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Cellular Analysis Of Hepg2 Cells On Gastrin Releasing Peptide (Grp) Nanostructure

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Cellular Analysis of HepG2 Cells on Gastrin Releasing Peptide (GRP) Nanostructure

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Dedication

This thesis is dedicated to my wife, Sharmista Kosaraju, my parents, Krishna Prasad Kosaraju and Lakshmi Kumari Kosaraju, and my sister, Vennela Kosaraju for their love, support and encouragement.

Biographical Sketch

Karshak Kosaraju was born on November 11, 1986 in Nidubrolu, Andhra Pradesh, India. He received his Bachelor's degree in Pharmacy from Jawaharlal Nehru Technologial University, India in 2008. He joined the Master's program in Chemistry at North Carolina Agricultural and Technical State University in the Fall of 2009. He conducted research on cell culture studies using an immobilized peptide and a growth factor on SAM-coated ITO. In parallel to the cell culture work he carried out steam reforming of methanol to produce hydrogen using bi-metallic nanocatalysts in mesoporous silica. While his work on cell culture has been selected for an oral presentation, while his work on hydrogen production will be part of a poster presentation at the ACS national meeting (August 19-23, 2012) in Philadelphia, Pennsylvania.

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Table of Contents

List of Figures

List of Tables

List of Nomenclature

GRP	Gastrin Releasing Peptide
CCP	Cell Culture Platform
ECM	Extra-cellular Matrix
ITO	Indium Tin Oxide
SAM	Self-Assembled Monolayer
LDH	Lactose Dehydrogenase
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide
AFM	Atomic Force Microscopy
ATR	Attenuated Total Reflectance
FT-IR	Fourier Transform-Infrared
3-APTES	3-aminopropyltriethoxysilane
HepG2	Human Hepatocellular Carcinoma Cell line
PET	Polyethyleneterephthalate

Abstract

Artificial juxtacrine stimulation arises from the covalent attachment of growth factors onto biomaterials. Incorporation of growth factors onto cell culture substrates has been found to enhance the functionality of cells in vitro. In this study, we describe how the immobilization of Gastrin Releasing Peptide (GRP) enhances the viability of hepatocarcinoma cell line, HepG2 up to 4 days.

The biomaterial for immobilization of GRP was prepared using indium tin oxide (ITO) sputtered on polyethylene terephthalate (PET). A self-assembled monolayer (SAM) of 3-aminopropyl triethoxysilane (3-APTES) on ITO was covalently attached to GRP. Characterization of the substrates before and after immobilization of GRP was carried out using contact angle measurements, FTIR and AFM techniques. HepG2 cells were cultured on immobilized GRP-SAM-ITO substrate for 24, 48, 72 and 96 hours and compared to cell viability of soluble GRP under similar conditions. The cell viability on immobilized GRP-SAM-ITO after 48 hours was less than that of soluble GRP by 19%. Lactate dehydrogenase (LDH) production after 48 hours was significantly reduced by 44% for immobilized GRP when compared to that with soluble GRP. After 96 hours, cell viability increased and cytotoxicity decreased for HepG2 cells on GRP-SAM-ITO substrates, suggesting viability was successfully extended. A similar experiment (LDH assay) with immobilized epidermal growth facor (EGF), obtained by covalent attachment of EGF shows that the cell viability can be extended to 5 days. These data may provide insight towards the development of bioreactors for drug toxicity screening.

CHAPTER 1

Introduction

1.1 Extracellular matrix

Cells play a major role in building tissues and maintaining tissue function in their respective microenvironments. Significant advances in cell and tissue engineering have been attributed to the successive recreation of the cellular microenvironment *in vitro.* When cells are removed from their microenvironment and placed in an *in vitro* environment, they lose their normal *in vivo* behavior. Cellular microenvironments play an important role in maintaining cell differentiation, cell function, cell aging and cell degradation. One of the principle goals of cell or tissue engineering is to understand the factors that control and regulate cell function and behavior.

The extracellular matrix (ECM) plays an important role in regulating a number of cellular properties and functions like cell viability and differentiation (Jauregui, 1987). The ECM is made up of a complex mixture of structural proteins (collagen, elastin), specialized proteins (fibrin, laminin) and proteoglycans. Each of these components has its own specialized function contributing to the entirety of the ECM (Jauregui, 1987).

Development of *in vitro* models of the ECM is challenging as they require several characteristics pertinent in cell biology. These models should be able to present a homogenous environment of ligands on the surface and be able to resist non-specific adsorption of proteins, which could render the ligand surface inactive (Milan Mrksich, 2000). Also, the surface should be compatible with cell culture and studies of cellular response (Richert et al., 2002). Significant studies have been carried out to study cell-cell, cell-ECM and cell-substrate interactions (Milan Mrksich, 2000). These provide inputs for the development of micro and nano scale technologies,

which define and control the *in vitro* cellular microenvironment. Extensive research is directed towards developing biomimetic surfaces, which exert control over spatial properties, defining the microenvironment for different cell types (Whitesides, Mathias, & Seto, 1991). An understanding of interactions of cells in culture with their environment could help in understanding the properties of the surfaces to which the cells are attached. Thus, developing *in vitro* cell culture platforms (CCPs) based on nanofabrication techniques is a promising area of research.

1.2 Cell culture platforms

The bottom up approach of manipulating cellular environments has brought about a new generation of 2D and 3D biomaterials which are useful in various applications. Bioactive and biocompatible materials such as ceramics, metals, natural products, composites, and polymers have been utilized in blood and tissue compatible applications both internally and externally (Whitesides, et al., 1991). The bioactivity of these materials may be enhanced through the use of adhesion peptides (RGD), growth factors (EGF), or genetic material (siRNA). The technique of artificial juxtacrine signaling utilizing covalently attached or immobilized growth factors to biomaterials has been used to enhance viability, direct differentiation, and support prolonged functionality (Sharon & Puleo, 2008). Many studies have shown the value and efficacy of using immobilized peptides over the free or soluble growth factors. Recently, researchers have utilized a method of immobilizing nerve growth factor (NGF) to glass cover slips to induce differentiation in PC12 cells which also provided prolonged cell culture efficiency (Suk Ho Bhang & Yun Hee Kim Kwon, 2009). Various immobilized cell adhesion molecules or CAM, such as fibronectin, have been found useful in long term expansion of progenitor cells and providing co-stimulatory signals to other growth factors (Qi Feng, 2006).

In recent years, isolated rat hepatocytes have been increasingly used as a model in various culture systems to study pharmacological and toxicological responses of drugs, environmental toxicants, and other compounds (Lee et al., 2008). However, after cells are removed from their microenvironment and placed within an *in vitro* environment, they typically lose some or all of their normal *in vivo* behavior (Hengstler, Godoy, & Bolt, 2009). Although immortalization solves the problem of cell longevity, they suffer in the cell quality. So, it is necessary to continue drug screening with primary hepatocytes and it is important to find ways of making these primary cells perform longer. Efforts to understand the interaction of cells in culture with their environment could benefit from a better understanding of the molecular structure of the surface to which the cells are attached (M Mrksich & Whitesides, 1996; Scotchford, Cooper, Leggett, & Downes, 1998). Thus, the development of cell culture platforms (scaffolds) using micro/nanofabrication is a promising area of research (Milan Mrksich, Dike, Tien, Ingber, & Whitesides, 1997). They are beneficial in the fields of toxicology and drug development due to increased accuracy of *in vitro* predictions, the simplification of testing procedures, and reduction of cost.

In this thesis, the immobilization of an important regulatory peptide, gastrin releasing peptide (GRP) to conductive polymer two dimensional substrates was performed using conjugation chemistry. The purpose of this study is to evaluate GRP (as a model) and EGF immobilized on a conductive substrate that may be useful in development of a bioreactor. Previous findings in our lab showed great promise for the employment of a SAM-linker that enhances functionality and viability of primary liver cells (Aithal, 2007). Our goal within this project is to determine how the immobilized peptides or growth factors would interact with seeded hepatocarcinoma cell line, HepG2. In order for most growth factors or peptides to

activate or affect the cell's functioning, the peptide in most cases must be internalized through receptor mediated endocytosis. Receptor mediated endocytosis is a mechanism in which ligands or peptides are recognized by specific receptors on the cellular surface. This recognition causes a series of pathways to become activated and therefore initiating cellular events such as viability or apoptosis. We have used immobilized GRP to analyze the effect of inhibiting the uptake and internalization of the peptide and monitor the outcome using various cellular based assays. Cellular based assays were utilized to observe the effect of immobilized proteins on cellular viability (MTT) and cellular cytotoxicity (LDH).

There are many substrates (gold, silicon, etc..,) that are available for conducting cell culture studies. Cell culture studies on SAMs of alkanethiols on gold (Au) surfaces has been extensively studies over the decades and studies on other surfaces such as silicon are limited. Even though ITO has been extensively studied for different applications in electrical engineering fields, the use of ITO for cell culture applications has been limited (Yousaf, 2009). We have chosen indium oxide-tin oxide (ITO) because of its transparent and conductive properties that make it a very good candidate for development of biosensor.

First, indium oxide-tin oxide (ITO) substrates were modified with self assembled monolayers (SAMs) containing amino end group (NH_2) as shown in Figure 1. These modified substrates were characterized using contact angle measuring goniometry, surface infrared (IR) spectroscopy and atomic force microscopy (AFM). The modified substrates with the NH_2 end group were then covalently coupled to GRP. The substrates immobilized with GRP were again characterized to confirm immobilization of the peptide. Then, HepG2 cells were cultured on GRP immobilized ITO substrates. Cellular activity was measured using spectrophotometric assays. The quantitative measurement of the conversion of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-

diphenyl-tetrazolium bromide (MTT) to formazan by viable cells was used as a quantitative measure of HepG2 cells viability and proliferation. Cytotoxicity profiles of HepG2 were determined by measuring lactate dehydrogenase (LDH) activity in media. LDH is a stable cytoplasmic enzyme, released into the cell culture supernatant by dead or damaged cells.

The suitable choice of a platform that supports optimal cell viability and culture may be useful in understanding the interactions between cell line and immobilized peptide. This will give a better understanding for conducting experiments using growth factors and primary hepatocytes which will help in the development of artificial liver devices. Thus, CCPs should be useful in drug screening and toxicology as they can increase the accuracy of *in vitro* predictions, reduce the costs and simplify testing procedures of those tests.

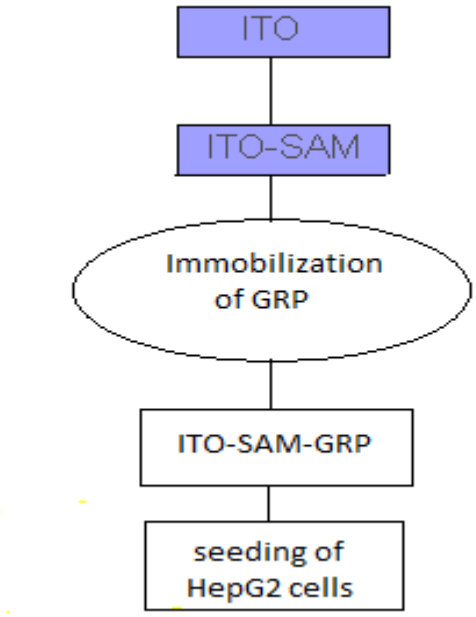


Figure 1. Schematic representation for immobilization of GRP on SAM-ITO and cell culture studies.

CHAPTER 2

Literature Review

2.1 Cell-substrate interactions

Extensive research in the areas of drug discovery and toxicity studies has lead to the development of CCPs which facilitate a thorough understanding of cell-substrate interactions (Pancrazio, Whelan, Borkholder, Ma, & Stenger, 1999). Cell culture platforms control cellular attachment and growth as a function of space and time. They are incorporated in developing cell based biosensors, which monitor physiological change due to exposure to different antigens (Pancrazio, et al., 1999; Wink, J. van Zuilen, Bult, & P. van Bennekom, 1997).

The cellular microenvironment (ECM) plays a major role in controlling cell behavior *in vivo* ensuring proper tissue function (Lampin, Warocquier-Clérout, Legris, Degrange, & Sigot-Luizard, 1997; Zhang et al., 1999). Cellular attachment is a consequence of protein adsorption on substrates, but how the cell receives information about the nature of the substrate is still under investigation. Cell attachment on substrates forms an important prerequisite for the development of bioimplants, cell culture platforms (CCPs) and cell colonization on tissue engineering scaffolds (Zhang, et al., 1999). Mammalian cells are anchorage dependant, requiring an underlying matrix to attach and carry out their regular metabolic, proliferative and differentiation functions (Williams & Wick, 2005).

The ECM is made up of three classes of macromolecules: glycosaminogens, polysaccharide chains covalently linked to proteins forming proteoglycans and fibrous proteins. There are two functional types of fibrous proteins, structural proteins (collagen, elastin) and adhesive proteins (laminin, fibronectin). The glycosaminoglycans intermesh and form a hydrated

gel like substance, in which the fibrous proteins are embedded (Singhvi et al., 1994; Ulman, 1996; Williams & Wick, 2005). The collagen fiber provides strength to the matrix while the elastin fibers provide resilience. The adhesive proteins help the cells attach to the ECM. These proteins provide specific receptor surfaces and ligands, which are identified by the cell surface receptors. Without adhesion, cells enter into apoptosis, eventually causing cell death (LeBaron & Athanasiou, 2000; Williams & Wick, 2005).

The need to recreate a suitable microenvironment for cell proliferation, growth and viability has resulted in the development of a large number of bioengineered substrates (Itle, Koh, & Pishko, 2005; Leoni, Attiah, & Desai, 2002; M. Mrksich, 1998; Milan Mrksich, 2000; T. H. Park & Shuler, 2003). As presented in the sections that follow, almost all of these surfaces have certain common properties like the ability to adsorb protein, uniformity, consistency in surface topography and minimal cytotoxic effect on cells.

2.2 Bioengineered surfaces

Cell attachment occurs due to the interaction of cells with the ECM through specific interaction sites called focal adhesion sites (X. H. Wang et al., 2003). Cell surface receptors identify specific protein domains containing peptide sequences like RGD (Arginine-Glycine-Aspartic acid) and bind to them. In order to mimic the ECM properties in-vitro, bioengineered surfaces coated with ECM components like collagen, fibronectin, vitronectin and fibrinogen have been developed to promote cell-surface interactions by spatially directing attachment of specific cell lines to substrates.

