A Novel Bioengineering Platform Using Functionalized Self-Assembling Peptides to Enhance CYP3A2 Activity in Modified Rat Hepatocyte Sandwich Cultures

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A Novel Bioengineering Platform using Functionalized Self-Assembling Peptides to Enhance CYP3A2 Activity in Modified Rat Hepatocyte Sandwich Cultures

by

Jonathan Wu

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ABSTRACT

Isolated hepatocytes removed from their microenvironment soon lose their hepatospecific functions when cultured. Highly oxygen-demanding hepatocytes are commonly maintained under oxygen-deficient culture conditions, limited by culture medium thickness as well scaffold thickness. Thus, the cells are forced into anaerobic metabolic states that degenerate liver specific functions. Furthermore, cells separated from their extracellular matrix and disconnected from the synergistic interactions between other hepatic cells types further exacerbate hepatocellular function. This study aims to improve hepatospecific activity, especially CYP3A2 – a biomarker that is notoriously known to quickly lose expression in primary cultures, by creating a platform based on collagen sandwich cultures. The modified sandwich cultures are substituted with self-assembling peptide, RAD16-I, combined with integrin-binding sequence RGD or laminin receptor binding sequence YIGSR functional peptide motifs to create a cell-instructive peptide scaffold. To facilitate oxygen and nutrient diffusion and exchange, plasma modification technology is employed to control peptide layer dimension. We have successfully shown that plasma engineering can be used to optimize peptide thickness. Likewise, we have shown that the incorporation of the functional motifs enhanced hepatospecific activity. CYP3A2 expression from cultures on our platform improved over 256 times the levels found in collagen sandwich cultures, the current standard for hepatocyte cultures. This study demonstrates the capability of sandwich cultures with modified instructive self-assembling peptides and the importance of thinner cultures scaffolds to promote better oxygen and nutrient exchange. We believe that our novel bioengineered platform has the potential to greatly improve existing hepatocyte culture methods and be invaluable to future *in vitro* hepatocyte studies as well as toxicity tests.

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1.0 INTRODUCTION

1.1 Liver

1.1.1 Significance

The liver is an important and complex organ that is located in the upper right quadrant of the abdomen. The liver plays a vital role in metabolism and is responsible for many important functions of the body including glycogen storage, plasma protein production, drug detoxification, and xenobiotics metabolization. Due to the importance of this organ in many of the body's daily processes, liver malfunction often leads to death. Despite its ability to regenerate from mild trauma or toxic shock there are many diseases that continue to manifest the liver. According to the Center for Disease Control and Prevention, the 10^{th} highest cause of death in the United States was chronic liver diseases and cirrhosis. Therefore, studying how the liver works will better our understanding of this complex organ to create more effective treatments which could save countless lives.

1.1.2 Hepatocytes

The liver is composed of several main cell types each with its unique and important function – hepatocytes, sinusoidal endothelial cells, Kupffer cells, and stellate cells (Figure 1). Hepatocytes are the main functional cells in the liver. These cells are involved in protein storage, synthesis of protein, cholesterol, phospholipids, and bile salts, transformation of carbohydrates, detoxification, modification and excretion of exogenous and endogenous material, and formation and secretion of bile. Most of the activity of the liver can be attributed to hepatocytes. They make up 60-80% of the cytoplasmic mass of the liver (Michalopoulos et al., 1997; Kimiec et al., 2001). Loss of hepatocyte function can result in acute or chronic liver disease and, as a result, substantially compromise the rest of the organ and the body. Thus, hepatocytes are an important key to the understanding of the complex liver.

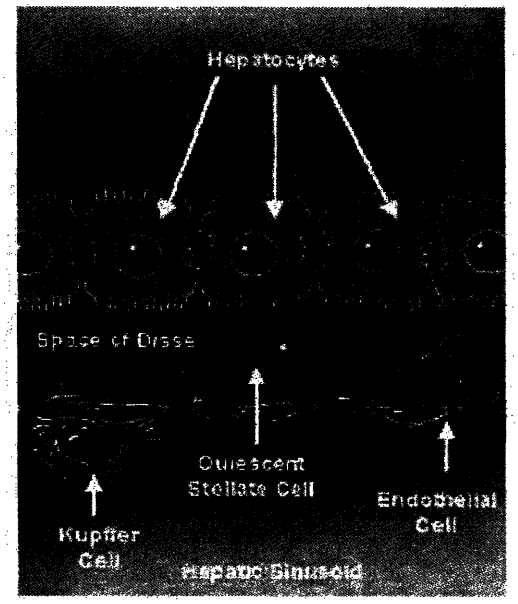


Figure 1: Cell types of the liver and their physical relationship to one another. Endothelial, Kupffer, and stellate cells contain many cell-cell contacts between the various cell types that are important for liver function.

1.2 *In vitro* Hepatocyte Cultures

Many previous strategies have been implemented to maintain these hepatocyte functions in *in vitro* cultures, including the use of extracellular matrices such as the current standard, Collagen (Ze'ev et al., 1988; Dunn et al., 1989; Dunn et al., 1991; Berthiaume et al., 1996), Matrigel (Silva et al., 1998), or liver derived basement membrane matrix (Zeisberg et al., 2006). However, the liver carries out and regulates numerous biochemical reactions that require the combined effort of specialized cells and tissues. As a result, isolated hepatocytes removed from their microenvironment, comprised of the extracellular matrix and the synergistic interactions between the other hepatic cells types, soon lose their hepatospecific functions. Therefore, it is important for *in vitro* cultures to provide a system that closely simulates the local environment of an intact liver.

1.3 Self-Assembling Peptide Scaffolds

More recently, the use of self-assembling peptides has been implemented and verified to be an excellent scaffold for cell cultures (Genove et al., 2005; Semino et al., 2004 and 2003; Kisiday et al., 2002). Not only does it provide an excellent three dimensional microenvironment but also it allows for the design and preparation of a tailor-made scaffold (Figure 2). This represents a

novel approach to tissue engineering which traditionally has relied on materials that were unknown in composition, like Matrigel, or not possible to design and alter, such as Collagens. Furthermore, the versatility of the modification of this material allows for the introduction of functionalized peptide motifs, such as integrin and laminin receptors (Genove et al., 2005). We have employed in our system the use of these self-assembling peptides to create a functionalized instructive peptide scaffold.

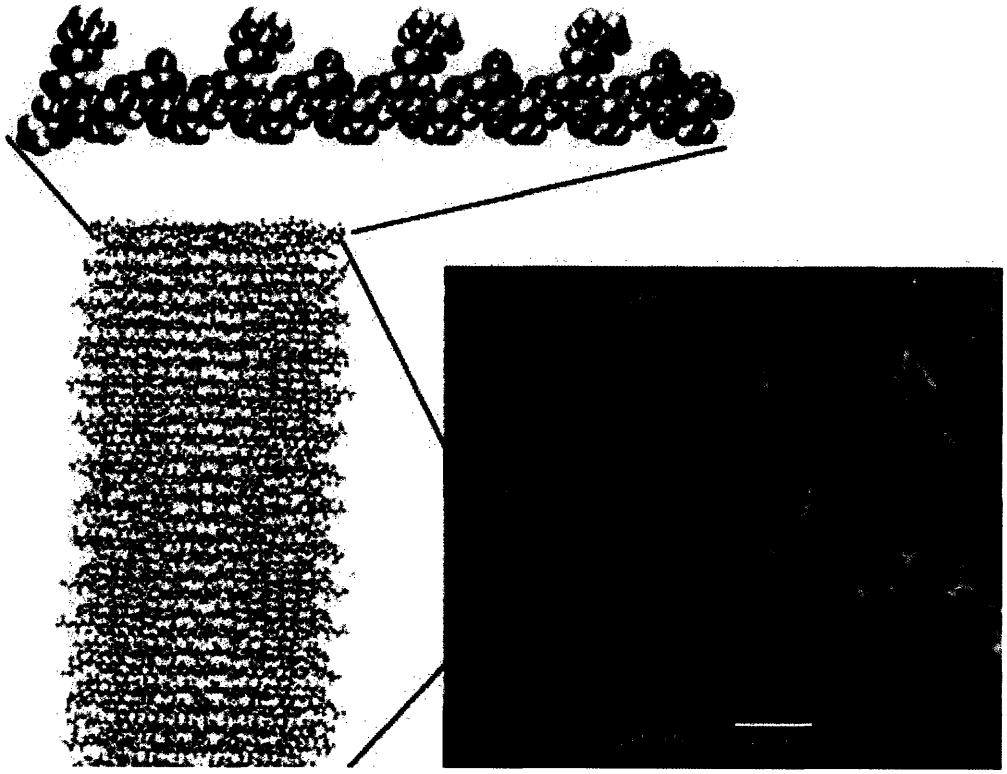


Figure 2: A single self-assembling peptide (~6 nm) of RAD16-I. Thousands of peptides self-assemble to form a single nanofiber. These hydrogel scaffolds contain approximately 99% water and 1% peptide material. (Zhang et al., 2007).

1.4 Sandwich Culture Method

Hepatocyte morphology is known to be closely linked to the functional output of the cells (Semler and Moghe, 2001; Singhvi et al., 1994a). Standard cell cultures that seed cells on top of a monolayer of extracellular matrix have been used in the past to successfully passage these hepatocytes; however, in certain instances hepatocellular functions become compromised because the cell no longer resembles a natural hepatocyte from a live liver. In many cases, specific cellular phenotypes are directly related to the cellular functions including cell survival,

proliferation, differentiation, motility, and gene expression (Huang et al., 1998; Singhvi et al., 1994b). Morphogenesis and assembly have been well established to be pertinent in the functional performance of liver-derived cells *in vitro* (Hansen et al., 1998; Singhvi et al., 1994a; Torok et al., 2001; Yuasa et al., 1993).

The double-gel "sandwich" method has been shown to improve morphology by embedding the cells between two layers to resemble *in vivo* conditions (Figure 3). Typically, one layer is set on the bottom of a culture dish and an additional layer is placed on top of the hepatocyte monolayer (Dunn et al., 1989; Ryan et al., 1993; Knop et al., 1995). Under these conditions, hepatocytes have been shown to maintain some function and differentiation for up to several weeks. Verification of hepatocyte function was shown by specific mRNA expression (Chen et al., 1998; Dunn et al., 1992) and protein secretion into culture media (Pahernik et al, 1996; Ryan et al., 1993).

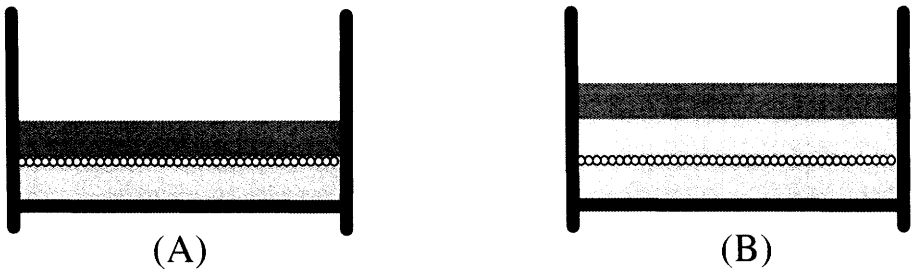


Figure 3: Monolayer culture method versus sandwich culture method in a tissue culture insert. (A) Monolayer cultures involve a layer of peptide (blue), followed by a layer of seeded cells (yellow circles), submerged under media (pink). (B) A typical sandwich culture method uses an additional layer of peptide over the seeded cells.

1.5 Sandwich Culture using Self-Assembling Peptide Scaffolds

A recent study by Genove et al. (2007) builds a hepatocyte culture system based on collagen sandwich cultures. The collagen component of the culture is substituted with the self-assembling peptide, RAD16-I, combined with modified self-assembling peptide scaffolds containing the integrin-binding sequence RGD or the laminin receptor binding sequence YIGSR (YIG) in order to create a microenvironment that resembles the extracellular matrix *in vivo*. Results showed that upon juxtaposition of the peptide system against the collagen system (gold standard), they both acquired similar morphology, levels of protein secretion and gene expression. Interestingly, the YIG modified peptide system did enhance certain hepatic gene markers. Likewise, in the

same study, Genove repeated the experiment for peptide layers of 1 mm and 500 um thick and found that the thinner layer fostered much better hepatic *in vivo* resemblance. Thus, this study demonstrated the potential for sandwich cultures with modified self-assembling peptides and the importance of thinner culture scaffolds to promote better oxygen and gas exchange.

1.6 Oxygen Diffusion and Gas Exchange Problems

In primary hepatocyte cultures oxygen supply is a crucial issue since it is known that the highly oxygen-demanding hepatocytes are commonly maintained in Petri dishes under oxygen-deficient culture conditions and, thus, the cells are forced into anaerobic metabolic states (Wallach et al., 1976). Generally, in these culture methods oxygen consumption is no longer dependent upon hepatocellular uptake rates but is limited by culture medium thickness as well as ambient oxygen concentrations. However, regardless of these constraints hepatocytes are able to tolerate the hypoxic conditions by satisfying energy requirements through anaerobic glycolysis.

A previous study has shown that hepatospecific functions are oxygen-dependent, especially demonstrated in the poor production of albumin, urea, and drug metabolites over a 14 day study period in common Petri dish models compared to enhanced oxygen delivery cultures on gas-permeable films (Bader et al., 1999). Furthermore, it was shown as early as 1968 that commonly used medium depths of 2-5 mm in Petri dishes rapidly produced hypoxic conditions when hepatocytes respired at their physiological rate (McLimans et al, 1968). Therefore, because plastic walls and culture medium are efficient barriers of oxygen diffusion, it is important create a system in which a physiological oxygen supply is maintained (Bader et al., 1996; Catapano et al., 1996). By building upon the culture system used by Genove et al (2006), we hope to minimize the oxygen and gas exchange barrier by controlling the layer of the sandwich peptide scaffold and reducing the medium depth.

1.7 Modified Sandwich Culture

In this work, we attempt to address the concerns of current traditional hepatocyte culture methods by combining current tissue engineering technologies. Our sandwich culture method is adjusted from the traditional double gel layer "sandwich" technique to address the oxygen and gas diffusion issue. Instead of culturing the hepatocytes under a thick second layer of peptide,

the cells are instead entrapped under a biocompatible porous membrane (PEEK-WC-PU or PTFE) that is modified through plasma processes to allow dimensional control of a thin peptide coating layer. The controlled layer and porous membrane provide much less of a barrier to oxygen diffusion (Figure 4). The dimensionally controlled self-assembling peptide layer, which analogs the basement membrane *in vivo*, contains signaling peptide sequences to promote specific activity, which analogs the cell-cell interactions that are lost when the hepatocytes are isolated. By combining the most modern technologies and methods, we hope to use a synergistic approach to improve upon the existing methods for hepatocyte culture. Therefore, our ultimate goal is to modify the use of self-assembling peptides to create thin fiber layer dimensions that will not only be biochemically favorable for the growth and development of hepatocytes, but also be biophysically conducive for oxygen diffusion and gas exchange to better achieve ideal hepatocyte culture conditions.

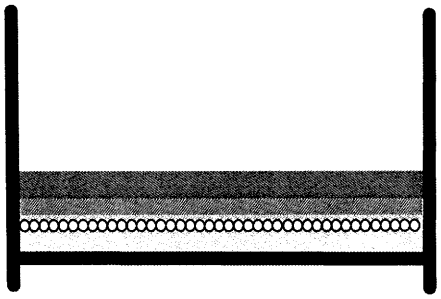


Figure 4: Modified sandwich culture provides a better oxygen and gas exchange. This method involves a layer of peptide (blue), followed by a layer of seeded cells (yellow circles), a thin network of peptide attached to a porous membrane (grey), submerged under media (pink).

2.0 BACKGROUND

A major challenge exists in designing materials suitable for supporting liver-derived cell cultures. To attain organ-equivalent levels of tissue function from cultures, the growth substrates must be both biochemically sensitive and biophysically hepatospecific to achieve a highly differentiated cell phenotype. Ideally, an *in vitro* culture should be able to closely simulate every aspect of a live environment from the simpler details of tuning culture temperature and humidity conditions to more complex details of providing cell-instructive surroundings and facilitating oxygen and gas diffusion. Thus, we attempt to create a platform in which the substrate's materials and properties are carefully selected to optimize both biochemical and biophysical attributes. The following supports our material choice, modification methods, and verification methods.

2.1 Membranes

The development of a novel polymeric biomaterial designed to promote specific cellular responses requires the sophistication of a biocompatible substrate that is physically, chemically, and biologically stable and not only capable of maintaining a culture environment for hepatocytes but also able to directly stimulate hepatospecific functions.

2.1.1 PEEK-WC

One such platform material that has already been shown to be successful in the culture of hepatocytes is the modified PEEK-WC polymeric mix (De Bartolo et al., 2004a). Previous studies have shown that modified PEEK-WC or poly(oxa-1,4-phenylene-oxo-1,4-phenylene-oxa-1,4-phenylene-3,3-(isobenzofurane-1,3-dihydro-1-oxo)-diyl-1,4-phenylene) membranes are capable of sustaining liver-specific functions in isolated hepatocytes (De Bartolo et al., 2004b). Different from native PEEK, PEEK-WC is soluble in various solvents due to the lack of crystallinity from the addition of the isobenzofurane-1,3-dihydro-1-oxo- group. It is a chemically stable polymer with great thermal and mechanical resistance. The amorphous character and solubility in solvents allows for membrane preparation with various properties

through phase inversion, an inexpensive and flexible method (Kimmerle et al., 1990), creating an asymmetric structure with undesired areas of low mechanical resistance.

2.1.2 PEEK-WC-PU

Recent studies have found that the structural and mechanical properties of PEEK-WC porous membranes can better be improved with the addition of aliphatic polyurethane, PU (De Bartolo et al., 2005). The addition of polyurethane gives the PEEK-WC membrane a more symmetric and homogeneous porous structure (Figure 5). Likewise, the PU addition reinforces the low mechanical resistance that causes the asymmetric PEEK-WC membranes to develop cracks that can propagate. Consequently, the PEEK-WC-PU polymeric mix shares the advantageous properties of its constituents to create a biocompatible, thermal and mechanical resistant, and elastic membrane. As a result, PEEK-WC-PU membranes have achieved long-term maintenance of human liver differentiation (De Bartolo et al., 2005).

