Bioactive glass nanofiber–collagen nanocomposite as a novel bone regeneration matrix

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Abstract: Nanoscale organized organic–inorganic nanocomposite systems have great potential in the development of biomaterials with advanced properties. Herein, we developed a novel nanocomposite biomaterial consisting of bioactive glass nanofiber (BGNF) and collagen reconstituted fibrous matrix for bone regenerative medicine. A sol–gel derived glass with a bioactive composition ($58SiO_2$·$38CaO$·$4P_2O_5$) was electrospun to a nanoscale fiber with an average diameter of ~320 nm. The BGNF was subsequently hybridized with type I collagen, which is the main organic constituent of bone matrix. The BGNF and self-assembled collagen sol were combined in aqueous solution, and then crosslinked to produce a BGNF-collagen nanocomposite, in the form of either a thin membrane or a macroporous scaffold, by adopting appropriate processing conditions. The BGNF was observed to be distributed uniformly within the collagen reconstituted nanofibrous matrix. The nanocomposite matrices induced rapid formation of bone-like apatite minerals on their surfaces when incubated in a simulated body fluid, exhibiting excellent bioactivity *in vitro*. Osteoblastic cells showed favorable growth on the BGNF-collagen nanocomposite. In particular, the alkaline phosphatase activity of the cells on the nanocomposite was significantly higher than that on the collagen. This novel BGNF-collagen nanocomposite is believed to have significant potential in bone regeneration and tissue engineering applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 698–705, 2006

Key words: bioactive glass; nanofiber; collagen; nanocomposite; bone regeneration

INTRODUCTION

Over the past few decades, bioactive materials including calcium phosphates (hydroxyapatite and tricalcium phosphate) and glasses/glass ceramics have gained widespread clinical acceptance in dentistry and orthopedics.[1–3] First among these, silica-based bioactive glasses are considered as promising bone substitutes and tissue regeneration matrices, because of their bioactivity, tissue compatibility (both hard and soft tissues), osteoconductivity, and possibly even osteoinductivity.[2,3]

A number of studies have been directed toward understanding the biocompatibility mechanism of bioactive glasses *in vitro* and *in vivo*, in which their biocompatibility was attributed to the induction of a bone-like mineral phase at the glass surface, which is directly in contact with the host tissues.[3]

Among the different types of bioactive glasses, sol–gel derived glasses have more recently been developed.[4,5] Compared with their melt-derived counterparts, these sol–gel glasses have generally been made over wider composition range (up to higher SiO_2 content), allowing the modification of solubility and bioactivity. Researchers have used these sol–gel glasses in the form of powders, coatings, and porous foams for the purpose of bone replacement, and reported their excellent bioactivity and cell responses *in vitro*, as well as their good bone forming ability *in vivo*.[6–9] In particular, the sol–gel approach has various technological benefits, such as allowing the sol–gel precursor to be prepared in required formulations and enabling the scale reduction of the final products.[10]

By making use of the sol–gel precursor of a bioactive glass, the authors recently developed a bioactive glass nanofiber (BGNF) via the electrospinning (ES) process.[11] The diameter of the BGNF could be kept extremely small (tens to hundreds of nanometers, depending on the processing conditions), in striking contrast to that of conventional melt-spun fibers (normally tens to hundreds of micrometers). Our recent observations suggested that the glass nanofiber retained excellent bioactivity *in vitro* and exhibited favorable cellular responses.[11]

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In this study, we combined this BGNF with a biological polymer to produce a hybridized nanocomposite. As a polymeric source, collagen was chosen, because it is the main organic constituent of the bone matrix and as such is one of the most widely used biomaterials.[12] When considering that the bone extracellular matrix is essentially an organic–inorganic composite organized on the nanoscale, this BGNF-Col nanocomposite can be considered as a promising system in the bone regeneration field.

In particular, the BGNF-collagen nanocomposite was produced both in the form of a thin membrane and a macroporous scaffold. The methodologies used for the synthesis of the nanocomposite are described, and the *in vitro* bioactive and cellular responses of the newly developed material are briefly discussed.