2.2.1 ECM modified surfaces. Collagens are a significant component of the ECM and collagen coated surfaces support growth and viability of different mammalian cell lines (Zavan

et al., 2005). Collagen is a major component of the hepatocyte basal membrane and promotes hepatocyte adhesion and growth. Collagen surfaces are preferred for *in vitro* studies due to their biocompatibility, biodegrability, mechanical integrity and widespread availability (Fukuda, Sakai, & Nakazawa, 2006; Zavan, et al., 2005). Hepatocytes and epithelia cultured on collagen films expressed phenotypes similar to that observed *in vivo*. Osteoblast culture on patterned collagen films to study cellular alignment and viability showed a high degree of phenotypic expression and cellular viability when the surfaces were stabilized using calcium phosphate deposition (Scotchford, Cascone, Downes, & Giusti, 1998).

Modification of collagen films by cross linking and blending with other polymer increase their mechanical stability for cell culture studies. Collagen-chitosan matrices have been used to culture hepatocytes towards the development of artificial livers and shown to support hepatocyte adhesion and division over extended periods of time (X. H. Wang, et al., 2003).

2.2.2 Polyelectrolyte multilayers. Polyelectrolytes are polymers whose units contain an electrolyte group. These polymers dissociate in solution as charged species. Since polyelectrolytes are soluble in water, they have been being investigated extensively for a number of biomedical applications like implant coatings, controlled drug release, biosensor fabrication and cell culture applications (Jewell & Lynn. 2008).

Polyelectrolyte multilayers contain alternating layers of oppositely charged polyelectrolytes. These multilayers are prepared using the layer-by-layer (LBL) assembly technique, where suitable growth substrates are immersed in dilute baths of positive and negative charged polyelectrolyte solutions (Mendelsohn, Yang, Hiller, Hochbaum, & Rubner, 2002). During every immersion, a small amount of polyelectrolyte is adsorbed onto the surface and

charge reversal occurs, thereby allowing the gradual buildup of electrostatically cross linked polyanion-polycation films. The thickness of these films can be controlled up to the nanometer scale. Various types of polyelectrolytes can be used to make these films, including polypeptides (poly-l-lysine), natural polyelectrolytes (chitosan, hyaluronan), proteins and DNA. The morphology of the films and thickness largely depend on the buildup conditions like the ionic strength and pH. Figure 2 shows the process by which polyelectrolytes of alternating charge are deposited on a substrate by the LBL technique. Polyelectrolyte multilayers are ideal candidates for biomaterial applications due to their biocompatibility and inert nature, ability to incorporate biological molecules and control film composition and thickness.

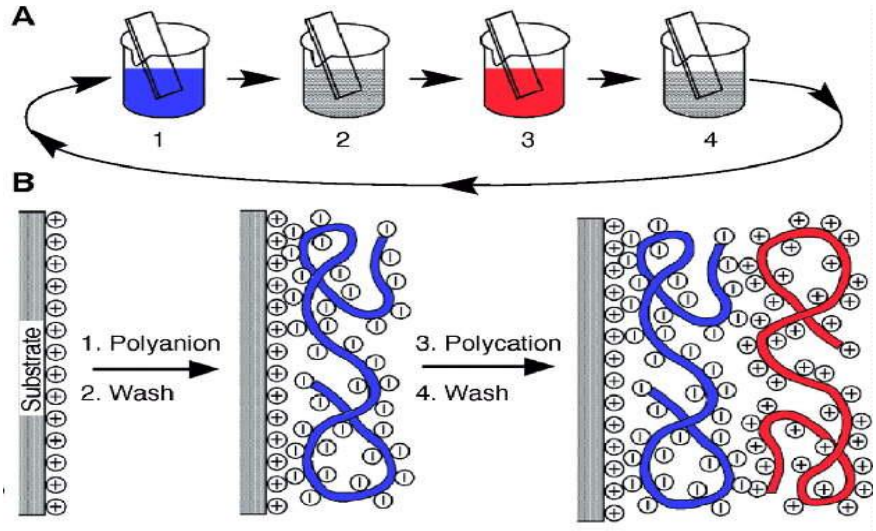


Figure 2. Mechanism of layer by layer assembly of polyelectrolytes (Jewell & Lynn, 2008).

The ability of these multilayers to be patterned effectively using microfabrication techniques like soft lithography and micropatterning gives rise to complex 3 D surfaces for biomedical applications (Ito, 1999). Cell interactions are influenced by the nature and charge of the outermost layer, protein adsorption and thickness of layers (Ito, 1999). Chondrosarcomas cultured on alternating layers of poly-l-lysine (PLL) and poly-glycolic acid (PGA) showed

increased cellular adhesion on PLL ending films when compared to PGA ending films, upon measurement of the adhesive forces. Protein adsorption was observed on PLL terminated films (Richert, et al., 2002).

Primary hepatocytes, which are selective in their attachment *in-vitro*, have been reported to attach, spread and exhibit differentiated functions on PEM multilayers. The PEM used were alternating layers of poly (diallyldimethylammonium chloride) (PDAC) and poly (4-styrenesulfonic acid (SPS). The hepatocytes exhibited characteristic cell patterns upon adhesion on PEM surfaces and showed increased urea and albumin production which are indicators of cellular viability (Kidambi, Lee, & Chan, 2004).

2.2.3 Nanofilms. The LBL technique is used in the assembly of ultrathin films with precise control over specific properties like biocompatibility, thickness and surface wettability. One can form thin films of specific nature by choosing ideal components for the bilayers. Nanoparticle thin films find important applications in material sciences forming corrosion resistive surfaces, polymer coatings and biosensors (Vautier et al., 2002).

More recently, researchers have investigated cell culture on titanium dioxide nanofilms. These nanofilms were prepared by alternating TiO_2 nano particles with polystyrenesulfonate (PSS) on glass, polydimethylsulphonate (PDMS) and polymethylmethacrylate (PMMA) substrates of specific thicknesses. Fibroblasts cultured on these nanofilms showed a higher degree of attachment and biocompatibility towards TiO_2/PSS surfaces (Kommireddy, Patel, Shutava, Mills, & Lvov, 2005).

2.2.4 Hydrogels. Hydrogels are networks of water soluble polymer chains. They are present in the form of colloidal gels having water as the dispersion medium. Hydrogels have been widely used for cell culture studies due to their high water content, pliability, and

biocompatibility and easily controlled mass transfer properties. These properties of hydrogels resemble those of biological tissue (Nettles, Vail, Morgan, Grinstaff, & Setton, 2004; Van Vlierberghe, Dubruel, & Schacht, 2011).

PEG hydrogels have been used to encapsulate mammalian cells like rat osteoblasts, rat cortical neurons and human hepatocytes. These hydrogels can be microfabricated and modified using peptide sequences. Rat hepatocytes encapsulated in PEG hydrogels maintained high cell viability, indicated by increased protein production over a period of time (Itle, et al., 2005).

2.3 Microfabrication techniques

Microfabrication is a process of constructing physical objects having dimensions in the millimeter to micrometer range, incorporating well known procedures in semiconductor fabrication to augment processes for microfabrication. Microfabrication technologies have been incorporated in the development of cell based biosensors, cell culture analogues and scaffolds for tissue engineering applications (Onoe et al., 2012; Voldman, Gray, & Schmidt, 1999). The advantages of using microfabrication are the control of size and integration of numerous processes on a single substrate. These techniques have been used to understand fundamental cell biology by studying cell-cell, cell-substrate and cell-media interactions (T. H. Park & Shuler, 2003; Voldman, et al., 1999).

2.3.1 Photolithography. The most predominantly used microfabrication techniques are photolithography and microcontact printing. In photolithography, the substrate is illuminated with UV light passed through a mask having defined transparent and opaque regions. Upon exposure to light, the substrate can be modified accordingly. This technique has been used extensively to create monolayers of alkylsiloxanes on the surfaces of glass and silicon dioxide (Folch & Toner, 2000; T. H. Park & Shuler, 2003; Pimpin & Srituravanich, 2011).

2.3.2 Microcontact printing. Micro contact printing uses a rubber stamp to print a patterned monolayer of alkanethiols onto a substrate like gold. In this procedure, a rubber stamp is fabricated using photolithography. The material of interest is transferred from the stamp onto the substrate. The areas where the stamp has not made contact can be exposed to another coating material. Thus, a single substrate can be used for multiple cell growth. The advantages of this technique include the casting of multiple stamps from a single master and the long term usage of each stamp (Bhatia, Yarmush, & Toner, 1997; T. H. Park & Shuler, 2003). Figure 3 highlights the principle of microcontact printing.

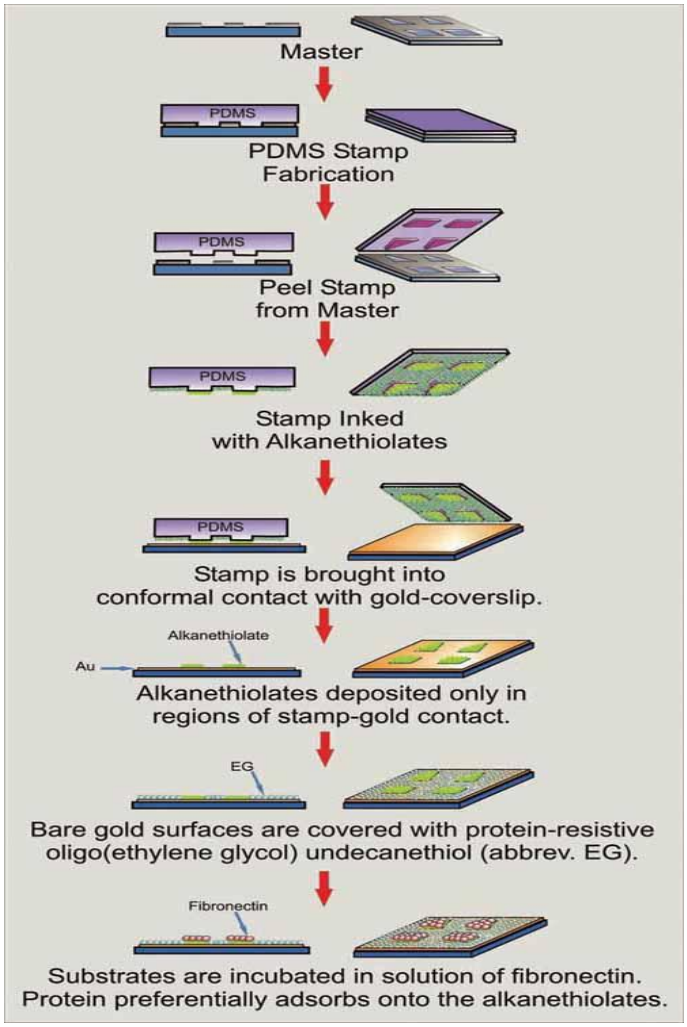


Figure 3. Microcontact printing (www.chem.wuslt.edu).

2.4 Self-assembled monolayers

Molecular self assembly is the spontaneous assembly of molecules under specific conditions to form stable aggregates. Self- assembled monolayers (SAMs) are ordered molecular assemblies of an active surfactant in the liquid phase or gaseous phase onto solid or liquid surfaces (Milan Mrksich, 2000). These adsorbates organize themselves into crystalline or semi-crystalline structures containing specific head groups. Substrates show affinity to specific head groups. These monolayers can displace previously adsorbed materials (Whitesides, et al., 1991).

The field of self-assembled monolayers (SAMs) has seen tremendous growth in synthetic sophistication and characterization, over the past 20 years. This field began when Zisman and coworkers published the formation of a monolayer by adsorption ofaa surfactant on a clean metal surface in 1946 (Bigelow, Pickett, & Zisman, 1946). The real interest in this field started when Nuzzo and Allara published their work in 1983 on preparation of alkanethiolates on gold (Nuzzo & Allara, 1983), even though many other works were done after 1946. SAMs can be prepared with different types of molecules on different substrates. Examples are alkylsiloxane monolayers, fatty acids on oxidic materials, alkanethiolate monolayers. Some of the typical applications of SAMs have in molecular recognition, development of biomimetic surfaces to study cell-substrate interactions, optical coatings, development of biosensors and optoelectronic devices (Milan Mrksich, 2000; Whitesides, et al., 1991).

The first monolayer formation, observed in 1983 was the assembly of disulphides on gold, followed by adsorption of alkanethiolates on gold. The high affinity of thiols for noble metal surfaces forms uniform monolayers whose physical and chemical properties can be altered accordingly (M Mrksich & Whitesides, 1996). SAMs are highly ordered and can be formed easily by dipping the substrates in solutions of desired composition. Patterning of SAM

deposited substrates create localized areas for manipulation (Milan Mrksich, et al., 1997). SAM functionality can be altered according to need, by simply modifying their end group (Figure 4) (Cooper et al., 2000). These properties make SAMs an ideal choice for development of cell culture platforms. Figure 5 shows the molecular organization of surfactants on the substrate forming a uniform monolayer.

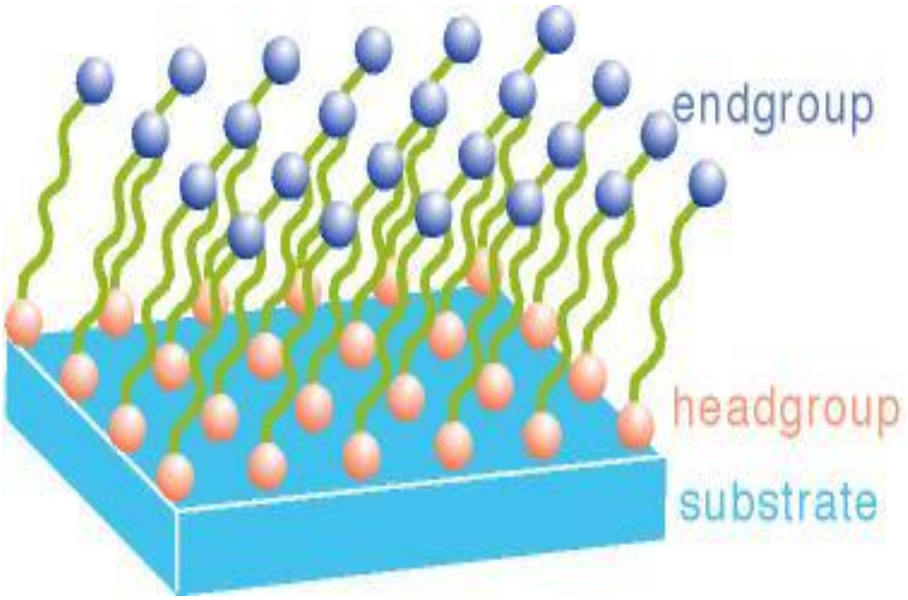


Figure 4. A typical molecular self-assembly (www.ipfdd.de).

Alkanethiols, fatty acid derivatives, organosilicon and organosulphur derivatives and alkyl monolayers are commonly used in preparation of self-assembly. Alkylchlorosilanes, alkylaminosilanes and alkyloxysilanes need the presence of hydroxylated surfaces for monolayer formation (Ulman, 1996). These molecules arrange themselves by forming polysiloxane *in situ* which connects to surface silanol groups through Si-O-Si bonds. SAMs of organosilicon derivatives have successfully been prepared on gold, silicon oxide and glass substrates (Faucheux, Schweiss, Lützow, Werner, & Groth, 2004).Controlling the amount of water in solution and temperature are important requisites for self-assembly.

