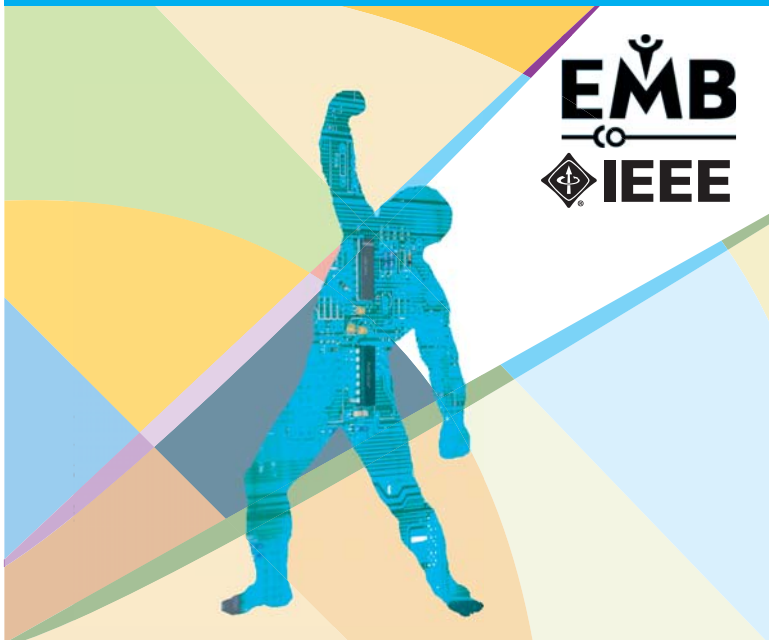
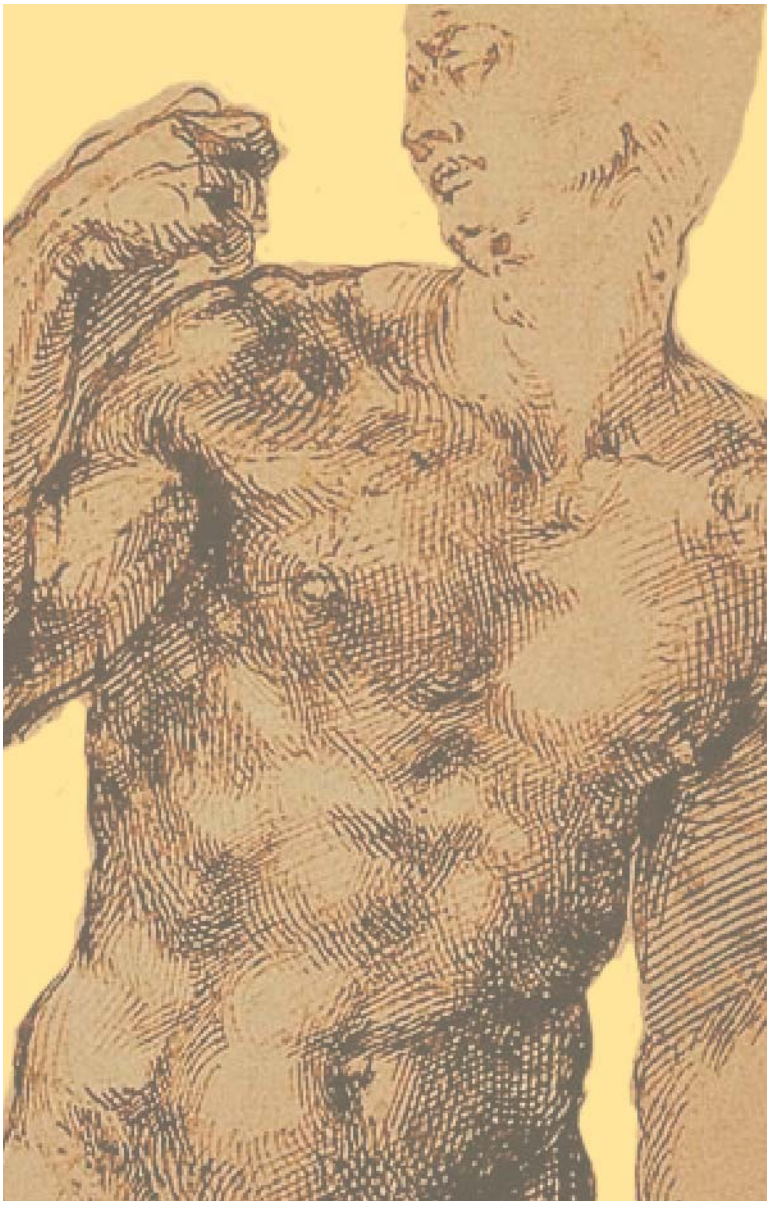
2007 IEEE Dallas Engineering in Medicine and Biology Workshop





