Research Article

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Bioactive glass-polycaprolactone fiber membrane and response of dental pulp stem cells *in vitro*

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Abstract: The study reports the fabrication and *in vitro* biological evaluation of a sol-gel derived bioactive glass (BG) / polycaprolactone (PCL) composite fiber membrane, as a potential candidate for bone regeneration applications. The non woven composite mats were prepared by introducing the glass particles into the electrospinning process. Adding the glass improved the homogeneity of the fibers. The apatite forming ability of the membranes in simulated body fluid were evaluated and showed that hydroxyapatite had formed within 21 days in SBF and completely covered the surface of the membrane. In cell culture, dental pulp stem cells adhered proliferated and produced mineralized matrix on the PCL/BG fiber membrane.

Keywords: Bioactive glass/polycaprolactone fibers, Bone tissue engineering, Dental pulp stem cells, Electrospinning

1 Introduction

Bioactive glasses (BGs) are frequently used, but not limited to, orthopedic applications for the repair and reconstruction of damaged or diseased bone tissue. BGs have the ability to bond with living tissue through the formation of hydroxyapatite (HA) following exposure to physiological environment and also their ionic degradation products have shown to stimulate osteogenic potential of osteoblast cells [1–5]. However, due to the brittle nature and fast degradation rate of BGs, they are often incorporated into a polymeric matrix to produce porous biodegradable composites that can act as a temporary 3D template for cell adhesion, proliferation and differentiation in bone tissue engineering applications [6–9].

Biodegradable polycaprolactone (PCL) has been studied extensively for use in soft and hard tissue engineering applications but the low degradation rate, hydrophobicity and lack of bioactivity limits its biomedical applications [10–13]. Combination of this polymer with BG could compensate for its intrinsically hydrophobic nature with poor cell adhesion and could offer a promising approach to overcome the limitations associated with the use of PCL [14–17].

A composite of BG/PCL would mean that the ionic products of BGs are released at an effective and controlled manner and the resultant composite fiber membranes can be applied as a multifunctional scaffolds for bone tissue engineering [12, 14, 16, 18, 19]. In addition, upon the dissolution of BGs, the controlled release of ionic products creates new functionalities, such as osteogenesis, osteoconductive, and osteoinduction effects [20–25]. To date, some studies have been carried out on BG contained PCL [26–28]. However, the main challenge associated with the produced fibers was the incorporation of 10-20 wt % melt-derived as lower amounts slowed down the production of HA. In addition, biological evaluations on the proposed structures were not evaluated. Therefore, in the current study, the aim was to utilize ternary sol-gel derived BG particulates, at lower concentration, for uniform fiber preparation.

Dental pulp stem cells (DPSCs) receive great attention for tissue regeneration applications due to their relative ease of isolation and expansion from dental pulp of molars with great differentiation potential to chondroblasts, neurons, endothelial odontoblast and osteoblast cells [29–32]. There are a great number of studies that are focused on PCL composites containing different forms of bioactive

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materials [28, 33]. However, to the best of our knowledge, there are limited studies that have reported the potential of this structure for the proliferation and differentiation of stem cells. Hence, the current study utilizes electrospinning, a simple and versatile processing method, to prepare composite of PCL/BG fiber membrane and then evaluate its bioactivity and biological behavior following exposure to dental pulp stem cells *in vitro*.

2 Materials and methods

2.1 Bioactive glass powder synthesis

BG powders of 60 mol% SiO_2, 12 mol% P_2O_5 and 28 mol% CaO composition were synthesized via sol-gel processing route [34]. This composition was chosen as it releases phosphate in addition to calcium ions, which can reduce the pH burst associated with bioactive glasses. All reagents were purchased from Sigma-Aldrich (UK). The compositions of the glass samples were based on 58S composition (60 mol% SiO_2, 36 mol% CaO and 4 mol% P_2O_5), which was made by hydrolysis of tetraethyl orthosilicate (TEOS) according to previous studies [35, 36]. Bioactive glass particles below 38μm were used

2.2 Electrospining of fiber membrane

PCL was dissolved in a co-solvent of methanol:chloroform in the ratio of 1:9 at room temperature for 4 h. Then, 5 wt% of BG, selected based on previous study [37], was added to the solution and left stirring for 1 h in an ultrasonic bath. The as-prepared solutions were electrospun at a voltage of 25 kV, flow rate of 7 ml/h and a collecting distance of 10 cm. Electrospinning procedure was performed under ambient conditions. The fibers were collected on an aluminum foil and then transferred to a desiccator prior to further investigations.