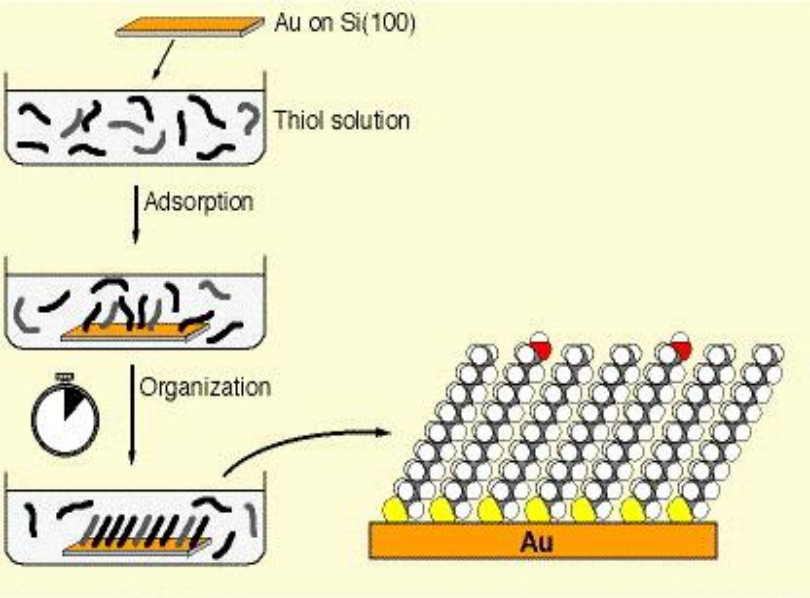


Figure 5. A general scheme for steps involved in the formation of self-assembled monolayers on gold (http://soft-matter.seas.harvard.edu/index.php/Insoluble_monolayers).

2.4.1 Cell culture on SAM modified surfaces. Cellular adhesion on substrates follows the interaction of specific substrate molecules with surface patterns. In a biological environment, cells attach and spread on the ECM. As with polymeric resins, hydrogels, nanofilms, lipids and self-assembled monolayers have been used to mimic ECM properties *in-vitro* to facilitate cell attachment (Hudalla & Murphy, 2011). Recently, it has been reported that SAMs can be used to model ECM (Milan Mrksich, 2009). SAMs are preferred since they have a well defined structure, and permit a wide range of ligands to attach (Pulsipher & Yousaf, 2011). The terminal or end group of the molecules (Figure 4) from which the monolayer is derived, controls the nature of the SAM surface (Faucheux, et al., 2004).

SAMs are also well studied in nanoscience and nanotechnology because 1) they are easy to prepare and do not require any specialized equipment, 2) they can be formed on surfaces of all shapes and sizes and are critical components for stabilizing and adding function to nanometer-scale objects such as thin films, nanowires, colloids, and other nanostructures, 3) they can couple

the external environment to the electronic and optical properties of metallic surfaces, and 4) they link molecular-level structures to macroscopic interfacial phenomena like as wetting, adhesion and friction. In addition, SAMs also provide necessary flexibility both at molecular and material levels on the surface and stability of two-dimensional assemblies can be later applied to three-dimensional structures (S. S. Kim et al., 1998). The simple process involved in the preparation of SAMs makes it attractive for surface engineering and building superlattices. SAMs also help in understanding the fundamentals of self-organization, structure-property relationships and interfacial phenomena. The ability to tailor both head and tail groups of the molecules makes SAMs an excellent system in understanding intermolecular, molecule-substrate and molecule-solvent interactions.

Two main groups of SAMs have been used in cell culture studies: a) alkanethiols on gold and b) alkylsiloxanes on hydroxylated surfaces (Milan Mrksich, 2002). Alkylsiloxanes are preferred due to their ability to adsorb on a number of surfaces, including glass and other polymers. However, they do not form ordered monolayers. Monolayer formation is difficult and a limited number of end groups are available (Schwartz, 2001). Cellular attachment on SAMs is governed by the nature of the terminal end group and length of the alkyl chain (Milan Mrksich, 2002; Stenger, Pike, Hickman, & Cotman, 1993). Fibroblasts, neuroblastoma cells, osteoblast viability and endothelial cell growth have been studied on SAMs. Variations in cellular response have been attributed to surface wettability, charge and variable protein adsorption (Lampin, et al., 1997).

Human Dermal Fibroblasts (HDFs) cultured on SAM modified surfaces having different wettabilities showed that the cells attached and spread on SAMs terminating with $–NH_2$ and –COOH groups are better than to $–CH_3$, PEG and –OH groups. Cell viability was significantly

higher on $–NH_2$ and –COOH as compared to the rest. Increased cell attachment and growth was attributed to enhanced integrin activity, which was confirmed by the analyses of focal adhesion complexes (Faucheux, et al., 2004). Human corneal epithelial cells cultured on SAM modified glass substrates showed better adhesion on amine and carboxylic acid terminated SAMs as compared to other end groups. Cell viability was more on –COOH terminated end groups (Franco, Nealey, Campbell, Teixeira, & Murphy, 2000).

Murine 3T3 fibroblasts and osteoblasts cultured on alkanethiol modified gold substrates to study the effect of chain length and nature on cellular attachment, showed better cell attachment and spreading on carboxylic terminated SAMs. Short chain methyl terminated SAMs showed less cell attachment and lower growth. Fibroblasts showed poor attachment on hydroxyl terminated SAMs, while osteoblasts grew well on them, showing preferences to SAMs having a longer chain (Cooper, et al., 2000). These studies show that the presence of a higher surface energy contributes to better cellular attachment as in the case of carboxyl and amine terminated end groups. It is commonly observed that cell proliferation, attachment and viability largely depend on the nature, end group and chain length of the deposited monolayer. Some of the properties like ordering, growth, wetting, adhesion, lubrication and corrosion have been studied previously.

2.5 Co-cultures

In recent years, isolated rat hepatocytes have been increasingly used as a model in various culture systems to study pharmacological and toxicological responses of drugs, environmental toxicants and other compounds. However, after cells are removed from their microenvironment and placed within *in vitro* environment, they typically lose some or all of their normal *in vivo* behavior (Bhandari et al., 2001). Co-culturing of hepatocytes with different cell

types has been studied extensively for toxicology as a means of maintaining specific hepatocyte functionality.

The liver is one of the most important organs of the body performing a number of metabolic and endocrine functions. Hepatocytes form the primary parenchymal cells of the liver and constitute 75-80% of the total liver volume (Peter & Nikolaus, 2003). Isolated hepatocytes closely model the liver, as they have been shown to exhibit specific liver functions, including drug metabolism under specific chemical conditions (Gomez-Lechon, Donato, Castell, & Jover, 2003). However, these cells are affected by significant loss of functionality and hepatocyte specific function over long term cultures *in vitro* (Gomez-Lechon, et al., 2003; Peter & Nikolaus, 2003). Hydrogels (Itle, et al., 2005), biodegradable polymer films (Kidambi, et al., 2004) and natural ECM components (Y. J. Wang, Liu, Guo, Wen, & Liu, 2004) have been studied as platforms for hepatocyte attachment and growth. Hepatocytes have distinct characteristics such as presence of binucleate cells and cell aggregation to form clusters.

Hepatocytes have been co-cultured with fibroblasts, stellate cells, liver endothelial, liver epithelial cells. Co-cultures aid in retaining the polymeric shape of isolated hepatocytes upon adherence. Hepatocyte co-cultures have been shown to express high levels of liver specific proteins like albumin. However, the exact mechanism of cell-cell interaction in these co-cultures is not yet understood and is being investigated by a number of research groups (Kaji, Camci-Unal, Langer, & Khademhosseini, 2011; Kang, Kim, Kwon, & Ito, 2004).

2.6 Model biological surfaces

A primary challenge in developing *in vitro* surfaces is to develop methods that will allow precise control of the composition and structure of the surface while permitting natural biological

interactions to occur. These interactions should be helpful in interpreting the results in relation to those *in vivo*.

SAM-modified surfaces are one of the useful systems for studying biological and biochemical processes because, like biological surfaces, they are nanostructured and are formed by self-assembly. They present a wide range of organic functionalities like the ones that can resist the adsorption of the proteins. SAM functionalized with large, delicate ligands needed for biological studies is easy to prepare just by either synthesizing molecules with the ligands attached to form the SAM or, more commonly, by attaching the ligands to the surface of a preformed SAM. These prepared SAMs are also compatible with a number of techniques such as surface plasmon resonance (SPR) spectroscopy (Milan Mrksich, Sigal, & Whitesides, 1995), optical ellipsometry (Tengvall, Lundström, & Liedberg, 1998), RAIRS (Reflectance-absorption infrared spectroscopy) (Tengvall, et al., 1998), QCM (Quartz crystal microbalance) (Marx, 2003), and mass spectroscopy (Min, Tang, & Mrksich, 2004), for analyzing the composition and mass coverage of surfaces as well as the thermodynamics and kinetics of binding events.

One disadvantage of SAMs is the static nature of the structure of SAM. This characteristic differs from that of biological membranes, which rearrange dynamically. Langmuir-blodgett films (Bhaumik et al., 2004) and bilayers of lipids on solid supports present two alternative technologies for creating dynamic models of biological surfaces. Instrument complexity and non-reproducibility makes this area of study limited. Studies have shown the patterning of lipid regions on solid supports but, thses are in the beginning stages (Burridge, Figa, & Wong, 2004).

The first method of attaching cells on surfaces is the use of mixed SAMs composed of a ligand-presenting molecule and a SAM-forming molecule, where on terminatal with functional

group can resist protein adsorption. The variation in type, density, and accessibility helps in understanding the interactions taking place at the surface and also at the cell (Kato & Mrksich, 2004; Roberts et al., 1998). A second method for attaching cells on surfaces is provided by SAMs prepared by microcontact printing. These patterned surfaces make it possible to study the biochemical responses of cells to mechanical stimuli (Parker et al., 2002). Electrochemical methods have also been utilized to modify SAMs to relate the cells from the confinement originally imposed by the pattern of the SAM.

The structure and properties of SAMs immersed in a solvent are not clearly understood compared with that of SAMs in vacuum or in air. The use of SAMs as substrates for studies in biology requires extended contact between SAMs and an aqueous environment containing high concentrations of salts and biomolecules (J. C. Love, Estroff, Kriebel, Nuzzo, & Whitesides, 2005). The structure and dynamics of the exposed surface of a SAM under these conditions have not been studied completely. Also the effect of physiological conditions on long-term stability of SAMs is not clearly understood. Langer and co-workers have shown that SAMs terminated with EG develop substantial defects after immersion in phosphate buffer solution or in calf serum in 4-5 weeks (Flynn, Tran, Cima, & Langer, 2003). The presence of cells at the surface also accelerates the process and ability of EG-terminated SAMs to prevent cell adhesion (Jiang, Bruzewicz, Thant, & Whitesides, 2004).

The flexibility in using SAMs along with the advantages make them ideal to be used as model biological surfaces even though, many factors have to be considered and studied before SAMs can be used for tissue engineering with full potential. The robustness, order and homogenous assembly, flexibility to modify both, head and tail, ability to couple a growth factor

or peptide depending on the end group make SAM a good candidate for developing cell culture platforms for toxicity screening (Whitesides, et al., 1991).

2.7 Cell culture platforms

Cell culture platforms (CCPs) have been developed to study cell interactions within their *in vitro* microenvironment. This provides opportunities to obtain a thorough understanding of the properties of the surface to which the cells are attached (Wu, Huang, & Lee, 2010). This offers a valuable area of research for engineering biomedical devices (Leoni, et al., 2002). CCPs would be useful in the fields of toxicology and drug testing as they can reduce costs and simplify testing procedures (Pancrazio, et al., 1999). Researchers have studied the use of ECM proteins (Jauregui, 1987), biodegradable polymers (Richert, et al., 2002), hydrogels (Koh, Revzin, & Pishko, 2002) and nanofilms (Kommireddy, et al., 2005) to develop CCPs. Microfabrication techniques like microcontact printing and photo-lithography have also been employed to control cell-cell and cell-substrate interactions and define specific areas for cell growth and attachment (Singhvi, et al., 1994). However, most of these methods have been limited in controlling prolonged cell viability and functionality.

2.7.1 SAMs on gold. SAMs of alkanethiolates on noble metals form ordered and uniform monolayers and have been widely used as models for cell culture studies (J. Christopher Love et al., 2003). Mammalian cell lines cultured on SAM modified surfaces have indicated higher cell viability, maintenance of functionality and expression of cell specific functions (M. Mrksich et al., 1996; Schreiber, 2004). The end group of the monolayer plays an important role in defining its surface properties. Wettability, charge and cell adhesion are associated with the nature of the end group (M. Mrksich, 1998). Growth of cells on SAMs of alkanethiols on gold (Au) surfaces has been extensively studies over the decades. Previous studies on fibroblast cell growth on

SAMs modified Au have indicated that SAMs with carboxylic end group are more favorable for cell attachment followed by methyl and hydroxyl groups. Similarly, the effect of the length of alkyl chain on cell attachment has been studied, and it has been indicated that cell attachment decreases with increase in alkyl chain length (Cooper, et al., 2000).

2.8 Indium tin oxide

In contrast to the various cell culture studies done on SAM-modified Au surfaces, very few studies have been done on other surfaces such as indium tin oxide, silicon and gallium arsenide. The focus of theis thesis is conducting cell culture studies on modified ITO substrates. ITO has been widely used as an electrode for studying electrochemistry of biomolecules due to its conductive and transparent properties (Yousaf, 2009). Indium Tin Oxide is a mixture of Indium (III) Oxide [In_2O_3] and Tin (IV) Oxide [SnO_2] typically 9:1 ratio by weight. Although, the common applications of ITO are coatings for electronic displays, gas sensors, and anti-static windows, ITO is also used as an electrode for biochemical studies, due to its transparent and conductive nature (Yang & Kleijn, 1999). This dual nature and the stability that ITO offers under physiological conditions make it ideal for tissue engineering.

It has already been shown that acids, amines and proteins specifically adsorb on ITO. Interdigitated microelectrodes made of ITO were also used for synapse formation by neuronal differentiation of rat pheochromocytoma cell and murine embryonic stem cells (Yousaf, 2009). ITO is of considerable interest in SAM studies, since monolayer formation on ITO has been relatively less investigated. The transparency of ITO makes it easier to observe and image cell attachment. The surface chemistry of ITO is similar to that of silicon; therefore alkyloxysilanes are used for monolayer formation (Oh, Yun, Kim, & Han, 1999).

