BMP-2-loaded silica nanotube fibrous meshes for bone generation

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Abstract
Silica nanotube fibrous meshes were fabricated as multiple functional matrices for both delivering bone morphological protein-2 (BMP-2) and supporting osteoblast attachment and proliferation. The meshes were fabricated via a collagen-templated sol–gel route and consisted of tubular silica with open ends. BMP-2 was loaded to the meshes by soaking in BMP-2 solution. The meshes effectively enabled the attachment and proliferation of osteoblast MC3T3-E1 cells and delivered bioactive BMP-2 to stimulate cell differentiation. These results demonstrate the potential use of the meshes in bone generation applications.

Keywords: silica nanotube, bone morphological protein-2 (BMP-2), osteoblasts, bone

1. Introduction
In recent years, considerable attention has been paid to the development of multifunctional fibrous matrices that combine cell-supporting and drug delivery capacities to promote tissue generation [1, 2]. These matrices mimic the nanoscale features of an extracellular matrix to stimulate cell attachment and proliferation, while simultaneously providing active sites for the adsorption and release of various biological factors owing to their large surface-to-volume ratio [3]. Special interest has been paid to the fabrication of fibrous nanotube matrices because their hollow structures provide large spaces for the storage and release of biological factors [4, 5].

Of the primary fibrous nanotube matrices, inorganic fibrous nanotubes have attracted considerable attention for their potential application as bone regenerative substitutes. For example, Balasundaram et al immobilized morphogenetic protein-2 on electrochemically grown TiO2 tubular arrays to enhance osteoblast adhesion [5]. Tsuchiya et al reported the induction of biological bone like apatite on anodic TiO2 tubes that had been soaked in Kokubo’s simulated body fluid (SBF) [6]. Akasaka et al reported on the deposition of apatite on carbon nanotubes [7], while Boccaccini et al coated carbon nanotubes on highly porous bioglass scaffolds to promote bone tissue generation [8].

Silica-based materials have been widely used for repairing and replacing defective bone tissue. Their silanol groups not only serve as active sites for the nucleation and growth of bone like apatite, which directly bonds to bone tissue [9–12], but are also well suited for osteoblast cell attachment and proliferation [12, 13]. For these reasons, silica-based materials have been processed in the form of particles, membranes, scaffolds and nanofibers to stimulate bone generation. However, until now, little attention has been paid to the fabrication and utilization of drug-loaded...
fibrous and multifunctional silica nanotube matrices for bone generation.

Bone morphological proteins (BMPs) have been extensively used in dental and orthopedic biomaterials to stimulate bone generation because of their strong osteogenic activity [4, 13]. However, the direct delivery of a BMP to defective sites is challenging because of the short half-lives of BMPs. To overcome this challenge, many delivery systems based on polymeric and inorganic matrices have been explored for BMPs [4, 13]. However, little attention has been paid to using silica nanotubes as BMP-2 delivery systems.

In this study, we report on BMP-2-loaded silica nanotube fibrous meshes as multifunctional matrices for bone generation.

2. Experimental details

We fabricated silica nanotube fibrous meshes using a method described in our previous study [12]. Reassembled type I porcine collagen fibrils were soaked in a Stöber-type sol–gel precursor mixture of ethanol (30 ml), tetraethoxysilane (TEOS, 5 ml), water (5 ml), and ammonium hydroxide (0.3 ml, 25%) for 24 h at room temperature to yield silica-coated collagen fibrils. These fibrils were then calcined at 600 °C for 2 h to produce silica nanotube meshes.

BMP-2 (Peprotech, Rocky Hill, NJ, USA) was loaded to the silica nanotube meshes by soaking them in BMP-2/phosphate buffer saline (70 ng ml⁻¹, pH 7.4). After 24h, the meshes were removed from the solution and washed once with PBS. The unloaded BMP-2 was quantified by enzyme-linked immunosorbent assay (ELISA, R&D Systems). BMP-2 release was performed by soaking the BMP-2-loaded meshes in phosphate buffer saline (PBS) for 2 weeks. The amount of released BMP-2 was measured by ELISA. BMP-2-loaded silica nanotube meshes were incubated with osteoblast MC3T3-E1 cells and the in vitro biological activity of BMP-2 was measured by alkaline phosphatase activity (ALP) assay, which detected the change in ALP concentration in the osteoblast.

