The Applications of Comb Polymer to the Study of Liver Cell Adhesion and Signaling

by

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Masters of Engineering in Biomedical Engineering

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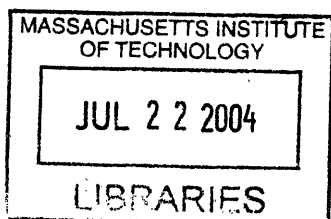May 24, 2004

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Abstract

Comb polymer, which consists of a hydrophobic poly(methyl methacrylate) (PMMA) backbone with hydrophilic hydroxy-poly(ethylene oxide) (HPOEM) side chains, is a tool that has many possible applications for the study of liver cell adhesion and signaling. This polymer has the unique properties of being cell resistant and chemically versatile such that various cell ligands can be coupled to its side chains. These properties allow adhesion through specific cell receptors to be studied without the effect of background adhesion to adsorbed proteins. By taking advantage of the ability to target specific receptors the comb polymer could be used as a powerful sorting tool. Sorting could be accomplished by finding cell type specific adhesion ligands. Several possible such ligands were screened. A ligand containing the tripeptide sequence RGD was found to elicit a strong cell adhesion response. However, this ligand is adherent to many cell types of the liver and would not be suitable for sorting purposes. Other cell type specific ligands tested showed little to no affinity for liver cell adhesion.

Additionally, the comb was utilized to study $\alpha_5\beta_1$ integrin-specific hepatocyte adhesion and the effect of Epidermal Growth Factor on adhesion. $\alpha_5\beta_1$ integrin adhesion was mediated using a novel branched peptide, SynKRGD. This peptide consists of a linear peptide sequence containing RGDSP and the synergy site sequence PHSRN connected by the sequence GGKGGG. By utilizing the amine side group of Lysine a GGC branch was added. The terminal cysteine was used to conjugate SynKRGD to comb polymer surfaces using N-(p-Maleimidophenyl) isocyanate (PMPI) chemistry. EGF has a great potential to benefit the field of tissue engineering due to its influence on cell proliferation, migration, and differentiation. EGF is also known to have a de-adhesive effect in some cell types. Hepatocytes were studied on comb surfaces of variable SynKRGD densities with and without the presence of EGF in the media. Distinct morphological differences were observed for hepatocytes on substrates of varying adhesivity with and without the presence of EGF. EGF was found to have a de-adhesive effect on $\alpha_5\beta_1$ integrin adhesion in hepatocytes. This effect became more pronounced as substrate adhesiveness increased.

Thesis Supervisor: Linda G. Griffith
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Chapter 1. Introduction and Background

1.1 Objective

The primary goal of this thesis was to elucidate adhesion and signaling properties in various types of liver cells through application of the comb polymer system.

1.2 Liver

1.2.1 Significance

The liver is an important and complex organ. It is located in the right upper quadrant of the abdomen and is central to the processes of metabolism, digestion, detoxification, and elimination of substances from the body. Because the liver is vital to so many of the body's key processes, death is often the end result of liver malfunctions. While the liver is highly regenerative and has the capacity to recuperate from moderate levels of trauma or toxic shock there are still many diseases of liver function which plague patients all over the world. In 2001 chronic liver diseases and cirrhosis were the 12th highest cause of death in the United States accounting for 9.4 deaths per 100,000 people (2003). Studying the liver to gain understanding of how its processes are carried out on a cellular level could lead to the preservation of countless lives.

1.2.2 Structure and Function

The vasculature of the liver is complex and unique. The liver is divided into many lobules. At the center of each lobule is a central vein. Blood enters the lobule from sinusoids at the periphery. These sinusoids draw blood from two sources, the portal vein and hepatic artery. Blood flows down the sinusoids towards the central vein between plates of hepatocytes that are one to two cells thick. The endothelial cells that line the sinusoids are distinctive in that they contain large fenestrations, or holes, that allow direct contact between the blood and the hepatocytes. The liver's ability to effectively clear blood of many classes of compounds depends on the hepatocyte surface exposure to sinusoidal blood. Within the hepatic plates, between adjacent hepatocytes, lie biliary canaliculi, which drain opposite to the blood flow into bile ducts at the periphery of the lobule. See Figure 1.
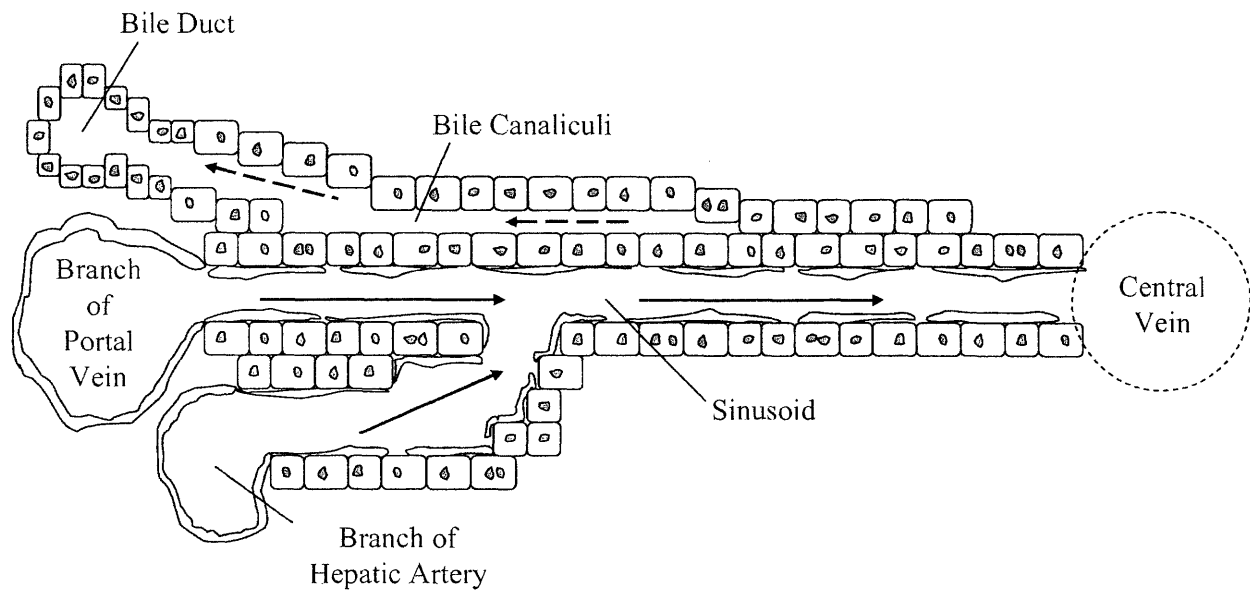
Figure 1. Structure of the Liver Lobule. Blood flows from the portal vein and the hepatic artery towards the central vein. Bile flows in the opposite direction from blood down the bile canaliculi.

The liver is a highly studied organ in the field of biotechnology due to its broad range of functions. The main contribution of the liver to digestion is bile secretion. Bile emulsifies lipids and is the only mechanism for excreting most heavy metals. Liver also regulates the metabolism of carbohydrates, lipids, and proteins. It is one of the two major storage sites for glycogen and is also the major site for gluconeogenisis, the conversion of amino acids, lipids and simple carbohydrates to glucose. When proteins are metabolized the amino acids become deaminated forming ammonia. Ammonia cannot be metabolized by most tissues and quickly becomes toxic to cells. However, ammonia is removed from the system by conversion to urea, which also occurs mainly in the liver. Synthesis is another key role of the liver. All the nonessential amino acids are synthesized by the liver as well as many plasma proteins such as albumins, globulins, and fibrinogens. In addition to metabolism and synthesis the liver is a vital storage site for iron and vitamins A, D, and B12. Finally, the liver plays crucial roles in hormone degradation, drug metabolism and toxin removal (Berne 1993).

1.2.3 Cell Types

There are several different cell types in the liver each with its own distinct and important functions. The following are brief descriptions of the major cell types (Michalopoulos and DeFrances 1997; Kimiec 2001). See Figure 2.
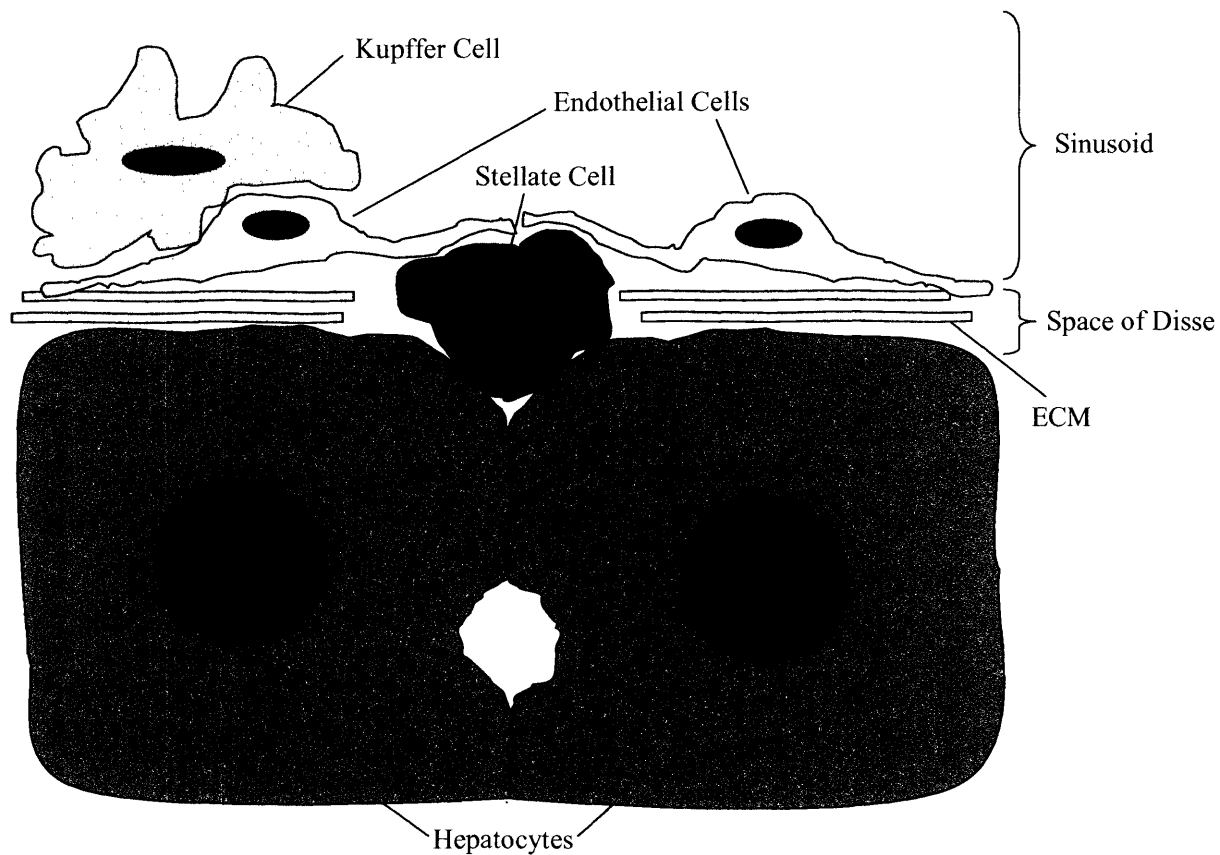
Figure 2. Various Cell Types of the Liver and Their Physical Relationship. Stellate, Kupffer, and endothelial cells reside in-between the hepatocyte plates. Many cell-cell contacts between various cell types are important for liver function.

Hepatocytes are the main functional cells of the liver. Most of the activity of the liver can be attributed to these cells. Loss of hepatocyte function due to injury by biological or chemical agents leads to acute or chronic liver disease. Hepatocytes make up about 60% of the liver in terms of cell number.

Sinusoidal endothelial cells are unique in many structural and functional characteristics from other endothelial cells of the body. They lack the typical basement membrane and are often in complexes with stellate cells. Additionally, these cells contain large cytoplasmic gaps called fenestrations which allow direct contact between the blood and hepatocytes. In terms of cell number, sinusoidal endothelial cells make up about 19% of the liver.

Kupffer cells are the macrophages of the liver. They clear the blood of gut-derived bacteria and bacterial toxins such as endotoxins or peptidoglycans. Additionally, these cells secrete many paracrine factors that influence hepatocytes and stellate cells. In terms of cell number, Kupffer cells make up about 15% of the liver.

Stellate or Ito cells are a fibroblast like cell that are unique to the liver. They have a distinctive morphology and surround hepatocytes with long processes. Stellate cells have several functions consisting of vitamin A storage, synthesis of connective tissue proteins, and secretion of several growth factors. In terms of cell number Stellate cells make up about 6% of the liver.

1.3 Comb Polymer

1.3.1 Structure and Protein Resistant Properties

Studying specific receptor-ligand interactions of cell adhesion can be difficult due to high levels of nonspecific protein absorption to surfaces. Adsorbed protein can lead to uncontrolled cell adhesion through many different receptor-ligand systems. A comb polymer was utilized in order to study liver cell adhesion in a specific and controlled manner. The comb polymer consists of a hydrophobic poly(methyl methacrylate) (PMMA) backbone with hydrophilic hydroxy-poly(ethylene oxide) (HPOEM) side chains. When coated onto a surface and introduced to an aqueous environment the backbone and side chains segregate at the liquid-substrate interface (see Figure 3). Through hydrophobic interactions the backbone is attracted to the substrate surface, while the hydrophilic side chains reach out towards the bulk liquid. This segregation of polymer components creates a PEO brush on the substrate surface. The hydrophilic side chains of the brush are mobile due to the free energy of the system. Because of the constant side chain motion proteins are unable to reach the substrate surface and therefore unable to adsorb, creating a protein free surface environment. A protein free surface is ideal in that it is resistant to cell

adhesion. To maintain this cell resistant property the polymer must consist of 30-35% HPOEM. This percentage ensures that there are enough side chains to effectively resist proteins, while maintaining a high enough hydrophobic polymer content such that the bulk polymer is not water soluble. The synthesis of this polymer has been previously described by (Irvine, Mayes et al. 2001).
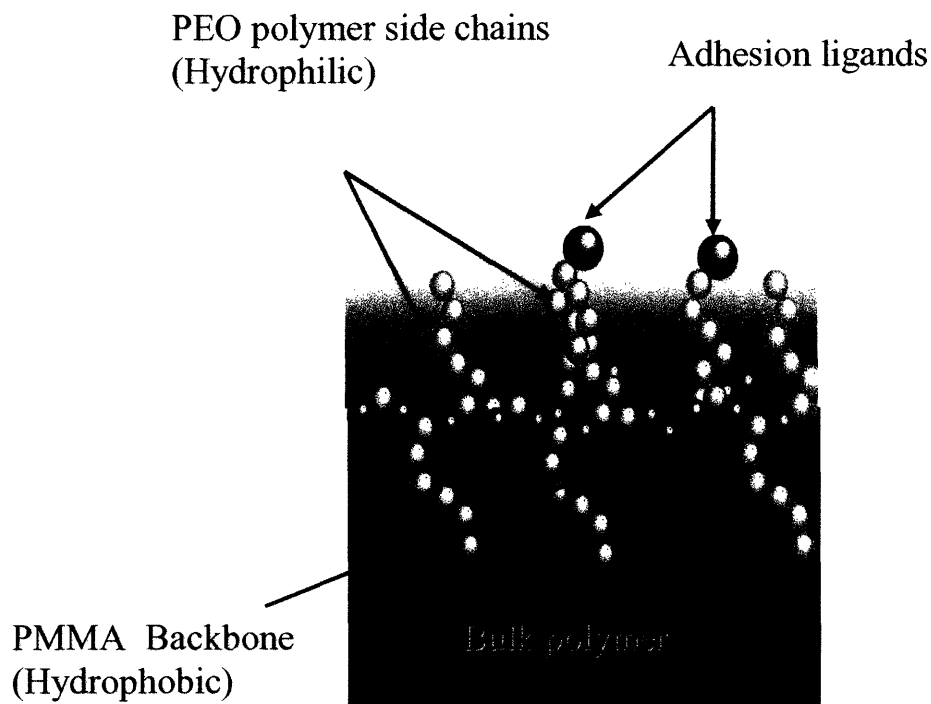
Figure 3. Structure of Comb Polymer (Koo, Irvine et al. 2002). The hydrophilic side chains move freely in the aqueous environment and prevent protein adsorption to the surface.

