NOVEL PREPARATION OF POLYMERIC SCAFFOLDS FOR TISSUE ENGINEERING USING PHASE SEPARATION WITH PROTEIN MICROBUBBLE INCORPORATION

by

ASHWIN MOHAN NAIR

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ABSTRACT

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Ashwin Mohan Nair, MS

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Supervising Professor: Liping Tang, Ph.D.,

Poly (lactic-co-glycolic acid) (PLGA) has been extensively used as a biodegradable and porous tissue scaffold due to their biocompatibility and degradability. Several techniques have been developed for the fabrication of polymeric scaffolds but they all have some limitation or the other which has in turn hampered the growth of cells seeded on them. Salt leached scaffolds have good porosity and large pores but poor mechanical strength and the problem of residual salt even after leaching. Similarly gas foaming scaffolds have shown large pores but poor interconnection with residual ammonium salts. Other methods like thermally induced phase separation and

fiber bonding do not involve the use of porogens and have yielded porous scaffolds although with small pores. To overcome these common problems, in this study, the use of protein microbubbles as a porogen and drug/protein carrier to produce polymeric scaffolds with good porosity was conceptualized. PLGA scaffolds were prepared by thermally induced phase separation with the incorporation of protein microbubbles. Two types of protein microbubbles, BSA and gelatin, were evaluated for their suitability as a porogen. The microbubbles which were incorporated in to the scaffolds at the time of fabrication were analyzed for their size and the scaffolds were characterized by SEM analysis and histological techniques. SEM revealed the synthesis of open pores measuring around 100 to 120 μm regardless of the type of protein used for synthesizing the microbubbles.

This novel technique provides two distinct advantages. First, microbubbles are made of biological materials which have no toxicity. Second, apart from having produced scaffolds with larger pores compared to conventional methods, our novel scaffold also has the potential to function as a delivery mechanism for chemokines and drugs in to the polymeric matrix. The scaffold can be degraded in a controlled manner to release the desired molecules to effect the desired cellular response.

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LIST OF ABBREVIATIONS

PLLA …….	Poly L Lactic Acid
PLGA …….	Poly L Lactic-co-Glycolic Acid
FDA ……	Food & Drug Administration
ECM …….....	Extra Cellular Matrix
BSA …….	Bovine Serum Albumin
SEM …….	Scanning Electron Microscope

CHAPTER 1

INTRODUCTION

Man has always endeavored to understand the nature of the physical world around us. From the ancient Greeks to modern day scientists, chemists have investigated natural materials and devised ways to synthesize new materials. The quest for developing the best technique is a never ending process and has been the driving force behind this project.

1.1 Need for Tissue Engineering

The loss or failure of an organ or tissue is a frequent, devastating and costly problem in health care, occurring in millions of patients every year. In the United States itself approximately 9 million surgical procedures are performed annually to treat these disorders and 40 to 90 million hospital days are required. The total national health care costs for these patients exceed $500 billion per year [1, 2, 29].

1.1.1 Existing Treatment & Limitations

Some of the existing ways to treat organ or tissue loss is by transferring the tissue from one location in the human body to the diseased site which is commonly done in clinics [1]. Autografts often have great survival rate. However, the availability of these tissues is limited. Another way is by transplanting organs from one individual to another or by surgical reconstruction. The major problem with allografts is availability of donors, and if a donor is available, chronic immune rejection limited the

success of the procedure. During 1996, only 20,000 donor organs were available for 50,000 patients in need. According to the United Network for Organ Sharing, as of February 15, 2006 there were 90,952 patients waiting for a transplant. Hence, many patients would die waiting for an organ donor.

Stem cell therapy is considered to be an alternative but is limited by the availability of cells and their lack of mechanical strength. The high cost associated with stem cell therapy is also a drawback [6, 14].

Yet another treatment method involves providing an artificial device which takes over the function of the damaged organ or tissue. However, mechanical devices cannot take over the functions of an organ and cannot prevent progressive patient deterioration [1, 4, 6].

Although these therapies have saved many lives, they are imperfect solutions [1]. This dilemma gave birth to the field of tissue engineering which carries on its shoulders the responsibility of providing a viable alternative for the countless medical problems facing mankind today.

1.2 Tissue Engineering

Tissue engineering can be defined as "the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function [5]."

Efforts to develop bioartificial tissues and organs for therapeutic purpose dates back to more than 30 years, but they have appeared to be more realistic and achievable in the last decade. Tissue engineering is a relatively new interdisciplinary field applying a set of tools at the interface of biomedical and engineering sciences that use living cells or attract endogenous cells to aid tissue formation or regeneration, to restore or maintain tissue function [6, 9].

The underlying concept of tissue engineering is the belief that cells can be isolated from a patient and associated with a scaffold, which guides tissue development and is subsequently implanted. Such scaffolds can be natural, man-made or a composite of both. The resulting tissue engineering construct is then grafted back in to the same patient to function as the introduced replacement tissue [10, 11, 12, 13]. It is advantageous in comparison with cell transplantation in that organized three dimensional tissues are designed and developed.

1.3 Production of a Tissue Engineered Product

The key components of a tissue engineered product are cells, scaffold, signals, bioreactor and biocompatibility. It is well established that one of the major challenges for tissue engineering is the creation of degradable scaffolds. To permit the growth of cells into 3-D structure, scaffolds are often porous and made of degradable polymers. The use of degradable polymer allows the slow disintegration of the building block along with the displacement of new tissue in the patients. Scaffolds can also be designed to release signals that induce cell growth and differentiation [16, 19].

CHAPTER 2

SCAFFOLDS

Scaffolds play a critical role in tissue engineering by acting as a temporary tissue construct or building block for cell accommodation, proliferation, and differentiated function as well as serving as three dimensional templates for neotissue/organ formation. While cells could be, ideally, autologous, the most important parameter for scaffolds is that they should be biodegradable and biocompatible. Biodegradability implies that the degradation products should be harmless to the surrounding environment.

The scaffolds should stay long enough to facilitate this but not long enough to hinder the formation of tissue. They should degrade in a timely manner so that they degrade as the new tissue takes its place [1, 7]. When the scaffold is implanted in situ, its biocompatibility becomes a vital parameter, which means that the scaffold should not elicit a host immune response.

The selection of the material for a scaffold still remains a key factor in the design and development of tissue engineering constructs, especially if it is considered that the biomaterial employed must produce controlled and predictable interactions with cells.

2.1 Types of Polymers

Two properties are used to classify the polymers used for synthesizing scaffolds. First the material is categorized based on its ability to break down in vivo as either absorbable or non absorbable. Second, the source of the material is used to classify it as either natural or synthetic [22]. From these two broad categories one can deduce four classes of polymeric biomaterials. Figure 2.1 summarizes the classification.

Natural Absorbable	Synthetic Absorbable
Natural Nonabsorbable	Synthetic Nonabsorbable

Fig 2.1 Classification of polymeric biomaterials.

2.1.1 Absorbable and Nonabsorbable Polymers

Considerable focus is being laid on absorbable polymers which typically take the form of fibrous meshes, scaffolds or hydrogels. The polymer degrades at a controlled rate and the cells infiltrate the matrix and replace the polymer space with natural tissue.

Absorbable polymers [22]:

- Provide less risk of infection than non absorbable polymers.
- Can be optimized for specific applications. For example, porosity, pore size, degradation , etc., can be controlled depending upon the specific need

- Minimizes the need for surgical removal after the purpose has been served.

2.1.2 Natural and Synthetic Polymers

Natural polymers like proteins and polysaccharides were among the first used materials for tissue engineering scaffolds and have been used since the 1970s. They have found wide application in the field of tissue engineering.

They are advantageous, in that, they contain information like amino acid sequence, which facilitates cell attachment or maintenance of differentiated function. They do not induce a host response, and may enhance the biological recognition in the growing neo-tissue, encouraging the normal cellular functions [27]. However, many natural materials suffer batch to batch variations and their purity is questionable. They, also, have poor mechanical strength.

Synthetic materials like biodegradable polyesters have gained a lot of recognition over the years and have also been approved for human use by the FDA [8, 15]. Poly (glycolic acid) (PGA), poly (lactic acid) (PLA) and their copolymer poly (lactic-co-glycolic acid) (PLGA) have been widely applied in bone and cartilage repair [28, 29, 30]. Poly (ε-caprolactone) (PCL), which degrades at significantly lower rates than PLA, PGA, PLGA, is less attractive for tissue engineering applications.

Synthetic materials are advantageous as their chemistry and material properties like biodegradation profile, microstructure, and hydrophobicity can be well controlled [4]. They do not suffer from many of the problems encountered with natural materials. The thermoplastic aliphatic polyesters like poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactide-co-glycolide) (PLGA) have generated a lot of interest over the past

two decades because of their properties of biocompatibility and biodegradability [15]. Synthetic polymers have the following advantages over natural polymers [22]:

- Synthetic polymers can be fabricated by molding, extrusion, solvent processing in to reproducible shapes and sizes with minimal batch-to-batch variations.
- Synthetic polymers can be designed to degrade at a controlled rate than in the variable rate observed in the case of enzymatic degradation of natural materials.
- Properties like molecular weight, hydrophobicity can be controlled in the case of synthetic polymers unlike natural polymers.
- Synthetic polymers are more versatile as their properties can be adjusted.

2.2 Scaffold Fabrication Techniques & Limitations

Pores are routinely created in scaffolds to promote three-dimensional tissue growth, nutrient diffusion, and vascularization. The size of pores must be large enough to allow the circumferential attachment of cells, yet small enough to encourage migration and proliferation [20, 21]. Porous scaffolds are fabricated using a variety of techniques. The fabrication technique plays an important role in implant properties and degradation characteristics. Some of the common scaffold fabrication techniques and their limitations are described in this section and summarized in table 2.1. It should be noted that solvent casting & particulate leaching, and gas foaming are the most popular techniques.

- **Solvent casting and particulate leaching** [31, 34]: The polymer is dissolved in chloroform or methylene chloride and then cast onto a Petri dish filled with the porogen (NaCl, etc.,). After evaporation of the solvent, the polymer/salt

composite is leached in water for two days to remove the porogen. The pore size can be controlled by controlling the size and amount of the porogen. With 70 weight percent salt and above the pores exhibited high connectivity [35].

