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Blood compatible N-maleyl chitosan-*graft*-PAMAM copolymer for enhanced gene transfection

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ABSTRACT

To improve transfection efficiency, we prepared N-maleyl chitosan-*graft*-polyamidoamine (NMCTS-*graft*-PAMAM) copolymer. Self-assembled NMCTS-*graft*-PAMAM/pDNA complexes were prepared by complex coacervation method at different N/P (nitrogen to phosphate ratio) ratios. The copolymer effectively formed complexes with pDNA at lower N/P ratio (N/P ratio 1.0) than that of unmodified chitosan (N/P ratio 2.0) and the complexes were spherical with particle size of 100–150 nm. The copolymer showed significant protection of DNA from nuclease attack with lower toxicity against HeLa cell. The copolymer also showed no noticeable hemolytic effects up to 10 mg/mL indicating no detectable disturbance of the red blood cell membranes. The transfection efficiency of the copolymer was increased significantly compared to that of chitosan and reached up to 36 ± 2% at N/P ratio 7.0 which was higher than that of PEI (30 ± 3% at N/P ratio 10). Therefore, the copolymer may be a strong alternative candidate as effective nonviral vector.

1. Introduction

Currently, safe and efficient DNA carriers are the main hurdle to the success of gene therapy. Several nonviral vectors have been developed for gene delivery with several advantages over viral vectors, including simplicity of production, low immune response, low cost, high flexibility for modification and the ability to transfer large DNA molecules. Cationic polymers are an important class of nonviral gene delivery vectors. Among the cationic polymers, polyethyleneimine (PEI) (Kircheis, Wightman, & Wagner, 2001; Lungwitz, Breunig, Blunk, & Gopferich, 2005) and polyamidoamine (PAMAM) dendrimer (Nanjwade, Bechra, Derkar, Manvi, & Nanjwade, 2009; Zhong et al., 2008) are the most effective nonviral vectors. Although, these polymers are synthetic and not biocompatible, and severe toxicity limits their clinical applications (Bergen, Park, Horner, & Pun, 2008; Schmidt-Wolf & Schmidt-Wolf, 2003). As, low toxicity is a major concern in the design of new gene delivery systems, researchers have been concentrated on biocompatible and natural polymers including polysaccharide such as schizophyllan (Matsumoto et al., 2004), glycopolymers (Tranter et al., 2012; Zhoua et al., 2012) or degradable synthetic polymers (O'Rorke, Keeney, & Pandit, 2010; Xu et al., 2006) to develop efficient nonviral vector.

Among the biopolymers, Chitosan (CTS), linear and partly acetylated (1 → 4)-2-amino-2-deoxy-β-D-glucan has received increasing attention as a nonviral gene delivery vector (Koping-Hoggard et al., 2001; Kundu & Sarkar, 2011; Mohammadi et al., 2011; Muzzarelli, 2010a, 2010b). Chitosan has gained considerable attention as a gene delivery vector due to its favorable biological properties such as low toxicity, good biocompatibility and biodegradability (Lee, 2007; Mao, Sun, & Kissel, 2010). The p*Ka* value of chitosan is 6.5 and therefore, it becomes cationic at pH < 6.5 due to protonation of the primary amine groups. So, the positively charged chitosan can form polyplex with negatively charged DNA due to electrostatic interaction and it also can effectively protect DNA from nuclease degradation (Sato, Ishii, & Okahata, 2001; Strand et al., 2010). However, native chitosan usually suffers from poor water solubility at physiological pH and it showed very low transfection efficiency. To overcome these problems, researchers have developed chitosan derivatives including oligoamine-grafted-chitosan (Lu et al., 2009), urocanic acid modified chitosan (Kim, Ihm, Choi, Nah, & Cho, 2003), glycosylated chitosan (Strand, Issa, Christensen, Varum, & Artursson, 2008), pegylated chitosan (Jiang et al., 2006) and trimethylated chitosan (Gao, Zhang, Chen, Gu, & Li, 2009). But, the transfection efficiency of these chitosan derivatives was still relatively low. Therefore, the development of chitosan derivatives with high transfection efficiency is still critically important for clinical applications.

Poly(amidoamine) (PAMAM) dendrimer has been attracted interest as nucleic acid delivery vectors (Boas & Heegaard, 2004;

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Dufes, Uchegbu, & Schatzlein, 2005; Guillot-Nieckowski, Eisler, & Diederich, 2007; Smith, 2008) due to its uniform in size with high density of primary amine groups at the surface, high water solubility and stability in aqueous solution. Full generation cationic PAMAM dendrimers (amine terminated PAMAM dendrimer) can also bind to negatively charged DNA or RNA molecules by electrostatic interaction. However, cytotoxicity of high generation PAMAM dendrimers is a marked problem which limits their widespread use in drug and gene delivery applications (Janaszewska et al., 2012; King Heiden, Dengler, Kao, Heideman, & Peterson, 2007). Therefore, chemical combination of chitosan with PAMAM dendrimers may provide novel biomaterial with improved water solubility, higher charge density and enhanced transfection efficiency with lower toxicity. Although, Sashiwa, Shigemasa, & Roy, 2002 prepared chitosan-*graft*-PAMAM copolymers through tetraethylene glycol spacer by reductive N-alkylation but they did not observe gene delivery applications.

In our previous report (Sarkar & Kundu, 2012), we prepared N-maleated chitosan-*graft*-PAMAM copolymer with different generation of PAMAM dendrimer (G1, G2 and G3). We used simple Michael addition reaction to prepare NMCTS-*graft*-PAMAM copolymer by reacting the amine groups of PAMAM dendrimers with the double bond of maleyl group of NMCTS to enhance the physiological property of chitosan. Here, we used NMCTS-*graft*-PAMAM (G2) copolymer due to its better solubility in water and low toxicity compared to that of NMCTS-*graft*-PAMAM (G3) copolymer and we investigated the size and morphology of self-assembled nanoparticles of NMCTS-*graft*-PAMAM (G2)/pDNA for gene transfection efficiency in cancerous cell, HeLa cell line. We also checked blood compatibility with this copolymer.

2. Experimental

2.1. Materials

Low molecular weight chitosan (25 kDa and DDA 84%) was prepared by oxidative degradation using $NaNO_2$. PAMAM dendrimers were prepared by Michael addition reaction between ethylene diamine (EDA) as core molecule and methyl acrylate (MA) followed by amidation reaction between the ester terminated half generation PAMAM dendrimers and EDA. DNase I (1 unit/μl) was obtained from Fermentas, Thermo Scientific, India. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, trypsin, fetal bovine serum (FBS) were purchased from Himedia Laboratories Pvt. Ltd., India. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and agarose were obtained from Sisco Research Laboratories Pvt. Ltd., India. pEGFP-N1 control vector (4.7 kb containing SV-40 promoter) was kindly donated by Dr. Gopal Chakroborty, Department of Biotechnology, University of Calcutta. All other reagents were analytical grade and were used directly without further modification.

