Carbohydrate Polymers 83 (2011) 898–904

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis of lactobionic acid-grafted-pegylated-chitosan with enhanced HepG2 cells transfection

Wen Jen Lin[a,*], Tze Dan Chen[a], Chia Wen Liu[a], Jiin Long Chen[b], Fu Hsiung Chang[c]

[a] Graduate Institute of Pharmaceutical Sciences, College of Medicine, National Taiwan University, F 12 NO. 1 Jen Ai Rd. Sec. 1, Taipei 100, Taiwan
[b] Institute of Preventive Medicine, Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan
[c] Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

ARTICLE INFO

Article history:
Received 11 June 2010
Received in revised form 23 August 2010
Accepted 31 August 2010
Available online 6 September 2010

Keywords:
Chitosan
Methoxy poly(ethylene glycol)
Lactobionic acid
DNA
Nanoparticle complex
Transfection

ABSTRACT

The aim of this study was to develop a functional nanoparticulate carrier with an enhanced transfection in asialoglycoprotein receptor overexpressed HepG2 cells. A series of chemical modifications of chitosan were conducted by grafting a hydrophilic methoxy poly(ethylene glycol) (MPEG) and a receptor ligand, lactobionic acid (LA). The pegylation efficiency of deacetylated-depolymerized-chitosan (DADP-CS) was 26% (w/w). Increase in feed molar ratio of lactobionic acid from 0.25 to 0.5 increased LA grafting degree of lactobionic acid-grafted-pegylated-chitosan (DADP-CS-(O-MPEG)-(*N*-LA)) from 10% to 28%. Plasmid DNA was more compacted by DADP-CS-(O-MPEG)-(*N*-LA) than by pegylated-chitosan (DADP-CS-(O-MPEG)) in terms of smaller particle size of DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex. The bearing of receptor ligand of DADP-CS-(O-MPEG)-(*N*-LA) (45.3%) showed better transfection efficiency than ligand-free DADP-CS-(O-MPEG) (19.8%) in asialoglycoprotein receptor overexpressed HepG2 cells.

1. Introduction

Polymeric nanoparticles play an important role on controlling drug release and passively or actively delivering drug to the desirable action site. 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. These induce the development of cell-specific targeting carriers especially for gene therapy and chemotherapy.

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 (Wagner, 1999; Weigel & Yik, 2002). 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 (Stockert, 1995). ASGPR is an integral membrane protein expressed on the surface of parenchymal cells of liver with high density of $1–5 \times 10^5$ receptors (Weigel & Yik, 2002). Nano-sized carriers (e.g., nanoparticles) with surface modification are usually necessary for specific targeting purpose. Several sugar ligands (e.g., galactose, *N*-acetylgalactosamine, mannose, lactose, fructose, etc.) interact to various extents 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 prevented dextran-modified polysaccharides from protein opsonization and further avoided liver recognition (Gref, Rodrigues, & Couvreur, 2002). Hashida, Nishikawa, Yamashita, and Takakura (2001) 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 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 (Hashida et al., 2001).

Chitosan is a relatively low toxic, biocompatible, and biodegradable polysaccharide with immunological, antibacterial and wound-healing activities. The strategies of chemical modification of chitosan by using different substitutes have been demonstrated for several different purposes, and it is usually through its C2-amino group or C6-hydroxyl group (Alves & Mano, 2008; Gao et al., 2009; Gorochovceva & Makus, 2004; Kato, Onishi, & Machida, 2001; Laurentin & Edwards, 2003; Lin & Chen, 2007; Liu et al., 2009; Morimoto et al., 2001; Park et al., 2001, 2003; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009; Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998). The chemically modified

* Corresponding author. Tel.: +886 2 23123456x88396; fax: +886 2 23916126.
E-mail address: wjlin@ntu.edu.tw (W.J. Lin).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.08.072

chitosan materials are applied for drug delivery, tissue engineering, and other biomedical applications (Alves & Mano, 2008). The free amino group of chitosan is feasible to complex with negatively charged DNA as a gene delivery carrier.

The aim of this study was to develop a functional nanoparticulate carrier with enhanced transfection efficiency in asialoglycoprotein receptor (ARGPR) overexpressed HepG2 cells. A series of chemically modified chitosans were designed by grafting a hydrophilic methoxy poly(ethylene glycol) (MPEG) and an asialoglycoprotein receptor (ASGPR) recognized sugar molecule, lactobionic acid (LA). Lactobionic acid is an endogenous substance present in the human body (Yu & van Scott, 2004). The chemical structure of lactobionic acid contains a galactose unit and a gluconic acid unit linked via an ether linkage. The carboxyl group of gluconic acid unit can be used to react with the amino group of chitosan via an amide linkage. The bearing of galactose unit of lactobionic acid acts as a specific targeting ligand for asialoglycoprotein receptor (ASGPR) on hepatocytes as mentioned above (Hashida et al., 2001). The coating of nanoparticle surface with poly(ethylene glycol) (PEG) has been proven to prolong their circulation time. The hydrophilic PEG polymer chains sterically stabilize nanoparticles against opsonization and subsequent phagocytosis, which reduce the clearance of nanoparticles by circulating macrophage of the liver and promote the enhanced permeation and retention (EPR) effect (Avgoustakis, 2004; Betancourt et al., 2009; Gref et al., 1994; Ioele, Cione, Risoli, Genchi, & Ragno, 2005; Kataoka, Kwon, Yokoyama, Okano, & Sakurai, 1993; Lu et al., 2009; Peracchia et al., 1999). The similar result has been reported by van Vlerken et al. They found that PLGA nanoparticles with a surface modification by PEG avoid uptake by the reticuloendothelial system, thereby improving circulation time of the nanoparticles, and the nanoparticles are retained in the tumor for prolonged period of time (van Vlerken, Duan, Little, Seiden, & Amiji, 2008).

