Abstract

Tissue engineering scaffolds require a controlled pore size and interconnected pore structures to support the host tissue growth. In the present study, three dimensional (3D) hybrid scaffolds of poly lactic acid (PLA) and poly glycolic acid (PGA) were fabricated using solvent casting/particulate leaching. In this case, partially fused NaCl particles were used as porogen (200-300 µm) to improve the overall porosity (≥90%) and internal texture of scaffolds. Differential scanning calorimeter (DSC) analysis of these porous scaffolds revealed a gradual reduction in glass transition temperature (Tg) (from 48°C to 42.5°C) with increase in hydrophilic PGA content. The potential applications of these scaffolds as implants were further tested for their biocompatibility and biodegradability in four simulated body fluid (SBF) types in vitro. Whereas, simulated body fluid (SBF) Type1 with the optimal amount of HCO$_3^-$ ions was found to be more appropriate and sensible for testing the bioactivity of scaffolds. Among three combinations of polymer scaffolds, sample B with a ratio of 75:25 of PLA: PGA showed greater stability in body fluids (pH 7.2) with an optimum degradation rate (9% to 12% approx). X-ray diffractogram also confirmed a thin layer of hydroxyapatite deposition over sample B with all SBF types in vitro.

Keywords: poly (lactic-co-glycolic acid) scaffolds, simulated body fluid, solvent immersion, polymer degradation, hydroxyapatite

INTRODUCTION

The core idea of tissue engineering is to allow the cells to repair and regenerate damaged tissues and organs by promoting cell growth and differentiation over the scaffolds$^{[1,2]}$. These scaffolds are biodegradable matrices designed to support cell proliferation, which finally provides a functional tissue$^{[3]}$. Since scaffolds are temporary matrices, the degradation performance of the scaffolds must correspond to the regeneration rate of the affected tissues$^{[4]}$. In this regard, the selection of scaffold material is very important to facilitate the cells to behave in the desired manner to generate tissues or organs of our requirement$^{[5]}$. 

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The materials used to synthesize biodegradable scaffolds for bone tissue engineering applications ranges between inorganic materials such as ceramics to synthetic polymers. Among them, synthetic polymers have the ability to tailor mechanical properties and degradation kinetics of scaffolds to suit various tissue engineering applications\textsuperscript{[6]}. The intrinsic properties of the polymer materials play a strategic role in the morphology, texture and performances of the scaffold\textsuperscript{[7]}. To be an artificial scaffold, the structure and surface morphology of the scaffolds have to meet general requirements specific for the targeted tissue: i) three-dimensional architecture; ii) interconnected pores to ensure cell growth, diffusion of nutrients and metabolic waste; iii) suitable surface chemistry; iv) suitable mechanical properties; v) controllable biodegradation and bioresorbability\textsuperscript{[8,9]}. In the current study, three combinations of hybrid scaffolds were prepared by blending polylactic acid (PLA) and polyglycolic acid (PGA) at the ratios of 80:20, 75:25 and 70:30 and these polymers are known for their cell-based tissue engineering approaches. These polymers have been shown to be degraded mainly by hydrolysis of ester bonds into acidic monomers, which can be removed from the body by physiological metabolic pathways\textsuperscript{[10,11]} (Fig. 1). The process which we adopted for preparation of microporous biodegradable scaffolds was solvent casting/particle leaching, where we used non-dispersed sodium chloride (NaCl) as particulate porogen for improved pore interconnectivity\textsuperscript{[12]}. In this case, pores were interconnected via fused salt particles prior to the synthesis of three-dimensional (3D) polymer scaffolds. Thus, dissolution of this fused porogen matrix leaves a highly interconnected pore structure in the polymer scaffolds\textsuperscript{[13]}. Once the polymer scaffolds are made, there is an immediate need to test the scaffolds for both in vivo and in vitro in order to consider them for human applications\textsuperscript{[14,15]}. These studies include their physical, chemical and mechanical properties helpful for assessing their bioavailability\textsuperscript{[16]}. In case of in vitro studies, scaffolds were exposed to a group of model solutions simulating the inorganic portions of blood plasma to study their surface interaction and changes. The composition of the most used simulated body fluids differs from that of human blood plasma by high content of Cl\textsuperscript{-} and lower content of HCO\textsubscript{3}\textsuperscript{-} ions. Considering the composition of bone like apatite, which contains carbonate ions, the test results could be influenced by this difference\textsuperscript{[17,18]}. In this study, we prepared four different simulated body fluids with varied concentrations of the above said ions and we monitored the influence of these simulated fluids on the physico-mechanical properties of polymer scaffolds.

