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ORIGINAL RESEARCH

The sustained-release behavior and in vitro and in vivo transfection of *pEGFP*-loaded core-shell-structured chitosan-based composite particles

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Yun Wang[1]
Fu-xing Lin[2]
Yu Zhao[1]
Mo-zhen Wang[2]
Xue-wu Ge[2]
Zheng-xing Gong[1]
Dan-dan Bao[1]
Yu-fang Gu[1]

[1]Department of Plastic Surgery, First Affiliated Hospital of Anhui Medical University, [2]CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, Anhui, People's Republic of China

Abstract: Novel submicron core-shell-structured chitosan-based composite particles encapsulated with enhanced green fluorescent protein plasmids (*pEGFP*) were prepared by complex coacervation method. The core was *pEGFP*-loaded thiolated N-alkylated chitosan (TACS) and the shell was pH- and temperature-responsive hydroxybutyl chitosan (HBC). *pEGFP*-loaded TACS-HBC composite particles were spherical, and had a mean diameter of approximately 120 nm, as measured by transmission electron microscopy and particle size analyzer. *pEGFP* showed sustained release in vitro for >15 days. Furthermore, in vitro transfection in human embryonic kidney 293T and human cervix epithelial cells, and in vivo transfection in mice skeletal muscle of loaded *pEGFP*, were investigated. Results showed that the expression of loaded *pEGFP*, both in vitro and in vivo, was slow but could be sustained over a long period. *pEGFP* expression in mice skeletal muscle was sustained for >60 days. This work indicates that these submicron core-shell-structured chitosan-based composite particles could potentially be used as a gene vector for in vivo controlled gene transfection.

Keywords: gene therapy, gene transfection, hydroxybutyl chitosan, thiolated N-alkylated chitosan, *pEGFP*, complex coacervation

Introduction

Gene therapy is a promising technology for medical treatment because it aims at treating or eliminating the causes of disease, whereas most current drugs treat symptoms.[1–6] The success of gene therapy largely depends on the development of the gene delivery vector. An ideal gene delivery vector should satisfy certain conditions: 1) the capacity of combining with deoxyribonucleic acid (DNA) and protecting DNA from nuclease digestion; 2) good biocompatibility and biosafety; 3) high transfection efficiency; and 4) good biodegradability.[7] Currently, viral vectors are widely used due to their high transfection efficiency.[8] However, many problems exist in common biological viral vectors, such as immunogenicity, gene mutation, potential pathogenesis, and other problems[9,10] For example, viral vectors were reported to be somewhat successful in the treatment of children suffering from the fatal form of X-linked severe combined immunodeficiency disease ([SCID]-X1 syndrome) in 2000.[1] The immune systems of these children were rendered functional by stem cell gene therapy using a retrovirus vector. Unfortunately, five of these patients subsequently developed a leukemia-type disease owing to insertional mutagenesis.[11] Such major side effects seriously hamper the clinical application of viral vectors. Therefore, researchers have for many years been devoted to looking for new vectors to replace viral vectors and to thereby avoid side effects, and to consequently advance the development of gene therapy.[12,13]

Correspondence: Yu Zhao
Department of Plastic Surgery, First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei, Anhui 230032, People's Republic of China
Tel +86 551 6292 3519
Email zhaoyuzj@aliyun.com

Mo-zhen Wang
CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, 96 JinZhai Road, Hefei, Anhui 230026, People's Republic of China
Tel +86 551 6360 0843
Fax +86 551 6360 1592
Email pstwmz@ustc.edu.cn

Chitosan (CS) is a cationic polysaccharide widely found in nature, and is well-known for its low toxicity, good biocompatibility, and good biodegradability.[14–17] Therefore, it has been widely used in controlled drug delivery.[18–21] Recent research indicates that CS can also be used as a gene carrier because, similar to other cationic carriers, it can form a complex with genes through its strong electrostatic interactions, which package and protect the carried gene from digestion by deoxyribonuclease I (DNase I).[22,23] However, the question remains whether this strong electrostatic interaction also hinders the gene from dissociation, and further limits transfection efficiency of the complex.[24] It had been reported that electrostatic interactions could be weakened by introducing long hydrophobic alkyl chains onto the CS chain, which favors endocytosis and gene dissociation, and thus effectively improves transfection efficiency.[25–27] In addition, thiolated polymers are alternative potential gene vectors, because thiol groups can transform into stable disulfide bonds in the extracellular environment and return to thiol groups in the intracellular environment.[28] For example, Schmitz et al prepared new-type sulfhydryl CS nanoparticles to load genes.[29,30] The results showed that the transfection efficiency of the sulfhydryl CS nanoparticles was higher than that of raw CS. Yu et al also introduced thiol for CS alkylation to improve its water solubility and transfection efficiency.[31]

Hydroxybutyl CS (HBC), synthesized by conjugating hydroxybutyl groups to CS hydroxyl and amino groups, has unique temperature sensitivity in addition to good biocompatibility.[32] At physiological temperature (37°C), HBC can rapidly transform from a liquid into a gel. This means that HBC can form gel in situ when it is injected into the body, which in turn can control the release of the loaded active species in HBC, and can improve active species' bioavailability. In certain conditions, genes are expected to be released slowly and thereby achieve long-term expression, eg, the expression of tumor-suppressor genes in the cancer model, or the expression of bone morphogenetic protein (BMP) genes in the bone defect model.[33] Therefore, in the present study, we designed and prepared a novel core-shell-structured, CS-based gene vector, which has a gene-loaded thiolated N-alkylated CS (TACS) core and a pH- and temperature-responsive HBC shell to control the release of genes in the body. We used enhanced green fluorescent protein plasmids (*pEGFP*) as the model gene, because *pEGFP* expression can be conveniently observed by laser as a green fluorescence. The sustained-release behavior of *pEGFP* was investigated in vitro, and a series of transfection experiments in vitro and in vivo, were also conducted to evaluate the performance of *pEGFP*-loaded core-shell-structured TACS-HBC composite particles, prepared in-house, as a safe and effective gene vector.

Materials and methods

Reagents and materials

CS (MW =2.0×10^5; 91% deacetylation) was purchased from Aladdin Chemistry Co., Ltd., Shanghai, People's Republic of China. Thioglycolic acid was provided by Alfa Aesar, Heysham, UK and used as received; 99% 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) was supplied by Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, People's Republic of China. DNA Marker 2000 (DNA standard sample containing 100~2000 bp) was supplied by Shanghai Mengry Bio-Technology Co., Ltd. (Shanghai, People's Republic of China). Agarose and ethidium bromide were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Other reagents and solvents were of analytical grade. Human embryonic kidney (HEK) 293T cells and human cervix epithelial (Hela) cells were provided by the Laboratory of Immunology, Anhui Medical University, Hefei, People's Republic of China.

Preparation of *pEGFP*

pEGFP-3.0, referred to simply as *pEGFP* elsewhere in this manuscript, provided by the Laboratory of Immunology, Anhui Medical University, People's Republic of China, was transformed in *Escherichia coli* and amplified in lysogeny broth media (Oxoid Ltd, Basingstoke, United Kingdom) at 37°C overnight. The plasmid was purified using an EndoFree plasmid purification system (Plasmid Maxi Kit (10); Qiagen NV, Hilden, Germany). The concentration of *pEGFP* was 350 ng/μL, as measured by a NanoDrop 2000 Micro ultraviolet (UV) spectrophotometer (Thermo Electron Corporation, Marietta, OH, USA) set to a ratio between the readings at 260 nm and 280 nm (OD260/OD280) of 2.0. Prepared *pEGFP* was stored at −20°C before being used.

Preparation of *pEGFP*-loaded core-shell-structured CS composite particles

Before any experiments were conducted, all materials and instruments used were sterilized by autoclaving.