2. Experimental

2.1. Materials

Low molecular weight chitosan was from Aldrich Chemical Company Inc. (Mw 260 kDa, WI, USA). Methoxy poly(ethylene glycol) (MW 5000) 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). Plasmid encoding enhanced green fluorescent protein (pEGFP-N1, 4.7 kb) was kindly provided by Professor Jiin Long Chen from National Defense Medical Center in Taiwan. HepG2 cancer cell line was a gift from Dr. Hui-Lin Wu in Hepatitis Research Center of National Taiwan University Hospital in Taiwan.

2.2. Synthesis

Scheme 1 shows the procedures to synthesize lactobionic acid-grafted-pegylated-chitosan (DADP-CS-(O-MPEG)-(*N*-LA)).

2.2.1. Phthaloylation of deacetylated-depolymerized-chitosan (DADP-CS)

Chitosan was deacetylated in 50% (w/v) NaOH aqueous solution at 140 °C for 4 h, and following depolymerized in 0.1 M sodium bisulfate acetic solution at room temperature for 3 h. The obtained deacetylated-depolymerized-chitosan (DADP-CS) 1.0 g was dissolved in 8.3 mL formic acid. Phthalic anhydride 1.3 g in 35.5 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 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.

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

DADP-PhACS was reacted with pre-activated methoxy poly(ethylene glycol) iodide (MPEGI) in the feed molar ratio of 1:0.5. DADP-PhACS 0.5 g, MPEGI 1.7 g and Ag_2O 0.2 g were mixed in 3 mL toluene under vacuum. DMF 11.5 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, and the mixture was centrifuged. The precipitate was collected and freeze dried, and DADP-PhACS-(O-MPEG) was obtained and characterized by FTIR and ^{1}H NMR. In order to remove phthalic anhydride from C2 position of chitosan, DADP-PhACS-(O-MPEG) 1.0 g was dissolved in 27 mL DMF, and 1.4 mL hydrazine was added and reacted under N_2 at 90 °C for 6 h. Finally DMF was removed, de-ionized water was added, and the concentrated solution was dialyzed. The mixture was then freeze dried, and pegylated-chitosan (DADP-CS-(O-MPEG)) was obtained and characterized by ^{1}H NMR as well as differential scanning calorimeter (DSC, PerkinElmer Inc., USA). The degree of pegylation was determined by calorimetric analysis (Nag & Ghosh, 1996), and calculated from Eq. (1).

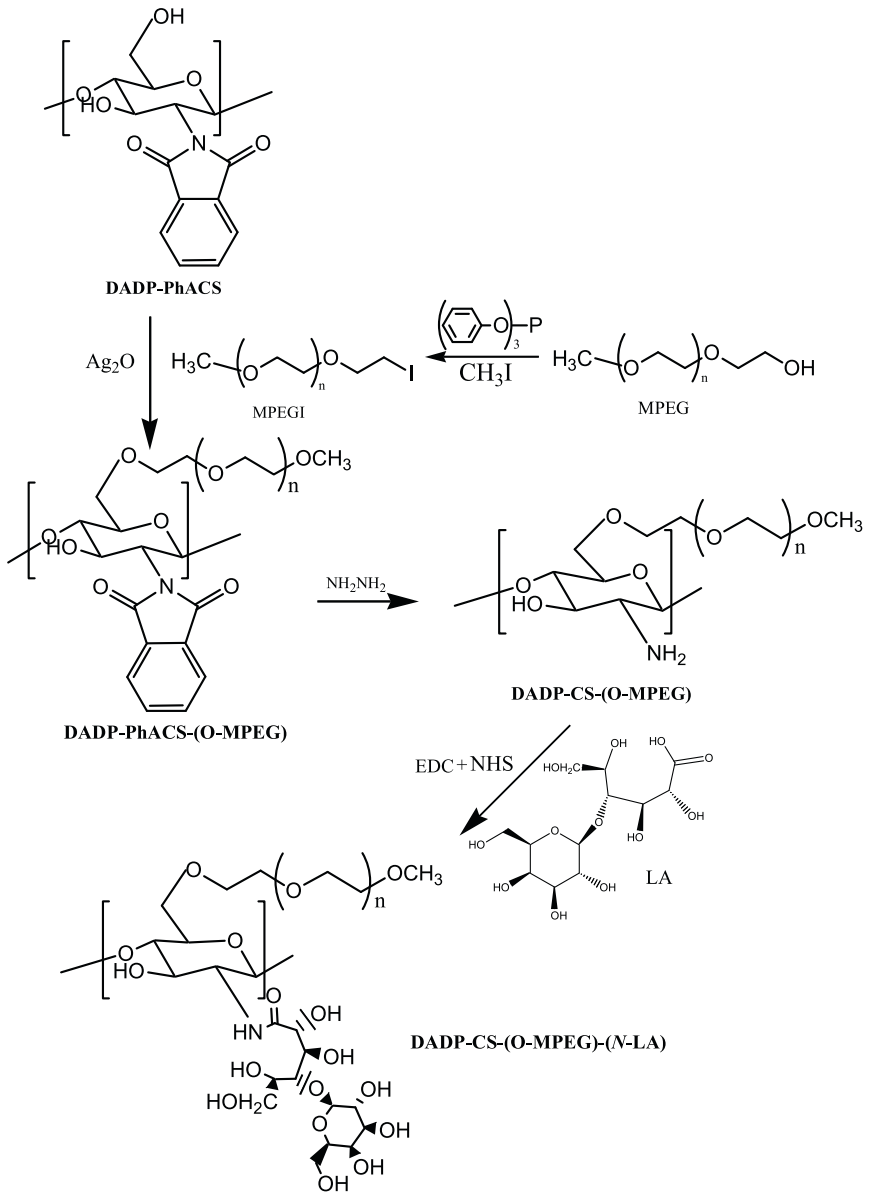
$$\text{MPEG}\,(\%\text{w/w}) = \left[\frac{(\text{measured PEG weight in the sample})}{(\text{sample weight})}\right] \times 100\% \quad (1)$$