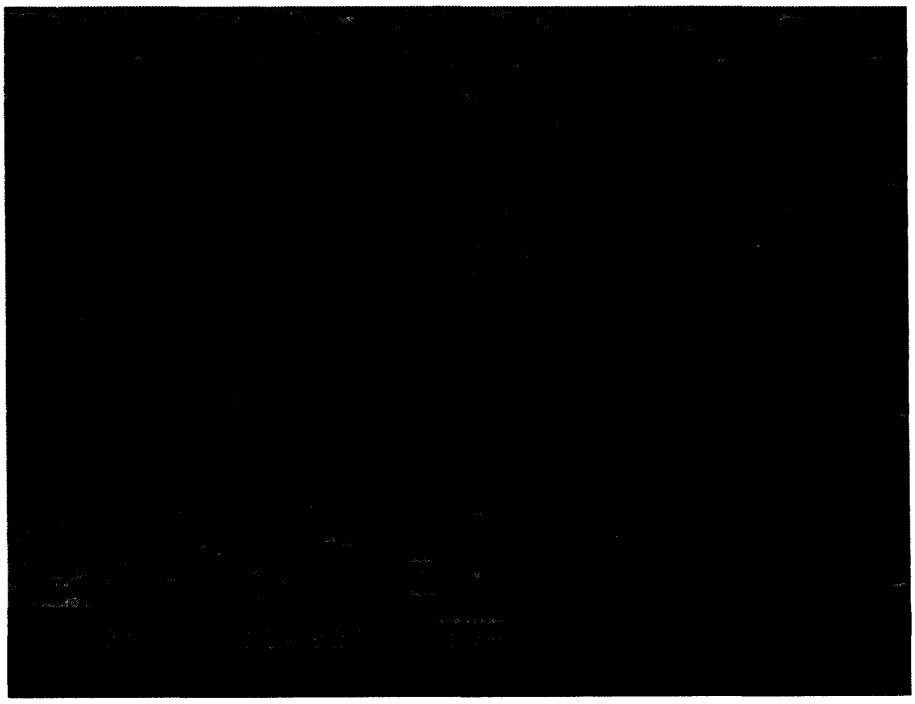


Figure 5: SEM image of the PEEK-WC-PU native membrane at x10,000 magnification.

2.1.3 PTFE

Another promising membrane material used in our studies was polytetrafluorethylene (PTFE) (Figure 6). PTFE has been widely applied as a biomaterial in many areas due to its excellent bulk properties, including physical and chemical inertness, low frictional coefficient, high stability against heat and chemical reagents, and high electrical resistance (Kang et al., 2000; Huanga et al., 2001; Godino et al., 1996; Diez et al., 1999).

Figure 6: SEM image of the PTFE native membrane at x2,500 magnification.

2.2 Plasma Modification

It has been shown in many studies that cellular attachment to extracellular matrix (ECM) molecules is not only crucial to cell survival and proliferation but also important to the determination of the cell into a differentiated phenotype, which in result affects the respective cellular function. To better create a more favorable environment for the hepatocytes, surface modifications were performed to better simulate the *in vivo* environment of a liver through the use of plasma.

The surface of the membranes were modified by non-equilibrium plasma-chemical processes, Plasma Deposition of thin films (PECVD – Plasma Enhanced Chemical Vapor Deposition) and Plasma Treatments (grafting of functional –COOH and $-NH_2$ groups from the plasma to the substrate). Plasma processes for surface modifications can be used to tune surface properties, including electric charge, wettability, free energy, surface chemistry, and morphology. This ability to optimize surface conditions can affect cellular behavior and attachment either directly, for instance, through guided cell spreading (Ratner et al., 1997), or indirectly, for example, through controlled protein adsorption of the surface (Favia et al., 2002). Membrane samples in this study were modified by plasma deposition of acrylic acid (hereby abbreviated as "PdAA") or by plasma grafting of pentafluorophenyl methacrylate, PFM (hereby abbreviated as "PgPFM").

2.2.1 Biomolecule Immobilization

Of the many uses of plasma, the oldest of uses consists of grafting polar groups onto the hydrophobic polystyrene Petri dishes to better allow the adhesion and growth of cells as compared to the native untreated surface. The more recent and advanced uses involve the immobilization of biomolecules onto biomaterial surfaces to promote specific cellular responses at the molecular and cellular levels (De Bartolo et al., 2005; Lopez et al., 2005). The biomolecules are immobilized through hydrophilic spacer molecules to allow the greatest conformational motion. In our system, the biomolecule sequences used for immobilization were purposefully identical to the peptide sequence of the RAD16-I self-assembling peptide used to coat the surface (Figure 7).

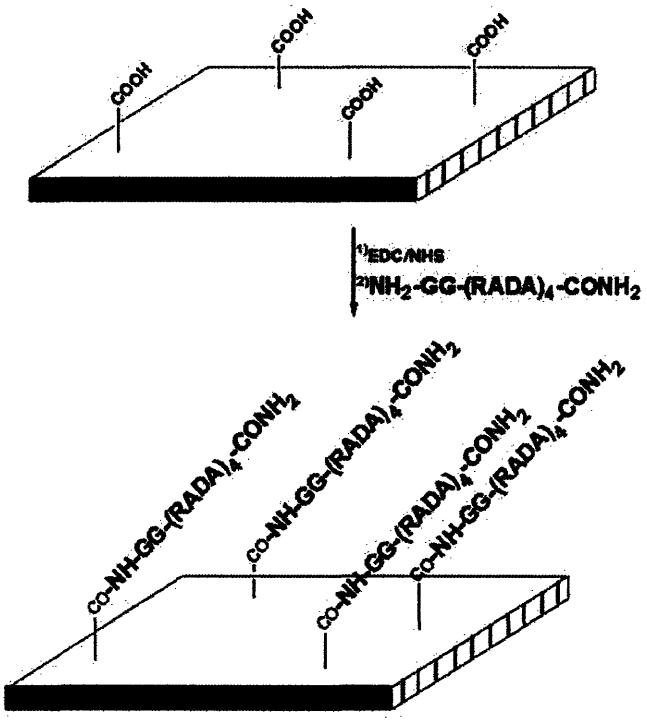


Figure 7: (A) Plasma deposition of acrylic acid onto membrane surfaces. (B) RAD16-I peptide sequences are immobilized to the deposited –COOH.

2.3 Self-Assembling Peptides

Self-assembling peptides, RAD16-I, were implemented in our culture system as a synthetic three-dimensional nanofiber network scaffold to simulate the extracellular matrix. The modification versatility of this material ensures a confidence in variable control that is not achieved in previous methods, such as materials that are not tunable (i.e. Collagens) or are of unknown composition and vary from lot to lot (i.e. Matrigel). These self-assembling Hydrogel scaffolds contain more than 99% water content (Semino et al., 2003; Caplan et al., 2000; Zhang et al., 1993) and gellify when exposed to physiological pH or salt concentrations. Fibers of 4-7 nm thickness develop into networks with average pore sizes of 50-200 nm, providing a matrix with similar dimensions to natural extracellular matrices. Self-assembling peptides have successfully been used to culture a variety of cell types, including endothelial cells, neurons, hepatic stem cells, chrondrocytes, and hepatocytes (Genove et al., 2006; Semino et al., 2004; Semino et al., 2003; Kisiday et al., 2002; Holmes et al., 2000).

2.3.1 Functional Peptide Motifs

The ability to strategically design the self-assembling peptide permits the incorporation of functional peptide motifs into the peptide scaffold (Genove et al., 2005) (Figure 8). The signaling motifs RGD from collagen and YIGSR (YIG) from laminin are incorporated to target integrin receptors and the 67 kDa laminin receptor, respectively (Graf et al., 1987). Functional peptide motifs have been shown to be crucial in the activation of numerous vital cell functions including migration, proliferation, and cell attachment (Nomizu et al., 1997; Tsilibary et al., 1990). In one study, grafted adhesion peptides RGD and YIG proved to promote hepatocyte adhesion to the surface by 60% (Carlisle et al., 2000). Also, RGD-containing synthetic peptides coated on plastics promoted hepatocyte adhesion and differentiated function (Bhadriraju et al., 2000). And recently, YIG motifs incorporated into self-assembling scaffolds has upregulated certain gene expression compared to the gold standard, collagen (Genove et al., 2007).

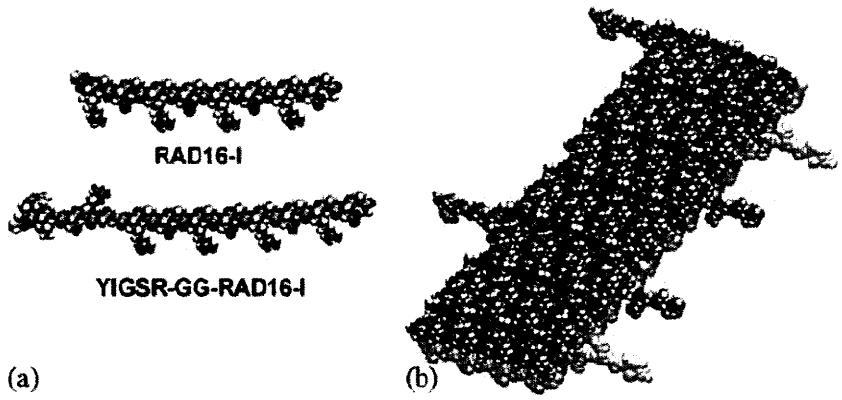


Figure 8: (A) Molecular models represent peptide RAD16-I (top) and peptide YIG (bottom). (B) Molecular model represents a β-sheet tape of a self-assembled peptide mix composed of RAD16-I and YIG at a ratio of 9:1. Known functional peptide motifs from proteins of the basement membrane are incorporated onto the ends of self-assembling peptides (as seen extending from the β-sheet tape) to facilitate specific *in vivo* responses. (Genove et al., 2005)

The functional peptide motifs were added onto the base RAD16-I sequence through solid phase peptide synthesis (Table 1). Two glycines (G) were added in between the self-assembling peptide sequence and the functional motifs to allow better conformational access to cellular receptors.

Sequence Name	Peptide Sequence	Function
RAD16-I	AcN-RADARADARADARADA-$CONH_2$	Base Sequence
RGD	AcN-G**RGD**SPGGRADARADARADARADA-$CONH_2$	Integrin Binding
YIG	AcN-**YIGSR**GGRADARADARADARADA-$CONH_2$	Laminin Binding

Table 1. Self-assembling peptide sequences. Functional peptide motifs, denoted in bold, are inserted onto the N-terminal of the base sequence of RAD16-I. (R=Arginine; A=Alanine; D=Aspartic Acid; G=Glycine; Y=Tyrosine; I=Isoleucine; S=Serine)

2.3.2 Controlling the Layer Dimension

Our strategy to control the peptide layer dimension to within a few nanometers scale is made possible by employing all of the methods above. The biocompatible membranes chosen are first plasma modified and then immobilized with sequences of RAD-I biomolecules through solid state chemistry. With the RAD16-I biomolecules immobilized onto the membrane surfaces, the self-assembling peptides can assemble from the anchored base sequences (Figure 9). Thus, the self-assembling peptides can securely assemble into a microscopic mesh without the need for a thick gellified layer for stability.

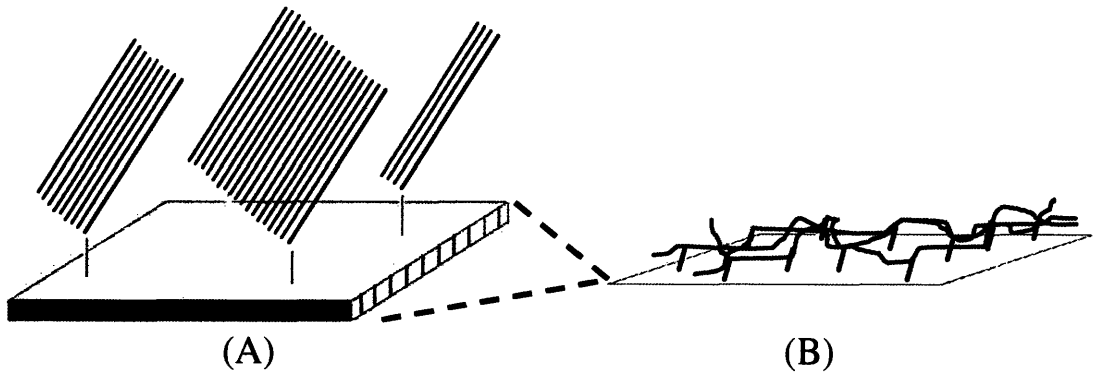


Figure 9: (A) Self-assembling peptide strands (blue) attach and assemble from the immobilized base RAD16-I peptide sequences (dark blue). (B) The self-assembling peptides strands form a mesh of nanofibers across the surface from the anchored peptide sequences.

2.4 Hepatospecific Biomarkers

2.4.1 Albumin

Liver-specific functions can be assessed in primary hepatocyte culture by analyzing hepatospecific biomarkers that should be present and expressed in fresh hepatocytes. One

standard liver-specific protein examined in this study is albumin. Albumin is one of the major plasma proteins found in blood that assists in maintaining blood volume and serves as a transport protein to carry fatty acids, hormones, some drugs, and other molecules throughout the body. Albumin is primarily produced in the liver and, thus, provides an important marker for healthily differentiated hepatocyte cultures.

2.4.2 HNF4-α

An additional biomarker observed was the hepatocyte nuclear factor 4 alpha (HNF4-α). HNF4-α is a protein that plays an essential role in development, organogenesis, and maintenance of organ function (Hayhurst et al., 2001). Again, it offers a vital benchmark for long-term primary hepatocyte cultures.

2.4.3 CYP3A2

The main hepatospecific biomarker of interest in this study is CYP3A2. Cytochromes P450 (CYP) form a gene superfamily that function in the metabolism of a wide array of chemical substances from endogenous substances to xenobiotics including drugs, carcinogens, and pollutants. The CYP3A family has been investigated extensively due to its vital role in pharmacology and toxicology. Specifically, in rats, the CYP3A2 gene is critical to hepatospecific metabolism. It is well known that primary cultured hepatocytes often lose expression of specific liver functions, including P450s, as they adapt to culture conditions over a length of time (Schuetz et al., 1988).

Various attempts have been made to maintain *in vivo* CYP3A2 levels and differentiated function of hepatocytes in culture without having to rely on artificial induction through drugs or hormonal supplementation. Studies have tried cocultures of hepatocytes with other cells such as fibroblasts with the idea that nonparenchymal cell factors may promote and induce specific hepatocyte expression (Morin et al., 1986; Baffet et al., 1982). Others have tried to achieve *in vivo* level induction by focusing on culture substratum using complex matrices including fibronectin (Johansson et al., 1984), extracts from liver (Reid et al., 1980), and Matrigel (Li et al., 1987). Currently, the best culture conditions for preserving constitutive CYP3A2 expression in primary hepatocytes are still unresolved.

Therefore, utilizing the membranes with the controlled self-assembled peptide layer in a sandwich culture system we hope to alleviate or reduce the oxygenation issue in traditional culture methods and at the same time employ modified self-assembled peptides with functional peptide motifs to enhance hepatocyte activity *in vitro*. It is our goal to specifically improve upon the results of current methods and maintain physiological levels of CYP3A2 in primary hepatocyte culture for a longer period of time up to seven days.

3.0 MATERIAL AND METHODS

3.1 Hepatocyte Isolation and Culture Medium

Hepatocytes were isolated from male Fisher rats weighing 150-180 gm using a modification of the Seglen 2-step collagenase perfusion procedure (Powers et al. 2002). Cell yield and viability were determined via Trypan blue exclusion and hemocytometry. Typically, 250-300 million hepatocytes were harvested per rat liver with viability ranging from 85-92%. Following isolation, cells were initially suspended in Hepatocyte Growth Medium (HGM) and were subsequently diluted for culture seeding. HGM was prepared in 50 ml allotments to maintain freshness. 1 ml Bovine Serum Albumin (BSA), and 50 ul aliquots of Epidermal Growth Factor (EGF), Gentomycin, Insulin, Trasferin, Ascorbic Acid, and Hydrocortisone were added to 50 ul Hepatocyte Basal Medium.

3.2 Membranes

PEEK-WC-PU membranes were manufactured by the De Bartolo Lab (Institute on Membrane Technology, National Research Council of Italy). PEEK-WC-PU membranes were prepared from 9% (w/w) PEEK-WC polymer, 5% (w/w) PU and 2% tetrahydrofurane (THF) (w/w) in dimethylformamide (DMF). Solid medical grade Pellethane (Dow Chemical Company, 2363-80AE) was dissolved in DMF at 50°C. Thereafter, PEEK-WC polymer and THF were mixed to PU solution. After casting the polymeric films were coagulated in a bath constituted of 30% DMF and 70% EtOH. Membranes were then washed extensively with water and dried at room temperature. PTFE membranes were purchased (Biopore, BCGM00010).

3.3 Plasma Modification of Membranes

PEEK-WC-PU membranes were modified with a plasma deposition (PE-CVD) process fed with acrylic acid (AA) vapors in the Favia Lab (Department of Chemistry, University of Bari, Italy). Membranes were placed in vacuum conditions (0.02 mbar) in a stainless-steel parallel plate plasma reactor. A schematic layout is shown in Figure 7. Acrylic acid vapors were fed into the chamber at an internal pressure of 0.2 mbar and samples were exposed at 100 W for 5 min (PEEK-WC-PU/PdAA). PdAA-coated substrates were then immersed in 1-ethyl-3-(3-

dimethylamino-propyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in morpholine ethane sulfonate (MES) buffer to activate –COOH groups. After plasma treatment, membranes were incubated in an aqueous solution of 1% RAD16-I at 37°C (PEEK-WC-PU/PdAA/RAD16-I).

PTFE membranes were modified with a graft polymerization of pentafluorophenyl methacrylate (PFM) in a two-step process at the Barcelona Bioengineering Center (Institut Quimic de Sarria, University Ramon Llull). The membranes were placed in a cylindrical Pyrex reactor equipped with a copper coil that generates plasma under vacuum conditions (0.02 mbar). Argon was fed into the chamber increasing the pressure to approximately 0.06 mbar and samples were exposed to argon plasma at 50 W for 5 min. Afterwards, argon inflow was closed and PFM vapor was introduced to the reactor and allowed to polymerize for 1 min (PTFE/PgPFM). After treatment, membranes were soaked overnight in an aqueous solution of 1% RAD16-I at 37°C (PTFE/PgPFM/RAD16-I).

