

Research Article

Effect of Nano-HAP on Proliferation of Hepatocellular Carcinoma Cells

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Abstract Recently, the treatments of hepatocellular carcinoma with nano-hydroxyapatite (nano-HAP) particles have been given much attention. In this study, a new kind of nano-HAP particles was prepared by a homogenous method, and these particle diameters and their distribution, crystallization degree, chemical bondings, and morphology were characterized by laser scattering particle analyzer, X-ray diffraction, FTIR spectrum, transmission electron microscope, and electron diffraction. The results showed that the average particle diameter of nano-HAP was 59.9 nm, the prepared nano-HAP was amorphous and having a low crystallization degree. Furthermore, the characteristic absorption peaks of HAP appeared in FTIR spectra and the electron diffraction annuli was also clear. The prepared nano-HAP particles were used for the treatment of hepatocellular carcinoma. The inhibition effect was determined in vitro for three different concentrations by MTT methods. The results demonstrated that the prepared nano-HAP with a size of 59.9 nm most effectively inhibited the proliferation of hepatocellular carcinoma cells and the inhibition rate was more than 70%.

Keywords nano-hydroxyapatite; hepatocellular carcinoma cells; proliferation inhibition; MTT; inhibition rate

1 Introduction

In recent years, there is a growing interest in nanoparticles (1 ~ 100 nm) due to their special physical and chemical characteristics. Nano-technology is one of the backbones that help develop new medical treatment techniques, and much research work focused on the aspects of the prevention and treatment of tumors. In fact, nano-HAP has already gained some significant attentions recently because of an increased interest in the treatment of hepatocellular carcinoma [2,3,4,5]. The purpose of this study is to develop a new kind of nano-HAP and exam the inhibition rate of that kind of nano-HAP to the proliferation of hepatocellular carcinoma cells.

2 Materials and methods

2.1 Materials

Ca(OH)₂ (AR), RPMI-1640 medium (Gibco, USA), MTT (Fluka, USA), NBCS (Gibco, USA), trypsinase (sigma, USA), Bel-7402 human hepatocellular carcinoma cell line (CCTCC GDC 035).

2.2 Preparation of nano-hydroxyapatite

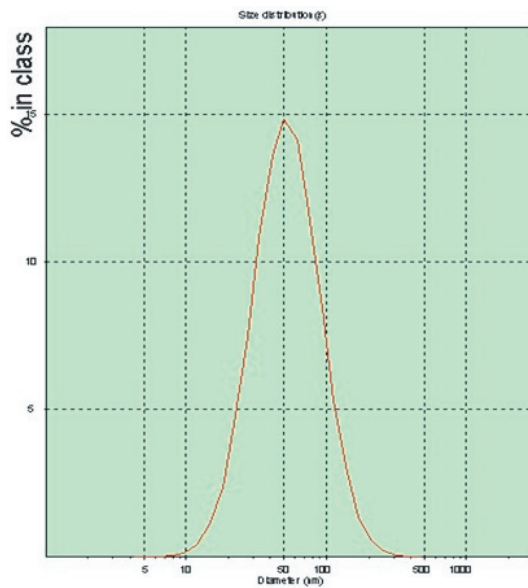
Ca(H₂PO₄)₂·H₂O solution was added into Ca(OH)₂ saturated solution to ensure a final Ca/P mole ratio slightly greater than 1.67 under magnetic stirring continuously, and a stabilizer of polysaccharide was also added to the system. Then, the sol was treated by ultrasonic at regular intervals for 15 min until the sol was stable.

2.3 Characterization of nano-hydroxyapatite

The sample was treated and determined the average particle diameter and particle diameter distribution with laser scattering particle analyzer (Zetasizer 3000HS, Malvern, England). The structural and chemical bonding analyses of sample were employed using X-ray diffraction (XRD) (D/MAX-III A, RIGAKU, Japan) and Fourier transform infrared spectroscopy (FTIR) (Nexus, Thermo Labsystems Nicolet, America) respectively, and the particle morphology was examined by transmission electron microscope (TEM) (H-600 STEM/EDX PV9100, Hitachi, Japan) and analyzed with electronic diffraction (ED).

2.4 Test of cell inhibition rate

Human hepatocellular carcinoma cells Bel-7402 were treated with different concentrations of nano-HAP and cultured for 7 days, then numbers of surviving cells were tested using the Methyl Thiazolyl Tetrazolium (MTT) method at an interval of 24 hours [1]. So the cell inhibition rate was obtained.



