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Hydroxylapatite nanorods: An efficient and promising carrier for gene transfection

Guo-Jun Wu [a], Lin-Zhu Zhou [a], Ke-Wei Wang [b], Feng Chen [b], Ying Sun [c], You-Rong Duan [c,*], Ying-Jie Zhu [b,*], Hong-Chen Gu [a,*]

[a] Med-X Institute, Shanghai Jiao Tong University, Shanghai 200030, PR China
[b] State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences,1295 Dingxi Road, Shanghai 200050, PR China
[c] Cancer Institute of Shanghai Jiao Tong University, Shanghai 200032, PR China

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ABSTRACT

Calcium phosphate (CaP) has been used as the vector for gene transfection in the past three decades with the characteristics of excellent biocompatibility and biodegradability. However, clinical application of calcium phosphate is still not popular due to poor-controlling of DNA/CaP complex preparation and its low transfection efficiency. In this study, block copolymer (PLGA–mPEG) assisted synthesis of hydroxylapatite (HAP) nanorods and DNA post-adsorbing method for transfection in vitro have been reported. By hydrothermal treatment, HAP nanorods with relatively uniform sizes of ~100 nm in length and ~25 nm in diameter and high crystallinity were prepared, which were characterized by TEM, XRD and FTIR measurements. In the presence of Ca^{2+} (0.2 mol/L), HAP nanorods showed ultra-high DNA loading capacity, which was significantly enhanced by one or two magnitude compared with the recently reported high loading capacity mesoporous silica vectors. HAP nanorods, therefore, have a great potential as the gene vector to deliver DNA into the cells effectively and safely.

1. Introduction

Calcium phosphate (CaP) was initially introduced as a type of non-viral gene vectors by Graham and van der Eb [1], and now it has been used for gene transfection due to its excellent biocompatibility and biodegradability. However, in most of the previous researches, CaP was precipitated with DNA to form the transfection complex [2–4]. The preparation of DNA/CaP complex by the above co-precipitation method was difficult to control and had spontaneous tendency to form CaP bulk precipitate. One reason is that the reaction must conduct strictly under physiological conditions in order to protect the structure and activity of DNA molecules. Due to the difficulty in controlling their properties at the nanoscale, the method of conventional in situ co-precipitation hence suffers from low transfection efficiency [5,6].

Recently, nanomaterials have received more attention in the fields of gene delivery and therapy for DNA manipulation. With the characteristics of huge surface area, nanomaterials exhibit much higher capacity of loading DNA than bulk materials and hence raise transfection efficiency [7,8]. CaP nanoparticles are efficient to bind DNA as complex for gene transfection in vitro. Actually, some researches have been reported on the study of the synthesis of CaP nanostructures [9,10]. However, those CaP nanoparticles reported are mostly amorphous, and it is difficult to control the shape and prevent agglomeration. Therefore, it is very necessary to synthesize shape-controllable CaP nanoparticles as the vector to elevate efficiency of gene transfection. Hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$, HAP), a kind of calcium- and phosphate-containing compound, is fundamental inorganic component of human tissue including bone and tooth and has been utilized as biomaterial in the field of bone tissue engineering and drug delivery vehicle due to its high binding affinity with a variety of molecules [11,12]. However, few researches have been reported of HAP nanoparticles as the gene vector [13]. Hence, the study on HAP nanostructures is necessary for its application in DNA loading and gene transfection.