**MATERIALS AND METHODS**

**Scaffold preparation**

PLA (2.9 kg/mol) and poly glycolic acid (PGA) (IV= 1.2 dL/g) were procured from Sigma (St. Louis, MO, USA). Porous polymer scaffolds were prepared by solvent casting/particle leaching where we used NaCl salt as particulate porogen (200-300 μm). PLA and PGA with higher molecular weight/inherent viscosity were used in our studies to ensure that the scaffolds would hold adequate mechanical integrity despite their relatively high porosity ( ≥ 90%). Briefly, NaCl matrices were prepared by subjecting NaCl particles to 95% humidity for 12 hours prior to solvent casting. PLA and PGA were blended at the ratios 70:30, 75:25 and 80:20 and dissolved in Hexafluoro-2-propanol (Sigma). This molten polymer blends were poured in to non-dispersed NaCl matrices (or) scaffold before solvent evaporation. Then, these scaffolds were vacuum dried for 48 hours before NaCl particles were further leached out by immersing scaffolds in de-ionized water.

**Scaffold characterization**

**Electron microscopy**

The transverse sections of NaCl scaffolds were imaged using scanning electron microscope (SEM) prior to solvent casting to monitor NaCl crystal fusion. In

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*Fig. 1 Structure of PLGA molecule.* The figure shows hydrolytic degradation to PLA and PGA monomers in the presence of physiological fluids. PLGA: poly (lactic co glycolic acid).
addition, transverse sections of polymer scaffolds after salt leaching were also imaged using SEM (Zeiss EVO® MA15).

**Determination of glass transition temperature (Tg)**

Glass transition temperature (Tg) of all three scaffold types was determined as per ASTM D7426 standard by differential scanning calorimeter (DSC) equipped with liquid nitrogen cooling system (Auto Q20, TA instruments). Ten mg of polymer samples were quantitatively transferred to sealed aluminum pans and subjected to cooling and heating cycles from 0°C to +200°C with cooling and heating rates of 5°C/min. During experiment, DSC cell was purged with dry nitrogen at 40 mL/min. The baseline correction was performed by recording a run with empty pans.

**Preparations of simulated body fluids**

We tested the polymer degradation as per ASTM F1635-04a standard with four simulated body fluid types by varying Cl⁻ and HCO₃⁻[19]. The composition of simulated body fluids is shown in Table 1. SBFs were prepared in polypropylene beakers by dissolving NaCl, NaHCO₃, KCl, K₂HPO₄, MgCl₂, 1M HCl, CaCl₂, Na₂SO₄, Tris HCl in double distilled water and pH was adjusted to 7.4 and the fluids were further incubated at 37°C for 3 to 4 days and monitored for hydroxyapatite (HA) deposition.

**In vitro degradation of scaffolds by immersion method (ASTM F1635-04a)**

Polymer scaffolds were cut to uniform sizes and each sample was weighed before the immersion test[20]. Then, scaffolds were placed in separate polypropylene beakers and fully immersed in simulated body fluids (0.2 mL of SBF/mm³ of scaffold) and incubated for 21 days at 37°C. These polymer scaffolds were taken out at preferred time intervals (at 14 and 21 days) and rinsed with distilled water and dried further for studying the morphological changes and weight loss; simultaneously, we checked for pH shift in simulated body fluids due to polymer degradation.

**Analysis of the scaffold surface by X-ray diffraction**

The interaction of polymer scaffolds with body fluids was evaluated by studying surface modifications over the scaffolds by X-ray diffraction (XRD) analysis. Precisely, polymer scaffolds immersed in four different simulated body fluids for 21 days were further vacuum dried in order to test HA deposition over the scaffold surface using XRD (Shimadzu) with a 2 theta (20) angle between 10 to 80 degrees at a scan speed of 5°/min.

**RESULTS**

**Scaffold preparation**

Highly porous (> 90%) hybrid poly (lactic co glycolic acid) (PLGA, scaffolds (3 mm thickness) were prepared by the solvent casting/particulate leaching method. These scaffolds were cut to uniform sizes for further characterization. For our convenience, scaffolds with 80:20, 75:25 and 70:30 of PLA: PGA are denoted as Sample A, Sample B and Sample C respectively.

**Scaffold characterization**

**Electron microscopy**

Initial incubation of NaCl crystals in a humidifier (95%) resulted in fusion of salt crystals, creating interconnected matrices (Fig. 2A). This fused NaCl crystals prior to addition of molten polymer mixture increased pore interconnectivity, which improved the overall porosity of scaffolds (> 90%) (Fig. 2B).

