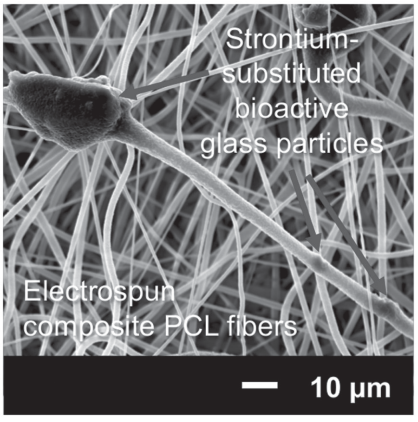
Preparation of Composite Electrospun Membranes Containing Strontium-Substituted Bioactive Glasses for Bone Tissue Regeneration

Martin E. Santocildes-Romero, Rebecca L. Goodchild, Paul V. Hatton,* Aileen Crawford, Ian M. Reaney, Cheryl A. Miller

Barrier membranes used for the treatment of bone tissue defects caused by periodontitis lack the ability to promote new bone tissue regeneration. However, the addition of an osteogenic component to membranes may enhance their regenerative potential. Here the manufacturing of composite membranes made of poly(caprolactone) and strontium-substituted bioactive glass is described using the solution-electrospinning technique, with particles located both inside and on the surface of the fibers. All membranes are characterized using scanning electron microscopy and energy dispersive X-ray spectroscopy, and glass dissolution from within the fibers is investigated in water. In vitro material cytotoxicity is determined using a rat osteosarcoma cell line. Electrospun fibers exhibit porous surfaces and regions of increased diameter where the particles are accumulated. The glass dissolves after immersion in water, releasing dissolution products that are associated with increased pH. Further evidence suggests accelerated polymer degradation due to interactions between both components, which may provide the additional benefit of reducing the pH changes associated with glass dissolution. All compositions are biocompatible in vitro, with the exception of membranes with >50 μg of glass on their surface. In conclusion, these membranes show great potential for bone healing applications, including guided bone regeneration and scaffolds for musculoskeletal tissue engineering.



1. Introduction

Membranes are widely used in periodontology and dental implantology to assist bone healing in an intervention commonly termed guided bone regeneration (GBR). The principle of this therapy is to physically exclude local soft tissues from a defect site, creating a relatively isolated environment where bone can heal. Membranes made using nonbioresorbable polymers, such as polytetrafluoroethylene, have been associated with an increased risk of infection and usually require a second surgical intervention for their removal.[1,2] Bioresorbable materials, in particular collagen, have therefore become more widely employed. However, the association of animal-derived collagen with increased risks of disease transmission, as well as some ethical objections, has limited the clinical use of these types of barrier membranes. Furthermore, all of the materials used to date have lacked the ability to actively promote the formation of new bone tissue beyond their

Dr. M. E. Santocildes-Romero, Dr. R. L. Goodchild, Prof. P. V. Hatton, Dr. A. Crawford, Dr. C. A. Miller
School of Clinical Dentistry
University of Sheffield
19 Claremont Crescent, Sheffield S10 2TA, UK
E-mail: Paul.Hatton@sheffield.ac.uk
Prof. I. M. Reaney
Department of Materials Science and Engineering
University of Sheffield
Sir Robert Hadfield Building
Mappin Street, Sheffield S1 3JD, UK

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physical barrier function. Therefore, in order to further improve bone healing in terms of rate and consistency, an ideal barrier membrane should be both resorbable and capable of stimulating bone tissue regeneration within the defect.

Electrospinning is a versatile manufacturing technique that has been used to fabricate porous membranes for numerous biomedical applications, including drug delivery systems, wound dressings, scaffolds for tissue engineering, and barrier membranes for GBR.[3–6] By selecting appropriate components and manufacturing conditions, it is possible to control the diameter of electrospun fibers (i.e., diameters ranging from a few micrometers to hundreds of nanometers), resulting in the fabrication of structures with high surface areas. Additionally, a wide range of polymers can be processed, which may be combined with other components in order to generate composite fibers.[7] Therefore, electrospinning is a very attractive technique for the fabrication of biocompatible composite membranes incorporating materials with osteogenic properties. For example, the addition of hydroxyapatite to electrospun fibers has been previously investigated, showing promising results.[8–10] However, other bioceramics, such as bioactive glasses, may be preferred instead, mainly due to their superior osteogenic properties. Discovered by Hench in the late 1960s, bioactive glasses are able to form a strong bond with bone and soft tissues post-implantation, and can encourage osteogenesis through the stimulatory effect of their dissolution products.[11–17] More recently, bioactive glass compositions in which calcium was substituted by strontium have been described.[18–20] There is good evidence suggesting that these modified bioactive glasses exhibit superior regenerative properties compared to conventional 45S5 bioactive glass, with strontium encouraging osteoblast proliferation and differentiation,[21,22] as well as the upregulation of genes associated with the osteogenic differentiation of bone marrow mesenchymal stromal cells.[23]

Considering the potential of electrospinning and the reported benefits of strontium-substituted bioactive glasses, it is very likely that the combination of these two approaches will result in the fabrication of membranes with enhanced properties for bone tissue regeneration. Only the one report by Ren et al.[24] about this approach has been published, in which melt-electrospinning was combined with a single strontium-containing bioactive glass composition. Unfortunately, this study did not include 45S5 bioactive glass as a reference material, so it is uncertain whether the final device benefitted from the substitution of calcium with strontium. Moreover, the diameter of the fibers reported by Ren et al. was in the range of several tens of micrometers as a result of electrospinning method selected. Fibers with smaller diameters, ideally in the range of a few micrometers to hundreds of nanometers, may be preferred for bone tissue regeneration due to the closer similarity in size with collagen fibrils naturally present in bone tissue,[25] an outcome that may be achieved through solution-electrospinning.

