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Evaluation of collagen and methylated collagen as gene carriers

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Abstract

This study explores the potential of DNA complexes prepared with methylated collagen (MC) and unmodified native collagen (NC) to deliver genes into cells. The physicochemical properties and transfection abilities of these two types of complexes are studied in parallel. MC was prepared by methylation of the carboxyl groups of collagen, rendering the collagen net positively charged at neutral pH. NC/DNA complexes were prepared at pH ~3, but aggregated rapidly at neutral pH. These complexes did not confer significant protection to DNA due to its poor stability in serum. MC carried a positive charge at neutral pH and formed complexes with DNA in PBS; therefore MC improved DNA binding ability and the stability of the complexes at physiological conditions. MC/DNA complexes were smaller and more stable than NC/DNA complexes in PBS, and sustained released of DNA from MC/DNA complexes was observed for up to 3 weeks in PBS at 37 °C. In contrast, NC/DNA complexes released almost all the DNA within 6 h under the same condition. In vitro gene transfection experiments revealed that MC mediated a higher gene expression than NC, although the level of gene expression was still much lower than that achieved with polyethyleneimine/DNA complexes. In contrast to in vitro results, NC/DNA complexes yielded a 3.8-fold higher gene expression than naked DNA and MC/DNA complexes ($P < 0.05$) at week 2 following intramuscular injection at a DNA dose of 3 μg per muscle and a weight ratio of 1. Higher weight ratios resulted in significant decrease of transfection efficiency, particularly for MC/DNA complexes. The results suggested that gene delivery via the intramuscular route followed a different mechanism that demands a different set of physiochemical properties of the carrier from other parental routes. The potential of these collagen-based gene carriers for other administration routes remain to be further investigated.

Keywords: Collagen; Cationic gene carrier; Transfection; Gene expression in muscle

1. Introduction

Corrective gene therapy and DNA vaccination will be the future therapeutic and prophylactic treatments for many genetic diseases, infectious diseases and cancers. To realize these concepts, safe and effective carriers are needed to allow the dose titration and repeated administration. A range of gene carriers would eventually be needed for various applications, as the

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optimal carrier characteristics may vary depending on the therapeutic gene product, target cells, and tissues and the administration route. Non-viral carriers have been increasingly proposed as alternatives to viral vectors because of their potential advantages, such as its low immunogenicity, ease of synthesis and unrestricted plasmid size. An ideal carrier should have high gene transfer efficiency while maintaining low toxicity. Carriers derived from natural polymers/macromolecules have been investigated because they generally exhibit low toxicity. Examples of natural polymeric carriers include chitosan, dextran, gelatin, atelocollagen and collagen.

Collagen is a main extracellular matrix protein and accounts for 20–30% of the total body protein. It is bioabsorbable and non-toxic, and has low antigenicity. Its use as a gene carrier has been explored recently. In the form of hydrogel or pellet matrix, collagen has been shown to deliver DNA to cells. Samuel et al. prepared a porous, crosslinked collagen–glycosaminoglycan (GAG) matrix to encapsulate plasmid DNA. Bioactive plasmid could be released from this collagen–GAG matrix over a period of 28 days in a biphasic pattern. Canine articular chondrocytes seeded into this matrix have been transfected by DNA incorporated in the matrix (Samuel et al., 2002). Ochiya et al. demonstrated that the atelocollagen (produced by elimination of the telopeptide moieties) pellets containing DNA yielded a sustained local and systemic circulation of gene products when delivered to the skeletal muscle (Ochiya et al., 2001).

Collagen has p*I* of 5.8 and carries net negative charge at neutral pH (Randell, 1953); therefore, they cannot form complexes with DNA at neutral pH. Although it could form complexes with DNA at low pH, these complexes aggregate rapidly at neutral pH and do not confer significant protection to DNA due to its poor stability in serum. Crosslinking has been shown to be an effective way to stabilize the complexes and improve transfection efficiency in vitro and in vivo (Leong et al., 1998; Truong et al., 1999). However, the crosslinked structure is difficult to characterize and it might increase antigenicity.

Collagen can be methylated to yield a positively charged collagen by partial esterification of the carboxyl groups in collagen using acidified methanol (Chia et al., 2000). These methyl ester groups can be hydrolyzed in vivo releasing collagen. This positively charged methylated collagen (MC) can form complexes with DNA at neutral pH. MC should be able to bind DNA at neutral pH and improve the stability of the complexes at physiological conditions. In this study, we will explore the potential of MC/DNA and NC/DNA complexes to deliver genes. The physicochemical properties and the transfection efficiencies of MC/DNA and NC/DNA complexes are studied in parallel to correlate their transfection ability.

2. Materials and methods

2.1. Materials

Polyethylenimine (branched PEI, average molecular weight of 25 kDa), iodoacetic acid, collagenase VII, Hoechst 33258, Dulbecco's Modified Eagle's Medium (DMEM), streptomycin and penicillin were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was a product of HyClone (Logan, Utah). Type I collagen (Vitrogen 100, 3 mg/ml in 0.012N hydrochloric acid (HCl)) was purchased from Cohesion Technologies, Inc. (Palo Alto, CA). DNase I, L-glutamine, Terrific Broth and ampicillin were purchased from Gibco BRL (Grand Island, NY).