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A NOVEL PREPARATION OF DEGRADABLE SCAFFOLDS USING BSA MICROBUBBLES AS POROGEN

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Abstract— **Biodegradable scaffolds play a key role in contemporary medicine for tissue replacement as well as regeneration. Many degradable scaffold fabrication techniques have been developed. However, these scaffolds often hamper the growth of cells due to material hydrophobicity and/or lack of biocompatible protein coating. To overcome the 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. Albumin bubbles were produced by sonicating bovine serum albumin in the presence of nitrogen gas. PLGA scaffolds were then prepared by thermally induced phase separation with the incorporation of protein microbubbles as porogens. SEM and cryosectioning of scaffold revealed the presence of open interconnected pores measuring around 100 to 150 μm size, which is suitable for cell migration into scaffold. This novel technique provides two distinct advantages. First, microbubbles are made of biological materials which can provide biocompatible protein coating on the pores throughout the scaffold. 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 delivering chemokines and drugs into the polymeric matrix.**

I. INTRODUCTION

Scaffolds play a critical role in tissue engineering by acting as a temporary tissue construct or building block for cell accommodation, proliferation, and differentiation. Several approaches, such as solvent casting/particulate leaching, emulsion freeze drying, gas foaming and thermally induced phase separation, have been reported for the preparation of these polymeric matrices [1-3]. In order to render pores to the scaffolds, various porogens have been used. More commonly used techniques like solvent casting/particulate leaching makes use of inorganic salt and sugars as porogens. However, the major problem associated with the use of porogens is that the complete removal of porogens is vital so as to avoid cell toxicity. Even if the porogens yield high porosity, lack of pore interconnectivity and poor surface hydrophilicity hinder the migration of cells in to the matrix. Furthermore, most degradable polymers are hydrophobic and discourage cell attachment and growth. 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 albumin microbubbles, which are currently used in the field of medicine, have many unique characteristics making them suitable candidates for serving as porogens. Protein microbubbles have been successfully used as contrast agents for medical imaging [4,5] and to carry genetic materials (DNA/RNA) for gene therapy [6]. A protein microbubble comprises of a gas filled core and a protein shell to stabilize the microbubble. Gases with low diffusivity, like nitrogen or perfluorocarbon, are typically used.

We have developed a novel technique wherein scaffolds were fabricated by phase separation of polymer solution mixed with BSA microbubbles as a porogen. Polymer solutions were quenched in liquid nitrogen and freeze dried. Scaffolds were characterized by Scanning Electron Microscopy (SEM) as well as histological techniques. The scaffold sections were stained with a protein binding dye to show the distribution of protein along the pores. The porosity was determined by ethanol displacement method.

II. MATERIALS AND METHODS

A. Synthesis of porogen microbubbles

A solution of Bovine Serum Albumin (40% or 5%) (Sigma, St Louis, MO) was overlaid with nitrogen gas. The mixture was sonicated using a probe sonicator (Ultrasonix, Bothell, WA) at 20 kHz for 10 seconds to yield BSA microbubbles. The microbubbles were pipetted out into 5ml glass tubes placed on a cold water bath. A small droplet of microbubbles was placed on a hemocytometer and imaged under a microscope (Leica, Wetzlar, Germany). The microbubble size was quantified using microscopic images.