Surface properties of the deposited monolayer are obtained by its characterization. The presence and nature of the monolayer is determined by measuring this angle. SAMs vary in their wettability from hydrophilic (water loving) to hydrophobic (water hating) surfaces. Hydrophobic surfaces are produced by SAMs containing $–CH_3$, bromine end groups while hydrophilic surfaces are produced by SAMs containing hydroxyl and amine groups. Hydrophobic surfaces form contact angles which are greater than 90°, and contact angles formed by hydrophilic surfaces are less than 90° (Lampin, et al., 1997).

2.9 Different scaffolds used for hepatocyte culture

A variety of culture methods have been developed to retain most of the hepatocytic functions such as culture on basement membrane gels. Co-culture with other liver-derived (Begue, Guguen-Guillouzo, Pasdeloup, & Guillouzo, 1984) and non-liver cell types (Hirose et al., 2000), culture in collagen gel sandwiches and polymers. Collagen is an important component of the hepatocyte basal membrane and promotes attachment of hepatocytes *in vitro*. Cells cultured on collagen-coated surfaces demonstrated increase in urea production and low LDH release (Y. J. Wang, et al., 2004). Collagen was observed to provide the closest alternative to hepatocyte architecture *in vitro*.

However, biodegradability and cross-link formation are some of the limitations of using ECM modified surfaces. High molecular weight polymers like polyethylene glycol (PEG) (K.-H. Park et al., 2005), polyglycolic acid (PGA) (Fiegel et al., 2004) and polylactic acid (PLA) (K.-H. Park, et al., 2005) were used to develop scaffolds for hepatocyte culture. However, these polymers lose their strength upon long culture periods. Primary rat hepatocyte culture on porous poly-tetrafluoroethylene (PTFE) showed increased protein secretion and polygonal morphology

over culture periods varying from 24-49 h. The lack of stability of polymer scaffolds over extended culture periods make them ineffective for cell culture (S. S. Kim, et al., 1998).

Other alternatives used to increase hepatocyte viability include culture on polyelectrolyte multilayers (Kidambi, et al., 2004), hydrogels (Itle, et al., 2005), gold substrates (Gu et al., 2004)and culture as spheroids (Dilworth, Hamilton, George, & Timbrell, 2000). Primary rat hepatocytes cultured on polyelectrolyte multilayer films with poly(4-styrenesulfonic)acid as the topmost layer attached and spread on the PEM surface and liver specific functions like urea and albumin production showed an increase with time in culture (Kidambi, et al., 2004). Porcine hepatocytes immobilized on gold colloids showed increased protein and albumin production upon culture. The LDH release was minimal which indicated that the cells suffered limited damage upon culture (Gu, et al., 2004).

Murine hepatocytes entrapped within PEG hydrogels were assessed for cell viability and total protein production over a period of seven days. The results indicated that the cellular viability was not affected by the hydrogel concentration, but total protein production decreased with increase in PEG concentration. The use of growth factors and cytokines like hepatocyte growth factor (HGF) (Michalopoulos & Zarnegar, 1992), epidermal growth factor (EGF) (Mitaka, Mikami, Sattler, Pitot, & Mochizuki, 1992), transforming growth factor (TGF)-alpha and beta (Nakamura et al., 1985) and norepinephrine (Cruise & Michalopoulos, 1985) have been shown to promote cellular viability, but the cells eventually de-differentiate and lose their functionality.

2.10 Growth factors

Growth factors are commonly present in soluble form during *in vitro* cell cultivation experiments. They provide signals for stimulation of viability, migration, and differentiation.

Several factors, including the EGF (Nakamura, et al., 1985), have been reported to regulate cell function in the transmembrane form by "juxtacrine stimulation" (Ito, Chen, & Imanishi, 1998). This phenomenon has led researchers to develop what is called "artificial juxtacrine stimulation" through the covalent coupling of growth factors to artificial substrates. This method to simulate the cell's natural environment would provide a controlled and sustainable influence on cell behavior over that of soluble or slow released proteins. A study utilizing these techniques reported that an immobilized growth factor induced a different signal transduction compared to a soluble growth factor (Ogiwara, Nagaoka, Cho, & Akaike, 2006).

2.10.1 Hepatocyte growth factor (HGF). HGF is an important growth factor which is a crucial cytokine during liver development and important for mechanisms of liver regeneration (Nakamura & Mizuno, 2010). It is produced primarily by mesenchymal cells and interacts with its receptor c-MET, a proto-oncogene gene product, which is present on most epithelia. HGF is a multifunctional polypeptide with morphogenic, motogenic, angiogenic, and proliferative capabilities (Hengstler, et al., 2009). HGF has the ability to induce several effects such as stimulation of reepithialization during wound healing, angiogenesis stimulation, extracellular matrix deposition, and modulates immune function (Jones et al., 2010). In fact, HGF was found to have dual purposes of initiating early viability of hepatocytes and liver progenitor cells (LPCs) as well as differentiation of LPCs into hepatocytes (Lee, et al., 2008).

Other studies report that HGF has cytoprotective, mitogenic and anti-apoptotic effects on hepatocytes in culture (Riehle, Dan, Campbell, & Fausto, 2011). In 2010, Revzin and coworkers investigated non-covalent immobilization of HGF on ECM where HGF was mixed in solution with ECM proteins which suggests that non-covalent matrix-bound HGF is sufficient to enhance and maintain phenotype expression of primary hepatocytes for a long period of time (Jones, et

al., 2010). Several systems such as hollow fiber systems, collagen films, collagen sandwiches, liver spheroids, and three-dimensional bioreactors are under development as a means to extend viability and improve cell functionality for isolated liver cells or hepatocytes in culture (Griffith & Naughton, 2002).

However, these systems lack smoothness and precise control of the surface for cell viability studies. Our choice of ITO for SCCP is unique for several reasons. ITO has been widely used as an electrode for studying electrochemistry of biomolecules due to its transparent and conductive properties (D. W. Kim, Sung, Park, & Yeom, 2001). This dual property and stability under physiological conditions make them ideal for clinical applications (Brewer, Brown, & Franzen, 2002; D. W. Kim, et al., 2001). Further, SAMs on ITO are well packed, homogeneous and can precisely control the surface properties for cell adhesion. The ability to covalently link the terminal group of SAM to EGF, HGF and GRP, a model protein, allows the bioactive substrate interacting and influencing cells at the molecular level.

In this study, we have studied the culture of HepG2 cells on SAM modified and GRP modified substrates in an attempt to mimic the cellular microenvironment. The long term objective of this study is the development of SAMs based CCPs using primary hepatocyes and immobilized growth factors like hepatocyte growth factor (HGF) and epidermal growth factor (EGF). The experiments done using HepG2 cell line and a model peptide, GRP, will provide a preliminary understanding on the ability of the cells to proliferate and be viable in presence of immobilized peptides, which can further be used to do studies with immobilized growth factors in order to build a bioreactor for drug toxicity screening. The use of a cell line (HepG2) eliminates the expenses and complication involved with the use of primary hepatocytes.

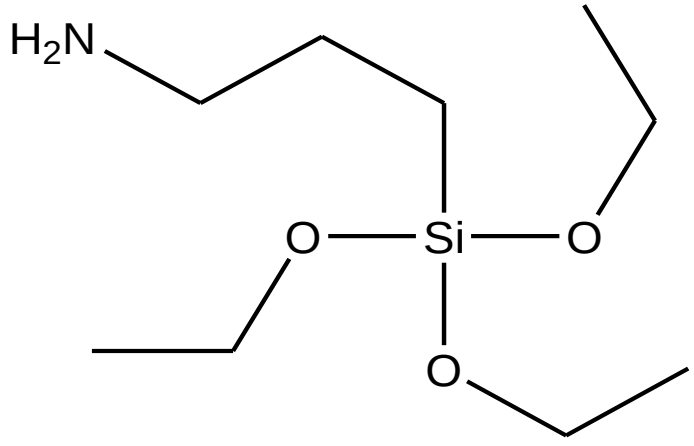
CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 ITO substrates. The ITO substrates with a resistance of 45 Ω were purchased from Sigma-Aldrich. These substrates are prepared by sputtering ITO on one side of a polyethylene terephthalate (PET) film. The side with ITO will be conductive and the other side is non-conductive. The side with ITO was modified with APTES and subsequently with GRP. During characterization and experiments the conductive side was always identified using a potentiostat.

3.1.2 3-APTES. 3-APTES (Structural formula NH_2-$(CH_2)_3$-Si $(OC_2H_5)_3$) is a silanizing agent used to modify surfaces using layer by layer assembly. It was purchased from sigma-aldrich. It is highly hygroscopic. 3-APTES binds to ITO by the formation of siloxane bonds leaving a free $–NH_2$ end group. This causes the SAM coated surface to be hydrophilic. This free amine $–NH_2$ can be coupled to the carboxyl terminus of a peptide or growth factor.



3-APTES

Figure 6. Structure of 3-aminopropyl triethoxysilane.

3.1.3 Gastrin releasing peptide (14-27). GRP (14-27) is a peptide derived from amino acid residues 14 to 27 of porcine and human GRP with a length of 14.8 nm. It is involved in the

regulation of numerous functions of gastrointestinal and the central nervous systems. Some of the other functions of GRP (14-27) include the release of gastrointestinal hormones, smooth muscle contraction and epithelial cell viability. GRP (14-27) is widely used as model peptides in cell culture studies due to its mitotic ability. It was purchased from American Peptide (Product No. 46-4-32). GRP can be immobilized to various SAMs on different surfaces suing different techniques, one of which is the immobilizing the carboxylic terminus of GRP to an amino end group of a SAM (which is 3-APTES in the study).

3.2 Methods

3.2.1 SAM deposition on ITO substrates. 3-APTES binds to ITO by the formation of siloxane bonds leaving a free $–NH_2$ end group. This causes the SAM coated surface to be hydrophilic. The ITO substrates were cleaned by sonication in toluene, acetone and ethanol for five minutes each and 30 minutes in DI water. The substrates were dried using dry N_2 gas, following which they were dipped in a 5 % 3-APTES solution in ethanol for 2-24 hours followed by rinsing in ethanol and N_2 drying. The substrates were sterilized in 100% ethanol for a day prior to use. The reaction by which SAM of 3-APTES in formed on ITO is shown below.

$$ITO\text{-}OH + NH_2\text{-}Si(OEt)_3 \longrightarrow ITO\text{-}O\text{-}Si\text{-}NH_2$$

3.2.2 Immobilization of gastrin releasing peptide on ITO-SAM. The amine modified ITO substrate was derivatized by using crosslinkers, EDC (carbodiimide) and NHS (N-hydroxysuccinimide). GRP (14-27) (10 µg/ml) was dissolved in a 2:5 molar ratio of EDC and NHS in 0.1M MES Buffer at pH 6. The amine-ITO substrates were then placed into the growth factor solution for 24 hours at 4°C. Prior to cell culture studies, the substrates were rinsed four times with 1X phosphate buffered saline (PBS). Figure 8 shows the schematic representation of the process of immobilization of GRP on ITO.

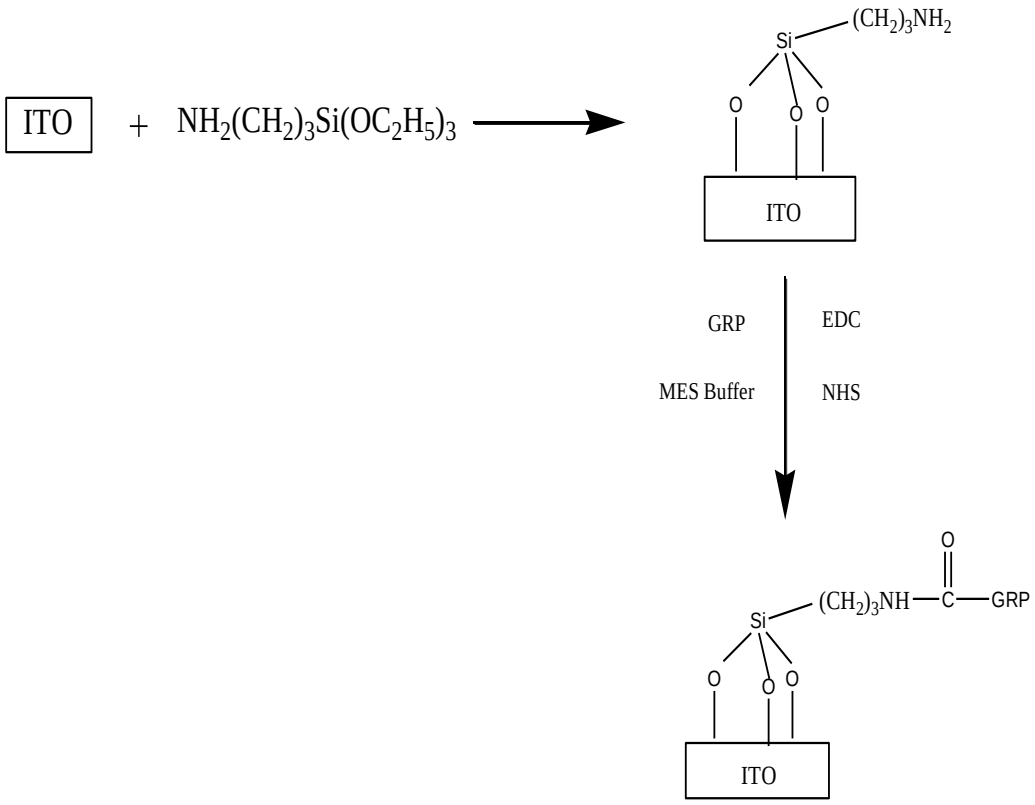


Figure 7. Schematic representation for immobilization of GRP on ITO substrate.

3.3 Characterization of modified ITO substrates

3.3.1 Contact angle measurements. It is necessary to characterize molecules on different surfaces before their use for cell culture studies. SAM on metal and semiconductor surfaces can be characterized by different techniques. Contact angle offers an easy-to-measure indication of the modification of the uppermost surface layers of a solid. The measurement determines wettability and adhesion and also allows prediction of coating properties and detection of trace surface contaminants. Contact angle is a physical manifestation of the more fundamental concepts of surface tension and surface energy. This technique is easy and is also used in determining the hydrophilic or hydrophobic nature of a SAM. The instrument used for these purposes is called a goniometer.

When a tangent line is drawn from the surface of the droplet to the solid surface, the contact angle (Θ_c), as shown in Figure 9, is the angle between the tangent line and the solid surface. If the contact angle is acute ($\Theta < 90^0$), the surface is considered hydrophilic and, if it obtuse ($\Theta > 90^0$), the surface is considered as hydrophobic.

