Evaluation of Layer-by-Layer Assembly of Polyelectrolyte Multilayers in Cell Patterning Technology

by

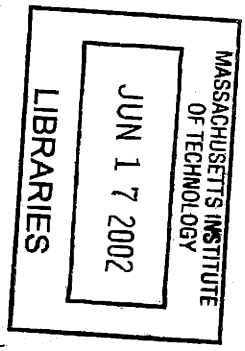
Aleksandr John White

B.S. Materials Science and Engineering
University of Michigan, 2001

SUBMITTED TO THE DEPARTMENT OF MATERIALS SCIENCE AND ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENGINEERING IN MATERIALS SCIENCE AND ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2002



Signature of Author: _____
Department of Materials Science and Engineering
May 10, 2002

Certified by: _____
Michael F. Rubner
TDK Professor of Polymer Materials Science and Engineering
Thesis Supervisor

Accepted by: _____
Harry L. Tuller
Professor of Ceramics and Electronic Materials
Chair, Departmental Committee on Graduate Students

Evaluation of Layer-by-Layer Assembly of Polyelectrolyte Multilayers in Cell Patterning Technology

by

Aleksandr John White

Submitted to the Department of Materials Science and Engineering
on May 10, 2002 in Partial Fulfillment of the
Requirements for the Degree of Master of Engineering in
Materials Science and Engineering

ABSTRACT

The layer-by-layer assembly of polyelectrolytes into multilayered films is an attractive approach for fabricating novel biomaterials, as it offers tremendous control over the internal composition and surface properties of their layered architectures. In this work, polyelectrolyte multilayers (PEMs) were evaluated as a platform for applications in controlling the spatial adhesion of living cells. An overview is presented on current developments and competing technologies within research and industry with respect to cell patterning and cell-based devices. Interviewed individuals in research and industry suggested a variety of potential applications of PEMs in cell patterning technology. A patent search on the core technologies (i.e. PEMs and patterning methods) and on applications in cell patterning, cell-based screening, and cell-based biosensors revealed ample opportunity for starting a new venture with a platform based on the layer-by-layer assembly of PEMs. A brief business plan for starting a new venture with a platform based on the layer-by-layer assembly of PEMs is proposed to initially target the high-throughput screening and cell-based biosensor markets.

Thesis Supervisor: Michael F. Rubner
Title: Professor of Polymer Materials Science and Engineering

ACKNOWLEDGMENTS

To Dr. Gene Fitzgerald –
For the DMSE MEng conception.

To the personnel that participated in this research –
For their investment in time and innovative suggestions.

To Dr. Michael Rubner and the Rubner group –
For your enthusiasm and assistance.

To Abel Hastings, Allison Hernandez, David Kupferer, and Larry Ong –
For your humor and friendship.

CONTENTS

1.0 Introduction

Advancements in biology and biotechnology often require new materials in elucidating complex biological phenomena and for the development of novel biomedical tools and devices. Since most biological phenomena occur on a molecular level, materials that can be tailored on a molecular scale are ideally suited. Control over surface and interface properties is particularly important in bioactive or bioresponsive materials. For example, specific surface functionality is necessary in order to study cells in a controlled manner and to manipulate their arrangement for applications in assays and sensor devices. Additionally, the ideal biological material should be easy to fabricate and customizable for a wide range of applications. The layer-by-layer assembly of polyelectrolytes into multilayer films is a technique that is well suited for these tasks. This process provides a means to build up polyelectrolyte complexes one molecular layer at a time, with unprecedented control over composition and surface functionality.

In this work, polyelectrolyte multilayers (PEMs) are evaluated relative to their application in cell patterning technologies. First, an overview of the fundamental technology is presented, including means to pattern the physicochemical properties of PEM surfaces. Some applications of the assembly of these multilayer films are discussed to illustrate the versatility of this technique.

The second section evaluates PEMs with respect to the ability to create surfaces and structures to control cell behavior, and to their application in cell-based systems. An overview of recent developments in cell patterning and cell-based devices taking place in research and in industry is presented. This section also includes a description of potential applications from the perspective of individuals in research and industry. The primary competing technology, i.e. self-assembled monolayers, is compared with the layer-by-layer assembly of polyelectrolyte multilayers outlining the pros and cons of each.

A patent search was conducted to assess the opportunity to market this technology and to develop novel products. Patents relative to the core technology and patents relating to applications in cell patterning and cell-based biosensors were evaluated with respect to their potential blocking power.

Lastly, a rough business plan is presented for a start-up venture to develop and market this technology. The high throughput screening market for drug discovery is chosen as the initial market to target.

2.0 Layer-by-layer assembly of polyelectrolyte multilayers and means of patterning their surfaces

In the midst of the nanotechnology boom, fabrication of more complex multicomponent nanostuctures and devices will require means to precisely control the arrangement of their elements on a nanoscale. There currently exists a simple and viable method of consecutively stacking single molecular layers, onto solid supports with high precision along the layer normal. Decher and co-workers,[1] in the early 1990's, developed a technique to construct multilayer thin films, one molecular layer at a time, using polyelectrolytes. Polyelectrolytes are polymers, which when dissolved in a polar solvent, spontaneously acquire or can be made to acquire a number of elementary charges distributed along their chain.[2] In the assembly process, a substrate is sequentially immersed into aqueous solutions of a positively charged polyelectrolyte (polycation) followed by a negatively charged polyelectrolyte (polyanion), with a rinsing step in between (Figure 1). Strong electrostatic attraction between polyanion and polycation is

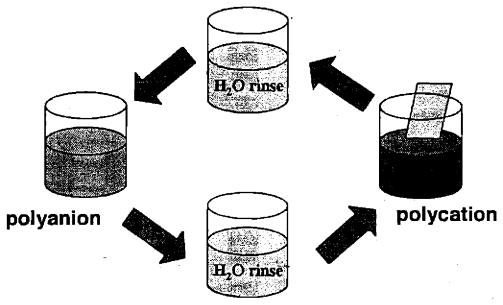


Figure 1. Schematic of the multilayer film deposition process using a slide and beakers

the driving force for assembly. In the first step, the adsorbing polyion creates a new surface atop the original substrate surface. This new surface repels other polyelectrolytes of equal charge, thereby permitting only one molecular layer at the surface. This newly

charged surface, however, promotes the adsorption of oppositely charged polyelectrolytes onto the first layer in a second step. Repeating these steps an arbitrary number of times enables the formation of multilayer structures.

PEM assemblies may be built using either strong or weak polyelectrolytes, or combination of the two. Strong polyelectrolytes, such as poly(styrene sulfonic acid) (SPS) and poly(dimethyldiallylammonium chloride) (PDAC), become fully ionized spontaneously independent of the solution pH, while charge densities of weak polyelectrolytes vary with pH. In the layer-by-layer assembly process, strong polyelectrolytes adsorbing onto one another tend to form molecularly thin (~ 5 Å) layers in which all of the anionic and cationic groups are coupled.[3,4] Salts (e.g. NaCl, $MgCl_2$) added to strong polyelectrolyte dipping solutions screen the charges of the polyions, inhibiting electrostatic bonding between oppositely charged groups, and ultimately result in thicker layers that are less tightly knit together. This means that it is possible to control the molecular organization of strong polyelectrolytes by adjustments of the ionic strength of the dipping solutions. But, at high ionic strengths of solution, water becomes a poorer solvent as salt ions "salt out" the polyelectrolytes. Therefore, control over bilayer thickness and composition is fairly limited using this approach.

This limitation can be overcome by using weak polyelectrolytes, where adsorption behavior is controlled by the dipping solution pH rather than the salt concentration.[4,5] The degree of functional group ionization in weak polyelectrolytes is extremely sensitive to the dipping solution pH. This means that it is possible to systematically vary the linear charge densities of both an adsorbing polyelectrolyte and previously adsorbed polyelectrolyte with simple pH adjustments. This method provides a greater range over which the linear charge density of a polyelectrolyte may be varied and an unprecedented ability to control the blending of a polycation and polyanion at the molecular level. Accordingly, more control over layer thicknesses and molecular organization may be achieved.

This principle may be further demonstrated by examining the behavior of the two weak polyelectrolytes, Poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) (Figure 2). At neutral pH, both of these polymers are in their fully charged state.

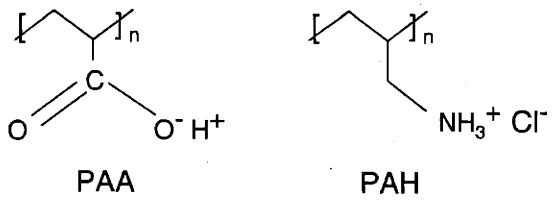


Figure 2. Structural formulas for poly(acrylic acid) PAA, and poly(allylamine hydrochloride) (PAH)

As the solution pH of PAA (polyanion) is lowered, its charge density decreases as its acid groups become protonated. As the solution pH of PAH (polycation) is raised, its charge density decreases as its ammonium groups become protonated. Work by Shiratori and Rubner[6] demonstrates control over molecular organization with pH adjustments. In their study, one layer of polycation, PAH, and one layer of polyanion, PAA, were deposited over the pH range of 2.5 to 9.0 to find out how pH effects layer thickness and organization. At different levels of pH, thicknesses varied from greater than 120 Å, to less than 10 Å, or adsorption was completely prevented. Figure 3 shows the variation of the layer thickness of PAH and PAA with changes in pH of the dipping solutions.

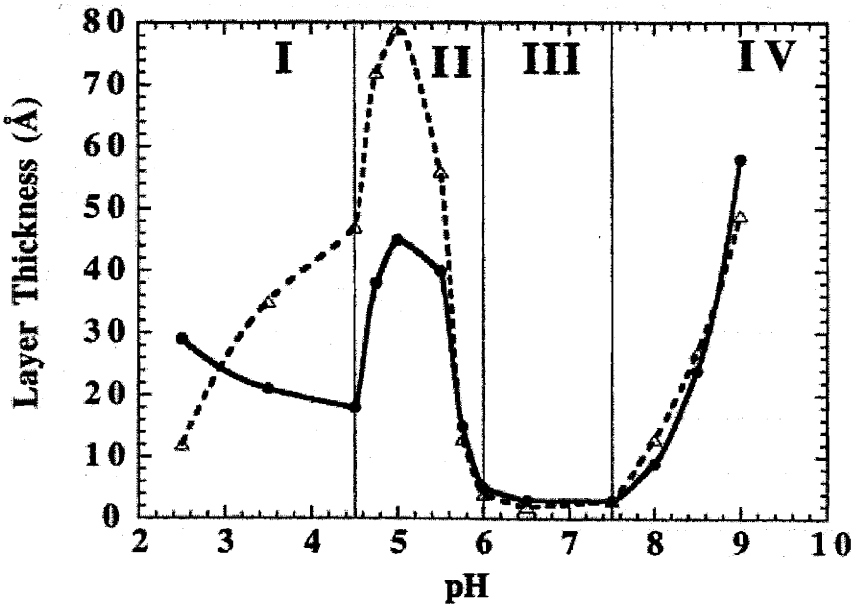


Figure 3. Plot of average incremental thickness of a PAA and PAH adsorbed layer with varying solution pH. PAA and PAH dipping solutions were always at the same pH level. Solid line represents PAA and the dashed line represents PAH. Reproduced from reference 6 with permission from corresponding author .

In this particular case, the pH was the same for both PAH and PAA. At neutral pH these fully charged polyions deposit as highly ionically cross-linked layers. The polymer chains stretch out and adopt flattened conformations to optimize interaction with ionic sites of opposite charge. The polymer chains form molecularly thin, well-interpenetrated layers, with a thickness ranging from 3-5 Å. An increase or decrease in pH results in dramatic changes in layer thickness of both PAH and PAA. For example, at a pH of 5.0 the thicknesses are 80 and 50 Å for PAH and PAA, respectively. In this case, polymer chains adopt more energetically favorable loopy conformations, as the charge density of PAA drops below the fully charged state. Figure 4 illustrates the molecular organization of a PAH/PAA bilayer at pH levels of 6.5/6.5 and at pH levels 5.0/5.0. At a pH level of 5.0, PAA is less than fully-ionized and has a small concentration of carboxylic acid functional groups along its chain.