B. Fabrication of scaffolds

Poly (d,l-lactic-co-glycolic acid) (PLGA, 113kDa, Medisorb Inc., Birmingham, AL) (10% or 7.5% w/v) was dissolved in 1,4-Dioxane (Aldrich, Milwaukee, WI) by vortexing for about 20 minutes till the polymer completely dissolved in the solvent. The polymer solution was poured in to 20ml glass vials. The BSA microbubbles, prepared as described earlier, were pipetted out and added to the polymer solution. For the preliminary study 40% BSA microbubbles were added to 10% w/v PLGA solution in different ratios (1:5; 1:2.5 and 1:1 v/v,). The solution was mixed by gentle agitation of the glass vial followed by

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quenching in liquid nitrogen to induce phase separation. The control scaffolds were prepared by dissolving 10% w/v PLGA in 1,4 dioxane without any BSA microbubbles followed by quenching in liquid nitrogen. The frozen mixtures were then freeze dried for three days to extract the solvent. Based on the observations from these scaffolds, the concentrations of the BSA solution and PLGA were optimized. Microbubbles were synthesized using 5% w/v BSA. The microbubbles were added in a 1:1 ratio to 7.5% w/v PLGA solution. Control scaffolds were fabricated using 7.5% w/v PLGA alone as described above.

C. Characterization of Scaffolds

Post freeze drying, the surface morphology of the scaffolds were characterized under scanning electron microscopy (SEM, JEOL JSM – IC 845A).

Internal structure of the scaffolds was visualized by embedding square sections of the scaffold in a plastic mold in freezing medium OCT (Optimum Cutting Temperature). The scaffolds were sectioned in the cryostat at a thickness of about 20μm and collected on glass slides. The sections were stained with Coomassie Brilliant Blue, a dye which stains protein [11]. After the staining process, the cross-sections of the PLGA scaffolds were imaged under a Leica microscope (Leica, Wetzlar, Germany) equipped with a Nikon camera. The size range of pores seen in the scaffold was determined using the cross-sectional images.

The porosity of the scaffolds was determined by ethanol displacement method [10]. Statistical comparison of the porosity of the control scaffolds and BSA microbubble-embedded scaffolds was conducted using Student's t-test. Differences were considered statistically significant at $p \leq 0.01$.

III. RESULTS AND DISCUSSION

In our preliminary experiments, the BSA microbubbles were synthesized by sonicating a 40% w/v bovine serum albumin (BSA) solution overlaid with nitrogen gas. Sonication of the BSA protein solution results in its emulsification and cavitation. When a low diffusivity gas like nitrogen is dispersed in the BSA solution, it results in the deposition of BSA over the gas bubble. The microbubble comprises of a nitrogen gas core and albumin shell (Fig. 1A). The size of these BSA microbubbles ranges from 50 to 150 μm and the entire range of sizes can be seen in Fig. 1B. Average size of the microbubbles was found to be 76 μm.

The scaffolds were fabricated by incorporating different amounts of these BSA microbubbles to PLGA solution (1:5, 1:2.5, 1:1) and quenching the mixture in liquid nitrogen. In our preliminary studies by varying quenching temperature from -20°C to -196°C, we confirmed that the quenching temperature has no effect on the porosity of the scaffold (data not shown), which was in agreement with earlier studies [9,10].

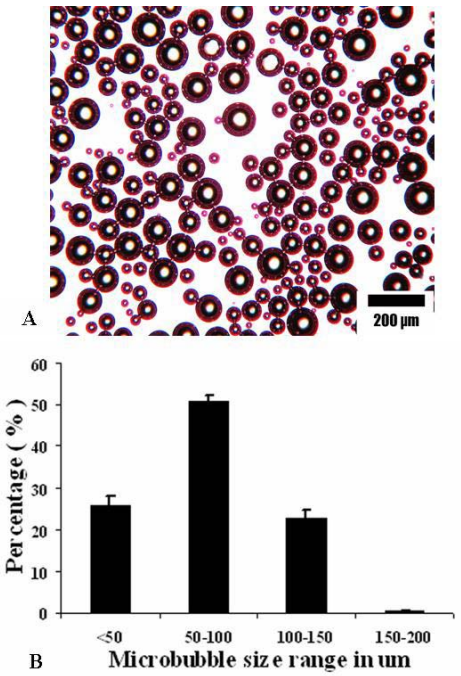

Fig. 1. Microscopic image of the 40% w/v BSA microbubbles reveals a core-shell structure. The gas filled core is surrounded by the protein shell (Mag 10X; Scale Bar 200μm) (A). Microbubble size distribution shows that the BSA microbubbles have an average size in the range of 50 to 100 μm (B).