Therefore, the aim of this study was to develop composite electrospun membranes made of a bioresorbable polymer and particles of strontium-substituted bioactive glasses, and to assess their potential for bone tissue regeneration. To the best of our knowledge here we report, for the first time, the successful fabrication of membranes made of poly(caprolactone) and particles of strontium-substituted bioactive glasses of demonstrated osteogenic potential[23] using the solution-electrospinning technique. Furthermore, the novel buffering capability of these composite materials was demonstrated, although via an alternative process than that previously suggested in the literature.[12,26]

2. Results

2.1. Fabrication and Characterization of Electrospun Membranes

Scanning electron microscopy (SEM) images of noncomposite electrospun materials (Figure 1a) showed that the fibers exhibited generally regular diameters with no apparent electrospinning defects (e.g., beading). Greater magnification images (Figure 1b) showed that the surface of the fibers was porous, and the porosity appeared to be uniformly distributed throughout the sample. In the case of the composite electrospun membranes (Figure 1c–h), the fibers exhibited regions of increased diameter and variable morphology that appeared to be distributed uniformly throughout the surface of samples. An apparent increase in variability of fiber dimensions in the composite materials was observed, with no significant differences between the three compositions. Energy dispersive X-ray spectroscopy (EDS) analyses (Figure 2) detected the presence of the chemical elements carbon and oxygen in the noncomposite materials, and of sodium, silicon, phosphorus, calcium, and strontium in addition to carbon and oxygen in the regions of increased diameter in the composite materials. Mean diameter of the noncomposite fibers was measured to be 1.99 ± 0.36 μm, while mean diameter of the regions of increased diameter in the composite fibers was measured to be 14.02 ± 7.67 μm. Previous work by the authors[23] showed that there were no significant differences in the size of the particles obtained from the three bioactive glass compositions. Therefore, the three composite electrospun materials were grouped together for the purpose of measuring the dimension of the fibers.

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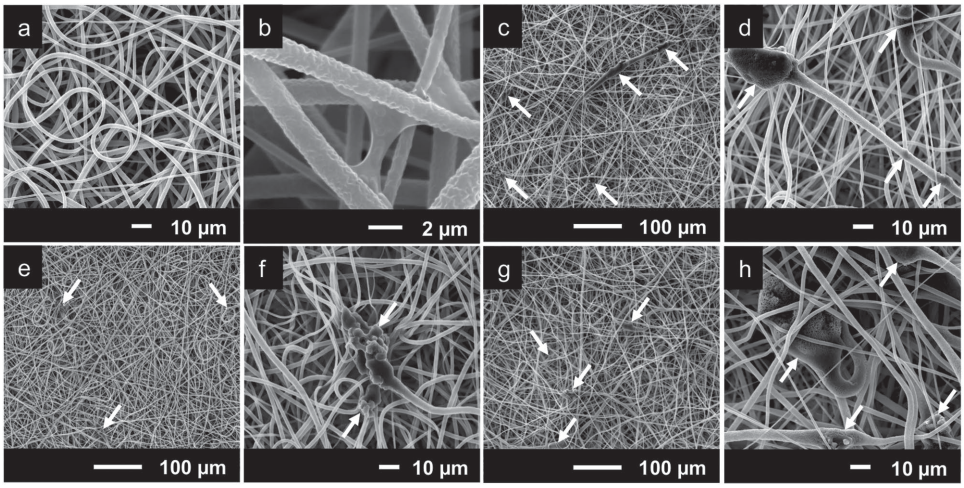

Figure 1. Scanning electron microscopy images of electrospun membranes made of a,b) poly(caprolactone) (PCL); c,d) PCL and particles of Sr0 bioactive glass (PCL/Sr0); e,f) PCL and particles of Sr50 bioactive glass (PCL/Sr50); and g,h) PCL and particles of Sr100 bioactive glass (PCL/Sr100). The white arrows indicate regions of increased diameter in the composite electrospun fibers where the bioactive glass particles were embedded.

2.2. Study of the Dissolution of the Bioactive Glass Component in Composite Membranes

A rapid increase of pH was observed during the initial 24 h in the vials containing deionized water and 1 mg of bioactive glass powders (Figure 3a). At 0 h, the pH values increased to around 8.3 for Sr0, 9.2 for Sr50, and 9.3 for Sr100, peaking at values around 10 at 24 h. Then the pH decreased slowly to values around 8.0 by the end of the study (14 d). The pH of the control samples, composed of deionized water only, was around 5.9 at 0 h and 6.5 at 24 h, and remained approximately constant for the remainder of the study. In the case of the vials containing deionized water and composite electrospun materials (Figure 3b) a similar increase of pH was observed during the initial 24 h. At 0 h the pH values were around 8.0 for poly(caprolactone) (PCL)/Sr0, 9.1 for PCL/Sr50, and 9.3 for PCL/Sr100, and peaked at values around 9.9 at 24 h. However, the subsequent decrease of pH occurred at a significantly faster rate than in the samples containing bioactive glass powder only, reaching values between 8.0 and 9.9 after 168 h, and then stabilizing at values around 7.0 by the end of the study. Similar to the control, the pH of the sample containing noncomposite PCL remained approximately constant throughout the whole experiment. Statistically significant differences (two-tailed Student's t-test, $p < 0.05$) were observed between the pH values of samples containing PCL/Sr0 and PCL/Sr100 in the period between 48 and 192 h, and were also observed between the pH values of the samples containing PCL/Sr50 and PCL/Sr100 in the period between 72 and 168 h. PCL/Sr0 and PCL/Sr50 presented statistically significant differences only at the 24 h time point.

