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Internalization of hydroxyapatite nanoparticles in liver cancer cells

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Abstract Hydroxyapatite (HAP) is the main inorganic component of hard tissues and shows excellent biocompatibility and osteoconductivity properties. Nanoparticles of HAP can be synthesised by the precipitation method in distilled water. The needle shaped particles are below 100 nm in size with low-crystallinity and high-surfacial activation. Recent studies showed toxic effects of HAP nanoparticles on cancer cells. Other studies focus on the application of HAP nanoparticles as drug and gene delivery system or cell marker. However, to date, the exact internalization pathway of HAP nanoparticles into cells has not been determined. When HAP nanoparticles were added to cell culture medium, the particles immediately became instable and formed agglomerates with a size of about 500–700 nm. Hence, cells seldom encounter single HAP nanoparticles in the environment of cell culture or body fluid. The TEM showed internalized HAP captured by vacuoles in the cytoplasm of the hepatocellular carcinoma cells. The invaginations in the cell membrane before nanoparticle uptake suggested endocytic pathways as internalization mechanism. This study revealed that agglomerated HAP nanoparticles were internalized by cells through the energy-dependent process of clathrin-mediated endocytosis. Depletion of intracellular potassium arrested the formation of coated pit, which inhibited the uptake of HAP.

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Introduction

Hydroxyapatite (HAP) is a widely used biomaterial for tissue engineering with excellent biocompatibility and osteoconductivity properties [1, 2]. Sintered porous HAP is used as scaffold material in bone repair [3, 4]. Orthopedic metals and implants are coated with hydroxyapatite to improve the biocompatibility [5, 6]. HAP particles are applied in bone cement as filler particles in the organic matrix [7]. Nanoparticles of HAP are low crystalline with highly active surfaces and used as carrier in drug and gene delivery systems as well as for protein separation [8–10]. Moreover, recent studies showed toxic effects of HAP nanoparticles on liver cancer cells [11]. In all these new applications of HAP nanoparticles, the knowledge of the exact internalization pathway into the cells represents the first necessary step towards the detailed investigation and optimization of the functional mechanism. The main groups of pathways into the cell are diffusion, passive and active transport, as well as a number of endocytic mechanisms. Bigger particles of far above 10 nm are internalized by eukaryotic cells through the endocytic pathways including phagocytosis, macropinocytosis, clathrin-mediated endocytosis and non-clathrin-mediated endocytosis such as internalization via caveolae [12]. Each endocytic mechanism is used by cells to accomplish different tasks. While phagocytosis is mainly practiced by specialized cells to simply internalize strange particles or bacteria, pinocytosis is the process of the uptake of soluble material (cell

drinking). The caveolae that facilitate non-clathrin-mediated endocytosis are invaginated, flask-shaped plasma membrane domains, which contain the integral membrane protein caveolin [13]. As caveolae internalized substances are not undergoing lysosomal digestion, the caveolae-mediated pathway may provide a safe gateway for viruses and bacteria into the cell [14–16]. Macromolecules that bind to specific receptors on the cell surface, which are concentrated in specialized regions of the plasma membrane, called clathrin-coated pits, are internalized via clathrin-mediated endocytosis. After entering the cytoplasm, the endocytotic vesicle loses its clathrin coat and quickly fuses with other such vesicles (endosomes). The endosome fuses or converts into an early endosome with reduced pH value of 5.9–6.0 and thereby releases the ligands from the receptors. The receptor containing parts of the endosomal membrane bud off and are sent as vesicles back to plasma membrane for recycling. The ligands are then either send in large vesicles to late endosomes or the early endosome converts into a late one along with a further pH drop to 5.0–6.0 due to lysosomal enzymes filled vesicles from the golgi apparatus. The late endosome finally fuse together with lysosomes (pH 5.0–5.5) to digest the enclosed substances and released them into the cytoplasm [17]. Potassium depletion or hypertonicity removes clathrin-coated pits from the plasma membrane in a reversible manner without affecting caveolae-mediated vesicular transport [18, 19]. The successful application of potassium free buffer for the exclusively inhibition of clathrin-mediated endocytosis has been described in many studies [13, 20–22]. Rejman et al. [13] identified clathrin-mediated endocytosis as internalization pathway of latex microspheres with sizes between 50 nm and 200 nm into murine melanoma cell. In contrast to the organic and spheric latex particles, HAP nanoparticles are inorganic and needle shaped. Recent studies intensively documented the internalization of HAP nanoparticles into hepatoma cells via TEM and suggested phagocytosis as possible entrance pathway [23]. In this paper, the TEM analysis of the uptake of HAP nanoparticles in liver cancer cells and the verification of clathrin-mediated endocytosis by its inhibition via potassium depletion is presented.