3.4 Scaffold Preparation for Modified Peptide Sandwich

Peptide scaffolds were prepared using the peptide hydrogel RAD16-I (PuraMatrix, BD Biosciences, 354250). In cases where modified peptides (RGD or YIG) were used, the modified peptides were mixed in a 95:5 proportion with the prototypic peptide, RAD16-I. The self-assembling peptide solution becomes a hydrogel through contact with salt-containing buffers or media (Zhang 1993). Therefore, 250 ul of peptide were loaded into the bottom of a Millicell tissue culture insert (Millipore, PICM 03050) and 1.5 ml of HGM were added underneath the insert membrane to induce gelation, forming a 1 mm-thick gel. Following the gelation of the peptide, 400 ul of HGM were added into the insert and the gel was allowed to equilibrate for 30 min in an incubator at 37°C.

3.5 Membrane Sterilization

All samples, including the native PEEK-WC-PU, PEEK-WC-PU/PdAA, PEEK-WC-PU/PdAA/RAD16-I, native PTFE, and PTFE/PgPFM/RAD16-I membranes, were sterilized using a 70% EtOH sterilization. Following the EtOH rinse was an autoclave sterilization using a 20 min, 120°C step followed by a 10 min drying step.

3.6 RAD16-I Peptide Layer Coating of Membrane

PuraMatrix RAD16-I Peptide Hydrogel was used to coat the surface of the membranes. 50 ul of peptide was used to thinly cover the surface of the 0.5 in x 0.5 in square membrane samples. In order to control the thickness of the peptide layer, gelation was not initialized through the introduction of salt-containing buffers or media. The soluble peptide was allowed to incubate for an hour to permit any self-assembling to occur with the immobilized peptide strands. Following the incubation included a rinse step that entailed dipping the coated membranes into deionized H_2O ten times in succession to remove any non-assembled peptide.

3.7 Modified Peptide Sandwich Preparation

The prepared peptide-coated membranes were next incubated with a volume of hepatocyte cell suspension at a final density of 65,000 cells/cm^2 and left to attach in a 37°C incubator for 8 hr. Following the attachment period, the medium was changed to remove dead cells. To complete the modified peptide sandwich, the peptide-coated modified membrane containing the attached cells was inverted on top of the gel layer in the tissue culture insert (Figure 10).

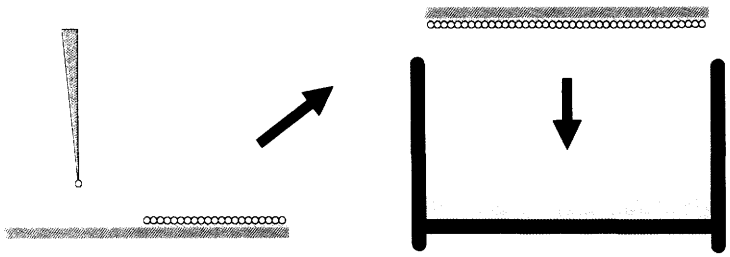


Figure 10: Self-assembling peptide-coated membranes are seeded with hepatocytes, incubated for several hours, and then inverted onto a layer of peptide in a tissue culture insert.

300 ul of HGM was added to the inside of the insert. Cultures were maintained in a water-jacketed incubator at 37°C and 5% CO_2. Media was changed every day so that a fresh reservoir of 1.6 ml surrounded the outside of the insert and 300 ul replaced the inside of the insert (Figure 11).

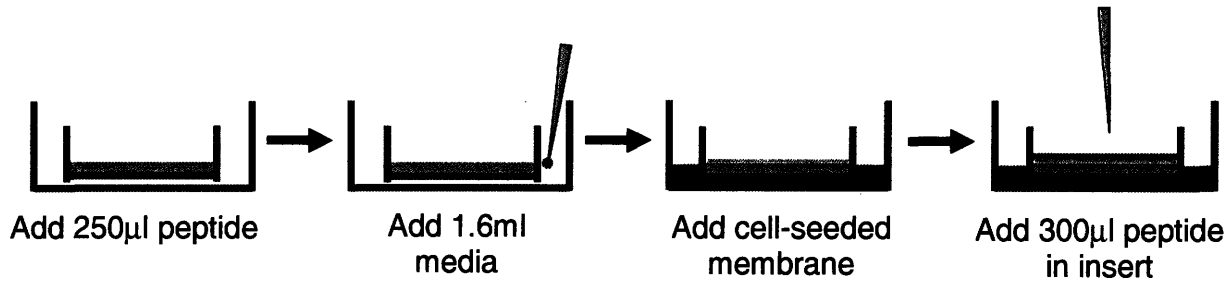


Figure 11: Modified sandwich culture system assembly. A tissue culture insert is coated with peptide and gellified with the addition of media. The inverted cell-seeded membrane is placed on top and then covered with media.

3.8 SEM Sample Preparation

Membrane samples for all conditions (treated/not treated with soluble peptide, incubated/not incubated with hepatocytes) were submerged for 20 min in a fixative mixture containing 2% glutaraldehyde (Sigma, G7526) + 3% paraformaldehyde (Sigma, P6148) in PBS (Invitrogen, 14040). Following the fixing was a series of ethanol dehydration steps that included incubating the samples for 15 min in 50% EtOH, then 30 min in 75% EtOH, then 60 min in 90% EtOH, followed by two changes of 100% EtOH in which the samples were kept in the 100% EtOH until the next step. Using a Tousimis Super Critical Point Autosamdri815 Dryer, the ethanol-saturated samples were put through a process in which the EtOH was slowly exchanged with CO_2 and then dried at the critical pressure and temperature of CO_2. The dried samples were subsequently sputter-coated in a vacuum (Denton Vacuum, LLC) with gold (30 sec, approximate thickness 4-5 nm). SEM was carried out using a JEOL JSM 6060 Scanning Electron Microscope at an accelerating voltage of 5 kV.

3.9 Rhodamine Phalloidin and DAPI Staining

Rhodamine Phalloidin and DAPI staining were used to observe the actin cytoskeleton and visualize the nucleus of the cells, respectively. Media was gently removed from the culture wells. The cells were then fixed in 4% paraformaldehyde for 20 min. The fixative was aspirated and the cells were washed with PBS. 300 ul of 160nM rhodamine-phalloidin (Sigma, 77418) in PBS was added to each sample and left to incubate for 5 min. Additionally, 1x DAPI (Sigma, 32670) was added to each sample for a 5 min incubation. The samples were then rinsed twice with PBS prior to imaging.

3.10 RNA Isolation and Quantification

RNA of samples were obtained by treating cells with Trizol Reagent (Invitrogen, 15596-026) and storing samples in a -80°C freezer until use. Samples were homogenized through pipetting with a 20G needle. Chloroform (Mallinckrodt Chemicals, 4440-04) was mixed thoroughly with each sample and then spun to separate the protein and RNA. The protein supernatants of the samples were individually stored in a -20°C freezer for further assays. The "clear" RNA supernatants of the samples were transferred to separate tubes with equal volumes of 70% ethanol. Using the RNEasy Mini Kit (Qiagen, 74104), the RNA mixture was extracted using a series of reagents and spin cycles. RNA of freshly isolated hepatocytes was extracted and used as a control. The obtained RNA samples were quantified and the quality assessed using a NanoDrop ND-1000 Spectrophotometer. RNA samples of poor quality (260/280 and 260/230 ratios not approximately 2.0) were cleaned up using the Qiagen RNA Clean Up procedure included in the RNEasy Mini Kit.

3.11 cDNA Preparation

The RNA samples were treated with DNAse Buffer and DNAse I (Invitrogen, 18068) to remove contaminant DNA. 150 ng of RNA for each sample was reversed transcribed with an Omniscript Reverse Transcription Kit (Qiagen, 205111). EDTA was added to each sample and then incubated at 65°C for 10 min. Next, a master mix of DEPC H2O, dNTP, Random Hexamers, RNase Inhibitor, RT Buffer, and Omniscript RTO Reverse Transcriptase was added to each sample and incubated at 37°C for 1.5 hrs.

3.12 RT-PCR

Quantitative PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, 204143) in a MJ Opticon Monitor instrument (Applied Biosystems). PCR primers were designed to obtain 150-200 base pair product. The PCR protocol entailed: incubation at 95°C for 15 min, followed by 45 cycles of incubating the samples at 94°C for 15 sec, annealing at 51-55°C for 30 sec and extending at 72°C for 30 sec. A melting curve was performed to assess the purity of the products formed. The primer sequences in Table 2 were used.

Primer Name	Primer Sequence
Albumin forward:	5'-GGTGCAGGAAGTAACAGACTTTG-3'
Albumin reverse:	5'-TAACTTGTCTCCGAAGAGAGTGTG-3'
HNF4-α forward	5'-CTGAGACTCCACAGCCATCA-3'
HNF4-α reverse	5'-CTAGATGGCTTCCTGCTTGG-3'
CYP3A2 forward	5'-GTAGTACTCTTTCCATTCCTCACCC-3'
CYP3A2 reverse	5'-GGTGCTTATGCTTAGAATCCAGAC-3'
18s forward	5'-GCAATTATTCCCCATGAACG-3'
18s reverse	5'-GGCCTCACTAAACCATCCAA-3'

Table 2. RT-PCR primers of albumin, HNF4-α, CYP3A2, and the housekeeping gene, 18s.

Using the $2^{-\Delta\Delta Ct}$ method, relative gene fold enrichments were determined relative to the ribosomal unit 18s as a housekeeping gene. Samples were then compared to the gene expression in freshly isolated hepatocytes.

3.13 SDS-Electrophoresis and Western Blotting

Supernatant medium was collected each day and maintained at -20°C until analyzed. Sample volumes of 12 ul were each mixed with 4 ul β-mercaptoethanol 4x (Bio-Rad Laboratories, 161-0710), incubated in boiling hot water for 10 min, and loaded into a NuPAGE 12% Bis-Tris gel (Invitrogen, NP0341BOX). The gel was run in NuPAGE MES SDS Running Buffer (Invitrogen) for 1 hr at 200 V. The proteins were then transferred to a polyvindyliden difluoride membrane (Invitrogen, LC2002) for 2 hr in transfer buffer consisting of Tris Base (Roche, 604205), Glycine (Sigma, G8898), and Methanol (Mallinckrodt Chemicals, 3016-16), diluted in deionized water. The membranes were first blocked overnight with blocking buffer, consisting of 5% dry skim milk in PBST buffer. Then they were incubated for an hour at 4°C with antibodies diluted in the same blocking buffer: sheep anti rat albumin-HRP conjugated antibody (1:20000 dilution, Bethyl Laboratories, A110-134P). Each membrane was then washed three times for 30 min with blocking buffer. The western blots were developed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce Biotechnology, 34080). The protein bands were visualized using a FluorChem gel imaging system (Alpha Innotech).

3.14 Proteomic Analysis

Media samples from the hepatocyte membrane sandwich culture were electrophoresed using a NuPAGE 12% Bis-Tris gel electrophoresis system and NuPAGE MES SDS Running Buffer (Invitrogen). Hepatocyte culture media was loaded as a control. The gels were stained with coomasie blue staining solution. The differential bands of the samples and control were excised and identified using a proteomic approach. Bands were desalted and digested with trypsin, and eluted using an Agilent 1100 Nanoflow HPLC system connected to a Thermo Electron LTQ Ion Trap mass spectrometer. Ions were scanned in a molecular weight range between 300-3500 Da. A modified SEQUEST algorithm that matched sample fragmentation patterns with theoretical fragmentation patterns of tryptic peptides in the Uniprot Rat protein database was used as a probability-based validation for protein identification. Ions were considered valid when Xcorr was 2.0 for peptides with m/z=1; 2.5 for peptides with m/z=2; and 3.5 for peptides with m/z=3.

4.0 RESULTS & DISCUSSION

4.1 Dimensional Control of Self-Assembling Peptide Layer

The first initiative was to create a thin homogenous layer of self-assembling peptide that was orders thinner than layers found in typical sandwich cultures. To find an optimal method, two variables were examined: varying the deposited amount of soluble peptide and changing the deposited peptide concentration. It was determined early on that the deposited amount of soluble peptide was already at its lowest, which was just enough volume to cover the entire surface of a dish. Therefore, attention was given to the peptide concentration. Peptide solutions were serially diluted from the stock 1% to 0.125%. It was observed that after gelation from salt-containing media contact that the 1% and 0.5% concentrations produced too thick of a layer (~500 um). Meanwhile, the lower concentrations were too dilute and produced unstable layers, even with introduction to salt solutions. Therefore, the plasma-modified membranes were implemented to allow for a new method that did not require gelation of the self-assembling peptides for stability. As demonstrated back in Figure 7, this new method relies on anchoring the self-assembling peptides on the RAD16-I immobilized sequences on the surface. By this method, self-assembling peptide is introduced by incubating the plasma-modified, biomolecules-immobilized substrates with 1% soluble RAD16-I peptide, followed by a water rinse to remove unbound and unassembled peptide.

4.1.1 Peptide Formation on PEEK-WC-PU

Figure 12 displays the self-assembling peptide layer formation on the PEEK-WC-PU membranes. As controls, native PEEK-WC-PU membranes and acrylic acid plasma-deposited PEEK-WC-PU membranes (PEEK-WC-PU/PdAA) without biomolecule immobilization were prepared. The data is arranged in a matrix format, juxtaposing the initial pre-incubated membrane to the RAD16-I peptide-incubated membrane.

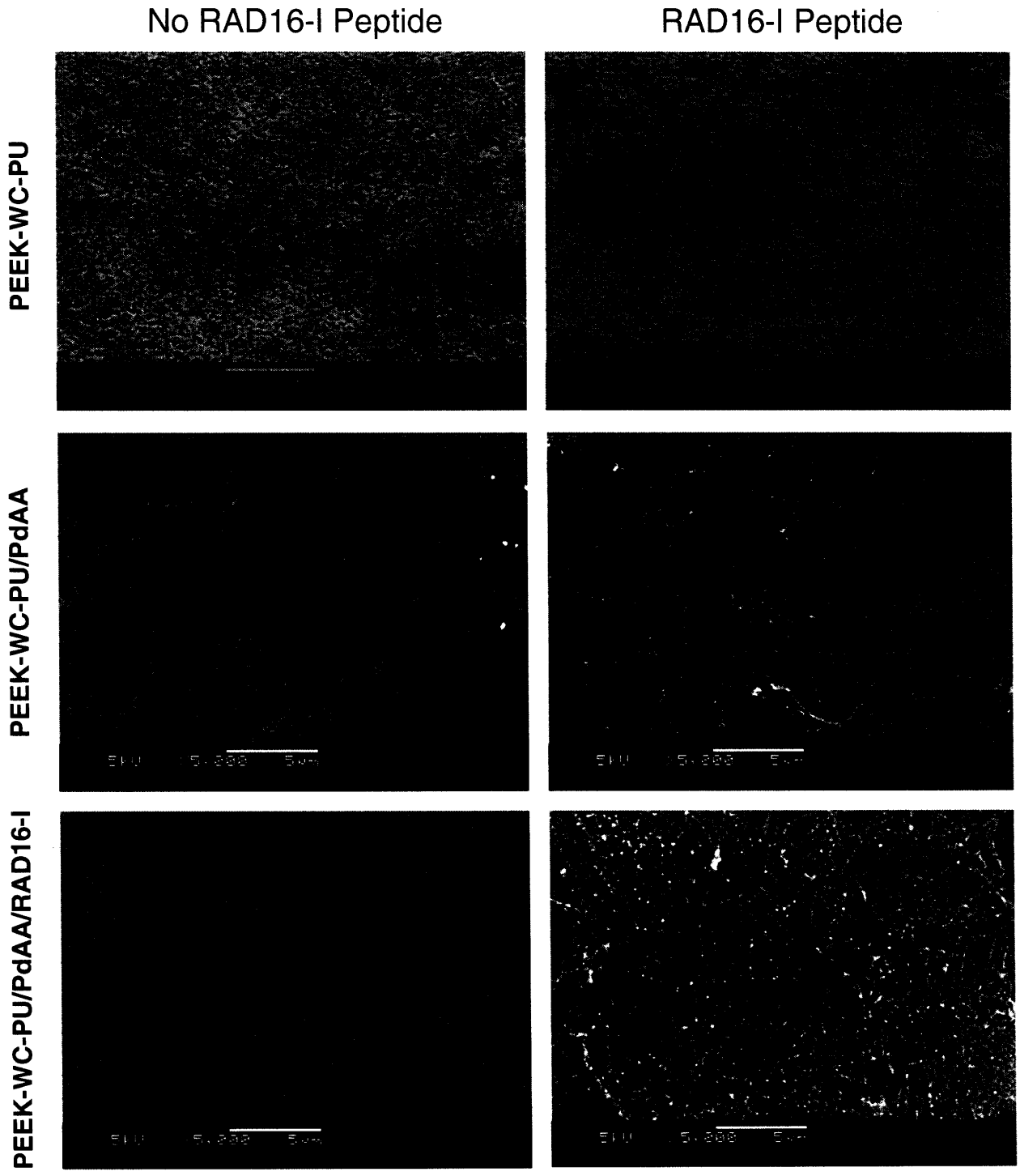


Figure 12: SEM images of fiber formation of RAD16-I self-assembling peptide on unmodified (top row), plasma-deposited acrylic acid (middle row), and plasma-modified RAD16-immobilized (bottom row) PEEK-WC-PU membranes.

PEEK-WC-PU, PEEK-WC-PU/PdAA, and PEEK-WC-PU/PdAA/RAD16-I membranes all had nearly identical visible surfaces. Alterations due to acrylic acid plasma-deposition or RAD16-I

biomolecule immobilization were not visibly apparent, as expected at this magnification. After the one hour self-assembling peptide incubation, it became apparent which membranes were modified. The native PEEK-WC-PU membrane showed no peptide attachment in Figure 7. The PEEK-WC-PU/PdAA membrane displayed some peptide fiber attachment. However, the peptide layer was not homogenous throughout the surface and certain areas were patchy containing more attached fibers than others.

