrinsic sites which are highly hydrated and thus the film swells more and become softer. On the other hand when PSS “layer” adsorbs, it compensates the positive extrinsic sites and converts them into intrinsic ones, thus we loose the hydrated extrinsic sites and the multilayer become
more dry and stiffer. As a result PSS ending films has similar mechanical properties to the bulk of the multilayer that is stoichiometric 1:1 compensation of PDADMA and PSS. It is known that a polyelectrolyte complex is softer when compensated extrinsically rather then intrinsically, as intrinsic compensations has more crosslink (via ion pairs) and are less hydrated, on the other hand extrinsic compensation has more counterion content that results in more hydration and thus soften the film.\textsuperscript{46} So it can be deduced that a PSS ending film is significantly intrinsic while a PDADMA terminated film is extrinsic. This comparison is represented in Figure 5.11, for PDADMA ending films you can see an excess of polymer charge, and counterions all over the surface while for PSS ending film the surface is mostly intrinsic with 1:1 compensation of PSS and PDADMA. This suggested that adding a PSS layer on the surface might result in an incomplete charge reversal on the surface which actually had been seen under some conditions.\textsuperscript{193} So it can be deduced that the growth of PDADMA/PSS multilayer is “asymmetric”. Asymmetric growth contradicted what had been proposed in literature where people always assumed and equal compensation of both polyelectrolytes on the surface.\textsuperscript{30-31}

![Figure 5.11](image1.png)

\textbf{Figure 5.11} Multilayer ending in PDADMA (left) showing the presence of extrinsic sites; the addition of PSS converts almost all extrinsic sites into intrinsic ones.

The asymmetric growth can be best witnessed by comparing AFM images of wet and dry PEMU. Figure 5.12 showed AFM images of a dry PDADMA/PSS film composed of 30 layers with PSS as the final layer and another image on the same film under wet conditions where the film was immersed in 10 mM NaCl during imaging. Under wet conditions the surface features
showed some increase as a result of swelling but generally the surface roughness was preserved between the dry and wet images (rms roughness was 66 nm and 70 nm for dry and wet PEMU, respectively). On the other hand adding a PDADAMA layer made the surface much smoother if imaged under dry conditions as compared to the dry PSS ending surface and this is shown in Figure 5.13. However, the wet (in 10 mM) PDADMA terminated films was difficult to image being much softer and showed highly swollen features and was much rougher (Figure 5.13) (rms roughness was 16 nm for the dry PEMU and 106 nm for the wet). This difference in surface roughness correlated well with the mechanism proposed in Figure 5.11 where PSS ending films were highly intrinsic and this resulted in minor swelling whereas the PDADMA topped surface is compensated extrinsically and therefore highly hydrated and much softer.
Figure 5.12 AFM images of dry and wet (PDADMA/PSS)$_{15}$ multilayer and their 3D projection. Also shown a graph of height versus distance which representing the morphology along a contour line through the image. The images of wet PEMU were taken while the multilayer was immersed in 10 mM NaCl. The surface roughness was 66 nm and 70 nm for dry and wet respectively,
Figure 5.13 AFM images of dry and wet (PDADMA/PSS)$_{15}$PDADMA multilayer and their 3D projection. Also shown a graph of height versus distance which representing the morphology along a contour line through the image. The images of wet PEMU were taken while the multilayer was immersed in 10 mM NaCl. The surface roughness was 16 nm and 106 nm for dry and wet respectively.
5.3.7 Contact angle measurements

The odd-even phenomenon was earlier seen in the swinging of the contact angle values on PEMUs, depending on the last layer. The explanation assumed that one of the polyelectrolytes is more “hydrophobic” than the other. Measuring the contact angle of PEMU is not often done under equilibrium conditions. To further explore the surface properties of PDADMA/PSS multilayer, three different contact angle measurements were performed as a function of end layer, the advancing and the receding angle by using the Wilhelmy plate technique and the static contact angle using the sessile drop method. Contact angle measurements provide information on the outermost surface of a few angstroms. The static contact angle measurements was performed at the solid/liquid interface that is not in motion and the dynamic contact angle, both advancing and receding angle, were measured when the liquid was in motion relative to the solid interface.

The static contact angle fluctuated based on the final layer as shown in Figure 5.14 and this was commonly observed in multilayers. These large oscillations in static contact angle might lead to a conclusion that there is a big difference in hydrophobicity between the polyelectrolytes. So based on the static contact angle data obtained, it can be deduced that PSS is more hydrophilic than PDADMA and this contradicts the swelling data obtained in Figure 5.3 and 5.4 that showed clearly that PDADMA ending layers were more hydrated and thus expected to be more hydrophilic. However by comparing the dynamic advancing, $\Theta_a$, against receding, $\Theta_r$, angle the non-equilibrium, or history-dependent, nature of the contact angle measured here could be revealed. PSS ending films showed no contact angle hysteresis between $\Theta_a$ and $\Theta_r$. On the other hand there was a big difference in advancing and receding angles for PDADMA terminated films. This difference can be explained by the glassier nature of PSS-topped surface concluded from the AFM and modulus measurements shown above and thus glassy PSS ending surface showed only little rearrangements on the surface (no difference before and after equilibrium). On the other hand, the PDADMA terminated surface experienced a big volume transition between the wet and dry AFM images indicating the presence of some sort of surface rearrangements and this can result in the strong hysteresis in the advancing and receding contact angle (Figure 5.14).

