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Synthesis and characterization of lactobionic acid grafted pegylated chitosan and nanoparticle complex application

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ABSTRACT

A series of chemical modifications of chitosan were conducted by grafting a hydrophilic methoxy poly(ethylene glycol) (MPEG) and a target sugar molecule lactobionic acid (LA). The MPEG was grafted onto C6–OH position of chitosan, and the grafting degree was reduced for chitosan with high degree of depolymerization. The lactobionic acid was proposed to graft onto C2–NH_2 position of chitosan. The LA grafting ratio was dependent on pegylation degree of chitosan, where the flexibility and shielding effect of MPEG hindered LA grafting onto chitosan. The lactobionic acid grafted pegylated chitosan, DADP-CS-(O-MPEG)-(*N*-LA), successfully provoked DNA condensation into nanoparticle complexes due to electrostatic compaction. The presence of MPEG on DADP-CS-(O-MPEG)-(*N*-LA) played an important role on preventing nanoparticle aggregation.

1. Introduction

Polymeric nanoparticles play an important role on controlling drug release and passively delivering drug to the desirable action site. Polyethylenimine (PEI) has been reported to possess significant potential as a gene delivery carrier. The cationic PEI electrostatically interacts with negative charge plasmid DNA (*p*DNA) to form stable nanoparticle complexes. Salem et al. found that branched PEI has a stronger electrostatic interaction with *p*DNA than linear PEI in terms of greater compaction, higher zeta potential and smaller nanoparticle size. Increasing the *N*:*P* ratio of branched PEI/*p*DNA nanoparticles increased luciferase activity in HepG2 cells, however, the same effect was not generated by linear PEI-*p*DNA nanoparticles [1]. Huang et al. investigated the potential of linear and branched ε- and α-oligo(L-lysines) and their ε-substituted homologues as a gene delivery carrier. The improvement of DNA compaction and transfection efficiency was successfully demonstrated via chemical modification of pendent group on oligo-(L-lysines) [2]. The performance and mechanism of provoking DNA condensation in nanoparticles by oligo(L-lysines) and poly-(L-lysines) was quite different. The EC_{50} and hydrodynamic radii of the oligo(L-lysines) nanoparticles increased with sodium concentration in the medium. In addition, the temperature significantly affected the size and stability of oligo(L-lysines)/DNA nanoparticles, whereas no such phenomenon was observed for poly(L-lysines) [3].

The advantages of targeting delivery of drugs have been addressed a lot, which include the accumulation of drug in the action site, reduction of therapeutic dose, increase in therapeutic efficacy, and reduction of toxicity, etc. These induce the development of cell-specific targeting carriers especially for gene therapy and chemotherapy, where the carriers are preferred to targeting via receptor-mediated endocytosis. Nano-sized carriers (e.g., nanoparticles) with surface modification are usually necessary for specific targeting purpose. Asialoglycoprotein receptor (ASGPR) receives much attention in gene targeting and also plays as a model system for studying receptor-mediated endocytosis due to its high affinity and rapid internalization rate [4,5]. The presence of asialoglycoprotein receptor on hepatocytes provides a membrane-bound active site for cell-to-cell interactions and allows the active targeting of chemotherapeutic agents and foreign genes on it [6]. ASGPR is an integral membrane protein and express on the surface of parenchymal cells of liver with high density of $1–5 \times 10^5$ receptors [5]. It is responsible for the clearance of desialylated galactose-terminal glycoproteins from the circulation by receptor-mediated endocytosis. Several sugar ligands (e.g., galactose, *N*-acetylgalactosamine, mannose, lactose, fructose, etc.) have been demonstrated possessing different extent of interaction with ASGPR. Gref et al. found that galactose-modified oligosaccharides show a high affinity for ASGPR in liver tumor cells. In opposite, the hydrophilicity and mobility characters of dextran modified polysaccharides prevented protein opsonization and further avoided liver recognition [7]. Hashida et al. have demonstrated that liposomes with different types of glycosylation possess different cell-specific targeting properties. The galactosylated cationic liposomes were able to be taken up by the

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ASGPR in liver parenchymal cells, and expressed higher gene expression than liposomes without galactose. However, replacement of galactose by mannose residues in carriers resulted in specific delivery of genes to non-parenchymal liver cells [8]. Chemical modification of chitosan to allow it bearing specific liver targeting function has been reported, and it is usually proposed through C2-amino groups of chitosan [9–13]. Gorochovceva et al. first proposed to modify chitosan through its C6-hydroxyl group rather than C2-amino group [14]. The free amino groups of chitosan play an important role on gene delivery, where it is feasible to complex with negatively charged DNA and allow DNA compacted inside nanoparticle carriers.

The aim of this study was to conduct a series of chemical modifications by using chitosan as the polymer backbone. Chitosan was deacetylated and depolymerized first, and its C2-amino group was protected by phthalic anhydride before grafting with hydrophilic methoxy poly(ethylene glycol) (MPEG). MPEG was designed to graft onto C6-hydroxyl group of chitosan via a S_N2 reaction. After pegylation of chitosan, phthalic anhydride was removed, and a proposed ASGPR recognized sugar molecule, lactobionic acid, was further introduced into C_2-amino group of pegylated chitosan via an amide linkage. The synthesis product for each step was identified by FTIR, NMR, etc. The final product, lactobionic acid grafted pegylated chitosan, DADP-CS-(O-MPEG)-(*N*-LA), was further characterized by GPC and DSC, and the graft contents of MPEG and lactobionic acid were determined by calorimetric analysis and ^{1}H NMR, respectively. The ability of condensing negatively charged plasmid DNA by DADP-CS-(O-MPEG)-(*N*-LA) was evaluated. The particle size and the zeta potential of nanoparticle complexes were measured, and the morphology was observed by transmission electron microscope.

2. Experimental section

2.1. Materials

Low molecular weight chitosan and tripolyphosphate were from Aldrich Chemical Company, Inc. (WI, USA). Phthalic anhydride was from Showa Chemical Co. LTD. (Tokyo, Japan). *N*,*N*-Dimethylformamide was from Tedia Company Inc. (Fairfield, USA). Methoxy poly(ethylene glycol) was from Fluka Chemical Company Inc. (Buchs, Switzerland). Lactobionic acid (4-O-β-D-galactopyranosyl-D-gluconic acid) was from Acros Organics, Fisher Scientific Co. Inc. (Leicestershire, UK). Poly(ethylene glycol) standards were from Scientific Polymer Products, Inc. (Ontario, USA). Plasmid DNA (pEGFP-N1, 4.7 kb) was kindly provided by Professor Jiin Long Chen from National Defense Medical Center in Taiwan.