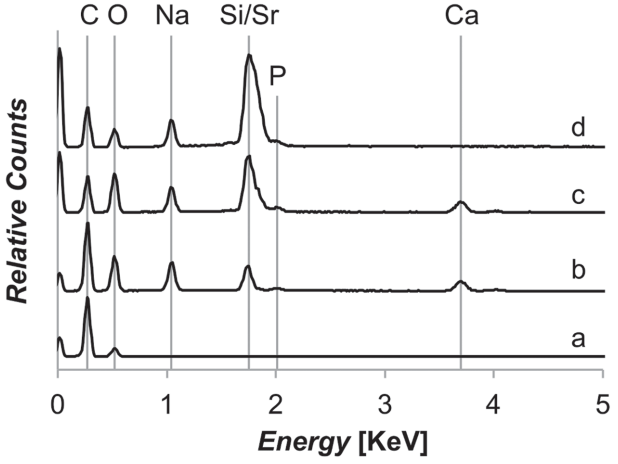

Figure 2. Energy dispersive X-ray spectroscopy patterns of noncomposite membranes made of a) electrospun poly(caprolactone), and of composite electrospun membranes made of PCL and particles of b) Sr0, c) Sr50, and d) Sr100 bioactive glass. The peaks attributed to the detected chemical elements are identified using their corresponding symbols (i.e., C, O, Na, Si, Sr, Ca).

2.3. Study of the "Loading" and "Filtering" Methods: Effect of Suspension Composition and Concentration

The "loading" and "filtering" methods were developed in order to add supplementary bioactive glass particles on the surface of the electrospun materials. The effects of the composition and concentration of the bioactive glass suspensions, as well as the efficiency of both methods, were investigated. Figure 4a shows the mean mass variation of electrospun samples processed using both methods and 1% w/v suspensions of Sr0, Sr50, and Sr100 bioactive glass powders, and Figure 4b shows the mean mass variation of electrospun samples processed using both

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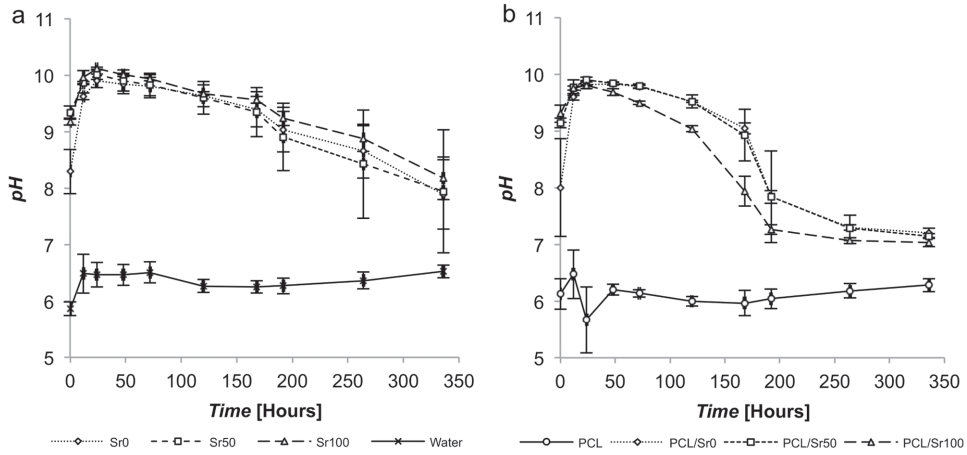

Figure 3. Plots showing the variation of media pH for a) 1 mg of Sr0, Sr50, and Sr100 bioactive glass powders immersed in 20 mL of deionized water; and b) 10 mg of electrospun poly(caprolactone) (PCL) and 10 mg of composite electrospun membranes (PCL/Sr0, PCL/Sr50 and PCL/Sr100) immersed in 20 mL of deionized water. The control samples contained 20 mL of deionized water only.

