APPLICATION OF POLYELECTROLYTE MULTILAYERS FOR PHOTOLITHOGRAPHIC PATTERNING OF DIVERSE MAMMALIAN CELL TYPES IN SERUM FREE MEDIUM

by
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ABSTRACT

Integration of living cells with novel microdevices requires the development of innovative technologies for manipulating cells. Chemical surface patterning has been proven as an effective method to control the attachment and growth of diverse cell populations. Patterning polyelectrolyte multilayers through the combination of layer-by-layer self-assembly technique and photolithography offers a simple, versatile and silicon compatible approach that overcomes chemical surface patterning limitations, such as short-term stability and low protein adsorption resistance.

In this study, direct photolithographic patterning of poly(acrlic acid)(PAA)/poly(acryl amide (PAAm) and PAA/poly(allyl amine hydrochloride) (PAH) polyelectrolyte multilayers was developed to pattern mammalian neuronal, skeletal and cardiac muscle cells. For all studied cell types, PAA/PAAm multilayers behaved as a negative surface, completely preventing cell attachment. In contrast, PAA/PAH multilayers have shown a cell-selective behavior, promoting the attachment and growth of neuronal cells (embryonic rat hippocampal and NG108-15 cells) to a greater extent, while providing a little attachment for neonatal rat cardiac and skeletal muscle cells (C2C12 cell line). PAA/PAAm multilayer cellular patterns have also shown a remarkable protein adsorption resistance. Protein adsorption protocols commonly used for surface treatment in cell culture did not compromise the cell attachment inhibiting feature of the PAA/PAAm multilayer patterns. The combination of polyelectrolyte multilayer patterns with different adsorbed proteins could expand the applicability of this technology to cell types that require specific proteins either on the surface or in the medium for attachment or differentiation, and could not be patterned using the traditional methods.

Dedicated to

My parents, grandmother and Sucheta

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LIST OF ACRONYMS/ ABBREVIATIONS

Acronym	Definition
PAH	Poly(allyl amine hydrochloride)
PDAC	Poly(diallyl dimethylammonium chloride)
PEI	Poly(ethylene imine)
SPS	Poly(sodium styrenesulfonate)
PVS	Poly(sodium vinylsulfonate)
PAA	Poly(acrylic acid)
PVP	Poly(vinyl pyrrolidone)
PEO	Poly(ethylene oxide)
PAAm	Poly(acrylamide)
PVA	Poly(vinyl alcohol)
PMA	Poly(methacrylic acid)
PDMS	Poly(dimethyl siloxane)
PEG	Poly(ethylene glycol)
DMSO	Dimethyl Sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)
RGD	arginine-glycine-aspartate
DETA	Trimethoxysilylpropyldiethlenetriamine
HBSS	Hanks Balanced salt solution

DMEM	Dulbecco’s modified eagle medium
UV	Ultraviolet
XPS	Xray photoelectron spectroscopy

CHAPTER ONE: INTRODUCTION

Manipulation of mammalian cells has attracted a lot of attention due to its potential application in tissue engineering, biosensors and drug screening devices. Numerous methods, including patterning through surface modifications, [1] have been developed to generate proper position and interaction of cells. Various approaches, such as UV lithography [2], laser ablation [3], soft lithography [4, 5] and laminar flow patterning in microfluidic channels [5], and materials, such as photoresists [2], polylysine [3], alkanethiolates [4, 6], elastomeric PDMS membrane [7], phospholipid bilayers [8], PEO terminated triblock copolymer [9], hyperbranched poly(acrylic) acid films [10], grafted polyethylene oxide [11], polyethylene glycol hydrogels [12], polyelectrolyte multilayers [13], interpenetrating network of polyacrylamide and polyethylene glycol [14], polyglycolic acid [15], functionalized poly-p-xylylenes [16], hyaluronic acid [17], etc., have been successfully used as cell attachment supporting or inhibiting surfaces and consequently for cell patterning. The limitation of these materials in actual devices is the low stability of the patterns [18]. The patterns are usually destroyed in a few days after plating, as cells start to grow in the cell resistant areas. The short life of the cellular micropatterns is due to the following reasons 1) the degradation of the coating material through oxidation or other mechanisms [19] and 2) a slow build up of an adsorbed protein layer, originating from the culture medium (serum) or secreted by the cells themselves, on the top of the surface patterns [20-22].

Some of the surfaces like interpenetrated networks of PAA- PEG- and polyelectrolyte multilayers have been successfully used to form stable cell patterns [23-25]. So far

polyelectrolyte multilayers have been the most promising candidates for cell patterning. The cell adhesion resistance of polyelectrolyte multilayers is based on the compliance, molecular architecture and other physical properties like swellability of the film [24]. Therefore, they are more resistant to the modifying effect of adsorbed proteins. Moreover, polyelectrolyte multilayers are highly stable after crosslinking and their deposition is a simple process, very similar to biological systems with nanoscale control over thickness, compositions and molecular structure [13].

Polyelectrolyte multilayers can be either cell attachment resistive or promoting depending upon their properties and the cell type [13, 24-28], this makes them promising candidates for patterning diverse cell populations. Although the patterning of several cell types such as NR6 fibroblast [19], neuron [29-31], primary hepatocytes [32], chondrosarcoma cells[33], microvascular endothelial cells [34] and smooth muscle cells [35] has already been demonstrated using polyelectrolyte multilayers, a comparative study with more than two cell types has not been done. Moreover, the combination of polyelectrolyte multilayers with different adsorbed proteins as well as the investigation into the protein resistance limits of the patterns could expand the applicability of this technology to cell types which require specific proteins for attachment or differentiation either on the surface or in the medium, and could not be patterned using the traditional methods.

CHAPTER TWO: LITERATURE REVIEW

The assembly of layers of charged particles from solution was described for the first time by Iler in 1966. The films were prepared on a hydrophilic glass surface from alumina fibrils, which were positively charged with a diameter of 5-6 nm, and silica colloid, which was negatively charged with particle size of 100 nm, with a resulting thickness ranging from 50 – 500 nm in diameter. [(36, 37)]. Ultrathin polyelectrolyte multilayer assembly deposition by the layer-by-layer technique was first described by Decher et. al. in 1991 [(38)]. They synthesized anionic and cationic bipolar amphiphilic compounds. The compounds were then dissolved in a mixture of DMSO & water and pure water. The positively and negatively charged amphiphiles in the solution were then alternatively deposited in layers on a substrate with water rinsing between the layers. Deposition of 35 alternating layers of positive and negative charges led to a film with a thickness of 170 nm [(39)]. The layer by layer deposition of polyelectrolytes and their properties have gained much interest since then. As shown in Figure 1, there has been a tremendous increase every year in the number of publications in this area of research [(40)].

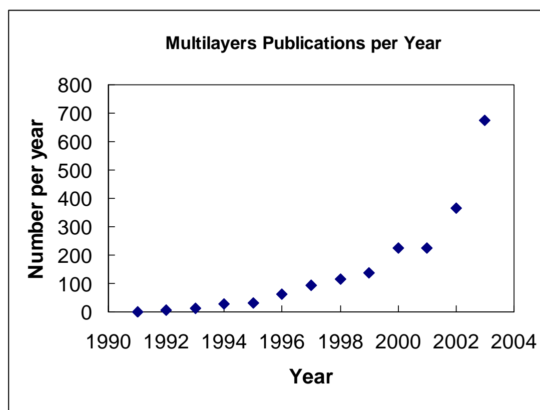


Figure 1: Multilayer publications per year

2.1. Polyelectrolytes

Polyelectrolytes are highly charged macromolecules or aggregates formed in the aqueous solution by dissociation of charged units of these macromolecules [41]. Some examples of polyelectrolytes found in nature are DNA, RNA, lipids and proteins. DNA and RNA form negatively charged anions in a solution surrounded by small counter ions. Proteins have polar groups which can dissociate in solution to form an anion or a cation. The charges present on these natural polyelectrolytes makes them suitable for layer by layer deposition[42]. There are many synthetic polyelectrolytes such as Poly(allyl amine hydrochloride) (PAH), Poly(diallyl dimethylammonium chloride) (PDAC), Poly(ethylene imine) (PEI), Poly(sodium styrenesulfonate) (SPS), Poly(sodium vinylsulfonate) (PVS), Poly(acrylic acid) (PAA), Poly(vinyl pyrrolidone) (PVP), Poly(ethylene oxide) (PEO), Poly(acrylamide) (PAAm), Poly(vinyl alcohol) (PVA), Poly(methacrylic acid) (PMA), Polyaniline etc [42, 43], which can also be deposited by the layer by layer technique. The structures of some of the polyelectrolytes are shown in Figure 2. For the past one and a half decade polyelectrolytes have been explored for many new applications by depositing them using the layer by layer technique.

