Hydroxyapatite nanoparticles modified by branched polyethylenimine are effective non-viral vectors for siRNA transfection of hepatoma cells *in vitro*

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Abstract. Small interfering RNA (siRNA) technology is a powerful tool in biomedical research and holds great potential for RNA interference-based therapies for HIV, hepatitis and cancer. However, the absence of a safe and efficient method for the delivery of siRNA has become a bottleneck for their development. Nanocrystallized hydroxyapatite (nHAP) appears to be an optimal candidate non-viral gene vector for several reasons, including its good biocompatibility and ease of production, however, nHAP microemulsions cannot remain monodispersed for long periods of time. Due to their high surface energy, nHAP particles gradually aggregate into large ones that are difficult for the cell to take up. To overcome this we modified nHAP with polyethylenimine (PEI) to generate a compound (MnHAP) with a tight size-distribution of <200 nm. The positive surface potential of MnHAP inhibited particle aggregation and thus made it easier to conjugate more siRNA. The transfection efficiency of MnHAP/fluorescent FAM-labeled siRNA complex was tested using flow cytometry, and the transfected cells were observed using fluorescence microscopy. The cytotoxicity of MnHAP/siRNA complexes to the human liver cancer cell line BEL-7402 was assessed *in vitro* by a formazan dye assay. Our results show that the *in vitro* transfection efficiency of MnHAP/siRNA was equivalent to that of the commercially available transfection agent Lipofectamine® 2000, but with decreased cytotoxicity. The MnHAP nanoparticles were also able to deliver siRNA for silencing of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in BEL-7402 cells, which supports that MnHAP might be a promising non-viral vector for biomedical research and gene delivery.

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Introduction

In the past decade, RNA interference (RNAi), a post-transcriptional gene silencing technology, has been used widely in biomedical research (1,2). However, the progress of *in vivo* studies using small interfering RNA (siRNA) has been slow because of the lack of safe and efficient gene delivery vehicles. Viral vectors are efficient for gene transfection, although they present risks, including inflammation and immunogenicity (3). Non-viral vectors, in contrast, are less toxic and immunogenic, but their transfection efficiency and the resulting gene expression changes are lower (4). In recent years, nanomedicine carboxy incorporating the advantages of nanotechnology in medicine, has attracted great deal of research interest. Hydroxyapatite (HAP) with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$, is the main inorganic component of bones and teeth (durapatite), and has been used as a repair material for a long time in medicine (5,6). HAP nanoparticles (nHAP) have, owing to their good biocompatibility (7), found utility in many applications, such as diagnostic probes (8), cancer treatments (9), delivery vectors for drugs, genes and other agents (10-14). Unfortunately, the use of nHAP for gene delivery is still limited due to particle aggregation, which results from the high interfacial free energy (13).

Aggregation of nHAP results in poorer cellular uptake (4) and the larger size of nanoparticles may trigger immunogenic responses in the body (15). This problem also impairs the colloid stability of nHAP, such that good transfection efficiency is possible for a short time only (16). Despite numerous fabrication processes being tested (17-19), it is still difficult to produce homogeneous and monodispersed nHAP. The positive charge of nHAP can improve the viability and proliferation of transfected cells, and can also make the colloid stable (20,21).

Some scientists have modified the surface charge of nHAP with Ca^{2+}, Mg^{2+} and poly-L-lysine (PLL) (10,22), however, the results indicated that the transfection efficiency remained low. PEI has been widely used to modify non-viral vectors for gene delivery and has led to high transfection efficiencies (23-27). Inspired by these findings, we modified nHAP using branched PEI in this study to increase the strength of heighten its surface charge and therefore improve its stability and the transfection efficiency. Our compound was also evaluated *in vitro* for its transfection efficiency and cytotoxicity.