2.2. Plasmid DNA

pRELuc plasmid is an 11.9 kb pcDNA encoding firefly luciferase driven by Rous sarcoma virus promoter inserted into an Invitrogen (San Diego, CA) pREP4 vector (a gift from Dr. R.G. Ulrich, NCI, Frederick, MD). VR1225 plasmid is a 6413 bp plasmid complementary DNA (pcDNA) encoding firefly luciferase driven by hCMV promoter inserted into an Invitrogen (San Diego, CA) pREP7 vector (a gift from Dr. C.J. Wheeler, Vical, San Diego, CA). All plasmids were amplified in *Escherichia coli* DH5α and purified by the endotoxin-free QIAGEN Giga plasmid purification kit (Hilden, Germany) according to the manufacturer's protocol. The purified plasmids were dissolved in saline and kept in aliquots at a concentration of 1–2 mg/ml. Naked DNA used in this study refer to pcDNA.

2.3. Modification of collagen

Methylated collagen was prepared according to a method described previously (Chia et al., 2000). Briefly, collagen solution (20 ml, 3 mg/ml) was first precipitated with 400 ml of acetone. Precipitated collagen was then dissolved in 200 ml of 0.1 M hydrochloric acid in methanol (Merck), stirred at 4 °C for 6 days under sterile conditions. The mixture was dialyzed against distilled water for 4 days at 4 °C, followed by freeze-drying. The lyophilized modified collagen was stored at −20 °C under desiccation.

2.4. Cell culture

Human embryonic kidney 293 cells (HEK293) were obtained from ATCC and maintained in DMEM supplemented with 10% FBS, L-glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37 °C in a humidified 5% CO_2-containing atmosphere.

2.5. Animals

Six to ten weeks old female Balb/c mice were obtained and housed in National University of Singapore Animal Holding Unit. Mice were maintained on ad libitum rodent feed and water at room temperature and 40% humidity. All animal procedures were approved by the National University of Singapore Faculty of Medicine Animal Care and Use Committee.

2.6. Preparation of native collagen (NC)/DNA and modified collagen (MC)/DNA complexes

Type I collagen solution from Cohesion Technologies is dissolved in 0.012N HCl. The solution (3 mg/ml) was diluted with saline to several working concentrations ranging from 60 to 480 μg/ml with final pH ranging from 2.8 to 3.6. NC/DNA complexes were prepared by mixing pRELuc plasmid DNA (60 μg/ml in saline) with the diluted collagen solutions at the same volumes to achieve various weight ratios (NC to DNA) of 1 to 8. The complexes were incubated for 30 min before use or analysis. DNA binding capacity at different weight ratios were qualitatively analyzed directly by gel electrophoresis (0.8% agarose gel).

The methylated collagen was dissolved in PBS to a concentration of 1 mg/ml. The solution was then diluted with PBS to working concentrations ranging from 60 to 480 μg/ml. MC/DNA complexes were prepared by mixing pRELuc plasmid DNA (60 μg/ml in saline) with MC working solutions at the same volumes to achieve various weight ratios (MC to DNA) of 1 to 8. The complexes were incubated for 30 min before use or analysis. DNA binding capacity at different weight ratios were analyzed by gel electrophoresis (0.8% agarose gel).

2.7. DNA release from the complexes

MC/DNA or NC/DNA complexes were incubated in PBS (0.1 M, pH 7.4) at 37 °C. Aliquots of the mixtures were sampled at various time points and subjected to the gel electrophoresis (0.8% agarose gel). Naked DNA incubated in the same buffer at 37 °C was used as a control. The amounts of DNA released from the complexes were determined by measuring DNA concentration in the supernatant using Hoechst 33258 dye. MC/DNA and NC/DNA complexes of different weight ratios were centrifuged for 30 min at 5000 × *g*. Fifty μl of supernatant was mixed with 150 μl of 1.3× TNE buffer containing 1.5 μg/ml Hoechst dye in a well of a 96-well black plate (NUNC). The fluorescence intensity at 538 nm with cut-off at 530 nm ($\lambda_{ex} = 485$ nm) was measured on a Gemini XS fluorometer (Molecular Devices Corp., Sunnyvale, CA). The measurement was performed in duplicates.

2.8. Cytotoxicity of MC and NC

The cytotoxicity of MC and NC was evaluated using methyl thiazole tetrazolium (MTT) assay in comparison with polyethylenimine and poly (L-lysine) (PLL). Human embryonic kidney 293 cells were seeded in a 96-well plate (Becton–Dickson, Lincoln Park, NJ) with 50 μl of complete DMEM at a density of 20,000 cells per well. Cells were incubated for 24 h at 37 °C. The medium was replaced with fresh medium containing PEI, PLL, MC or NC at concentrations ranging from 0.01 to 1 mg/ml. After 24-h incubation, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well. The plates were subsequently incubated at 37 °C for an additional 2 h, followed by adding 100 μl of extraction buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) to each well. The plate was incubated overnight at 37 °C. The opti-

cal density at 570 nm was measured on a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) using wells without MTT addition as the blank.

2.9. Characterization of MC/DNA and NC/DNA complexes

The size and zeta potential of MC/DNA and NC/DNA complexes were measured in their preparation media, respectively, by photon correlation spectroscopy and laser Doppler anemometry on a Zetasizer 3000 (Malvern Instruments, Southborough, MA). The size measurement was performed at 25 °C at a 90° scattering angle and recorded for 180 s for each measurement. The hydrodynamic diameter was generated by cumulative assay. Zeta potential measurements were performed using an aqueous dip cell in the automatic mode.