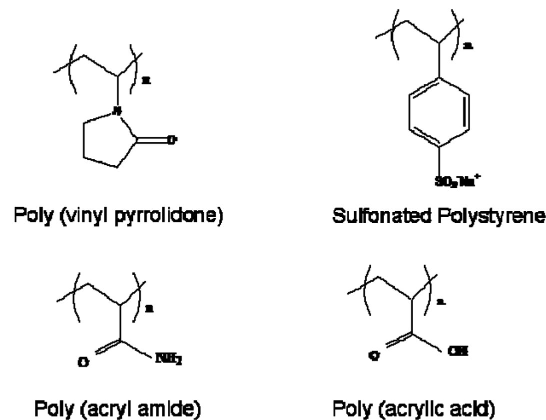


Figure 2: Structure of some common polyelectrolytes

2.2. Layer by layer deposition is a versatile bottom up fabrication technique

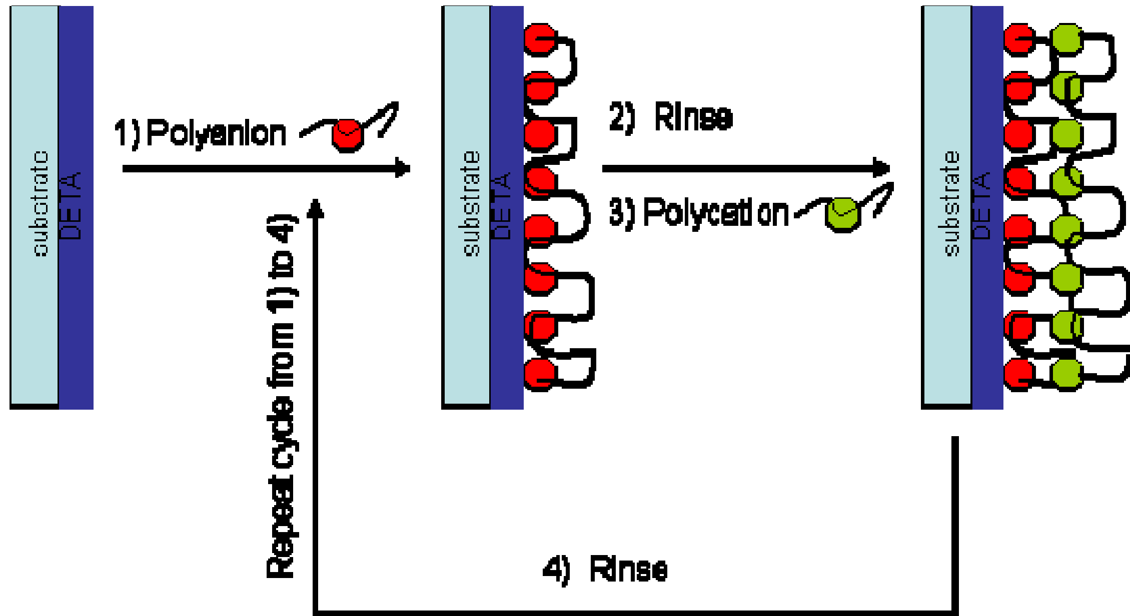


Figure 3: layer by layer deposition process

Figure 3 illustrates the layer by layer deposition technique. It is a simple process in which multilayers are constructed one layer at a time by alternate dipping of the substrate in a positively charged polymer solution and a negatively charged polymer solution to deposit polymer from aqueous solutions. These charges on the polymers can be from the delocalized charged defects along the backbone of a doped conjugated polymer or ionic charges on the pendant sidegroups (44). There are many features of the layer by layer process which make it an interesting and useful technique: 1) This method is suitable for the fabrication of nanostructures with accurate placement of the materials in desired geometry(42). Ultrathin films can be created with nanoscale control over thickness and molecular level manipulation and processing of polymer layers(43, 44). There is a precise control of the structure and properties at a nanoscale level. 2) The technique is environmentally friendly as the processing is water-based and can be automated at a low

cost and can be easily integrated with current technologies[45]. 3) A combination of two polymers can be used to deposit thin and thick layers depending on the charged state of the polymers [46]. 4) Polyelectrolytes of both synthetic and of natural origin can be deposited into multilayer films by the layer by layer technique.

2.3. Properties of polyelectrolyte multilayer films

2.3.1. Bonding

The driving force behind the deposition of multilayers is the bonding between the polymers in the multilayers. Multilayers have been deposited using various types of bonding forces such as ionic, hydrogen, donor acceptor, covalent, stereoregularity based etc. The bonding forces depend on the nature of the polymers and on the pH of the solution used for deposition. The polymer pairs that are used at fully charged state are assembled via electrostatic or ionic attraction. By changing the degree of ionization of these polymers the thickness and nanoarchitecture of the multilayers can be adjusted at a molecular level [45]. Multilayers have also been assembled with hydrogen bonding as driving force. Polyaniline paired with PVP, PVA, PAAm and PEO assemble by hydrogen bonding forming interpenetrating layers of polyaniline and the hydrogen bond forming polymer [43]. Other types of bonding for multilayers include donor acceptor or charge transfer type, [47]. stereoregularity based [48] and covalent type [49]. Therefore, multilayers can be deposited with diverse types of interactions acting as a driving force for the deposition. The hydrogen and ionically bonded polyelectrolyte multilayers are the ones that have been most frequently used for micropatterning the cells. The type of bonding and hence the effective nanoarchitecture affects the cell adhesion characteristics of the multilayers. For example the weak polyelectrolytes PAA (pKa = 5) and PAH (pKa = 9)

when deposited at different pH yield different types of nanoarchitectures and bonding between the multilayers due to different degree of ionization of the polymers at different pH. Mendelsohn et-al [(24)] studied the effect of bonding between the layers on cell attachment behavior. They studied cell attachment behavior of PAA/PAH multilayers deposited at pH 2.0/2.0, 3.5/7.5 and 6.5/6.5 using murine NR6WT Fibroblast cells. The layers deposited at pH 2.0/2.0 were lightly ionically bonded as most of the COOH groups in the PAA were in protonated state. At pH 3.5/7.5 the polyelectrolytes were partially ionized, therefore the polymers absorb in loop rich confirmation with high degree of internal charge pairing. The polymers did not form well blended multilayers at this pH, rather were deposited as almost discrete layers. At pH 6.5/6.5 both polymers were almost fully ionized forming thin multilayers of equal blending of both polymers. The cell attachment behavior was found to vary with the configuration of the multilayers deposited at different pH. The coatings deposited at pH 2.0/2.0 were resistant to cell growth however the coatings deposited at pH 3.5/7.5 and 6.5/6.5 promoted cell growth of NR6WT fibroblast cells. They carried out similar experiments with PMA/PAH at 6.5/6.5 and 2.5/2.5 which yielded completely and lightly ionically stitched multilayers respectively. The results obtained were similar to the PAA/PAH system, the cells adhered to the ionically stitched system whereas the lightly ionically bonded multilayers were cell resistant. This shows that the cell adhesion behavior is not only specific to the PAA/PAH system, it depended on the bonding between the multilayers. They studied cell adhesion properties of SPS/PAH systems deposited at pH 2.0/2.0 and pH 6.5/6.5. The SPS/PAH system forms highly ionically crosslinked multilayers at both of these pH conditions, it was observed that the multilayers assembled at both pH conditions promoted cell

attachment. The layers deposited at pH 10.0/10.0 were lightly ionically crosslinked and cell resistant, supporting the conclusion that the ionically crosslinked multilayers and their nanoarchitecture promote cell adhesion and not the deposition pH. They also studied system of two fully ionizable polymers PDAC/SPS at pH of 6.5/6.5. It formed highly ionically crosslinked multilayer hence promoting cell adhesion. However, when 0.25 M NaCl was added to the polymers solutions, due to the charge shielding effect of ions from the salt, the formed layers were less ionically bonded and more loopy thus becoming cytophobic in behavior. Thus, it was observed that for both weak and fully ionizable polyelectrolytes the layers with high degree of ionic bonding were cytophilic in nature and ones with low degree of ionic bonding and loopy architecture were cytophobic in nature. The cytophobicity of the lightly crosslinked multilayer was related to the high degree of swelling (~ 250 - 400%) of these multilayers in buffered conditions as compared to the significantly less (only 105-130%) swelling of the highly ionically bonded multilayers [(24)].

2.3.2. Thickness

The molecular organization and the thickness of the layers of the polymers which are bonded by ionic forces can be controlled by adjusting the ionic strength of the dipping solution and degree of ionization of the polymers. However, the high molecular weight polyelectrolytes have poor solubility in solutions of high ionic strength, consequently there is only a small ionic strength adjustment range for controlling thickness and composition. Therefore, weak polyelectrolytes have been studied, as the thickness and organization of the multilayers is strongly dependent on the pH of the dipping solution. The linear charge density of weak polyelectrolytes varies considerably with the change in

the pH near the pKa of the polyelectrolyte therefore the formation of the multilayer can be determined by controlling the degree of ionization of the polyelectrolyte [(45)]. By controlling the pH of the weak polyelectrolytes the bonding between the layers can be controlled from being completely ionic to completely hydrogen bonding. It has been observed that the thickness of the hydrogen bonded multilayers were greater than that of the ionically bonded multilayers. The thinner layers in the ionically bonded polyelectrolytes are due to the segmental repulsion of the ions distributed along the chains and providing less surface area for the attachment of the next layer. The thicker layers are formed in the case of the hydrogen bonded multilayers are due to the absorption of the polymer with a high density of chains extending from the surface providing a large surface area for the absorption of the next layer [(43)]. For cell patterning applications the thickness of the multilayers plays an important role as there is critical thickness of the multilayers which is required to effectively cover the substrate to make it either cell resistant or cell adhesive. For example it was observed that up to ~11 layers of the PAA/PAH 2.0/2.0 system the surface was cell adhesive, more than 15 layers were required to completely cover the substrate and make it cell resistant. Similarly for the PAA/PAH assembled at pH 6.5/6.5 at least 20 layers were required to make the surface completely cell adhesive and up to 11 layers it was cell resistant.

2.3.3. Mechanical properties

Recently, there has been lot of interest in the mechanical properties of the polyelectrolyte multilayers due to their potential applications as cell and tissue scaffolding materials and in biosensor applications. The attachment of cells to a substrate is dependent on the stiffness or modulus of the substrate. The cells very minutely experience the mechanical

cues from the substrate and respond accordingly. Overall the mechanical properties of the substrate influence adhesion, differentiation, migration and spreading of the cells [50].

Due to the small thickness and practically two dimensional nature of the polyelectrolyte multilayers, measurement of the mechanical and viscoelastic properties is difficult by conventional methods. There are many sophisticated techniques used for the measurement of the mechanical properties of the multilayers for example quartz crystal microbalance [37, 51], capillary wave technique [37, 52], point load nanodeflection experiments [37, 53] and nanoindentation of films on planar substrates[37, 54, 55]. It has been found that the Young's modulus of the multilayers varies between 10^{-5} to 12 GPa [37, 50, 56]. This big range in the Young's modulus of the polyelectrolyte multilayers can be used to create multilayers with tunable compliance and use the surfaces for differential attachment of cells. It has been observed that a surface with low Young's modulus is resistant to the attachment of cells. In contrast, surfaces with higher young's modulus are cell adhesive. For example a study was conducted to correlate compliance of PAA/PAH multilayer with their cell attachment response to the human dermal microvascular endothelial cells [34]. It was found that the cell attachment number increased linearly with the increase in modulus of the fully hydrated multilayer. The modulus of the multilayer increased with the increase in ionic bonding in the multilayers. As mentioned in the previous sections the ionic bonding character between the PAA/PAH layers increases with the increase in the pH of deposition. PAA/PAH multilayers, assembled at pH 6.5/6.5 had $2X10^5$ cells on a 60 mm diameter area and a modulus of ~ $1.5X10^8$ Pa, whereas those assembled at pH 4.0/4.0 had $1.5X10^5$ cells and a modulus of ~ $5.5X10^7$ Pa and the ones assembled at pH 2.0/2.0 had $0.5X10^5$ cells and a modulus of ~ $4X10^5$ Pa. Also, after 7

days no cells remained attached to the compliant 2.0/2.0 PAA/PAH multilayer whereas the high modulus 6.5/6.5 PAA/PAH still had ~ 130 cells\mm^2 attached to it [(34)]. In another study, the mechanical properties of multilayer films based on poly(l-lysine)/hyaluronan were correlated with their cell adhesion response to HCS-2/8 human chondrosarcoma cells [(50)]. The films were prepared at pH 6.5, and then their elastic modulus was varied by crosslinking the films at different concentrations of EDC, the crosslinking agent. The films showed an exponential increase in the elastic modulus with increase in the EDC concentration and obtained a plateau value after a critical concentration of 50 mg/mL. The cell attachment number as well as spreading area increased with increase in the EDC concentration. When the cell surface area were correlated with the elastic modulus of the film it was found that it increased with the modulus and started to plateau after the modulus reached a value of ~ 450 KPa.

