Chitosan based oligoamine polymers: Synthesis, characterization, and gene delivery
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A R T I C L E   I N F O

Article history:
Received 26 November 2008
Accepted 9 March 2009
Available online 18 March 2009

Keywords:
Chitosan
Oligoamine
Gene vector
Transfection efficiency

A B S T R A C T

A series of chitosan-based oligoamine polymers was synthesized from N-maleated chitosan (NMC) via Michael addition with diethylenetriamine (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and linear polyethyleneimine (Mw 423), respectively. The resulted polymers exhibited well binding ability to condense plasmid DNA to form complexes with size ranging from 200 to 600 nm when the polymer/DNA weight ratio was above 7. The polymer/DNA complexes observed by scanning electron microscopy (SEM) exhibited a compact and spherical morphology. The cytotoxicity assay showed that the synthesized polymers were less toxic than that of PEI(25 K). The gene transfection effect of resulted polymers was evaluated in 293T and HeLa cells, and the results showed that the gene transfection efficiency of these polymers was better than that of chitosan. Moreover, the transfection efficiency was dependent on the length of the oligoamine side chains and the molecular weight of the chitosan derivatives.

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1. Introduction

The development of safe and efficient gene delivery vectors is a prerequisite for the success of gene therapy [1–3]. Non-viral gene vectors such as cationic liposomes and cationic polymers are being sought as alternatives for viral vectors since non-viral gene vectors have several advantages over viral counterparts, including low immune response, ease of production, the ability to transfer large DNA molecules and potential cell targeting property [3,4]. Among non-viral vectors, chitosan which is derived from natural sources has been considered as a safer alternative to other non-viral gene vector candidates since chitosan is known as a biocompatible, biodegradable, and low toxic material with high cationic potential [5]. Due to the p-glucosamine residue in chitosan having a pKa of around 6.2–7.0, the amines in chitosan could become positively charged at acidic pH below pKa. Therefore, these protonated amines enable chitosan to bind negatively charged DNA and condense it into particles. Besides, it was reported that chitosan has shown a promise to protect DNA from DNase I and II degradation and transfect DNA into different cell types [6]. To date, although many encouraging results have demonstrated chitosan as non-viral gene carriers [7–9], a main drawback is the low gene transfection efficiency for the chitosan-based gene delivery systems. It has been suggested that the low transfection efficiency was attributed to the strong interactions between chitosan and DNA, resulting in highly stable particles, thereby preventing dissociation within the cell and ultimately precluding translation of the DNA [10].

In addition, the poor water solubility of chitosan at physiological pH also has the negative influence on the transfection. To overcome these drawbacks, many chitosan derivatives have been developed in the last few years. For example, chemical modifications of chitosan using hydrophilic [11,12], hydrophobic [13,14] moieties were reported to enhance the water solubility of chitosan. To improve gene transfection efficiency, pH-sensitive [15], thermosensitive [16,17] and cell-specific [18–20] polymers or groups grafting with chitosan were also reported.

Polyethyleneimine (PEI), a cationic polymer, has been demonstrated as an effective non-viral vector and widely used in gene transfection study [21–23]. It is widely accepted that the good transfection ability of PEI is due to its high buffer capacity over a broad pH, which is called “the proton sponge effect” [24,25]. Although PEI(25 K) has many advantages such as high condensation capability and strong buffer capacity [26,27], the high toxicity and lack of biodegradability of PEI(25 K) remain to be the drawbacks, especially preventing repeated administration in vivo gene delivery [28]. Further studies showed that the cytotoxicity of PEI is dependent on its molecular weight. A lower molecular weight PEI has a lower cytotoxicity but these lower molecular weight compounds suffer from lower transfection ability [27,29].

