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Synthesis and characterization of chitosan-*g*-poly(ethylene glycol)-folate as a non-viral carrier for tumor-targeted gene delivery

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Abstract

Poor water solubility and low transfection efficiency of chitosan are major drawbacks for its use as a gene delivery carrier. PEGylation can increase its solubility, and folate conjugation may improve gene transfection efficiency due to promoted uptake of folate receptor-bearing tumor cells. The aim of this study was to synthesize and characterize folate-poly(ethylene glycol)-grafted chitosan (FA-PEG-Chi) for targeted plasmid DNA delivery to tumor cells. Gel electrophoresis study showed strong DNA binding ability of modified chitosan. The pH_{50} values, defined as the pH when the transmittance of a polymer solution at 600 nm has reached 50% of the original value, suggested that the water solubility of PEGylated chitosan had improved significantly. Regression analysis of pH_{50} value as a function of substitution degree of PEG yielded an almost linear correlation for PEG-Chi and FA-PEG-Chi. The solubility of PEGylated chitosan decreased slightly by further conjugation of folic acid due to the relatively more hydrophobic nature of folic acid when compared to PEG. In addition, the chitosan-based DNA complexes did not induce remarkable cytotoxicity against HEK 293 cells. FA-PEG-Chi can be a promising gene carrier due to its solubility in physiological pH, efficiency in condensing DNA, low cytotoxicity and targeting ability.

Keywords: Chitosan; PEGylation; Folate conjugation; Water solubility; Folate receptor targeting; Gene delivery

1. Introduction

Numerous vectors have been explored for gene delivery [1,2], however many applications suffer from their hydrophobicity and low stability, which is reflected in poor biopharmaceutical properties. Many gene carriers undergo rapid clearance from the body, which takes place by combination of proteolysis, glomerular ultrafiltration, liver excretion and depletion by the immune system [3]. Gene delivery vehicles are generally classified into two categories: recombinant viruses and synthetic vectors. Safety is the major concern for viral carrier systems, and viral vectors are also limited by non-replicative, immunogenic, and target-cell specificity problems. Synthetic vectors can be divided into two categories: cationic polymers and cationic lipid carriers. The advantages of using synthetic vectors include no-restriction on size of DNA that they can carry, ability to administer repeatedly with minimal host immune response, targetability, stability in storage, and easy production in large quantities. Cationic polymer/DNA complexes tend to be more stable than cationic lipid/DNA complexes [2].

Chitosan is a biodegradable polysaccharide derived by partial deacetylation of chitin, which is a copolymer of glucosamine and *N*-acetyl-D-glucosamine linked together by $\beta(1,4)$ glycosidic bonds. Chitosan can be degraded into *N*-acetyl glucosamine by general lysozymes in the body, which is subsequently excreted as carbon dioxide via the glycoprotein synthetic pathway. Chitosan has been widely used in pharmaceutical and medical areas, due to its favorable biological properties such as biodegradability, biocompatibility, low toxicity, hemostatic, bacteriostatic, fungistatic, anticancerogen, and anticholesteremic properties, as well as reasonable cost [4]. Due to its unique cationic nature, chitosan is able to interact electrostatically with negatively charged polyions such as indomethacin, sodium hyaluronate, pectin and acacia polysaccharides; it has been found to interact with negatively charged DNA in a similar fashion [5]. Chitosan has been shown to condense

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DNA effectively and protect DNA from nuclease degradation [6,7]. However, the use of chitosan in the biomedical field is restricted. In particular, the poor solubility of chitosan is a major drawback for its use as a gene delivery carrier. Chitosan is insoluble in a neutral or basic pH range, and it can only be dissolved in some specific organic acids such as formic, acetic, propionic, lactic, citric and succinic acid, and in a few inorganic solvents including hydrochloric, phosphoric and nitric acid. The development of water-soluble chitosan is a prerequisite to successful implementation for gene delivery [8]. The water solubility of chitosan can be improved by various approaches such as quaternization of the amino group, *N*-carboxymethylation, and PEGylation [9,10], among which PEGylation is the most popular one.

Poly(ethylene glycol) (PEG) is a polyether diol, which is amphiphilic and can be dissolved in aqueous and organic solvents. It is non-toxic, and shows reduced reticuloendothelial system (RES) clearance, and has been approved by FDA for human intravenous, oral, and dermal applications. In addition, PEG is known for low deposition of proteinaceous material, making it ideal for prevention of bacterial surface growth, decrease of plasma protein binding and erythrocyte aggregation, and prevention of recognition by the immune system. PEG has been often used as a soluble polymeric modifier in organic synthesis; it is also widely used as a pharmacological polymer with high hydrophilicity, biocompatibility and biodegradability. Enormous research has been carried out on PEGylation of biomolecules to improve their water solubility and prolong blood circulation time [11].

Polycationic carrier that incorporates cell-specific ligands such as galactose has been shown to improve transfection efficiency [12]. Folate receptors (FR) are known to be overexpressed in various cancer cells, but rarely found on normal cell surface, or they are localized to the apical surfaces of polarized epithelia [13]. It is well known that folate conjugates, which are covalently derivatized via its γ-carboxyl moiety, can retain the high-affinity ligand binding property of folate ($K_d \sim 10^{-9}$ M), and the kinetics of cellular uptake of conjugated folate compounds by folate receptors is similar to that of free folate. Recycling of folate receptors in target cells can lead to further accumulation of folate conjugates [14]. Folate conjugated liposomes are shown to be internalized by receptor-bearing tumor cells via folate receptor-mediated endocytosis [15]. Other folate conjugates such as protein toxins, anti-T-cell receptor antibodies, radioimaging agents, chemotherapy agents, and gene transfer vectors have also demonstrated receptor-specific delivery properties [13]. In particular, polylysine, poly(dimethylaminomethyl methacrylate), and polyethylenimine modified with folate via a PEG linker have exhibited both long systemic circulation time and efficient folate-selective gene delivery [15]. The objective of this study was to synthesize and characterize folate-conjugated chitosan with a spacer of PEG. The potential of using this chitosan-based carrier for tumor targeted gene delivery was explored. The water solubility, DNA binding ability and cytotoxicity of the modified chitosan were studied. The results showed that chitosan-*g*-PEG-folate would make a promising non-viral vector for targeted gene delivery to tumors.