For further illustration, when immersing the glassy immobile PSS in water the hydrophobic part (aromatic part) would be directed towards the bulk of the film away from the
water and the hydrophilic sulfonate groups that has the hydrated Na\(^+\) ions would reside near the surface and thus the surface would appear more hydrophilic. This was also shown in the case of PAA ending films\(^{113}\) which are also glassy and compensated by sodium ions and had a lower static contact angle.\(^{155}\) The similarity in the receding contact angle between PSS and PDADMA ending films indicated that there is no difference in hydrophobicity at equilibrium wetting, thus when reporting a difference in cells behavior based on the final layer identity of PEMUs, it cannot be attributed to difference in hydrophobicity, it has to be related to other surface properties such as mechanical properties, surface chemistry, or charge density.

![Figure 5.14](image)

**Figure 5.14** (◊, □, ▲) the advancing, static and receding contact angle of PDADMA/PSS multilayer as a function layer number. Odd layers are PDADMA and even layers PSS.

### 5.4 Conclusions

Nano-indentation is a reliable technique to study surface and bulk properties of nanometer thin films. It provided significant insight into the regarding the build-up mechanism and surface topography of PDADMA/PSS multilayers. We found that the stiffness of the film under wet conditions depended on the type of polyelectrolyte on the surface where PDADMA ending films had a lower modulus then PSS ending films and this correlated well with the
swelling measurements where a films ending in PDADMA was more swollen. This observation was explained as the “asymmetric growth” of PDADMA/PSS multilayer. From the oscillation in hydration and surface modulus we deduced that PDADMA ending films are extrinsically compensated while PSS ending film are compensating intrinsically, also PSS ending films have mechanical properties similar to the bulk of the multilayer and there is 1:1 stoichiometric compensation between PDADMA and PSS. The theory of “asymmetric” growth contradicts the early literature regarding the build up of polyelectrolyte multilayer where people always assumed equal compensation between the polyelectrolytes. Surface roughness of PEMUs which always create a debate concerning its emergence, and some people assumed that it is due to phase separation, here mapping the surface and keeping in mind that the modulus depends on surface composition, force curves were performed on the hills and valleys and showed no difference, so we were able to show that surface roughness is not due to phase separation. For the future radioactivity measurements using radiolabelled counter ions are being used to actually quantify the number of extrinsic sites for PDADMA and PSS ending films and the preliminary results correlate well with what we saw in this work.
CHAPTER SIX

STUDYING THE ROLE OF WATER AS A PLASTICIZER IN POLYELECTROLYTE MULTILAYER

6.1 Introduction

The obvious way to describe the association between the oppositely charged polyelectrolytes during multilayer build-up is “electrostatics”. This theory had been used by the people in the field for long time. Inspired by Michaels and co-workers who explained the complexation of polyelectrolytes to be driven by the “escaping tendency of microions”, Bucur et al. showed that the association of the polyelectrolytes was driven by the release of water and counterions to the solution in an “entropy driven assembly”. As we saw in chapter 5 and in a lot of studies conducted in our lab on PEMUs and the macroscopic polyelectrolytes complexes that their mechanical properties is controlled by the ionic content of the film, decreasing the salt concentration of the medium will increase the number of crosslinks between the monomers of the oppositely charged polyelectrolytes, this will decrease the free volume available for polyelectrolyte chains to rearrange thus decreases the viscoelasticity of the material, and the vice versa is true. This outcome was explained as salt induced plasticization and the material was termed “Saloplastic”. As water plays a major role in polyelectrolyte multilayer assembly, it is expected that it has a strong effect on the mechanical properties of the multilayer.

To enhance the flexibility of materials, people used small molecules that are capable of decreasing the glass transition temperature of the system, these molecules are called plasticizers. A plasticizer can enhance the viscoelasticity by 3 ways: 1) Lubricity theory where the plasticizer acts as a lubricant reducing the intermolecular friction between the polymer chains, 2) The gel theory, in this case the plasticizer act by breaking or masking polymer-polymer interaction leading to reduction in the stiffness of the materials, and 3) In this case the plasticizer work in increasing the free volume available for polymer chains making them more mobile and enhancing their viscous flow. Usually a good plasticizer is the one that comprise the three effects collectively. It was found that water acts in plasticizing polymer systems not only by the gel/lubricating theory but also by providing free volume to the polymer chains and enhancing their mobility and therefore softening the material similar to the effect of
temperature.\textsuperscript{220-222} Recently Hariri et al. showed that water plays an important in controlling the viscoelasticity of macroscopic complexes of PDADMA/PSS.\textsuperscript{60} In this chapter we present a study illustrating the role of water in controlling the elasticity and viscoelasticity of PEMUs. We used an osmotic stress technique by applying poly(ethylene glycol) (PEG) as the osmolyte to control the amount of water within the polyelectrolyte films while keeping the same doping level. We showed that water plays an important role in plasticizing the PDADMA/PSS multilayers

\section*{6.2 Materials and Methods}

Poly(4-styrenesulfonic acid) ($M_w = 7.5 \times 10^4$ g mol\(^{-1}\)), poly(diallyldimethylammonium chloride) ($M_w = 40 \times 10^4 - 50 \times 10^4$ g mol\(^{-1}\)) were used as received from Aldrich. NaCl (ACS grade) from Aldrich was used to adjust the ionic strength of the polyelectrolyte solutions and for the doping solutions. Polyethylene glycol (PEG $M_w = 8000$ g mol\(^{-1}\)) was purchased from Alfa Aesar. All solutions were prepared using deionized water (Barnstead, 18 MΩ E-pure).

