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Enhance immune response to DNA vaccine based on a novel multicomponent supramolecular assembly

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Abstract

DNA vaccination has tremendous potential for treating or preventing numerous diseases for which traditional vaccines are ineffective but the technique can be limited by low immunogenicity. Current synthetic DNA delivery systems are versatile and safe, but substantially less efficient than viruses. Here, a novel multicomponent supramolecular system involving the preparation of mannose-bearing chitosan oligomers microspheres with entrapping complexes of DNA vaccine and polyethylenimine was developed to mimic many of the beneficial properties of the viruses. After delivery by intramuscular immunization in BALB/c mice, the microspheres induced an enhanced serum antibody responses two orders of magnitude greater than naked DNA vaccine. Additionally, in contrast to naked DNA, the microspheres induced potent cytotoxic T lymphocyte responses at a low dose. Consequently, formulation of DNA vaccines into multicomponent vectors is a powerful means of increasing vaccine potency.

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Keywords: Mannose-bearing Chitosan oligomers microspheres; Supramolecular assembly; DNA vaccine; Artificial virus; Immunogenicity

1. Introduction

Immunization with DNA vaccines results in humoral and cellular immune responses that protect against disease in preclinical models of infectious diseases, cancer, and autoimmunity [1]. Intramuscular (i.m.) injection has been the primary route of administration for DNA vaccines, and has been shown to elicit protective and therapeutic immune responses in many animal models. However, optimal

antibody and cytotoxic T lymphocyte (CTL) responses were induced with high doses of DNA in nonhuman primates and human volunteers [2–4]. Although it is difficult to quantitate the amount of plasmid DNA that enters cells or is degraded before they enter the nucleus and initiate gene expression, it is believed that $\geq 90\%$ of the DNA injected never gets into the cytoplasm and of the $\approx 10\%$ that does enter the cell, $\leq 1\%$ enters the nucleus where gene expression occurs [5,6]. This lower bioavailability may be due to major cellular barriers such as plasma, endo/lysosomal and nuclear membranes, nuclease degradation *in vivo*, and the fact that DNA has an overall net negative electrostatic charge [7,8]. Although the use of DNA vaccines at high doses is feasible, it would impose serious limitations on the number of constructs that could be included in a vaccine. In addition, the use of very high doses of DNA is less favorable from a process economics standpoint [9]. There is, therefore, a clear need to induce

Abbreviation: MBCO microspheres, mannose-bearing chitosan oligomers microspheres; PEI, polyethylenimine; CTL, cytotoxic T lymphocyte; i.m., intramuscular; TA, anterior tibialis; APCs, antigen presenting cells; DCs, dendritic cells; HSPGs, heparin sulfate proteoglycans; DS, degree of substitution; LDH, lactate dehydrogenase; DLS, dynamic light scattering.

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effective immunity in humans with lower and fewer doses of DNA, as well as to increase the magnitude of the immune response obtained.

There are a number of strategies available that have the potential to improve the potency of DNA vaccines. Several groups have attempted to improve the delivery using cationic microparticles [9], chitosan [10], non-ionic block co-polymers [11] and cationic liposomes [12,13]. Although such approaches have had some success, they are far from ideal because gene delivery is too complicated to be achieved with a single carrier molecule [14]. The viruses, having evolved in parallel with their hosts, have been employed in over 85% of gene therapy clinical trials because of their incredible gene delivery efficiency; however, few have proven to be immunogenic. A more promising direction would be to develop safer delivery vectors that would mimic key properties of viruses, a multi-component polymer, with each component performing a viral function [15,16].

Here we have designed novel mannose-bearing chitosan oligomers (MBCO) microspheres with entrapping polyethylenimine (PEI)/DNA complexes to mimic the beneficial properties of viruses:

- (i) *DNA condensation*. For viruses, the nucleic acids are protected by a protein coat or capsid, which can form a hollow, symmetrical structure enclosing coiled genetic materials. The core of the microsphere is a polycation (PEI) capable of inducing DNA condensation. Conversion of a filiform molecule into a compact particle improves both chemical stability and physical properties.
- (ii) *Cell entry—targeting*. To enter their hosts, viruses divert the function of cell–matrix and cell–cell anchoring proteins, such as heparin sulfate proteoglycans (HSPGs) and integrins, which are responsible for cell entry by receptor-mediated endocytosis. Condensed DNA particles also require a specific surface for cell entry. For DNA vaccine, the activation of T-cell responses requires presentation of antigen by professional antigen presenting cells (APCs), and it has been shown by a number of groups that the presentation indeed occurs by professional bone marrow-derived APCs rather than by transfected muscle cells themselves [17,18]. In our approach, the surface of the microspheres is responsible for binding and entry to APCs. We decided to conjugate mannose to the surface of the microspheres because APCs, such as dendritic cells (DCs), express high levels of mannose receptor and mannose-related receptor, which are used for endocytosis and phagocytosis of a variety of antigens having exposed mannose and fucose residues.
- (iii) *Endosome escape*. After internalization, DNA microspheres must escape from the formed intracellular vacuoles. Viruses that do not fuse at the plasma membrane exploit endosome acidification as an escape signal, by means of sophisticated conformational

changes of fusion proteins. In MBCO microspheres, PEI shares the ability to buffer the acidity of endosomes. The vacuolar pH should therefore decrease more slowly, with a large concomitant ionic concentration increase. Osmotic swelling due to water entry may then burst the parvacuole and release the complexes into the cytoplasm. This is the famous ‘proton sponge’ hypothesis. After preparation and characterization, the microspheres were administered to experimental animals and the immune responses induced were compared with immunization with naked DNA. A significant improvement in immunogenicity over naked DNA was achieved for both CTL and antibody induction of the MBCO microspheres. This technology offers an improved way of delivering DNA vaccine to the nucleus of cell, by mimicking viruses in their ability to do the same.

