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Solvent Based 3D Printing of Biopolymer/Bioactive Glass Composite and Hydrogel for Tissue Engineering Applications

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Abstract

Three-dimensional (3D) bioprinting is an emerging technology in which scaffolding materials and cell-laden hydrogels may be deposited in a pre-determined fashion to create 3D porous constructs. A major challenge in 3D bioprinting is the slow degradation of melt deposited biopolymer. In this paper, we describe a new method for printing poly-caprolactone (PCL)/bioactive borate glass composite as a scaffolding material and Pluronic F127 hydrogel as a cell suspension medium. Bioactive borate glass was added to a mixture of PCL and organic solvent to make an extrudable paste using one syringe while hydrogel was extruded and deposited in between the PCL/borate glass filaments using a second syringe. The degradation of the PCL/borate glass composite scaffold with and without the presence of hydrogel was investigated by soaking the scaffold in minimum essential medium. The weight loss of the scaffold together with formation of a hydroxyapatite-like layer on the surface shows the excellent bioactivity of the scaffold.

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Keywords: 3D Bioprinting; Biopolymer/bioactive glass composite; Pluronic hydrogel; Borate bioactive glass

1. Introduction

The main role of a scaffold in tissue engineering is to create a three-dimensional environment to recruit appropriate cells and to repair the specific tissue while providing temporary structural integrity at the defect site. Three-dimensional bioprinting processes can create such a scaffold with programmed spatial arrangements of cells. One of the most essential characteristics for implant integration with the rest of the animal body is the vascularization of the implant (formation of blood vessels). Factors such cell type, materials, and method of fabrication play an important role in the vascularization of the scaffold. Incorporating materials such as bioactive glass in the scaffold can transform the surrounding 3D environment with its dissolution products by up-regulating cell-cell and cell-matrix interactions, which promotes vascularization.

Fused deposition modeling (FDM) is one of the widely used processes in 3D bioprinting to fabricate the scaffolding (not including hydrogels/bio-inks). In the FDM method, the polymer is melted and deposited as filaments using an orifice in a layer-by-layer fashion. The deposited filaments solidify upon cooling. However, FDM fabricated biopolymer scaffolds are only biocompatible and not bioactive. Because of the high temperatures involved in melting biopolymers such as polylactic acid (PLA), with a melting point of 160°C, polycaprolactone (PCL) has become one of the most widely used polymers owing to its low melting point of 60°C, even though it takes several months to a couple of years for its complete degradation [1]. Several studies have considered the

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addition of bioceramics to a polymer matrix to improve the bioactive properties of the polymer composite [2]. However, no significant improvement in FDM scaffolds was reported with little to no dissolution of the bioactive glass into the surrounding environment [3]. This could be because of the dense filaments with no significant porosity for the glass to interact with the surrounding media.

Bioactive glasses elicit a specific biological response at the interface of the materials, which results in a bond between tissue and the implant [4]. The advantage of bioglasses over other bioceramics is their amorphous structure and high resorbability. More specifically, glasses with a strong and chemically durable network such as SiO_2 (e.g., 45S5 glass and 13-93 glass) are not as bioactive in comparison to glasses with a weak network such as B_2O_3. The compositions of the above glasses are given in Table 1. Bioactive borate glass (13-93B3), which degrades faster (a few hours to days in a simulated body fluid) in comparison to silicate glass, has found applications in repairing both soft and hard tissues [4]. This glass is FDA approved and has been shown an excellent candidate for soft tissue regeneration and vascularization, wound repair. 13-93B3 glass in the form of submicron fibers (resembling cotton candy) is manufactured by MO-SCI Corporation (Rolla, MO) for use in wound healing [5]. The release of ions from 13-93B3 glass in a controlled manner is the reason for its wound healing properties, and the release of boron ions contributes to its highly angiogenic nature [6,7]. This makes 13-93B3 glass an excellent candidate for use in 3D bioprinting to investigate its effect on different cell types including stem cells on generating vascular networks.

Table 1. Compositions (in wt.%) of 13-93, 13-93B3, and 45S5 glasses.