Material and methods

Preparation of PEI modified nHAP (MnHAP). HAP nanoparticles (nHAP) were purchased from Nanjing Emperor Nano Material Co. Ltd. (Nanjing, China). To overcome the disadvantages of nHAP as a gene delivery vector, we modified nHAP using branched PEI to prepare a new nanoparticle (which we named MnHAP), according to the protocol described by Ashokan *et al* (28) with some modifications. In brief, 200 mg nHAP powder was dissolved in double distilled water and stirred at 1200 rpm/min for 12 h. Then, PEI (MW 25 kDa, Sigma-Aldrich, St. Louis, MO, USA) was added to the nHAP microemulsion and stirring was continued for another 12 h, all at room temperature. The generated MnHAP microemulsion was centrifuged at 5000 rpm for 30 min after ultrasonification at 20 KHz for 5 min. The supernatant was collected for 48 h dialysis to remove the unreacted PEI. The resulting sample was lyophilized and stored at room temperature.

Characterization of MnHAP. The particle size distribution and zeta potential of MnHAP preparations were measured by Malvern Zetasizer Nano ZS90 (Malvern Instruments, Ltd., Malvern, UK). The morphology of the nanoparticles was examined using a Hitachi H-7650 transmission electron microscope at 80 kV.

Gel retardation assay. Different amounts of MnHAP (μg) were mixed with a fixed amount (0.25 μg) of negative control siRNA (sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3', Shanghai GenePharm Co., Ltd) according to the MnHAP:siRNA weight ratios (0:1, 0.2:1, 0.4:1,1:1,2:1,4:1) by gentle pipetting for 30 min at room temperature. The above six mixtures were then electrophoresed on a 3% agarose gel at 100 V for 20 min. The bands were visualized under a UV transilluminator.

Cell culture. The human liver cancer cell line BEL-7402 was obtained from the Laboratory Animal Center of Sun Yat-sen University of China. The cells were grown in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) under 5% CO_2 in air at 37˚C.

Transfection efficiency of MnHAP conjugated with FAM-labeled siRNA. For transfection efficiency analysis, BEL-7402 cells were plated in 6-well plates at a density of $5x10^5$/well and allowed to adhere for 24 h. Green fluorescent negative-control siRNA (FAM-siRNA) (2.5 μg) and 5.0 μg MnHAP, or 5 μl Lipofectamine 2000 were used per well (sense: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3', Shanghai GenePharm Co., Ltd). At 4 h after transfection with FAM-siRNA, MnHAP/FAM-siRNA complexes and Lipofectamine 2000/FAM-siRNA complexes, cells were harvested and analyzed by flow cytometry to determine the percentage of FAM-positive cells. A Nikon Eclipse TE300 fluorescent microscope (Nikon, Tokyo, Japan) was used for cell observations and recording images after washing cells 3 times with PBS to reduce background fluorescence.

Cytotoxicity evaluation. A formazan-based assay (MTT assay, Sigma-Aldrich) was employed to determine the cytotoxicity of the MnHAP/siRNA mixture (weight ratio at 2:1). BEL-7402 cells were seeded in 96-well plates at a density of 10^4/well with 100 μl culture medium. Cells were incubated for 24 h to allow complete adherence then the medium was replaced with fresh medium containing siRNA alone, MnHAP/siRNA complexes and Lipofectamine/siRNA complexes respectively; control wells received fresh medium. For each well, the amount of siRNA was 0.25 μg and the amount of MnHAP was 0.25 μg or 0.5 μl Lipofectamine. Four replicates of each condition were present and analyzed simultaneously.

To assess how treatments affected the cell number, medium in each well was aspirated after 4 h incubation and 100 μl fresh medium was added, followed by a further 24 h incubation after which time 10 μl MTT (5 mg/ml, MP Biomedicals, USA) was added to each well and the cells were again incubated in a CO_2 incubator for 4 h. The supernatant containing MTT substrate was aspirated and 100 μl DMSO added to dissolve the formazan deposits. The absorbance at 495 nm was measured on a spectrophotometer (Spectra Max M5, Molecular Devices), and the relative cell viability (%) was calculated as the percentage of absorbance of the treated cells sample relative to that of the untreated control cells.