MATERIALS AND METHODS

Generation of nanofibrous bioactive glass

The glass composition ($58SiO_2{\cdot}38CaO{\cdot}4P_2O_5$) and the sol–gel processing conditions used in this study were based on and modified from those of previously developed bulk glasses that exhibit bioactivity *in vitro*.[4,6] The glass precursors (tetraethyl orthosilicate, calcium nitrate, and triethyl phosphate, all from Aldrich) were added at appropriate ratios in a mixture of ethanol and water containing 1 *N* HCl as a catalyst. The sol mixture was stirred for 48 h and aged at 40°C for 48 h followed by a further 12 h at 70°C. The acidic catalyst was necessary to produce a clear sol during the prolonged aging time. The aged sol was mixed with 7% poly-vinyl-butyral (from Aldrich) at an appropriate ratio to adjust the rheological properties of the sol, so that they would be fit for the fiber generation during ES. For the ES process, the sol mixture was loaded in a syringe and injected into a rotating mandrel under predetermined conditions (DC electric field strength of 1.5 kV/cm and injection speed of 0.1 mL/h). The electrospun fibers were subsequently thermal-treated at 700°C for 3 h in air at a heating rate of 2°C/min, to completely eliminate the organic sources and to stabilize the glass.

Fabrication of BGNF-collagen nanocomposite

The collagen solution was prepared prior to combining with the generated BGNF. Type I collagen (MW 300,000, RegenMed, South Korea) was first dissolved in distilled water containing 50 m*M* acetic acid to produce a final concentration of 1% w/v. The reconstitution of the collagen to a native fibrillar structure was initiated by adding the collagen solution to phosphate-buffered saline (PBS) solution at a concentration of 0.2% w/v, followed by incubation at 37°C for 24 h.[13] After the reconstitution process, the collagen solution became cloudy, as observed by the turbidity change at an absorbance of 313 nm. The presynthesized BGNF mesh was dispersed in a 1:1 mixture of PBS and distilled water by gentle vortexing, followed by its addition to the collagen reconstituted solution to produce final BGNF:collagen ratios of 40:60 and 20:80 by dry weight. The mixture was vigorously vortexed for 3 min to produce a cloudy but homogeneous suspension. The suspension was then subjected to two different procedures designed to produce either a thin membrane or a macroporous scaffold. For the preparation of the thin membrane, the solution was poured into a designed poly(tetrafluoroethylene) vessel, and dried under a laminar flow for 24 h, followed by freeze-drying and mold-pressing to a uniform thickness. Alternatively, the macroporous scaffold was produced by quenching the solution in a freezer at −70°C, and then evaporating the ice crystals under high vacuum (~5 mmHg) in the frozen state. The fully dried membranes and scaffolds were crosslinked in a solution {EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) 100 m*M* with NHS (*N*-hydroxysuccinimide) 100 m*M* in 95% ethanol} at room temperature for 12 h, washed fully with ethanol/water (at least five times), and then dried. Samples were cut to appropriate dimensions and stored at 4°C for further tests.

Characterization

The morphology of the produced BGNF and BGNF-Col nanocomposite was characterized with field-emission scanning electron spectroscopy (FESEM, JSM6330F, JEOL). In particular, the average diameter of the BGNF was measured on arbitrary 10 different areas from SEM image. The internal organization of the BGNF and collagen fibril was observed with transmission electron microscopy (TEM, CM20, Philips). The *in vitro* bioactivity of the nanocomposite was assessed by incubating the sample in a medium simulating body fluid (namely SBF, which contains similar ion concentrations to those of human body plasma).[14] Typically, 50 mg of the macroporous-type nanocomposite was immersed in 20 mL SBF at 37°C for up to 7 days without refreshing. The surface morphology and structural change of the nanocomposite during the SBF treatment were analyzed with FESEM and Fourier transform infrared spectroscopy (FTIR, System 2000, Perkin-Elmer), respectively.

In vitro cell tests

The osteoblastic cellular responses to the BGNF-Col nanocomposite were assessed using human osteoblastic MG63 cells. To observe the effect of BGNF concentration on the cellular responses, two different compositions (BGNF:Col = 40:60 and 20:80) were produced. The cells were maintained as previously described in detail.[15] In brief, the cells, subcultured in a complete medium consisting of Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 50 IU/mL of penicillin, and 50 μg/mL of streptomycin, were plated on both types of samples (membrane: ~10 mm × 10 mm × 180 μm and porous scaffold: ~ϕ10 mm × 5 mm). Prior to seeding cells, the test specimens were sterilized with 70% ethanol and washed

with PBS and cell-free culturing medium twice. The cell seeding densities on the membranes and scaffolds were 2×10^4 and 6×10^5 cells/mL, respectively. Aliquots of 1.5 mL of medium were seeded on all specimens and cultured in a humidified atmosphere. The cell morphology grown on the samples for 3 days was observed with FESEM, after fixing and dehydrating the cells, followed by gold coating.