Materials and methods

Preparation of HAP nanoparticles

The HAP nanoparticles were synthesized through the precipitation method in distilled water. Solution A was prepared by dissolving 0.226 g monocalcium phosphate $Ca(H_2PO_4)_2$ in 120 mL distilled water. Solution B consisted of a 100 mL calcium hydroxide $Ca(OH)_2$ in saturated solution at 4 °C. The calcium phosphate precipitation was obtained by mixing solution A and B while stirring at room temperature. Ultrasound was applied for several minutes until the HAP sol became stable. The stabilizer, a blood-compatible anionic polysaccharide, was added to the sol system. [24]. Before adding to the cell culture, the nanoparticle sol was steam sterilized for 20 min at 120 °C in an autoclave. The HAP crystals were verified by X-ray diffraction (XRD) using Rigaku X-ray diffractometer. The nanoparticle size distribution was measured by Malvern Instruments Zetasizer 3000 HS.

TEM of nanoparticles

One drop of the nanoparticle sol of each sample was given on a hydrophobic plate. Two grids were placed on each drop for 5 min. After drying in air, the samples were observed under TEM.

Preparation of cell culture

Hepatocellular carcinoma cell lines were supplied by the China Center for Type Culture Collection (CCTCC) at Wuhan University. The cells were cultured in RPMI-1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), and kept at 37 °C in the presence of a humidified atmosphere of air (95%) and CO_2 (5%).

Intracellular potassium depletion

The potassium-free buffer (PFB) containing 140 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 1 mM, $MgCl_2$, 1 mg/mL D-glucose was calibrated to pH 7.4. The potassium-containing buffer (PCB) as control consisted of PFB with additional 10 mM KCl. To accomplish the intracellular depletion of potassium, the hepatocellular carcinoma cells were rinsed twice with PFB or PCB and washed again 2 times for 10 min. The HAP nanoparticle sol with a concentration of 1.4 mg/mL was added to the cells in PFB or PCB at a ratio of 2:3. After 1 h incubation of the cells with the nanoparticles, the samples were analysed via TEM.

Results

The prepared HAP nanoparticles in aqueous solution showed an average particle size of below 100 nm. Immediately after adding the particle sol to the cell culture, the measured particle size increased to above 500 nm in average with strong deviation among the samples (Fig. 1).

The TEM observation of the HAP nanoparticles revealed that the particles in the environment of cell culture

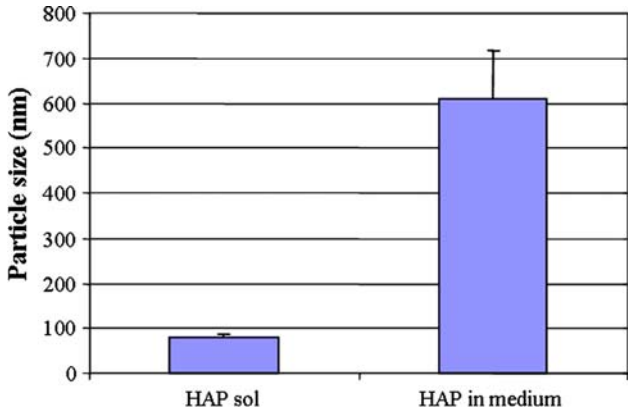

Fig. 1 The average size of HAP nanoparticles in different media measured by laser scattering

medium attached to each other, formed agglomerations, and became instable (Fig. 2a, c). While the HAP nanoparticles in aqueous sol revealed a comparatively uniform particle size, the size distribution of the agglomerated nanoparticles in the cell culture medium was found to be significantly wide (Fig. 2b, d).

After one hour of incubation with HAP nanoparticles, most cells that were treated and cultured in potassium-containing buffer exhibited internalized HAP nanoparticles enclosed in intracellular vacuoles (Fig. 3). These vesicles showed a diameter of 100–800 nm with most particles attached to the vesicle membrane (Fig. 3b). Some of the nanoparticles outside of the cells were attaching to the cell membrane and the characteristic concave shape of a clathrin coated pits bud [17] could be identified (Fig. 3a).

