Quantitative Analysis on Cellular Uptake of Hydroxyapatite Nanoparticles in the Primary Osteoblasts in Vitro

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ABSTRACT

With the fast development of nanotechnology, the nanomaterials have raised questions concerning its potential toxic effects on human health. In this study, the effects of hydroxyapatite nanoparticles on the primary osteoblasts were investigated. As an indicator of membrane damage, lactate dehydrogenase was quantitatively assessed. The quantitative analysis on cellular uptake of hydroxyapatite nanoparticles could be detected by flow cytometer and inductively coupled plasma mass spectrometry, respectively. The results demonstrated that hydroxyapatite nanoparticles can enter cells through cell membrane and the nanoparticles taken up in the cells followed dose and time dependent manner. The methods could be used for the initial screening of the uptake potential of nanoparticles as an index of nanotoxicity.

INTRODUCTION

Nanomaterials are the materials which size range from 1 to 100 nm at least one dimensions. Compared with bulky or microscopic structured materials, nanomaterials typically exhibit novel physical and chemical properties, such as a large specific surface area and greater chemical reactivity [1, 2]. Hydroxyapatite (HA) nanoparticles have attracted much attention due to its excellent qualities such as bioactivity, adequate mechanical properties and similar composition to bone mineral [3, 4]. Therefore HA nanoparticles are gaining interest for biomedical applications in the field of drug delivery, biological imaging and other therapeutic agents [5-8]. The widely potential medical application prospects of HA nanoparticles have raised questions concerning its potential extraordinary hazards on human health. Although HA nanoparticles are currently being widely used in biomedical field, there is a serious lack of information concerning the human health implications of HA
nanoparticles [9,10]. The assessment of its potential toxicity is critical before it can be a suitable candidate for biomedical applications.

In this report, HA nanoparticles were prepared by sol-gel method. The size, morphology, structure and chemical composition of HA nanoparticles were characterized using scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and dynamic light scattering (DLS) techniques. Furthermore, the cellular uptake and potential toxicity of HA nanoparticles were evaluated using cultured primary osteoblasts (OBs) in vitro. The analytical method using flow cytometer and inductively coupled plasma mass spectrometry can accurately reflect the amounts change of HA nanoparticles taken up into cells. This method would be available for the initial screening of uptake into cells as an index of nanotoxicity.

EXPERIMENTAL

Materials and Reagents. Calcium nitrate tetrahydrate (Ca(NO$_3$)$_3$•4H$_2$O), Diammonium hydrogen phosphate (NH$_4$)$_2$HPO$_4$, Ammonia solution (NH$_3$•H$_2$O) and Anhydrous ethanol (CH$_3$COOH) were purchased from Kemiu Chemical Reagent Co. Ltd. (Tianjin, China). Kunming (KM) mice were purchased from the Animal Center of Hebei Medical University. Dulbecco’s modified Eagle’s medium (DMEM) and trypsin were purchased from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Polyethylene glycol (PEG), penicillin, streptomycin and cetlypyridium chloride were from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Organism Engineering Institute. A LDH kit was obtained from the Nanjing Jiancheng Biological Engineering Institute (Jiangsu, China). All chemicals used in this experiment were of analytical grade reagents and used without further purification.

Preparation of HA Nanoparticles. The HA nanoparticles were prepared by an optimized sol-gel method. 2.0 mmol of Ca(NO$_3$)$_3$•4H$_2$O, and certain amount of NH$_3$•H$_2$O to adjust the pH value (10.0) were dissolved in 20 mL of anhydrous ethanol which contains PEG to form solution 1. Then, 1.2 mmol of (NH$_4$)$_2$HPO$_4$ was added into 20 mL H$_2$O to form solution 2. Solution 2 was introduced into solution 1, after vigorously stirring for 2 h at 25 °C, the obtained collosol was transferred into a water bath kettle, and maintained at 60 °C for 24 h. The precipitates were separated by centrifugation, washed with deionized water and ethanol in sequence, and then dried in air at 100 °C to obtain the intermediate products. The obtained products were heat treated at 800 °C for 2 h to obtain the final samples.