2.2. Preparation of NMCTS-graft-PAMAM copolymer

NMCTS-*graft*-PAMAM (G2) copolymer was prepared according to our previous report (Sarkar & Kundu, 2012). Briefly, 1.0 g chitosan was dissolved in 50 mL of 1% acetic acid solution, precipitated with 1 M NaOH solution and the precipitate was collected by filtration and then washed with water to pH 7. Chitosan obtained from the above was dispersed in 75 mL of DMSO with constant stirring. 1.75 g maleic anhydride in DMSO solution was added into above solution. The mixture was then reacted in a 60 °C oil bath for 8 h. The product was precipitated in 250 mL of acetone, filtered, washed with acetone and ether and then vacuum dried at 50 °C to obtain N-maleated chitosan. The degree of substitution (DS) was calculated from ^{1}H NMR spectrum and the DS value was 0.41.

To graft PAMAM (G2) dendrimer with NMCTS, 0.2 g NMCTS was dissolved in 20 mL of 0.25% sodium hydroxide solution. The aqueous solution of 3.0 g PAMAM (G2) was then added into the above solution. The mixture was stirred and the reaction was carried out at room temperature for 4 days. Then hydrochloric acid was added to the mixture with stirring until pH value reached 7.0. The product was then dialyzed (MWCO: 3500 Da) against distilled water for 3 days and then lyophilized for 3 days. The DS value of NMCTS-*graft*-PAMAM (G2) copolymer was 0.42. The synthetic route of NMCTS-*graft*-PAMAM (G2) copolymer is shown in Fig. 1.

2.3. Plasmid DNA isolation

The plasmid was propagated in XL1B *Escherichia coli* bacteria and isolated by the QIAGEN Plasmid Purification Midi Kit (QIAGEN, Chatsworth, CA) according to the manufacturer protocol. The isolated pDNA was washed with 70% ethanol at room temperature, air dried for 5–10 min and then, re-suspended in MQ water. The purity and concentration of pDNA were determined by measuring UV absorbance at 260 and 280 nm. The obtained pDNA was stored at −20 °C for further use.

2.4. Preparation of polymer/pDNA complexes

Chitosan and NMCTS-*graft*-PAMAM (G2) copolymer were dissolved in 1% acetic acid solution and distilled water with a concentration of 2 mg/mL, respectively and both the solutions were filtered by Millipore 0.45 μm filter paper. pDNA was dissolved (100 μg/mL) in 25 mM of sodium sulfate solution. The polymer solutions were diluted with acetic acid/sodium acetate buffer (pH 5.5) to get the desired concentration for polyplex formation. Both the polymer and DNA solutions were preheated separately at 50–55 °C for 10 min. Then, polymer/pDNA complexes (polyplexes) at various charge ratios (amino group to phosphate group ratio, N/P ratio) were prepared by immediate mixing of equal volume of polymer and pDNA solution and subsequently vortexing for 15–30 s with cyclomixer (REMI, India). After that, the resulting mixtures were incubated at room temperature for 30 min to completely form the polyplexes.

2.5. Agarose gel retardation assay

The binding ability of chitosan or its copolymer with pDNA was confirmed by agarose gel electrophoresis. The complexes containing 0.5 μg of DNA at various N/P ratios were loaded into individual wells of 0.8% agarose gel in 1× Tris–boric acid–EDTA buffer containing 10 μg/mL ethidium bromide as a DNA visualizer. The gel electrophoresis was carried out at 100 V for 40 min and then, the gel was illuminated on an UV illuminator to show the location of the pDNA. The picture of the gel was subsequently captured by BIOTOP gel doc system (Shanghai, China).

2.6. Determination of particle size and zeta potential

The particle size and distribution as well as surface charge of polymer/pDNA nanoparticles at different N/P ratios were determined by dynamic light scattering (DLS). The DLS measurement was carried out by means of an argon ion laser system tuned at 514 nm. To avoid the influence of dust on the reliability of results, the solutions of the polymer/pDNA complex were filtered through a 0.45 μm filter (Millipore) directly into a freshly cleaned 10 mm diameter cylindrical cell. The intensity of autocorrelation was measured at a scattering angle (θ) of 173° with Zetasizer Nano ZS

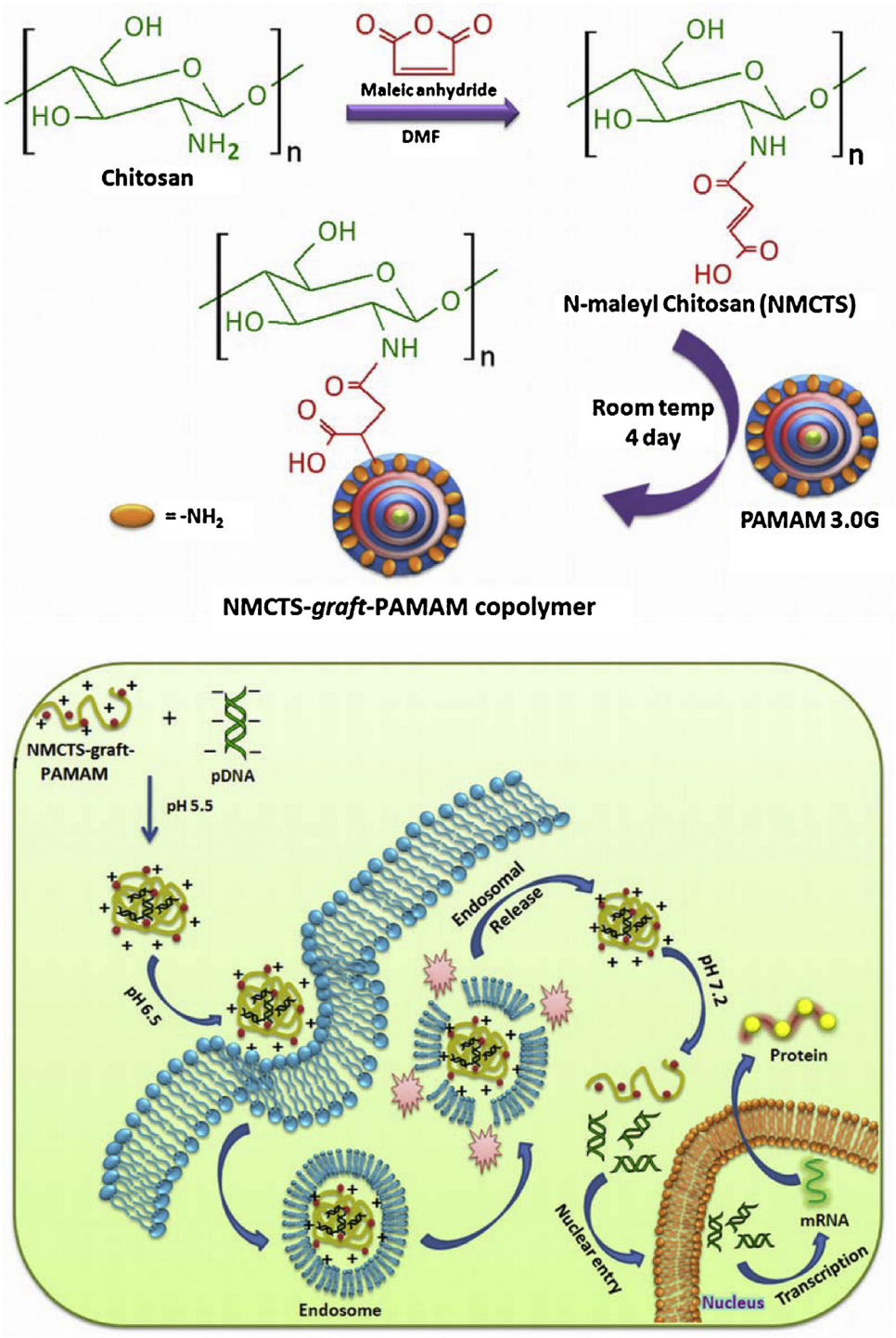

Fig. 1. Schematic representation of NMCTS-*graft*-PAMAM copolymer synthesis and in vitro gene transfection.