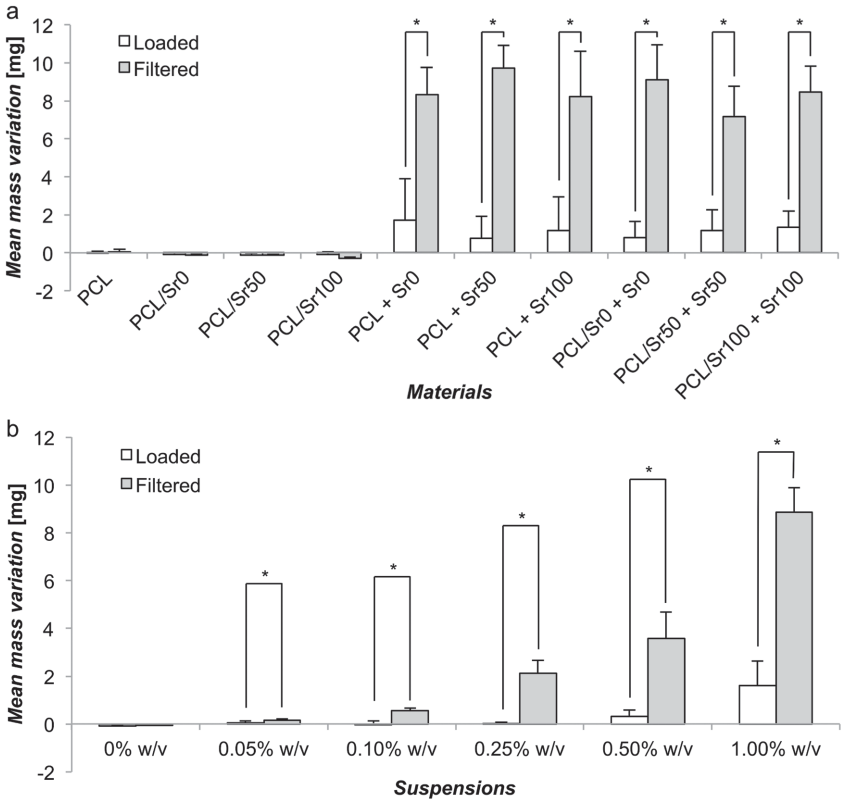

Figure 4. Mean mass variation of samples of electrospun poly(caprolactone) (PCL) and of composite electrospun membranes (PCL/Sr0, PCL/Sr50, and PCL/Sr100) processed using the "loading" and "filtering" methods of adding bioactive glass particles to the surface of the materials: a) samples processed using a 1% w/v suspension of Sr0, Sr50, and Sr100 bioactive glass powders in DMEM; and b) samples processed using a series of suspensions of increasing content of Sr0 bioactive glass powder. Only Sr0 was used in (b) because particle size analyses[23] showed that there were no significant differences in particle size between the three glass compositions. The lines indicate significant differences between the mean mass variation of the control and the samples (*$p < 0.05$).

methods and 0.05, 0.10, 0.25, 0.50, and 1.00% w/v suspensions of Sr0 bioactive glass powder. Results showed that all the samples exhibited some degree of mass increase, which was significantly greater in the case of the samples processed with the "filtering" method. The study of the effect of suspension composition showed that the "loading method" was able to add an average of 1.16 mg of glass particles (i.e., ≈12% of the glass content present in 1 mL of the suspension) and the "filtering method" was able to add an average of 8.51 mg of glass particles (i.e., approximately 85% of the glass content in 1 mL of the suspension). Significant differences between the results of the methods in both studies were reported (two-tailed Student's t-test, $p < 0.05$). The control samples, which were processed using cell culture medium without any glass content, exhibited small losses of mass (≤0.3 mg). SEM images of the samples (Figure 5a–d) demonstrated the presence of glass particles on the surface of the electrospun fibers, showing the blockage of the pores in the mesh (Figure 5c) or the complete coverage of the surface (Figure 5d) depending on the concentration of the suspension used. Apparent differences between the results of both methods were also observed, with samples processed using

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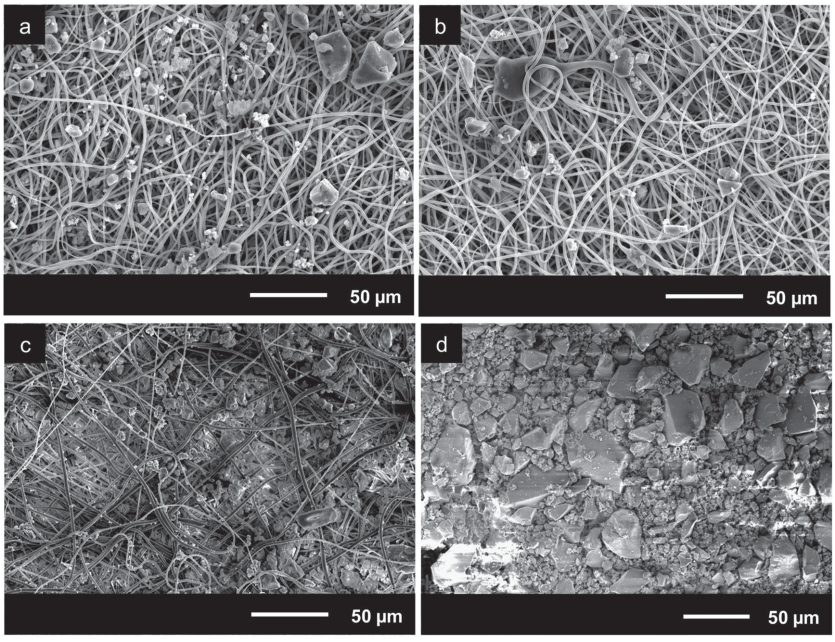

Figure 5. Scanning electron microscopy images of a,b) electrospun poly(caprolactone) (PCL) processed using the "loading" method and 1% w/v suspension of Sro bioactive glass powder; c,d) electrospun PCL processed using the "filtering" method and 0.05% w/v and 0.25% w/v suspensions of Sro bioactive glass powder.

the "filtering" method showing a significantly greater amount and a more homogeneous distribution of particles, even when suspensions with a low content of bioactive glass particles were used.

