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Preparation and characterization of nano hydroxyapatite sol①

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Abstract: Nano hydroxyapatite has special biological effects when it interacts with cells. The method of preparation of nano hydroxyapatite crystals in water and the stability of hydroxyapatite sol are reported. Nanometer sized hydroxyapatite crystals were synthesized by precipitation with monocalcium phosphate and calcium hydroxide. The size of the crystals is 30－50 nm as determined by laser light scattering and transmission electron microscopy (TEM). The shape of the crystals particles is either sphere or rod-shaped. Beijing Synchrotron Radiation Facility (BSRF) micro-probe X-ray fluorescence analysis and TEM analysis reveal that hydroxyapatite crystals can pass human liver cancer cell membrane in the form of particles.

Key words: hydroxyapatite; nano crystals; disperse characteristics; stability
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1 INTRODUCTION

Among inorganic biomaterials, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) is a very important material and it is widely used as substitute or filling material for sclerous tissues because of its good biocompatibility and osteoconductivity[1,2]. It is also used in drug delivery system and protein separation[3-5].

As a new technology, nanotechnology has advanced rapidly since 1990s. The dimension of nanostructure unit ranges from 1 nm to 100 nm, which matches some characteristic length such as de Broglie wavelength, superconduct coherent length and ferromagnetism critical dimension. Because of this, physical and chemical properties of nano materials are different from atoms, microcosmic molecules and macroscopic block substitute[6].

Recently, nano technology has gained extensive attention. It is applied to the biomedical fields such as nano biomaterials[7,8], DNA nano technology[9,10], nano particles target medicine[11], gene therapy[12], analytical and evaluation testing technologies[13]. Nano HAP was also used as materials in hard tissue substitute or rebuilding and as drug deliver carrier. In 1996, Deb et al[14] successfully prepared HAP nano composite materials which simulated sub-structure of bone. In 1993, Hideki[15] used HAP as carrier for drugs, doxorubicin hydrochloride, mitomycin C and fluoroyracil. In this paper, we report our study in exploitation of the bioeffects of nano HAP in controlling the proliferation of cancer cells. In vitro experiments showed that nano HAP had obvious inhibition effects on liver cancer cells but no effects on normal cells. The preparation of nano HAP sol, the size of particles, the morphology of the crystals and the stability of the sol were also discussed in the paper.

2 MATERIALS AND METHODS

2.1 Preparation of monocalcium phosphate

Ten grams of calcium hydroxide powder were added with stirring into 250 mL of thrice distilled water to obtain $Ca(OH)_2$ suspension. Mixture of 80 mL of 85% phosphoric acid with 20 mL of thrice distilled water was slowly dripped into $Ca(OH)_2$ suspension with stirring until the solution became transparent. The solutions was dried under 120－140 ℃ for 7－14 h and then slowly cooled. White crystals were precipitated and dried from the viscous solution at 85－95 ℃. The crystals were washed by anhydrous ethanol and acetone several times. Samples of the powder were analyzed by X-ray diffraction (XRD, D/MAX-YB, RIGAKU, Japan, scanning speed: 10 (°)/min).

2.2 Preparation of calcium hydroxide saturated solution

The $Ca(OH)_2$ powder was placed in thrice distilled water and kept at about 0 ℃ for more than 12 h. Then the $Ca(OH)_2$ suspension was filtered quickly and vacuum filtered again when its temperature was raised to room temperature. The transparent filtrate obtained was kept in hermetical for further use. The concentration of Ca^{2+} of the filtrate was titrated accurately in order to prepare stoichiometric HAP.

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2.3 Preparation of HAP sol

The $Ca(H_2PO_4)_2 \cdot H_2O$ was dissolved in distilled water and the concentration of Ca^{2+} in the solution was accurately calibrated. The solution was added while stirring into $Ca(OH)_2$ saturated solution at 60 – 80 ℃ to obtain a final Ca/P mole ratio of 1.67. Ultrasonic was used to disperse the sol. A stabilizer of blood compatible polysaccharide was added to the sol system. The sol was treated 4 – 5 times with ultrasonic at 1 h interval with each treatment lasting for 5 – 10 min until the sol was stable. The sol was sterilized under 115 – 121 ℃ before its medical application.

2.4 Test of HAP property

The preparation process of HAP powder is similar to that of HAP sol except that the stabilizer was not added. The HAP was lyophilized to obtain HAP powder. The physical property of the HAP powder was then analyzed by XRD.

Human liver cancer cells Bel-7402 and normal human liver cells L-02 were treated with HAP sol for 24 h and tested for a number of surviving cells with the Methyl Thiazolyl Tetrazolium (MTT) method[16]. Then the cell growth curve and inhibition rate were obtained. The sample for BSRF microprobe X-ray fluorescence (XRF) analysis was prepared as follows: The cells cultured in RPMI-1640 media (Gibco, USA) were fixed with glutaraldehyde and spread as a thin layer on Malyer film. The sample was then dried in vacuum at room temperature.