1.3.2 Surface Versatility

The hydrophilic side chains of the comb polymer are hydroxy-terminated. These hydroxyl groups can be exploited as a way to conjugate a variety of small molecules such as short peptide sequences. Conjugation to comb polymer can be accomplished through a variety of chemistries such as 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) activation, 4-nitrophenyl chloroformate (NPC) activation, and N-(p-Maleimidophenyl) isocyanate (PMPI) activation. See Figure 4.
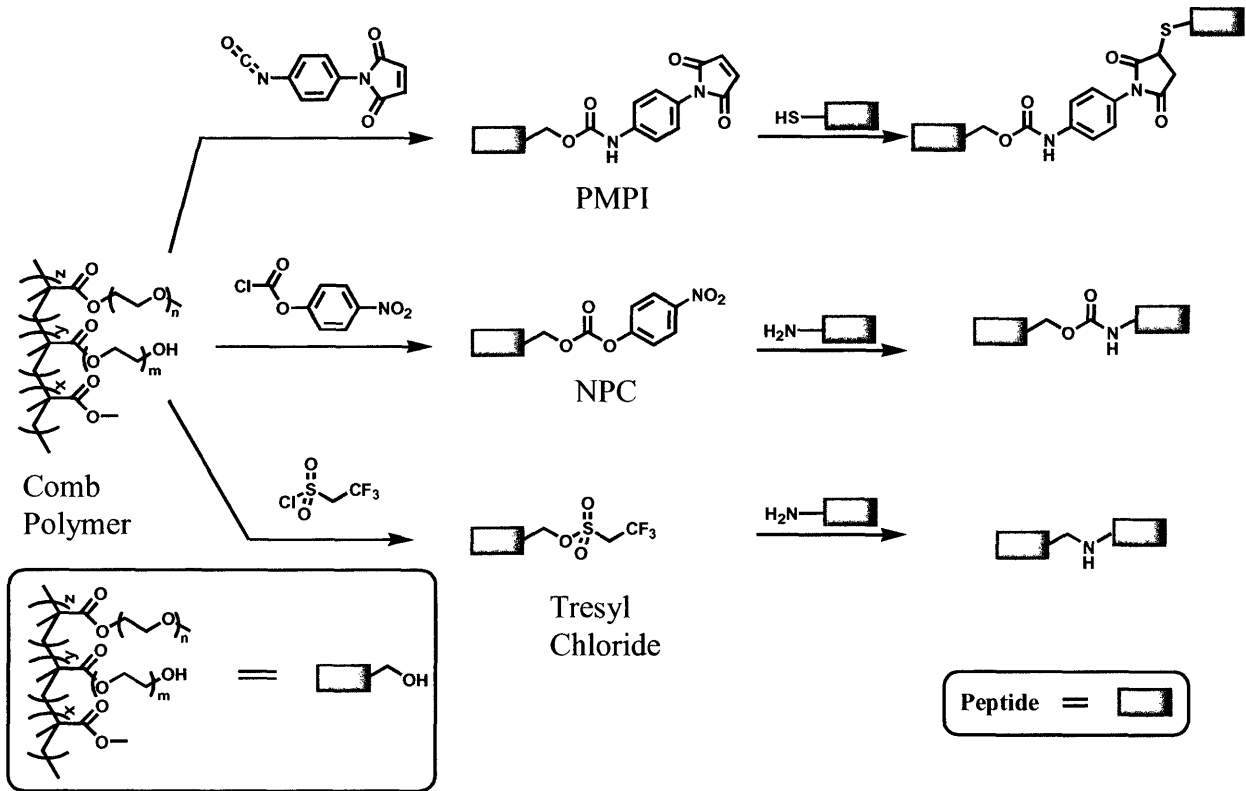
Figure 4. Different Comb Polymer Activation Chemistries (Courtesy of Maria L. Ufret Ph.D.). These activation chemistries are commonly used to conjugate various peptides to comb polymer.

1.4 Integrin Adhesion

Integrins are the major class of cell adhesion receptors that mediate cell-matrix interactions in metazoans (Hynes 2002). Integrins are heterodimeric cell adhesion molecules that consist of an α and a β subunit. These subunits contain both extracellular and intracellular domains though the intracellular domains are typically small (30-50 amino acids). In humans there are 18 α and 8 β subunits. The different combinations of α and β subunits result in 24 specific integrins with nonredundant functions. Most integrins recognize and bind to relatively short peptide sequences. For example, a subset of integrins recognize the tripeptide sequence Arginine-Glycine-Aspartic Acid (RGD) which can be found in many extracellular matrix (ECM) molecules such a as fibronectin, vitronectin, and fibrinogen (Koivunen, Wang et al. 1995). The ability of integrins to bind ECM requires the presence of Mg^{+2} in the cellular environment. This necessity is due to a characteristic metal ion dependent adhesion site motif (MIDAS) found in the integrin structure (Plows 2000). While the diversity of integrins increases as organisms become more complex the structure and function is conserved from sponges to humans (Hynes and Zhao 2000). Integrins

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are expressed by a variety of cells and each cell type expresses several integrins allowing cells to bind several matrix molecules. Integrins not only adhere cells to surfaces but are transmembrane mechanical connections of the extracellular environment to the cytoskeletal intracellular structure. After integrins adhere to their ligands, they cluster and recruit various cytoskeletal and cytoplasmic proteins (Miyamoto 1995), which eventually lead to the formation of specialized adhesive structures called focal adhesions. Because of their interactions with the intracellular environment, integrins play an important role in triggering various cell processes such as proliferation, differentiation, apoptosis, and cell migration (Flier 2001). Integrins are key to the phenomena of anchorage dependent cell survival. Integrins generally exhibit low ligand affinities (K_D equals 10^{-6} -10^{-8} mol/liter) compared to the affinities of cell surface hormone receptors (K_D equals 10^{-9} -10^{-11} mol/liter) (Lodish 2000). However, each cell creates hundreds of thousands of integrin interactions with extracellular matrix allowing them to remain attached to the ECM. These weaker interactions are beneficial to behaviors such as cell migration where the ability to break contacts with the extracellular matrix would be essential.

1.5 Applications of Comb Polymer to Liver Cell Adhesion and Signaling

As stated above, most integrins recognize and bind to relatively short peptide sequences. Many such peptide sequences have been identified in the literature and are easily synthesized. Once obtained, these peptides can be coupled to comb polymer surfaces through one of the many conjugation chemistries. Because the comb polymer is inherently cell resistant when integrin specific peptides are coupled to the surface, cells should only adhere *via* the desired integrin of study. Additionally, comb polymer is ideal for the presentation of integrin ligands because surface clustering can be achieved (Koo, Irvine et al. 2002). It is well characterized in the literature that integrin clustering allows cells to adhere in a more effective manner (Maheshwari, Brown et al. 2000).

1.5.1 Liver Cell Sorting

Different cell types in the liver express varying levels and kinds of integrins. In order to sort cells of similar size and density these differences in integrin expression can be exploited. It is well known that cell substrate interactions can be used to separate mixed populations of cells into

subpopulations by taking advantage of varying adhesivities (Wysoki 1978; Hammer 1987). This thesis explores possible adhesion ligands and their effectiveness for sorting cells of the liver.

1.5.2 Effects of EGF Signaling on Hepatocyte Adhesion

Epidermal Growth Factor (EGF) affects many cell types including epithelial and mesenchymal lineages. EGF can elicit a wide range of cellular responses depending on cell type such as mitogenisis, apoptosis, migration, protein secretion, differentiation or dedifferentiation (Wells 1999). Because EGF can stimulate proliferation, migration, and differentiation it has been highly studied in the field of tissue engineering and has the potential for many clinical applications. In addition to these cellular processes, EGF is also known to have a de-adhesive influence (Xie, Pallero et al. 1998; Glading, Chang et al. 2000). The crosstalk between EGF receptor signaling and integrins is an important phenomena to understand for bioengineers because the use of EGF as a mitogen would then effect the cellular interaction with biomaterials perhaps leading to undesired results. The de-adhesive effect of EGF on hepatocytes in culture has been observed in the literature (Kuhl and Griffith-Cima 1996). However, through the use of comb polymer the interaction between EGF signaling and specific integrins can now be elucidated.

Chapter 2. Cell Sorting by Adhesion

2.1 Introduction

2.1.1 Why Sort Cells?

Rat livers are typically perfused with collagenase and then the cells are purified through a series of centrifugation steps to yield a parenchymal and a nonparenchymal cell (NPC) fractions (Seglen 1976; Powers and Griffith-Cima 1996), yielding liver cells for study. The parenchymal fraction contains about 95% hepatocytes and 5% other liver cell types (Powers, Janigian et al. 2002). The NPC fraction however is not well characterized and the percentages of the various cell types are unknown. Information about the remaining 5% of the parenchymal cell fraction and the total break down of the NPC fraction would be invaluable to tissue engineers. One motivation for sorting and identifying cells efficiently is this lack of data. Another motivation for cell sorting would be to purify small hepatocytes from the NPC fraction. It has been hypothesized that these smaller hepatocytes might have a higher proliferative potential in which case they would be a better target for tissue engineering use. Finally, due to the high level of cell cooperativity in the liver, effective in vitro study would require that all the different cell types be present in the chosen culture system (Bhatia, Balis et al. 1999). Thus, the ability to sort cells and add them back to the culture system in known quantities would be critical.

2.1.2 Previous Sorting Techniques

There are many sorting techniques that are currently used to separate liver cell types. The following is a brief description of methods that are widely used and their drawbacks.

Percoll is a commercially available gradient material that consists of a colloidal suspension of silica particles coated with polyvinyl pyrrolidine (Alpini, Phillips et al. 1994). Centrifuging cells in the presence of a Percoll gradient allows cells to be separated by size and density (Leo, Mak et al. 1985; Smedsrod, Pertoft et al. 1985). However, no method based exclusively on size and density can yield a cell population of high purity from complex mixtures of hepatic cells (Alpini, Phillips et al. 1994). This limitation is due to the overlap in size and density of many cell types. This method is fairly effective but has the drawback of being time consuming. Liver cells require signals from substrate adhesion to survive thus the long periods spent in suspension cause cell viability to drop dramatically.

Elutriation is a process where fluid flow is forced counter to the force of centrifugation. This allows futher separation of particles by size and density. Elutriation has been used through out the literature to separate liver cells (Alpini, Lenzi et al. 1989; Janousek, Strmen et al. 1993; Valatas, Xidakis et al. 2003). However, this process like density gradient separation is time consuming. Additionally, the elutriation process can lead to physical damage of cells resulting in cell death.

Florescence activated cell sorting (FACS) is a commonly used method for sorting cells. FACS takes advantage of cell differences that can be detected by fluorescent fluorescently labeled antibodies. Cells are labeled and then sent individually through a florescence detector which then statically charges cells based on their fluorescent intensity and color. While this method is effective it is not widely used to isolate specific liver cell subpopulations. FACS has a relatively low cell yield and has a slow rate of sorting (10^7 cells/hr). Additionally, FACS instruments are extremely expensive and require highly trained personnel (Shapiro 1983; Alpini, Phillips et al. 1994). Furthermore, the FACS process is generally very species specific because antibodies are typically utilized as the fluorescent label. Thus, if the process were optimized for sorting rat liver cells whole new sets of antibodies would have to be generated for human liver cell sorting. Generating new antibodies would difficult, time consuming, and expensive.

2.1.3 Proposed Sorting Technique

By utilizing the comb polymer and its ligand conjugation versatility it is possible to generate a cell selective surface. Specific cells could be selected from a mixed population of cells based on their adhesive properties. Once non-adherent cells are washed away, selected cells could then be removed from the surface through receptor competition using soluble ligand (see Figure 5). There are several benefits to sorting cells in this way. Sorting cells by adhesion would not require that cells remain in suspension for long periods of time, thus increasing cell viability. Moreover, this method would not depend on size and density differences leading to higher cell type resolution. If integrin adhesion was used to sort cells, as opposed to antibodies, the process could easily be applied to different species due to the evolutionary conservation of integrin structure and function.
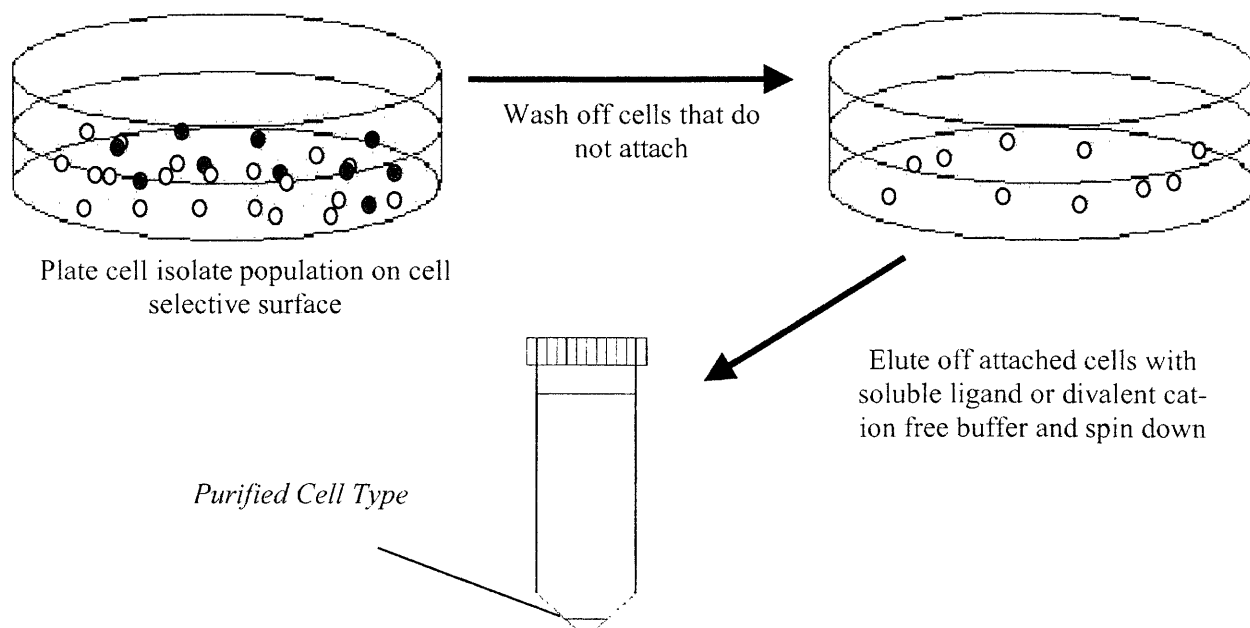
Figure 5. Proposed Adhesion Based Sorting Process. Sorting by integrin adhesion would require that cells spend less time in suspension and could be readily applied to different species.

2.1.4 Targeted Receptors for Functional Sorting

In order to create a cell selective surface the literature was reviewed for possible ligand candidates that might be specific to a particular cell type. While the focus of the project was on integrin adhesion other adhesion ligand candidates were also tested.

Integrin Candidates

$\alpha_9\beta_1$ integrin is a candidate for hepatocyte selection. The $\alpha_9\beta_1$ integrin is only expressed by the hepatocytes of the liver (Palmer, Ruegg et al. 1993). While not much is known about this integrin, its exclusive hepatocyte expression makes a good possible candidate for selection. Several short peptide sequences have been described in the literature as having $\alpha_9\beta_1$ specificity. The ones studied in this thesis are PLAEIDGIELTY (Schneider, Harbottle et al. 1998; Yokosaki, Matsuura et al. 1998) and SVVYGLR (Yokosaki, Matsuura et al. 1999).

$\alpha_4\beta_1$ integrin is a candidate for endothelial cell selection. In the literature a ligand for $\alpha_4\beta_1$ integrins, REDV was found to be endothelial cell specific (Hubbell, Massia et al. 1991; Massia

and Hubbell 1992). Another $\alpha_4\beta_1$ integrin specific ligand, IDAPS, (Mould and Humphries 1991) was also tested.

Nonintegrins Candidates

Asialoglycoprotein Receptor (ASGP-R) is a candidate for hepatocyte selection. The ASGP-R has been highly characterized in the literature. This receptor is uniquely expressed in hepatocytes and binds to galactose terminal oligosaccharides. The physiological function of the ASGP-R is to remove damaged proteins from the blood. It has also been shown in previous studies that selective immobilization of hepatocytes using the ASGP-R is possible (Weigel, Schmell et al. 1978; Oka and Weigel 1986; Lopina, Wu et al. 1996). Galactose molecules coupled to the ends of the HPOEM side chains of comb polymer could mimic the structure of galactose terminal oligosaccharides.

Neural Cell Adhesion Molecule (NCAM) is a candidate for stellate cell selection. NCAM is found in most nerve tissue and some non-neural tissues. In the literature activated stellate cells have been shown to exclusively express NCAM in the liver (Knittel, Aurisch et al. 1996). NCAM is typically involved in homophilic binding. However, a peptide sequence that shows NCAM specific adhesion, ASKKPKRNIKA, has been reported in the literature (Knittel, Aurisch et al. 1996).