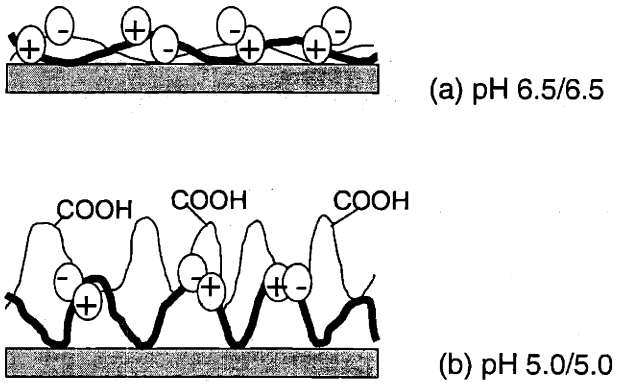


Figure 4. Schematic representation of the molecular organization of PAH/PAA multilayer films assembled with the dipping solution at different pH levels: (a) pH 6.5/6.5 (b) pH 5.0/5.0. PAA is the outermost layer represented by the thin line; the thick line represents PAH.

This technique provides a remarkable capability to customize the physicochemical properties of the thin film surfaces. Surface properties can be made to range widely while maintaining the same two polymer constituents. In one example, the wettability of PEM thin film surfaces were adjusted such that advancing water contact angles could be made to vary from essentially 0° (completely wettable surface) to as high as 50°, using the same polycation/polyanion combination at different pH conditions.[7]

Likewise, PAA/PAH films may be made to have cytophilic surfaces that adhere cells or cytophobic surfaces that resist cell attachment. This can be accomplished by simply changing the pH assembly conditions.

In addition to controlling the charge density, the dipping solution pH may be used to control the number of free uncharged functional groups of weak polyelectrolytes present in a multilayer film. These nonionized groups may be utilized for subsequent chemistry in developing novel structures and devices. As a polyanion chain adsorbs onto a surface containing an oppositely charged polycation, most of the ionized anionic groups end up forming ion pairs with the cationic groups. The remaining nonionized groups, however, are available for subsequent chemistry. For example, free carboxylic acid groups of PAA can be used to bind various inorganic ions by exchanging protons for metal cations. The metal cations may subsequently be converted into nanoparticles. Joly *et al.* utilized PEMs, based on PAH and PAA, as nanoreactors for metallic (Ag, Pb) and semiconductor (PbS) nanoparticles.[3] Inorganic nanoparticles were synthesized within

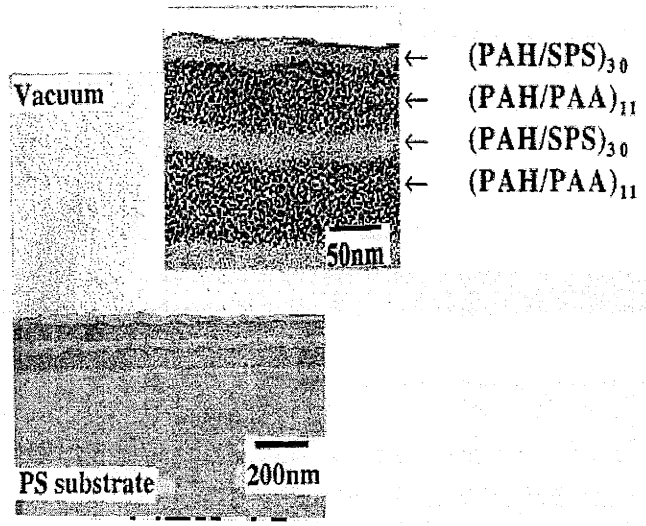


Figure 5. Cross-sectional TEM image of a multilayer thin film comprised of PAH/PAA bilayer blocks alternating with PAH/SPS bilayer blocks. Silver nanoparticles are created within the two PAH/PAA blocks. Reproduced from reference 3 with permission from corresponding author.

PEM thin films, in which nanoparticle growth was restricted to specific regions of the films by spatially controlling the relative amount of free carboxylic acid groups within the multilayers (Figure 5). In other recent works, free carboxylic acid groups were used to bind Pd-complexes within PEM thin films or on their surfaces to promote electroless

nickel plating.[8,9] These multicomponent nanocomposites have potential applications in electronics, photonics, and magnetic information storage.

This principle has also been employed in loading and releasing molecules in multilayer films. Methylene blue, a cationic dye bound to available negatively charged carboxylate groups of PAA layers in a PAA/PAH film. Films subsequently immersed in water at appropriate pH conditions prompted the release of the dye.[10]

Functional groups at surfaces (e.g. carboxylic acid, amine groups, etc.) can act as physical or chemical binding sites to anchor other molecules and polymers with a specific affinity for these functional groups. Once these molecules anchor to the surface of the PEM, the surface acquires the physicochemical properties of the newly attached species. In a recent study, PAA/PAH multilayers were modified with amphiphilic block copolymer, polystyrene-*block*-poly(acrylic acid) (PS-PAA).[11] The surface adsorption of PS-PAA was driven primarily by secondary interactions, such as hydrogen bonding. Hydrophilic PAA blocks anchor onto the surface, while the PS blocks dangle out from the surface. Depending on the deposition sequence of the PAA/PAH multilayer film and the processing pH conditions, the PEM surface could be made to be either highly promoting or highly blocking for PS-PAA adsorption. Once attached, the surface exhibited wettability properties similar to that of pure PS surfaces.

Light emitting diodes (LEDs) based on electroactive polymers have been fabricated using the layer-by-layer assembly of polyelectrolytes. Green light emitting diodes were fabricated using PAA as the polyanion in conjunction with the cationic precursor polymer to poly(p-phenyl vinylene).[12,13] Red light emitting diodes were fabricated with a water soluble polycation containing Ru(II) complex.[14] In these studies, external quantum efficiencies and luminance levels were varied by changing the pH of the dipping solutions. Improved hole injection and transport have been demonstrated with graded electrically conductive multilayers.[15] Electrical characteristics of PEM thin films were made to vary by changing the deposition conditions. In another recent study, Durstock and co-workers showed a decrease in dielectric constant of PAH/PAA films when increasing the pH of the dipping solutions from 3.5 to 6.5.[16]

Perhaps the greatest advantage of layer-by-layer adsorption from solution is that many different functional materials (e.g. conductive, photoresponsive, or cell-resistive) may be incorporated into multilayer films by including them in the deposition sequence.

The layer-by-layer assembly process can be integrated with various methods of planar patterning such that PEM surfaces may exhibit multiple surface functionalities. Means by which PEM surfaces have been patterned include microcontact printing (μCP), ink jet printing (IJP), and photolithography.

In one recent study, subtractive patterning approaches were used on multilayer films comprising PAA and polyacrylamide (PAAm).[17] PAA /PAAm multilayers may be assembled via hydrogen bonding interactions at low pH levels. Exposing these films to water at neutral pH, disrupts the hydrogen bonding as acid groups of PAA ionize and introduce electrostatic repulsive forces between the layers. Thermal and photochemical means, however, may be used to render these films stable under neutral pH conditions. Thermally treating the film induces cross-links between the PAA/PAAm bilayers, rendering the film stable at neutral pH conditions. Introducing a photoinitiator-labeled PAA copolymer into the film, also enables cross-linking between PAA/PAAm bilayers upon exposure to UV irradiation. These principles have been utilized to pattern multilayer surfaces consisting of PAA/PAAm bilayers using ink jet printing and photolithography techniques. In the first method PAA/PAAm bilayers are built up via layer-by-layer adsorption. An ink jet printer deposits pH 7.0 water onto designated regions of the PAA/PAAm multilayer film, rendering these segments non-cross-linkable. Subsequent heating of the PAA/PAAm multilayers induces cross-linking in non-printed regions. The films are then rinsed to remove non-cross-linked regions. In the latter approach, photoinitiator-labeled PAA copolymer is incorporated as the outermost layer of a PAA/PAAm multilayer film. The film is irradiated through a mask with UV light, then rinsed to remove non-cross-linked regions. Figure 6 shows a PAA/PAAm multilayer film patterned via IJP and photolithography. PAA/PAAm multilayer films have been patterned with surface regions exhibiting cytophilic and cytophobic regions.[18] These patterning approaches have also been used in creating polymer LEDs.

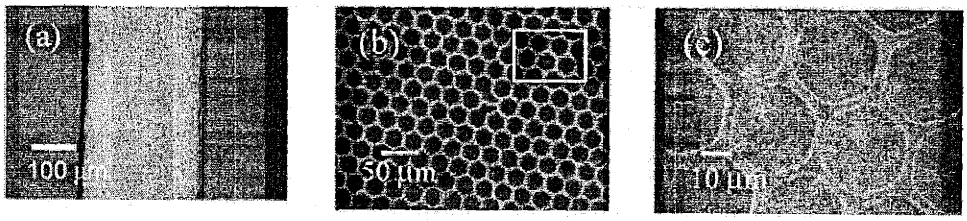


Figure 6. Optical microscope images of micropatterned multilayer films of PAA/PAAm. (a) by ink jet printing, (b) photolithography through a TEM grid (c) magnified image of boxed area in b. Reproduced from reference 17 with permission from corresponding author.

In μCP a patterned polydimethylsiloxane (PDMS) stamp is used to transfer self-assembling molecular species from the stamp surface to the substrate, providing the substrate surface with the pattern of molecules. This technique was introduced by the Whitesides group[19] to form chemical patterns on gold using alkanethiols or on silicon oxide using triethoxysilanes. In this technique, a stamp is molded in a silicon master mold created using photolithography. The stamp and mold may be used numerous times, rendering this patterning technique simple and inexpensive. Surface features may have resolutions of 1 μm or greater. Recently, this technique has been utilized to pattern features as a template for the selective deposition of polymers.[20] In this approach a substrate is patterned with alternating regions that allow or resist the adsorption of polyelectrolytes. The Hammond group[20] used carboxylic acid (COOH) as a functionalized monolayer surface to promote polyion adsorption, and oligoethylene glycol (EG) functionalized regions to inhibit deposition. This technique enables layers of oppositely charged polyelectrolytes to be stacked upon COOH regions while completely avoiding EG regions. This approach was performed using SPS and PDAC as the adsorbing polyelectrolytes. In another approach, polymers are loaded directly onto the stamp and transferred to a surface that may consist of PEMs.[20]

3.0 Cell patterning

Clearly applications for PEMs are far-reaching, extending into areas such as optoelectronics, photonics, and biomedicine. Polyelectrolyte complexes are particularly well suited for biomedical applications because of their similarities to protein structures:

ionic charge characteristics, hydrogel structure, and permeabilities to water and to solutes existing in body fluids.[21] The ability to adjust the physicochemical properties of surfaces using PEMs and the capacity to integrate functionality vertically are also no doubt invaluable for customizing biomaterials. It is little wonder, therefore, that the layer-by-layer assembly of polyelectrolytes is finding its way into more and more biomedical applications. For example, CIBA Vision recently began marketing contact lenses in Europe that exhibit a hydrophilic PEM coating to enable extended wear. In other current studies, multilayer complexes composed of biopolymers and synthetic polyelectrolytes were fabricated via layer-by-layer assembly. Multilayer films comprising DNA and synthetic polycations like poly(ethylenimine) (PEI), polylysine, or PAH have attracted interest for gene therapy applications. [22] Enzymes, proteins, and lipid bilayers have been incorporated in multilayer films assembled layer-by-layer.[22,23,24,25] Such films can enable functional properties of proteins to be exploited. For instance, Sun and co-workers[25] fabricated an enzyme multilayer film to be used as a maltose sensor. Additionally, PEMs served as encapsulation membranes for enzymes where the release of enzymes could be controlled by adjusting the thickness and composition of the polyelectrolyte walls.[26]

Currently there is a desire to be able to manipulate cells in a controlled manner, and to arrange them in specific and predetermined positions. The ability to accomplish this holds promise in applications such as cell culturing, cell-based biosensors, cell screening, cell sorting, toxicology, tissue engineering, cell differentiation and proliferation studies, and in other fundamental studies of cell behavior.