Preparation of *pEGFP*-loaded TACS core particles

TACS was synthesized by conjugating thiol and dodecyl groups to the hydroxyl and amino groups of CS, respectively. The detailed preparation process is presented in the Supplementary materials section. *pEGFP* was loaded into a TACS solution (with a 26.5% degree of alkylation and a 7.1% degree

of thiolation) by complex coacervation. Briefly, an aqueous solution of 0.2 mg/mL TACS was prepared by dissolving TACS particles in a 5 mM acetic acid solution. The pH of the solution was adjusted to 5.5 with sodium hydroxide. A specific amount of *pEGFP* was dissolved in an aqueous solution of 50 mM sodium sulfate. The concentration of *pEGFP* was controlled at 76 μg/mL. Then, pre-prepared TACS and *pEGFP* solutions (total volume, 100 μL) were vortex mixed for 30 seconds. The N-P ratio (the molar ratio of CS amine groups to *pEGFP* phosphate groups) in the mixture was 10. Particles produced were collected by centrifugation at 15,000 rpm for 5 minutes. The concentration of *pEGFP* in the supernatant was detected by using the NanoDrop 2000 Micro UV spectrophotometer (Thermo Electron Corporation). The percentage of *pEGFP* loading was calculated by the following equation:

$$\text{Encapsulation efficiency} = \left(1 - \frac{c_1}{c_0}\right) \times 100\%. \quad (1)$$

In this equation, c_0 is the original concentration of *pEGFP* in the solution before being loaded in TACS particles; and c_1 is the concentration of *pEGFP* after being loaded in TACS particles.

Preparation of *pEGFP*-loaded core-shell-structured CS-based composite particles

First, HBC was dissolved in a solution of sodium acetate at a slightly acidic pH of 5.5~6. The detailed HBC preparation process is described in the Supplementary materials section; the degree of substitution was 1.22. The HBC solution was subsequently added into the aqueous solution containing pre-prepared *pEGFP*-loaded TACS core particles. The mixture was shaken for 30 minutes at 25°C on a thermostatic shaker. When the pH of the mixture was adjusted to neutral, eg, pH 7.4, HBC aggregated and precipitated from the solution onto the surface of the *pEGFP*-loaded TACS core particles; this reaction was driven by the mutual attraction between TACS and HBC, based on their possession of identical CS structural units, and as described in the Supplementary materials section. Finally, reaction products were passed through a 0.22 μm filter to separate the products.

Characterization of *pEGFP*-loaded CS-based composite particles

Size and size distribution of *pEGFP*-loaded CS-based particles

Particle size and size distribution in solution were analyzed by light scattering method, using a Nano Particle Analyzer (NanoSight LM10; Malvern Instruments, Malvern, UK).

Nuclease protection assay

The stability of *pEGFP* loaded in 1) TACS core particles; and 2) TACS-HBC composite particles, both in the presence of DNase I, was evaluated. Naked *pEGFP*, *pEGFP*-loaded TACS core particles, and TACS-HBC composite particles were incubated with DNase I (130 U/mL; 650 U/μg DNA) separately at 37°C for 30 minutes. All samples contained the same mass of *pEGFP*, ie, 2 μg. A solution without DNase I was used as the control. Gel electrophoresis was subsequently conducted by using 1% agarose gel bound with 0.5 μg/mL ethidium bromide at room temperature and 180 V in 1× tris-acetate-EDTA (ethylenediaminetetraacetic acid) buffer (Sangon Biotech [Shanghai] Co., Ltd., Shanghai, People's Republic of China). To check the integrity of treated *pEGFP*, the digestion of *pEGFP* in TACS core particles by DNase I solution was stopped by adding 2 μL of 0.5 M EDTA, and the resultant solution was incubated for another 10 minutes. Gel electrophoresis was performed after 10 μg of releasing agent heparin was added.

Cell toxicity assay

Toxicity of prepared *pEGFP*-loaded CS-based particles to cells in vitro was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay. HEK 293T cells and Hela cells were seeded in 96-well plates at a density of 5,000 cells/well, and allowed to grow overnight. The cells were incubated then for 48 hours in separate 200 μL samples of complete Dulbecco's Modified Eagle's Medium (DMEM) containing either naked *pEGFP*, pre-prepared *pEGFP*-loaded TACS core particles, TACS-HBC composite particles, or *pEGFP*-loaded Lipofectamine® 2000 transfection reagent (Lip2000) (Thermo Fisher Scientific, Waltham, MA, USA); 200 μL of fresh, complete medium was used as a control. Cells were incubated for another 4 hours after 20 μL of 5 mg/mL MTT solution was added to each well. The medium in each well was then replaced with 150 μL of dimethyl sulfoxide. The plates were shaken for 10 minutes to ensure formazan crystals dissolved completely. Absorbance spectra were read at a wavelength of 490 nm in a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability is characterized by the following equation:

$$\text{Cell viability} = \frac{\text{The absorbance of the experimental group}}{\text{The absorbance of the control group}} \times 100\%. \quad (2)$$

In vitro sustained-release behavior of *pEGFP*-loaded TACS-HBC composite particles

In vitro release of *pEGFP* from TACS-HBC composite particles was determined by the dialysis method; 175 μg *pEGFP*-loaded TACS-HBC composite particles dispersed in tris-EDTA (TE) solution (Qiagen NV, Hilden, Germany) were placed in Spectra/Por® cellulose ester dialysis tubing (300,000 MWCO) (Spectrum® Laboratories, Inc., Rancho Dominguez, CA, USA). The tubing was placed in a flask filled with 50 mL TE buffer solution. The flask was shaken at 37°C; 100 μL of the TE solution (with a *pEGFP* concentration of 3.7 ng/μL) was sampled at 24 and 48 hours, and 3, 5, 7, and 15 days, and an equivalent volume of fresh TE solution was added to the flask after each sampling. The amount of *pEGFP* released was determined by UV-visible absorption method, as measured with the NanoDrop 2000 Micro UV spectrophotometer. The release rate was characterized as

$$\text{Release rate} = \frac{C_{sample}}{C_{total}} \times 100\%, \quad (3)$$

where C_{sample} is the concentration of *pEGFP* in the sample; and C_{total} is the theoretical concentration of *pEGFP*, provided that all *pEGFP*-loaded in particles have been released into the TE buffer solution.

In vitro transfection of *pEGFP*-loaded CS-based particles

Cell culture

A HEK 293T cell line was cultured in high glucose DMEM (HyClone™; Thermo Fisher Scientific) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Harbin Pharmaceutical Group Holding Co., Ltd., Helongjiang, People's Republic of China).

Transfection

In vitro transfection of *pEGFP*-loaded TACS core particles and *pEGFP*-loaded TACS-HBC composite particles in HEK 293T cells seeded in 24-well plates at a density of 3×10^4 cells/well was investigated. The seeded cells were maintained overnight at 37°C in a humidified 5% CO_2 incubator (Dan Ding Shanghai International Trade Co., Ltd., Shanghai, People's Republic of China). The following day, the medium was removed from the wells, and each well was washed with 0.5 mL serum-free DMEM; 100 μL of serum-free DMEM containing naked *pEGFP* or *pEGFP*-loaded particles (*pEGFP* mass =4 μg) was then added to each well. *pEGFP*-Lip2000 complexes were adopted as the positive control. Another 100 μL of fresh serum-free DMEM was set as the untreated control sample. Four hours after the addition of the serum-free DMEM, the media in all wells were replaced with fresh DMEM mixed with 10% FBS. *pEGFP* expression was qualitatively evaluated by fluorescence microscope (Olympus, Tokyo, Japan), and quantitatively measured by flow cytometer (BD FACSVerse; BD, Franklin Lakes, NJ, USA) after 72 hours' incubation.