In an alternate form of the particulate leaching method, the fabrication of PLLA and PLGA scaffolds with up to 87% porosity and pores well over 100 μm in diameter using waxy hydrocarbons as porogens has been reported [36]. After mixing the porogen and polymer (dissolved in methylene chloride or chloroform) into a paste, the composite is packed in a Teflon mold. The mold is immersed in a hydrocarbon solvent (pentane or hexane) to remove the wax without dissolving the PLLA/PLGA. The remaining foam is vacuum-dried for several days to extract any solvents. Thick samples (up to 2.5 cm) with interconnected pores can be created using this technique. This method also offers the possibility of adding a particulate phase to the paste to increase the strength or electrical conductivity of the final structure. When blended with polyethylene glycol (PEG) and seeded with bovine chondrocytes for four weeks, formation of cartilage-like tissue is seen in these foams, demonstrating their biocompatibility.

Advantages:

These scaffolds are highly porous.

One can control the pore size by controlling the size of the porogen.

Disadvantage:

However this method yields scaffolds which are structurally weak, thin and the presence of remnant salt can trigger immunogenic response upon implantation.

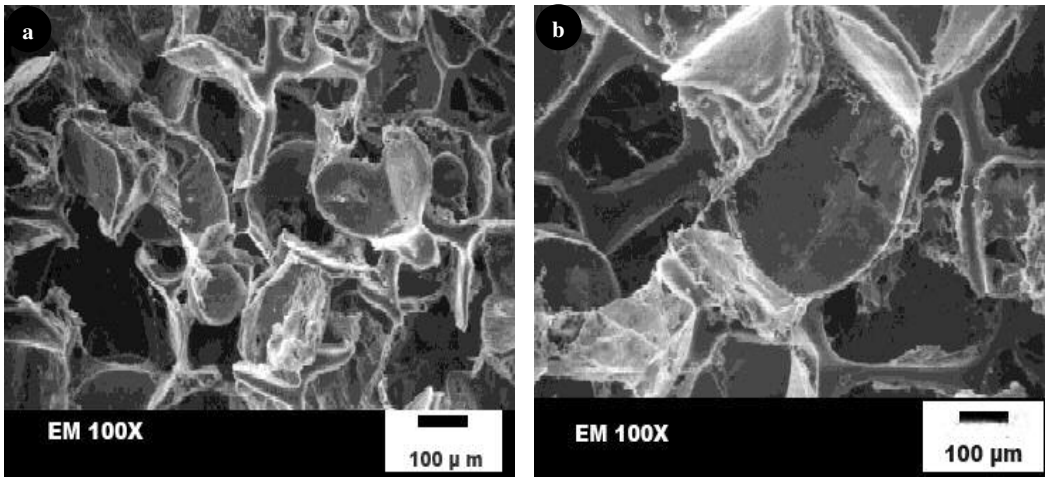

Fig 2.2 SEM image of PLGA scaffolds prepared by solvent casting and particulate leaching with (a) porogen salt particles of size 150-300 µm; (b) porogen salt particles of size 300-500 µm. [69]

The leaching step for waters-soluble porogens significantly increases the scaffold preparation time.

Also, the incorporation of biomolecules is very difficult.

- **Gas foaming** [34]: In order to eliminate the need for organic solvents, a new technique was developed [37]. Solid discs of PLLA or PLGA are prepared using compression molding with a heated mold. The discs are placed in a chamber and exposed to high pressure CO_2 (5.5 MPa) for three days, at which time the pressure is rapidly decreased to atmospheric pressure. Porosities of up to 93% and pore sizes of up to 100 mm can be obtained. However, the pores were unconnected. Although, no leaching was required and no harsh chemical

solvents were used, the high temperature involved in the disc formation made incorporation of cells and bioactive molecules almost impossible.

Another approach involved combining gas foaming and solvent leaching [38, 39]. Ammonium bicarbonate is added to a polymer solution in methylene chloride or chloroform, which is then shaped in a mold. The solvent is evaporated and the composite is immersed in warm water which leads to gas evolution and particle leaching; thereby, yielding large pores. Rat liver cells remained viable for one week in culture, suggesting that the scaffolds were biocompatible and facilitated nutrient exchange [38].

Advantage:

Scaffolds prepared by gas foaming were very porous and showed the presence of large pores.

Disadvantage:

However these scaffolds show very poor interconnection between the pores.

Residual ammonium salts can have catastrophic effects on cells when seeded.

- **Fiber Bonding:** Scaffolds based on the use of PGA fibers were some of the earliest constructs proposed in tissue engineering [40]. These fibers, when bonded together in three-dimensions, can provide large surface area for cell interaction and growth. The fibers can be attached to each other via two different techniques. In the first technique PGA fibers are immersed in a PLLA solution. When the solvent evaporates, the network of PGA fibers is embedded in PLLA. The composite is then heated to above the melting temperature of both

polymers. The PLLA melts first and fills all voids left by the fibers. This helps retain the spatial arrangement of fibers so that when the PGA begins to melt, the fiber structure does not collapse. Instead, in order to minimize interfacial energy, fibers at the cross-points become "welded" (melted) together, forming a highly porous foam. The PLLA is then removed by dissolution with methylene chloride. This fabrication technique results in foams with porosities as high as 81% and pore diameters of up to 500 μm [41]. Hepatocytes cultured for one week in these foams remained alive and began to interact with each other to form clusters [35].

In the second method PLLA or PLGA is dissolved in chloroform and sprayed onto the PGA fibers [42]. Since PGA is only weakly soluble in chloroform, the fibers remain unchanged during this process. The solvent is then evaporated, leaving the fibers glued with PLLA or PLGA. Although porosities were not reported, pore sizes similar to those of the previous technique were attained. When tubes made in this manner were implanted in rats for 17 days, fibrous tissue ingrowth was observed, indicating that constructs with these physical properties could encourage neotissue formation [42].

Advantage:

This technique yields scaffolds with good porosity.

Interconnected pores were observed.

Disadvantage:

Although fiber bonding techniques produce highly porous scaffolds with interconnected pores that are suitable for tissue regeneration [40, 41, 42] it methods involve the use of solvents that could be toxic to cells if not completely removed.

In order to extract these chemicals, the constructs must be vacuum dried for several hours, making it difficult to be used immediately in a clinical setting. In addition, the first method involves heating to high temperatures.

The combination of toxic chemicals and extreme temperature presents difficulties if cells or bioactive molecules, such as growth factors, are to be included in the scaffold during processing.

- **Membrane Lamination:** A contour plot of the three-dimensional anatomical shape is first prepared [43]. Highly porous PLLA or PLGA membranes were then manufactured using the solvent-casting and particulate leaching technique. The adjacent membranes are bonded together by coating chloroform on their contacting surfaces, thereby yielding three-dimensional structures [31].

  Advantage:

  Scaffolds of desired porosity can be fabricated.

  High porosity of scaffolds is observed.

  Disadvantage:

  Toxic solvents are harmful when cells are seeded.

- **Thermally Induced Phase Separation** [34, 57, 58, 59, 60]: In the thermally induced phase separation (TIPS) process, the polymer, PLGA, is dissolved in a

solvent with a low melting point that is relatively easy to sublime, like 1, 4-dioxane at a low temperature. Subsequently, liquid-liquid or solid-liquid phase separation is induced by cooling the solution below the melting point of the solvent (polymer poor phase). The temperature can be lowered by either refrigerating at -20^{o}C or by quenching in liquid nitrogen to -196^{o}C. The cooling parameters for the solution play an important role in determining the morphology of the resultant scaffold. At temperatures just below the critical temperature (or cloud point in the case of polydisperse polymers), such as the one used in this study, the phase separation occurs via a nucleation and growth mechanism. At lower temperatures, the phase separation occurs via spinodal decomposition. While the nucleation and growth mechanism results in spheroidal domains, spinodal decomposition causes the formation of interconnected cylinders. The scaffolds are freeze-dried at -55°C, resulting in the removal of the solvent by sublimation. The freeze-drying process converts frozen solid solvent directly into gas phase, skipping the liquid phase entirely. The basic idea of freeze-drying is to "lock in" the composition and structure of the material by drying it without applying the heat necessary for the evaporation process, thereby, preserving the shape, texture and composition of the material. The removal of the frozen solvent rich phase by sublimation yields scaffolds with a microporous structure and porosity as high as 90%.

Advantage:

These scaffolds are highly porous and show interconnected pores.

Table 2.1 Scaffold fabrication techniques - Advantages and Limitations. [31, 33]

Technique	Advantage	Limitation
Fiber weaving	• Easy process. • High porosity.	• Poor structural stability.
Fiber bonding	• High porosity.	• Poor mechanical strength. • Residual organic solvent. • High processing temperature limits biomolecules incorporation.
Solvent casting and particulate leaching	• High porosity. • Large pores. • Crystallinity can be tailored.	• Very thin membranes (about 3 mm). • Poor mechanical strength. • Residual solvent. • Immunogenic remnant salt. • No incorporation of biomolecules.
Membrane lamination	• 3-D structures can be obtained.	• Poor mechanical strength. • Limited pore interconnectivity. • Residual solvent.
Gas Foaming	• High porosity.	• Poor pore-interconnection. • Residual solvent and salts.
Thermally Induced Phase Separation	• Highly porous. • Incorporation of biomolecules without loss of activity. • Good solvent extraction.	• Small pore size. • Difficult to control internal scaffold architecture.

Disadvantage:

The limitation of this procedure is that the pore size has been found to be very

small in the range of 13 to 35 μm.

2.3 Requirements of Ideal Scaffolds

Regardless of the type of polymer used, all tissue engineering scaffolds should meet the following requirements [22, 31, 57, 58, 59, 60]:

- Easy processability and ease of handling
- Surface properties that promote cell adhesion, proliferation, and differentiation.
- Mechanical properties to withstand stress.
- Large surface area to volume ratio to allow cell seeding.
- Biocompatibility so that degradation products are not harmful and are expelled out of the body through normal metabolic pathways.
- Controllable rate of degradation of the scaffold.