Therefore, it is attractive for the combination of multiple lower molecular weights PEI fragments and biocompatible polymers for gene vectors. Up to now, low molecular weight PEI grafted to dextran, chitosan, polyaspartamine or cyclodextrin as effective gene vector has been reported [30–33]. Our group also developed low molecular weight PEI grafted chitosan with higher transfection efficiency and lower cytotoxicity [34]. However, a clear relationship between structure and transfection efficiency was not elucidated. In this
study, a series of chitosan based oligoamine polymers was synthesized and the effect of the oligoamine side chains on gene vectors was evaluated systematically.

2. Materials and methods

2.1. Materials

Diethylenetriamine (DETA), triethylenetetramine (TETA), tetra-ethylenepentamine (TEPA) were obtained from Shanghai Chemical Reagent Co., China. Chitosans ($M_w = 5 K, 10 K, 50 K$ with deacetylation degree 85.5%, 85.3%, 85.3%, respectively) were purchased from Haidebei Marine Bioengineering Co. Ltd., Jinan, China. Linear polyethylenimine (Mn, 423) and branched PEI(25 K) were provided by Aldrich-Sigma Chemical Co. Dimethyl sulphoxide (DMSO) obtained from ShangHai Chemical Reagent Co., China. Chitosans were obtained from Haidebei Marine Bioengineering Co. Ltd., Jinan, China. Linear polyethylenimine (Mn, 423) and branched PEI(25 K) were obtained from Aldrich-Sigma Chemical Co. Dimethyl Sulphoxide (DMSO) obtained from Shanghai Chemical Co., China. Ethylenepentamine (TEPA) were obtained from Shanghai Chemical Co., China. D-glucosamine unit, H-3, H-4, H-5, H-6, H-6′

2.2. Synthesis of N-maleated chitosan (NMC)

NMC was synthesized according to our previous study[34]. In brief, NMC was synthesized by acylation of chitosan with maleic anhydride. The mixture was placed in a 60 °C oil bath for 8 h. The product was precipitated in 500 mL acetic acid, washed with acetone and ether three times, and then dried to obtain NMC.

NMC(5 K): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.33–3.54$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 5.68–5.72$ and $\delta = 6.17-6.20$ (CH = CH–); $\delta = 1.80$ (COCH3). Grafting degree (GD): 65.6%.

NMC(10 K): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.35–3.58$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 5.72–5.76$ and $\delta = 6.18–6.22$ (CH = CH–); $\delta = 1.82$ (COCH3). GD: 60.4%.

NMC(50 K): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.35–3.58$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 5.70–5.75$ and $\delta = 6.18–6.22$ (CH = CH–); $\delta = 1.82$ (COCH3). GD: 60.0%.

2.3. Synthesis of NMC with oligoamine side chains

To graft oligoamine onto NMC, 0.2 g NMC was dissolved in 20 mL 0.25% sodium hydroxide solution. The aqueous solution with 3 g of oligoamine was added into the above solution. The mixture was stirred at 60 °C for 24 h. Then hydrochloric acid was added in the mixture with stirring until pH value reached 7.0. The product was dialyzed (MWCO: 3.5 K) against distilled water for 3 days and then lyophilized for 3 days. The products are designated as NMC($X$)-g-OEI (X refers to the molecular weight of raw chitosan, OEI refers to DETA, TETA, TEPA and PEI(423)).

NMC(5 K)-g-DETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.28–3.60$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.53–2.90$ (−NHCH2CH2–); $\delta = 1.85$ (COCH3). GD: 41.3%.

NMC(10 K)-g-DETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.30–3.61$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.52–2.91$ (−NHCH2CH2–); $\delta = 1.86$ (COCH3). GD: 40.7%.

NMC(50 K)-g-DETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.32–3.6$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.50–2.99$ (−NHCH2CH2–); $\delta = 1.89$ (COCH3). GD: 39.3%.

NMC(5 K)-g-TETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.29–3.60$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.51–2.88$ (−NHCH2CH2–); $\delta = 1.86$ (COCH3). GD: 39.7%.