(a)

Size(nm)	Intensity	Volume	Number
4.3	0.0	0.3	8.4
5.3	0.0	0.8	10.9
6.5	0.0	1.7	13.9
8.0	0.0	3.3	15.4
9.8	0.1	5.8	15.1
12.0	0.4	8.8	13.0
14.7	1.1	11.8	9.8
18.0	2.4	13.8	6.5
22.1	4.5	14.3	3.8
27.1	7.5	13.0	1.9
33.3	10.8	10.3	0.9
40.8	13.6	7.3	0.3
50.1	14.8	4.5	0.1
61.4	14.1	2.4	0.0
75.3	11.7	1.2	0.0
92.4	8.4	0.5	0.0
113.3	5.3	0.2	0.0
138.9	2.9	0.1	0.0
170.4	1.4	0.0	0.0
209.0	0.6	0.0	0.0
256.3	0.2	0.0	0.0
314.4	0.1	0.0	0.0
385.6	0.0	0.0	0.0
472.9	0.0	0.0	0.0

(b)

Peak Analysis by intensity			
Peak	Area	Mean	Width
1	100.0	59.9	72.1

Peak Analysis by volume			
Peak	Area	Mean	Width
1	100.0	24.8	30.4

Peak Analysis by number			
Peak	Area	Mean	Width
1	100.0	10.6	11.5

Figure 1: Average diameter and size distribution of nano-HAP. (a) Distribution curve of intensity percent, (b) statistic data of number, volume and intensity.

3 Results and discussion

3.1 Average particle diameter and distribution

Laser scattering particle analyzer was used to determine the average particle diameter and particle diameter distribution of nano-HAP. The analysis results showed that the average particle diameter analyzed by intensity is 59.9 nm, the particle diameter is distributed between 9.8 nm and 314.4 nm and concentrated between 33.3 nm and 75.3 nm (as shown in Figure 1). The average particle diameter is 24.8 nm analyzed by volume and 10.6 nm analyzed by number, respectively. The size of particle depends

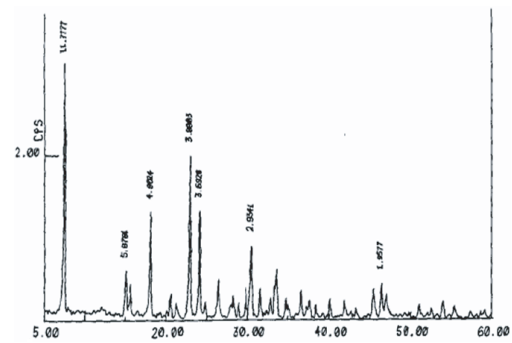


Figure 2: X-ray diffraction spectra of monocalcium phosphate.

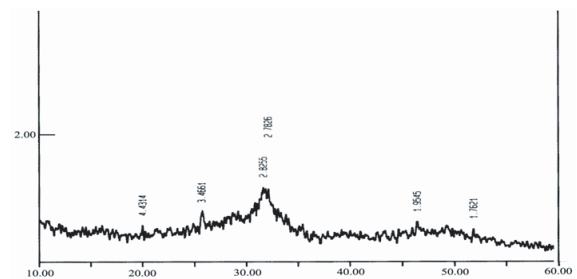


Figure 3: X-ray diffraction of nano-HAP.

on the relative velocity of nucleation and crystal growth. If nucleation rate is faster, but crystal growth velocity is slower even close to zero, the nano-HAP is obtained; otherwise, the particle diameter would become larger. On the other hand, aggregation of small particles may cause the forming of a larger size particle. Due to using ultrasonic technology during the preparation of nano-HAP, the particle were dispersed steadily. Additionally, the process of preparation is under the room temperature, which avoided agglutination under the high temperature.

3.2 XRD analysis

The XRD pattern of the synthesized monocalcium phosphate is shown in Figure 2. The crystallinity of $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ is high and no impurity was found. It can be seen from Figure 3 that the peak intensity of the prepared nano-HAP is low and the peak distribution is wide, which illustrated that the nano-HAP is amorphous and that of crystallinity is low.

3.3 FTIR analysis

Figure 4 shows the FTIR spectra with characteristic absorption peaks of HAP. The peak at 1040 cm^{-1} is stretching vibration peak of P-O. Two absorption peaks appeared at 603 cm^{-1} and 566 cm^{-1} , which correspond to

	Cell culture time (day)						
	1	2	3	4	5	6	7
H ₁₋₁	0.9 ± 0.2	6.2 ± 0.5	17.0 ± 1.6	15.5 ± 3.6***##	14.7 ± 6.3***#	12.4 ± 5.9**	8.2 ± 0.1
H ₁₋₂	4.9 ± 1.1	24.2 ± 11.7	37.9 ± 7.6	39.7 ± 4.4**	33.4 ± 3.2**	27.6 ± 5.4**	16.3 ± 1.9
H ₁₋₃	23.1 ± 7.1	36.4 ± 5.1	55.7 ± 2.5	72.5 ± 4.4	72.2 ± 4.5	68.4 ± 4.0	47.6 ± 4.9

Compared with H₁₋₃ group: ** P < .01

Compared with H₁₋₂ group: # P < .05, ## P < .01

Table 1: The inhibition rate of Bel-7402 cells treated with nano-HAP (%) ($\bar{x} \pm s$, n = 18).