2.2.3. Synthesis of lactobionic acid grafted DADP-CS-(O-MPEG)

Lactobionic acid-grafted-pegylated-chitosan, DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5), with feed molar ratio of DADP-CS-(O-MPEG) to LA 1:0.5 was synthesized. DADP-CS-(O-MPEG) 1.0 g was dissolved in 24 mL 0.2*N* HCl. Lactobionic acid (LA) 40.3 mg, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) 21.5 mg and *N*-hydroxysuccinimide (NHS) 13.3 mg were added and reacted at room temperature for 24 h. The mixture was dialyzed following freeze dried. The lactobionic acid grafted-pegylated-chitosan in terms of DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5) was obtained. Additional batch of DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25) with feed molar ratio of DADP-CS-(O-MPEG) to LA 1:0.25 was synthesized by adding half of feed molar ratios of LA, EDC, and NHS according to the reaction compositions mentioned above. The LA grafting degree (DS_{LA}(%)) was measured by ^{1}H NMR, and the molecular weight of DADP-CS-(O-MPEG)-(*N*-LA) was determined by gel permeation chromatography. The gel permeation chromatography was equipped with a refractive index detector (Shimadzu RID-10A, Japan). Two linear columns (Ultrahydrogel, 7.8 mm × 300 mm, Waters) were applied, and acetate buffer solution (pH 5.0) was used as the eluting solvent at a flow rate of 0.8 mL/min at 35 °C.

2.3. Preparation of polymer/pDNA nanoparticle complex

DADP-CS-(O-MPEG), DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25), and DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5) were used to complex with negatively charged DNA in the weight ratio of 1:1. Each polymer (50 μg) was dissolved in 1.0 mL 0.25% acetic acid aqueous solution (pH 3.1). Plasmid DNA 50 μg was dissolved in 1.0 mL sterile distilled water, and slowly added into equal volume of polymer solution and stirred for 3 min. The resulting nanoparticle complex was allowed to stand at room temperature for 1 h. The particle size was measured at 25 °C by using Zetasizer nano analyzer



Scheme 1. The procedures to synthesize lactobionic acid-grafted-pegylated-chitosan (DADP-CS-(O-MPEG)-(*N*-LA)).

(Malvern Instruments Ltd., Worcestershire, UK). The morphology of polymer/pDNA nanoparticle complex was observed by using transmission electron microscope (Hitachi H-7100, Tokyo, Japan). The stability of nanoparticle complex was further evaluated their particle size change after storage at 4 °C for 5 weeks.

2.4. Transfection of polymer/pDNA nanoparticle complex

The transfection of DADP-CS-(O-MPEG)/DNA and DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5)/DNA nanoparticle complexes was evaluated in ARGPR overexpressed HepG2 cancer cell line. HepG2 cancer cells were seeded in a 6-well plate at a density of 5×10^5 cells/well overnight. After that the cells were cultured in 2 mL complete MEM medium and incubated for 24 h in the incubator at 37 °C in 5% CO_2. The medium was changed to MEM medium and the cells were incubated for an additional 30 min. The medium was removed and 1 mL MEM medium containing polymer/*p*DNA nanoparticle complex or naked *p*DNA (15 μg *p*DNA/mL) was added into each well and incubated for 24 h. After that the MEM medium was removed, and the fresh complete MEM medium 2 mL was added to each well. Cells were incubated for an additional 24 h. Finally, the cells were collected and resuspended in phosphate buffer solution (pH 7.4). The relative transfection efficiency of HepG2 cells was measured by using a flow cytometry (FACS Calibur, Becton Dickinson, NJ, USA) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The transfected cells were also examined by confocal microscope (Leica TCS SP2, Leica Microsystems CMS GmbH, Mannheim, Germany).

3. Results and discussion

3.1. Deacetylated and depolymerized chitosan

The FTIR spectrum of deacetylated-chitosan (DA-CS) was analyzed. The peaks at ~1653.66 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). 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

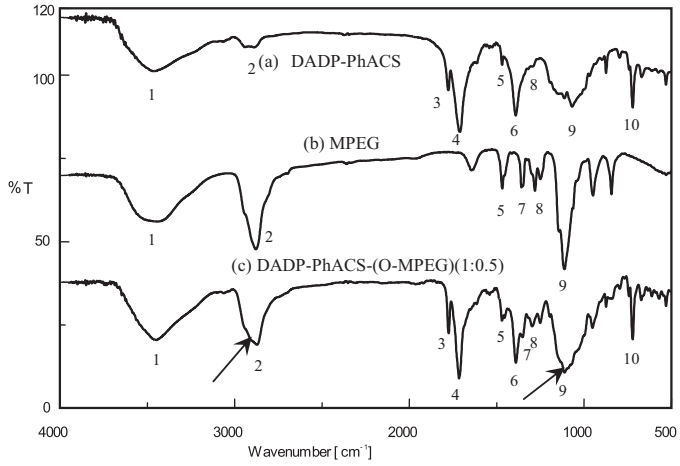


Fig. 1. The FTIR spectra of (a) phthalic anhydride protected chitosan (DADP-PhACS), (b) methoxy poly(ethylene glycol) (MPEG), and (c) phthalic anhydride protected pegylated-chitosan (1:0.5) (DADP-PhACS-(O-MPEG) (1:0.5)).