2. Materials and methods

2.1. Materials

The 25 kDa PEI (branched), NaBH(CN)₃, NaNO₂, Mannobiose, Chitosan (MW 102 kDa; 85% deacetylated) were purchased from Sigma-Aldrich. All reagents used were of the highest purity available. The HBV S/V1012 plasmid (CMV promoter, 5.7 kb), pLuc/V1012 plasmid (CMV promoter, 6.4 kb) and pEGFP/V1012 plasmid (CMV promoter, 5.8 kb) were constructed by our Lab and obtained by transforming *Escherichia coli* strain top10 and cultured under defined growth conditions. The plasmids were purified by using a Qiagen Plasmid Giga kit, and the final product was endotoxin free (<2.5 units/mg). Permission to do animal work was obtained from the Laboratory Animal Ethics Committee of Jilin University, China.

2.2. Synthesis of mannose-bearing chitosan oligomers

Mannose-bearing chitosan (M-chitosan) was obtained by reductive amination with mannobiose, as described in detail elsewhere [19]. The purified mannose-bearing chitosan was chemically treated with sodium nitrite in 1% acetic acid at 25 °C for 30 min to produce chitosan oligomers of lower molecular weights, according to a method described by Peniston [20]. The oligomers were purified, lyophilized and used without further modification. The degree of substitution of mannosyl units on the chitosan was obtained from elementary analysis and distortionless enhancement of polarization transfer (DEPT) (135°) ¹³C NMR. Water solubility of mannose-bearing chitosan oligomer was estimated using the transmittance of the chitosan derivatives solution [21].

2.3. Preparation and characterization of MBCO microsphere

MBCO microspheres were prepared following the report of Aral and Akbuga [22] with slight modification. Briefly, PEI/DNA complexes (preparation and characterization in supporting information) were added to 50 mM sodium sulfonate solution, and this mixture was quickly dropped into the mannose-bearing chitosan oligomers solution and vortexed for 15–30 s. Formed microspheres were washed with deionized water and separated by centrifugation at 11000g, then freeze-dried. The size distribution of the MBCO microspheres was determined by dynamic light scattering (DLS) with a Zetasizer 3000 HS (Malvern Instruments, UK) and the value was calculated by volume measurement. The loading level of

the DNA in the microspheres was determined by assaying the supernatant after centrifugation and measuring DNA by absorbance at 260 nm. The encapsulation efficiency of microspheres was calculated. The Zeta potential of the microspheres was measured on the same apparatus as DLS. Selected batches of microspheres were evaluated by transmission electron microscopy (TEM) and atomic force microscopy (AFM) for size and shape. AFM images of the microspheres were taken with a Nanoscope IIIa AFM Multimode (Digital Instruments, Santa Barbara, CA) under ambient conditions. AFM was operated in the tapping mode with an optical readout using Si₃N₄ cantilevers (Nanoprobes, Digital Instruments). By replacing mannose-bearing chitosan oligomers with chitosan or M-chitosan, Chitosan and M-chitosan microspheres with encapsulated PEI/DNA can be prepared in the same method as MBCO microspheres.

2.4. *In vitro* and *in vivo* release

In vitro release studies of DNA from MBCO microspheres were carried out by suspending 10 mg of microspheres in 1 ml of FBS at pH 7.4 (120 mM NaCl, 2.7 mM KCl, 10 mM PBS, 10% FCS) at 37 °C under stirring. At predetermined time intervals, the suspension was centrifuged and replaced with the same volume of fresh medium. The DNA concentration in the supernatant was determined by UV absorbance at 260 nm. Each experiment was performed in triplicate. Release profiles of chitosan and M-chitosan microspheres with encapsulated PEI/DNA were performed as a control according to the same operation.

Initial burst release of DNA vaccine from microspheres is favorable to vaccination [9]. *In vitro* burst release studies of DNA from MBCO microspheres were carried out in COS-7 cells (simian virus 40-transformed kidney cells of an African green monkey) using the GFP as reporter gene. Cells were grown at 37 °C in humidified air containing 5% CO₂ and passaged every 2–3 days. The cells (1 × 10⁵ per well) were plated on 24-well tissue-culture plates 24 h before use. Immediately before the initiation of the experiments, the medium was removed from each well, and the cells were washed once with DMEM without serum and antibiotics and treated with a dilution of MBCO, M-chitosan and chitosan microspheres. GFP expression was directly viewed under a fluorescence microscope (OLYMPUS America).

In vivo release of DNA from MBCO microspheres were carried in BALB/c mice, which were bred and cared in the animal facilities of the vaccine center of Jilin University, Changchun. Three groups of female BALB/c mice ($n = 6$) were injected with either 50 µg of pCMVLuc DNA, M-chitosan microspheres containing 50 µg of pCMVLuc DNA or the MBCO microspheres containing 50 µg of pCMVLuc DNA. Three groups of mice were injected i.m. in the anterior tibialis (TA) muscle on one leg. The muscle from each mouse was harvested either at day 1, 7, or day 14 and stored in a –80 °C freezer. The muscles were ground up with a mortar and pestle on dry ice. The powdered muscles were collected in Eppendorf tubes with 0.5 ml of 1 × reporter lysis buffer. After freeze/thawing three times, the samples were spun at 14000 rpm for 10 min. Twenty microliters of the supernatants were assayed by using the luciferase assay and normalized to the total content in the sample.