\subsection*{6.2.1 Polyelectrolyte multilayer preparation}

Multilayers made from PDADMA and PSS were built with the aid of a robot (StratoSequence V, nanoStrata Inc.) on silicon wafer substrates that were cleaned with “piranha” reagent (70\% H\(_2\)SO\(_4\) and 30\% H\(_2\)O\(_2\)). Caution: piranha is a strong oxidizer and should not be stored in closed containers), rinsed vigorously with distilled water and followed by drying with a flow of N\(_2\). Polyelectrolyte solutions were 10 mM based on the monomer repeat unit, and the ionic strength was fixed using NaCl at 1.0 M. The substrates were mounted on a shaft that rotated at 300 rpm. The dipping time in the polymer solutions was 5 min followed by three 1 min rinsing steps in water after each layer. (A/B\(_x\)) and (A/B\(_y\)A indicate a multilayer ending in B and A respectively, where x is the number of layer pairs, A represents the polycation and B the polyanion.

\subsection*{6.2.2 Force spectroscopy}

The stiffness of the films, represented by the apparent modulus, was determined by force-indentation measurements, or force curves, using an atomic force microscope (AFM). An MFP-3D AFM unit was equipped with an ARC2 controller (Asylum Research Inc., Santa Barbara, CA) and Igor Pro software. Multi75AI tips were used, with spring constant $\sim 2$ N m\(^{-1}\). The optical lever sensitivity (OLP) of the tip was first calibrated in air and then its spring constant
was determined by the thermal fluctuation technique. The sample was then immersed in salt solution at a given concentration and the OLP of the tip was recalibrated. Force maps of 4 x 5 points were performed on the PEMU in situ with a scan size of 20 x 20 µm. The distance from the surface and the velocity of the tip were set to 500 nm and 1 µm s\(^{-1}\) respectively unless stated otherwise. The term “apparent” modulus is used to describe the modulus recorded at a specific tip velocity. For viscoelasticity studies, force curves were performed at indentation velocities ranging from 50 nm/sec to 3.17 µm/sec on the PDADMA/PSS multilayer. The applied force and the resulting indentation are given by Equations 2.2 and 2.3.

All force curves were analyzed using Hertzian contact mechanics for a punch model as in Equation 4.1 which is valid for linear relations between force and indentation, and in this work the relation between the force and indentation were all linear.

By fitting the force vs. indentation curves, \(E_c\), can be obtained and related to the apparent modulus \(E_1\) of the indented material and \(E_2\) (of the indenter) using Equation 4.2. The radius of the AC240-TS tip was 10 nm as provided by the manufacturer.

### 6.2.3 Thickness measurements and water content in different PEG solutions

Thickness measurements were carried out by nano-indentation using the Multi75AI tips with a spring constant of ~2 N m\(^{-1}\). AC and contact mode imaging were used to measure the thickness of the dry and wet films respectively. The cantilever was tuned to resonate below its resonance frequency by 10%. The films were scratched gently using a tweezer, and the thickness was measured by scanning the AFM tip over the scratch to obtain an edge profile (step height). The scan size over the scratch was 90 x 90 µm\(^2\) and the scan rate was 0.5 Hz. The thickness was then deduced by the difference in height between the film and the scratch.

All freshly prepared multilayers were undoped in 10 mM NaCl for 12 hours, 10 mM NaCl was used to prevent the films from annealing. Following, the wet thickness and force curve were measured on the multilayer in 10 mM NaCl was obtained. Next, the films were dipped in different PEG weight percentages from 5 wt% to 50 wt%. PEG solutions were prepared with equivalent salt activity to 10 mM. The multilayers were kept in the different PEG wt% for no less then 24 hours after which the wet thickness in the corresponding PEG and force curve measurements were conducted also by AFM. Kinetic experiments showed that 24 hours were enough for all PEG percentages to dehydrate the multilayer.
Immersing the film in PEG solutions lead to water removal from the film. Following the percentage of water remaining was calculated from the difference in film thickness before (in 10mM NaCl) and after dipping in PEG. All thickness values were measured by AFM as described before. The difference in thickness of the films in PEG and the films when dry correspond to the water content measured as percent volume in different PEG percentages:

\[
\%H_2O_{(v/v)} = \frac{t_{PEG} - t_d}{t_{PEG}} \times 100
\]

(6.1)

Where \( t_{PEG} \) and \( t_d \) are the thickness of the films in PEG and in the dry state respectively. PSS/PDADMA were found to have 2.4 water molecules per ion-pair at room temperature conditions (30% RH), so to calculate an accurate water content this amount was subtracted from dry thickness of the multilayer measured at room temperature condition to acquire the thickness of the dry network, \( t_d \), as follows:

So, the weight ratio of water to dry matrix at ambient conditions \( R_d (w/ w) \):

\[
R_d (w/ w) = \frac{m_{H_2O}}{M_d} = \frac{2.4 \times 18}{318.69} = 0.1355
\]

(6.2)

Where \( M_d \) is the dry molar mass of PSS/PDADMA PEMU and is equal to:

\[
M_d = 1 \times M_{PSS} + 1 \times M_{PDADMA} + 0.06 \times (M_{PDADMA} + M_{Cl^-}) = 318.69\text{ g mol}^{-1}
\]

(6.3)

\( M_{PSS} \) is 183 g mol\(^{-1} \), \( M_{PDADMA} \) is 126 g mol\(^{-1} \), \( M_{Cl^-} \) is 35.45 g mol\(^{-1} \), and 0.06 corresponds to ratio of extrinsic PDADMA in PSS/PDADMA PEMUs.\(^{201} \)

The average density of PEMU matrix can be taken as 1.2 g/cm\(^3\), weight fraction of water is converted to volume ratio \( R_d (v/ v) \):

\[
R_d (v/ v) = \frac{V_{H_2O}}{V_d} = R_d (w/ w) \times 1.2 = 0.1626
\]

(6.4)
Volume ratio is equivalent to thickness ratio of water, therefore:

\[ t_{\text{H}_2\text{O}} = 0.1626 \times t_d \]  \hspace{1cm} (6.5)

Thickness of the multilayer at ambient conditions \((t_a)\) is the sum of dry matrix thickness and water contribution:

\[ t_a = t_d + t_{\text{H}_2\text{O}} = 1.1626 \times t_d \]  \hspace{1cm} (6.6)

t\(_a\) was measured for PEMUs at room conditions by AFM, and the above equation was used to determine \(t_d\) which was used in calculating water content of the PEMUs.

### 6.3 Results and discussion

#### 6.3.1 Osmotic pressure of PEG in solution

Osmotic stress technique had been widely used in biochemical studies for quantifying conformation changes in enzymes\(^{223}\), measuring changes in hydration\(^{224}\) and to the study the interaction between synthetic polymers\(^{225}\) and colloids\(^{226}\). Osmotic stress technique is based on controlled removal of water from the system by applying osmotic stress on the system\(^{227-228}\).

PEG is a neutral hydrophilic polymer with high solubility in water\(^ {229}\) and is capable of exerting high osmotic pressure in aqueous solutions. It has a big size in solution due to its huge hydration shell\(^ {230}\), and this make it capable of excluding other molecules in solution. The osmotic pressure exerted by PEG depends on molecular weight. Here PEG of molecular weight 8000 was used, because it can exert high osmotic pressure and was big enough not to diffuse into the system studied. Vapor pressure osmometry was used to measure the osmotic pressure at different weight percentages. The experiments were conducted at room temperature and the results are shown in Figure 6.1. The results showed that the osmotic pressure exerted by PEG is strong function of the weight percentage of PEG in solution.
6.3.2 Correlating the modulus of PEMU and water content

The water content in polyelectrolyte multilayers is controlled by the ionic strength of the surrounding medium. At high salt concentration the ionic-interactions between the monomers of the oppositely charged polyelectrolyte (intrinsic sites) within the film split leading to the formation of more extrinsic sites which are monomer unites compensated by counter ions. These extrinsic sites are highly hydrated and thus increase the amount of water within the film and make it soft. Jaber et al. showed that at high salt concentration, the modulus of PDADMA/PSS films depend on the crosslinking density and thus followed the theory of rubber elasticity, however at low salt concentration, the crosslinking density increased and the crosslinks became shorter and stiffer thus decreasing the degrees of freedom available for the chains. In the latter case the modulus followed the smith theory for the non-Gaussian distribution of chain length. It was concluded by Jaber et al. that the mechanical properties of PEMUs was only affected by the crosslinking density of the film. Recently, Porcel et al. and Hariri et al. showed that macroscopic polyelectrolyte complexes or hydrogels behaved as viscoelastic materials under uniaxial testing and shear deformation. They showed that water plays an important role in controlling the viscoelasticity of polyelectrolyte complexes.
Dehydration by PEG was used to study the water content inside PDADMA/PSS films while keeping the number of crosslinks between the polyelectrolytes intact and leaving water as the only variable. This technique will make it possible to test if factors other than the number of crosslinks affect the mechanical properties of PDADMA/PSS multilayer and here we were interested in seeing the effect of water content of the film. (PDADMA/PSS)$_{15}$ were prepared as described before at 1.0 M NaCl. These films were then dipped in 10 mM NaCl for 12 hours. In 10 mM NaCl, PDADAMA/PSS films were known to “hyperswell” as a result of osmotic pressure exerted by the residual extrinsic charge.$^{42,45}$ The PEMUs were dipped in the different PEG solution prepared at different weight percentages from 5 wt% to 50 wt% each having NaCl activity equal to 10mM. AFM was used to determine the wet thickness of the films before (in 10mM) and after dipping in the different PEG solutions and water content was estimated using Equation 6.5. Figure 6.2 showed a significant decrease in water with increasing PEG percentages. In 50 % PEG, 26 % by volume of water was lost from the film, which corresponds to 24 wt%. The theoretical prediction of the weight percent of bound water (water bound to the chains) in PDADMA/PSS is 29 wt%. In this experiment the remaining 24 wt% is less than what was predicted for bound water suggesting that at high PEG and thus at high osmotic pressure some bound water might be lost from the PEMUs.
To see the effect of water on the stiffness of PDADMA/PSS multilayer, the modulus of the films was determined in each PEG solution using force curve measurements conducted by nano-indentation and the change in modulus was then correlated with the change in water content. Force curves measurements were conducted at different indentation rates for all the PEMUs. Apparent modulus at each indentation rate was obtained by fitting the force vs. indentation graph to 10% indentation in each PEG solution. Following apparent modulus vs. indentation rate was plotted for all the samples and then extrapolated to zero indentation rate to obtain the equilibrium modulus. Equilibrium modulus was then plotted as a function of PEG (wt %) as shown in Figure 6.2. All samples were immersed in their corresponding salt or PEG solutions for 12 hours before testing. All force curve measurements were performed in situ in the test solutions. A kinetic experiment was carried out to decide the required time needed to equilibrium dehydration and shown in Figure 6.3. It was found that the dehydration time was so fast, in PEG 30% 7 minutes was more then enough to reach equilibrium dehydration (Figure 6.3). Figure 6.2 showed a strong correlation between the water content of the film and the equilibrium modulus, the water content of the film decreased with increasing PEG while the
equilibrium modulus increased and interestingly, there was an exponential increase in modulus with only a small decrease in water content clearly seen after PEG 20 wt%.