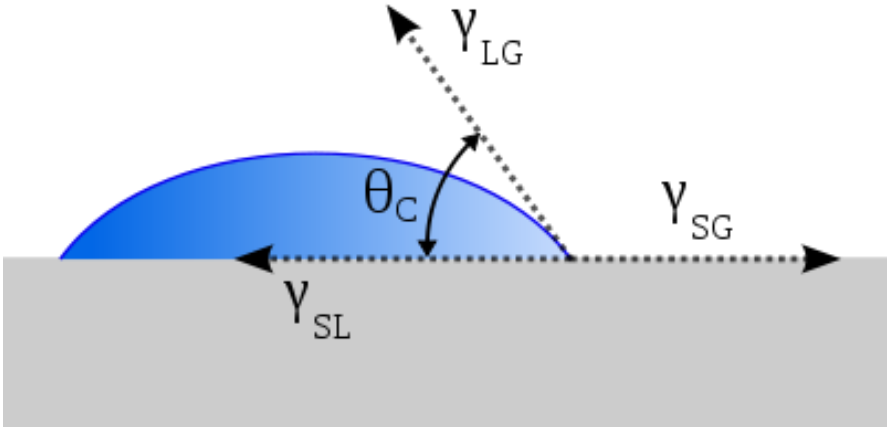


Figure 8. Definition of contact angle (Kwok & Neumann, 1999).

The operation of a goniometer is very simple. A droplet of a liquid is dispersed onto the surface, and a CCD camera is used to capture the image of the droplet. The software calculates the tangent to the shape of the droplet and then calculates the contact angle. All this process can be done both manually and automatically on the same instrument. Data and images are collected at various points, as required, analyzed and stored in a computer.

Contact angle measurements of liquids on SAM modified ITO is used to measure its wettability and adhesive properties by calculating the solid-vapor surface tension (Giannoulis & Desai, 2002). Further investigations have shown specific adsorption of amines and proteins on ITO coated surfaces (King, Hawkridge, & Hoffman, 1992; Margalit & Vasquez, 1990). The contact angle is the angle produced by drawing a tangent to the curvature of the water droplet positioned on the surface. Figure 9 shows experimental data of a contact angle formed by positioning a water droplet on the surface of a substrate.

Figure 9. Micrograph of water contact angle on SAM surface (experimental data).

Advanced contact angle measurements are carried on SAM coated substrates using the sessile drop method on a contact angle goniometer.

3.3.2 Infrared spectroscopy. Infrared spectroscopy is one of the powerful tools for identifying types of functional groups. The wavelength of IR-light absorbed is characteristic of the chemical bond. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be inferred. IR spectra of pure compounds are generally unique in that they behave like a molecular fingerprint of the molecule. Table 1 shows an illustration of IR absorbance of common organic functional groups. Alcohols and amines display strong broad O-H and N-H stretching bands in the region 3400-3100 cm^{-1} as shown in the figure. Carbonyl stretching bands wich are generally strong and broad occur in the region of 1800-1700 cm^{-1}. Carbon-carbon double bond stretching occurs in the region around 1650-1600 cm^{-1}. The bands are generally sharp and of medium intensity. Aromatic compounds will typically display a series of sharp bands in this region. Carbon-oxygen single bonds display stretching bands in the region of 1200-1100 cm^{-1} and these bands are generally strong and broad. Attenuated Total Reflectance Infrared (ATR-IR) Spectroscopy technique was used to characterize the ITO substrates. Table 1 shows characteristic IR absorption frequencies in molecules

Table 1

Characteristic infrared absorption frequencies of some common organic functional groups

Bond	**Compound**	**Frequency Range, cm^{-1}**
C-H	Alkanes	2850-2960 1350-1470
C-H	Alkenes	3020-3080 675-1000
C-H	Aromatic ring	3000-3100 675-870
C-H	Alkynes	3300
C=C	Alkenes	1640-1680
C-O	Alcohols, ethers, carboxylic acids, esters	1080-1300
C=O	Aldehydes, ketones, carboxylic acids, esters	1690-1760
O-H	Alcohols, phenols	3610-3640 3200-3600
N-H	Amines	3300-3500
C-N	Amines	1180-1360

ATR-IR spectroscopy also known as internal reflection spectroscopy works on the principle that by pressing small pieces of membrane against an internal reflection element (IRE), e.g., zinc selenide (ZnSe) or germanium (Ge) mid-infrared spectra spectra can be obtained. IR radiation is focused onto the end of the IRE. Light enters the IRE and reflects down the length of the crystal. At each internal reflection, the IR radiation actually penetrates a short distance (1 mm) from the surface of the IRE into the polymer membrane. It is this unique physical phenomenon that enables one to obtain infrared spectra of samples placed in contact with the IRE. RAIRS is also employed to characterize SAM deposition on ITO. The principle of RAIRS involves the study of molecular vibration on a surface using infrared light incident on it. The

presence of a dipole causes the molecule to absorb infrared at fixed frequencies. Thus, an infrared spectrum of light incident on the surfaces exhibits characteristic peaks of absorption which are specific to a molecule and its nature of binding (Roucoules, Gaillard, Mathia, & Lanteri, 2002). The IR spectra obtained for this thesis were obtained using ATR FT-IR. The nanometer size of molecules attached on the substrates results in very low signal due to which the ATR chamber has to purged with nitrogen.

3.3.3 Atomic force microscopy (AFM). Atomic force microscope was used to measure the roughness of the SAM- modified ITO and GRP- modified ITO surfaces. The AFM (NT-MDT, Japan) available at IRC was used for all the experiments. It has a profiler unit, an electronics interface, computer to run the software and store the data, and a heating unit for the substrate. The probe has a silicon cantilever at its end, and the stylus tip is at the bottom of the cantilever. The profiler unit has a stepper motor for downward and upward movements. A feedback signal stops the unit from crashing onto the substrate at a minimum distance. The stylus moves from left to right laterally during scanning of the sample. A laser in the profiler unit hits the cantilever and is reflected back. The profiler unit and the sample stage are placed in the vibration-free chamber. For all the samples, the scan head of imaging was initiated at the lowest magnification.

The imaging was performed in the non-contact mode or wave mode with a NSC16 silicon cantilever. Initially, the scan head was brought down towards the sample and the region of interest was brought into the camera view. Then the scan parameters were set using the SPM configuration menu. An integral gain of 250 and a proportional gain of 300 were used throughout the imaging process. For the initial scans, a scan size of 5µm x 5µm, a scan rate of 3 Hz and a scan resolution of 500 lines per scan were used. Once these large area scans were complete and

the images were stored, a smaller region of interest was chosen with the previously scanned region using the hard zoom option.

The roughness of the samples were measured (using already stored image) using the histogram analysis window in AFM. The height of the histogram shows the statistical distribution of Z-heights of all the points in the image and, in addition, calculates several measure of surface roughness from different measurements and displays them in the surface characterization panel. We are interested in the average roughness (R_a): average deviation from the mean surface plane.

3.4 Cell culture studies

3.4.1 Sterile techniques for cell culture. Sterile technique refers to procedures by which cultures may be manipulated without infecting the worker or contaminating the cultures or the laboratory environment. Good sterile technique is the first and most important step in ensuring consistent results with cell culture. Working with cells take place within a laminar flow hood, either Class I (use with animal tissues), or Class II (use with human tissues). The purpose of these hoods is to minimize the risk of infection entering from the outside environment, as well as protect the user against potential pathogens being transmitted from the culture into their environment. Some of the common sterile techniques followed in the labs are wiping the work area and hands with 70% ethanol before starting the experiments, keeping sterile pipettes in their wrappers prior to use and not using the same pipette to draw media from different bottles.

3.4.2 Cell line and culture conditions. HepG2 liver hepatocarcinoma cell line was purchased from American Type Culture Collection (Rockville, MD). The cells were maintained in Eagle's Minimum Essential Media (EMEM) (Cellgro) supplemented with 10% fetal bovine serum, 2% penicillin/streptomyocin, and 1 % amphotericin. Cells were fed every two-three days

and subcultured every 5 days. Cells were seeded at a density of 1 x 10^5 cells per well on each experimental surface in EMEM supplemented with 1% fetal bovine serum. Soluble growth factors were added after initial seeding and evaluated after 24 hours. HepG2 cells were cultured on various test surfaces which could be divided into four separate groups: (i) HepG2 cells cultured on polystyrene (control) (ii) HepG2 cells cultured on amine modified ITOP (APTES-ITOP) (iii) HepG2 cells cultured on covalently coupled GRP and iv) HepG2 cells cultured with soluble GRP

3.4.3 Cell morphology. Morphological observations of cell culture were performed using Miotic inverted microscope. The cells observed periodically for any changes in morphology and visual indications of cellular damage. Observations were recorded using the connected digital camera. The images were captured in a computer attached to the microscope using Miotic software for further image analysis.

3.4.4 Cell viability analysis. MTT assay was used to quantitatively evaluate the cell viability of HepG2 cells. The principle of MTT assay is the conversion of (3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromibe) to purple formazan crystals in the presence of mitochondrial reductase enzyme which is present in viable cells. The amount of formazan produced is thus directly proportional to cell viability and can be measured using a spectrophotometer.

The MTT assay kit was purchased from Promega. HepG2 cells were seeded with densities of 100,000/substrate. The cells were treated with MTT reagent and incubated for four hours. After four hours, purple formazan crystals were formed. The crystals were dissolved using the MTT solvent and the absorbance was measured at 590 nm. MTT activity was measured after

48 hours of seeding. A standard curve was established by measuring MTT activity as a function of cell density.

3.4.5 Cellular cytotoxicity analysis. LDH assay was used to evaluate the cellular cytotoxicity of HepG2 cells cultured on different substrates. Cytotoxic responses of hepatocytes cultured on these substrates were evaluated by measuring the amount of Lactate Dehydrogenase leakage from seeded cells. Lactate Dehydrogenase (LDH) is a stable cytoplasmic enzyme present in most cell types. LDH catalyses the oxidation of lactate to pyruvate reducing nicotinamide adenine dinucleotide (NAD) to NADH. The activity of LDH is determined by measuring the absorbance at 340 nm to analyze the amount of LDH present in the cell culture media.

HepG2 cells were seeded on ITO-APTES and ITO-APTES-GRP modified substrates at densities of 100,000 cells/substrate. The substrates were maintained at 95% 0_2 and 5% CO_2 under normal humidified conditions. The media was removed after specific intervals of time over a 48 hour period and the LDH reagent was added to it.

3.4.6 Absorbance measurements. Absorbance is defined as the negative logarithm of the ratio of transmitted light intensity through a sample to incident light intensity on the sample.

$$A_\lambda = -\log_{10}(I / I_0)$$

Where A_λ= Absorbance of the sample.

I= Intensity of light transmitted through the sample.

I_0= Intensity of light incident on the sample.

Absorbance measurements is based on the principle of Beer-Lambert law which states that, when light passes through a particular sample, the absorbance is proportional to the concentration of light-absorbing molecules in the sample at a given wavelength. Spectroscopy

techniques are used to measure the absorbance of the given samples. The absorbance plate reader and the UV vis spectrophotometer was used to measure the absorbance of the sample.

The ELX 800 plate reader was used to conduct cell viability and cytotoxicity analyses on HepG2 cells cultured on GRP modified ITO substrates as it involves the measurement of absorbance at a particular wavelength. Initially, the necessary measurement and reference filters were selected and fitted in the filter carriage. The samples were taken in 6 well plates and absorbance measurements were carried out using the quick mode where the measured optical density was printed on the printer.

CHAPTER 4

Results and Discussion

4.1 Surface characterization

4.1.1 Contact angle measurements. The contact angle of amine modified and growth factor modified surfaces were determined to verify the presence of amine functional groups using an optical contact angle measuring device (OCA15) purchased from Future Digital Scientific Corporation (Bethpage, NY). Pre-cleaned bare or unmodified ITO substrates were used as control. The contact angle of each substrate was analyzed at three different regions to generate an average contact angle for each substrate. The sessile drop method provided a contact angle measurement of 74.1^0 on bare unmodified ITO substrates. After modification with APTES, the substrates exhibited significantly lower contact angles $40.1^0 \pm 5^0$ which indicates the hydrophilicity of the amine end group of the SAM (3-APTES). These amine modified substrate contact angle values correlate with values reported in literature (Aithal, 2007).

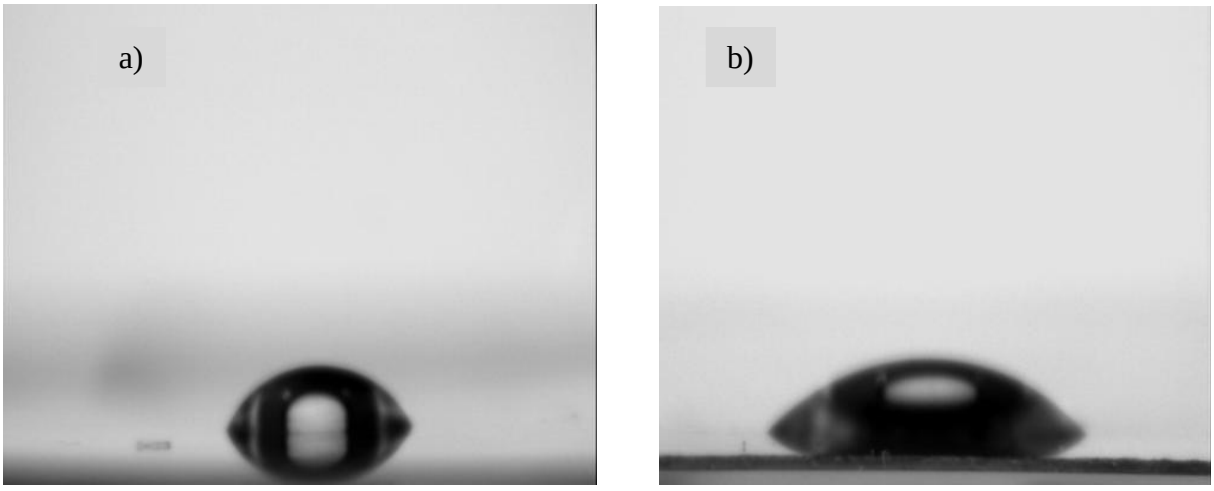


Figure 10. Contact angle measurement on a) ITO substrate and b) ITO modified with APTES.

4.1.2 ATR FT-IR spectroscopy. To confirm surface modification further, ATR-FT-IR was carried on all the substrates. FT-IR was utilized to identify the functional groups created by the conjugation of GRP to the amine surface.

Figure 11 shows the IR spectrum of APTES on ITO in the range of 1800-700 cm^{-1}. The strong to medium peak at 1578 cm^{-1} corresponds to the primary amine N-H bending. The peak at 972 cm^{-1} in Figure 11 indicates the stretching of Si-O bond representing the siloxane part of APTES. The weak band at 1685 cm^{-1} is due to primary amide stretching indicating the covalent bond between the amino end group of APTES and carboxylic end group of GRP. The asymmetric and symmetric bands of methylene (CH_2) are observed at 1470 cm^{-1} in Figure 11. The peak at 968 cm^{-1} in Figure 11 indicates the stretching of Si-O bond.