Whole cells are the smallest biological entity that is self-sustaining. Each cell contains a number of reacting chemicals, is laden with environmental sensors, and allows heat and certain chemicals to pass through its walls.[27] Whole cells, in screening and sensor applications, offer the distinct advantage of providing an integrated physiological response, as opposed to other non-cell-based biosensors that may only respond to a specific analyte.[28,29] To date, living animals have been the only cell-based biosensors to be successfully used in the field,[30] though developments are currently underway for such sensors in military applications; these should appear in 2002.[31] Canaries, for example, were used over a hundred years ago to detect lethal levels of methane gas, and more recently, in the 1995 police raids of the Aum Shin Rikyo compound.[30] Fish have been

used to monitor water quality. One obstacle in the development of cell-based biosensors is the ability to maintain and store cells for an extended period. Such cell-based detectors require a stable environment to sustain the life and physiology of the cells.

In drug discovery, cell-based high-throughput screening (HTS) systems are already emerging in the marketplace. HTS refers to the methods for performing rapid assays of thousands of compounds in search of biological activity in identifying target biomolecules (i.e. proteins, DNA) and in discovering drug compounds. HTS wet assay methods fall into two categories: binding assays and functional cell-based assays.[32]

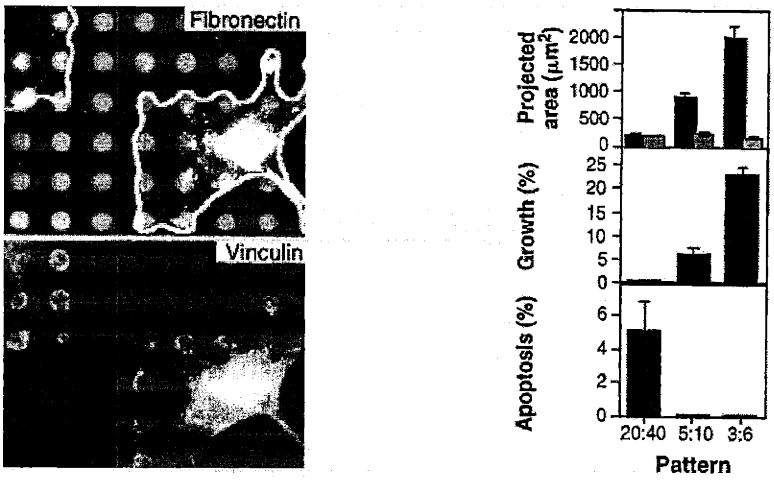
Binding assays are used to determine potential targets among compounds. In these assays, an antibody or specific receptor is covalently bound to a solid surface, typically a microtiter plate or occasionally plastic beads.[32] The compounds in solution are brought into contact with the receptors such that binding will occur if configurations between compounds and receptors or antibodies match. Binding is typically observed by fluorescent indicator molecules that may be subsequently tagged onto the compounds. Binding indicates potential pharmacological activity, but is no definite indicator. For instance, such binding may be non-specific, or may not even produce a biological response (i.e. antagonist compounds). Another problem associated with binding assays is that a positive signal obtained may be the result of one compound or a multitude of compounds binding. Thus, it is possible that compounds in the reaction mixture may be structurally quite similar but bind to the receptor or antibody in varying degrees. These limitations in sensitivity render binding assays essentially useless in optimization processes of compound validation.

Pharmaceutical scientists are pursuing functional cellular assays to provide more accurate information.[32] Cells can be modified to express specific receptors for assay compounds such that compounds binding to these receptors induce physiological changes (e.g. cellular pH, morphology, growth rate) in the cells. In fact, under normal circumstances, most drugs act at the level of cell membrane receptors.[28] These functional assays can separate agonists (compounds that elicit a physiological response) from antagonists. Additionally, the degree to which cells respond can be used to indicate levels of toxicity and carcinogenicity.

The proceeding two subsections review some of the current developments taking place in research and industry with respect to cell patterning and other cell-based systems and devices. Subsequently, progress on the use of PEM based systems with respect to controlling the behavior of cells is discussed. The perspectives of individuals within research and industry on potential applications for PEM based devices were gathered and are presented in a fourth subsection. Lastly, the layer-by-layer assembly of polyelectrolyte multilayers is compared with the self-assembly of molecules into monolayers – the primary competing technology.

3.1 Developments in research

In one study, micropatterned surfaces were created to study the effects of cell shape on cell life and death. [33] The object was to determine whether cell shape or integrin binding, in and of themselves, governs life and death in capillary cells. Understanding how cell death, or apoptosis, is triggered in capillary cells has important clinical implications, because the development of new capillary blood vessels, in a process called angiogenesis, is a prerequisite for tumor growth. Human and bovine capillary endothelial cells were switched from growth to apoptosis by using substrates that contained extracellular matrix-coated adhesive islands of decreasing size. Significantly more cells entered apoptosis on smaller sized islands than on larger ones demonstrating that increased cell spreading leads to cell survival and growth. Additionally, they evaluated cell death and growth in single cells by spreading them across multiple cytophilic islands interspersed among cytophobic regions. Thus, cell spreading could be varied while maintaining the extracellular matrix contact area constant by adjusting the spacing between multiple adhesion islands (Figure 7). Their results showed that the extent of spreading (the projected surface area of the cell), and not the area of adhesive contact, controlled cell life and death. Cell shape was found to determine cell growth and apoptosis regardless of the type of surface adhesive protein.



(a) (b)

Figure 7. (a) Immunofluorescence micrographs of cells stretching to cytophilic islands of a micropatterned substrate stained for fibronectin (top) and vinculin (bottom). (b) Graphs of projected cell area (black) and total extracellular matrix contact (ECM) areas (gray) per cell (top), cell growth index (middle), and cell apoptotic index (percentage of cells that die) (bottom) when cells cultured on circular islands having diameters of 20, 5, and 3 μm separated by 40, 10, and 6 μm, respectively. Results indicate that cell growth and apoptosis depend on the projected area of the cell and not on the ECM contact area. Reproduced from reference 33 with permission from corresponding author.

Zhang and co workers[34] used self-assembling oligopeptides and μCP to fabricate a variety of surface patterns consisting of areas either supporting or resisting cell adhesion. Their intent was to observe differences in cell migration behavior for a given surface using a variety of cell types. Cell types tested included mouse fibroblast 3T3 cells, human epidermoid carcinoma cells, and bovine aortic endothelial cells. Cytophilic linear array tracks were patterned on surfaces to observe cell compliance along the tracks. The human epidermoid carcinoma cells were well confined to the peptide tracks while some mouse fibroblast cells would cross over inhibitory EG_6SH tracks. In a second experiment, surfaces were designed to address how a community of cells may communicate through inter-cell connectors. Cell suspensions were added to culture dishes containing patterns of square islands connected with narrow tracks of variable width and length. All cell types formed readily defined patterns. Endothelial cells were completely confined to the printed areas since they do not have elongated processes.

Their findings show that different cell types may respond disparately to a given surface with respect to their migration behavior, even if they exhibit positive adhesive interactions with the same particular surface-coated molecules.

In another study, films were modified with biologically active peptide amphiphiles to control cell behavior. Pakalns *et al.*[35] formed these amphiphiles by covalently linking the amino-terminus, carboxyl-terminus, and both termini of the cell recognition sequence Arg-Gly-Asp (RGD) peptides to dialkyl lipid tails. The peptide sequence RGD is particularly relevant in cell recognition since it is found in many proteins located on cell surfaces, within extracellular matrices, and in blood. RGD is often incorporated onto biomaterial surfaces to activate cellular response due to its ability to bind to a variety of cells through ligand-receptor interactions. Cell spreading, cell adhesion, and cell cytoskeletal reorganization were observed on surfaces composed of self-assembled monolayers of the three RGD amphiphiles. The three RGD amphiphiles were separately mixed with analog RGE (a biologically inactive sequence) amphiphiles in varying concentrations and deposited onto glass slides via the Langmuir-Blodgett method to investigate how spatial orientation and molecular concentration would influence the properties of these films with respect to their ability to promote cellular response. Cellular response varied depending on the film uniformity, packing density, and the conformation of the peptide sequences. Melanoma cells spread on looped RGD amphiphiles in a concentration-dependent manner, spread indiscriminately on carboxyl-coupled RGD, and did not spread on amino-coupled RGD amphiphiles. Well-packed amphiphiles with the RGD sequence in its flexible loop conformation were most effective in promoting cellular response. RGD sequences in their loop conformation were shown to be most accessible to cells. Peptide amphiphiles containing the control sequence RGE were most effective at inhibiting cellular response with the flexible loop conformation. Thus cell response through receptor-ligand interactions (in this case RGD is the ligand and the extracellular matrix of the cell contains receptor sites) was shown to depend on sequence accessibility and on sequence conformation. This study demonstrates that ligand identity is not the sole factor in achieving cell response; factors such as ligand sequence conformation, and packing must also be considered in developing biomimetic coatings.

Wu and co-workers[36] developed a multi-light addressable potentiometric sensor (MLAPS) based on the Cytosensor® microphysiometer, a product put out by Molecular Devices Corporation. The device measures the acidification rate of cells' extracellular matrix under the effects of drugs, and thereby enables the ability to correlate, in real-time, cell metabolism in the presence of drugs. During energy metabolism cells release acidic products into their microenvironments. The cytosensor put out by Molecular Devices Corp. measures the rate of proton excretion from cells. This is accomplished using a light-addressable potentiometric sensor (LAPS). Wu and coworkers essentially enhanced the microphysiometer system by enabling simultaneous measurement of several extracellular ion concentrations. The MLAPS system is setup such that compounds in solution or suspension flow over cells seeded in a microvolume reaction chamber. The drugs may induce a physiological response of the cells, thereby changing the variance ratio of the concentration of extracellular ions (H^+, K^+, and Ca^+). The fluid, with cell-excreted ions, subsequently flows over three ion sensitive membranes in parallel, illuminated by three infrared light sources at different frequencies. A photocurrent is produced in an underlying chip, where the magnitude of the current is dependent on the ion concentration and then converted into a readable signal.

3.2 Development in industry: companies and their technologies

This section presents recent developments on cell-based platforms in industry. A few companies are involved in developing cell-based HTS systems for the drug discovery market. Others are developing cellular biosensors for hazardous reagent testing.

Surface Logix[37] is a recent start-up company combining surface chemistry and microfabrication techniques including microcontact printing, micromolding, and stenciling in developing cell-based assays. Their technology focuses on targeting molecular interactions relevant in drug discovery including signal transduction pathways, receptor/ligand interactions, enzyme function, cell morphology, and cell-surface and cell-cell interactions. Their initial portfolio of discovery capabilities includes three cell-based assays: a motility assay, a chemotaxis assay, and cell assembly and co-culture assays. These Cell Mosaic™ assays enable control over cell location, the microenvironment and phenotype as well as the ability for continuous monitoring of changes in phenotype at the

cellular or molecular level. Their Cell Motility Assay™ is designed to measure adhesion and movement of cells from a predetermined pattern on surfaces with biospecific ligands. The motility of cells is important in the progression of many diseases, especially in oncology and immunology. In these assays cell spreading elucidates cell activity. The assays are created by patterning biospecific ligands onto a surface via microcontact printing. Then a sheet-like 96-well device is placed over the surface, where each well presents a thin membrane containing microscopic holes. Cells are patterned onto the biosurface through the microscopic holes and interact with the surface ligands. The sheet is removed enabling the cells to spread from the pattern. The surface is then analyzed visually using an optical scanner or microscope, and the spreading behavior can be quantified using proprietary algorithms. This system will offer new insights into new potential inhibitory pathways, cell-cycle synchronization by non-chemical means, non-chemical apoptotic control, and cell specific adhesion surfaces.

Cellomics, Inc.[38] is a privately held company developing methods for HTS and for biowarfare detection. The company is currently supplying pharmaceutical companies with their ArrayScan High Content Screening System to analyze the effects of potential new drugs on various aspects of cellular targets. This device scans cells through the bottom of transparent microplates and measures and analyzes spatial and temporal events in cellular targets of organelles at the single cell level or as a population average over a field of cells. Cellomics is also cooperating with the Defense Advanced Research Projects Agency (DARPA) on a cell-based biosensor for the detection, classification, and identification of chemical and biological warfare reagents.[31] They claim to finish this device sometime in 2002.

