ORIGINAL RESEARCH

A nonviral pHEMA+chitosan nanosphere-mediated high-efficiency gene delivery system

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Abstract: The transport of DNA into eukaryotic cells is minimal because of the cell membrane barrier, and this limits the application of DNA vaccines, gene silencing, and gene therapy. Several available transfection reagents and techniques have been used to circumvent this problem. Alternatively, nonviral nanoscale vectors have been shown to bypass the eukaryotic cell membrane. In the present work, we developed a unique nanomaterial, pHEMA+chitosan nanospheres (PCNSs), which consisted of poly(2-hydroxyethyl methacrylate) nanospheres surrounded by a chitosan cationic shell, and we used this for encapsulation of a respiratory syncytial virus (RSV)-F gene construct (a model for a DNA vaccine). The new nanomaterial was capable of transfecting various eukaryotic cell lines without the use of a commercial transfection reagent. Using transmission electron microscopy, (TEM), fluorescence activated cell sorting (FACS), and immunofluorescence, we clearly demonstrated that the positively charged PCNSs were able to bind to the negatively charged cell membrane and were taken up by endocytosis, in Cos-7 cells. Using quantitative polymerase chain reaction (qPCR), we also evaluated the efficiency of transfection achieved with PCNSs and without the use of a liposomal-based transfection mediator, in Cos-7, HEp-2, and Vero cells. To assess the transfection efficiency of the PCNSs in vivo, these novel nanomaterials containing RSV-F gene were injected intramuscularly into BALB/c mice, resulting in high copy number of the transgene. In this study, we report, for the first time, the application of the PCNSs as a nanovehicle for gene delivery in vitro and in vivo.

Keywords: pHEMA+chitosan nanoparticles, nonviral vector, RSV-DNA vaccine

Introduction

Cellular uptake of nucleic acids (deoxyribonucleic acid [DNA] or small interfering ribonucleic acid [siRNA]) into the cytosolic or nuclear compartment is essential for efficient gene transfection in gene therapy, gene silencing, and DNA vaccine applications. The major impediments in DNA vaccine delivery are the limited transport of DNA through the mucosal membranes and the low bioavailability of delivered DNA arising from degradation and instability.[1,2] Several studies have used nonviral delivery systems because of their safety, minimal immunological reactions, and ease of production for DNA vaccine.[3,4] In addition, for enhanced transfection, negatively charged nucleic acids have been encapsulated into cationic lipids and polymers, which easily pass through cell membranes.[1] Several commercial transfection reagents (for eg, Lipofectamine® 2000 [Life Technologies, Carlsbad, CA, USA], ExGen 500 [Thermo Fisher Scientific, Waltham, MA, USA], etc) and techniques (electroporation, etc) are currently used for the transfection of nucleic acids in vitro. Recent studies have

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shown that DNA can be transfected efficiently into the cells using biocompatible polymers[1,3,5–8] or metallic particles[9–12] in place of liposomal-based transfection reagents for gene delivery. Hu et al[2] proposed that within the acidic conditions of endosomes, polycations could attract protons and act as a "proton sponge" that could disrupt the endosomal vesicles, causing the therapeutic molecules to be released from the cations into the cytosol. Thus, the limited transportation capabilities of recombinant vaccines can be improved by their incorporation into nanostructures that protect them from enzymatic degradation and provide controlled delivery in vivo.[13] Since encapsulated DNA or proteins are protected, this leads to increased stability, targeted delivery, controlled release of molecules and enhanced host immune response.[4,14] In keeping with this, we developed a unique nanomaterial, poly(2-hydroxyethyl methacrylate) (pHEMA)+chitosan nanospheres (PCNSs), comprised of pHEMA nanospheres surrounded by a chitosan cationic shells, and this was used for encapsulation of a respiratory syncytial virus (RSV)-F gene construct (a model for DNA vaccine). We tested the application of PCNSs as a promising nanomaterial for improving the delivery of a DNA vaccine against Respiratory Syncytial Virus (RSV).

RSV infection leads to severe pneumonia and repeated infections in young infants. Despite being the leading cause of lower respiratory tract infections in infants, there is no vaccine against RSV. The antiviral drug Ribavirin (a nucleoside analog) was developed to target hepatitis C and other viruses, including RSV. However, Ribavirin is expensive and not very effective. A safe and effective vaccine conferring long-lasting immunity would be preferred over use of this expensive drug.[15] Vaccine development has been hindered by the antigenic variations of RSV, which lead to escape from immune memory, resulting in frequent reinfections. RSV F protein is highly conserved among the different strains of RSV and highly immunogenic; thus, it has been targeted for vaccine development.[16,17] Based on these characteristics, we used a RSV-F DNA vaccine as a model for nanoparticle mediated delivery.

PCNSs are unique in comparison with other nanomaterials, since they are biologically safe, biodegradable, and inexpensive.[18] Due to its nanoscale size, the positively charged chitosan can electrostatically bind to negatively charged sialic groups in mucin cell membranes and enter into the deep tissues. Also, cationic chitosan molecules can ionically form inter- and intramolecular cross-linkages with multivalent polyanions.[19,20] pHEMA is one of the currently used synthetic nanomaterials that have received safety approval by the US Food and Drug Administration (FDA) for use in industrial, biomedical and pharmaceutical applications. The abundance of hydroxyl groups in its structure makes the attachment of biomolecules, such as DNA or protein, possible. It is one of the most promising drug and vaccine delivery vehicles because of its controlled-release capacity, natural degradability, and inertness in living systems.[21,22] Thus, the pHEMA+chitosan composite nanomaterial might provide a unique, biocompatible, and robust nanovehicle with enhanced penetration efficiency and high stability under various physiological conditions.

Materials and methods
Materials
Medium molecular weight (MMW) chitosan (50,000–190,000 Da), 2-hydroxyethyl methacrylate (HEMA), ammonium cerium (IV) nitrate (CAN), and nitric acid were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Restriction enzymes NotI and BamHI, T4 DNA ligase, Eagle's minimal essential medium (MEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine (100 mM), antibiotics, TrypLE™, 7-aminoactinomycin D (7-AAD), Lipofectamine 2000, TaqMan® Master Mix 2× (Life Technologies), real-time probe, primers, SuperScript® II Reverse Transcriptase, and RNAlater® Tissue Collection: RNA Stabilization Solution were all obtained from Life Technologies. All DNA and ribonucleic acid (RNA) isolation kits were purchased from Qiagen (Germantown, MD, USA). The MEM was supplemented with 10% FBS (MEM-10), penicillin (45 μg/mL), streptomycin (100 μg/mL), kanamycin (75 μg/mL), and L-glutamine (1 mM). Human epithelial type 2 (HEp-2) and monkey kidney (Vero and Cos-7) cells were obtained from American Type Culture Collection (ATCC®, Manassas, VA, USA). The cell toxicity assay was performed using the Cell Titer96® AQueous One Solution Cell Proliferation Assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye-based cell proliferation assay kit from Promega Corp (Madison, WI, USA). All chemicals used in experiments were analytical grade.