The alkaline phosphatase (ALP) activity of the cells was measured to observe their functional activity. After culturing for 7 days, the cell layers were gathered and disrupted by treating them with 0.1% Triton X-100 and a further cyclic freezing-and-thawing process. Aliquots of the cell lysates were normalized to the total protein content, which was obtained from a DC protein assay kit (BioRad, Hercules, USA). The ALP activity was assessed by using the *p*-nitrophenyl phosphate substrate to measure the ALP level colorimetrically. The enzyme ALP expressed by the cells hydrolyzes the substrate to *p*-nitrophenol and an inorganic phosphate, and the *p*-nitrophenol was converted to a yellow product under alkaline conditions. The absorbance of the eluted yellow product was measured at 410 nm, and converted to the ALP activity level based on a protein standard curve, which was obtained with bovine serum albumin.

The ALP assay was performed on four replicate samples ($n = 4$) and data were compared using one-way ANOVA analysis with statistical significances at $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSION

Morphology and organization

In Figure 1, the typical morphology of the BGNF generated through the ES process and subsequent thermal treatment is shown. Continuous fibers on the nanoscale were successfully generated without the formation of any beads. The average diameter of the fibers was measured 320 nm (±87 nm). During the thermal treatment, the nanofibrous morphology was observed to be well preserved, while the diameter of the fiber was reduced by a factor of ~2.

The nanofiber mesh was subsequently hybridized with collagen as described in the experimental section. Figure 2(a–c) shows images of the solutions prepared at each processing stage. The BGNF solution dispersed in PBS by gentle agitation gave the appearance of an optically cloudy but homogeneous suspension [Fig. 2(a)], while the collagen sol reconstituted in PBS at 37°C for 24 h in a separate experiment was also cloudy and homogeneous [Fig. 2(b)]. Both suspensions were directly mixed and homogenized to produce a composite sol [Fig. 2(c)]. The composite sol maintained its initial emulsion state over a period of several hours without being segregated or any sedimentation of the nanofiber being observed. On the basis of these observations, the PBS was believed to fill in the BGNF interspacings well and to disperse the BGNF effectively. Moreover, the collagen-reconstituted sol was considered to be well intermixed with the nanofiber-dispersed solution. The maintenance of the emulsion state of the mixture observed herein was attributed to the nature of the ultrafine-scaled nanofiber structure of both the BGNF and collagen components. As such, the composite sol could be kept in static drying conditions for an extended period of time (~24 h) without inducing any compositional variation along the product during the preparation of the dense membrane (refer to the experimental section).

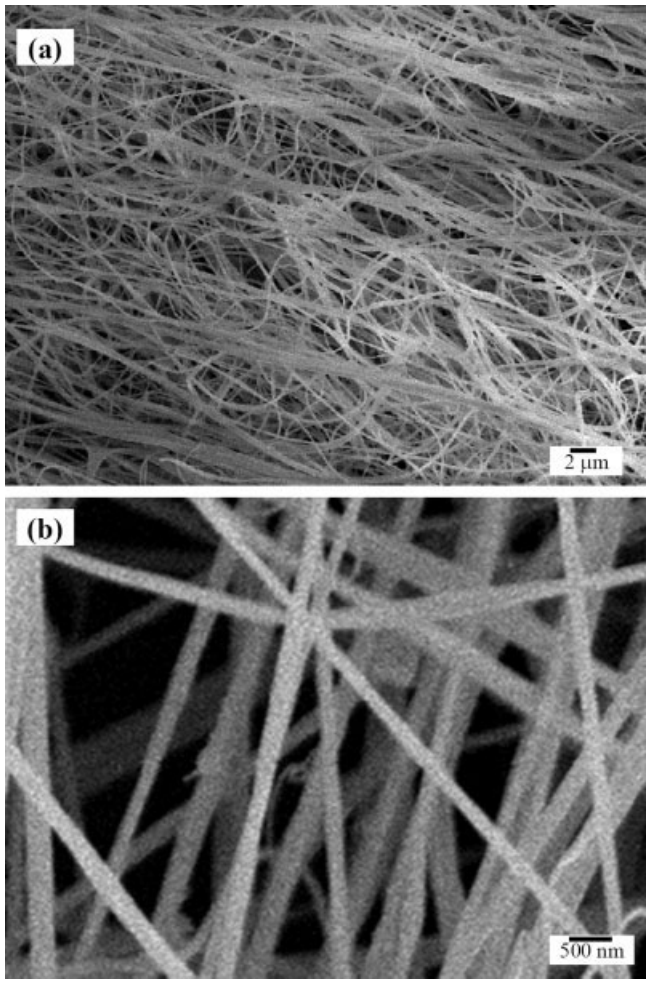

Figure 1. Morphological feature of the BGNF electrospun and subsequently heat treated at 700°C: (a) low and (b) high magnification.

