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Effect of Tb/Mg doping on composition and physical properties of hydroxyapatite nanoparticles for gene vector application

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Abstract: Transfection efficiency of hydroxyapatite nanoparticles (HAnps) is relative to the particle size, morphology, surface charge, surface modifier and so on. This study prepared HAnps with doped Tb/Mg by hydrothermal synthesis method (HTSM) and investigated the effects of different Tb/Mg contents on the morphology, particle size, surface charge, composition and cellular endocytosis of HAnps. The results showed that Mg-HAnps possessed better dispersion ability than Tb-HAnps. With increasing doping content of Tb/Mg-HAnps, the granularity of Tb-HAnps increased, while that of Mg-HAnps declined. Both particle size and zeta potential of Mg-HAnps were lower than those of Tb-HAnps. 7.5% Mg-doping HAnps presented relatively uniform slender rod morphology with average size of 30 nm, while 10% Mg-doping HAnps were prone to agglomeration. Moreover, Mg-HAnps-GFP (green fluorescent protein) endocytosed by MG63 cells was dotted in the perinuclear region, while Tb-HAnps were more likely to aggregate. In conclusion, as gene vectors, Mg-HAnps showed enhanced properties compared to Tb-HAnps.
Key words: hydroxyapatite nanoparticles; gene vector; endocytosis; doping; fluorescence labeling

1 Introduction

Transfection, extensively used in laboratory and promising for clinical therapeutic application, is introducing functional foreign DNA into cell nucleus to repair missing cell function and enhance or silence gene expression. Naked DNA and siRNA are negatively charged, thus the electrostatic repulsion with the anionic cell membrane reduces their transfection efficiency [1]. Rapid clearance from the body and extracellular enzymatic degradation by plasma nucleases are the major reasons that make direct gene delivery an inefficient process [2,3]. Therefore, a suitable delivery vehicle is necessary for effective transfection.

Many different vectors have been investigated for transfection including viral [4], polymeric [5,6], liposomes [7] and inorganic vectors [8]. Among the inorganic vectors, hydroxyapatites (HAs) are able to encapsulate negatively charged genetic material by chelating calcium ions while forming calcium phosphate crystals [9,10]. HAs are not prone to enzymatic degradation in the physiological environment, comparing with organic or polymeric gene delivery systems [11,12]. Regardless of the Ca/P ratio, phase and crystallinity, HAs are relatively insoluble at physiological pH of 7.4, but soluble in acidic environment, e.g. below pH 6.5 [13], such as in endocytic vesicles [14], lysosomes [15] or around solid tumors [16]. Therefore, HAs are promising for gene delivery, due to their biocompatibility, biodegradability and encapsulating ability.

However, HAs' utility in vivo is profoundly limited by the lack of tissue specificity and the uncontrollable growth in a physiological solution. Nanoparticles with 20−200 nm in diameter can be taken up by endocytosis, while phagocytosis is thought to take the predominance if their particle size is above this range [17]. Nanoparticles are prone to agglomerate, which changes their effective size "seen" by the cells. Currently, the preparation of HAnps is mainly carried out in liquid

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media including chemical co-precipitation, sol−gel and microemulsion method, etc. These methods have some shortages such as complex process, poor controllability and intermediate calcinations causing the agglomeration of HAnps. However, hydrothermal synthesis method (HTSM) can directly generate HAnps in a closed reactor, which has good controllability and can prevent particles from agglomerating in the course of high-temperature calcination [18]. Moreover, adjusting the process parameters of HTSM enable the growth of HA crystals controllable.

The gene loading efficiency of HAnps is closely related to chemistry, surface area, surface charge, crystallinity and local micro-environment [19]. In the synthesis process, the calcium element was relatively active, and could be easily replaced by other elements to generate different doped hydroxyapatite compounds [20]. The ionic radius and biological activity of terbium (Tb) and magnesium (Mg) were similar to those of calcium ion (Ca^{2+}). Tb^{3+} doping played an important role in controlling the growth of hydroxyapatite and contributed to an especially strong luminescence which was beneficial to the study of the behavior of cellular endocytosis [21]. HANIFI et al [22] showed that Mg^{2+} doping into HAs increased the surface positive charge of the HAnps and hence increased their DNA loading capacity. Although doped HAnps are widly used as in vitro transfection agents, little attention has been paid to the composition and physical properties of the hydroxyapatite precipitate and their effects on transfection.