(Malvern Instrument, UK) digital autocorrelator at 37 °C. The correlation function was accepted when the difference between the measured and the calculated baselines was less than 0.1%. The mean diameter was obtained by the Stokes–Einstein relationship.

2.7. Atomic force microscopy measurement

The size and morphology of NMCTS-*graft*-PAMAM/pDNA nanoparticles were characterized by AFM (Nanoscope IV Bioscopet, Digital Instruments, Veeco). One to two microliters of samples containing complexes in acetate buffer with a final DNA concentration of 100 μg/mL was deposited onto the center of a freshly split untreated mica disk. The mica surface was dried at room temperature before imaging. The imaging was conducted with silicon nitride tip in tapping mode and a scan speed of 1 Hz at ambient condition.

2.8. Scanning electron microscopy measurement

The morphology of NMCTS-*graft*-PAMAM/pDNA nanoparticles was also characterized by scanning electron microscopy (SEM). About 10 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 (Hitachi, Japan, Model-3400N). Before the SEM observation, the samples were fixed on an aluminum stub and coated with gold by ion sputter coater (Hitachi, Japan, Model-E1010) for 7 min.

2.9. DNase I digestion assay

The digestion of naked pDNA and polymer/pDNA complexes against DNase I (1 unit/l μg of DNA) was assayed in 2 mL of 10 mM phosphate buffered saline (PBS) containing 5 mM $MgCl_2$ at 37 °C. At first, polymer/pDNA complexes and naked pDNA were incubated with 1 μL of DNase I for 10 min at 37 °C and then, DNase I was inactivated by adding of 0.5 M EDTA. The degradation of DNA was investigated by 0.8% agarose gel electrophoresis.

2.10. Blood compatibility

Whole human blood was collected from healthy male adults (25–31 years old). A total of 200 μl of the whole blood was added to different amounts of polymer solutions and the volume was adjusted to 1 mL with sterile PBS solution (pH 7.4). The blood compatibility test in PBS solution and in Triton X-100 solution (1%, v/v) were used as negative and positive controls, respectively. After incubation at 37° C for 90 min, the solutions were centrifuged at 2000 rpm for 10 min. A total of 200 μl of the supernatant was collected and seeded in each well of 96-well plate. The absorbance was recorded on ELISA micro plate reader (Multiskan EX, Labsystems, Helsinki, Finland) at a wavelength of 543 nm. The percentage hemolysis (PH%) was calculated using the following equation:

$$PH\% = \frac{A_s - A_n}{A_p - A_n} \times 100,$$

where A_s, A_n and A_p are the absorbance of sample, negative and positive controls, respectively. All data are presented as the mean of three measurements (±SD).

2.11. Cell culture

HeLa (human cervical epithelial carcinoma cell line) was maintained in DMEM-HG (high glucose) medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U mL^{-1}) at 37° C in a humidified atmosphere containing 5% CO_2.

2.12. In vitro cytotoxicity assay

The cytotoxicity of chitosan and NMCTS-*graft*-PAMAM (G2) copolymer was determined on HeLa cells by MTT assay. HeLa cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well. After 24 h incubation, the cells were treated with CTS, NMCTS-*graft*-PAMAM (G2) copolymer, PAMAM dendrimer (G2) and polyethyleneimine (PEI) (as positive control) with final concentrations of 5, 10, 25, 50, 75, 100, 150, 200, 250 and 300 μg/mL for 24 h. Untreated cells in growth media were used as the blank control. Then, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) in PBS buffer was added to each well. After further incubation for 4 h, the media was removed and replaced with 100 μL DMSO to dissolve the MTT formazan crystals. The absorbance was recorded at 570 nm by an ELISA microplate reader (Multiskan EX, Labsystems, Helsinki, Finland). The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability}(\%) = \frac{OD_{570(\text{sample})}}{OD_{570(\text{control})}} \times 100$$

where $OD_{570(\text{sample})}$ represents measurement from the wells treated with polymer and $OD_{570(\text{control})}$ from the wells treated with DMEM only. All data are presented as the mean of six measurements (±SD).

2.13. In vitro transfection studies

For in vitro transfection, the transfection media (DMEM) was prepared containing 5 mM MES and 10 mM $NaHCO_3$ at a pH of 6.5. Transfection media containing 10% fetal bovine serum was equilibrated overnight at 37 °C in 5% CO_2 incubator and the pH was adjusted to 6.5 using sterile HCl (1 M) prior to transfection. HeLa cells were seeded in 6-well plates at the density of 1×10^3 cells/well in 1 mL of complete medium (DMEM high glucose supplemented with 10% FBS) and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 until the cells were approximately 80% confluent. The polymer/DNA complexes were prepared at different N/P ratios according to the conditions described above (containing 2 μg DNA in each N/P ratio) prior to the transfection. Two hours before transfection, the complete growth media was replaced with fresh media without FBS and antibiotics. The polymer/DNA complexes containing 2 μg DNA at different N/P ratios were added into each well and were incubated at 37° C in a CO_2 incubator for another 4 h. Then, the transfection media were replaced with complete growth media and incubated for 24 h. All transfection studies were carried out in triplicate. Naked DNA and PEI/DNA (N/P = 10) were used as negative control and positive control, respectively. The cells were analyzed after 24 h post incubation for enhanced green fluorescence protein (pEGFP-N1) expression with a fluorescence microscope (OLYMPUS IX70, Japan).

For flow cytometric analysis, pEGFP-N1 transfected cells were harvested through trypsinization. Cells were resuspended into 1X PBS buffer, pH 7.4 and the cell suspensions were then transferred to 5 ml flow cytometry tubes. EGFP expression in the transfected cells was quantified using a Becton-Dickinson FACS scan instrument and the data were analyzed using Cell Quest program from Becton-Dickinson. For each sample, 10,000 events were counted and the green fluorescence of EGFP was detected through 525/30 nm (FL1) band pass filter.

2.14. Statistical analysis

All experiments were repeated in triplicate unless otherwise noted. Statistical analysis was performed using Student's *t*-test and differences were judged to be significant at $p < 0.05$.