2. Materials and methods

2.1. Materials

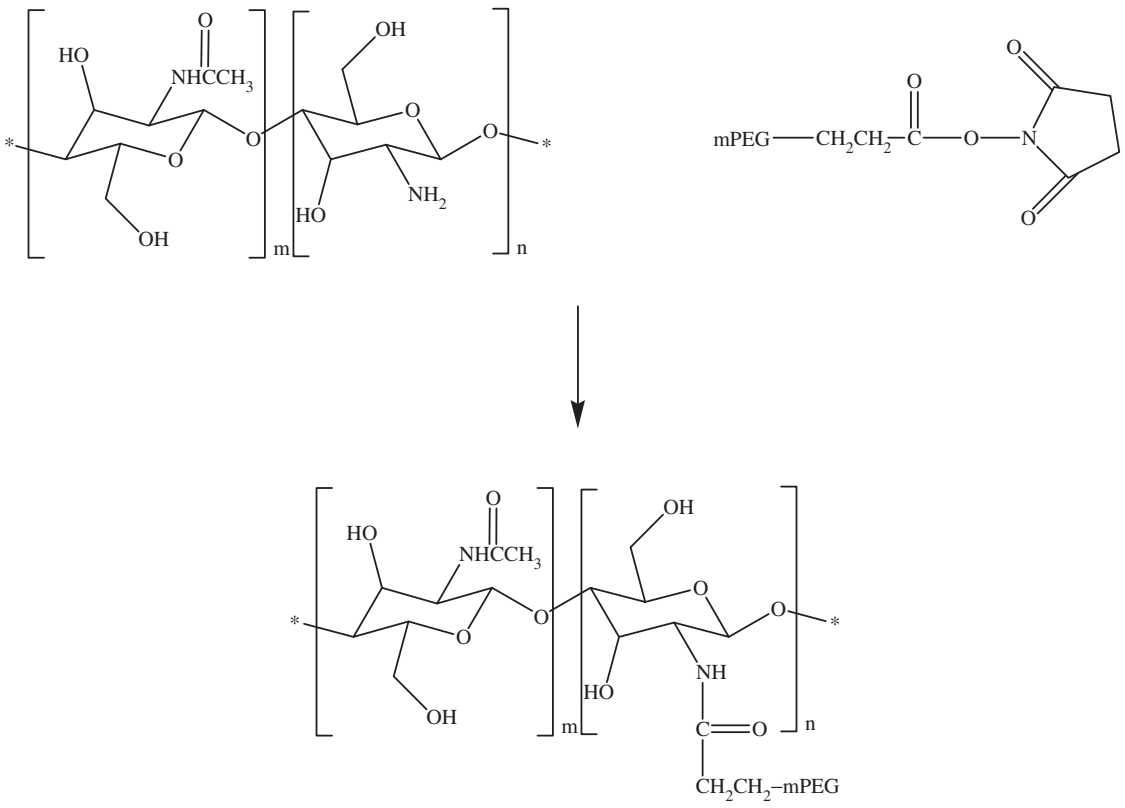
Chitosan (Mw ~255 kDa, viscosity: 200–800 cps in 1% acetic acid) was purchased from Sigma-Aldrich (Singapore). *N*-hydroxylsuccinimide-PEG-Maleimide (NHS-PEG-MAL, Mn 3400 Da) and succinimidyl ester of PEG propionic acid (mPEG-SPA, Mn 5000 Da) were purchased from Nektar (NOF Corporation, Tokyo, Japan). 2-Aminoethanethiol, and DNA Col EI were bought from Wako (Wako reagents, Sematec Pte Ltd, Singapore). Lipofectamine™ 2000 reagent, cell culture media and supplements were obtained from Gibco (Invitrogen, Singapore). Fetal bovine serum (FBS) was purchased from Hyclone (Research Instrument, Singapore). TransFast™ transfection reagent was obtained from Promega (Research Instruments, Singapore). AlamarBlue® was bought from TREK Diagnostic Systems (England). Folic acid dihydrate, and other generally used chemicals were purchased from Sigma-Aldrich (Singapore). Unless stated otherwise, all reagents and solvents were of biological grade, and were used without further purification.

2.2. Synthesis of PEG grafted chitosan

It was reported that chitosan-*g*-PEG with long PEG side chain (e.g. Mn 2000 or 5000) were soluble in water, whereas chitosan-*g*-PEG with shorter PEG side chain (e.g. Mn 550) were insoluble in water [11]. In the present study, PEG polymers with Mn of 5000 and 3400 were used to modify chitosan at various feed ratios of PEG to chitosan. Chitosan-*g*-PEG (PEG-Chi) was prepared as depicted in Scheme 1. In brief, 100 mg of chitosan with 82% degree of deacetylation (DD) was dissolved in 50 ml of 20% acetic acid solution; pH of the solution was adjusted to 6 with gradual addition of sodium hydroxide. mPEG-SPA was then added to the solution, and the reaction was conducted for 24 h at room temperature. The synthesized product was dialyzed against de-ionized (DI) water using a dialysis tubing with Mw cut-off of 12,000 Da (Spectrum Laboratories, USA) for 48 h, followed by freeze drying.

2.3. Synthesis of chitosan-g-PEG-folate

N-Hydroxysuccinimide ester of folic acid (NHS-FA (γ)) was prepared in accordance to a reported procedure as shown in Scheme 2 [16]. In brief, folic acid (1.0 g) was added into a mixture of anhydrous dimethyl sulfoxide (DMSO, 40 ml) and triethylamine (TEA, 0.5 ml), and folic acid was allowed to dissolve in the stirring mixture under anhydrous conditions and in the dark overnight. Folic acid was mixed with dicyclohexylcarbodiimide (DCC, 0.5 g) and *N*-hydroxysuccinimide (NHS, 0.52 g), and stirred in the dark for further 18 h. The side product dicyclohexylurea (DCU) precipitated was removed by filtration. DMSO and triethylamine were evaporated under vacuum. Vacuum dried FA-NHS was then dissolved into 1.5 ml of the mixture of DMSO and TEA with a volume ratio of 2:1. An equal molar amount of 2-aminoethanethiol was added to the mixture, and the reaction was conducted under an anhydrous condition overnight. Therefore, a thiol group was introduced into folic acid to form FA-SH by nucleophilic substitution as depicted in Scheme 2. Chitosan was deacetylated to obtain 82% DD (determined by ^{1}H-NMR) according to a procedure reported in the literature [17]. About 100 mg of chitosan was dissolved in 50 ml of 20% acetic acid solution; the solution was adjusted to pH 6 with gradual addition of sodium hydroxide. About 100 mg of NHS-PEG-Mal was then added to chitosan solution at room temperature, and the pH was adjusted to 7 after 3 h. The reaction was performed overnight