NMC(10 K)-g-TETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.31–3.61$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.49–2.90$ (−NHCH2CH2–); $\delta = 1.88$ (COCH3). GD: 38.5%.

NMC(50 K)-g-TETA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.30–3.62$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.51–2.87$ (−NHCH2CH2–); $\delta = 1.83$ (COCH3). GD: 36.5%.

NMC(5 K)-g-TEPA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.35–3.54$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.50–2.87$ (−NHCH2CH2–); $\delta = 1.85$ (COCH3). GD: 34.7%.

NMC(10 K)-g-TEPA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.40–3.63$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.58–2.95$ (−NHCH2CH2–); $\delta = 1.93$ (COCH3). GD: 35.0%.

NMC(50 K)-g-TEPA: $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.32–3.75$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.53–2.89$ (−NHCH2CH2–); $\delta = 1.82$ (COCH3). GD: 30.7%.

NMC(5 K)-g-PEI(423): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.28–3.74$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.56–2.85$ (−NHCH2CH2–); $\delta = 1.79$ (COCH3). GD: 21.6%.

NMC(10 K)-g-PEI(423): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.28–3.50$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.57–2.86$ (−NHCH2CH2–); $\delta = 1.79$ (COCH3). GD: 20.6%.

NMC(50 K)-g-PEI(423): $^{1}H$ NMR (D2O, ppm) d: $\delta = 3.29–3.48$ (multiplet, p-glucosamine unit, H-3, H-4, H-5, H-6, H-6′); $\delta = 2.47–2.82$ (−NHCH2CH2–); $\delta = 1.82$ (COCH3). GD: 16.2%.

2.4. Buffer capacity

The buffer capacity of cationic copolymers was determined by acid-base titration assay as described by Benns et al.[35,36]. Briefly, 0.2 mg/mL of each sample solution was prepared in 30 mL 150 mM NaCl solution. The sample solution was firstly titrated by 0.1 M NaOH to a pH of 10, and then 0.1 M HCl solution with particular volume was added to the solution to obtained mixtures with different pH values which were determined using a microprocessor pH meter.

2.5. Preparation of NMC-g-OEI/DNA complexes

NMC-g-OEI was dissolved in NaCl solution (150 mM, which is mimicking physiological saline environment) with a concentration of 2 mg/mL and then filtered using a 0.22 μm filter. A plasmid DNA stock solution (120 ng/μL) was prepared in 40 mM Tris–HCl buffer solution. Nanoparticles were prepared by adding copolymer solution to equal volumes of DNA solution (containing 1 μg DNA) at various weight ratios with gentle vortexing and incubated at 37 °C for 30 min before use.

2.6. Cell viability assay

Evaluation of the cytotoxicity of polymers as well as polymer/DNA complexes was performed by MTT method [37]. Cells were seeded in the 96-well plate at a density of 6000 cells/well in 100 μL DMEM containing 10% FBS. After incubation for 24 h, polymer solutions (2 mg/mL) were added to the culture medium, while polymer/DNA
complexes (w/w = 20) and PEI/DNA complexes (N/P = 10, equal to PEI(25 K)/DNA at weight ratio of 1.3. Here, N/P ratio refers to the ratio of moles of the amine groups of copolymer to moles of phosphates of DNA) were added to each well of the culture medium at 1 μg DNA/well. Cell viability was tested after the addition of polymer or polymer/DNA complexes for 48 h. After incubation, 20 μL MTT (5 mg/mL) solution in phosphate-buffered saline (PBS, pH = 7.4) was added to each well and further incubated in 5% CO2 incubator at 37 °C for 4 h. After removal of the MTT containing medium, 150 μL DMSO was added to dissolve the formazan crystals formed in live cells. Finally, the absorbance was measured at 570 nm using a microplate reader (BIO-RAD, Model 550, USA). The relative cell viability was calculated as: Cell viability (%) = (ODsample)/(OD control)×100, where ODcontrol was obtained in the absence of polymers or polymer/DNA complexes and ODsample was obtained in the presence of polymers or polymer/DNA complexes. Each value was averaged from 4 parallel experiments.