The PEEK-WC-PU/PdAA/RAD16-I membrane demonstrated the best fiber formation of the three conditions. The peptide layer was both thin and homogenous in thickness, creating a mesh that clearly did not obstruct the pores of the native membrane beneath. Visually, we are able to see the peptide fiber network and, thus, can estimate that the sheath thickness is on the orders of nanometers. This favorable fiber formation successfully addresses and greatly reduces the problem of peptide scaffolds being efficient barriers of oxygen and gas diffusion in hepatocyte cultures.

4.1.2 Peptide Formation on PTFE

Figure 13 exhibits the self-assembling peptide formation on the Biopore PTFE membranes. As a control, the native PTFE membrane was used to compare against the modified PTFE (PTFE/PgPFM/RAD16-I). Again, the data is arranged in a matrix format, juxtaposing the initial pre-incubated membrane to the RAD16-I peptide-incubated membrane.

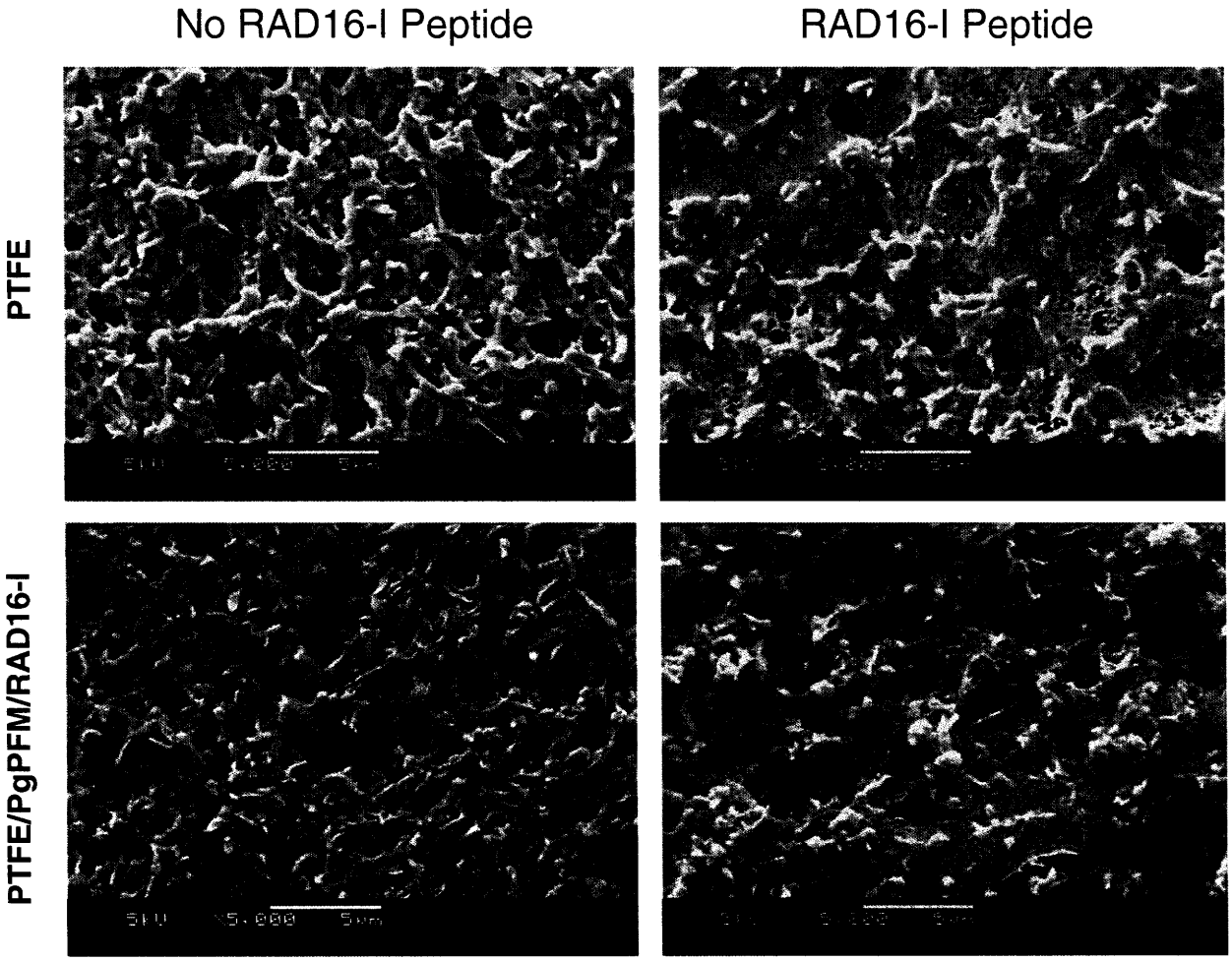


Figure 13: SEM images of fiber formation of RAD16-I self-assembling peptide on unmodified (top row) and plasma modified RAD16-I immobilized (bottom row) PTFE membranes.

The native PTFE and the modified PTFE membranes looked nearly identical. Again, plasma modifications to the PTFE membranes were not visible at this magnification. After the one hour incubation, both the native and modified PTFE membranes presented the same fiber formation pattern of self-assembling peptide. The peptide-covered surfaces of both the native and modified samples appeared nearly identical. The peptides seemed to have assembled a very thin web layer using the protruding features of the membrane. Closer examination revealed a mesh of individual fibers in the membrane pores.

Unlike the unmodified PEEK-WC-PU, the PTFE membranes clearly showed a thin layer of peptide that covered the entire surface. Also important to note is the difference in the texture of the membrane surface between the PEEK and PTFE. The PEEK surface was smoother with

smaller pores as seen in Figure 12. The PTFE membranes clearly have a rough contour which may have assisted in the peptide attachment despite the lack of plasma modification on the surface. Again, both the native and modified PTFE exhibited fiber formation that successfully reduces the problem of peptide scaffolds being efficient barriers of oxygen and gas diffusion in hepatocyte cultures.

4.2 Hepatocyte Attachment on Controlled Peptide Layer

The next objective was to assess the attachment of hepatocytes onto the self-assembling peptide-coated modified membranes. This was performed by seeding hepatocytes at a density of 65,000 cell/cm^2 proceeding the self-assembling peptide incubation and water rinse. To determine whether cellular attachment was specifically enhanced by the presence of the self-assembling peptide layer, the hepatocytes were given several hours to incubate before a media change was done. This media change was executed to not only remove dead cells but to also eliminate cells that might have attached due to settling or through the production of endogenous adhesion proteins for attachment. The following data for both membrane materials was attained 24 hours post cell-seeding.

4.2.1 Hepatocellular Attachment on PEEK-WC-PU

Figure 14 shows SEM images of the hepatocellular attachment as a result of the self-assembling peptide conditions on PEEK-WC-PU (Light microscopy was not possible due to the opaqueness of the PEEK-WC-PU membranes). Because it was shown earlier that native PEEK-WC-PU failed to adhere any self-assembling peptide fibers, only the PEEK-WC-PU/PdAA membrane was used as a control. The images are arranged in the same layout as above with the addition of the seeded hepatocyte.

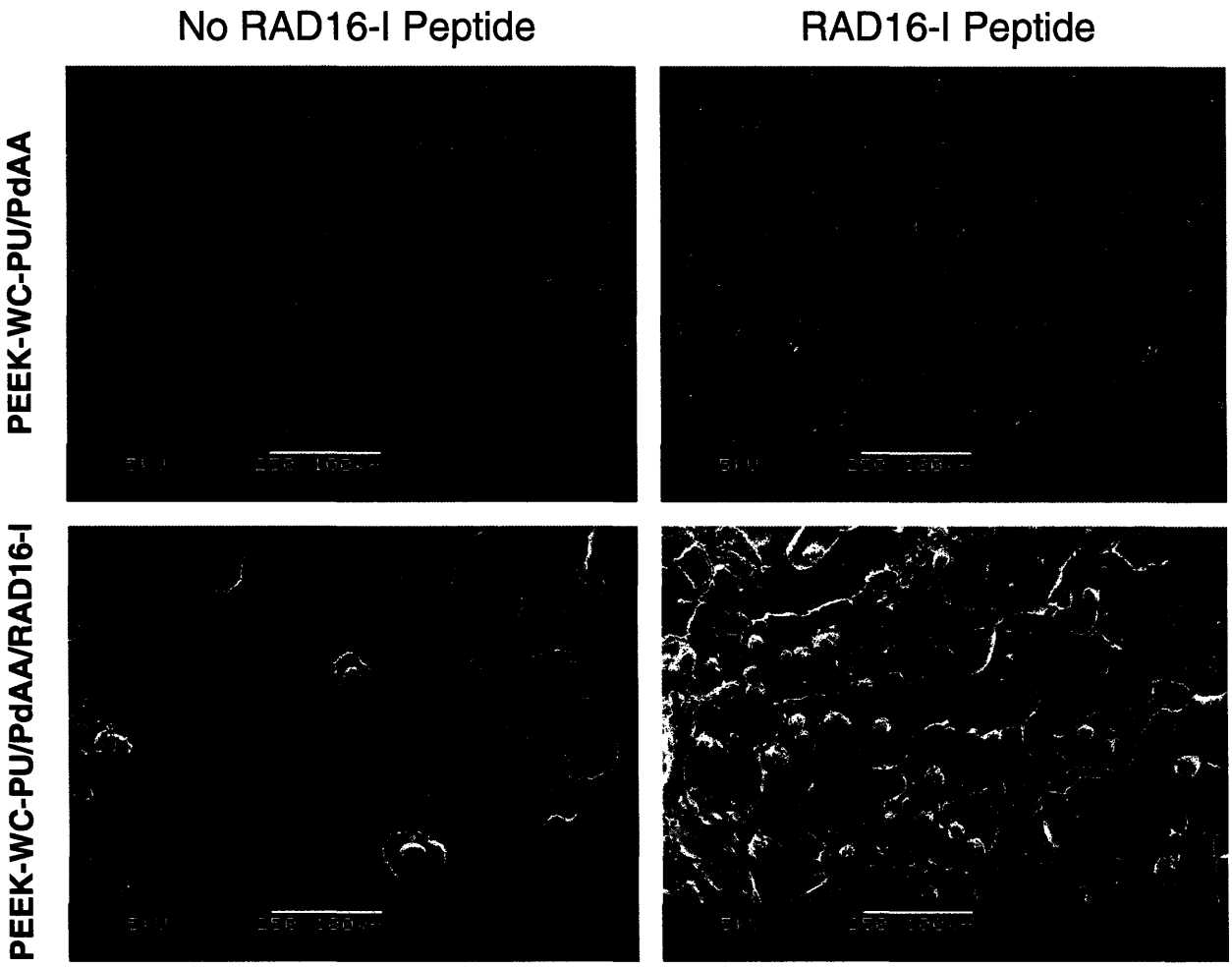


Figure 14: SEM images of hepatocyte attachment with (left column) and without (right column) RAD16-I incubation on plasma-deposited acrylic acid (top row) and plasma-modified RAD16-immobilized (bottom row) PEEK-WC-PU membranes.

As expected, the PEEK-WC-PU/PdAA that was not incubated with peptide did not bind any cells. Likewise, there was no cellular attachment apparent on the PEEK-WC-PU/PdAA that was incubated with peptide. In addition, there was no visible fiber formation on the membrane. As noted before, the PEEK-WC-PU/PdAA condition yielded a patchy, variable, and unreliable fiber formation, which explained the absence of the peptide fibers in this trial.

On the other hand, the PEEK-WC-PU/PdAA/RAD16-I membranes demonstrated cell binding in both conditions. Without the peptide incubation, a few cells unexpectedly still attached to the surface. There was no fiber matrix present, however, the immobilized RAD16-I peptides might have provided a more favorable cell-attaching surface than the PEEK-WC-PU/PdAA substrates. With the peptide incubation, the surfaces were completely filled with hepatocytes. It is apparent

that the self-assembling peptide fiber network vastly improves hepatocellular attachment. A close-up of one of the hepatocytes reveals an intricate cellular attachment with the substratum (Figure 15). Upon closer examination, cytoplasmic projections visibly adhere to individual fibers for anchorage.

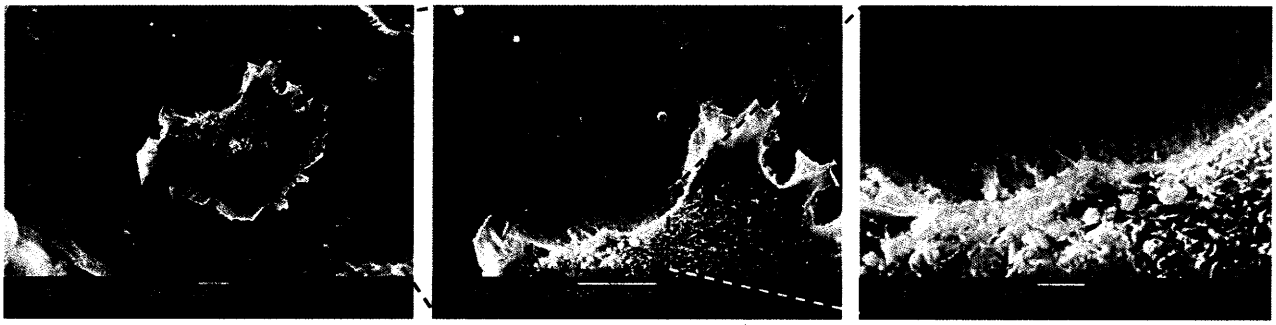


Figure 15: Close-up SEM images of a single hepatocyte on PEEK-WC-PU/PdAA/RAD16-I. At closer magnifications, cytoplasmic projections visibly adhere to the self-assembling fibers.

To verify cellular attachment, DAPI and Rhodamine Phalloidin stainings were performed to observe the actin cytoskeleton and visualize the nucleus of the cell, respectively (Figure 16). Rhodamine Phalloidin staining was unsuccessful for all conditions. The photo-bleached results were a result of auto-fluorescence of the PEEK-WC-PU material.

DAPI staining confirmed the earlier results (Figure 16). No cells were observed on the PEEK-WC-PU/PdAA that was not incubated. However, the incubated counterpart showed some cells that sparsely attached in small patches – agreeing with our initial observation of the spotty peptide fiber patches. Again, a small amount of cells uniformly attached to the PEEK-WC-PU/PdAA/RAD16-I sample that was not incubated with peptide. Moreover, the RAD16-incubated PEEK-WC-PdAA/RAD-16-I membrane prominently displayed a cell-covered surface.

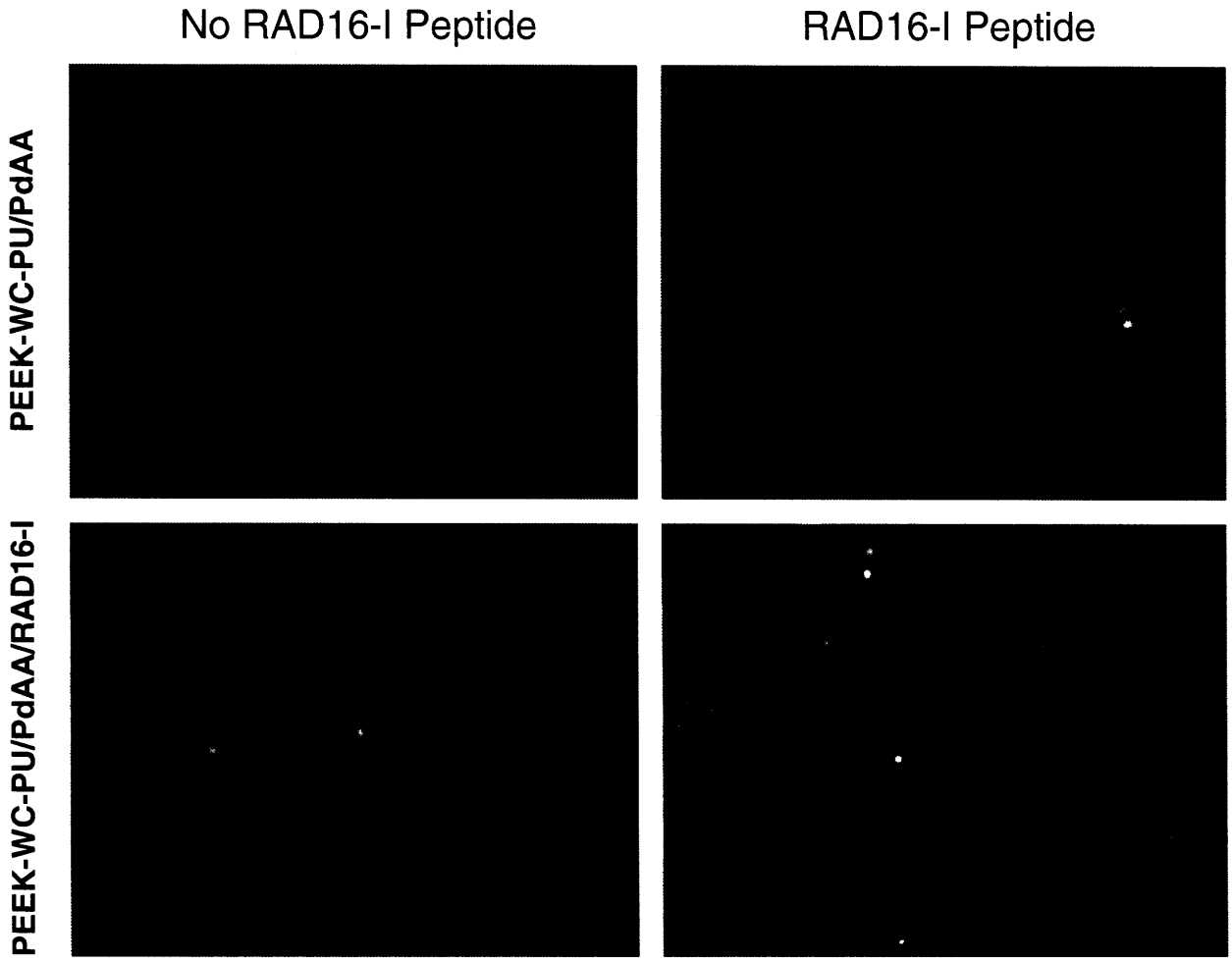
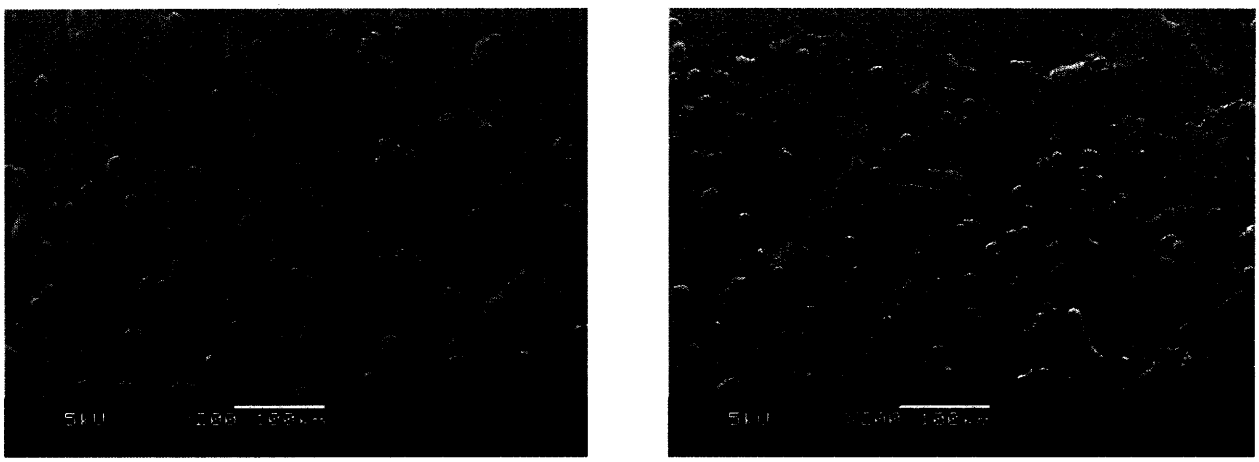


Figure 16: DAPI staining of hepatocyte nuclei on PEEK-WC-PU with (left column) and without (right column) RAD16-I incubation on plasma-deposited acrylic acid (top row) and plasma-modified RAD16-immobilized (bottom row) membranes.