2.2. Synthesis

2.2.1. Deacetylation of chitosan

The synthesis procedure and structure of chitosan (CS) and deacetylated chitosan (DA-CS) are shown in Scheme 1. Chitosan 7 g was placed in 140 mL 50% w/v NaOH aqueous solution, and refluxed at 140 °C in an oil bath for 4 h. After cooling to room temperature, large volume of de-ionized water was added to neutralize the mixture. The mixture was filtered, and the solid was collected and dried in the oven at 40 °C overnight. Finally, the deacetylated chitosan (DA-CS) was obtained and characterized by FTIR. The deacetylation degree was determined by potentiometric titration [15], and the molecular weight and distribution were determined by gel permeation chromatography.

2.2.2. Depolymerization of deacetylated chitosan

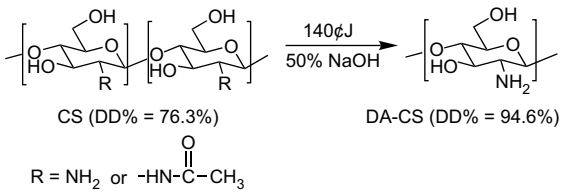
DA-CS 1 g was previously dissolved in 100 mL 1% acetic aqueous solution, and 0.1 M sodium bisulfate was slowly added into DA-CS solution drop by drop. The reaction was continuously stirred at room temperature for 3 h. The mixture was filtered, and 1 N NaOH aqueous was added to precipitate the product. The mixture was centrifuge, and the precipitate was collected and freeze dried. Finally, the deacetylated depolymerized chitosan (DADP-CS) was obtained and characterized by FTIR and gel permeation chromatography. The solubility of DA-CS before and after depolymerization was evaluated by determination of its turbidity [16]. The polymer sample 200 mg was dissolved in 100 mL 0.25% acetic aqueous solution, and then titrated with 1 N NaOH. The pH value and the transmittance at 600 nm were recorded after each titration. The transmittance versus pH value was plotted, and the pH values corresponding to 50% polymer precipitated ($pH_{50\%}$) in terms of 50% transmittance was determined.

2.2.3. Phthaloylation of DADP-CS

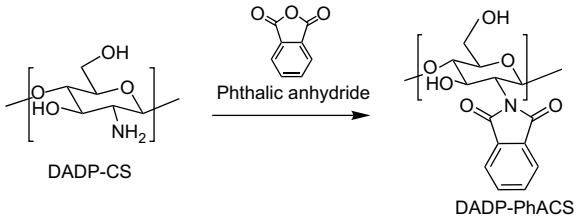
The synthesis procedure is shown in Scheme 2. DADP-CS 0.8 g was dissolved in 6.7 mL formic acid. Phthalic anhydride 0.4 g in 35.6 mL DMF was added and reacted at 130 °C under N_2 for 8, 12, and 24 h, respectively. The mixture was cooled to room temperature and precipitated by acetone. The precipitate was collected by filtration and washed with methanol several times following dried in an oven at 40 °C. Finally, the phthalic anhydride protected deacetylated–depolymerized chitosan, DADP-PhACS, was obtained and characterized by FTIR and ^{1}H NMR. The *N*-phthaloylation degree was further calculated based on ^{1}H NMR with decoupling technique.

2.2.4. Synthesis of pegylated chitosan DADP-CS-(O-MPEG)

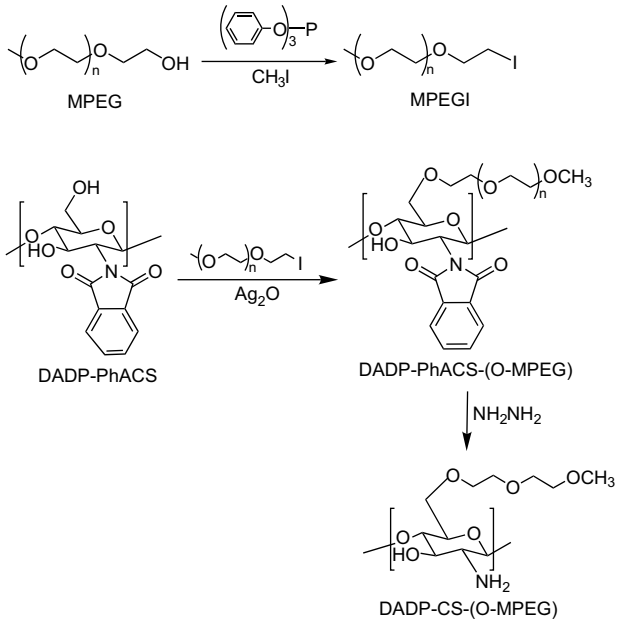
The synthesis procedures are shown in Scheme 3. DADP-PhACS was reacted with pre-activated methoxy poly(ethylene glycol) iodide (MPEGI) in the feed molar ratio of 1:0.8 and 1:1.6, respectively. DADP-PhACS (0.3 and 0.6 g), MPEGI 3.5 g and Ag_2O 0.2 g were mixed in 3 mL toluene under vacuum. DMF (60 and 120 mL) was added and the mixture was reacted at 60 °C under N_2 for 16 h. Ag_2O was removed by Celite and DMF was removed by rotary evaporation. De-ionized water was added to precipitate the product. The mixture was centrifuged, and the supernatant was discarded. The precipitate was collected and freeze dried, and DADP-PhACS-(O-MPEG) was obtained and characterized by FTIR. In order to remove phthalic anhydride from C2 position of chitosan, DADP-PhACS-(O-MPEG) 0.1 g was dissolved in 25 mL DMF, and 0.7 mL hydrazine was added and reacted under N_2. Finally DMF was



Scheme 1. Deacetylation of chitosan.



Scheme 2. Phthaloylation of depolymerized deacetylated chitosan.



Scheme 3. Iodinization of methoxy poly(ethylene glycol) and synthesis of pegylated chitosan DADP-CS-(O-MPEG).

