J Mater Sci: Mater Med (2007) 18:2167–2172
DOI 10.1007/s10856-007-3229-9

Stimulating effect of silica-containing nanospheres on proliferation of osteoblast-like cells

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Received: 31 March 2006 / Accepted: 19 June 2007 / Published online: 15 August 2007

Abstract Silica and silica-based materials have been found widespread application for medical purposes, especially in the fields of bone tissue engineer. Nano-sized silica has been developed too and been considered to be used in bone regeneration. In this study, we observed the biological response of osteoblast-like cells to three kinds of silica nanospheres: SNs-A (30–40 nm), SNs-B (70–80 nm), and Silica/OCP (70–80 nm). Cells treated with three kinds of nanospheres always showed higher cell viability than control cells. ALP activity of cells treated with three kinds of silica nanospheres was higher than that of control cells at early time. Both of the two effects were not in a concentration-dependent manner. Silica/OCP had better effect on MG-63 cell activity and proliferation than SNs-A and SNs-B. The three kinds of silica nano material all have biological validity on osteoblast-like cells, especially Silica/OCP.

Introduction

Bone loss due to traumatic injury, orthopedic surgery, or tumor removal is a significant clinical problem. The limitations of donor sources of natural bone tissue for both autografts and allografts have led to the search for synthetic alternatives. Silica-based bioactive glasses have attracted a great deal of interests as promising bone substitutes [1]. Silica can inhibit connective tissues invading into bone defects and improve bone regeneration. Many authors reported that the silica-containing bioactive materials have the potential ability to activate bone-related gene expression and stimulate cell proliferation [2].

It is suggested that nano-phase materials can be synthesized to possess similar nanometer dimensions to components of bone tissue to promote new bone formation, compared with conventional orthopedic implant materials [3]. Nano silica-calcium phosphate composite can serve as a delivery system for cells and biological molecules and an alternative to autologous bone grafting [4]. In this study, we prepared two kinds of silica nanospheres with different nanometer dimensions. Their effects on viability of osteoblast cells were compared.

The ion of silicon can be released from silica. Silicon has been recognized as an essential element in young bone calcification. The release of soluble ion of silicon can stimulate osteoblast cells to produce bone [5]. In vitro studies have shown that the material itself and the soluble ionic species released by network dissolution may participate in producing an osteoinductive effect [6]. But excessive dose of silicon becomes toxic or inhibitory. Generally, the initial ion release rate of biomaterials is large. Control of silicon ion with desirable sustained-release is pivotal for the efficacy of silica-based materials [7]. So we prepared another silica nanosphere packed with octacalcium phosphate (OCP) shell which was used to control the release of silicon.

The present study aims to evaluate the suitability of three silica nanospheres for supporting osteoblast cells

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growth and metabolism using the MG63 cell line model in vitro.

Materials and methods

Preparation of silica nanospheres

The silica nanospheres (SNs) were obtained by improved Stŏber method [8]. Tetraethyl orthosilicate, ammonia and deionized water were added to 180 mL dehydro-ethanol with the molar ratio of 1:(2–3):(2–5). The resulting mixture was hold at 35 °C with stirring at 200 rpm for 6 h, forming silica-based nanoparticles suspending solution. SNs-A, SNs-B nanospheres were made after the solution was filtrated and dried. Synthesis of OCP shell was carried by dropwise addition of calcium nitrate and sodium phosphate solution in the SNs suspending solution at pH 6.5 and the resulting mixture was hold at 40 °C with stirring for 3 h. Silica/OCP was made after the solution was filtrated and dried.

Characterization measurement of the nanospheres

Size distribution of three kinds of nanospheres was determined by particle analyzer (Marvin 2000). The phase composition and morphology of the nanospheres were tested separately by X-ray diffraction (XRD, Rigaku D/max-rA) and transmission electron microscope (TEM, JEM 2010).