Construction of recombinant RSV-F DNA vaccine model
The RSV-F DNA sequence originally published by Collins et al[23] was synthesized by Epoch Life Science Inc (Missouri City, TX, USA) and amplified by polymerase chain reaction (PCR) using forward and reverse primers (Table 1). Both the RSV-F gene and the phCMV1 DNA vector were digested with BamHI and NotI restriction enzymes. The purified DNA

(QIAquick Gel Extraction Kit (Qiagen)) pieces were sealed by T4 DNA ligase enzyme and transformed into competent cells of *Escherichia coli* DH5α. For selection, competent cells were grown on kanamycin supplemented luria broth (LB) agar. Positive clones were verified by RE (BamHI and NotI) digestion and DNA sequencing. Recombinant clones containing the RSV-F gene in the phCMV1 vector were named PF. The recombinant PF vector was purified using the Qiagen EndoFree Plasmid Giga kit, and the purified PF aliquots were stored at −80°C until use for encapsulation into the PCNSs and transfection into eukaryotic cells.

Construction of the phCMV1 vector containing GFP gene

The GFP gene was amplified by PCR, using forward and reverse primers (Table 1), with *BamH*I and *Not*I restriction sites, respectively. The amplified GFP gene was inserted into the phCMV1 vector following the same protocol and conditions as described above. The clones containing the GFP gene in the phCMV1 vector were named PG.

Purification of chitosan and synthesis of PCNSs

Chitosan nanoparticles were prepared by alkaline deacetylation of the chitosan. Purification of the chitosan nanoparticles was performed using the protocols of Gan et al[19] with slight modifications. Medium molecular weight chitosan powder (5 g) was dissolved in 70 mL of 1 M sodium hydroxide (NaOH), with continuous stirring at 50°C for 2 hours. The supernatant was removed after centrifuging the sample at 2100 RCF for 5 minutes. The chitosan pellet was washed twice with deionized water, and the supernatant removed by centrifugation. The chitosan pellet was then dried overnight in an incubator, at 40°C. The following day, the dried chitosan was completely dissolved in 0.1 M acetic acid, and the insoluble particles were removed by centrifugation.

The pH of the supernatant was slowly adjusted to 8.0 with 1 M NaOH until pure chitosan appeared as white precipitate. The precipitated chitosan was washed twice with deionized water, and then vacuum-dried overnight at room temperature. The final pellet was kept at room temperature until needed.

The complex coacervation method described by Atyabi et al[18] was followed, with some modifications, to prepare the PCNSs. The purified medium molecular weight chitosan (75 mg) was dissolved in 10 mL 0.2 M nitric acid solution by vigorous stirring at 40°C and filtered to remove the insoluble particles. The clear chitosan nanoparticle solution was stirred at 40°C for an additional 10 minutes, and then, CAN (0.08 M) and HEMA (0.16 M) were slowly added into 10 mL of the chitosan solution while gently stirring, at 40°C for up to 40 minutes. The solution at room temperature was adjusted to a final pH of 5.0 with 5 M NaOH.

Encapsulation of PF DNA into PCNSs

The PCNSs were synthesized as described in the previous section and used for DNA encapsulation. The PF DNA was mixed with HEMA and then added to the chitosan solution. Then, the HEMA-DNA mixture was transferred into the chitosan-CAN solution (pH 4.0) dropwise with continuous stirring. The final DNA concentration in the PCNS-DNA solution was 100 μg/mL. All the PCNS or PCNS-DNA solutions were stored at pH 5.0 in the refrigerator until further use.

Characterization of PCNSs with/without DNA

Transmission electron microscopy (TEM), Fourier transform infrared (FTIR) spectroscopy, ultraviolet (UV) visible spectroscopy, and zeta potential analyses were used to verify the synthesis of PCNSs and their encapsulation of DNA. Cytotoxicity was done to test cell toxicity while the release assay determines amount of DNA released at various time intervals and conditions.

Table 1 The names and sequences of probes and primers that were used to generate RSV-F and GFP genes for cloning in phCMV1 vector

Name of the primers	Sequence of the primers
1. phCMV1-F full *BamH*I Frw	GGATCCACCATGATGGTCCTCAAAGCAAATGCAATTACCAC
2. phCMV1-F full *Not*I Rev	CCACCGCGGCCGCTTATCATTGTCGACCAATATTATTTATACCACTC
3. phCMV1-F *Not*I Rev	CCACGCGGCCGCTTGTCGACCAATATTATTTATACCACTCAG
4. phCMV1 GFP *BamH*I Frw	GGATCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGG
5. RS-F1 Forward primer	AACAGATGTAAGCAGCTCCGTTATC*
6. RS-F2 Reverse primer	CGATTTTTATTGGATGCTGTACATTT*
7. RS-F3 Probe	TGCCATAGCATGACACAATGGCTCCT*

Notes: *According to the sequences published by Mentel et al.[24] All sequences are given 5′-3′ direction.
Abbreviations: GFP, green fluorescence protein; qPCR; quantitative polymerase chain reaction; RSV-F, respiratory syncytial virus-fusion gene.

In vitro DNA release from the PCNS-DNA complex

PCNS or PCNS-DNA solutions (1 mL each) were centrifuged at 18,000 RCF for 10 minutes. The pellet was washed once in 1 mL of 1 × phosphate-buffered saline (PBS) (pH 7.0) and suspended in 1 mL 1 × PBS, followed by incubation at 37°C with shaking at 200 rpm. The sample collection was performed by repeating the steps described above. Supernatants were collected and saved at the end of each incubation time, for up to 68 days. The concentrations of released DNA were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), at 260 nm wavelength. In order to confirm the integrity of plasmid DNA, the supernatants, collected at time periods (days 0.5, 1, 10, 21, 31, 42, and 56) during the DNA-release study, were run on 1% agarose gel. The PF DNA (60 ng/well) was used as the positive control.

TEM analysis

A Zeiss EM 10C 10CR TEM (Carl Zeiss Meditec, Oberkochen, Germany) was used to examine the morphology and size of the PCNSs and DNA-encapsulated PCNSs. The nanoparticle solutions were diluted in distilled water to 1/50 (v/v) before dispersion onto the copper grid (carbon-coated copper grid, 200 mesh). After a 5-minute incubation, the samples were stained with 1% phosphotungstic acid. The morphology and size of the nanospheres were verified by two independent experiments, with at least ten scans of randomly chosen areas.

Determination of zeta potential

The zeta potential of chitosan-alone, HEMA-alone, and PCNSs with/without DNA was measured using a Zetasizer (Nano-ZS; Malvern Instruments Ltd, Malvern, UK). The nanoparticle solutions were diluted in distilled water to 1/10 (v/v), and three repeated measurements were taken from each sample. The average of two independent experiments was taken as the final value.

FTIR spectroscopy

FTIR spectra were recorded for chitosan-alone, HEMA alone, and for PCNS complexes, in attenuated total reflectance (ATR) mode using an infrared (IR) spectrophotometer (Nicolet 380 FT-IR; Thermo Fisher Scientific). The spectra were obtained with 64 scans per sample, ranging from 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1}. The sample chamber was purged with dry N_2 gas.

UV visible spectroscopy

Chitosan-alone, HEMA-alone, and PCNS samples were diluted in distilled water 1/10 (v/v), and the absorption spectra were recorded using a Beckman Coulter DU® 800 (Beckman Coulter, Inc, Brea, CA, USA) at the end of wavelength ranging from 280–400 nm.

In vitro cytotoxicity of the PCNSs

The cytotoxicity of the PCNSs was tested on Cos-7 cells with an MTT toxicity assay and flow cytometric method as per manufacturer's guidelines.