As shown in figure 6.2 that the equilibrium modulus increased with decreasing water content, the decrease in the amount water should be associated in an increase in crosslinking density. As explained previously by Jaber et al. who showed that at high salt concentration the modulus of PDADMA/PSS films was linearly proportional to the crosslinking density and thus followed the theory of rubber elasticity. On the other hand at low salt concentration the curve deviated from the rubber theory and the change in modulus with crosslinking density was fitted to the Smith model for the non-Gaussian distribution of chains length and this theory is presented in Equation 6.11.231

\[
\frac{\nu RT}{G} = \frac{1}{\Phi} - \frac{6\bar{C}_v}{5q^2n}
\]  

(6.7)
Where $\bar{n}$ is the geometric mean number of bonds in the network chain, $\bar{C}_n$ is a characteristic ratio given by $\bar{C}_n = n^{0.57}$, and $q$ is a dimensional factor determined by bond angles and length of the backbone chain, for a network containing only carbon-carbon single bonds, a value of 0.83 can be assigned to $q$. Jaber et al. and Calculated $\Phi$ to be 0.55 at 0 M NaCl for highly crosslinked network and is estimated to stay the same in 10 mM NaCl and this was in agreement with what was reported by Smith for highly crosslinked network. $\Phi$ (\(\nu\)) is the number of moles of subchains per unit volume and can be estimated according to Smith who used the following Equation 12.\(^{231}\)

$$\nu = 3\Omega Ip$$

(6.8)

Where $Ip$ is the ion pair density and is calculated according to Equation 6.14. $\Omega$ is network fraction or the fraction of ion pairing that exist in the network mode and is used here as 0.3 according to Jaber et al.\(^{46}\)

Here, to model the change in modulus, the ion pair density ($Ip$) at each PEG wt% was calculated according to the water content as follows:

The number of water per ion pair was estimated as the moles of water in the films to the moles of polymer matrix. The water content of the film can be obtained from the ratio of the wet thickness of the film to the dry matrix. Solving these equations together and given that the molar mass for the matrix is 318.69 g mol\(^{-1}\) and the density is 1.2 g cm\(^{-3}\) we can deduce:

$$\# H_2O = \frac{n_{H_2O}}{n_{\text{matrix}}} = \frac{t_{H_2O}}{t_{\text{matrix}}} \times \frac{318.69}{18 \times 1.2}$$

(6.9)

The Ion pair density in 1 mole of PEMU was measured as the moles of crosslinks (1\(-y\)), where $y$ in the doping level (extrinsic sites) per unit volume of PEMU (1.1 g cm\(^{-3}\) was used as the density for the wet PEMU). The Ion pair density ($Ip$) at each PEG percentage can be computed from the following equation:
The effective crosslinking density can be then calculated from Equation 6.11.

\[ ECLD = \Omega \eta \]  \hspace{1cm} (6.11)

Where \( ECLD \) is the effective crosslinking density, and \( \Omega \) is equal to 0.3

Given that the equilibrium shear modulus is \( G_0 = E_0 / 3 \), where \( E_0 \) is the equilibrium elastic modulus, the equilibrium shear modulus was plotted as a function of effective crosslinking density \( (ECLD) \) and shown in Figure 6.4.