2.7. Agarose gel electrophoresis

To assess DNA condensation ability of the copolymers, electrophoresis was performed. The NMC-g-OEI/DNA complexes with different weight ratios ranging from 4 to 7 were formed according to the conditions described above. Then 6 μL complexes suspension containing 0.1 μg DNA was electrophoresed on the 0.7% (w/v) agarose gel containing GelRed™ and with Tris-acetate (TAE) running buffer at 80 V for 80 min. DNA retardation was observed by irradiation with UV light and assayed with Cam2com software.

2.8. Measurement of particle size and zeta-potential

The particle size and zeta-potential measurements were performed at pH 7.0 in triplicates by Nano-ZS ZEN3600 (MALVERN Instrument) at 25 °C. The NMC-g-OEI/DNA complexes were prepared by adding appropriate volume of copolymer solution (150 mM NaCl solution) to 1 μg of DNA (in 40 mM Tris–HCl buffer solution) at weight ratios ranging from 5 to 11. The solution containing complexes was diluted by 150 mM NaCl solution for particle size measurement or diluted by distilled water for zeta-potential measurement to 1 mL, and then incubated at 37 °C for 30 min.

2.9. Scanning electron microscopy (SEM)

The polymer/DNA complexes were prepared as described above. 100 μL of complex suspension was deposited onto a glass slide. After drying at room temperature, the morphology of the sample was observed by a scanning electron microscope (SEM, FEI-QUANTA 200, Holland). Before the SEM observation, the samples were fixed on an aluminum stub and coated with gold for 7 min.

2.10. In vitro transfection

Cells were seeded in 24-well plates at an initial density of 6 × 10⁴ cells/well with 1 mL DMEM containing 10% FBS and incubated at 37 °C for 24 h in 5% CO2 (to reach 70% confluence at the time of transfection). The polymer/DNA (pEGFP-C1 or pGL3-Luc) complexes were formed at different weight ratios ranging from 10 to 40 according to the conditions described above (containing 1 μg DNA in each weight ratio). Before transfection, the cells were washed by phosphate buffered saline (PBS, 0.1 M, pH 7.4), and the cells were incubated with the complexes in 10% serum-containing culture medium for 4 h at 37 °C. Then the medium was replaced with fresh medium containing serum and incubated for 48 h. And the cells were analyzed for green fluorescence protein (pEGFP-C1) expression with a fluorescence microscope (OLYMPUS IX70, Japan).

To assay the expression of luciferase, the medium was removed and the cells were rinsed gently by PBS. After thorough lysis of the cells with reporter lysis buffer (Promega) (200 μL/well), the luciferase activity was determined by detecting the light emission from an aliquot of cell lysate incubated with 100 μL of luciferin substrate (Promega) in a luminometer (Lumat LB9507, Berthold). The protein content of the cell lysate was determined by BCA protein assay kit [38]. All the experiments were carried out in triplicate to ascertain the reproducibility.

2.11. Confocal laser scanning microscopy

8 × 10⁴ HeLa cells were seeded per well in 6 well chamber slides and allowed to grow for 24 h. Polymer/DNA complexes were prepared as described above using 2.5 μL of 10 μM YOYO-1 (Invitrogen) labeled 1.0 μg DNA/well and incubated with the cells for 4 h at 37 °C in DMEM with 10% FBS. Subsequently, cells were washed twice with PBS and

Scheme 1. Schematic illustration of the synthesis of N-maleated chitosans with oligoamine side chains.
incubated with fresh DMEM with 10% FBS for 10 min. The cells were directly visualized on confocal laser scanning microscopy (C1-si, Nikon, Japan). The micrographs were obtained at the magnification of 400×. For excitation of YOYO-1 fluorescence, an argon laser with an excitation wavelength of 488 nm was used.