In contrast, the potassium-depleted cells that were treated and incubated in potassium-free buffer did not show any internalized nanoparticles (Fig. 4). Although some particles attached to the cell membrane, no invaginations could be found among all PFB treated cells during the TEM observation. This result clearly reveals that the intracellular potassium depletion prohibited the internalization of HAP nanoparticles via clathrin-mediated endocytosis.

Discussion

In aqueous solution, the stabilizer was successfully separating the single nanoparticles from each other. In the presents of cell culture medium, the organic molecules and macromolecules such as proteins quickly formed an adhesion layer on the highly reactive surface of the low crystalline HAP nanoparticles (Fig. 2c). This organic coating diminished the effect of the stabilizer and resulted in the formation of comparatively big agglomerations of attached nanoparticles. Since the particle size can influence the pathway of internalization [13, 25], the increase of the

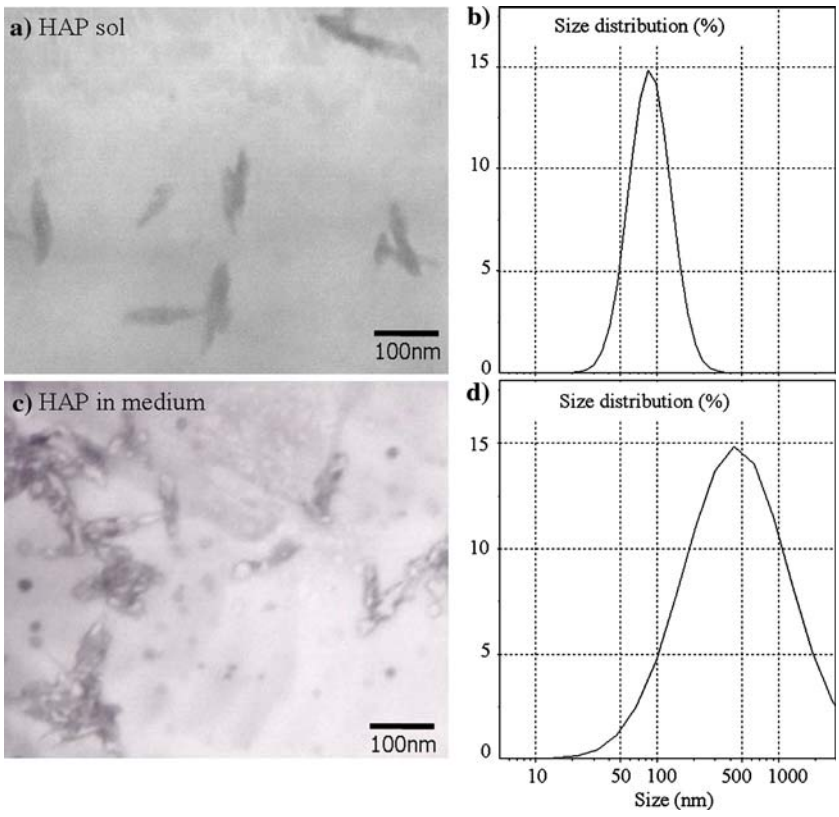

Fig. 2 TEM of HAP nanoparticles in (**a**) aqueous sol and (**c**) cell culture medium with the correlated particle size distribution (**b**, **d**)

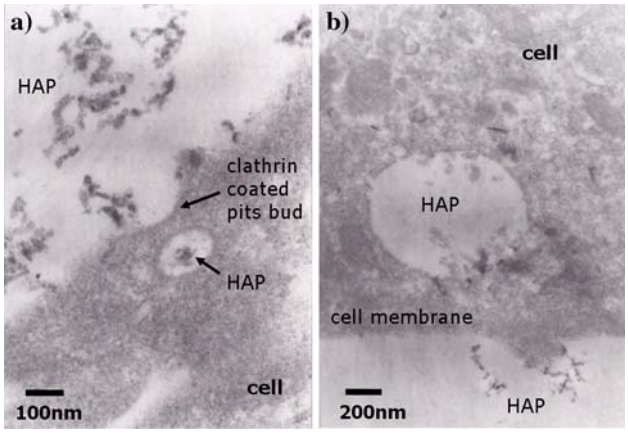

Fig. 3 Cancer cells treated with potassium containing control buffer (PCB) internalized HAP nanoparticles