In vivo transfection of *pEGFP*-loaded CS-based particles

Forty-two male KM mice (weighing 20–30 g each) at 4~5 weeks of age were randomly divided into three groups for in vivo transfection of *pEGFP*-loaded TACS and TACS-HBC composite particles. Group A was transfected with *pEGFP*-loaded TACS core particles. Group B was transfected with *pEGFP*-loaded TACS-HBC composite particles, and Group C, the control group, was transfected with saline. The mice were first anaesthetized by intraperitoneal injection with 3% chloral hydrate according to a standard of 0.01 mL/g/mouse to make the skeletal muscles loosen and to prevent *pEGFP* from being extruded by muscle contractions. Both hind legs of each mouse were sterilized with alcohol after the hair was shaved, and 50 μL of 25 g/L hypertonic sucrose (China Otsuka Pharmaceutical Co., Ltd., Tianjing, People's Republic of China) was injected into the quadriceps muscle. Fifteen minutes post-injection, the solutions containing *pEGFP*-loaded particles (20 μg *pEGFP*) were injected into the quadriceps slowly. The needle remained in the muscle 5~10 seconds, and was then removed slowly to prevent injected solutions from exuding. *pEGFP* expression was detected by laser scanning confocal microscopy (Leica Microsystems, Wetzlar, Germany) after quadriceps muscle sections were cut and frozen.

Results and discussion

Morphological characterization of *pEGFP*-loaded TACS-HBC composite particles

The synthesis route of *pEGFP*-loaded TACS-HBC composite particles is illustrated in Figure 1. First, positively charged CS or TACS forms a complex with negatively charged *pEGFP* through strong electrostatic interactions. Figure 2A shows size distribution and morphology of complexes consisting of TACS and *pEGFP*, which were formed through complex coacervation. The particles are spherical and have a uniform size distribution with a mean diameter of 90 nm, as detailed in the Supplementary materials section.

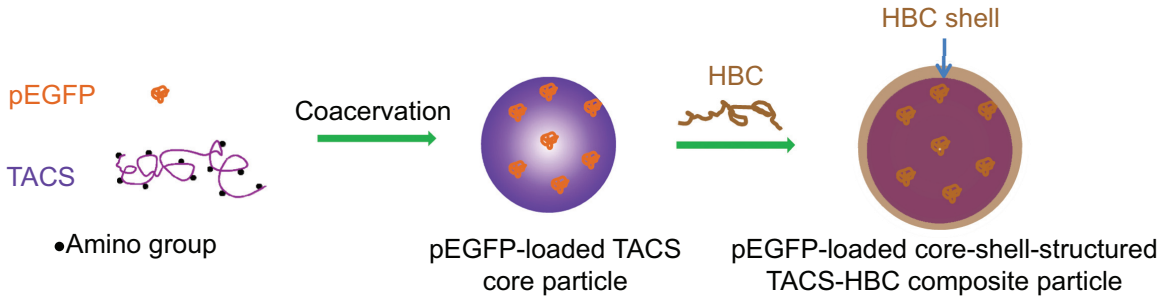

Figure 1 *pEGFP*-loaded core-shell-structured TACS-HBC composite particle synthesis.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan.

After HBC solution was added to the *pEGFP*-loaded TACS complex, 74.2% of particles presented with a mean diameter of 120 nm, as indicated in Figure 2B. The calculation of particle ratios is shown in the Supplementary materials section. The large particle diameter was a result of the fact that most HBC precipitates on the surface of *pEGFP*-loaded TACS particles to form a shell around the particles, which causes an increase in particle size. In other words, core-shell-structured *pEGFP*-loaded TACS-HBC composite particles are mainly produced after the addition of HBC. It should be noted that a few smaller particles (8.0%) with a mean diameter of 64 nm also appeared. These smaller particles were probably aggregates of pure HBC, because HBC readily self-aggregates in solution to form particles with a mean diameter of 64 nm (Figure 2C). Only 17.8% of *pEGFP*-loaded TACS particles remained unencapsulated with HBC. Gel retardation assays of the prepared particles before and after they were digested by DNase I, shown in Figure 3, indicate that nearly all *pEGFP* combined with TACS because no *pEGFP* can be detected in line c, which represents *pEGFP*-loaded TACS core particles. In Figure 3, line b indicates that most *pEGFP* was protected in TACS-HBC composite particles.

Cell toxicity assay

The toxicity of the pre-prepared CS-based particles was quantitatively estimated through investigating cell viability by MTT assay. Such assay depends on the detection of purple-colored formazan crystals,[34] which were formed by the reduction of MTT by succinate dehydrogenase in living cells. These purple crystals may be dissolved in a variety of organic solvents, and the optical density of the resulting solution we obtained was measured on a multi-well spectrophotometer.

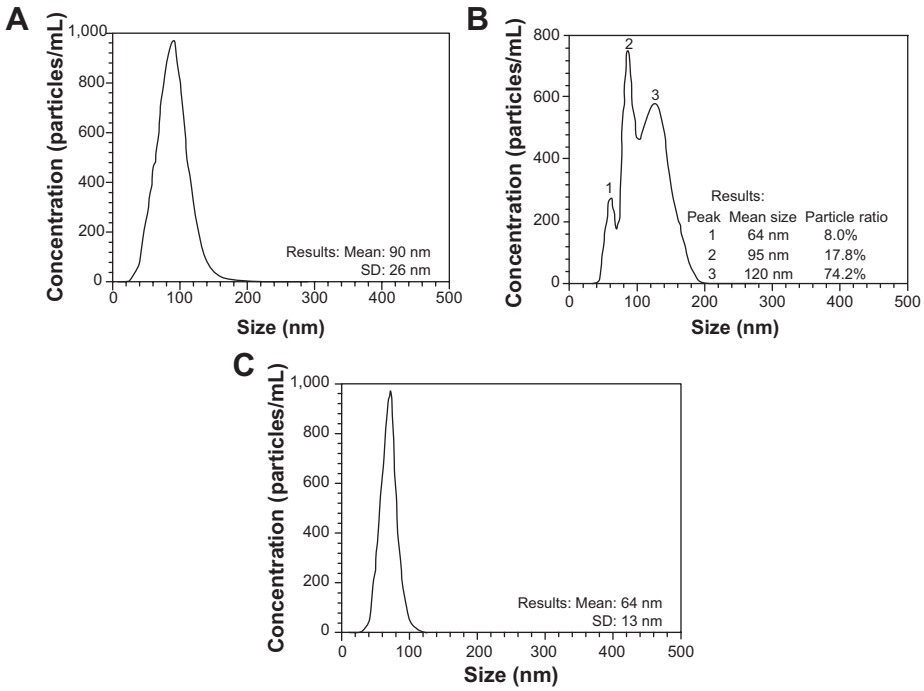

Figure 2 Particle sizes and size distributions.
Notes: Particle size and size distribution, represented by size/concentration peaks, of *pEGFP*-loaded TACS core particles (**A**); *pEGFP*-loaded TACS-HBC composite particles (**B**); and pure HBC particles formed by self-aggregation in 5 mM acetic acid solution (**C**).
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; SD, standard deviation.

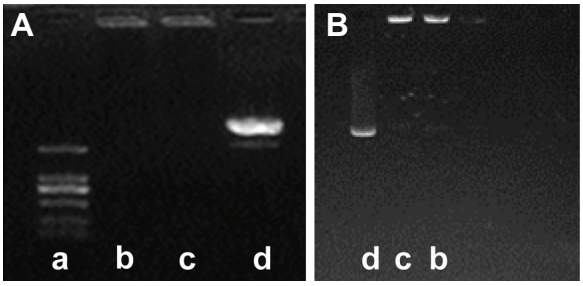

Figure 3 Gel electrophoresis of *pEGFP*-loaded particles before (**A**), and after (**B**) being digested by DNase I.
Notes: Gel line (**a**) represents DNA marker 2000; line (**b**) represents *pEGFP*-loaded TACS-HBC composite particles; line (**c**) represents *pEGFP*-loaded TACS core particles; and line (**d**) represents naked *pEGFP*. Line identification scheme applies to both (**A**) and (**B**).
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; DNase I, deoxyribonuclease I; DNA, deoxyribonucleic acid; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan.