2.3 Characterization

X-ray diffraction (XRD) patterns of the scaffolds samples were obtained using a Phillips PW1050 diffractometer (PANalytical, NL). The structure and morphology of the samples were observed with a JEOL JSM6300 scanning electron microscope (SEM) equipped with energy dispersive x-ray (EDX).

2.4 Bioactivity testing

Bioactivity testing was carried out in simulated body fluid (SBF) in accordance to an existing protocol for 21 days [37]. Fiber mats were cut into 1x1 cm sections and immersed in 10 ml SBF. A falcon tube containing SBF as a control was incubated throughout the study to monitor the stability of the testing solution. At the end of each time point, samples were removed from the falcon tube, dried and analysed using XRD and SEM to check for HA formation.

2.5 Cell culture studies

Isolation and cell culture of DPSCs

Intact human impacted third molars with immature roots were collected, under the approved guidelines by the Ethical committee of Royan institute as previously described by Labbaf *et al.* [35, 36]. The pulp was separated from the crowns and roots, minced into small pieces and digested in 3 mg/mL collagenase type I (Sigma-Aldrich). The cell suspensions were collected and seeded onto a culture plate containing DMEM with 10% fetal bovine serum (Gibco, USA), 1% penicillin (Gibco, USA) and 1% streptomycin (Gibco, USA) at 37°C in 5% CO_2.

Scaffold sterilization

The as-prepared scaffolds were sterilized with 96% ethanol for 30 minutes and washed twice with PBS and were then placed in pencilin/ Streptomycin and PBS^- for 15 minutes. Finally wash with PBS and put in a 24-well plate. To avoid the floating of the scaffolds, plastic rings were placed on top of the scaffolds. Rings were sterilized with 96% ethanol for 3 days and washed with PBS and put in pencilin/ Streptomycin and PBS for 1 day.

Cell morphology assessments

The DPSCs were cultured at a density of 30,000 cells/scaffold in a 24-well plate. After 4 hours, cells were fixed with 2.5% gluteraldehyde (Sigma, UK) in PBS for 40 minutes at 4°C. Cells dehydration was achieved through increased ethanol concentration (0, 25, 50, 75 and 100%). Samples were sputter coated with gold and viewed using scanning electron microscope (SEM, type of Philips XL30).