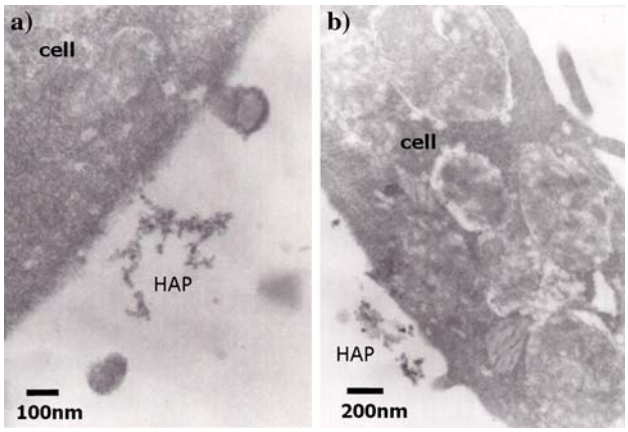

Fig. 4 Cancer cells treated with potassium-free buffer (PFB) did not internalize HAP nanoparticles

particle size due to the nanoparticle agglomeration in the presents of organic medium has to be taken into account for the discussion of the internalization mechanism. As earlier mentioned, Rejman et al. [13] found that the uptake of latex microspheres with a diameter smaller than 200 nm involved clathrin-coated pits while caveolae-mediated internalization became the predominant pathway of entry for particles of 500 nm in size. Although the agglomerations of HAP nanoparticles were in average bigger than 500 nm, only particle agglomerations of below 500 nm were observed inside the internalized vesicles and most of them were even smaller than 200 nm. Furthermore, the structure and shape of these agglomerations were quite different from single, solid, and spheric latex particles (Fig. 2c). Therefore, the found clathrin endocytosis pathway does not contradict to Rejman's results. Since the clathrin endocytosis pathway has been verified to be responsible for the uptake of HAP nanoparticles in the cancer cells, the range of possible reasons for the reported inhibition effect on the proliferation activity can be reduced. Internalized HAP particles are captured in endosomes and transported to the lysosomes for digesting by the means of degradative enzymes at pH 5.0–5.5. Due to the higher metabolism of cancer compared to normal cells, cancer cells internalized HAP particles at a higher rate with less repulsion. Hence, the origin of the toxic effects can be derived to basically two possible mechanisms. First, the number of accumulating, big sized, and with HAP particles filled endosomes themselves were damaging the cell structure and thereby causing the cell degeneration. Second, the HAP nanoparticles were partially dissolved during lysosomal digesting and the obtained solutes such as Ca^{2+}, PO_4^{3-} or OH^- ions diffused into the cytoplasm and reached toxic concentrations. Looking at the use of HAP nanoparticles as drug and gene delivery system, the endosomal membrane does not permit the attached drugs or genes to enter the cytoplasm unless these substrates are digested. Neukamm et al. investigated the transfection of mammalian cells by endocytosis and concluded that either a transport blockade before the fusion with early endosomes or the presence of adjuvant for transfection such as chloroquine were required for successful transfection [26, 27]. Similar studies discussed the hampered DNA release of gene delivery systems based on endocytic internalized cationic lipid nanoparticles. Only due to the destabilization of the endosomal membrane, a few macromolecules were able to enter the cytoplasm while most remained aggregated in the vesicular compartments [28–31]. Hence, the successful use of HAP nanoparticles as drug and gene carrier requires the use of adjuvants for the destabilization of the endosomal membrane.

Conclusions

In the presence of cell culture medium, the needle shaped hydroxyapatite (HAP) nanoparticles of below 100 nm average size were destabilized by the medium ingredients. This leaded to the formation of particle agglomerations with an average size of above 500 nm in a wide distribution. The internalization mechanism of these HAP particle agglomerations into hepatocellular carcinoma cells was investigated by TEM and the blockage of the clathrin endocytosis pathway through intracellular potassium depletion. The clathrin-mediated endocytosis was found to be responsible for the uptake of HAP nanoparticles. Since the clathrin endocytic pathway keeps the particles captured in the endosomes for lysosomal digestion, the reported toxic effect of HAP nanoparticles could only be caused by the cell structure damages due to accumulating HAP filled endosomes or the toxic effect of lysosomal degraded HAP solutes in the cytoplasm. For the application of HAP

nanoparticles as drug and gene delivery system, adjuvants that destabilize the endosomal membrane would improve the release of drugs or genes into the cytoplasm.

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