Herein, we report a block copolymer-assisted strategy for the preparation of HAP nanorods, which shows very high DNA binding capacity and good transfection efficiency. The block copolymer poly(lactide-co-glycolide)-block–monomethoxy (polyethyleneglycol) (PLGA–mPEG) and polyvinyl pyrrolidone (PVP) are both safe materials for tissues and cells, which can affect the growth of HAP and lead to the formation of relatively uniform HAP nanorods. This method involves two steps: first, the precipitation reaction of calcium phosphate takes place in the solution of PLGA–mPEG and PVP at a comparatively low temperature (40 °C); second, the resulted suspension is treated under a hydrothermal condition to

* Corresponding authors. Fax: +86 021 62932907 (H.-C. Gu), +86 021 52413122 (Y.-J. Zhu), +86 021 64178312 (Y.-R. Duan).
E-mail addresses: yrduan@sci.shmu.edu.cn (Y.-R. Duan), y.j.zhu@mail.sic.ac.cn (Y.-J. Zhu), hcgu@sjtu.edu.cn (H.-C. Gu).

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obtain the relatively uniform HAP nanorods with a high crystallinity. This approach can effectively avoid the bulk precipitation of CaP and obtain nano-sized and well-dispersed HAP nanorods. The adsorption and elution effects of salmon sperm DNA and plasmid DNA on as-prepared HAP nanorods are explored. Meanwhile, transfection and cytotoxicity are assayed with HAP nanorods/DNA complex in COS-7 cells. The experimental results indicate that HAP nanorods can act as an excellent candidate as DNA vector with high DNA loading capacity, good transfection capability and low toxicity.

2. Experimental

2.1. Materials

DL-lactide and glycolide were recrystallized twice in ethyl acetate and dried under vacuum at room temperature before use. Monomethoxypoly (ethylene glycol) (MeO-PEG, molecular weight 2000) and stannous octoate were obtained from Sigma Co. Other chemicals were purchased from Sinopharm Chemical Reagent Co. and used as received without further purification.

All media and supplements used for cell culture were obtained from GIBCO. Salmon sperm DNA was purchased from Sigma–Aldrich. Plasmid DNA (pEGFP-C1 and pGL3-control vector) was extracted from cultured *Escherichia coli*. with plasmid extraction Kit (Invitrogen). Agarose gel (0.9%) electrophoretic analysis showed that the plasmid was mainly in supercoiled form. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco. All media and supplements used for cell culture were obtained from GIBCO. Other chemicals were purchased from Sinopharm Chemical Reagent Co. and used as received without further purification. Mill-Q water with resistance >18 MΩ/cm was used throughout the experiments.

2.2. Synthesis of PLGA–mPEG

Poly (lactide-co-glycolide)-block-monomethoxy(polyethyleneglycol) (PLGA–mPEG) was synthesized according to the previous publication with a little optimization [14]. The molecular weight of block copolymer PLGA–mPEG was Mw = 15,000, the molecular weight of the mPEG segment was 2000 and the molar ratio of DL-lactide to glycolide was 50:50.

2.3. Preparation of HAP nanorods

1.838 g of $CaCl_2$, 0.46 g of PLGA–mPEG and 0.15 g of PVP (K30) were added to 75 ml of de-ionized water, the mixture was stirred at 40 °C to form a clear solution, and the pH was adjusted to 9.5 by adding ammonia. Then 25 ml of phosphate solution containing 0.99 g of $(NH_4)_2HPO_4$ was added drop-wise to the above mixture and the pH was maintained at 9.5 by slow addition of ammonia. When the addition of phosphate solution was completed, 20 ml of the resulted milky suspension was transferred into a Teflon lined stainless steel autoclave and heated at 160 °C for 18 h. Samples before and after the hydrothermal treatment were respectively collected and washed through centrifugation and re-dispersion with distilled water and dried at 60 °C for 24 h.

2.4. Evaluation of DNA loading capacity on HAP nanorods

Salmon sperm DNA was suspended in sterile water, diluted to 0.1% (w/v) and kept at 4 °C. Plasmid DNA, extracted and purified from bacteria, was diluted to 0.177 mg/ml. HAP nanorods were suspended in water with a concentration of 2 mg/ml.