DARPA has a Tissue Based Biosensor program in which they are developing single cell and multi-cellular tissue-based sensors as part of their biological warfare defense program.[39] Theses sensors could incorporate cells to be used as physiological based biosensors that functionally respond to known and unknown biological, chemical, or physical stimuli. Some of the current issues in the fabrication of these sensors include nutrient requirements to cells, efficient fluid transport of nutrients and wastes, spatial requirements of cells within the matrix, signal processing and information extraction from electrical, optical, mechanical, or other outputs from cells. This research is being

performed under collaboratoions with other Universities and Companies including Cellomics Inc., Cornell University, Harvard University, Life Technologies Inc., the Massachusetts Institute of Technology, the Naval Research Laboratory, Promega Corp., the Univerisity of California Irvine, and Univerisity of Southern California.

Medtronics and Carmel Biosensors are collaborating together in an effort to develop cell-based biosensors for various medical technology applications. [83] Living cells are used as the sensing element for measuring levels of various constituents in the human body, such as glucose, blood gases, etc. One sensor under the last stages of development continuously monitors diabetics' glucose level within the human body. The sensor has a self-adhesive patch from which a miniature probe, the size of a human hair, protrudes into the skin. Pancreatic cells, encapsulated within the probe, generate electrical signals in response to blood glucose levels. The signals are picked up by tiny electrodes and transferred to the skin patch where they are processed by microelectronic circuitry to determine the blood glucose level.

Applied BioPhysics[41] is a small company based on a product that uses electrical methods to study the activities of human cells grown in tissue cultures. Their product, Electric Cell-substrate Impedance Sensing (ECIS), measures the impedance of cells as a function of time under a low AC current. This enables the morphology and motion of cells cultured upon small gold electrodes to be measured with high sensitivity. They claim that ECIS can impact areas of fundamental cell research including wound healing, metastasis, and the dynamics of cell attachment and spreading.

Biotrove[42] is another recent start-up founded in 1997 as Advanced Instrumentation Systems by Professor Ian Hunter, of MIT, and some of his colleagues. The company focuses on HTS tools for drug discovery and has two core technology platforms in this area: The Living Chip™, and a microfluidic screening system under development with a Pfizer, Inc. The Living Chip™ enables massively parallel synthesis, storage, and screening of up to 100,000 samples simultaneously. The device consists of high density arrays of micro-channels, or bottomless wells, in a plate. Each chip has 10,000 channels where each channel is 250 μm square with a depth of 500 μm. The exterior surfaces of the chips have hydrophobic coatings while the interior channels surfaces are made to be hydrophilic. Small volumes (50 nl) may be loaded into the chips

by dipping them into a solution. Upon removing the chip from solution, the surface tension at the interface between the coated channel surfaces and the liquid holds the liquid within the channels. Reagents are mixed simultaneously by aligning and stacking one chip on top of another. Screening of substances using living bacteria and mammalian cells has been demonstrated using this system. For example, an array of 10,000 small molecules from chemical libraries can be loaded into a Living Chip. Mammalian cells may be cultured in a second Living Chip, and a third chip is filled with Fura-2, a fluorescent calcium-chelating agent. The Fura-2 is loaded into the cells by stacking the second and third chips upon one another. The molecular compounds in solution are released into the cells as the first chip is stacked onto the other two. Intracellular calcium is released in response to small molecules binding to the cellular receptors. Fluorescence may be observed with a CCD camera.

Aurora BioSciences[43] is yet another company that designs and develops drug discovery technologies. The company was founded in 1995 and was later acquired by Vertex Pharmaceuticals. In 2000 the company made nearly $64 million and it currently has over 250 employees.[44] Aurora's platform includes capabilities in cell-based and biochemical assays development; generating assays to rapidly measure target activity in living cells; HTS systems incorporating microfluidics, and compound management; and analytical instrumentation. Aurora's fluorescent assay technologies are performed in live mammalian cells and are designed to enable screening of compounds against nearly all major classes of human drug targets, including receptors, ion channels and enzymes. One example of one of their fluorescence systems is the GeneBLAzer™ Reporter System, which is used to develop cell-based assays for a wide variety of drug targets. This system facilitates the study of intracellular signals, cell differentiation pathways and proliferation events in living cells. First, individual genes within cells are randomly tagged with the fluorescent enzyme reporter, β-Lactamase. Activated genes inside the cells give off blue fluorescence while inactive ones fluoresce green. The change in fluorescence from green to blue indicates that the β-Lactamase reporter has been activated (it has attached to an active gene). This enables a portion of genes to be identified based on their response to specific stimuli. The "target set" of stimuli-responsive cells are subsequently sorted by fluorescence-activated cell sorting (FACS), a flow cytometry technique. This enables the

creation of libraries of potential target genes. These cells may then be directly used for identification of the activated gene, assay development, and additional high-throughput screening for drugs that may either increase or decrease gene expression. Aurora is currently using these libraries for internal and external programs in inflammation, cancer, and metabolic diseases. Aurora has similar reporting systems as well such as their FlAsH Protein Labeling System. In this system non-fluorescent tags applied to the outside of cells, cross the cell membrane, find and bind to proteins intracellularly, and then may become fluorescent. Cells are monitored by measuring the increase in fluorescence.

3.3 PEMs and cells

The physicochemical properties of PEM surfaces may vary drastically, depending on factors such as charge density, density of exposed functional groups, degree of interpenetration with previously adsorbed layers, porosity, and roughness. Likewise, the membranes of cells may differ considerably in both structure and composition. The differing surface attributes of PEMs and cell membranes can promote a cooperative binding between the two and may enable PEM surfaces to be tailored to interact only with specific cell types. PAA, for instance, stimulates activity in B-lymphocytes, but has no effect on T cells.[21] The implementation of layer-by-layer assembly of PEMs for applications in controlling cell behavior is only in its infancy, however, results thus far forecast a promising future.

Researchers in the Rubner group, at the Massachusetts Institute of Technology, have fabricated PEM films with several combinations of polyelectrolytes at varying levels of pH.[18,45,46] Cells placed on the surface of these films responded in different manners ranging from highly cytophobic to highly cytophilic in behavior. In a truly remarkable demonstration of this technology, multilayer films comprised of the same polycation/polyanion combination exhibited cytophilic and cytophobic properties by adjusting only the pH conditions. The interaction that induced these varying responses is not completely understood, but is likely dependent on a number of factors including surface roughness, chain conformation, degree of ionized/nonionized functional groups, swellability, and the degree of hydrophilicity of the multilayer films.

In another demonstration of the versatility of PEMs, ligands were patterned on cell resistive PEM surfaces using microcontact printing. A cross-linker was made to bind to the head groups of ligands anchored to the surface. Subsequently cross-linkers were activated such that RGD peptide sequences could be attached to provide cell adhesive character. The net result was a film patterned with cytophilic islands surrounded by cytophobic regions.[46] Other PEM systems show indications of bacterial resistance. Currently, no material exists that can completely resist the adhesion of bacteria.[47] Photolithography, microcontact printing, and ink-jet printing have all been employed in patterning PEM surfaces with regions of cytophilic or cytophobic character.

3.4 Individual perspectives on PEMs

Several people in research and industry were interviewed for their perspectives on the potential of layer-by-layer assembly of PEMs and planar patterning methods for the arrangement of cells. The technology and capabilities of PEMs were briefly described to each interviewee, after which interviewees were asked questions pertaining to the relevance and applicability of the technology, including questions regarding current limitations and needs that exists with respect to biosensors, high-throughput screening, cell cultures, and tissue engineering. Responses varied: some interviewees were not sure how PEMs could be useful; other contacts refused interviews due to confidentiality issues because the technology was too close to that of their own; most interviewees agreed that the technology offers great potential. Some interviewees provided their opinions of potential applications. Some of the suggested applications are discussed here.

Tara Heitner, a chemist at BioTrove Inc., and Kelly Cassutt, a development scientist on the cell pharmacology team at Biacore Inc., both mentioned current weaknesses in cell lines and suggested potential solutions using PEMs. Cell lines are permanently established cell cultures that will proliferate indefinitely given appropriate fresh medium and space. It is critical that cell lines maintain a stable environment to promote growth and to prevent differentiation so that cells maintain their phenotype. Some cell lines are weakly adherent and therefore do not grow very easily (e.g. huvec cells). Establishing cytophilic areas for such cell lines would create an environment more stable for the cells.[48,49] Some cells lines need to grow in two dimensions along a plane,

rather than being restricted to lines. In such cases, PEM surfaces could be patterned to control the growth of the cells in specific directions.[48] Customized matrices could be made according to a cell line's needs.[49]

Cell survival is optimized in a tissue-like atmosphere. Surfaces that mimic a tissue environment can extend cell life in some applications.[48] This may involve attaching peptide sequences (e.g. RGD) or proteins (e.g. fibronectin) to surfaces. Or perhaps there exist some combination of polyelectrolytes at proper pH conditions that enhance cell life. Heitner suggested creating a system that could somehow provide nutrients to cells. It has been recently demonstrated that functionalized channels within multilayer thin films can bind chemical species and release them through multilayer membranes in a controlled manner. Cells could be made to sit above chambers containing nutrients, then be fed these nutrients through a membrane.

Both Darrel Irvine, in the Department of Biomedical Engineering at MIT, and Heitner posed the idea of depositing PEMs onto living cells. This would be important in tissue engineering applications such as the realization of 3D scaffolds comprised of a variety of cells types seeded within different layers.[50] Tissue engineering may require that cells be placed in specific locations to create organized structures.[51] The ability to place cells within PEMs could also be useful in applications where the interest lies in what a cell excretes and not in the cell itself. This would require membranes permeable to cell excretions.[49]

Surfaces that are able differentiate between different cell types was recommended by Cassutt, and Amer Elhage, an engineer at Molecular Devices Corp. For instance, it would be desirable to have a surface that would bind to either red or white blood cells, so that one or the other could be separated.[50] Distinguishing physiological differences between live and dead cells is also important.[50]

One current technology uses paramagnetic dyna beads with cell-specific coatings that can be used to selectively extract cells from a source comprising multiple cell types. Cells are separated based on the expression of some receptor. For example, if the cells express something on the surface, i.e. a protein only present in infected cells, then an antibody with the paramagnetic bead will bind onto the cell. The paramagnetic bead-with the antigen and cell are attracted to a magnet, and the cells are then separated from the

uninfected cells. This technology could be replaced by designing a surface with a matrix consisting of regions where antibodies are bound to the matrix. Infected cells would stick to the surface while healthy cells are easily separated. This could be used to immediately separate cells in a large-scale format.[49]

Dr. Wang, a scientist working at the Biosensor National Special Laboratory in Zhejiang, China and also at the Micro System Center for Research and Development at Zheijiang University, pointed out some difficulties with cell based biosensors. Intimate attachment of cells in whole cell sensors is critical in detecting clear signals from cells. Individual cells are typically separated from one another by gaps of 10 - 20 nm in their natural state. When growing cells on artificial substrates, e.g. silicon, cells maintain this distance between themselves, however the distance between substrate and cells is not very intimate. No one has succeeded in making the distance between cells and substrates less than 40nm so far.[52] Doing so, however, would enhance the signal tremendously. Dr Wang said that perhaps this distance could be decreased using a surface composed of polyelectrolytes. A conductive polymer could be incorporated to transduce an electric signal from the cells.