The results are shown in Figure 4, from which it can be seen that the influence of both *pEGFP*-loaded TACS core particles and *pEGFP*-loaded TACS-HBC composite particles on the viability of HEK 293T and Hela cells is insignificant. After 48 hours exposure, cell viability of both HEK 293T and Hela cells in the wells containing *pEGFP*-loaded TACS core particles and TACS-HBC composite particles was over 95%. In contrast, cell viability in *pEGFP*-loaded Lipo2000 was only 60.98%±2.95% for HEK 293T cells, and 38.34%±4.61% for Hela cells. These figures imply that both TACS and HBC will provide good biosafety as gene vectors.

In vitro sustained release and transfection of *pEGFP* loaded in CS-based particles

pEGFP release from pre-prepared CS-based particles in a dialysis tube put on the shaking bed at 37°C is illustrated in Figure 5. *pEGFP* release from both TACS core particles and

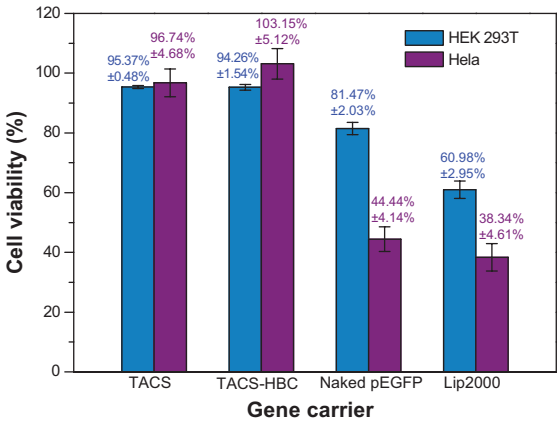

Figure 4 The relative growth rates of HEK 293T and Hela cells in the presence of different gene carrier particles.
Note: Cell growth was assessed 24 hours after introduction of particles.
Abbreviations: HEK 293T, human embryonic kidney cell line 293T; Hela, human cervix epithelial; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; *pEGFP, enhanced green fluorescent protein plasmids*; Lip2000, Lipofectamine® 2000 transfection reagent.

TACS-HBC composite particles is evidently controlled. At the first 24 hours after loading, *pEGFP* release rate from TACS core particles was 51.4%. Release rate increased gradually, reached an 88.6% maximum after 5 days, and then leveled off. In contrast, the release rate from core-shell-structured TACS-HBC composite particles was much lower than that in TACS core particles. It was only 25.7% at the first 24 hours after loading, and 42.9% at 15 days. These results support the assumption that HBC shells can be used to protect loaded *pEGFP* from dissociation to a certain degree.

After being stored at 37°C for 30 days, stability and integrity of *pEGFP* were investigated by gel electrophoresis (Figure 5). *pEGFP* released by heparin from TACS core particles treated at 37°C for 30 days appears at the same location on the gel band as naked *pEGFP* (Figure 5). This result implies that *pEGFP* is stable after forming a complex with TACS.

Since release of the *pEGFP* gene from CS-based particles was sustained, in vitro transfection of *pEGFP* loaded in those particles was investigated subsequently by fluorescence microscopy. If transfection is successful, bright green fluorescence induced by EGFP should be observed. Transfection results in HEK 293T cells, using the three particle types, are illustrated in Figure 6; *pEGFP* expressions in 1) Lipo2000 complex, and 2) TACS core particles were more rapid than in 3) core-shell-structured TACS-HBC composite particles. However, the intensity of the green fluorescence increased gradually with time, which means that *pEGFP* expression in TACS-HBC composite particles is time-dependent. After 72 hours, green fluorescence could be seen in all three groups. Figure 6 also shows the percentage of cells in each group containing fluorescent protein, ie, the transfection efficiency of *pEGFP*-loaded particles. The transfection efficiency of *pEGFP*-loaded Lip2000 complex, naked *pEGFP*-loaded TACS core particles, and TACS-HBC composite particles was 60.32%, 38.99%, and 8.00%, respectively. These results are in accord with results obtained from fluorescence microscopy. The sustained expression of *pEGFP* in TACS-HBC composite particles can be ascribed to the existence of HBC shells, which limit the rapid release of loaded *pEGFP* after particles enter cells (Figure 7). At first, the release rate of *pEGFP* was very slow from particles encapsulated in an HBC shell. In living cells, *pEGFP* release will increase slowly as the HBC shell becomes progressively thinner due to degradation by enzymes. After the HBC shell degrades totally, *pEGFP* release also become progressively faster, because degradation of TACS cores starts so that the maximum *pEGFP* release rate will be achieved at this time. Based on the above results and analysis, it can be concluded that prepared core-shell-structured TACS-HBC composite

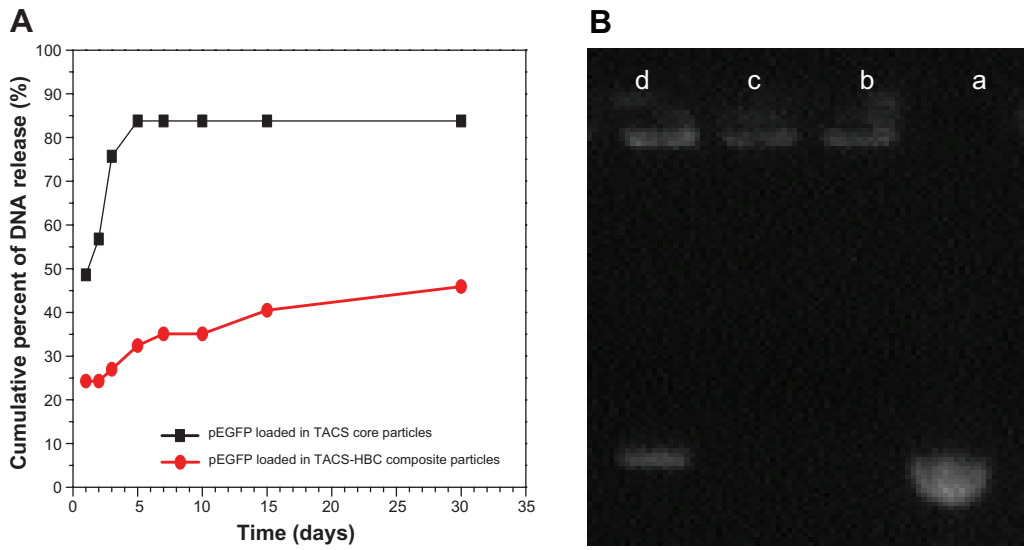

Figure 5 In vitro release behavior of *pEGFP* loaded in TACS core and TACS-HBC composite particles in TE solution (**A**), and gel electrophoresis of *pEGFP*-loaded particles after 30 days' release at 37°C (**B**).
Notes: In (**B**), line (**a**) represents naked *pEGFP*; line (**b**) represents *pEGFP*-loaded TACS core particles; line (**c**) represents *pEGFP*-loaded TACS core particles kept at 37°C for 30 days; line (**d**) represents *pEGFP* released by heparin from the particles in line (**c**).
Abbreviations: DNA, deoxyribonucleic acid; *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; TE, tris-ethylenediaminetetraacetic acid.

particles can be used as gene carriers in cases requiring controlled transfection of a loaded gene.