2.4 Development of Better Porogens

Porogens have been used to render pores to the scaffolds and takes advantage of the fact that inorganic salts and sugars which are used as porogens do not dissolve in organic solvents, which dissolve the polymer, and hence they can be extracted by water later on in the fabrication process leaving the polymer behind. This is the basis of solvent casting and particulate leaching and gas foaming techniques. However, the major problems associated with the use of porogens is that their incorporation does not guarantee homogenous pore distribution in the scaffold and if they can yield high porosity the pores are not big enough to facilitate the migration of cells in to the matrix. Furthermore, the residual porogens in scaffolds can lead to cell death, as has been observed in the case of scaffolds prepared by salt leaching and gas foaming. Another

disadvantage is that during porogen leaching, continuous exposure of polymeric scaffold to water may lead to its degradation [68]. Hence, there is an urgent need to develop porogens with low or no toxicity and improved biocompatibility. Apart from these properties, it would be a major advantage if porogens can also serve as a drug delivery device. Recently, we have identified that protein microbubbles, which are currently used in the field of medicine, have many unique characteristics making them suitable candidates for serving as porogens.

CHAPTER 3

PROTEIN MICROBUBBLES

3.1 Creation of Microbubbles

Microbubbles had been first used in the 1980s to image angiogenesis in animal models chemically engineered to grow new blood vessels. They functioned as contrast agents to improve ultrasound scanning. Contrast agents have been used in the field of imaging for a long time but had little place in the field of ultrasonography [46, 48]. However, the introduction of microbubbles, typically around 3 μm, by an intravenous injection was found to improve the quality of ultrasound images. Also, clinical experience has shown that the tiny volume of air in the microbubbles doesn't pose any danger to the subject and in fact microbubbles have been found to be safer than the conventional contrast agents used in radiography and magnetic resonance imaging (MRI) [44]. Although microbubbles were originally designed to improve ultrasound scanning they have also opened up a plethora of emerging applications.

3.2 Evolution of Microbubbles

Early day contrast agents consisted of a dispersion of air-filled microbubbles in biocompatible liquids like saline or viscous dextrose [46, 52]. Air was dispersed in to the medium immediately prior to injection in the organism. These first generation microbubbles however resulted in bubbles of very poor stability and burst as soon as they rose to the top of the solution [46, 52, 53]. However, even these short lived

microbubbles improved the quality of the images obtained and hence researchers worked on improving the lifetime of these microbubbles.

In order to achieve this, a shell was placed around the gas core creating the second generation of ultrasound contrast agents [52]. A thin shell, usually a polymer, protein or lipid-based surfactant was prepared. These air-filled microbubbles had extended storage ability but were yet not very stable in vivo as they were rapidly destroyed in the bloodstream. The destruction of microbubbles occurred as a result of gas loss from the core. Thus the second generation of microbubbles came in to being with perfluorocarbons which had a very low permeability, being used. This is the type of microbubbles currently being used in the field of imaging [46, 52].

The third generation microbubble contrast agents were designed to have high reflectivity; however, it still remains to be evaluated [67]. In this study, the possibility of using gas filled microbubbles with a protein shell as a porogen to render pores in a polymeric scaffold and at the same time deposit the protein it carries along the pores of the scaffold, has been conceptualized.

3.3 Clinical Application of Microbubbles

The microbubbles which were predominantly used as contrast agents have come a long way and today have applications in the field of radiology, cardiology [45] and, as will be seen in the following sections, in tissue engineering scaffolds as porogens.

- **Microbubbles in general radiology:** Microbubbles increase the intensity of Doppler signals from blood for several minutes after their injection, and this effect can be prolonged by infusing them [51]. For example, they can improve detection of

flow in the intracranial arteries by transcranial Doppler in adults, where the skull greatly attenuates the ultrasound signal [52]. Another use is in detecting flow in smaller vessels, such as in the circulation of malignant tumors [53].

- **Specific diagnostic applications:** Imaging the liver is the most promising application of microbubbles in radiology. Some, but not all, microbubbles are taken up by the liver and spleen. The precise mechanism is unclear, but the reticulo-endothelial system is probably involved. The liver is particularly well seen with microbubble-specific imaging modes such as harmonic imaging. Another promising application is in the imaging of heart valve stenoses [53] and left ventricular function. Microbubbles will soon be used to assess myocardial perfusion [54].
- **Therapeutic applications** [55]: The use of microbubbles in treatments may eventually be even more important than their diagnostic uses [55, 56]. Microbubbles can aid drug delivery and function as agents to carry drugs for site-specific treatment. Their most exciting application is in the emerging area of gene therapy, where delivery of genetic material to a chosen site is difficult [56].

3.4 Mechanism of Microbubble Synthesis [48, 49]

A gas with a low permeability, like perfluorocarbons, nitrogen etc., is dispersed in a liquid which contains all the essential building blocks to form the shell that will cover the gas core. The shell forming liquid upon sonication undergoes cavitation and the protein deposits around the gas - liquid interface and reduces surface tension [46].

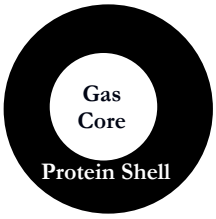

Fig 3.1 Illustration of a gas filled protein microbubble.

At the interface they partially unfold and associate to produce an intermolecular cohesive film with some degree of elasticity [63]. This process yields microbubbles which are more stable than the first generation microbubbles as the protein shell and the low permeation gas ensures that the microbubbles last for longer durations Fig 3.1.

CHAPTER 4

OVERALL HYPOTHESES AND EXPERIMENTAL DESIGN

4.1 Assumption

The thermally induced phase separation technique yields scaffolds with high porosity and good extraction of solvent although with very small pores. Incorporation of a suitable porogen can enable the formation of bigger, open, interconnected pores.

4.2 HypothesisI

Polymeric scaffolds with open interconnected pores can be fabricated using thermally induced phase separation by incorporating bovine serum albumin microbubbles as a porogen.

4.3 HypothesisII

Other protein microbubbles that mimic the ECM can also be used as a porogen for manufacturing polymeric scaffolds.

4.4 Experimental Design

Working on our assumption that protein microbubbles could be used as porogens to fabricate scaffolds with larger open pores using the process of thermally induced phase separation, we made protein microbubbles and incorporated them in different concentrations in to Poly (l-lactic-co-glycolic) acid (PLGA). The scaffolds that were synthesized were analyzed for their surface morphology using Scanning Electron Microscopy (SEM), histological studies to assess the internal architecture of the

scaffolds and to determine the role of the protein microbubbles in rendering porosity to the scaffolds.

CHAPTER 5

MATERIALS AND METHODS

5.1 Selection of Polymer

Selection of a suitable polymer is a very important step and for this study PLGA is used. Poly (L-lactic-co-glycolic) acid is a copolymer of poly (l-lactic) acid and poly (glycolic) acid as shown in figure 5.1.

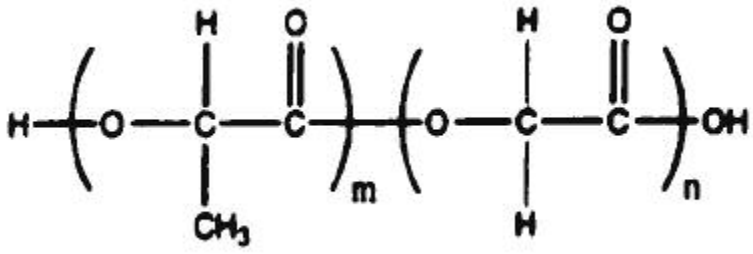

Fig 5.1 Structure of PLGA. [30]

PLGA has a glass transition temperature of 37^{o}C and is glassy in physiological conditions. They have a rigid chain structure and have good mechanical strength [26]. They degrade slowly and can be controlled by adjusting the ratio of lactide to glycolide. PLGA degrades in to lactic acid and glycolic acid; lactic acid is expelled from the body through the tricarboxylic acid (TCA) cycle while glycolic acid is eliminated through the kidneys unchanged and also through the TCA cycle [64].

5.2 Preparation of Bovine Serum Albumin (BSA) Microbubbles

The shell forming liquid consisted of 1ml PBS, 2ml 40% glucose (Sigma, St Louis, MO), and 2ml 40% BSA (Sigma, St Louis, MO) in a glass test tube. Nitrogen gas was bubbled through the mixture, while being sonicated using a probe sonicator (Ultrasonix, Bothell, WA) at 20 kHz for 10 seconds. This procedure resulted in the formation of nitrogen gas filled microbubbles which were surrounded by a BSA protein shell. The microbubbles were pipetted out and placed in glass tubes placed on a cold water bath. They were incorporated in to the polymer-solvent mixture prepared as will be described in section 5.5.

5.3 Preparation of Gelatin Microbubbles

The shell forming liquid consisted of 1ml PBS, 0.40 ml 40% glucose (Sigma, St Louis, MO), and 0.40 ml 70% Gelatin (Sigma, St Louis, MO) in a glass test tube. Nitrogen gas was bubbled through the mixture while being sonicated using a probe sonicator (Ultrasonix, Bothell, WA) at 20 kHz for 10 seconds. This procedure resulted in the formation of nitrogen gas filled microbubbles which was surrounded by a gelatin protein shell just as was observed in the case of BSA microbubbles. The microbubbles were pipetted out and placed in glass tubes placed on a hot water bath maintained at 45^{o}C. They were incorporated in to the polymer-solvent mixture prepared as described in section 5.5.

5.4 Measurement of the Size of BSA Microbubbles and Gelatin Microbubbles

A small droplet of the microbubbles solution was pipetted on the hemocytometer covered with a cover slip. They were observed under microscope (Leica, Wetzlar, Germany) to estimate the average size of the bubbles.

5.5 Fabrication of Scaffolds

The 75:25 Poly (d, l-lactic-co-glycolic acid) PLGA with a molecular weight of 113 kDa was purchased from Medisorb (Lakeshore Biomaterials, Birmingham, Al). 10% w/v PLGA was dissolved in 1, 4-dioxane (Aldrich, Milwaukee, WI) by vortexing, on a Thermolyne type 16700 mixer, for 30 minutes. The PLGA - dioxane mixture was poured in to Petri-dishes. The bovine serum albumin microbubbles and gelatin microbubbles were prepared as described in sections 5.2 & 5.3 respectively. The protein microbubbles were incorporated in to the PLGA solution in different concentrations of 21.5%, 35% and 45% which translated to 1ml, 2ml, and 3ml protein microbubble in 5ml PLGA-1, 4 dioxane solution.