Western blot analysis. BEL-7402 cells were harvested 48 h after siRNA transfection and lysed with lysis buffer for 30 min on ice. Total protein concentration was determined using a BCA protein assay. For western blotting, protein extracts were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk powder, the membrane was first incubated overnight at 4˚C with a mouse monoclonal antibody against GAPDH (Abcam). The blots were then washed and incubated with a goat anti-mouse IgG secondary antibody (Abcam). Bands were visualized using an ECL detection kit (Millipore) and intensities analyzed using Image J software. The relative expression of GAPDH was determined by dividing the densitometric value of the GAPDH band by that for its control (Tubulin).

Statistical analyses. All *in vitro* assays were performed at least twice independently, with 3 or 4 replicates of each data point. Student's t-test was employed for statistical analysis using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Preparation and characterization of MnHAP. To prevent nHAP aggregation and enhance transfection efficiency, nHAP was coated with branched PEI using published reaction conditions

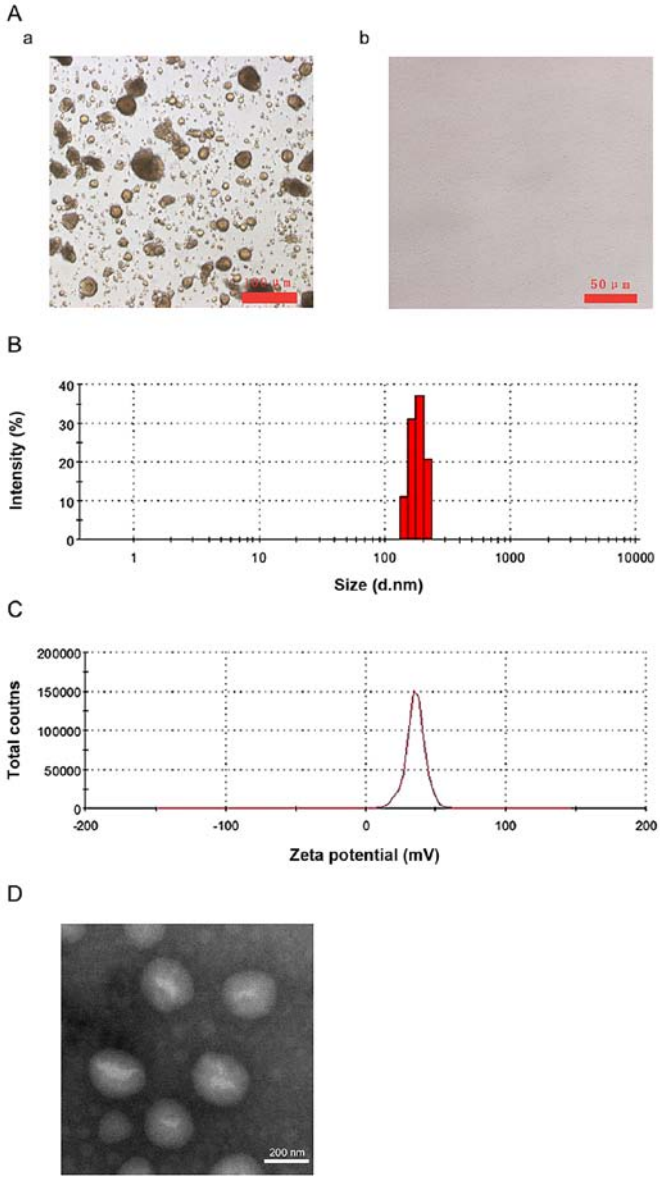

Figure 1. Characterization of MnHAP. (A) (a) Light microscopy (x200 magnification) showed that nHAP particles were of different sizes, and aggregated immediately in form of solution. (b) Light microscopy (x400 magnification) 24 h after the MnHAP microemulsion was prepared, showed that MnHAP particles remained evenly dispersed. (B) The size distribution of MnHAP particles was <200 nm, and the mean diameter was 184.5 nm. (C) Zeta potential measurement of MnHAP in double distilled water. The surface of MnHAP showed a high positive charge of +35.4 mV. (D) Transmission electron microscopy (TEM) at x1,5000 magnification demonstrated that MnHAP particles were of uniform size, spherical morphology and with smooth surfaces in accordance with the data in (A and B).