By adopting appropriate techniques, the composite suspension was easily formulated to either a thin membrane or a macroporous scaffold, as shown in Figure 2(d,e), respectively. These two types, explored herein as exemplary formulations of the nanocomposite, can find explicit uses in bone tissue regenerative medicine. Specifically, the thin membrane type should be of great utility in guided bone regeneration, while the porous form should act as an effective tissue scaffolding matrix by providing enough space for the vasculization and cell migration.[16]

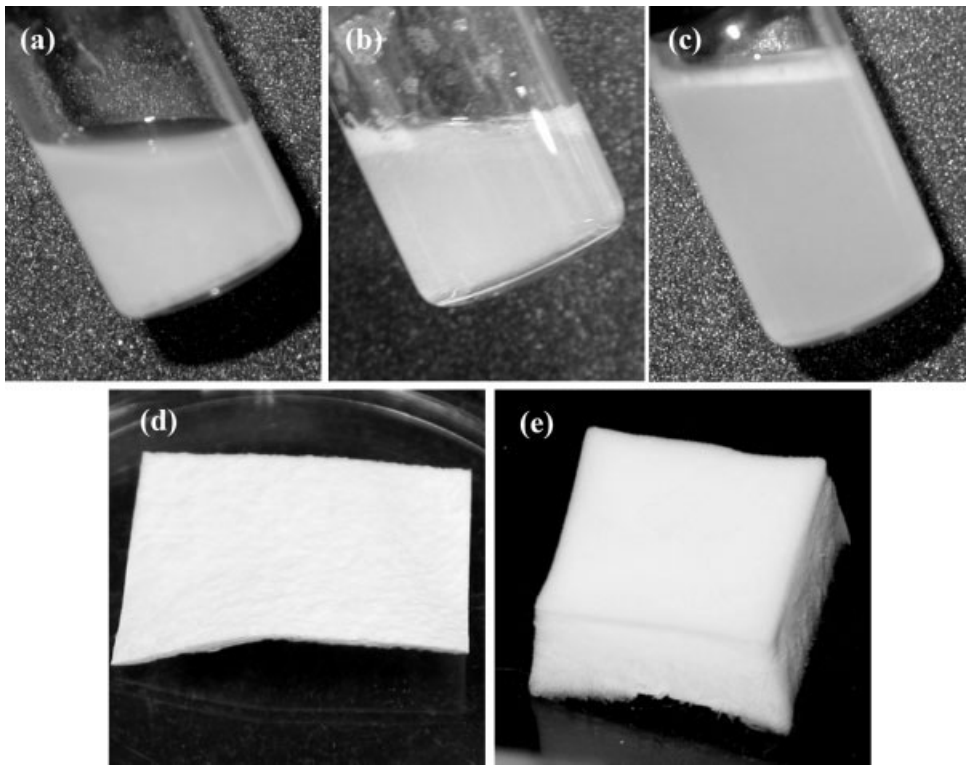

Figure 2. Optical images, showing (a–c) the sols in a glass vessel during the nanocomposite synthesis: (a) BGNF, (b) collagen, and (c) BGNF-collagen mixture in PBS, and the (d,e) produced nanocomposites formulated as thin membrane (d) and porous scaffold (e).