MTT dye-based cell proliferation assay

Following the manufacturer's protocol, 1×10^4 cells/well in 100 μL MEM-10 were seeded into a 96-well plate. The cells were allowed to grow overnight in an incubator at 37°C and 5% CO_2 humidified atmosphere. After overnight incubation, the media from the 96-well plate were replaced with the serial dilutions of PCNSs in cell culture media (MEM-10). The treated cells were further incubated at 37°C and 5% CO_2 for 24, 48, and 72 hours. At the end of the corresponding incubation time, 15 μL of MTT dye was added into the each well and the plate was allowed to incubate again for the next 4 hours, in darkness. The reaction was then stopped with 100 μL of stop solution. The absorbance of the plate was measured at 570 nm on a TECAN Sunrise™ enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan US, Inc., Morrisville, NC, USA). Nontreated cells, in growth media, were used as a control.

Flow cytometry cytotoxicity assay

Cos-7 cells at 2×10^5 cells/well were seeded into 12-well plates. The nanoparticle concentrations, incubation intervals, and growth conditions were similar to those of the MTT assay. At the end of the incubation time, the cells were collected by trypsinization and centrifugation and were stained with 7-AAD (1 μg/mL) dye solution for 5 minutes at room temperature prior to flow cytometric analysis on a Becton Dickinson FACS Canto™ II (BD Biosciences, San Jose, CA, USA). Dead and live cells were analyzed using FlowJo 7.6 software (Tree Star Inc©, Ashland, OR, USA).

Proof of encapsulation of DNA into pHEMA core

To prove the stability of coated DNA and the encapsulation of DNA into the pHEMA rather than chitosan, the PCNS-DNA complex (1 μg DNA/reaction) was digested in the presence/

absence of chitosanase. Naked PF DNA (1 μg DNA/reaction) was used as a positive control. The protocol used was slightly modified from that described by Boyoglu et al.[5] The digestion reaction was carried out by incubating the samples in a 37°C water bath for 3 hours. Later, the samples were visualized on 1% agarose gel and imaged using ChemiDoc™ XRS (Bio-Rad Laboratories Inc, Hercules, CA, USA) system.

PCNS-mediated gene transfection for immunofluorescence microscopy

The PG construct was transfected into Cos-7 cells in order to show the uptake of PCNSs and expression of the GFP protein in Cos-7 cells. A specific protocol was developed for delivery of the PG construct via PCNSs. Briefly, 2×10^5 Cos-7 cells/well were seeded into a 12-well plate and grown overnight; the next day, the medium was replaced with HBSS, followed by mixing with 2.5% solution of the PCNSs/DNA complex. The mixture was incubated for 1 hour until the PCNSs settled down and attached to the cell membrane. At the end of the PCNS treatment, the HBSS was replaced with MEM-10, and the cells were incubated for 3 days. After 3 days of incubation, the uptake of the PCNSs with/without GFP was observed using an immunofluorescence microscope. PCNSs/phCMV1 DNA was used as a negative control.

TEM ultramicrosection showing the uptake of PCNSs in Cos-7 cells

Cos-7 cells, transfected using PCNSs as described above, were trypsinized, centrifuged, fixed, and processed for ultramicrotome sectioning. Collected cells were fixed in primary fixative (10% glutaraldehyde, 25% paraformaldehyde in 0.1 M phosphate buffer) for 3 hours, followed by secondary fixative (1% OsO_4 diluted in 0.1 M phosphate buffer) for 2 hours, in darkness, and final dehydration steps, including serial incubations in 30%, 50%, and 70% ethanol. The cells were washed with 0.1 M phosphate buffer (pH 8.0) and centrifugated between each step. The processed cells were embedded into epoxy resin, and ultrathin sections were taken before staining with uranyl acetate and lead citrate, and the cells were then observed under a TEM microscope.

Quantitative PCR (qPCR) analysis for comparing transfection efficiencies in Cos-7 cells

In order to analyze the transfection efficiency, Cos-7 cells were transfected with the PF construct using PCNSs- and Lipofectamine 2000–mediated gene transfection. The PCNS-mediated gene transfection was described in an earlier section. The Lipofectamine 2000 transfection reagent was used for in vitro transfection, following the manufacturer's instructions.

qPCR analysis

Following the transfection experiment, the total RNA was isolated from harvested cells and 1 μg of total RNA was converted into complementary (c)DNA using superscript reverse transcriptase enzyme, following the manufacturer's protocols. The RSV-F gene-specific primers, probe, and the experimental protocol for qPCR were adapted from Mentel et al.[24] The qPCR reaction was carried out with 20 μL total reaction mixture containing 2 μL of cDNA, 1 μL of 5 μM reverse and forward primers (Table 1), 2 μL of 2.5 μM probe, and 10 μL of TaqMan master mix 2X, using the Applied Biosystems® ViiA™ 7 real time PCR (Life Technologies). Each qPCR reaction was run in duplicate along with water as a negative control. Dilutions of the RSV-F gene amplicon (10^0–10^8 copy numbers) were used to prepare the standard curve. Each experiment was repeated twice from the transfection step.

In vivo quantification of RSV-F gene in BALB/c mice

Animal studies were performed according to the National Institutes of Health (NIH) guidelines following a protocol approved by the Alabama State University Institutional Animal Care and Use Committee. The animals were housed under standard approved conditions and provided daily with sterile food and water ad libitum. Six- to 8-week-old female BALB/c mice (Charles River Laboratories Inc, Wilmington, MA, USA) were injected with the negative controls, PBS, PCNSs/phCMV1 DNA, naked PF DNA (50 μg/mouse), or PCNSs-PF (30 μg/mouse) to show the expression of RSV-F gene in vivo. The DNA or PCNSs-DNA solutions were brought to the final volume of 300 μL with $1 \times$ PBS. BALB/c (three mice per group) were injected intramuscularly into the skeletal muscle (150 μL of solution/muscle) and reared for 1 week, prior to being sacrificed by CO_2 inhalation. Aseptically collected skeletal muscles were kept in RNAlater solution until the RNA was isolated and converted into cDNA, followed by qPCR analysis. The RNeasy Fibrous Tissue Mini Kit (Qiagen) was utilized for RNA isolation, and the other reagents and protocols for cDNA synthesis and qPCR were the same as described in earlier sections.

Statistical analysis

Statistical analysis of the qPCR data was performed using Minitab® 15 statistical software (State College, PA, USA). Differences between the means of four experimental groups were determined using one-way analysis of variance (ANOVA) with the significance level of 5%.

Results
Synthesis and characterization of PCNSs

In this study, we developed PCNSs and used these to encapsulate a DNA vaccine for efficient transfection of eukaryotic cells without the need for transfection reagents. The PCNSs were characterized using different microscopic