3. Results and discussion

3.1. Preparation of NMCTS-graft-PAMAM/pDNA complex

The DNA binding property of NMCTS-*graft*-PAMAM (G2) copolymer was assessed by monitoring the electrophoretic mobility of DNA using agarose gel electrophoresis. Positively charged polymers were mixed with negatively charged plasmid DNA (pDNA) to form self-assembled polymer/DNA complexes at different charge ratios (N/P ratios). As shown in Fig. 2, the amount of free DNA decreased with increasing the N/P ratio. It was found that unmodified CTS showed complete retention of pDNA at N/P ratio 2.0. But, the DNA retention by CTS was significantly improved after grafting of PAMAM (G2) dendrimer with chitosan backbone, suggesting that amine groups from not only CTS but also PAMAM dendrimer were contributive to DNA condensation. The migration capability of DNA was found to be completely retarded at lower N/P ratio (N/P = 1.0) by modified chitosan, which indicated that all pDNA was trapped inside the polyplex particles at N/P ratio

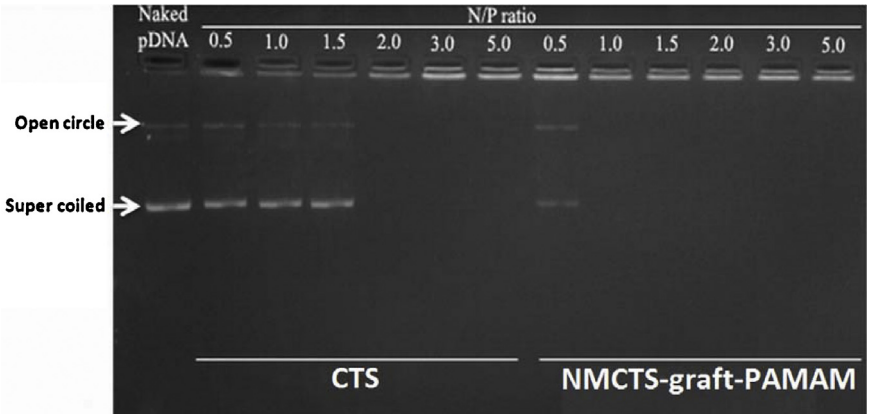

Fig. 2. Agarose gel electrophoresis assay of chitosan/pDNA and NMCTS-*graft*-PAMAM/pDNA at different N/P ratios.

1.0. A complete retardation assures strong complexation between DNA and NMCTS-*graft*-PAMAM (G2) copolymer. In our previous study (Sarkar, Debnath, & Kundu, 2013), we obtained similar results where DNA condensation capability of chitosan was singinficantly increased after conjugation of polyethyleneimine with chitosan. Yang, Li, Goh, & Li, 2007 prepared cationic cyclodextrin having oligoethyleneimine arms and the compounds showed stronger DNA condensation capability due to presence of primary amine groups.

3.2. Particle sizes, morphology and zeta potential of polymer/pDNA complexes

Appropriate particle size of polycation/DNA complexes is important for cell uptake and efficient transfection. It was previously reported that cells typically uptake particles ranging from about 50 to several hundred nanometers (Liu & Reineke, 2005). The diameters of the polymer/pDNA complexes at various N/P ratios were measured by dynamic light scattering (DLS) technique and the results are shown in Fig. 3a. As shown in Fig. 3a, the size of all polymer/DNA complexes was measured at pH 5.5 and it was found that both chitosan and NMCTS-*graft*-PAMAM copolymer formed large particle with pDNA at lower N/P ratio because of inefficient binding with pDNA and aggregation effect. When, the N/P ratio increased to 3.0, both polymers formed smaller particles (198 nm by copolymer and 246 nm by CTS). Further augmentation of the N/P ratio caused a slight increase of the average particle sizes for both polymers and this phenomenon might be attributed to the repulsion of excessive positive charge provided by molecules.

The zeta potential of the polyplexes with different N/P ratios at pH 5.5 is shown in Fig. 3b. As shown in Fig. 3b, the zeta potential of the polyplexes increased with N/P ratio of the polyplexes. The zeta potential was almost neutral (0 mV) at N/P ratio of 1.0 and 2.0 for NMCTS-*graft*-PAMAM and CTS, respectively. Because at these N/P ratios, all negative charges on the pDNA molecules were neutralized by the positive charges on the polymer molecules. The zeta potential values of both CTS/pDNA and NMCTS-*graft*-PAMAM/pDNA complexes were increased with increasing the N/P ratios and then reached plateau above N/P ratio of 3.

In previous study (Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001), it was found that chitosan formed nanoparticles with DNA ranging from 200 to 500 nm and other polymeric systems have also been reported ranging from 100 to 700 nm (MacLaughlin et al., 1998; Truong-Le, August, & Leong, 1998). In this study, the size of nanoparticles was similar to those in the literature. However, most mammalian cells can uptake polycation/DNA nanoparticles through endocytosis, which is limited to particles size less than about 150 nm in diameter (Guy, Drabek, & Antoniou, 1995). But, the zeta potential of the polymer/DNA complexes plays an important role for biodistribution (Mahato, Takakura, & Hashida, 1997) and transfection efficiency (Nomura et al., 1997) because a positive surface charge allows an electrostatic interaction between negatively charged cellular membranes and positively charged complexes (Mansouri et al., 2006). As shown in Fig. 3b, the positive values of zeta potential were obtained in the present study at N/P ratio of 1.5 for copolymer and at 2.0 for chitosan.

The size and morphology of NMCTS-*graft*-PAMAM/pDNA complexes were observed by AFM as well as SEM. Fig. 4 shows

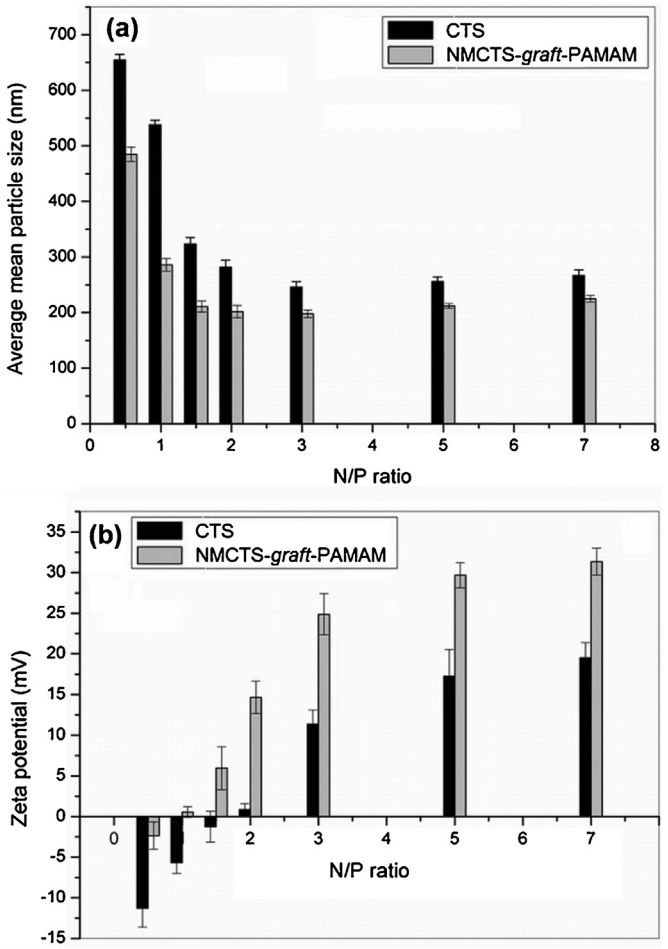

Fig. 3. DLS particle size of CTS/pDNA and NMCTS-*graft*-PAMAM/pDNA complexes at pH 5.5 (a) and zeta potential of CTS/pDNA and NMCTS-*graft*-PAMAM/pDNA complexes at pH 5.5 (b). Data are shown as mean ± SD ($n = 3$).