Compositions	13-93	13-93B3	45S5
Na_2O	6	6	24.5
K_2O	12	12	0
MgO	5	5	0
CaO	20	20	24.5
SiO_2	53	0	45
P_2O_5	4	4	6
B_2O_3	0	53	0

Pluronic F127 hydrogel was used in this study for two purposes: (i) to fabricate a porous scaffold using the PCL/13-93B3 glass composite, and (ii) to act as a medium to disperse cells and print them alongside the PCL/13-93B3 glass composite to create a cellularized scaffold. Pluronic F127 is a thermo-sensitive and widely investigated hydrogel in bioprinting due to its ease of printing, reverse thermal gelation, and compatibility with multiple cell types [8]. In addition, Pluronic F127 is FDA approved and inexpensive. Pluronic is a poly (ethylene oxide) - poly(propylene oxide) - poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymer, which undergoes sol-to-gel transition when increasing the temperature above 20°C (the lower critical gelation temperature). Researchers have used Pluronic hydrogel as a supporting material during the fabrication of a complex shaped part or a porous structure because it is easy to rinse away Pluronic by decreasing the temperature below the critical point [9]. Alternatively, it can also be used for cell encapsulation purposes which would be beneficial for 3D bioprinting processes [10].

The aim of this study is to create a bioactive polymer composite scaffold for use in tissue engineering applications. To investigate the feasibility of printing a bioactive scaffold using a two syringe system, a PCL/13-93B3 glass composite was used in the first syringe as a scaffolding material and Pluronic hydrogel was used in the second syringe either as a supporting material to create a 3D porous structure or as a cell-carrier to create a cellularized scaffold.

2. Materials and Methods

2.1. PCL/Borate bioactive glass paste preparation

PCL (Sigma-Aldrich, St. Louis, MO) was dissolved in chloroform (Sigma-Aldrich, St. Louis, MO) in a covered glass container with the help of a stirrer at ~50°C. Chloroform in the amount of 4ml was used to dissolve 5g of PCL for the best possible ratio between them for printing. Then, 5g of 13-93B3 borate bioactive glass (MO-SCI Corp., Rolla, MO) of size less than ~20µm was added to the PCL:chloroform mix after complete dissolution of PCL. The 50:50 PCL/13-93B3 glass paste was continuously stirred for about another 30min after adding the glass particles to obtain a uniformly mixed composite paste with no glass particle precipitate before transferring the required quantity to a 3ml syringe barrel (Loctite® Henkel North America, Rocky Hill, CT) with a dispensing tip (25G) for printing. More details on paste preparation can be found in Murphy et al. [11].

2.2. Hydrogel preparation

Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) was dissolved in Dulbecco's modified Eagle's Medium (DMEM) (Lonza). The weight percentage of F-127 was 1:4 (grams of F-127 to ml of DMEM). This ratio was based on the results by Gioffredi et al. [8] as a 25% weight to volume ratio of the F-127 solution provided suitable gelation and extrusion parameters for 3D printing applications. Chilled DMEM was used to prepare the F-127 solution. F-127 was slowly added to avoid clumping of the particles. The glass container was covered and kept in ice bath maintained at ~4°C, while being stirred. After F-127 was fully dissolved, the glass container was kept in a vacuum chamber for about ~2mins to remove air trapped in the solution while stirring. The F-127 solution was covered and stored in the ice bath. Before printing, the F-127 solution was poured into a 3ml syringe barrel with a dispensing tip (30G). The syringe was then placed in an incubator at 37°C for about 10 minutes for complete gelation before moving it to the printer.