2.5. Gene expression *in vitro*

RAW264.7 cells (murine monocyte-macrophage cells) were cultured in DMEM medium containing Gln supplemented with 10% heated-inactivated FCS (from GIBCO) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin from Sigma). The cells (1 × 10⁵ per well) were plated on 24-well tissue-culture plates 24 h before transfection. Immediately before the initiation of transfection experiments, the medium was removed from each well, and the cells were washed once with DMEM without serum and antibiotics and treated with a serial dilution of MBCO, M-chitosan and chitosan microspheres. A competition assay was carried out to confirm the uptake of MBCO microspheres mediated by mannose-receptor by adding 20 mM of mannose to MBCO microspheres suspension. Transfection with lipofectamineTM2000-DNA complexes was performed as a positive control according to the manufacturer's protocol. Luciferase

gene expression was monitored 48 h later by using a commercial kit (Promega). Each transfection experiment was performed in triplicate and is expressed as mean light units per mg of cell protein ± SD.

2.6. Measurement of antibody responses and T-cell responses

Female BALB/c mice in groups of 8 were immunized with either the MBCO microspheres, naked DNA or control PBS at different doses. The immunization protocol was performed at weeks 0, 2, 4 and 6. Sera samples were collected from all mice by tail bleeding at different times points and HBsAg specific serum IgG titers were confirmed by enzyme-linked immunosorbent assay (ELISA).

Spleens from immunized mice were harvested 2 weeks after the fourth immunization and used in pools of five. Spleen cells were cultured with RPMI 1640 phenol red-free medium. Approximately 1 × 10⁶ P815/BALB target cells were sensitized with synthetic epitope peptide at a concentration of 1 mM for 2 h at 37 °C. Cytotoxicity was measured by a standard lactate dehydrogenase (LDH)-release assay with a CTL kit (Promega) according to the instructions provided by the manufacture. The HBsAg-specific T cells in the spleen were also indirectly determined using cytokine enzyme-linked immunosorbent spot (ELISPOT) by measuring HBsAg induced IFN-γ production, which was performed according to the instruction of the murine IFN-γ ELISPOT kit (BD, USA).

2.7. Statistical analysis

Means and SD were obtained, Student's *T*-test was conducted to analyze differences, and a *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. MBCO microspheres evaluation

We produced a low-molecular-weight chitosan by random depolymerization of M-chitosan, with an average molecular weight of 12 kDa (Table 1). In this study, PEI/DNA complexes were encapsulated into the chitosan, M-chitosan and MBCO microspheres (Table 2). DLS measurements of MBCO microspheres showed a unimodal particle size distribution between 200 and 300 nm. The zeta potential of MBCO microspheres was approximately 4.12 mV at pH 5.5 and close to neutral at pH 7.2. The low surface potential of MBCO microspheres is suitable for use *in vivo* due to the low reaction with components of the *in vivo* milieu. The MBCO microspheres obtained under these conditions appeared fairly spherical and polydisperse nature, as revealed by TEM and AFM image (Fig. 1).

3.2. *In vitro* and *in vivo* release study

The *in vitro* release of DNA from the MBCO microspheres showed initial burst release. In the first day, about 27.1% of the PEI/DNA complexes were released from MBCO microspheres, and no obvious release was detected for chitosan and M-chitosan microspheres at the first day. The molecular weight of chitosan and chitosan derivatives significantly affected the drug release (Fig. 2a). The *in vitro* burst release ability of MBCO microspheres was also evaluated in COS-7 cells using the pEGFP plasmid. In the experiment we observed that a higher

Table 1
The characteristics of chitosan, mannose-bearing chitosan (M-chito) and depolymerized mannose-bearing chitosan (DM-chito)

Formulation	M_w (kDa)	Formula ^a	pH ^b
Chitosan	102	$[(C_8H_{13}NO_5)_{0.15}(C_6H_{13}NO_4)_{0.85}] \cdot 0.23H_2O$	7.0 ± 0.5
M-chito		$[(C_8H_{13}NO_5)_{0.05}(C_6H_{11}NO_4)_{0.65}(C_{18}H_{33}NO_{14})_{0.3}] \cdot 0.72H_2O$	9.0 ± 0.5
DM-chito	12		≥ 12.0

^aObtained from elementary analysis.

^bThe highest pH that can dissolve the polymer; the pH of the polymer solution in 0.1 N HCl was adjusted by adding stepwise 6 N NaOH. The polymer was considered as an insoluble one when the transmittance of the polymer solution was lower than 50%, compared to that of a control solution (0.1 N HCl). All data were expressed as mean \pm SD ($n = 3$).

Table 2
Chitosan, M-chitosan and MBCO microspheres encapsulated PEI/DNA within it

Formulation	Mean size (nm) ^a	Encapsulation efficiency (%)	Zeta potential (mV) ^b
Chitosan	415.2 ± 44.2	(94 ± 5.4)	19.44 ± 2.78
M-chitosan	326.3 ± 31.9	(89 ± 2.6)	10.19 ± 3.23
MBCO	246.9 ± 30.8	(82.5 ± 3.4)	4.12 ± 0.64
PEI/DNA (N/P = 6)	86.6 ± 30.2		36.64 ± 1.77

^aThe mean size of microspheres was measured by DLS.

^bThe zeta potentials of chitosan derivatives microspheres was measured at pH 5.5 and PEI/DNA complexes were measured at pH 7.2. All data were expressed as mean \pm SD ($n = 3$).

