Acta Biomaterialia 9 (2013) 7527–7535

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Mechanism of the uptake of cationic and anionic calcium phosphate nanoparticles by cells

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ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form 15 February 2013
Accepted 19 February 2013
Available online 26 February 2013

Keywords:
Calcium phosphate
Nanoparticles
Endocytosis
Inhibitors

ABSTRACT

The uptake of calcium phosphate nanoparticles (diameter 120 nm) with different charge by HeLa cells was studied by flow cytometry. The amount of uptaken nanoparticles increased with increasing concentration of nanoparticles in the cell culture medium. Several inhibitors of endocytosis and macropinocytosis were applied to elucidate the uptake mechanism of nanoparticles into HeLa cells: wortmannin, LY294002, nocodazole, chlorpromazine and nystatin. Wortmannin and LY294002 strongly reduced the uptake of anionic nanoparticles, which indicates macropinocytosis as uptake mechanism. For cationic nanoparticles, the uptake was reduced to a lesser extent, indicating a different uptake mechanism. The localization of nanoparticles inside the cells was investigated by conjugating them with the pH-sensitive dye SNARF-1. The nanoparticles were localized in lysosomes after 3 h of incubation.

1. Introduction

Nanoparticles are widely used in cell biology and medicine for transfection, gene silencing, photodynamic therapy and drug delivery [1–8]. Large molecules alone often cannot penetrate the cell membrane; therefore, an efficient carrier is needed [9,10]. It was shown that nanoparticles with a size up to several hundred nanometers can enter the cells in membrane-bound vesicles by endocytosis [11]. Endocytosis consists of three major steps: formation of membrane vesicles with the cargo, endosomal delivery of the cargo inside the cell and the distribution to various organelles inside the cell [9,11–14]. It is generally distinguished between clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin- or caveolin-independent endocytosis and macropinocytosis (Fig. 1) [12,15].

Calcium phosphate nanoparticles are well known as nanoscopic mineral of bone [16–18], and are also used as carriers for the transport of genes (DNA; siRNA) [8,19,20] and drugs [21,22] into cells. Calcium phosphate is also used as bone substitution material as a paste in the form of water-dispersed nanoparticles [23,24]. Concerns have been raised about the potential problems of an increased intracellular calcium level after cellular uptake [25,26]. A cytotoxic increase of the intracellular calcium level was observed for DNA/calcium phosphate precipitates [27] which are formed with the classical calcium phosphate transfection method [28]. However, in the case of functionalized calcium phosphate nanoparticles in the colloidal state, no adverse effects were found [27], probably due to the much lower dose of calcium in this case. If calcium phosphate nanoparticles are used to deliver biomolecules into a cell, a cellular uptake is necessary. Thus, it is important to address the question of the uptake mechanism and the final fate of the nanoparticles within a cell. Here we studied the uptake of fluorescent calcium phosphate nanoparticles with a diameter of ~120 nm, both cationic and anionic, to provide a better understanding of the mechanisms and in turn to optimize their application as gene and drug delivery vehicles. Their intracellular pathway was also followed by covalent functionalization with the pH-sensitive dye SNARF-1 (carboxyseminaphthorhodafluor-1).

2. Materials and methods

2.1. Preparation of anionic triple-shell CaP/CpG/CaP/CpG nanoparticles

The synthesis was carried out as described previously [29]. Aqueous solutions of calcium nitrate (6.25 mM; Merck p.a.) and diammonium hydrogen phosphate (3.74 mM; Merck p.a.) were rapidly mixed by pumping them into a glass vessel. The pH of both solutions was adjusted beforehand to 9 with NaOH (0.1 M; Merck, p.a.). A few seconds after mixing, 1 ml of the calcium phosphate nanoparticle dispersion was taken with a syringe and mixed with 0.2 ml of a 1:3 mixture of fluorescent CpG (labelled either with Alexa 488 or with Alexa 555) and non-fluorescent CpG (total

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http://dx.doi.org/10.1016/j.actbio.2013.02.034

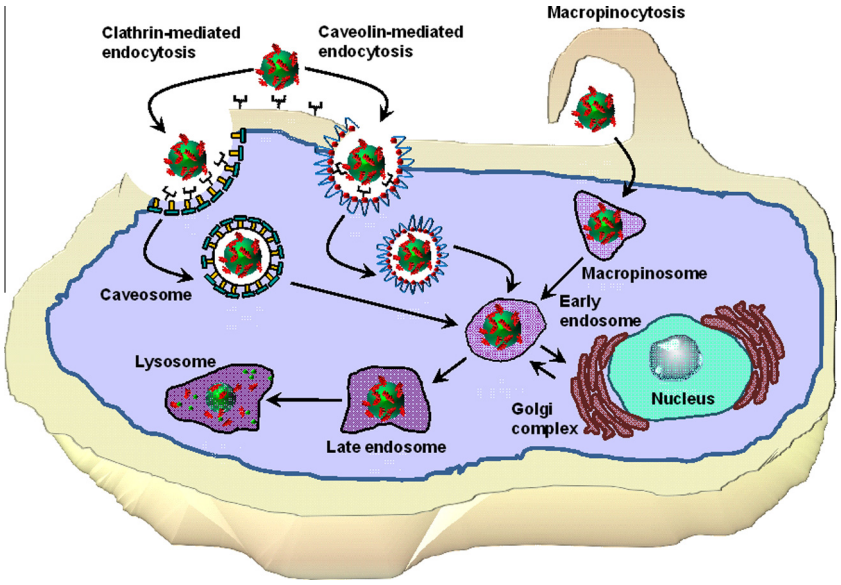

Fig. 1. Schematic representation of the different internalization mechanisms for nanoparticles, including clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis.

concentration of CpG 0.4 mg ml^{-1}). The oligonucleotides CpG, CpG-Alexa 488 and CpG-Alexa 555 were obtained from Life Technologies (Germany) and had the sequence 5′-TCCATGACGTTCCTGAC GTT-3′.