2.4. Applications of polyelectrolyte multilayers

The nanotechnology has brought revolution in the world by miniaturizing devices like computers, storage devices, displays, cellular phones etc. All these devices are based on the top – down fabrication techniques of the silicon industry. However, to further go down in the size scale of the devices, the bottom up type nanofabrication driven by self assembly has recently gained interest. The layer by layer deposition technique provides a simple and versatile means of the bottom up fabrication process. The applications of the layer by layer based systems can be divided into mainly two groups such as physical & device applications and chemical & biological applications [(42)]

2.4.1. Physical and device based applications

Layer by layer process offers a simple fabrication method along with great control over thickness which makes it attractive technique for modern device fabrication. Many applications such as solar cells, fuels cells, electrochromic devices, chemical sensors and semiconducting devices have been explored by the layer by layer technique. Photoactive layer by layer films have been prepared by depositing semiconducting CdSe nanoparticles onto polyelectrolyte multilayer films with a goal to develop devices with high light energy conversion efficiency, low cost and ease of preparation [(57)]. All solid state electrochromic devices have been prepared by depositing electrochromic polymers by layer by layer technique. The electrochromic properties could be tailored depending on the layer sequence therefore making layer by layer an easy way for tailoring the properties of the device [(58)]. Layer by layer method has also been used in preparing photovoltaic cells. A rhenium-containing hyperbranched polymer and poly [2- (3-thienyl)ethoxy-4-butylsulfonate] (PTEBS) were deposited into thin films by layer by layer technique and their photocurrent responses were measured. Although the efficiency of the device was low, the layer by layer technique provided a simple versatile approach of preparing photovoltaic cells by solution process [(59)]. The layer by layer technique can prepare donor – acceptor films with precise molecular level control over the structure and energies of the active layers of photovoltaic cells. Fuel cells are another potential application which has been explored by layer by layer technique [(42)]. The crossover of vanadium ion through the proton exchange membrane such as Nafion results in low efficiency of the redox flow batteries. Layer by layer assembly of polyelectrolytes PDAC and SPS has been used to prepare a barrier layer on the surface of the Nafion membrane

which can prevent the crossover of Vanadium ions. Therefore, the batteries exhibit higher coulombic efficiency, energy efficiency and slower discharge rate without affecting the important properties of the Nafion membrane [(60)]. Optical chemical sensors have also been fabricated using layer by layer immobilization of a fluorescence indicator in polymer [(61)]. Superhydrophobic surfaces have been used for many applications like water repellency, contamination prevention, self cleaning and antifouling. Biomimetic superhydrophobic surfaces have been prepared by forming a rough surface on the smooth cellulose acetate nanofibers using the layer by layer technique [(62)]. Antireflective and antifogging coatings have been prepared by assembling silica nanoparticles and a polycation by layer by layer technique. The surfaces formed were superhydrophilic in nature [(63)].

2.4.2. Biological applications

Layer by layer technique has been used for preparing materials for biological applications like controlled release/ permeation substrates for drug delivery, bioinert coatings, odorant removal filters, surfaces for biosensors and drug screening devices [(42)]. The polyelectrolyte microcapsules prepared by layer by layer technique have been suggested to be used as microcontainers. The microcontainers such as liposomes, micelles and microemulsions have been used for containing DNA, drugs, enzymes, catalysts etc for applications in diagnostics, therapeutics, bio engineering catalysis etc. As compared to the previously used microcontainers such as liposomes, micelles etc., the polyelectrolyte microcontainers offer advantage of chemical multifuctionality which allows the modification of the shell at the nanoscale level. The polyelectrolyte capsules with gas core can potentially be used as ultrasonic contrast agent, gas dispersing container etc. [(64)].

The electroactive FDA approved material, Prussian blue has been deposited into thin films by layer by layer technique. These films undergo remotely controlled dissolution in the presence of a small applied voltage (+1.25 V) to release precise quantities of chemical agents. The films can find applications in drug delivery and tissue engineering [(65)]. The layer by layer technique can be used for encapsulation of DNA. DNA was encapsulated by first absorption of DNA onto amine functionalized silica. Then thiolated PMA and PVP were deposited on the DNA absorbed silica particle to form multilayers. The thiol groups of the PMA(SH) in the multilayers were crosslinked into disulfide linkages and silica particles were removed. The DNA could be controllably released by the degradation of the multilayer. These capsules can find potential applications in gene therapy and diagnostic applications [(66)]. The polyelectrolyte multilayer films can be functionalized with different molecules to manipulate cell behavior on surface. For example peptide PGA-NDPMSH chains were embedded in PAH/SPS multilayers and plasmid DNA (pDNA-PEI) complexes were deposited on top of the film. It was found that melanoma cells (B16-F1) could be transfected with the plasmid after 24h of contact with functionalized films. When the films were kept in contact with Huh-7 cells which do not express the NDPMSH peptide receptors, the films having the peptide significantly increased the transfection rate as compared to the film without the peptide, showing that it is possible to enhance the transfection process by incorporating specific peptides into multilayer films [(67)]. Chitosan/alginate multilayer films prepared by the layer by layer technique have been used for pH controlled loading of antibody in the multilayer films and pH controlled binding of the antigen to the immobilized antibody in the films. This kind of films can find applications as high sensitivity immunosensors [(68)]. Antibacterial

coatings have been prepared using the layer by layer technique by incorporating silver ions in the polyelectrolyte multilayers. Bactericidal chemicals have been incorporated in the multilayers and at the same time the multilayers were capped with nanoparticles functionalized with immobilized bactericides thus the coatings functioned in two ways by releasing the bactericide and by contact bacteria killing. [(69)]. Polyelectrolyte multilayer coatings have been used as bioinert material for controlling cell adhesion behavior. The main application of bioinert materials is coating implants and medical devices, which requires precise control of cell and protein adhesion properties. The uncontrolled adhesion of the cells to the device or insert can lead to devise encapsulation which may further lead to poor device performance or immunorejection. The advantage of polyelectrolyte multilayers is the possibility of conformal coating any substrate material of any shape and size, along with providing excellent control on the cell and protein adhesion behavior [(24)]. Polyelectrolyte multilayers have also been extensively explored for cell patterning applications. The patterns generated can have potential use in high throughput drug screening and biosensor applications. Multilayers based on polyelectrolytes like PAA/PAAm [(13)] and PEI/PAH have been used as substrates for cell patterning [(30)]. The patterns generated by polyelectrolyte multilayers have long term stability and high resistance to proteins and to the extracellular matrix components secreted by the cells [(13)]. The patterning of mammalian cells has been discussed in detail in the next sections.

2.5. Patterning mammalian cells on a synthetic surface

Patterning of mammalian cells has attracted a lot of attention due to its emerging applications in tissue engineering, biosensor design and cell based high throughput drug

screening devices. Cell patterning is a method where adjacent cytophobic and cytophilic surfaces are created so that cell attachment and growth can be controlled. There are three approaches to cell patterning (i) seeding cells on chemically patterned surface of different cell attachment properties, (ii) seeding cells on topographically patterned surface (iii) directed delivery of cells onto discrete regions of the substrate. The most common approach is the first one [(70)]. So far, a lot of surfaces and patterning methods have been used for patterning mammalian cells. The materials and methods commonly used for patterning are discussed in the following section.

2.5.1. Materials used for cell patterning

There are two kinds of materials that can be used for cell patterning, one which resist cell adhesion and other which promote cell adhesion. Kodak photoresists have been used for patterning cardiac myocytes. The photoresist patterning did not have an adverse effect on the growth and functionality of the myocytes [(2)]. However, the solvents used for the photoresist deposition and development may not be completely non hazardous and the residual solvents can create problems in the biological systems. There are chemically active photoresist materials reported for cell patterning which do not require the developing step. However, these materials require Photoacid generator (PAGs) for pattern generation, making the UV exposed region contain strong acid rendering the surface unsuitable for tissue engineering purposes. Generally, nitrobenzyl groups are the photocleavable groups in the photoresists, consequently they require high UV exposure and release aromatic aldehydes after cleavage which makes these photoresists less environment friendly. Diazoketo-functionalized aliphatic polymers have been reported as an alternative to the photoresists as they do not require PAG to generate patterns. These

polymers have been used to pattern mouse fibroblast cells and DNA [71]. Immobilized RGD peptide sequence on oligo(ethylene glycol) alkanethiolate has also been used for patterning cells. The efficacy of the pattern for controlling cell adhesion was varied by controlling the density of the RGD peptide on the pattern [72]. The problem with using the oligo(ethylene glycol) is low stability towards oxidation and the base coat of alkanthiolate has a restriction of using gold and silicon surface only. Also due to complicated multiple steps related to the preparation of these alkanethiolates the uniform coverage of the substrate is difficult to ensure [24]. Microfabricated elastomeric PDMS membrane stencils have been used for controlling cell attachment. The stencil was first placed on the cell culture substrate and then the cells were seeded through the stencil. After cells have attached the stencil was peeled off, leaving the cells in the form of islands in on the substrate [73]. The method of using stencils is simple and inexpensive but the surface on which cells are present and the adjacent surface are essentially the same, therefore the method is not suitable for long term cell patterning. Patterning of endothelial cells was carried out by patterning proteins, fibronectin islands laid on a substrate by microcontact printing. The remaining area was filled by a directed self assembly of phospholipid bilayers. The endothelial cells attached to the fibronectin only [74]. Hydrogels have been used for many years as material for micropatterning cells. Microgrooved collagen hydrogels have been used for aligning and growing human dermal fibroblasts and human umbilical artery smooth muscle cells [75]. Neurite outgrowth was guided in hyaluronic acid hydrogel where channels were formed in the hydrogel by an ultraviolet laser. Concentration gradient of immobilized glycine-arginine-glycine-aspartic acid-serine (GRGDS) oligopeptides was created in the channel

facilitating the neurite outgrowth [76]. Photocrosslinkable chitosan hydrogel was patterned by UV photolithography, cardiac fibroblasts, cardiac myocytes and osteoblasts patterned in the hydrogel showed their normal physiological properties [77]. Patterned scaffolds, based on poly(ethylene glycol) acrylate, have been demonstrated to mimic the complex tissue environment, such systems can be useful in studying cell behavior in a complex microenvironment [78]. One obstacle, which limits the application of these novel technologies in actual devices, is the relatively short lifetime of the created cellular patterns [18]. In many cases the patterns are destroyed a few days after plating, as cells start to grow in the cell resistant areas. The most possible causes for this short-term stability of the chemical surface patterns are 1) degradation of the coating material through oxidation or other mechanisms [19] and 2) a slow build up of an adsorbed protein layer, originating from the culture medium (serum) or secreted by the cells themselves, on the top of the surface patterns [20-22]

In earlier experiments, the interpenetrated networks of PAA PEG and polyelectrolyte multilayers have shown encouraging results in terms of high pattern stability [23-25]. The reason for the high stability of patterns on polyelectrolyte multilayers is that in contrast to chemical surface patterns, cell adhesion resistance of polyelectrolyte multilayers is not based on the hydrophobicity of the surface, but on the molecular architecture and physical properties of the film [24]. Therefore, they are more resistant to the modifying effect of adsorbed proteins. Moreover, polyelectrolyte multilayers are highly stable and their deposition is a simple process, very similar to biological systems with nanoscale control over thickness, compositions and molecular structure [13].