Scanning electron microscopy (SEM) analysis of the scaffold cross sections showed that the technique yielded scaffolds with large pores when protein microbubbles were incorporated (Fig 2). The cross-sections were cryosectioned and stained with Coomassie Brilliant Blue, dye binds to arginine and lysine chains on the protein [11]. Staining revealed the deposition of the BSA protein along the pores (Fig 3). This shows that the protein microbubbles seat themselves in the polymeric matrix and act as porogens. The average pore size of scaffold with various amounts of BSA microbubbles are 10.55 ± 2.3 μm (no microbubble), 216.11 ± 49 μm (1:5 v/v), 406.92 ± 81.4 μm (1:2.5 v/v) and 405.62 ± 81.5 μm (1:1 v/v), respectively. The difference in pore size between the scaffolds with BSA microbubbles as porogen and control scaffolds is significant at $p \leq 0.01$. However, increasing the proportion of microbubbles in the polymer solution, from 1:2.5 to 1:1 did not have a significant effect on the pore size. We believe that BSA microbubble embedded scaffolds possess superior cell affinity, since it is generally accepted that a pore size of around 100 to 150 μm is required to achieve optimal cell infiltration into scaffold. This pore size has been suggested for skin and bone regeneration [12,13],

However, during the fabrication procedure it was observed that the protein microbubbles did not disperse easily in to the polymer solution. This could have resulted in the isolated areas in the scaffold with very large pores that can be observed in figures 2 and 3. We believe that the high concentration of the PLGA solution would impede the dispersion of the microbubbles. This coupled with the high concentration of BSA protein used lead to microbubble clumping upon contact with the polymer-organic solvent

solution. The concentrations of BSA and polymer were then optimized to have minimal clumping of protein.

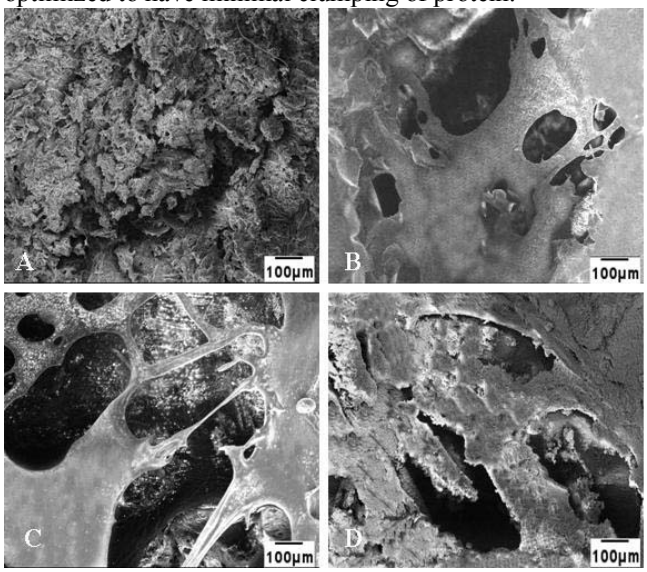

Fig 2. Scanning electron microscopy (SEM) analysis shows the microporous structure in control scaffolds (A). Increasing the amount of porogen, 1:5 (B) 1:2.5 (C) and 1:1 (D) results in larger pores.