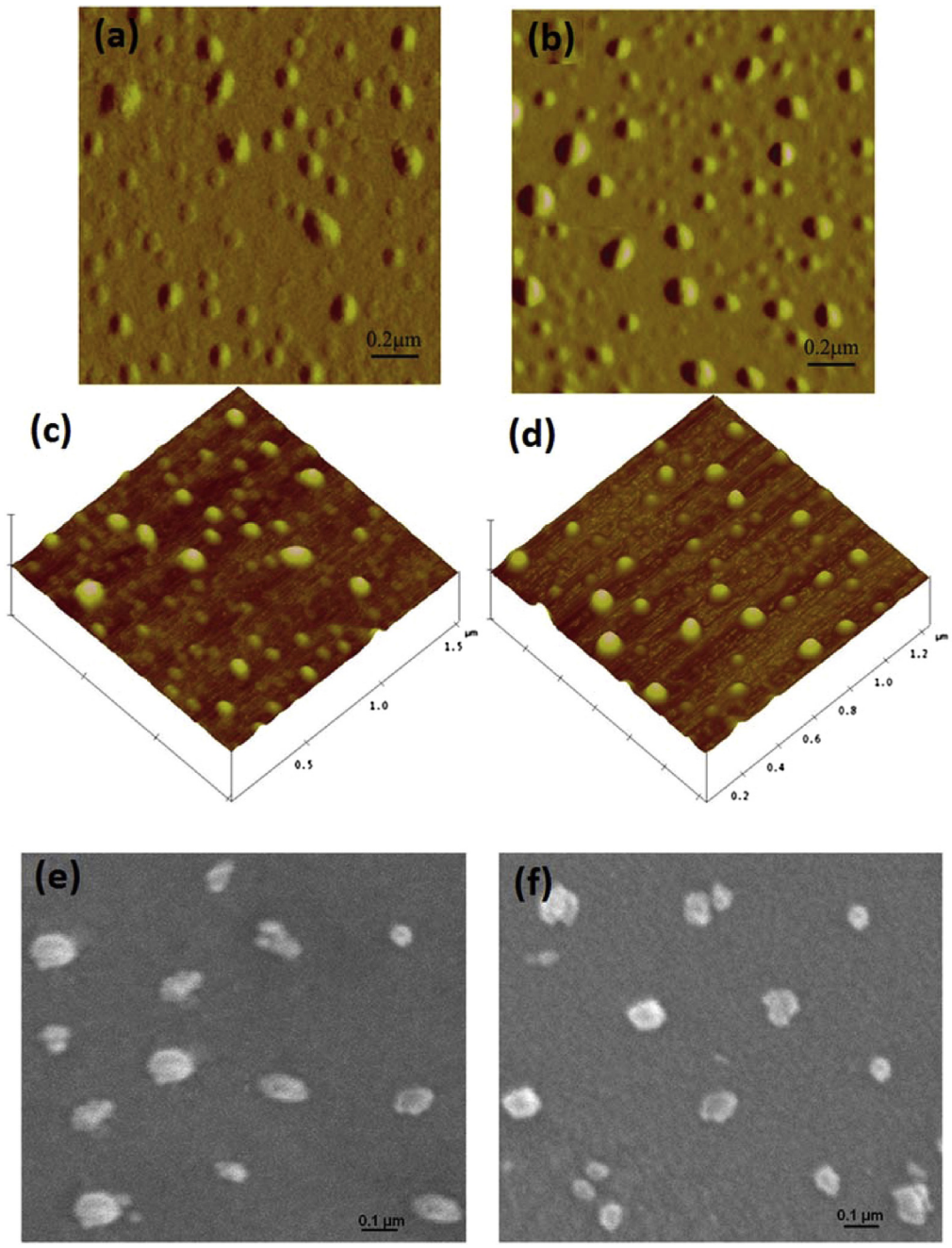

Fig. 4. AFM 2D (a) and (b) and 3D (c) and (d) micrographs of NMCTS-*graft*-PAMAM/DNA complexes at N/P ratio of 2 and 3. SEM images of NMCTS-*graft*-PAMAM/DNA complexes at N/P ratio 2 (e) and 3 (f).

representative taping mode AFM (Fig. 4a–d) and SEM images (Fig. 4 e and f) of NMCTS-*graft*-PAMAM/pDNA nanoparticles at N/P ratios 2.0 and 3.0. As shown in Fig. 4, the polyplexes were found to have a spherical shape and compact structure. Moreover, the AFM images demonstrated that the size of the nanoparticles was roughly within the range of 100–150 nm at N/P ratio 3 (Fig. 4b and d) whereas the complexes at N/P ratio 2 were slightly larger (Fig. 4a and c), which was consistent with the results measured by dynamic light scattering method. At higher N/P ratio, the number of cationic charge offered by polymer increased and consequently condensed the negatively charged DNA more tightly and formed smaller particles. Our previous report showed the similar results (Sarkar, Srivastava, Chatterji, & Kundu, 2011). SEM images of NMCTS-*graft*-PAMAM/pDNA complexes also showed that the polyplexes were spherical in shape and the particle size was in the range of 100–150 nm, which was also in good agreement with the AFM and DLS results. These results also support the previous results (Xu et al., 2008; Li et al., 2007). The above results confirm that NMCTS-*graft*-PAMAM copolymer is capable to condense the pDNA into nanoparticles within the size suitable for cellular uptake.

3.3. DNase I digestion assay

DNA stability against degradation by nucleases is an important issue for successful gene therapy application. Because, Deoxyribonuclease I (DNase I) or DNase I like enzymes exist in the extracellular space and cellular cytoplasm and they play an important role in DNA degradation and disruption. Therefore, it is necessary to investigate the ability of synthetic vector to protect the genetic material from degradation during its transport to the

