Applied Surface Science 255 (2008) 357–360

Contents lists available at ScienceDirect

Applied Surface Science

journal homepage: www.elsevier.com/locate/apsusc

Preparation of nano-hydroxyapatite particles with different morphology and their response to highly malignant melanoma cells *in vitro*

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ARTICLE INFO

Article history:
Available online 26 June 2008

PACS:
81.07.Wx
81.10.Dn
81.16.Be

Keywords:
Hydroxyapatite
Morphology
Nano
Malignant melanoma cells

ABSTRACT

To investigate the effects of nano-hydroxyapatite (HA) particles with different morphology on highly malignant melanoma cells, three kinds of HA particles with different morphology were synthesized and co-cultured with highly malignant melanoma cells using phosphate-buffered saline (PBS) as control. A precipitation method with or without citric acid addition as surfactant was used to produce rod-like hydroxyapatite (HA) particles with nano- and micron size, respectively, and a novel oil-in-water emulsion method was employed to prepare ellipse-like nano-HA particles. Particle morphology and size distribution of the as prepared HA powders were characterized by transmission electron microscope (TEM) and dynamic light scattering technique. The nano- and micron HA particles with different morphology were co-cultured with highly malignant melanoma cells. Immunofluorescence analysis and MTT assay were employed to evaluate morphological change of nucleolus and proliferation of tumour cells, respectively. To compare the effects of HA particles on cell response, the PBS without HA particles was used as control. The experiment results indicated that particle nanoscale effect rather than particle morphology of HA was more effective for the inhibition on highly malignant melanoma cells proliferation.

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1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA), due to its excellent biocompatibility and bioactivity, is widely used in the clinic of orthopedic, dental, and maxillofacial applications [1–3]. However, previous studies demonstrated that HA ceramics could produce debris or particles which would deposit between the prosthetic interface and surrounding tissue [4,5]. Accumulated particles would act as a stimulus to irritate cells such as monocytes or macrophages to release inflammatory mediators, cytokines and matrix metalloproteinases for a long time [6,7], which would induce cytotoxicity, pathologic bone resorption and so on [8]. Different characteristics of HA particles, such as morphology, size and crystallinity, would cause apparently different consequences [9].

On the other hand, it was reported that nano- or micron HA particles had suppressive effect on the proliferation of tumor cells [10,11]. Malignant tumor cells, especially those with high malignancy, were characterized by their capability of rapid proliferation, local invasion and distance migration. Since it was almost impossible to excise malignant tumor completely, the influence of the degraded particles of HA with different morphology, size and ions substitution on tumor cells, especially highly malignant ones, was significant in clinic [9]. But to our best knowledge, few studies focused on the biological properties of nano-particles with different morphology, especially the response to highly malignant melanoma cells *in vitro*. In this study, a precipitation method with or without citric acid addition as surfactant was used to produce rod-like HA particles with nano- and micron size, respectively, and a novel oil-in-water emulsion method was employed to prepare ellipse-like nano-HA particles. The nano- and micron HA particles with different morphology were co-cultured with highly malignant melanoma cells. To compare the effects of HA particles on cell response, the PBS without HA particles was used as control. Immunofluorescence analysis and MTT assay were employed to evaluate morphological change of nucleus and proliferation of tumour cells, respectively.

2. Materials and methods

2.1. Particles preparation and characterization

The method of preparing rod-like HA particles under 100 nm was similar to the previous report [12], followed by freeze-drying method to get more uniform morphology. On the other hand, the

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0169-4332/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsusc.2008.06.114

previous experiment without citric addition was performed to produce micron size HA particles. A novel emulsion method was used to produce ellipse-like HA particles (which will be discussed in detail somewhere else, and we have submitted it to Materials Letters). To get the precise information of particle size and morphology, the dried powders were investigated by transmission electron microscope (TEM, JEM-100cx, JEOL, Japan). The powders were dispersed in deionized water and then a few droplets were put on copper grids coated with carbon film and observed under TEM. The particle size distribution was further characterized by dynamic light scattering technique (Malvern Co., UK). Generally, the as prepared HA powders were ultrasonically dispersed in deionized water to form a dilute sol, and then the sol was dropped into the sample cell to determine the particle size distribution. Each specimen was tested three times and the average value would be used. Prior to cell experiment, samples were dispersed in PBS to form sol.