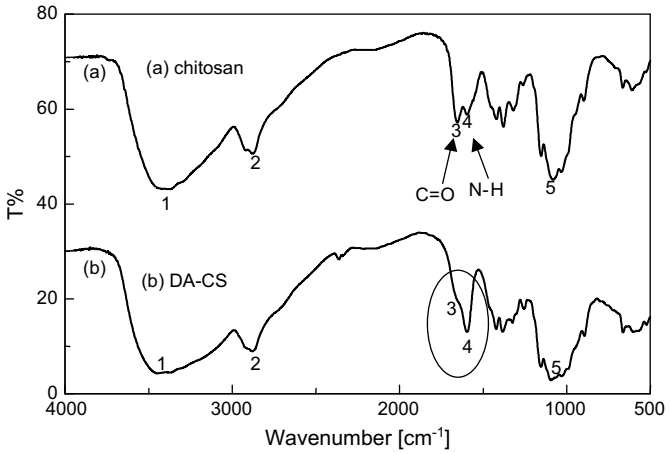
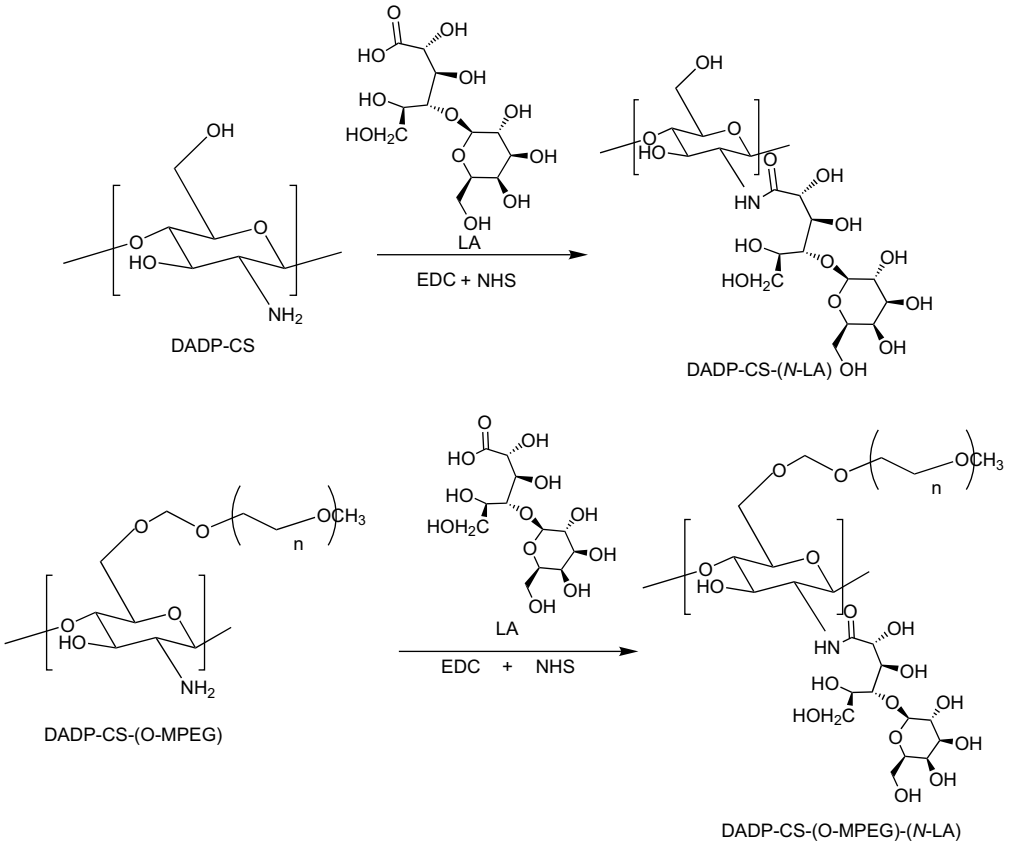


Fig. 1. FTIR spectra of (a) chitosan and (b) deacetylated chitosan (DA-CS).

removed by rotary evaporation. De-ionized water was added and the concentrated solution was dialyzed against large quantity of water to remove hydrazine residue. The mixture was then freeze dried, and DADP-CS-(O-MPEG) was obtained and characterized by FTIR and ^{1}H NMR. The melting temperature (T_m) and enthalpy of fusion (ΔH) of pegylated polymer was measured using differential scanning calorimeter (DSC, PerkinElmer Inc., USA). Sample was heated from 25 to 180 °C at a rate of 10 °C/min, cooled to 5 °C at a rate of 5 °C/min, and then reheated again from 5 to 180 °C at a heating rate of 10 °C/min. The T_m and the enthalpy of fusion were recorded from the DSC endotherm of second run.

2.2.5. Synthesis of lactobionic acid grafted chitosan with or without pegylation

The synthesis procedures are shown in Scheme 4. DADP-CS and DADP-CS-(O-MPEG) 150 mg were dissolved in 3.5 mL 0.2 N HCl separately. Lactobionic acid (LA) 12.2 mg, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) 6.5 mg and



Scheme 4. Synthesis of lactobionic acid grafted chitosan with or without pegylation.

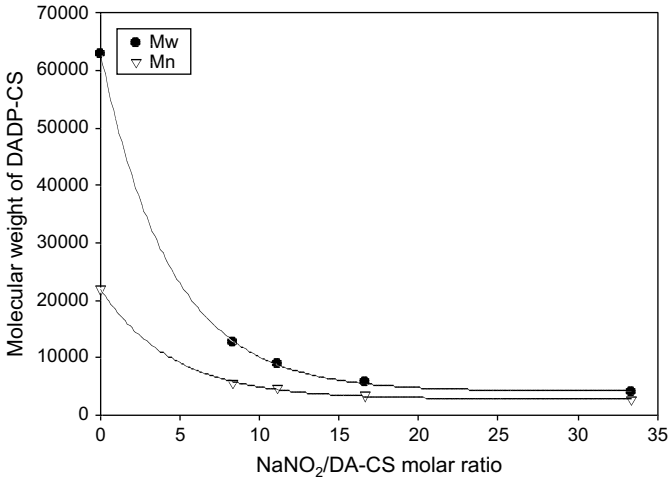


Fig. 2. The molecular weight of DADP-CS after depolymerization at various molar ratios of $NaNO_2$.

N-hydroxysuccinimide (NHS) 3.9 mg were added and reacted at room temperature for 24 h. The mixture was dialyzed against large quantity of de-ionized water following freeze dried. The lactobionic acid grafted DADP-CS, DADP-CS-(*N*-LA), and lactobionic acid grafted DADP-CS-(O-MPEG), DADP-CS-(O-MPEG)-(*N*-LA), were obtained and characterized by FTIR and ^{1}H NMR. The LA grafting degree (DS_{LA}(%)) was measured by ^{1}H NMR.

2.3. Gel permeation chromatography (GPC)

The molecular weight and molecular weight distribution in terms of polydispersity of CS, DA-CS, DADP-CS, and DADP-CS-(O-MPEG) were determined by gel permeation chromatography equipped with a refractive index detector (Shimadzu RID-10A, Japan). Two linear columns (Ultrahydrogel, 7.8 × 300 mm, Waters) were used and acetate buffer solution at pH 5.0 was used as the eluting solvent at a flow rate of 0.8 mL/min at 35 °C. The GPC procedure was calibrated using poly(ethylene glycol) standards of different molecular weights. The DMF instead of buffer system was used as the eluting solvent for phthalic anhydride protected polymers, and the elution rate was set at 1.0 mL/min at 35 °C and the linear column (Ultrastyragel HR 4E, 7.8 × 300 mm, Waters) was used.