2.10. DNase I and serum protection assay

NC/DNA complexes were neutralized to pH 7.4 with 0.1 M sodium hydroxide solution. MC/DNA complexes were prepared in saline. These complexes (40 μl) were then incubated at 37 °C with 0.2 and 0.4 μl of DNase I solution (0.05 U/μl) in 10× reaction buffer (Gibco BRL, Grand Island, NY) for 20 min. The samples were then mixed with 10 μl of stop solution (80 mM iodoacetic acid), followed by adding 10 μl collagenase solution (1 U/μl) and incubated at 37 °C overnight. The mixtures were analyzed by gel electrophoresis (0.8% agarose) for the integrity of DNA. To characterize the protection of plasmid in serum-containing medium, complexes were incubated with DMEM medium containing 10% fetal bovine serum for an hour, and analyzed by the same procedure.

2.11. In vitro transfection activity of the released DNA

Functional integrity of DNA samples released from the complexes at different weight ratios (0 to 8) were evaluated by examining their transfection efficiency using a commercial liposome (Transfast®) (Promega Co., Madison, WI) as a carrier. pRELuc DNA released from the complexes were complexed with Transfast® according to the manufacturer's protocol without purification, at a weight ratio of 2 (Transfast to DNA). HEK293 cells were seeded 24 h prior to transfection into a 24-well plate (Becton–Dickson) at a density of 8×10^4 cells per well with 1 ml of complete DMEM medium. At the time of transfection, the medium in each well was removed, followed by washing cells with 1 ml of PBS in each well. PBS was then replaced with 400 μl of Opti-MEM, followed by adding the Transfast®–DNA complexes containing 1 μg of DNA. The transfection was performed at 37 °C for 1 h followed by adding 1 ml of fresh complete DMEM medium. After additional 48-h incubation, cells were washed with PBS and permeabilized with 200 μl of cell lysis buffer (Promega Co.). The luciferase activity in cell extracts was measured for 10 s using a luciferase assay kit (Promega Co.) on a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). The light units (LU) were normalized against protein concentrations in the cell extracts, which were measured using a Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

2.12. Luciferase expression in mouse tibialis muscle

Bilateral injections of Luciferase, MC/VR1255 or NC/VR1255 complexes were given to the anterior tibialis muscle of balb/c mice at a dose of 3 μg of DNA (6–8 weeks old, eight mice per group). Complexes were prepared in saline (40 μl) at weight ratio of 1:1 and 3:1. NC/VR1255 complexes were neutralized to pH 7.4 before injection. The naïve control group ($n = 6$) received the same volume of saline injection. At days 3, 7, 14, 28 and 36, the injected muscles were isolated. The excised tissue was homogenized in 0.5 ml of 1× lysis buffer. The lysate was subjected to three freeze–thaw cycles. The supernatant was collected after centrifugation ($10{,}000 \times g$, 5 min). An aliquot of 10 μl of cell extracts was used to measure the light units for 10 s using the luciferase assay kit (Promega Co.) on a Lumat LB 9507 (EG&G Berthold).

3. Results and discussion

3.1. MC and NC show different DNA binding capacities

MC was synthesized by partial esterification of the carboxyl groups with methanol in the presence of hy-

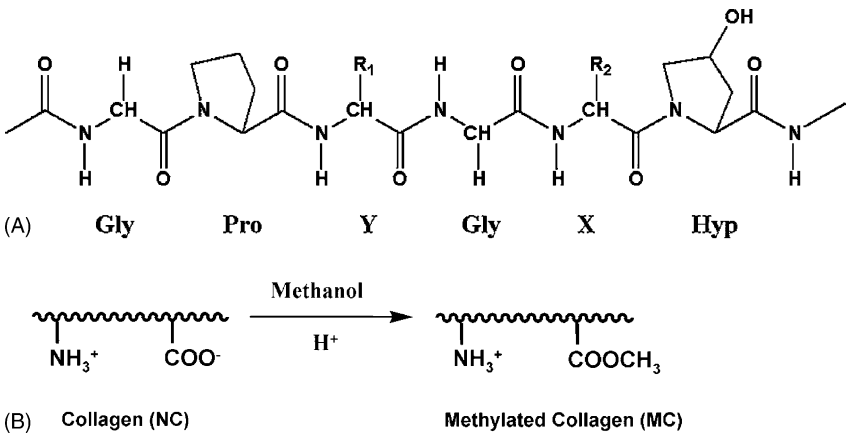

Fig. 1. (A) Representative sequence of native collagen (NC). (B) Preparation of methylated collagen (MC) from NC.

drochloric acid as a catalyst, resulting in a positively charged collagen derivative at neutral pH, shown in Fig. 1 (Chia et al., 2000), thus enhancing ionic association with the negatively charged phosphate backbone of DNA at neutral pH. We also hypothesized it would enhance the stability of the DNA in the complexes at physiological conditions.

MC/DNA complexes were prepared at neutral pH. In contrast, NC could only form complexes with DNA at low pH (<5) when it is soluble and assumes a net positive charge. At neutral pH as in PBS, NC assumes a zwitterionic form and phase separates from the solution. The binding ability of MC (pH 7.4) to plasmid DNA was compared with NC (pH 3) as shown in Fig. 2. The gel electrophoresis analysis suggested that MC could bind completely to the plasmid DNA at a weight ratio of 3 or above in PBS. We tested the NC/DNA complex formation at pH 3. NC could bind DNA completely at weight ratios of 4 and above.