Lectins are candidates for endothelial cell selection. Lectins are plant derived molecules that recognize carbohydrate moieties in glycoproteins, many of which are displayed on cell surfaces. These molecules have been used much like antibodies to identify and sort cells (Alpini, Phillips et al. 1994; Marelli-Berg, Peek et al. 2000; Ismail, Poppa et al. 2003). Several lectins have been known to display endothelial cell sensitivity. In the literature, certain lectins have been reported to have rat endothelial cell sensitivity such as Concanavalin A (ConA) and Lens culinaris (LCA) (Smolkova, Zavadka et al. 2001).

2.2 Materials and Methods

2.2.1 Liver Cell Isolation

Liver cell isolations were conducted by Emily Larson and Megan Whittemore. Cells were isolated using a modified two-step collagenase perfusion method from 150 to 230g male Fischer rats (Seglen 1976; Powers and Griffith-Cima 1996). Once isolated the cells are spun down at 50G for 3 minutes, 3 times. The pellets are about 95% hepatocytes and 5% NPC. The supernatants containing mostly NPC's are decanted or aspirated. Cells from the pellets were used for hepatocyte studies, and cells from the supernatant were used for NPC studies. See Appendix 1 for a more detailed perfusion protocol.

2.2.2 Cell Culture

Hepatocytes in all experiments were cultured using modified Hepatocyte Growth Medium (HGM) (Block, Locker et al. 1996). For full HGM preparation see Appendix 2.

NPC in all experiments were cultured in Endothelial Cell Growth Medium 2 (EGM-2) purchased from Cambrex (catalog #CC-3162).

2.2.3 Polymer Synthesis

The comb polymer used in all studies was a two component polymer consisting of PMMA with 10 mer HPOEM side chains of 526 molecular weight. These side chains are about 3.5nm in length. The same batch of polymer was used for all studies (Large Batch 003) and synthesized by Dan Pregibon (Summer 2003). NMR analysis indicated that this batch of polymer was 33% HPOEM, which is within the range for cell resistance and water insolubility. The synthesis of comb polymer has been previously described (Irvine, Mayes et al. 2001). For a detailed synthesis protocol see Appendix 3. Polymer composition and properties were analyzed using techniques outlined in Appendix 4. Before use in experiments cell resistance properties of polymer were tested using the protocol available in Appendix 8.

2.2.4 Polymer activation (NPC)

Evaluation of cell sorting ligands was carried out using NPC activated comb polymer. NPC activation allows ligands to be coupled through terminal amines (Veronese, Largajolli et al.

1985; Jo, Shin et al. 2001). See Appendix 6 for a detailed NPC activation protocol. NMR analysis indicated that NPC activation yielded 50% activated groups. For the chemical structure of NPC see Figure 6.
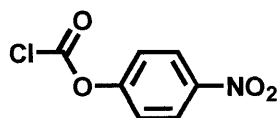
Figure 6. Structure of NPC (Courtesy of Maria L. Ufret Ph.D.). NPC is conjugated to the comb polymer through the chloroformate. The p-phenoxy then becomes a leaving group for peptide conjugation.

2.2.5 Surface Preparation

Substrates were prepared on 12mm diameter circular glass coverslips. In order to increase polymer affinity for the glass surface and reduce polymer delamination the coverslips were all silanized using 4% metacryloxypropyl-trimethoxysilane (MPTS) (Gelest Inc, cat #SIM6487.4), which increased surface hydrophobicity. See Appendix 5 for detailed coverslip silanization protocol.

Treated coverslips were spin coated with 20mg/mL comb polymer in methyl ethyl ketone (MEK). See Appendix 7 for spin coating protocol. The comb polymer used for coating is a 1 to 3 blend of NPC activated comb polymer to inactive comb polymer. Blending is done to obtain ligand clustering and to increase the efficiency of the conjugation reaction. Work by Ada Au (Griffith Lab, Massachusetts Institute of Technology) indicates that using only activated polymer yields a lower NPC conjugation reaction efficiency. This lower efficiency could be due to higher local concentrations of side product produced. Spin coated coverslips were left overnight in a vacuum oven before use.

Peptide coupling was done by leaving NPC activated comb surfaces for four hours covered with 1mg/mL peptide in coupling solution. Coupling coverslips were kept in a sealed humidified box at room temperature. After coupling coverslips were washed 3 times with coupling solution (0.1M sodium bicarbonate) and then covered with blocking solution (1 to 1, 0.5M sodium bicarbonate and 0.1M ethanolamine). Blocking solution was left on over night. While blocking, coverslips were left in a sealed humidified box. Blocking is done to deactivate any remaining

NPC groups. For a detailed NPC coupling protocol see Appendix 14. Surfaces are ready to use once blocked.

NPC activated surfaces were coupled with radiolabel RGD and quantified (Ada Au, unpublished data). Surfaces prepared from one to four blends of NPC comb and inactive comb were found to have about 14,000 RGD groups per square micron. This result indicates peptide coupling does occur. Because radioactive surface quantification is a time consuming and highly regulated process each peptide tested was not quantified. However, because peptide coupling has been validated by many members of the lab using different peptide sequences it is assumed with confidence that coupling has occurred.

2.2.6 Peptides
All peptides were ordered from either MIT Biopolymers Laboratory or Tufts University Core Facility. Exact sequences ordered were PLAEIDGIELTY, SVVYGLR, GREDVY, GIDAPSY, ASKKPKRNIKA, and GRGDSPY.

2.2.7 Carbohydrates
Amino terminal carbohydrate ligands were ordered so they could be coupled to be comb polymer using the same method as peptides. 1-amino-1-deooxy-β-D-galactose was ordered from Sigma (catalog #A-2267). A negative control carbohydrate 1-amino-1-deooxy-β-D-glucose was ordered from Indofine (Catalog #04-268).

2.2.8 Lectins
Fluorescently labeled Concanavalin A (catalog #C7642) and Lens culinaris (catalog # L9262) were ordered from Sigma to test endothelial cell specificity. Before attempting to conjugate lectins to comb surfaces, a live staining using fluorescently labeled lectins was conducted. Sinusoidal endothelial cells were purified using a percoll gradient (purifications were done by Albert Hwa, see Appendix 17) and then seeded onto collagen treated tissue culture plastic. Cells were allowed to spread overnight. Lectins were then dissolved in EGM-2 media (0.1, 1 and 5 mg/mL) and incubated on cells for 1 hour.

2.2.9 Selection Ligand Screening

To test cell adhesion substrates were placed in 24 well plates and then sealed down by silicone sealing rings. These rings cover some of the substrate surface reducing it from 12mm in diameter to 7mm. Hepatocytes and NPC were seeded at 15,000 cells per substrate in 150 uL of media. This concentration was selected such that there would be enough cells to adhere without overcrowding the surface. It was found that when hepatocytes were seeded on inactive comb substrates at concentrations above 50,000 cells per substrate, cells displayed nonspecific surface adhesion in large rounded clumps. This behavior could be attributed to an upper limit of comb polymer protein resistance. Cells were counted using a hemocytometer and trypan blue exclusion. Cells were incubated on surfaces for 24 hours before observation. All substrate conditions were done in triplicate and the experiment was repeated three times. Substrates prepared with only inactive comb were used as negative controls. For a detailed selection ligand screening protocol see Appendix 11.

2.2.10 Microscopy

All microscopy was done with an Axiovert 100. Pictures were taken with a Zeiss Axiocam (#412-312) and acquired using Open Lab 3.0.4 software.

2.3 Results and Discussion

2.3.1 Results

Hepatocytes are known to express integrins which have affinity for the tripeptide sequence RGD. Thus, surfaces conjugated to RGD were tested for hepatocyte adhesion. RGD induced a significant level of cell adhesion and spreading. See Figure 7.

Figure 7. Hepatocytes Adhered to RGD surfaces. Hepatocytes adhere and spread well on RGD conjugated surfaces. This result was consistently reproducible.

The $\alpha_9\beta_1$ integrin ligands tested, PLAEIDGIELTY and SVVYGLR, were not found to be good candidates for hepatocyte sorting use. Experiments were carried out using cells from the hepatocyte fraction. The sequence PLAEIDGIELTY showed some cell adhesion, but most cells were rounded and not well adhered. The sequence SVVYGLR showed little to no hepatocyte adhesion. Cells immobilized on the surface were all rounded. See Figure 8.
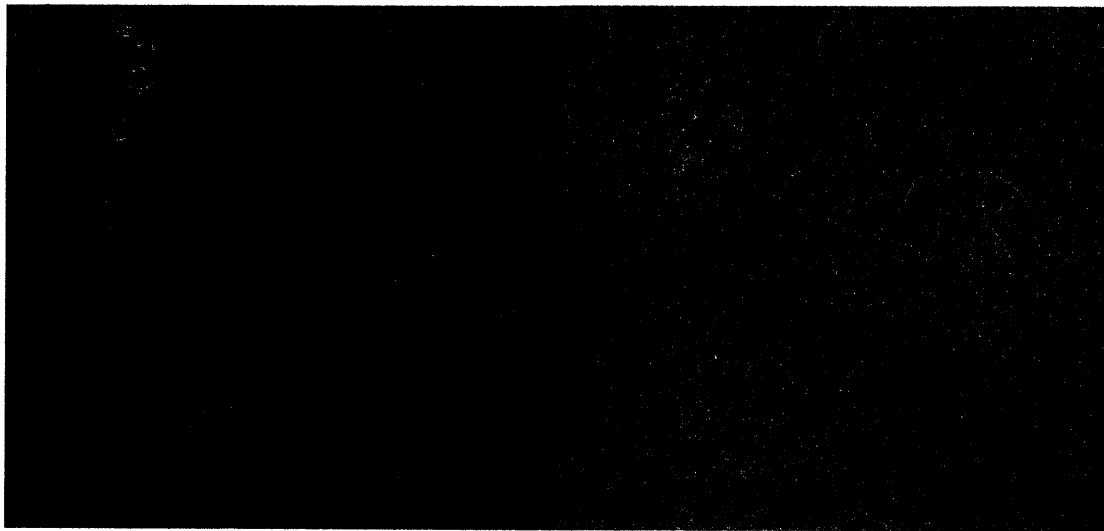
Figure 8. Hepatocyte Adhesion to PLAEIDGIELTY Surfaces. Few hepatocytes spread on PLAEIDGIELTY making it unsuitable for sorting purposes.

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The $\alpha_4\beta_1$ integrin ligands tested, REDV and IDAPS, were found not to be good candidates for endothelial cell sorting use. The REDV sequence showed some cell adhesion, but the results varied from isolation to isolation. Additionally, cells in these experiments were stained with fluorescent low density lipoproteins (DiLDL) to verify cell type (Biomed Tech, cat#BT-904). DiLDL specifically stains endothelial cells and Kupffer cells. Staining results indicated that adhered cells were neither endothelial cells nor Kupffer cells. Thus, ligand specificity was not achieved. It is thought that these unidentified cells could be small hepatocytes (see Figure 9). The IDAPS showed no significant cell adhesion for any experiments. All experiments were seeded with NPC fraction cells.
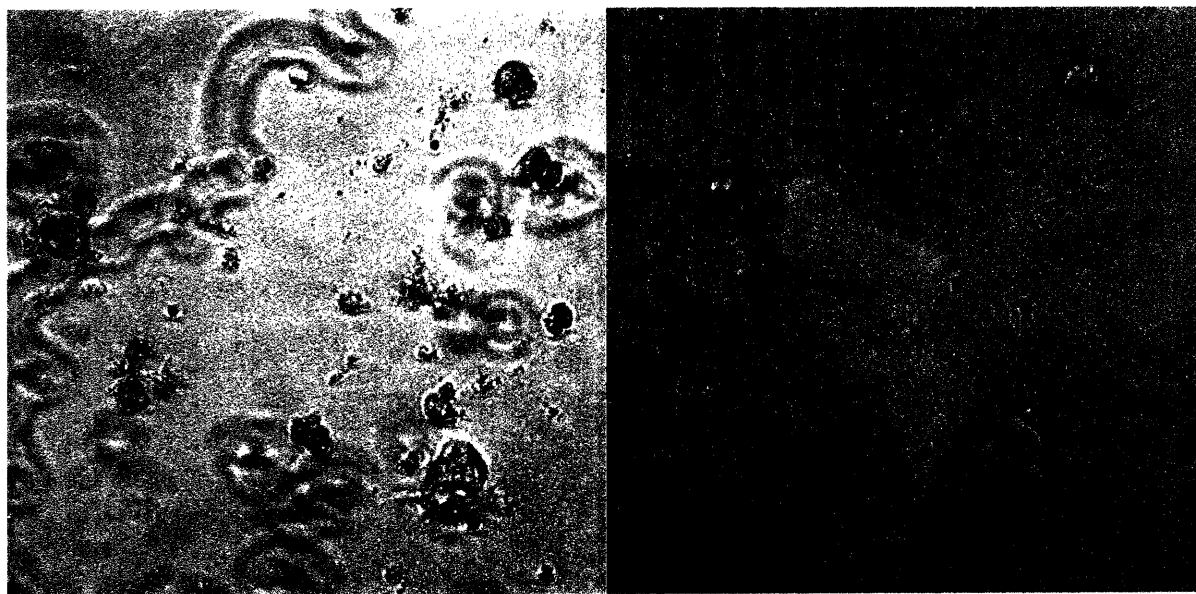
Figure 9. Unidentified NPC on REDV Surfaces. These cells are thought to be small hepatocytes present in the NPC fraction.

Previous literature has indicated that the asialoglycoprotein receptor was a probable ligand for hepatocyte specific adhesion. To target the asialoglycoprotien receptor 1-amino-1-deoxy-β-D-galactose was coupled to comb substrates using the same protocol as peptide coupling. All experiments were seeded with hepatocytes. None of these experiments exhibited hepatocyte adhesion to galactose conjugated comb.

Neural Cell Adhesion Molecule

Activated stellate cells are known to uniquely express NCAM in the liver. The peptide sequence ASKKPKRNIKA was used to prepare comb surfaces to target NCAM and encourage specific stellate cell adhesion. All experiments were seeded with NPC fraction cells. However, these peptide surfaces showed no cell adhesion.

Lectins

Endothelial cells were plated overnight and then stained with Concanavalin A (Con A) and Lens culinaris (LCA). Both lectins showed endothelial cell staining when compared to DiLDL staining.

2.3.2 Discussion

None of the ligands screened for $\alpha_9\beta_1$ integrin selection were deemed suitable for use in an adhesion based sorting procedure. The ligand PLAEIDGIELTY, specific for the $\alpha_9\beta_1$ integrin, showed levels of adhesion to low too be used for sorting purposes. The ligand SVVYGLR, also for the $\alpha_9\beta_1$ integrin displayed no visually detectable levels of cell adhesion. There are several reasons for which these ligands were not suitable. The level of $\alpha_9\beta_1$ integrin expression on hepatocytes is unknown and thus there may not be high enough expression to maintain cell adhesion via this integrin alone. Another possible reason is that the affinity for the $\alpha_9\beta_1$ integrin to these particular ligands may be too low to support cell adhesion. Additionally, in has been found in the literature that $\alpha_9\beta_1$ integrin appears to oppose cell spreading and stimulate cell migration (Liu, Slepak et al. 2001). This function of inducing migration could explain the poor levels of cell adhesion observed.

The ligands tested for $\alpha_4\beta_1$ integrin adhesion specificity, REDV and IDAPS, were not found to be suitable for an adhesion based sorting method. While REDV showed some cell adhesion the results were inconsistent from isolation to isolation and it was fount that adhered cells were not the desired endothelial cell type. These cells are thought to be small hepatocytes left in the NPC fraction. IDAPS was not found to display any visually detectable cell adhesion properties. As with the $\alpha_9\beta_1$ integrin, the level of $\alpha_4\beta_1$ integrin expression of sinusoidal endothelial cells is unknown and may be too low to support cell adhesion. All previous studies in the literature that

yielded positive endothelial cell adhesion to REDV substrates were done with a different endothelial cell type.

While other systems were able to use the asialoglycoprotein receptor to target hepatocyte specific adhesion there was no hepatocyte adhesion observed in these experiments. There are several possible explanations for this lack of adhesion. The coupling protocol used was directly adopted from peptide coupling. Thus, there is the possibility that no carbohydrates coupled to the surface. The literature was reviewed for a way to quantify the amount of carbohydrate on the surface. A possible method would be to use tritium labeled sugars. Furthermore, the asialoglycoprotein receptor binding affinity for galactose terminal oligosaccharides increases with increasing ligand valency with highest affinity occurring for a tribranched ligand (Lopina, Wu et al. 1996). The galactose presentation on the comb surface may have been too sparse to mimic this tribranched conformation.