**Figure 6.4** Change of equilibrium shear modulus \( (G_0) \) with effective crosslink density of PSS/PDADMA PEMUs in different PEG solutions. Also shown is the fit to smith model (red line)

The data in Figure 6.4 was fitted to the smith model that assumes non-Gaussian distribution of chains. Jaber et al. showed that the smith model was valid at high crosslinking density.\textsuperscript{46} In Figure 6.4 the smith model was not valid even at high crosslinking density, where only minor changes in crosslinking density caused large changes in modulus, keeping in mind
that in this experiment the number of extrinsic sites and number of crosslinks were the same for all the measurements at different PEG, and the only variable is the crosslinking density which is a function of water content of the film in each PEG solution as indicated in Equation 6.11, since the smith model did not fit the data even at high crosslinking density we could predict that factors other than crosslinking density was contributing to the change modulus, we think that upon the loss of water, the film was becoming more compact, thus less free volume was available, making the polymer chains less mobile and decreasing their degrees of freedom. These phenomena reduced the shielding between the polymer chains by the water molecules, thus enhancing polymer chains interaction. This demonstrated that water provide free volume to the polymer chains and act in lubricating the film which are two main characteristics of a plasticizer.

The effect of water on the mechanical properties of hydrogels was seen for a number of systems. The elastic modulus of poly(N-isopropylacrylamide) was shown to increase significantly when decreasing the water content. It was found that the effect of water in hydrogels is similar to the affect of temperature in elastomers. In Figure 6.3 there was a sharp transition in modulus with decreasing water content similar to glass-to-rubber transitions usually observed in elastomers when changing temperature.

To further explore and quantify the plasticizing efficiency of water, we followed the procedure used to measure the efficiency for a number of plasticizers common in industry. Sothornvit et al. showed that there was exponential decrease in modulus with increasing the plasticizer amount and the plasticizer efficiency was obtained from the exponential decay fit according to the following equation,

\[ E_p = E_{p0}e^{-kr} \]

where \( E_p \) is the modulus of the plasticized material, \( E_{p0} \) is the modulus of the material in absence of the plasticizer (absence of water in our case), and \( r \) is the mole ratio of the plasticizer. From the AFM thickness measurement (in dry state and in PEG with equivalent salt) we obtained the ratio of water to the polymer matrix. By plotting the modulus of the films at each PEG percentage versus the water to polymer ratio at each corresponding PEG solution we obtained the graph in Figure 6.5. The modulus showed an exponential decrease with increasing the water to polymer ration and by fitting this graph to the exponential decay explained before, \( E_{p0} \) and \( k_m \) were found to be 4200 MPa and 117 respectively. To double check for \( E_{p0} \) value obtained, a PDADMA/PSS multilayer was prepared and then heated in vacuum oven at 100°C to remove all the water in the film and this procedure
was followed before by Farhat et al.\textsuperscript{233} The film was then cooled under vacuum and transferred into a desiccator to prevent it from rehydration. Force curve measurements were performed on the film to obtain the modulus of the film when completely dry; a value of 4 GPa was obtained which was pretty close to what was obtained from the exponential fit. In comparison to other common plasticizers described in literature, water appeared to have higher plasticizing efficiency than (2-ethylhexyl phthalate) (DOP) which is used to plasticize poly(vinylchloride) (the plasticizing efficiency of DOP was 51).\textsuperscript{234-235} It is known that small molecules are more efficient in plasticization which gives water an advantage on other plasticizers that are commonly used.\textsuperscript{232}

The effect of water on the mechanical properties of the PDADMA/PSS was further extended to investigate any effect on the viscoelasticity of these films. Jaber et al. conducted dynamic mechanical testing and found that the crosslinking density affect the viscoelasticity of the films and not the hydration water.\textsuperscript{58} The data obtained in Figure 6.4 and 6.5 strongly suggested that water must have a big impact on the viscoelasticity of PDADMA/PSS films. Different indentation rates (equivalent to different strain rates) were made when doing force curve measurements on PDADMA/PSS films of increasing PEG percentages as shown in Figure 6.6. A change in the modulus of the films with different strain rates was due to the viscoelastic

![Figure 6.5](image_url)

**Figure 6.5** Change of equilibrium elastic modulus of PSS/PDADMA PEMUs with mole fraction of water to polymer matrix of the PEMU. The red line is fit to the equation $y = 4200 \times e^{-117x} + 17$
nature of the films. This change was found to decrease with increasing PEG percentage or with increasing dehydration indicating that the film is turning less viscoelastic i.e. more glassy and this was another indication strongly supporting the proposing made that water is plasticizing PDADMA/PSS multilayers.

**Figure 6.6** Change in modulus with increasing indentation rate for (PDADMA/PSS)$_{15}$ PEMUs in PEG 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50. The modulus is less affected by the indentation rate with increasing PEG weight percent as a result of water loss. The film becomes less viscoelastic (glassier) with increasing PEG.
6.4 Conclusions

Osmotic stress technique was used to study the effect of water on the mechanical properties of PDADMA/PSS multilayers. PEG was used as osmolyte to exert osmotic pressure on the film. Using osmotic stressor we were able to control the amount of water in the film while keeping the number of extrinsic and intrinsic sites the same, on the other hand the crosslinking density increased. The films were dipped in increasing PEG weight percentages. All PEG
solutions were prepared with equivalent NaCl concentration to 10mM. Increasing the PEG percentages lead to more water loss the films. Water content was estimated from the difference in thickness measured by AFM before and after dipping in PEG. Force spectroscopy using nano-indentation was used to study the mechanical properties of the film. We found a significant increase in modulus of the film as water was removed and the removal of water also affect the viscoelasticity of the multilayer. The multilayer was becoming less viscoelastic with increasing PEG weight percentages. This phenomena lead to the conclusion that water was plasticizing the PDADMA/PSS films and the plasticizing efficiency was found to be much higher then common plasticizers reported in literature.
CHAPTER SEVEN