Characterization of HA Nanoparticles. The morphology and size of HA nanoparticles were measured by field emission scanning electron microscope (JSM-7500F, JEOL, Japan). A minute drop of nanoparticles solution was cast on to a carbon-coated copper grid and subsequently drying in air before transferring it to the microscope. X-ray powder diffraction was performed on a Bruker D8 Advance X-ray diffractometer employing Cu-Kα radiation with 40 kV and 50 mA (D8 ADVACE, Bruker, Germany). The size distribution of the nanoparticles in medium was evaluated by dynamic light scattering (Delsa Nano C, Beckman, USA). Data were analyzed based on six replicated tests.

Isolation and Culture of Primary OBs. The primary OBs were prepared mechanically from three-days-old KM mouse calvarias following the sequential
enzymatic digestion method previously described [11]. Briefly, the skulls were
sliced, then the endostem and peristem were stripped off and the bone was cut
into approximately 1–2 mm² pieces and sequentially digested with trypsin (2.5
mg/ml) for 30 min and collagenase II (1.0 mg/ml) twice for 1 h each time. The cells
were collected and cultured in DMEM with 10% heat-inactivated fetal bovine serum,
benzylpenicillin (50 U/ml) and streptomycin (50 µg/ml) for 24 h in a humidified
atmosphere of 5% CO₂ in air at 37°C (Sanyo, Model MCO-18AIC, Japan).

**Cell Viability Assay.** The viability of OBs was measured according to MTT
method. In brief, OBs were seeded in 96-well culture plates at a density of 2 ×
10⁴/well and incubated for 24 h. After incubation, HA nanoparticles were added
to the wells at concentrations of 5, 10, 20, and 40 µg/ml and incubation continued for 24
and 48 h. Nanoparticles were sonicated and vortexed before being added to the cells.
Cells without nanoparticles treatment were used as control group. 10 μl of MTT
solution was added to each well and the plates incubated for 4 h. The supernatant was
removed and DMSO (100 μl) was added to solubilize the MTT. The absorbance at
570 nm of each well was measured with a microplate spectrophotometer (BioRad
Model 3550, USA). The cell viability was calculated according to the formula:
\[
\text{OD}_{\text{sample}} / \text{OD}_{\text{control}} \times 100.
\]

**LDH Measurement.** Lactate dehydrogenase (LDH) activity in the cell medium
was determined using a commercial LDH Kit. One hundred microliters of cell
medium was used for LDH analysis. Absorption was measured using a microplate
spectrophotometer (BioRad Model 3550, USA) at 340 nm. Released LDH catalyzed
the oxidation of lactate to pyruvate with simultaneous reduction of NAD⁺ to NADH.
The rate of NAD⁺ reduction was measured as an increase in absorbance at 340 nm.
The rate of NAD⁺ reduction was directly proportional to LDH activity in the cell
medium.

**Flow Cytometry Assay.** Cells were treated with HA nanoparticles at several
concentrations (5, 10, 20, and 40 µg/ml) for 24 h and 48 h. Subsequently, the cells
were washed three times with PBS, digested by trypsin, centrifuged and resuspended
in PBS. The amounts of particles taken up by the cells were analyzed using a flow
cytometer (FCM) (FACSCalibur, BD, USA). In FCM, the laser beam (488 nm)
illuminates cells in the sample stream which go through the sensing area. The laser
light scattered at about a 90° angle to the axis of the laser beam is called side scatter
(SSC) light. The intensities of SSC are proportional to the intracellular density.

**Calcium Content Analysis.** Cells treated with several doses of HA nanoparticles
were trypsinized, digested and analyzed for calcium content. Briefly, the cells were
digested in nitric acid overnight and heated at about 160 °C the next day. At the same
time, H₂O₂ solution was used to drive off the vapor of nitrogen oxides until the
solution was colorless and clear. At last, the remaining solutions were fixed to 3 ml
with 2% nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS,
Thermo Elemental X7, Thermo Electron Co.) was used to analyze the calcium
concentration in each sample. Indium of 20 ng/ml was chosen as an internal standard
element.

**Statistical Analysis.** Data were expressed as mean ± standard deviation (S.D)
from three independent experiments. Statistical evaluation was analyzed by a
one-way ANOVA, followed by Tukey post-hoc analysis was used for multiple group
comparisons. $P$ values less than 0.05 were regarded as indicating statistical differences.