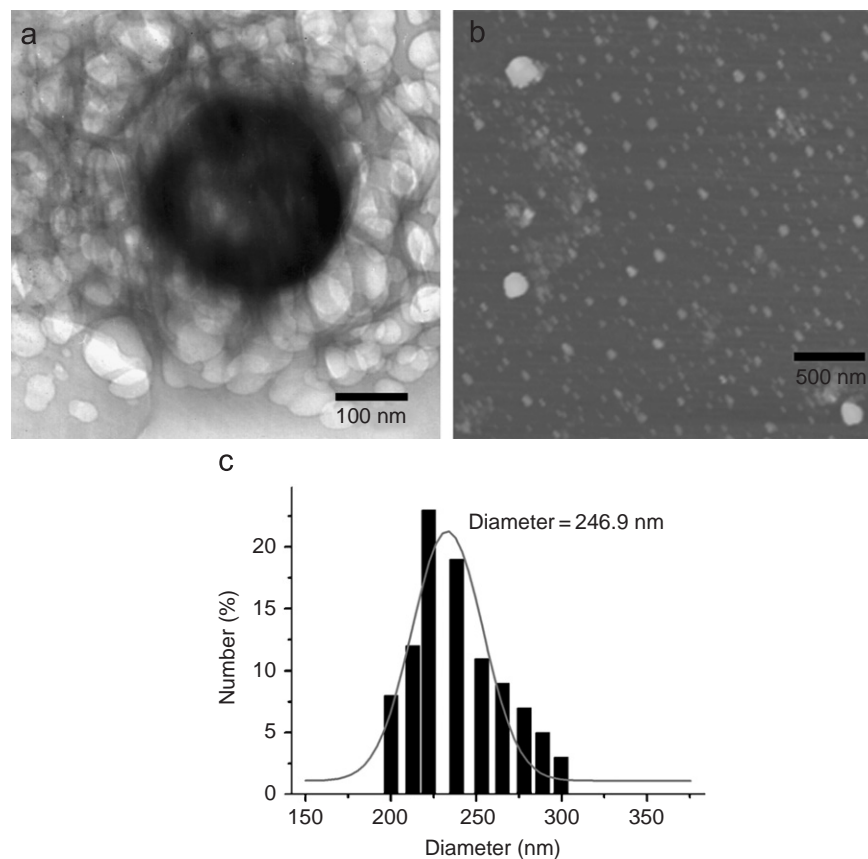


Fig. 1. Appearance and size of MBCO microspheres. (a) Transmission electron micrograph of MBCO microspheres. Scale bar represents 100 nm. (b) Atomic force micrograph of MBCO microspheres. Scale bar represents 500 nm. (c) Size distribution of freshly prepared microspheres. Size was measured using photon correlation spectroscopy (dynamic light scattering) and data were plotted as volume distribution.