NCAM coupled surfaces displayed no cell adhesion. NCAM is specifically expressed by activated stellate cells of the liver. The majority of stellate cells in the liver are quiescent. Thus, the number of activated stellate cells may be so small that the odds of capturing many using this type of surface sorting could be very low due to the low seeding density. If this process could be scaled and optimized the ability to characterize the number of activated stellate cells in a freshly isolated liver could be scientifically useful and give further insight into liver function on a cellular level.

Though lectins did stain the endothelial cells of the liver there was a high level of nonspecific background staining which could lead to nonspecific cell staining. Furthermore, the lectins tested were used in the literature as a stain on fixed cells. When used as a live cell stain they appeared to be toxic to cells. Due to this cell toxicity further lectin testing was not pursued.

When testing surfaces using NPC, experimental results were variable from isolation to isolation. There are several possible explanations for this phenomenon. During liver cell isolations the NPC are highly sensitive to the flow rate used to perfuse the liver and can cause variable cell viability. Further, cell death appeared to be sensitive to the seeding concentration. At higher cell

densities more cells appeared to die. This effect could be due to dying cells signaling surrounding cells to apoptose as well. Though cells were counted before seeding, it was difficult to maintain a constant seeding density. As a result of the isolation there is a significant amount of cell debris present when counting cells. The debris size can often be as large as cells making counting difficult and inaccurate. In order to more effectively utilize the NPC fraction without further purification a better method of counting cells must be developed.

Chapter 3. The Effects of EGF Signaling on Hepatocyte Adhesion

3.1 Introduction

3.1.1 EGF and the EGF Receptor

Many different growth factors have been discovered over the last few decades. Growth factors are generally proteins that stimulate a multitude of cell functions such as proliferation, migration, and differentiation. Epidermal Growth Factor (EGF) is a well characterized growth factor. It was first isolated from the submaxillary glands of adult male mice and has been found to stimulate proliferation, *in vivo* and *in vitro*, of many epithelial tissues (Ogiso, Ishitani et al. 2002). EGF has been shown to elicit a wide range of cellular responses depending on cell type such as mitogenisis, apoptosis, migration, protein secretion, de-adhesion, differentiation or dedifferentiation (Wells 1999). Human epidermal growth factor is a single chain polypeptide that is 53 amino acids long and contains three internal disulfide bonds (Lu, Chai et al. 2001; Ogiso, Ishitani et al. 2002). The structure and function of the EGF receptor is evolutionarily conserved from nematodes to humans (Burke, Schooler et al. 2001). The EGF receptor is a transmembrane glycoprotein that consists of 1186 amino acids. The EGF receptor is part of a family of receptors called Receptor Tyrosine Kinases. Each of these types of receptors binds a single ligand. Then these receptor-ligand complexes dimerize. Once dimerized the receptors phosphorylate each other in their cytoplasmic domains allowing them to then phosphorylate other proteins, beginning a complex signaling cascade resulting in phosphorylation of MAP Kinase and transcriptional modulation (see Figure 10) (Ogiso, Ishitani et al. 2002). Generally, once the EGF receptor is activated it is quickly internalized through coated pits into early endosomes and eventually transported to lysosomes where the receptor ligand complexes become degraded (see Figure 11). While the EGF receptor is known to signal at the cell surface there is data in the literature that indicates signaling from the endosomes as well (Wang, Pennock et al. 2002). The EGF receptor does not always follow the path to immediate degradation. Many times the receptor is recycled to the cell surface three to five times before it its ultimately degraded.(Clague and Urbe 2001)
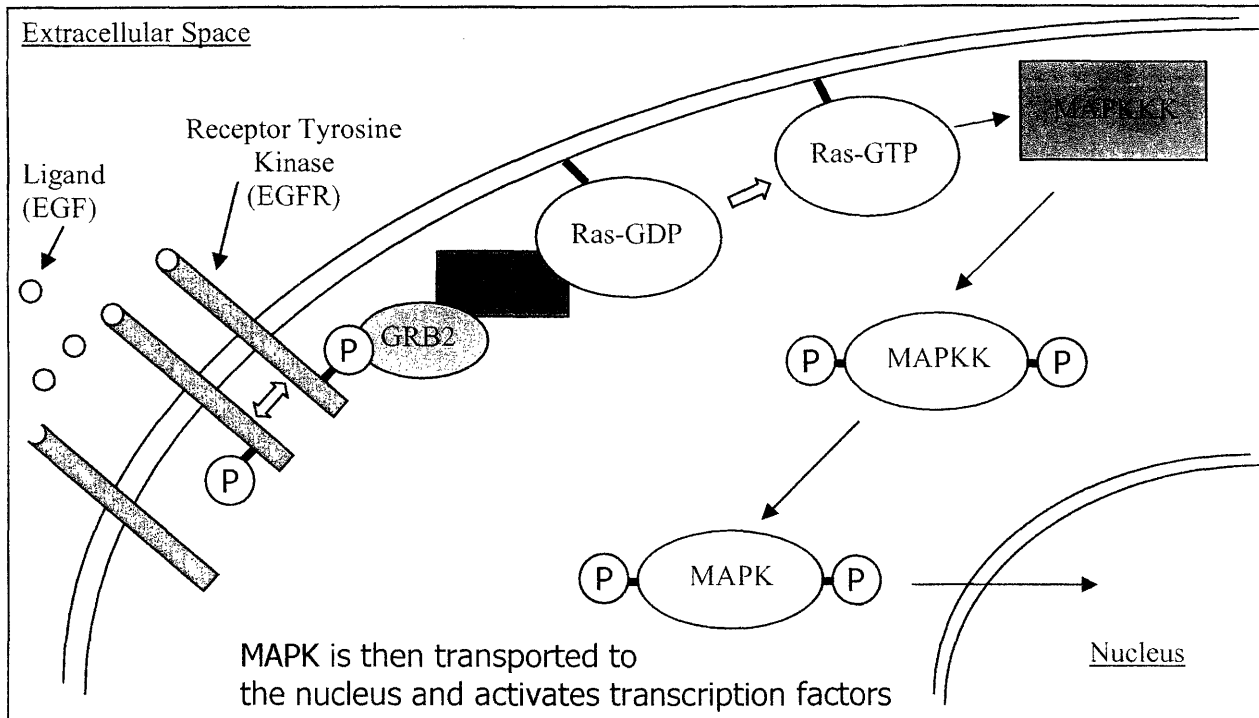
Figure 10. General Scheme of Receptor Tyrosine Kinase Signaling Cascade. EGF activates a complicated signally cascade that results in transcriptional modulation.
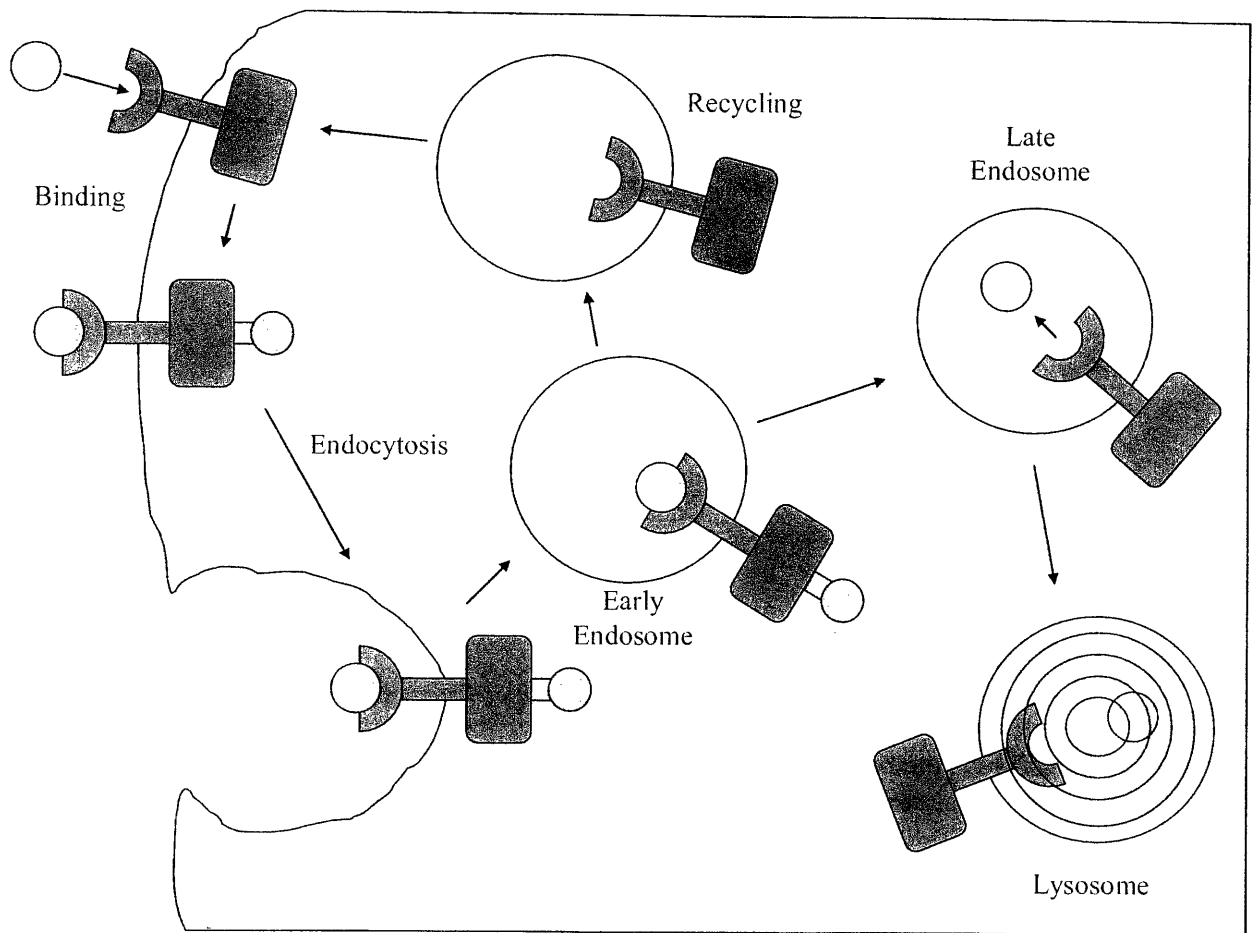
Figure 11. Summary of Trafficking and Signaling of EGFR. EGF signaling can continue after the EGF receptor complex has been endocytosed.

3.1.2 Previous Work

The effect of EGF on hepatocytes has been explored in the literature (Moriarity and Savage 1980; Gladhaug and Christoffersen 1987). (Kuhl and Griffith-Cima 1996) studied the effects of EGF on hepatocytes using both soluble and tethered presentations. In their studies hepatocytes were seeded on substrates that had been coated with polyethylene oxide (PEO) stars. However, the PEO stars utilized were relatively large and poorly packed and thus inefficient inhibitors of protein absorption. Because all of their experiments were done in serum free media their substrates adsorbed with 1:1 Type 1 Collagen and Cell Tak. Consequently, all cell spreading and adhesion was due to these adsorbed adhesion proteins. Soluble EGF at a concentration of 10 ng/mL was shown to completely inhibit the spreading of hepatocytes on these substrates.

3.1.3 The $\alpha_5\beta_1$ Integrin and Ligand

$\alpha_5\beta_1$ integrin is part of the subgroup of integrins that recognizes the tripeptide sequence Arginine-Glycine-Aspartic Acid (RGD) (Hynes 2002). This integrin has been shown to be important to hepatocyte adhesion to the extracellular matrix (Stamatoglou, Sullivan et al. 1990; Schaffert, Sorrell et al. 2001). Adhesion of hepatocytes to RGD substrates has been studied in the literature (Bhadriraju and Hansen 2000). However, it is known that RGD is only a minimal recognition motif for $\alpha_5\beta_1$. Higher affinity to $\alpha_5\beta_1$ can be achieved through the simultaneous presentation of RGD and a synergy site sequence, PHSRN, derived from 9th type III repeating unit of fibronectin (Dillow, Ochsenhirt et al. 2001). Peptides that contained both the RGD and PHSRN sequences were synthesized in order to study hepatocyte adhesion through the $\alpha_5\beta_1$ integrin. Peptides that contain both these sequences have been used throughout the literature. However, these have typically been incorporated into single linear peptide sequences (Kao and Lee 2001; Kao, Lee et al. 2001; Kim, Jang et al. 2002). In order for there to be synergistic activity of these sequences they must be correctly spaced. It has been noted in the literature that a spacer of six glycines between sequences results in a higher cell adhesion response than other glycine spacer lengths (Kao and Lee 2001). This work indicates that there are steric limitations to the function of activity of RGD with PHSRN. In order to further overcome these limitations Maria L. Ufret Ph.D. (Griffith Lab, Massachusetts Institute of Technology) designed a novel branched RGD-PHSRN peptide that would allow additional independent freedom of movement for each sequence, while approximately maintaining the six glycine spacer length. The branch peptide consists of the linear sequence PHSRNGGGKGGRGDSPY with a branch emanating from the lysine residue consisting of GGC (see Figure 12). This peptide will be referred to as SynKRGD. SynKRGD was tethered to PMPI activated comb polymer surfaces through the cysteine of the lysine branch. Surface tethering will be further discussed in the materials and methods section below.
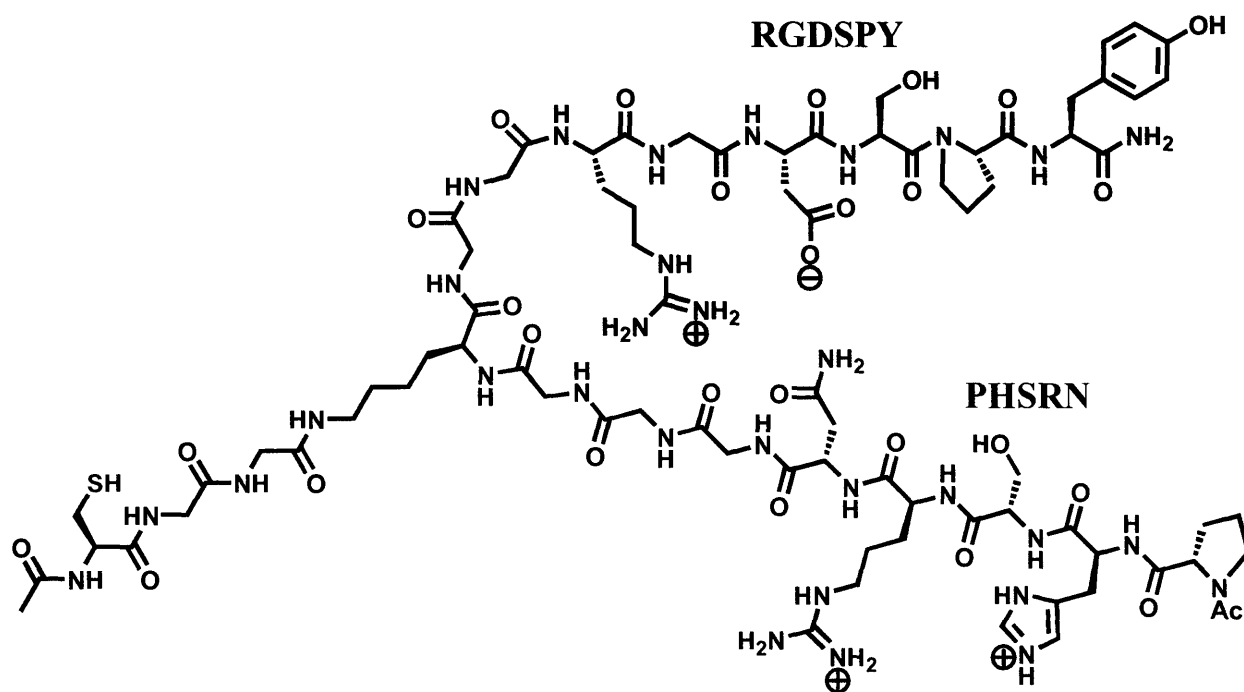
Figure 12. Structure of SynKRGD (Courtesy of Maria L. Ufret Ph.D.). The branching of SynKRGD provides freedom of movement such that each arm can find its optimal binding position.

3.2 Materials and Methods

3.2.1 Liver Cell Isolation

See liver cell isolation in Chapter 2, Materials and Methods, 2.2.1.

3.2.2 Cell Culture

All experiments were cultured using modified Hepatocyte Growth Medium (HGM) from (Block, Locker et al. 1996). For full HGM preparation see Appendix 2. EGF free media was also prepared and was otherwise identical to complete HGM.