titration method, and the corresponding values were 76.3 ± 2.1 for chitosan and 94.6 ± 0.1% for DA-CS. The Mw, Mn and polydispersity of $(chitosan)_{76.3\pm2.1}$ and $(DA\text{-}CS)_{94.6\pm0.1\%}$ were measured by GPC, and the corresponding values were 261 kDa, 72 kDa as well as 3.6 for $(chitosan)_{76.3\pm2.1}$, and 62 kDa, 21 kDa as well as 2.9 for $(DA\text{-}CS)_{94.6\pm0.1\%}$.

3.2. Phthalic anhydride protected chitosan (DADP-PhACS)

Three batches of DADP-PhACS with feed molar ratio of DADP-CS to PhA 1:1 were prepared after reaction for 8, 12, and 24 h, respectively. Two peaks at ~1710 and 1777 cm^{-1} corresponding to C=O of imide linkage appeared after protection of C2-amino groups of chitosan by phthalic anhydride. The peak at 721 cm^{-1} was assigned to the benzene ring of phthalic anhydride, which provided another evidence of phthaloylation of chitosan. The corresponding ^1H NMR spectra were further analyzed. 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. There was no significant difference in three batches of DADP-PhACS. The *N*-phthaloylation degree was calculated to be 93.0 ± 2.6%, which indicated the protection of C2-amino groups of DADP-CS by phthalic anhydride was feasible and acceptable under current reaction condition.

3.3. Pegylation of DADP-PhACS

Fig. 1 shows the FTIR spectrum of DADP-PhACS-(O-MPEG) which was synthesized at feed molar ratio of DADP-PhACS to MPEG 1:0.5. The peak at 2900 cm^{-1} of DADP-PhACS-(O-MPEG) FTIR spectra was prominently stronger than that of DADP-PhACS due to $-CH_2-CH_2-O-$ stretching of MPEG. Another evidence supporting pegylation of DADP-PhACS was based on the peak at 1100 cm^{-1}, and its intensity was prominently enhanced due to the formation of –C–O–C– ether linkage via C6–OH of DADP-PhACS and MPEG. The ^1H NMR spectrum of DADP-PhACS-(O-MPEG) (1:0.5) was further evaluated. The peaks at 3.5 and 3.2 ppm were assigned to the $-CH_2-CH_2-O-$ and $-OCH_3$ of MPEG. The peaks at 4.0–5.5 ppm and 7.5–8.2 ppm were assigned to DADP-PhACS. This result supported the pegylation evidence of DADP-PhACS.

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

Fig. 2 shows the ^1H NMR spectrum of DADP-CS-(O-MPEG) (1:0.5). The peaks at 3.6 ppm (#1) and 3.3 ppm (#2) were assigned

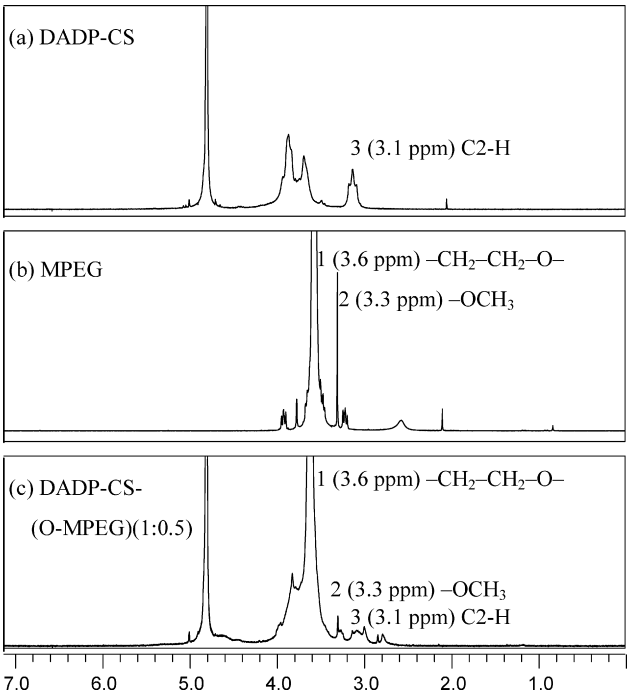


Fig. 2. The ^1H NMR spectra of (a) deacetylated-depolymerized-chitosan (DADP-CS), (b) methoxy poly(ethylene glycol) (MPEG), and (c) pegylated-chitosan (1:0.5) (DADP-CS-(O-MPEG) (1:0.5)).

to the $-CH_2-CH_2-O-$ and $-OCH_3$ of MPEG. The peak at 3.1 ppm (#3) was assigned to the C2-H of DADP-CS. The DSC thermal properties of MPEG and DADP-CS-(O-MPEG) were measured. The melting temperature and enthalpy of fusion of MPEG were 59.4 °C and 182.6 J/g. The melting peak corresponding to MPEG of DADP-CS-(O-MPEG) (1:0.5) was shifted to 56.1 °C, and the related enthalpy of fusion was reduced to 47.9 J/g. The pegylation efficiency in terms of the weight percentage of MPEG (MPEG (%w/w)) of DADP-CS-(O-MPEG) was calculated to be 26.2% based on the thermal analysis data which was further confirmed by calorimetric analysis. The values corresponding to weight percentage of MPEG (MPEG (%w/w)) of DADP-CS-(O-MPEG) was calculated to be 26.3% based on Eq. (1). This result was consistent with that obtained from the DSC thermal analysis.