For the DNA loading on HAP nanorods, each component was added and mixed as described in Table 1. The mixture was continuously shaken for 30 min, and then was centrifuged for 5 min with 10,000 rpm. For the elution of DNA, 100 μl Na_2CO_3 was then added into the tube, re-suspending nanorods. After incubation for 10 min at 37 °C, the sample was separated from the mixture by centrifugation. The supernatant and eluted products were analyzed by UV–visible spectrophotometer to determine the UV absorption at 260, 280 and 320 nm.

2.5. Preparation of DNA/HAP nanorods complexes

Complexes of DNA and HAP nanorods were prepared by adding 200 μl of mixture, comprised of 8 μl (0.2, 0.5 or 0.8 μg/μl) of DNA, 100 μl of 40 mM $CaCl_2$ and 92 μl of normal saline, to an equal volume of HAP nanorods solution (1.6 μg in de-ionized water). Immediately, the tube was put on a vortexer and the complex in it was mixed in several seconds.

2.6. In vitro transfection and cytotoxicity assay

COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/l glucose) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere. Confluent cells were sub-cultured every 3 days in standard procedure. For transfection studies, cells were seeded at a density of 10^4cells/well in 96-well plates and incubated for 16–24 h until 60–70% confluent. Before transfection, the medium was immediately removed, and cells were washed and replaced with 50 μl of fresh and pre-warmed DMEM in the absence of FBS.

DNA/HAP nanorods complexes (50 μl, corresponding to 0.2, 0.5 or 0.8 μg plasmid per well) were added to each well and cells were incubated at 37 °C for 4 h. The medium was then replaced with fresh DMEM supplemented with 10% FBS and incubated for additional 48 h. The luciferase was assayed according to manufacturer's protocol (Promega). Relative light units (RLU), measured with Wallac 1420 multi-label counter (Perkin Elmer, USA), were normalized with respect to protein concentration in the cell extract determined using the BCA protein assay kit (Beyotime, China).

For cytotoxicity assay, the cells were washed with PBS at the end of 4 h's transfection experiment with DNA/HAP nanorods and 50 μl of 2 mg/ml MTT solution in DMEM was added to each well. Plates were incubated for an additional 2 h at 37 °C. Then, MTT-containing medium was removed and 100 μl of DMSO were added to dissolve the formazan crystals formed by living cells. Absorbance was measured at 490 nm by a Wallac 1420 multi-label counter.

2.7. Characterization

The transmission electron microscopy (TEM) micrographs were taken by a JEOL JEM 2100 field emission electron microscope. X-ray powder diffraction (XRD) patterns were recorded by a Rigaku D/max 2550 V X-ray diffractometer with Cu Kα radiation (λ = 1.54178 Å) and a graphite monochromator. The Fourier transform infrared (FTIR) spectra were taken on a Nexus FTIR spectrometer

Table 1
Binding scheme of DNA and HAP nanorods.

	Total volume 500 (μl)			
	DNA	Nanorods	$CaCl_2$	ddH_2O
Salmon sperm DNA	200	20	50 (0.2–2.0 M)	230
Plasmid DNA	226	5	50 (0.2–2.0 M)	219

(Thermo Nicolet). The thermogravimetric analysis (TGA) was taken at a heating rate of 10 °C min^{-1} in air with a STA 409/PC simultaneous thermal analyzer (Netzsch, Germany). NanoDrop 1000 spectrophotometer (Thermo Scientific) was utilized to measure the amount of DNA which was determined by absorbance at 260 nm. DNA absorbs light of certain wavelength so that light of 260 nm through DNA at 50 μg/ml concentration (in water) has an absorbance of 1.0.