2.3. Scaffold fabrication

Fabrication was performed with an assembled DIY 3D printer (Geeetech, Prusa I3 A Pro), which was modified to have two syringes connected to external digital syringe dispensers (Loctite® Henkel North America, Rocky Hill, CT). The dispensers are computer controlled. The schematic of the 3D

printer set-up is shown in Fig. 1a. The scaffold (10x10x1mm^3) was printed with 0-90° orientation of the filaments in alternate layers on a heated bed plate maintained at ~37°C. A customized g-code was written for the printer for directing the nozzle movement. The printing parameters such as filament spacing, layer height, printing speed, etc. were identified based on the optical microscopic images after a two-layer extrusion of both materials. A printing speed of 8mm/s was used for both materials. Needle tips with internal diameter of 260µm and 160µm were used for PCL+13-93B3 glass composite paste and hydrogel respectively. Air pressure of 40psi was required to extrude the composite paste while 30psi was found to be suitable for the hydrogel extrusion. The printing parameters were tuned so as to obtain similar filament deposition characteristics such as filament width and height for both materials during fabrication. Filament spacing of 0.8mm was used for both the PCL+13-93B3 glass composite and the hydrogel filaments during fabrication. A dwell time of ~2min was required to fabricate scaffolds with the PCL+13-93B3 glass composite. However, printing hydrogel filaments alongside the polymer composite filament meant an increased dwell time of ~4 min for each layer. The wait time was compensated by printing multiple parts [12] in a single run.

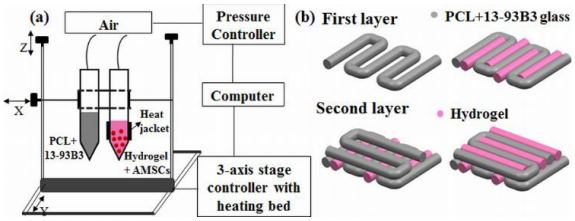

Fig. 1. (a) schematic of the 3D printer; (b) schematic of the printing with alternate filaments of PCL+13-93B3 glass composite and hydrogel.

2.4. Scaffold degradation

PCL+13-93B3 glass composite degradation was studied on scaffolds measuring (10 x 10 x 1) mm^3. The printed scaffolds were dried overnight and weighed before immersion in minimum essential medium alpha modified (α-MEM). 300 mL of α-MEM was used for 1g of the scaffold for soaking and the bottles were stored in an incubator maintained at 37°C for different time intervals ranging from 1 day to 7 days. After removal, scaffolds were gently washed with de-ionized (DI) water, and again dried overnight. The dried scaffold was weighed to calculate the weight loss percentage and the results were reported as mean ± standard deviation. At least three samples were used for each data point. Scaffolds were sputter coated with gold/palladium (Au/Pd) for 60s before performing scanning electron microscopy (SEM). SEM (Hitachi S-4700 FESEM, Hitachi Co., Tokyo, Japan) images were taken to evaluate the surface morphology of scaffolds before and after the immersion. PCL+13-93B3 glass composite scaffolds fabricated with and without the hydrogel were used for the degradation tests.

3. Results and Discussion

3.1. Scaffold fabrication

A filament width of 397±100µm and pore size of ~400µm was measured for scaffolds printed with the PCL+13-93B3 glass composite paste with a filament spacing of 0.8mm. Similar filament width and pore size was also observed for the hydrogel (~400µm). Fig. 2 shows the pictures of sample scaffolds. Food color was added to hydrogel for contrast in Fig 2c and 2d. It has to be noted that the tip size used for the composite paste is 260µm and 160µm for the hydrogel. Though tips with smaller diameter (210µm and 160µm) can be used to extrude the polymer composite paste with higher air pressure, frequent clogging was noticed with their use during fabrication. A 160µm tip was used to extrude the hydrogel to achieve a larger filament to fill the space between polymer composite filaments even though it was possible to extrude hydrogel filaments using a smaller 110µm tip. It is reported that the F-127 solution behaves as a Newtonian-fluid having a constant viscosity with increasing shear rate whereas the hydrogel viscosity decreases with shear rate as a function of power law [8]. In our results, we noticed the difficulty in maintaining the circularity of the hydrogel filament extruded using a 160µm dispensing tip. A smaller tip (110µm) and reduced air pressure generated a more circular filament because of the quick viscosity recovery of the hydrogel which is less likely with a larger tip and higher air pressure. Also, another reason for not being able to obtain a near circular filament could be due to the current syringe heating set-up which only covers the syringe barrel maintaining the hydrogel at 37°C but does not cover the dispensing tip which leaves a certain amount of hydrogel at reduced temperature (30°C) just before printing. As the aim of the study was to create a PCL+13-93 glass composite scaffold and not a hydrogel alone scaffold, the issue of filament circularity was not addressed in this study.