2.4. Determination of PEG content

The MPEG grafting degrees of DADP-CS-(O-MPEG) and DADP-CS-(O-MPEG)-(*N*-LA) were determined by calorimetric analysis [17]. The ammonium ferrothiocyanate aqueous solution was prepared by dissolving 674.5 mg $FeCl_3 \cdot 6H_2O$ and 760 mg NH_4SCN in 25 mL de-ionized water. DADP-CS-(O-MPEG) or DADP-CS-(O-MPEG)-(*N*-LA) 4 mg was dissolved in 1% acetic aqueous solution previously. Freshly prepared ammonium ferrothiocyanate aqueous solution 2 mL and equal volume of chloroform were added into the tube and 200 μL of sample solution was added. The mixture was mixed for 5 min, and then shaken at 200 rpm for 30 min at 25 °C. After that the mixture was centrifuged, and the supernatant was removed. The chloroform layer was collected and measured by spectrophotometer at 510 nm. The absorbance was recorded, and the corresponding concentration of MPEG was recalculated from the established standard curve, which was constructed by several concentrations of methoxy poly(ethylene glycol). The amount of MPEG grafted onto DADP-CS-(O-MPEG) and DADP-CS-(O-MPEG)-(*N*-LA) in terms of the weight percentage of MPEG (MPEG(%w/w)) and the MPEG grafting degree (DS_{MPEG}(%)) were further calculated from Eqs. (1) and (2), respectively.

$$\text{MPEG}\ (\%\text{w/w}) = (\text{measured PEG weight in the sample}) / (\text{sample weight})] \times 100\% \quad (1)$$

$$DS_{MPEG}\ (\%) = (\text{MPEG}(\%)/M_{n,\text{MPEG}})/[(100 - \text{MPEG}(\%)) / \text{MW}_{\text{chitosan monomer}}] \times 100\% \quad (2)$$

2.5. Preparation of DNA complexes

DADP-CS and DADP-CS-(O-MPEG)-(*N*-LA) were used to complex with negatively charged DNA. Various weight ratios of polymer to DNA 5:1, 4:1, 3:1, 2:1, and 1:1 were investigated. DADP-CS and DADP-CS-(O-MPEG)-(*N*-LA) (50–250 μg) were dissolved in 1.0 mL 0.25% acetic acid aqueous solution (pH 3.1), respectively. Plasmid DNA 50 μg was dissolved in 1.0 mL de-ionized water, and slowly added into equal volume of polymer solution under magnetic stirring. After that the nanoparticle complex system was stirred for 3 min and incubated for 1 or 24 h at room temperature, respectively. The size of nanoparticles was measured by using COULTER N4 Plus particle size analyzer (Beckman Coulter, Inc., Fullerton, CA) at 25 °C at a 90° scattering angle, and the zeta potential was measured by using ZetaPlus zeta potential analyzer (Brookhaven Instruments corporation, Holtsville, NY). The morphology of DNA complexes was observed using transmission electron microscope (JEOL, JEM-1010, Japan).

3. Results and discussions

3.1. Deacetylated chitosan (DA-CS)

Fig. 1 shows the FTIR spectra of chitosan before and after deacetylation. The peaks 3 and 4 at ~1653.66 cm^{-1} and ~1597.73 cm^{-1} were assigned to the C=O stretching vibration of acetyl group (amide I) and N–H bending vibration (amide II) of deacetylated chitosan (DA-CS), respectively. After deacetylation, the intensity of amide I peak was reduced, which provided the evidence of loss of acetyl group of chitosan. The degree of deacetylation was determined by potentiometric titration method, and the corresponding values were 76.3 ± 2.1 for chitosan and 94.6 ± 0.1% for DA-CS. The M_w, M_n and polydispersity of chitosan and DA-CS were measured by GPC, and the corresponding values were 261,000 Da, 72,000 Da as

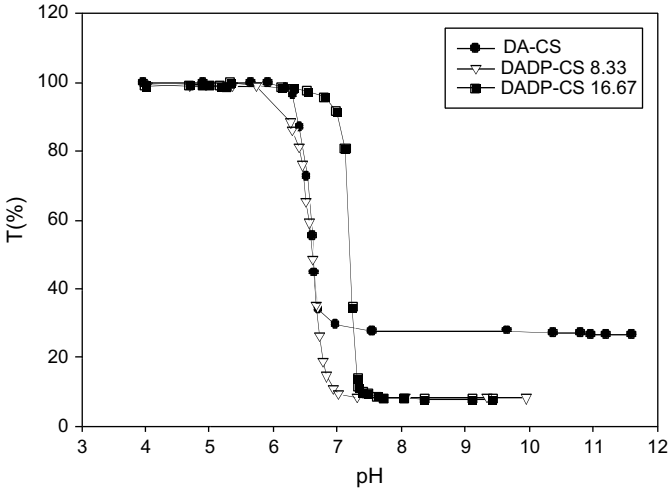


Fig. 3. The transmittance of DA-CS, DADP-CS8.33 and DADP-CS16.67 at various pH.

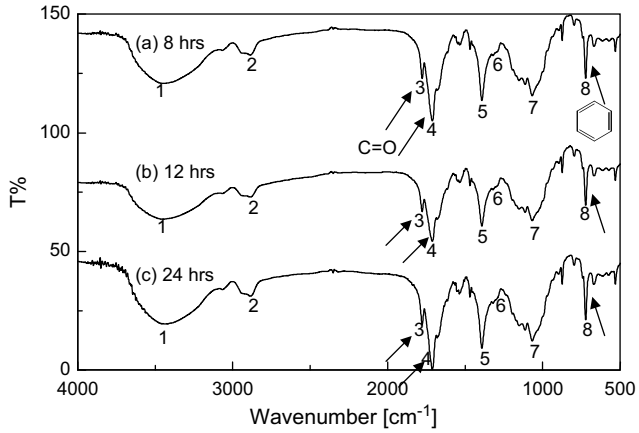


Fig. 4. FTIR spectra of DADP-PhACS (3:1) after reaction for 8, 12, and 24 h, respectively.