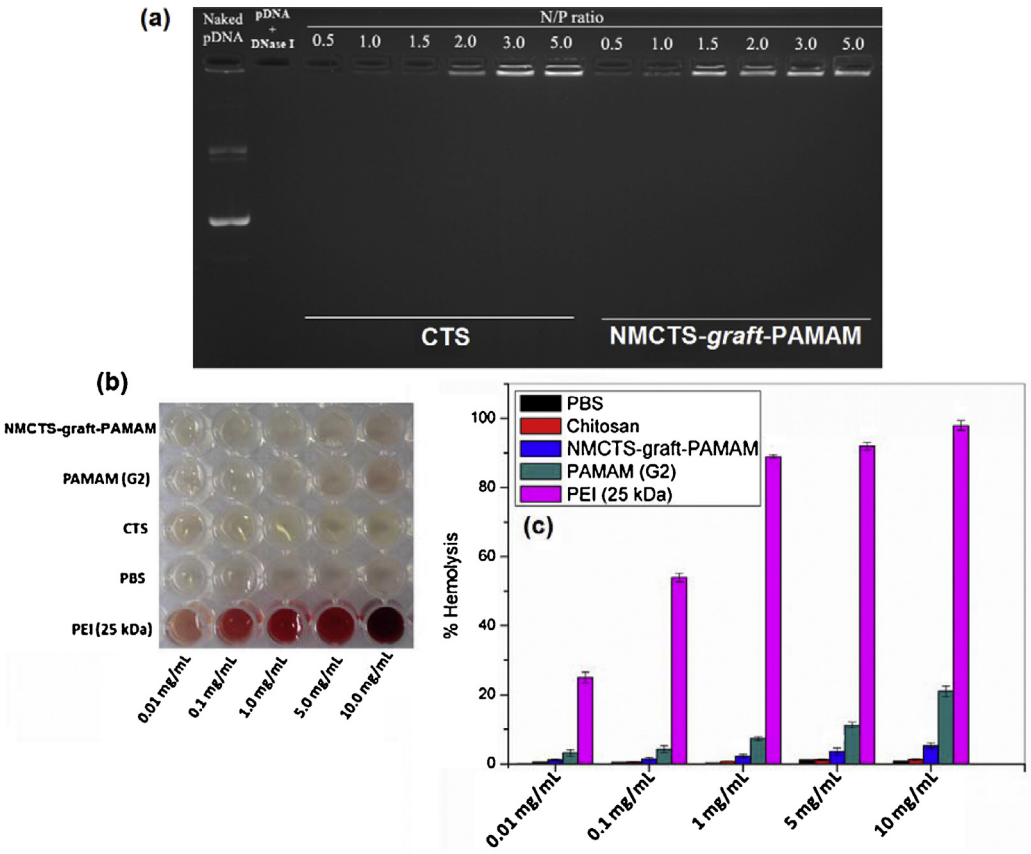

Fig. 5. DNase I assay of CTS/pDNA and NMCTS-*graft*-PAMAM/pDNA complexes at different N/P ratios (a), visual observation of hemolysis caused by NMCTS-*graft*-PAMAM, PAMAM (G2), CTS, PBS and PEI (25 kDa) in phosphate buffer at pH 7.4 (b) and bar graph of % hemolysis by different polymers at concentration range 0.01–10 mg/mL (c). PBS buffer at pH 7.4 was used as negative control and Triton X-100 (1%, v/v) was used as positive control. Data are shown as mean ± SD ($n = 3$).

target cells. DNase I protection assay is a general procedure to evaluate the nuclease protection of pDNA. Fig. 5a shows the protection capability of chitosan and NMCTS-*graft*-PAMAM (G2) copolymer against pDNA degradation by DNase I. As illustrated in Fig. 5a, naked plasmid DNA (lane 2) was completely digested by DNase I at 1 U/μg DNA within 15 min of incubation, confirming the activity of nuclease. In contrast, NMCTS-*graft*-PAMAM copolymer efficiently protected pDNA from nuclease digestion at or above N/P ratio 1.0, whereas CTS protected the pDNA at or above N/P ratio 2.0. Our studies showed that NMCTS-*graft*-PAMAM could protect DNA efficiently against degradation by DNase I, which is one of the crucial factors for efficient gene delivery in vitro as well as in vivo.

3.4. Blood compatibility

The instability of polymer/DNA complexes in blood due to non-specific interactions of cationic polymers with blood components is one of the serious limitations to the in vivo application of cationic polymers (Vader, Van der Aa, Engbersen, Storm, & Schiffelers, 2012). Cationic polymers have been known to interact with negatively charged cell surface (Karchalsky, Danaon, & Nevo, 1995; Yu, Suh, Koh, & Kim, 2001). Similarly, they can do so with negatively charged red blood cells (RBCs), so we investigated the release of hemoglobin from RBCs. In this study, we determined the percentage hemolysis of chitosan, NMCTS-*graft*-PAMAM (G2) copolymer, PAMAM (G2) and PEI (25 kDa) with different concentrations in the range 0.01–10 mg/mL. Triton X-100 (1%, v/v) and phosphate buffered saline solution (pH 7.4) were used as positive control and negative control, respectively. As shown in Fig. 5b, chitosan and NMCTS-*graft*-PAMAM (G2) copolymer showed no significant hemolytic effects up to 10 mg/mL indicating no detectable disturbance of the red blood cell membranes. Fig. 5c shows the percentage hemolysis of chitosan and NMCTS-*graft*-PAMAM (G2) copolymer in phosphate buffer at pH 7.4. PEI was found to be the most membrane damaging polymer reaching complete hemolysis of the hemoglobin at concentration ≥1 mg/mL and PAMAM (G2) dendrimer caused 21% hemolysis at concentration 10 mg/mL. Dekie et al. (2000) showed that the presence of primary amines has a significant toxic effect on red blood cells causing them to agglutinate. In this study, PEI was found to be the polymer with the highest cytotoxicity and this is attributed to the large molecular size as well as the high number of charges. Due to the high degree of branching, PEI has a higher charge density compared to equally sized polycations with a linear structure. Although, both chitosan and NMCTS-*graft*-PAMAM copolymers are linear, flexible polymers of comparable or higher molecular weights, they showed differences in their cytotoxicity. This result demonstrates that NMCTS-*graft*-PAMAM (G2) copolymer is highly blood compatible.

3.5. Cytotoxicity of polymers

Minimum cytotoxicity of polymeric vectors is an important requirement for in vivo application. To investigate the cytotoxicity of the polymers, cell viability was determined by MTT assay using the polymers at various concentrations (5, 10, 25, 50, 75, 100, 150, 200, 250 and 300 μg/mL) on HeLa cells (Fig. 6). Cells without treatment of polymer were used as a control with cell viability assumed to be 100%. As shown in Fig. 6, it was observed that the cell viability of HeLa was above 90% in presence of chitosan even at high concentration (300 μg/mL), which is in good agreement with the previous

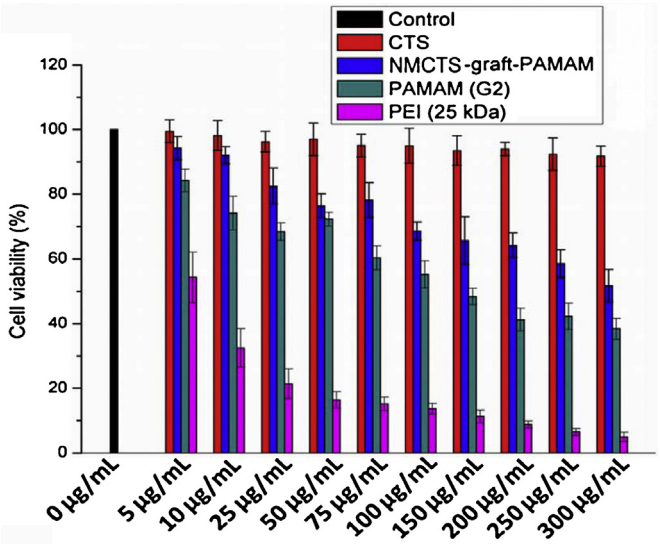

Fig. 6. Cell viabilities of CTS, NMCTS-*graft*-PAMAM (G2) copolymer, PAMAM (G2) dendrimer and PEI at different concentration in HeLa cell line. Data are shown as mean ± SD ($n = 6$).