3.2.3 Peptide Synthesis

The linear portion of the SynKRGD peptide and linear RGD with synergy site peptide (CPHSRNGGGGGGRGDSPY) were synthesized using an Advanced ChemTech 396Ω and standard 9-fluorenylmethyloxycarbonyl (FMOC) chemistry. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and N-hydroxybenzotriazole (HOBt)

were used as activating agents. NovaSyn® TGR resin (catalog #01-64-0060) was used and purchased from Novabiochem (http://www.emdbiosciences.com). All amino acids were also purchased from Novabiochem. The additional branch of SynKRGD was added by hand. The methoxytrityl (Mtt) protecting group of the lysine was removed using 1% trifluoroacetic acid (TFA), resulting in a free amine. This amine was utilized to add the GGC the branch of SynKRGD using FMOC chemistry. The amino terminus was capped using acetic anhydride. Solid phase peptide synthesis is detailed in Figure 13 below. Once the peptide was synthesized it was cleaved from the resin using TFA:triisopropylsilane (TIS):H_2O:Ethanedithiol (EDT) (92.5:2.5:2.5:2.5) and then precipitated using ice cold ether, spun down, and resuspended in ice cold ether several times. The peptide was then lyophilized overnight and subsequently purified by High Pressure Liquid Chromatography (HPLC). See Appendix 12 and 13 for detailed protocols on peptide synthesis and cleavage from resin.
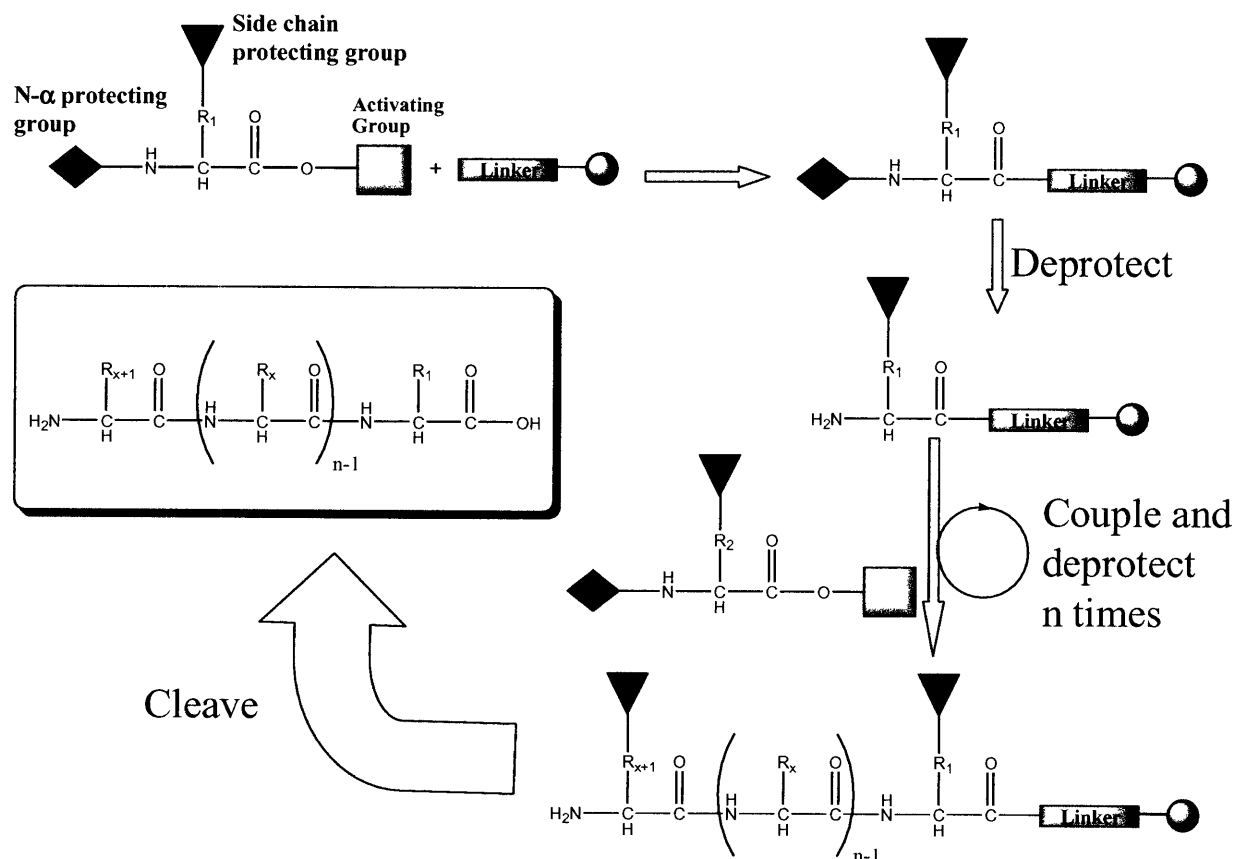
Figure 13. Schematic of Solid Phase Peptide Synthesis (Courtesy of Maria L. Ufret Ph.D.). Coupling and deprotecting is repeated for each amino acid added to the peptide. After peptides are cleaved from the resin they are purified using HPLC.

3.2.4 Polymer Synthesis

See Chapter 2, Materials and Methods, 2.2.3.

3.2.5 Polymer activation (PMPI)

Studying the effect of EGF on $\alpha_5\beta_1$ integrin adhesion was carried out using PMPI activated comb polymer. PMPI activation allows ligands to be coupled through cysteine residues (Annunziato, Patel et al. 1993). See Appendix 9 for a detailed PMPI activation protocol. NMR analysis indicated that PMPI activation yielded about 25% activated groups. For the chemical structure of PMPI see Figure 14.
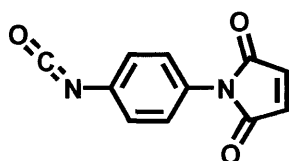
Figure 14. Structure of PMPI (Courtesy of Maria L. Ufret Ph.D.). PMPI is conjugated to comb polymer through the isocyanate group. The cysteines of peptides then bind to the maleimide during conjugation.

3.2.6 Surface Preparation

Substrates were prepared on 10mm diameter circular glass coverslips. In order to increase polymer affinity for the glass surface and reduce polymer delamination the coverslips were all silanized using 4% metacryloxypropyl-trimethoxysilane (MPTS) (Gelest Inc, cat #SIM6487.4). This treatment increases surface hydrophobicity. See Appendix 5 for detailed coverslip silanization protocol.

Treated coverslips were spin coated with 20mg/mL comb polymer in methyl ethyl ketone (MEK). See Appendix 7 for spin coating protocol. The comb polymer used for coating was a blend of PMPI activated and non-activated. Surfaces made were either 10% or 25% PMPI activated comb polymer. Blending is done to obtain ligand clustering and control surface concentration of ligand. Spin coated coverslips were left overnight in a vacuum oven before use.

Peptide coupling was done by leaving PMPI activated comb surfaces for four hours covered with 125 μM peptide in 7.4 pH phosphate buffer. While coupling, coverslips were kept in a sealed humidified box at room temperature. After coupling, coverslips were washed 3 times with pH 7.4 PBS. For a detailed PMPI coupling protocol see Appendix15. Surfaces are ready to use at the end of the coupling process.

Coupled peptides were quantified using radiolabeled SynKRGD and it was determined that for a 4 hour coupling 10% SynKRGD surfaces displayed 228,000 peptides/μm^2, while 25% SynKRGD surfaces displayed 577,000 peptides/μm^2(Ley Richardson, Griffith Lab, Massachusetts Institute of Technology unpublished data).

3.2.7 Spreading Experiments

Spreading area was used as a measurement of cell adhesion. It is assumed that the larger the spread cell area the higher the affinity of the cell for the substrate. To test cell adhesion to 10% and 25% SynKRGD substrates with and without the presence of EGF, substrates were placed in 24 well tissue culture plates. Hepatocytes were seeded at 15,000 per substrate in 500 uL of media. The seeding density was selected such that there would be enough cells to adhere without overcrowding the surface such that cell spreading area could be more easily calculated. Cells were counted using a hemacytometer and trypan blue exclusion. Once seeded, cells were incubated for 27 hours with or without EGF before analysis. At 27 hours, live cells were fluorescently stained with 5μl/ml Vybrant DiI (Molecular Probes) and 1μl/mL Hoechst for plasma membrane and nuclei, respectively. After staining, nine different fields were taken for each coverslip. Each field was photographed three times for a bright field, florescent spread area, and fluorescent nuclei. All substrate conditions were done in triplicate and each experiment was repeated at least once. For a detailed spreading experiment protocol see Appendix 10.

3.2.8 Microscopy

All microscopy was done with an Axiovert 135. Photos were taken with Hamamatsu Digital Camera (#C4742-95) and saved using Open Lab 2.2.5 software. All images were taken at 10x.

3.2.9 Image analysis

Images for spreading were analyzed using the Scion Image software (version Beta 4.0.2) obtained from www.scioncorp.com. Scion Image was used to calculate the total spread cell area in μm^2 per image field. The conversion used was 0.745 pixels per micrometer. The number of nuclei per field are also counted. For each field the total spread cell area is then divided by the number of nuclei. The areas/nuclei for all the fields of each condition are then averaged to obtain an average area/nuclei. See Figure 15 below for examples of field images. See Appendix 16 for a detailed Image Analysis Protocol.
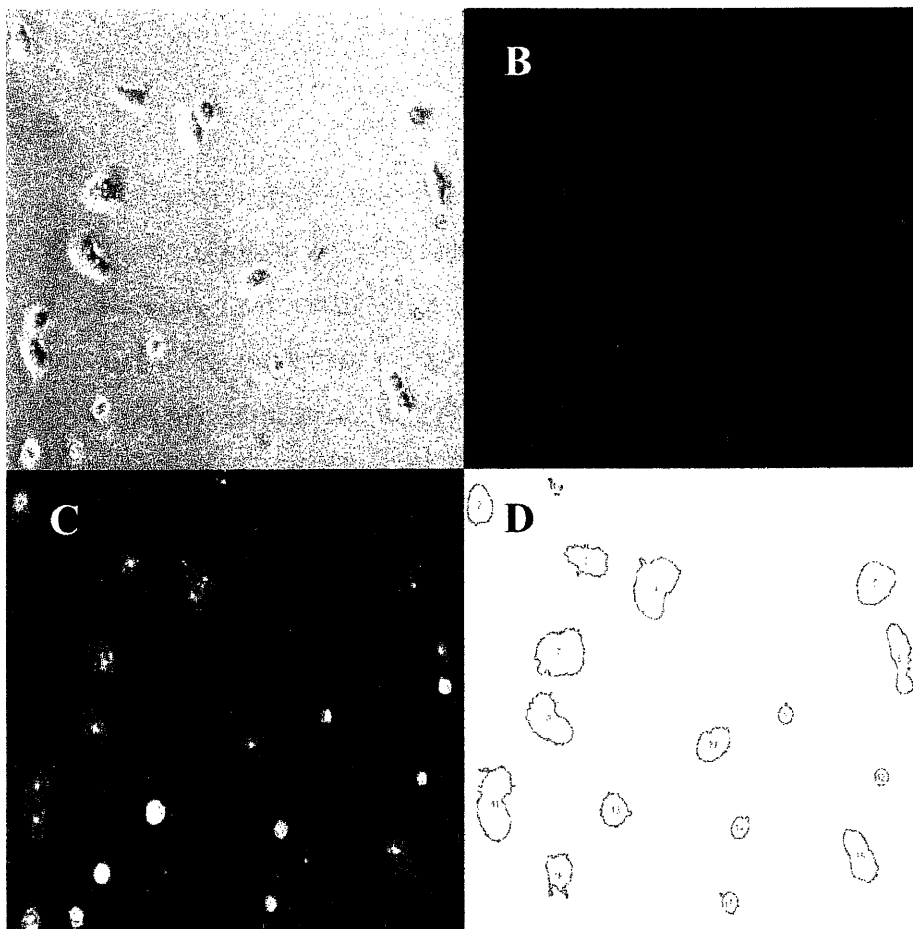
Figure 15. Examples of Image Analysis. A) Bright field. B) Nuclear stain. C) Cell area stain. D) Image analyzed using Scion Image with traced cell area.

3.3 Results and Discussion

3.3.1 Results

To test the adhesivity of SynKRDG versus linear RGD with synergy site, 100% PMPI polymer surfaces were coupled with each peptide and seeded with 20,000 hepatocytes per substrate (see Figure 16). Greater spreading of hepatocytes was observed on SynKRGD than linear RGD peptide with synergy site.
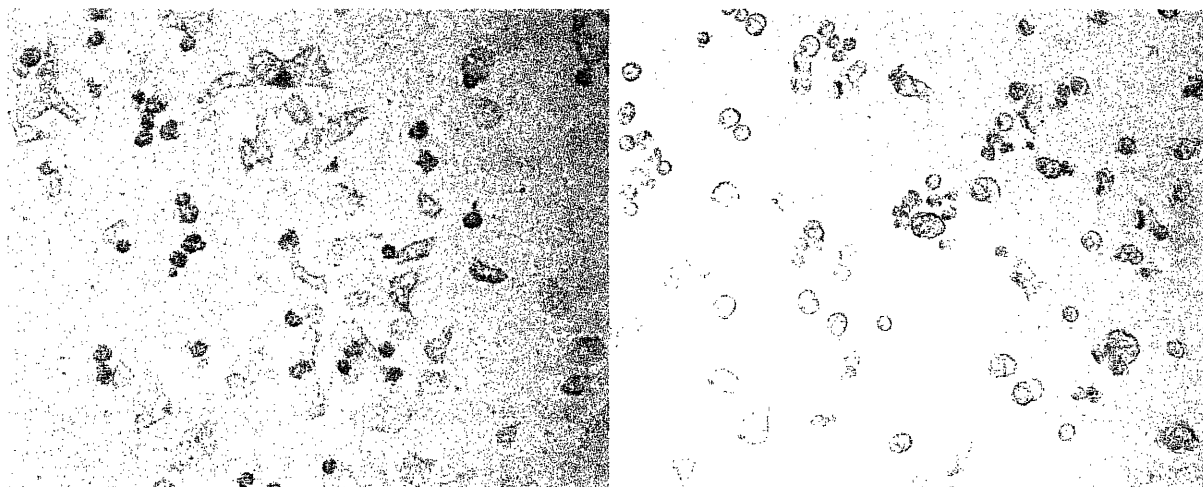
Figure 16. Comparison of Hepatocyte Adhesion on SynKRGD and Linear RGD with Synergy Site Surfaces. Peptides were coupled to 100% PMPI surfaces. Hepatocytes were seeded at 20,000 cells per substrate in HGM. A) Hepatocytes spread on SynKRGD. B) Hepatocytes spread on linear RGD with synergy site.

Experiments conducted compared the adhesion of hepatocytes on two concentrations of SynKRGD and then with and without the presence of 20 ng/mL of EGF. To ensure that the adhesive properties of the SynKRGD surfaces was due to coupled ligand and not nonspecifically adsorbed peptide, surfaces prepared from inactive comb were carried through the coupling procedure. Hepatocytes were seeded on the peptide adsorbed surfaces for 24 hours and no adhesion was observed (see Figure 17).
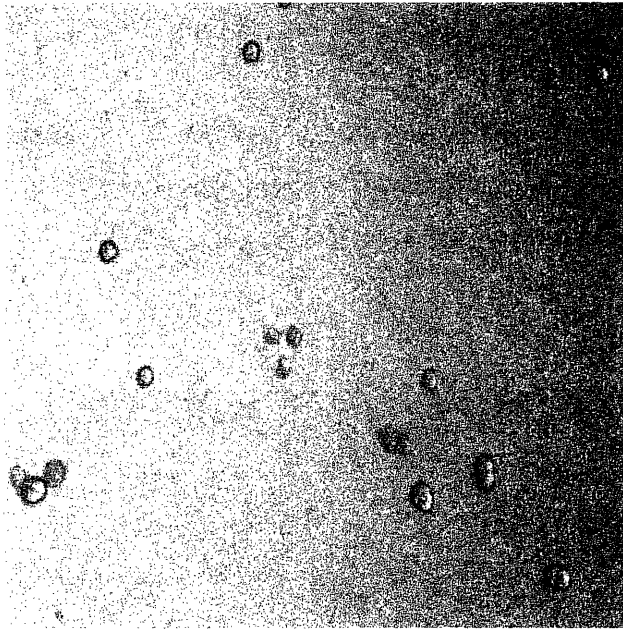
Figure 17. Hepatocytes on Inactive Comb Absorbed with SynKRGD Peptide. Hepatocytes showed no adherence or spreading on SynKRGD surfaces.