In vivo sustained transfection of *pEGFP* loaded in CS-based particles

pEGFP-loaded TACS core particles and TACS-HBC composite particles, as well as a saline control, were injected into mice skeletal (quadriceps) muscle. Skeletal muscle was sampled at specific time points (10, 30, and 60 days), cut into frozen sections, and observed with a fluorescence microscope. Transfection results from these muscle sections are shown in Figure 8. In the *pEGFP*-loaded TACS core particle group (Group A), there was a small amount of green fluorescence at 10 days; fluorescence intensity reached its highest point at 30 days, and gradually weakened between 45–60 days. However, in the *pEGFP*-loaded TACS-HBC composite

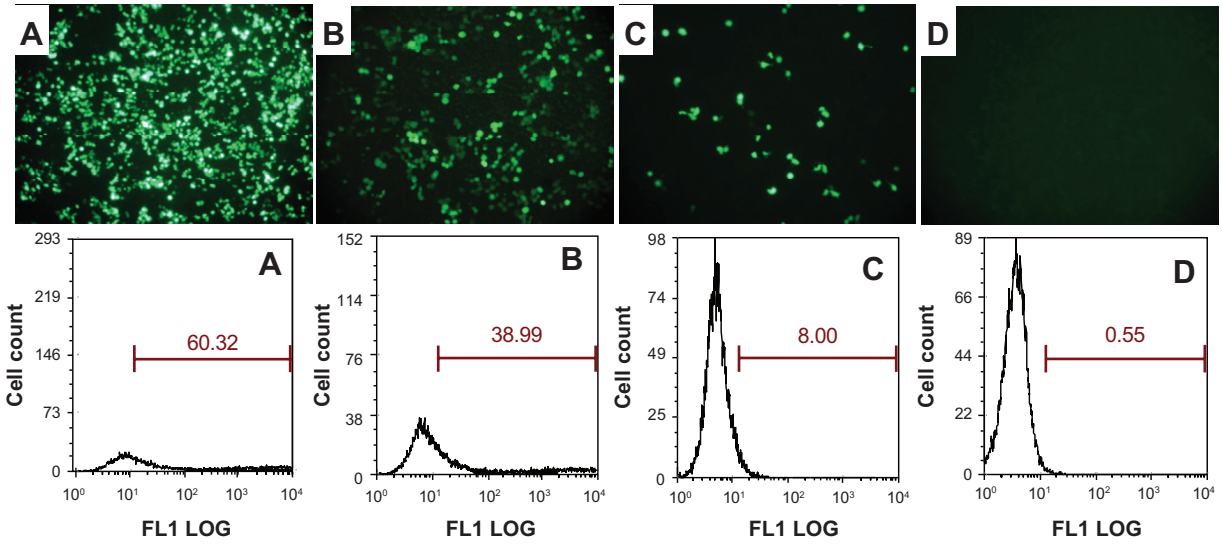

Figure 6 In vitro gene expression efficiency of *pEGFP* loaded in different gene carriers.
Notes: (**A**) Lip2000; (**B**) TACS particle; and (**C**) TACS-HBC composite particle expression of *pEGFP* in HEK 293 cells after 72 hours. Expression efficiency was detected by fluorescence microscopy (images, top row) and flow cytometry (graphs, bottom row). Image and graph (**D**) represent the control, which was DMEM without any particles.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; Lip2000, Lipofectamine® 2000 transfection reagent; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; HEK 293T, human embryonic kidney cell line 293T; FL1, green fluorescence; LOG, logarithmic relative intensity of green fluorescence; DMEM, Dulbecco's Modified Eagle Medium.

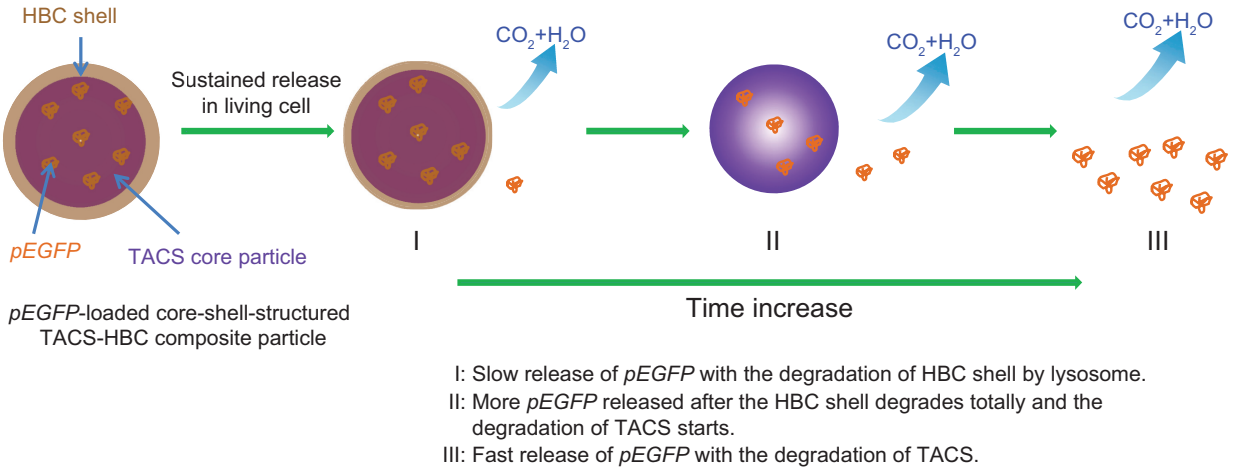

Figure 7 Sustained release of *pEGFP*-loaded core-shell-structured TACS-HBC composite particles in living cells.
Notes: Sustained release is represented pictorially at three different time scales: slow release; release after HBC shell degrades; and fast release upon degradation of TACS.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan.

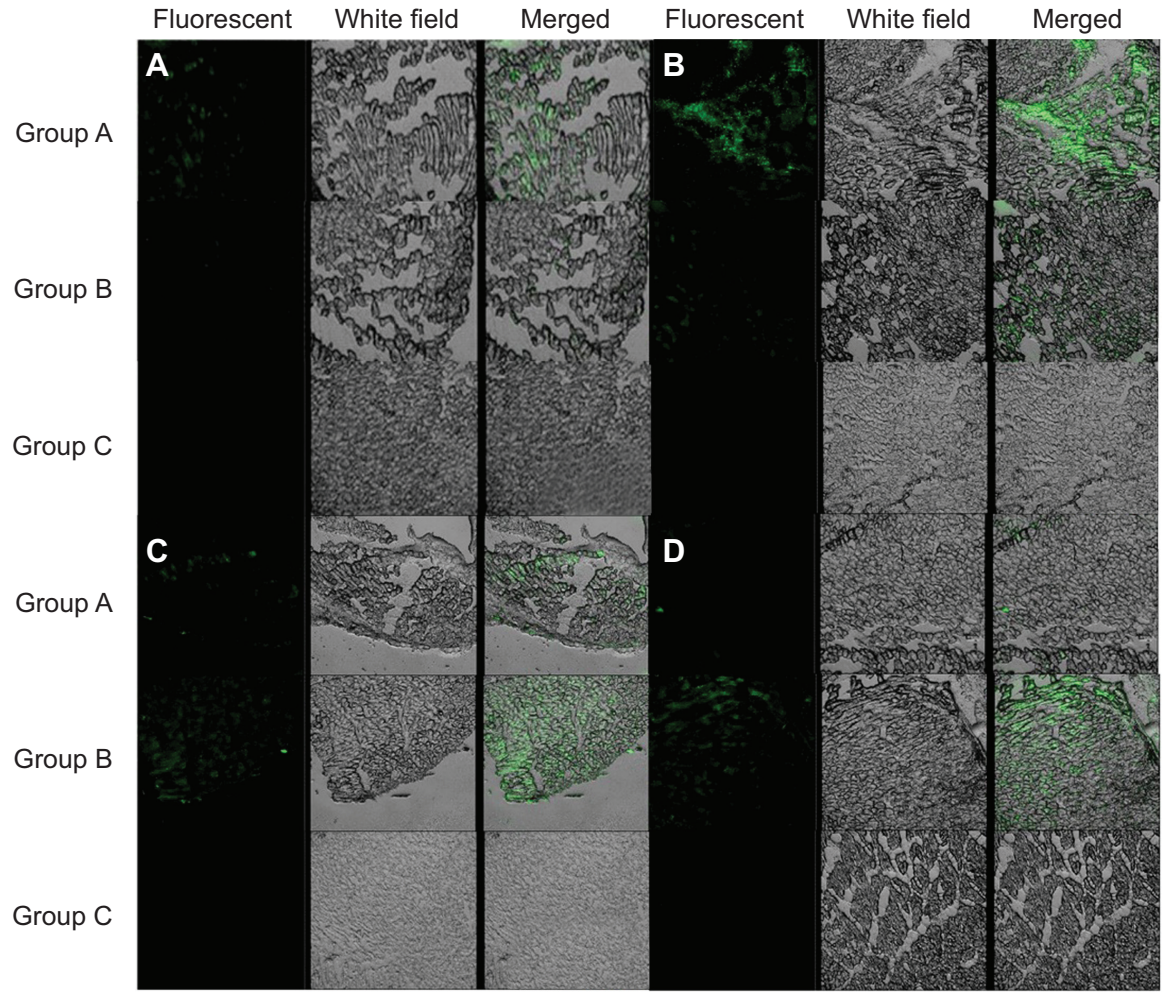

Figure 8 I In vivo expression in mice skeletal muscle of *pEGFP* loaded in different gene carriers.
Notes: Group A, TACS particles; Group B, TACS-HBC composite particles; Group C, saline control. In vivo expression is shown in fluorescent, white field, and merged light conditions at 10 (**A**), 30 (**B**), 45 (**C**), and 60 (**D**) days. Expression at different time points clearly illustrates superior sustained release of *pEGFP* by TACS-HBC composite particles.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; d, days.