Polyelectrolyte multilayers can be either cell attachment resistive or promoting depending upon their properties and the cell type [24-27, 50], this makes them promising candidates for patterning diverse cell populations. The patterning of several cell types has been demonstrated using polyelectrolyte surfaces. NR6 fibroblast have been patterned on PAA PAAm multilayers, the patterns were stable for one month [19]. Neurons have been patterned using three bilayers of PDAC/ SPS and subsequently assembling biopolymers on the multilayer. It was observed that the polyelectrolyte precursor layers also affect the cellular response [29]. Retinal cells i.e., neurons have been patterned on PEI/SPS and PEI/PAH lines created on a PDMS microfluidic network. The patterns were created by flowing the polyions through the microfluidic channels. A total of 13 layers were deposited with positively charged polymer PEI and PAH on the top allowing the attachment of the retinal cells on the positively charged polymer vs. PDMS [30]. Primary rat hepatocytes have been patterned on PDAC and SPS by stamping PDAC (negative surface) on SPS (positive surface) [32]. pH dependent molecular architecture of poly(L-lysine) and poly(L-glutamic acid) was used to study the selective growth of chondrosarcoma cells. The cells grow on the films built at high pH whereas films built at low pH were cell resistant [33]. In other experiments microvascular endothelial cells were cultured on PAA\PAH multilayers and the cell adhesion behavior was studied by varying the mechanical compliance of the multilayer by assembling the multilayer at varying pH. The cells did not attach to the multilayer with low elastic modulus assembled at low pH. In contrast, the cells attached to the multilayer with high elastic modulus assembled at high pH [34].

2.5.2. Methods used for micropatterning cells

For creating patterns on the surface primarily two methods are used 1) Photolithography, 2) Soft lithography.

1) Photolithography: In this process the features of the photomask are transferred to the substrate using UV light or Laser ablation. In case of UV photolithography, the substrate is coated with a light sensitive photoresist/polymer. After the UV exposure of the photoresist/polymer through the photomask, the exposed region either becomes crosslinked or uncrosslinked depending on whether the photoresist is positive or negative. After UV exposure the uncrosslinked region is washed away with solvent or water [(70)]. In laser ablation the polymer/surface is covered with the photomask and the exposed region is ablated by laser pulses.
2) Soft lithography: In soft lithography elastomeric stamp is used for making a patterned relief. Photolithography is used for making the master. Replica stamps can be made by molding against the master stamp. The main advantage of the process is that it is low cost and unlike photolithography it does not require clean room environment. There are two kinds of commonly used soft lithography techniques microcontact printing and microfluidic patterning [(70)].

   Microcontact printing: Microcontact printing uses PDMS stamps fabricated from the photolithographically patterned master. The elastomeric stamp is then dipped in the material to be stamped, for example fibronectin. The material is then transferred by contact to the substrate [(70)].

   Microfluidic patterning: In microfluidic patterning a PDMS mold having the microfluidic network is stamped to the substrate. The solution of interest is then

injected through the microfluidic channel resulting in a pattern on the substrate. Microfluidic patterning can also be used for directed delivery of cells to the substrate [70].

2.5.3. Applications of micropatterned cells

There are three important potential application areas of cell patterning. 1) Cellular analysis, 2) Cell based sensing, 3) Tissue engineering.

1) Cellular analysis: Micropatterning provides a straight forward and inexpensive tool to reintroduce the *in vivo* structure into *in vitro* systems. For example organized synaptic connections are important for the appropriate functioning of neurons. When primary neurons are isolated from the tissue, the neuronal networks are destroyed. Patterning of the neurons is carried out to partially regain the functional interconnectivity in the original network. These patterned neurons help to study higher level functioning of the brain. Also patterning has been used to study cells other than neurons as it provided an easy way to control the location, shape, attaching area and number of attaching cells on a surface. Micropatterning has been used for distributing single cells on a surface for the study of gene expression kinetics in individual cells [70].

2) Cell based sensing: Cell patterning can also find potential applications in high throughput cell based sensing and cell based drug testing. Cell based microarrays can be used for high throughput drug screening. Neurons and cardiac myocytes have been developed for functional drug screening based on extracellular multielectrode recording of their action potential. The patterning

of the cells has improved the proximity of the cells to the electrode increasing the sensitivity of the method [70].

3) Tissue engineering: Micropatterning has been used to mimic the cellular microenvironment *in vitro*. Constructs can be prepared on patterned substrates for transplantation. The patterned substrates can direct the cell attachment, growth, differentiation and migration of cells. Such guiding of cell behavior is very important to obtain a functional tissue in vitro. The limitation of this approach is that the constructs are difficult to detach from the surface [70].

CHAPTER THREE: METHODS

3.1. Materials

3.1.1. Coatings

Poly(arylic acid) (PAA) (MW 90,000, 25 wt% solution) was purchased from Polysciences. Poly(acryl amide) (PAAm) (MW 10,000, 50 wt% solution) and Poly(allyl amine hydrochloride) (MW 70,000) were purchased from Sigma Aldrich. Trimethoxysilylpropyldiethylenetriamine (DETA) was obtained from United chemical Technologies Inc.

3.1.2. Cardiac myocyte culture

Calcium and magnesium free Hank's balanced salt solution (HBSS), Trypsin, Trypsin inhibitor, Collagenase and Leibovitz medium (dissolved in cell culture grade water and filtered through a 0.2 μm filter) were obtained from Worthington Biochemical Corporation together in their neonatal cardiomyocyte isolation system. Ultraculture (general purpose medium) was obtained from Bio Whittaker Cambrex. Dulbecco's modified eagle medium (DMEM) (containing high glucose 1X, 4.5 g/L D-glucose, L-glutamine, 110/mg/L sodium pyruvate), L- glutamine (200 mM, 100X), Penicillin (10,000 units/mL), Streptomycin (10,000 μg/mL), B-27, non essential amino acids (MEM NEAA) (100X), HEPES buffer (1M) and Fetal Bovine serum were obtained from Gibco/ Invitrogen. Dextrose was obtained from Fisher Scientific. Growth factors L-thyroxine and Epidermal growth factor (EGF) were purchased from Sigma and Hydrocortisone was purchased from BD.

3.1.3. Hippocampal cell culture

Hibernate E medium was purchased from BrainBits (Springfield, IL). Neurobasal E medium, B27, Glutamax and Antibiotic/Antimycotic supplement were obtained from Invitrogen.

3.1.4. NG and C2C12 cell culture

DMEM by HyQ (containing 4 mM L-Glutamine, 4500 mg/mL glucose and sodium pyruvate), Fetal Bovine Serum, HAT (100X) and B-27 supplement were obtained from Gibco.

3.1.5. Patch clamp electrophysiology

All chemicals were purchased from Sigma Aldrich. Borosilicate glasses (BF150-86-10) were obtained from Sutter (Novato, CA).

3.1.6. Calcium imaging of cardiac myocytes

Calcium imaging dyes Fluo-4 and Fura Red were purchased from invitrogen. Pluronic was purchased from invirogen and DMSO was purchased from sigma.

3.2. Multilayer deposition on coverslips

DETA coverslips were prepared as described by Das et al. [79, 80]by cleaning glass coverslips (VWR, 22 X 22 mm^2) with O_2 plasma cleaner (Harrick, Ithaca, NY) for 30 minutes at 100 mTorr. DETA was deposited on clean coverslips by dipping them in 0.1% (v/v) mixture of DETA in freshly distilled toluene. The DETA coverslips were heated to just below the boiling point of toluene for 30 minutes, rinsed with toluene and again heated to just below the boiling point of toluene. The coverslips were then dried in an oven overnight. The DETA coverslips were coated with the multilayers on an automatic dipping machine (StratoSequence Slide Stainer). 0.01 M solutions of PAA and PAAm

were prepared in deionized (DI) water by taking the molecular weight of the repeat unit of each polymer into consideration; in addition, their pH was adjusted to 3.0 using a 1 M HCl aqueous solution. First, the coverslips were immersed in the PAA solution for fifteen minutes and then rinsed with pH 3.0 water three times in separate beakers for 2 minutes, 1 minute and 1 minute, respectively. The coverslips were then immersed in PAAm solution for fifteen minutes and then rinsed with pH 3.0 water three times as described above. This cycle was repeated 20 times to deposit 20 bilayers of PAA and PAAm. The coverslips were then kept in an oven at 140°C for eight hours for cross linking.

Similarly, PAA/PAH multilayers were deposited by using 0.01 M solutions of PAA and PAH at a pH of 3.5 and 8.5, respectively. The coverslips were immersed in the PAA solution first for fifteen minutes and then rinsed with DI water three times in separate beakers for 2 minutes, 1 minute and 1 minute, respectively. The coverslips were then immersed in the PAH solution for fifteen minutes and then rinsed with DI water three times as described above. This cycle was repeated 20 times to deposit 20 bilayers of PAA and PAH.

3.3. Patterning of coverslips

The coverslips were patterned using a deep UV (193 nm) excimer laser (LambdaPhysik) at a pulse power of 230 mW and a frequency of 10 Hz for 2 minutes through a quartz photomask (Bandwith Foundry, Eveleigh, Australia). Patterns were visualized by phase contrast microscopy or with an epifluorescent microscope after fluorescent tagging, namely the PAA/PAAm patterned coverslips were dipped in fluorescently tagged PAH at a pH of 8.5.

3.4. X-ray photoelectron spectroscopy

The bare glass, PAA/PAAm, PAA/PAH coatings, Laser ablated PAA/PAAm, PAA/PAH coatings and protein incubated coverslips were examined by X-ray photoelectron spectroscopy (XPS) using a Kratos (Manchester, UK) Axis 165 equipment according to established protocols [79, 80]. XPS survey scans as well as high resolution C 1s, N 1s, O 1s and Si 2p were obtained using monochromatic Al kα excitation.

3.5. Contact angle measurement

Contact angle measurements were performed according to published protocols [79, 80]. Briefly, contact angle of a static, sessile drop (5 µL) of deionized water was measured using a CAM 200 digital goniometer (KSV Instruments, Ltd.). Three measurements were taken and averaged.