Cells on a surface can induce a stress field as they "grasp" the surface with cell receptors. Heajee Kim,[53] a Ph.D. candidate at MIT suggested that piezoelectrics underneath a multilayer surface could be used to monitor cell motility and adhesion. Piezoelectrics could like wise be used as actuators to induce cellular response. For example, an external stimulus (i.e. chemical, electrical, or mechanical) from the surface could in theory induce cell secretion (e.g. insulin from pancreas cells), and thus serve as a bioreactor.[52] A surface modified with photoisomers that exhibit a change in surface tension upon illumination with UV or blue light, could also be used to invoke cell response.[54]

3.5 PEMs versus SAMs

In this section, an analysis of PEMs and their primary competing technology is presented. The object of both of these technologies with respect to cell patterning is to be able to modify surfaces to enable the manipulation of cells in a manner such that they may be used in devices for screening and detection applications, and in elucidating their behavior

for fundamental research. A comparison between polyelectrolyte multilayers and self-assembled monolayers relative to their capability to adjust surface properties is useful in assessing which of these technologies is more appropriate relative to a given application.

Self-assembled monolayers[55,56] (SAMs) is the term used to describe the molecular self-organization of molecules on surfaces. The driving force for this assembly is the minimization of the free energy of the substrate surface and the surface-anchor groups of the organic molecules, such that molecules in solution will bind to the substrate and align themselves into crystalline-like domains, resulting in ultrathin organic films. Binding can occur via physical means (i.e. hydrogen-bonding, dipole-dipole interactions) or through chemical means (i.e. coordination and covalent bonds). Van der-Wall interactions between the aligned molecules provides for tight packing and extra stability of the monolayer. This method of self-assembly is accomplished by immersing a substrate into a dilute solution (~0.1 M) of the adsorbing molecules.[55] Like PEMs, this can be accomplished at room temperature and under normal atmospheric pressure.

There are several limitations associated with the self-assembly of molecules on surfaces. For instance, only certain classes of organics are able to form self-assembled films via coordination or covalent chemistry. Furthermore, substrates capable of forming such bonds with these organics are generally restricted to metal and silicon. High quality multilayer formation comprising self-assembled molecules are generally less stable and cannot be reliably obtained. This is likely due to the high steric demands of covalent bonds, and the inability to consistently complete reactions with 100% yield. Achieving high yield is necessary in retaining functionality with each succeeding layer.[1] On the other hand, electrostatic attraction between oppositely charged molecules or polymers has the least steric demands of all chemical bonds.[1] Electrostatic binding of polyelectrolytes provides good surface coverage and enables assembly to proceed largely independent of the nature and topology of the substrate; there is essentially no limit in substrate choice.

Strong adhesion at the substrate-polyelectrolyte interface is provided by the enthalpic bonus of multiple ionic binding sites along individual polymer chains. Neighboring, well-interpenetrated polyelectrolytes of opposite charge provide additional stabilization in these layered films. Though single molecules may form strong covalent

bonds at the surface, they are typically tethered at only one site, and interactions with neighboring molecules are generally weaker Van der-Waal forces.

Crystallinity of the substrate is important in achieving uniform, compact films of self-assembled molecules. Monolayers of molecules can completely cover metal surfaces, but some pinhole defects are inevitable because of substrate surface defects and the limited number of chemical reactions with 100% yield. In fact, the crystallinity of a substrate can be quantitatively estimated by the pin hole distribution.[34] Pin holes allow direct contact of redox active molecule with the electrode surface, and in effect can cause a "short circuit". In contrast, polymers simply bridge over underlying defects. Conformations at the surface are primarily dependent on the properties of the polyelectrolyte, its linear charge density, and the adsorption conditions; the substrate and the substrate charge density play a much more minor role. Observations show that polyelectrolyte multilayers have similar surface roughness, irrespective of the roughness of the substrate surface.[1] Adsorption conditions of polyelectrolytes can be adjusted to create loopy chain conformations providing a high surface area, which can be important in establishing intimate contact with biomolecules or cells.

Surface properties of films comprising self-assembled molecules are primarily determined by the head groups of the molecules. Significant changes in surface properties, therefore, often require the use of disparate molecules. However, some leeway in varying their surface properties may be achieved by adjusting the packing or chain length of the molecules. Packing may be controlled by finding appropriate concentrations of mixed monolayers that will deposit without any preferential segregation.[55] This strategy can prevent steric hindrance between bulky molecules (e.g. proteins) and their binding counterparts. Pakalns and co-workers demonstrated a change in cellular response through adjustments in the packing of monolayers by mixing RGD peptide amphiphiles with methyl ester amphiphiles.[35] Since methyl ester amphiphiles have smaller head groups, they could be mixed in with the RGD amphiphiles to separate the larger, cell-responsive head groups of the latter molecules. Chain lengths of molecules may be extended or shortened to induce different cell responses,[35] but this requires additional synthesis steps. Polyelectrolyte multilayers, on the other hand, have demonstrated remarkable variance in surface properties using the *same* two polyelectrolytes.

The ability to form highly organized structures on surfaces can be important in certain cases. Some applications may require a surface that mimics the nature of lipid layers. For instance, films containing peptide sequences may require high orders of organization to enable their ability to assume well-defined secondary and tertiary conformations that effectively promote cell adhesion and other activities such as spreading, migration, growth, and differentiation.[35] In such cases, SAMs may be more appropriate. The high degree of order and dense nature of some long chain molecules of SAMs mimics the cellular structure of lipid bilayers providing an ideal environment for immobilizing biomolecules (antibodies, enzymes, nucleic acids) and biological systems (receptors, whole cells).[55] PEMs may exhibit high order in the direction of the substrate normal, but not along the plane of the film. The functional groups of the outermost layers of PEMs can be modified to incorporate ligands that can bind other biological species, however, highly ordered and well-packed layers are unlikely since the outermost polymer layers exhibit disorder with respect to roughness, and a non-uniform distribution of available functional groups.

Perhaps the most obvious advantage of layer-by-layer assembly of PEMs is that many different materials can be incorporated within these films, thus enabling the ability to integrate functionality vertically. This is important for materials that require specific functionality in three dimensions, but also enables variability in surface properties. Surface properties of PEMs are not solely conditional upon the single outermost adsorbed polymer layer, but rather on the outermost few layers and on the degree of their interpenetration. Functional or ionic groups present on polyelectrolyte layers and their density along the polymer chains may also provide variance in surface properties. Conformation (loopy versus flat layers), roughness, and porosity are additional attributes that factor into surface properties. Precise control over these factors is achieved through simple pH adjustments of polyelectrolyte dipping solutions.

Finally, assembly of ionic-interacting macromolecules may be carried with water as the solvent. This is environmentally-friendly and it also permits the use of charged biopolymers such as DNA, proteins, natural polyelectrolytes, colloids, and other charged or chargeable materials.[1] The ability to incorporate such biomolecules certainly makes polyelectrolyte multilayers attractive for biosensors and applications in medicine.

4.0 Intellectual property issues

An intellectual property (IP) search was conducted to identify relevant patents relating to the technology and the application of the intended market. In this section, a number of patents are presented and analyzed with respect to their potential to block PEM technology and the applications thereof. A detailed description of the device or method presented in each of the patents is beyond the scope of this work. Instead, this analysis will focus on some aspect of the patent relating to cell patterning technology, or cell-based systems for screening or biosensors.

4.1 Patents on layer-by-layer assembly of polyelectrolytes

The core technology of interest is the pH-controlled layer-by-layer assembly of polyelectrolyte multilayers. Secondary to this are the methods of patterning including microcontact printing, ink-jet printing, and photolithography.

Several patents exist that relate to the layer-by-layer assembly of polyelectrolyte multilayers. Most of them are quite specific in function for a given application of layer-by-layer assembly and do not focus on the fundamental assembly process itself. These inventions include: (1) nonlinear optical structures assembled with polyelectrolytes that include nonlinear optical chromophoric side chains;[57] (2) optical chemical sensor probes based on PEM thin films with dyes incorporated into the layers that react and exhibit color changes in the presence of corresponding chemicals;[58] (3) Multilayered films formed with a cationic polyelectrolyte and anionic sheets of silicate clay;[59] (4) multilayer functional films incorporating functional molecules such as enzymes and other proteins, pigments and dyes admixed with polymer ions;[60] (5) durable hydrophilic surface coatings fabricated comprising a polyelectrolyte bilayer[61]; (6) molecular self-assembly of p-doped conjugated polymers.[62,63] These patents are all quite specific and would only prohibit one from developing systems similar to those described within the patents.

One invention implements PEMs for an optical solid phase biosensor labeled for the detection of analyte molecules in liquid phase.[64] Two complementary fluorescent dyes are attached to the uppermost layer of a PEM film. The first is electrostatically

bound to the PEM surface; a complementary dye labels antibody or antigen receptors that are anchored to the PEM surface. The spacing between the donor/acceptor pair fluorescent dye molecules permits a radiationless Förster energy transfer. When the PEM coated support contacts an analyte solution, a matching analyte molecule will bind to a receptor. The concentration of bound analyte molecules is measured as a function of change in fluorescence intensity.

The only patent with potential blocking power is the patent by Gero Decher and co-workers[66] issued in 1993 on the layer-by-layer assembly of oppositely charged organic materials. Bayer Corporation holds the license to this patent. Claims of the patent describe in detail the procedure and refer to specific substrates, ionic molecules and polymers in building multi-layered films of oppositely charged molecules. Overall the claims of the patent are broad and could block others from implementing similar techniques. The patent claims "a layer element applied to a support" that comprises "one or more layers made of organic materials which in each layer contain ions of the same charge, the ions of the first layer having the opposite charge of the modified support," and each additional layer "having a charge opposite that of the previous layer." The organic material may be either ionic or ionizable monomeric substances, or polyelectrolytes. The materials are applied from an aqueous or nonionic organic solution. These claims are fairly broad and would include the use of both strong and weak polyelectrolytes. However, the extent to which the patent may block others from using this technology is limited since the claims specify that the substrate must be modified with "ions or ionizable compounds of the same charge over the entire surface area of the support." It further stipulates that the support is selected from metal surfaces covered with a single layer of thiol, or silicon supports treated with silane, or polymers carrying ionic or ionizable functional groups on the surface. Therefore, this patent cannot prohibit the practice of assembling films of charged organic materials on supports that are not modified. Furthermore, control over the adsorption process of weak polyelectrolytes using pH adjustments was not performed until after this patent had been issued.

4.2 Patents on surface patterning methods

The three patterning methods of interest are photolithography, ink jet printing, and microcontact printing. A patent search revealed 23,946 patents on photolithography, and 5,689 patents on ink jet printing between the years 1986 and 2002. The majority of these patents are highly specific in nature, and those that are sufficiently broad as to provide any significant blocking capability have long since expired. Therefore, most of these patents can be ignored and attention is instead focused on those patents that implement photolithography and ink jet printing in the application of patterning surfaces for cell arrangement. These patents are discussed in the proceeding subsection (section 4.3). Seventy patents on microcontact printing were issued between 1996 and 2002. No patents on microcontact printing were issued before 1996. The majority of these patents are quite specific and do not pose any threat to inhibit the use of microcontact printing on PEM surfaces. Only the first patent is sufficiently broad to provide any potential blocking power.

In 1996 Kumar and Whitesides came out with their patent on microcontact printing entitled, "Formation of Microstamped Patterns on Surfaces and Derivative Articles".[67] Thirty-nine claims of the patent cover a variety of uses of μCP and would likely block attempts of others to use μCP in most respects. Claims include methods of reorienting the surface and stamping the surface a second time with the same or different molecular species; creating a plurality of discrete regions; stamping the surface a second time such that portions of self-assembled monolayers intersect with each other; using multiple stamping patterns; stamping on nonplanar surfaces; stamping on electrically conductive surfaces; coating a patterned surface with a second set of molecular species; depositing self-assembled monolayers on unstamped regions such that the surface comprises dual functionalities (i.e. hydrophobic and hydrophilic; optical recording material and optically inert material); reacting unstamped regions with other species that are chemically inert with respect to the molecular species of stamped regions; and plating unstamped regions with a plating reagent. Methods of fabricating the stamp including molding or micromaching procedures are also mentioned. No restrictions are imposed on the substrate or stamp material. Claims do however, specify that the method of patterning comprises "coating said stamping surface with molecular species terminating at a first

end in a functional group selected to bind to said material." This clause poses limitations to using only molecules terminated with functional groups. Additionally, the term "self-assembled monolayers" is used throughout the claims. Hence, the patent leaves open the opportunity for stamping with polymers instead of molecules. Paula Hammond, in the Chemical Engineering Department at MIT, has implemented polyelectrolytes in μCP in a polymer on polymer stamping (POPS) method. No patents, however, exist on this technique. Implementing μCP with self-assembled monolayers in a start-up venture would almost certainly require obtaining a license from the owners (in this case the President and Fellows of Harvard College) of this patent. The use of polymers with μCP might require obtaining a "competent opinion of counsel" for patent-clearance.