particle group (Group B), there was little green fluorescence at 10 days, but fluorescence intensity gradually increased between 30–60 days. At 45 days, fluorescence intensity in Group B exceeded that in Group A. In the saline control group (Group C), there was no green fluorescence at any time.

Conclusion

In this work, we prepared a novel nanosized core-shell-structured CS-based composite particle as a gene carrier. *pEGFP* could then be efficiently combined with a TACS core, which was enwrapped by an HBC shell. *pEGFP*-loaded TACS-HBC composite particles are relatively stable and nontoxic. In vitro, they exhibited sustained release and transfection of loaded *pEGFP*. In vivo expression of loaded *pEGFP* was also sustained for up to 60 days. The design and production of *pEGFP*-loaded TACS-HBC composite particles as a gene carrier in this study has theoretical and practical significance for gene therapy with sustained transfection effect. Further applications for core-shell-structured TACS-HBC composite particles loading BMP genes are currently being researched.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Preparation of thiolated N-alkylated chitosan

Thiolated N-alkylated chitosan (TACS) was prepared in two steps as described in Figure S1. First, chitosan (CS) was N-alkylation modified with 1-bromododecane (Aladdin Chemical Co., Ltd., Shanghai, People's Republic of China); TACS was then further modified with thioglycolic acid.

N-alkylated modification of CS

CS (1 g) was dispersed in 15 mL isopropanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, People's Republic of China), followed by the addition of 2.5 g NaOH. The subsequent reaction proceeded at 70°C for 30 minutes, with continuous stirring; 3 mL 1-bromododecane was then added and left to react with CS for 10 hours. The mixture was subsequently poured through filter paper. The product remaining on the filter paper was collected and washed by ethanol until the filtrate was neutral, followed by washing thoroughly with acetone, ether, and water, in that order. TACS obtained from this process was dried at 60°C for 5 hours.

Thiolated modification of TACS

First, 0.25 g of pre-prepared TACS was dissolved in 2 mL of 1 M hydrochloric acid (HCl). The resulting solution was diluted with distilled water to obtain a 1% solution of CS hydrochloride. Next, 0.24 g 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) was added to activate the carboxylic acid moieties of the subsequently added 0.5 g thioglycolic acid. The solution was stored in the dark for 3 days after the pH was adjusted to 4.5 using 1 M NaOH. The reaction mixture was dialyzed in the dark sequentially in three solutions, ie 5 mM HCl solution for 12 hours, 5 mM HCl solution containing 1% NaCl for 12 hours twice, and the 1 mM HCl solution for 24 hours. Finally, the product was lyophilized, and TACS was obtained.

Preparation of hydroxybutyl CS

Hydroxybutyl CS (HBC) was prepared as shown in Figure S2; 4 g of CS was first alkalinized with 40 mL of 50% w/v NaOH under the protection of N_2 for 24 hours. Subsequently, the solution was filtrated through filter paper to remove excess NaOH. Pre-prepared CS was then dispersed in 80 mL of a 9:1 mix of isopropanol and water, under vigorous stirring for 8 hours. The resultant solution was left for 4 days after 80 mL of 1,2-epoxybutane was added. Excess 1,2-epoxybutane was removed by means of bubbling N_2. Afterward, the solution was vacuum filtrated to remove the unreacted species. Filter residue was washed with acetone until the pH of the filtrate achieved neutral (pH =7). Finally, the product was dried in a vacuum oven at 50°C.

To investigate pH and temperature responsiveness of HBC, HBC in aqueous solution was observed at different pH values and temperatures. Briefly, HBC was first dissolved in an acetic acid solution (pH =5.5) at a concentration of 2 mg/mL at room temperature. The solution was then divided into two portions, hereafter named groups one and two. The pH of three separate batches within group one was adjusted to 6.0, 6.5, and 7.0 by adding an appropriate amount of 1% NaOH. For group two, one of the solutions was heated to 37°C and reserved for 2 hours. The other was kept at room temperature (25°C). Digital photos were taken to record changes in solution appearance, as shown in Figure S3. It is clear from these photos that HBC transformed into a gel when the pH was adjusted to 7, or the temperature was increased to 37°C.

Size distribution analysis of TACS-HBC composite particles

Figure S4 shows particle size distribution after HBC was added into the solution containing pEGFP (enhanced green fluorescent protein plasmids)-loaded TACS core particles, as measured by the Nano Particle Analyzer (NanoSight LM10; NanoSight Ltd., UK Malvern Instruments, Malvern, UK), after HBC was added to *pEGFP* (enhanced green fluorescent protein plasmids)-loaded TACS core particles. To investigate

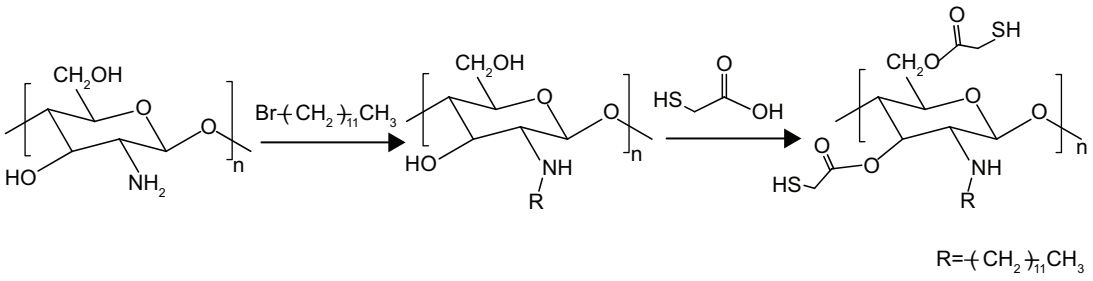

Figure S1 TACS synthesis.
Abbreviation: TACS, thiolated N-alkylated chitosan.

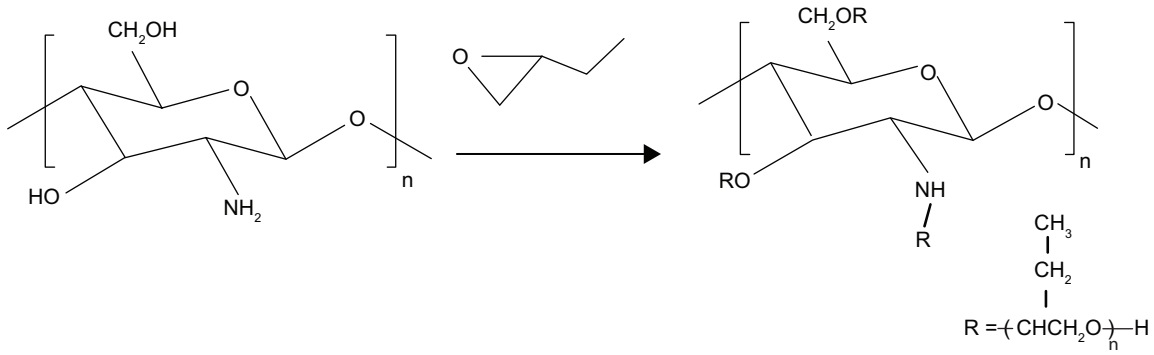

Figure S2 HBC synthesis.
Abbreviation: HBC, hydroxybutyl chitosan.

the size distribution of HBC-wrapped *pEGFP*-loaded TACS core particles the NanoSight LM10 was also used for nanoparticle tracking analysis. Three peaks can be seen in Figure S4. The mean particle diameters corresponding to peaks one, two, and three are 64 nm, 95 nm, and 120 nm, respectively.