3.6. Plating cells on the patterns

3.6.1. Cardiac myocytes

Two-day-old rat pups were euthanized with Halothane. Hearts were dissected and minced in ice cold HBSS. Cardiac myocytes were dissociated by incubation in trypsin (1000 µg in 10 ml HBSS) for 20 h at 2-8°C followed by collagenase (1500 units in 5 ml L15 medium) treatment for 45 min at 37°C and mechanical trituation. The cell solution was then centrifuged at 50 g for 5 minutes at 25°C. The cells were resuspended in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin streptomycin and preplated in Petri dishes and kept in an incubator at 37°C and 5% CO_2 for 45 minutes. The preplating step was carried out to separate fibroblasts from myocytes. The supernatant from the Petri dishes were centrifuged at 50 g for 5 minutes at 25°C. The cells were resuspended in the plating medium consisting of: 100 ml Ultraculture medium

supplemented with 10 mL B-27, 1 mL L-glutamine, 1 mL Penicillin Streptomycin, 0.375 g dextrose in 800 µL water, 1 mL non essential amino acids and 1 mL HEPES buffer. Growth factors were added in following concentrations to the medium, L-thyroxine 0.1 µg/mL, EGF 10 ng/mL and Hydrocortisone 0.5 µg/mL. Cells were plated at a density of 10^5 cells/ cm^2 on the coverslips. Non-coated clean glass coverslips were also plated at the same density in serum containing medium as described above as a positive control for the experiment. The medium was changed after 24 hours of plating. Subsequent changing of the medium was carried out every fourth day.

3.6.2. Hippocampal cells

Embryonic rat hippocampal cells were cultured according to established protocols [(81)]. Briefly, the hippocampus was dissected from E18 rat embryos in ice-cold Hibernate E medium. Tissue was minced and mechanically dissociated using a 1 ml pipette. Cells were centrifuged at 300g, 4°C for 2 min and resuspended in the plating medium consisting of Neurobasal E medium supplemented with B27, Glutamax and Antibiotic/Antimycotic. Cells were plated at a density of 200 cells/mm^2. Cultures were maintained in an incubator at 5% CO_2 and 37°C.

3.6.3. NG 108-15 cells

About 1 million frozen NG 108-15 cells were thawed and centrifuged at 300 g for 5 minutes in DMEM medium containing 2% HAT and 10% FBS. The cells were resuspended in the same medium and plated in a 75 cm^2 culture flasks for proliferation. Upon confluency the cells were plated on the PAA/PAH patterned coverslips at a density of 100 cells/mm^2 in the serum-free differentiating medium consisting of DMEM and 2% B-27.

3.6.4. C2C12 cells

C2C12 cells were cultured and plated according to the same protocol as the NG108-15 cells [82, 83] on PAA/PAAm patterned coverslips. The coverslips were incubated with different proteins before plating the C2C12 cells. The plating density was 300 cells/mm^2.

3.7. Statistical analysis of cell resistance of the coatings

The cell resistance and cell adhering properties of the coatings were evaluated statistically by plating all the above cell types on PAA/ PAH, PAA/ PAAm, Glass and DETA. The cells were counted in each frame at a magnification of 10X and the count was averaged over 10 frames for two coverslips of each type. The counting was done on days 3 and 6 of the culture.

3.8. Patch clamp electrophysiology

Patch clamp experiments on hippocampal and cardiac cells were performed according to published protocols [79, 80]. In brief, whole-cell patch clamp recordings were performed in a recording chamber on the stage of a Zeiss Axioscope 2FS Plus upright microscope at room temperature, in the culture medium, where the pH was adjusted to 7.3 with HEPES. Patch pipettes were prepared from borosilicate glass with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, $MgCl_2$ 2, Na_2ATP 2, Hepes 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon, Union City, CA). Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were performed with pClamp 10 software (Axon). Action potentials were evoked with 1s depolarizing current injections from a -70 mV holding potential.

3.9. Calcium imaging in cardiac myocytes

10% pluronic stock solution was prepared in DMSO. 2 mM stock solutions of Fluo-4 and Fura red in DMSO were prepared by adding 20 μL of DMSO to 50 μg of Fluo-4 and 23 μL of DMSO to 50 μg of Fura red. 1mM stock solution of Fluo-4 and Fura red were prepared by adding 20μL of 10% pluronic stock solution to Fluo-4 stock solution and 23 μL of 10% pluronic stock solution to Fura red stock solution. 2μL of 1mM Fluo-4 and 20 μL of 1mM Fura red were added to 2 mL of L-15 media. The coverslip was then incubated in the dye containing media for 1 hour at 37°C. The cells were then washed with L-15 media. The cells were then observed under confocal microscope. The fluorophore excitation and emission of 488/10nm-525/50nm and 568/10nm-600/45nm were used for Fluo-4 and Fura red, respectively.

CHAPTER FOUR RESULTS AND DISCUSSION

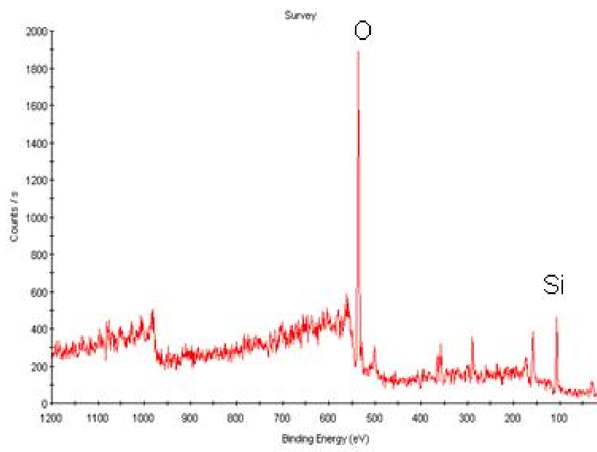
4.1. Results

4.1.1. Polyelectrolyte deposition, patterning and visualization

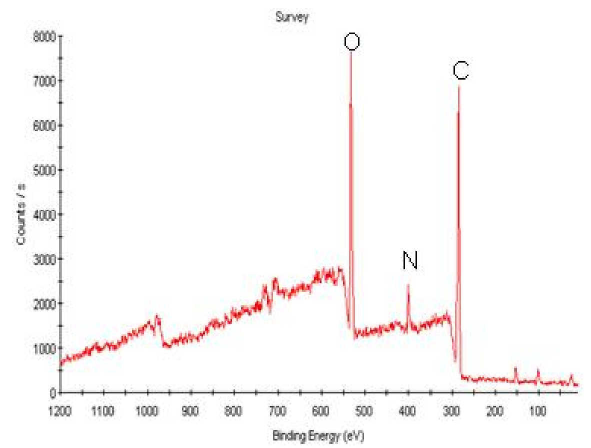
For the initialization of the first layer of the polyelectrolyte, we used trimethoxysilylpropyldiethylenetriamine (DETA) covalently – modified glass coverslips as the substrates. DETA coverslips have a strong inherent positive charge on the surface, which makes the uniform deposition of the multilayers easier in comparison to deposition onto clean glass substrates.

Formation of polyelectrolyte multilayers were verified by contact angle measurements, X-ray photoelectron spectroscopy (XPS) and visual inspection. Static contact angle values for PAA/PAAm and PAA/PAH multilayers were 101.4±5.2 and 67.1±3.3, respectively. The appearance of large carbon and nitrogen peaks in the XPS spectra verified the formation of thick, uniform polyelectrolyte multilayers.

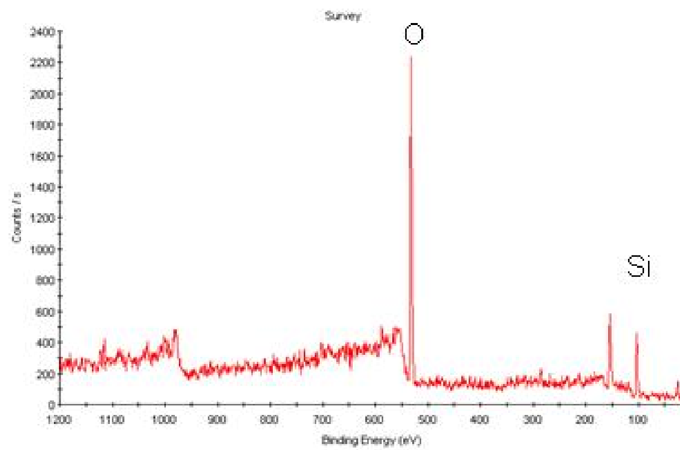
A) Clean glass



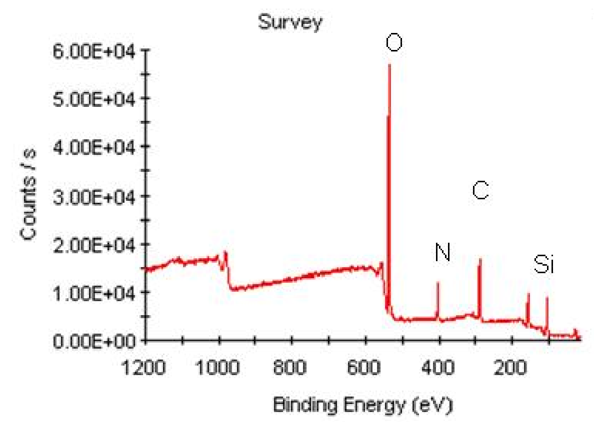
B) PAA/PAAm



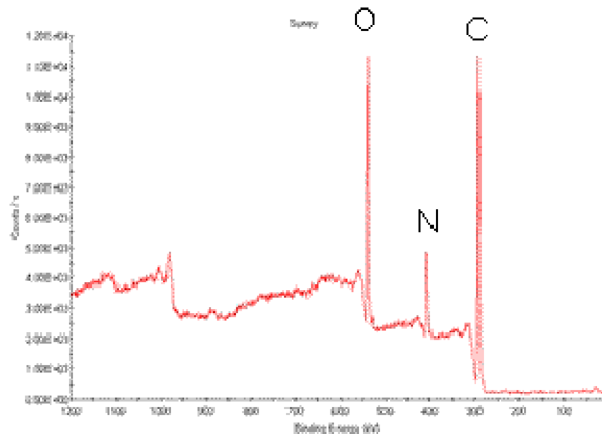
C) Ablated PAA/PAAm



D) Ablated PAA/PAAm + Fibronectin



E) PAA/ PAH



F) Ablated PAA/PAH

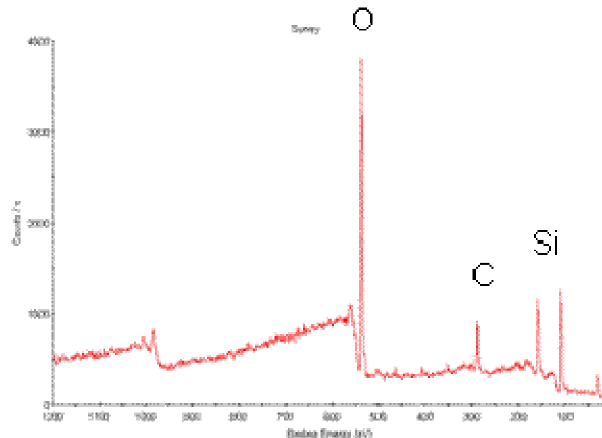


Figure 4: XPS survey scans of A) Clean glass, B) PAA/PAAm coating, C) Ablated PAA/PAAm D) Ablated PAA/PAAm after fibronectin incubation, E) PAA/PAH, F) Ablated PAA/PAH

The ablation time for patterning the polyelectrolyte multilayers was set to remove all measurable traces of the film, which was proven by XPS measurements on the coated and ablated coverslips. The XPS survey spectra obtained on the glass substrate, after the deposition of PAA/PAAm multilayers and after ablation of the film, are shown in Figure 4 The carbon and nitrogen peaks, characteristics of the PAA/PAAm film, were also observed on the PAA/PAAm coated glass substrates, but not observed on the bare glass substrates or the PAA/PAAm coated glass substrates followed by laser ablation. Similarly, large carbon and nitrogen peaks were observed on PAA/PAH modified coverlips, whereas after ablation, there were only traces of carbon present and nitrogen was totally absent.