The Petri – dishes containing the polymer solution with the microbubbles were quenched by two methods:

(1) at -20 ^{o} C (by placing in a refrigerator overnight), &

(2) to -196 ^{o} C by incubating in liquid nitrogen.

The control sample for each quenching method consisted of just the PLGA-dioxane mixture in a Petri – dish. The quenched experimental and control samples were then placed in a freeze dryer for 3 days, in order to remove the solvent by sublimation as described in section 2.2. Fig 5.2 summarizes the technique.

5.6 Characterization of Surface Morphology of Scaffolds

5.6.1 Scanning Electron Microscope (SEM) Analysis

Scanning electron microscopy analysis was done to characterize the surface of the PLGA scaffolds prepared by the above mentioned procedure. Small square – sections of the scaffold were sectioned using a razor blade and bonded to a steel stub using a colloidal silver adhesive.

The sections were coated with carbon using a sputter coater (CRC – 100 Sputtering Systems, Plasma Sciences Inc) for 15 minutes at an Argon (Ar) gas pressure of 8 mTorr and 50 mA, in order to minimize overcharging. They were subsequently observed under a JEOL JSM – IC 845A SEM which was operated under an accelerating voltage of 12 kV.

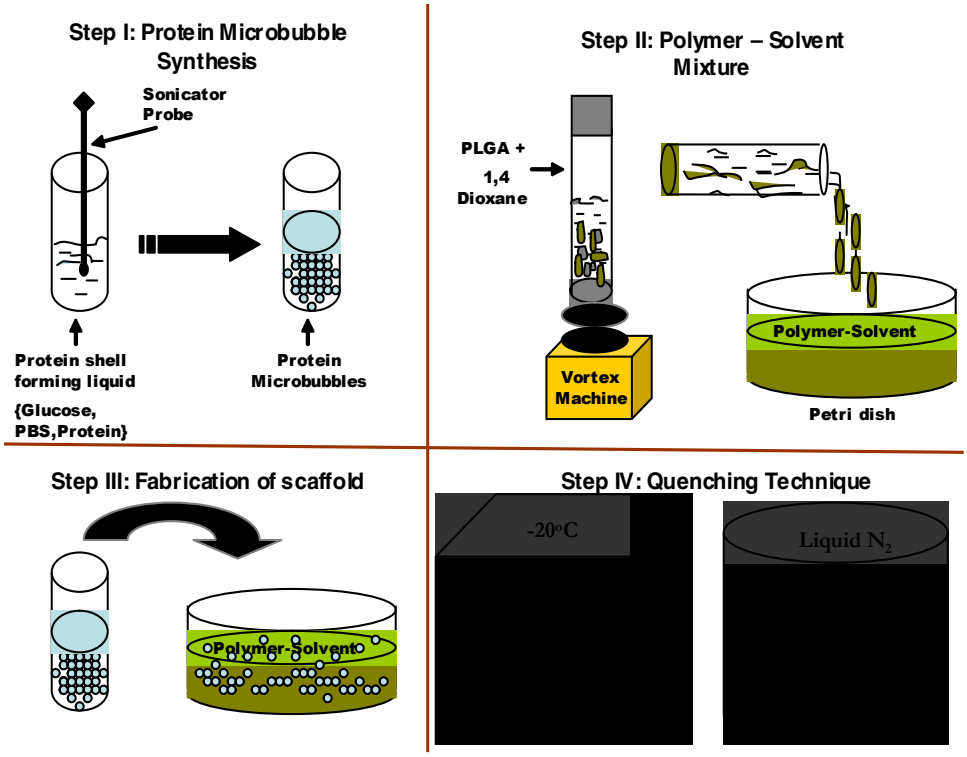

Fig 5.2 Steps involved in the fabrication of protein microbubble incorporated scaffolds.

5.6.2 Frozen Sectioning and Coomassie Brilliant Blue Staining

Square sections of the scaffold were cut using a razor blade and placed in a plastic mold in freezing medium OCT (Optimum Cutting Temperature). The molds were placed under vacuum at -70 kPa, for 60 minutes to facilitate the perfusion of the freezing medium through the pores of the scaffold. The molds were then placed in a cryostat (Leica, Wetzlar, Germany) and allowed to freeze overnight [58]. The scaffolds were sectioned in the cryostat at a thickness of about 10µm. The sections were collected on poly (l-lysine) treated positively charged slides.

Coomassie Brilliant Blue Staining

The Coomassie Brilliant Blue Assay is commonly used in biochemical and clinical laboratory tests. CBB exists in three forms, cationic (red), neutral (green) and anionic (blue). The blue dye form is generally considered to be the form that complexes the protein, since it's negative charge argues in favor of electrostatic attraction to arginin and lysine chains on the protein. In order to bind with CBB anion, the compound must possess both macromolecular form and an active basic or aromatic functional group [61, 62]. The domain two of bovine serum albumin contains a high number of basic amino acids that bind to the dye [63]. The number of Coomassie dye reagent ligands bound to each protein molecule is approximately proportional to the number of positive charges on protein. This dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues. Albumins are characterized by a high content of cystine and the charged amino acids, aspartic and glutamic acids, lysine, and arginine.

The PLGA scaffolds were sectioned in a cryostat as described in section 5.7. Coomassie Brilliant Blue staining solution was prepared by mixing 0.1% (w/v) of Coomassie blue dye (Gibbstown, NJ,USA); 45% (v/v) methanol (B&J ,Muskegon , MI); 45%(v/v) distilled water (AquaSolutions Inc,Jasper ,GA); 10% (v/v) glacial acetic acid (Mallinckrodt Baker Inc,Paris , KY).

The sections were first stained with Eosin for 2 minutes to create a pink background. The sections on the slides were then stained with CBB for 5 min. Coomassie blue destaining was accomplished by dropping the destaining solution which

consisted of 10 %(v/v) methanol, 10 %(v/v) glacial acetic acid & 80% (v/v) distilled water on to the scaffold sections for 5 minutes. The sections were then observed under phase contrast microscope (Leica, Wetzlar, Germany) and images were captured.

CHAPTER 6

EVALUATION OF BOVINE SERUM ALBUMIN MICROBUBBLES AS POROGENS - RESULTS AND DISCUSSION

6.1 Physical Characteristics of BSA Microbubbles

BSA when heat-treated, goes through two structural stages. The first stage is reversible whilst the second stage is irreversible but does not necessarily result in a complete destruction of the ordered structure [63]. Heating up to 65^{o}C results in a reversible change in structure. Sonication produces heat and excessive heat can result in the decomposition of BSA. Hence it was ensured that at any given point, the duration of sonication never exceeded 10 seconds. The mechanism responsible for forming the proteinaceous microbubbles is a combination of two phenomena: emulsification and cavitation. As nitrogen gas dispersed in the shell-forming liquid, sonication resulted in the deposition of BSA over the air bubble. The BSA microbubbles were observed under the microscope and showed a structure as shown in figure 6.1 (a). The figure reveals the presence of core and a shell. The core comprises of nitrogen gas while the shell mainly consists of BSA. To determine the size of these BSA microbubbles, several such images were captured and more than 2500 microbubbles were characterized on the hemacytometer to yield a size distribution as shown in figure 6.1 (b). The average size of the BSA microbubbles was observed to be in the range of 76µm. Almost, 70% of the microbubbles were in the range of 40 to 100 µm.

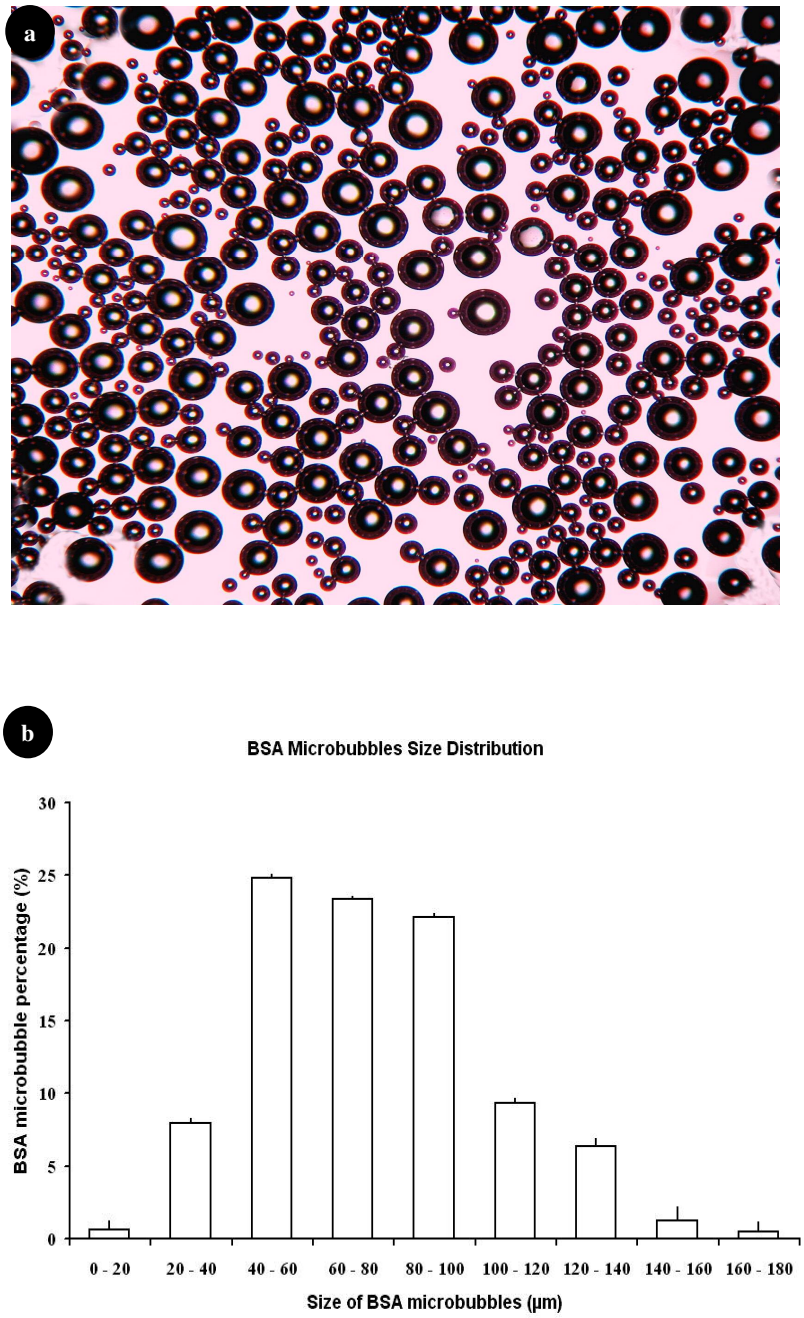

Fig 6.1 BSA microbubbles (a) structure observed at 10X magnification; (b) Graph showing BSA microbubble size distribution.