3. Results and discussion

3.1. Synthesis of N-maleated chitosan with oligoamine side chains

N-Maleated chitosan with different oligoamine side chains was prepared as illustrated in Scheme 1. The products were synthesized via Michael addition by the reaction between amino groups of oligoamine with the double bonds of N-maleated chitosan. The Michael-type addition of the amine to the conjugated double bonds of N-maleated chitosan is compatible with a diversity of functional groups, and this reaction allows the introduction of a large variety of side groups into the chitosan. In this study, we used excessive oligoamine to avoid gelation. The reaction was carried out in basic environment with following advantages. First, NMC could be completely dissolved in basic water. Secondly, the basic environment is in favor of Michael addition reaction. The synthesis route is simple and can be carried out conveniently.

The 1H NMR spectra of NMC, and NMC-g-OEI are shown in Fig. 1. In the spectrum of NMC-g-OEI, the peak ascribed to –CH = CH– disappears, and the proton peaks of OEI (–NHCH2CH2–) appear at 2.5–3.0 ppm, indicating that OEI was grafted to NMC chain success fully. All polymers showed good water solubility and their 1H NMR spectra were in accordance with the expected structures. The grafting degree of oligoamine decreased from DETA to PEI(423), calculated by 1H NMR spectra. The reason was attributed to the reduced reaction activity of the increased length of oligoamine.

3.2. Agarose gel electrophoresis

The formation of complexes between DNA and NMC-g-OEI was observed by agarose gel electrophoresis. The complexes of polycation/DNA at a weight ratio ranging from 4 to 7 were electrophoresed separately in agarose gel. Naked DNA was used as a control. As shown in Fig. 2, all polymers showed good DNA binding ability and migration of DNA in agarose gel was completely retarded when the NMC-g-OEI/DNA weight ratio was higher than 7. Among the series, NMC-g-PEI(423) have the strongest binding ability, as the DNA mobility is completely retarded at weight ratio of 4. In addition, the binding ability of polymer increased when the molecular weight of chitosan increased from 5 K to 50 K. The results indicated that the binding ability to DNA increased with the increasing of length and charge of the corresponding polymer, which was in agreement with the literature [39].

3.3. Particle size and zeta potential of polycation/DNA complexes

Proper particle size and positive surface charge of polycation/DNA complexes are important for cell uptake and efficient transfection. It was reported that cells typically uptake particles ranging from about 50 to several hundred nanometers [40]. The diameters of the complexes formed at various weight ratios were measured by dynamic light scattering (DLS). As shown in Fig. 3(a), it was found that the particle size of the complexes tended to decrease with the increase of weight ratio. The morphology of NMC-g-OEI/DNA complexes was observed by SEM. As shown in Fig. 3(c–d), the typical image showed that the complexes had a spherical shape. Moreover, the SEM images demonstrated that the sizes of the complexes were roughly 200–400 nm, which were consistent with the results measured by DLS.

The zeta potential of polymer/DNA complexes in Fig. 3(b) showed that all complexes had positive zeta potential at the weight ratios above 5 and the zeta potentials of complexes increased with the increasing weight ratios. Once an excess of amines was available for particle formation, the final zeta potential gradually approached a plateau. Besides, the surface charge of the complexes gradually increased with the increasing length of oligoamines, and the zeta potentials of the complexes were also found to increase with an increase of molecular weight of polymers. It is also in agreement with the literature that higher molecular weight chitosan had a higher zeta potential than lower molecular weight chitosan [41].

![Fig. 1]( Typical 1H NMR spectra of (a) NMC(10 K)-g-TEPA, (b) NMC(10 K)-g-TETA and (c) NMC(10 K) in D2O.)