2.4. Cytotoxicity of Electrospun Membranes

There were no statistically significant differences reported between the levels of fluorescence emission from cell culture media used in the study of the cytotoxicity of the electrospun materials and the control samples (Figure 6). Additionally, there were no statistically significant differences between the levels of fluorescence emission obtained from cell culture media used in the analysis of electrospun samples processed using the "filtering" method and 0.05% w/v suspensions of bioactive glass powders and the control samples (Figure 7). However, statistically significant differences were reported between the control samples and those processed using 0.25% and 1.00% w/v suspensions (one-way ANOVA and two-tailed Student's t-test, $p < 0.05$).

3. Discussion

In this study, composite electrospun materials made of PCL and particles of strontium-substituted bioactive glass were successfully fabricated. The electrospun fibers forming these materials showed regions of increased diameter and variable morphology (Figure 1c–h) where the bioactive glass particles accumulated, as demonstrated by EDS analysis (Figure 2). It is possible that this significant increase in fiber diameter may have a biological effect in applications such as tissue engineering, where dimensions similar to those of fibers present in the native tissues may be preferred for biomimetic purposes. However, the increase mainly occurred in regions containing bioactive glass particles, with the diameters of glass-free regions in the composite membranes exhibiting dimensions comparable to those of noncomposite PCL fibers. Furthermore, the average diameter of those regions (14.02 ± 7.67 µm) was significantly smaller than the diameter of noncomposite PCL fibers reported by Ren et al.[24] (30.6 ± 1.8 µm), suggesting that the impact of the increased diameters on the osteogenic properties of the material and on cell viability may be minimal. No regions of the composite fibers were identified where the particles may have preferentially accumulated, suggesting a potentially uniform distribution throughout the material. However, as the methods employed to study the presence and locations of the glass particles (i.e., SEM imaging, EDS analysis) were only able to examine the surface of the membranes, it was not possible to determine their distribution throughout the full thickness of the membranes. The presence of the glass particles did not result in apparent fiber discontinuities despite the

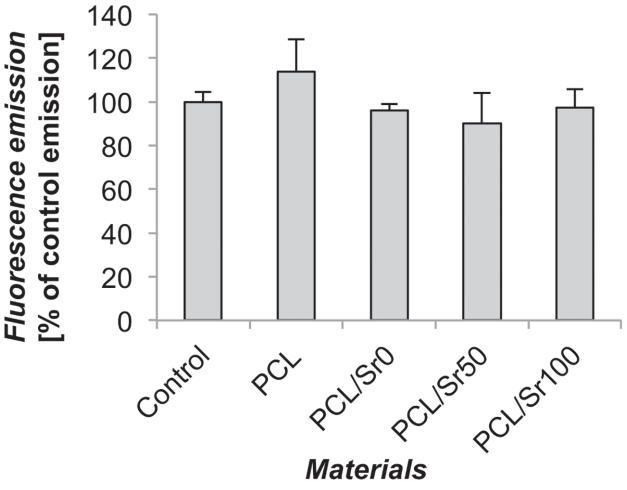

Figure 6. Fluorescence emission measured from cell culture media used in the study of the in vitro cytotoxic effect of the electrospun membranes: electrospun poly(caprolactone) (PCL) and composite electrospun membranes (PCL/Sro, PCL/Sr50, and PCL/Sr100).

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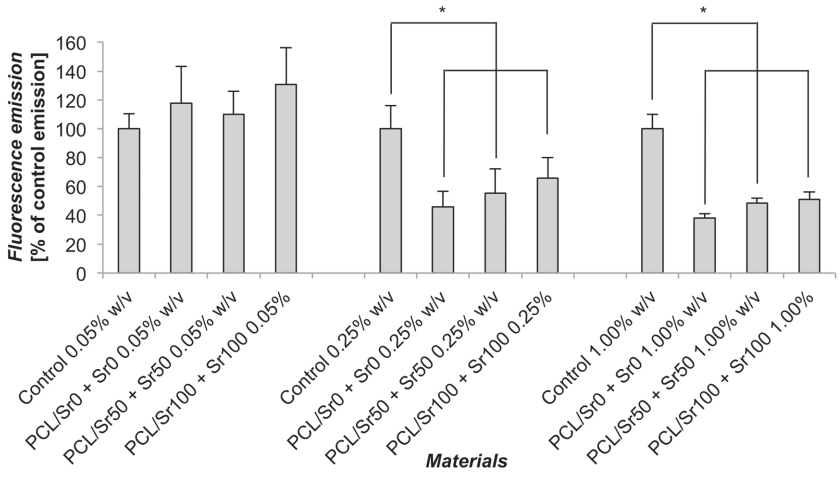

Figure 7. Fluorescence emission measured from cell culture media used in the study of the in vitro cytotoxic effect of composite electrospun membranes (PCL/Sr0, PCL/Sr50, and PCL/Sr100) containing added bioactive glass particles to the surface of the samples using the "filtering" method. The lines indicate significant differences between the mean fluorescence emission of the control and the samples (*$p < 0.05$).

significant increase in diameter this caused, suggesting that their inclusion does not disrupt the formation of a continuous electrospinning jet. The surface of the fibers was generally porous, creating a surface topography that may potentially increase fiber surface area and enhance the material's ability to absorb water and release the bioactive glass dissolution products. This feature may also be of importance for tissue engineering applications, as it has been shown that substrate topography may influence cell behavior.[27] The pores may be formed by the rapid evaporation of dichloromethane from the polymer solution, causing it to become thermodynamically unstable.[28] This results in a reduction of the temperature of the electrospinning jet as it travels toward the collector, promoting the condensation of water droplets on the surface of the forming fibers. As water is a nonsolvent in this particular system, its presence can induce phase separation and the creation of polymer-rich regions that solidify shortly after fiber deposition, as well as polymer-poor regions that form the pores after the water droplets evaporate.