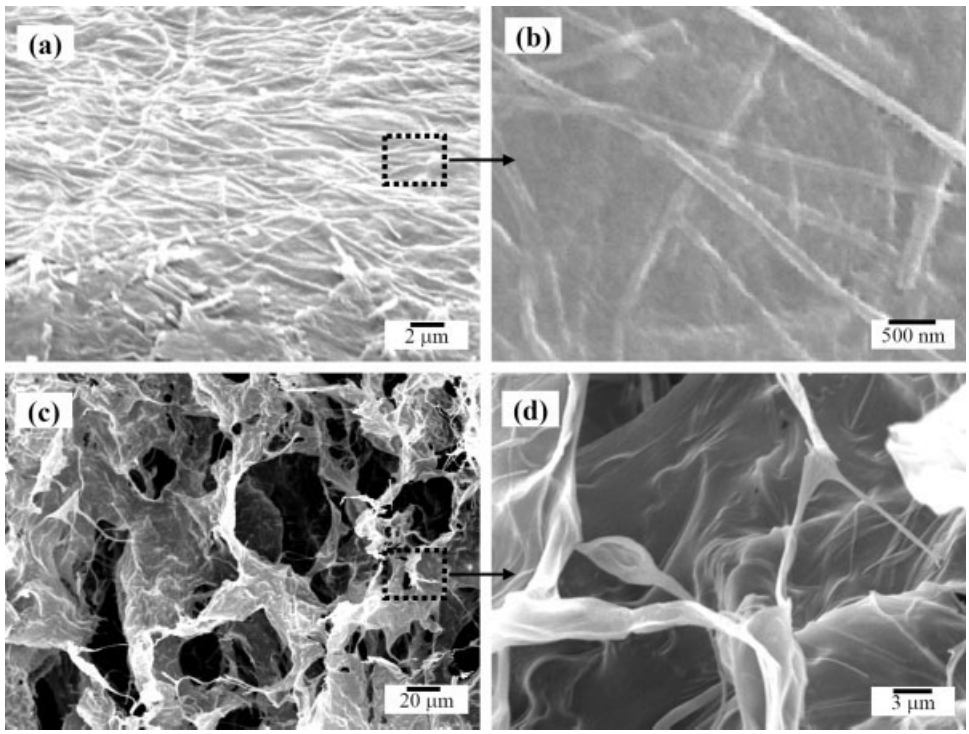

Figure 3. SEM morphology of the BGNF-Col nanocomposite, formulated as (a,b) thin membrane and (c,d) porous scaffold. Parts of (a) and (c) are enlarged in (b) and (d), respectively.

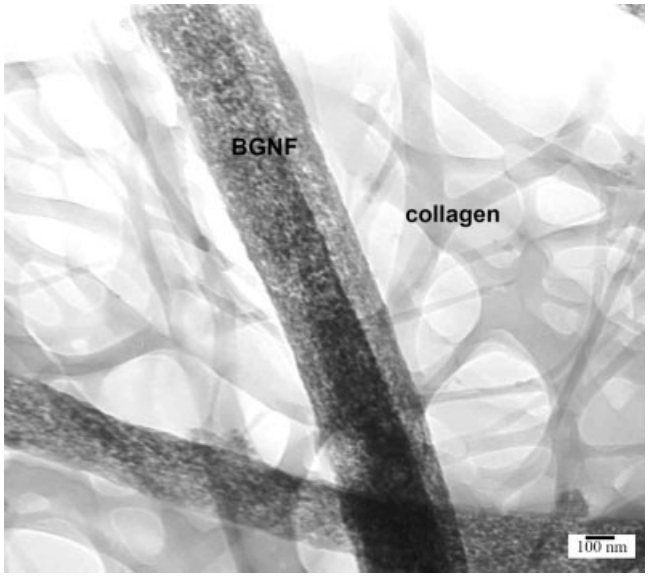

Figure 4. TEM image of the BGNF-Col nanocomposite, showing the mesoporous BGNF nanofibers (~300–400 nm in diameter) organized with collagen self-assembled nanofibrils (less than ~100 nm in diameter).

The morphology of the BGNF-collagen nanocomposite was observed with SEM, as presented in Figure 3. The membrane type, produced by drying the composite sol under a laminar flow, was quite dense and had an average thickness of 181 μm (±64 nm) [Fig. 3(a)]. The surface of the membrane clearly revealed the existence of the nanofibrous glass with a corresponding diameter (average of 320 nm), as observed in the as-generated BGNF [Fig. 3(b)]. The BGNF was well-separated without being entangled and was uniformly distributed within the collagen matrix. On the other hand, the porous scaffold form showed the development of macro–pores with sizes of the order of ~tens to hundreds of micrometers [Fig. 3(c)]. The examination of the surface of the scaffold's framework revealed a similar morphology to that observed in the dense membrane, that is, the BGNF nanofiber was well separated and uniformly distributed in the collagen dense matrix.

The organization level of the BGNF and collagen was more clearly characterized with TEM. Figure 4 shows the TEM image of the nanocomposite sample prepared directly on a copper grid by dropping the composite sol and subsequent drying under vacuum. Along with the existence of BGNF nanofibers (~300 nm in diameter), collagen nanofibers with smaller diameters (approximately less than 100 nm) were also observed. Clearly, the collagen nanofibrillar structure was created through the reconstitution process. The BGNF was intact in its organization with the collagen and preserved its initial morphology prior to hybridization. Practically, this native fibrillar form of collagen could not be visualized well on the SEM image, which was partially attributed to the fact that the collagen nanofibrils were more densely packed in the products, when compared with the relatively loose collagen fibrous mesh in the TEM sample. On the basis of this TEM observation, the BGNF-collagen nanocomposite is believed to be organized with nanofibrous components of both the inorganic glass and organic collagen. The diameter of the glass nanofiber can be changed by altering the ES conditions (from tens to hundreds of nanometers), while that of the collagen fibrils can be tuned by controlling the reconstitution level, for example, adjusting such parameters as the solvent pH, ionic concentration, and the incubation temperature and time.[13,17] The regulation of this organization level is regarded as another interesting area for further research.