3. Results and discussion

3.1. Preparation and characterization of HAP nanorods

The influence of hydrothermal treatment on the shape and size of the as-prepared HAP samples was investigated through the transmission electron microscopy (TEM). As shown in Fig. 1a and b, two kinds of HAP nanorods can be obtained. The sample prepared without hydrothermal treatment consists of nanorods with lengths of about 60 nm and diameters of about 5 nm. In contrast, the shape and size of the sample prepared via hydrothermal treatment are quite different. This type of material is composed of relatively uniform HAP nanorods with lengths of approximately 100 nm and diameters of about 25 nm. And the sizes in both diameter and length of this sample are much larger than those of the sample prepared without hydrothermal treatment (Fig. 1c and d). Such differences illustrate that nanorods with larger sizes tend to be formed under hydrothermal condition. The high-resolution TEM images are displayed in Fig. 1e and f, by careful observation, one can see a very thin amorphous layer of the polymer on the surface of the HAP nanorod. This result is in agreement with the FTIR result which will be discussed below.

The crystal phases of the samples prepared with and without hydrothermal treatment were characterized by X-ray powder diffraction (XRD). As shown in Fig. 2, two samples prepared with and without hydrothermal treatment are both hydroxyapatite (JCPDS No. 09-0432). Compared with the sample prepared at low temperature, the nanorods obtained via hydrothermal treatment exhibit higher crystallinity.

The FTIR spectrum of the sample prepared through hydrothermal treatment is presented in Fig. 3. The adsorption peaks at 3570 and 633 cm^{-1} are assigned to the characteristics of hydroxyl bond (–OH) in HAP nanorods [15]. In addition, the bands at 1090, 1040, 962, 603 and 564 cm^{-1} are attributed to PO_4^{3-} ions (ν_1 – 962 cm^{-1}, ν_3 – 1040 and 1090 cm^{-1}, ν_4 – 564 and 603 cm^{-1}) [16,17]. These results confirm the formation of HAP and are consistent with the XRD characterization. Furthermore, the weak bands at 2920 and 2854 cm^{-1} can be ascribed to the stretching vibration of C–H, while the adsorption peak at 1460 cm^{-1} is caused by the asymmetrical bending vibration of $-CH_3$. Moreover, the characteristic adsorption peak of C=O caused by its stretching vibration can also be found at 1640 cm^{-1}. These results indicate the adsorption of the block copolymer on the surface of HAP nanorods and are in agreement with the HRTEM result. The TGA result reveals that the polymer was about 3 wt.% in the HAp nanorod product, indicating that the polymer molecules were adsorbed on the surface of HAp nanorods. In addition, the BET specific surface area of HAP nanorods was 49.28 m^2/g.

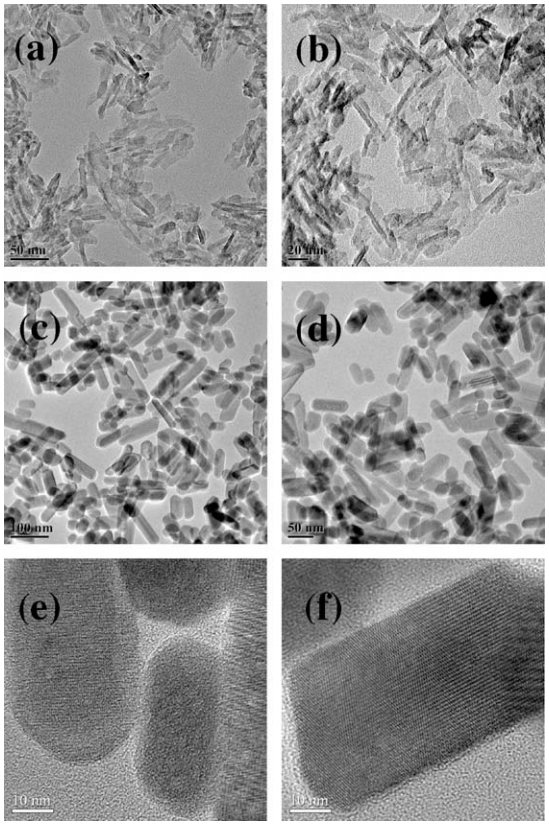

Fig. 1. (a) and (b) TEM micrographs of the sample precipitated at 40 °C and collected immediately after the phosphate solution is added; (c) and (d) TEM micrographs of the sample prepared at 160 °C for 18 h via hydrothermal treatment; (e) and (f) HRTEM images of the sample synthesized at 160 °C for 18 h via hydrothermal treatment.