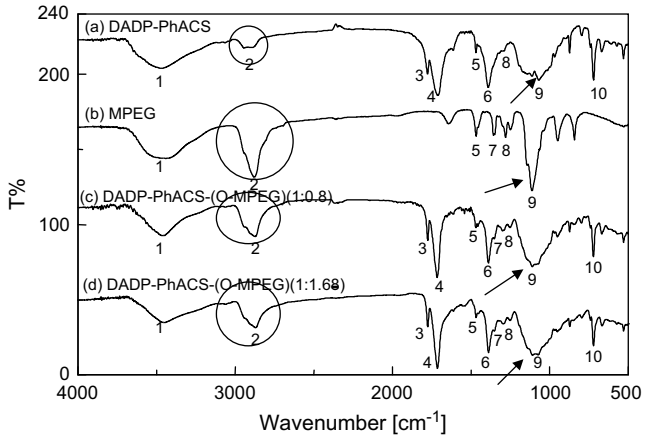


Fig. 6. FTIR spectra of (a) DADP-PhACS, (b) MPEG, (c) DADP-PhACS-(O-MPEG) (1:0.8), and (d) DADP-PhACS-(O-MPEG) (1:1.6).

well as 3.6 for chitosan, and 62,000 Da, 21,000 Da as well as 2.9 for DA-CS. This result indicated that during deacetylation process not only removed acetyl group from chitosan but also caused some extent of depolymerization which resulted in the molecular weight of DA-CS was smaller than that of chitosan.

3.2. Deacetylated depolymerized chitosan (DADP-CS)

The deacetylated chitosan (DA-CS) was further depolymerized at various molar ratios of $NaNO_2$ in the range of 8.33–33.33, and the corresponding molecular weight of deacetylated depolymerized chitosan (DADP-CS) is shown in Fig. 2. The molecular weight of DADP-CS was decreased exponentially as increase in $NaNO_2$ molar ratio. The solubility of deacetylated chitosan (DA-CS) and two batches of deacetylated–depolymerized chitosan (DADP-CS8.33 and DADP-CS16.67), which were depolymerized at molar ratios of $NaNO_2$/DA-CS 8.33 and 16.67 respectively, were determined as a function of pH. The result is shown in Fig. 3. Both DA-CS and DADP-CS were dissolved at pH < 6.0, and the transmittance was decreased rapidly at pH higher than 6.0. The pH values corresponding to 50% transmittance ($pH_{50\%}$) in terms of 50% polymer precipitated was determined, and the corresponding values for DA-CS, DADP-CS8.33 and DADP-CS16.67 were 6.56, 6.58 and 7.19, respectively. The $pH_{50\%}$ value of DADP-CS8.33 was similar to DA-CS but less than DADP-CS16.67. The M_w and M_n of DADP-CS8.33 were 12,000 and 5000 Da, and of DADP-CS16.67 were 5000 and 3000 Da, respectively. The result indicated that a prominent change in solubility property of

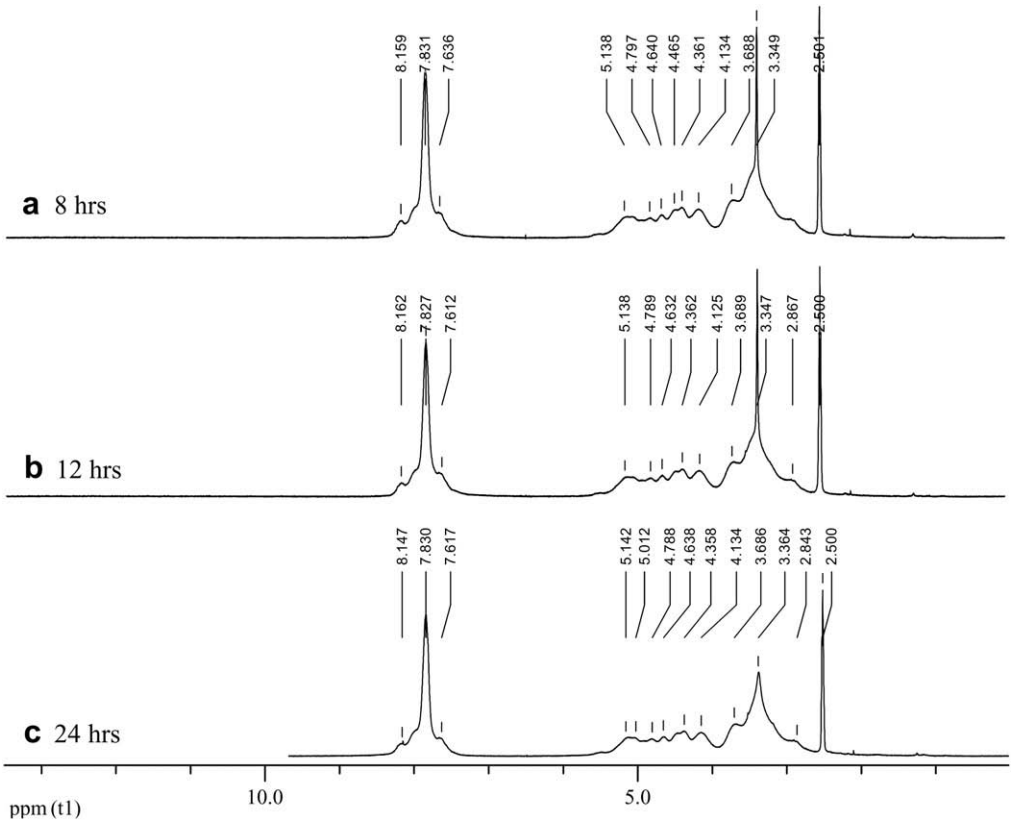


Fig. 5. ^1H NMR spectra of DADP-PhACS (3:1) after reaction for 8, 12, and 24 h, respectively.

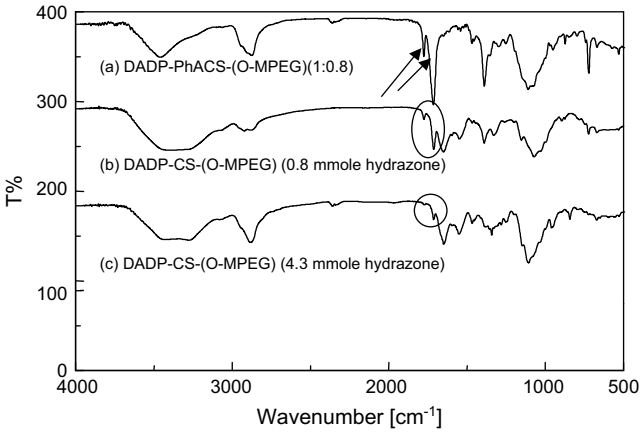


Fig. 7. The FTIR spectra of (a) DADP-PhACS-(O-MPEG) (1:0.8) and DADP-CS-(O-MPEG) after de-protection by (b) 0.8 mmol and (c) 4.3 mmol of hydrazine for 15 h.

chitosan was occurred when the M_w and M_n reached the critical values ~5000 and ~3000 Da, respectively.