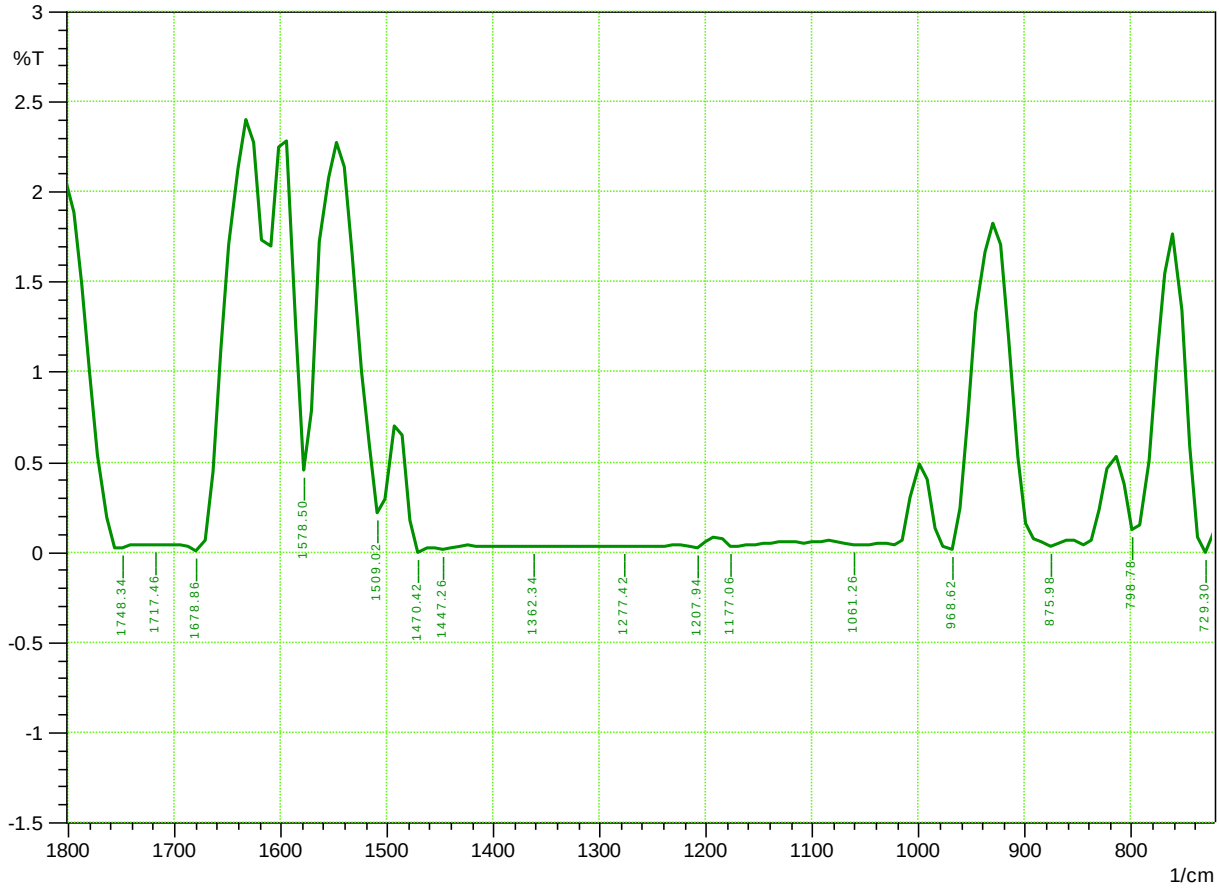


Figure 11. ATR FT-IR spectrum of APTES on ITO in the range of 1800-700 cm^{-1}.

Figure 12 shows the IR spectrum of APTES on ITO in the range of 3800-2800 cm^{-1}. The strong to medium peaks observed between 3500 and 3300 cm^{-1} correspond to primary amine N-H stretching. The asymmetric and symmetric bands of methylene (CH_2) are observed at 2966 and 2906 cm^{-1}, respectively.

Figure 12. ATR FT-IR spectrum of APTES on ITO in the range of 3800-2800 cm^{-1}.

Figure 13 shows the IR spectra of GRP coupled to APTES on ITO in the range of 2250 to 700 cm^{-1}. The medium peaks at 1612 and 1577 cm^{-1} in Figure 13 correspond to the primary amine N-H bending. The strong peak at 790 cm^{-1} in Figure 13 represents N-H wagging. The

asymmetric and symmetric bands of methylene (CH_2) observed between 1485 and 1444 cm^{-1} in Figure 13 indicate the propyl chain of APTES. The peaks between 1475 and 1435 cm^{-1} correspond to the asymmetric bending of alkanes.

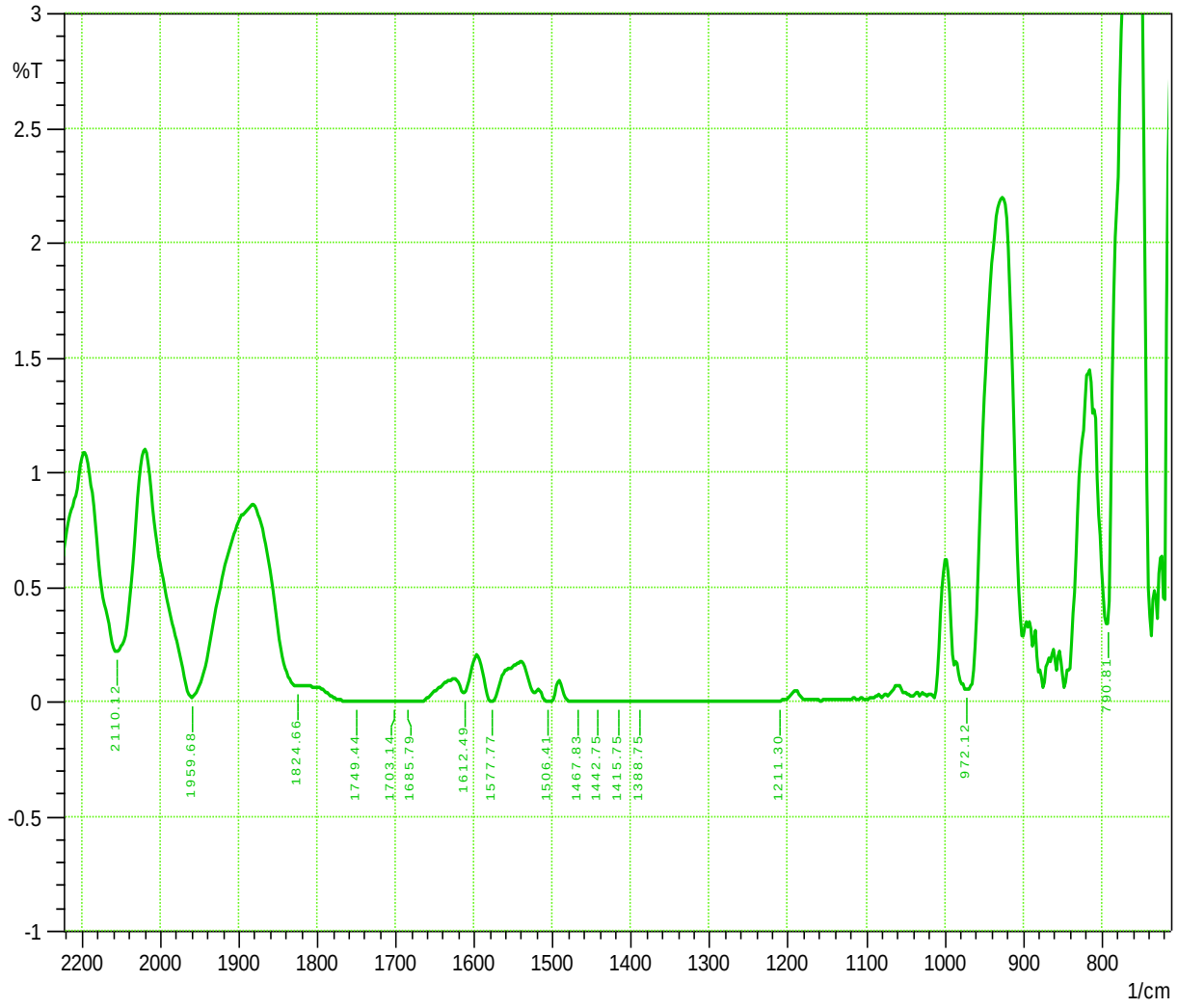


Figure 13. ATR FT-IR spectrum of GRP immobilized on ITO in the range of 2250-700 cm^{-1}.

Figure 14 shows the IR spectra of GRP coupled to APTES on ITO in the range of 3700-2250 cm^{-1}. The strong to medium peaks observed between 3500 and 3300 cm^{-1} correspond to primary amine N-H stretching. The asymmetric and symmetric bands of methylene (CH_2) are observed at 2968 and 2906 cm^{-1}, respectively. The peaks between 1475 and 1435 cm^{-1} correspond to the asymmetric bending of alkanes.

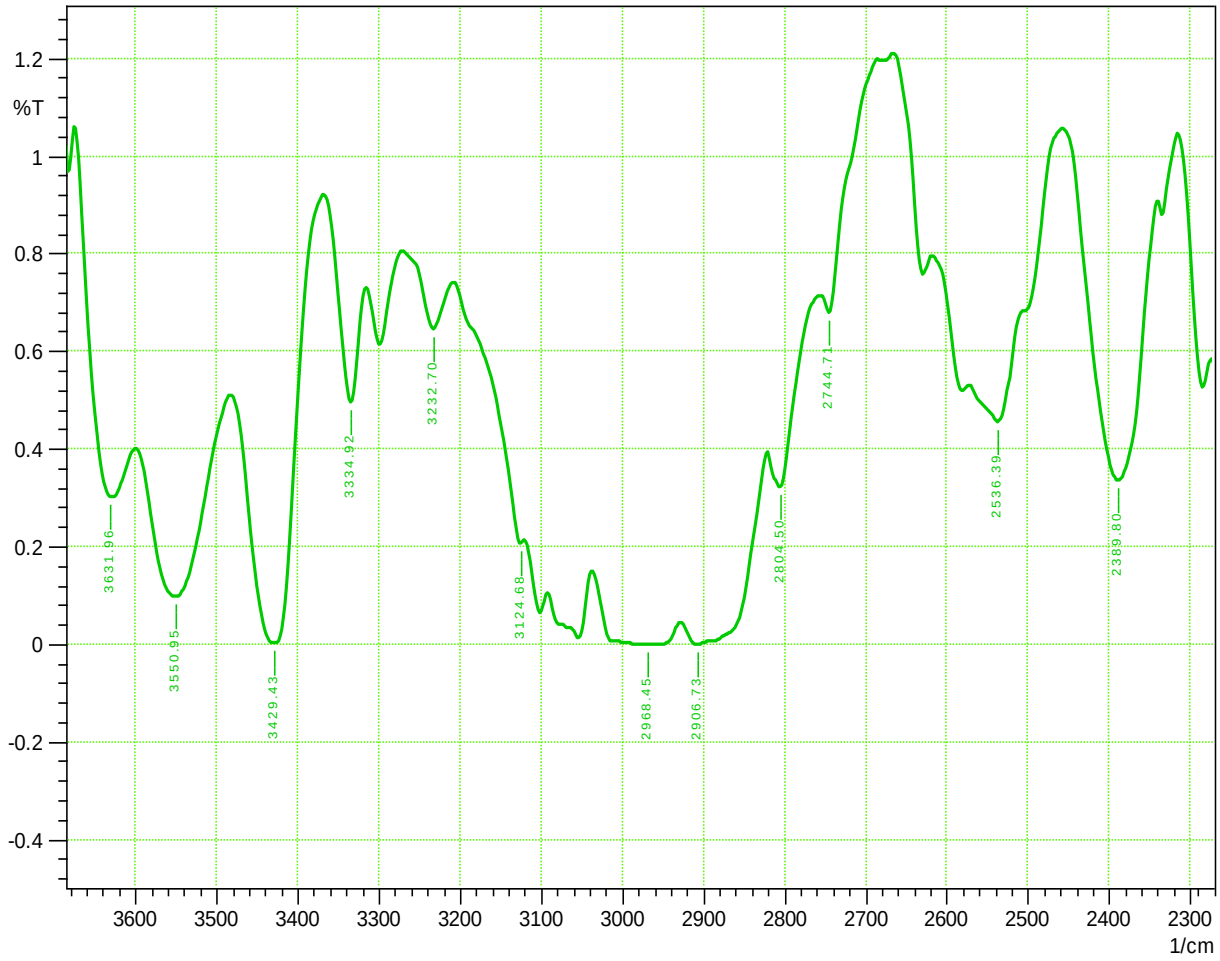


Figure 14. ATR FT-IR spectrum of GRP immobilized on ITO in the range of 3700-2350 cm^{-1}.

4.1.3 AFM analysis. Atomic force microscopy (AFM) was used to confirm the presence of immobilized molecules on ITO substrates microscopically. AFM of the substrates shows the difference in the size of molecules on ITO substrates after each modification.

A NT-MDT AFM was used to confirm the presence of immobilized APTES and GRP on ITO substrates. Figure 15 shows the 2D images of cleaned ITO substrates showing a characteristic roughness between 0.8 to 1.2 nm which is observed on ITO sputtered over a PET substrate (Lin, Li, & Yen, 2008).

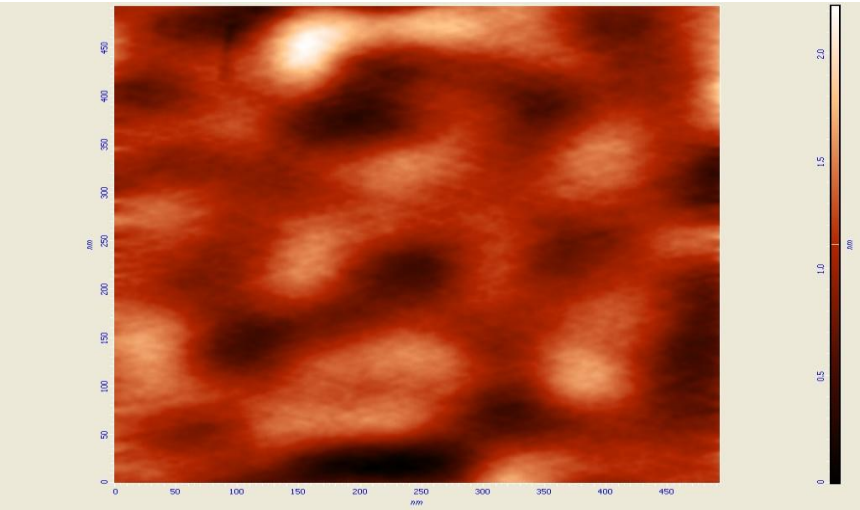


Figure 15. AFM image of a cleaned ITO substrate.