Under these conditions (pH 7.4 and weight ratio >3 for MC, and pH 3 and weight ratio >4 for NC), MC and NC formed uniform complexes with plasmid DNA, respectively. Temperatures between 25 and 55 °C did not affect the complex formation and quality of complexes. For MC/DNA complexes, using 5% glucose or saline or HEPES as the preparation medium did not significantly affect complex formation and the degree of aggregation. But the use of high concentration of carriers or large volumes of MC/NC or DNA solution caused visible aggregation (data not shown). In the following studies, the volumes of the DNA and MC or NC solutions were kept to less than 200 μl during the preparation to minimize aggregation.

3.2. Characterization of MC/DNA and NC/DNA complexes

The effect of weight ratio on the particle size and surface charge of complexes was examined using the Zetasizer. MC/DNA complexes prepared in saline with the weight ratio of 3 through 80 had the average particle size ranging from 300 nm to 2 μm. The largest MC/DNA complexes were formed at weight ratio of 9 and the average size dropped rapidly as weight ratio increased, shown in Fig. 3(A). NC/DNA complexes formed with a larger average size than MC/DNA complexes in general, ranging from 800 nm to 4 μm, shown in Fig. 3(A). The degree of aggregation was more severe in NC/DNA formulation at higher weight ratios.

Results from Figs. 2 and 3 suggest that the total number of positive charges per collagen molecule is lower in NC at pH 3 compared with MC at pH 7.4. This lower positive charge density of NC (pH 3) led to less efficient complexation/binding between DNA and NC. The NC/DNA particles appeared two to five times larger than MC/DNA particles. This could be an indication that the particles are less condensed due to lowered binding affinity between DNA and NC. An alternative explanation is that each DNA molecule requires more collagen for efficient condensation, resulting in higher collagen payload per particle. Further analysis is needed to explain the size and zeta

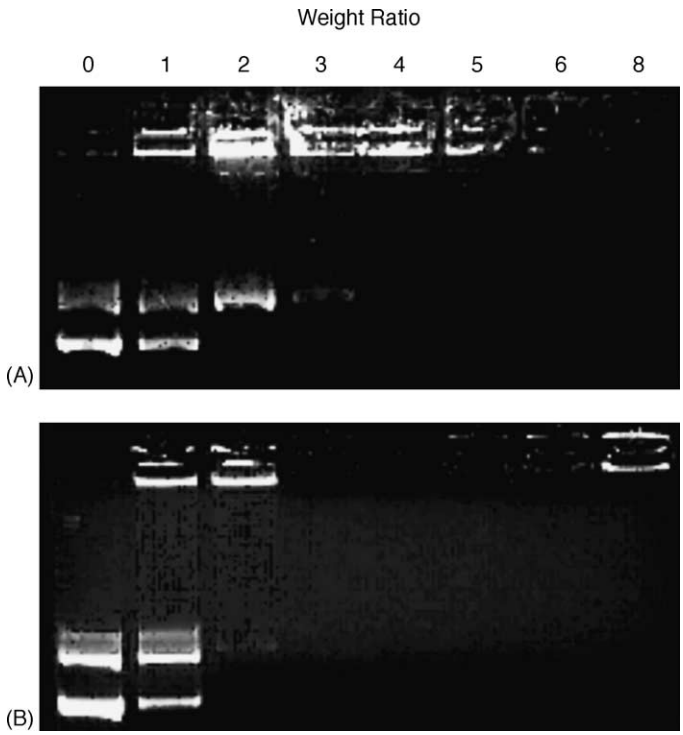

Fig. 2. Gel electrophoresis images showing DNA-binding abilities of (A) NC/DNA and (B) MC/DNA complexes at different weight ratios. The weight ratio at which NC and MC completely bind DNA is 4 and 3, respectively.

potential differences between MC/DNA and NC/DNA complexes.

It is worth emphasizing that the particle size and surface charge for NC/DNA complexes were measured in the preparation medium (pH 3). When these particles were neutralized by adding to PBS or cell culture medium, significant aggregation occurred and particles became nearly neutral. Data are not shown as the

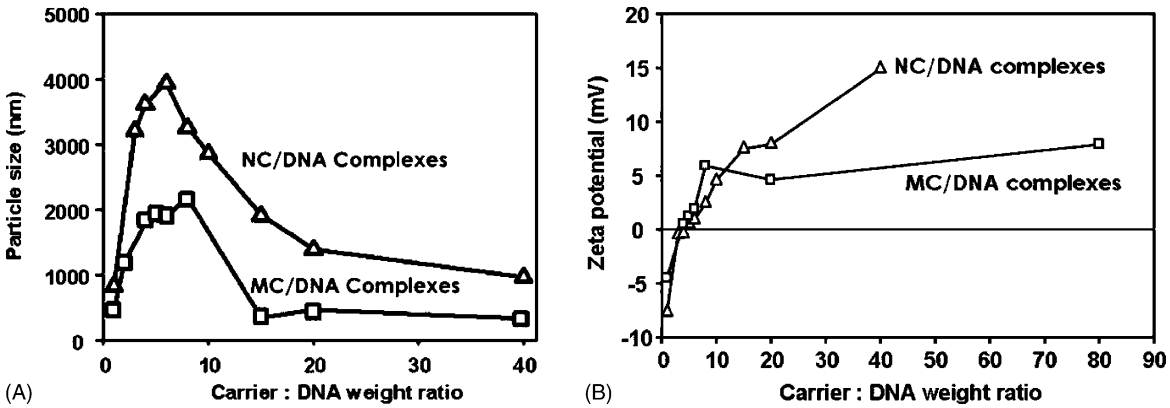

Fig. 3. The mean particle size (A) and zeta potential (B) of NC/DNA and MC/DNA complexes as a function of weight ratio. NC/DNA complexes were prepared and characterized at pH 3, whereas MC/DNA complexes were prepared and characterized in PBS at pH 7.4. Values represent the average of three measurements with 10 subruns for each measurement.