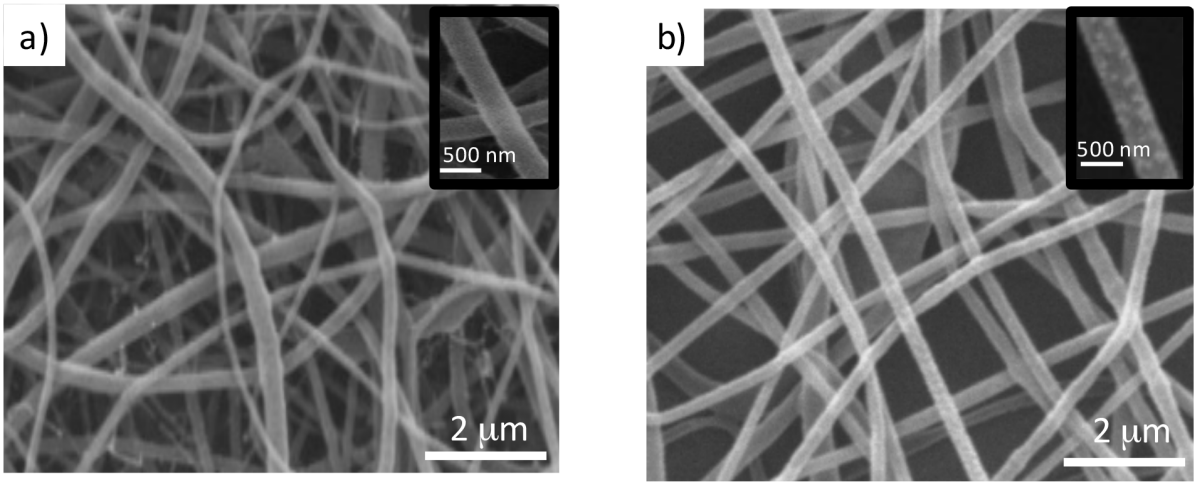

Figure 1: SEM images of as-spun fibers of a) PCL and b) PCL/BG.

Cell attachment and viability assessments

DPSCs were cultured at a density of 30,000 cells/scaffold. Cell attachment was evaluated after 4 hours and cell viability was conducted at 1, 3, 5, 7 and 14 days following culture. At the end of each time point, for viability studies, Alamar blue cytotoxicity/proliferation assay was conducted. Samples were incubated for 2 h in 500 µl of Alamar blue solution and then measured at 570 nm using the microplate reader (Bio-Rad, USA).

Matrix mineralization

The DPSCs were cultured at a density of 30,000 cells/scaffold in a 24-well plate for 21 days and the medium was changed every three days. It must be noted that the tests were carried out in culture medium in the absence of osteogenic supplements. Following 21 days of culture, cells were washed and fixed in 96% ethanol and then stained with 0.2% alizarin red solution in water (pH 6.4) at for 1 hr. Alizarin red was dissolved in a solution of 20% methanol and 10% acetic acid in water for 15 min. The plate was read at 450 nm using the micro plate reader (Awareness Technology Stat Fax 2100 Microplate Reader, USA).

Statistical analysis

The results were reported as mean ± standard deviation (SD) of two individual experiments each performed in duplicate. Differences between groups were determined by SPSS, one-way analysis of variance (ANOVA) with $p<0.05$ considered significant.

3 Results and Discussion

The main focus of this study is the assessment of the feasibility of PCL/BG fiber mat for bone tissue engineering applications through evaluation of bioactivity and cellular behavior. Figure 1 represents SEM images of PCL and PCL/BG fiber membrane. Interestingly, PCL/BG fibers appear to have a more uniform structure compared to PCL fiber membrane. Also, with the addition of BG particles, the resultant fiber diameter increased due to increase in solution viscosity, in agreement with previous studies [18, 28, 38]. Nevertheless, the fiber diameter in PCL fiber membrane is extremely random and varies from 200 nm to 1 µm. Also, from SEM image (Figure 1b- inset) it is evident that particles also appear on the surface of the fibers giving it a unique surface topography. It is believed that BG-PCL interaction is mainly through interlocking and/or physical interactions to combine the properties of the organic PCL matrix with the reinforcement of the inorganic BG phase embedded inside the fibers.

It is well known that the bioactivity phenomenon of biomaterials is an important parameter for assessing their ability to bond with living bone tissue through the formation of hydroxyapatite (HA) [39]. The mechanism and detailed analysis of HA formation on BG structures has been extensively reported by Hench *et al.* [40]. In the current study, PCL/BG fiber membranes were immersed in SBF for 21 days to test their bioactivity. Table 1 demonstrates the changes in pH with time. In the first few hours there is an

increase in pH, which is related to the dissolution of BG particulate following exposure to SBF. This increase in pH reaches a steady phase at day 7, suggesting that ions have begun to precipitate on fiber surface. At day 14, there is a drop in pH of 6.83, which could be due to the degradation of PCL through breakage of ester bond cleavage through hydrolysis by SBF penetration [41]. PCL degradation leads to the release of acidic by-products, making the solution highly acidic (Table 1). However, due to the presence of BGs ionic dissolution products from the composite fiber membrane, the solution pH is balanced [42].