**Determination of glass transition temperature (Tg)**

The DSC analysis of PLGA scaffolds revealed their amorphous nature identified by the presence of glass transition temperature (Tg) and by the absence of melting temperature (Tm). The PLGA scaffolds showed increased polymer degradation with increases in the PGA proportion in the scaffolds. We also noticed a gradual reduction in Tg of polymer scaffolds A, B and C to 48°C, 44.5°C and 42.5°C, respectively.

**In vitro degradation of scaffolds by the immersion method (ASTM F1635-04a)**

1) Morphological variations with time

   After 14 days in simulated body fluids, the mor-
Properties of PLGA scaffolds fabricated by salt fusion

By the end of 30 days of incubation, Fig. 3 shows the pH shift in the simulated body fluids over time at 37°C. Sample A showed a slight increase in pH from 7.4 to 7.6, which might be due to slower polymer degradation and continuous release of \((\text{PO}_4)^{3-}\) ions which acts as conjugate bases. Sample C, due to its accelerated degradation to lactic and glycolic acids; showed higher decline in pH from 7.4 to 6.7, making this sample vulnerable for in vivo applications whereas sample B was found to be stable with a shift in pH from 7.4 to 7.2.

4) Analysis of scaffold surface by XRD
XRD analysis using the X-ray diffractometer revealed the presence of straight base line and semi-sharp peaks (Fig. 4), suggesting the semi crystalline

phological changes were found to be irregular, with increase in pore size over the scaffold surface. After 21 days, regular and recognizable morphological changes were detected in PLGA scaffolds. We also compared the morphological variations with weight loss in PLGA scaffolds during degradation studies.

2) Weight loss
Sample C had shown accelerated weight loss when compared to the other two samples; this may be due to higher PGA content in the scaffold, which is hydrophilic in nature. Though the percentage degradation was high in case of samples C, the degradation rate was not even (approximately 11% to 22%). This uneven degradation property makes this combination inappropriate for in vivo application. Comparatively, sample B had a stable and optimum degradation rate (approximately 9% to 12%), whereas sample A exhibited a low degradation rate (approximately 4% to 8%) compared to the other two samples. These observations indicated that increase in the PLA content made the scaffolds more hydrophobic and denser, thus making them tougher to be degraded both in vivo and in vitro.

3) pH shift in the simulated body fluids over time

Fig. 2 Scanning electronic microscopic images of fused salt crystals (A) and porous polymer scaffolds (B).

Fig. 3 Time dependence on pH of simulated body fluids. A: 80:20 PLGA; B:75:25 PLGA; C: 70:30 PLGA. And 2, 3 and 4 represents four SBF types.
nature of our PLGA scaffolds. The XRD patterns also clearly indicated the deposition of HA traces over the scaffold surface. Compared to other SBF types, the XRD spectrum of SBF1 showed better HA deposition whose carbonate content and phosphate contents are more similar to human blood plasma compared to other SBF solutions.

**DISCUSSION**

Porous hybrid polymer scaffolds (PLGA) were prepared by the solvent casting/particle leaching method, where we fused NaCl particles by prolonged exposure to rich moist environment (95% humidity), resulting in enhanced pore interconnectivity in the PLGA scaffolds ([Fig. 5A](#)). In this study, fused NaCl particles resulted in the creation of holes on the walls of the scaffolds, which increased the comprehensive modulus of the polymer scaffolds\(^{[21,22]}\). Improved pore interconnectivity is also helpful in a variety of tissue engineering applications, particularly those requiring close cell to cell contact\(^{[23]}\).

**Fig. 5B** depicts the transverse section of PLGA scaffolds prepared using fused salt particles as porogen. Scanning electron micrographs illustrate the polymer scaffolds with highly porous and well interconnected network. The microstructures of the scaffold determine its interaction with the cells and molecular transport of nutrients and biological wastes from within the scaffold\(^{[24]}\). Exclusively, the pore size of the scaffolds determines the cell seeding efficiency into the scaffold; small pores prevent the cells from piercing into the scaffold, while very large pores prevent cell adhesion due to reduced area to colonize cells\(^{[25,26]}\). Therefore, scaffold with an open and interconnected pore network and high degree of porosity (≈90%) is described as a perfect model to integrate with the host tissue\(^{[27]}\).

PLGA scaffolds prepared by salt fusion also showed irregular pore sizes, ranging between few microns to 300 \(\mu\)m. This variation in porosity may be due to a phenomenon known as solid-liquid phase separation which is attributed to solvent crystallization\(^{[28]}\). When the temperature of the polymer solution is lower than the solvent freezing point (crystallization temperature), solvent crystallizes and the polymer phase is expelled as impurity. A continuous polymerrich phase is formed by the aggregation of polymer fractions excluded from solvent crystals\(^{[29]}\). After solvent crystals have been sublimated, the scaffold is produced with a micro-porosity similar to the geometry of solvent crystals.