A second patent, by Whitesides and co-workers on microcontact printing issued in 2001, describes and protects more derivative articles of this technique. [68] In particular, the claims of the patent go into more detail on the fabrication of isolated regions of self-assembled monolayers, their surface properties, shapes, and dimensions. Claims state that the stamp "includes an elastomeric stamping surface", and specify that the substrate materials may consist of a range of metals or semiconductors, their alloys and oxides, or of glass. As in their previous patent, the material transferred with the stamp is limited to self-assembling monolayers of molecular species.

4.3 Patents on cell patterning and its applications

The primary applications in cell patterning include assays for cell-based HTS, cell-based biosensors, and other patterned cellular templates for drug discovery and fundamental research studies on cellular behavior.

In 1992, Robert Klebe,[69] at The University of Texas – Austin, invented an apparatus that positions cells into organized tissue structures using ink jet printing. The device and apparatus consist of four parts: a solid support, a substratum on the solid support that is separable from the support, a container with cell adhesion material, and an apparatus that is able to deposit the cell adhesive material at selected points on the substratum. Cells or cell adhesion material are transferred from their container through a "housing material" attached to a mechanically controlled arm and deposited on the cytophobic substratum. The patent describes the cytophobic substratum as a surface

coated with heat-inactivated proteins. Claims limit the cell adhesion material to fibronectin, laminin, chondronectin, epinectin, epibolin, uromorulin, or an antibody with an affinity for cell attachment. The substratum is selected from the group consisting of collagen, polylactide, pyrex glass, carnmauba wax, silicone aluminum, polytetrafluoroethylene, polyvinyl acetate, polyvinyl chloride and polystyrene. Claims mention that the substratum comprises a collagen sheet in the form of a wafer or sheet, but do not specify how it is deposited. Claims say that the housing material for the apparatus comprises is an ink-jet printer or graphics plotter head. Overall the patent is specific with respect to the materials used, and therefore, poses no major blocking threats.

Matsuda and coworkers[70] patented a device for control over cell arrangement in 1993. The patent is assigned to Kanegafuchi Kagaku Kogyo Kabushiki Kaisha in Oaska, Japan. All other patents presented in this section relating to cell patterning cite this work. Photolithography is used to pattern the surface with cytophilic and cytophobic regions. The patent presents three methods that utilize cytophilic or cytophobic photosensitive polymers in creating such a surface. First a cytophilic or cytophobic polymer is deposited onto a substrate. A photosensitive polymer having the reverse property is deposited onto the previously deposited polymer and is subsequently irradiated with UV light through a patterned mask. The irradiated portion is removed leaving a pattern of the cytophilic or cytophobic polymer. In a second method, a cytophobic polymer surface is irradiated through a mask such that functional groups are introduced that enable adhesion at specific regions of the surface. A third method involves photoirradiation to introduce polymerization initiation sites available for grafting other cytophilic or cytophobic molecules. The claims only provide a brief description of the first method stating specifically that the cytophobic photosensitive polymer applied to a cytophilic surface is irradiated to leave the irradiated portion on the cytophilic surface. The reverse procedure (cytophilic surface atop a cytophobic surface) is not mentioned in the claims. The claims also specify that the photosensitive polymer is a "cell non-adhesive polymer having at least one azido group", or that the polymer is "a composition containing a cell-adhesive polymer and a compound having at least two azido groups". The patent leaves ample opportunity to create a device similar in function using polymers without azido groups.

Miyamoto patented another method of arranging cells, utilizing photolithography.[71] The patent was issued in 1997 and is assigned to NEC Corporation in Tokyo Japan. A photoresist layer applied to a substrate is irradiated such that irradiated regions are removed. The substrate is subsequently spin coated with culture medium containing enzyme substrates (i.e. glucose oxidase with glutal-aldehyde cross-linking membrane of bovine serum albumin as its base) such that an immobilized enzyme membrane forms on the bare substrate surface regions. Finally, the substrate is soaked in acetone to dissolve the remaining photoresist exposing the bare substrate surface (i.e. quartz). The glucose oxidase inhibits cell adhesion, while cells readily adhere to the quartz substrate. The claims describe the process in detail but do not specify the composition of the immobilized enzyme membrane.

Since 1998, three patents, on devices for adhering cells in a controlled manner, have been issued from the same seven individuals, namely, Rahul Singhvi, Amit Kumar, George Whitesides, Donald Inberg, Gabriel Lopez, Daniel Wang, and Gregory Stephanopoulus. [72,73,74] The first was issued July 7th, 1998, the second a year later, and the third was recently issued April 9th, 2002. The first two are shared by the President and Fellows of Harvard College, the Massachusetts Institute of Technology, and the Children's Medical Center Corporation; the latter belongs solely to the President and Fellows of Harvard College. The latter two are essentially the same device as the first, but provide additional capabilities. The patents describe a device that comprises a substrate surface with cytophilic islands isolated by cytophobic regions. The islands are composed of SAMs, and can be configured to take on any size or configuration and may be adapted to bind only selected cell types. The device is applicable for use in cell culturing, cytometry, toxiclogy, cell screening, immobilization of cells, microinjection, and influencing cell differentiation. Claims focus on the parameters of the device itself, and on applications pertaining to cell culturing, cytometry, microinjection, and in controlling the shape of the cell. The patent provides no substrate limitations mentioning only that the device comprises "a plate defining a surface". Additionally, the claims mention nothing of the procedure in patterning the surface, but do specify that the "cytophilic islands" and "cytophobic regions are formed of self-assembled monolayers". These clauses restrict the patent to self-assembling molecular species, and would not, for

instance, block a similar device in which the cytophilic and cytophobic regions are composed of polymers. Claims of the second patent provide one addition, namely, that the islands bind only selected cell types. Claims in the most recent issue add the clause: "discrete cytophilic regions promote adherence of a single cell type", and that cytophilic regions include "at least one region having a size chosen such that only an individual cell is able to adhere thereto." Of all the patents presented in this section, these three patents impose the greatest blocking capability on patterning surfaces for cell arrangement. However, similar devices based on polyelectrolytes can be achieved without any risk of infringement. In any case, it could prove beneficial to obtain a license on these patents, as the incorporation of self-assembling molecular species with PEMs would certainly provide greater latitude in the types of devices that could be realized.

Zhang and co-workers[75] were recently issued a patent on the invention of a film comprising a self-assembled monolayer of reactive linear peptides on a substrate where the molecules may be designed to exhibit an affinity for cell adhesion. The assignees of this patent are the Massachuesetts Institute of Technology, and the President and Fellows of Harvard College. The patent focuses on the composition of the peptides, and the method of assembling them onto the surface. Claims specify that the peptide comprises a terminal group, a central linker, and a presenting group, where each of these segments may be selected from other specific groups of molecules. The presenting group may consist of antigens, antibodies, antibody fragments, cellular adhesion motifs, high chain alkyls, hydrophilic blocked amino acids or ligands. The terminal amino acid group may be comprised of scrine, aspartic aid, glutamic acid, or cysteine. The central linker comprises between 2 to 50 amino acids selected from the group of an oligoclycine or oligoalanine. The peptides bond directly to the substrate through a terminal amino acid. The 'Detailed description of the invention' section of the patent explains that the film is deposited via microcontact printing. The first claim is fairly specific in detailing the steps of microcontact printing to assemble the SAM onto the surface. The claims also specify use of an elastomeric stamp. Other claims are more broad stating merely that the film "comprises a printed pattern [of] self-assembled monolayers". This patent would likely block anyone from using these SAMs regardless of the method of deposition.

Two patents exist on cell-based sensor devices intended for harmful reagent detection. The first was patented by Nakamura *et al.*[76] in 1992 and assigned to Fuji Electric Co. and Public Works Research Institute (both are located in Japan). The patent describes a device that detects toxic substances in water by use of nitrous acid-producing bacteria. The device is set up such that bacteria cells are fixed in a nitrocellulose membrane, where the membrane separates a water intake channel and a dissolved oxygen electrode. A separate channel feeds a buffer solution containing ammonium hydroxide (NH_4^+) and nutrients such as calcium chloride ($CaCl_2$) and magnesium sulfate ($MgSO_4$) to the bacteria. Test water enters the channel, is saturated with dissolved oxygen, then flows over the membrane enabling toxic substances (if present) to interact with the bacteria. The bacteria consumes the NH_4^+ and O_2, producing NO_2^-. If toxic substances are present in the water, the oxygen respiration activity of the bacteria is reduced. This decreased amount of dissolved oxygen is detected by the dissolved oxygen electrode through a gas-permeable membrane. The claims of the invention focus on the setup and operation of the device and say nothing of how the membrane is fabricated, how it is secured to the chamber walls, or how cells are fixed within the membrane. In addition to details relating to device set-up, the claims do specify a "toxic substance-detecting device...wherein nitrous acid-producing bacteria are fixed on [a] fixed-microorganism membrane...". The claims state that the fixed-microorganism membrane is a nitrocellulose membrane, and that the cells are nitrous acid-producing bacteria. This patent leaves open the possibilities for the creation of a device similar in function, choosing from a wide variety of cells other than the bacteria cells mentioned. These cells could also be fixed within a membrane composed of some material other than a nitrocellulose membrane.

More recently, Simpson *et al.*[77] patented a biosensor device that incorporates yeast, bacteria, fungus, or animal or plant cells as a bioreporter. UT Battelle, LLC, and The University of Tennessee Research Corporation hold the license to this patent. Bioreporter cells are genetically engineered with a nucleic-acid sequence encoding a gene that expresses a light-emitting polypeptide such that the bioreporter emits light as it metabolizes a particular substance. Cells are encased in a clear polymer matrix that is permeable to the substance detected. The polymer matrix lies on an integrated circuit that

includes a photodiode that generates an electrical signal in response to the light. The polymer matrix is separated from the integrated circuit by a bioresistant/biocompatible material such as silicon nitride. As in the previous patent, the claims focus on the operation and setup of the apparatus, and leave out any description of the how the cells are seeded into the polymer matrix, what the polymer matrix consists of, or how it is fabricated.

Cellomics Inc. holds two patents on cell-based HTS devices. The first, patented in November 23, 1999 by Dunlay *et al.*,[78] describes a computer controlled optical-mechanical system for determining the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds. Cells are placed in an array within a microtiter plate or a microplate where a microfluidic system delivers compounds to the cells. The cells are scanned to obtain fluorescent signals from fluorescent reporter molecules in subcellular compartments, such as the cell nucleus or cytoplasm. Changes in fluorescence intensity are analyzed. The patent focuses on the automated method of analyzing the cells including the method to image the cells, converting the optical information into digital data, and the analysis and interpretation of the data. No attention is given to the method of patterning cells on the microtiter plates and microplates. The microplates are described as follows in the 'Detail Description of the Invention' section: "Microplates may consist of coplanar layers of materials to which cells adhere patterned with materials to which cells will not adhere, or etched 3-dimensional surfaces of similarly patterned materials." The claims provide no information on the method or apparatus in which cells are placed.

Their second patent, issued August 15, 2000, discloses a device and method that allows multiple types of cell interactions to be studied simultaneously by combining multicolor luminescence reading, microfluidic delivery, and environmental control of living cells in uniform micro-patterned arrays.[79] The device is intended for high throughput screening of the physiological response of cells to compounds. This device is unique since it incorporates an array of multiple cell types. It also enables the treatment of cells with one or more reagents via its microfluidic system. The system also includes a luminescence reader instrument for detecting luminescence signals from the luminescent reporter molecules, a digital detector for receiving data, and a computer for processing

data. The claims, however, mention very little about patterning. Two clauses are present: the method comprises "preparing a micro-patterned chemical array" and the second states that "the micropatterned array is then modified such that it exhibits multiple different cell binding sites on the surface that interact with different cell types." The remainder of the claims focus primarily on the microfluidic system. The description section mentions photolithography for patterning the surface, but the claims say nothing.