Cell culture

MG-63 cell (purchased from American Type Culture Collection), a human osteoblast-like cell line, was used to assess the cellular responses to the nanospheres. Cells were cultured in 25 cm^2 flask in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When digested, the cells were washed with phosphate-buffered saline (PBS, Gibco), detached with trypsin-EDTA solution (0.25% trypsin, Gibco) at 37 °C for 5 min, and centrifuged and resuspended for further cell tests.

Cell morphology

Cells were dispensed in 96-well plate at a density of 2×10^4 cells per well. SNs-A, SNs-B, Silica/OCP were respectively added in the culture medium with concentrations of 2×10^{-2} mg/mL, 4×10^{-3} mg/mL, 8×10^{-4} mg/mL, 1.6×10^{-4} mg/mL, 3.2×10^{-5} mg/mL. After fully vibration and mixing, the medium containing nanospheres was added to the well (300 μL each well). After incubated with SNs-A, SNs-B, and Silica/OCP nanospheres for 2, 4, 7 days, cells were put in the light path of phase-contrast microscope. The condenser turret was rotated to be aligned with the phase contrast lens. Then cell morphology was observed with the optimized phase effect.

MTT assay

Following incubation of cells with three kinds of silica nanospheres solution for indicated time in 96-well plate, 20 μL of PBS containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, sigma) was added to each well. Plates were incubated at 37 °C for 4 h, then medium was replaced with 150 μL of dimethyl sulfoxide (DMSO, Sigma). After 10 min incubation absorbance in control and treated wells was measured in a plate reader at 490 nm.

ALP test

Alkaline phosphatase (ALP) activity was measured colorimetrically by using disodium phenyl phosphate as the substrate. The enzyme ALP expressed by the cells hydrolyzed the substrate to phenol and an inorganic phosphate. Under alkaline conditions, the phenol was converted to a red product and its absorbance was subsequently measured at 520 nm using a spectrophotometer. The absorbance was directly converted to ALP activity level based on a bovine serum albumin standard level.

Statistical analysis

Collected data were expressed as means ± SD. Statistical analysis was performed by Student's t test to express the difference between two groups. And $p < 0.05$ was considered significant.

Results

Characterization of silica nanospheres

Particle size analysis showed that the dimensions of the SNs-A, SNs-B, Silica/OCP nanospheres were 30–40 nm, 70–80 nm, 70–80 nm respectively (Fig. 1A). Figure 1B shows XRD patterns of SNs-B and Silica/OCP. Phase of OCP (presented by O) was detected in Silica/OCP, but not in SNs-B. Figure 1C shows Silica/OCP had a layer of thin shell (~5 nm) observed under transmission electron microscope in comparison with SNs-B. These results reveal that when Ca^{2+} and PO_4^{3-} contained salt titrants were