3.3. Phthaloylated deacetylated depolymerized chitosan (DADP-PhACS)

Phthalic anhydride (PhA) was used to protect C2-amino groups of DADP-CS. The reaction time for phthaloylation of DADP-CS was investigated. Three batches of DADP-PhACS with feed molar ratio of DADP-CS to PhA 3:1 were prepared after reaction for 8, 12, and 24 h, respectively, and the corresponding FTIR spectra are shown in Fig. 4. Two peaks at ~1710 cm^{-1} (#3) and 1777 cm^{-1} (#4) corresponding to the C=O of imide linkage were appeared after protection of C2-amino groups of chitosan by phthalic anhydride. The peak (#8) at 721 cm^{-1} was assigned to the benzene ring of phthalic anhydride, which provided another evidence of phthaloylation of chitosan. Fig. 5 shows the corresponding ^1H NMR data. The peaks at 7.5–8.5 ppm were assigned to the phthaloyl group and the peaks at 2.7–5.5 ppm represented the protons of chitosan. The *N*-phthaloylation degree was calculated to be 109%, which indicated complete protection of C2-amino groups of DADP-CS by phthalic anhydride in the current reaction condition.

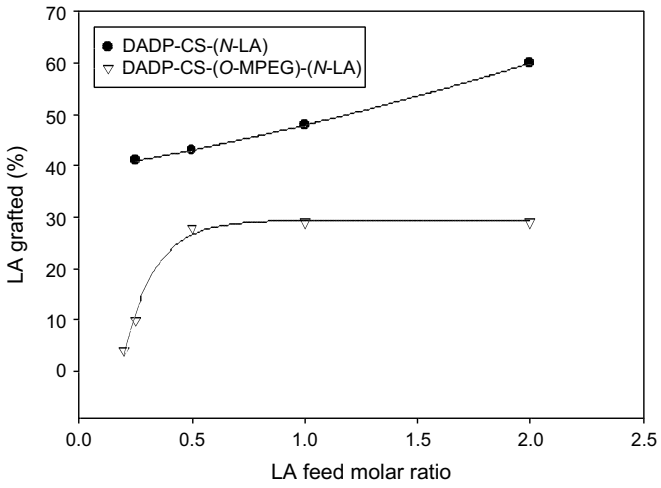


Fig. 9. The LA grafting percentage of DADP-CS-(*N*-LA) and DADP-CS-(O-MPEG)-(*N*-LA).

3.4. Pegylation of DADP-PhACS

Fig. 6 shows the FTIR spectra of DADP-PhACS-(O-MPEG) which were synthesized under feed molar ratios of DADP-PhACS to MPEG 1:0.8 and 1:1.68, respectively. These two batches of DADP-PhACS-(O-MPEG) have similar FTIR spectra. The peak at 2900 cm^{-1} of DADP-PhACS-(O-MPEG) was prominently stronger than that of DADP-PhACS due to $-CH_2-CH_2-O-$ stretching of MPEG. Another evidence supported pegylation of DADP-PhACS was based on the peak at 1100 cm^{-1}, and its intensity was prominently enhanced due

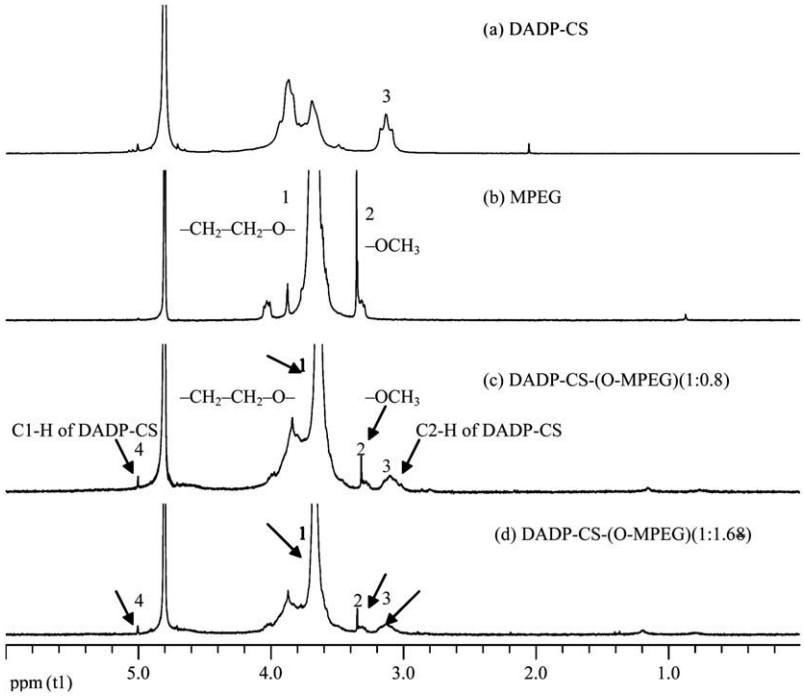


Fig. 8. The ^1H NMR spectra of (a) DADP-CS, (b) MPEG, (c) DADP-CS-(O-MPEG) (1:0.8), and (d) DADP-CS-(O-MPEG) (1:1.6).

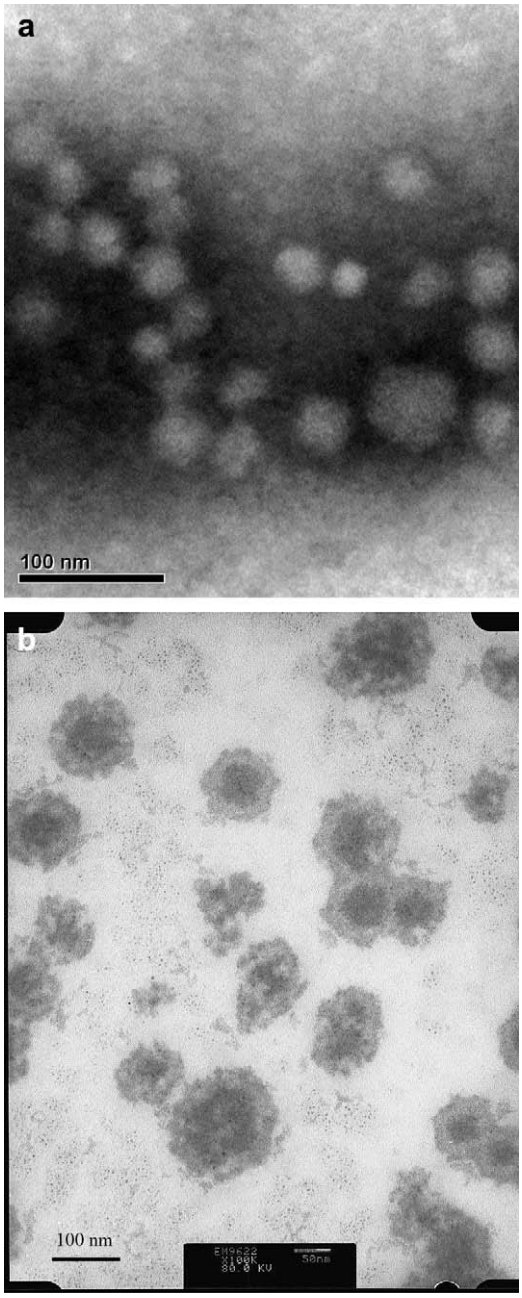


Fig. 10. TEM micrographs of (a) DADP-CS/DNA (5:1) and (b) DADP-CS-(O-MPEG)-(*N*-LA)/DNA (3:1) nanoparticle complexes.

to the formation of –C–O–C– ether linkage via C6–OH of DADP-PhACS and MPEG.