Two methods were developed to add bioactive glass particles to the surface of the electrospun materials: the "loading" method and the "filtering" method. Results demonstrated that both methods were successful, although the "filtering" method consistently showed a significantly greater efficiency. Glass particles were observed to be trapped by the electrospun mesh as the suspensions were pumped through (Figure 5c), completely covering the surface of the samples in some cases (Figure 5d). Results suggested that suspensions with a concentration of 0.05% w/v or lower of bioactive glass particles should be used in order to minimize pore blockage. The impact of such blockage will depend on the intended application of the membranes. For example, this may prove problematic for tissue engineering as the obstruction may likely impair the diffusion of nutrients and waste products, and eventually may prevent cell migration and the formation of new blood vessels into the scaffold. However, this may not be a significant problem for the treatment of bone tissue defects caused by periodontal disease, such as those described earlier. As the main function of the membrane in GBR is to create a barrier between defects and the surrounding tissues, pore blockage using an osteogenic component may even be useful to enhance bone tissue regeneration. It was also apparent that a significant proportion of the deposited particles were not in direct contact with the fibers, separating with ease from the sample after drying due to the lack of a stable attachment to the membrane. It is possible that a mechanical attachment may be achieved if the glass particles bond to each other due to the formation of a layer of hydroxycarbonate apatite after glass dissolution begins. This may occur fewer than 2 h after exposure to body fluids,[29] but this effect was not clearly observed on the samples here described.

The study of the dissolution of the bioactive glass component in the composite fibers showed that the particles were able to dissolve within the polymeric fibers after immersion in water, and that their dissolution products could be released to the local environment, as evidenced by the increase in pH observed. Composite samples containing strontium-substituted bioactive glasses were able to induce greater initial variations of pH (Figure 3b) due to the increased solubility of these compositions compared with unmodified glasses.[23,30] Additionally, the faster decrease of pH observed in the case of the electrospun samples suggested the existence of an interaction between the polymer and the bioactive glass, which may be associated with the accelerated degradation of PCL by the severely alkaline conditions created by glass dissolution. If this was followed by the release of acidic degradation products from the polymeric fibers, it is expected that the pH would decrease. As PCL degradation initially occurs due to the hydrolytic breakage of ester bonds in the amorphous regions of the polymer,[31,32] this process may have been further accelerated by the lower levels of crystallinity observed in electrospun fibers of semicrystalline polymers.[33,34] Additionally, the presence of small-sized glass particles within the polymeric matrix may affect the arrangement of the surrounding polymer chains, further reducing the overall level of crystallinity.[35] The

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alkalinization induced by bioactive glass dissolution has been proposed as a potential way of reducing the impact of inflammation responses due to the acidic degradation of implanted bioresorbable polymers.[12,26] Although there is some evidence pointing to this potential buffering effect,[36] the results of this study suggested that the system may actually work in a different way to what has been proposed elsewhere. The accelerated degradation of the polymer appears to diminish the impact of the rapid initial increase of pH caused by glass dissolution, reducing it to neutral levels by the end of the experiment. This may prove useful for applications where the use of nonpreconditioned bioactive glass may be preferred or necessary.

The inclusion of bioactive glass particles into the electrospun fibers did not induce a significant detrimental effect on cellular metabolic activity (Figure 6), suggesting that cells seeded on the composite membranes could retain viability after 3 d of culture. This is supported by the study by Fabbri et al.,[37] in which there were no appreciable differences in proliferation between cells cultured in scaffolds made of PCL and those cultured in scaffolds made of PCL and particles of 45S5 bioactive glass. Although cellular proliferation was not studied in these experiments (i.e., the cytotoxicity assays were directly associated with metabolic activity and not cellular growth), the results reported on this manuscript and by Fabbri et al.[37] suggest that the combination of PCL and bioactive glass generally has good in vitro biocompatibility. Fabbri et al.[37] also observed that proliferation on the scaffolds was significantly lower than on tissue culture plastic, likely due to the low wettability of PCL. Electrospun PCL also showed a high degree of hydrophobicity, an effect that was reduced by the immersion of the samples in isopropyl alcohol during sterilization. However, this apparent increase of surface hydrophilicity simply occurred due to the introduction of water by the alcohols. This change was temporary and only effective as long as the membranes remained wet. Regarding the study of the cytotoxic effect of bioactive glass particles added to the surface of the electrospun fibers, all materials were processed using only the "filtering method" because it had proved to be a significantly more efficient addition technique than the "loading method" (Figure 4). Results (Figure 7) suggested that the addition of more than 0.5 mg of particles of the three bioactive glass compositions might have a significant cytotoxic effect, an outcome that appears to contradict previous studies. For example, Isaac et al.[22] reported no significant differences in cell viability and metabolic activity of cells exposed to 4 mg mL^{-1} of preincubated strontium-substituted bioactive glasses. In the present study, the glass may also be considered as preincubated because the processing of the materials using the "filtering method" results in the potential loss of a considerable amount of the glass ionic content before exposure to the cells. Furthermore, as the solubility of Sr50 and Sr100 is greater than in Sr0,[23] the release may be expected to be faster during that period of time. However, cytotoxicity may be increased in the electrospun materials due to the direct exposure of the glass particles to the cells on the surface of the samples, potentially inducing an enhanced effect compared to that from particles embedded within the fibers, as well as possibly reducing the availability of suitable substrates for cell attachment.