To add the second layer (calcium phosphate), 0.5 ml of an aqueous solution of $Ca(NO_3)_2$ (6.25 mM) and 0.5 ml of an aqueous solution of $(NH_4)_2HPO_4$ (3.74 mM) were consecutively added. For the third layer (CpG), 0.2 ml of a 1:3 mixture of fluorescent CpG and non-fluorescent CpG was added.

The functionalized triple-shell CaP/CpG/CaP/CpG nanoparticles were collected by ultracentrifugation for 30 min at 66,000*g*. The supernatant was taken with a syringe to quantify the amount of Ca^{2+} by atomic absorption spectroscopy (AAS). The concentration of Ca^{2+} in the supernatant was 50.4 μg ml^{-1}. Because the theoretical concentration of Ca^{2+} in the triple-shell nanoparticles was 104 μg ml^{-1}, 53.6 μg ml^{-1} of Ca^{2+} were still present in the particles and 2.4 ml × 53.6 μg ml^{-1} = 128.6 μg Ca, corresponding to 323 μg calcium phosphate (calculated as hydroxyapatite; $Ca_5(PO_4)_3OH$) were incorporated in the nanoparticles.

The particles were dispersed in the original volume of water (2.4 ml) by ultrasonication (UP50H, Hielscher, Ultrasound Technology; sonotrode 3, cycle 0.8, amplitude 60%, 10 s). The final concentration of calcium phosphate was therefore 323 μg/2.4 ml = 135 μg ml^{-1}.

2.2. Preparation of cationic CaP/PEI/SiO_2/NH-FITC and CaP/PEI/SiO_2/NH-SNARF nanoparticles

Polyethylenimine-stabilized calcium phosphate nanoparticles (CaP/PEI) were prepared as previously described [30]. Aqueous solutions of calcium lactate (18 mM, pH 10), $(NH_4)_2HPO_4$ (10.8 mM, pH 10) and PEI (2 g l^{-1}; Sigma–Aldrich; branched; 25 kDa) were simultaneously pumped in a volume ratio of 5 ml: 5 ml: 7 ml into a stirred glass vessel containing 20 ml of ultrapure water during 1 min at room temperature. After 20 min stirring, 10 ml of the CaP/PEI-nanoparticle dispersion was added to a mixture of 40 ml ethanol, 50 μl tetraethylorthosilicate (TEOS; Sigma–Aldrich) and 26 μl aqueous ammonia solution (30–33%) for coating with a thin layer of silica. This reaction mixture was stirred for 16 h at room temperature. Then the particles were isolated by ultracentrifugation and redispersed in the original volume of water (10 ml) (UP50H, Hielscher, Ultrasound Technology; sonotrode 7, cycle 0.8, amplitude 70%, 15 s). The covalent functionalization of CaP/PEI/SiO_2 nanoparticles with amino groups was carried out as follows: 50 μl (3-aminopropyl)triethoxysilane (APTES; Sigma–Aldrich) were dissolved in 40 ml ethanol. 10 ml of the CaP/PEI/SiO_2 nanoparticle dispersion was added, and the mixture was stirred for 8–10 h at room temperature. Then the particles were dispersed in 10 ml ethanol under ultrasonication as described above. 100 μl of fluorescein isothiocyanate (FITC; Merck), dissolved in ethanol (1 mg ml^{-1}), were added to the amino-functionalized nanoparticles for the covalent attachment of FITC. After stirring at room temperature for 18–20 h, the particles were purified several times by consecutive ultracentrifugation and redispersion in pure water by ultrasonication as described above to remove unreacted reagents. The final volume after redispersion was 7 ml of water. The concentration of calcium in the nanoparticle dispersion was determined to 82.2 μg ml^{-1} by AAS, corresponding to 206 μg ml^{-1} calcium phosphate (as hydroxyapatite).

For covalent attachment of SNARF-1 (carboxylic acid, acetate, succinimidyl ester; Life Technologies), we added 100 μl of SNARF-1 DMSO solution (0.5 mg ml^{-1}) to 10 ml of the dispersion of CaP/PEI/SiO_2/NH_2 nanoparticles. Then the mixture was stirred for 18–20 h at room temperature and purified by ultrasonication/redispersion as described above for FITC. We assumed the same nanoparticle concentration as with FITC-conjugated nanoparticles because of the identical synthetic route.

2.3. Characterization

Scanning electron microscopy was performed with an ESEM Quanta 400 instrument with gold/palladium-sputtered samples. Dynamic light scattering and zeta potential determinations were performed with a Zetasizer nanoseries instrument (Malvern Nano-ZS, laser: λ = 532 nm) using the Smoluchowski approximation and taking the data from the Malvern software without further correction. The particle size data refer to scattering intensity distributions (*z*-average). Ultracentrifugation was performed at 25 °C with an Optima XL-I instrument (Beckman–Coulter). The cells were analysed by flow cytometry with a LSR II instrument using the DIVA software (BD Biosciences). Confocal laser scanning microscopy was performed with a Zeiss LSM 510 Axiovert 200 instrument.

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