3.5. Lactobionic acid-grafted-pegylated-chitosan DADP-CS-(O-MPEG)-(N-LA)

Pegylated-chitosan, DADP-CS-(O-MPEG), was further conjugated with a functional ligand, lactobionic acid (LA). Two batches of DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25) and DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5) were synthesized at feed molar ratios of LA to DADP-CS-(O-MPEG) 0.25:1 and 0.5:1, respectively. The corresponding Mw, Mn and polydispersity were 9300 Da, 5200 Da, and 1.79 for DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25), and 11,700 Da, 5500 Da, and 2.13 for DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5). Fig. 3 shows the ^1H NMR spectrum of DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25). The peak *a* at 2.9 ppm was assigned to the C2-H of chitosan, and peak *b* at 3.9 ppm was assigned to LA. The LA grafting degree (DS_{LA}(%)) was calculated based on the integrated area of peak *b* to peak *a*, which was 10% for DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25) and 28% for DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5), respectively. Increase in feed molar ratio of LA increased LA grafting degree of DADP-CS-(O-MPEG)-(*N*-LA). Although MPEG polymer chain played an important role on preventing nanoparticles aggregation, its flexibility and shielding effect might hinder lactobionic acid grafting

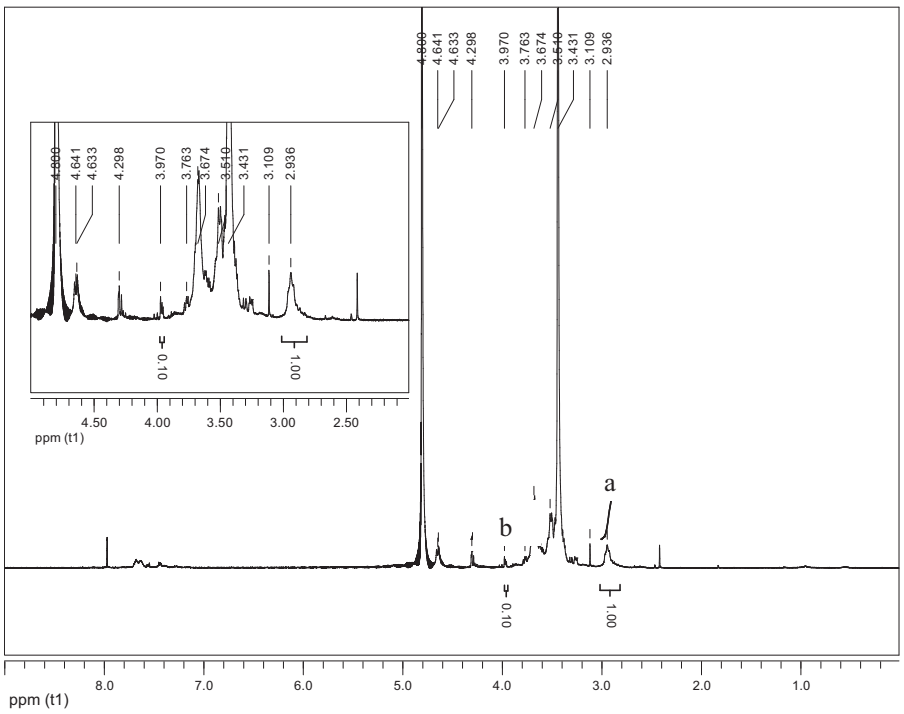


Fig. 3. The ^{1}H NMR spectrum of lactobionic acid-grafted-pegylated-chitosan (1:0.25) (DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25)) (D_2O, 4.8 ppm).

onto DADP-CS-(O-MPEG) in terms of LA grafting degree not so high as expected (Lin, Chen, & Liu, 2009).

3.6. Characterization of polymer/DNA nanoparticle complex

The morphology of DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex was observed by transmission electron microscopy (Fig. 4). Mixing cationic chitosan with negatively charged plasmid DNA spontaneously formed complex in nano-size due to electrostatic compaction. The particle size of fresh prepared DADP-CS-(O-MPEG)/DNA nanoparticle complex was 210.7 ± 30.5 nm, and the corresponding values of DADP-CS-(O-MPEG)-(*N*-LA)/DNA (1:0.25) and DADP-CS-(O-MPEG)-(*N*-LA)/DNA (1:0.5) nanoparticle complexes were 115.2 ± 22.1 and 129.2 ± 17.4 nm, respectively. More compaction of DNA by DADP-CS-(O-MPEG)-(*N*-LA) than by DADP-CS-(O-MPEG) resulted in smaller particle size of DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex. The particle size of DADP-CS-(O-MPEG)/DNA, DADP-CS-(O-MPEG)-(*N*-LA) (1:0.25)/DNA, and DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5)/DNA nanoparticle complexes after storage at 4 °C for 5 weeks were 203.2 ± 16.4, 140.3 ± 22.5, and 101.8 ± 16.9 nm. There was no significant change in particle size after storage regardless of pegylated-chitosan with or without lactosylation. This result indicated that pegylated-chitosan was not only capable of condensing plasmid DNA but also forming stable nanoparticle complex. The presence of MPEG on DADP-CS-(O-MPEG) and DADP-CS-(O-MPEG)-(*N*-LA) played an important role on preventing polymer/DNA nanoparticle complex from aggregation and maintaining their stable nature. According to the reports, it seemed that the hydrophilic PEG polymer chains act as the outer shell to