In this study, we took advantage of HTSM to prepare HAnps and adjusted different doping amounts of Tb/Mg to control the growth of HA crystals under the same reaction temperature, reaction time and dropping rate of $(NH_4)_2HPO_4$. The processing parameters were optimized by doping different amounts of Tb/Mg, and their influence on dispersion, particles size, morphology, surface charge and biological characterization was also investigated. The effects of doped HAnps on cytotoxicity and endocytosis were assessed.

2 Experimental

2.1 Preparation of Tb/Mg-HAnps

1 mol/L $Ca(NO_3)_2·6H_2O$, 1 mol/L $Tb(NO_3)_3$, 1 mol/L $Mg(NO_3)_2$ and 1 mol/L $(NH_4)_2HPO_4$ solutions (Yacoo Chemical Reagent Co., Ltd., Suzhou, China) were prepared in deionized water respectively. HAnps were prepared by HTSM, with Ca/P molar ratio of 1.67. Tb/Mg-HAnps with different Tb/Mg-doped amounts were prepared by HTSM, with molar ratios of (Ca+Tb)/P and (Ca+Mg)/P of 1.67, and mass fractions of Tb/(Ca+Tb) and Mg/(Ca+Mg) of 0, 2.5%, 5.0%, 7.5% and 10.0%, respectively. Meanwhile, surfactant PEG−2000 (4%, mass fraction) (Yacoo Chemical Reagent Co., Ltd., Suzhou, China) was added to 1 mol/L $Ca(NO_3)_2·6H_2O$ solution. The mixed solutions of $Ca(NO_3)_2·6H_2O$ and $Tb(NO_3)_3·6H_2O$ or $Ca(NO_3)_2·6H_2O$ and $Mg(NO_3)_2·6H_2O$ were prepared, followed by drop-wise addition of 1 mol/L $(NH_4)_2HPO_4$ solution at a feeding rate of 0.12 mL/min during the stirring. Both suspensions were sonicated in ultrasonic oscillator (B200, Branson, USA) for 1 h. The two resulting suspensions were adjusted pH to 10 with ammonium solution, and then transferred to the kettles for hydrothermal synthesis at 170 °C for 3 h (Guang Ying Instrument Co., Ltd., Shanghai, China). After the reaction, the two resulting suspensions were naturally cooled to room temperature, washed with ethanol and centrifuged at 1000 r/min for 5 min. The precipitate nanoparticles were freeze-dried at −50 °C for 12 h in vacuum using a freeze-dryer (FD-1A−50, Boyikang Corp., Beijing, China). All of the chemicals used in the sample preparation were analytical grade, and used as received without further purification.

2.2 Characterization of samples

The nanoparticles were firstly characterized using XRD for phase analysis. The X-ray analysis was carried out using a X-ray powder diffractometer with Cu K_α radiation (λ=1.5418 Å) over a range of $20°<2\theta<80°$. The operational voltage and current were kept at 40 kV and 250 mA, respectively. The as-prepared particles were morphologically characterized by transmission electron microscopy (TEM). For TEM, HAnps were applied to copper grids covered with a Formvar support film. Micrographs were recorded at 80000 magnification using a JEOL JEM−3010 TEM (JEOL, Tokyo, Japan). The zeta potential measurements (n=6) were made in deionized water solutions using a ZetaSizer Nano ZS (Malvern Instruments, MA, USA) fitted with a red laser (633 nm). Size data were analyzed for significance using the Mann–Whitney test (SPSS v15.0).

2.3 Preparation of Mg-HAnps-GFP

A standard curve was drawn according to the gradient concentration and the absorbance of the standard solution of green fluorescent protein (GFP). 10 mg Mg-HAnps were added to 5 mL phosphate-buffered saline (PBS, pH 7.4), mixed with 1 mL GFP standard solutions with different concentrations and incubated for 1 h. The aqueous suspension was centrifuged at 5000 r/min for 5 min to precipitate the nanoparticles and then the supernatant was removed. The optical density (OD) value of unabsorbed GFP of the supernatant was measured using a automatic microplate spectrophotometer (ELX800, BioTek Instruments, Inc., USA) at 505 nm. The experiment was repeated in