Figure 16 shows AFM image of amine-modified ITO substrate showing the distribution of crests and troughs ranging mainly between 2 to 4 nm. The length of molecules on APTES modified ITO is a lot higher than indicated in literature which could be attributed to accumulation of 3-APTES rather than forming a monolayer.

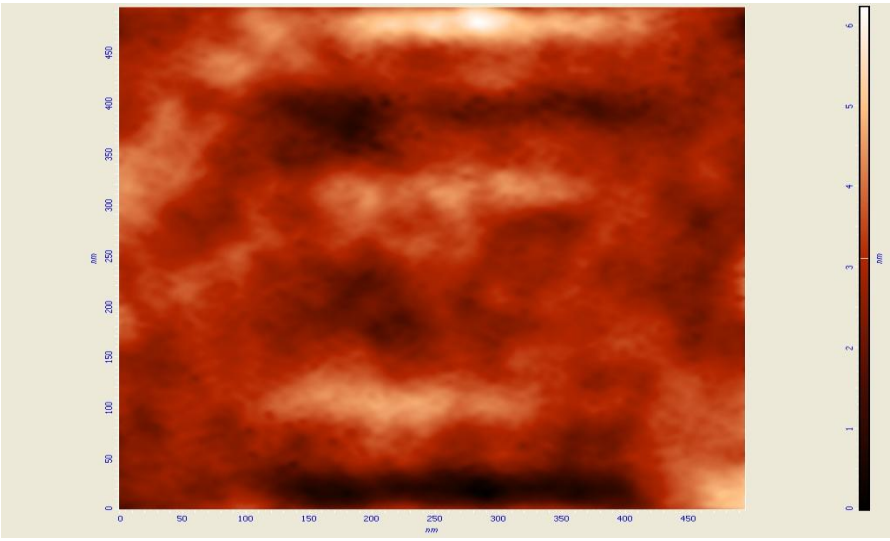


Figure 16. AFM image of ITO modified with 3-APTES.

Figure 17 represents the distribution of molecules mainly between 14-17 nm suggesting the presence of a longer molecule, GRP, compared to APTES on ITO. The length of GRP in 14.8

nm as indicated in the literature. The length of molecules in case of GRP modified on ITO substrate shows a good indication of the immobilization of GRP on ITO.

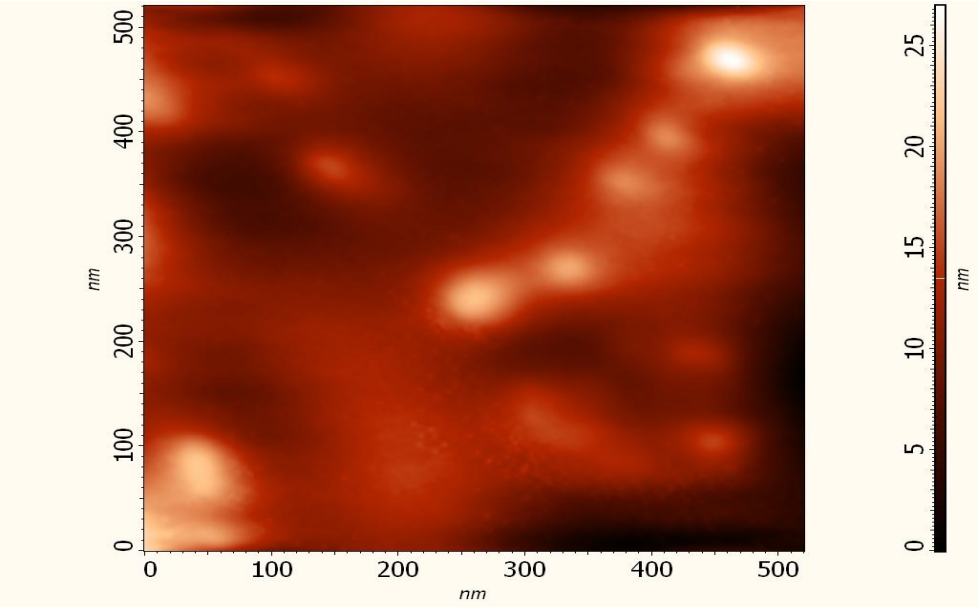


Figure 17. AFM image of GRP immobilized on ITO substrate.

AFM images suggest the presence of expected molecules and GRP on ITO. The increase in height of molecules after each attachment is clearly observed (Table 2). The abrupt height in molecules observed in Figure 16 with APTES on ITO could be attributed of agglomeration. The height of molecules ranging from 14-17 nm in Figure 17 in case of GRP immobilized on ITO indicates the length of GRP (14.8 nm).

Table 2

Height of SAM and immobilized GRP molecules on ITO from AFM analysis

Substrate	**Height observed (nm)**
ITO substrate	0.8-1.3
ITO with APTES	2-4
ITO with immobilized GRP	14-17

4.2 Cell culture studies

4.2.1 Cellular morphology. Cells are observed under the microscope at a magnification of 40x at regular intervals to record and study cellular morphology. Figure 18a) shows the morphology of the cells phase contrast images of HepG2 cells on the control. Figure 18b) shows the morphology of HepG2 cells seeded with soluble growth factor after 24 hours of seeding and Figure 18c) shows the HepG2 cells seeded with soluble growth factor after 48 hours of seeding. A few number of cells can be observed in case of the Figure18b) compared to Figure18c) as it should be expected in case of soluble growth factor showing good viability. Moreover, the morphology of HepG2 cells is also maintained after 24 and 48 hours of seeding. HepG2 cells after 48 hours of seeding with soluble GRP (Figure 18c) display increase in size which could be because of accumulation and cellular contact that most cell types tend to have in order to facilitate growth of each other by mutual sharing of growth facotrs and other components responsible for differentiation and maintaining cell functionality.

a) b) c)

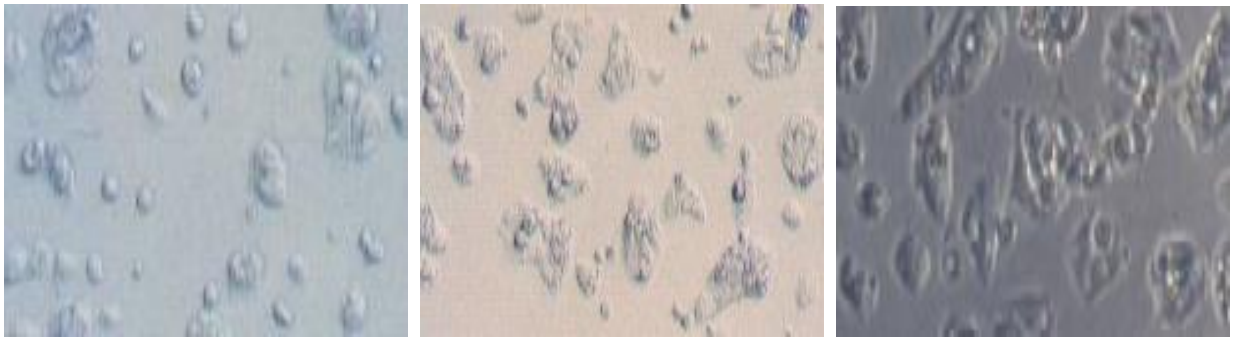


Figure 18. Morphology of HepG2 cells a) on a control 24 hours after seeding, b) seeded with soluble GRP 24 hours after seeding and c) seeded with soluble GRP 48 hours after seeding.

Figure 19 shows the phase contrast images of HepG2 cells that are seeded on GRP modified ITO substrates at 24 hours of seeding (Figure 19a) and 48 hours of seeding (Figure

19b). The cell density is higher for cells after 48 hours of seeding in Figure 19a) when compared to cells after 24 hours of seeding in Figure 19b). This indicates that the cell viability is maintained in cells seeded on GRP modified ITO substrates. There is change in morphology of HepG2 cells cultured with immobilized GRP when compared to those cultured with soluble GRP and on control which could be because of change in cell functionality. This could be a reason for immobilized GRP to show similar cytotoxicity as soluble GRP, shown in the results that follow.

a) b)

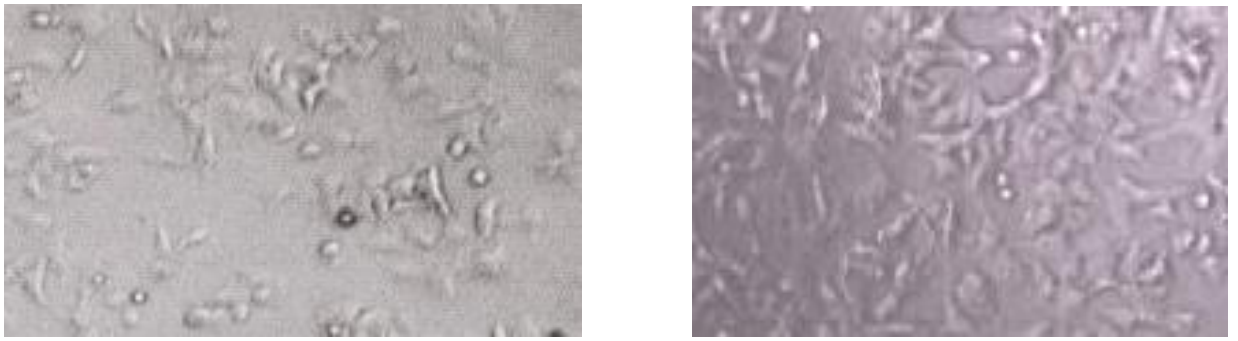


Figure 19. Morphology of HepG2 cells on immobilized GRP a) 24 hours after seeding and b) 48 hours after seeding.

4.2.2 Cell viability analysis. HepG2 cell viability was evaluated by performing the MTT assay on cells seeded on immobilized GRP substrates, cells seeded with soluble GRP and cells seeded on amine modified surfaces, each seperately. The principle of MTT assay is based on the conversion of yellow colored MTT to purple formazan crystals by mitochondrial reductase present in viable cells. The absorbance of formazan at 590 nm is the measure of viable cells in culture and it is directly proportional to the number of cells during the experiment.

Initially, cells with a density of 100,000 cells per substrate were seeded in each well. The cells were maintained under normal humidified conditions of 95% moisture and 5% CO_2 for a period of 24 hours to allow the cells to attach to the surfaces and the wells. The cells were treated

with the MTT reagent and incubated for four hours during which the purple formazan crystals were produced. These purple crystals were dissolved using a MTT solvent and the absorbance was measured at 590 nm and a reference absorbance at 630 nm. The viability of the HepG2 cells was assessed after 24, 48, 72 and 96 hours of culture. A calibration curve of variation in absorbance as a function of cell density was first obtained. Figure 20 shows the plot of optical density versus HepG2 cell density. The optical density increased with increase in cell density.

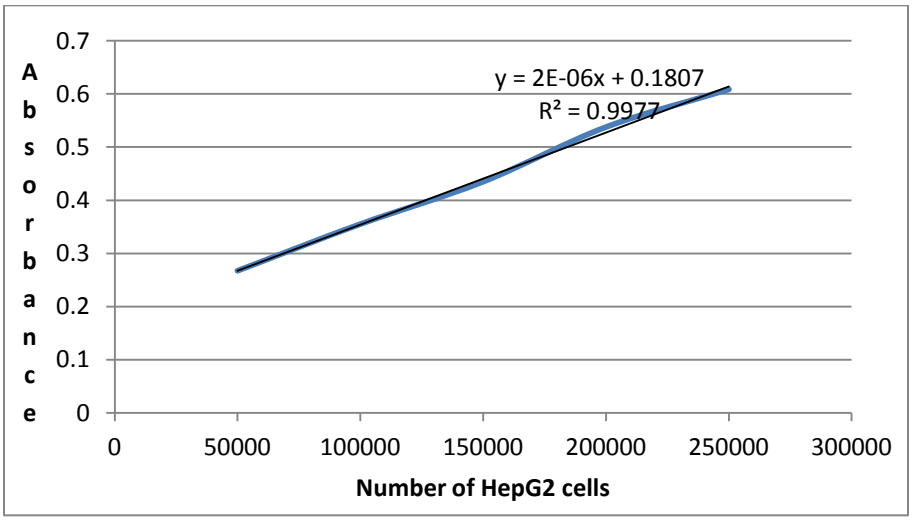


Figure 20. Calibration curve for MTT assay.

Figure 21 shows a plot of absorbance at different time intervals (24, 48, 72 and 96 hours) obtained from the MTT assays. The absorbance measured at 590 nm is a measure of comversion of MTT (yellow colour) to formazan crystals (purple) in the presence of mitochondrial reductase present only in viable cells. So, the absorbance due to foramzan formation directly represents number of viable or living cells. These cell densities were obtained by comparing the corresponding absorbances (mean of 3 values) with the standard curve (Figure 20). The results reveal that cell viability on GRP modified substrates after 48 hours was less than soluble GRP by 19%. In contrast, after 96 hours, cell viability increased in case of cells seeded on GRP modified

surfaces when compared to cells seeded with soluble GRP. This suggests that cell viability was successfully etended in case of immobilized GRP up to 96 hours.

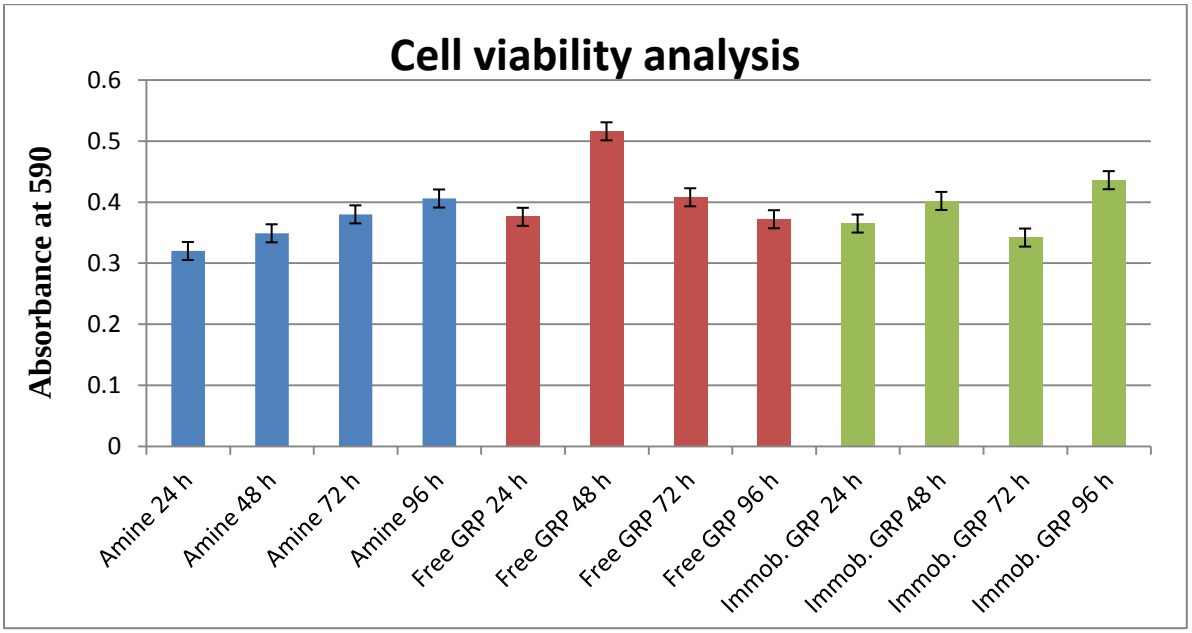


Figure 21. Cell viability studies of HepG2 cells on immobilized GRP after 24, 48, 72 and 96 hours of cellular culture.