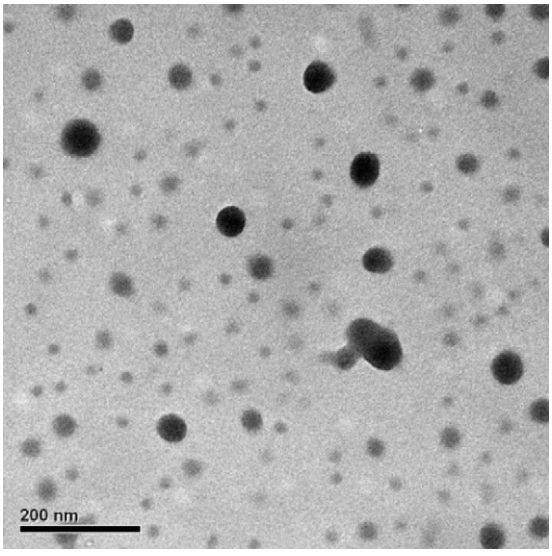


Fig. 4. TEM micrograph of lactobionic acid-grafted-pegylated-chitosan/DNA (1:1) (DADP-CS-(O-MPEG)-(*N*-LA)/DNA (1:1)) nanoparticle complex.

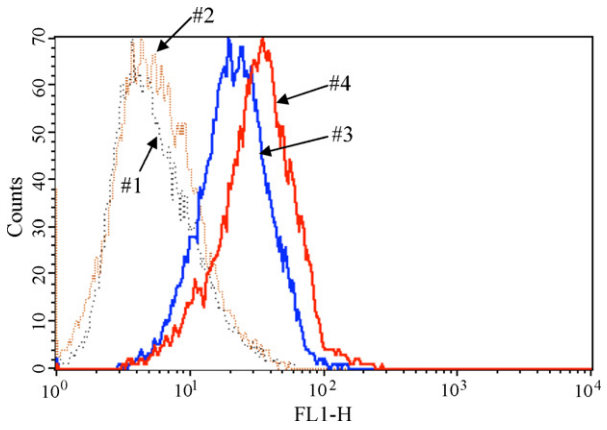


Fig. 5. Transfection of (−) control (#1), naked plasmid DNA (#2), pegylated-chitosan/DNA (DADP-CS-(O-MPEG)/DNA) nanoparticle complex (#3), and lactobionic acid-grafted-pegylated-chitosan (1:0.5)/DNA (DADP-CS-(O-MPEG)-(*N*-LA) (1:0.5)/DNA) nanoparticle complex (#4) in asialoglycoprotein overexpressed HepG2 cells.

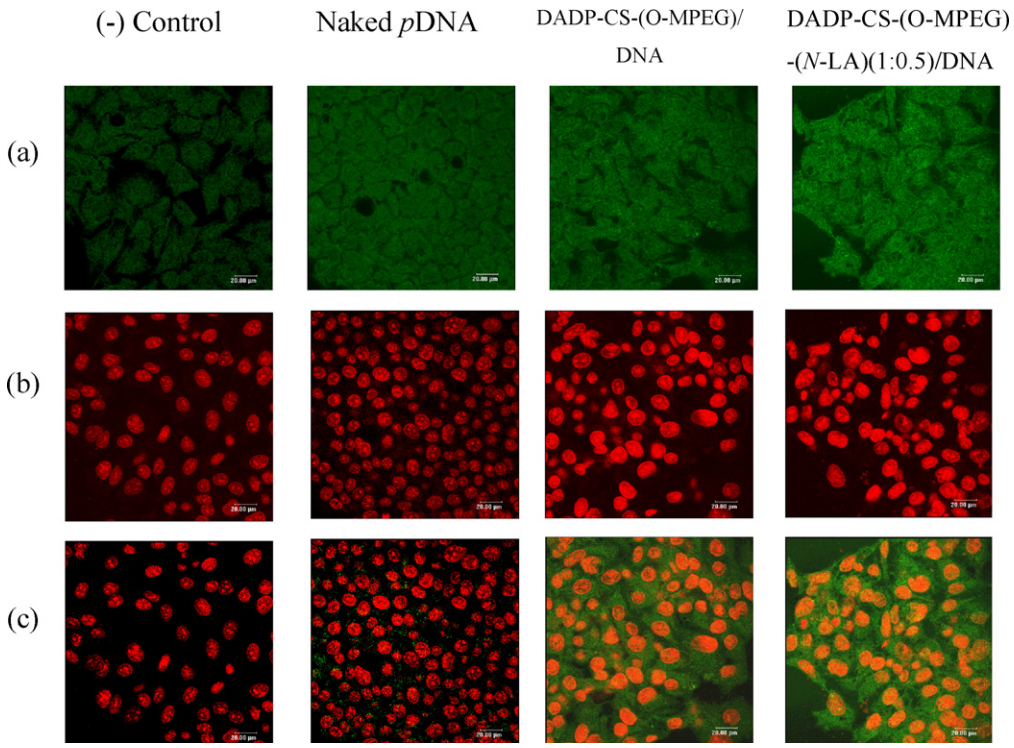


Fig. 6. The confocal fluorescence images for *p*EGFP-N1 expression in HepG2 cells. (a) The green-fluorescent image represented the signal of encoding enhanced green fluorescent protein (EGFP), (b) the red-fluorescence image represented the location of cell nucleus, and (c) the overlap of two images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

extend in the aqueous environment to prevent nanoparticle complex from aggregation. In other words, the shielding effect of PEG polymer chain was able to maintain nanoparticle complex stable (Avgoustakis, 2004; Betancourt et al., 2009; Ioele et al., 2005; Lin et al., 2009; Lu et al., 2009; van Vlerken et al., 2008).