5.0 Business plan for a start-up venture

In commercializing this technology, a preliminary business plan is essential in determining what markets to attack, how to penetrate them, and how acquire profits from them. Initiating a new venture requires a clear focus on what the main research and development efforts will be. A thorough market analysis is also critical in determining a comprehensive strategy. This section presents a brief overview of the intended markets and a rough outline of a business plan for a new venture proposing to use the layer-by-layer assembly of PEMs as a platform in the biotech industry. A detailed market analysis and business plan is beyond the scope of this work.

The layer-by-layer assembly of PEMs clearly poses options for developing products in a wide range of applications. In biotechnology alone, there are applications in biosensors, drug delivery, cell-based assays, bioinert and biocompatible coatings, tissue engineering, and more. Some of these applications fall into the categories of therapeutics or in-vivo diagnostics. Jeff Volk, Manager of Marketing Services at Cascade Biologics, said that breaking into these markets is often extremely difficult, even with a great product, since the markets are predominantly profit-based.[80] Entering these markets would require strong venture capitalist backing. One of the major obstacles in getting a product to these markets involves approval from the Food and Drug Administration (FDA). Elhage referred to the FDA as "the Great Wall of China".[50] Venture capitalists (VCs), outside the high risk biotech areas, are especially hesitant about investing in companies that target these markets. Therefore, a venture with the PEM platform will more likely succeed by targeting other markets, such as high-throughput screening for

drug discovery or in-vitro biosensors, where such barriers may be avoided. An investigation of these markets reveals great potential within both.

Currently, pharmaceutical and biotech companies are under pressure to increase productivity and decrease their time to market while simultaneously decreasing drug discovery costs. Wall Street is requiring at least 10% growth in annual revenues, and the top firms must triple their success rates to remain viable.[81] This pressure is forcing companies to find more efficient drug discovery methods such as HTS. Pharmas and biotech companies worldwide spent $42 billion in research and development in 1997. $32 billion was spent in the US alone. The portion of the worldwide R&D expenditure solely on HTS was $5.88 billion. Most of the drug screening is being performed internally by pharmas, but a significant portion is being spent on outside products and services, and the market for HTS products and services is growing as HTS directors intend to expand their screening operations. The demand for HTS equipment is driven by demands of pharmas and biotech companies for miniaturized, more efficient, and easier-to-use detection assays. The HTS market is forecasted to increase in revenues growing at a compound annual growth rate of 22.5%. Thus, HTS suppliers involved in developing new assays are expected to experience remarkable growth.[32]

The biosensor market includes biosensors used in medical, environmental, industrial and military applications. A good portion of the devices produced in these segments are portable immunobiosensors and applications for their use are expected to grow. Medical segments include applications in research and diagnostics. The demand for biowarfare sensors in the military has especially increased in light of recent terrorist acts. For these reasons the market is expected to grow from $413 million in 1996 to over $500 million in 2002.[82]

In starting a new venture, efforts would focus first on developing cell-based assays for the HTS market. Once a product is finally developed and being marketed, then efforts would be broadened to develop cell-based biosensors for the biowarfare and environmental sectors. Once the venture is achieving high profits, applications in therapeutics such as tissue engineering would be explored.

The business plan for the first five to six years would include four stages: an early technical phase, a second technical phase, an execution phase, and a commercial launch

stage.[81] The early technical phase would involve developing basic samples to demonstrate the proof of principle and thereby establish a proprietary technology. This would be accomplished in six months or less and would require approximately $250,000 of seed-round financing. Having demonstrated the principle, additional funds would be necessary for further development in the second technical phase. In this stage, roughly $4 million would be needed to lease lab space, purchase laboratory equipment, hire scientists and engineers, and begin making the cell-based assay for HTS. Research and development efforts would be divided into three teams: a biology group, a chemistry group, and a microfabrication group. Capitol from first round VC funding would be used to build a sustainable company and prove that the technology will work in an industrial setting. In this time a portfolio of various niche patents would be built up. Reaching milestones such as completing the design of a product and having it ready to be manufactured would be critical in order to obtain any further funding from investors. This second phase is anticipated to take up to two year.

The company would enter its execution phase after procuring second round financing from early stage investors. As the company enters this phase, it would likely need an additional $10-15 million over the course of the next year to do early product development. The company would expand its work force to include additional scientists and engineers, technicians, computer specialists and business people. Additionally, developing a joint venture with a company specializing in automated and analytical instrumentation would be largely beneficial at this stage. This would enable co-development and distribution of novel products to serve the market. Moreover, strategic partners would provide an in-depth knowledge of the customers' needs, and an opportunity to access market and product expertise. Cash funding could be secured in the form of both equity and cost sharing. Preparations for an Initial Public Offering (IPO) would also be underway during the execution phase.

Over the course of the next two years the product would be fine-tuned for the market. Funding from later-stage mezzanine investors would be sought, and an IPO would be considered. Final product development and manufacturing would likely require anywhere from $10-20 million. Cell-based assay kits for HTS currently sell for $500-1,000 each. Environmental and biowarfare biosensors will likely sell for the same price.

Costs for manufacturing assays based on PEMs would certainly be less than that of current methods. Thus the new venture would aim to sell its HTS assays for the same range in price and acquire greater profits than other current systems.

After the first six years of start-up and once profits are being realized, the company would expand its R&D efforts to include therapeutics, i.e. tissue engineering, and in-vivo diagnostic devices. Broad patents would also be licensed to other companies in markets that the new venture is not pursuing. For instance, patents on bioinert or biocompatible coatings could be licensed to companies specializing in surgical tools, implants, and prosthetics. License agreements for such patents would specify that the rights for the use of the patents are restricted to the field of the designated application.

6.0 Conclusions and future prospects

The assembly of weak polyelectrolyte multilayers offers enormous control over molecular organization and surface properties by simple adjustments of the dipping solution pH. Additionally, PEMs overcome some of the limitations and problems surrounding SAMs, including limitations in substrate choice, surface defects (i.e. pin holes), and stability. Moreover, the ionic charge characteristics of polyelectrolytes and their permeabilities to water render them naturally compatible with other charged biopolymers such as DNA and proteins. These compiled attributes make PEMs attractive for novel biomaterials. The ability to control the adhesion of cells and their arrangement on PEM surfaces offers tremendous possibilities for applications in cell-based screening and sensing, tissue engineering, and for fundamental studies in cell behavior.

In the past few years, interest in cell-based HTS systems in industry has grown as a result of increased market pressure for pharmaceutical companies to increase productivity while simultaneously reducing costs. The market for cell-based biosensors is also expected to grow, especially in military applications. Professionals interviewed suggested that PEMs could be employed in providing better cell adhesion and stable environments for the improved maintenance of cell lines and for more intimate contact in cell-based biosensors. Additional suggested applications included cell sorting techniques, and the incorporation of cells within multilayers for applications in tissue engineering.

A US patent search on the core technology revealed no patents that would significantly block one from developing products using a technology platform based on the layer-by-layer assembly of polyelectrolytes. The patent owned by Bayer Corporation[63] can be bypassed without infringement risks, because it is limits the assembling technique to the use of a charged substrate. The patents on μCP would inhibit one from using self-assembling molecular species, but poses no restrictions on the use of polymers. It may however be wise in this case to seek a "competent opinion of counsel" for patent-clearance, before using μCP to develop similar devices based on PEMs. A competent clearance opinion will also satisfy investors that the company is proceeding in a prudent manner, thus reducing the risk of investment. In any case, the use of molecular species in μCP with polyelectrolytes would certainly provide greater latitude in developing novel structures. Therefore, obtaining a license to the two patents on μCP could prove to be highly beneficial. In such a case, royalty payments would be made to the owner of the patent. Three patents block the ability to pattern surfaces with cytophilic and cytophobic regions composed of self-assembled monolayers,[69,70,71,] but do not restrict the use of polyelectrolytes and other polymers. As in the case of the patents on μCP, it may be advantageous to obtain licenses on these patents, as this would provide tremendous flexibility in the range of systems possible. Other patents on cell patterning, cell-based high-throughput screening, and cell-based biosensors are either too far removed from PEM technology or specific enough that they pose no major threat in blocking the ability to create similar devices with PEMs.

A new venture with a platform based on layer-by-layer assembly of polyelectrolytes for the HTS market would likely take six years to become profitable. Funding over the course of these six years would require anywhere from $25 - 40 million in funding to develop and manufacture cell-based screening kits. Soon after the company begins marketing its cell-based screening kits, research and development efforts would include cell-based biosensors. Once profits are reached, other markets in therapeutics would be pursued including cell-based tissue engineering, and in-vivo sensors.

Future research with respect to the manipulation of cells using PEMs should seek to further identify mechanisms by which cells may interact with multilayer substrates, and to further exploit opportunities of integrating functionality along the layer normal.

This could include developing substrates that direct cell behavior or respond to cellular processes, or providing cells with a suitable environment within multilayer films. Ultimately, biomimetic materials should mimic the behavior of natural substrates that respond to nearby cells and refashion themselves accordingly.

Bibliography

1. G. Decher,"Fuzzy Nanoassemblies: Toward Layered Polymeric Mulitcomposites", *Science* **277**, 1232 (1997)
2. M. Mandel, "Polyelectrolytes", *Encyclopedia of Polymer Science and Engineering* **11**, John Wiley & Sons, New York,NY, (1988) p. 739
3. S. Joly, R. Kane, L. Radzilowski, T. Wang, A. Wu, R. E. Cohen, E. L. Thomas, M. F. Rubner, "Multilayer Nanoreactors for Metallic and Semiconducting Particles", *Langmuir* **16**, 1354 (2000)
4. J. D. Mendelsohn, C. J. Barrett, V. V. Chan, A. J. Pal, A. M. Mayes, M. F. Rubner, "Fabrication of Microporous Thin Films from Polyelectrolyte Multilayers", *Langmuir* **16**, 5017 (2000)
5. M. F. Rubner, "pH-Controlled Fabrication of Polyelectrolyte Multilayers: Assembly and Applications", submitted for publication
6. S. S. Shiratori, M. F. Rubner, "pH-Dependent Thickness Behavior of Sequentially Adsorbed Layers of Weak Polyelectrolytes", *Macromolecules* **33** , 4213 (2000)
7. Yoo D., Shiratori S. S., Rubner M. F., "Controlling Bilayer Composition and Surface Wettability of Sequentially Adsorbed Multilayers of Weak Polyelectrolytes", *Macromolecules* **31**, 4309 (1998)
8. T.C. Wang, B. Chen, M. F. Rubner, Cohen R. E. , "Selective Electroless Nickel Plating on Polyelectolyte Multilayer Platforms", *Langmuir* **17**, 6610 (2001)
9. Wang T.C., Rubner M. F., R. E. Cohen submitted for publication
10. A. J. Chun, M. F. Rubner, "Methods of Loading and Releasing Low Molecular Weight Cationic Molecules in Weak Polyelectrolyte Multilayer Films", *Langmuir* **18**, 1176 (2002)
11. J. Choi, M.F. Rubner, "Selective Adsorption of Amphiphilic Block Copolymers on Weak Polyelectoyte Multilayers", *Journal of Macromolecular Science – Pure and Applied Chemistry* **A38**, 1191 (2001)
12. O. Onitsuka, A. C. Fou, M. Ferreira, B. R. Hsieh, M.F. Rubner, "Enhancement of light emitting diodes based on self-assembled heterostructures of poly(*p*-phenylene vinylene)", *Journal of Applied Physics* **80**, 4067 (1996)
13. A. C. Fou, O. Onitsuka, M. Ferreira, M.F. Rubner, B. R. Hsieh, "Fabrication and properties of light-emitting diodes based on self-assembled multilayers of poly(phenylene vinylene)" *Journal of Applied Physics* **79**, 7501 (1996)
14. A. Wu, D. Yoo, J. K. Lee, M.F. Rubner, "Solid-State Light-Emitting Devices Based on the Tris-Chelated Ruthenium(II) Complex: 3. High Efficiency Devices via a Layer-by-Layer Molecular-Level Blending Approach" *Journal of the American Chemical Society* **121**, 4883 (1999)
15. P. K. H. Ho, J. S. Kim, J. H. Burroughes, H. Becker, S. F. Y. Li, T. M. Brown, F. Cacialli, R. H. Friend, "Molecular-scale interface engineering for polymer light-emitting diodes", *Nature* **404**, 481 (2000)
16. M. F. Durstock, M. F. Rubner, "Dielectric Properties of Polyelectrolyte Multilayers", *Langmuir* **17**, 7865 (2001)
17. S. Y. Yang, M. F. Rubner, "Micropatterning of Polymer Thin Films with pH-sensitive and Cross-linkable Hydrogen-Bonded Polyelectrolyte Multilayers" *Journal of the American Chemical Society* **124**, 2100 (2002)
18. Sung Yun Yang, converation with author, 25 March 2002