The effect of EGF on hepatocyte spreading on 10% and 25% SynKRGD was studied over three experiments. Distinct morphological differences of hepatocytes could be observed as surface ligand concentration decreased and in the presence or absence of EGF. As surface ligand density decreases the spread cell area also decreases. Additionally, cell shape changes from flat and circular to a more amorphous morphology. Cells in the presence of EGF are much less spread than cells in the absence of EGF. There are a higher number of rounded unspread cells attached to surfaces in the presence of EGF. Furthermore, in the presence of EGF many cells also take on a long slender morphology (see Figure 18).
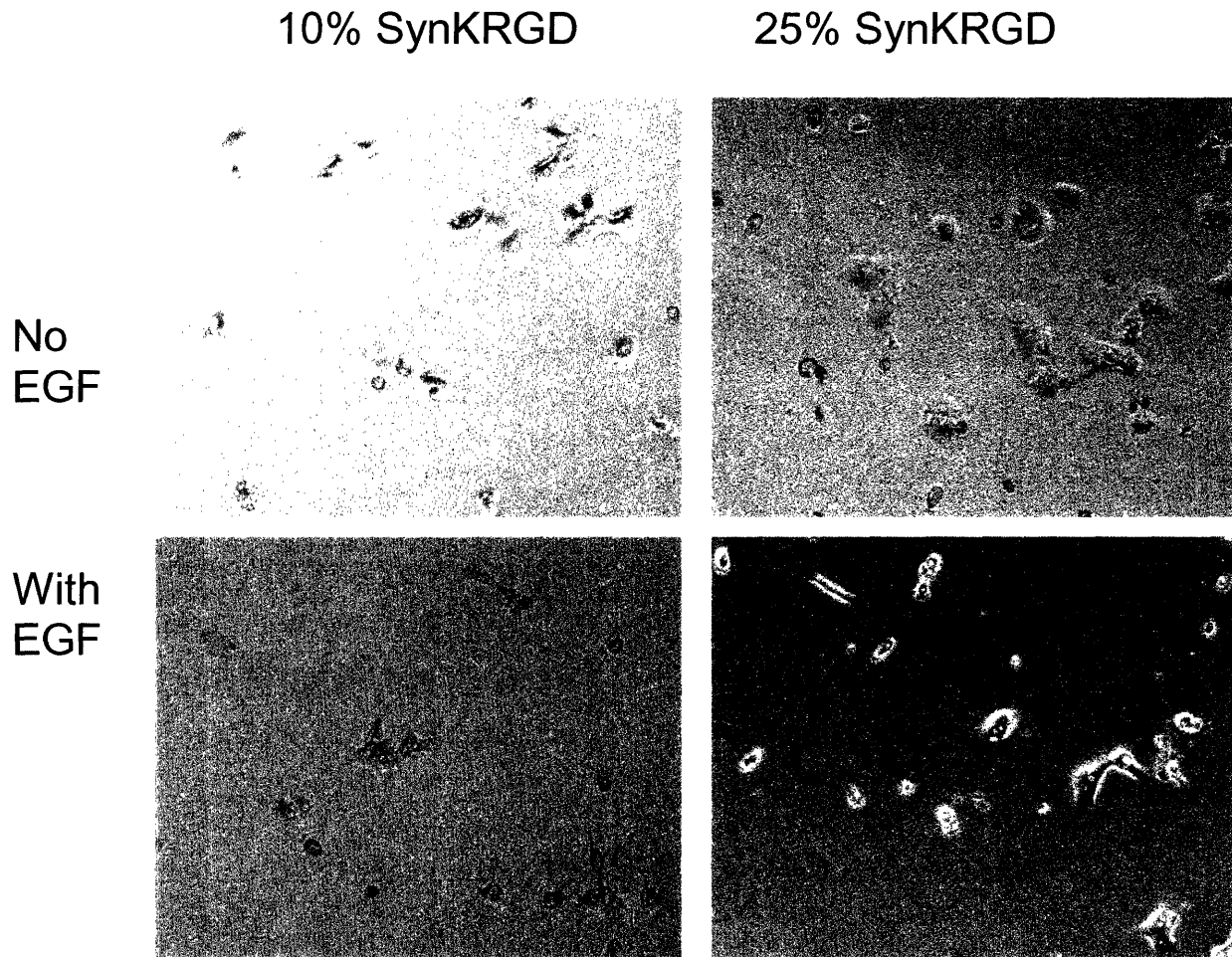
Figure 18. Examples of Cell Spreading Under the Various Experimental Conditions. Cells are most spread on 25% SynKRGD without EGF and least spread on 10% SynKRGD with EGF.

While studying hepatocyte adhesion, occasionally cells of distinctly different morphologies would be observed. These cells were thought to be NPC that had not been purified from the hepatocytes during isolation. The morphology of these unidentified cells is similar to that of hepatic stellate cells, due to their long thin extensions. Stellate cells are the fibroblast like cells of the liver and are known to express $\alpha_5\beta_1$ integrins. Thus, these cells would adhere to the SynKRGD surfaces (see Figure 19).
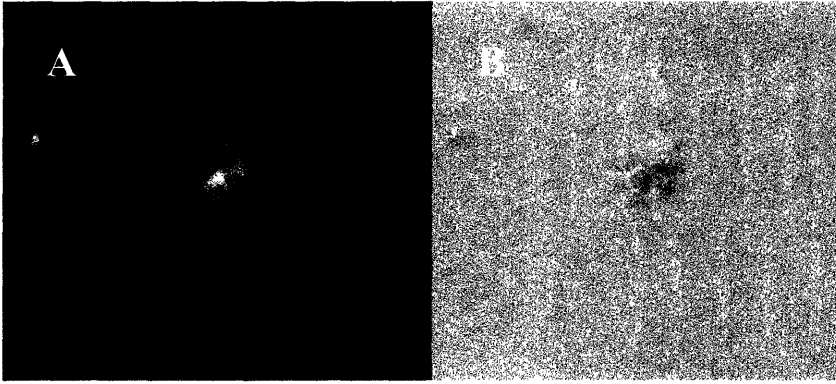
Figure 19. Non-hepatocyte Cells Adhered to SynKRGD Surfaces. These cells are thought to be stellate cells due to their morphology. A) Cell membrane stained with DiI. B) Bright field image of same cell.

Hepatocytes were seeded on 10% and 25% SynKRGD surfaces with and without the presence of EGF in the media. The following three Figures (20, 21, and 22) are the results from each of the three experiments. A similar trend was observed over three experiments. All error bars are the mean standard error, standard deviation divided by the square root of the number of fields taken for each condition.
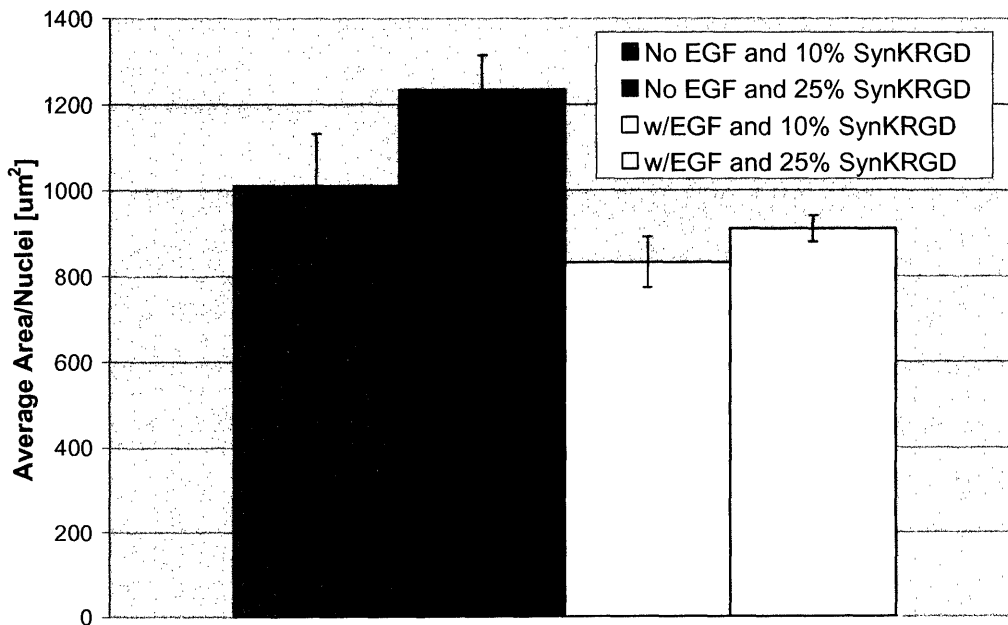
Figure 20. Data from Hepatocyte Adhesion Experiment 1. Hepatocytes were seeded at 15,000 cells per substrate in HGM and incubated for 27 hours before staining.

Hepatocyte Spreading on SynKRGD: Experiment 2
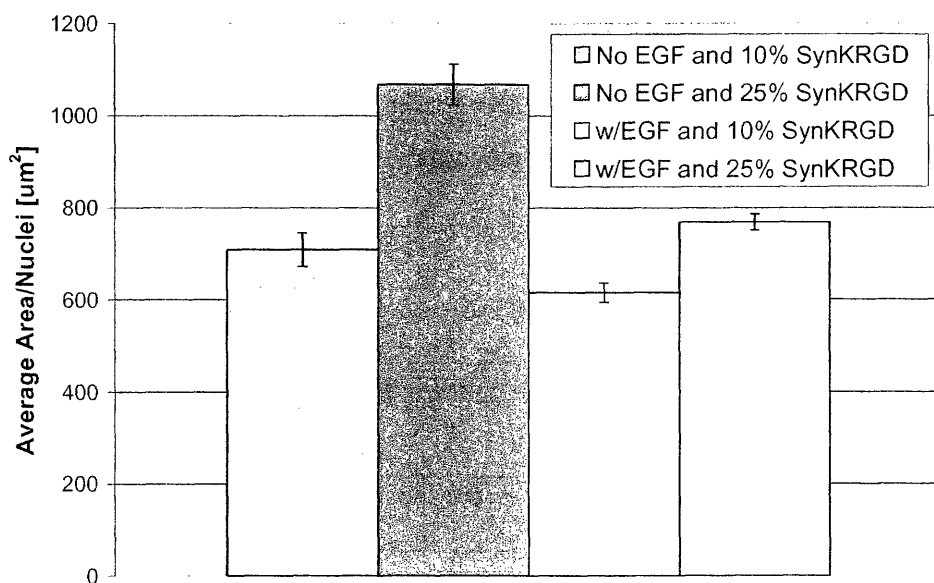
Figure 21. Data from Hepatocyte Adhesion Experiment 2. Hepatocytes were seeded at 15,000 cells per substrate in HGM and incubated for 27 hours before staining.

Hepatocyte Spreading on SynKRGD : Experiment 3
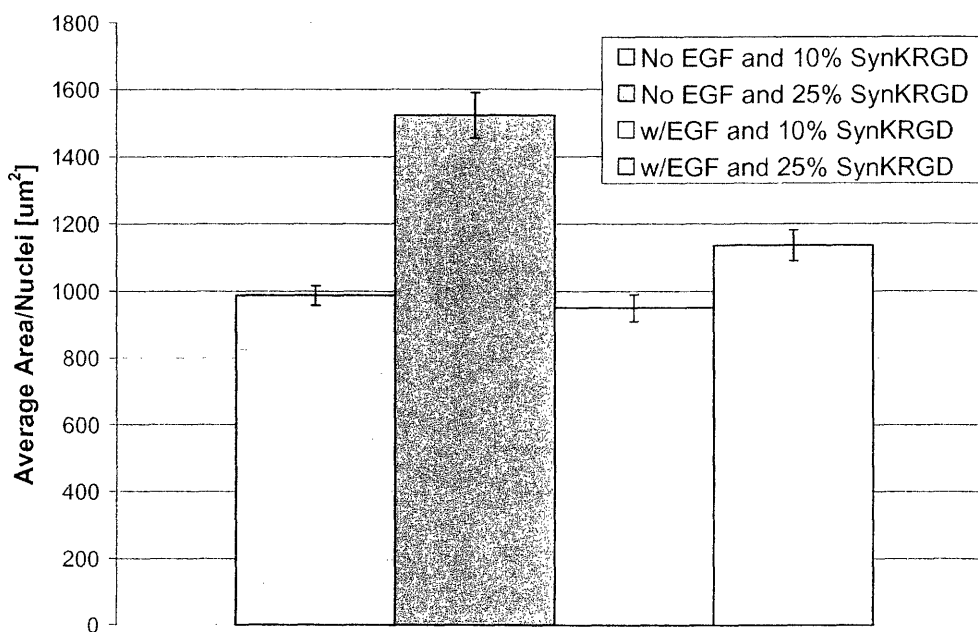
Figure 22. Data from Hepatocyte Adhesion Experiment 3. Hepatocytes were seeded at 15,000 cells per substrate in HGM and incubated for 27 hours before staining.

Greater cell spreading for 25% SynKRGD than for 10% SynKRGD was obtained with and without EGF, as expected. Also, cells seeded on 10% and 25% SynKRGD surfaces in the absence of soluble EGF spread more than cells seeded on similar surfaces in the presence of EGF. These results are consistent with the de-adhesive properties of EGF. Statistical analysis was done on all sets of data to determine p values and statistical significance. Table 1 contains the *p* of two conditions being statistically the same.

Table 1. Probability Values for Each Experiment.

Compared Conditions	Experiment 1	Experiment 2	Experiment 3
No EGF, 10% SynKRGD vs. No EGF, 25% SynKRGD	0	0	0.0426
No EGF, 10% SynKRGD vs. w/EGF, 10% SynKRGD	0.4497	0.0283	0.0543
No EGF, 25% SynKRGD vs. w/EGF, 25% SynKRGD	0	0	0.0004
w/EGF, 10% SynKRGD vs. w/EGF, 25% SynKRGD	0.0036	0	0.2408

Table 1 shows that almost all conditions are statistically different up to 95% confidence. The values that are below 95% confidence can be attributed to experimental variability when compared to the other experiments. In order to reduce the effect of experimental variability the data from all three experiments was compiled and the total data is shown below in Figure 23. The error bars are the mean standard error.

Total Hepatocyte Spreading Data
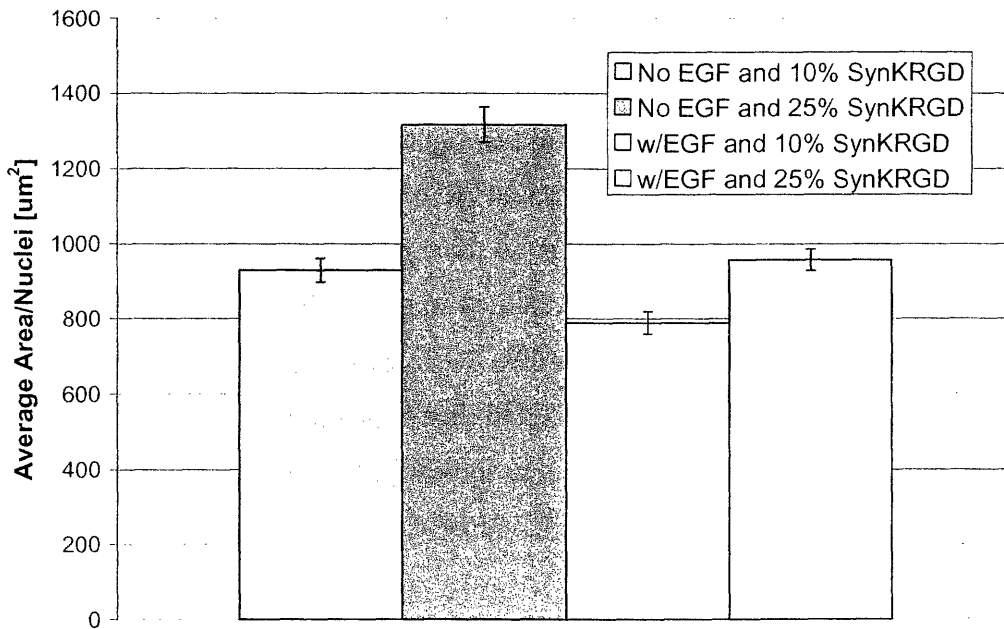
Figure 23. Total Result Over All Three Experiments. Hepatocytes were seeded at 15,000 cells per substrate in HGM and incubated for 27 hours before staining. The combined data shows more distinct trends.

The combined data shows more distinct trends. Statistical analysis was performed on the total data set yielding the p values listed in Table 2.

Table 2. Probability Values Over All Three Experiments.

Compared Conditions	p values
No EGF, 10% SynKRGD vs. No EGF, 25% SynKRGD	0
No EGF, 10% SynKRGD vs. w/EGF, 10% SynKRGD	0.002
No EGF, 25% SynKRGD vs. w/EGF, 25% SynKRGD	0
w/EGF, 10% SynKRGD vs. w/EGF, 25% SynKRGD	0.001

Table 2 shows that all conditions are statistically significant with greater than 95% confidence.

