Different Inhibitory Effect and Mechanism of Hydroxyapatite Nanoparticles on Normal Cells and Cancer Cells In Vitro and In Vivo

Yingchao Han1, Shipu Li1,2, Xianying Cao1*, Lin Yuan1 †, Youfa Wang1, Yixia Yin1, Tong Qiu1, Honglian Dai1 & Xinyu Wang1

1Biomedical Materials and Engineering Center, Wuhan University of Technology, 122 Luoshi Road, Wuhan, 430070, P.R. China, 2State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, 122 Luoshi Road, Wuhan, 430070, P.R. China.

Hydroxyapatite (HAP), similar to inorganic phase in bones, shows good biocompatibility and bioactivity as bone defect repairing material. Recently, nanoscaled HAP shows the special properties differing from bulk HAP in physics, chemistry and biology. This paper demonstrates that HAP nanoparticle (nHAP) possesses the ability for inhibiting cancer cell growth in vitro and in vivo. In vitro, after treatment with nHAP for 3 days, proliferation of human cancer cells are inhibited by more than 65% and by less than 30% for human normal cells. In vivo, injection of nHAP in transplanted tumor results in significant reduction (about 50%) of tumor size. The anticancer effect of nHAP is mainly attributed to high amount by endocytosis in cancer cells and inhibition on protein synthesis in cells. The abundant nHAP internalized in cancer cells around endoplasmic reticulum may inhibit the protein synthesis by decreasing the binding of mRNA to ribosome due to its high adsorption capacity for ribosome and arrest cell cycle in G0/G1 phase. nHAP shows no ROS-involved cytotoxicity and low cytotoxicity to normal cells. These results strongly suggest that nHAP can inhibit cancer cell proliferation and have a potential application in cancer treatment.
Results and Discussion

We compare the inhibitory effects of nHAP on the cell proliferation of several normal cells with several cancer cells. Three human cancer cell lines - MGC-803, Os-732, Bel-7402, and three human normal cell lines - hepatocytes (L-02), lung fibroblast (MRC-5), keratinocyte (HaCaT) are investigated using MTT assay. Results show that nHAP can inhibit the proliferation of both human cancer cells and normal cells (Table 1). However, the inhibitions of cancer cell proliferation are much greater than those of normal cell proliferation at low and high nHAP concentration respectively. Differences in inhibition degree of cell proliferation between cancer cells and normal cells are over 30% and up to 70% depending on cell lines. In addition, there are differences in inhibition degree within different cancer cell lines and within normal cell lines. For example, the inhibitions for MGC-803, Os-732 and Bel-7402 are 54.3–65.0%, 60.6–75.8% and 76.3–88.0% respectively. These results suggest that the inhibitory effect of nHAP may be cell type related or cell-type specific.

The inhibitory effects of nHAP on cell proliferation are also dependent on treatment time. Two comparable cell lines, Bel-7402 (cancer) and L-02 (normal) are determined at different time points. These cells are both from the human liver, and their sensitivities to nHAP have been shown to be typical among cancer cells and normal cells. nHAP results in different inhibitions of cell proliferation of Bel-7402 and L-02 cells during 3 days treatment. The inhibition of cell proliferation of Bel-7402 is quickly increased from about 25% to 76% along with a little increase of proliferation inhibition from about 15% to 24% for L-02 (Figure 1). The inhibitory effect of a traditional cancer drug, sodium caranthidin (SCA), is also tested. It is observed that there is no significant difference in the inhibitory effects of SCA on L-02 cells and Bel-7402 cells, which are all quickly increased from about 40% to over 80%. At the third day, SCA only shows a little higher inhibitory effect on Bel-7402 cells (about 10%) than that of nHAP. However, SCA shows a strong toxic effect on L02 cells, which is about 2 times larger than that of nHAP. These results indicate that nHAP treatment is safer than SCA used in cancer chemotherapy.