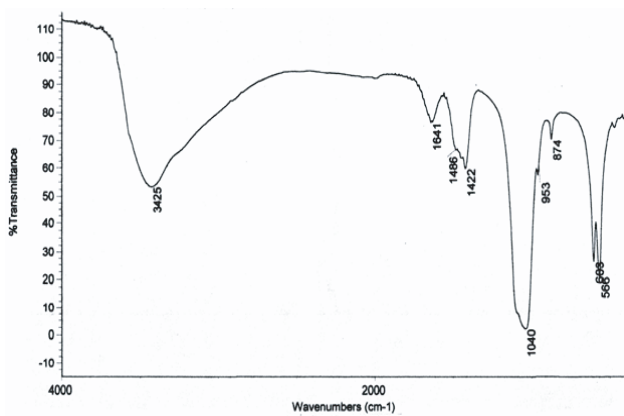


Figure 4: Infrared spectrum of nano-HAP.

the bending vibration peaks of P-O. The peaks at 3425 cm^{-1} and 1641 cm^{-1} correspond to the stretching vibration and bending vibration peak of H-O, respectively. The peak at 1422 cm^{-1} is stretching vibration peak of C=O, and the peak at 874 cm^{-1} is bending vibration peak of C=O.

3.4 TEM and ED analysis

The morphology of nano-HAP was analyzed by TEM, and chemical components were analyzed by ED. The TEM pattern and electronic diffraction of nano-HAP are shown in Figure 5. From Figure 5(a), the particles of nano-HAP are in the form of rods and are uniformly distributed with a size of $10\text{ nm} \times 30\text{ nm}$. From Figure 5(b), the electron diffraction annuli was coincide with that of HAP, but more wider, which showed that the crystallinity is low.

3.5 Cell inhibition rate

The MTT tests were performed after different concentration of nano-HAP (group 1–0.14 mmol/L, group 2–0.35 mmol/L, group 3–0.56 mmol/L) was added in the culture medium. Then the cell inhibition rate was obtained. The result was listed in Table 1.

Figure 6 showed that the highest inhibiting effects of nano-HAP on human hepatocellular carcinoma cells Bel-7402 appeared on the 4th day. The IR (%) of experimental groups increases with increasing concentration

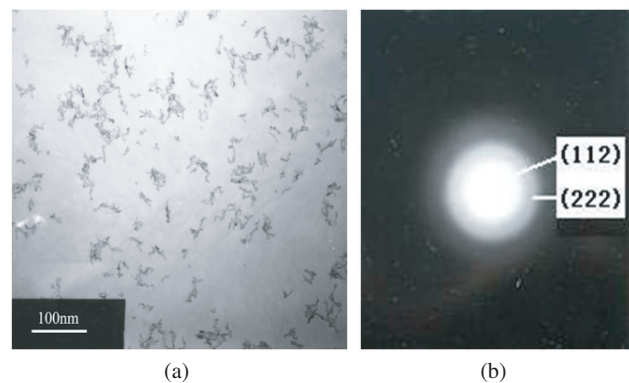


Figure 5: TEM photo and electronic diffraction of nano-HAP. (a) TEM photo, (b) ED of particles in photo (a).

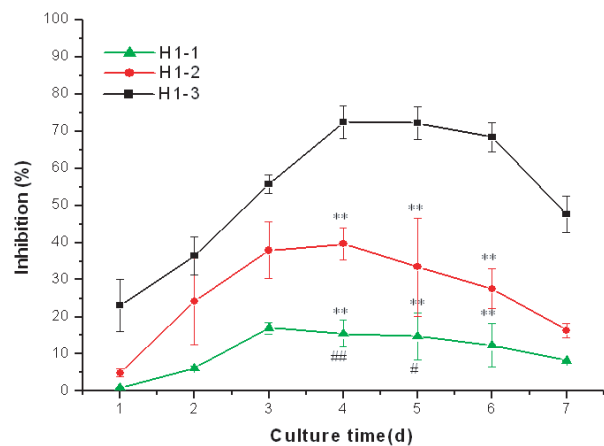


Figure 6: Inhibition of proliferation of Bel-7402 cells with nano-HAP. H₁: 59.9 nm; H₁₋₁: 0.14 mmol/L, H₁₋₂: 0.35 mmol/L, H₁₋₃: 0.56 mmol/L.

of nano-HAP. At first 24 hours, the IR of each group ($0.9 \pm 0.2\%$, $4.9 \pm 1.1\%$, $23.1 \pm 7.1\%$) had no significant difference ($P > .05$). On the 2nd day, the IRs of each group were $6.2 \pm 0.5\%$, $24.2 \pm 11.7\%$, $36.4 \pm 5.1\%$. On the 4th day, the IR of each group ($15.5 \pm 3.6\%$, $39.7 \pm 4.4\%$, $72.5 \pm 4.4\%$) had a significant difference ($P < .01$). The results demonstrated that the nano-HAP particles (group 3) have amazing anticarcinogenic properties.

4 Conclusions

The homogenous nano-HAP particles with an average particle diameter of 59.9 nm can be prepared successfully using a homogenous precipitation method. The prepared nano-HAP particles significantly inhibited the proliferation of Bel-7402 human hepatocellular carcinoma cells in vitro. Specially, optimum concentration of the nano-HAP particles is 0.56 mmol/L. At that time, the inhibition rate is more than 70%.

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