2.2. Cell culture

Malignant melanoma cells (A875, received it as a gift from Dr. Z. Hao, Department of Pathology, Sichuan University, China) were cultured in RPMI1640 medium (Gibco, USA) supplemented with 100,000 units/l penicillin G, 100 mg/l streptomycin, and 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Immunofluorescence analysis

Malignant melanoma cells were placed into 6-well plates (2×10^5/well) and incubated with the HA particles. Nuclear morphology was evaluated by immunofluorescence analysis after 48 h in culture. The samples were fixed in 4% paraformaldehyde at 4 °C and washed with PBS for 5 min and 3 times. Then the incubated cells were counterstained with DAP (4′,6-diamidino-2-phenylindole, dihydrochloride, molecular probes, USA) for 5 min at 37 °C, and controls were obtained by omitting the nuclear counterstain. After washing with PBS for 5 min and 3 times, samples were evaluated by fluorescence microscope (TE2000-U, Japan).

2.4. Cell proliferation

Malignant melanoma cells proliferation rate was evaluated by MTT assay. Cells, seeded in 96-well plate, were cultured with three kinds of HA particles described above. PBS was used as control and four parallel samples were used. After day 1, 2, 3, and 4, 20 μl MTT solution (5 mg/ml) was added to each well, and plates were placed in incubator at 37 °C for 4 h. Then, 150 μl dimethyl sulfoxide (DMSO, Sigma) was added after supernatant medium was removed. The absorbency value (O.D. value) was recorded at a wavelength of 570 nm.

2.5. Statistical analysis

Significant differences between the groups were identified by an analysis of *t*-test with SPSS10.0 and a level of $P < 0.05$ was considered significant.

3. Results

3.1. HA particles analysis

The different morphology of nano-HA particles is shown in Fig. 1. Fig. 1(a) exhibits the morphology of the as prepared HA powders with citric acid addition as surfactant. The TEM micrograph confirms that the HA particles are rod-like with the width and length of 10–20 nm and 50–70 nm, respectively. Fig. 1(b) shows that ellipse-like HA particles with the width of 20–40 nm and the length of 50–60 nm were produced with emulsion route. The particle size distribution of the three samples was further analyzed by dynamic light scattering technique. From Fig. 2, it is examined that the particle size of rod-like and ellipse-like HA particles is uniform. The particle size distribution of rod-like and ellipse-like HA is about 20–120 nm, and is concentrated at 65 nm approximately. In terms of the micron HA particles produced by co-precipitation method without citric acid, particle size analysis shows that size distribution is from 500 to 1100 nm, and is concentrated at about 1000 nm. It could be seen that the results of particles size distribution were in accord with TEM micrographs mostly since the particle size distribution measured with this method is the size of agglomerate.

3.2. Immunofluorescence analysis

The effects of HA particles on malignant melanoma cell nucleus were investigated by immunofluorescence analysis. In blank control and micron sized groups, the cell nucleus are smooth and the chromatin is uniform, as can be seen from Fig. 3(c and d). When nano-sized HA particles were used, the morphology of malignant melanoma nucleus changed greatly compared with the blank control. The membrane of nucleus contracted and some cell nucleus broke into debris, as could be seen from Fig. 3(a and b).

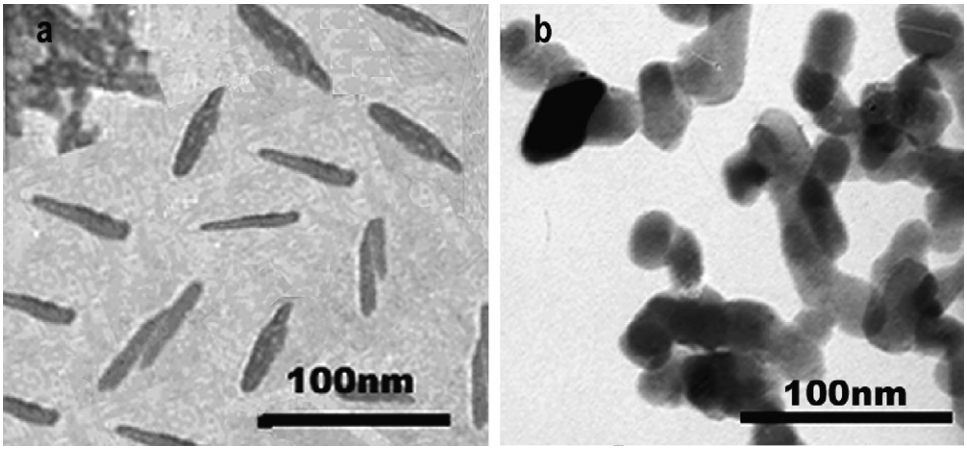

Fig. 1. TEM micrographs of rod-like (a) and ellipse-like (b) HA particles.

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