It is also observed that the inhibitory effect of nHAP on cancer cells is dependent on the particle size and the dosage (Figure 2). Greater degree of inhibition is observed with smaller HAP particles. At the concentration of 0.56 gL⁻¹, along with the decrease of particle size from about 290 nm to about 170 nm and 60 nm, the inhibition of HAP particles on Bel-7402 is significantly increased from about 20% to about 40% and 75% respectively. Moreover, the inhibitory effect of nHAP on Bel-7402 shows strong dependent on dosage of nHAP. The inhibition is almost linearly increased from about 15% to 24% for L-02 (Figure 1). The inhibitory effects of nHAP on cell proliferation are also increased from about 40% and 75% with the rising of concentration (0.14 gL⁻¹ to 0.35 gL⁻¹, 0.56 gL⁻¹).

Table 1 | Degree of inhibition of nHAP treatment on human cancer cells and normal cells. The cancer cells (MGC-803, Os-732, and Bel-7402) and normal cells (L-02, MRC-5, and HaCaT) were treated with low (0.14 gL⁻¹) and high (0.56 gL⁻¹) concentrations of nHAP for 3 days. The data represent the average of 3–5 measurements (x ± SE).

| Cell type     | Low (0.14 gL⁻¹) | High (0.56 gL⁻¹) |
|---------------|----------------|-----------------|
| Cancer cells  |                |                 |
| MGC-803       | 54.3 ± 1.5     | 65.0 ± 2.6      |
| Os-732        | 76.3 ± 4.3     | 88.0 ± 3.5      |
| Bel-7402      | 60.6 ± 1.9     | 75.8 ± 2.5      |
| Normal cells  |                |                 |
| L-02          | 12.8 ± 1.8     | 23.8 ± 3.2      |
| MRC-5         | 14.3 ± 2.9     | 18.0 ± 2.6      |
| HaCaT         | 22.0 ± 4.0     | 29.0 ± 4.0      |

Figure 1 | Degree of inhibition of nHAP on cell proliferation. Comparative effect of inhibition of nHAP and SCA on Bel-7402 and L-02 cells. Each column represents the mean of 3-5 separate experiments; bar represents SE.

The in vivo anticancer effect of nHAP is tested by directly injecting into nude mice transplanted tumors (Figure 3). Results show the vacuolization in the cytoplasm and chromatin condensation induced by nHAP but not in the untreated control. More than 50% decrease in the tumor size is observed on the 7th day after nHAP treatment. In addition, the survival time of the infected nude mice is increased from 72.3 ± 7.3 days to 104.9 ± 6.2 days.

Generally, nanoparticles could enter into cancer cells and distribute in different organelles depending on the characteristics of the nanomaterials. In this study, there is a significant difference in the number of internalized nHAP between cancer cells and normal cells (Figure 4). In order to compare the number of internalized nHAP by cancer cells and normal cells, nHAP was doped with Eu⁺ ions to have fluorescence signal for the observation under confocal laser scanning microscopy (CLSM). A greater number of nHAP in treated cancer cells is observed than that in treated normal cells, which may explain the observed difference in the degree of inhibition by nHAP between cancer cells and normal cells. This phenomenon should be attributed to the characteristics of nHAP as well as cancer cells. As we know, HAP possesses two different binding sites on the crystal surface, which are C (Ca²⁺) site arranged on ac or bc crystal faces for binding the acidic groups of the biomolecules and P (PO₄³⁻) site arranged hexagonally on the ab crystal face for attaching to the basic groups of the biomolecules. Abundant negatively charged groups on the surface of cancer cells generate more negative charge of cancer cells than normal cells, which are mainly deriving from the sialic acid residues protruding from the apical surface of the plasma membrane. Our previous study demonstrated that nHAP showed high adsorption capacity for sialic acid than HAP microparticles and could attach to the membrane of RBCs (non-phagocytic cells) with abundant sialic acid residues. Consequently, nHAP can be expected to have higher adhering capacity to cancer cells than normal cells on the basis of the electrostatic interactions between the negatively charged sites on the cell membrane and the positive binding sites on the HAP surface. In addition, relative experiments show that nHAP enters into the cells through endocytosis. Since the nHAP prepared in this study was not loaded with cancer cell-specific drug or cytoplasm membrane-binding molecule, the difference in the numbers of nHAP entering into cells between L-02 normal cells and Bel-7402 cancer cells can be attributed to the harvest pathway of the cells. Since the endocytosis activity of cancer cells is greater than...
that of normal cells\textsuperscript{20–23}, the observed much more nHAP in Bel-7402 cells than that in L-02 cells can be expected.