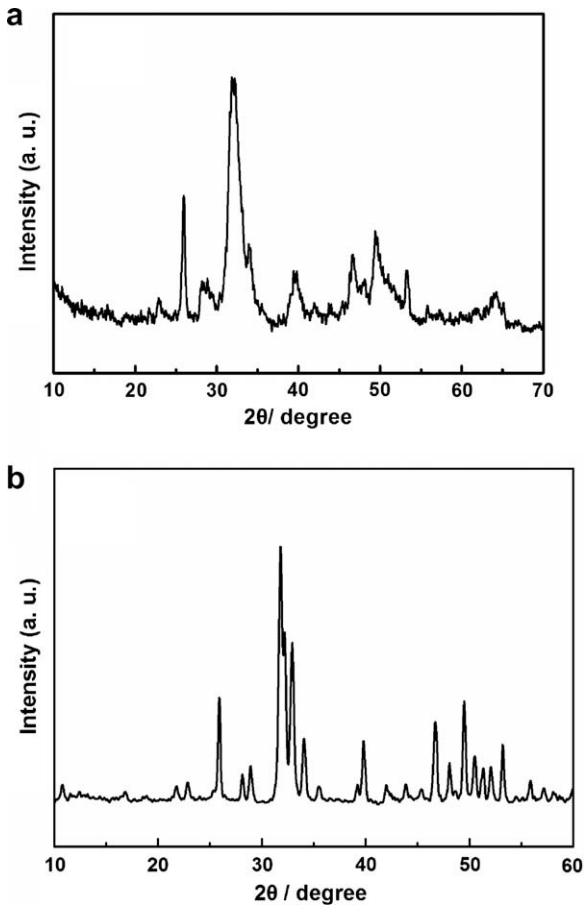

Fig. 2. XRD patterns of HAP nanorods. (a) The sample precipitated at 40 °C and collected immediately after the phosphate solution was added and (b) the sample synthesized at 160 °C for 18 h via hydrothermal treatment.

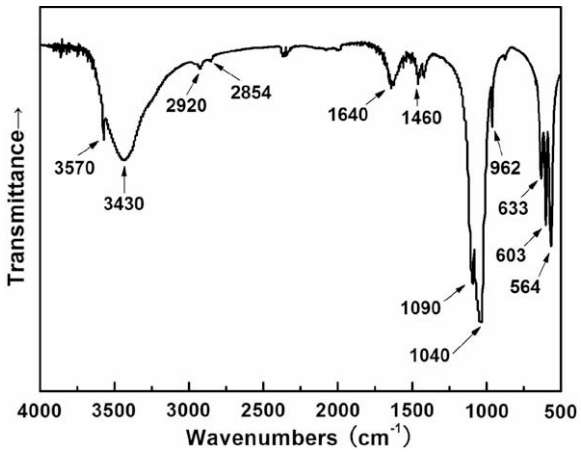

Fig. 3. FTIR spectrum of HAP nanorods prepared at 160 °C for 18 h via hydrothermal treatment.