6.2 Characterization of Surface Morphology of Scaffolds

6.2.1 Scanning Electron Microscope (SEM) Analysis

Effect of BSA microbubble concentration on porosity of scaffolds quenched at -20°C:

PLGA in a 10% (w/v) proportion was dissolved in 1, 4 - dioxane to yield the polymer solution in to which the BSA microbubbles were incorporated. As described earlier, the scaffolds were quenched to -20°C and by liquid nitrogen to -196°C to induce phase separation. Thermally induced phase separation is based on the principle that a single homogenous polymer solution made at elevated temperature is converted to two-phase separated domains of a polymer-rich and a polymer-poor phase by removal of heat [57, 58, 59]. Figure 6.2 shows the surface morphology of PLGA scaffolds that were quenched to -20°C. Looking at the overall surface, the control scaffold, figure 6.2 (a), shows a microporous structure with pores measuring around 10 to 20 µm scattered scantily over the surface. In contrast, the scaffolds with 21.5% and 35% BSA microbubbles, figures 6.2 (b) & (c) respectively, show an open porous structure throughout the surface. The size of the pores is typically observed to be around 100 to 150 µm. Surprisingly, figure 6.2 (d) which shows PLGA scaffolds with 45% BSA microbubbles, did not reveal a surface which was as porous as the 21.5% or 35% BSA incorporated scaffolds. A closer look at each of these scaffolds under higher magnification, figure 6.2 (lower panel), revealed more circular pores in the case of 21.5% microbubble, figure 6.3 (f) and irregular pore shape in the case of 35% microbubbles, figure 6.2 (g). Close inspection of the scaffold with 45% BSA

microbubbles, figure 6.2 (h,) shows the presence of some open pores with interspersed grooves.

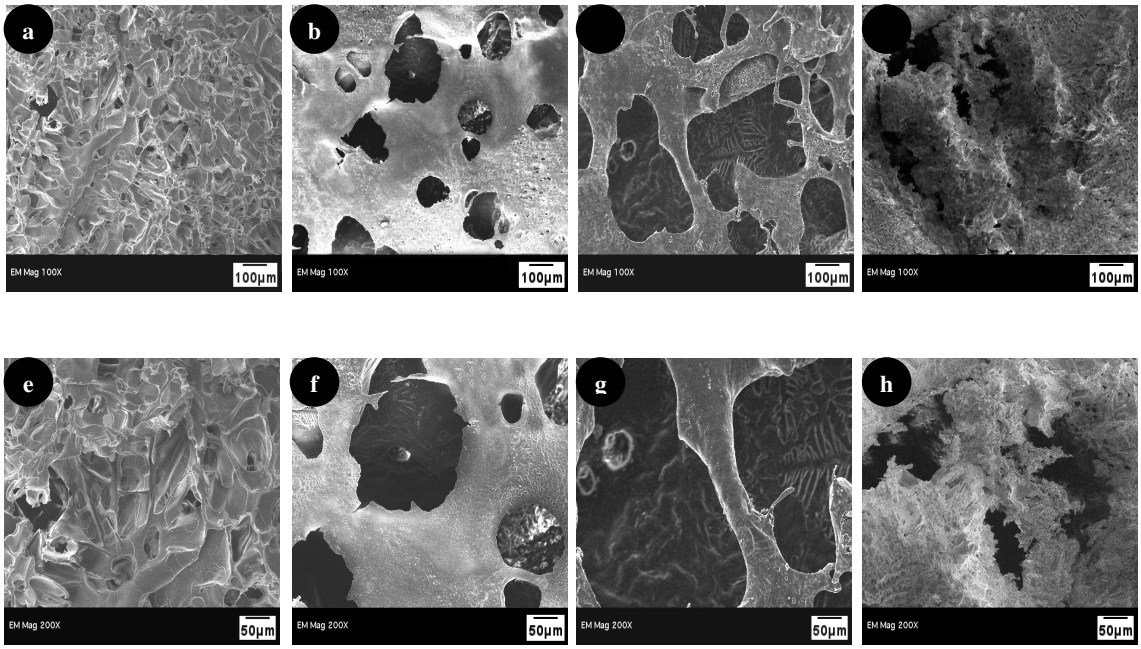

Fig 6.2 SEM image of PLGA scaffolds, quenched to -20^{o}C:
Low mag (upper panel) (a) Control; (b) 21.5% BSA; (c) 35% BSA; (d) 45% BSA.
High mag (lower panel)(e) Control; (f) 21.5% BSA; (g) 35% BSA; (h) 45% BSA.

Effect of BSA microbubble concentration on porosity of scaffolds quenched by liquid N_2:

This, interesting finding, led to an increased interest in the characterization of the scaffolds that were quenched by liquid nitrogen. Figure 6.3 (upper panel) shows the overall surface morphology of scaffolds prepared by quenching in liquid nitrogen. Similarities between pore morphology of the 21.5%, fig 6.3 (b), and 35%, fig 6.3 (c), are observed. The control scaffold, fig 6.3 (a), revealed small pores and a rougher surface morphology than its -20^{o}C quenched counterpart. However, if one were to compare the scaffolds prepared by the two quenching methods, the common factor was

the similarity in scaffold pore morphology for lower concentration of microbubbles. However, in contrast to the scaffolds with 45% BSA microbubbles, prepared by quenching to -20^{o}C, quenching in liquid nitrogen yielded a porous surface. Figure 6.3 (lower panel) gives a closer view of the pore morphology of liquid nitrogen quenched scaffolds.

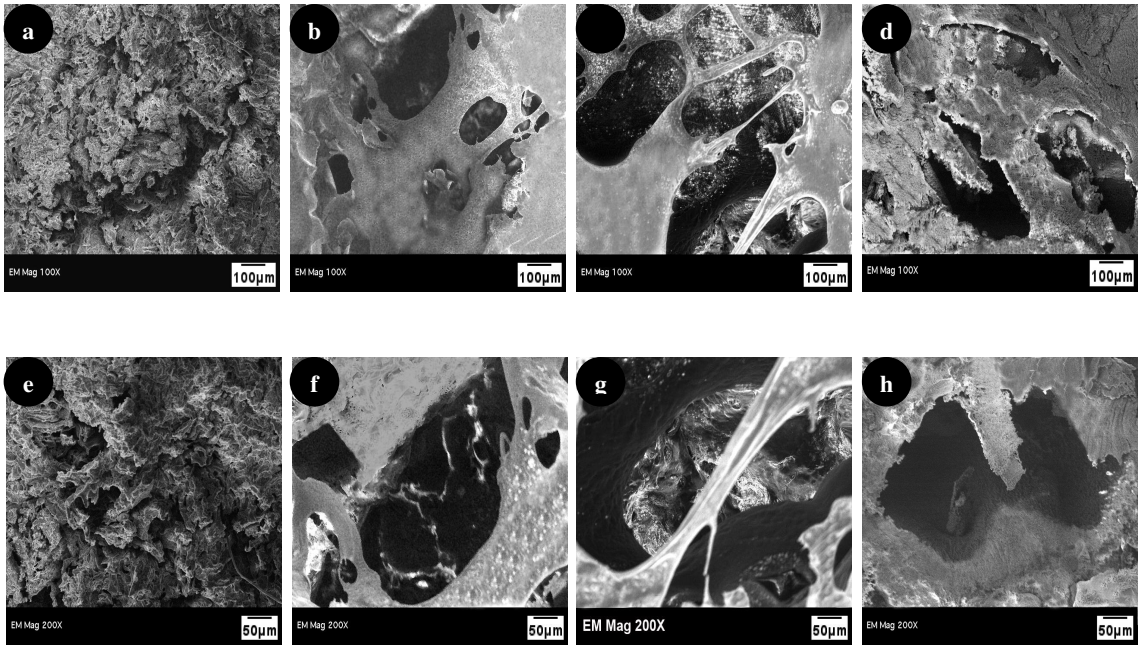

Fig 6.3 SEM image of PLGA scaffolds, quenched by liquid N_2:
Low mag (upper panel) (a) Control; (b)21.5% BSA; (c) 35% BSA; (d) 45% BSA.
High mag (lower panel)(e) Control; (f) 21.5% BSA; (g) 35% BSA; (h) 45% BSA.

Earlier we saw that the BSA microbubbles that were synthesized measured around 76 µm. Regardless of the quenching technique, the pore size was observed to be in the range of 100 to 150 µm. The SEM images validate the claim that the microbubbles seat themselves in the polymer matrix and they maintain their shape and form as they are quenched to very low temperatures. Once the solvent is extracted, the bubbles burst rendering porosity to the scaffold. Though, the surface morphology of

scaffolds quenched to -20^{o}C with high concentration of BSA microbubbles (45%) showed lesser pores at the surface, the scaffolds with the same concentration of microbubbles but quenched in liquid nitrogen did not show a similar feature. This is where the role of quenching comes in to picture. It has been observed in earlier studies [58, 59, 60, 61] that quenching to very low temperatures yields a highly interconnected porous structure. Quenching in liquid nitrogen is a rapid process, where in the polymer solution with the microbubbles freezes from room temperature to -196^{o}C very rapidly whereas quenching to -20^{o}C is a gradual process. In liquid nitrogen, the microbubbles retained their form and were frozen in the form of microbubbles itself, but in the slow quenching (-20^{o}C) a few bubbles at the surface burst to deposit a dense layer of BSA at the surface. This resulted in the slightly lesser porosity in the slow quenching technique.