The distribution of nanoparticles in the cell structure is closely related to their biological activities. In this study, nHAP is internalized into cancer cells and mostly distributed around endoplasmic reticulum (ER) (Figure 4 and 5a), the organelle responsible for protein and lipid synthesis. Also, it is observed that after nHAP treatment, the percentage of cancer cells in different phases of cell cycle changes considerably. There is a 24.2\% increase of G0/G1 cells, while 38.2\% and 12.9\% decrease of S and G2/M cells, respectively (Figure 6). During the cell proliferation, the cell cycle is divided into major events occurring during different phases: G0, G1, S, G2, M. Cell divides in the M phase, while the major substances preparation such as protein synthesis and DNA duplication mainly happens in

![Figure 2](image2.png)

**Figure 2** | Size and dosage effects of HAP particles on Bel-7402 cancer cell proliferation. The average sizes of HAP particles were 60 nm (a), 170 nm (b), 290 nm (c), respectively. All the particles were applied at the concentrations of 0.14 g L\textsuperscript{−1}, 0.35 g L\textsuperscript{−1} and 0.56 g L\textsuperscript{−1}, respectively.

![Figure 3](image3.png)

**Figure 3** | Inhibitory effect of nHAP treatment on nude mice with transplanted tumor. TEM images of (a) nHAP treated tumor tissues, (b) control. The scale bars represent 1 μm. (c) Tumor growth in volume.
G1 phase and S phase, respectively. The progress of cell cycle depending on different environmental conditions can be significantly changed by the exogenous substance treatment. Accordingly, these results indicate that the inhibition of cancer cell proliferation may be due to the decrease of protein synthesis or lack of key proteins for cell cycle progression after nHAP treatment.

In order to prove this hypothesis, the synthesis of transferrin receptor (TR) and enhanced green fluorescence protein (EGFP) by cell-free protein synthesis system were detected after nHAP treatment. A decrease in the TR amount (differing in amounts) occurs on the membranes of both the cancer cells and the normal cells. The reduction is 55.6% in the cancer cells and 23.2% in the normal cells (Figure 7a). Moreover, after nHAP treatment, EGFP produced by cell-free protein synthesis system in the cancer cell is decreased by 71.0% similar to that of the normal cells (Figure 7b). These results suggest that nHAP has similar inhibition effect on the translation process of cancer cells to that of normal cells in vitro. However, since there are different amounts of nHAP internalized into the cells, the different levels of the decrease of TR amounts between the cancer cells and normal cells can be expected. This generates the difference in the degree of inhibition between normal cells and cancer cells.

The effect of biomaterials on the local cells is determined by the amount and activity of the proteins in the interface. Ribosome is mainly composed of proteins and RNA, and can be purified by HAP with high adsorption activity. Our results show that HAP particles display different adsorption capacity for BSA, DNA and RNA (Table S1). Moreover, HAP particles with varied size show different adsorption capacity. Along with the decrease of size from 290 nm to 170 nm and 60 nm, the adsorbed DNA amount on HAP particles (per g) is much increased from 0.43 g to 1.36 g and 1.12 g; the adsorbed BSA amount is raised from 33.33 mg to 42.40 mg and 55.47 mg; however, for RNA the amount on nHAP (60 nm) is decreased compared to those of larger HAP particles.