and spectroscopic methods. The hydrophobic pHEMA core of the PCNSs was surrounded by a positively charged hydrophilic chitosan coating (Figure 1A). DNA was encapsulated in the pHEMA core and surrounded by a chitosan shell which had affinity for the negatively charged cell membrane (Figure1B). According to the TEM images, the diameter of the PCNSs ranged between 150–250 nm (Figure 1C). The size and shape of the composite nanospheres remained unchanged after encapsulation of the PF gene construct at a concentration of 100 µg/mL, indicating that the DNA was encapsulated in the pHEMA core and not adsorbed on the chitosan shell (Figure 1D). The size of the nanospheres was in accordance with those of other studies.[18,21] The synthesized nanosphere
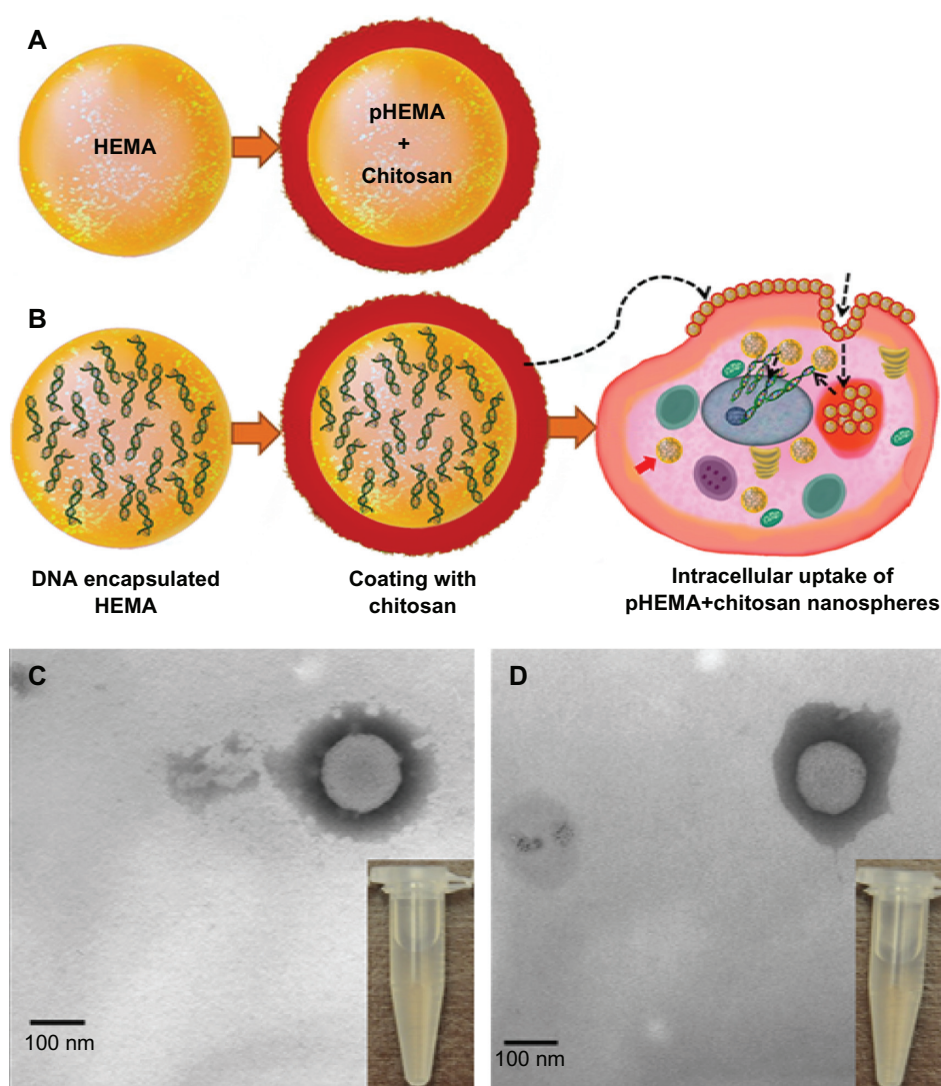
Figure 1 Development of the PCNS complex. (**A**) Schematic diagram of PCNSs showing pHEMA surrounded by chitosan shell. (**B**) Schematic diagram of PCNSs loaded with RSV-F DNA and the process of intracellular uptake. (**C**) TEM image of the PCNSs, and the synthesized PCNSs in a tube, showing no agglomeration. (**D**) TEM image of PCNSs encapsulated with RSV-F DNA, and the synthesized nanospheres in a tube, showing no visible agglomeration.
**Note:** The shape of the PCNSs was spherical, with approximately 200 nm diameter; encapsulation of DNA did not change either the shape or the diameter of the PCNSs.
Abbreviations: HEMA, 2-hydroxyethyl methacrylate; PCNSs, pHEMA+chitosan nanospheres; pHEMA, poly(2-hydroxyethyl methacrylate); RSV, respiratory syncytial virus; TEM, transmission electron microscopy.

solutions were colorless (Figure 1C and D inset) and stable over a month, at 4°C.

The chemical interaction and bond formation between the chemical groups of HEMA and chitosan were confirmed with FTIR analysis (Figure 2A). The characteristic peak of the chitosan is at 1559.17 cm⁻¹, due to the stretching vibration of the amino group of chitosan. Another peak at 3367.1 is due to amine NH symmetric vibration. HEMA shows specific peaks at around 1000–1500 cm⁻¹, with a band appearing at 1405 cm⁻¹ that is attributed to =CH2, and asymmetric methyl bending CH3 at 1458 cm⁻¹. However, the newly formed composite PCNSs had a transmittance behavior similar to that of chitosan alone, along with faint peaks at ≈1000–1500 cm⁻¹, apparently arising from the pHEMA. The hazy peaks for PCNSs were probably related to the chemical bond formation between chitosan molecules and functional groups found in the chemical