3.5. Pegylated chitosan DADP-CS-(O-MPEG)

After pegylation, DADP-PhACS-(O-MPEG) (1:0.8) was further de-protected by hydrazine in order to remove phthalic anhydride and leave C2-amino group free. The effect of concentration of hydrazine on this process was investigated, and the corresponding FTIR spectra are shown in Fig. 7. Fig. 7(a) is the FTIR spectrum of DADP-PhACS-(O-MPEG) and the peaks at ~1776 and ~1771 cm^{-1} were assigned to the phthalic anhydride as mentioned before. Fig. 7(b)–(c) shows the FTIR spectra of DADP-CS-(O-MPEG) after de-protected by 0.8 and 4.3 mmol hydrazine for 15 h, respectively. The intensities of these two indicating peaks were prominently decreased after de-protection process especially at high concentration of hydrazine. Fig. 8 shows the ^{1}H NMR spectra of two batches of DADP-PhACS-(O-MPEG), and both have similar result. The peaks at 3.6 ppm (#1) and 3.3 ppm (#2) were assigned to the $–CH_2–CH_2–O–$ and $–OCH_3$ of MPEG. The peaks at 3.1 ppm (#3) and 5.0 ppm (#4) were assigned to the C2–H and C1–H of DADP-CS, respectively. The weight percentage of MPEG (MPEG(%w/w)) and the MPEG grafting degree (DS_{MPEG}(%)) of DADP-PhACS-(O-MPEG) were further determined by calorimetric analysis, and the corresponding values were calculated based on Eqs. (1) and (2), respectively. The MPEG(%w/w) values for DADP-PhACS-(O-MPEG) (1:0.8) and DADP-PhACS-(O-MPEG) (1:1.68) were 45.31 and 35.83%, respectively, and the corresponding DS_{MPEG}(%) values were 6.68 and 4.50%. The DSC thermal properties of MPEG and DADP-CS-(O-MPEG) were determined. The melting temperature and enthalpy of fusion of MPEG were 59.4 °C and 182.6 J/g, respectively, and of DADP-CS-(O-MPEG) were decreased to 56.2 °C and 47.1 J/g, respectively.

3.6. Lactobionic acid grafted chitosan with or without pegylation

Chitosan with or without pegylation in terms of DADP-CS-(O-MPEG) and DADP-CS were further conjugated with a sugar molecule lactobionic acid (LA). A series of DADP-CS-(*N*-LA) were synthesized under several feed molar ratios of LA to DADP-CS 0.25, 0.5, 1.0, and 2.0, respectively. The corresponding M_w, M_n and polydispersity of these batches were in the range of 12,000–18,000 Da, 7000–8000 Da, and 1.7–2.4, respectively. The same procedure was applied to synthesize several batches of DADP-CS-(O-MPEG)-(*N*-LA) at feed molar ratios of LA to DADP-CS-(O-MPEG) 0.20, 0.25, 0.5, 1.0, and 2.0, respectively. The corresponding M_w, M_n and polydispersity were in the range of 12,000–14,000 Da, 5000–6000 Da, and 2.5–2.6, respectively. The LA grafting degrees (DS_{LA}(%)) of DADP-CS-(*N*-LA) and DADP-CS-(O-MPEG)-(*N*-LA) were measured by ^{1}H NMR. Fig. 9 shows the percentage of LA grafted as a function of LA feed molar ratio. Increase in the feed molar ratio of LA to DADP-CS from 0.25 to

Table 1
The particle size, polydispersity and zeta potential of DADP-CS/DNA and DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complexes.

Complex	Polymer/DNA weight ratio	Particle size at 1 h (nm)	PDI at 1 h	Particle size at 24 h (nm)	PDI at 24 h	Zeta potential (mV)
DADP-CS/DNA	1:1	115.6 ± 74.5	0.267	99.5 ± 42.3	0.301	−0.35 ± 0.21
	2:1	97.8 ± 53.7	0.237	76.1 ± 29.0	0.522	−0.69 ± 0.92
	3:1	103.7 ± 58.7	0.423	84.4 ± 29.4	0.730	29.61 ± 3.77
	4:1	75.6 ± 31.7	0.146	74.9 ± 10.2	0.423	34.73 ± 6.82
	5:1	74.9 ± 24.9	0.198	73.9 ± 26.0	0.768	44.48 ± 5.30
DADP-CS-(O-MPEG)-(*N*-LA)/DNA	1:1	179.0 ± 25.7	0.255	167.8 ± 38.0	0.280	−0.81 ± 1.84
	2:1	178.1 ± 20.8	0.121	177.6 ± 20.8	0.140	2.05 ± 1.44
	3:1	178.2 ± 31.9	0.254	168.7 ± 45.1	0.233	−1.46 ± 1.07
	4:1	183.2 ± 56.7	0.333	171.7 ± 38.0	0.304	−3.06 ± 0.91
	5:1	140.8 ± 47.8	0.310	136.0 ± 62.8	0.262	2.55 ± 1.61