Scheme 1. Synthesis of chitosan-*g*-PEG.

under an argon atmosphere (Scheme 3). The conjugation of FA-SH to PEGylated chitosan through a heterobiofunctional NHS-PEG-Mal spacer was performed as depicted in Scheme 4. The long PEG spacer served two purposes (1) to improve the water solubility of chitosan; (2) to extend the distance between folate and the amino group to reduce steric hindrance, and to increase accessibility of folate to the receptors [18]. In brief, FA-SH was added slowly to PEGylated chitosan solution with stirring as depicted in Scheme 4, and the mixture was adjusted to pH 6.5–7.5 using 6 M sodium hydroxide. FA-PEG-Chi conjugates with various degrees of substitution (DS) of PEG and folic acid were synthesized by changing the feed ratio of NHS-PEG-Mal and FA-SH to chitosan. The synthesized product was dialyzed against DI water using a dialysis tubing with Mw cut-off of 12,000 Da (Spectrum Laboratories, USA) for 48 h, followed by freeze drying.

2.4. Characterization of PEG-Chi and FA-PEG-Chi

The DD or percentage of free amine groups ($-NH_2$) on chitosan, and degree of substitution (DS) of PEG to monosaccharide residue of chitosan were calculated based on ^1H NMR spectra of chitosan, PEG-Chi and FA-PEG-Chi, which were obtained using a Bruker AVANCE 400 spectrometer (400 MHz) [19]. The NMR samples were prepared according to the procedure reported previously [11]. Folic acid content of the final product was determined by a UV/Vis spectrophotometer (V-570, Jasco Corporation, Tokyo, Japan) using the molar extinction coefficient value of 6197 $mole^{-1}\,cm^{-1}$ at $\lambda = 363$ nm [20].

2.5. Solubility measurement

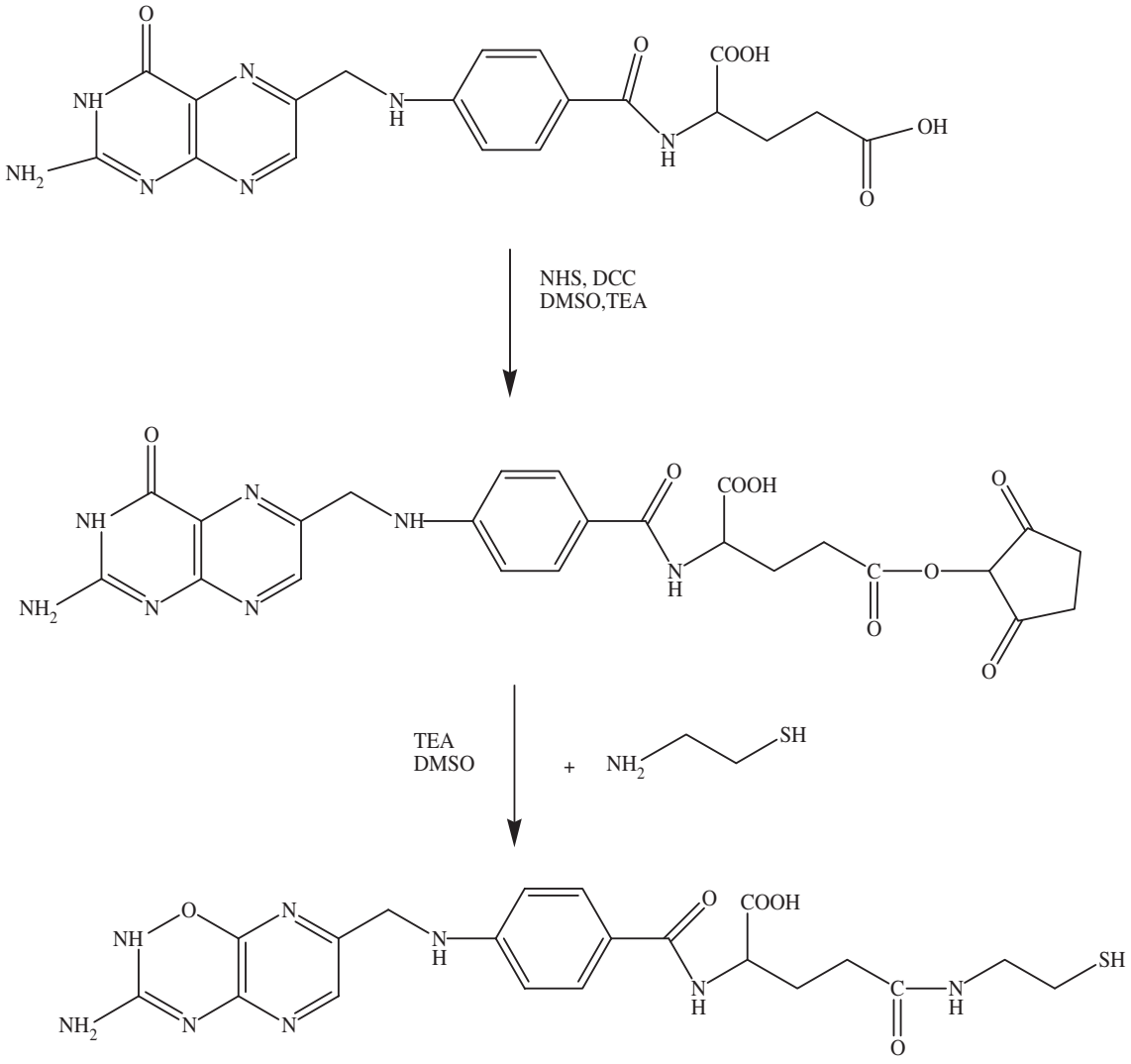
The solubility of the chitosan and chitosan derivates was evaluated at different pH values according to the method described previously [6]. Polymers were dissolved in 0.2% acetic acid solution (2 mg/ml), the pH of the solution was adjusted by drop wise addition of 1 M NaOH solution. The transmittance of the solution was recorded as a function of pH on the UV/Vis spectrophotometer using a quartz cell with an optical path length of 1 cm at $\lambda = 600$ nm. The pH_{50} values defined as the pH when the transmittance reached 50% of the original value, were determined.

