JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 1105– 1109

Microsphere of apatite-gelatin nanocomposite as bone regenerative filler

HAE-WON KIM*, BYUNG-HO YOON, HYOUN-EE KIM
School of Materials Science and Engineering, Seoul National University, Seoul, 151-742, Korea
E-mail: hwkim@snu.ac.kr

In this study, we developed novel microspheres comprised of an apatite-gelatin nanocomposite. This microsphere formulation is considered to be useful as a bone regenerative filler, either directly or combined with polymeric matrices. Using the water-in-oil emulsion technique, the apatite-gelatin viscous nanocomposite solution was successfully formulated into microspheres with an average diameter of $\sim$110 μm. The microspheres were composed of apatite nanocrystallines precipitated within the gelatin matrix, revealing a typical nanocomposite internal structure. This nanocomposite structure contrasted markedly with that of the conventional composite microspheres which were obtained by directly mixing gelatin with apatite powder. Initial cellular assays showed that the microspheres maintained the adhesion and proliferation of the osteoblastic cells, suggesting the usefulness of the apatite-gelatin nanocomposite microspheres in the bone regeneration field.

1. Introduction

The composite approach to developing artificial materials represents one of the most promising strategies in bone reconstructive surgery [1, 2]. This approach is mainly based on the mimicking of the composition and structure of human hard tissues, which is a real typical nanocomposite comprised of collageneous proteins and apatite nanocrystallines. A number of composite systems, such as powders, dense bodies and porous scaffolds, have been created from apatite and collagen-based proteins [1–6]. In these earlier studies, composite systems have been shown to improve the osteoblastic cellular responses and *in vivo* bone forming ability. In particular, compared to micron-scale mixture composites, nanoscale organized composites have proven to be more effective, because of their structural similarities to real bones and their higher mechanical and biological properties [5, 6].

Recently, the authors produced a series of nanocomposites made up of apatite and natural proteins (collagen and gelatin), in the forms of dense body and porous scaffold [4–6]. The precipitation methodology adopted in these studies was highly effective in producing the uniform nanocomposite structure of apatite nanocrystals within protein matrices and generating a well-developed macrostructure, such as a pore configuration [6].

In this study, we utilized an apatite precipitated nanocomposite solution to produce microspheres. These microspheres have the benefit of being able to be used as fillers in bone defects, either directly or formulated with polymeric sources. Moreover, the microsphere form can hold and release bioactive moieties in a more controllable manner than other formulations, and is thus suitable for use as a drug delivery system. To date, a significant number of studies have been carried out on the production of microspheres using polymeric sources [7, 8]. However, very little is known about composite microspheres. The inorganic powders are sometimes directly added to the polymeric solutions, in order to produce conventional composite microspheres [9–11]. Herein, the authors produced microspheres consisting of apatite-gelatin from a precipitated nanocomposite solution, and assessed their properties and preliminary cellular responses.

2. Materials and methods

2.1. Production of apatite-gelatin microspheres

As a precursor for the microspheres, an apatite-gelatin nanocomposite viscous sol was prepared by the precipitation of apatite within a gelatin solution. Type B gelatin (Sigma) was dissolved at 10 w/v% in distilled water at 60 °C. As the calcium and phosphate sources for the apatite, $Ca(OH)_2$ (Sigma) and H_3PO_4 (Sigma, 85 wt%(aq.)), respectively, were dissolved separately ([Ca]/[P] $\sim$ 1.67) in distilled water at 60 °C for 24 h with vigorous stirring. The Ca-containing solution was added to the gelatin solution and stirred for 3 h. The

*Author to whom all correspondence should be addressed.

P-containing solution was added dropwise to the Ca-gelatin mixture solution and stirred for 24 h at 60 °C. The amount of apatite was considered to be ∼20 wt% with respect to the gelatin when the Ca and P that were added reacted completely to form apatite. This consideration was based on our preliminary study on the phase and thermal analyses. For the purpose of comparison, a conventional composite sol was also prepared by mixing the pure gelatin solution with 20 wt% HA commercial powder (Alfa Aesar, USA).