To determine the particle ratios, particle size distribution in Figure S4 was successfully analyzed by peak-fit processing using Origin software (OriginLab, Northampton, MA, USA). Each peak was regarded as a Gaussian distribution. The areas under each peak could be calculated easily; results are shown in Figure S5, in which particle ratios from Figure S4 equate to the ratio of the areas under the peaks.

Transmission electron microscopy observation of *pEGFP*-loaded CS-based composite particles

Morphologies of *pEGFP*-loaded TACS core particles and TACS-HBC composite particles were observed using transmission electron microscopy (100 kV; Hitachi-7650, Hitachi Ltd., Tokyo, Japan). Samples were prepared by dispensing one drop of each particle type suspended in aqueous solution on copper grids, followed by air-drying. Results are shown in Figure S6. *pEGFP*-loaded TACS core particles (Figure S6A) have an average diameter of 80 nm, while *pEGFP*-loaded TACS-HBC composite particles (Figure S6B) average ~130 nm.

In vitro transfection of cells by *pEGFP* loaded in CS-based particles

In vitro transfection behavior of *pEGFP* loaded in CS-based particles in human cervix epithelial (Hela) cells was investigated for comparison with transfection of human embryonic kidney (HEK) 293T cells.

Cell culture

A Hela cell line was cultured in a high-glucose solution of Dulbecco's Modified Eagle's Medium (DMEM; HyClone™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Harbin Pharmaceutical Group Holding Co., Ltd., Helongjiang, People's Republic of China).

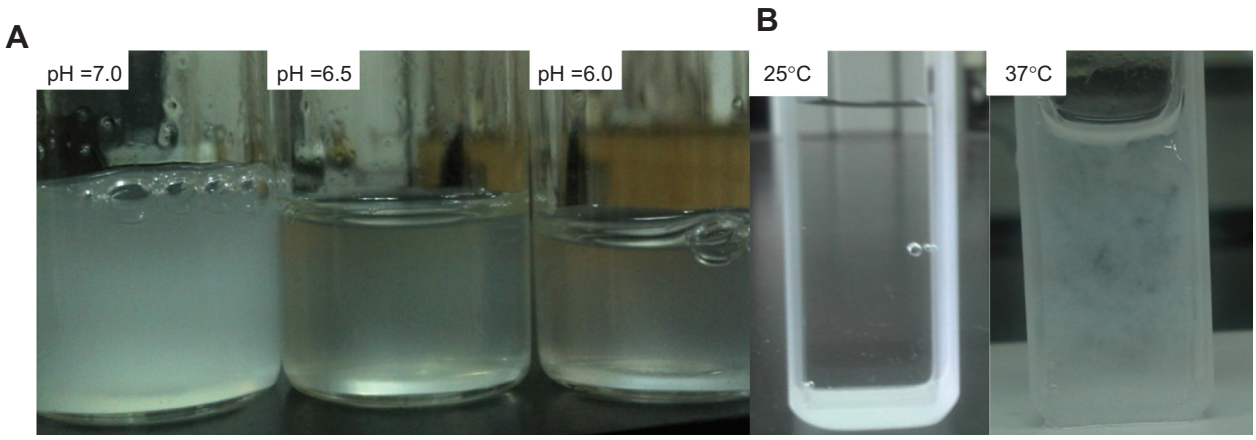

Figure S3 Digital photos of HBC in aqueous solution.
Notes: Photos in (**A**) are of three solutions (group one) mixed at different pH values; photos in (**B**) are of two solutions (group two) at different temperatures.
Abbreviation: HBC, hydroxybutyl chitosan.

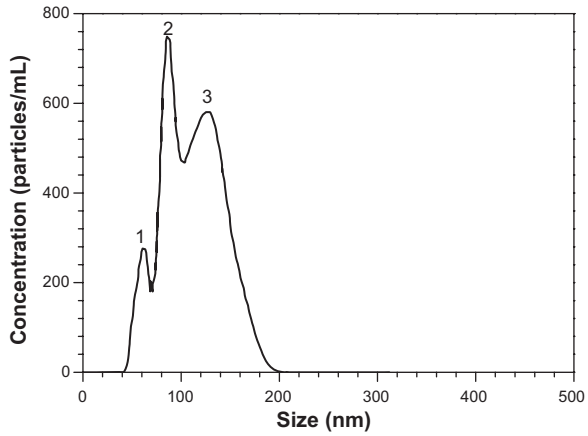

Figure S4 Size and size distribution of *pEGFP*-loaded TACS-HBC composite particles.
Notes: Three peaks (1, 2, and 3) can be seen at 64 nm (the pure HBC particles), 95 nm (*pEGFP*-loaded TACS core particles), and 120 nm (*pEGFP*-loaded TACS/HBC composite particles), respectively. These peaks correspond to mean diameters of particles.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan.

Results

Transfection efficiency of *pEGFP*-loaded Lip2000 complex, *pEGFP*-loaded TACS core particles, and TACS-HBC composite particles in Hela cells was 15.82%, 14.64%, and 5.53%, respectively, as shown in Figure S7. Evidently, transfection efficiency of *pEGFP* in Hela cells is much worse than in HEK 293T cells on average, even when *pEGFP* is loaded in Lip2000. However, the transfection behavior of all three gene carriers in both Hela and HEK 293T cells is very similar.

Reverse transcription polymerase chain reaction analysis

Transfection efficiency of *pEGFP*-loaded TACS core, and *pEGFP*-loaded HBC-TACS composite particles prepared under various N-P ratios (2.5:1, 5:1, 10:1, and 20:1), and Lip2000, was also tested in HEK 293T cells seeded in 24-well plates at a density of 3×10^4 cells/well. *pEGFP* expression was confirmed by semiquantitative reverse transcription polymerase chain reaction (RTPCR) analysis after 3 days transfection. Total cell ribonucleic acid (RNA) was harvested after 7 days with a Takara RNA PCR kit (Takara Bio Inc., Otsu, Japan). TAQ polymerase and primer (5′-GTGGGCTTCCTGGTGA-3′ and 5′-CTTTGGAGCCAGCTGGA-3′, respectively) were used in an RTPCR amplification reaction in which RTPCR products were visualized on a 1% w/v agarose gel (Sigma-Aldrich Co., St Louis, MO, USA) by staining with ethidium bromide (Sigma-Aldrich Co.). Relative levels of messenger RNA expression were estimated by the brightness of each gel line (Figure S8).

It can be seen in Figure S8 that the brightest gel bands correspond Lip2000 (gel band C, far right), and TACS core particles prepared at an N-P ratio of 10:1 (gel band B_{10},

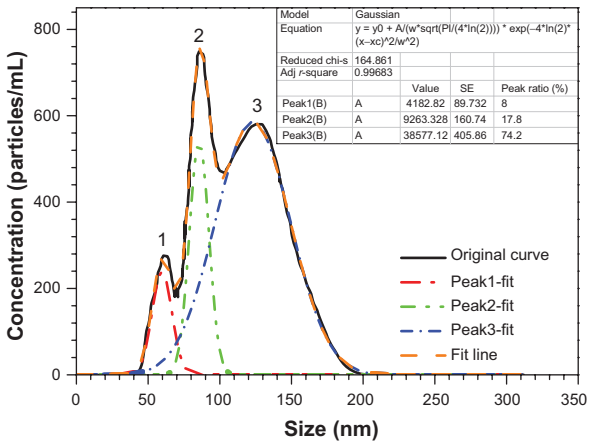

Figure S5 The peak-fit processing of particle size distribution measured by dynamic light scattering.
Abbreviations: SE, standard error; Adj *r*-square, adjusted determination coefficient; chi-s, chi-square.