4.2.2 Hepatocellular Attachment on PTFE

Figure 17 displays SEM images of hepatocellular attachment on native and modified PTFE incubated with RAD16-I. As we saw above, the modified PTFE did not seem to show any difference in peptide formation and attachment over the native PTFE. However, hepatocellular attachment between the two conditions was strikingly dissimilar.



(A) PTFE (B) PTFE/PgPFM/RAD16-I

Figure 17: SEM images of hepatocyte attachment with RAD16-I incubation on (A) native PTFE and (B) plasma-grafted PFM RAD16-immobilized PTFE membranes.

The native and modified PTFE membranes both supported hepatocyte attachment. Interesting to note is the morphology of the cells on the respective membranes. On the native membrane, the hepatocytes remained round and spherical throughout the entire surface. Likewise, the cells tended to clump and form spheroids. The modified PTFE on the other hand had some cells with similar morphology but also contained cells with a flat and extended morphology. The cells on this membrane tended not to cluster and form spheroids. The spread and extended morphology is more favorable for hepatocytes for cell-cell interactions and tissue assembly. This morphology promotes polarization and the formation of bile canilicular spaces between neighboring cells.

The plasma-grafted RAD16-I peptides seemed to have had an effect on the overall attachment of the cells and, as a result, the morphology of the cell. Although visibly identical, we suspect that the immobilized RAD16-I created an anchor for the peptide layer on the modified PTFE. In this case, the hepatocytes were able to naturally pull the substrate and spread along the peptide surface. To the cell, the immobilized peptides generate a more "rigid" surface. The native PTFE does not provide a tether for the peptide layer in which the cells are attached to and, thus, the biomechanics are changed. Without the immobilized fibers, the cells appear to pull off surrounding unanchored peptide without being able to interact with the substrate, and instead interact with surrounding cells to form clusters (Figure 18).

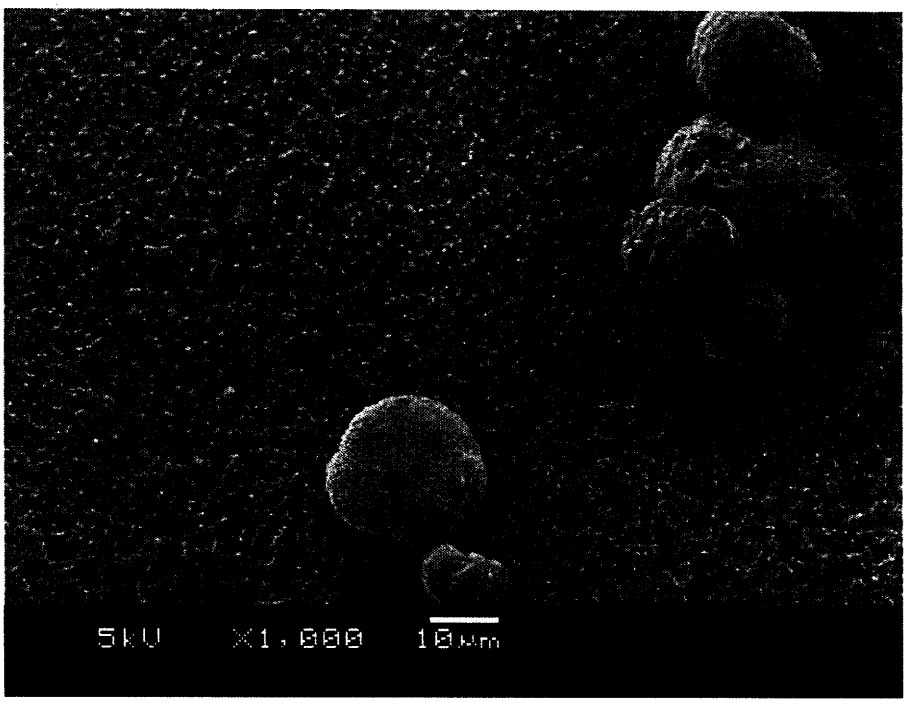


Figure 18: SEM image of hepatocyte attachment on native PTFE membrane. Hepatocytes appear to pull off surrounding peptide without the anchorage of immobilized peptides and form clusters. The cells are unable to interact with the rigid substrate beneath the peptide and, thus, do not achieve a flat morphology.

DAPI and Rhodamine Phalloidin stainings were used to verify cellular attachment to the membranes (Figure 19). Slight background signal was noticed from the peptide layer during the Rhodamine staining. Regardless, the stainings confirmed the previous observations. DAPI staining verified the presence of hepatocytes on both the native and modified surfaces. The nuclear stain also illustrated the cluster tendency of the hepatocytes on the native membrane. On the same membrane, the Rhodamine stain revealed the same spherical morphology of the cells that was seen under the SEM. Likewise, the stain also verified the expanded flat morphology of the hepatocytes on the modified PTFE membrane.

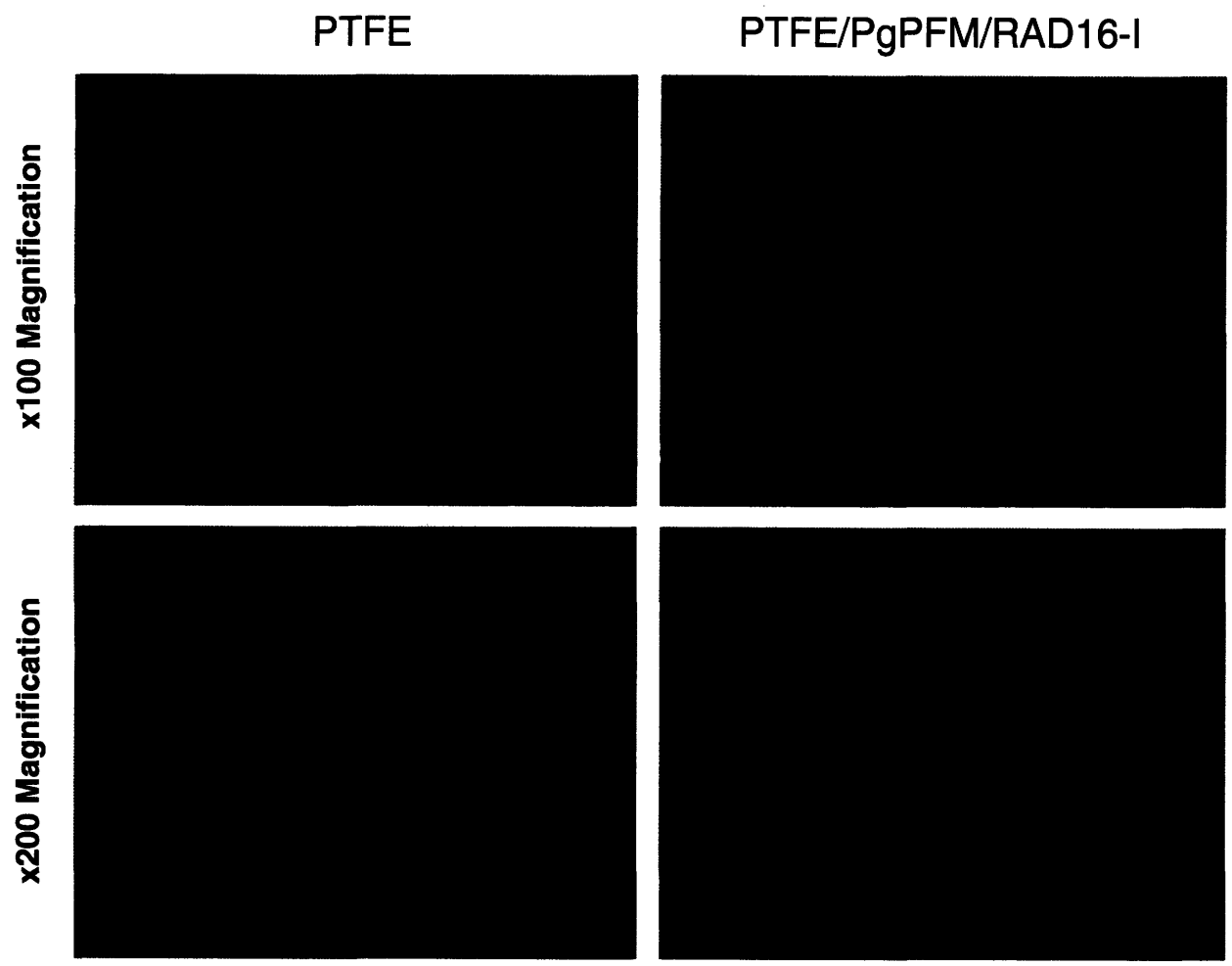


Figure 19: DAPI (blue) and Rhodamine (yellow) stainings of hepatocytes on RAD16-incubated native and plasma-modified RAD16-immobilized PTFE membranes.

4.3 Modified Sandwich Culture of Primary Hepatocytes

After demonstrating that our substrates were successfully able to promote cell attachment and healthy morphology, the following objective was to determine if and to what extent the self-assembling peptides and the increased oxygen diffusion enhanced hepatocellular function, especially CYP3A2 activity. Modified sandwich cultures were prepared similar to typical sandwich cultures except the top layer of soluble peptide was substituted with the inverted cell-seeded modified membrane as shown in Figure 8. Cultures were observed over a week long period.

4.3.1 PEEK-WC-PU in Modified Sandwich Cultures

Quantitative PCR was performed to measure hepatospecific biomarkers that should be present and expressed in fresh hepatocytes. Figure 20 displays the gene expression profile of albumin, CYP3A2, and HNF4-α relative to gene expression in freshly isolated hepatocytes over a period of seven days for modified sandwich cultures using PEEK-WC-PU. Results were attained in three separate experiments and then combined. Data is presented on a log base 2 scale. Therefore, a 2-fold downregulation is equivalent to a x4 ($=2^2$) decrease in expression. Values between -1 and +1 are considered excellent and on par with fresh hepatocyte levels.

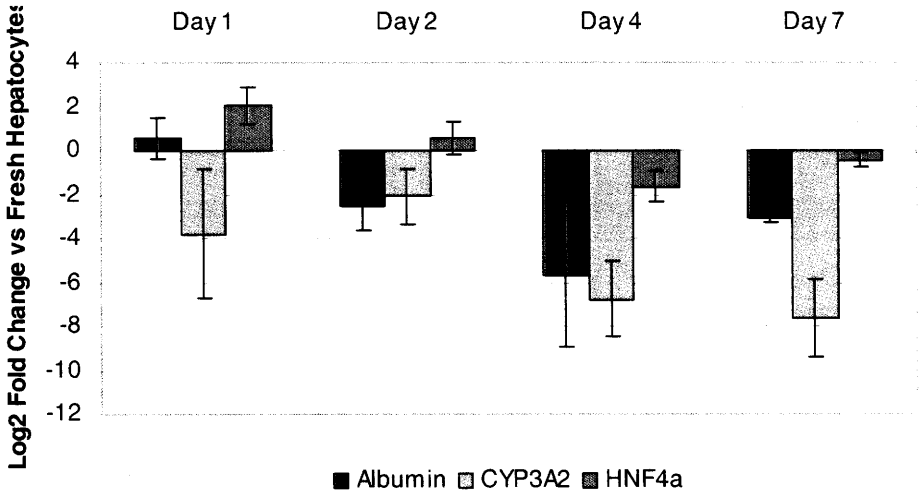


Figure 20: Gene expression profile of albumin, CYP3A2, and HNF4-α obtained by quantitative PCR relative to gene expression in freshly isolated hepatocytes. Cells cultured on modified PEEK-WC-PU membranes incubated with RAD16-I.

After 24 hours post-seeding, the cells expressed great levels of albumin and HNF4-α. Albumin expression was close to fresh levels after a day and then began to slightly decline up to day 4. However, the variation in albumin expression on day 4 made it hard to judge to what extent the culture was downregulated. On the other hand, by day 7 the albumin expression appeared to have improved back to a -3-fold downregulation. HNF4-α expression stayed within a close range to fresh cell levels. CYP3A2 was downregulated after a day and slightly evened off around a -7-fold downregulation after a week.

In comparison to the results of Genove et al (2006), our data appears to be on par (Figure 21). There is no significant improvement over Genove's system. However, our system is still about 1.5-fold better than the current gold standard method of culturing hepatocytes with collagen.

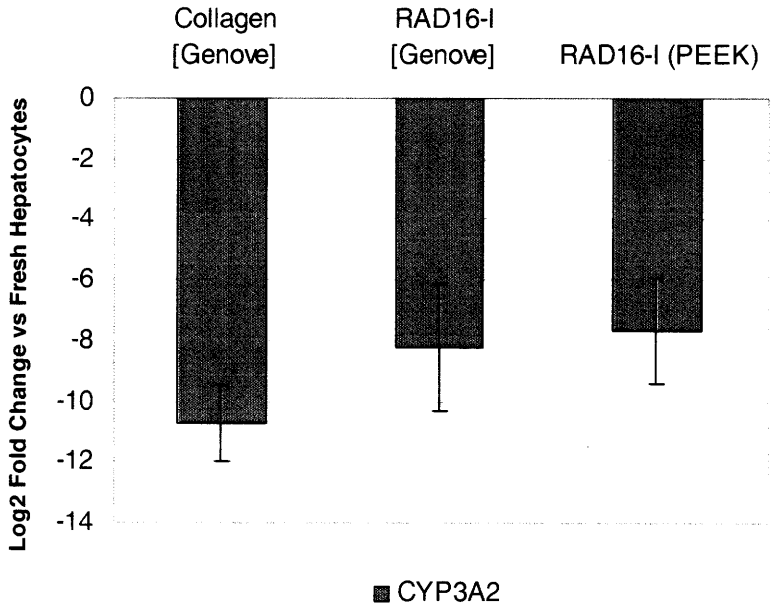


Figure 21: Comparison of CYP3A2 gene expression relative to freshly isolated hepatocytes at day 7 by quantitative PCR with previous results by Genove et al of collagen cultures and self-assembling peptide sandwich cultures.

Interesting to note, during one of the trials, the cell-seeded top membrane/peptide layer failed to adhere to the bottom peptide layer after the initial placement and continued to remain afloat after each daily media change. To examine what effect this defect would have on overall gene expression, the cultures were carried out to completion under identical treatment as the normal samples. Figure 22 illustrates the remarkable difference between sandwiched culture and the freely floating inverted modified membrane.

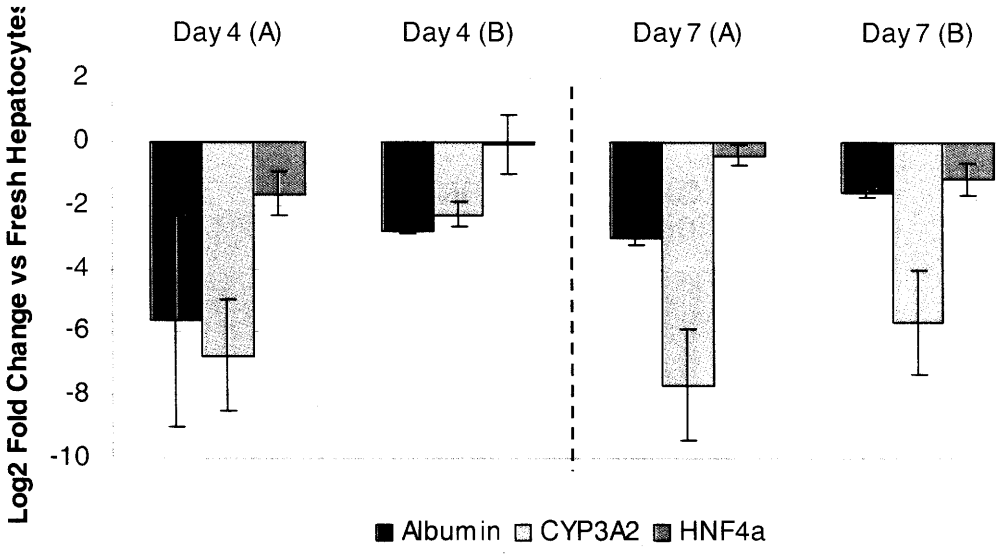


Figure 22: Gene expression profile 4 and 7 days after initial cell-seeding. "(A)" samples were normally functioning modified sandwich cultures. "(B)" samples exhibited an irregularity in which the top layer membrane portion failed to attach and floated in the surrounding media. Cells cultured on peptide-incubated modified PEEK-WC-PU membranes.