structure of pHEMA. Similarly, the UV visible spectra of these nanoparticles supported the idea that the pHEMA core was surrounded by chitosan (Figure 2B). pHEMA alone has a specific absorption maxima at 286 nm, while chitosan displays an absorption maxima at 300 nm. The fact that the PCNS composite nanomaterials showed the exact same absorption maxima as chitosan at 300 nm could be due to the chitosan shell. The zeta potential values of chitosan alone, pHEMA alone, and the PCNSs with and without DNA were +20.82, −16.27, +38.42, and +38.15, respectively (Figure 2C). Similar values were obtained when the PCNSs were encapsulated with gene constructs. As expected, the zeta potential value of chitosan alone (+20.82) was positive, but pHEMA alone had a negative zeta potential value (−16.27) due to the chemical nature of the respective nanomaterials. Plasmid DNA was confirmed to have been successfully incorporated into the PCNSs because there was
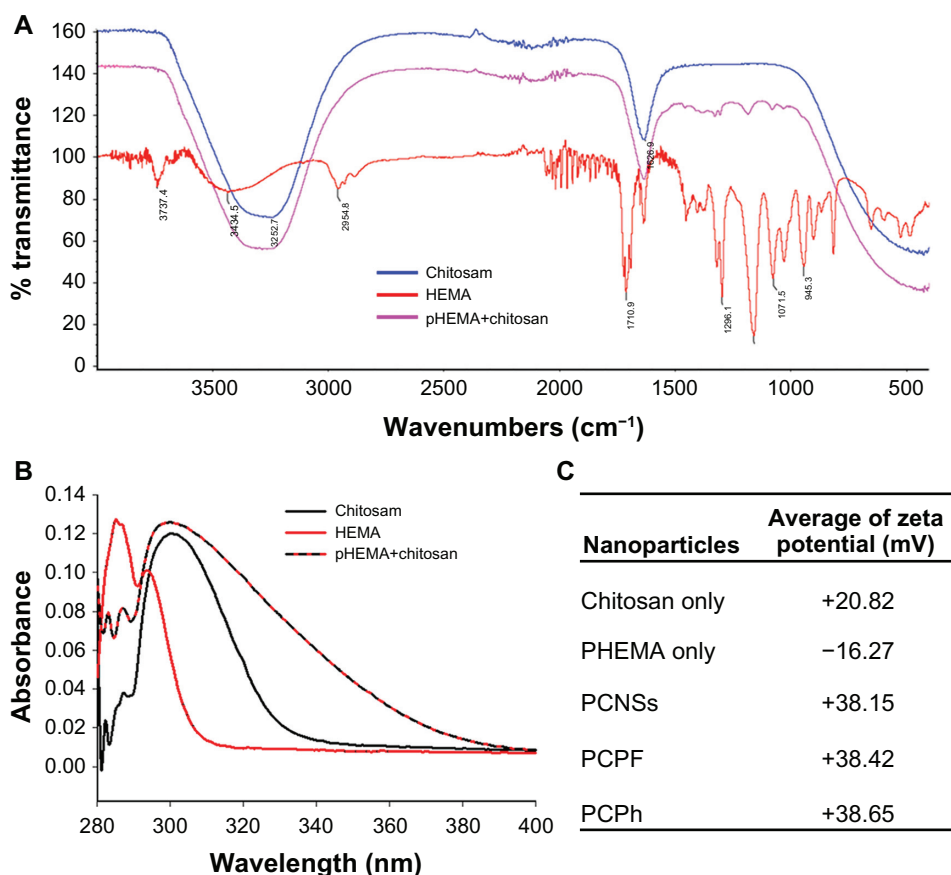
Figure 2 Characterization of PCNSs. (**A**) FTIR spectra of chitosan, HEMA, and PCNSs. HEMA displayed specific peaks between ≈1000–1500 cm⁻¹. However, the transmittance characteristic of the PCNSs was more similar to chitosan only, with noise (≈1000–1500 cm⁻¹) from pHEMA. (**B**) UV visible spectra of chitosan, HEMA, and PCNSs. The HEMA displayed specific absorption maxima at 286 nm, whereas chitosan showed a clearly distinct absorption peak at 300 nm. The PCNSs also exhibited an absorption maxima at 300 nm, the same as chitosan only. (**C**) Zeta potential values of chitosan nanoparticles, HEMA, PCNSs, and PCNSs encapsulated with PF gene and Ph.
Notes: The chitosan nanoparticles were positively charged while HEMA was negatively charged. The PCNSs with/without DNA were also positive, since the positively charged chitosan shell wrapped around the HEMA core in which the DNA was loaded. The zeta potential values are another example of the core–shell relationship between the pHEMA and chitosan.
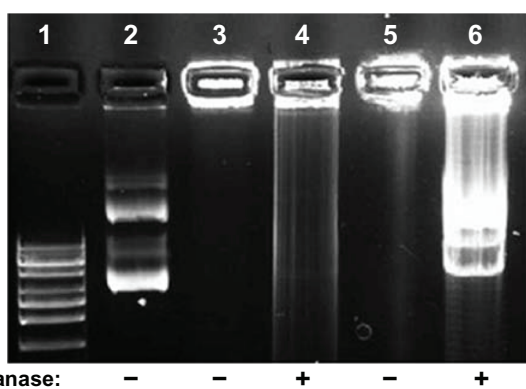**Abbreviations:** FTIR, Fourier transform infrared spectroscopy; HEMA, 2-hydroxyethyl methacrylate; PCNSs, pHEMA+chitosan nanospheres; PCPF, pHema Chitosan Plasmid F gene; PCPh, pHema Chitosan pHCMVI plasmid; PF, phCMVI+respiratory syncytial virus-fusion gene; Ph, phCMVI control DNA; pHEMA, poly(2-hydroxyethyl methacrylate); UV, ultraviolet.

no DNA detected in the supernatant after centrifugation and separation of the PCNSs from the suspension.

According to our hypothesis, the target DNA was encapsulated into the pHEMA core instead of the chitosan shell, increasing the release time into the cytoplasm and the transfection efficiency. In a previous study, the interaction of DNA with chitosan was revealed by showing the release of DNA from the chitosan-DNA complex that was digested with chitosanase enzyme.[5] Similarly, we substantiated the encapsulation of DNA into the pHEMA core. After the PCNSs/ DNA complex was treated with chitosanase, no DNA band was observed on the agarose gel (Figure 3). This showed the encapsulation of DNA into the pHEMA core, and not into the chitosan shell. In a recent report, the encapsulation of a DNA vaccine into pHEMA nanoparticles was shown to be highly efficient.[13]
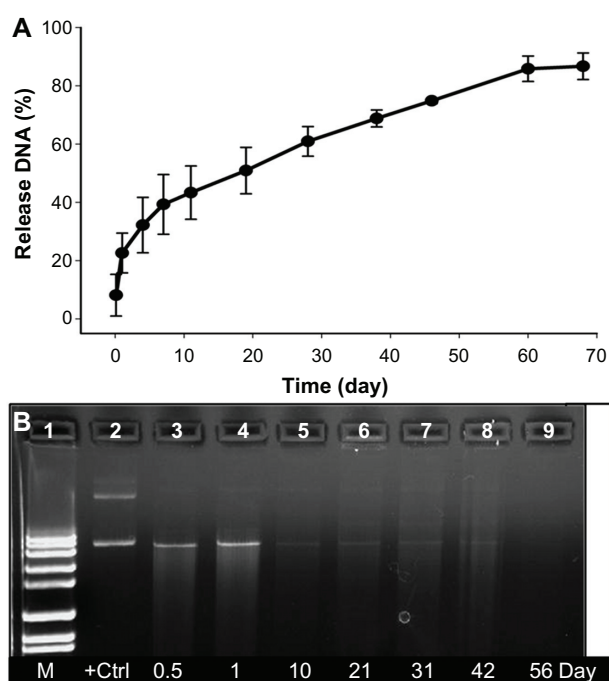
A DNA release assay was performed in $1 \times$ PBS for 68 days, resulting in the release of DNA. The DNA release was highest on day 1, with 22% release on that day, followed by a continuous slow release until day 60. About 85% of DNA had been released by the end of day 60 (Figure 4A). In contrast, in another study, a DNA vaccine encapsulated into chitosan alone was released in a week.[5] The fast release of DNA from the chitosan nanoparticles was due to the weak interactions between the DNA and chitosan. Our intent was to load DNA into the pHEMA-core to extend the release time of the DNA plasmid. The quantity of DNA released upon