Two main possibilities may lead to the inhibitory effect of HAP on the protein synthesis. The interaction of the ribosome with HAP inhibits the further binding of ribosome with mRNA, or mRNA bound by HAP cannot reach its proper binding site in the ribosome. Generally, HAP has poor binding activity to mRNA. It is also observed in this study that there is almost no mRNA binding with HAP (Table S1). However, the binding of ribosome with mRNA is decreased significantly after nHAP treatment (Figure 7c). Because the ribosome can be purified by HAP, these results indicate that nHAP may inhibit the protein synthesis by interacting with the ribosome and decreasing the binding of mRNA to its proper binding site in the ribosome, which further induces the inhibition of cell proliferation. Based on the higher fraction of C and P sites of nHAP due to its higher specific surface ratio than that of larger HAP particles, the greater binding activity of nHAP with ribosome can be achieved. In addition, cancer cells show much higher internalization of nHAP than that of HAP microparticles (Figure 5). Consequently, the higher inhibition of nHAP on the proliferation of cancer cells can be expected.
Recently, it was reported that some active nanomaterials, such as carbon nanotubes and metal oxides nanomaterials can release reactive oxygen species (ROS) from their surfaces, and treatment with these nanomaterials induced an increase of cellular ROS and promoted cell death\cite{29}. While such treatment can be used to kill cancer cells, it was also shown to be equally toxic to normal cells\cite{26,30}. In this study, the activities of succinate dehydrogenase and superoxidase dismutase, the key enzymes responsible for ROS generation and scavenging, respectively, are significantly decreased and remained unchanged after nHAP treatment (Table S2). Moreover, the apoptotic/live cell ratios in the control cells and the treated cells are almost same. These results suggest that the mechanism for the inhibitory effect of nHAP treatment is not explained by ROS release. Instead, it may be concluded that the inhibitory effect of nHAP treatment on cell proliferation is due to the blocking of cell cycle by decreasing the protein synthesis.

Malignant tumor is one of the most serious life-threatening human diseases. Presently, chemical and radiation therapies remain the basic treatments for cancer. However, these therapies have serious side effects, including toxicity and complications. There is therefore an urgent need for a safer cancer treatment, which will inhibit the growth of cancer cells but not of the normal cells. The development of nanotechnology provides novel techniques in cancer diagnosis and treatment. Our results in vitro and in vivo demonstrate that nHAP has much greater inhibitory effect on cancer cell proliferation than that of normal cells and extends the longevity of animals with tumors. nHAP treatment can decrease protein synthesis directly, block cell cycle, and thus inhibit cell proliferation. The difference in the ingestion activity of nHAP between normal cells and cancer cells leads to a greater inhibition of cancer cell proliferation than that of normal cells. Considering the biocompatibility and biodegradation, as anticancer agent or drug carrier, nHAP or nHAP combined with chemotherapeutic drugs is hopeful to be safe and effective alternative for cancer treatment.

**Methods**

### Preparation of nHAP

nHAP was prepared referring to Ref. 31. According to the molar ratio of Ca/P of 1.67, Ca(H_2PO_4)_2?H_2O aqueous solution was rapidly mixed with saturated Ca(OH)_2 aqueous solution with rapid stirring. Then, the stabilizer of sodium heparin was added into the above suspension with a final concentration of 0.4 g L\(^{-1}\). Next, the turbid dispersion was ultrasonically irradiated for 8 min by a high-intensity ultrasonic probe to be transparent suspension. Finally, the transparent suspension was treated by moist-heat sterilization at 121°C for 1 h. The details for preparing large HAP particles\cite{32,33} and characterization were shown in Supplementary Information.
Scherrer’s formula as follows: \( X_{hkl} \) was utilized to identify the crystalline phase. The crystallite size was calculated by X-Ray Diffraction.

Medium, 10% FBS, and different amounts of nHAP at the final concentrations of were replaced with treatment media. All treatment media contained 90% culture fluid.

Measurement of the binding of ribosome with mRNA

Total RNA purified from Bel-7402 or L-02 cells was mixed with the reaction solutions and different amounts of nHAP, and the binding of ribosome with mRNA was performed.

Protein expression assay

The cells treated with nHAP were collected and fixed with 2.5% glutaraldehyde and 1% osmic acid. Both samples were dehydrated in graded ethanol, embedded in epoxy resin, and sectioned using an ultramicrotome (LRB-V). The sections were double stained with lead citrate and uranyl acetate, and thereafter observed with transmission electron microscopy (TEM, H-600, Hitachi).