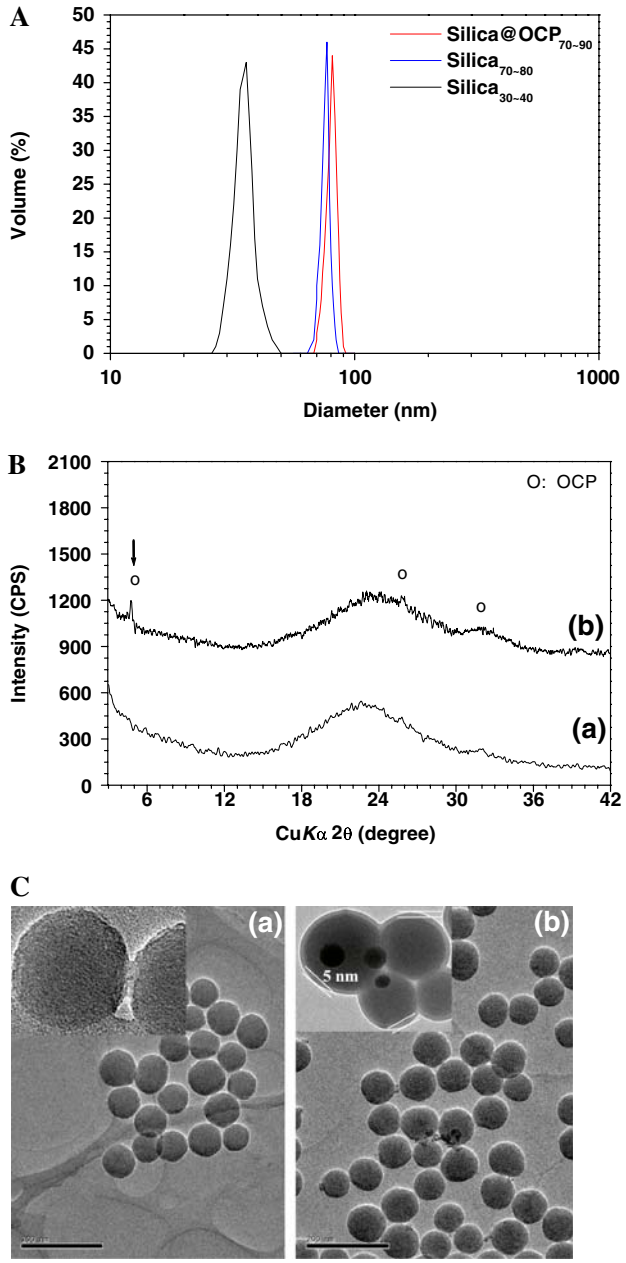

Fig. 1 Characterization of silica nanospheres. (**A**) Particle size distribution of three kinds of silica nanospheres. (**B**) XRD of SNs-B (**a**) and Silica/OCP (**b**). O represents the phase of OCP. (**C**) TEM morphologies of SNs-B (**a**) and Silica/OCP (**b**). Scale bar represents 200 nm

added dropwise in the suspension solution, OCP shells were obtained.

Cell morphology

As shown in Fig. 2, cells treated with three kinds of silica nanospheres presented similar morphology changes. With time lapse, cell gap decreased and cell density increased gradually. At the 2nd and the 4th day, cells were spreading on the substrate. At the 7th day, cells were growing in a multi-layer and cell shape changed from spindly to spherical.

Cell viability

At the 2nd, 4th, and 7th day, cells treated with the three kinds of silica nanospheres all showed higher optical density (OD) value than control and the difference was significant mostly (Fig. 3). At the 4th day, treated cells and control cells all showed highest optical value. The viability of treated cells was independent of silica nanospheres concentration. The highest optical value of SNs-A was extracted from 4×10^{-3} mg/mL at the 4th day. The highest optical value of SNs-B was extracted from 3.2×10^{-5} mg/mL at the 4th day. The highest optical value of SNs-A was extracted from 2×10^{-2} mg/mL at the 4th day.

ALP activity

ALP activity of cells treated with three kinds of silica nanospheres was about 2-fold of control cells at the 2nd day, about 1-fold at the 4th day, about 0.5-fold at the 7th day (Fig. 4). ALP activity of treated cells was also independent of silica nanospheres concentration. Cells treated with 8×10^{-4} mg/mL SNs-B for 2 days showed the highest ALP activity.

Discussion

Silicon is believed to be essential in skeletal development. It was uniquely localized in the active areas of young bone and involved in the early stage of bone calcification [9]. Considerable amounts of silica gel-based bio-/nano-materials are developed as biomedicine and regeneration biomedical application with the development of the molecular biological techniques. Examples of such applications include encapsulation of functional genes, living cells, drugs, and functional nanoparticles. In this study, we prepared three kinds of silica nanospheres and observed cell response to them.

The dimensions of three kinds of silica nanospheres SNs-A, SNs-B, Silica/OCP were 30–40 nm, 70–80 nm, and 70–80 nm respectively, measured by particle size analyzer.