Table 1: Changes in pH with time of SBF solution for PCL/BG fiber mat sample.

pH	Time
7.5 ± 0.3	0
7.67 ± 0.01	30 min
7.67 ± 0.01	1hr
7.73 ± 0.02	2hr
7.78 ± 0.02	4hr
7.66 ± 0.04	24hr
7.62 ± 0.02	Day 3
7.60 ± 0.05	Day 5
6.83 ± 0.015	Day 14
6.75 ± 0.02	Day 21

Figure 2 represents an XRD pattern for samples before and after immersion in SBF. There are two diffraction peaks that appear in both samples and are related to the crystalline phase of semi-crystalline polymer of PCL (around 21° and 23°) [43, 44]. Following 21 days immersion in SBF, the two additional peaks appear around 26° and 32° which correspond to crystalline HA phase. Therefore, it is believed that the incorporation of BG particles into polymeric matrix greatly enhanced HA formation. This is consistent with previous studies. For instance, in a study by Lin *et al.* [45] it was found that the incorporation of mesoporous BG (MBG) into a PCL nanofibrous matrix significantly enhanced its apatite-formation ability in SBF compared with a PCL nanofibrous matrix. Li *et al.* reported that PCL-MBG scaffold prepared through solvent casting and particulate leaching technique had an improved hydrophilicity and induced significant level of apatite formation after soaking in SBF for 3 weeks [39].

To further confirm HA formation, SEM images were also conducted following 21 days soaking in SBF. It is evident there is a great area of white deposits covering PCL/BG fiber (Figure 3). The appearance of the deposits is typical morphology of HA which is in accordance with

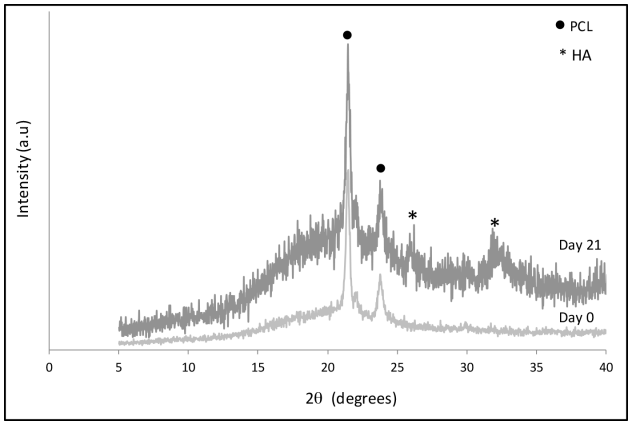

Figure 2: XRD patterns of PCL/BG fibers before (d0) and after (d21) immersion in SBF.

previous studies which have reported such depositions to grow on the surface of PCL/BG fiber structures [38, 46]. It is also shown that the inclusion of BG in PCL matrix would increase its bioactivity and promote calcium phosphate precipitation on the scaffold which is also favorable for cells [39, 47].

Cell-material interaction occurs as a result of a sequence of chemical and physical interactions that modulates cell proliferation, differentiation and extracellular matrix deposition. Cell attachment is hence a crucial process in determining the fate of cell-material interactions. In this regard, DPSC interaction with fiber membranes was qualitatively and quantitatively evaluated. SEM images show the attachment and spreading of DPSCs on PCL/BG composite fiber membrane (Figure 4). The addition of BGs is thought to increase the surface hydrophilicity, roughness and provide anchor points (where the bioactive glass breaches the surface of the fibers), making it more favorable for the cell attachment [18, 28, 33, 48, 49]. Figure 5 demonstrates that cell attachment is greater on tissue culture plastic (TCP- control) than the fiber membrane. This finding is expected as the topography and surface chemistry differs from that of the ideal condition in control group. However, the attachment is greater on PCL/BG membrane than PCL alone. Nevertheless, the cells appear to adopt/adjust and proliferate on the fiber membranes at the following days (Figure 6).