Bioactivity and cellular responses

The bioactivity of the BGNF-collagen nanocomposite was examined *in vitro* by incubating the samples in SBF, to determine whether it would induce bone-like apatite minerals. Figure 5 shows the SEM morphology of the nanocomposite (scaffold type) after the SBF-treatment for 2 [Fig. 5(a)] and 7 days [Fig. (b,c)]. Numerous precipitates formed islands on the nanocomposite surface after incubation for 2 days [Fig. 5(a)]. However, the surface was not fully covered by the precipitates. After incubation for 7 days, the scaffold surface showed complete coverage by the precipitates, resulting in the scaffold having a thicker stem [Fig. 5(b)]. On closer examination, the precipitates revealed a morphological feature typical of elongated CaP nanocrystallines (~100 nm × 20–30 nm), which were often observed on the surfaces involving bioactive moieties after the SBF treatment.[18,19]

The chemical bonding structure of the mineralized product on the nanocomposite (with 20% BGNF in a scaffold type) was characterized with FT-IR spectroscopy, as shown in Figure 6. The data of the nanocomposite treated with SBF for 2 days exhibited additional phosphate bands (~600 and 1030 cm^{-1}), which were associated with the calcium phosphate precipitate. However, these bands did not correspond to the structural feature of an apatite. When the nanocomposite sample was treated with SBF for 7 days, the phosphate bands observed at 2 days changed notably, with the appearance of a strong peak at ~1030 cm^{-1} and separate peaks being observed at 570 and 605 cm^{-1}, along with an additional carbonate band at ~870 cm^{-1}.[20] This illustrates the typical evolution of an apatitic structure on the SBF-treated BGNF-collagen. From the *in vitro* SBF test, the BGNF-collagen nanocomposite is considered to possess a high degree of bioactivity, on the basis of the rapid induction of CaP precipitates on

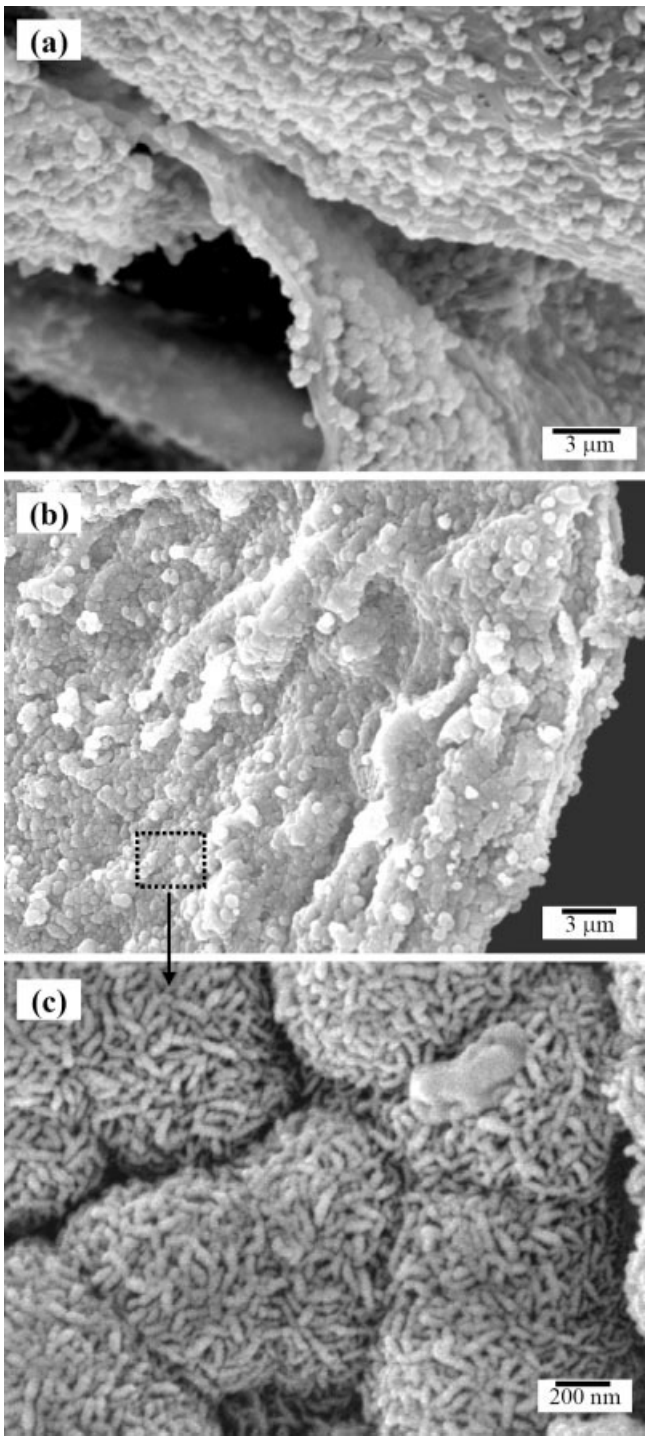

Figure 5. SEM morphology of the BGNF-Col nanocomposite (porous scaffold form) after treating it with SBF for (a) 2 and (b,c) 7 days, showing the precipitation of calcium phosphate minerals on the nanocomposite surface.

its surface (less than a few hours), and the evolution of carbonated-apatite and its rapid coverage over the nanocomposite surface within several days. Practically, from a comparison test on the collagen scaffold alone, we could not observe any indication of the precipitation of CaP products with SBF treatment even for 2 weeks.