3 RESUITS

3.1 Analysis of precursor

The XRD pattern of the synthesized monocalcium phosphate is shown in Fig. 1. The pattern coincides with that of $Ca(H_2PO_4)_2 \cdot H_2O$ shown on JCPDS card 9 – 347. The d value is similar to that shown on the card($\Delta d \leqslant 0.001$ nm). The relative intensities also match those shown on the card. The $Ca(H_2PO_4)_2 \cdot H_2O$ crystals were plate-like with high crystallinity. No impurity was found.

3.2 Qualitative analysis of HAP

The product prepared by homogeneous precipitation method was analyzed through XRD. The diffraction pattern is shown in Fig. 2. The pattern verified that the specimen was HAP. However, the crystallinity is low, which may lead to low stability of the obtained material. On the other hand, bioactivity of the material is high, which is favorable to its nano bioeffects.

3.3 Dimension measurement of HAP particles

Fig. 3 shows the particle size of HAP measured by dynamic light scattering method (Zeta PALs, Brookhaven Instruments Corp, USA, wave length: 635 nm). The result reveals that the effective diameter of the particles is about 70 nm. The dimension of the nano crystals normally distributes between 40 nm and 110 nm. The dead size of the particles measured by TEM analysis varies from 30 nm to 50 nm(Fig. 4). The particles are rod-shaped and they are homodispersed in the sol.

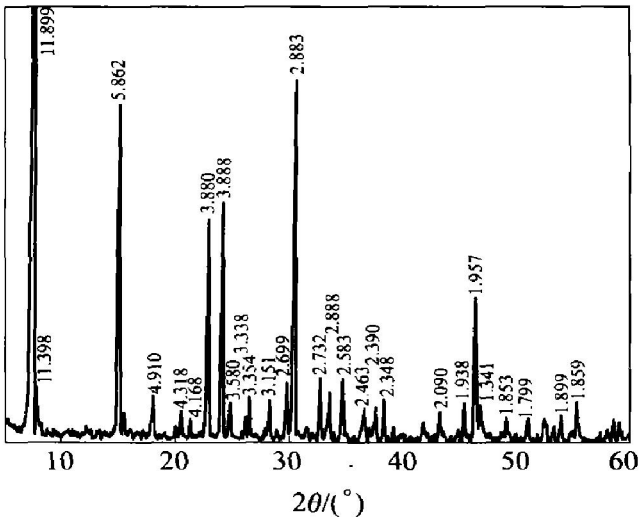


Fig. 1 XRD pattern of MCPXRD pattern of MCP

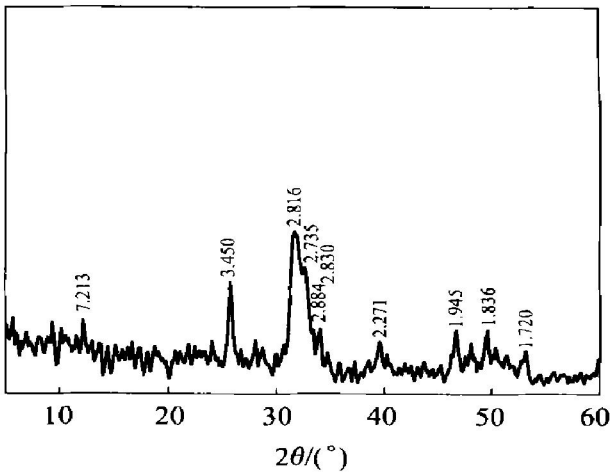


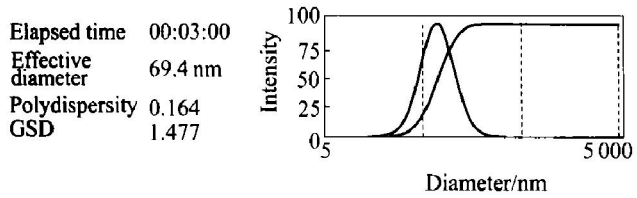
Fig. 2 XRD pattern of nano HAP

3.4 Stability of HAP sol

Zeta potential reflects the stability of the sol. The Zeta potential of the HAP sol is about – 32.5 mV, which is in the stable zone of suspend system (Fig. 5). In fact, the sol was prepared 3 years ago. No coacervation or demixing occurred. The particle size and Zeta potential are almost the same as those as determined 3 years ago. The FESEM analysis reveals that the morphology of the particles is slightly changed.