2.6. Amplification and purification of DNA plasmid

The plasmid pCAGLuc (a gift from Dr. Shu Wang, Institute of Bioengineering and Nanotechnology, Singapore, originally developed by Yoshiharu Matsuura, National Institute of Infectious Diseases, Tokyo, Japan) encoding firefly luciferase driven by a CAG promoter was amplified in *Escherichia coli* and purified using HiSpeed Plasmid Maxi kit (Qiagen gmbH, Germany). The plasmid was quantified and qualified by PicoGreen assay (Molecular Probes, Inc., Eugene, OR), and by electrophoresis in 1% agarose gel, respectively.

2.7. Polymer/DNA complex preparation

Chitosan, PEG-Chi and FA-PEG-Chi/DNA complexes were prepared at various charge ratios. The charge ratios (N/P) of chitosan or its derivative/DNA complexes were expressed as the molar ratios of amine group of chitosan or its derivative to phosphate group of DNA. The average mass 330 Da per charge for DNA was used for the calculation of charge ratios [21]. The molecular weight of repeating unit of chitosan was used as the mass per charge for the polymer. Complexes were induced to self-assemble by incubating DNA with the polymer. First, DNA and various amounts of chitosan or its derivative were diluted separately in 0.2 M sodium acetate/acetic acid buffer (pH 5.6), and an appropriate amount of polymer solution was then added to the solution of plasmid,



Scheme 2. Activation of folate.

and the resulting solution was mixed by vortex and left for 20 min at room temperature.

2.8. Agarose gel electrophoresis

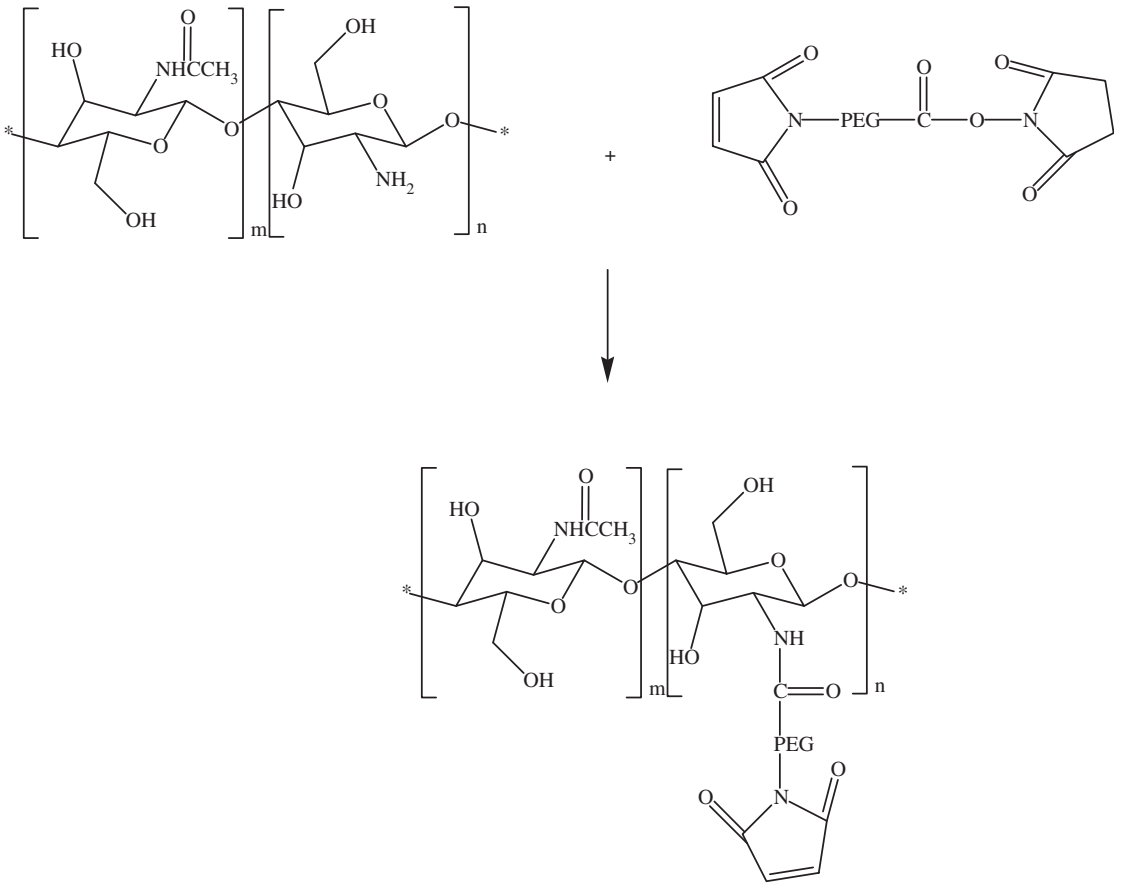
The DNA binding ability of chitosan or its derivatives was evaluated by agarose gel electrophoresis. The complexes containing 0.5 μg of DNA at various N/P ratios were loaded into individual wells of 1.0% agarose gel in 1 × Tris–boric acid–EDTA buffer, electrophoresed at 80 V for 45 min, and stained with 0.5 μg/ml ethidium bromide. The resulting DNA migration pattern was revealed under UV irradiation (Chemi Genius, Evolve, Singapore).