In in vitro degradation studies, PLGA scaffolds were degraded by hydrolysis of their ester linkages\(^{[30]}\).
The presence of methyl side chain in PLA makes it more hydrophobic and denser than PGA and hence lactide-rich PLGA copolymers are less hydrophilic, absorb less water and are subsequently degraded at a lower rate\cite{31,32}. Additionally, reduced molecular weight with increased PGA content influences the reduction in “Tg” of the PLGA scaffolds (from 48°C to 42.5°C, which was quite near the incubation temperature of 37°C)\cite{33,34}. All the above discussed features made sample B (PLA: PGA (75:25)) as a favorite in comparison with other scaffolds. Also, controlled degradation (9-12%) of sample B might provide the room for tissue growth both in vivo and in vitro as biodegradable or restorable material.

X-ray diffraction spectra of polymer scaffolds after interaction with different model solutions are explained in Figure 5. After 21 days of immersion in simulated boy fluids, a thin layer of HA deposition was observed over the scaffold surface. Comparatively, SBF1 showed characteristic peaks for HA with all three scaffold types. This fact indicates that SBF1 with carbonate content similar to the human blood plasma could be more suitable and sensitive for in vitro testing of bioactivity and the diffusive character of observed peaks might be the result of poor crystallinity of the precipitated product due to the relative short time of exposure in the simulated body fluids and/or thinner precipitated layer\cite{35}. The slower apatite deposition with SBF1 in comparison with other body fluids could enable the more sensitive in vitro testing of bioactive materials. Moreover, during interaction with human blood plasma, the creation of carbonated hydroxyapatite could be awaited rather than pure HA precipitation. Therefore, the content of carbonate ions in the solutions can be important for the plausibility of in vitro test.

In conclusion, in vitro degradation behaviors of PLGA scaffolds in three different formulations were tested systematically with four simulated body fluids for 30 days. Detailed quantitative studies on the physiological features of scaffolds in wet environment along with other material parameters were tested. During these studies, sample B was found to be more appropriate with better physiological characteristics for further
in vivo studies. The composition of SBF1 also proved as a better source for further optimization studies.

References

[1] Tabata Y. Significant role of cell scaffolding and DDS technology in tissue regeneration: tissue engineering strategies. Int Congress Ser 2005; 1284: 257-65.

[2] Mekala NK, Baadhe RR, Parcha SR, Prameela DY. Osteoblast Differentiation of Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Enhanced Cell Adhesion by Fibronectin. Tissue Eng Regen Med 2012; 9: 259-64.

[3] Stevens B, Yang Y, Mohandas A, Stucker B, Nguyen KT. A review of materials, fabrication methods, and strategies used to enhance bone regeneration in engineered bone tissues. J Biomed Mater Res B 2007; 85: 573-82.

[4] Yeo A, Rai B, Sju E, Cheong JJ, Teoh SH. The degradation profile of novel, bioresorbable PCL:TCP scaffolds: an in vitro and in vivo study. J Biomed Mater Res B 2007; 84: 208-18.

[5] Flanagan TC, Wilkins B, Black A, Jockenhoevel S, Smith TJ, Pandit AS. A collagen-glycosaminoglycan co-culture model for heart valve tissue engineering applications. Biomaterials 2006; 27: 2233-46.

[6] Willows A, Fan Q, Ismail F, Tomlins PE, Mikhalovska Li, et al. Assessment of tissue scaffold degradation using electrochemical techniques. Acta Biomater 2008; 4: 686-96.

[7] Olah L, Borbas L. Properties of calcium carbonate-containing composite scaffolds. Acta Bioeng Biomech 2008; 10(1): 61-6.

[8] Chung HJ, Park TG. Surface engineered and drug releasing pre-fabricated scaffolds for tissue engineering. Adv Drug Deliv Rev 2007; 59: 249-62.

[9] Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 2006; 27: 3413-31.

[10] Hasirci V, Berthiaume F, Bondre SP, Gresser JD, Tran-tolo DJ, Toner M, et al. Expression of liver-specific functions by rat hepatocytes seeded in treated poly (lactic-co-glycolic) acid biodegradable foams. Tissue Eng 2001; 7: 385-94.

[11] Kuo YC, Leou SN. Effects of composition, solvent, and salt particles on the physicochemical properties of poly-glycolide/poly(lactide-co-glycolide) scaffolds. Biotechnol Prog 2006; 22: 1664-70.