We used an in vitro model of evaluating osteoblast cell response to biomaterials as a preliminary screen for biocompatibility [10, 11]. Using this model, we tested the hypothesis that silica nanospheres were favorable

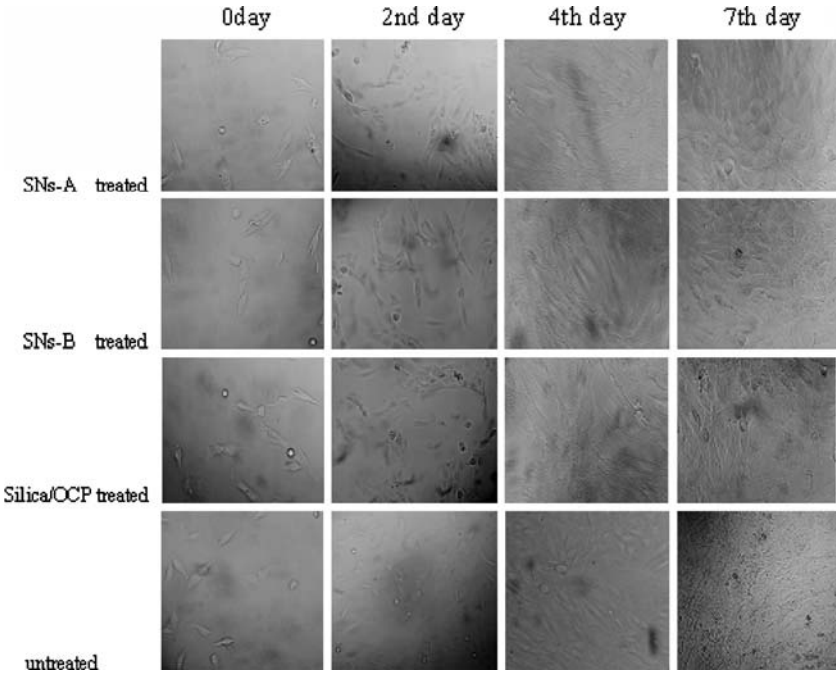

Fig. 2 Morphology of cells treated with three kinds of silica nanospheres. Cells were treated with medium containing 2×10^{-2} mg/mL SNs-A, SNs-B, Silica/OCP respectively for 2, 4, 7 days and were observed under phase-contrast microscope

biomaterial in vitro for the proliferation and function of MG63. The MG-63 cell line exhibits osteoblast-like characteristics such as production of osteocalcin and collagen type I. With its osteoblast-like phenotype, the MG-63 cell line serves as a useful model to test the biological performance of various materials [12, 13].

Observed by phase-contrast microscope, cells treated with three kinds of silica nanospheres all presented spreading cytoplasm, which indicated good cell adhesion. At the 4th day, visual field was full of cells and cell density was much higher than that at the 2nd day. It suggests that three kinds of silica nanospheres despite of the nano size are not cytotoxic but biocompatible. At the 7th day, cells were spherical and growing in a multi-layer, which meant cells began aged.

MTT test presents not only cell number but also cell activity. The test is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product, then the absorbance was measured. So only alive cells can deoxidize MTT and number of alive cells can be quantified. Higher OD value means more alive cells. OD value of cells treated with three kinds of silica nanospheres were always higher than control at any time, which indicates that three kinds of silica nanospheres can induce osteoblast-like cell proliferation. OD value was highest at the 4th day. It was in accordance with the result observed by microscope. At the 4th day, cell number was large and cell activity was high. At the 7th day, the OD value decreased because cells were aged and the apoptotic or necrotic cells increased, though the cell number was still large.

The OD value of three kinds of silica nanospheres treated cells was independent of silica nanospheres concentration, which was accordance with the ALP result. ALP activity of cells treated with three kinds of silica nanospheres was also not related with nanospheres concentration. At the 2nd day, ALP activity was highest in cells treated with silica nanospheres comparable to control. It may be because the release of silicon ion affected ALP activity.