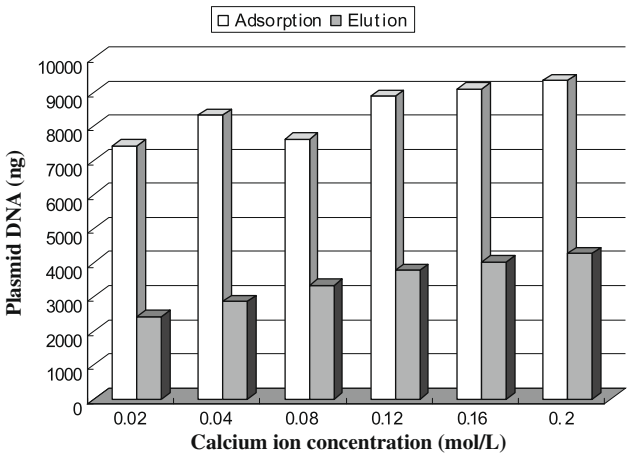

Fig. 4. DNA adsorption (white column) and elution (gray column) capacity by HAP nanorods for plasmid DNA was studied with the above methods. 40 μg plasmid DNA and 10 μg HAP nanorods were added into each tube to the final volume of 500 μl and the final Ca^{2+} concentration was regulated from 0.02 to 0.20 mol/L. 100 μl Na_2CO_3 solutions were added to elute DNA from the complex.

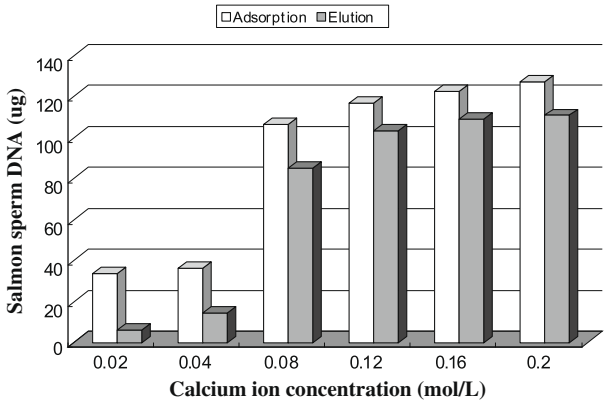

Fig. 5. Salmon sperm DNA adsorption and elution capacity by HAP nanorods was studied with the above methods. 200 μg salmon DNA and 40 μg HAP nanorods were added into each tube to the final volume of 500 μl and the final Ca^{2+} concentration was regulated from 0.02 to 0.20 mol/L. 100 μl Na_2CO_3 solution was used to elute DNA from the complex. Adsorption (white column) and elution (gray column) were illustrated.

The template-based synthesis technique has been proven to be an effective way to prepare calcium phosphate nanoparticles because the polymer template can be adsorbed on the surface of CaP nanoparticles and hence prevent their aggregation. For example, Kataoka et al. [18,19] modulated the crystal size of CaP by block copolymer of poly-(ethylene glycol)-block-poly (aspartic acid) (PEG-PAA) or poly (ethylene glycol)-block-poly (methacrylic acid) (PEG-PMA) and then obtained CaP/polymer/DNA hybrid nanoparticles, which exhibited high DNA or siRNA loading capacity and conspicuous DNA transfection efficiency. In this study, during the formation process, HAP crystals first nucleate from the precursor solution, and at the same time the polymer chains are attached to the HAP crystal surface through both the hydrogen bond formed between –OH and C═O, and the interaction between C–O–C groups and calcium ions. The adsorption of the polymer hampers the aggregation of HAP crystals. In addition, the PVP molecules exhibit a linear structure, and the capping effect will make the HAP crystals preferentially grow alone the long chains of PVP molecules. Thus, the smaller nanorods are obtained. Finally, during the hydrothermal treatment, the larger nanorods are formed through the Ostwald ripening and oriented attachment [20].