The nanocomposite microspheres were produced using the water-in-oil emulsion technique. 10 ml of the prepared sol was added to 300 ml of an olive oil (Sigma) bath dropwise with a stirring speed of 500 rpm maintained at 60 °C to form a cloudy micro-emulsion. After stirring for 30 min, the oil bath was quenched to 4 °C and maintained at this temperature for 60 min, followed by the addition of 300 ml acetone to solidify the microspheres. The microspheres were re-dispersed and washed with acetone and 95% ethanol solution twice. The microspheres were further cross-linked with water soluble 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Aldrich, USA) dissolved at 0.1% w/v in ethanol/water solution for 12 h at 4 °C with gentle stirring at 80 rpm. The microspheres were washed fully with ethanol/water at least five times.

2.2. Characterization

The phase of the apatite-gelatin nanocomposite was evaluated with X-ray diffraction (XRD; Philips) at a scanning speed of 0.5°/min. The bonding structure of the nanocomposite was analyzed with Fourier transformed infrared spectroscopy (FT-IR; System 2000, Perkin-Elmer) over the range of 400–4000 cm^{-1} using KBr as a reference. The morphology of the microspheres was observed with field emission scanning electron microscopy (FESEM; JSM 6330F, JEOL) at an accelerating voltage of 5–10 kV after Pt coating. The internal structure of the microspheres was analyzed with transmission electron microscopy (TEM; CM20, Philips) after embedding the microspheres with spurr-resin, slicing them to ∼80 nm using a microtome and supporting them on a copper grid and carbon coating. For the determination of their size, 10 mg of the microspheres was suspended in 10 ml ethanol under sonification and mild stirring, and then analyzed with laser diffractometry using a particle size analyzer (Mastersizer 2000, Malvern Instruments).

2.3. Preliminary cellular responses

The cell growth morphology on the nanocomposite microspheres was observed using osteoblast-like MG63 cells. The cells were maintained in a medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50IU/ml of penicillin, and 50 mg/ml of streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C. 100 μl aliquots of the microsphere solutions which were pre-dispersed in 70% ethanol at a concentration of 0.1% w/v were distributed into 24-wells and then dried overnight under a laminar flow. The cells were seeded on plates containing the microspheres as well as on blank plates (used as a control) at a density of 4×10^4 cells/ml, and then incubated for up to 7 days. After culturing for predetermined time periods (1, 3, and 7 days), the cell proliferation level was measured using an MTT assay as described previously [6]. The MTT test was performed on three replicate samples and the data were compared using one-way ANOVA.

3. Results and discussion

The apatite-gelatin microspheres produced by means of the water-in-oil emulsion technique using the nanocomposite solution showed the size distribution presented in Fig. 1. The average diameter of the microspheres was found to be 112 μm. This size distribution obtained was specific to the conditions used in this study, particularly the concentration of the apatite-gelatin in water, the loading ratio of the solution in oil and the stirring speed. Normally, as the concentration of the material and the loading ratio of solution increase and the stirring speed decreases, the size of the microspheres increases. Using this water-in-oil technique, previous studies on the microspheres of various polymers including gelatin have resulted in the production of microspheres with a wide range of sizes (∼several micrometers to several hundreds of micrometers) [8–10]. Although no systematic examination of this size variance was performed herein, it is thought that the size of the apatite-gelatin microspheres can be adjusted according to the requirements of specific applications. The size of the microspheres produced in this study renders them useful as bone defect fillers, either directly or as bone regeneration matrices when formulated with other polymeric sources.

The typical morphologies of the nanocomposite microspheres are presented in Figs. 2(A)–(C) at different magnifications. The micron-scale image revealed a well-developed isotropic sphere morphology Fig. 2(A). The surface of the microspheres was rumpled, but

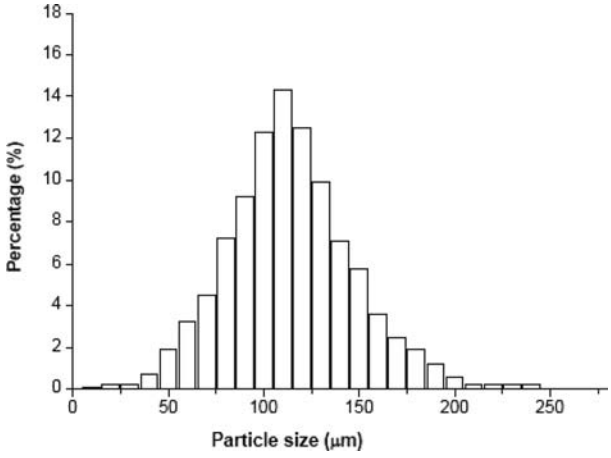

Figure 1 Particle size distribution of the apatite-gelatin nanocomposite microspheres obtained by the water-in-oil emulsion technique. The average diameter (d_{ave}) was 112 μm.