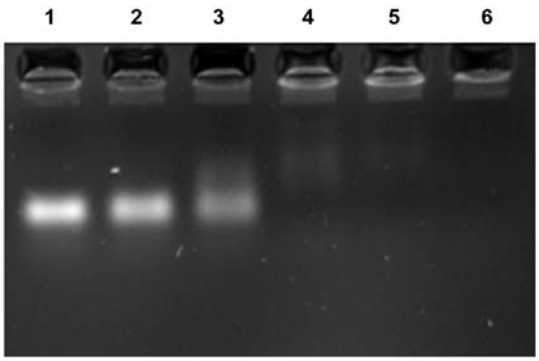

Figure 2. Gel retardation assay of MnHAP/siRNA complexes. Lane 1, free negative control siRNA (0.25 μg); lane 2, MnHAP/siRNA complex with a weight ratio of 0.2:1; lane 3, MnHAP/siRNA at 0.4:1; lane 4, MnHAP/siRNA at 1:1; lane 5, MnHAP/siRNA at 2:1; lane 6, MnHAP/siRNA at 4:1.

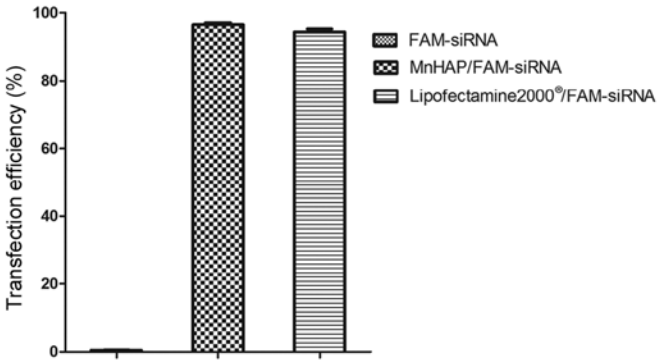

Figure 3. Comparison of gene transfection efficiency of various FAM-siRNA formulations after 4 h by flow cytometry. The amount of FAM-siRNA used in each group was 2.5 μg and the ratio of MnHAP to FAM-siRNA was fixed at 2:1.

to generate MnHAP (28). The appearance of the newly formed MnHAP microemulsion was clear/milky and appearance was stable for more than 7 days without formation of precipitates, whereas nHAP alone sank quickly from the solution and formed aggregates. As seen using light microscopy, the particle dispersion of MnHAP was better than that of nHAP (Fig. 1A) and their average size was 184.5±20.5 nm (Fig. 1B). Surface charge is an important property of nanoparticles. When it exceeds ±30 mV, particles in suspension could be kept well-dispersed and stably suspended (21). The surface charge of nHAP without modification is -10.47 mV (data not shown), which implies that it is difficult for the dispersed particles to remain stable. In the present study, the measurement of surface zeta potential of MnHAP revealed a positive surface charge of +35.4 mV, which not only enables the particles to avoid agglomeration but also facilitates binding to the negatively charged siRNA to form MnHAP/siRNA complexes (Fig. 1C). Moreover, when imaged at x1,5000 magnification using transmission electron microscopy (TEM) MnHAP particles were of uniform size and spherical with smooth surfaces (Fig. 1D).