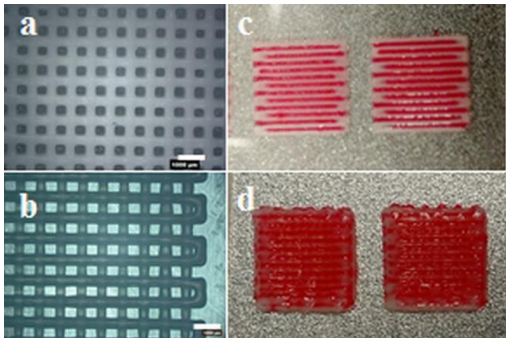

Fig. 2. (a-b) optical microscopic images after printing two layers of (a) polymer composite, (b) hydrogel, (c) picture taken after first layer printing of scaffolds measuring 10x10mm, and (d) picture after 8 layers (scale bar – 1mm).

The scaffold printed with both PCL+13-93B3 glass composite and hydrogel was soaked in a glass container with chilled DI water and kept in ice-bath maintained at ~4°C. Fig. 3a shows an optical image of the top view of the scaffold dried

overnight before dissolution of the hydrogel while Fig. 3c shows the cross-section. The hydrogel was rinsed away revealing the pores in the scaffold as shown in Fig. 3b with a top view and Fig. 3d with a cross-sectional view. The pores in Z-direction are much smaller (tens of microns as opposed to hundreds of microns) in comparison to pores in X-Y direction.

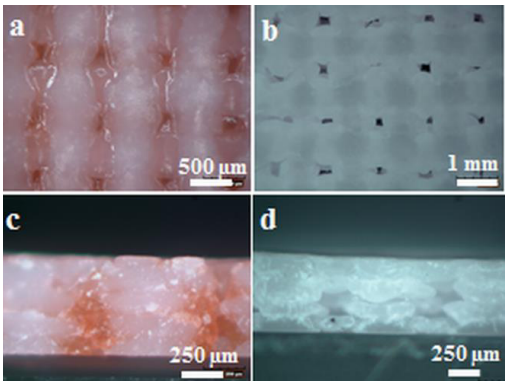

Fig. 3. Optical microscopic images of top view (a) before hydrogel removal, (b) after hydrogel removal; cross-sectional views of the scaffold, (c) before hydrogel removal, and (d) after hydrogel removal.

3.2. Degradation and bioactivity

It has been reported that even cell culture media can serve as an alternative to conventional simulated body fluid (SBF) to evaluate the bioactivity of the material in terms of its capability to precipitate hydroxyapatite [12,13]. In our study, α-MEM was used to soak the scaffolds for the weight loss experiments. The degradation of the PCL+13-93B3 glass scaffolds showed no significant weight loss during the first 3 days (~1%) and ~11% after 7 days. As PCL takes a longer time to degrade, the weight loss measured is attributed to the ionic dissolution of the 13-93B3 glass. Fig. 4 shows the weight loss comparison of the scaffolds fabricated with and without the hydrogel. For PCL+13-93B3 glass scaffolds fabricated with hydrogel, the weight loss was ~14% after 1 day and 16% after 3 days.

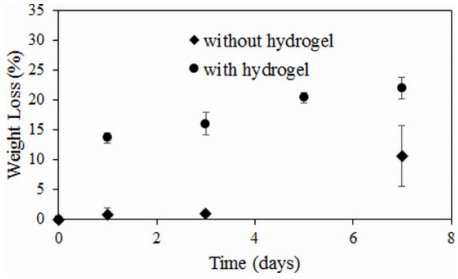

Fig. 4. Weight loss percentage profiles of the PCL+13-93B3 scaffolds fabricated with and without hydrogel after soaking in α-MEM at 37°C.