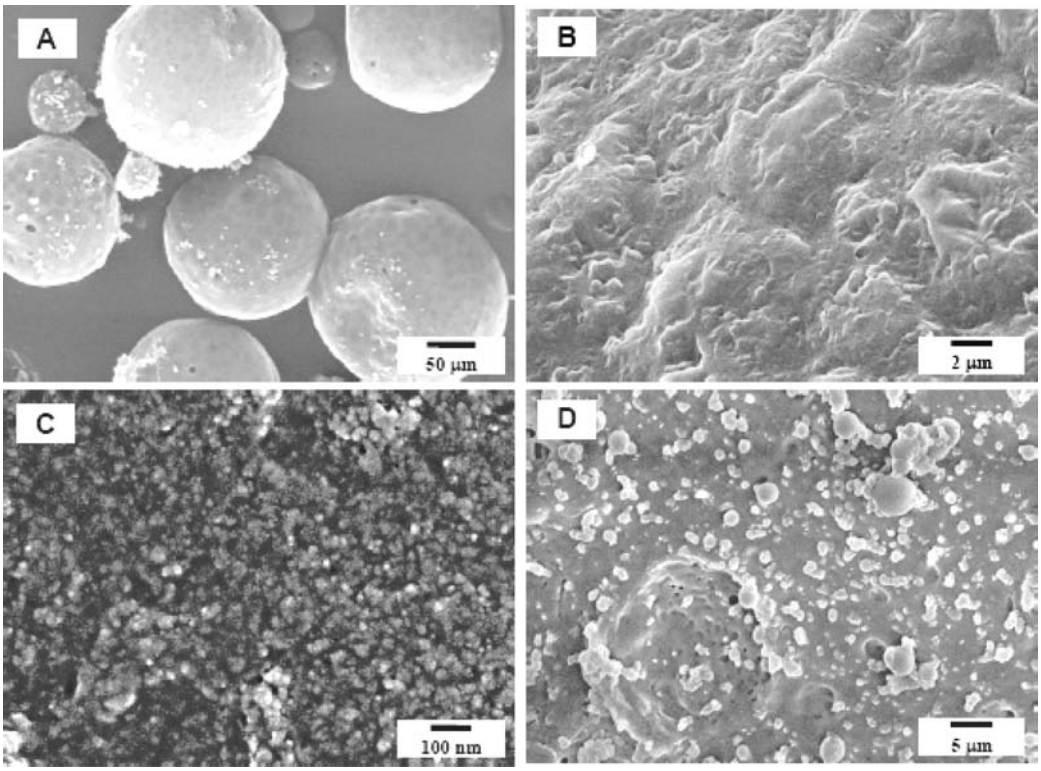

Figure 2 FESEM morphology of the (A)–(C) apatite-gelatin nanocomposite microspheres at different resolutions and (D) conventional composite microsphere. Note the difference in the scale bar. A nanoscale organized structure was revealed on the nanocomposite sphere (C), whilst apatite agglomerates with sizes of a few micrometers were readily observed on the conventional composite sphere (D).

appeared to be smooth on the micron-scale (Fig. 2(B)). However, a higher magnification image of (B) revealed an interesting structure constituted of nanoscale organized components (Fig. 2(C)). This morphological feature was not created in the conventional composite microspheres when produced by directly mixing the gelatin solution with apatite powders. In the comparison shown in Fig. 2(D), the conventional composite showed a number of apatite agglomerated particles with sizes of several micrometers. This was attributed to the fact that the directly mixed apatite powders were not effectively dispersed within the gelatin solution, but were agglomerated as secondary particles, leading to the nonuniform morphology. However, the apatite crystals precipitated in-situ within the gelatin solution were able to be effectively distributed within the gelatin network, because the nucleation and grain growth of the apatites are controlled on the molecular level through the amino acid backbone of gelatin. Similar results were previously observed in the production of a macroporous structure consisting of apatite-gelatin [5, 6].

The phase and chemical status of the apatite-gelatin nanocomposite microspheres were evaluated and the results are shown in Figs. 3(A) and (B), respectively. The XRD pattern of the nanocomposite showed poorly crystallized apatite peaks at the characteristic *d*-spacings, as well as the broad regime derived from gelatin (Fig. 3(A)) [5]. Moreover, the IR spectrum (Fig. 3(B)) of the nanocomposite revealed well-developed bands associated with apatite (noted as open circle symbols at 562, 601, and 1030–1050 cm^{-1}), when referenced with the HA-gelatin mixed composite [6]. The gelatin amide bands (dashed lines) in the nanocomposite were similar to those of pure gelatin.