Gel retardation assay. To assess the siRNA binding efficiency of MnHAP, a gel retardation assay was carried out at six weight ratios of MnHAP to siRNA while maintaining the amount of siRNA. Fig. 2 illustrates that with the increase of MnHAP/siRNA weight ratio, the migration of siRNA bands was significantly retarded. When the ratio of the MnHAP/siRNA was ≥2:1, no free siRNA could be detected, which indicated that the siRNA was completely confined to the sample well. Therefore, the 2:1 ratio could be the minimal ratio for siRNA being totally bound by MnHAP and this ratio was used in the subsequent experiments.

Transfection efficiency determined by FAM-labeled control siRNA. The *in vitro* transfection studies on BEL-7402 cells

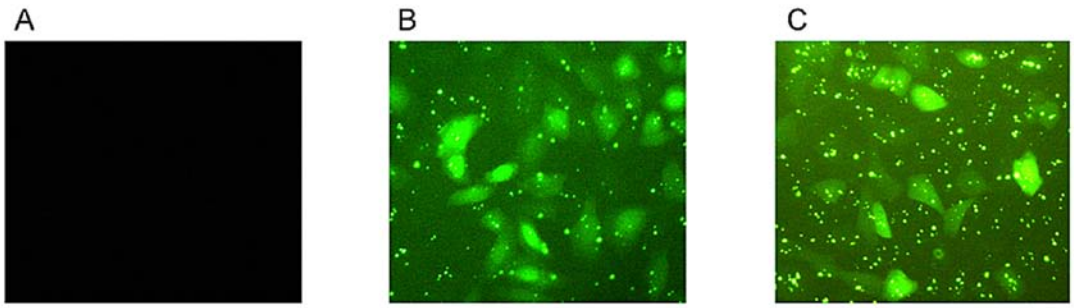

Figure 4. Observation of FAM-positive cells with fluorescence microscope at x200 magnification. The FAM-positive cells were directly visualized and the transfected cells with green granules in cell cytoplasm and intact cellular structures were counted in the same field. (A) FAM-siRNA, (B) MnHAP/FAM-siRNA, (C) Lipofectamine® 2000/FAM-siRNA.

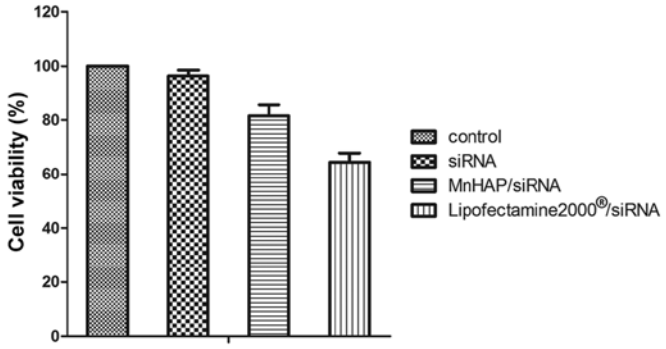

Figure 5. Cell viability analysis. BEL-7402 cells were treated with siRNA alone, MnHAP/siRNA complexes or Lipofectamine® 2000/siRNA complexes for 4 h and incubated for 24 h. The experiments were done with four replicates and the mean and standard error are shown.

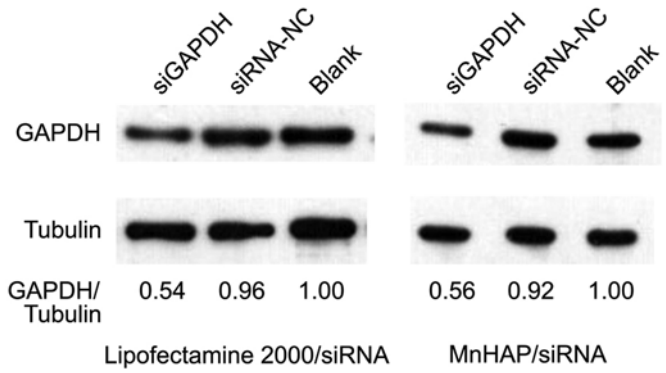

Figure 6. Western blot analysis showing the expression of GAPDH protein at 48 h after a 4 h exposure to MnHAP/siRNA-GAPDH, MnHAP/siRNA-NC, untransfected cells (Blank) or Lipofectamine 2000/siRNA-GAPDH, Lipofectamine 2000/siRNA-NC, untransfected cells (Blank), respectively. Total tubulin levels were used as loading controls.