3.3.2 Discussion

Hepatocytes were found to adhere specifically to comb surfaces presenting the SynKRGD peptide, which has a high affinity for $\alpha_5\beta_1$ integrin. The experiments performed indicated that EGF signaling down regulates adhesion through the $\alpha_5\beta_1$ integrin of hepatocytes. Distinct morphological changes were observed as substrate ligand concentration was decreased and EGF was added to the system. The data obtained suggests that there is a greater effect of de-adhesion through EGF signaling on more adhesive surfaces. In all three experiments the difference between 25% SynKRGD surfaces, with and without EGF was greater than the difference between 10% SynKRGD surfaces, with and with out EGF. Thus, as the surface becomes less adhesive, cells adjust their sensitivity to the effect of EGF on adhesion. These results provide insight into the direct interactions between $\alpha_5\beta_1$ integrins and EGF signaling. There appears to be lower $\alpha_5\beta_1$ integrin sensitivity to EGF signaling as a function of substrate properties.

Chapter 4. Conclusions and Future Work

<u>4.1 Conclusions and Future Work</u>

Several methods of applying comb polymer to the study of liver have been attempted during this project. Comb polymer has the potential to be a powerful sorting tool that could be used to characterize cells from the liver as well as separate cells for further culture. Additionally, comb polymer has been used to study the effects of EGF signaling on hepatocyte adhesion through a specific integrin.

4.1.1 Liver Cell Sorting

Sorting cells using comb polymer would require further study and further review of the literature. The ligands tested during this project did not yield any viable ligands for a cell sorting process. All ligands tested exhibited little to no cell adhesion. Ligands that did exhibit some cell adhesion were not specific for the desired cell type. When testing ligands for NPC sorting results were variable from isolation to isolation. This variability was attributed to two major sources. First the NPC fraction is sensitive to the flow rate used to perfuse the liver. Recently, when lab members require NPC, lower flow rate perfusions are conducted. Second, counting of NPC is difficult due to high levels of cell debris from the isolation. Much of the debri is the size of cells increasing error during cell counting. The current method for counting cells is trypan blue exclusion. A better more accurate method should be explored. While the ligands tested here were not viable candidates for an adhesion based sorting process there are still other possible ligands in the literature. A possible candidate for endothelial cell screening is the peptide sequence LALERKDHSG, which is specific to $\alpha_6\beta_1$ integrins (Calzada, Sipes et al. 2003)

4.1.2 Effects of EGF Signaling on Hepatocyte Adhesion

The application of comb polymer to study the effect of EGF on $\alpha_5\beta_1$ adhesion in hepatocytes yielded interesting results. It appears that as the substrate becomes less adhesive cells down regulate de-adhesive signaling from EGF. The peptide used to study $\alpha_5\beta_1$ integrin adhesion was a novel branched peptide consisting of RGD and the synergy site PHSRN. Further studies are currently being conducted on hepatocyte adhesion to only RGD peptide with and without the presence of EGF for comparison to the results reported here. Additionally, studies should also be conducted on the effect of $\alpha_5\beta_1$ integrin adhesion in the presence of surface tethered EGF.

Cells would be unable to endocytose surface tethered EGF which is known to prolong EGF signaling and have different effects on cell function. The PMPI surfaces used in these experiments are currently being tested for the potential to co-couple adhesion peptides and EGF. Another interesting study would be to co-couple $\alpha_9\beta_1$ integrin specific peptides with $\alpha_5\beta_1$ integrin specific peptides. $\alpha_9\beta_1$ integrins are known to be expressed by hepatocytes and are also known to induce de-adhesion and cell migration. It was shown during sorting ligand studies that hepatocytes responded to $\alpha_9\beta_1$ peptide sequences. However, that peptide sequence alone may not have been enough for cells to adhere properly for migration. Combining $\alpha_9\beta_1$ and $\alpha_5\beta_1$ specific peptide sequences on a single surface could encourage hepatocyte migration. Migration is an important cell process for wound healing and the progression of cancers. Very little is known about hepatocyte migration.

Appendices

Appendix 1. Perfusion Protocol
Appendix 2. Hepatocyte Growth Medium (HGM) Preparation
Appendix 3. Comb Polymer Synthesis
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Appendix 1

Perfusion Protocol

Procedure:
1. Male Fischer rats between 175-210 grams given an IP injection of Pentobarbital. We inject according to weight and inject and equal volume of Pento with 1x PBS.
2. The rat is taped onto a surface. The incision area is shaved and washed with Ethanol.
3. An "I" incision is made, the internal organs are pushed aside and the Portal Vein is identified. The tissue is teased away from the Inferior Vena Cava and a loose suture is tied just above the branch to the kidney.
4. A catheter is put just below the suture and connected to the pump. We make sure there is a fluid-fluid connection before the pump is started. The suture is then tied off. A calcium-free buffer is first perfused (sodium chlorides, potassium chloride, Hepes, sodium hydroxide, water)
5. Immediately after the flow begins (25 ml/min) the portal vein is cut as well as the IVC below the catheter to decrease back-flow. The diaphragm is cut and the SVC is tied off.
6. The liver does turn a caramel color is about 2-3 seconds, but the buffer is perfused for 6 minutes with the idea that it takes that long at a Calcium free environment to permanently sever the desmosomes.
7. The second solution which carries the collagenase (in our case, Blendzyme), is perfused for about 11 minutes or until 250 ml has been pumped through at 25 ml/min. This solution is 222 ml of the calcium free buffer, 28 ml of a calcium buffer (water and calcium chloride...10x), and whatever volume of enzyme for the desired concentration. I don't know why we perfuse all 250 ml, it's just what's been done in the past. The liver never looks that broken up until then, either.
8. the liver is cut out and places in a centrifuge tube of DAPS (D-MEM, BSA, Penn/Strep) media that has been on ice. The rest of the isolation is performed on ice or at 4 C.

ISOLATION:
1. The liver and media are poured into a Petri dish and the capsule is pulled away. The liver is gently swirled to shake out the cells.
2. The liver is placed on a 100 um filter and the media is pipetted over the liver and into a centrifuge tube. The liver is places back into the Petri dish and washed in more DAPS before being filtered again. These two tubes of cells are equilibrated together and spun at 50G for 3 min. 100 ul are taken before the spin for a live/dead count.
3. The NPC fraction is drawn off the pellet and it is resuspended in more DAPS. There are two to three spins before the hepatocytes are counted for the final viability.

Appendix 2

Hepatocyte Growth Medium (HGM) Preparation
Original protocol last modified May 2003
Reference: Block et al., J Cell Biol. (1996) 132(6):1133-1149

Base Medium:
DMEM, low glucose, pyridoxine HCL, sodium pyruvate, no glutamine, no phenol red;
      Gibco catalog #11054-020 (500mL)- stored at 4°C.

Add to Base Medium:

1) 0.015g L-Proline   0.03g/L in medium   Sigma Catalog #P-4655

2) 0.05g L-Ornithine   0.1g/L in medium   Sigma Catalog #O-6503

3) 0.153g Nictonamide   0.305g/L in medium   Sigma Catalog #N-0636

4) 0.5g D-(+)-Glucose   2.0g/L in medium   Sigma Catalog #G-7021
                   (base medium already contains1g/L)

5) 1.0g D-(+)-Galactose   2.0g/L in medium   Sigma Catalog #G-5388

6) 1.0g Bovine Serum Albumin   2.0g/L in medium   Sigma Catalog #A-9647

7) 5uL of each of the following trace metal solutions:
      a) 5.44 mg/mL $ZnCl_2$ in MilliQ H_2O
      b) 7.5 mg/mL $ZnSO_4 \cdot 7\ H_2O$ in MilliQ H_2O
      c) 2.0 mg/mL $CuSO_4 \cdot 5\ H_2O$ in MilliQ H_2O
      d) 2.5 mg/mL $MnSO_4$ in MilliQ H_2O

STERILE FILTER MEDIUM AFTER STEP 7

8) 5mL Penicillin/Streptomycin (sterile)   Sigma Catalog #P-0781
      (Stored at -20°C)

9) 2.5mL L-Glutamine (sterile)   5.0mM in medium   Gibco Catalog #25030-081

10) 500uL Insulin-Transferrin-Sodium Selenite (sterile)
                        5mg/L-5mg/L-5ug/L in medium
      Roche Catalog #1074-547 (50mg); #1213-849 (250mg);
      (dissolve 50mg or 250mg powder in 5mL or 25mL sterile MilliQ H_2O, store at -20°C)

11) 400uL dexamethason (sterile)   0.1uM in medium   Sigma Catalog #D-8893
      (dissolve 1mg EtOH using sterile syringe and needle, after powder is dissolved add 19mL
      PBS, mix thoroughly. Stored at -20°C. Expires 3 months from date of reconstitution)

Add to Medium Immediately Prior to First Use:

12) 200uL Epidermal Growth Factor (EGF) (sterile)   20ng/mL in medium
      Collaborative Catalog: #40001
      (Dissolve 100ug powder in 2mL sterile MilliQ water, dispense into 205uL aliquots, store
      at -20°C, expires 3 months from date of reconstitution)

Appendix 3

Comb Polymer Synthesis
By Dan Pregibon
8/25/03

Materials:
    Chemicals:
        Toluene
        Methyl Methacrylate
        Poly(Ethylene Glycol) Methacrylate Monomers
        AIBN
        Hydroquinone
        Hexane (or Petroleum Ether)
        Methanol
        Tetrahydrafuran
    Chemware:
        500ml round-bottom flask
        Rubber septum
        500ml graduated cylinder
        Pipette-man and glass pipettes
        Football-shaped stir bar
        Cork flask stand
        Long metal syringe needle
        Small disposable syringe needle
        Hotplate w/ oil bath
        Large re-crystallization dish
        Large Stir Bar
        50ml glass syringe w/ large metal needle
        300ml beaker

Procedure:
    Solution Preparation
1. Place small football-shaped stir bar in flask
2. Measure 300ml Toluene in graduated cylinder and add 200ml to flask
3. Add methyl methacrylate and poly(ethylene glycol) methacrylate monomers using glass pipettes (should be 30g total of monomer)
4. Add AIBN
5. Use remaining 100ml toluene to rinse flask opening, eventually pouring all toluene into the flask
6. Seal flask with rubber septum and place on stir plate using cork flask stand
7. Stir

Degassing
1. Insert small disposable syringe needle in rubber septum
2. Turn Ag gas on and adjust until flow from hose is just more than detectable on skin
3. Connect long metal syringe needle to hose
4. While the solution is stirring, insert metal needle in septum and all the way into the solution – a steady flow of bubbles should be rising
5. After ~20 Degassing, remove disposable needle and then metal needle from septum. Turn off Ag flow

Reaction
1. Set oil bath temperature to 68-70°C
2. Suspend flask in Oil Bath so oil level is above solution level in the flask
3. Label reaction and allow to proceed for ~10-18 hours depending on tendency for polymer to crosslink (10-12 hours is sufficient for 2-component 526 synthesis)
4. After reaction, remove flask from bath, dry, and place on stir plate using cork stand
5. Remove septum. Stir. Add hyrdroquinone and allow to dissolve
6. Remove stir bar from flask
7. Before purifying, assure that there is indeed polymer in the solution. Drip a few drops of the reaction solution into a small amount of pure hexane – if polymer is present, a precipitate will be seen

Rotovap
1. Rotovap cooling pump should be given ½ - 1 hour to cool water before using the rotovap (setpoint should be ~5°C)
2. Add dry ice/acetone mixture (preferred) or liquid nitrogen to cold trap cooling container, and submerge cold trap into the liquid. Cover top of container with tin foil if desired
3. Set rotovap water bath to ~60-70°C.
4. Connect flask to rotovap using plastic clasp. Little or no vacuum grease is needed (and can act as possible contaminant in polymer product)
5. Adjust the speed control so the flask spins rapidly (near full speed is typical)
6. Open relief valve at top of rotovap condenser (glass knob)
7. Turn on vacuum
8. Close relief valve and allow a few second for pressure to drop in the rotovap chamber (the solution may bubble)
9. Once the solution has stopped bubbling, slowly submerge the rotating flask into the water bath, being careful not to boil the solution too rapidly.
10. The toluene solvent should drip at a steady rate into the waste flask. Adjust the submersion depth and/or bath temperature to achieve this.
11. Rotovap ~1/3 of the total solution off (for optimal precipitation). Slight or no change should be noticeable in the viscosity of the solution. If the solution is too thick, the precipitation will not be as effective

12. Turn off the vacuum and immediately REMOVE THE COLD TRAP FROM THE COOLING CONTAINER
13. Open the relief valve and turn the rotating speed to zero
14. Remove the flask from the rotvap and place in hood
15. Discard waste toluene in the appropriate waste container

Precipitation
1. Pour ~1.5L hexane or petroleum ether into the large re-crystallization dish with a large (preferably football shaped) stir bar at the bottom.
2. Stir the precipitation solvent and add methanol (~35ml) to bring the methanol to ~2.5% solution. Solution should be hazy at first and turn clear within a few seconds.
3. Pour reaction solution from round-bottom flask into 300ml beaker
4. Using the 50ml syringe, draw up a full syringe of reaction solution and eject it rapidly into the precipitation solvent uniformly across the dish. Turbulent flow is desirable
5. Continue until all reaction solution has been spent. If necessary, rinse (or soak in large dish) syringe and needle in tetrahydrofuran to avoid clogging and immobilization of plunger
6. Allow the precipitated polymer to sit in precipitation solvent (still stirring) for at least a few minutes
7. Decant solvent into appropriate waste container

Re-dissolving in THF
1. Use THF from syringe soaking to rinse the round-bottom flask of residual solution and add rinse to the beaker (or use fresh THF)
2. Scrape polymer from bottom of re-crystallization dish, chop or rip into smaller pieces, and add it to the THF in the beaker (~150ml total of THF/polymer solution is desirable)
3. Stir until polymer is dissolved (user stir bar if desired)

Repeat precipitation
1. Re-precipitate as before in 2.5% Methanol in hexane (or pet. ether) as before
2. Re-dissolve in 150ml THF as before
3. Re-precipitate in pure hexane (or pet. ether) as before
4. Decant hexane

Drying Polymer
1. Chop polymer, and allow polymer to dry overnight in hood
2. Place polymer in vacuum oven for at least a few hours
3. When polymer is sufficiently dry (and doesn't smell like solvent) grind polymer in coffee grinder and collect in small jar
4. Tighten the lid on the jar and freeze it in the –20°C freezer
5. Remove the lid, and cover the jar with a Kimwipe, fastening it using a rubber band
6. Lyopholize overnight

Appendix 4

Comb Polymer Analysis Protocols
By Dan Pregibon
8/25/03

Water Solubility Testing

Add a few flakes of polymer to a small vial of Milli-Q water. Vortex, sonicate, and allow to sit overnight. Solubility is assessed visually.

Molecular Weight Analysis

Use Gel Permeation Chromotography (GPC) to determine the molecular weight and polydispersity index (PDI) of the polymer. Use a 0.2 micron-filtered solution of 5mg of polymer in 1ml GPC-grade THF. See GPC instructions for further detail.

Composition Analysis

Use NMR spectroscopy to determine the molar (and weight) composition of the polymer, as well as the purity. Use a 0.2 micron-filtered solution of 20mg polymer in d-Chloroform. NMR performed by Will Kuhlman.

Appendix 5

Coverslip Silanization
By Dan Pregibon
8/25/03

Materials:
    Chemicals:
        100% Ethanol
        Metacryloxypropyl-trimethoxysilane (MPTS)
        95% Ethanol in Milli-Q water (pH 4.7 – 5.2)
    Chemware
        250ml Erlenmeyer Flask
        Medium size re-crystallization dish
        Orbital Shaker
        Pipette-man with glass pipette

Procedure:
    Cleaning Coverslips

1. Pour ~100ml pure ethanol into Erlenmeyer flask. Add desired amount of coverslips
2. Sonicate coverslips in ethanol for ~20 minutes

    Preparation of silanizing solution

3. Make up a 4% solution of MPTS in 95% Ethanol/5% H_2O in the re-crystallization dish. Usually, 50 ml of solution is sufficient (2ml TPMS, 48ml Ethanol/Water)
4. Stir gently and allow to sit for ~5 minutes

    Silanization

5. Decant most of the ethanol from the coverslips, and pour coverslips (and remaining ethanol) into the silanizing solution
6. Cover the dish with tin foil and place on the orbital shaker for ~20 min stirring at 150 rpm

    Rinsing

7. After silanization, decant the solution from the slips and rinse 5 times with pure ethanol, decanting after each rinse

    Drying Slips

8. Coverslips can be dried between layers of Kimwipes overnight, or air-dried individually
9. To air dry, separate the slips on a bed of Kimwipes, hold each one with a pair of tweezers and dry both sides using a moderate air stream
10. After drying overnight, or by air, place the slips in a glass petri dish, and place in the vacuum oven until use

Appendix 6

NPC Activation

By Dan Pregibon
8/25/03

Materials:

    Chemicals

        Comb Polymer

        4-nitrophenly chloroformate (NPC)

        Triethylamine (TEA)

        Anydrous tetrahydrofuran

        Hexane (or petroleum ether)

        Ice

    Chemware

        Round-bottom flask (variable size depending on amt.)