After selective ablation of the PAA/PAAm multilayers through a photomask (patterning), the border between the ablated and non-ablated regions were clearly visible through a

standard phase-contrast microscope (Figure 5A). For a more reliable visualization of the electrically charged multilayers, fluorescently- tagged PAH was used to bind with the multilayers and make the films fluorescent (Figure 5B).

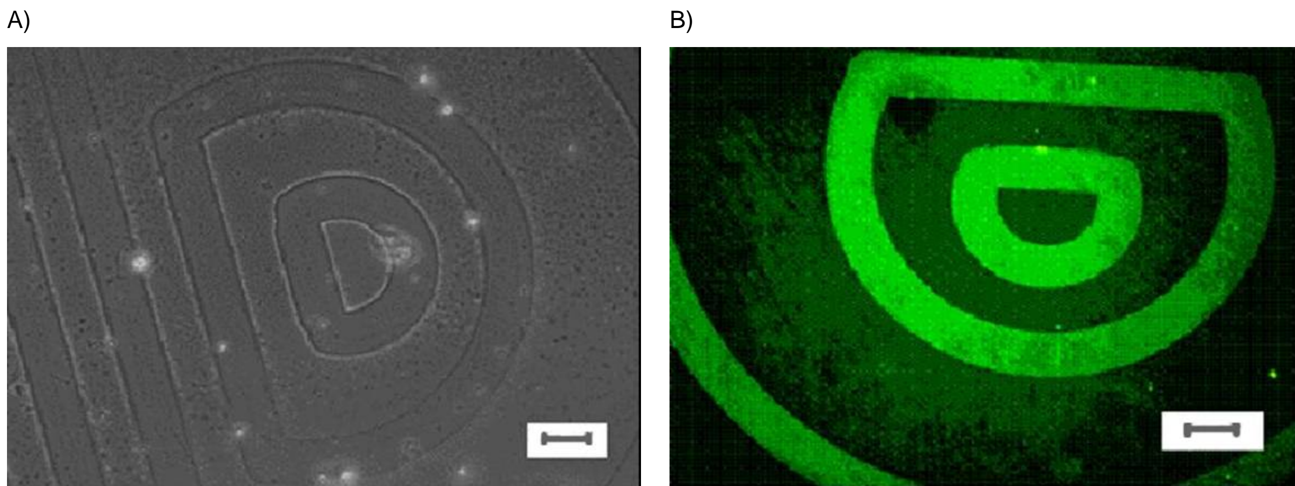


Figure 5: Visualization of patterned polyelectrolyte multilayers by A) phase contrast microscopy B) fluorescently tagged PAH. Scale bar depicts 100 µm.

The stability of the polyelectrolyte multilayers was greatly enhanced by crosslinking when exposed to 140°C for 8 hours according to Yang et al (84). According to the Fourier Transform Infrared Spectroscopy (FTIR) measurements, the thermal crosslinking resulted in the formation of imide bonds between PAA and PAAm, making the multilayer insoluble in water at a higher pH.

4.1.2. Cell attachment and growth on polyelectrolyte multilayers and patterns

This study investigated the applicability of polyelectrolyte multilayers for the patterning and manipulation of different mammalian cell types. Cell patterning usually requires two types of surfaces; one promotes cell attachment and growth, while the other prevents cell attachment and growth. In our experiments, two types of polyelectrolyte multilayers,

PAA/PAAm and PAA/PAH were used as the patterned substrates, and four cell types, embryonic rat hippocampal cells, neonatal rat cardiac cells, the skeletal muscle C2C12 cell line and the neuroblastoma / glioma NG108-15 neuronal cell line, were studied. In all cases, the cells were cultured in serum-free medium to prevent the cover up of the surface patterns by proteins generally adsorbed from the serum containing medium.

For all studied cell types, the PAA/PAAm multilayer behaved as a negative surface, completely preventing cell attachment and growth. In contrast, the PAA/PAH multilayer showed a cell-selective behavior by promoting the attachment and growth of neuronal cells (embryonic rat hippocampal and NG108-15 cells) and, to some extent, skeletal muscle cells; whereas it prevented the attachment and growth of neonatal rat cardiac cells. The statistical data on the attachment of the various cell types on the different surfaces is provided in Table 1 and Table 2

Table 1 A) Statistical analysis, Day 3 of the culture

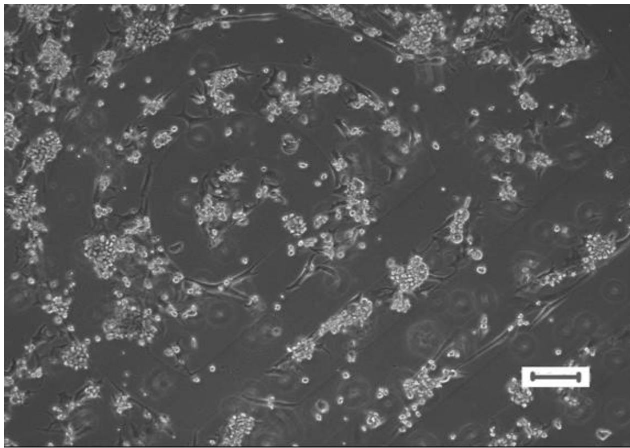
Surface	Cardiac	C2C12	Hippocampus	NG
PAA PAAm	1.9 ± 1.3	0 ± 0	0 ± 0	0 ± 0
PAA PAH	10.9 ± 5.4	10.6 ± 2.3	25 ± 6.8	27.5 ± 7.3
Glass	82.7 ± 15.6	24 ± 4.7	3.8 ± 2.2	1.4 ± 1

Table 1 B) Statistical analysis, Day 6 of the culture

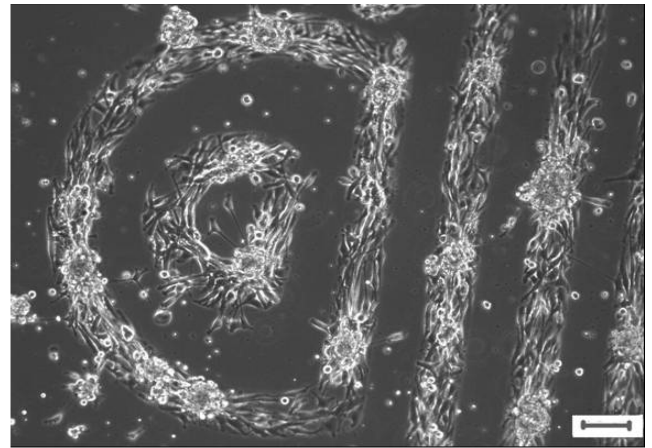
Surface	Cardiac	C2C12	Hippocampus	NG
PAA PAAm	1.2 ±1.1	0 ± 0	0 ± 0	0 ± 0
PAA PAH	27± 11.6	3.6 ± 2.4	19.1 ± 5.3	19.1 ± 5.7
Glass	89 ± 16.8	31.4 ± 6.1	1.5 ± 1.3	1 ± 1.9

Unfortunately, the technical difficulties associated with depositing and patterning more than one type of polyelectrolyte multilayers on the same coverslips prevented us from using this method to enhance the contrast between cell growth enabling and resisting areas. For this a clean glass was used as the alternative surface for cell patterning. Glass was utilized as a positive surface for cardiac myocytes and C2C12 skeletal muscle cells with s PAA/PAAm negative background; however, it was used as the negative surface for neurons with PAA/PAH as the positive surface.

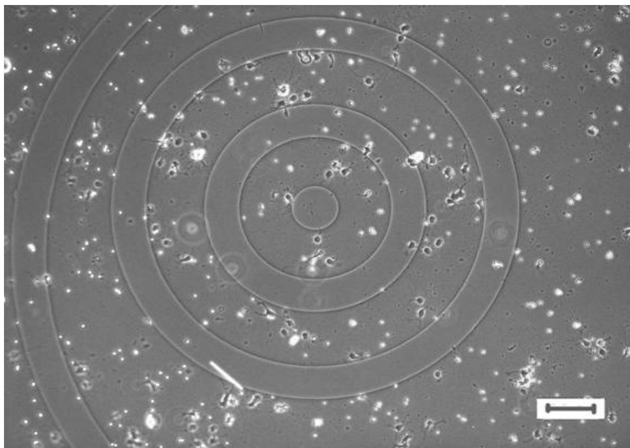
A) Cardiac Cells on a PAA/PAAm Pattern



B) C2C12 Cells on a PAA/PAAm Pattern



C) Hippocampal Cells on a PAA/PAH Pattern



D) NG108-15 Cell on a PAA/PAH Cells

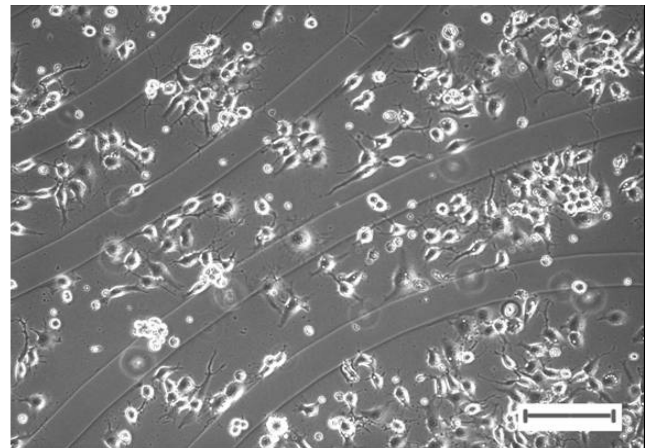


Figure 6: Cell attachment and growth on patterned polyelectrolyte multilayers PAA/PAAm was negative for all cell types. PAA/PAH was positive for neurons and negative for cardiac myocytes and skeletal muscle cells. Glass was negative for neurons, but allowed attachment and growth of cardiac myocytes and skeletal muscle cells. Scale bar depicts 100 μm