Figure 4 Release of DNA from the PCNS complex. (**A**) spectrophotometer measurements. As incubation progressed, DNA release increased and reached 85% by day 60. (**B**) A 1% agarose gel electrophoresis showing DNA released from the PCNS complex.
Notes: (1) DNA marker; (2) DNA alone; (3–9) DNA collected upon day 0.5, 1, 10, 21, 31, 42, and 56 incubations, respectively.
Abbreviations: DNA, deoxyribonucleic acid; PCNS, pHEMA+chitosan nanosphere; pHEMA, poly(2-hydroxyethyl methacrylate).
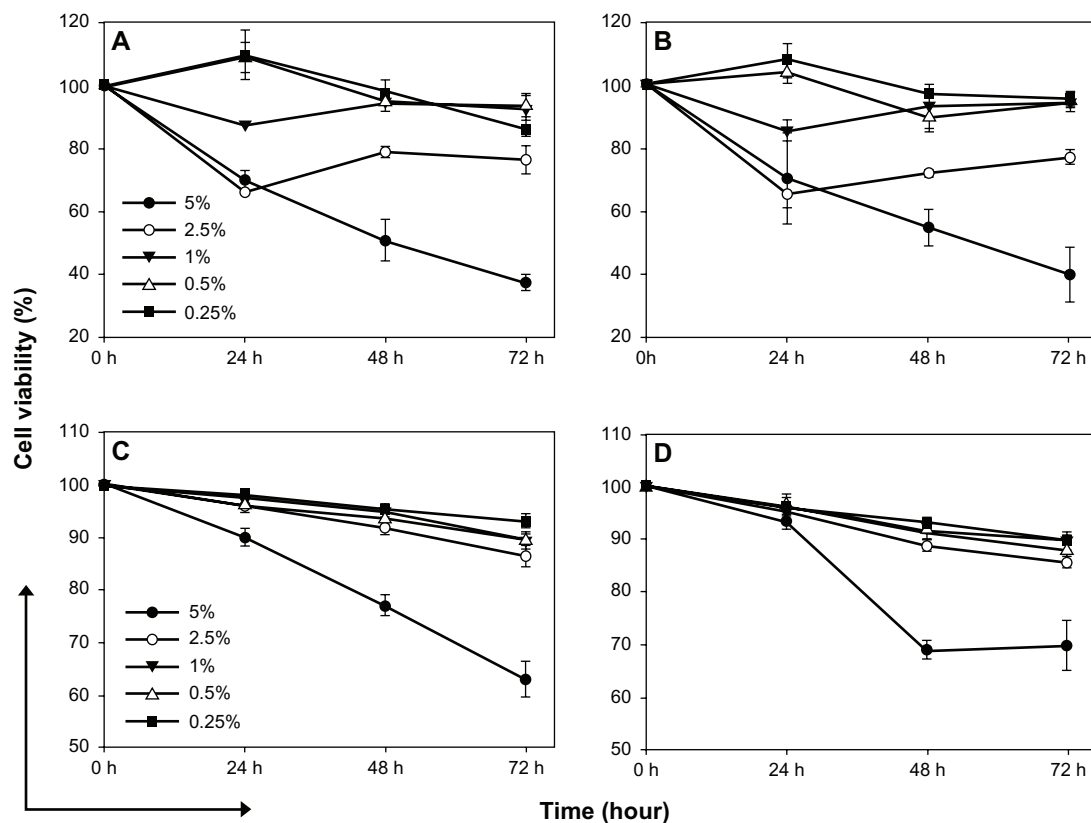
different incubation times was determined using a NanoDrop spectrophotometer (Figure 4A), which corresponds to the intensity of DNA bands visualized on 1% agarose gel (Figure 4B). The method of DNA encapsulation into PCNSs was also critical. Our preliminary experiments showed that DNA can be rapidly degraded if it is not encapsulated into PCNSs under proper pH conditions (data not shown).

The cytotoxicity of PCNSs was tested in Cos-7 cells using MTT cytotoxicity and flow cytometry. Concentrations ranging from 0.25% to 5% (w/v) were used for both PCNSs alone and PCNSs/DNA. The PCNSs with/without DNA did not display significant cytotoxicity (Figure 5). Up to 80% of Cos-7 cells were viable at a 2.5% PCNS concentration, which decreased to <60% at a 5% PCNS concentration. As the length of treatment was prolonged, a higher cytotoxic effect was observed.

Uptake of PCNSs in Cos-7 cells using TEM

TEM was performed to study the translocation and mechanism of endocytosis of PCNSs into Cos-7 cells. The cells were mixed with DNA-encapsulated PCNSs

Figure 3 Chitosanase digestion of PCNSs-PF and chitosan-PF nanoparticles to demonstrate the encapsulation of DNA into pHEMA.
Notes: (1) DNA marker; (2) DNA control; (3) PCNSs-PF without chitosanase treatment; (4) PCNSs-PF treated with chitosanase; (5) Chitosan-DNA nanoparticles without chitosanase treatment; and (6) Chitosan-DNA nanoparticles treated with chitosanase. The chitosanase treatment degraded chitosan, and DNA was released from chitosan nanoparticles (lane 6); when PCNSs-PF was treated with chitosanase (lane 4), DNA was not released, since the DNA was encapsulated into pHEMA. The DNA did not migrate on the agarose gel.
Abbreviations: chitosan-PF, chitosan-respiratory syncytial virus-fusion gene; DNA, deoxyribonucleic acid; PCNSs-PF, pHEMA+chitosan nanospheres-phCMVI+respiratory syncytial virus-fusion gene; pHEMA, poly(2-hydroxyethyl methacrylate).

Figure 5 Cytotoxicity of PCNSs with/without DNA. (**A**) MTT cytotoxicity assay of PCNSs alone and (**B**) PCNSs encapsulated with PF DNA. (**C**) Flow cytometry cytotoxicity assay of PCNSs alone and (**D**) PCNSs encapsulated with PF DNA.
Notes: Both techniques showed the same cytotoxicity pattern on Cos-7 cell. The highest concentration (5% v/v) of PCNSs was determined as cytotoxic for Cos-7 cells.
Abbreviations: DNA, deoxyribonucleic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNSs, pHEMA+chitosan nanospheres; PF, phCMV1+ respiratory syncytial virus-fusion gene; pHEMA, poly(2-hydroxyethyl methacrylate).

in serum-free conditions and were incubated for 3 days. Compared with the nontreated control (Figure 6A), the positively charged PCNSs were observed to have attached to the negatively charged cell membrane. These PCNSs were found accumulated on the cell membrane and caused membrane invagination, as was evident in the TEM image (Figure 6B and C). The PCNSs were endocytosed and were dispersed in the cytosol (Figure 6C and D).

In vitro analysis of PCNS uptake using immunofluorescence microscopy and flow cytometry

In order to verify the integrity of the DNA and the transfection efficacy of the PCNS nanospheres carrying the DNA cargo, we performed in vitro expression using Cos-7 cells, as described earlier. A DNA plasmid containing GFP as a reporter gene was constructed for expression in cells and their subsequent analysis using immunofluorescence microscopy (Figure 6E and F). A clear expression of GFP protein in the cells indicated the stability of DNA and its ability to be transcribed (Figure 6F). The expression of the GFP gene was

also analyzed using flow cytometry and confirmed robust expression of the GFP genes (Figure 6G) compared with the control cells.