measurements were not stable when aggregation occurred. This observation highlights the advantage of MC as a gene carrier with regard to the preparation and stability of the complexes.

The zeta potential of MC/DNA complexes reached a plateau of 6 mV at the weight ratio of 9, shown in Fig. 3(B). This is typical of polymer–DNA complexation. On the other hand, NC/DNA complexes showed increasing surface charge as the weight ratio increased, no plateau was observed up to weight ratio of 40, suggesting that the complexation/condensation continued as N/P increased from 1 to 40. This observation is consistent with the notion that NC is a weaker polycation.

3.3. DNA release kinetics from MC/DNA and NC/DNA complexes

When MC/DNA and NC/DNA complexes were incubated in PBS at 37 °C, both complexes released DNA gradually, as shown by gel electrophoresis in Fig. 4. It indicated that MC/DNA complexes had a sustained release of DNA, and the release rate was weight ratio-dependent. A sustained release over a period of 3 weeks was achieved for complexes with a weight ratio between 4 and 8. This sustained release of DNA is most likely due to the hydrolytic cleavage of ester groups of MC, which decreased the positive charges in the gene carrier.

NC/DNA complexes prepared at low pH were neutralized with 2× PBS. The mixture was incubated at 37 °C. A rapid DNA release was observed for NC/DNA complexes. All DNA was released from NC/DNA complexes within 6 h versus 7 days for MC/DNA complexes.

The DNA release rate from the MC/DNA complexes for the first 3 days was quantified using the Hoechst 33258 dye. The conditions for this assay were optimized for the concentration and volume of Hoechst 33258 solution, centrifugation time, reaction time and the volume ratio of sample to TNE buffer. Fig. 4(C) was obtained using the optimized conditions described in the Section 2. Partial binding of DNA occurred with weight ratios between 1 and 3. For example, at a weight ratio 1, only 44% of the DNA was incorporated into the complexes, and 12, 3 and 6% of the DNA were released from the complexes on days 1 through 3, respectively. Release profiles for MC/DNA complexes with weight ratios from 3 to 8 were similar. A burst release of DNA was observed on day 1 for all complexes, followed by a steady release of ∼5% DNA per day. As discussed earlier, the release rate was most likely controlled by the cleavage rate of the methyl groups from the MC side chains.

3.4. DNA released from complexes retains bioactivity

The conformation and bioactivity of DNA released from the complexes could be assessed by gel electrophoresis and transfection ability, respectively. As revealed in Fig. 4, no significant degradation of DNA was observed, although certain degree of nick was present. To determine the transfection activity of DNA released from the complexes, DNA samples recovered on day 12 were complexed with Transfast® at a dose of 1 μg DNA at a Transfast® to DNA ratio of 2. These complexes were applied to transfect HEK 293 cells. Plasmid DNA incubated at the same condition was used as a control. The transfection results indicated that the DNA released from the MC/DNA complexes at weight ratios of 3 and 8 exhibited a transfection activity comparable to the original DNA, whereas DNA released from NC/DNA complexes had a slightly lower transfection activity (data not shown). These results suggest a better preservation of the bioactivity of DNA in MC/DNA complexes.

3.5. MC partially protects plasmid DNA against DNase I and serum degradation

The protection of plasmid DNA in MC/DNA against DNase I degradation was demonstrated in a gel electrophoretic analysis, as shown in Fig. 5. Complexes were subjected to DNase I for 15 min and then to collagenase overnight at 37 °C to release DNA from the complex. MC/DNA complexes could be degraded by collagenase and therefore release DNA completely as shown in Lanes 4 and 5 of Fig. 5. This suggested that this methylation modification does not alter collagen's susceptibility to collagenase degradation. Incubation of naked DNA in 10 U/ml of DNase I at 37 °C for 15 min led to complete degradation of DNA (Lane 6). MC/DNA complexes at a weight ratio of 10 or less than 10 (data not shown) subjected to the same treatment offered little protection to the plasmid, resulting in a similar degree of degradation (Lane 7). Complexes at a weight ratio of 20 rendered a higher