4. Conclusions

Composite membranes made of PCL and particles of strontium-substituted bioactive glass with known osteogenic potential were successfully manufactured using solution electrospinning. The materials consisted of fibers showing regular diameters and porous surfaces, and the bioactive glass particles were located within the polymeric fibers in regions of increased diameter. Furthermore, the particles were able to dissolve from within the fibers after immersion in water, releasing their dissolution products into the local environment. There was some evidence that the presence of the glass particles within the fibers may accelerate the degradation of PCL due to the rapid alkalinization of the medium, resulting in the release of acidic degradation products and the reduction of pH to neutral levels. All the electrospun materials showed good in vitro biocompatibility, except when more than 0.5 mg of bioactive glass particles was added to their surface. In conclusion, results demonstrated that composite electrospun materials made of PCL and particles of strontium-substituted bioactive glasses can be fabricated and show great promise to be used as membranes for bone tissue regeneration.

5. Experimental Section

5.1. Production of Bioactive Glass Powder

Bioactive glasses based on the 45S5 bioactive glass composition were produced where 0% (Sr0), 50% (Sr50), and 100% (Sr100) of the calcium was replaced by strontium on a molar basis (Table 1). Analytical grade SiO_2, $CaCO_3$, Na_3PO_4 (Fisher Scientific, UK), and $SrCO_3$ (Sigma-Aldrich, UK) were mixed and melted in platinum crucibles (1350 °C) for a total time of 180 min, including 120 min of homogenization using a rotating platinum paddle (60 rpm). Bioactive glass powders (<45 μm particle size) were then produced by milling and sieving the glass frits obtained after quenching the melt in distilled water.

5.2. Electrospinning

Electrospun membranes were fabricated using solutions of 10 wt% poly(caprolactone) (PCL) (Average M_w 80 000;

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Table 1. Batch composition of Sr0, Sr50, and Sr100 bioactive glasses, presented in weight percentage (wt%) and molar percentage (mol%).

Oxide	Bioactive glasses		
	Sr0 (45S5)	Sr50	Sr100
Wt%			
SiO_2	45.0	40.8	37.3
Na_2O	24.5	22.2	20.3
CaO	24.5	11.1	0.0
SrO	0.0	20.5	37.5
P_2O_5	6.0	5.4	4.97
Mol%			
SiO_2	46.1	46.1	46.1
Na_2O	24.4	24.4	24.4
CaO	26.9	13.5	0.0
SrO	0.0	13.5	26.9
P_2O_5	2.6	2.6	2.6

Sigma-Aldrich, UK) in a blend of dichloromethane (DCM) (Fisher Scientific, UK) and dimethylformamide (DMF) (Fisher Scientific, UK) (DCM/DMF 90/10 v/v). Polymer and bioactive glass solutions were produced by adding the glass powders to the PCL solutions (10:1 PCL:glass weight ratio) under continuous stirring. All solutions were thoroughly mixed (2 h) and spun using an electrospinning system composed of a KDS200 syringe pump (KdScientific, USA) and an Alpha IV Brandenburg power source (Brandenburg, UK). Plastic syringes (1 mL; Becton Dickinson, UK) were used to contain and drive the solutions into 20 gauge blunt metallic needles (Intertronics, UK). The applied voltage was 17 kV, the flow rate was 2–3 mL h^{-1}, and the distance from the tip to the collector was 21 cm.

5.3. Addition of Glass Particles to the Surface of Electrospun Materials Using the "Loading Method"

The "loading method" of adding bioactive glass particles to the surface of the electrospun fibers, based on work by Dinaryand et al.,[38] consisted in immersing samples of the electrospun materials in suspensions of bioactive glass powders in order to allow the particles to deposit due to gravitational force. Discs (13 mm diameter) were produced from each electrospun mat, placed in 24-well plates, sterilized by immersion in isopropyl alcohol (Fisher Scientific, UK) for 20 min, washed twice in sterile phosphate buffered saline (PBS; Sigma-Aldrich, UK), and then transferred to a new plate. A volume (1 mL) of bioactive glass suspensions prepared in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK) was added to each electrospun disc, and all plates were incubated at 37 °C. After 24 h the suspensions were removed, the samples transferred to a new plate and washed twice in deionized water. Finally, the samples were dried overnight at room temperature.