The cellular responses to the BGNF-collagen nanocomposite were assessed using human osteoblastic cells. The electron morphologies of the cells cultured on both types of nanocomposites for 3 days are shown in Figure 7. The cells cultured on the dense membrane showed a favorable growth morphology [Fig. 7(a)]. A higher magnification image of the samples showed that the cells spread in intimate contact with the underlying nanocomposite surface and that the cell membranes exhibited active cytoplasmic extensions [Fig. 7(b)]. The cells also grew favorably on the porous scaffold, [Fig. 7(c)], spanning the pore spaces by forming active cytoskeletal extensions [Fig. 7(d)].

In particular, the osteoblastic activity of the cells was evaluated by measuring their expression of ALP. ALP is regarded as an important marker of the bone forming ability of cells, and has been used in the evaluation of osteoblast activity on various engineered biomaterials.[21] After culturing for 7 days, the cells grown on the membrane matrices were subjected to an ALP enzymatic assay. Data normalized to the total protein content are presented in Figure 8. The cells on the BGNF-collagen nanocomposites (both 20 and 40% BGNF) expressed significantly higher ALP levels than those on collagen alone ($p < 0.05$ for 20% and $p < 0.01$ for 40% BGNF). Comparing the two nanocomposites, 40% BGNF-Col showed slightly higher level than 20% BGNF-collagen although the difference was not statistically significant. This finding allowed us to conclude that BGNF played a crucial role in recruiting the osteoblastic cells so as to exhibit enhanced functional

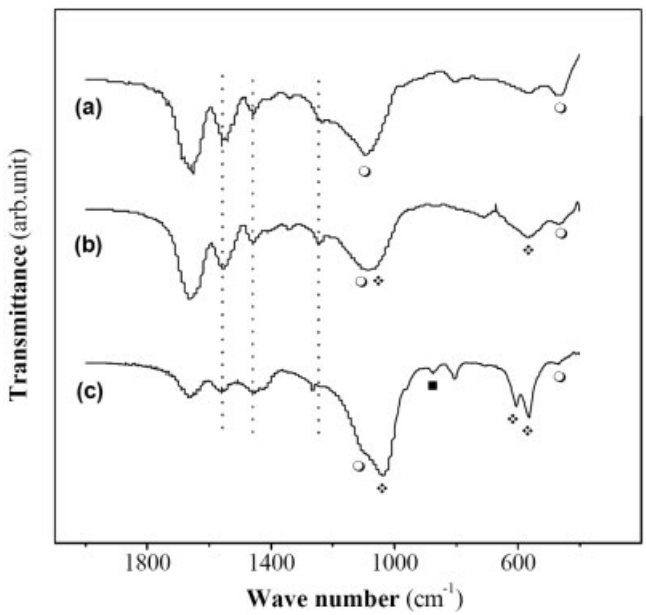

Figure 6. FTIR spectra of the BGNF-Col nanocomposite (a) before and after SBF treatment for (b) 2 and (c) 7 days. Symbols are bands related to calcium phosphate precipitates ((❖) phosphate and (■) carbonate) and (○) glass. Dashed lines indicate amide bonds associated with collagen.