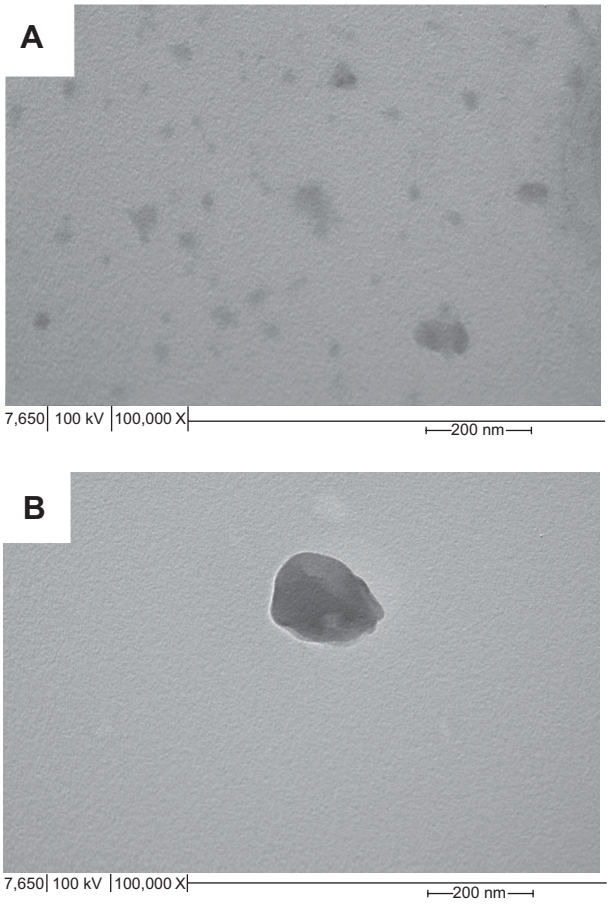

Figure S6 TEM images of *pEGFP*-loaded particles.
Notes: TACS core particles (**A**), and TACS-HBC composite particles (**B**).
Abbreviations: TEM, transmission electron microscopy; *pEGFP, enhanced green fluorescent protein plasmids*; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan.

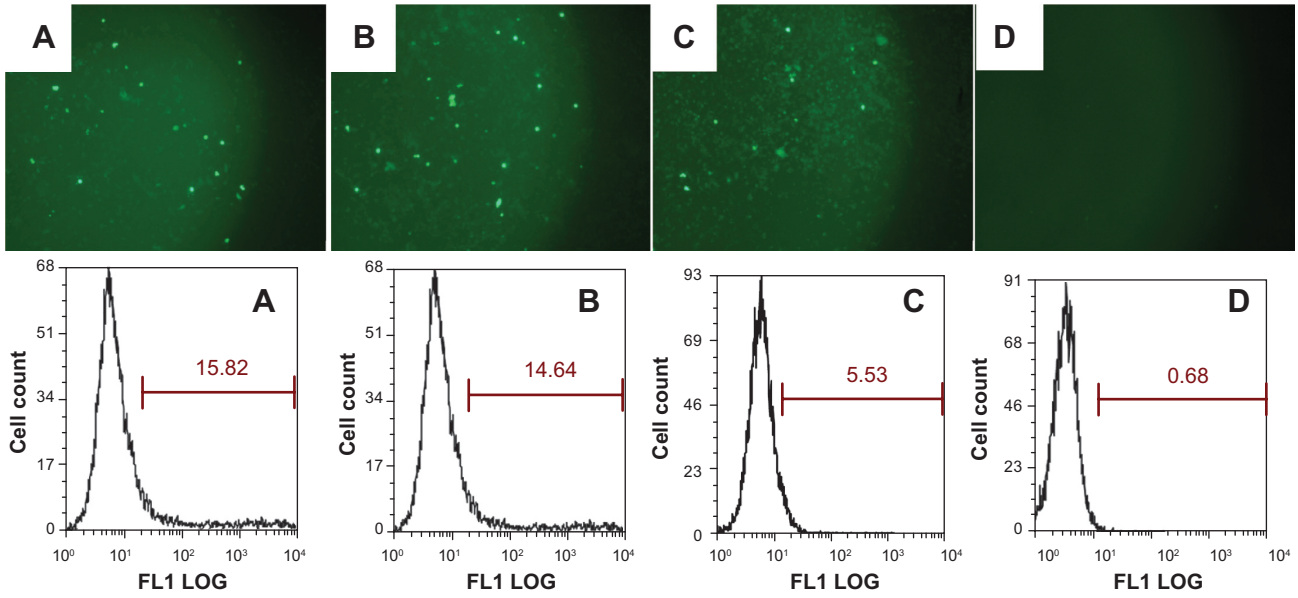

Figure S7 In vitro gene expression efficiency of *pEGFP* loaded in different gene carriers.
Notes: Gene carriers tested include Lip2000 (**A**); TACS particles (**B**); and TACS-HBC composite particles (**C**). Expression was measured in cultured Hela cells after 72 hours, as detected by fluorescence microscope and flow cytometer. Figure portion (**D**) represents the control, which was DMEM without any particles.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; Lip2000, Lipofectamine® 2000 transfection reagent; TACS, thiolated N-alkylated chitosan; HBC, hydroxybutyl chitosan; Hela, human cervix epithelial; FL1, green fluorescence; LOG, logarithmic relative intensity of green fluorescence; DMEM, Dulbecco's Modified Eagle Medium.

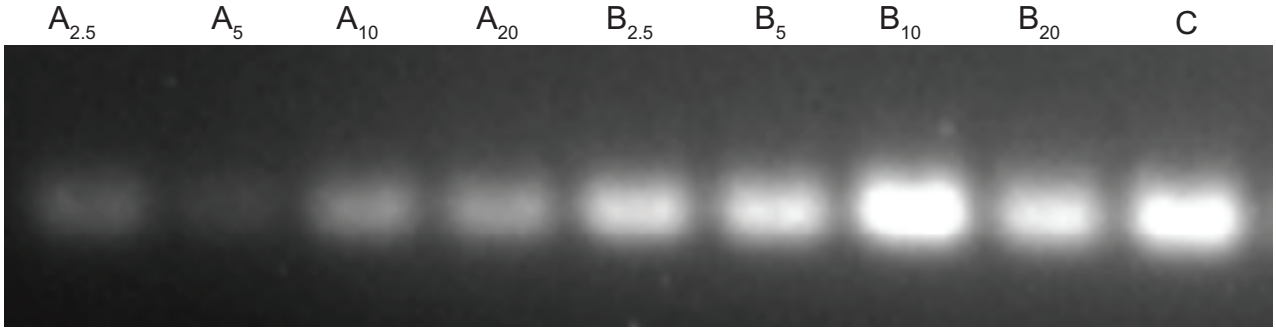

Figure S8 Transfection efficiency analysis by gel electrophoresis.
Notes: Expression *of pEGFP* loaded in HBC/TACS composite particles (gel lines $A_{2.5}$, A_5, A_{10}, and A_{20}); TACS core particles (gel lines $B_{2.5}$, B_5, B_{10}, and B_{20}); and Lip2000 (gel line C) using RT-PCR. Footnotes represent N-P ratios, where the number 10 indicates an N-P ratio of 10:1, for example.
Abbreviations: *pEGFP, enhanced green fluorescent protein plasmids*; HBC, hydroxybutyl chitosan; TACS, thiolated N-alkylated chitosan; Lip2000, Lipofectamine® 2000 transfection reagent; RTPCR, reverse transcriptase polymerase chain reaction.

second from right), which indicates that *pEGFP*-loaded TACS core particles with a NP ratio of 10:1 have a superior transfection efficiency that is comparable with Lip2000. The presence of the HBC shell delays the release rate of *pEGFP*-loaded TACS core particles, which results in lower brightness.