CONCLUSIONS AND FUTURE REMARKS

Directing the fate of cells was achieved by varying the mechanical properties of their underlying substrate. In Chapter three we presented a system composed of poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) grafted with a photosensitive benzophenone (PAABp). The grafting procedure was followed using the well know carbodiimide coupling agent (DCC). The synthesis was then characterized by NMR, FTIR, and UV-Vis spectroscopy. A multilayer was then prepared from PAH as the polycation and PAABp as the polyanion. The presence of a photosensitive molecule embedded in the film allowed to systematically tune the stiffness of the film by UV-irradiation. The mechanical properties of the film varied over a wide range by adjusting the irradiation time. We also showed that other surface properties that affect the cells behavior such as surface topography and surface wettability remained intact after photocrosslinking. This was a promising system to be used as substrate for cell culture and to study the effect of substrate mechanical properties on the behavior of cells.

The PAH/PAABp prepared in chapter three was put into application in chapter four. Substrates made from PAH/PAABp were prepared and irradiated with UV at increasing time to form uniform substrates of increasing stiffness. Rat aortic smooth muscle cells (A7r5) were plated on the uniform substrates. These cells showed higher spread area and higher number of focal adhesion on the stiffest substrate and kept a non-adhesive morphology on the softer substrate. Then, we wanted to test the behavior of cells on the same substrate that has variation in stiffness (gradient). Two gradients were prepared using different procedures; a continuous gradient prepared using a neutral density filer, and an edge (steepest) gradient prepared by covering half of the film with a black shield while irradiating the other half. On the continuous gradient A7r5 cells adhered better on the stiffest side of the gradient in agreement with the trend on uniform substrate also A7r5 cells that happened to be on the soft side of the gradient were oriented and polarized towards the stiff side showing their ability to detect the gradient. On the edge gradient both A7r5 a U2OS cells were plated and showed some difference in behavior, focusing on a small area at the gradient, A7r5 cells that were on the soft side were oriented to the stiff side and some of the cells were able to cross the edge and migrate towards the stiff side where they adhered better, on the other hand U2OS cells that were on the soft side did not adhere.
at all and undergone apoptosis and died and they only lived and adhered on the stiff side. These results showed how cells are able to detect variation of mechanical properties of their substrates and how we can direct their destiny by changing the stiffness of the underlying substrate. So PAH/PAABp is a promising system for the improvement of biomedical devices, and studies are still on the way to coat some of the common implants such as titanium and characterize the behavior of cells on them and testing if this can make them better. Windrose plots and software analysis are still on the way to study the polarity and orientation of the cells on the gradient and to track their migration. Finally, PAH/PAABp system could be used in membrane ion separations.

In chapter five, using nano-indentation and performing swelling experiments we showed that films ending in poly(diallyldimethylammonium chloride) (PDADMA) swelled more then a film ending in poly(styrene sulfonate) (PSS), this correlated well with the change in modulus where PDADMA terminated films were softer then PSS ending films. The odd-even effect observed in the swelling and modulus of PDADMA/PSS encouraged us to think about the build-up mechanism of these films. To support our thoughts AFM images of PSS and PDADMA ending film were taken under wet and dry condition, only minimal difference in surface feature between the dry and wet conditions was observed for PSS ending film, on contrary the dry PDADMA ending film was very smooth and the film got much rougher when imaged under wet atmosphere as a result of increased swelling. The minimal difference in swelling for PSS ending films suggested a surface was more stoichiometric (intrinsic) which was evaluated by viscoelasticity study which showed that the equilibrium modulus of PSS ending film was equal to the bulk of the film. This lead us to put forward a mechanism describing the build-up of PDADMA/PSS films, when a PDADAMA layer is added, the surface was more extrinsic and thus hydrated. On the other hand when PSS was deposited it compensated the positive extrinsic sites and thus the surface became intrinsic and i.e. less hydrated and stiffer. As surface modulus appeared to be a strong function of surface composition, mapping the surface of PDADMA/PSS multilayer and measuring the modulus of the “hills” and “valleys” showed that they had equal modulus concluding that roughness that appear on the surface of PDADMA/PSS films was not due to phase separation as some literature suggested. Radioactivity studies by employing radiolabelled counter ions are being performed to quantify the amount of extrinsic sites for
PDADMA and PSS ending films and the results are in good agreement with what we proposed in this chapter.