[12] Po KY, Hui S, Vasily NG, Katherine AF. Three-dimensional interconnected microporous poly(dimethylsiloxane) microfluidic devices. Lab on a Chip 2011; 11: 1541-4.

[13] William LM, Robert GD, Joel LK, David JM. Salt Fusion: An Approach to Improve Pore Interconnectivity within Tissue Engineering Scaffolds. Tissue Eng 2002; 8: 43-52.

[14] Wang M. Composite Scaffolds for Bone Tissue Engineering. Am J Biochem Biotechnol 2006; 2: 80-4.

[15] Agarwal CM, Athanasiou KA. Technique to control pH in vicinity of biodegrading PLA-PGA implants. J Biomed Mater Res 1997; 38:103-14.

[16] Shinoka T, Shum TD, Ma PX, Tanel RE, Isogai N, Langer R, et al. Creation of viable pulmonary artery autographs through tissue engineering. J Thorac Cardiovasc Surg 1998; 115: 536-45.

[17] Ito A, Maekawa K, Tsutsumi S, Ikekaki F, Solubility product of OH-carbonated hydroxyapatite. J Biomed Mater Res 1997; 36:522-8.

[18] Helebrant A, Jonasova L, Sanda L. The influence of simulated body fluid composition on carbonated hydroxyapatite formation. Ceramics 2002; 46: 9-14.

[19] Kokubo T, Kushitani H, Sakka S, Katsuki T, Yamamuro T. Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. J Biomed Mater Res 1990; 24: 721-34.

[20] Chandra R, Renu R. Biodegradable Polymers. Prog Polym Sci 1998; 23: 1273-335.

[21] Kaufmann PM, Heimrath S, Kim BS, Mooney DJ. Highly porous polymer matrices as a three-dimensional culture system for hepatocytes. Cell Transplant 1997; 6: 463-8.

[22] Mekala NK, Baadhe RR, Parcha SR. Study on osteoblast like behavior of umbilical cord blood cells on various combinations of PLGA scaffolds prepared by salt fusion. Curr Stem Cell Res Ther 2013; 8: 253-9.

[23] Murphy WL, Kohn DH, Mooney DJ. Growth of continuous bone-like mineral within porous poly(lactide-co-glycolide) scaffolds in vitro. J Biomed Mater Res 2000; 50: 50-8.

[24] Chen Y, Zhou S, Li Q. Microstructure design of biodegradable scaffold and its effect on tissue regeneration. Biomaterials 2011; 32: 5003-14.

[25] O Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen GAG scaffolds. Biomaterials 2005; 26: 433-41.

[26] Rossella D, Claudia C, Ida G, Tiziana M, Bice C. Effect of porogen on the physico-chemical properties and degradation performance of PLGA scaffolds, Polym Degrad Stabil 2010; 95: 694-701.

[27] Freyman TM, Yannas IV, Gibson LJ. Cellular materials as porous scaffolds for tissue engineering. Prog Mater Sci 2001; 46: 273-82.

[28] Zhang R, Ma PX. Poly(α-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology. J Biomed Mat Res A 1999; 44: 446-55.

[29] Wan Y, Fang Y, Wu H, Cao X. Porous polylactide–chitosan scaffolds for tissue engineering. J Biomed Mat Res A 2006; 80: 776-89.

[30] Yoo JY, Kim JM, Seo KS, Jeong YK, Lee HB, Khang G. Characterization of degradation behavior for PLGA in various pH condition by simple liquid chromatography
Properties of PLGA scaffolds fabricated by salt fusion

[31] Houchin ML, Topp EM. Physical properties of PLGA films during polymer degradation. J Appl Polym Sci 2009; 114: 2848-54.

[32] Hirenkumar KM, Steven JS. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. Polymers 2011; 3: 1377-97.

[33] Kim K, Yu M, Zong X, Chiu J, Fang D, Young SS, et al. Control of degradation rate and hydrophilicity in electrospun non-woven poly (d,l-lactide) nanofiber scaffolds for biomedical applications. Biomaterials 2003; 24: 4977-85.

[34] Ramakrishna S, Mayer J, Wintermantel E, Leong KW. Biomedical applications of polymer-composite materials: a review. Compos Sci Technol 2001; 61: 1189-224.

[35] Bigi A, Boanini E, Panzavolta S, Roveri N, Rubini K. Bonelike apatite growth on hydroxyapatite-gelatin sponges from simulated body fluid. Inc J Biomed Mater Res 2002; 59: 709-14.

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