Figure 6 represents DPSCs proliferation and hence viability following culture on PCL/BG fiber membrane. At days 1 and 3 the proliferation rate is significantly lower than the control (TCP); this is in consistent with Fig 5, where a lower cell attachment was observed. The reduced proliferation rate and cell viability at days 1 and 3 could also be because at the first few hours and days following degradation of BGs, there is often a burst release of ions

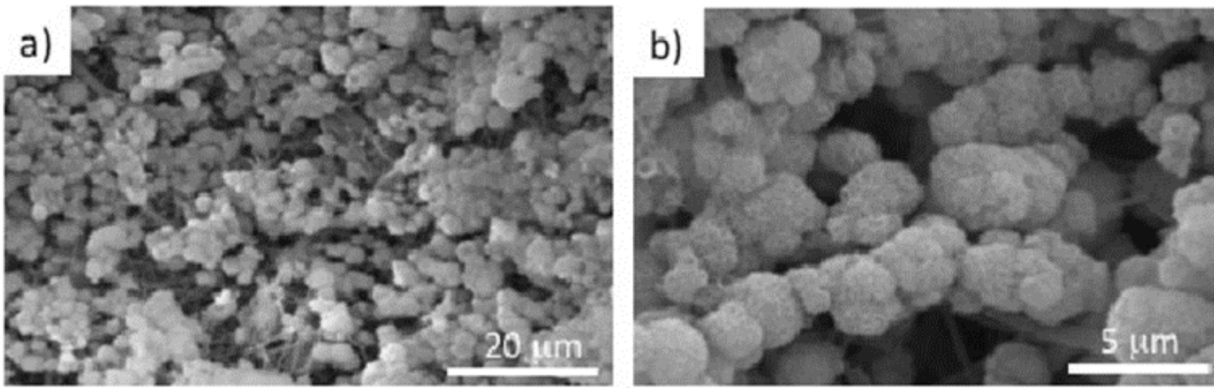

Figure 3: SEM micrograph of PCL/BG after 21 days immersion in SBF, presented at a) low and b) higher magnification.

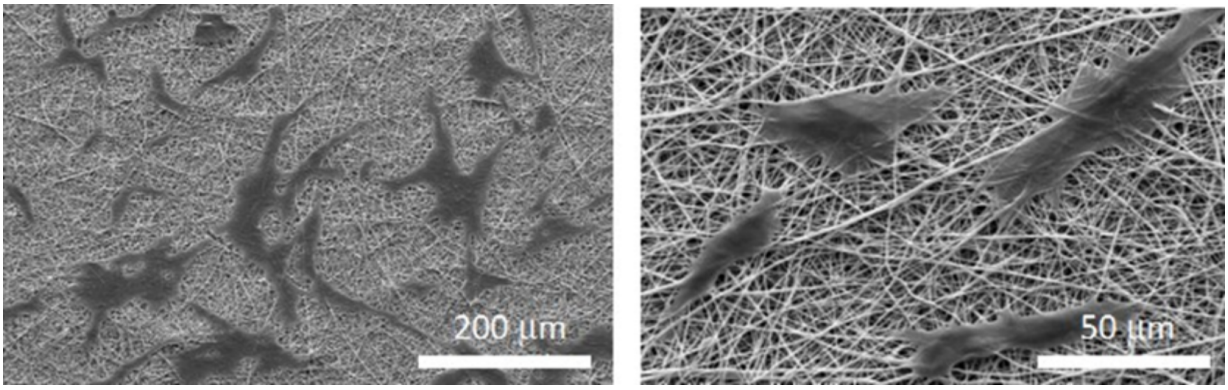

Figure 4: SEM micrographs of DPSCs attachment on PCL/BG surface following 24 h of culture.