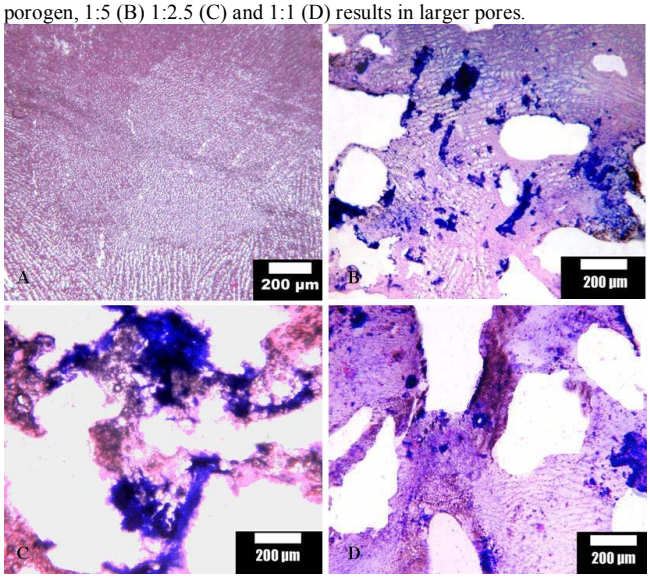

Fig 3. Internal cross-sections show control scaffolds (A) with a microporous structure. Scaffold stained with Coomassie Blue showed the presence of protein (blue staining) along the pores of the scaffold when loaded with BSA microbubbles in the ratio of 1:5 (B), 1:2.5 (C) and 1:1 (D).(Mag 10X).

We used 5% w/v BSA instead of 40% w/v BSA to synthesize the microbubbles. The microbubbles were analyzed to determine their size distribution (Figs 4A & B). They were incorporated into 7.5% w/v PLGA solution in a 1:1 ratio in order to have a high amount of protein delivered in to the scaffold. This was followed by quenching in liquid nitrogen and freeze drying for 3 days to extract the solvent completely.

SEM analysis of the scaffold cross-sections showed a microporous structure in the control scaffolds with dense small pores in the range of 10 to 20 µm (Figs. 5A and B). Our observation is in accordance with earlier studies involving scaffolds fabricated by phase separation [9,14]. The small pores form due to the crystals of the solvent after freeze drying. But, this pore size is too small for cell immigration or infiltration. It is likely that most of the seeded cells would grow on top of, rather than inside the scaffold.

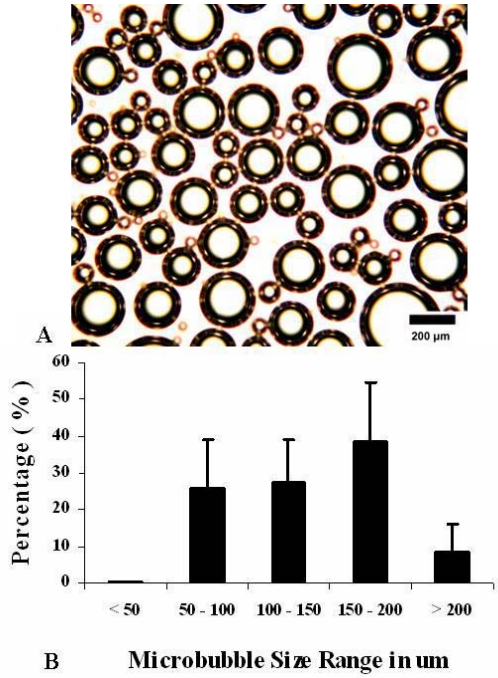

Fig 4. Microscopic image of 5% w/v BSA microbubbles (Mag 10X; Scale Bar 200µm) (A). Microbubble size distribution shows that the BSA microbubbles have an average size in the range of 50 to 200 µm.with an average size close to 142 µm.