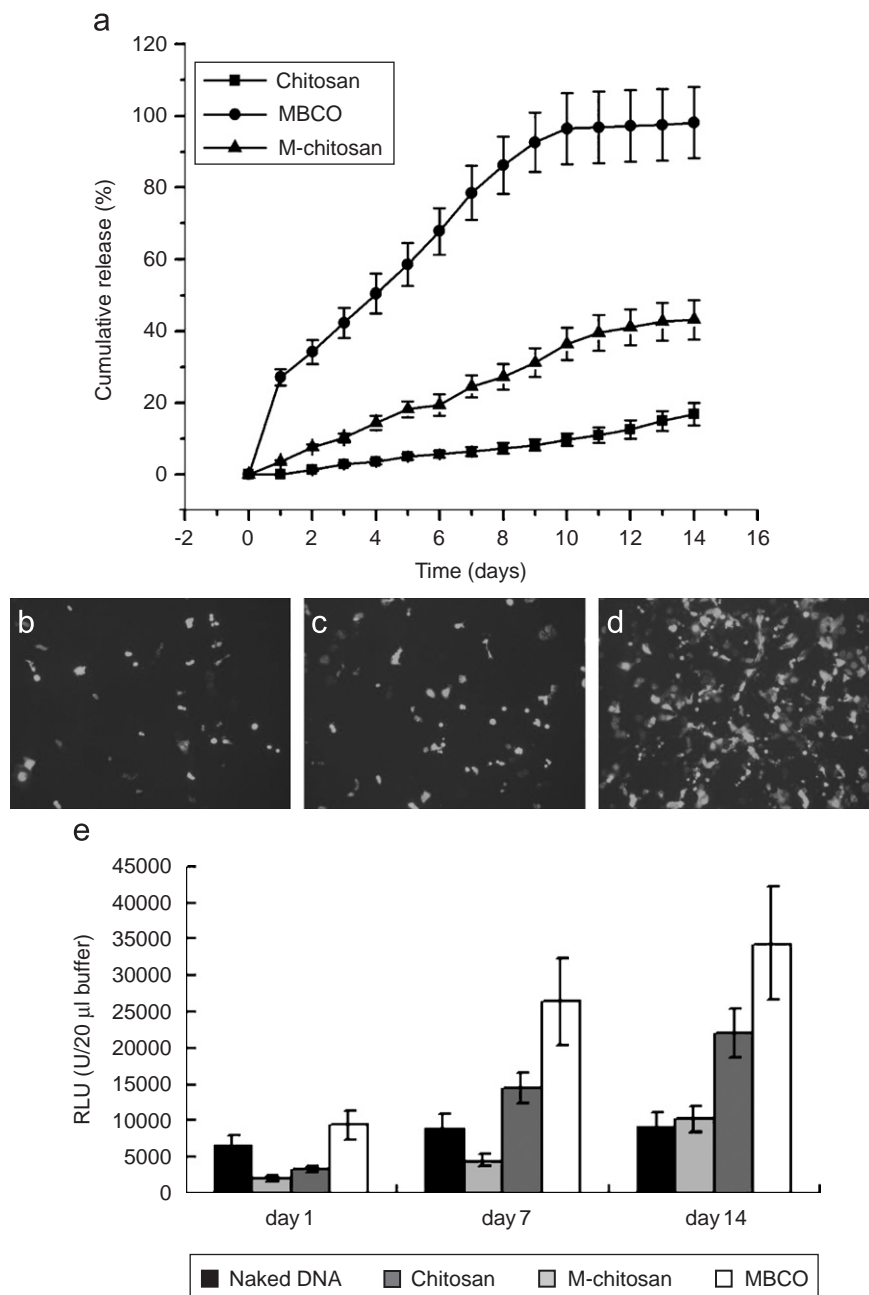


Fig. 2. *In vitro* and *in vivo* release profiles of chitosan, M-chitosan and MBCO microspheres containing PEI/DNA complexes. (a) *In vitro* release profiles. The microspheres (10 mg) were suspended in 1 ml of PBS at pH 7.4 (120 mM NaCl, 2.7 mM KCl, 10 mM PBS) at 37 °C under stirring. At predetermined time intervals, the suspension was centrifuged and replaced with the same volume of fresh medium. The DNA concentration in the supernatant was determined in triplicate by UV absorbance at 260 nm. All data were expressed as mean \pm SD ($n = 3$). (b–d) *In vitro* burst release studies of DNA from MBCO microspheres. The experiments were carried out in COS-7 cells using the GFP as reporter gene. GFP expression was directly viewed under a fluorescence microscope (OLYMPUS America). (e) *In vivo* release of luciferase plasmid was performed in mice at days 1, 7, and 14 with either naked DNA, M-chitosan, or MBCO microspheres. The TA muscles of injection site were peeled off and treated by using a normal chemiluminescence assay. Mean average values \pm standard deviation of triplicates are given in relative light units (RLU), correlated to the total volume content.

percentage of transfected cells producing higher amounts of transgene product for MBCO (Fig. 2d) microspheres than for chitosan (Fig. 2b) and M-chitosan (Fig. 2c) microspheres. The faster release rate seems entirely appropriate, given evidence that most DCs die within 7 days after activation and migration to draining lymph nodes [23].

The *in vivo* release of DNA from MBCO microspheres was detected by the expression of luciferase after injection into TA muscle in BALB/c mice. The level of *in vivo* expression of luciferase was higher for MBCO microspheres than for naked DNA, chitosan and M-chitosan microspheres at the day of 1, 7 and 14 time point (Fig. 2e). The uptake and expression of naked plasmid in muscle cells

may be related to muscle physiology and function, most likely via a receptor-mediated event involving the T tubules and caveolae. For naked DNA, peak level of gene expression in muscle was found between 7 and 14 days. And for chitosan, M-chitosan and MBCO microspheres, peak level of gene expression was found over 14 days post-injection, possibly explained by the controlled release of DNA. The MBCO microspheres based on chitosan oligomers dissociated more easily than the high-molecular-weight chitosan microspheres. The more easily dissociated microspheres mediated a faster onset of action and gave a higher gene expression after i.m. administration *in vivo*.

3.3. Transfection efficiency of MBCO microspheres *in vitro*

The *in vitro* transfection efficiency of MBCO microspheres was evaluated in RAW264.7 cells using the pCMV luciferase plasmid. RAW264.7 cells are well-known model cells of monocyte-macrophage cells, having rich mannose receptors on the cell surface. The results of the luminescence assay to detect the luciferase activity in the transfected cells indicated relatively high transfection efficiencies (Fig. 3). The expressed luciferase activity of MBCO was about three orders of magnitude higher than the background level (DNA transfection), and it was only about five to ten times lower than that of the LipofectamineTM2000. The expressed activity of chitosan and M-chitosan microspheres was about ten-fold lower than MBCO microspheres, and showed only a slight transfection efficiency. Furthermore, the decreased transfection activity by the addition of superfluous mannose demonstrated that MBCO microspheres might be internalized by endocytosis or phagocytosis via recognition of the mannose receptor on the surface of macrophages (Fig. 3).

3.4. Enhanced humoral and cellular immune responses with MBCO Microspheres

Though mouse bone marrow-derived DCs showed relatively lower mannose receptor levels than human DC,

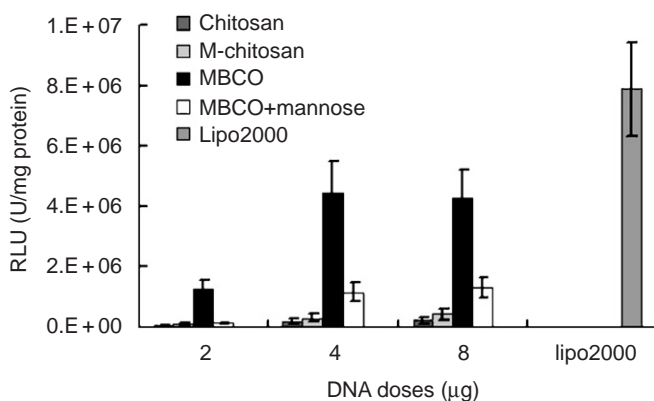


Fig. 3. Transfection efficiency of MBCO microspheres in RAW264.7 cells compared with chitosan and M-chitosan microspheres. The luciferase values have been normalized to total protein content ($n = 3$).