3.2. Evaluation of DNA adsorbing and desorbing performance of HAP nanorods

In order to test the DNA adsorption capability by HAP nanorods, we mixed HAP nanorods with salmon DNA or plasmid DNA and then regulate the Ca^{2+} concentration from 0.02 to 0.2 mol/L. In the presence of Ca^{2+} ions, salmon DNA was adsorbed by HAP nanorods to form hybrid compound (DNA-calcium phosphate). The amount of adsorbed DNA was calculated by subtracting the quantity of DNA in supernatant from the initially added DNA amount. The results are shown in Figs. 4 and 5. Compared with traditional materials universally used for DNA extraction such as silica, Chelex or spin column, the DNA adsorption capacity is enormously elevated to 930 mg/g (plasmid DNA) and 3170 mg/g (salmon sperm DNA), i.e. 18.87 mg/m^2 and 64.32 mg/m^2, respectively. The value is approximately 40–130 times higher than the recently reported high loading capacity mesoporous silica vectors (less than 30 mg/g) [21], and is also 80 times higher than the mesoporous silica–magnetite nanocomposite [22]. Interestingly, in addition to the very high DNA loading capacity of HAP nanorods, a high percentage of the loaded DNA can also be released under the action of Na_2CO_3 (Fig. 5).

Silica based materials have been used widely as the vector to separate DNA or RNA from several kinds of cells or tissues and many commercial products are available with silica particles as one important component in their kits [21,22], although the mechanism of DNA adsorbing to silica is not completely clear. In this experiment, as demonstrated by Figs. 4 and 5, it is really surprising to observe the tremendous enhancement of DNA adsorption capacity of HAP nanorods in the presence of Ca^{2+} ions compared with the conventional method using silica materials even mesoporous silica. The gigantic elevation of DNA adsorption in HAP nanorods suggests that the procedure maybe have a different mechanism compared with universally used silica materials or other materials. It seems difficult to explain our results by classic Langmuir adsorption model. We suggest that DNA molecules may self-assemble with Ca^{2+} ions as the bridge to form a multilayer adsorption complex with the HAP nanorod as the central and initial core. Beside the high DNA loading capacity, we also noticed the difference on the adsorption capacity between the plasmid and salmon sperm DNA. This difference maybe induced by the different molecular conformation of these two kinds of nucleic acid. However, the detailed mechanism needs to be further investigated.

3.3. Transfection and cytotoxicity assay of HAP nanorods in vitro

HAP nanorods were used to deliver pGL3-control vectors (luciferase expressing plasmid) in COS-7 cells. The results are shown in Fig. 6. The HAP nanorods are able to deliver DNA into the cells effectively, and the efficiency can be dramatically enhanced in the presence of Ca^{2+} ions. These results are in accordance with the research of DNA loading capacity. The performance of HAP nanorods is much better than the traditional calcium phosphate. And transfection efficiency increases obviously when more DNA is used.

To determine the cytotoxicity of HAP nanorods, an MTT assay with the complexes of DNA and HAP nanorods was employed (Fig. 7). A slight reduction by about 6–8% in cellular viability was observed for the complexes of DNA and HAP nanorods in COS-7 cells, while about 15% for custom calcium phosphate transfection. The results show that HAP nanorods were safe and cell growth was barely affected.

In our transfection approach, the HAP nanorods are prepared with the assistance of a block copolymer and then plasmid DNA is adsorbed onto the pre-synthesized HAP nanorods. This method is easy to handle and reproducible. Therefore, uncontrollable crystal growth is effectively avoided unlike the custom calcium phosphate transfection and furthermore, the efficiency is even better. Many factors may attribute to the elevated transfection efficiency. Anyway, the characteristic of HAP nanorods and the contribution of calcium ion condition can not be ignored and maybe important agents to upgrade the plasmid DNA loading capacity of HAP and then boost DNA amount in transfected cells.