2.9. Cytotoxicity assays

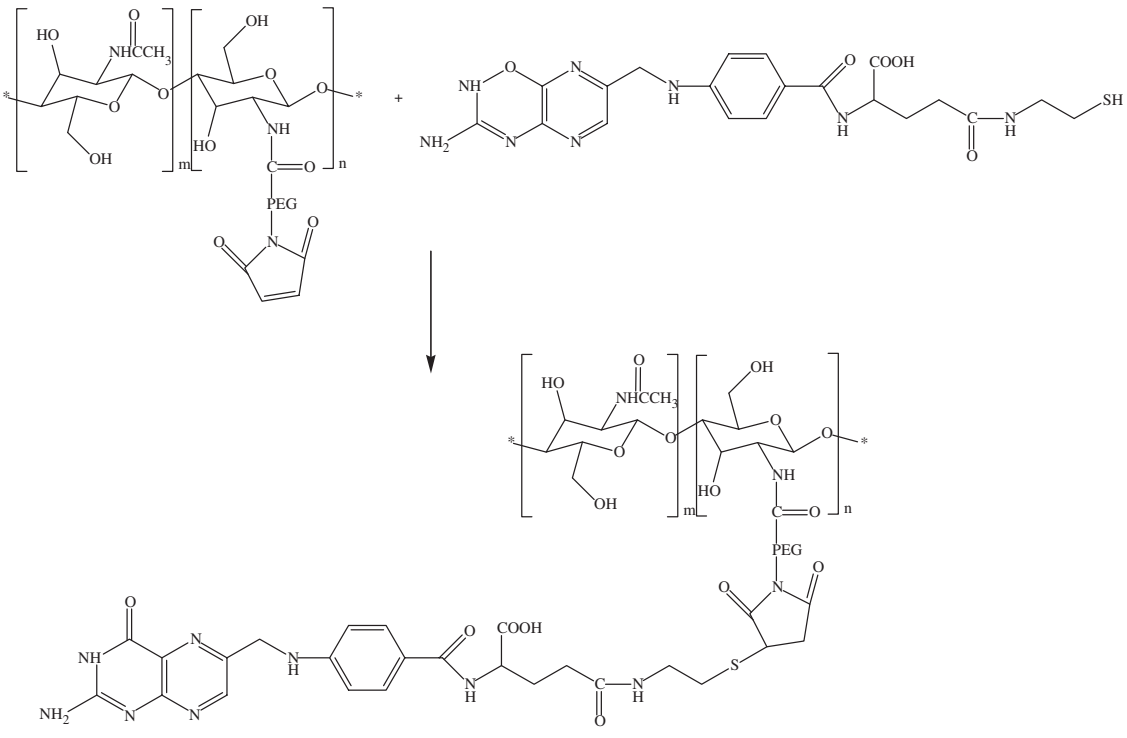
Human embryonic kidney (HEK 293) cells (Invitrogen) were maintained in RPMI 1640 medium, supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, and 50 units/ml penicillin-streptomycin at 37 °C in a humidified 5% carbon dioxide incubator. Cells were seeded at 1×10^4 cells per well in 96-well plates and incubated for 18–24 h to obtain 75–80% confluence. The spent media were removed from each well, and then washed with PBS prior to 4 h exposure to polymer/DNA complexes. The complexes prepared at different N/P ratios were diluted with folic acid-free RPMI 1640 containing 1% FBS, 2 mM L-glutamine, 50 units/ml penicillin-streptomycin, and added to each well with a final amount of 0.5 μg plasmid. Cells without treatment with the complexes and cells treated with naked DNA, lipofectamine^{TM}2000/DNA complexes, or TransFast^{TM}/DNA complexes were used as controls. After 4 h incubation, media were replaced with fresh complete media, and cultured for another 72 h. Each well received 1 μg lipofectamine^{TM}2000 or TransFast^{TM} reagent complexed with 0.5 μg DNA. The cytotoxicity of polyplexes was evaluated as relative viability in HEK 293 cells using Alamar blue assay (Serotec, UK) [22]. The fluorescence was recorded on a microplate reader (GENios Pro, Tecan, Austria) with excitation wavelength at 530 nm and emission wavelength at 590 nm.

2.10. Statistical analysis

All experiments were repeated at least three times, and measurements were collected in triplicate. Data are expressed as mean ± standard deviations. Statistical analysis was performed using Student's *t*-test. Differences were considered statistically significant with $p < 0.05$.



Scheme 3. Synthesis of chitosan-*g*-PEG-Mal.



Scheme 4. Synthesis of chitosan-*g*-PEG-folate.

3. Results and discussion

3.1. Synthesis and characterization of PEG-Chi and FA-PEG-Chi

For PEGylation of chiotsan (PEG-Chi), mPEG-succinimidyl propionate ester (mPEG-SPA) (Mw 5000 Da) was used to react with amine groups of chitosan to provide a physiologically stable amide linkage (Scheme 1). Methoxy poly(ethylene glycol) (mPEG) was used for PEGylation of chitosan instead of PEG in order to avoid cross-linking of the copolymers [23].

FA-PEG-Chi was prepared with a three steps procedure: (1) activation of folic acid with NHS and 2-aminoethanethiol to yield FA-SH; (2) grafting of chitosan with NHS-PEG-Mal to form Mal-PEG-Chi; (3) conjugation of Mal-PEG-Chi with NHS-FA to produce FA-PEG-Chi. Heterobiofunctional NHS-PEG-MAL, which contains a thiol selective maleimide group and the amine reactive hydroxysuccinimide ester, was used as a selective cross-linker for conjugating folic acid onto chitosan. NHS-PEG-MAL was first grafted onto chitosan by coupling of primary amino group of chitosan to NHS to form amide bond, followed by coupling thiol group of FA-SH to the maleimide. *N*-hydroxysuccinimide released from the MAL-PEG-NHS spacer can be easily removed by dialysis in a later step. The carbodiimide-activated folic acid can couple either via the α or γ carboxyl group of the glutamate residue [20], therefore reaction conditions were selected to favor linkage of the distal γ carboxyl residue. Due to the steric hindrance constraints imposed by the folic acid chain, the folate derivatives were expected to consist mostly of the γ-linked isomer. Coupling of maleimide to thiol group is a useful preparation method for PEGylation of folic acid, as the reaction is highly site-specific and convenient to control, and the reaction with thiol moiety generates a stable 3-thiosuccinimidyl ether linkage.

Successful synthesis of PEG-Chi and FA-PEG-Chi was confirmed by ^{1}H NMR spectra. Typical ^{1}H spectrum of FA-PEG-Chi is showed in Fig. 1. The bond between chitosan and PEG corresponding to the signal of –NH–CH_2CH$_2$O– appeared at $\delta = 2.45$–2.60 ppm (Signal a). The DD of the starting chitosan was evaluated with Signal b at $\delta = 2.0$–2.1 ppm from the acetyl group (–COCH_3) and Signal c at $\delta = 2.95$–3.10 ppm from the monosaccharide residue (C*H*–NH–), being about 82%. DS value of PEG was determined from the relative peak area of methylene group of PEG (Signal a) to acetyl group (Signal b) of the monosaccharide residue. The grafting degrees of PEG and folate of PEG-Chi and FA-PEG-Chi are listed in Table 1.

Coupling of the folate residue to PEG grafted chitosan was confirmed by the appearance of signals at δ 6.0–8.1 ppm in the ^{1}H NMR spectrum of FA-PEG-Chi (Fig. 1), which corresponded to the aromatic protons of folic acid. The relevant signals of folate are much weaker than the broad and strong proton signals of PEG and chitosan residues. Therefore, for more accurate evaluation, the DS of folate was assessed from UV spectroscopy.