3.5 HAP sol's effect on cell growth

The BSRF's XRF analysis(Table 1) verified that the concentration of element calcium in cancer cells



d/nm	G(d)	C(d)	d/nm	G(d)	C(d)	d/nm	G(d)	C(d)
36.5	26	5	62.9	97	40	90.3	80	75
42.1	44	10	66.1	99	45	96.4	70	80
46.3	58	15	69.4	100	50	104.0	58	85
50.0	70	20	72.9	99	55	114.4	44	90
53.4	80	25	76.6	97	60	131.8	26	95
56.6	87	30	81.5	92	66			
59.7	93	35	85.1	87	70			

Fig. 3 Size distribution of HAP particles

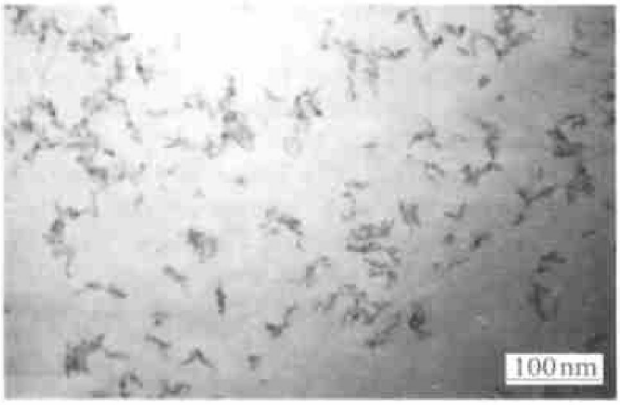
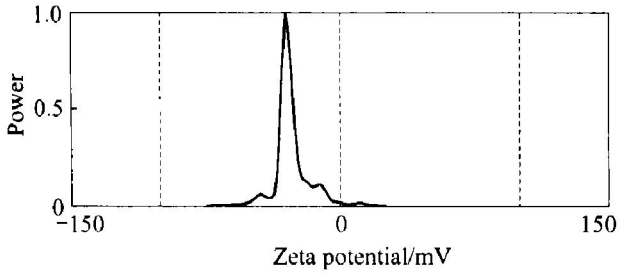


Fig. 4 TEM photo of HAP



Run	Zeta potential/mV	Half width/mV	Sample quality
1	−29.94	3.77	33%
2	−32.16	5.32	33%
3	−36.35	4.68	33%
Mean	−32.48	4.59	33%
Standard error	8.59	0.45	

Fig. 5 Zeta potential of HAP sol

group was much higher than that of the control group. Further TEM detection confirmed the HAP penetrated into the cells in the form of particles (Fig. 6). HAP nanoparticles could inhibit DNA synthesis of the cancer cells. It had ability to stop the cell cycle at the G_1 phase, preventing the cells from entering the S phase. The HAP particles also have more or less effects on morphology, structure, cell cycle and the expression of c-myc gene of the Bel-7402 cells[17]. These result in

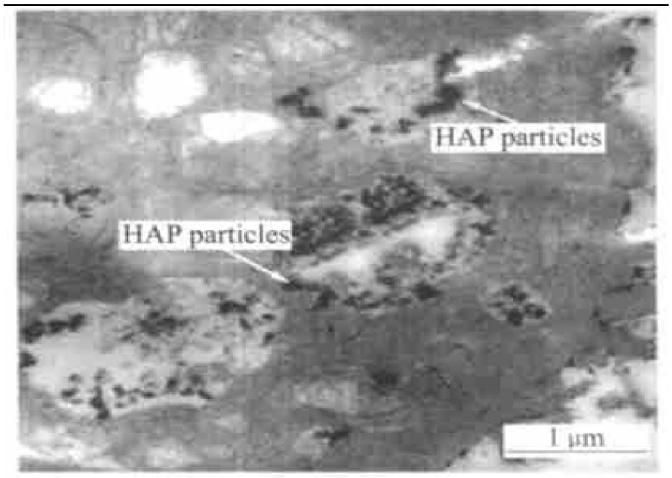


Fig. 6 TEM image of cell line Bel-7402 treated for 3 d with nano HAP

Table 1 Fluorescence intensity excited by element Ca (Ca counts/mA)

Time/d	Group 1	Group 2	Group 3
1	4.1	40.0	23.2
2	4.0	354.3	43.7
3	4.1	333.5	36.3

cancer cell death.

4 DISCUSSION

Hydroxyapatite sized 30 nm to 50 nm could be obtained while $Ca(H_2PO_4)_2 \cdot H_2O$ and $Ca(OH)_2$ were used as initial reagents. The crystals were rod-like with low crytallinity. The specific surface ranged from 150 m^2/g to 90 m^2/g. In vitro experiments revealed that the HAP nano crystals penetrated the liver cancer cells in the form of particles, changing the inner environment of cancer cell, which leads to cancer cell death. The high specific surface area of HAP nanoparticles and possibly their charge and composition, provided the surface with high bioactivity for attachment of the microvillus of the cancer cells. But their channel and mechanism of interaction are still unknown and need to be further studied.

The HAP sol is very dilute due to the low concentration of $Ca(OH)_2$ saturated solution. Therefore, the sol needs to be condensed before it is used as cancer killer. Both lyophilization and condensation in vacuum at room temperature are effective methods for condensation of HAP sol.

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