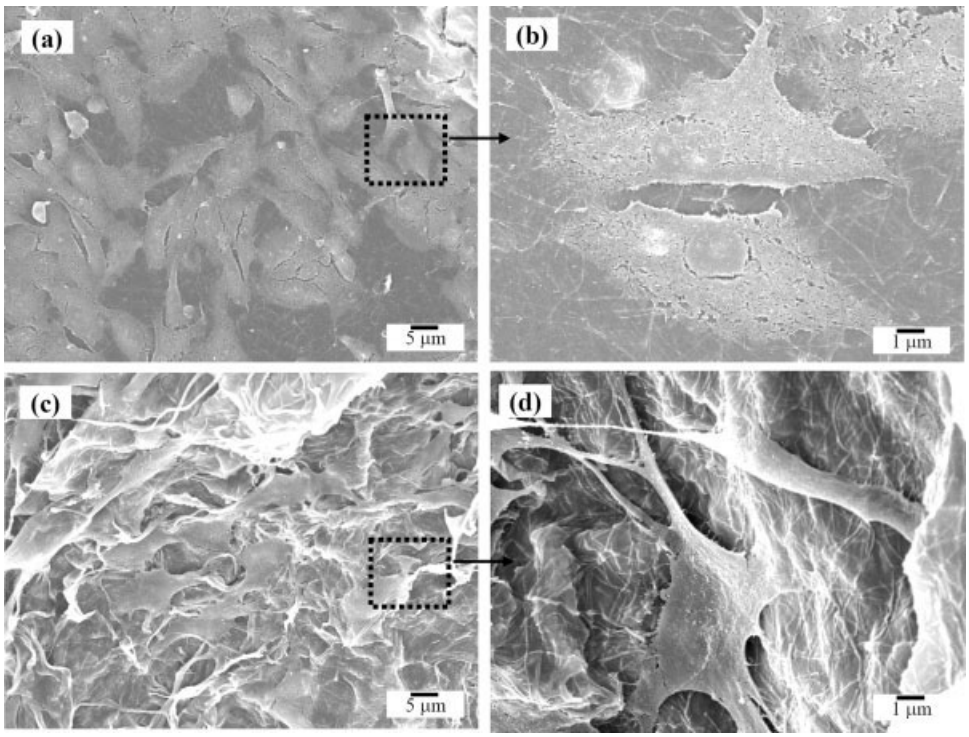

Figure 7. SEM image of the osteoblastic cells grown on the BGNF-Col nanocomposite (a,b) membrane and (c,d) macroporous scaffold after culturing for 3 days

activity on the substrate. The stimulation of ALP expression afforded by the bioactive inorganic component has been reported elsewhere in the literatures dealing with bioglass powder/synthetic polymer composites.[22,23] We could again confirm the effective role played by the bioactive glass phase in the composite approach with natural polymer, particularly in the nanoscale organized system.

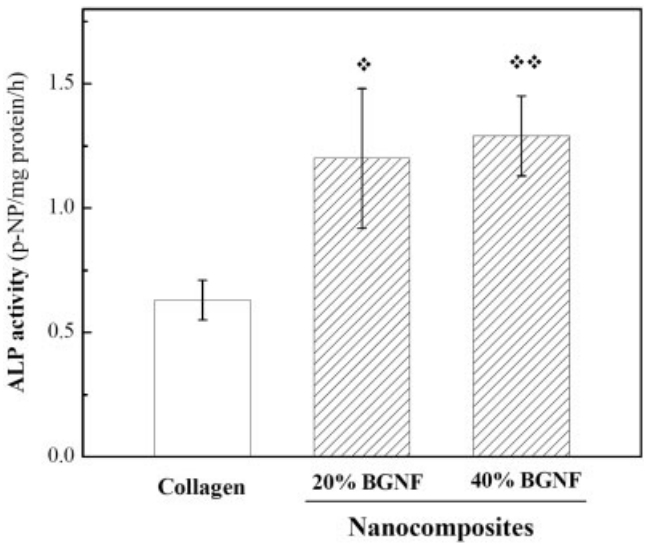

Figure 8. ALP activity of the cells on the BGNF-Col nanocomposites (20 and 40% BGNF) and collagen membrane after culturing for 7 days. ($^{*}p < 0.05$ and $^{**}p < 0.01$, for $n = 4$).

Clearly, the *in vitro* properties examined herein show the potential of the newly developed BGNF-collagen nanocomposite in the bone regeneration field. Most of all, the nanofibrous bioactive glass implemented in this study is believed to contribute significantly to the nanoscale organization level, which has been a daunting task in the bioglass–polymer composite system. In this manner, the BGNF is also expected to be used for diverse systems when hybridized with polymers in different formulations and compositions. As a further study on this BGNF-collagen system, *in vivo* biological performance and mechanical properties need to be elucidated.

CONCLUSION

A nanocomposite composed of bioactive glass nanofiber (BGNF) and reconstituted collagen was newly developed for use as a bone regeneration matrix. Within this nanocomposite, the BGNF was found to be well dispersed and distributed uniformly in the collagen nanofibrous matrix. The nanocomposite exhibited an active induction of apatite minerals on its surface under simulated body fluid conditions, showing excellent bioactivity *in vitro*. Human osteoblastic cells grew favorably on the nanocomposite. In particular, the cells on the nanocomposite expressed significantly higher ALP levels than those on collagen alone. These

findings are indicative of the strong potential of the novel biomaterial BGNF-collagen nanocomposite in bone regenerative medicine.

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