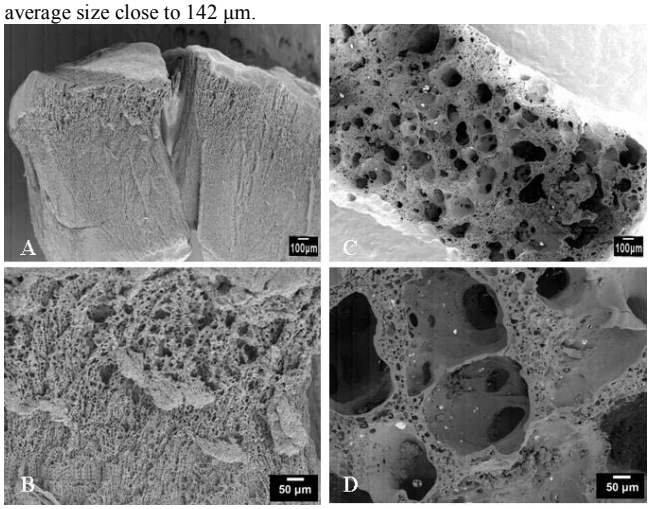

Fig 5. Scanning electron microscopy (SEM) analysis shows that the control scaffolds without the microbubbles have a microporous structure (A) and high magnification (B) images. Incorporation of BSA microbubbles as porogen results in scaffolds with larger interconnected pores as shown by the low magnification (C) and high magnification (D) images.

Interestingly, in the presence of BSA microbubbles, the polymer scaffolds form highly porous structure with much larger pores in the range of 150 µm to 200 µm (Figs. 5C and D). The pore size of BSA microbubble embedded scaffold is significantly larger than microbubble free control scaffold (average size: 150.5 ± 50 µm vs. control scaffold 13.3 ± 5.3 µm; $P \leq 0.01$). Close observation of the pores revealed improved pore inter-connectivity (Fig. 5D).

This porous structure is similar to salt leached scaffold [15], but with larger pore size. Lowering the concentration of the BSA solution to produce microbubbles and the polymer solution to manufacture scaffold ensured the better dispersion of the microbubbles in the polymer solution, thereby yielding a more uniform pore structure.

This pore structure is seen not just on the surface, but also internally. In case of the scaffolds prepared by incorporating the protein microbubbles, we found that the dye distribution is more dominant along the pores of the matrix (Fig 6). This supports the idea of the scaffold design, that the protein microbubbles yielded the pores in the scaffold. In contrast, the control scaffolds show a microporous structure internally. It should be noted that, during the drying process, albumin microbubble burst and provide a thick layer of protein coating on scaffold. This protein coating may serve as a biocompatible coating to improve cell seeding and growth as shown in previous works [16,17].

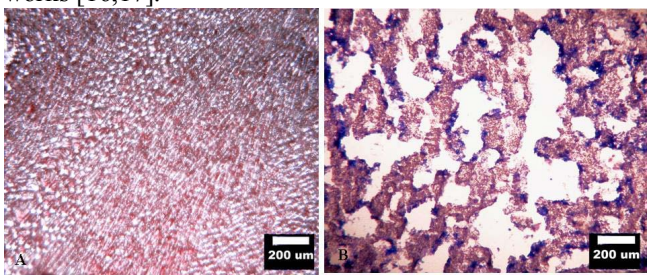

Fig 6. Scaffold stained with Coomassie Blue showed the presence of BSA along the pores of the scaffold. The internal cross-sections show an interconnected pore structure.(Mag 10X; Scale Bar 200μm).

The porosity of the scaffolds were determined by ethanol displacement method [10], and summarized in table 1. The scaffolds with the BSA microbubbles as porogen have a porosity of almost 95% and thereby validated the visual observations. The difference between the porosities of the scaffolds with BSA microbubbles as porogen and the control scaffold is not significant. However, the BSA microbubble scaffolds have a larger pore size.

The novel BSA 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. This novel technique provides two distinct advantages. First, microbubbles are made of biological materials which have no toxicity. It would be interesting to study the cell growth on our scaffolds. Second, our scaffold also has the potential to function as a delivery mechanism for chemokines and drugs in to the polymeric matrix. The protein shell would ensure that the bioactivity of encapsulated drug/growth factor is preserved.

TABLE I
SCAFFOLD POROSITY (N=4)

Scaffold Type	Porosity (%)
PLGA Control	91.35 ± 3.91
PLGA-Porogen Microbubbles	94.43 ± 4.2

In summary our novel scaffolds prepared by using protein microbubbles as porogens have the potential to be an excellent tissue engineering scaffold as well as a drug /growth factor delivering device.