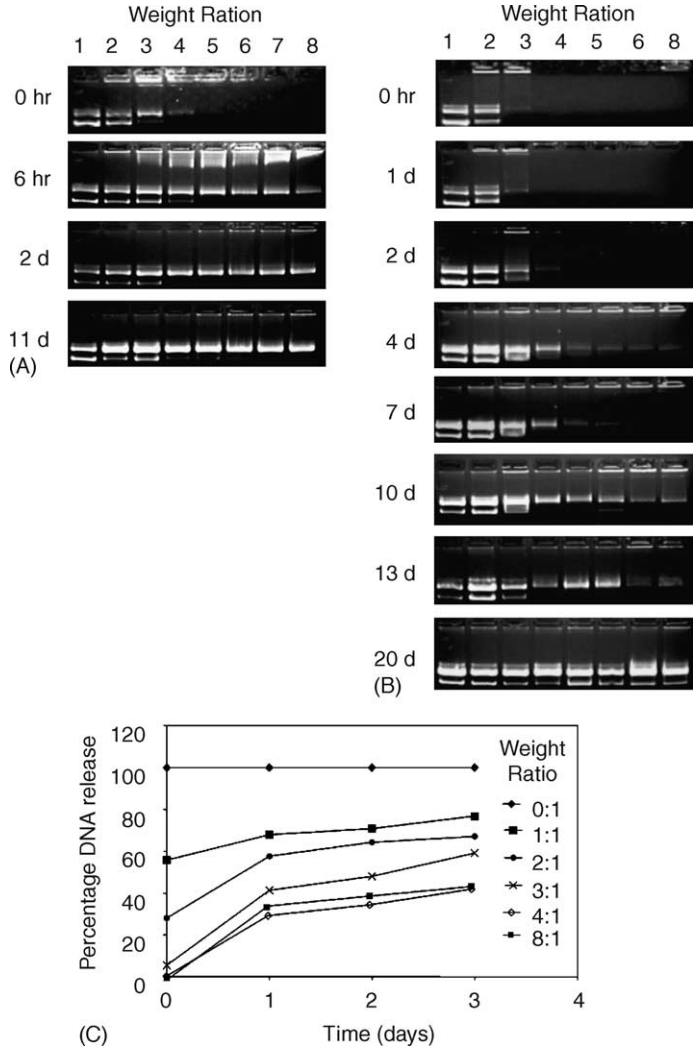

Fig. 4. Gel electrophoresis images showing DNA release from (A) NC/DNA and (B) MC/DNA complexes prepared at various weight ratios. Complexes were neutralized (in case of NC/DNA complexes) and incubated at PBS at 37 °C, and then analyzed on a 0.8% agarose gel at different time points. Most DNA was released from NC/DNA complexes in less than 6 h at all weight ratios (1–8) tested, while gradual release from MC/DNA complexes was observed over the period of two weeks depending on the weight ratio of the complexes. (C) DNA release from MC at different weight ratio was quantified using Hoechst 33258 dye.

degree of protection even though the DNA was still partially degraded (Lane 8). The degree of protection by MC/DNA complexes in complete medium containing 10% serum at 37 °C was also tested. Naked plasmid incubated with serum for 1 h showed significant degradation (Lane 9). DNA recovered from the MC/DNA complexes subjected to the same incubation had significant degradation as well, as shown in Lanes 10 and 11, although complexes with a weight ratio of 20 offered higher degree of protection. This indicated more room for improvement for the protection to DNA in the complexes. NC/DNA complexes, on the other hand, exerted no protection to DNA at the same weight ratios. DNA was completely degraded when subjected to 10 U/ml of DNase I treatment for 15 min at 37 °C (data not shown).

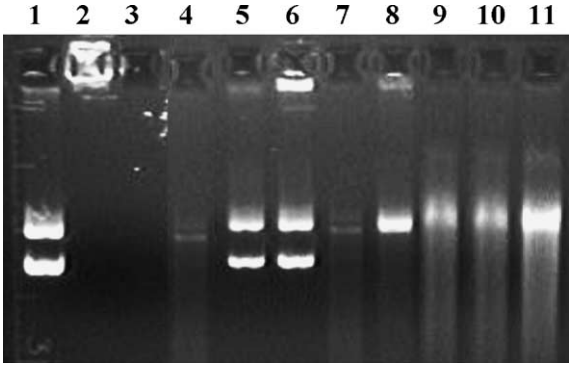

Fig. 5. DNA protection conferred by MC against DNase I degradation. Lane 1: plasmid DNA; Lanes 2 and 3: untreated MC/DNA complexes at weight ratios 10 and 20, respectively; Lanes 4 and 5: MC/DNA complexes at weight ratios of 10 and 20, respectively, were subjected to 10 μl collagenase overnight; Lanes 6–8: naked DNA and MC/DNA complexes at weight ratios of 10 and 20, respectively, were subjected to 0.01 U/μl DNase I at 37 °C for 15 min; Lanes 9–11: naked DNA and MC/DNA complexes at weight ratios of 10 and 20, respectively, were subjected to incubation with DMEM medium with 10% serum for 1 h.

These results highlighted the protection effect of MC carrier offered to DNA, and the degree of protection increased with the MC/DNA weight ratio. Although complexes formed at low weight ratio (≤10) showed no protection to DNA, at physiological conditions (for instance in muscle or skin) where nuclease concentration is significantly lower than the tested condition, complexes with lower weight ratios could likely confer protection to DNA.

3.6. MC exhibits minimal cytotoxicity

The cytotoxicity of MC was evaluated using a MTT assay in comparison with NC. COS-7 cells were incubated with different concentrations of MC and NC from 10 μg/ml to 1 mg/ml for 24 h. Two widely used polymeric carriers, PLL and PEI, were included as controls, and the results were shown in Fig. 6. As expected, no cytotoxicity was observed for both MC and NC with concentration up to 1 mg/ml. Neither the proliferation rate nor the morphology of the cells was different compared with untreated cells. In contrast, PEI and PLL both had a LD_{50} of less than 10 μg/ml.