![Fig. 2](Agarose gel electrophoresis retardation assay of the polymers at various weight ratios of NMC-g-OEI/DNA complexes.)
3.4. Buffer capacity

According to proton sponge mechanism, cationic polymers like PEI are assumed to induce facilitated endosomal escape due to the uptake of protons by the amino groups. The buffer capacity caused an increase of swelling of endocytic vesicles, escaping into the cytoplasm and overall gene transfection efficiency [25]. As shown in Fig. 4, the data indicated that the buffer capacities of chitosan based derivatives were improved in comparison with the one of chitosan, and the buffer capacity of the copolymers with longer length pendant oligoamine, is higher than that of the copolymers with shorter length pendant oligoamine. Besides, the buffer capacity of copolymers decreased with increasing molecular weight of NMC chain when the copolymers have the same oligoamine side chain. The observed increase in buffer capacity of NMC-g-OEI was attributed to the presence of oligoamine of the side chain. However, the titration curves of these copolymers decreased faster than that of PEI(25 K) due to following two reasons. First, in comparison with PEI(25 K), the number of primary, secondary, and tertiary amines groups which may be protonated in the copolymers was less, resulting in lower buffer capacity. Secondly, the un-reacted carboxyl groups of the copolymer would inhibit the protonation of the amine groups of the oligoamine. This neighboring effect of carboxyl groups of copolymers might also result in the lower buffer capacity.

3.5. Cell viability

The significant disadvantage of PEI(25 K) as gene vector is that PEI (25 K) shows high cytotoxicity to many kinds of cells. We used MTT assay to evaluate the cytotoxicity of chitosan based oligoamine polymers to 293T and HeLa cell lines. Fig. 5 shows the cytotoxicity of polymers at various concentrations. It was found that the cytotoxicity of the synthesized polymers was strongly affected by the side chain of oligoamine and the molecular weight of polymers. The results exhibited that cell viability increased with decreasing length of oligoamine side chain in the polymers or decreasing molecular weight of polymers in both cell lines. This indicated that the cytotoxicity was determined by the number of charge as well as the charge density of the polymers. It was previously reported that the cytotoxicity of cationic polymers is probably caused by the
interactions with the plasma membrane or interactions with negatively charged cell components and proteins [42,43]. Although the cytotoxicity increased with increasing concentration of the synthesized polymers, the IC50 of all resulted polymers are generally 1–2 orders of magnitude higher than that of PEI(25 K), which showed that all the polymers exhibited lower cytotoxicity. In addition, when combined with DNA, the synthesized polymers exhibited much lower toxicity on cell viability, and PEI(25 K)/DNA also showed decreased cytotoxicity. The reason may be that the positive charge of polymers was counterbalanced by negative charge of DNA, which minimized the direct contact of polycations to cell membrane.

3.6. In vitro transfection and cellular uptake

To investigate in vitro gene transfer capability of NMC-g-OEI copolymers, transfection studies were performed on 293T and HeLa cells by using pEGFP-C1 and pGL3-Luc as reporter genes. The transfection experiments were performed either in the presence or absence of 10% serum. The results exhibited that the transfection efficiency of chitosan based polymers was higher in the presence of serum than that without serum (data not shown), which was in agreement with our previous report [34]. Literature also reported the transfection efficiency of chitosan/DNA complexes in the presence of...
serum [44] and our results also well agreed with their findings. On the basis of these results, thereafter, all the transfection experiments here were carried out in the presence of 10% serum. Fig. 6(I) displayed the typical fluorescence images of the transfected 293T cells. It can be seen that NMC-g-PEI(423) copolymers possess good gene transfection activity, and the transfection activity of NMC-g-OEI decreased with the decreasing length of oligoamine of the pendant side chains.