5.4. Study of Bioactive Glass Solubility

The ability of the bioactive glass particles to dissolve and release their dissolution products from within the electrospun fibers was studied by immersing samples of the materials in water and then measuring media pH over time. Discs (10 mm in diameter, 10 mg in total) were produced from each electrospun mat and immersed in deionized water (20 mL) in vials maintained at 37 °C for 14 d. Additionally, vials containing bioactive glass powders only (1 mg) were prepared and maintained under the same conditions. Vials containing deionized water only were used as controls. All samples were prepared in triplicate, and the pH was measured at 0, 12, 24, 48, 72, 120, 168, 192, 264, and 336 h using a pH 211 Microprocessor pH meter (Hanna Instruments, USA).

5.5. Addition of Glass Particles to the Surface of Electrospun Materials Using the "Filtering Method"

The "filtering method" consisted in using samples of the electrospun materials as filters through which a suspension of bioactive glass powders was forced through, trapping the glass particles in the electrospun mesh. Discs (13 mm diameter) were produced from each electrospun mat as previously described, placed inside sterile reusable syringe filter holders (Whatman, UK), and a volume (1 mL) of bioactive glass suspensions prepared in DMEM was pumped through the filter holder using a sterile syringe. Afterward, the electrospun discs were placed in new 24-well plates, DMEM (1 mL) was added to each well, and the plates were incubated at 37 °C for 24 h so the process would be comparable with the "loading method." Then the discs were transferred to a new plate, washed twice in deionized water, and dried overnight at room temperature.

5.6. Study of the "Loading" and "Filtering" Methods: Effect of Suspension Composition and Concentration

The effect of the composition of bioactive glass suspensions on the "loading" and "filtering" methods was studied using 1% w/v suspensions of Sr0, Sr50, and Sr100 bioactive glass powders. The effect of the concentration of bioactive glass suspensions was studied using 0.05, 0.1, 0.25, 0.5, and 1% w/v suspensions of Sr0 bioactive glass powder. Only Sr0 bioactive glass was used because previous work by the authors[23] showed that there were no significant differences in the size of the particles obtained from the three bioactive glass compositions. All suspensions were prepared by adding the required amount of sterile glass powders to DMEM (Sigma-Aldrich, UK), and then placing the suspensions in an ultrasonic bath to facilitate particle dispersion. Samples of electrospun membranes ($n = 6$ for the study of suspension composition, and $n = 12$ for suspension concentration) were weighed and processed as previously described. Finally, all samples were reweighed after drying in order to calculate variations of mass.

5.7. SEM Imaging and EDS Analysis of Electrospun Materials

All electrospun membranes were imaged using a Jeol JSM6400 scanning electron microscope. The presence of the bioactive glass

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particles within the electrospun fibers was determined by performing EDS point analysis on the regions of increased diameter of the composite membranes. Discs (13 mm diameter) were produced from each electrospun mat, sputter coated with gold for SEM and with carbon for EDS, and then processed (9 and 20 kV emission currents). Mean fiber diameters were calculated by taking 60 measurements on SEM images of each membrane formulation using the ImageJ software (US NIH, USA). EDS patterns were processed using the INCAEnergy software (Oxford Instruments, UK).

5.8. Cytotoxicity of Electrospun Materials

The cytotoxicity of the electrospun materials was studied by evaluating cellular metabolic activity using a resazurin dye-based assay. Discs (13 mm diameter) were produced from each electrospun mat, placed in 24-well plates, sterilized by immersion in isopropyl alcohol (Fisher Scientific, UK), washed twice in sterile phosphate buffered saline (PBS; Sigma-Aldrich, UK), and transferred to a new plate. A volume of bioactive glass suspension (1 mL) was added to each sample, and all plates were incubated at 37 °C. After 24 h the suspensions were removed and the samples were transferred to a new plate. Additionally, the cytotoxic effect induced by additional surface bioactive glass particles was studied. The electrospun samples were processed using the "filtering method" and 0.05, 0.25, and 1% w/v suspensions of bioactive glass powder prepared in DMEM (Sigma-Aldrich, UK). Samples of the membranes were seeded with rat osteosarcoma (ROS 17/2.8; Merck Inc., USA) cells (2.5×10^4 cells) and a volume (1 mL) of fully supplemented cell culture medium (i.e., DMEM (Sigma-Aldrich, UK), 10 units mL^{-1} penicillin (Sigma-Aldrich, UK), 0.1 mg mL^{-1} streptomycin (Sigma-Aldrich, UK), 20×10^{-3} M L-alanyl-L-glutamine (Sigma-Aldrich, UK), and 10% v/v foetal calf serum (Biosera, UK)) was added to each well. After incubation (72 h), 10% v/v resazurin dye in fully supplemented cell culture medium was added to each well and the plates were incubated (40 min). Finally, two samples of cell culture medium (200 µL each) were taken from each well and transferred to 96-well plates for spectrophotometric analysis. Fluorescence emission intensities were calculated by measuring emission at 590 nm following excitation at 560 nm, and then using the following formula: FE = (FC – FCF), where FE is the intensity of fluorescence emission, FC is the mean value of the fluorescence emission obtained from the wells containing cells, and FCF is the mean value of the fluorescence emission obtained from the wells containing cell-free controls. A total of 4 replicates were used in each test.

5.9. Statistical Analysis

Statistical analyses were performed on Microsoft Excel 2010 software using one-way ANOVA, followed by two-tailed Student's t-test in order to determine significance. In all cases, p values <0.05 were considered as statistically significant.

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