The expression appeared to be improved substantially in the defective cultures, especially in day 4. What we suspected was the reason for the improvement was that the free-floating membrane allowed the hepatocytes better contact with nutrients from the surrounding media and, likewise, a better oxygen and gas exchange. We believed the bottom peptide layer, although already at a 0.5 mm to 1mm thickness, did not facilitate the type of exchange made possible by the controlled peptide layer found on the top surface.

Media was collected and changed each day to monitor the albumin production of the cultures. A western blot was run with the supernatant media of each time point to observe hepatospecific function upkeep by examining how the relative albumin production produced changed through the duration of the culture (Figure 23). Hepatocyte growth media was also examined as a control to ensure that albumin from the media was not interfering with the blot against the rat albumin.

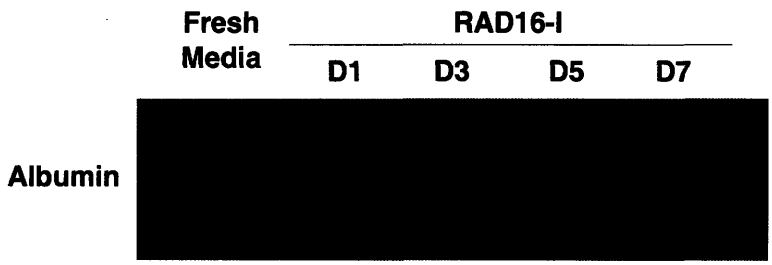


Figure 23: Albumin western blot of the supernatant media from hepatocytes sandwich-cultured on peptide-incubated modified PEEK-WC-PU membranes.

Although the hepatocyte growth media contained only bovine species albumin, the anti rat albumin antibodies apparently still bound the cross species as seen by the faint band in the western blot. The other days seemed to have produced albumin as seen by the relative intensity of the bands compared to the fresh media band, however confirmation was needed to ensure that production of albumin was from the rat hepatocytes. Therefore, rat albumin was confirmed in the media samples through Proteomic Analysis.

The albumin production appeared to have decreased during the duration of the culture, which is an indication that the cells are losing their hepatospecific functions. Using an image analysis we were able to estimate the relative drop in albumin production from Day 1 until Day 7 (Figure 24). The production of albumin drops to nearly a third of the level it was at 24 hours after initial cell seeding.

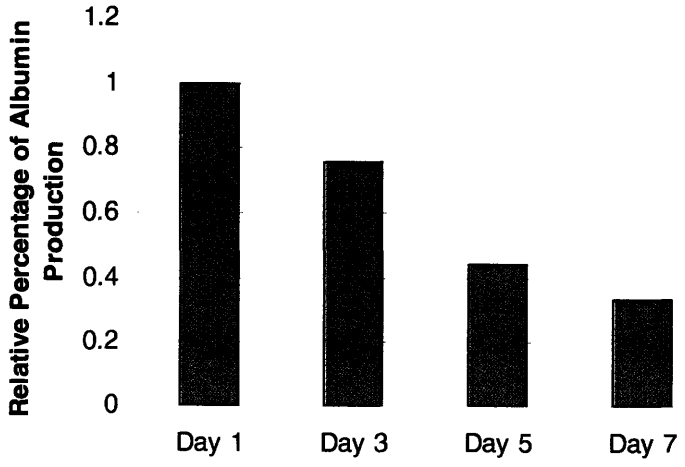


Figure 24: Relative proliferation of albumin production compared to the level after 24 hrs. Data extrapolated from image analysis of western blot.

4.3.2 PTFE in Modified Sandwich Cultures

Figure 25 presents the gene expression profile of albumin, CYP3A2, and HNF4-α relative to gene expression in freshly isolated hepatocytes over a period of seven days for modified sandwich cultures using PTFE. Varying culture conditions were tested using RAD16-I, 5% RGD in RAD16-I, 5% YIG in RAD16-I, and a mix of 2.5% RGD and 2.5% YIG in RAD16-I. RNA samples were collected at day 1, 4, and 7 to determine if and to what extent the peptides and the functional motifs enhanced hepatospecific functions.

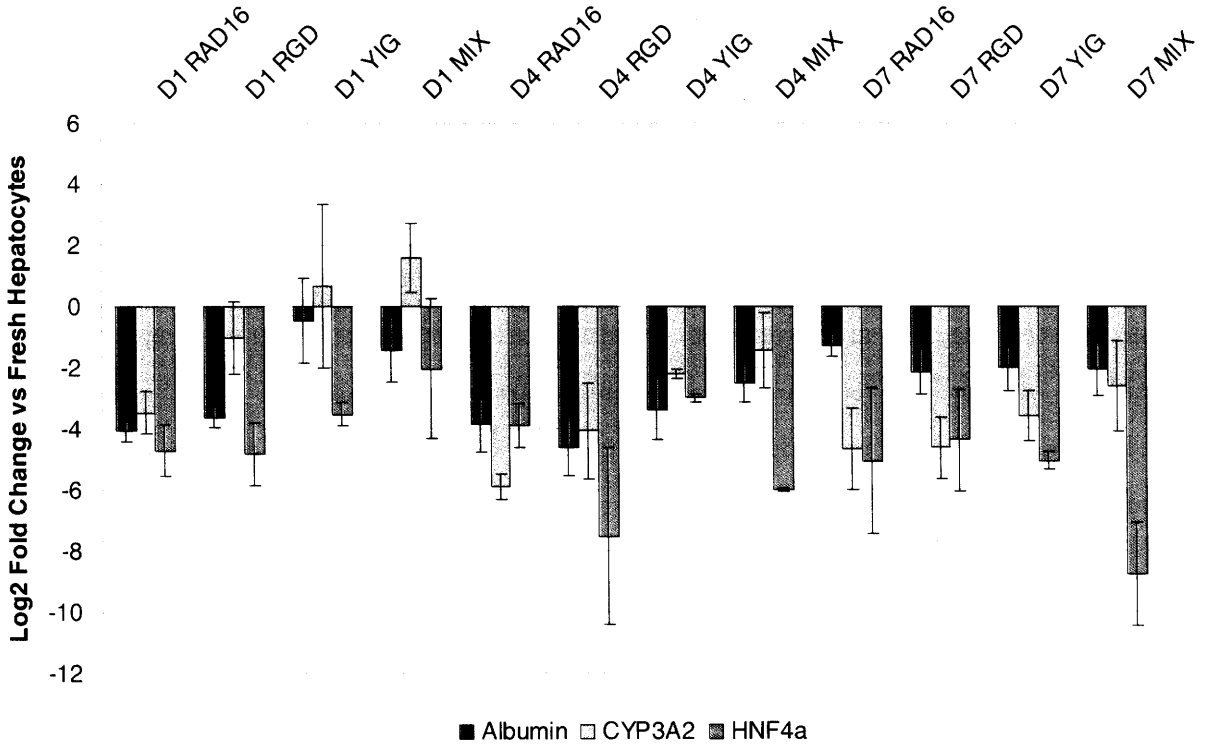


Figure 25: Gene expression profile of albumin, CYP3A2, and HNF4-α obtained by quantitative PCR relative to gene expression in freshly isolated hepatocytes at day 1, 4, and 7. Cells cultured on modified PTFE membranes incubated with RAD16-I, 5% RGD, 5% YIG, or a mix of 2.5% RGD and 2.5% YIG.

RAD16-I peptide cultures expressed a fairly stable level for each target gene. Albumin remained downregulated by about 4-fold after day 1 until day 4. Between then and day 7 it appeared that the albumin production was improved up to a great -1-fold downregulation. HNF4-α levels remained consistent and floated between -4-fold to -5-fold downregulation. The CYP3A2

expression fell from an initial -3.5-fold and a -6-fold downregulation. However, the condition does seem to slightly improve culture from day 4 to a final -4.7-fold downregulation.

RGD cultures (5% RGD / 95% RAD16-I) did not seem to improve upon the RAD16-I cultures significantly. Albumin levels were slightly improved from an initial -3.6- to a final -2.1-fold downregulation. HNF4-α levels started at -4.8-fold, then worsened to -7.5-fold, and finally improved to slightly better than initial levels of -4.4-fold downregulation. CYP3A2 levels initially were excellent at -1-fold. They eventually dropped to about -4-fold and slightly leveled off by day 7 at a -4.6-fold downregulation. Especially by day 7, results were extremely similar and were neither insignificantly better nor worse than the RAD16-I cultures.

YIG cultures (5% YIG / 95% RAD16-I) greatly improved short-term culture gene expression. Albumin levels were at fresh levels initially and then dropped to -3.4-fold by day 4 but improved to -2-fold by day 7. HNF4-α initial levels were improved over the RAD16 and RGD conditions by about 1.5-fold. However, by day 7, levels were similar to RAD16 and slightly lower than RGD. CYP3A2 levels were greatly improved. Though there was a high variation in signal, regardless, the levels were all higher than the levels found in RAD16. Levels dropped by day 4 to about a modest -2-fold and then by day 7 to a still reasonably good level of -3.6-fold.

The MIX cultures (2.5% RGD / 2.5% YIG / 95% RAD16-I) provided a vast improvement over the RAD16 condition. Again, short-term cultures were largely enhanced over RAD16 as seen in all three target genes. Albumin conditions started off -1.4-fold and by the end of the week resulted at a -2-fold downregulation. HNF4-α levels began the highest of the conditions at -2-fold but greatly deteriorated to the lowest of all conditions by day 7 at a -8.7-fold downregulation. CYP3A2 levels were the best of all conditions at each day point. Levels started on a successful 1.6-fold upregulation. Expression began to decline by day 4 to -2.2-fold and then to a respectable -2.6-fold by day 7. With the exception of the day 7 HNF4-α levels, the MIX cultures looked to be the most promising for short-term (few days) and mid-term (week long) cultures.

Important to note is the HNF4-α expression across all conditions and time points. Unexpectedly, the levels for this target gene were extremely downregulated from previous experiments. In earlier experiments or studies by Genove et al., HNF4-α is generally unproblematic to maintain to around fresh levels. Likewise, unpredictably the CYP3A2 expression was vastly improved. Therefore, certainty is reserved until the experiment can be repeated. On the other hand, downregulation for albumin expression seemed to be on par with previous results, which is a good indication.

Regardless, the results are extremely promising. Compared to the previous results by Genove et al. and the data from the PEEK-WC-PU membranes, the day 7 CYP3A2 levels of all conditions on the PTFE membranes are significantly better (Figure 26). With respect to the previous PEEK-WC-PU material, the RAD16-I condition on PTFE provided a 3-fold improvement. This was about an x8 ($=2^3$) increase in gene expression. We conjecture this enhancement to be due to physical differences between membrane geometry and topography. As seen in the SEM images of Figure 5 and 6, the porosity of the PTFE membrane is much higher and the pore sizes are much larger. Tying back to our hypothesis of culture improvement by reducing oxygen and nutrient diffusion barriers, the PTFE membranes provide much less of an obstruction and facilitate better exchange with the microenvironment.

In comparison to the other PTFE conditions, the RAD16-I and RGD cultures appear to be on par with each other. The YIG condition slightly improves upon the previous two. Likewise, the mix condition of RGD and YIG furthermore enhances the CYP3A2 levels to almost -2.5-fold. This is a tremendous improvement over the standard collagen method that produced about a -10.5-fold downregulation. This 8-fold difference translates to a x256 ($=2^8$) improvement of the MIX modified sandwich culture to the typical collagen sandwich culture.

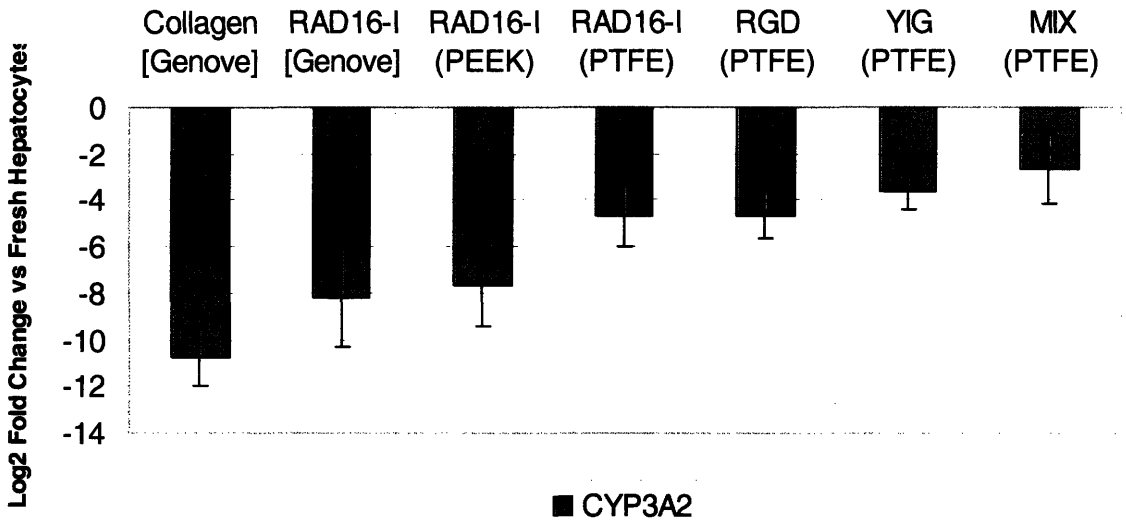


Figure 26: Comparison of CYP3A2 gene expression relative to freshly isolated hepatocytes at day 7 by quantitative PCR with previous results by Genove et al and results from PEEK-WC-PU membranes.

Again, media was collected at each time point over the seven days to monitor albumin production. A western blot was run for all four peptide conditions (RAD16-I, RGD, YIG, and MIX) with the supernatant media to assess degeneration in hepatospecific function (Figure 27). Hepatocyte growth media was also tested to determine the background signal due to bovine albumin. Bovine albumin was slightly detected through the western blot against rat albumin. However, rat albumin was detected and confirmed for each peptide condition at each time point through Proteomic Analysis.

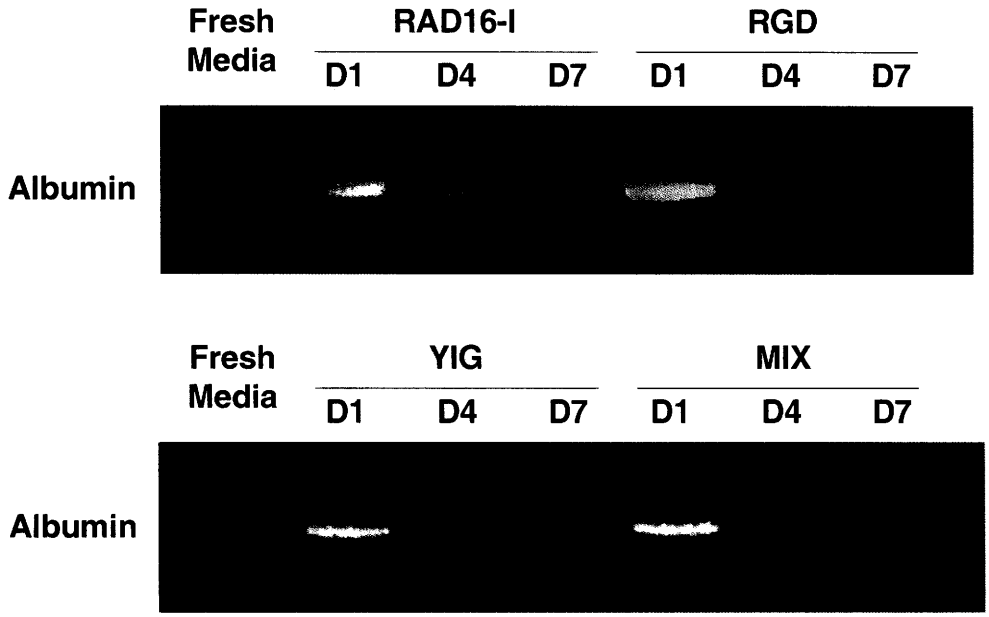


Figure 27: Albumin western blot of the supernatant media from hepatocytes sandwich-cultured on peptide-incubated modified PTFE membranes.

Albumin production was very strong at day 1 for all peptide conditions. The RAD16-I cultures exhibited the same degeneration seen in the previous results done on PEEK-WC-PU membranes. By day 7, the albumin production quickly fell to extremely low levels for RAD16-I. RGD cultures presented similar trends, however the day 4 levels appeared to have fallen lower comparatively to RAD16-I cultures based on the intensity of the band. By day 7 the overall production has decreased substantially. YIG and MIX cultures both demonstrated high levels at day 1 with a drop by day 4. However, these two conditions seem to yield healthier levels of albumin production by day 7 as determined by the band intensity difference compared to the RAD16-I and RGD conditions. Likewise, unlike the RAD16-I and RGD cultures, the band intensity differences between day 4 and day 7 are not as apparent strong indicating a better maintenance of culture using these peptide conditions.

It was observed on the western blots of the cultures on both PEEK-WC-PU and PTFE membranes that the albumin production levels drop during the week-long period. As we noticed from the qPCR, some of the cultures managed to maintain a healthy albumin gene expression. Thus, a contributing factor to the disparity between the greatly declining production levels after the first day and the slower deteriorating gene expression is most likely due to cells dying

through the duration of the culture (expected from cells freshly harvested and seeded, especially after initial seeding). Therefore, the drop in total albumin production found in the media is not necessarily due to loss of hepatospecificity of the cells but just production from less overall viable cells. Hence, we believe our system is capable of providing and enhancing some maintenance of liver-specific functions compared to current standard methods.

5.0 CONCLUSIONS & FUTURE DIRECTIONS

We have successfully shown that our novel bioengineering platform can successfully enhance CYP3A2 activity. This was ultimately done by improving the biophysical features of traditional sandwich cultures by optimizing the top peptide layer dimension to orders of nanometers to facilitate oxygen exchange and nutrient diffusion. Additionally, the biochemical aspects of typical cultures were enhanced by engineering the scaffold with the introduction of functional peptide motifs to strategically target certain cell receptors responsible for the activation of numerous vital cell functions.