3.2. Complex formation between chitosan/chitosan derivatives and plasmid DNA

The formation of polymer/DNA complexes is an important prerequisite for gene delivery using cationic polymers. Chitosan-based polymer is an attractive non-viral vector as

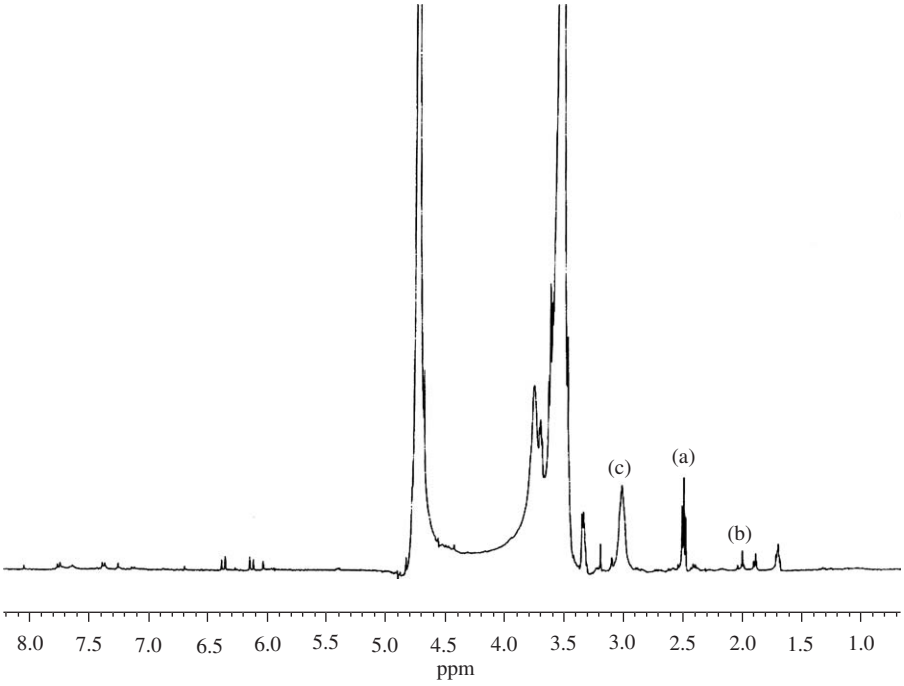


Fig. 1. ^{1}H NMR spectrum of FA-PEG-Chi with 11% DS of PEG, and 8% grafting degree of folate in D_2O containing one drop of 35 wt% DCl/D_2O.

it can form complexes with DNA based on electrostatic interaction between the positive amino groups of chitosan and negative phosphate groups of DNA.

The ability of both PEG-Chi and FA-PEG-Chi to form complexes with DNA was confirmed by gel retardation assay. Complexes containing 0.5 μg DNA were prepared at various N/P ratios in sodium acetate buffer. It was reported that chitosan by itself could complex DNA efficiently even down to a DNA/charge ratio of 1:0.01 [24]. As shown in Figs. 2 and 3, migration of DNA in agarose gel was completely retarded at N/P ratio of 1 and onward for the formulations of chitosan, PEG-Chi and FA-PEG-Chi, indicating that all the polymers could bind DNA strongly, and the introduction of PEG and FA-PEG did not affect its DNA binding ability.

3.3. Water solubility

The solubility of PEGylated chitosans was assayed to elucidate the effects of PEG substitution. All the chitosans were soluble in acidic pH. The relationship between pH_{50} and DS of PEG is shown in Fig. 4. It is observed that pH_{50} increased with increasing DS of PEG. The samples with DS values below 6% were poorly soluble. This is expected as the increase in water solubility of PEGylated chitosan is attributed to the decrease of intermolecular interactions, such as van der Waals forces and hydrogen bonding. As suggested by Yang et al. [25], the introduction of substitutes on amino groups can destruct the rigid crystalline domain of chitosan, thus disturbing the intra and intermolecular hydrogen bonding and leading to enhancement

Table 1
Properties of PEG-Chi and FA-PEG-Chi

Sample code	PEG/Mn	DS[a] (%)	FA[b] (%)	
Chi	—	—	—	—
PEG-Chi-3	mPEG-SPA	5000	3.2	—
PEG-Chi-4	mPEG-SPA	5000	4	—
PEG-Chi-7	mPEG-SPA	5000	6.9	—
PEG-Chi-8	mPEG-SPA	5000	8.2	—
PEG-Chi-15	mPEG-SPA	5000	15.1	—
PEG-Chi-16	mPEG-SPA	5000	15.6	—
FA-PEG-Chi-3	NHS-PEG-MAL	3400	5.4	3.1
FA-PEG-Chi-6	NHS-PEG-MAL	3400	16.2	6.3
FA-PEG-Chi-7	NHS-PEG-MAL	3400	11.6	7.2
FA-PEG-Chi-8	NHS-PEG-MAL	3400	11.0	8.0
FA-PEG-Chi-9	NHS-PEG-MAL	3400	16.5	8.9
FA-PEG-Chi-14	NHS-PEG-MAL	3400	27.9	13.9

[a]Degree of substitution of PEG to amino group of chitosan determined by ^{1}H NMR spectrum.
[b]Degree of grafting of folate to amino group of chitosan determined by UV–Vis spectroscopy at 363 nm.