REFERENCES

[1] S. Yang, K. Leong, Z. Du, C. Chua, "The design of scaffolds for use in tissue engineering. Part I. Traditional factors," *Tissue Engineering*, vol.7, pp.679-689, 2001.

[2] A.G. Mikos, J.S. Temenoff, "Formation of highly porous biodegradable scaffolds for tissue engineering," *Electronic Journal of Biotechnology*, vol. 3, pp.114-119, 2000.

[3] Y.S. Nam, J.J. Yoon, T.G. Park, "A novel fabrication method of macroporous biodegradable polymer scaffolds using gas foaming salt as a porogen additive," *Journal of Biomedical Materials Research (Applied Biomaterials)*, vol. 53, pp.1-7,2000.

[4] A.L. Klibanov, "Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging," *Advanced Drug Delivery Reviews*, vol. 37, pp.139–157, 1999.

[5] F. Ries, C. Honisch, M. Lambertz, R. Schlief, "A transpulmonary contrast medium enhances the transcranial Doppler signal in humans," *Stroke*, vol. 24, pp.1903-9, 1993.

[6] M.W. Miller, "Gene transfection and drug delivery," *Ultrasound Med Biol*, vol.26 (suppl 1), pp.S59-62, 2003.

[7] Y.S. Nam, T.G. Park, "Biodegradable polymeric microcellular foams by modified thermally induced phase separation method," Biomaterials vol. 20pp.1783-1790, 1999.

[8] C. Tu, Q. Cai, J. Yang, Y. Wan, J. Bei, S. Wang, "The fabrication and characterization of poly(lactic acid) scaffolds for tissue engineering by improved solid–liquid phase separation," *Poly. Adv. Tech.* vol.14, pp.565-573, 2003.

[9] F.J. Hua, G.E. Kim, J.D. Lee, Y.K. Son , D.S. Lee, "Macroporous poly (l-lactide) scaffold 1.Preparation of a macroporous scaffold by liquid-liquid phase separation of a PLLA-dioxane-water system," *Journal of Biomedical Materials Research: Applied Biomaterials,* vol.63, pp.161-167, 2001.

[10] C. Tu, Q. Cai, S. Wang, "The fabrication and characterization of poly (lactic acid) scaffolds for tissue engineering by improved solid-liquid phase separation," *Polymers for Advanced Technologies*, vol.14, pp.565-573, 2003.

[11] S.J. Compton, G.J. Clive, "Mechanism of dye response and interference in the Bradford protein assay," *Anal. Biochem*, vol.151, pp.369-374, 1985.

[12] S.L. Ishaug, G.M. Crane, M.J. Miller, A.W. Yasko, M.J. Yaszemski, A.G. Mikos, "Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds", Jour Biomed Mater Res, vol.36, pp.17-28, 1997.

[13] V. Macquet, R. Jerome, "Design of macroporous biodegradable polymer scaffolds for cell transplantation", *Mat. Sci. For*, vol 250, pp.15-42, 1997.

[14] Y.S. Nam, T.G. Park, "Biodegradable polymeric microcellular foams by modified thermally induced phase separation method," *Biomaterials*, vol.20, pp.1783-1790, 1999.

[15] S.L. Ishaug, G.M. Crane, M.J. Miller, A.W. Yasko, M.J.Yaszemski, A.G. Mikos, "Bone formation by three-dimensional stromal Osteoblast culture in biodegradable polymer scaffolds," *Jour Biomed Mater Res*, vol.36, pp.17-28, 1997.

[16] K. Kottke-Merchant, J.M. Anderson, Y. Umemura, R.E. Marchant, "Effect of albumin coating on the in vitro blood compatibility of Dacron arterial prostheses," *Biomaterials*, vol.10, pp.147-155, 1989.

[17] J.P. Ranieri, R. Bellamkonda, J Jacob, T.G. Vargo, J.A. Gardella, P. Aebischer, "Selective neuronal cell attachment to a covalently patterned monoamine on fluorinated ethylene propylene films," *Jour Biomed Mater Res*, vol.27, pp.917-925, 1993.