The cytotoxicity of polymer/DNA complexes is usually lower than the polycationic carrier alone (Wang et al., 2004). Under cell transfection condition

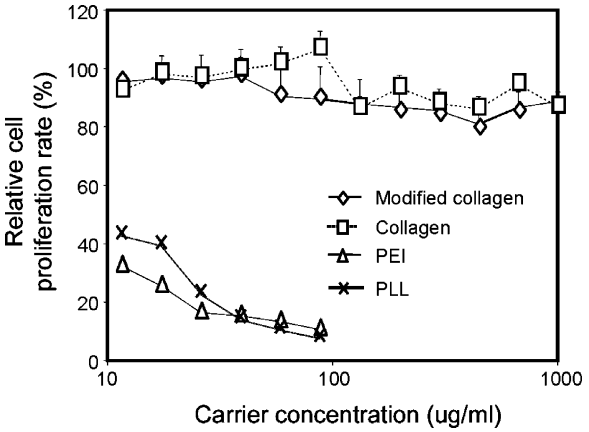

Fig. 6. Cytotoxicity of MC and NC in COS-7 cells in comparison with poly-L-lysine (PLL; Mw 27 kDa) and polyethyleneimine (PEI; Mw 25 kDa). Results represent mean ± S.D. ($n = 3$).

shown below, the doses of MC and NC carriers were in the range of 5–15 μg/ml. No sign of cytotoxicity was observed based on the observation of cell morphology and the total protein content reflecting cell proliferation rate (data not shown).

3.7. MC/DNA and NC/DNA complexes exhibit low transfection ability in HEK293 cells

In vitro transfection activity of MC/DNA and NC/DNA complexes in HEK293 cells using pRE-luciferase plasmid is shown in Fig. 7. PEI transfected HEK293 cells well, reaching 10^9 RLU/mg of protein. The presence of 100 μM of chloroquine (CQ) does not affect the transfection. This also confirms that PEI/DNA complexes could be released from endosomes efficiently due to the strong buffering capacity of PEI (Boussif et al., 1995). Naked VR1255 plasmid gave about 20 times higher transfection efficiency than the background level. Adding 0.1 M CQ in the transfection medium reduced the transfection efficiency by 80%. Surprisingly, although it formed complexes with DNA, NC did not improve the transfection efficiency at all. Quite the contrary, it hindered transfection at high weight ratios. This is most likely related to the severe aggregation of the NC/DNA particles in the medium. As expected, MC/DNA complexes yielded 18.9-fold higher transfection efficiency than naked DNA or NC/DNA complexes at a weight ratio of 5. This is likely due to

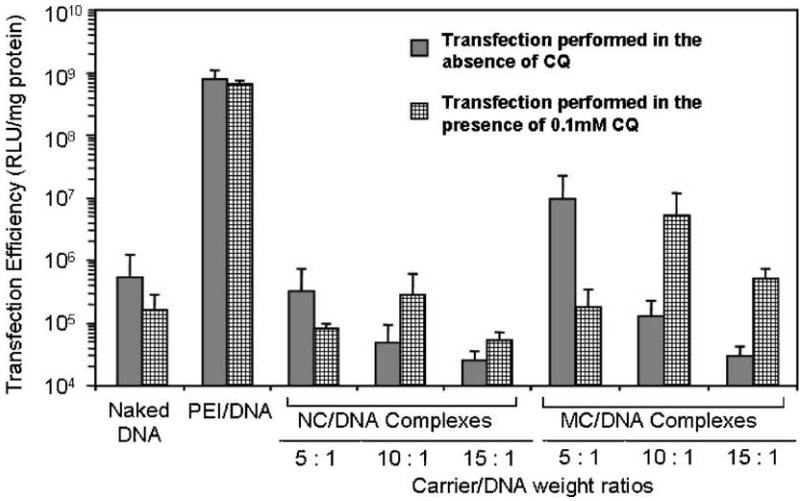

Fig. 7. Transfection efficiency of NC/DNA and MC/DNA complexes prepared at various weight ratios in HEK 293 cells in the absence and presence of chloroquine (CQ) using pRE-luciferase plasmid. Results represent mean ± S.D. ($n = 3$).

the improved protection of DNA and stability of the complexes as described earlier. However, increasing the weight ratio of MC/DNA gradually reduced the transfection efficiency to below the naked DNA transfection level. This might be the result of competition between the complexes and free NC or MC. Interestingly, supplementing CQ to the transfection medium markedly reduced the transfection efficiency mediated by MC/DNA complexes at a MC/DNA weight ratio of 5, whereas at higher weight ratios (10 or 15), transfection efficiency was significantly enhanced, even though the overall level of gene expression was still similar or lower than that of MC/DNA complexes at weight ratio of 5 in the absence of CQ. NC/DNA complexes followed the same trend except that the gene expression levels were much lower as shown in Fig. 7. In general, the results showed that MC mediated a higher gene transfection in culture than NC, although the level of gene expression was still much lower than that achieved with PEI/DNA complexes.