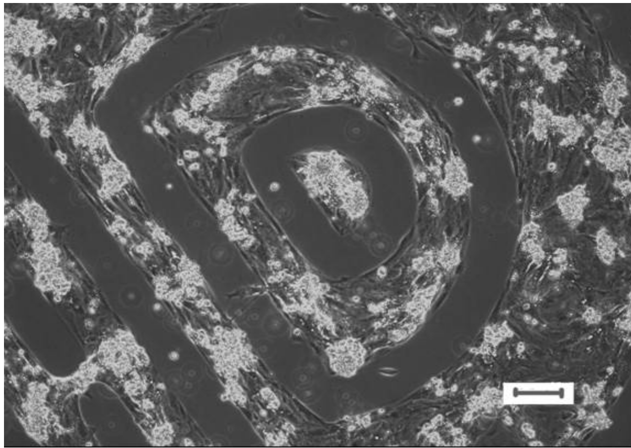
However, glass is not an ideal negative surface for promoting physiological development of certain cell types. For example, as reported earlier [83], C2C12 skeletal muscle cells do not form myotubes in serum-free medium without contact signaling that originated from the growth surface. Based on the fact that the attachment of cardiac myocytes is significantly better with fibronectin or serum on the surface, we have taken advantage of

the remarkable protein adsorption resistive feature of polyelectrolyte -based cellular patterns and used protein modified patterns to promote cell growth.

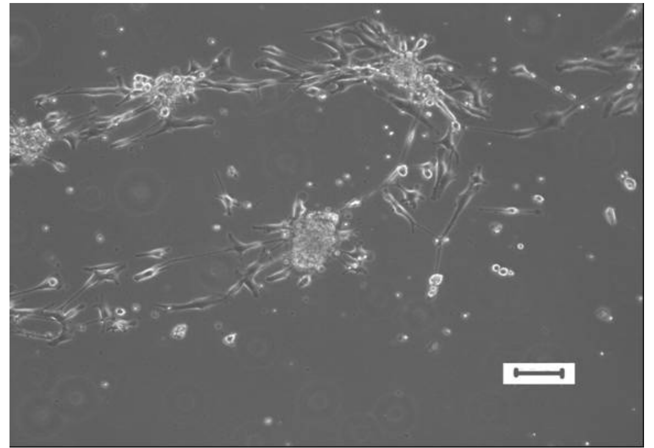
4.1.3. Cell growth on protein – modified polyelectrolyte patterns

In order to assess the protein adsorption resistance of polyelectrolyte based cellular patterns, we incubated the polyelectrolyte patterns in protein containing solutions for different durations of time before cell plating.

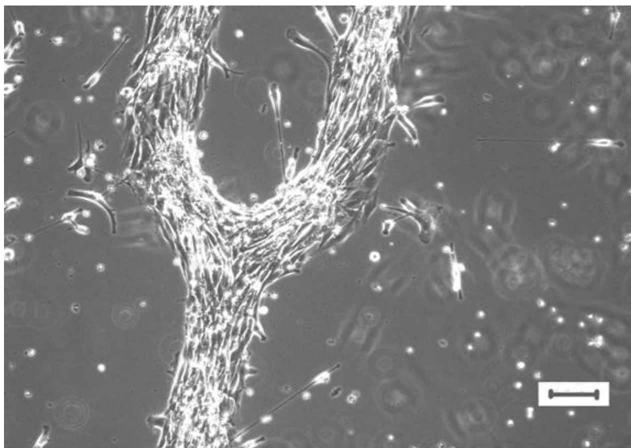
A) Cardiac Myocytes on glass vs. PAA/PAAm patterns, incubated with fibronectin



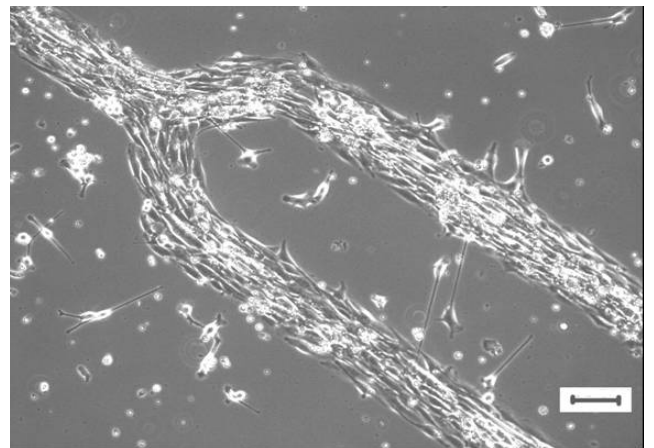
B) C2C12 cells on glass vs PAA/PAAm patterns, without incubation with 10% serum



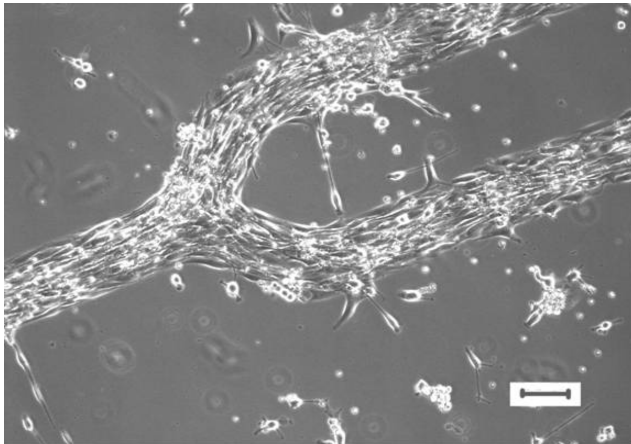
C) C2C12 cells on glass vs. PAA/PAAm patterns, incubated with 10% serum for 1 hour



D) C2C12 cells on glass vs. PAA/PAAm patterns, incubated with 10% serum for 2 hours



E C2C12 cells on glass vs. PAA/PAAm patterns, incubated with 10% serum for 4 hours



F) C2C12 cells on glass vs. PAA/PAAm patterns, incubated with 10% serum for 8 hours

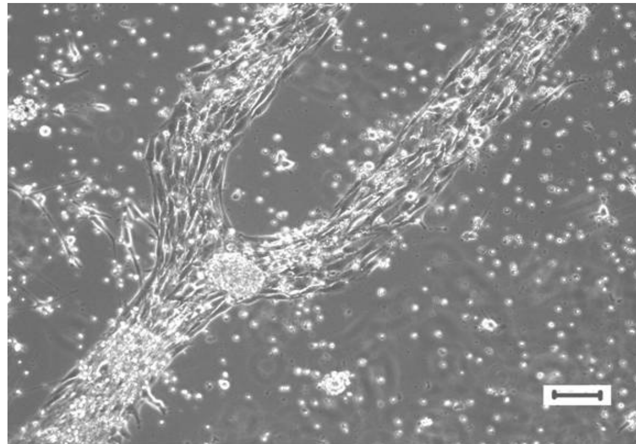


Figure 7: Effect of protein incubation on the patterns and cell morphology A) Cardiac myocytes on glass vs. PAA/PAAm surface patterns incubated with fibronectin B) C2C12 cells did not form myotubes without incubation with serum containing medium C)-F) C2C12 cells plated on glass vs. PAA/PAAm surface patterns incubated with 10% serum containing medium for 1 – 8 hours. Scale bar depicts 100 µm.

As noted in Figure 7, cardiac myocytes were complying with the patterns even after 1 h incubation in 0.2 g/L human plasma fibronectin solution. XPS data (Figure 4) show that a thick layer of fibronectin was adsorbed to the ablated portion of the PAA/PAAm coverslips during this time. This amount of protein adsorption on the PAA/PAAm multilayer did not change its cell-attachment and growth resistive properties. In the case of the C2C12 cells, protein absorbed on the glass significantly improved the cell growth and differentiation promoting properties of this surface, enabling the formation of C2C12 myotubes. In the experiments presented in Figure 7, the patterned PAA/PAAm coverslips were incubated with a 10% serum containing medium (NG proliferation medium) for different time periods. The patterns were observed on the second day of plating. The

pictures show that the pattern was formed without the protein incubation, but the formation of myotubes took place only after incubation with the serum containing medium.

4.1.4. Physiology of patterned cells

Visual inspection revealed no obvious morphological difference between the cells grown on the polyelectrolyte patterns and the cells grown on traditional control surfaces (PDL and DETA for neurons, fibronectin for cardiac myocytes). In order to evaluate the physiological properties of the excitable cells, whole-cell patch clamp recordings of spontaneous or evoked action potentials were performed in cardiac and hippocampal cells (Figure 8).

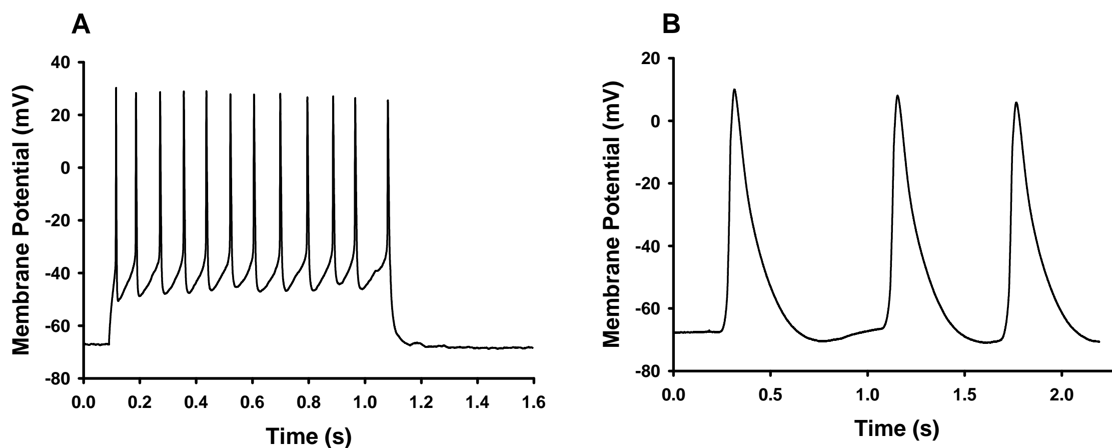


Figure 8: Electrophysiological characterization of hippocampal (A) and cardiac cells (B) grown on polyelectrolyte patterns for 2 weeks. Hippocampal cells were grown on PAA/PAH surfaces as a positive with clean glass as the background negative surface. Cardiac cells were cultured on fibronectin-treated clean glass as the positive and PAA-PAAm as the negative surface. Conventional whole-cell patch clamp recordings were performed on both cell types in current clamp mode. For hippocampal cells repetitive firing were evoked by 1s current injection. Cardiac cells were spontaneously active, thus,

no current was injected. Both cell types showed normal characteristic electrophysiological behavior on the patterns.