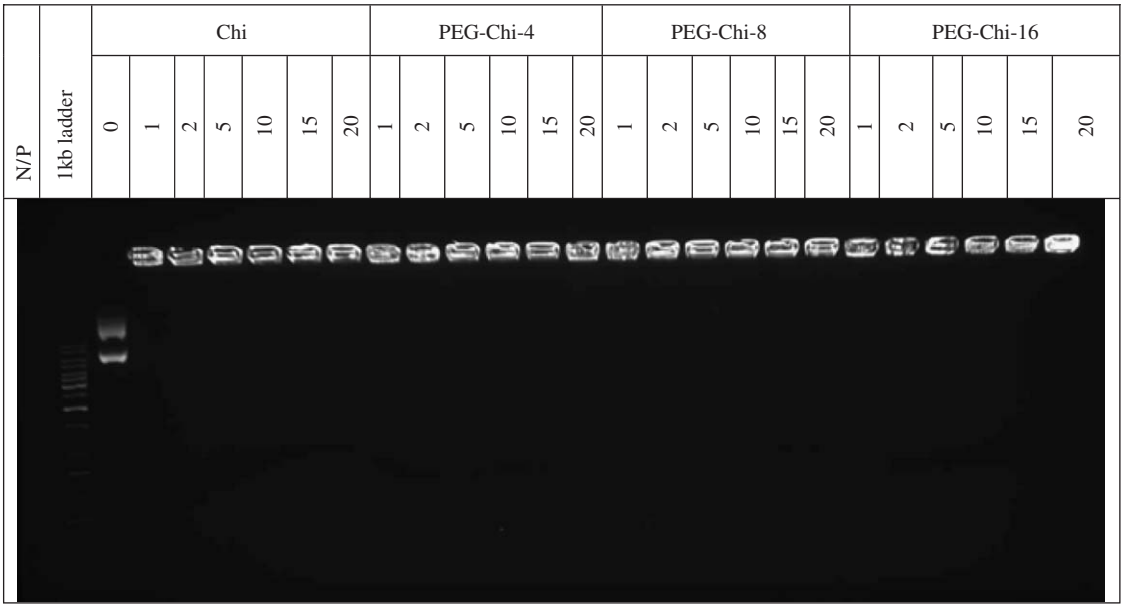


Fig. 2. Electrophoretic mobility of plasmid DNA (0.5 μg) complexed with Chi and PEG-Chi. Sample codes correspond to those in Table 1.

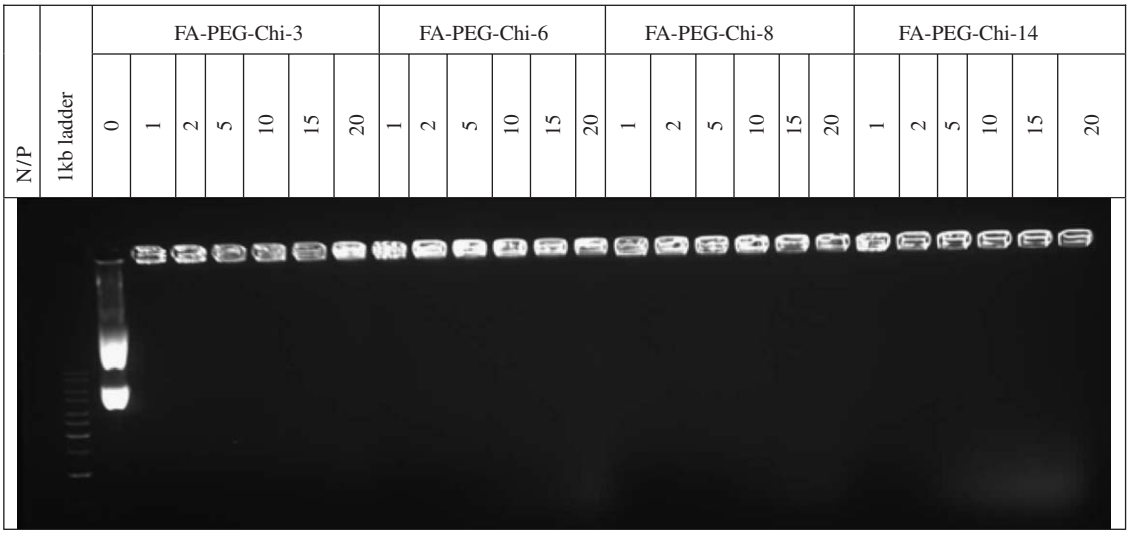


Fig. 3. Electrophoretic mobility of plasmid DNA (0.5 μg) complexed with FA-PEG-Chi. Sample codes correspond to those in Table 1.

in hydrophilicity. Therefore, the lower the DS, the higher did the intra and intermolecular attraction forces become. Due to the improvement of water solubility, the biological and physiological applications of PEGylated chitosans may develop dramatically.

Regression analysis of pH_{50} value as a function of DS of PEG yielded an almost linear correlation with correlation coefficients of 0.042 and 0.033 for PEG-Chi and FA-PEG-Chi, respectively. For the same DS of PEG, lower water solubility was observed for FA-PEG-Chi when compared to that of PEG-Chi, due to the presence of multiple hydrophobic groups in folic acid, thus influencing the overall solubility of the polymer. In addition, synthetic problems were encountered when the feed molar ratio of FA-SH was increased further, where precipitation of these conjugates started to take place.

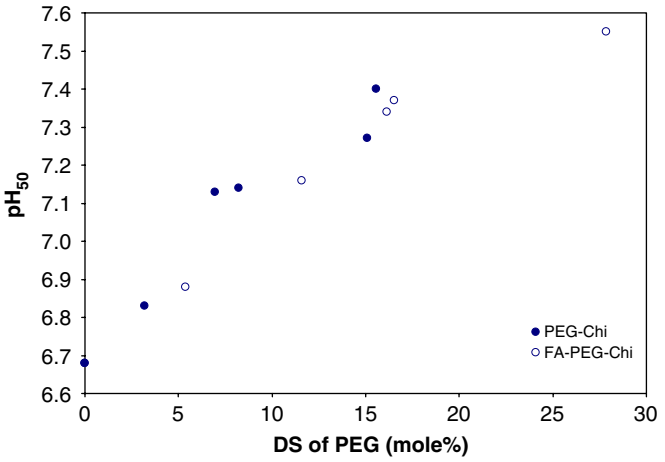


Fig. 4. Dependence of water solubility (measured as pH_{50}) of chitosan derivates on PEG content.

3.4. Cytotoxicity

For the concerns of efficient gene delivery and biocompatibility, polyplexes should exhibit minimal cytotoxicity. To investigate a potential cytotoxic effect of PEG-Chi/DNA complexes after conjugation of folic acid, the viability of HEK 293 cells was tested in the presence of Chi, PEG-Chi and FA-PEG-Chi/DNA complexes at various N/P ratios. Naked DNA, Lipofectamine™2000/DNA and TransFast™ reagent/DNA complexes were used as controls. Cells without treatment of polymer or polymer/DNA complexes were considered as a positive control with a cell viability of 100%.

Several studies [26–28] reported that no significant decrease in viability was observed for cells incubated with chitosan/DNA and folate grafted chitosan/DNA complexes. The results obtained in this study are in good agreement with those reported in the literature. Average cell viability over 90% was obtained with naked DNA and chitosan, PEG-Chi and FA-PEG-Chi/DNA complexes at various polymer doses (Fig. 5).