report (Huang, Khor, & Lim, 2004; Jiang et al., 2007; Sarkar et al., 2011). On the other hand, NMCTS-*graft*-PAMAM (G2) copolymer showed little toxicity toward the cell line at low concentrations up to 75 μg/mL where above 80% cells were viable. But, the cell viability decreased with further increasing the polymer concentration although, the cells were above 50% viable at higher concentration (300 μg/mL). The number of primary amine groups of cationic polymers was important (Ekrami & Shen, 1995) because the surface charge density of polymer was related to the number of primary amine groups. The cytotoxicity of polycations is probably due to strong electrostatic interaction with the plasma membrane (Guo et al., 2011; Leclercq, Boustta, & Vert, 2003; Sunoqrot et al., 2011; Wang, Lin, Jiang, He, & Zhuo, 2009), which results in destabilization by aggregation on the cell surfaces and ultimately impairs the cell membrane functions. On the contrary, branched PEI (25 kDa) showed significant toxicity to the cells and the cell viability reached to 20% at very low polymer concentration of 25 μg/mL whereas, NMCTS-*graft*-PAMAM copolymer showed above 80% cell viability at the same concentration and above 60% cell viability even at higher concentration of 100 μg/mL. It is also found from Fig. 6 that PAMAM (G2) dendrimer showed around 50% and 40% cell viability at concentrations of 100 μg/mL and 300 μg/mL, respectively which was much higher than PEI but lower than the NMCTS-*graft*-PAMAM (G2) copolymer. But, the toxicity of PAMAM dendrimer decreased after grafting it with chitosan. Our previous report showed the similar result where branched PEI was grafted with chitosan through naphthalimide moiety (Sarkar et al., 2013).

3.6. *In vitro* transfection

We compared the transfection efficiency of NMCTS-*graft*-PAMAM (G2)/pDNA with chitosan/pDNA, at different N/P ratios (N/P = 3.0, 5.0 and 7.0) to confirm whether the attachment of PAMAM (G2) dendrimer with chitosan could improve the transfection. The transfection experiments were carried out against HeLa cells by pEGFP-N1 plasmid encoding green fluorescent

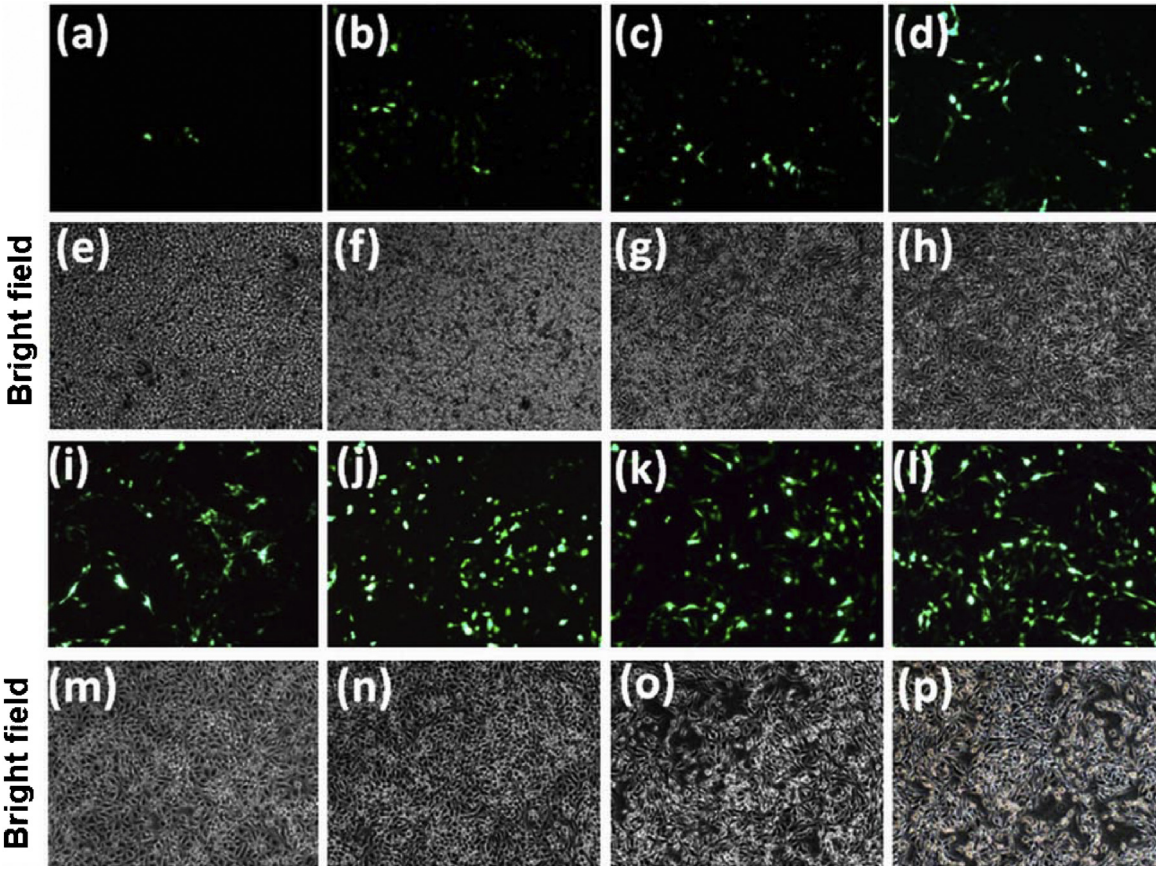

Fig. 7. Typical fluorescence images of HeLa cells transfected by chitosan/pDNA complexes at N/P ratios 3.0, 5.0 and 7.0 (b), (c) and (d); NMCTS-*graft*-PAMAM (G2)/pDNA complexes at N/P ratios 3.0, 5.0 and 7.0 (i), (j) and (k); pDNA only (a), and PEI (25KDa)/pDNA complex at N/P ratio 10.0 (l). In each transfection study 2 μg pDNA was used in every N/P ratio.