Current understanding of the potential toxicity of nano-particles is limited, but research indicates that some of these products may become toxic at the cellular level [14]. Cell proliferation and cell function can be inhibited by nano-particles. The impact of nano-particle interactions with the cell is dependent on their size, chemical composition, surface structure, solubility, shape and how the individual nano-particles amass together. In our study, the three kinds of silica nanospheres all improved cell viability and ALP activity of MG-63 cells. They are not cytotoxic but proliferation-stimulative to osteoblast cells.

The difference of the three kinds of nanospheres was shown in Tables 1 and 2. Since the concentration of nanospheres did not affect cell activity and cell number of MG-63 cell, the highest OD value and ALP activity of three kinds of nanospheres-treated cells at each time point were chosen to compare.

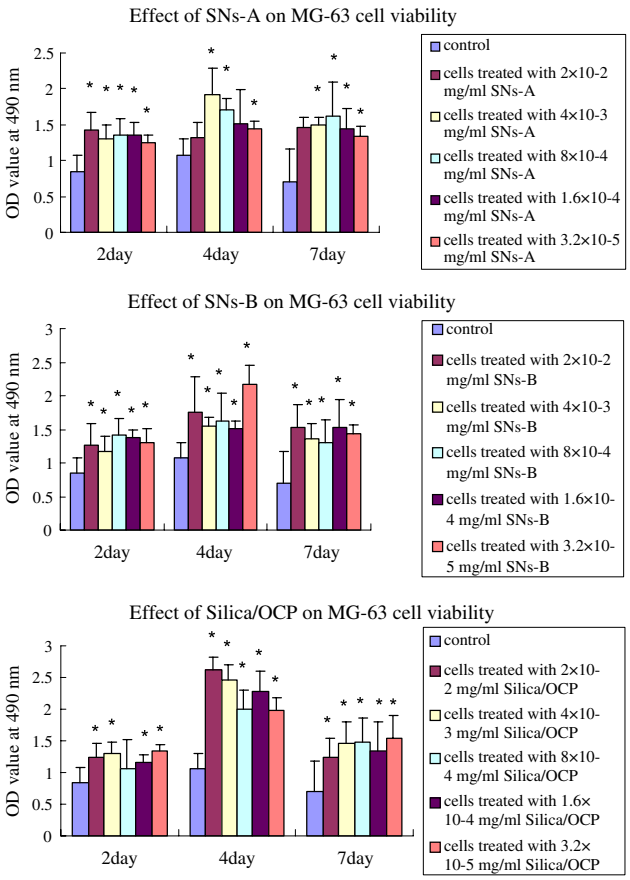

Fig. 3 Effects of three kinds of silica nanospheres on cell viability of MG-63. Cells were treated with a series of concentration of three kinds of silica nanospheres for 2, 4, 7 days. Cell viability was measured by MTT test separately. * means $p < 0.05$ compare to control