A consistently higher weight loss (about 11-13%) was measured for PCL+13-93B3 glass scaffolds printed with hydrogel in comparison to those printed without hydrogel over a period of one week of soaking in α-MEM. The unstable nature of the Pluronic in cell culture conditions was previously reported by Gioffredi et al. [8]. We have also noticed that Pluronic present on the outer layers separated itself from the scaffold and settled in the container. Therefore, the initial ~15% weight loss (first three days) of the scaffold can be almost entirely attributed to the hydrogel because the weight loss through glass dissolution studied using PCL+13-93B3glass scaffolds was less than 1% during the same time frame. With the exception of the initial weight loss, the weight loss trend of the scaffolds fabricated with and without hydrogel remained the same.

Formation of florets, which typically represent hydroxyapatite-like material, was observed on the filament surface as shown in Fig 5. 3D printing of scaffolds using the solvent dissolved PCL+13-93B3 glass composite provides internal porosity to the filament because of the chloroform evaporation. In comparison, scaffolds fabricated using the FDM process and other melt-derived scaffold fabrication techniques have dense filaments. The high scaffold porosity combined with a faster glass dissolution shows the solvent based 3D printing process potential for different tissue engineering applications. In fact, PCL+13-93B3 glass composite thin sheets have been investigated for nerve tissue repair [14], 13-93B3 glass nano fibers were found highly beneficial for faster wound healing and angiogenesis even in un-healable diabetic wounds [5,6], and 13-93B3 glass was investigated for bone repair using different manufacturing processes [7,15,16].

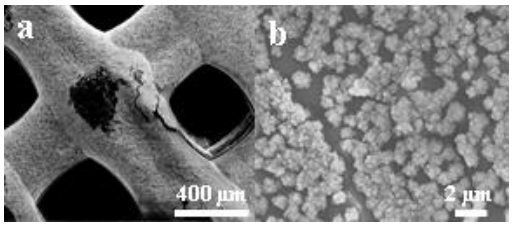

Fig. 5. SEM images of (a) surface of the scaffold printed without hydrogel after immersion in α-MEM for 14 days, (b) magnified image showing the hydroxyapatite-like crystal floret formation on the surface.

Fig. 6 shows the optical images of the scaffolds after soaking in α-MEM for 1 day, 5 days, and 7 days. It was observed that the pores on the outer surface of the scaffold revealed themselves as soon as the first day of soaking in the media. This is because the hydrogel on the outer layers of the scaffold separated itself from the rest of the scaffold in less than one day. This observation is also consistent with the weight loss data which shows a weight loss of ~14% after day 1 and then the rate loss slows down thereafter. The difficulties in performing long-term culture assays on scaffolds made with only Pluronic F127 hydrogel was reported because of the unstable nature of the hydrogel [8]. In our experiments, even as the hydrogel present on the outer layers of the scaffold separates itself at the beginning, hydrogel present in the inner layers of the scaffold seems to be present after 5 days and to a certain extent after 7 days. The hydrogel can be seen in the

optical images as a material in more contrasting white while the pores closed with the presence of hydrogel are marked using arrows in Fig. 6a and 6b. More through pores can be seen in the scaffold soaked for 7 days in the media. This result indicates that long-term culture assays (at least up to 7 days) can be performed on the scaffolds bioprinted with PCL+13-93B3 glass composite and cell dispersed Pluronic hydrogel.

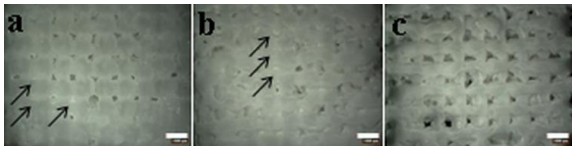

Fig. 6. Optical microscope images of the scaffolds after soaking in α-MEM for (a) 1 day, (b) 5 days, and (c) 7 days (scale bar – 1mm). More pores are revealed with increased soaking time.