3.7. Transfection of polymer/DNA nanoparticle complex

Fig. 5 shows the transfection efficiency of DADP-CS-(O-MPEG)/DNA and DADP-CS-(O-MPEG)-(*N*-LA)(1:0.5)/DNA nanoparticle complexes. The transfection of asialoglycoprotein receptor (ARGPR) overexpressed HepG2 cells by naked plasmid DNA (*p*EGFP-N1) was similar to the negative control. The transfection efficiency increased to 19.8% by pegylated DADP-CS-(O-MPEG)/DNA nanoparticle complex. The conjugation of a specific receptor ligand of DADP-CS-(O-MPEG)-(*N*-LA) further enhanced transfection efficiency to 45.3%. The transfection efficiency of un-modified chitosan/DNA complex was reported very low due to particle aggregation. Zhang et al. demonstrated that the conjugation of chitosan with α-methoxy-ω-succinimidyl PEG not only minimized particle aggregation but also improved transfection efficiency (Zhang et al., 2007). The similar aggregation phenomenon was observed in our un-pegylated DADP-CS-(*N*-LA)/DNA nanoparticle complex. Pegylation of chitosan resulted in DADP-CS-(O-MPEG)/DNA and DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complexes with stable nature. In other words, the pegylation of chitosan enhanced nanoparticle complex stability in terms of improving its transfection efficiency. Fig. 6 shows the confocal fluorescence images of *p*EGFP-N1 expression in HepG2 cells. The green-fluorescent image represented the signal of encoding enhanced green fluorescent protein (EGFP), and the red-fluorescence image represented the location of cell nucleus which was stained with propidium iodine (PI). It was clearly observed the appearance of EGFP protein signal in cytoplasm of cells, and the fluorescence intensity of cells transfected by DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex was the most prominent. Kim et al. have proved that the galactose-modified water-soluble chitosan had higher transfection efficiency in ASGPR-postive HepG2 cells than in ASGPR-negative HeLa cells, and the gene expression in terms of luciferase activity was affected by the charge ratio of polymer to DNA (Kim, Park, Nah, Choi, & Cho, 2004). In our study, we further demonstrated that receptor ligand conjugated DADP-CS-(O-MPEG)-(*N*-LA) was able to form stable complex with plasmid DNA, which was capable of producing better gene expression in HepG2 cells than ligand-free DADP-CS-(O-MPEG)/DNA nanoparticle complex.

4. Conclusions

Through this study, chitosan was chemically modified by grafting a hydrophilic methoxy poly(ethylene glycol) (MPEG) and a receptor ligand lactobionic acid (LA) onto C6-OH and C2-NH_2 positions. The pegylation efficiency of deacetylated-depolymerized-chitosan was 26% (w/w) based on both DSC thermal analysis and calorimetric analysis. Increase in feed molar ratio of lactobionic acid increased LA grafting degree of DADP-CS-(O-MPEG)-(*N*-LA). More compaction of plasmid DNA by DADP-CS-(O-MPEG)-(*N*-LA) than by DADP-CS-(O-MPEG) was observed in terms of smaller particle size of DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex. The bearing of receptor ligand led DADP-CS-(O-MPEG)-(*N*-LA)/DNA nanoparticle complex binding to asialoglycoprotein receptor overexpressed HepG2 cells and produced more enhanced transfection efficiency than ligand-free DADP-CS-(O-MPEG)/DNA nanoparticle complex.

Acknowledgement

This work was supported by National Science Council in Taiwan.

Appendix A. Appendix

AbbreviationsFull namesCSChitosanDA-CSDeacetylated-chitosanDADP-CSDeacetylated-depolymerized-chitosanDADPPhACSP-hthalic anhydride protected chitosanDADP-PhACS-(O-MPEG) Phthalic anhydride protected Pegylated-chitosanDADP-CS-(O-MPEG)Pegylated-chitosanDADP-CS-(O-MPEG)-(*N*-LA)Lactobionic acid-grafted-pegylated-chitosan

References

Alves, N. M., & Mano, J. F. (2008). Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. *International Journal of Biological Macromolecules*, *43*, 401–414.

Avgoustakis, K. (2004). Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: Preparation, properties and possible applications in drug delivery. *Current Drug Delivery*, *1*, 321–333.

Betancourt, T., Byrne, J. D., Sunaryo, N., Crowder, S. W., Kadapakkam, M., Patel, S., et al. (2009). PEGylation strategies for active targeting of PLA/PLGA nanoparticles. *Journal of Biomedical Materials Research*, *91A*, 263–276.

Gao, S., Dagnaes-Hansen, F., Nielsen, E. J., Wengel, J., Besenbacher, F., Howard, K. A., et al. (2009). The effect of chemical modification and nanoparticle formulation on stability and biodistribution of siRNA in mice. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, *17*, 1225–1233.

Gorochovceva, N., & Makus, ˇk. R. (2004). Synthesis and study of water-soluble chitosan-O-poly (ethylene glycol) graft copolymers. *European Polymer Journal*, *40*, 685–691.

Gref, R., Minamitake, Y., Peracchia, M. T., Torchilin, V., Trubetskoy, V., & Langer, R. (1994). Biodegradable long-circulating polymeric nanospheres. *Science*, *28*, 1600–1603.

Gref, R., Rodrigues, J., & Couvreur, P. (2002). Polysaccharides grafted with polyesters: Novel amphiphilic copolymers for biomedical applications. *Macromolecules*, *35*, 9861–9867.