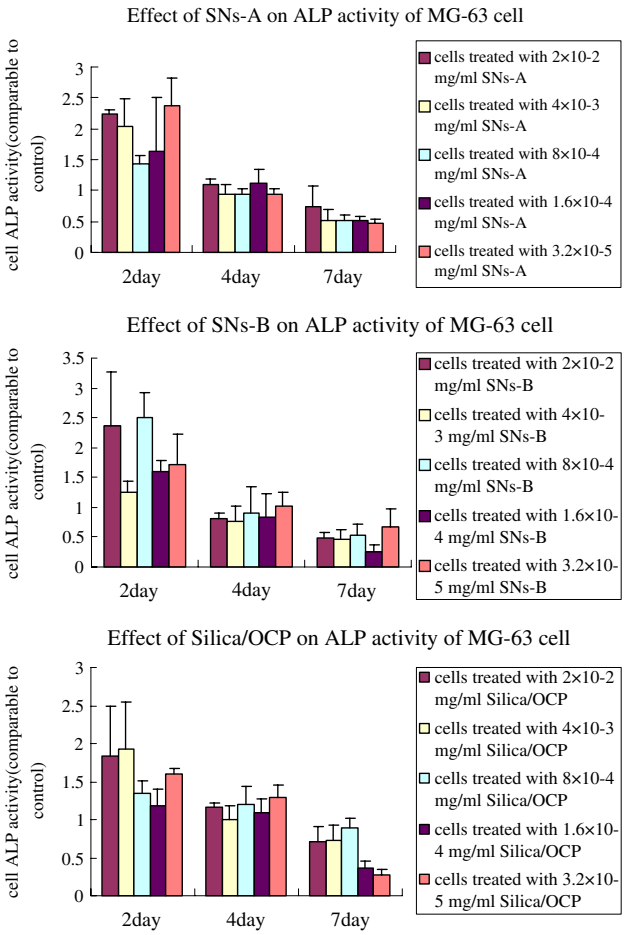

Fig. 4 Effects of three kinds of silica nanospheres on activity of ALP of MG-63 cell. Cells were treated with a series of concentration of three kinds of silica nanospheres for 2, 4, 7 days. Cell ALP activity was measured as described in Materials and methods separately

As shown in table, cells treated with SNs-A and SNs-B presented close OD value and ALP activity. Silica/OCP always showed lower value at the 2nd day, but higher value at the 4th and 7th day. OD value of Silica/OCP-treated cells was significantly higher than that of SNs-A, SNs-B-treated cells at the 4th day. ALP activity of cells treated with Silica/OCP was also higher than that of cells treated with SNs-A, SNs-B at the 4th day, but the difference was not significant. OCP is reported having osteointegration and osteoinductive potential and able to induce ectopic bone formation. OCP shell can increase bioactivity of silica nanospheres [15, 16]. Moreover, release of silicon ion was maybe inhibited by OCP shell. So Silica/OCP had better effect on MG-63 cell activity and proliferation than SNs-A and SNs-B. Further study will need to be carried out to demonstrate effect of the three kinds of silica nanospheres on bone-related gene expression of osteoblast cells. And animal experiments are also needed to test biocompatibility of the material in vivo.

Table 1 Comparison of highest OD value of three kinds of nanospheres-treated cells

Nanospheres	SNs-A (OD value)	SNs-B (OD value)	Silica/OCP (OD value)
2nd day	1.42 ± 0.25	1.41 ± 0.25	1.35 ± 0.10
4th day	1.91 ± 0.38	2.18 ± 0.28	2.61 ± 0.21*,#
7th day	1.61 ± 0.48	1.54 ± 0.40	1.55 ± 0.35

* Means $p < 0.05$ compare to SNs-A

Means $p < 0.05$ compare to SNs-B

Table 2 Comparison of highest ALP activity (comparable to control) of three kinds of nanospheres-treated cells

Nanospheres	SNs-A (OD value)	SNs-B (OD value)	Silica/OCP (OD value)
2nd day	2.38 ± 0.44	2.51 ± 0.42	1.93 ± 0.62
4th day	1.11 ± 0.23	1.03 ± 0.23	1.29 ± 0.17
7th day	0.73 ± 0.34	0.67 ± 0.30	0.89 ± 0.12

Conclusion

After all, both SNs-A (30 nm silica nanosphere) and SNs-B (70 nm silica nanosphere) were not cytotoxic but proliferation-stimulative to osteoblast-like cell. Toxicity induced by nano size effect was not observed. Silica/OCP had better bioactivity than SNs-A and SNs-B because of OCP shell. The three kinds of silica nano material all have the potential to be used safely and effectively in bone regeneration, especially Silica/OCP.

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