In vitro quantification of RSV-F gene transfected via PCNSs

We also demonstrated that use of the PCNSs resulted in high transfection of the RSV-F gene in vitro. We transfected three different cell lines (Cos-7, HEp-2, and Vero) with RSV-F gene that was encapsulated in PCNSs. These studies showed that the transfection yielded a high copy number of RSV-F transcript, with high efficiency. These results further supported our hypothesis that PCNSs can transfect different cell types, with the release of DNA from the nanospheres leading to enhanced transcription in vitro. During the transfection studies, PCNSs-DNA was incubated with cells without the use of cationic transfection reagent. In fact, we used one of the most preferred transfection reagents, Lipofectamine 2000, for comparison with PCNS-mediated gene transfection. The PCNSs were found to be as robust as the commercial transfection agent in all
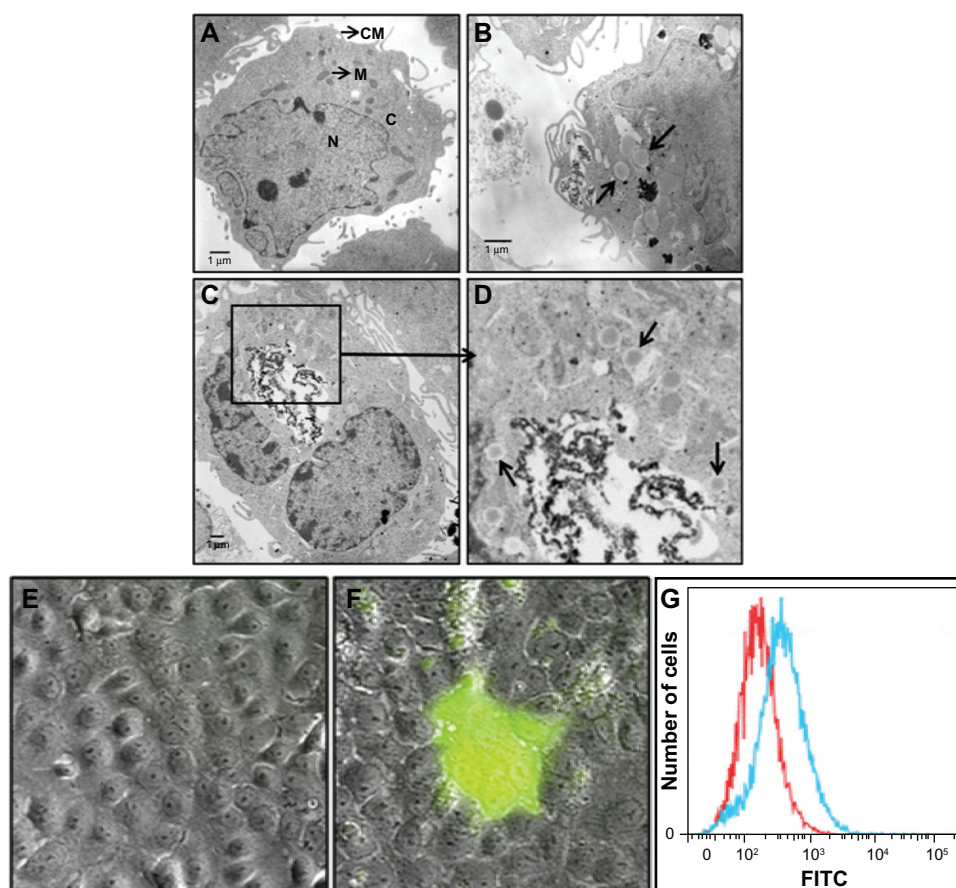
Figure 6 TEM, immunofluorescence, and FACS studies of Cos-7 cells transfected with PCNSs. TEM images showing uptake of PCNSs into Cos-7 cells. (**A**) Cells with PCNSs-Ph as a negative control. (**B–C**) TEM images showing the process of PCNS uptake into the Cos-7 cells through endocytosis. (**D**) Inset of image C, showing the escape of the pHEMA core into the cytoplasm. Immunofluorescence microscopy images of (**E**) cells only and (**F**) expression of GFP gene transfected using PCNSs. (**G**) Flow cytometry analysis showing the expression of the GFP gene (blue color) transfected using PCNSs, in comparison with the control cells (red color).
Abbreviations: C, cytoplasm; CM, cell membrane; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; M, mitochondria; N, nucleus; PCNSs, pHEMA+chitosan nanospheres; PCNSs-Ph, pHEMA+chitosan nanospheres-phCMVI; pHEMA, poly(2-hydroxyethyl methacrylate); TEM, transmission electron microscopy.

three cell lines studied, as evident from the quantitative real-time PCR (Table 2). We also used electroporation to transfect the most efficient cell line, Cos-7, with PF DNA, which yielded a similar transfection efficiency to that of Lipofectamine 2000- and PCNS-mediated transfection (Data not shown).

In vivo quantification of RSV-F gene encapsulated in PCNSs

RSV-F DNA vaccine encapsulated in the PCNSs was efficiently delivered in mouse skeletal muscles, resulting in higher level of gene expression of the DNA vaccine in comparison with the controls, as evidenced by qPCR analysis of the RSV-F gene (Figure 7). PCNSs were highly effective in delivering DNA in muscles, even at a lower concentration (30 μg), while naked DNA did not show any gene expression at 50 μg. The significant differences between the means of groups were analyzed using one-way ANOVA.

Discussion

Chitosan is also biologically safe, since it is naturally derived and biodegradable. Also, it specifically adheres to mucosal membranes, facilitating its selective delivery into mucosal cells.

Gene delivery systems have limited applications because most are either not effectively internalized or are degraded in the host cytoplasm. Nanoparticles have been widely used to enhance the delivery of the targeted gene. We devised, for the first time, a pHEMA-chitosan composite nanomaterial as a nonviral gene delivery vehicle. In our system, pHEMA serves as the core, while chitosan surrounds the pHEMA, forming the shell. The DNA is encapsulated in the pHEMA and in turn, in the chitosan, thus DNA is protected within the core–shell structure. We confirmed this structure by using TEM analysis, zeta potential value, FTIR, and UV visible spectra. TEM images clearly illustrated the pHEMA core was enveloped by the chitosan shell. The positive zeta potential value of the

Table 2 qPCR results representing copy numbers of RSV-F mRNA of cells transfected with Lipofectamine® 2000 (Life Technologies, Carlsbad, CA, USA) and PCNSs

Cell lines	Groups	Lipofectamine® 2000	PCNSs
Cos-7	Untreated cells	$9 \pm 4.E+01$	$2 \pm 1.E+03$
	Naked PF DNA	$4 \pm 2.E+03$	$2 \pm 08.E+04$
	PCNSs-Ph control	$2 \pm 1.E+03$	$2 \pm 1.E+03$
	PCNSs-PF	$\pm 2.E+08$	$2 \pm 1.E+08$
HEp-2	Untreated cells	$5 \pm 2.E+01$	$5 \pm 1.E+01$
	Naked PF DNA	$5 \pm 2.E+02$	$2 \pm 1.E+02$
	PCNSs-Ph control	$5 \pm 1.E+01$	$1 \pm 06.E+03$
	PCNSs-PF	$2 \pm 1.E+08$	$8 \pm 05.E+06$
Vero	Untreated cells	$3 \pm 1.E+01$	$2 \pm 03.E+00$
	Naked PF DNA	$1 \pm 03.E+03$	$1 \pm 07.E+03$
	PCNSs-Ph control	$3 \pm 06.E+00$	$6 \pm 06.E+01$
	PCNSs-PF	$5 \pm 03.E+07$	$3 \pm 1.E+06$

Abbreviations: Cos-7, monkey kidney cells; DNA, deoxyribonucleic acid; HEp-2, human epithelial type 2 cells; mRNA, messenger ribonucleic acid; PCNSs, pHEMA+chitosan nanospheres; PCNSs-PF, pHEMA+chitosan nanospheres-phCMV1+respiratory syncytial virus-fusion gene; PCNSs-Ph, pHEMA+chitosan nanospheres-phCMV1 control DNA; PF, phCMV1+respiratory syncytial virus-fusion gene; qPCR, quantitative polymerase chain reaction; RSV-F, respiratory syncytial virus-fusion gene; Vero, kidney epithelial cells.