The internal structure of the nanocomposite microspheres was further analyzed with TEM observation, as shown in Fig. 4. Samples were prepared after microtome-sectioning of the resin-embedded microspheres. The TEM image showed a relatively dark area with numerous elongated products (A, B). The selected area electron diffraction pattern of the products revealed diffused rings which were characterized as typical apatite crystallines, as normally observed in biomimetically produced apatites [6, 12].

The cellular responses to the nanocomposite microspheres were briefly assessed. Cells were seeded on the microspheres contained in tissue culture plates and cultured for up to 7 days. Fig. 5 shows the proliferation level of the cells as assessed using the MTT method at different culturing periods. The cell proliferation on the microspheres increased with increasing culturing time, but was slightly lower than that on the control (blank tissue culture plate). However, this does not directly mean that the microspheres had a harmful effect on the cell viability; rather, the similar pattern observed for both sets of data with respect to the culturing time suggests that the microspheres also maintain the viability of the cells over the testing periods used in this study. It is likely that certain parameters which were not considered fully in this preliminary study, such as the curvature of the substratum and the concentration of the microspheres, etc, complicated the *in vitro* cellular interaction with the microspheres. Therefore, further in-depth biological studies, including *in vivo* animal tests,

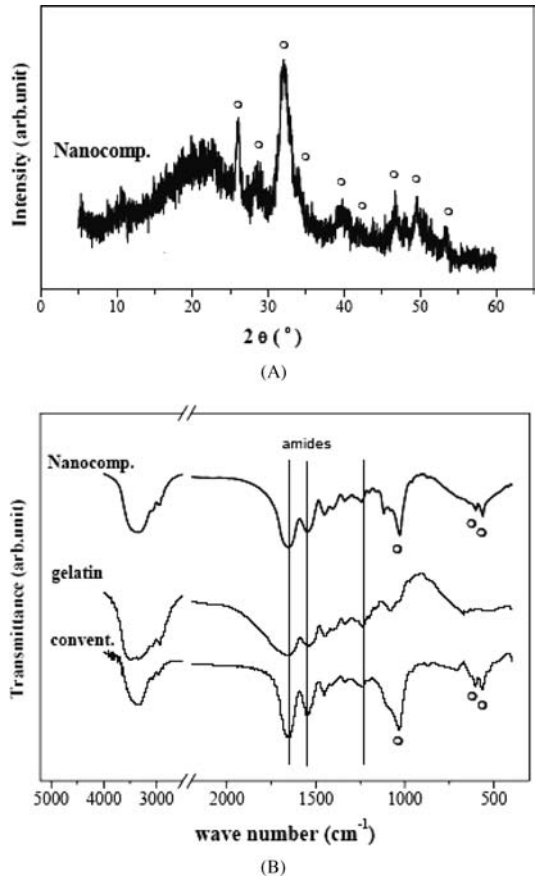

Figure 3 (A) XRD pattern and (B) FT-IR spectra of the apatite-gelatin nanocomposite microspheres. The apatite associated peaks and bands are indicated by symbols (<Wingdings_m_ND>). The spectra of pure gelatin and conventional composite are also presented as references in (B).