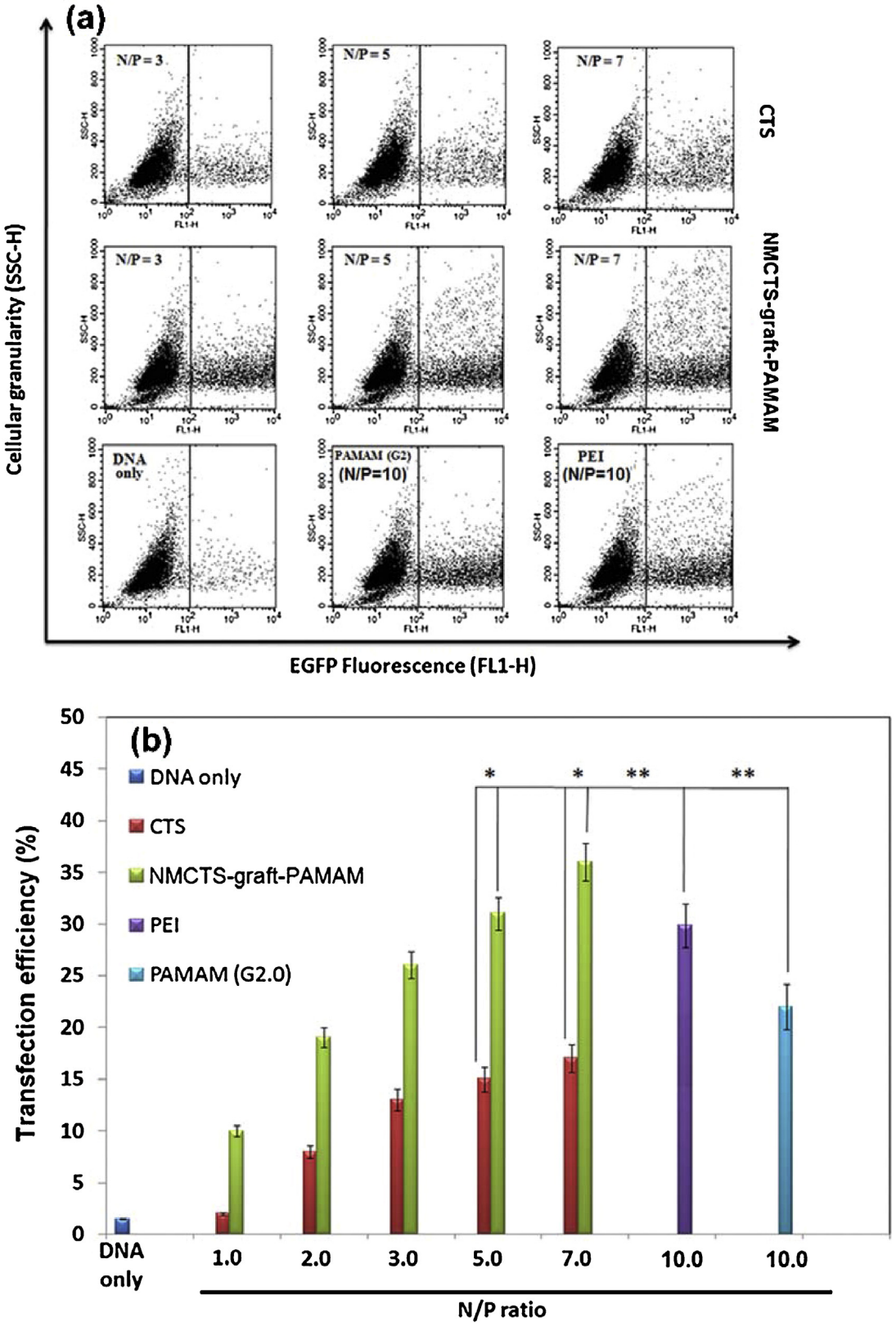

Fig. 8. Representative flow cytometric analysis of GFP-expressing cells after 48 h post transfection by CTS, NMCTS-*graft*-PAMAM (G2) copolymer at different N/P ratios (3.0, 5.0 and 7.0), PAMAM (G2) dendrimer (N/P ratio 10) PEI (N/P ratio 10) and pDNA only (a) and bar graph of transfection efficiency by CTS/pDNA complexes, NMCTS-*graft*-PAMAM (G 2)/pDNA complexes, PAMAM (G2)/pDNA complexes at N/P ratio 10.0 and PEI (25 kDa)/pDNA complex at N/P ratio 10.0 in HeLa cell lines (b). Data are shown as mean ± SD ($n = 3$). $^{*}p < 0.01$ and $^{**}p < 0.001$.

protein (GFP). Polyethyleneimine (PEI, 25 kDa) complexed with DNA at the N/P ratio 10.0 was used as positive control. Typical fluorescence images of the transfected HeLa cells are shown in Fig. 7. It was observed that transfection efficiency of NMCTS-*graft*-PAMAM/pDNA complexes was significantly increased than that of unmodified chitosan. The transfection efficiency of NMCTS-*graft*-PAMAM/pDNA complexes was increased with increasing the N/P ratios and optimum transfection efficiency was obtained at N/P ratio 7.0. After further increasing the N/P ratio (N/P ratio 10), the transfection efficiency was decreased (data not shown). For a comparison, the transfection efficiency of PAMAM (G2)/pDNA complex at N/P ratio 10.0 was also investigated. The copolymer/pDNA complexes at N/P ratio 7.0 surpassed the transfection efficiency of PEI/pDNA and PAMAM (G2)/pDNA complexes. The increased transfection efficiency of the copolymer can be attributed to the higher amine content from PAMAM dendrimer. The interaction of NMCTS-*graft*-PAMAM copolymer with DNA increased due to incorporation of PAMAM dendrimer onto chitosan backbone and the ability of cell uptake increased accordingly compared to that of chitosan.

The transfection efficiency of NMCTS-*graft*-PAMAM/pDNA complexes was further quantitatively determined through Flow cytometric analysis of the transfected HeLa cells. Fig. 8a shows the representative dot plots of cellular uptake with chitosan and the copolymer at different weight ratios (N/P = 3.0, 5.0 and 7.0) and PAMAM (G2)/pDNA (N/P = 10.0) complex. PEI/pDNA complex at N/P ratio 10.0 was used as positive control and pDNA as negative control. From Fig. 8b, it was found that the transfection efficiency of chitosan/pDNA complexes at N/P ratio 7.0 was only 17%. But, the transfection efficiency of NMCTS-*graft*-PAMAM/pDNA complex at the same N/P ratio (N/P ratio 7.0) was significantly increased to 36% and surpassed the transfection efficiency by PEI/pDNA complex (31%) at N/P ratio 10.0. In this study, the transfection efficiency of PEI/pDNA complex was optimized at N/P ratio 10.0 due its severe toxicity at higher concentration (Fig. 6). It was reported that the transfection efficiency of low generation PAMAM dendrimer is low and we also got similar type of result. In this study, PAMAM dendrimer of generation 2.0 showed transfection efficiency only 22% at N/P ratio 10.0. But, the transfection efficiency was increased significantly and reached up to 31% and 36% at N/P ratio 5.0 and 7.0, respectively. This phenomenon can be explained by the fact that the surface charge density of chitosan was increased by incorporation of PAMAM molecule onto chitosan backbone and the higher surface charge of the copolymer enhanced the complexation ability of the copolymer with pDNA and consequently increased transfection efficiency. Therefore, NMCTS-*graft*-PAMAM (G2) copolymer may be effective alternative nonviral gene carrier for gene therapy.

4. Conclusion

The complexation capability of NMCTS-*graft*-PAMAM (G2) copolymer with pDNA markedly increased after modification of chitosan with low generation (G2) PAMAM dendrimer and the copolymer formed nanoparticles with spherical shaped and an average size within 200 nm. The copolymer showed better protection capability of DNA against nuclease attack and showed no significant hemolytic effect up to 10 mg/mL indicating no detectable disturbance of the red blood cell membranes. In vitro cytotoxicity of NMCTS-*graft*-PAMAM copolymer was also markedly less compared to that of PAMAM dendrimer and most widely used nonviral vector PEI toward HeLa cell lines even at high polymer concentration, although the copolymer was little toxic than that of chitosan. The transfection efficiency of NMCTS-*graft*-PAMAM copolymer significantly increased compared to that of unmodified chitosan and surpassed the transfection efficiency of PEI at very low N/P ratio (N/P = 7). Therefore, NMCTS-*graft*-PAMAM copolymer may find application as an efficient alternative nonviral vector in gene therapy instead of PEI.

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