Action potential generation is a complex process and distinctive to the type and maturation state of the cells. Most of the recorded hippocampal cells fired repetitive action potentials upon prolonged depolarization, which is characteristic of mature pyramidal cells in culture. Cardiac cells fired spontaneous, short action potentials, which is characteristic of mature postnatal rat cardiac cells [80].

4.1.5. Calcium imaging of cardiac myocytes

Calcium imaging is carried out in the cells to detect the movement of calcium ions by using calcium indicators. The calcium indicators like Fura red and Fluo-4 chelate with the calcium ions in the cell and make them fluorescent so that they can be observed under confocal laser scanning microscope. The transfer of the action potential from one myocyte to the other was observed by the movement of calcium from one myocyte to the other. The snapshots of patterned myocytes showing the calcium movement are given in Figure 9

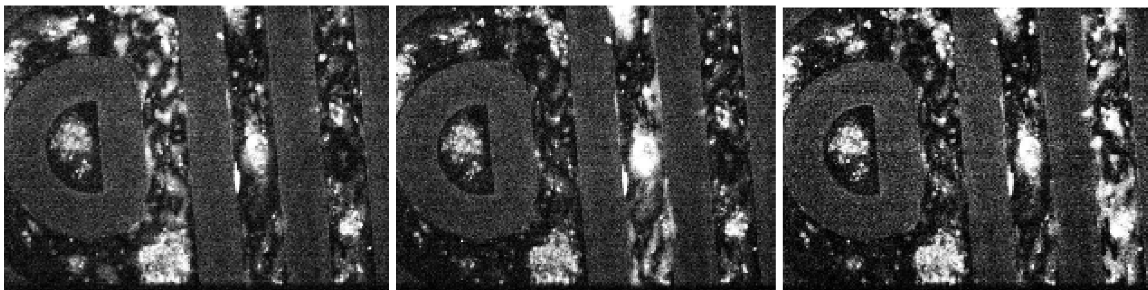
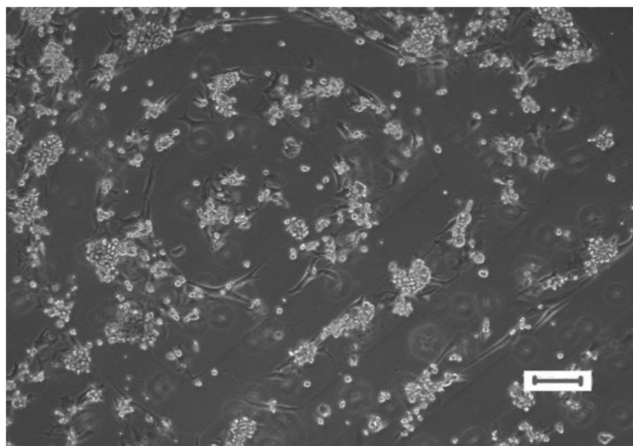


Figure 9: Calcium imaging of cardiac myocytes on a 100 day old pattern showing a spontaneous beating layer and no connectivity between the patterns

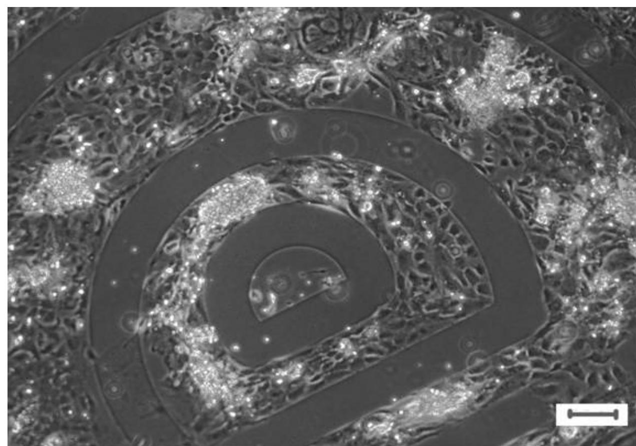
4.1.6. Long term stability of the pattern

The advantage of using the polyelectrolyte multilayers over other materials for patterning cells is the long term stability of the patterns. In our studies, polyelectrolyte-based cellular patterns were much more stable than the self-assembled monolayer-based patterns reported earlier [82, 83].

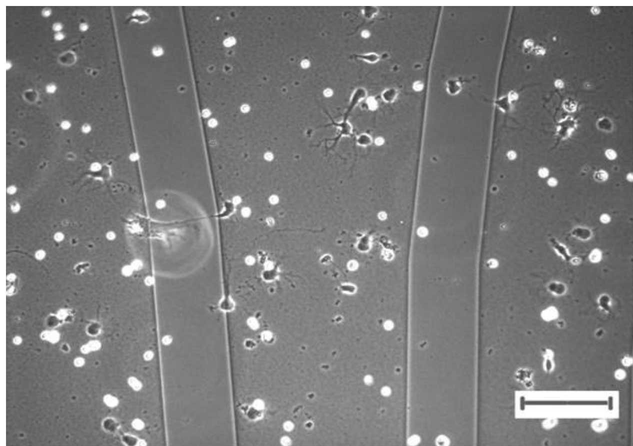
A) Day 4



B) Day 100



C) Day 3



D) Day 20

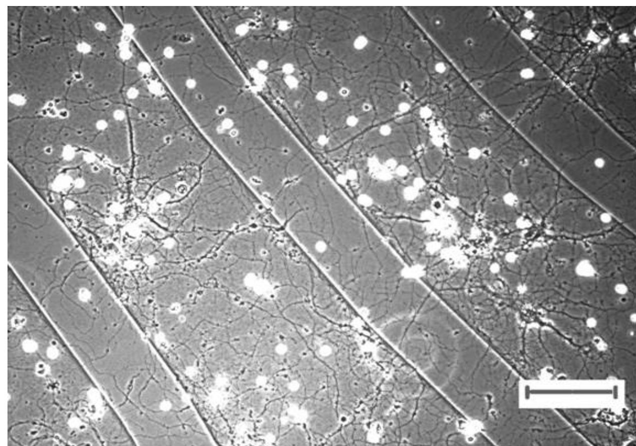


Figure 10: Long term stability of the polyelectrolyte-based patterns. Neonatal cardiac myocytes on day 3 (A) and day 100 (B). Hippocampal cells on day 3 (C) and day 20 (D) . Scale bar depicts 100 µm.

Figure 10 illustrates high fidelity cardiac myocyte patterns after 100 days in culture, which was not achievable with our earlier patterning methods. Moreover, our hippocampal patterns were stable for up to twenty days.

4.2. Discussion

In this study PAA/PAAm and PAA/PAH polyelectrolyte multilayers were patterned by laser ablation through a photomask in order to create cell attachment resistive and promoting areas on glass coverslips. The patterns were visualized by simple phase contrast microscopy or fluorescence contrast after the fluorescent tagging. PAA/PAAm multilayers prevented the attachment of all studied cell types. PAA/PAH was cell attachment promoting for embryonic rat hippocampal and NG108-15 cells, whereas it was inhibitory for neonatal rat cardiac myocytes and C2C12 skeletal muscle cells. Cellular patterns on the polyelectrolytes were exceptionally stable; cardiac myocytes did not overgrow the patterns and were beating for at least 100 days. Cellular patterns created with PAA/PAAm multilayers as the negative surface showed remarkable protein resistance, they tolerated standard, cell culture surface treatment protein adsorption protocols.

The Layer-by-Layer deposition of the polyelectrolyte multilayers was a simple, reliable process, did not require complex chemical procedures. In comparison with the commonly used covalent surface modification methods, it was simpler, less variable, robust and stable after crosslinking in cell culture conditions. Another advantage of the polyelectrolyte multilayers was the improved visualization; surface patterns were visible under a normal phase contrast microscope. In specific applications, such as time-lapse

imaging or repetitive multi-layer patterning, visualization of the patterns has been a challenging requirement.

Cell attachment inhibiting or promoting features of polyelectrolyte multilayers was determined by the molecular architecture and the physical properties of the layers. Therefore, the tunable and flexible properties of polyelectrolyte multilayers can be used to selectively pattern different cell types. For example, in our experiments PAA/PAH was positive for neurons and negative for muscle cells. Polyelectrolyte patterns combined with a simple and widely used protein adsorption surface treatment, which does not compromise the cell resistance of the background, could significantly enhance cell selectivity, as well as cell attachment promoting and physiological effects of the foreground. These unique properties could lead to various applications in many cell culture laboratories.

The origin of the cell resistance of the PAA/PAAm multilayers was based not on the chemical, but on the physical properties of the layers. The high degree of swelling (about 3.5 times) of PAA/PAAm in PBS, with the same ionic strength as the cell culture medium, makes the coatings soft and water like that they do not provide a rigid support for cell attachment [(13)]. PAA/PAH coatings deposited at the pH of 3.5/7.5, respectively, have been reported to be cell adhesive as they swell only to 130% of their original thickness in the buffered conditions [(24)].

Laser ablation through a photomask has proved to be a simple and effective way to create polyelectrolyte surface patterns, this method was high-throughput and compatible with standard silicon manufacturing process. Technical difficulties prevented the ‘backfill’ of the ablated areas with a second/different polyelectrolyte multilayer, instead, protein

modification of the background clean glass was utilized, as it is widely used in most cell culture practices.

CHAPTER FIVE: CONCLUSIONS

- Photolithographic patterning of polyelectrolyte multilayers is a simple, versatile and robust method to pattern cells with exceptional long-term stability and protein adsorption resistance.
- It has been reported in previous studies that depending on the mechanical compliance or degree of swelling the polylectrolyte multilayers can be cell adhesive or cell repelling. Our study also confirms that the PAA/PAAm multilayer which has shown high degree of swelling and mechanical compliance in other studies is cell resistant and PAA/PAH multilayer which has shown low swelling and mechanical compliance is cell adhesive.
- Different polyelectrolytes can be used for patterning different kind of cells. PAA/PAH multilayers can be used for patterning neurons and PAA/PAAm multilayers can be used for patterning skeletal muscle cells and cardiac myocytes.
- High fidelity beating patterns of neonatal rat cardiac myocytes were observed after 100 days on polyelectrolyte patterns in our novel serum-free medium.
- The patterned cells were physiologically active as shown by the electrophysiology and calcium imaging results.
- Pre-made polyelectrolyte patterns combined with commonly used protein adsorption, surface modification methods could extend the applications of the patterned cultures. For example the protein resistance of polyelectrolytes allows the use of a straight forward method for C2C12 derived myotube of protein

adsorption and myotube formation can be observed in the C2C12 culture after protein incubation.

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