Hashida, M., Nishikawa, M., Yamashita, F., & Takakura, Y. (2001). Cell-specific delivery of genes with glycosylated carriers. *Advanced Drug Delivery Reviews*, *52*, 187–196.

Ioele, G., Cione, E., Risoli, A., Genchi, G., & Ragno, G. (2005). Accelerated photostability study of tretinoin and isotretinoin in liposome formulations. *International Journal of Pharmaceutics*, *293*, 251–260.

Kataoka, K., Kwon, G. S., Yokoyama, M., Okano, T., & Sakurai, Y. (1993). Block copolymer micelles as vehicles for drug delivery. *Journal of Controlled Release*, *24*, 119–132.

Kato, Y., Onishi, H., & Machida, Y. (2001). Biological characteristics of lactosaminated N-succinyl-chitosan as a liver-specific drug carrier in mice. *Journal of Controlled Release*, *70*, 295–307.

Kim, T. H., Park, I. K., Nah, J. W., Choi, Y. J., & Cho, C. S. (2004). Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. *Biomaterials*, *25*, 3783–3792.

Laurentin, A., & Edwards, C. A. (2003). A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Analytical Biochemistry*, *315*, 143–145.

Lin, W. J., & Chen, M. H. (2007). Synthesis of multifunctional chitosan with galactose pendant as targeting ligand for glycoprotein receptor. *Carbohydrate Polymers*, *67*, 474–480.

Lin, W. J., Chen, T. D., & Liu, C. W. (2009). Synthesis and characterization of lactobionic acid grafted pegylated chitosan and nanoparticle complex application. *Polymer*, *50*, 4166–4174.

Liu, J., Teng, L., Liu, C., Hu, L., Wang, Y., Liu, H., et al. (2009). Augmented inhibitory effect of superoxide dismutase on superoxide anion release from macrophages by chemical modification with polysaccharide and attenuation effects on radiation-induced inflammatory cytokine expression in vitro. *Journal of Drug Targeting*, *17*, 216–224.

Lu, J. M., Wang, X., Marin-Muller, C., Wang, H., Lin, P. H., Yao, Q., et al. (2009). Current advances in research and clinical applications of PLGA-based nanotechnology. *Expert Review of Molecular Diagnostics*, *9*, 325–341.

Morimoto, M., Saimoto, H., Usui, H., Okamoto, Y., Minami, S., & Shigemasa, Y. (2001). Biological activities of carbohydrate-branched chitosan derivatives. *Biomacromolecules*, *2*, 1133–1136.

Nag, A., & Ghosh, P. C. (1996). A colorimetric assay for estimation of polyethylene glycol and polyethylene glycolated protein using ammonium ferrothiocyanate. *Analytical Biochemistry*, *237*, 224–231.

Park, I. K., Ihm, J. E., Park, Y. H., Choi, Y. J., Kim, S. I., Kim, W. J., et al. (2003). Galactosylated chitosan (GC)-graft-poly(vinyl pyrrolidone) (PVP) as hepatocyte-targeting DNA carrier: Preparation and physicochemical characterization of GC-graft-PVP/DNA complex (1). *Journal of Controlled Release*, *86*, 349–359.

Park, I. K., Kim, T. H., Park, Y. H., Shin, B. A., Choi, E. S., Chowdhury, E. H., et al. (2001). Galactosylated chitosan-graft-poly(ethylene glycol) as hepatocyte-targeting DNA carrier. *Journal of Controlled Release*, *76*, 349–362.

Peracchia, M. T., Fattal, E., Desmaele, D., Besnard, M., Noel, J. P., Gomis, J. M., et al. (1999). Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *Journal of Controlled Release*, *60*, 121–128.

Sajomsang, W., Tantayanon, S., Tangpasuthadol, V., & Daly, W. H. (2009). Quaternization of N-aryl chitosan derivatives: Synthesis, characterization, and antibacterial activity. *Carbohydrate Research*, *344*, 2502–2511.

Stockert, R. J. (1995). The asialoglycoprotein receptor: Relationships between structure, function, and expression. *Physiological Reviews*, *75*, 591–609.

Sugimoto, M., Morimoto, M., Sashiwa, H., Saimoto, H., & Shigemasa, Y. (1998). Preparation and characterization of water-soluble chitin and chitosan derivatives. *Carbohydrate Polymers*, *36*, 49–59.

van Vlerken, L. E., Duan, Z., Little, S. R., Seiden, M. V., & Amiji, M. M. (2008). Biodistribution and pharmacokinetic analysis of paclitaxel and ceramide administered in multifunctional polymer-blend nanoparticles in drug resistant breast cancer model. *Molecular Pharmaceutics*, *5*, 516–526.

Wagner, E. (1999). Application of membrane-active peptides for nonviral gene delivery. *Advanced Drug Delivery Review*, *38*, 279–289.

Weigel, P. H., & Yik, J. H. (2002). Glycans as endocytosis signals: The cases of asialoglycoprotein and hyaluronan/chondroitin sulfate receptors. *Biochimica et Biophysica Acta*, *1572*, 341–363.

Yu, R. J., & van Scott, E. J. (2004). Hydroxyacids and their topical use in the elderly. In *Skin diseases in the elderly*. New York: Marcel Dekker Inc.

Zhang, Y., Chen, J., Zhang, Y., Pan, Y., Zhao, J., Ren, L., et al. (2007). A novel PEGylation of chitosan nanoparticles for gene delivery. *Biotechnology and Applied Biochemistry*, *46*, 197–204.