The SEM images that have been shown reveal the story at the surface, but to determine whether the porogen indeed played a crucial role in enhancing the porosity the frozen sections were analyzed as explained in section 6.3.

6.2.2 Frozen Sectioning and Coomassie Brilliant Blue Staining

One of the main reasons why sectioning a scaffold is desirable is, because, as we section the scaffold and observe consistency in the pore distribution we can ascertain that indeed the microbubbles were successful as porogens. Scaffolds were sectioned in a cryostat as described in section 5.6.2. As discussed in section 6.2, SEM analysis revealed the surface morphology of the scaffolds, but to characterize the internal architecture of these novel scaffolds frozen section analysis was necessary. Frozen sectioning was determined as the most appropriate technique in this lab as the

scaffold would disintegrate when sectioned in paraffin wax. It was essential to keep the scaffold immersed in OCT under vacuum (-70 kPa) to facilitate the infiltration of the pores of the scaffold with OCT. The greater this infiltration the better the scaffold section integrity. To detect the presence of BSA protein in the scaffold, the scaffold sections were stained with Coomassie Brilliant Blue (CBB) dye.

Coomassie Brilliant Blue Staining

Frozen sections of the scaffolds stained with Coomassie Brilliant Blue (CBB) and then destained as described in section 5.6.2. The destained sections were observed under a phase contrast microscope after placing a cover-slip over the section. Figure 6.4 shows the images as observed at a magnification of 10X. The staining with Eosin helped create a pink background which helped create a contrast to discriminate the protein staining from the background. The control samples show the presence of very small pores but as one observes the images of the scaffolds with increasing concentration of microbubbles the size of the pores is found to increase. The pores reveal a dense deposition of the blue stain, which implies that the BSA has been deposited around the pores. The staining intensity for BSA is very high as CBB binds to arginine residues on proteins (which is a constituent of BSA), with 8 times more efficiency than any other amino acid. This deposition is found to increase with increasing microbubble concentration. In the light of the aforementioned findings, it can be concluded that the hypothesis of BSA microbubble serving as a porogen to render pores to scaffolds is validated.

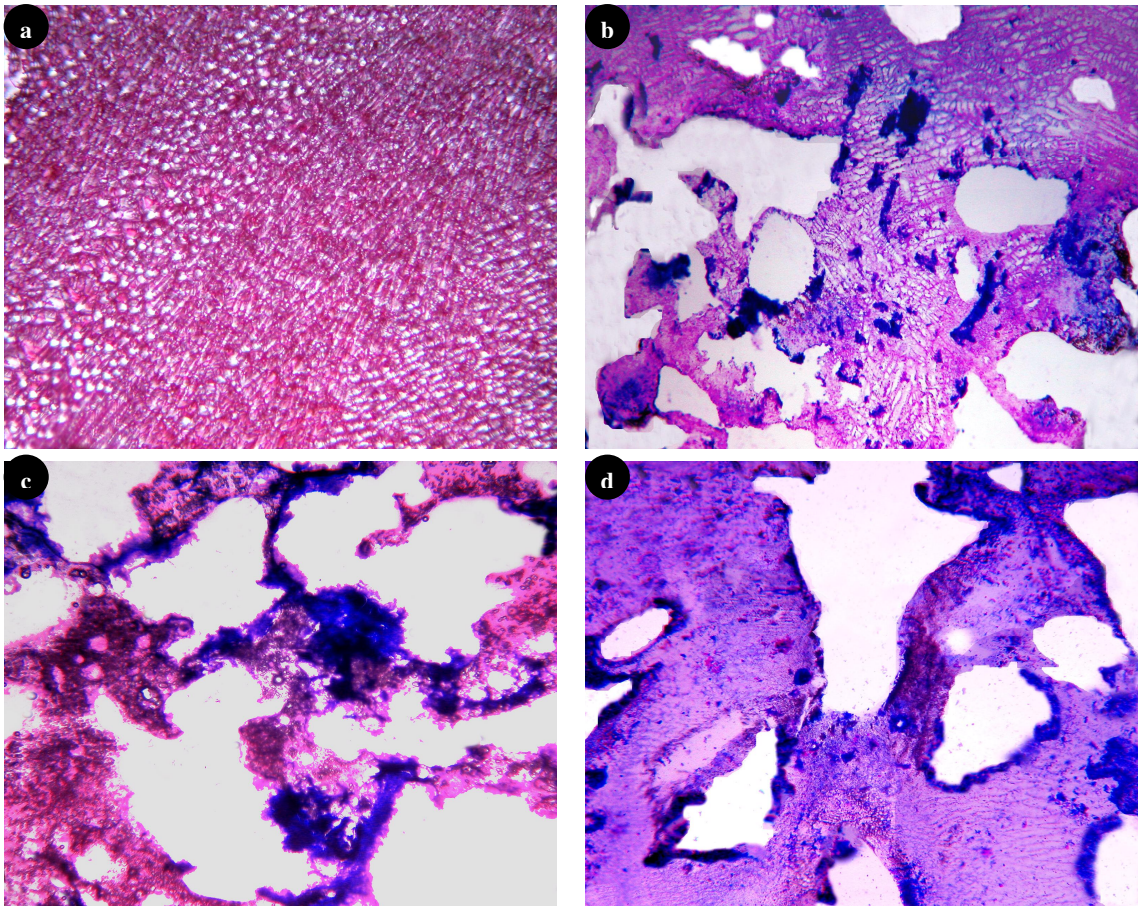

Fig 6.4 CBB Assay - Frozen section images of PLGA scaffolds with:
(a) Control; (b) 21.5% BSA microbubbles ; (c) 35% BSA microbubbles; (d) 45% BSA microbubbles.

Past studies [57, 58, 59, 60] have shown that by quenching scaffolds to temperatures lower than the melting point of the solvent induces phase separation which aids in forming interconnected pores. These studies also showed that the lower the quenching temperature the more microporous the morphology. Increasing the quenching temperature resulted in an increase in cell size, but created a closed cellular structure [57]. However, by using BSA microbubbles as a porogen, it has been shown that regardless of the quenching temperature, one can fabricate scaffolds with open

pores. The SEM images show that 35% BSA microbubble incorporated scaffolds showed good pore distribution at both quenching temperatures. The frozen section images upon staining revealed the presence of BSA around the pores and in the scaffold matrix. This demonstrates the efficacy of the microbubbles as a protein delivery system.

CHAPTER 7

EVALUATION OF GELATIN MICROBUBBLES AS POROGENS - RESULTS AND DISCUSSION

The previous study using BSA microbubbles as a porogen, yielded PLGA scaffolds with open pores. The novel technique of incorporating a microbubble porogen has succeeded in synthesizing scaffolds which have a pore diameter of around 120 μm. Apart from rendering large pores the microbubbles also served as a delivery mechanism to deliver the protein to the interstices of the polymeric matrix. Having ascertained that BSA microbubbles can be used as a porogen, we investigated the extensibility of the concept to a range of proteins.

7.1 Hypothesis II

The wider open pores observed using BSA microbubbles as a porogen is not specific to BSA and should be reproducible for an ECM protein.

7.2 Physical Characteristics of Gelatin Microbubbles

The gelatin microbubbles were prepared as described in section 5.3. The only major consideration while preparing gelatin microbubbles was that gelatin had to be maintained at a temperature of $45^{o}C$ in order to prevent it from becoming very viscous. Sonication of the gelatin solution resulted in the deposition of the protein over the nitrogen gas bubble. The gelatin microbubbles when observed under the microscope

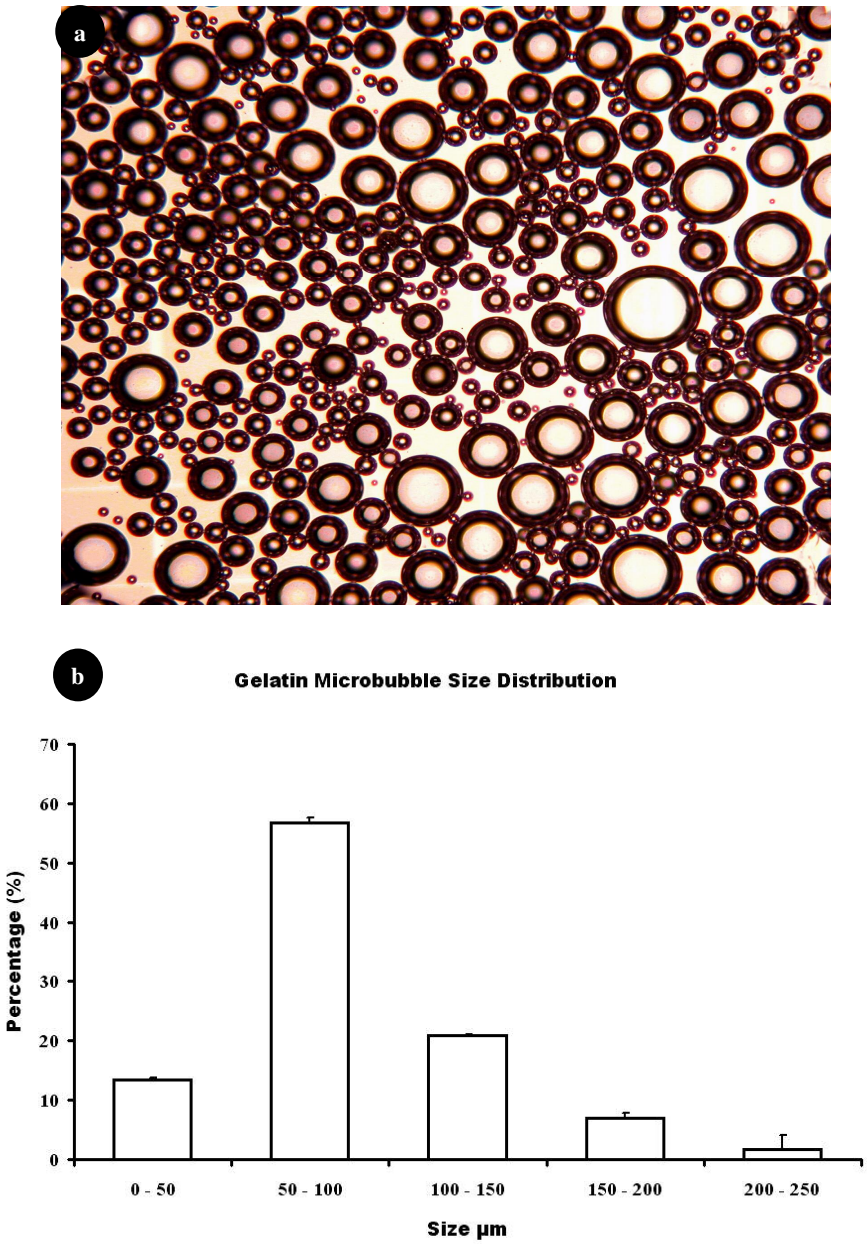

Fig 7.1 Gelatin microbubbles (a) structure observed at 10X magnification;
(b) Graph showing microbubble size distribution.

showed a core and shell structure as was observed in the case of BSA microbubbles (fig 7.1 (a)). The core comprises of nitrogen gas while the shell mainly consists of gelatin. The microbubble size was determined by measuring the size of more than 1800 microbubbles under the microscope.