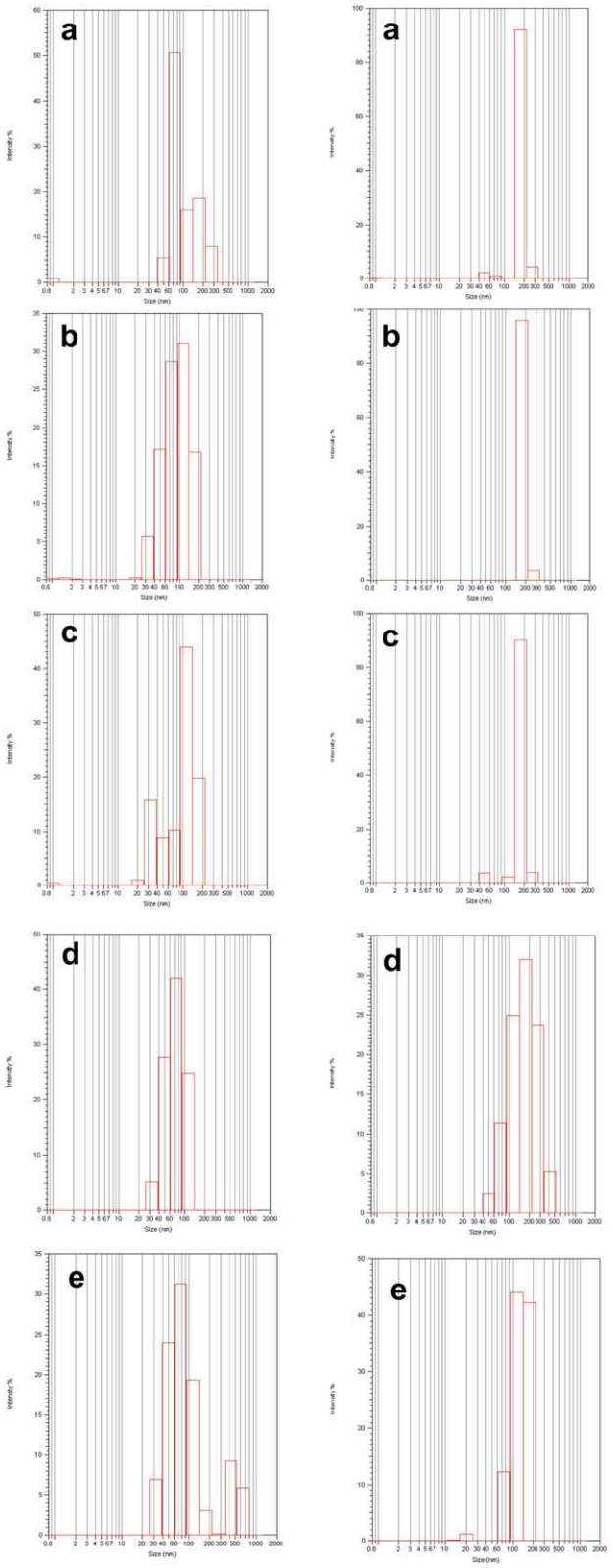


Fig. 11. The particle size distribution of DADP-CS/DNA (left column) and DADP-CS-(O-MPEG)-(*N*-LA)/DNA (right column) nanoparticle complexes at weight ratios of (a) 1:1, (b) 2:1, (c) 3:1, (d) 4:1, and (e) 5:1.

2.0 proportionally increased the LA grafting degree of DADP-CS-(*N*-LA) from 41% to 60%. However, increase in the feed molar ratio of LA to DADP-CS-(O-MPEG) from 0.2 to 0.5 increased the LA grafting degree of DADP-CS-(O-MPEG)-(*N*-LA) from 4% to 29%, after that further increase in LA feed molar ratio up to 2.0 did not increase LA grafting degree further. This phenomenon was quite different from the grafting of LA onto DADP-CS. The flexibility and shielding effect of MPEG polymer chain hindered LA grafting onto DADP-CS-(O-MPEG), and resulted in the LA grafting efficiency of DADP-CS-(O-MPEG)-(*N*-LA) was lower than that of DADP-CS-(*N*-LA). Nevertheless, the presence of MPEG on DADP-CS-(O-MPEG)-(*N*-LA) did play an important role on preventing nanoparticles aggregation which was elucidated as follow.

3.7. Characterization of DNA complexes

DADP-CS/DNA and DADP-CS-(O-MPEG)-(*N*-LA)/DNA complexes were prepared at various weight ratios of polymer to DNA. The morphology of DNA complexes was observed by transmission electron microscope, and showed a regular spherical shape (Fig. 10). Especially the DADP-CS-(O-MPEG)-(*N*-LA)/DNA complexes had a condensed center with brush outer shell, and this particular morphology did not occur to DADP-CS/DNA. The particle size, polydispersity and zeta potential of complexes are listed in Table 1, and the associated particle size distribution is shown in Fig. 11. The particle sizes of DADP-CS/DNA and DADP-CS-(O-MPEG)-(*N*-LA)/DNA were in the range of 74.9 ± 24.9–115.6 ± 74.5 nm and 140.8 ± 47.8–183.2 ± 56.7 nm, respectively, and the corresponding polydispersities were 0.146–0.423 and 0.121–0.333, respectively. Mixing cationic polymer with negatively charged plasmid DNA spontaneously formed complexes in nano-size due to electrostatic compaction. The polydispersities of DADP-CS-(O-MPEG)-(*N*-LA)/DNA complexes were maintained consistently in the range of 0.140–0.304 after incubation for 24 h. However, the corresponding values of DADP-CS/DNA complexes were prominently increased to 0.301–0.768 after the same incubation time. This result indicated that DADP-CS-(O-MPEG)-(*N*-LA) was capable of condensing plasmid DNA and formed a more stable complex than DADP-CS. In other words, MPEG played an important role on preventing DADP-CS-(O-MPEG)-(*N*-LA)/DNA complexes from aggregation. This result was supported by TEM morphology, where the hydrophilic PEG polymer chains acted as the outer shell to extend in the aqueous environment to protect nanoparticle complexes from aggregation. Hence, the PEG polymer chain possessed the advantage to maintain DADP-CS-(O-MPEG)-(*N*-LA)/DNA complexes with a stable nature. The zeta potentials of DADP-CS/DNA complexes were prominently decreased from 44.48 ± 5.30 to -0.35 ± 0.21 mV as decrease of polymer to DNA weight ratio from 5: 1 to 1:1, and this was due to neutralize positively charged polymer by negatively charged DNA. The chemical linkage of lactobionic acid onto amino group of chitosan further neutralized the positive charge of DADP-CS-(O-MPEG)-(*N*-LA), and in terms of the zeta potentials of the corresponding DNA complexes were close to neutral.

4. Conclusions

A series of chemical modifications of chitosan were conducted by grafting the hydrophilic methoxy poly(ethylene glycol) (MPEG) and a target sugar molecule lactobionic acid (LA) onto the C6–OH and C2–NH_2 of chitosan. The MPEG grafting degree was strongly affected by chitosan depolymerization, and the corresponding value was reduced for chitosan with high degree of depolymerization. The LA grafting ratio of DADP-CS-(*N*-LA) was proportionally increased with LA feed molar ratio. Nevertheless, the maximum value was achieved for DADP-CS-(O-MPEG)-(*N*-LA) irrespective of

continuously adding LA. The flexibility and shielding effect of MPEG polymer chain hindered LA grafting onto chitosan and resulted in lower grafting efficiency. The DADP-CS-(O-MPEG)-(*N*-LA) successfully provoked DNA condensation into nanoparticle complexes due to electrostatic compaction. The presence of MPEG on DADP-CS-(O-MPEG)-(*N*-LA) played an important role on preventing aggregation of nanoparticle complexes with a size in the range of 100–200 nm.

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