Our attempts to minimize peptide layer thickness proved successful; consequently, the next steps will be to focus on the impedance caused by the thick bottom layer of the sandwich culture. Of course it would be prudent to rerun the aforementioned experiments to ensure reproducibility; however, it would be a waste of resources and time since it has been established that minimizing peptide thickness is essential. As a result, we currently have several possible methods that we would like to execute. The first of methods would be to try to minimize the peptide layer by using dilutions of the peptide. This did not work for the top layer against the membrane because we were purposefully avoiding having to gel the self-assembling peptides since we believed the gelled thickness was worse than what we might be able to achieve through the immobilized RAD16-I peptides. However, dilutions of RAD16-I followed by gellification has been shown to produce stable layers up to 0.5% (Genove et al., 2006). Therefore, we will try gellifying dilutions of 0.5% and lower to try to obtain a thinner peptide layer.

Our second method to reduce the bottom peptide layer involved using the peptide-coated modified membranes as both the top and bottom surfaces. This would ensure that the self-assembling peptide surfaces are dimensionally controlled. In this system, the cells would be sandwiched between the two coated membranes and placed in a cell culture insert to take advantage of the media flowing from the inside of the insert, through the culture, and out the bottom membrane as opposed to a stagnant well with no flow whatsoever.

Likewise, our last method to be tested entails directly plasma-modifying the tissue culture insert membrane. This might improve upon the two membrane sandwich above. By modifying the insert membrane itself, we would be able to control the peptide layer directly on that surface. The only slight disadvantages that it might pose would be handling the entire tissue culture insert during the plasma modification and the peptide incubation steps. This might prove somewhat cumbersome but would definitely be feasible. Nevertheless, this method would seem to be the most effective way of controlling both top and bottom layer.

There are additional features we would like to introduce to our platform that might enhance the hepatocellular functions. One such proposal would be to culture cells on both the top and bottom layer and then stack after a day (long enough for cells to attach but short enough so cells do not start losing hepatospecific functions). This is inspired from a study that constructed stacked-up structures of hepatocytes to create highly differentiated 3D tissues, including functional bile canaliculi between the two stacked layers and RNA levels of hepatic-differentiation markers (Sudo et al., 2005). This could hypothetically be done on any of the three methods explained above. It would be an interesting future experiment since the tissue-like organization might help to improve CYP3A2 and other gene expression levels.

Another feature we would like to eventually implement will involve an automated media feeder for the sandwich culture system. As noted earlier, hypoxic conditions can be caused by typical medium depths of 2-5 mm (McLiman et al., 1968). In most cases, an abundant amount of media is placed in culture to ensure a plentiful amount of nutrients. An automated system will help to deliver an amount of media that is enough to thoroughly saturate the cells and provide a constant fresh source of nutrients. Additionally, the automation will allow a regulated flow of media that can easily be controlled to maintain a depth of below 2 mm.

This platform has greatly improved upon previous hepatocyte sandwich culture methods. We have shown that our bioengineered system allows control of both biophysical and biochemical parameters that have not been previously achieved in isolated hepatocyte culture studies. By improving both fronts we were able to successfully enhance the notoriously downregulated CYP3A2 gene. The potential of this system is noteworthy and can be further improved.

Through additional tuning and modifications, we hope to further enhance hepatospecific functions to achieve in vivo levels.

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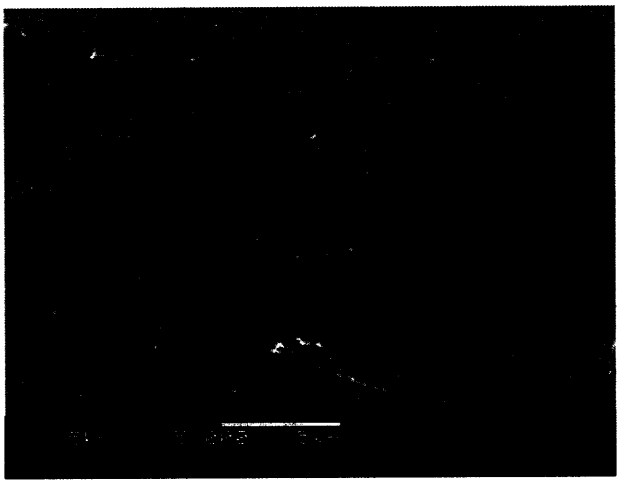
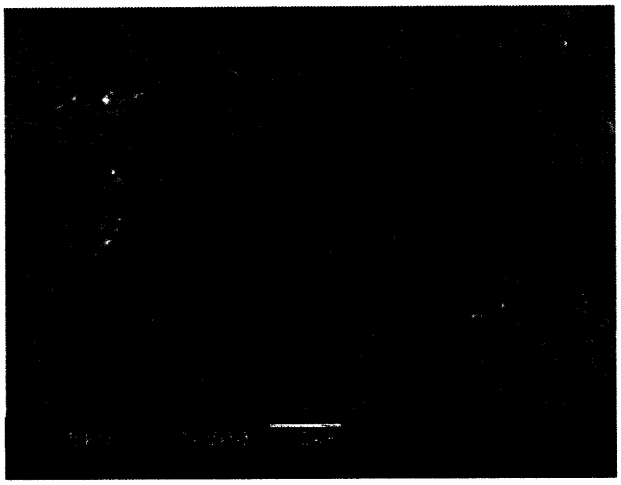
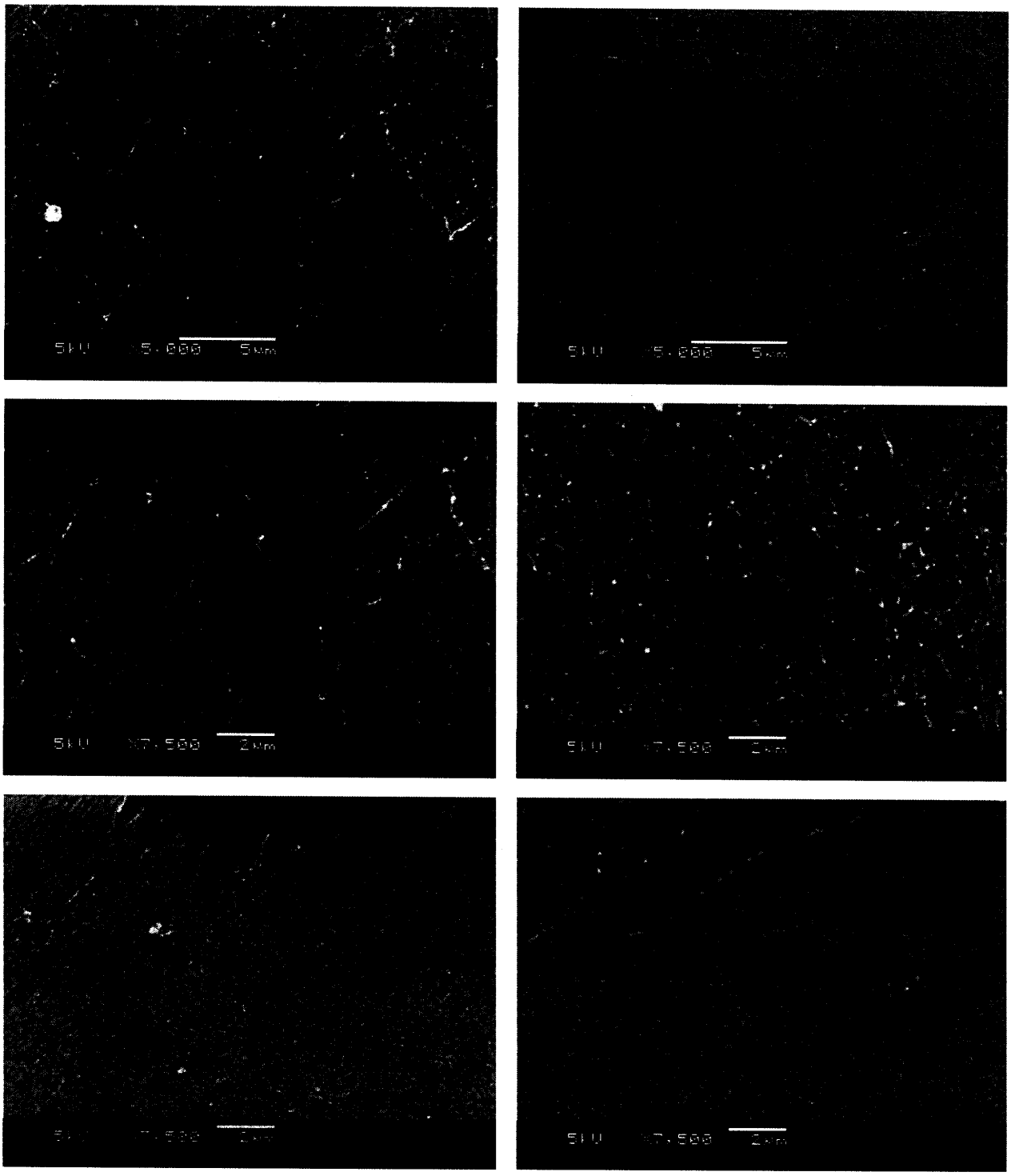
8.0 Appendix: Additional Images

8.1 PEEK-WC-PU/PdAA +RAD16-I Peptide





8.2 PEEK-WC-PU/PdAA/RAD16-I +RAD16-I Peptide



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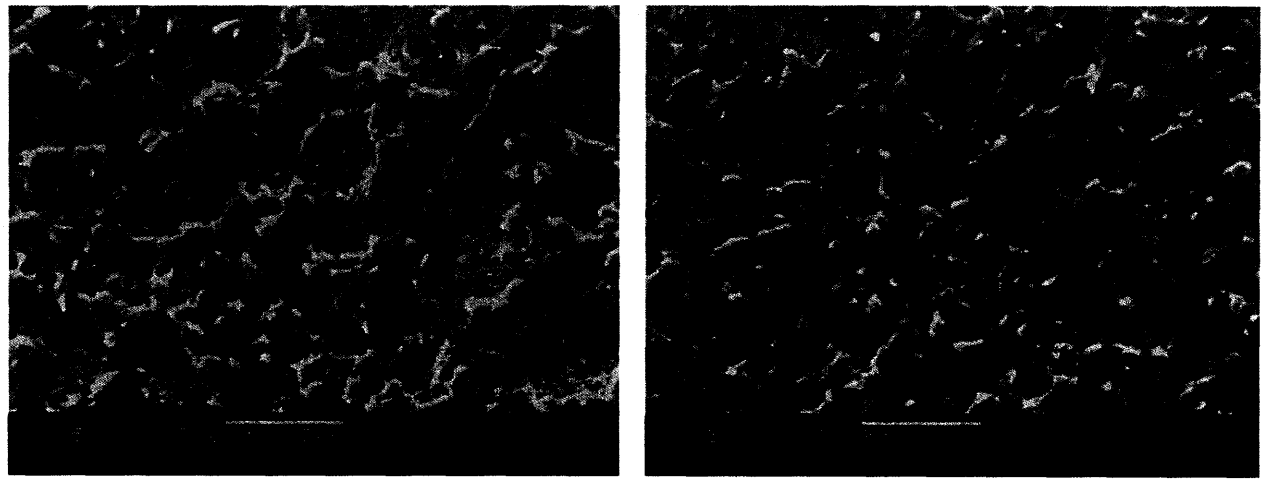
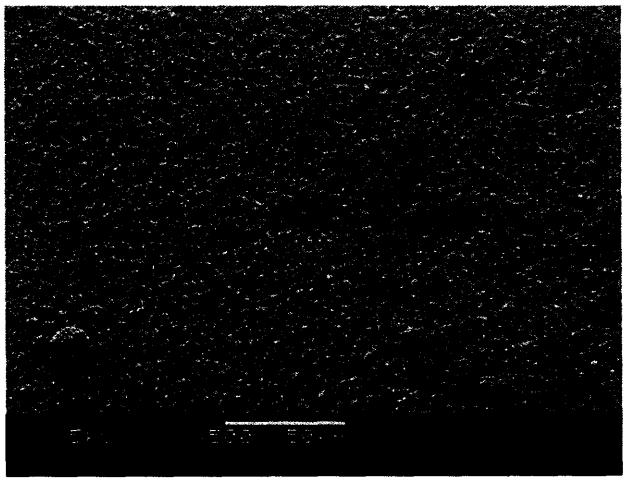
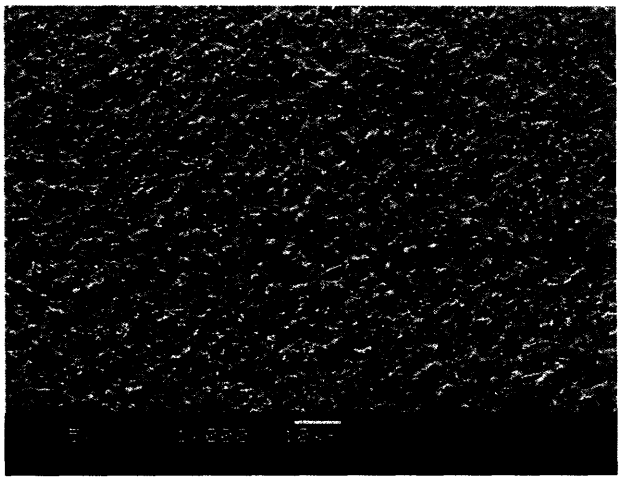
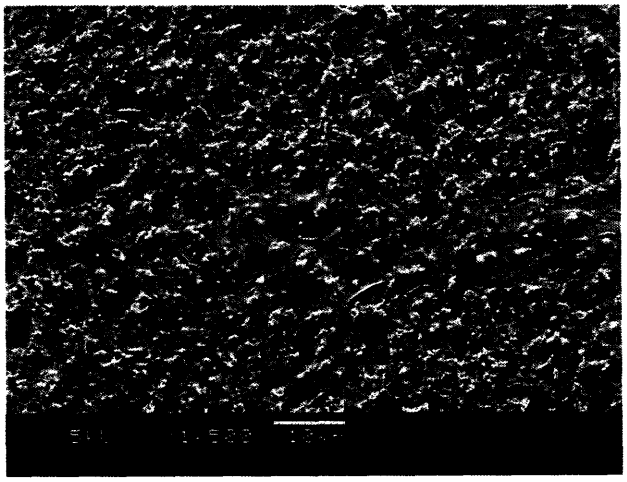
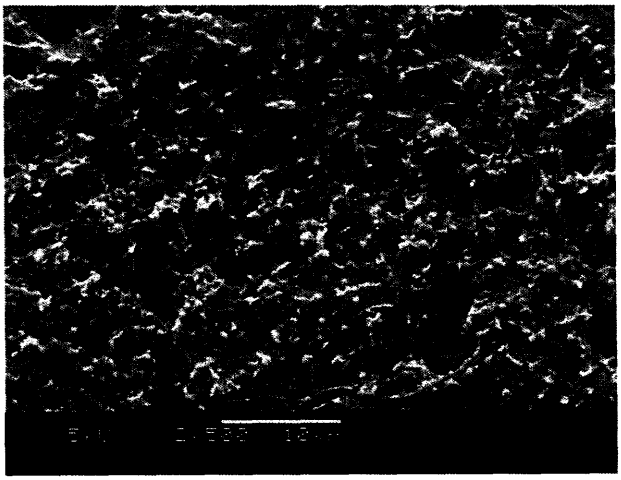