The average size of the gelatin microbubbles was observed to be in the range of 93.85 µm and the size distribution as shown in fig 7.1 (b). majority of the bubbles were found to range in size from 50 to 100 µm with more than 20 % measuring 100 to 150 µm.

7.3 Characterization of Surface Morphology of Scaffolds

7.3.1 Scanning Electron Microscope (SEM) Analysis

Effect of gelatin microbubble concentration on porosity of scaffolds quenched at -20^{o}C:

The SEM images of PLGA scaffolds, quenched at -20^{o}C, with gelatin microbubbles incorporated in different concentrations are shown in figures 7.2 and 7.3.

Overcharging was a commonly observed phenomenon while analyzing the non conducting scaffolds under the SEM and hence sputtering was done for more than 30 minutes to ensure that the overcharging was limited. Figure 7.2 shows the surface morphology of PLGA scaffolds quenched to -20^{o}C. The control scaffolds with no porogens, figure 7.2 (a), revealed a microporous structure whereas the 21.5% gelatin microbubble incorporated scaffold, figure 7.2 (b), showed large pores measuring around 100 µm scattered amidst smaller pores. Scaffolds with 35% microbubbles, figure 7.2 (c), showed a rough exterior surface with lots of pores. Unlike the 45% BSA microbubble incorporated scaffolds, the 45% gelatin microbubble scaffolds, fig 7.2 (d), showed porosity at the surface which was comparable to that seen in the lower concentration microbubbles. Higher magnification SEM images, figure 7.2 (lower panel), showed that the scaffolds' pore morphology was uniform at lower concentration of gelatin microbubbles, a feature that is distinctly comparable to that observed in the

case of BSA microbubbles. Though, evidently, 21.5% gelatin microbubble bearing scaffolds, figure 7.2 (f), showed non homogeneous pore distribution i.e. large pores interspersed with small pores. However, for 35% and 45% gelatin microbubble scaffolds, figures 7.2 (g) & (h) respectively, the pore sizes are consistent through out the surface.

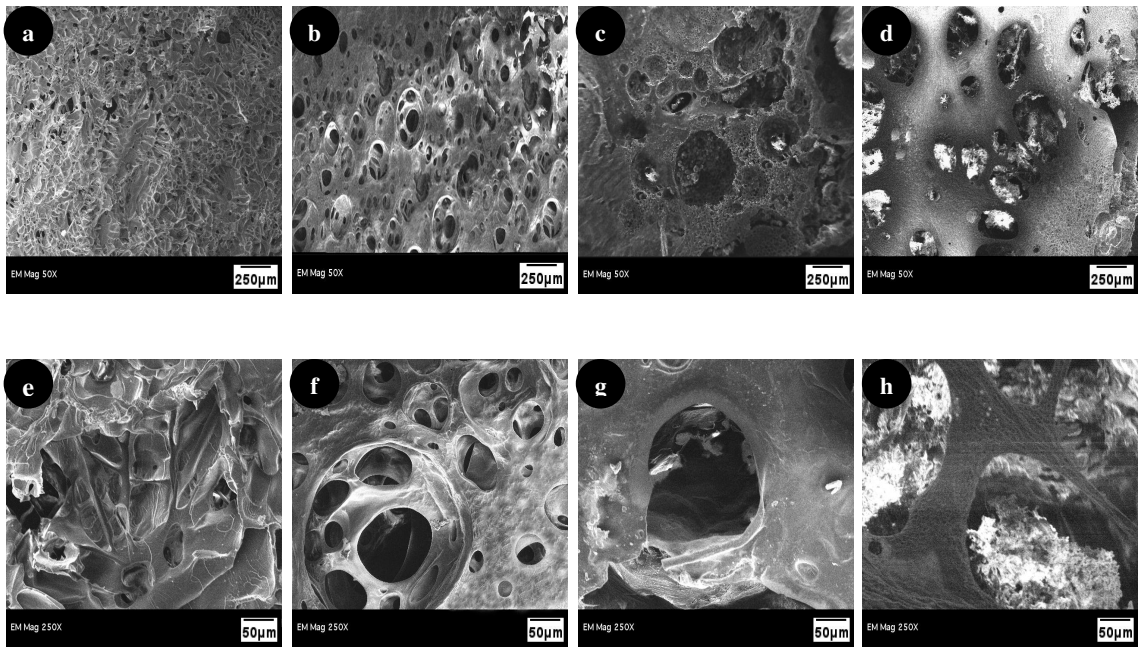

Fig 7.2 SEM image of PLGA scaffolds, quenched to -20^{o}C:
Low mag (upper panel) (a) Control; (b)21.5% Gelatin; (c)35% Gelatin; (d)45% Gelatin.
High mag (lower panel)(e) Control; (f) 21.5% Gelatin; (g) 35% Gelatin; (h) 45% Gelatin.

Effect of gelatin microbubble concentration on porosity of scaffolds quenched in liquid N_2:

The scaffolds quenched in liquid nitrogen were also characterized under the SEM. The low magnification images are shown in figure 7.3. As observed in the case of BSA microbubble scaffolds quenched in liquid nitrogen, the surface morphology of

these scaffolds was rough. High magnification images, figure 7.3 (lower panel), revealed increased porosity as the gelatin microbubble concentration increased. Unlike the BSA microbubble incorporated scaffolds, gelatin microbubbles rendered porosity at the surface to a greater extent.

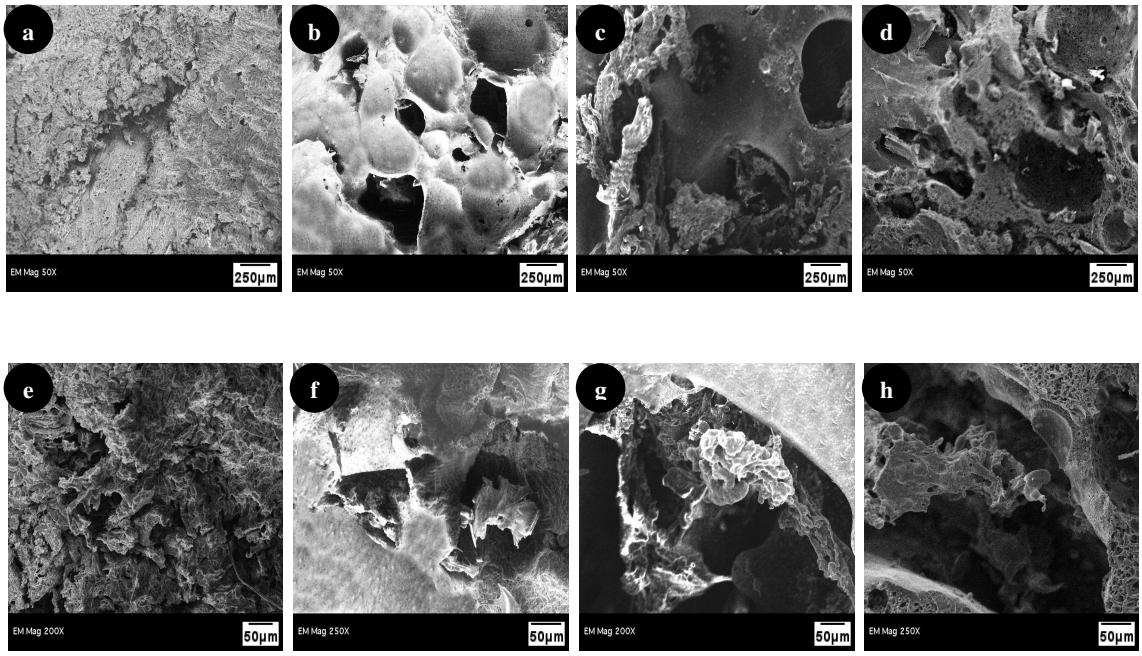

Fig 7.3 SEM image of PLGA scaffolds, quenched by liquid N2:
Low mag (upper panel) (a) Control; (b) 21.5% Gelatin; (c) 35% Gelatin; (d) 45% Gelatin. High mag (lower panel) (e) Control; (f) 21.5% Gelatin; (g) 35% Gelatin; (h) 45% Gelatin.

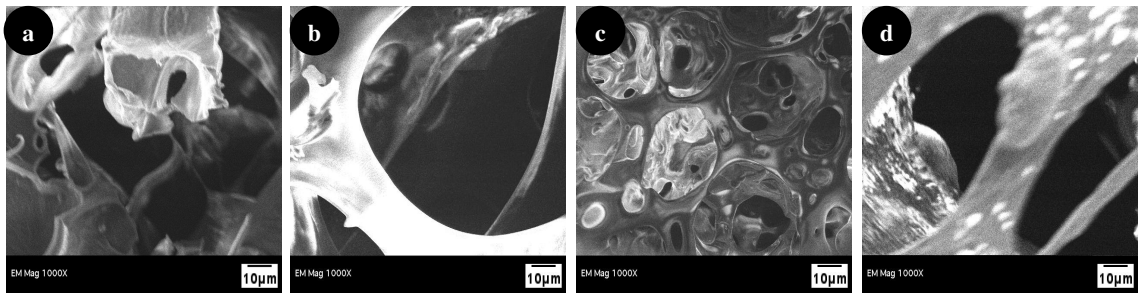

Fig 7.4 Pore morphology of PLGA scaffolds, quenched to -20^{o}C:

(a) Control; (b) 21.5% gelatin microbubbles; (c) 35% gelatin microbubbles; (d) 45% gelatin microbubbles.