In the previous chapters we discussed factors that affected the mechanical properties of PEMUs and appeared to be mostly controlled by crosslinking density. In chapter six we showed that water, present in the pores or bound to the chains, had a major contribution on the mechanical properties of PEMUs. An osmotic stress technique using poly(ethylene glycol) (PEG) as an osmotic stressor was used to systematically control the amount of water within the film while keeping the number of extrinsic and intrinsic sites unaffected. Decreasing the amount of water within the film significantly increased the elastic modulus and decreased viscoelasticity of the film. Water was found to provide free volume to the polymer chains and act in lubricating the film similar to the effect of plasticizers in polymeric materials.
REFERENCES


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BIOGRAPHICAL SKETCH

Professional Skills

- 5 years of experience in surface and structural characterization and mechanical testing of polyelectrolyte multilayer thin films (PEMUs) built by layer by layer fashion on silicon wafers or quartz and polyelectrolyte complexes.
- Experienced in organic synthesis and functionalization of polymers (mainly charged polymers).
- Extensive knowledge in the field of polymer-based materials behavior in solutions and at the solid interface.
- Cell Culture trained, Biosafety level 2 (BSL2), maintaining cells, subculturing, plating and studying the behavior of cells on thin polymer films (PEMUs) of variable mechanical and surface properties.

EXPERIENCE

Graduate research and teaching assistant, Department of Chemistry and Biochemistry, Florida State University, August 2007-Present

- Developed a new method to synthesize thiol-functionalized polyelectrolytes.
  - Thiol-based polyelectrolytes were used to tune the stiffness of PEMUs by oxidation to form disulfides crosslinks or breaking the disulfide crosslinks by reduction to thiols.
- Designed a method to synthesize fluorescently labeled polyelectrolyte used as surface coatings to observe the response of cells to viscoelasticity of the substrate.
- Developed new routes for the chemical modification of small photocrosslinkable molecules and coupling reactions to polyelectrolytes and synthesized zwitterionic N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) to enhance the efficiency of coupling and crosslinking in polymeric thin films.
- Designed films from photocrosslinkable polyelectrolytes with tunable mechanical properties and consistent surface chemistry used to monitor cell response to substrate stiffness for potential application as biocoatings for implants.
- Optimized a new method to prepare thin films with continuous gradient in stiffness by using neutral density filter that were used in exploring the effect of substrate mechanical properties on the adhesion, proliferation and differentiation of different cell types for medical applications.
- Designed photomask setup controlled with a step motor for consistent irradiation of photocrosslinkable PEMUs with UV light, used to prepare step-wise gradient in stiffness on PEMUs.
- Designed films from different polyelectrolytes to control surface chemistry (hydrophobicity, surface charge, etc.) using fluorinated and zwitter-ion functionalized polyelectrolytes.
- Optimized methods for controlling ion-permeability in polyelectrolyte thin films
- Developed a method to explore the viscoelasticity of polyelectrolyte thin films by nanoindentation using atomic force microscopy (AFM).
Developed a method to study the effect of nanometer surface structures and roughness on the mechanical homogeneity of PEMUs thin films.

Presented a new theory on the mechanism of polyelectrolyte multilayer build up from the behavior of individual polymers monitored by swelling and mechanical studies.

Designed a method to study structural parameters in PEMUs and polyelectrolyte complex hydrogels using osmotic stress from polyethylene glycol and established correlation between the structure and mechanical behavior of the materials studied by mechanical analysis techniques such as nanoindentation and tensile testing.

Enhancing the surface properties of catheters provided by a medical company by developing nonfouling surface coatings based on polyelectrolytes.

Experience in characterization techniques:
- Mechanical characterization of materials and thin polymer films: tensile and compressive testing, atomic force microscopy (nanoindention)
- Surface characterization techniques: optical/fluorescence microscopy, atomic force microscopy (surface imaging and force spectroscopy), ellipsometry, static and dynamic contact angle, plasma cleaning.
- Permeability studies of Redox ions using electrochemistry by rotating disc electrode.
- Spectroscopy: UV-Vis Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Fluorescence Spectroscopy.
- Nuclear Magnetic Resonance: $^1$H-NMR, Varian (300 MHz), Bruker (400, 600 and 700 MHz)
- Osmometry: Vapor Pressure Osmometry and Freezing Point Osmometry.

Enhanced research efficiency in my lab by.
- Organizing weekly meeting for literature discussions.

Professional Safety Training: Chemical waste management and lab safety (by Environmental Health and Safety Department (EHS) at FSU), radiation safety short course by EHS and biosafety levels 1&2.

TEACHING EXPERIENCE

- Undergraduate teaching at Florida State University of General Chemistry (laboratory and recitation) and Analytical Chemistry Laboratory (Introductory and Advanced levels). 2007-present
- Mentoring undergraduate research students aspiring to gain experience in their field of interest.
AFFILIATIONS

- American Chemical Society (ACS), member since 2009
- Lebanese Social Organization, Head of outreach and advertisement (2010-2011) and Treasure (2011-2012)

REPRESENTATIVE PUBLICATIONS

- Schlenoff, Joseph; Lehaf, Ali M.; Ghostine, Ramy; Al-Jisr, Rana “Mechanism of polyelectrolyte multilayer build-up” In preparation
- Hariri, Haifa H.; Lehaf, Ali M. and Schlenoff, Joseph B. “Mechanical properties of polyelectrolyte complexes and multilayers: Water as a plasticizer” Accepted in macromolecules

EDUCATION

- Ph.D., Materials/Polymer science and analytical Chemistry, The Florida State University, Tallahassee, Fl. Advisor, Prof. Joseph B. Schlenoff. Fall 2012
- B.Sc., Chemistry, Lebanese University, Beirut, Lebanon, 2005