19. A. Kumar, H. A. Biebuyck, G. M. Whitesides, "Patterning self-assembled monolayers: Applications in materials science", *Langmuir* **10**, 1498 (1994)
20. P. T. Hammond, "Chemistry Directed Deposition via Electrostatic and Secondary Interactions: A Nonlithographic Approach to Patterned Polyelectrolyte Multilayer Systems", submitted for publication.
21. J. Kötz, "Polyelectrolyte Complexes", *Polymeric Materials Encyclopedia*, CRC Press, Inc.: Boca Raton, FL, (1996), p. 5762, 5773
22. T. Salditt, U. S. Schubert, "Layer-by-layer self-assembly of supramolecular and biomolecular films", *Reviews in Molecular Biotechnology* **90**, 55 (2002)
23. M. Onda, Y. Lvov, K. Ariga, T. Kunitake, "Sequential actions of glucose oxidase and peroxidase in molecular films assembled by layer-by-layer alternate adsorption", *Biotechnology and Bioengineering*, **51**, 163 (2000)
24. K. Ariga, Y. Sasaki, H. Horiguchi, N. Horiuchi, J. Kikuchi, "Layered nanoarchitectures between cationic and anionic materials – Composite assemblies of polyions, lipid bilayers, and proteins", *Soft Chemistry Leading to Novel Materials Defect and Diffusion Forum*, **191**, 35 (2001)
25. Y. P. Sun, X. Zhang, C. Q. Sun, B. Wang, J. C. Shen, "Fabrication of ultrathin film containing bienzyme of glucose oxidase and glucoamylase based on electrostatic interaction and its potential application as a maltose sensor."
26. F. Caruso, D. Trau, H. Mohwald, R. Renneberg, "Enzyme Encapsulation in Layer-by-Layer Engineered Polymer Multilayer Capsules", *Langmuir* **16**, 1485 (2000)
27. G. M. Whitesides, B. Gryzbowski, "Self-Assemlby at All Scales" *Science* **295**, 2418 (2002)
28. Charles Keese (Applied Biophysics R&D VP), conversation with author, Mar. 14, 2002
29. J. J. Pancrazio, J. P. Whelan, D. A. Borkholder, W. Ma, D. A. Stenger, "Development and Application of Cell-Based Biosensors", *Annals of Biomedical Engineering* **27**, 697 (1999)
30. D. A. Stenger, G. W. Gross, E. W. Keefer, K. M. Shaffer, J. D. Andreadis, W. Ma, J. J. Pancrazio, "Detection of physiologically active compounds using cell-based biosensors", *Trends in Biotechnology* **19**, 304 (2001)
31. "Cellomics, Inc. Announces the Development of Biowarfare Detection Methods" *Cellomics News Archives Page* (21 November 2001)
32. R C. Smith, Jr., "Biomolecular Research and Development – High-Throughput Screening", *Medical & Healthcare Market Place Guide*, **1**, (2001) 16th ed., p. I-247
33. C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. E. Ingber "Geometric Control of Cell Life and Death", *Science* **276**, 1425 (1997)
34. S. Zhang, L. Yan, M. Altman, M. Lassle, H. Nugent, F. Frankel, D. Lauffenburger, G. M. Whitesides, A. Rich, "Biological surface engineering: a simple system for cell pattern formation", *Biomaterials* **20**, 1213 (1999)
35. T. Pakalns, K. L. Haverstick, G. B. Fields, J. B. McCarthy, D. L. Mooradian, M. Tirrell, "Cellular Recognition of synthetic peptide amphiphiles in self-assembled monolayer films", *Biomaterials* **20**, 2265 (1999)

36. Y. Wu, P. Wang, X. Ye, G. Zhang, H. He, W. Yan, X. Zheng, J. Han, D. Cui, "Drug evaluations using a novel microphysiometer based on cell-based biosensors", *Sensors and Actuators B* **80**, 215-221 (2001)
37. *Surface Logix Home Page* <http://www.surfacelogix.com/index.htm> (April 22, 2002)
38. *Cellomics Home Page* http://www.cellomics.com/ (22 April 2002)
39. *DARPA Tissue Based Biosensors* <http://www.darpa.mil/dso/thrust/sp/Tbb/index.html> (33 Apr. 2002)
40. *Medtronic Newsroom* <http://www.medtronic.com/newsroom/news_19991004.html> (Mar. 22, 2002)
41. *Applied BioPhysics Home Page*<http://www.appliedbiophysics.com/> (April 22, 2002)
42. *BioTrove Home Page*< http://www.biotrove.com/> (20 April 2002)
43. *Aurora Bioscinces Home Page* <http://www.aurorabio.com/> (Apr. 20 2002)
44. "Profiles: Aurora BioSciences", *Biospace* <http://www.biospace.com/company_profile.cfm?CompanyID=1781> (Apr. 29, 2002)
45. Jonas Mendelsohn, conversation with author, 5 February 2002
46. Michael Berg, conversation with author, 8 February 2002
47. E. Otsuni, R. G. Chapman, M. N. Liang, G. Meluleni, G. Pier, D. E. Ingber, G. M. Whitesides, "Self-Assembled Monolayers That Resist the Adsorption of Proteins and the Adhesion of Bacterial and Mammalian Cells" *Lamgmuir* **17**, 6336 (2001)
48. Tara Heitner, conversation with author, 12 April 2002
49. Kelly Cassutt, conversation with author, 9 April 2002
50. Amer Elhage, conversation with author, 4 March 2002
51. R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber, G. M. Whitesides, "Patterning proteins and cells using soft lithography", *Biomaterials* **20**, 2363 (1999)
52. Ping Wang, "Fwd: letter" 20 April 2002 [personal email with attachment] (19 April 2002)
53. Heejae Kim, conversation with author, 11 March 2002
54. K. Ichimura, S. Oh, M. Nakagawa, "Light-Driven Motion of Liquids on a Photoresponsive Surface", *Science* **288**, 1624 (2000)
55. N. K. Chaki, K. Vijayamohanan, "Self-assembled monolayers as a tunable platform for biosensor applications", *Biosensors & Bioelectronics* **17**, 1 (2002)
56. S. Flink, F. C. J. M. van Veggel, D. N. Reinhoudt, "Sensor Functionalities in Self-Assembled Monolayers", *Advanced Materials* **12**, 1315 (2000)
57. X. Wang, S. Balasubramanian, L. Li, S. Tripathy, J. Kumar, "Nonlinear Optical Structure and Methods of Making" *United States Patent* **6,106,948** Aug. 22, 2000
58. S. Luo, P. K. Lo, H. P Groger, R. J Churchill, "Optical Chemical Sensor Based on Multilayer Self-Assembled Thin Film Sensors for Aquaculture Process Control", *United States Patent* **6,051,437** Apr. 18, 2000
59. G. S. Ferguson, E. R. Kleinfeld, "Stepwise Formation of Multilayered Nanostructures from Macromolecular Precursors", *United States Patent* **6,022,590** Feb. 8, 2000

60. M. Onda, Y. Lvov, K. Ariga, T. Kunitake, "Multiple Layered Functional Thin Films", *United States Patent* **6,020,175** Feb. 1, 2000
61. Min-Shyan S., I. H. Loh, "Durable Hyrdophlic Surface Coatings", *United States Patent* **5,807,636** Sep. 15, 1998
62. M. F. Rubner, J. H Cheung, "Molecular self-assembly of electrically conductive polymers", *United States Patent* **5,518,767** May 21, 1996
63. M. F. Rubner, J. H. Cheung, "Molecular self-assembly of electrically conductive polymers", *United States Patent* **5,536,573** July 16, 1996
64. H.U. Siegmund, L. Heiliger, B. Lent, A. Becker, "Optical Solid-Phase Biosensor Based on Polyionic Layers Labelled with Flourescent Dyes", *United States Patent* **5,711,915** Jan. 27, 1998
65. G. Decher, J. Hong, "One- or multi-layered layer elements applied to supports and their production", *United States Patent* **5,208,111** May 4, 1993
66. A. Kumar, G. Whitesides, "Formation of Microstamped Patterns on Surfaces and Derivative Articles", *United Sates Patent* **5,512,131** Apr. 30, 1996
67. G. Whitesides, Y. Xia, J. L. Wilbur, R. J. Jackman, E. Kim, M. Mrksich, A. Kumar, C. B. Gorman, H. Biebuyck, K. K. Berggren, "Microcontact Printing on Surfaces and Derivative Articles", *United Sates Patent* **6,180,239** Jan. 30, 2001
68. R. Klebe, "Apparatus for the Precise Positioning of Cells", *United Sates Patent* **5,108,926** Apr. 28, 1992
69. T, Matsuda, K. Inoue, N. Tank, "Control of Cell Arrangement", *United Sates Patent* **5,202,227** Apr. 13, 1993
70. S. Miyamoto, "Method for Fabricating Substrate for Cell Culture and Method for Cell Arrangements", *United Sates Patent* **5,602,029** Feb. 11, 1997
71. R. Singhvi, A Kumar, G. M. Whitesides, D. E. Ingber, G. Lopez, D. I. C. Wang, G. N Stephanopoulos. , "Method of formation of microstamped patterns on plates for adhesion of cells and other biological materials, devices, and uses therefore", *United States Patent* **5,776,748** Jul. 7, 1998
72. R. Singhvi, A. Kumar, G. M. Whitesides, D. E. Ingber, G Lopez , D. I. C Wang. , G. N. Stephanopoulos, "Device containing cytophilic islands that adhere cells separated by cytophobic regions", *United States Patent* **5,976,826** Nov. 2, 1999
73. Singhvi R., Kumar A., Whitesides G. M., Ingber D. E., Lopez G., Wang D. I. C., Stephanopoulos G. N., "Adhering cells to cytophilic islands separated by cytophobi regions to form patterns and manipulate cells", *United States Patent* **6,368,838** Apr. 9, 2002
74. S. Zhang, A. Rich, L. Yan, G. Whitesides,"Self-Assembling Peptide Surfaces for Cell Patterning and Interations", *United Sates Patent* **6,368,877** Apr. 9, 2002
75. E. Nakamura, H. Tanaka, Y. Tanaka, T Iitake, H. Hoshikawa, "Toxic substance-detecting system with fixed microorganism membrane for water quality-monitoring", *United Sates Patent* **5,160,603** Nov. 3, 1992
76. M. Simpson, G.S. Saylor, M Paulus , "Bioluminescent bioreporter integrated circuit", *United Sates Patent* **6,117,643** Sep. 12, 2000
77. R. T. Dunlay, D. L. Taylor, "System for cell-based screening", *United States Patent* **5,989,835** Nov. 23, 1999
78. D. L. Taylor, "Miniaturized cell array methods and apparatus for cell-based screening", *United States Patent* **5,989,835** Aug. 15, 2000

79. Jeff Volk, conversation with author, 14 March 2002
80. T. Oeakman, Y. Bonduelle, "Steering a course through the technology maze", *Drug Discovery Today* 8, 337 (2000)
81. R C. Smith, Jr., "Biosensors", *Medical & Healthcare Market Place Guide*, 1, (2001) 16th ed., p. I-247
82. H. C. Birndorf presented a similar model in his article: "Rational financing", *Nature Biotechnology* 17, BE33 (1999)