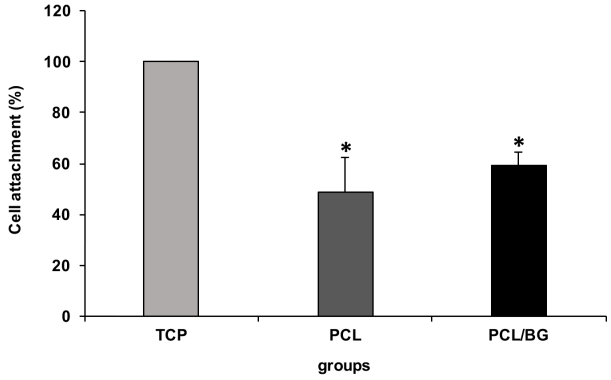

Figure 5: Cell attachment monitored using Alamar blue performed 4 h post seeding. Values represent the mean SD of two individual experiments each performed in triplicate presented as percentages to control.

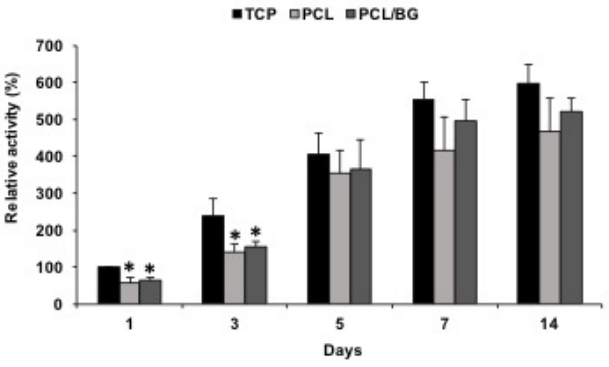

Figure 6: Alamar blue cytotoxicity assays. Values represent the mean SD of two individual experiments each performed in triplicate and presented relative to the control of day 1.

which may cause changes in cellular behavior [21, 35, 36]. Interestingly, at days 5, 7 and 14 the difference between the two groups of control and BG/PCL is negligible. This suggests that the cells have not detached from the surface of the fibers, in fact, have led to increased metabolic activity and proliferation. These changes then stabilize at the following time points. Here compared to In a study by Shirani *et al.* on *In vitro* cultivation of MG63 osteoblast cells, a better cell attachment and growth on the electrospun nanocomposite scaffold (PCL/Gel(50/50)/BG) compared to control of PCL/GEL scaffold was obtained attributed to the bioactive nature of BGs [50]. This pattern was also observed in the current study but it must be noted that the

cells utilized were DPSCs which are quote robust compared to the MG63 cell line

Alizarin red staining was conducted after 21 days of culture (Figure 6). Results demonstrated significant increase in the levels of matrix mineralization on BG/PCL fibers. Based on previous studies, it is believed that matrix mineralization is associated with the release of BG ionic products which are known to stimulate osteogenic differentiation of dental pulp stem cells [35, 36]; however, this requires further evaluation to confirm this finding.

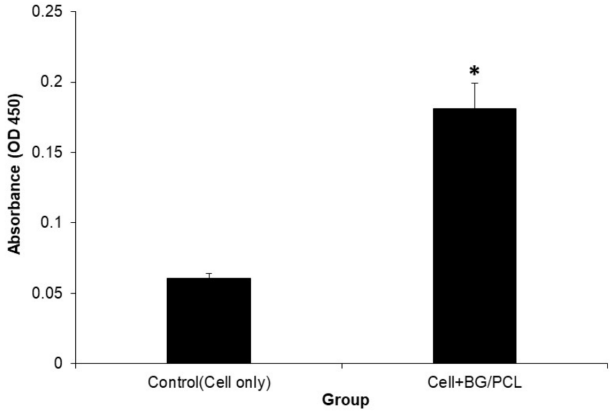

Figure 7: Normalized alizarin red staining absorbance following 21 days in culture.

4 Conclusion

BG/PCL fiber mats were successfully prepared through an electrospining technique. The structure of the fibers was more uniform following the addition of BG particles. It was found that BG-PCL structure, at the given composition of BG, leads to HA formation following 21 days of immersion in SBF. The fibers were shown to be non-cytotoxic, in fact, were found to show osteogenic potential. However, a more thorough and in depth study is required to further evaluate this finding.

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