The gene transfection activity of the copolymers was also evaluated in terms of luciferase assay, which is a more sensitive method than fluorescence intensity determination. The transfection efficiencies of different polymers were optimized as a function of polymer/DNA weight ratio, ranging from 10 to 40. A typical example of the effect of variation in polymer/DNA ratio in 293T cell is shown in Fig. 6(IIa), the transfection efficiency of chitosan was much enhanced after coupling with oligoamine and increased with increasing number of secondary aminoethylene groups in the pendant chains. The results were in agreement with the fluorescence images. The trend of transfection activity of NMC-g-OEI was further confirmed in the transfection of pGL3-Luc to HeLa cells in Fig. 6(IIb). However, the transfection efficiency in HeLa cells was lower than that in 293T cells, which was ascribed to the fact that transfection activity of the copolymers was dependent on cell lines.

In order to compare the gene transfection efficiency of NMC-g-OEI with other non-viral gene vectors, PEI(25 K) which has been considered to be the highly effective cationic gene vector was used as the control. Fig. 6(IIa) showed that in its optimum value, the gene transfer ability of NMC(10 K)-g-PEI(423) is similar to that of PEI(25 K) (N/P = 10) in 293T cell line. However, the NMC(10 K)-g-PEI(423) showed much lower cytotoxicity than that of PEI(25 K). Moreover, chitosan based gene vectors are considered to provide intracellular sustained release in vivo compared with PEI. It was reported that the level of luciferase in the cells transfected by chitosan/DNA nanoparticle remained nearly the same for 2 weeks [45].

As we know, efficient gene transfection involves multiple steps including DNA complexation, cellular uptake of the complexes, release of DNA from the vectors and transfer into the nucleus [22]. For better...
understanding of the improved gene transfection efficiency of NMC-g-OEI, the cellular uptake of polymer/DNA complexes was further investigated. The uptake of NMC-g-OEI nanoparticles by HeLa cells was confirmed using a confocal microscopy as shown in Fig. 7. The confocal microscopy results showed that fluorescent labeled complexes were observed to be internalized into the cells for all NMC-g-OEI/DNA complexes, and endocytosis of the complexes was gradually decreased from NMC(50 K)-g-PEI(423)/DNA to NMC(5 K)-g-DETA/DNA complexes. The results demonstrated that the pendant oligoamines did affect the cellular uptake of the NMC-g-OEI/DNA complexes, and the cellular uptake improved with the increasing length of pendant oligoamine.

4. Conclusions

In this paper, a series of chitosan based oligoamine polymers was synthesized as promising non-viral gene vectors. These polymers displayed good DNA binding ability. Structure difference of oligoamine side chains affected the buffer capacity, cytotoxicity, and transfection efficiency. In vitro cytotoxicity of the polymers was significantly lower than that of branched PEI(25 K). The transfection efficiencies of NMC-g-OEI/DNA complexes increased with increasing length of pendant oligoamine of side chains. The structure–activity relations of chitosan based oligoamine polymers might be helpful for the design of novel efficient and low toxic gene delivery vectors.

Fig. 7. The cellular uptake of NMC-g-OEI/DNA complexes formed with YOYO-1 labeled plasmid in HeLa cells in the presence of serum at optimized polymer/DNA weight ratio. a, NMC (5 K)-g-PEI(423) (20); b, NMC(10 K)-g-PEI(423) (20); c, NMC(50 K)-g-PEI(423) (20); d, NMC(50 K)-g-TEPA (10); e, NMC(10 K)-g-TEPA (20); f, NMC(5 K)-g-TEPA (20); g, NMC (50 K)-g-TETA (10); h, NMC(10 K)-g-TETA (20); i, NMC(5 K)-g-TETA (20); j, NMC(50 K)-g-DETA (10); k, NMC(10 K)-g-DETA (20); l, NMC(5 K)-g-DETA (20). Scale bar: 50 μm.
Acknowledgements

This work was financially supported by National Key Basic Research Program of China (2005CB623903) and Ministry of Education of China (Cultivation Fund of Key Scientific and Technical Innovation Project 707043).

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