No significant decrease in viability was found for HEK 293 cells treated with chitosan-based polyplexes when compared to naked DNA even at high N/P ratio (up to 20). In exception for FA-PEG-Chi-14/DNA polyplexes, the viability of HEK 293 cells was found to decrease slightly in relation to increased concentration ($p<0.05$ at N/P ratio of 20). De Laporte et al. [29] suggested that bigger complexes were likely to increase cytotoxicity. The slight decrease in viability found for FA-PEG-Chi-14/DNA complexes was possibly due to the higher molecular weight associated with high DS of PEG (28%).

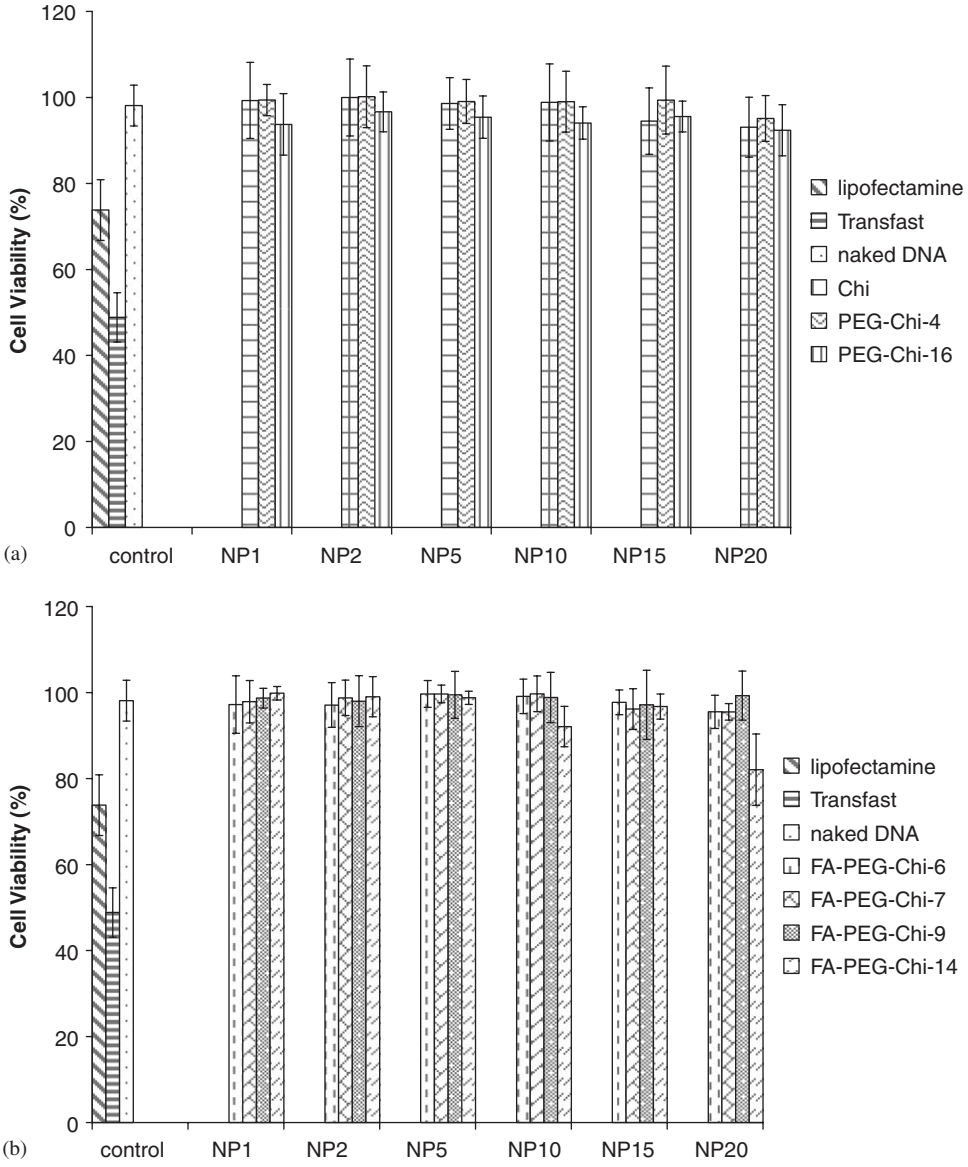


Fig. 5. Cytotoxicity of (a) lipofectamine^{TM}2000/DNA, TransFast^{TM}/DNA, naked DNA, Chi/DNA, PEG-Chi-4/DNA and PEG-Chi-16/DNA complexes; (b) lipofectamine^{TM}2000/DNA, TransFast^{TM}/DNA, naked DNA, FA-PEG-Chi-6/DNA, FA-PEG-Chi-7/DNA, FA-PEG-Chi-9/DNA and FA-PEG-Chi-14/DNA complexes at various N/P ratios after 72 h incubation with HEK 293 cells. Percent viability of cells is expressed relative to control cells. Sample codes correspond to those in Table 1. Results are expressed as mean values ± SD ($n = 5$) $p < 0.05$ (*).

Complexes derived from chitosan carried lower cytotoxicity than the commercial carriers such as Lipofectamine^{TM}2000 and TransFast^{TM} transfection reagents. These results are in agreement with those reported by Mansouri et al. [28]. The use of chitosan or folate modified chitosan as vectors showed lower cytotoxicity against HEK 293 cells when compared to Lipofectamine^{TM}2000. Beens et al. [30] also reported that by grafting folate-PEG-folate onto PEI, the cytotoxicity of PEI decreased even at excess positive charge ratios. Decrease in cytotoxicity was not found in chitosan by grafting folate-PEG due to the fact that the viability of cells treated with FA-PEG-Chi/DNA complexes were almost 100%. In general, chitosan-based polyplexes showed to induce no remarkable cytotoxicity against HEK 293 cells.

4. Conclusions

PEG and FA-PEG have been successfully grafted onto chitosan. The introduction of PEG and FA-PEG did not affect its DNA binding ability. The water solubility of chitosan increased as a function of PEG grafting degree. The water solubility of FA-PEG-Chi was slightly lower than that of PEG-Chi due to the introduction of hydrophobic groups in folic acid. In addition, the presence of PEG and FA-PEG did not add significant cytotoxicity to chitosan, and the complexes derived from chitosan and modified chitosan showed lower toxicity against HEK 293 cells than the commercial carriers such as Lipofectamine^{TM}2000 and TransFast^{TM} transfection reagents. FA-PEG-Chi can be a promising carrier for targeted gene

delivery to folate receptor-bearing tumor cells. The in vitro and in vivo studies of gene transfection using FA-PEG-Chi are being conducted in the laboratory to evaluate its gene transfection efficiency.

Acknowledgements

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biomaterials.2006.08.046

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