3.8. NC enhances luciferase expression in muscle compared with naked DNA

We tested the gene expression mediated by MC/DNA and NC/DNA complexes in a mouse muscle model. Naked DNA and different complexes were given at a dose of 3 μg of DNA per muscle in 40 μl of saline. A number of studies have shown that cationic DNA complexes do not transfect myofibers when injected intramuscularly. One of the plausible hypotheses is that the highly negatively charged extracellular matrix (Caron et al., 2001) in muscle tissue binds the cationic complexes or nanoparticles (Ruponen et al., 2001) and therefore hinders the transfection process. Our pilot study using MC/DNA complexes with weight ratios of 5 and 10 did not show any detectable transfection (data not shown). In this study, we tested complexes with low weight ratios (1 and 3). The surface charges of these complexes were negative or close to neutral (−8 to 0 mV).

The luciferase expression by naked DNA injection peaked at day 3 and maintained at the similar level for 4 weeks. MC/DNA complexes (1:1, w/w) yielded comparable level of gene expression as naked DNA throughout the experimental period. Increasing the weight ratio to 3 almost completely abolished the gene expression. Contrary to in vitro transfection results where MC/DNA complexes outperformed NC/DNA complexes, NC/DNA complexes showed similar or higher gene expression in mouse muscle. NC/DNA complexes yielded a 3.8-fold higher gene expression at week 2 than naked DNA or MC/DNA complexes ($P < 0.05$) as shown in Fig. 8. Increasing weight ratio of NC to DNA significantly reduced the gene expres-

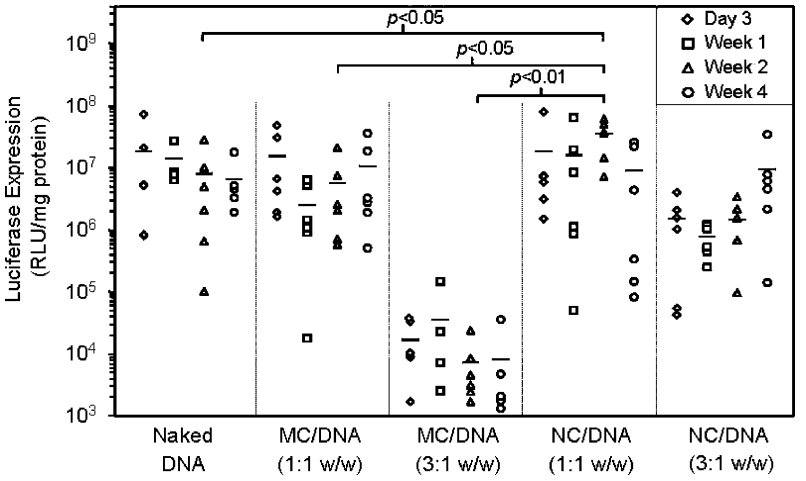

Fig. 8. Luciferase expression in mouse muscle after intramuscular injections of naked DNA, MC/DNA and NC/DNA complexes at weight ratios of 1 and 3. Naked DNA and complexes were given at a dose of 3 μg of DNA per muscle in 40 μl of saline. Gene expression in muscle was assayed after 3 days, 1, 2 and 4 weeks. Each symbol represents one experimental data, and the horizontal line in each column indicates the mean value ($n = 5$–6). *P*-values were calculated by student *t*-test.

sion level at day 3, but it increased gradually to about the same level as NC/DNA complexes at weight ratio of 1 on week 4.

It is worth noting that at low weight ratios there is significant amount of free DNA uncomplexed in the mixture. For example, at weight ratio of 1, 58% of the total DNA remained uncomplexed in MC/DNA mixture. The amount of free DNA in NC/DNA complexes would be slightly higher than that in MC/DNA complexes. Luciferase expression observed by the MC/DNA or NC/DNA complexes might be due to the residue amount of naked DNA, or the combination of naked DNA, sustained release of DNA from the complexes and the transfection by complexes. In particular, the mechanism for enhanced gene expression by NC/DNA complexes at week 2 is unclear.

This study further confirms that polymer–DNA complexes carrying positive surface charges inhibit DNA transfection in the muscle, even when the carrier is highly degradable. The fact that improved DNA protection and complexes stability did not translate to increased transfection efficiency suggested that gene delivery via the intramuscular route followed a different mechanism that demands a different set of physiochemical properties of the carrier from other parental routes. The transfection efficiency of MC/DNA complexes in other administration routes, where cationic carriers have shown enhanced gene transfer efficiency, should further be explored.

In summary, these collagen-based gene carriers possess some favorable characteristics, including the low toxicity, biodegradation property, sustained release of DNA and good cell adhesion property, which might facilitate cell binding and uptake of MC/DNA or NC/DNA complexes. Native collagen formed complexes with DNA at acidic pH, but conferred little protection to DNA due to its poor stability in serum. MC formed complexes with DNA at neutral pH and improved the stability of the complexes at physiological conditions. MC/DNA complexes released bioactive plasmid DNA for up to 3 weeks in PBS, whereas NC/DNA complexes released almost all the DNA within 6 h. In vitro gene transfection experiments revealed that MC mediated a slightly higher gene expression than NC, although the level of gene expression was still much lower than that achieved with PEI/DNA complexes. In a mouse muscle model using luciferase as a marker gene, NC/DNA complexes showed higher transfection efficiency than MC/DNA complexes, particularly at higher weight ratios. The good biocompatibility, biodegradability of collagen-based complex systems warrant more studies to improve the gene transfer efficiency, though opti-

mization of administration route and physiochemical properties of MC/DNA complexes.

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