Figure 7.4 is a very high magnification SEM image of the scaffolds as seen through a pore. This image gives a better view of the internal structure of the pores close to the surface and reveals the presence of open pores as seen from the surface. To assess the internal pore morphology of these scaffolds, frozen sections were analyzed by CBB assay for pore morphology and protein distribution.

7.3.2 Frozen Sectioning and Coomassie Brilliant Blue Staining

The scaffolds were sectioned in a cryostat, as described in section 5.6.2 and the sections were collected on poly (l-lysine) treated glass slides. Sections measuring 20 to 30 μm in thickness were then characterized for their pore morphology and protein deposition. The CBB assay was performed to detect the presence of gelatin around the pores.

Coomassie Brilliant Blue Staining:

Frozen sections were stained with CBB and destained. Subsequently, these sections were cover-slipped and observed under the microscope. Figure 7.5 shows the images as observed at a magnification of 10X. As performed in the case of BSA microbubble loaded scaffolds, Eosin staining was performed for 2 minutes to create a pink background to observe the Coomassie Blue stain. The control samples show the presence of very few pores but as one observes the images of the scaffolds with increasing concentration of microbubbles the porosity is found to increase. The scaffolds with 21.5% gelatin microbubbles had larger pores than 35% gelatin microbubble-bearing scaffolds. The pores reveal a dense deposition of the deep blue

stain, which implies that gelatin has been deposited around the pores. This deposition of CBB is found to increase with increasing microbubble concentration as was observed in the case of BSA microbubbles.

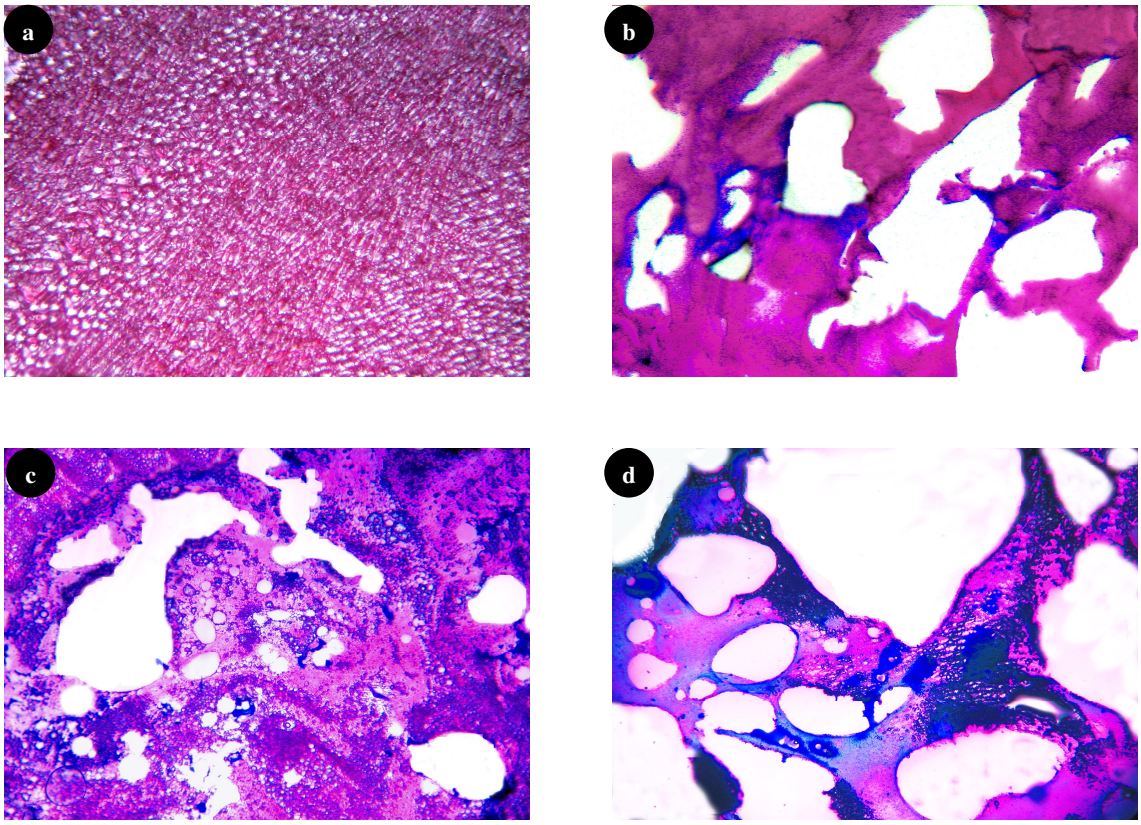

Fig 7.5 CBB Assay - Frozen section images of PLGA scaffolds with: (a) Control; (b) 21.5% gelatin microbubbles; (c) 35% gelatin microbubbles; (d) 45% gelatin microbubbles.

The size of the gelatin microbubbles was found out to be around 93 µm and hence the bursting of the microbubble upon solvent extraction yielded pores of around 100 to 150 µm. The frozen section images reveal that the scaffolds have a highly porous internal architecture.

Table 7.1 gives a qualitative summary of the porosity of the scaffolds observed with respect to different porogen type, concentration and quenching method.

Table 7.1 Qualitative summary of porosity.

Quenching Method	**Porogen**						
	No Porogen	BSA 21.5%	BSA 35%	BSA 45%	Gelatin 21.5%	Gelatin 35%	Gelatin 45%
-20^{o}C	Microporous 10-20µm	++	+++	+	+++	++	++
Liquid N_2	Microporous 10-30 µm	+	+++	++	+++	++	++

"+" indicates extent of porosity

Comparing the BSA microbubble incorporated scaffolds and gelatin microbubble incorporated scaffolds it was found that the optimal gelatin microbubble incorporated scaffolds (21.5%) yielded circular pores homogenously distributed over the surface of the scaffold. BSA has good biocompatibility but low cell adhesion properties and hence gelatin microbubble scaffolds which showed rounded pores hold a lot of promise for cell seeding experiments.

CHAPTER 8

ADVANTAGES & LIMITATIONS OF PROTEIN MICROBUBBLE POROGENS

One of the major advantages of using protein microbubble is that the common limitation of TIPS techniques of fabricating scaffolds is overcome. This novel technique makes it possible to incorporate active biomolecules and drugs in to the scaffold around the pores and elicit cells to infiltrate in to the pores of the matrix when used as a matrix for cell culture. The nature of the protein plays a very important role in rendering porosity to the scaffold, as has been observed in the case of BSA microbubbles and gelatin microbubbles. Regardless of the nature of the protein, this novel technique has yielded scaffolds with pore sizes in the range of 100 to 150 µm whereas; the conventional TIPS scaffolds had a pore size of 10 to 20 µm. In studies by various researchers [57, 58, 59, 60], a lot of emphasis has been laid on the role of quenching the scaffolds wherein it was found that quenching them to very low temperatures yields porous scaffolds.

One of the limitations of this technique is that the pore size is not found to be consistent throughout the surface of the scaffold. This is because microbubbles incorporated in the scaffold varied in size from as low as 10 µm to 180 µm. Hence the bursting of the microbubble yielded pores which ranged in size from small microporous range to as high as 300 µm. This limitation can be overcome by selectively

incorporating the protein microbubbles which meet a specific size requirement. Selection of protein microbubble of a specific size can be achieved either by freeze drying the bubbles and sieving them or by optimizing the microbubble synthesis conditions. This is an important part of the future work that will be done in order to expand this technique. The effect of solvent on the stability of protein can be investigated by High Performance Liquid Chromatography (HPLC) applications.

However, using protein microbubbles it has been found that regardless of the quenching temperature open porous scaffolds can be fabricated. Apart from serving as a porogen, the protein microbubbles also serve as a protein delivery mechanism. This technique holds tremendous potential in the advancement of tissue engineering.

CHAPTER 9

CONCLUSIONS

- The novel protein microbubble porogen incorporation technique coupled with phase separation could overcome the limitation of the conventional phase separation technique, in that, it yielded scaffolds with bigger, open interconnected pores.
- No salts are used and hence there is no potential risk post - implantation.
- The use of protein microbubbles as a porogen is a novel technique.
- Biomolecules like peptides, drugs and chemicals, growth factors etc., can be loaded in to the microbubbles and then incorporated in to the polymeric scaffold, thereby facilitating its controlled release.
- The biomolecules are in fact protected by the protein shell.
- The biomolecules that are incorporated are not destroyed or damaged during the process of fabrication unlike other methods.

CHAPTER 10

FUTURE RECOMMENDATIONS

- This technique synthesizes scaffolds with a favorable environment for cell growth and infiltration in to the pores of the scaffold. Hence, the growth of cells on these scaffolds should be investigated.
- Various chemokines can be incorporated in a non destructive manner using this novel technique and cell migration and proliferation can be assessed in vitro and in vivo.
- This technique can serve as a method to incorporate drugs in to the scaffold.
- The composition of the microbubble can be optimized to yield microbubbles of specific sizes so as to yield scaffolds with consistent pore sizes.

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BIOGRAPHICAL INFORMATION

Ashwin Mohan Nair received his Bachelor of Engineering in Instrumentation Engineering from the University of Mumbai (Bombay), India in November 2002. Ashwin was born in Bombay on April 21, 1980 and did his schooling and undergraduation there. He was accepted in to the Biomedical Engineering program at the University of Texas at Arlington in Spring 2004, and left for the United States of America to pursue what was closest to his heart.

Ashwin believes that tissue engineering is the future of medicine. He envisions a world where there are affordable tissue engineered solutions to the ailments plaguing mankind today, and this vision motivates him every single day to work tirelessly towards that ultimate goal.