The microstructure of the silica nanotube meshes was examined by scanning electron microscopy (SEM; JEOL-6500F, JEOL, Tokyo, Japan), while individual silica nanotubes were studied by transmission electron microscopy (TEM; JEM-2100F, JEOL, Tokyo, Japan). Infrared spectra were collected by a Fourier transform infrared spectrometry (FTIR; Model 300, JASCO, Tokyo, Japan). ²⁹Si magic angle spinning nuclear magnetic resonance (MAS-NMR) spectrum of the samples was recorded by FT-NMR spectrometry (UNITYINOVA300, Varian, Palo Alto, CA, USA).

3. Results

Figure 1 shows the microstructure of the silica nanotube fibrous meshes. The nanotubes have an average diameter of 175 ± 5 nm (figure 1(a)), a shell thickness of 33 ± 4 nm and an inner diameter of 168 ± 12 nm (figure 1(b)). Each nanotube had an open end (figure 1(c)). Similar results were reported in our previous study [12].

Figure 2 shows an FTIR spectrum of the silica nanotubes. The 1110 and 804 cm⁻¹ peaks can be attributed to Si–O–Si moieties, suggesting the hydrolysis and condensation of
Figure 3. $^{29}$Si MAS-NMR spectrum of silica nanotubes.

Figure 4. BMP-2 release profile from silica nanotube meshes over 2 weeks.

TEOS in the precursor solution. However, the calcination process led to the disappearance of a characteristic Si–OH peak at 950 cm$^{-1}$. Similar results were reported by Ahmed and Ramli who also found that Si-OH groups disappear after a mesoporous molecular sieve was calcined at 450 °C [14].

Figure 3 shows a $^{29}$Si MAS NMR spectrum of the silica nanotubes. There is only one peak at –110 ppm, which is normally assigned to $Q^2$, Si(O–Si)$_4$ [9, 10]. Its presence further confirms the hydrolysis and condensation of TEOS in the solution and the condensation of all Si–OH groups into Si–O–Si networks upon calcination. This result is consistent with the spectrum showing in figure 2.

By measuring the free BMP-2 in the BMP-2 solution, the amount of BMP-2 adsorbed in the silica nanotube meshes was calculated to be 86%. Furthermore, a BMP-2 releasing experiment was performed by soaking the BMP-2-loaded silica nanotube meshes in PBS for 2 weeks. Figure 4 shows the release kinetics of BMP-2 from the silica nanotube meshes. We found that BMP-2 exhibited sustained release behavior, and the amount of released BMP-2 with soaking time up to 2 weeks. Two models were considered for the BMP-2 release from the silica nanotube meshes. Fitting them to the experimental data showing in figure 4 yielded the equations $[\text{BMP-2}] = 1.9d$ for the reaction-controlled mechanism and $[\text{BMP-2}] = 6.6d^{1/2}$ for the diffusion-controlled mechanism. Here, $[\text{BMP-2}]$ is the amount of BMP-2 released from the silica nanotube meshes and $d$ is the soaking time.

Figure 5 shows SEM images of the osteoblast MC3T3-E1 cells on the silica nanotube fibrous meshes, revealing the effect of BMP-2 on cellular behavior. Despite the presence of BMP-2, all nanotube meshes supported the attachment and proliferation of osteoblast cells, indicating good biocompatibility, as we had found previously [12]. On the first day, the cells on the BMP-2-loaded meshes were much larger than those on the BMP-2-free meshes. After 1 week, cell sheets were visible for the meshes with and without BMP-2. This observation indicates that the silica nanotubes supported both the attachment and proliferation of osteoblast cells and that the released BMP-2 stimulated cell behavior owing to the larger sheets formed on the meshes with BMP-2. After 2 weeks, spherical P–Ca particles were deposited on the BMP-2-loaded meshes, but not on the BMP-2-free meshes, indicating that the released BMP-2 exhibited in vitro bioactivity and stimulated the differentiation of osteoblast cells. Furthermore, figure 6 shows that the ALP activity of the osteoblast cells on the BMP-2-loaded meshes was much higher than that on the meshes without BMP-2. This further confirms that the addition of BMP-2 stimulates osteoblast differentiation.