the mouse was a convenient model for DNA-based immunization. The formulated MBCO microspheres enhanced antibody responses to HBsAg protein over naked DNA at all time points (Fig. 4a). In an extensive dose–response titration assessed by ELISA, naked DNA primed HBsAg-specific antibody response after four continuous doses of DNA as high as 10 μg. In contrast, MBCO microspheres were effective at 100 ng, indicating a ~100-fold increase in DNA vaccine potency (based on the lowest dose of DNA required to prime a measurable response). In addition, MBCO microspheres accelerated the antibody responses. A detectable antibody responses to HBsAg were achieved within 5 weeks of injection of naked DNA at 10 μg doses, whereas only 3 weeks were required for MBCO microspheres at the same dose. This acceleration by 2 weeks is statistically significant, and is of clinical interest whenever a rapid immune response is critical.

HBsAg-specific CD8⁺ T cells were measured *in vitro* by assessing the death of P815/BALB target cells which had been sensitized with HBsAg CTL epitope peptide. Naked DNA primed mild HBsAg-specific CD8⁺ T cells only at the highest DNA dose of 100 μg, whereas MBCO microspheres were effective at a dose as low as 1 μg, indicating a ~100-fold increase in DNA vaccine potency, as judged by reduction of DNA vaccine dose (Fig. 4b).

During natural infection, HBV-specific Th1 cells, CTL, and the associated antiviral cytokines (IFN-γ, TNF-α, IL-2) may play key roles in virus resolution [24–26]. HBsAg-specific CD8⁺ T cells were measured *in vitro* by determining IFN-γ production in spleen cells in response to brief restimulation with CTL epitope, as measured by ELISPOT (Fig. 4c). MBCO microspheres immunization at 10 μg clearly corresponded to naked DNA at 100 μg doses, which indicated a ~100-fold increase in DNA vaccine potency, as judged by reduction of DNA vaccine dose.

4. Discussion

Viruses, having evolved in parallel with their human hosts, are a superior gene delivery system. There has been interest in designing “Artificial viruses” which mimic aspects of this delivery system [27, 28]. A virus contains a single genetic (DNA or RNA) molecule which is coated with a specific number of capsid proteins in a compact viral size (20–250 nm). Monomolecularity, stoichiometry, and size in addition to transfection ability are the golden criteria for artificial viruses. There is little doubt that viral characteristics can be incorporated into nonviral vectors to enhance delivery efficiency, but it is not necessary that delivery systems of the future must be “viruslike” particles. Instead, by understanding and incorporating the extremely efficient mechanisms of infection by viruses, DNA delivery systems will be viruslike in function, not necessarily in shape—just as our aviation systems mimic the function of birds, but not always their morphology [29]. Based on previous studies, the ideal “artificial virus” system should possess the following properties: ease of assembly; efficient

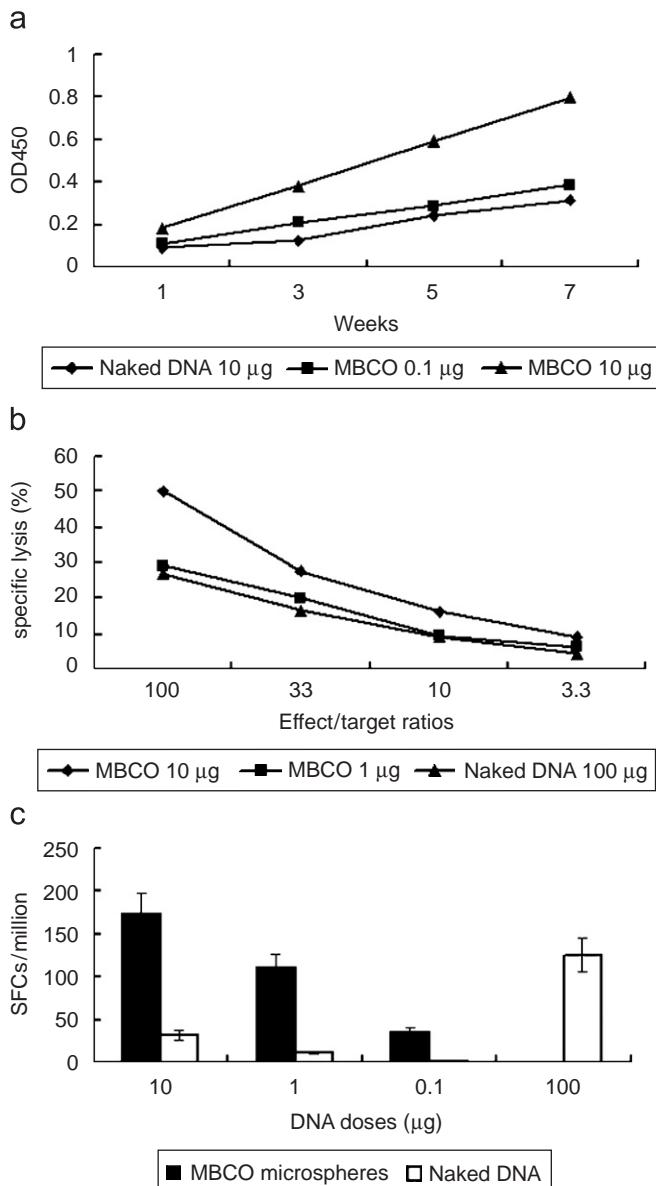


Fig. 4. (a) Serum HBsAg antibody assay in groups of BALB/c mice immunized either MBCO microspheres or naked DNA alone. The immunization protocol was performed at weeks 0, 2, 4 and 6. At the different time points, HBsAg-specific total antibody (as pool of five) was measured in serum from each group ($n = 5$) of mice. OD4500.2 represents positive value. (b) Analysis of cytolytic activities of splenic mononuclear cells harvested from mice immunized. P815 mastocytoma cells (as pool of five) pulsed with epitope peptides were used as targets. Percent specific lysis was determined by a 4-h LDH-release assay. (c) The HBsAg-specific, IFN- γ producing splenocytes were generated in mice by HBsAg DNA vaccine after intramuscular injection. Each bar represents the average number of cells secreting IFN- γ per 10^6 cells.