RESULTS AND DISCUSSION

**Nanoparticle Characterization.** The SEM results showed that HA nanoparticles were sphere-like with approximate diameters of 60 nm (Fig. 1). The crystal phase and structure of HA nanoparticles were examined by powder XRD method. All the diffraction peaks could be indexed to hexagonal crystal phase of hydroxyapatite which belonged to space group P63/m (JCPDS No. 01-074-0565). No other impurities can be detected (Fig. 2). It was also revealed that HA nanoparticles exhibited sharp diffraction peaks, indicating a high crystallinity. The SEM images provided information on the size and shape of nanoparticles, however, it could not provide information on whether the nanoparticles existed in single or aggregated forms in the culture medium. The size distribution in the culture medium, therefore, was investigated using a DLS method, which showed that the average size of HA nanoparticles in the culture medium were 66.3 ± 18.9 nm (Fig. 3). The DLS analysis showed that the HA nanoparticles were homogeneously dispersed in culture medium.

![Figure 1. SEM image of HA nanoparticles.](image1.png)

![Figure 2. XRD patterns of HA nanoparticles.](image2.png)

![Figure 3. Size distribution of HA nanoparticles in culture medium measured by DLS.](image3.png)

**Effects of HA Nanoparticles on the Cell Viability.** As shown in Fig 4, after the OBs exposure to HA nanoparticles at 10, 20, and 40 $\mu$g/ml for 48 h, cell viability was increased to 112.7%, 133.3%, and 148.3%, respectively, compared to the control. The promotion effects of HA nanoparticles at 24 h were lower than that of HA nanoparticles at 48 h. Cell viability was increased to 106.7%, 117.4%, and 119.2%, respectively, after exposure to HA nanoparticles at the same dose for 24 h. The promotion effects of HA nanoparticles followed dose and time-dependent manner.
**LDH Release after Exposure to HA nanoparticles.** The cell membrane damage was reflected in the elevated LDH levels in the cell medium. After cells were exposed to HA nanoparticles for 48 h, the LDH levels in the cell culture were increased in all groups. As shown in Fig 5, the LDH levels were increased by 21.0%, 36.4%, 61.5%, and 84.1% after exposure to HA nanoparticles at 5, 10, 20 and 40 μg/ml, respectively, compared with the control.

**Flow Cytometry Analysis of HA Nanoparticles Uptake.** The uptake of HA nanoparticles was measured by quantitative analysis of the intracellular side scatter signal by flow cytometry. As shown in Fig 6, after cells were treated by nanoparticles, the scatter intensity increased markedly compared with untreated group. The intensities of SSC reflect inner cell density and higher concentrations of HA nanoparticles resulted in higher intensities of SSC. That is, the cells which took up higher doses of nanoparticles showed higher intensities of SSC. This result suggested that the determination of SSC is a good way to judge the uptake potential of HA nanoparticles. Using this experimental approach, a time and dose-dependent increase in cellular uptake of HA nanoparticles was detected at doses from 5 to 40 μg/ml.

**ICP-MS Analysis of the Contents of Calcium.** ICP-MS analyses were employed to further verify the uptake of HA nanoparticles in OBs at different time.
points and doses. The contents of calcium in cells exposure to HA nanoparticles were shown in Fig. 7. Calcium could not be detected in controls. However, a dose- and time-dependent accumulation of HA nanoparticles could be measured in OBs at 24 and 48 h (Fig. 7b). After cells were exposed to HA nanoparticles at 5, 10, 20 and 40 μg/ml for 24 h, the calcium content was 11.9 ± 0.4, 15.7 ± 0.8, 2.8 ± 1.1 and 30.6 ± 1.2 ng/mm², respectively. When the exposure time extended to 48 h, the calcium content was increased to 12.8 ± 0.2, 21.1 ± 0.7, 33.0 ± 1.1 and 35.0 ± 1.2 ng/mm² at the same dose.

CONCLUSION

In summary, sphere-like HA nanoparticles were synthesized successfully using optimized sol-gel method. The results showed that HA nanoparticles can promote the proliferation of primary osteoblasts. HA nanoparticles can enter cells follow dose and time-response effect. At present, accurate, sensitive and cost-effective measurement techniques for characterizing them do not exist. Usage of nanomaterials will increase with the development of nanotechnology, and assessments of their risks to the environment and human health also will be required. Academia, industry, and regulatory governmental agencies should seriously consider the view that nanomaterials have new and unique biologic properties and the potential risks are not the same as those of bulk materials of the same chemistry. The simple method introduced in this study was useful for the initial screening of the uptake potential of insoluble nanomaterials in biological tissues and cells.

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