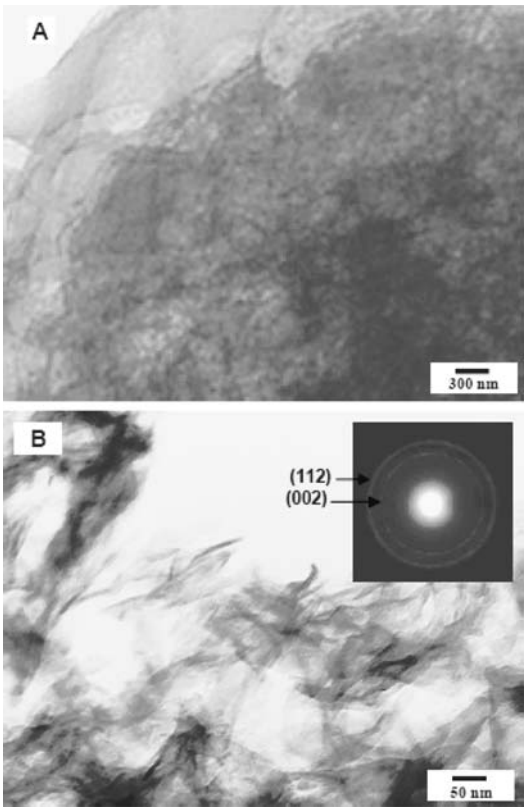

Figure 4 TEM analyses of the apatite-gelatin nanocomposite microspheres: image at low (A) and high (B) magnification, and diffraction pattern of the apatite crystallines (inset in (B)). The specimen was prepared after microtome sectioning of the resin-embedded microsphere.

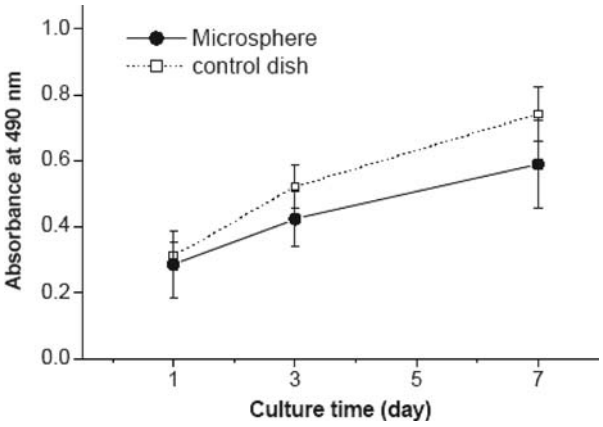

Figure 5 Cell viability measurement on the nanocomposite microspheres as assessed by the MTT method for various culturing times. A blank tissue culture plate was used as a control. Data are represented as means $\pm$ 1 std for $n = 3$. Significant difference (ANOVA, $p < 0.05$) was observed only at day 7.

are warranted, in order to confirm the performance of the nanocomposite microspheres.

4. Concluding remarks

Based on the present findings, the apatite-gelatin microspheres developed herein are regarded to preserve the structural and morphological features typical of an organic-inorganic nanocomposite. Of special importance is the fact that the apatite-gelatin nanocomposite gel was successfully converted into the microsphere form, which should enable it to find diverse and specific uses in the bone regeneration field. Further studies are required for the biological assessment of the nanocomposite microspheres, in order to verify their usefulness.

Acknowledgments

This work was supported by grants from the Korea Health 21 R&D Project of the Ministry of Health and Welfare (02-PJ3-PG6-EV11-0002) and from the Korea Research Foundation (R08-2004-000-10192-0). The authors are grateful to S.Y. Chae for her experimental assistance.

References

1. P. M. AJAUAN , L. S. SCHADLER and P. V. BRAUN, in "Nanocomposite Science and Technology" (Wiley-VCH, 2003).
2. S. MANN and G. A. OZIN, *Nature* **365** (1996) 499.
3. A. BIGI, S. PANZAVOLTA and N. ROVERI, *Biomater* **19** (1998) 739.
4. B. H. YOON, H. W. KIM, S. H. LEE, C. J. BAE, Y. H. KOH, Y. M. KONG and H. E. KIM, *ibid.* **26** (2005) 2957.
5. H. W. KIM, J. C. KNOWLES and H. E. KIM, *J Biomed Mater Res A* **72A** (2004) 136.
6. H. W. KIM, H. E. KIM and V. SALIH, *Biomater* **26** (2005) 5221.
7. R. CORTESI, C. NASTRUZZI and S. S. DAVIS, *ibid.* **19** (1998) 1641.
8. M. A. VANDELLI, F. RIVASI, P. GUERRA, F. FORNI and R. ARLETTI, *Int J Pharm* **215** (2001) 175.

9. T. J. WU, H. H. HUANG, C. W. LAN, C. H. LIN, F. Y. HSU and Y. J. WANG, *Biomater* **25** (2004) 651.
10. F. I. HSU, S. C. CHUEH and Y. J. WANG, *ibid.* **20** (1999) 1931.
11. M. SIVAKUMAR and RAO K. PANDURANGA, *ibid.* **23** (2002) 3175.
12. S. EIDEN-ASSMANN, M. VIERTELHAUS, A. HEIß, K. A. HOETZER and J. FELSCHE, *J Inorg Biochem* **91** (2002) 481.

Received 30 June
and accepted 19 August 2005