delivery leading to total transfection; stabilization of DNA before and after uptake; capability of traversing the presumed barriers to gene delivery (e.g., by incorporating viral components or mimicking viral characteristics); efficient decomplexation or “unpackaging” (e.g., intracellular controlled release) and efficient nuclear targeting. While no current nonviral systems have all these properties,

biopolymers provide versatile chemistry with a wide variety of different functionalities. In addition, they are relatively cheap to produce. So, biopolymers are excellent candidates for construction of artificial viruses.

In this study, we have designed novel MBCO microspheres to specially address these desirable characteristics.

(i) The mannose-bearing chitosan oligomers were chosen as an inert coat to enclose the DNA vaccine. Chitosan has been proposed as non-toxic biodegradable polycationic polymers with an extensive record of safe use for gene delivery systems [30, 31]. In most of these studies, commercially available chitosans of high-molecular weight (100–400 kDa) were used. Unfortunately, these chitosans biodegrade slowly, which delays the release of the DNA. Another pharmaceutical drawback is their low solubility at physiological pH and their viscosity-enhancing properties at concentrations suitable for *in vivo* gene therapy [32]. We therefore produced a low-molecular weight version with an average molecular weight of 12 kDa chitosan by random depolymerization of chitosan (Table 1). The more easily dissociated chitosan oligomers gave a 10-fold higher gene expression in RAW264.7 cells *in vitro* as compared to the more stable high-molecular weight chitosan (Fig. 3). In addition, MBCO microspheres induced a higher luciferase expression *in vivo* than stable high-molecular weight chitosan microspheres at day of 1, 7 and 14 time points. Both our *in vitro* and *in vivo* data suggest that dissociation of the chitosan oligomer microspheres is a fast process for the release of DNA from the microspheres and the subsequent onset of action, compared to a biodegradation-dependent release [33]. Additionally, covalent attachment of mannose to the primary amine function of chitosan can target APCs and be internalized via recognition of the mannose receptor on the surface of APCs such as M Φ s (Fig. 3). The mechanism of the adjuvant effect achieved with MBCO microspheres is not currently known, but we believe that efficient delivery to APCs may be an important contributing factor.

(ii) The core of the MBCO microspheres is a polycation (PEI) capable of inducing DNA condensation. PEI has been used for gene and oligonucleotide transfer into cells in culture and *in vivo*. It can, however, be rapid inactivation due to undesired interactions with components of the *in vivo* milieu. Polycations are generally less immunogenic than viruses: however, their activation of innate defense mechanisms is a major problem. Complement system activation by PEI has been demonstrated, with highly positive complexes being the most active stimulators [34]. In current study, activation of the complement cascade might be avoided by shielding the positive particles with mannose-bearing chitosan oligomers (Table 2). In addition, PEI possesses the ability to buffer the acidity of endosomes and is able to escape from them, based on

the ‘proton sponge’ hypothesis. The former chitosan-based systems were the inefficient release from endosomes into the cytoplasm, causing low transfection efficiency [35]. In our system, a synergistic effect on transfection efficiency was clearly observed by the combined use of chitosan and PEI (data not shown). Hence, the MBCO microspheres mimic the following five desirable characteristics of a virus: (i) DNA condensation; (ii) cell entry—targeting; (iii) endosome escape; (iv) compact viral size (200–300 nm) and (v) efficient decomplexation.

Biopolymer-based artificial viruses have the potential to become highly efficient, versatile, and safe DNA vaccine vectors. In doing so, they may replace recombinant viruses as the standard vehicle for genetic vaccine. However, we must first engineer the polymers to improve the delivery of genes from outside the target cell to the nucleus. Genetic vaccine vectors of future will be constructed much like viruses, multifunctional supramolecular systems that self-assemble around DNA, each component performing a function imitative of viral characteristics.

5. Conclusion

These studies have demonstrated that MBCO microspheres were potent delivery systems for DNA vaccines and are capable of inducing the enhanced humoral and cellular responses (about 100-fold) after i.m. immunization with HBsAg plasmid. Another key advantage of this system is its speed of immune induction. In recent years, outbreaks of Ebola virus, severe acute respiratory syndrome, and influenza A have caused concern that conventional DNA vaccination strategies are too slow at inducing immunity [36,37]. This study has served to highlight the exciting potential of multifunctional supramolecular systems as a method of induction of a rapid immune response to DNA vaccines. In subsequent studies, several new biomaterials have been designed which overcome specific important gene delivery barriers. Further enhancements are needed to reach the gene delivery efficiency obtained by viruses, but results are encouraging and ultimately this technology holds great promise for use in humans.