Measurement of HepG2 cell viability on SAM modified surfaces provided quantitative validation of the influence of the immobilization of GRP on cellular properties. Even though there are no reports on the immobilization of GRP, soluble form of GRP has been reported to stimulate lung cancer cells (Carney, Cuttitta, Moody, & Minna, 1987). MTT conversion indicated that cell viability on GRP modified substrates after 48 hours was less than soluble GRP by only 19% which is an indicative of effect of complete availability of GRP in soluble form, unlike in immobilized form. However, MTT activity was observed to increase at 96 hours in case of immobilized GRP where as it decreased in case of soluble GRP indicating that biocompatibility was extended in case of immobilized GRP.

4.2.3 Cellular cytotoxicity analysis. The substrate influence on cellular cytotoxicity is assessed by measuring the LDH release by dead or damaged cells. LDH release was measured as

a function of duration in culture. The results obtained were expressed as mean of 3 independent trials. Control cultures were always established on tissue culture plastics (TCPS) and bare ITO surfaces.

Figure 22 shows a plot of LDH activity at different time intervals (24, 48, 72 and 96 hours) obtained from assays conducted on HepG2 cells seeded with SAM-modified substrate, GRP-modified substrate and soluble or free GRP. LDH production or plasma membrane damage after 48 hours was significantly reduced by 44% using growth factor modified substrates as compared to soluble growth factor, which suggests good biocompatibility. The study of LDH activity (Figure 22) indicated a decrease in LDH activity with increase in time in culture. LDH activity was higher initially due to cytotoxicity caused by placing cells in a foreign environment. Subsequent adaptation to the new environment resulted in decreased cytotoxicity as reflected by LDH activity.

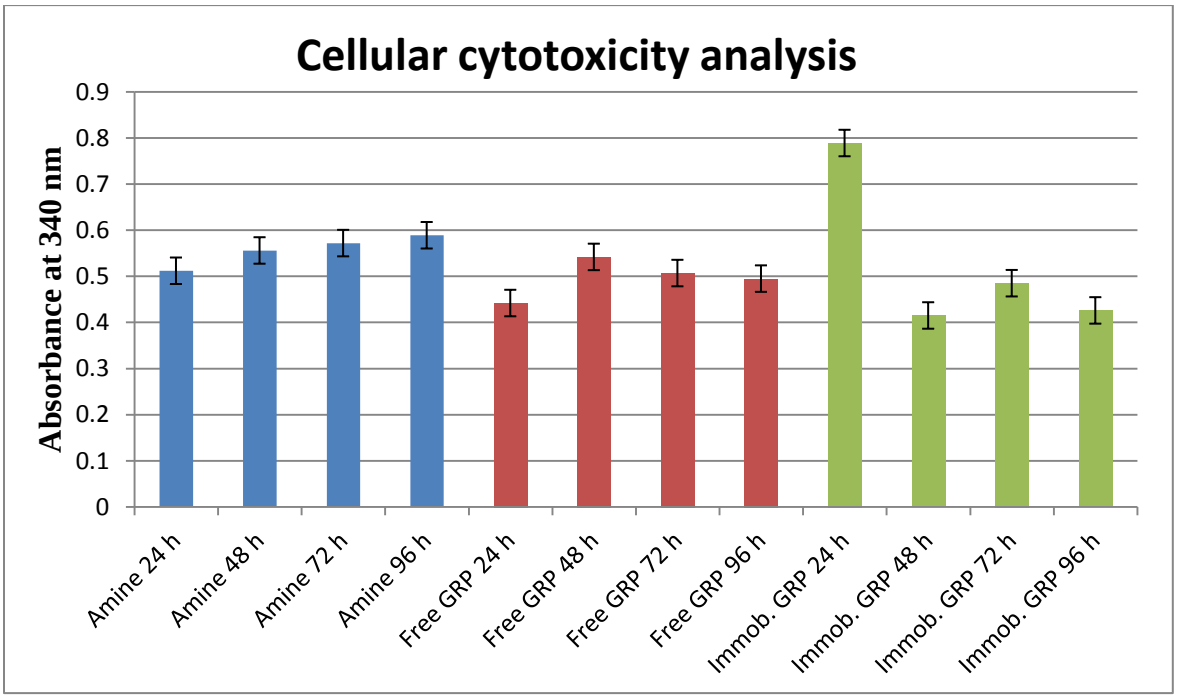


Figure 22. Cellular cytotoxicity of HepG2 cells on immobilized GRP compared to soluble GRP (10 µg/ml) and amine modified surface after 24, 48, 72 and 96 hours of cell culture.

The cytotoxicity analysis using immobilized GRP indicated that LDH production or plasma membrane damage after 48 hours was significantly reduced by 44% using growth factor modified substrates as compared to soluble growth factor, which suggests good biocompatibility. After 48 hours, LDH production decreased in case of soluble GRP and immobilized GRP substrates where as it increased in case on amine modified substrate.

4.2.4 Cellular cytotoxicity analysis using immobilized EGF. The results obtained from the experiments using immobilized GRP substrates encouraged us the investigate the culture of HepG2 cells on immobilizaed EGF substrates. The immobilization of EGF on ITO was carried out in the same way as immobilization of GRP (mentioned in chapter 3). Limited cell culture experiments were carried out with immobilized EGF. Cytotoxicity of soluble EGF is used to compare the cytotoxicity of immobilized EGF as in case of GRP. Cells cultured on ITO-SAM of 3-APTES and on tissue culture plastic were used as controls. The EGF modified substrates were placed in ethanol for 24 hours prior to cell culture studies. HepG2 cells were seeded on ITO-APTES and EGF-modified ITO substrates at densities of 100,000 cells/substrate.

LDH production or plasma membrane damage after 48 hours decreased by 26% in case on EGF-modified substrates, where as for both soluble EGF and amine modified substrate the cytotoxicity increased, indicating good biocompatibility of immobilized EGF at 48 hours. The study of LDH activity (Figure 23) indicated a decrease in LDH activity with increase in time in case of soluble and immobilized EGF where as LDH activity increased in case of amine-modified substrate indicating that biocompatibility further extended with immobilized EGF up to 5 days.

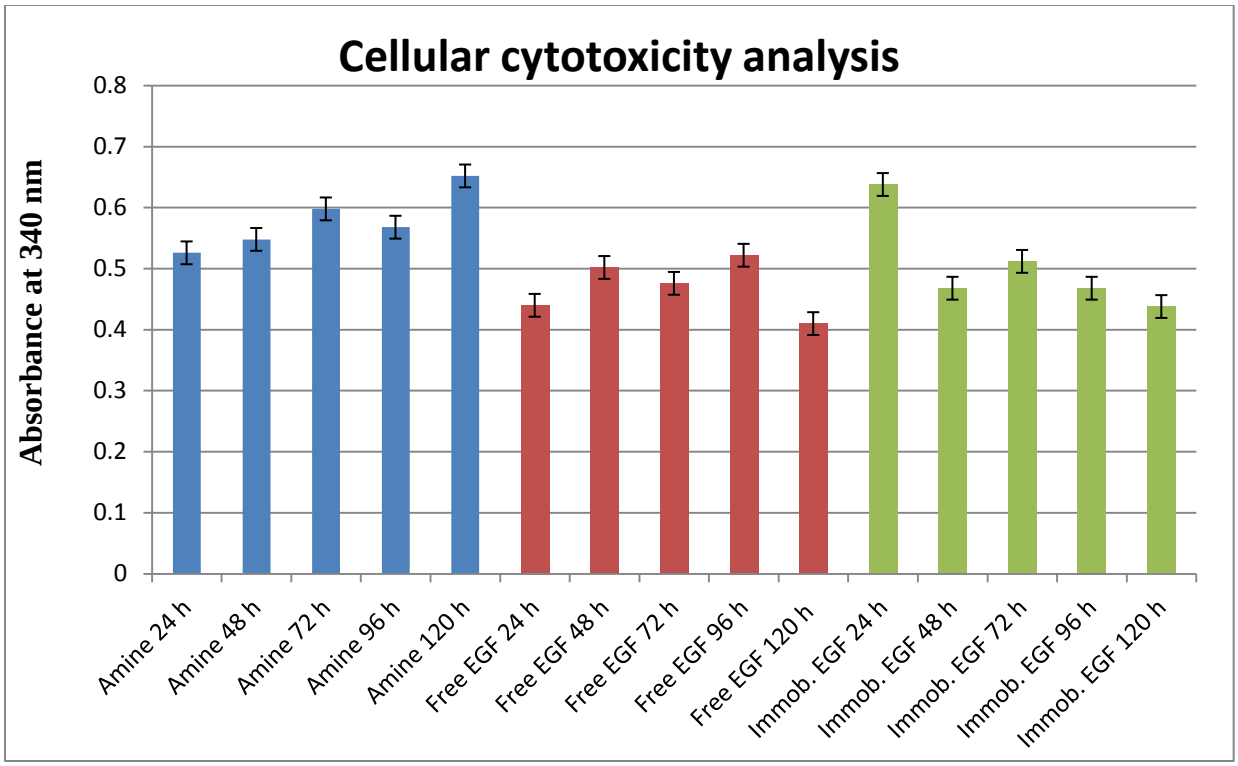


Figure 23. Cytotoxicity analysis of HepG2 cells using immobilized EGF substrates up to 5 days of cell culture.

CHAPTER 5

Conclusion

The experiments presented in this thesis were carried out with an objective of evaluating cellular response of HepG2 cells upon culture mainly on GRP modified ITO substrates. GRP was coupled to the amino end group of 3-APTES SAM immobilized on ITO by using standard deposition techniques. The modified substrates were characterized using contact angle measurement, ATR FT-IR, and AFM techniques. The characterization using ATR FT-IR showed the immobilization of 3-APTES SAM and the immobilization of GRP on the amino end group showing amine-carboxy linkage. The AFM images provided evidence for immobilization of 3-APTES SAM followed by GRP coupling on ITO.

A study of HepG2 cell morphology on SAM modified ITO surfaces showed that cells exhibited characteristic cell clustering, which increased with increase in culture period. HepG2 cell viability was evaluated using MTT assay at intervals of 24, 48, 72 and 96 hours. Spectrophotometric measurement of MTT conversion indicated that cell viability on GRP modified substrates after 48 hours was less than soluble GRP by 19%. A study of cellular cytotoxicity by measuring LDH release from cultured hepatocytes verified the same. LDH production or plasma membrane damage after 48 hours was significantly reduced by 44% using immobilized GRP substrates as compared to soluble GRP, which suggests extended biocompatibility.

Cells cultured on GRP modified ITO substrates showed similar cytotoxicity profile when compared to soluble GRP and less cytotoxicity when compared to amine modified ITO substrates after 96 hours. These results show that the influence of immobilizing GRP does not have a significant decrease in the cellular response after 96 hours when compared to soluble

GRP. However, after 96 hours, cell viability increased and cytotoxicity decreased for HepG2 cells cultured on GRP modified substrates suggesting that viability was extended.

The studies with GRP were extended to cytotoxicity analysis of HepG2 cells on EGF-modified surfaces. Preliminary results with EGF modified ITO substrates showed similar trends in LDH activity as GRP-modified substrates when compared with soluble and immobilized forms of EGF. The results obtained from these experiments would be used to design and fabricate SAM based CCPs.

CHAPTER 6

Future Recommendations

Immobilization of peptides and growth factors on SAMs is a promising area of research to develop a cell culture platform for drug toxicity screening. However, several studies need to be conducted to have a better understanding of immobilized growth factors to mimic ECM *in vitro*. Western blot analysis and enzyme-linked immunosorbent assay may be done to investigate active cellular pathways responsible for cellular interactions in the above mentioned systems. Studies on immobilization of EGF and HGF should be done by immobilization on SAMs for enhanced cell viability. Cell culture studies should be done with primary cells (like primary hepatocytes) to investigate the effect of immobilized growth factors. This will provide an understanding to mimic the components of the ECM and will help in developing artificial juxtacrine stimulation. This will aid in the cultivation of primary cells *in vitro* for prolonged periods of time. These systems can then be used for dug toxicity screening in the preclinical stages of drug development.

The change in morphology of HepG2 cells cultured on immobilized GRP may result in change in functionality. This is another aspect of the thesis that could be addressed. The immobilized GRP system could be used to study the effect of cell death intiators on cancer cell lines to investigate whether it has any effect on cell death.

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Appendix

Protocols employed

Immobilization of GRP on ITO

1) Trace and cut the ITO-PET (ITO sputtered on polyethyleneterephthalate) sheet into 2 cm diameter circular pieces.
2) The ITO substrates are cleaned by sonication in toluene, acetone and methanol for five minutes each and 30 minutes in DI water.
3) Dry the substrates using N_2 gas.
4) Dip the ITO substrates in a solution of 5% 3-APTES in ethanol for 24 hours followed by rinsing in ethanol and N_2 drying to yield the SAM of APTES on ITO.
5) Sterilize the substrates in 100% ethanol for a day prior to immobilization of GRP.
6) Place the ITO-SAM substrates in a solution containing 2:5 molar ratio of EDC:NHS in 0.1 M MES buffer for 24 hours at refrigerated conditions (4^0 C).
7) Sterilize the ITO-GRP substrates in 100% ethanol for a day prior to cell culture and then dry them using N_2 gas.

Contact Angle Measurements to measure Hydrophilicity or Hydrophobicity of substrates

1) Suspend a drop of water (0.5µL) using a microliter syringe positioned above the sample stage.
2) Move the syringe towards the stage so that the water droplet makes contact with it.
3) Retract the syringe, leaving the sample on the substrate.
4) Record the image is recorded using a CCD camera.
5) Measure the contact angle using the SCA20 software provided.