PCNS complex was another proof of the core–shell interaction between the pHEMA and chitosan, since the negative charge of the pHEMA molecule was probably buried in the chitosan shell, which imparted the positive charge to the PCNS complex. Also, the zeta potential value of the PCNSs remained the same after the loading of DNA, since DNA was loaded in the pHEMA core and not in the chitosan shell. The
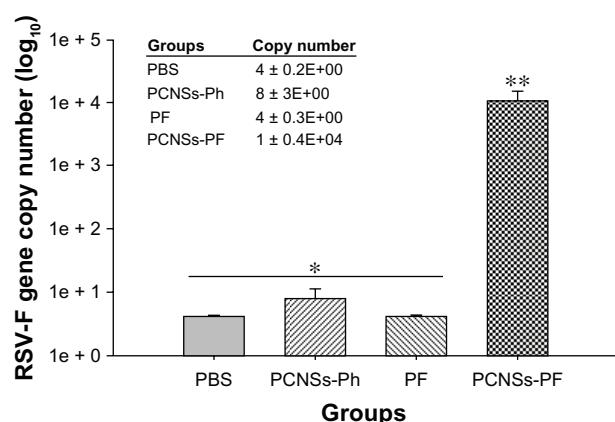
Figure 7 qPCR analysis for the RSV-F gene copy numbers from mice thigh muscle tissues injected with PBS, PCNSs-Ph, naked PF, or PCNSs-PF.
Notes: The PCNSs-PF showed the highest gene copy number compared with other groups. Statistical comparisons between the means of the four groups were analyzed by one-way ANOVA, with the significance level of 5%.
Abbreviations: ANOVA, analysis of variance; PBS, phosphate-buffered saline; PCNSs, pHEMA+chitosan nanospheres; PCNSs-PF, pHEMA+chitosan nanospheres-phCMV1+respiratory syncytial virus-fusion gene; PCNSs-Ph, pHEMA+chitosan nanospheres-phCMV1; PF, phCMV1+respiratory syncytial virus-fusion gene; qPCR; quantitative polymerase chain reaction; RSV-F, respiratory syncytial virus-fusion gene.

encapsulation of DNA into the pHEMA core rather than the chitosan shell was confirmed using the chitosanase digestion experiment. The same findings were confirmed using different techniques, such as FTIR and UV visible spectra.

Our PCNSs nanomaterial can be used effectively for drug or vaccine delivery because of its intracytoplasmic delivery and slow-release properties. The endocytosis mechanism observed is consistent with known biological endocytosis processes. Moghaddam et al[20] previously demonstrated that pHEMA+chitosan nanoparticles have mucoadhesive properties and are permeable in vitro. The significance of the positive surface charge of the nanoparticles to their uptake, along with nucleic acids, by host cells has been described earlier.[18,21,22] The chitosan shell of the PCNSs enables them to enter tissues or organs. Once inside, the DNA encapsulated in pHEMA core is released. The slow release of drugs or vaccines is preferred in order to extend the efficacy of the drug or vaccine. Taking advantage of slow release, Jadav et al[25] tested cationic PLGA nanoparticles loaded with a DNA vaccine, to achieve protective immunity, in a guinea pig model. It was found that the extended release of DNA vaccine could activate adaptive T cell-mediated acquired immunity. DNA vaccines have been known to elicit a long term and stable immune response via memory cells, as the expression of the antigen coincides with antibody production. Thus, DNA vaccines not only enhance the immune response but also provide long-term immunity.[26–28]

The stability and retention time of nanomaterials in vivo is a major consideration in the development of an efficient delivery system, since these materials are degraded by lysosomal enzymes and cleared through host secretory mechanisms. The biodegradation of chitosan in mice takes place via enzymatic degradation, resulting in excretion through urinary system within 14 hours of chitosan administration.[29] Chitosan is dissociated and excreted rapidly from the host body.[29] Conversely, pHEMA, a synthetic hydrogel, can resist hydrolytic and enzymatic degradation[30] and persists in the system for longer time. Therefore, we believed the development of a novel nanocomposite made from pHEMA (a strong depot core) surrounded by a chitosan shell (with target specificity) could provide a new, nonviral delivery system for biological applications.[31] In order to evaluate the efficacy of PCNSs for gene delivery, we used a RSV-F DNA model vaccine.

The similarities in the expression of RSV-F gene obtained using PCNSs and Lipofectamine 2000 for transfection is evidence of the uniqueness of PCNSs and their capability to deliver DNA and facilitate gene expression. Since the cells could not be cultured beyond a few days, we could

not validate the long-term DNA release in the cells. In similar studies, Sunshine et al[3,32] reported augmented gene delivery with positively charged poly(B-amino ester) nanoparticles into Cos-7 cells and retinal pigment epithelial cells in vitro compared with Lipofectamine 2000-mediated gene transfection. Green et al[33] showed identical result in Cos-7 cells. The superior transfection capability of PCNSs may be attributed to the cationic nature of chitosan shell that can interact with negatively charged cell membranes, allowing it to be taken up by cells via endocytosis. It is suggested that positively charged nanoparticles are generally taken up into the cells through endocytosis mechanism.[33] Our TEM experiments also support a similar mechanism and the high transfection of RSV-F gene via PCNSs, due to the high endocytosis efficiency of PCNSs (through cell membranes).

A role of for the core–shell structure in a nanoparticle system, similar to our PCNSs, has been described by Hu et al[2], where the hydrophilic shell mediates specific targeting, leading to intracellular uptake (while the proton-absorbing core pacifies the acidity and resists endosomal degradation). Biological materials such as vaccines or drugs could be delivered directly into the cytosol or nucleus using PCNS-like nanomaterials. Hu et al prepared a hydrophilic shell of aminoethyl methacrylate that surrounded a pH-sensitive core of diethylamino ethyl methacrylate, to efficiently deliver small molecules or proteins into dendritic cells in vitro. In a similar study, Green et al[33] demonstrated a nonviral polymeric gene delivery system that employed poly(B-amino ester) as a proton sponge, facilitating endosomal escape for high-throughput gene transfection. Also, Guo et al[9] proposed a similar endosomal escape mechanism leading to enhanced intracytoplasmic uptake of GFP gene and siRNA by exploiting gold nanoparticles functionalized with charge-reversal polyelectrolyte.[9] Our findings are in accordance with these studies, as we showed the degradation of chitosan shell resulting in the escape of the pHEMA core from the endosomes into the cytoplasm (Figure 6D). Previous studies have shown that modifications to the structure of polymers not only ameliorate transfection efficiency, but also decrease the cytotoxic effects of polymeric nanomaterials.[3] We expect that chemical modification or functionalization of the chitosan molecules in the shell of the PCNS system could improve its gene transfection efficiency as well as making it less toxic. Furthermore, target specificity can be enhanced by surface modifications and by attaching specific ligands, for successful vaccine or drug delivery. Considering the slow release of DNA from the PCNS complex, long-term transcription and expression of the target gene is anticipated, and it is expected that PCNS-mediated gene delivery can

be a promising model for DNA vaccine development, gene silencing, or gene therapy.

Our in vitro results corroborate those of previous studies.[34,35] Based on previous studies and the preferred route of DNA vaccine injections, we used the intramuscular route for our studies.[36] In one previous study, a plasmid DNA nanopolymer vaccine showed elevated transgene expression and antigen presentation when injected via the skeletal muscle.[26] In another study, improved transgene expression and systemic immune response was achieved, with localization at the site of injection, using nanoparticle-mediated DNA vaccine delivery via intramuscular injections, as compared with naked DNA.[37] We also assessed other organs (lung, liver, heart, spleen, and kidney) for the presence of RSV-F gene, but none showed any significant levels of the gene expression (data not shown). This could be due to the localization of the DNA vaccine in the muscle tissues and the duration of the animal studies.

In summary, we developed a novel nanovehicle for the delivery of a DNA vaccine model in vitro and in vivo based on pHEMA+chitosan nanospheres, exploiting their core–shell interaction, which showed high transfection capability through interaction with the cell membrane. The PCNS-DNA vaccine achieved highly efficient transfection without the use of a transfection reagent. The encapsulated DNA showed robust gene expression, and PCNSs entered cells using a endocytosis mechanism. To our knowledge, this is the first study showing the successful incorporation of DNA into PCNs nanospheres and the successful delivery of a model DNA vaccine via PCNSs, in cells and animals. The PCNS system can be used as model to develop other novel nanomaterials for vaccine, drug, antibiotic delivery, and many other biomedical applications.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

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