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References

- [1] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccine. *Ann Rev Immunol* 1997;15:617–48.
- [2] Letvin NL, Montefiori DC, Yasutomi Y, Perry HC, Davies ME, Lekutis C, et al. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc Natl Acad Sci USA* 1997;94:9378–83.
- [3] Calatota S, Bratt G, Nordlund S, Hinkula J, Leandersson AC, Sandstrom E, et al. Cellular cytotoxic responses induced by DNA vaccination in HIV-1-infected patients. *Lancet* 1998;351:1320–5.
- [4] Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998;282:476–80.
- [5] Boutorine AS, Kostina EV. Reversible covalent attachment of cholesterol to oligodeoxy-ribonucleotides for studies of the mechanisms of their penetration into eukaryotic cells. *Biochimie* 1993;75:35–41.
- [6] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther* 1999;10:2461–80.
- [7] Hasan UA, Abai AM, Harper DR, Wren BW, Morrow WJ. Nucleic acid immunization: concepts and techniques associated with third generation vaccines. *J Immunol Methods* 1999;229:1–22.
- [8] Pouton CW, Seymour LW. Key issues in non-viral gene delivery. *Adv Drug Deliv Rev* 2001;46:187–203.
- [9] Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: a potent delivery system for DNA vaccines. *Proc Natl Acad Sci USA* 2000;97:811–6.
- [10] Illum L, Jabbal-Gill I, Hinchcliffe M, Ficsher AN, Davis SS. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev* 2001;51:81–96.
- [11] Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002;415:331–5.
- [12] Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem* 1994;269:2550–61.
- [13] Gregoriadis G, McCormack B, Obrenovic M, Saffie R, Zadi B, Perrie Y. Vaccine entrapment in liposomes. *Methods* 1999;19:156–62.
- [14] Somia N, Verma IM. Gene therapy: trial and tribulations. *Nat Rev Genet* 2000;1:91–9.
- [15] Ogris M, Wagner E. Targeting tumors with non-viral gene delivery systems. *Drug Discov Today* 2002;7:479–85.
- [16] Hood JD, Bednarski M, Frausto R, Guccione S, Reisfeld RA, Xiang R, et al. Tumor regression by targeted gene delivery to the neovasculature. *Science* 2002;296:2404–7.
- [17] Corr M, Lee DJ, Carson DA, Tighe H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med* 1996;184:1555–60.
- [18] Doe B, Selby M, Barnett S, Baenziger J, Walker CM. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc Natl Acad Sci USA* 1996;93:8578–83.
- [19] Mansur Y, Laurance DH. Some chemical and analytical aspects of polysaccharide modifications. 3. Formation of branch-chain, soluble chitosan derivatives. *Macromolecules* 1984;17:272–81.
- [20] Peniston QP. 1975; USP, 3922260.
- [21] Park JH, Cho YW, Chung H, Kwon IC, Jeong SY. Synthesis and characterization of sugar-bearing chitosan derivatives: aqueous solubility and biodegradability. *Biomacromolecules* 2003;4:1087–91.

- [22] Aral C, Akbuga J. Preparation and in vitro transfection efficiency of chitosan microspheres containing plasmid DNA: poly(L-lysine) complexes. *J Pharm Pharm Sci* 2003;6:321–6.
- [23] Garg S, Oran A, Wajchman J, Sasaki S, Maris CH, Kapp JA, et al. Genetic tagging shows increased frequency and longevity of antigen-presenting, skin-derived dendritic cells in vivo. *Nat Immunol* 2003;4:907–12.
- [24] Deml L. Purification and characterization of hepatitis B virus surface antigen particles produced in *Drosophila Schneider-2* cells. *J Virol Methods* 1999;79:205–17.
- [25] Rico MA, Quiroga JA, Subira D, Castanon S, Esteban JM, Pardo M, et al. Hepatitis B virus-specific T-cell proliferation and cytokine secretion in chronic hepatitis B e antibody-positive patients treated with ribavirin and interferon alpha. *Hepatology* 2000;33:295–300.
- [26] Seder RA, Hill AV. Vaccines against intracellular infections requiring cellular immunity. *Nature* 2000;406:793–8.
- [27] Nakai T, Kanamori T, Sando S, Aoyama T. Remarkably size-regulated cell invasion by artificial viruses. Saccharide-dependent self-aggregation of glycoviruses and its consequences in glycoviral gene delivery. *J Am Chem Soc* 2003;125:8465–75.
- [28] Zuber G, Zammuto-Italiano L, Dauty E, Behr JP. Targeted gene delivery to cancer cells: directed assembly of nanometric DNA particles coated with folic acid. *Angew Chem Int Ed Engl* 2003;42:2666–9.
- [29] Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol* 2000;18:33–7.
- [30] Lee MK, Chun SK, Kim JK, Choi SH, Kim A, Ounqbho K, et al. The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer. *Biomaterials* 2005;26:2147–56.
- [31] Onishi H, Machida Y. Biodegradation and distribution of water-soluble chitosan in mice. *Biomaterials* 1999;20:175–82.
- [32] Koping-Hoggard M, Tubulekas I, Guan H, Edwards K, Nilsson M, Varum KM, et al. Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Ther* 2001;8:1108–21.
- [33] Lavertu M, Methot S, Tran-Khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials* 2006;27:4815–24.
- [34] Plank C, Mechtler K, Szoka Jr FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther* 1996;7:1437–46.
- [35] Kim TH, Kim SI, Akaike T, Cho CS. Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. *J Control Release* 2005;105:354–66.
- [36] Sullivan NJ, Sanchez A, Rollin PE, Yang ZY, Nabel GJ. Development of a preventive vaccine for Ebola virus infection in primates. *Nature* 2000;408:605–9.
- [37] Sullivan NJ, Geisbert TW, Geisbert JB, Xu L, Yang ZY, Roederer M, et al. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 2003;424:681–4.