        Rubber septum

        Football-shaped stir bar

        50ml syringe

        5ml syringe and long needle

        Plastic centrifuge tubes

        Vacuum flask with filter and filter paper

        Medium re-crystallization dish

        Large re-crystallization dish

        Beaker (variable size depending on amount activating)

Procedure:

    Solution preparation

1. Calculate the amount of NPC and TEA to be used reaction (use 2 molar equivalents NPC to polymer active OH group, and 2 molar equivalent TEA to NPC)
2. Add polymer, NPC, and stir bar to flask. Place in vacuum oven with top open for a few hours
3. Fill the re-crystallization dish with ice and place on stir plate
4. Remove flask and immediately seal with septum
5. Burry flask up to the neck with ice in the re-crystallization dish and secure with a clasp
6. Using the 50ml syringe, add anhydrous THF to the flask so the polymer is a 10% solution (ex: 50ml THF for 5g polymer). Be sure to use anhydrous techniques when working with the THF (i.e. use the syringe to inject Ag into the THF container before taking THF out)
7. Degass with Ag as described in "Polymer Synthesis" as the solution cools on ice

Reaction

1. Using the 5ml syringe, add calculated amount of TEA drop-wise to the reaction solution
2. Once all of the TEA has been added, allow the reaction to proceed on ice for 4-6 hours

Purification

1. Pour the reaction solution into the centrifuge tubes and centrifuge at ~3000rpm for 7-8 minutes.
2. Set up the vacuum filtering system and filter the non-settled centrifuge tube contents
3. Pour the filtered solution from the vacuum flask into a beaker
4. Pour 1-1.5L hexane or petroleum ether into the large re-crystallization dish, with large stir bar
5. Precipitate using 50ml syringe as described in "Polymer Synthesis"
6. Decant hexane and re-dissolve polymer in THF (anhydrous not necessary)
7. Centrifuge, filter, and re-precipitate as before (a total of 3 precipitations is usually necessary)
8. Dry polymer as described in "Polymer Synthesis"

Appendix 7

Coverslip Coating

By Dan Pregibon
8/25/03

Materials:
 Chemicals
  Comb Polymer
  Methyl ethyl ketone
 Chemware
  Two 4ml vials
  Pipette-man and glass pipette
  5ml syringe and 2 micron filter
  100ml or 200ml pipette w/ tips
  Tweezers

Procedure:
 Glove-box preparation
  1. Turn on air stream for glove-box and allow time for humidity to drop below 30% (if possible). This may take a couple hours.

 Solution Preparation
  1. Prepare 20mg/ml solutions of comb polymer in methyl ethyl ketone (in 4ml vial).
  2. Sonicate the solutions for ~10 minutes, or until polymer is completely dissolved
  3. Filter the solution, using the syringe and 0.2 micron filter, into a clean 4ml vial

 Spin-Coating
  1. Turn the glove-box vacuum on
  2. Turn the spin-coater on a set it to recipe 8.
  3. Place all materials (pipette, tips, coverslips, polymer solution, tweezers, hands, etc) in glove-box
  4. Use tweezers to place coverslip on spin head
  5. Step on green pedal and hold
  6. Use the pipette to add desired amount of polymer solution onto center of slip (16μl is sufficient for 12mm slips, 10μl is sufficient for 10mm)
  7. Release pedal

Appendix 8

Cell-Resistance Testing

By Dan Pregibon
8/25/03

Materials:
    Chemicals
        Phosphate Buffered Saline (PBS)
        Complete NR6 Media
    Chemware
        24-well culture plate
        Sealing rings (cut silicon tubing)

Procedure:

Coverslip Preparation
1. Spincoat 12mm coverslips with 20mg/ml polymer in MEK as described in "Coverslip Coating," placing the slips in the wells of the culture plate
2. Fix the slips to the bottom of the plate with silicon sealing rings
3. Place in plate in vacuum oven for at least 4 hours
4. Place the plate (with lid off) in the hood under UV for 5 minutes

Cell seeding
1. In ½ ml media, seed ~25,000 cells per culture plate well
2. Place plate in incubator overnight
3. Aspirate media
4. Rinse each well with ½ ml PBS
5. Add ½ ml media to each well
6. View under microscope

Appendix 9

PMPI Activation
by Will Kuhlman

Material:
  Comb Freeze dried from benzene
  p-Maleimidophenylisocyanate (PMPI) (VWR# 80053-760)
  DMSO Anhydrous (VWR# EM-MX1457-6)
  Flask and stir bar dried in oven overnight

Procedure:

PMPI Activation:
1. Remove flask and stir bar from oven, cap with septum and cool with dry nitrogen. (Alternately, you can use the same flask used for freeze-drying.)
2. Add a measured amount of comb and re-attach stopper. Add enough DMSO to make a ~2% solution via syringe. Stir with low heat until comb dissolves. Protect from light with aluminum foil.
3. Once dissolved, add at least 1.5 molar equivalents of a ~1% PMPI (214.2 g/mol) solution in DMSO via syringe, drop-wise with rapid stirring. The reaction is complete in about two hours, as indicated by a change in color from light yellow to nearly orange. Yeild: ~50% conversion of OH groups.

To purify at this stage:
Precipitate in diethyl ether (10ml ether per ml DMSO) and centrifuge (3.4k RPM for 5 min) to isolate product. Wash product with diethyl ether to remove residual DMSO and dry in hood.

Characterization by GPC:
GPC will show a UV signal at 258 nm that is not present in the original material. Conversion can be quantified from the GPC using a known value of dndc (0.077) and the Beer's law coefficient for PMPI. (~0.686 g/cc estimated using CMSE UV/Vis).

Characterization by NMR
Dissolve 30 mg/ml in DMSO-d6. Look for: Broad singlet (NH), δ~9 Broad doublets, δ ~7.5, 7.2 (2H ea, phenyl), singlet δ ~7.1 (maleimide, 2H) and a broad triplet (2H, CH2OCON) somewhere around δ = 5. Unreacted PMPI shows up as narrow peaks in the δ = 7-6.5 range.

Appendix 10

Spreading Experiment Protocol

Materials:
>   Vybrant DiI
>   Hoechst
>   24 well tissue culture plate
>   forceps

Procedure:
Seeding
1. Remove substrates from vacuum oven and transfer coverslips to 24 well plates.
2. Place coverslips under UV light for 15 minutes to sterilize.
3. Seed hepatocytes at 15,000 cells per well in 500 µL HGM with or without EGF. (Make sure when seeding to drip cell suspension on top of coverslip first)
4. Surfaces with hepatocytes were then incubated for 27 hours.

Staining:
1. 30 minutes before cells are ready, prepare a 24 well plate with one well of plasma membrane stain Vybrant DiI (5 µL/mL, 500 µL total), one well of Hoechst (1µl/mL, 500 µL total), and two wells of HGM (500 µL each) per coverslip. Make sure to maintain the media conditions with or without EGF. Both Vybrant DiI and Hoechst are light sensitive. Keep plate covered with aluminum foil.
2. Transfer coverslips to the wells of Vybrant DiI and place in incubator for 45 minutes.
3. Transfer coverslips to the well of Hoechst and place in incubator for 15 minutes.
4. Transfer coverslips to the first well of HGM and place in incubator for 5 mins.
5. Transfer coverslips to the second well of HGM and take to microscope for imaging.

Appendix 11

Selection Ligand Screening Protocol

Materials:
    24 well plate
    Silicone sealing rings
    Forceps

Procedure:
1. Coverslips are removed from vacuum oven and transferred into a 24 well tissue culture plate.
2. Each coverslip is sealed down in the well with a silicone ring. These silicone rings are cut from silicone tubing.
3. The plate is placed under UV light for 15 minutes for sterilization.
4. The plates are seeded with 15,000 cells per well in 150 μL of media(either hepatocytes or NPC depending on the ligand tested)
5. The plates are left to incubate overnight.
6. Plates are taken for microscopy the next morning.

Appendix 12

Peptide Synthesis

Materials:

      Resin
      Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)
      N-hydroxybenzotriazole (HOBt)
      9-fluorenylmethyloxycarbonyl (FMOC) amino acids
      N,N-dimethylformamide (DMF)
      N-Ethyldiisopropylamine (DIPEA)
      Acetic anhydride
      Dichloromethane (DCM)
      20% Piperinidine in DMF
      10% DIPEA in DMF
      2,4,6-Trinitrobenzenesulfonic acid (TNBS)
      Round bottom flask with frit
      Vacuum line with solvent trap
      Pasture pipettes with bulbs
      PCR Eppendorfs
      Small spatula

Procedure:

All steps should be done in fume hood.

1. Measure out desired amount of resin into a round bottom flask with frit.
2. Measure out 4 molar equivalents of each amino acid desired in to 4mL vials.
3. Measure out 4 molar equivalents of PyBOP and HOBt for each amino acid and add to each 4mL vial.
4. Make sure amino acids are in order to be coupled from carboxyl terminus to amine terminus.

Deprotecting amines
5. Add 20% Piperidine in DMF to the resin and shake on low rpm for 5 mins.
6. Suck off piperidine and repeat the 5 min piperidine rinse 2 more times.
7. Suck off excess solvent through the frit.
8. Rinse at low rpm with DMF and suck dry through the frit.
9. Repeat DMF rinse 4 more times.

Coupling
10. Dissolve the next amino acid to be coupled in DMF.
11. Add 8 molar equivalents of DIPEA to the resin then add the dissolved amino acid to the resin.
12. Shake the flask at low rpm, just enough to see freely moving resin, for 45 min.
13. Suck off excess solvent through the frit.
14. Rinse at low rpm with DMF and suck dry through the frit.

15. Repeat DMF rinse two more times.

Testing for free amines
16. Using a small spatula take a small amount of resin and place in a PCR Eppendorf tube.
17. Add 2 drops of 10% DIPEA in DMF and 2 drops of TNBS to the resin in the Eppendorf.
18. Shake and examine for red color on resin beads.
19. If red go back to step 10 and recouple the amino acid.
20. If clear continue to next step.

Capping unreacted amines
21. Mix 500 μL acetic anhydride and 700 μL DIPEA in a 4ml vial.
22. Add a small volume of DMF and DCM to make mixture components miscible.
23. Add mixture to the resin and shake at low rpm for 5 minutes.
24. Suck off excess solvent through the frit.
25. Rinse at low rpm with DMF and suck dry through the frit.
26. Repeat DMF rinse two more times.
27. Go to step 5 and repeat coupling with next amino acid.

Appendix 13

Peptide Cleavage

Materials:
    Trifluoroacetic acid (TFA)
    Triisopropylsilane (TIS)
    Ethanedithiol (EDT)
    MilliQ water
    Ice cold ether
    50 mL centrifuge tubes
    Pasture pipette

Procedure:
1. Once the peptide is complete rinse 3 times with DMF.
2. Rinse 3 times with cold ether to dry resin.
3. Dry Resin under high vacuum for 30 minutes.
4. Add TFA: TIS:H_2O: EDT (92.5:2.5:2.5:2.5) and shake for 90 minutes.
5. Filter solution in to a 50 ml centrifuge tube.
6. Blow nitrogen slowly through a pasteur pipette on to the surface of the solution. Leave blowing until most of the volume is evaporated
7. Add ice cold ether to precipitate cleaved peptide.
8. Spin down precipitate and carefully aspirate the supernatant
9. Resuspend pellet in cold ether and spin down.
10. Repeat aspiration and resuspention at least twice.
11. Place the 50 mL tube with cleaved peptide pellet on the lyophilizer overnight.

Appendix 14

NPC Coupling

Materials:
- Sodium bicarbonate
- Ethanolamine
- MilliQ water
- 24 well tissue culture plate lids
- Forceps
- Tupperware box
- Parafilm

Procedure:

Volumes are for 12mm coverslips

1. Make fresh coupling and blocking solutions. Coupling solution: 0.1M sodium bicarbonate. Blocking solution 1:1, 0.5M sodium bicarbonate: 0.1M ethanolamine.
2. Make 1mg/mL solutions of peptide to conjugate in coupling solution (total volume should be enough to cover surface of all coverslips. 150μL per 12mm coverslip).
3. Take spincoated coverslips out of vacuum oven.
4. Cover the inside of a 12 well tissue culture plate lid with a parafilm layer.
5. Carefully place cover slips face up on the parafilm covered lid.
6. Add 150μL peptide solution to each cover slip.
7. Place an additional tissue culture plate lid on top of the lid containing the coupling coverslips.
8. Place all coupling coverslip containers inside a Tupperware box.
9. Place wet paper towels inside the box with the coupling coverslips to keep box humidified, and make sure box is tightly closed.
10. Leave coverslips with peptide solutions for 4 hours.
11. Remove coverslip containers from humidified box and carefully aspirate off the peptide solution.
12. Add 150μL of peptide free coupling solution to each coverslip and aspirate off.
13. Repeat rinse two more times.
14. Add 150μL of blocking solution to each coverslip.
15. Once again cover coverslip containers and place back inside sealed humidified Tupperware box.
16. Leave coverslips with blocking solution overnight.
17. Remove coverslips from humidified box.
18. Aspirate off blocking solution.
19. Add 150μL of MilliQ water to each cover slip.
20. Aspirate off water.
21. Repeat rinse 2 more times.
22. Place coverslips in vacuum oven until ready to use.

Appendix 15

PMPI Coupling

Materials:
> 7.4 pH phosphate buffer
> Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)
> MilliQ water
> 24 well tissue culture plate lids
> Forceps
> Tupperware box
> Parafilm

Procedure:
> Values for 10mm coverslips
> 1. Make 125 µM solution of desired peptide in pH 7.4 phosphate buffer. Solution should be 10% TCEP (pH 7.5) by volume. Make enough of peptide solution for 40 µL per coverslip.
> 2. Prepare 24 well tissue culture plate lid(s) by covering the inside with parafilm
> 3. Take spincoated coverslips out of vacuum oven.
> 4. Place a 40 µL drop in each circle of the prepared lid per coverslip to be coupled.
> 5. Turn coverslips over face down on each drop of peptide solution.
> 6. Place an additional tissue culture plate lid on top of the lid containing the coupling coverslips.
> 7. Place all coupling coverslip containers inside a Tupperware box.
> 8. Place wet paper towels inside the box with the coupling coverslips to keep box humidified; make sure box is tightly closed.
> 9. Leave coverslips with peptide solutions for 4 hours.
> 10. Prepare a fresh 24 well tissue culture plate lid with layer of parafilm.
> 11. Remove coverslip containers from humidified box and place coverslips face up on new prepared plate lid.
> 12. Add 150 µL phosphate buffer to each coverslip.
> 13. Aspirate off water.
> 14. Repeat rinse two more times.
> 15. Place coverslips in vacuum oven until ready to use.

Appendix 16

Image Analysis

Image analysis is conducted using Scion Image software (version Beta 4.0.2) obtained from www.scioncorp.com.

1. Open image to be analyzed in scion.
2. Click "Analyze" menu and then choose "Set Scale".
3. Set the scale to .745 pixels per micron (scale for 10X objective).
4. Click the "Edit" menu and then choose "Invert".
5. After inverting image, click the "Options" menu and choose "Density Slice"
6. Open the bright field image for density slice comparison
7. Slide density bars on left of screen till whole cell area becomes red.
8. Click the "Analyze" menu and choose "Analyze Particles"
9. Once the "Analyze Particles" window is open choose 200 to be the minimum particle size, make sure all the options are checked, and click "ok".
10. Click the "Analyze" menu and choose "Show Results"
11. Hit "control-c" to copy the results.
12. Go to an Excel file and hit "control-v" to paste the results into Excel.
13. There should be two rows of numbers. Sum up the left column of numbers to obtain total spread cell area in the image field.
14. Open the nuclear field image and count nuclei.
15. Divide the total area per field by the total nuclei per field to obtain average spread area per nuclei.

Appendix 17

Percoll Endothelial Cell Purification Protocol
By Albert Hwa

Materials:
    Percoll
    50ml centrifuge tubes

Procedure:
1. Take NPC fraction (supernatant from first two hepatocyte spins), spin at 100g 5min
2. Take supernatant from spin, spin down again at 350g for 10min
3. Resuspend pellet in PBS.
4. Slowly and carefully layer 15ml of 50% percoll below 15ml of 25% percoll in two 50ml conical tubes.
5. Carefully pipette a layer of resuspended NPC on top of 25% percoll.
6. Spin at 900g for 20 min
7. Collect cell layer at 25/50% percoll interface.
8. Double the liquid volume by adding more PBS. Spin at 900g for 10 min
9. Resuspend pellet to obtain liver endothelium-enriched cell solution.

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