Analysis of cell cycle by flow cytometry

The cells treated with nHAP were collected and resuspended in PBS. Then they were stained with propidium iodide (PI, 50 mg L\(^{-1}\)) at 4°C for 30 min. The cell cycle distributions were measured by flow cytometry (BD FACSsort).

Transfection of pEGFP-C1 into Bel-7402 and L-02. Polyethylenimine-PEI mediated plasmid transfection was performed according to Bousif et al. The plasmid pEGFP-C1 was purified from DH5\(\text{a}\) and mixed with PI in salt solution. The cells were observed after 24 h by CLSM at Ex488/Em 510. The positive preparation was cultured for 2 days, total RNA was extracted and purified.

Protein expression assay. The measurement of TR expression in vivo by FITC labelled transferrin was performed according to Hedley et al. and the expression of EGFP was performed in vitro with the cell-free protein synthesis system (RTS 100 Wheat Germ CECF Kit, Roche). Briefly, total RNA purified from Bel-7402 or L-02 cells was mixed with the reaction solutions and different amounts of nHAP, and incubated at 24°C for 24 h. The fluorescence emission of the reaction solutions was then detected for EGFP at Ex488/Em510.

Measurement of the binding of ribosome with mRNA. mRNA was extracted and purified from rat liver. The extracted mRNA was oxidized and cross-linked with the fluorophore according to Proudnikov & Mirzabekov except with Alexa Fluor 488 hydrazide (AF488) replacing of tetramethylrhodamine hydrazide. The ribosomes containing extracts of Bel-7402 and L-02 cells were isolated from the cell lysates by collecting of the supernatants after centrifugation at 50,000 \(\times g\) for 4°C for 30 min. The AF488 mRNA was mixed with the ribosomes containing extracts and different amounts of nHAP, and incubated at 24°C for 24 h. Then the mixture was centrifuged at 150,000 \(\times g\) for 4°C for 2 h. The pellets were resuspended and the fluorescence signals were detected at Ex490/Em520.

Determination of succinate dehydrogenase and superoxide dismutase activity. The livers from health Wister rats (animal weight 160–200 g) were homogenized at 600 rpm in ice bath. The crude homogenate was centrifuged and centrifuged to sediment the mitochondrial fraction according to Kennes. Succinate dehydrogenase (SDH) activity was measured by 2.6-DFTP reduction with SDH Detection Kit (Nanjing Jiancheng Bioengineering Inst., China). And total superoxide dismutase (SOD) activity of mitochondria was determined by hydroxylamine assay with SOD Kit (Nanjing Jiancheng Bioengineering Inst., China).

Adsortion of DNA, RNA and protein on HAP. Plasmid DNA of pUC18 was purified from DH5\(\text{a}\) by Tinker mini plasmid purification Kit (Tiangen Biotech Co. Ltd., China). Total RNA was isolated from rat liver according to Chirgwin et al., 20 g of nHAP was respectively incubated with 200 g of bovine serum albumin (BSA), pUC18 DNA, and rat liver RNA at 37°C for 30 min. The mixtures were centrifuged at 12,000 \(\times g\) for 10 min and resuspended in ddH\(\text{2}\)O before the determination of the adsorption of these biomolecules with nHAP. The concentrations of BSA, DNA and RNA were measured by the absorbances at 280 nm and 260 nm.

In vivo animal experiment. The Bel-7402 human hepatocarcinoma cells were transplanted subcutaneously into the right side of the nape of every nude mouse (16–20 g) with a cell number of \(1 \times 10^6\). After the tumor grew to about 1 cm\(^3\) in volume, the nude mice were killed and the tumors were extracted. The extracted tumors were transformed into cell suspensions and injected subcutaneously into the right nape of new experimental nude mice. About 0.8 mm diameter of tumors were used for the study. The nude mice bearing cancer were divided randomly into two groups (n=13 in each group). The mice in the treated group obtained intra-tumor injection of nHAP at a dose of 0.050 g/kg body weight every day. The mice in the control group were given equal volume of 0.9% sodium chloride solution.

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