revealed that the proportion of FAM-positive cells increased remarkably following transfection with MnHAP/siRNA and Lipofectamine 2000/siRNA complexes compared to that with siRNA alone. According to the quantitative analysis shown in Fig. 3, the transfection efficiency of the group treated with FAM-labeled siRNA was <0.50% (0.48±0.02%), whereas the efficiency was increased significantly for the MnHAP/siRNA treatment group (96.49±0.34%), which is almost the same as that of Lipofectamine 2000/siRNA (94.42±0.50%). The FAM-positive cells in each group were observed and counted using fluorescence microscopy (Fig. 4A-C). The morphological features of the positive cells were confirmed and the positive cell percentages were in agreement with those from flow cytometry analyses.

Cytotoxicity of MnHAP/siRNA complexes. MTT assays were performed on BEL-7402 cells to evaluate the cytotoxicity of MnHAP. The results showed that the cell viability following exposure to MnHAP/siRNA complex was 81.60±2.00%, whereas for the commercially available transfection agent Lipofectamine 2000/siRNA complex, the cell viability dropped to 64.40±1.69% (Fig. 5). As siRNA itself had no obvious cytotoxic effect on BEL-7402 cells, the cytotoxicity of MnHAP/siRNA and Lipofectamine 2000/siRNA complexes were quite possibly due to MnHAP and Lipofectamine 2000 themselves. Even so, MnHAP was less toxic than Lipofectamine 2000 as demonstrated in Fig. 5.

The cytoxicity of MnHAP is similar to that of nHAP according to a previous study of BEL-7402 cells in which the cytotoxic effect was attributed to inhibition of proliferation and induction of apoptosis by nHAP (29). Therefore, our modification by PEI did not change the cytotoxicity of nHAP. However, in some studies, HAP without nano-processing was viewed as a non-toxic biocompatible biomaterial (5,30). It has been reported that HAP cytotoxicity seemed to be related to its various physical characteristics including size, shape, charge and surface area as well as to the cell lines used, and that the degree of cell toxicity is positively associated with the amount of HAP taken up into cells (31). Compared to micro-size HAP, nano-sized nHAP particles are much more easily taken up by cells. The intracellular nHAP results in the rapid release of calcium, and this is the main reason for nHAP cytotoxicity (32).

Detection of GAPDH protein expression by western blot. In this study, we found that MnHAP could bind siRNA to form nano-complexes capable of knocking down GAPDH expression in BEL-7402 cells *in vitro*. Fig. 6, shows western blot analysis using tubulin as an internal control; GAPDH protein levels were lower when MnHAP delivered siRNA-GAPDH compared to the control siRNA-NC. Lipofectamine 2000 was similarly effective. The strong gene silencing activity of MnHAP loaded with siRNA-GAPDH is probably due to the high stability of complexes in the culture medium.

In conclusion, we have successfully developed MnHAP, a novel transfection vector that can carry siRNA into BEL-7402 cells efficiently. This study shows that an MnHAP emulsion is stable for a longer time compared with that of nHAP, and this ensures its successful binding to siRNA for transfection. The high transfection efficiency and decreased cytotoxicity relative to Lipofectamine 2000 support that MnHAP may be a good candidate vector for biological and medical research. To further explore its biological properties and the potential medical applications, we will conduct further studies on the modification of MnHAP with ligands and on the delivery of other functional genes to target specific organs.

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