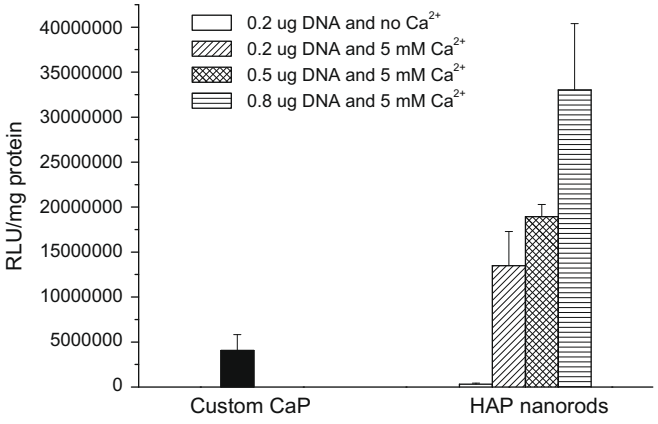

Fig. 6. Luciferase activity in COS-7 cells transfected with pGL3-control vector complexed with HAP nanorods. The amount of DNA in the legend is for a well of 96-well plate. The final concentration of Ca^{2+} was 5 mM. And the amount of HAP nanorods was 0.2 μg per well. Custom CaP transfection was carried out according to the literature.

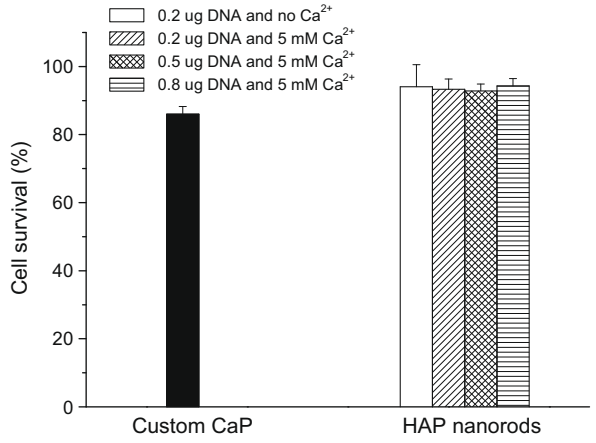

Fig. 7. Cell viabilities of COS-7 cells incubated with DNA/HAP nanorods complexes in the absence of FBS were determined by the survival cells per well in the transfected groups compared with that of untreated cells. Experiments were carried out in triplicate and error bars indicate standard deviations.

Although some progress has been made, the application of polymer assisted technique is still limited in the preparation of HAP as gene vectors because most of surfactants, used as polymer templates, are cytotoxic for tissues and cells. Only those polymers with both excellent biocompatibility and biodegradability can be used to fabricate HAP/polymer hybrid gene vectors for safety consideration [15]. In our study, as described in Figs. 6 and 7, HAP nanorods seem to have relatively high transfection efficiency while almost no cytotoxic effect on the cultured cells in the presence of Ca^{2+} ions. The cell safety maybe attributes to the biocompatible polymer templates and other agents. The components used in this research such as PEG and PLGA have been widely used in biomedicine and have possessed the safe approval of FDA [23–25]. Thus, HAP nanorods are promising gene delivery materials. This technique will certainly widen the application of calcium phosphate in gene transfection.

4. Conclusions

HAP nanorods with relatively uniform sizes and high crystallinity have been prepared via a block copolymer-assisted hydrothermal strategy. The shape and size of the sample can be adjusted by regulating the reaction conditions. Through hydrothermal treatment, the HAP nanorods with larger sizes and higher crystallinity are obtained compared with those prepared at a low temperature.

Plasmid DNA and salmon sperm DNA were used to test the DNA loading capacity of HAP nanorods in the presence of Ca^{2+} ions. These experiments demonstrated a huge enhancement of DNA adsorptive capacity of HAP nanorods compared with traditional DNA adsorbents. Another interesting result is that adsorbed DNA can be desorbed reversibly from HAP nanorods with high eluted percentage. The reversible procedure means that these kinds of materials may be promisingly applied in life science such as gene transfection. With the strategy of using PLGA–mPEG block copolymer and post-binding of nanorods with DNA, HAP nanorods act excellently as the gene vector to deliver DNA into the cells effectively and safely. Basing on the performance of DNA binding and gene transfection, HAP nanorods are undoubtedly an outstanding gene delivery vehicle.

Acknowledgments

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