4. Discussion

We previously demonstrated that silica nanotube fibrous meshes have good biocompatibility for supporting the attachment and proliferation of osteoblast cells [12]. However, the drug delivery capability of the meshes was not investigated. In this study, we introduced BMP-2 to fibrous meshes with the expectation that this would promote bone generation. Results showed that the meshes could be used not only as a supporting matrix for osteoblast cells but also as a sustained delivery system for bioactive BMP-2.

There have been a few reports on the interaction between BMP-2 and silica-based materials and some factors were found to affect the interaction. El-Ghannam et al. [15] considered that nanopores in bioactive resorbable silica–calcium phosphate nanocomposites provided a sufficiently large surface area for BMP-2 adsorption and protection. In our study, the meshes were fibrous and each silica nanotube had an open end allowing for the infiltration of BMP-2 (figure 1). However, the specific surface area of the silica nanotube fibrous meshes was small as 52 m$^2$ g$^{-1}$ [12]. Thus, the surface area is hardly a key factor for supporting the adsorption of BMP-2 in the silica nanotube meshes. On the other hand, Ehlert et al. pointed out that the immobilization of BMP-2 on amino-modified silica surfaces can be attributed to their electric interaction [16]. With an isoelectric point of approximately 9, BMP-2 is an almost neutral system, which is slightly positively charged in PBS [17]. The spectra showing in figures 2 and 3 suggest that the silica nanotubes were mainly composed of silicate networks (Si–O–Si) and were negatively charged when soaked in the BMP-2/PBS solution. Therefore, the electric interaction should have promoted the adsorption of BMP-2 in the silica nanotube meshes. Bae et al.
found that the amount of adsorbed BMP-2 was higher for an anodic oxidized than for a non-anodic oxidized nanotubular surface owing to the enhanced hydrophilicity [18]. Han et al pointed out that superhydrophilic carbon nanotubes retain a larger amount of BMP-2 than superhydrophobic ones [19]. We speculate that the strong hydrogen bonding due to the hydrated silicate networks in the BMP-2/PBS solution had another contribution to the adsorption of BMP-2 in the silica nanotube meshes. This strong hydrogen bonding led to the sustained release of BMP-2 over a period of as long as 2 weeks (figure 4). Two mechanisms might be responsible for the release of BMP-2 from silica nanotube fibrous meshes: the degradation of silica nanotubes [12] and the diffusion-controlled release from the pores between silica nanotubes. According to the fitting results, both mechanisms contributed to the release of BMP-2.

It is unclear whether the status of BMP-2 on the substrates has an effect on its biological activity. Yamachika et al reported that BMP-2 immobilized on collagen enhanced the cellular behavior of osteoblasts in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide [20], and according to Shi et al, the chemical immobilization of BMP-2 on the surface of titanium resulted in enhanced osteoblast functions [21]. Interestingly, BMP-2 released from nanotubes can significantly stimulate bone generation [4, 5, 14]. Irrespective of the status of BMP-2, we found that adding BMP-2 to the silica nanotubes enabled them to maintain their biological activity and indeed enhance the osteoblast functions (figures 5 and 6).

5. Conclusion

We demonstrated that silica nanotube fibrous meshes can not only support osteoblast attachment and proliferation but also deliver bioactive bone morphogenetic proteins. This result indicates that silica nanotube fibrous meshes can be applied to multifunctional matrices for bone generation.

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Figure 5. SEM images of osteoblast MC3T3-E1 cells on silica nanotube meshes with and without BMP-2 (scale bar: 10 µm).

Figure 6. ALP activity of osteoblast MC3T3-E1 cells on silica nanotube meshes with and without BMP-2.
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