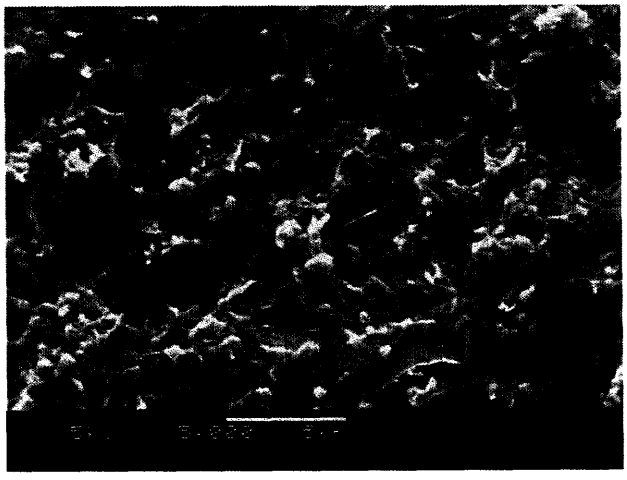
8.4 PTFE/PgPFM/RAD16-I +RAD16-I Peptide



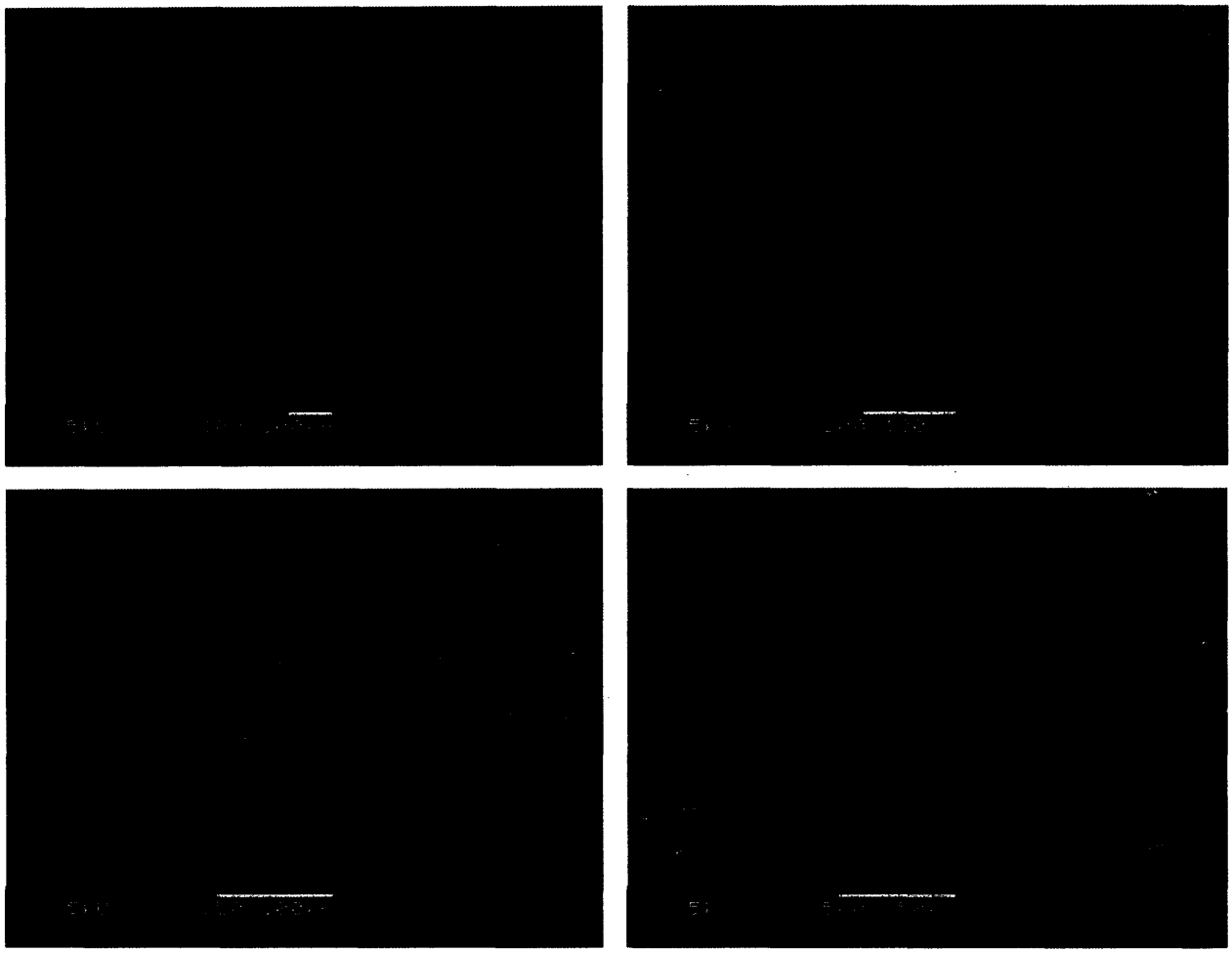




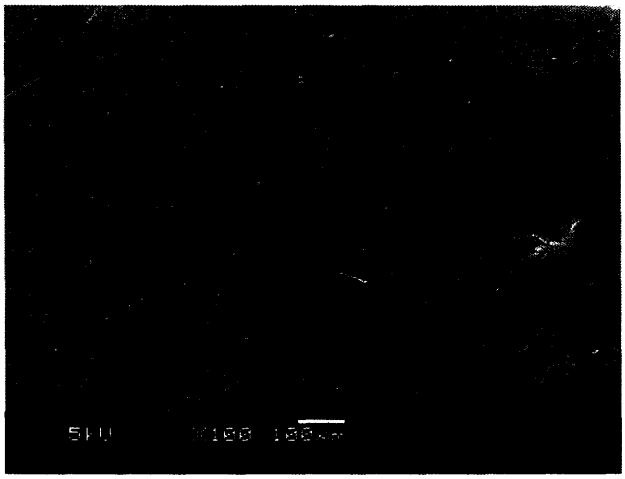


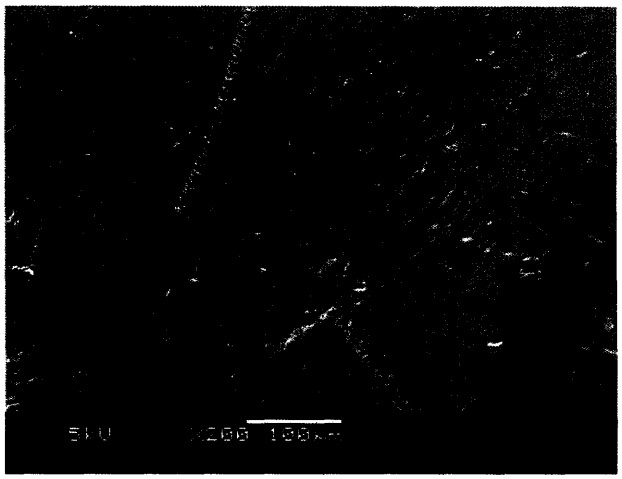


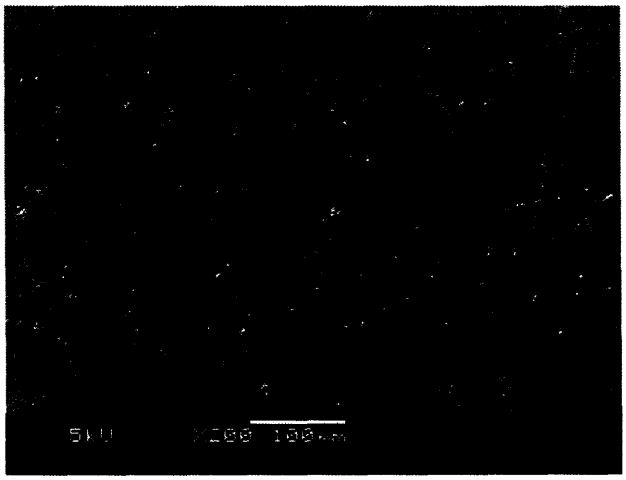
8.5 PEEK-WC-PU/PdAA –RAD16-I Peptide +Hepatocytes

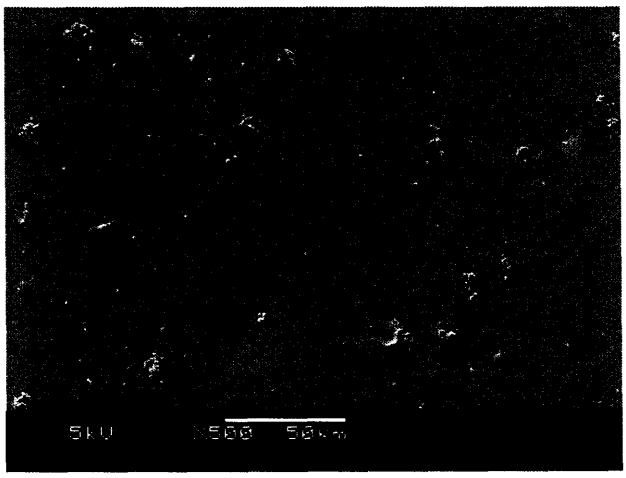


8.6 PEEK-WC-PU/PdAA +RAD16-I Peptide +Hepatocytes

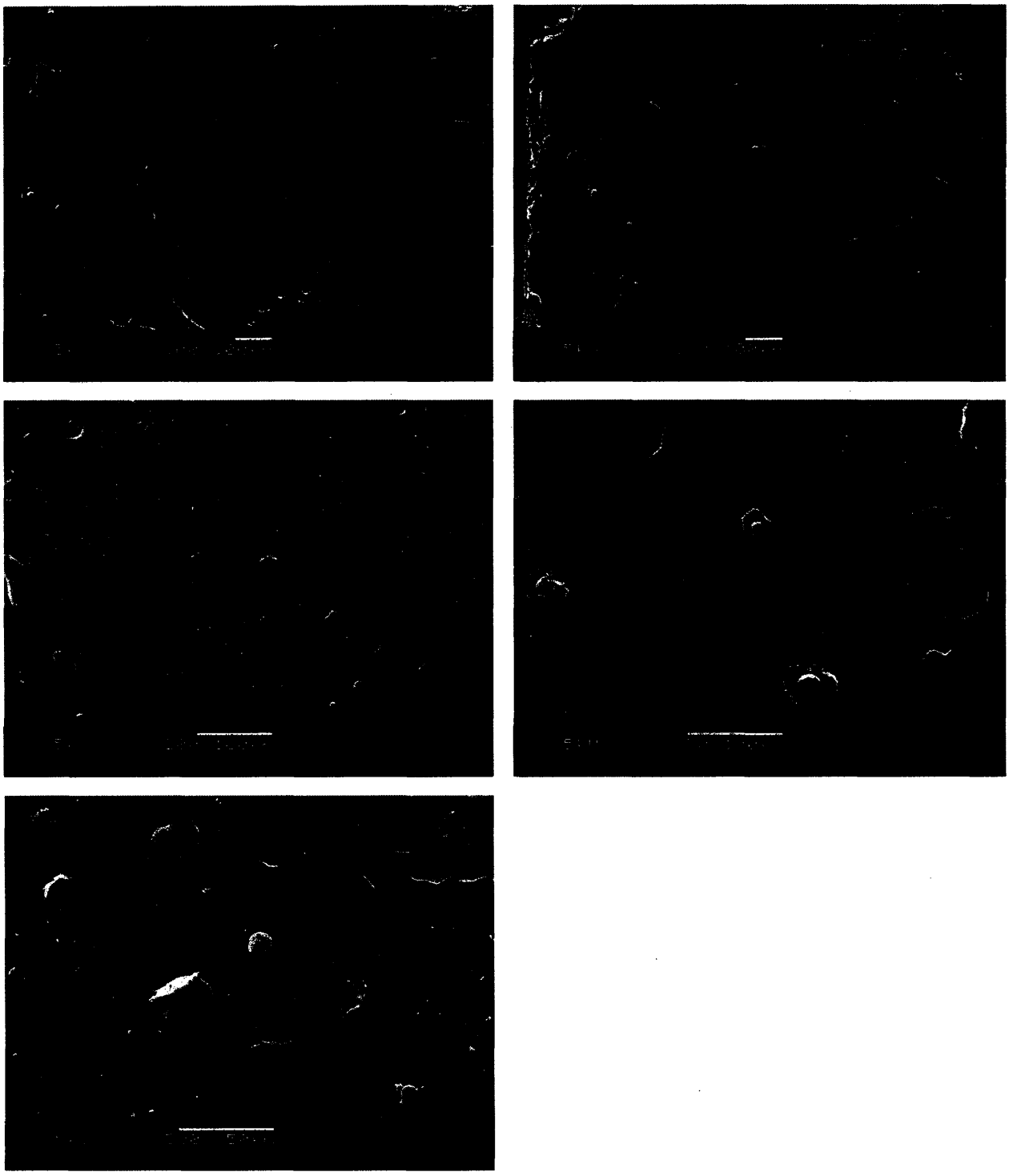




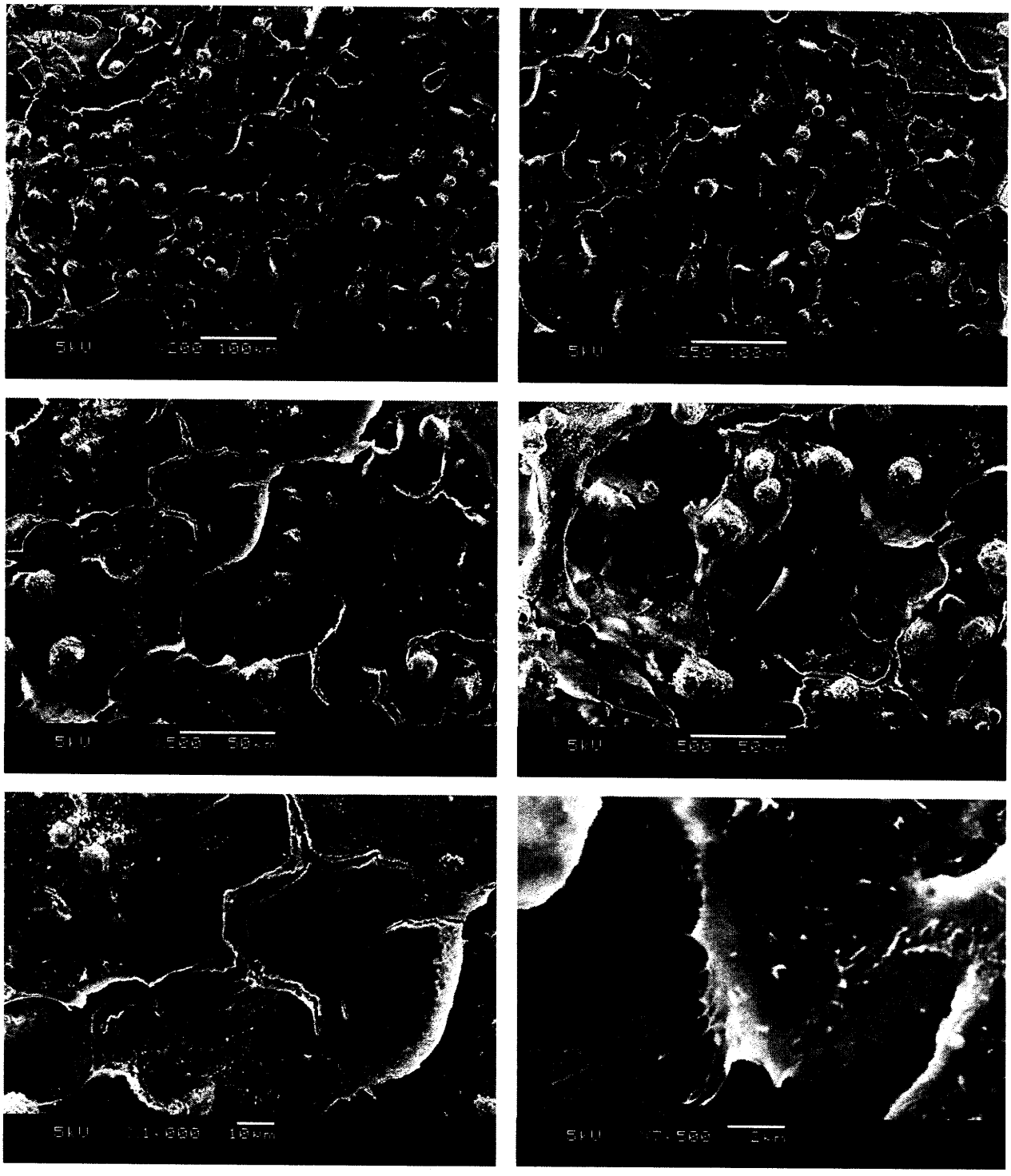




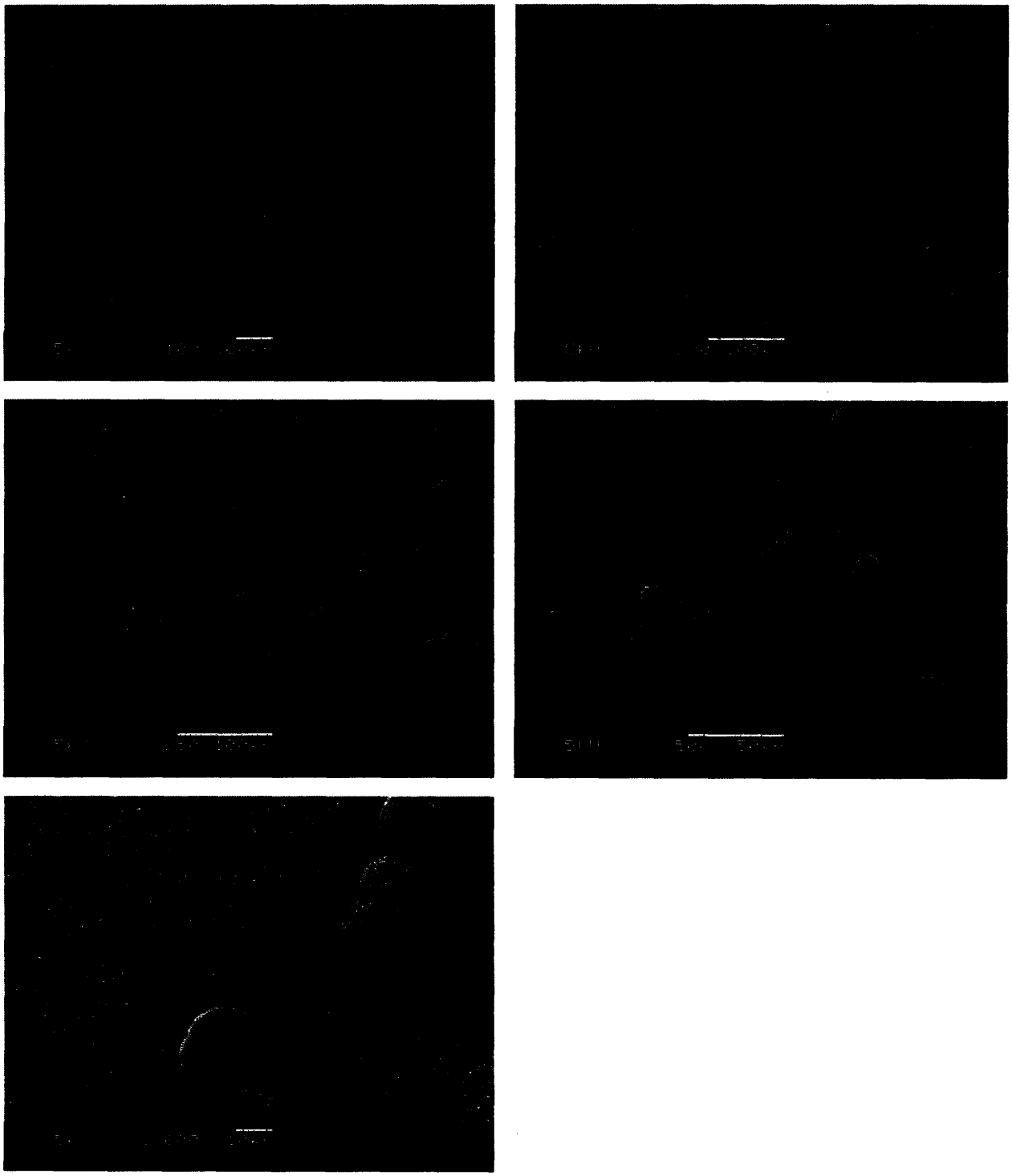
8.7 PEEK-WC-PU/PdAA/RAD16-I –RAD16-I Peptide +Hepatocytes



8.8 PEEK-WC-PU/PdAA/RAD16-I +RAD16-I Peptide +Hepatocytes



8.9 PTFE +RAD16-I Peptide +Hepatocytes



8.10 PTFE/PgPFM/RAD16-I +RAD16-I Peptide +Hepatocytes