The surface morphology of the scaffold before and after soaking in the media is shown in Fig. 7. Fig. 7a shows the scaffold printed with PCL+13-93B3 glass composite and hydrogel. The surface with no presence of macro pores indicates that the gaps between the PCL+13-93B3 glass composite filaments are completely filled with the hydrogel. Fig. 7b and 7c shows the pores of the scaffolds that are revealed after hydrogel separates from the scaffold. Deeper pores can be seen in the scaffolds soaked for 7 days (in Fig. 3c) and hydrogel was present in scaffolds soaked for 1 day (in Fig. 3b). The interface of the hydrogel-PCL+13-93B3 glass composite filament has a different surface morphology with more surface micro pores present in this region. This can be clearly observed in the magnified image of the region of the scaffold pore as shown in Fig. 7d. Such micro pores could aid in faster glass dissolution and thereby improve the scaffold bioactivity.

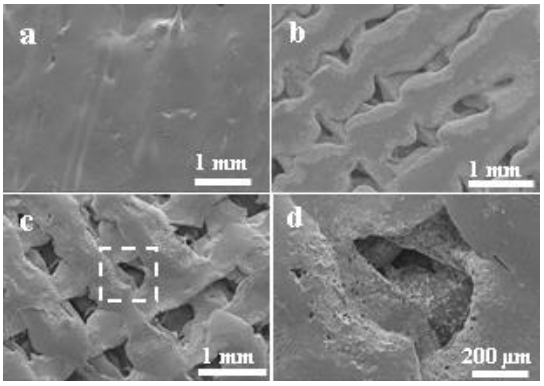

Fig. 7. SEM images of scaffold surface (a) after printing with hydrogel, (b) after soaking in α-MEM for 1 day, (c) after 7 days, and (d) magnified image of the region marked in (c) showing the pore surface morphology.

Different fabrication methods are used to create 3D scaffolds using polymer-glass composite for tissue engineering applications. These include freeze drying [17], particulate leaching [18], gas foaming [19], electrospinning [20], and 3D printing [21]. One of the major concerns in seeding cells on the fabricated scaffold is the low seeding efficiency and inhomogeneous distributions. In a static cell culture, cells on the scaffold outer surface could act as a barrier to diffusion of nutrients and oxygen to the inside thereby resulting in cell death in the scaffold interior [22]. Some researchers have studied the effects of scaffold architecture to improve the cell seeding and culturing with mixed results [23]. Though perfusion of media through scaffolds using a bioreactor is better than static culture, it has limitations in promoting cell migration and uniform formation of extracellular matrix [24]. In our bioprinting process, cell-laden hydrogels and scaffolding materials are deposited together to control the spatial distribution of cells in the scaffold.

Recently, electrospinning and 3D printing are being widely used to fabricate cell-laden scaffolds. The diameter of the electrospun fiber typically ranges from a few hundreds of nanometer to tens of micron and the weight loss of the polymer-bioglass scaffold is relatively high (18%) within 6 days, providing required bioactivity [20]. Researchers have combined electrospinning and 3D printing techniques to create new tissue engineering approaches [25]. Among different 3D printing processes, melt deposition and FDM are most commonly employed to fabricate a polymer composite scaffold. Bioactive glass particles added to the polymer melt is pneumatically extruded through a syringe in the melt deposition process and a polymer-bioactive glass wire is used in the FDM process to fabricate the scaffold. In both cases, the extruded filament will be dense with little to no filament porosity, limiting the dissolution of glass into the 3D environment and bioactivity [3]. The results in this paper show the bioactivity of the scaffold is maintained unlike the melt-deposition process and also the scaffold fabrication process is much faster than the electrospinning process, thereby demonstrating the potential of the process as a 3D bioprinting technique for tissue engineering applications.

4. Conclusion

This study investigated the feasibility of fabricating a 3D porous scaffold with PCL, 13-93B3 bioactive borate glass, and Pluronic F-127 hydrogel. Pluronic hydrogel was printed as filaments alongside the PCL+13-93B3 glass composite filaments. The weight loss measurements together with microscopic images of the scaffolds indicate the presence of hydrogel after 7 days of soaking in the culture media despite the unstable nature of Pluronic. The high scaffold porosity with the presence of macro and micro pores enables faster glass dissolution and the formation of hydroxyapatite-like layer on the surface shows the scaffold's bioactivity. The results demonstrate a high potential of the solvent based extrusion process in 3D bioprinting of a cellularized scaffold with bioactive properties for tissue engineering applications.

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