Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 67 (2007) 474–480
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Synthesis of multifunctional chitosan with galactose as a targeting ligand for glycoprotein receptor

Wen-Jen Lin *, Ming Hsiung Chen

School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan

Received 28 April 2006; received in revised form 13 June 2006; accepted 13 June 2006
Available online 7 September 2006

Abstract

Chitosan-*O*-PEG-galactose was synthesized through hydroxyl groups of chitosan, which followed several steps including protection of amino group of chitosan, pegylation of chitosan, galactosylation of pegylated chitosan, and final removal of protection to obtain chitosan-*O*-PEG-galactose. The synthesized intermediates and final product were characterized and confirmed by ^{1}H NMR and FTIR, and the amounts of PEG and galactose conjugated with chitosan were measured. The pegylated chitosan possesses amphiphilic property in terms of soluble in both neutral aqueous (e.g., water) and organic solvents (e.g., DMF, dichloromethane). The corresponding critical micelle concentration is measured to be 0.56 mg/mL, and the size of micelles is 294.5 ± 2.3 nm with polydispersity 0.123 ± 0.021. The contents of PEG and galactose conjugated in chitosan-*O*-PEG-galactose are $98.09 \pm 4.63\%$ w/w and $3.06 \pm 0.54\%$ w/w, respectively. In terms of the degree of *O*-substitution of chitosan by PEG (DS_{PEG}) and the degree of substitution of PEG by galactose (DS_g) are 177.69% and 86.7%, respectively. Exclusively high DS_{PEG} indicates both C6–OH and C3–OH of chitosan are conjugated with PEG polymer chains. Further prominent attachment of galactose onto hydroxyl end group of PEG allows chitosan-*O*-PEG-galactose to possess sufficient quantity of targeting moieties for asialoglycoprotein receptor on hepatocytes.

Keywords: Chitosan; Poly(ethylene glycol); Galactose

1. Introduction

Polymer plays an important role in control of drug release and passive delivery of drug to the desirable action site. The advantages of drug targeting have been addressed a lot, such as to accumulate drug in action site, to reduce therapeutic dose, to increase efficacy, and to reduce toxicity, etc. These induce the development of cell-specific targeting carriers especially in gene therapy and chemotherapy, where the carriers are preferred to targeting via receptor-mediated endocytosis system. Surface modification of nano-sized carriers like nanoparticles is usually necessary for specific targeting purpose. Asialoglycoprotein receptor receives much attraction 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 the 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).

Several sugar ligands (e.g., galactose, *N*-acetylgalactosamine, mannose, lactose, fructose, etc.) and oligosaccharides/polysaccharides (e.g., dextran) have been demonstrated possessing different extent interaction with asialoglycoprotein receptor. As Gref *et al.* mentioned before, galactose-modified oligosaccharides show a high affinity for asialoglycoprotein receptors in liver tumor cells. However, the hydrophilicity and mobility characters of dextran polysaccharides prevent protein opsonization and further avoid liver recognition (Gref, Rodrigues, & Couvreur, 2002). Except for specific targeting purpose,

* Corresponding author. Tel.: +886 2 23123456x8396; fax: +886 2 23916126.
E-mail address: wjlin@ha.mc.ntu.edu.tw (W.-J. Lin).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.06.017

polysaccharide has also been used to increase hydrophilicity of hydrophobic polymer. Poly(ε-caprolactone) (PCL) is a highly crystallized and hydrophobic polymer, and modification of PCL by copolymerizing with polysaccharides like starch or dextrose has been proved to improve PCL's hydrophilicity and to obtain copolymers with amphiphilic property (Choi, Kim, & Park, 1999). Hashida *et al.* have demonstrated that different glycosylated carriers possess different cell-specific targeting properties. Such as the galactosylated cationic liposomes and poly(amino acids) are able to uptake by the asialoglycoprotein receptor in liver parenchymal cells, and express higher gene expression than liposomes without galactose modification. However, replacement of galactose by mannose residues in carriers results in specific delivery of genes to non-parenchymal liver cells (Hashida, Nishikawa, Yamashita, & Takakura, 2001). The conjugation of biocompatible polyesters, such as poly(lactide-co-glycolide) or poly(ε-caprolactone), and functional sugars shows a special recognition function, which can be designed as a drug-targeting material (Gref et al., 2002).

Chitosan has been reported as a non-toxic, biocompatible, and biodegradable polysaccharide, however, its poor water solubility due to strong intermolecular hydrogen bonding of amino and hydroxyl groups limits the application of chitosan. One strategy by using different substitutes to chemical modification of chitosan can improve its water solubility or to allow it bearing specific targeting functions (Gorochovceva & Makuška, 2004; Laurentin & Edwards, 2003; Park et al., 2001). Different molecular weights of poly(ethylene glycol)s (PEGs) have been grafted onto chitosan to alter its water solubility (Sugimoto, Morimoto, Sashiwa, Saimoto, & Shigemasa, 1998). PEG is used as a main additive in the film coating material, where it acts as a pore-forming agent to create interconnected channels for drug release (Lin & Lee, 2003; Lin, Lee, & Wang, 2004). Functionalization of outer surface of polymeric micelles by PEG alters their physicochemical and biologic properties resulting in long-circulating characteristics and significant tumor accumulation (Lin, Wang, & Chen, 2005). Chemical modification of chitosan is usually proposed through C2-amino groups. Gorochovceva *et al.* first proposed to pegylate chitosan through its hydroxyl group rather than amino group (Gorochovceva & Makuška, 2004). The free amino groups of chitosan play an important role on gene delivery. It is feasible to complex with negative charge DNA and allows DNA compacted inside polymeric carriers. The aim of our study was to develop a functional polymer via modification of chitosan with a liver-targeting moiety, galactose. Two chemical modifiers, PEG and galactose, were included. PEG was used to increase water solubility of chitosan and galactose was acted as a liver-targeting moiety. PEG was grafted onto hydroxyl group of chitosan after the amino groups of chitosan were previously protected by phthalic anhydride. The functional polymer, chitosan-*O*-PEG-galactose, was synthesized through several steps including: (1) protection of amino group of chitosan; (2) pegylation of chitosan; (3) galactosylation of pegylated chitosan; and (4) final removal of protection from amino group. The synthesized intermediates and chitosan-*O*-PEG-galactose were characterized by ^{1}H NMR and FTIR, and the amounts of PEG and galactose conjugated with chitosan were measured by colorimetric assay using ammonium ferrothiocyanate and anthrone sulfuric acid, respectively.

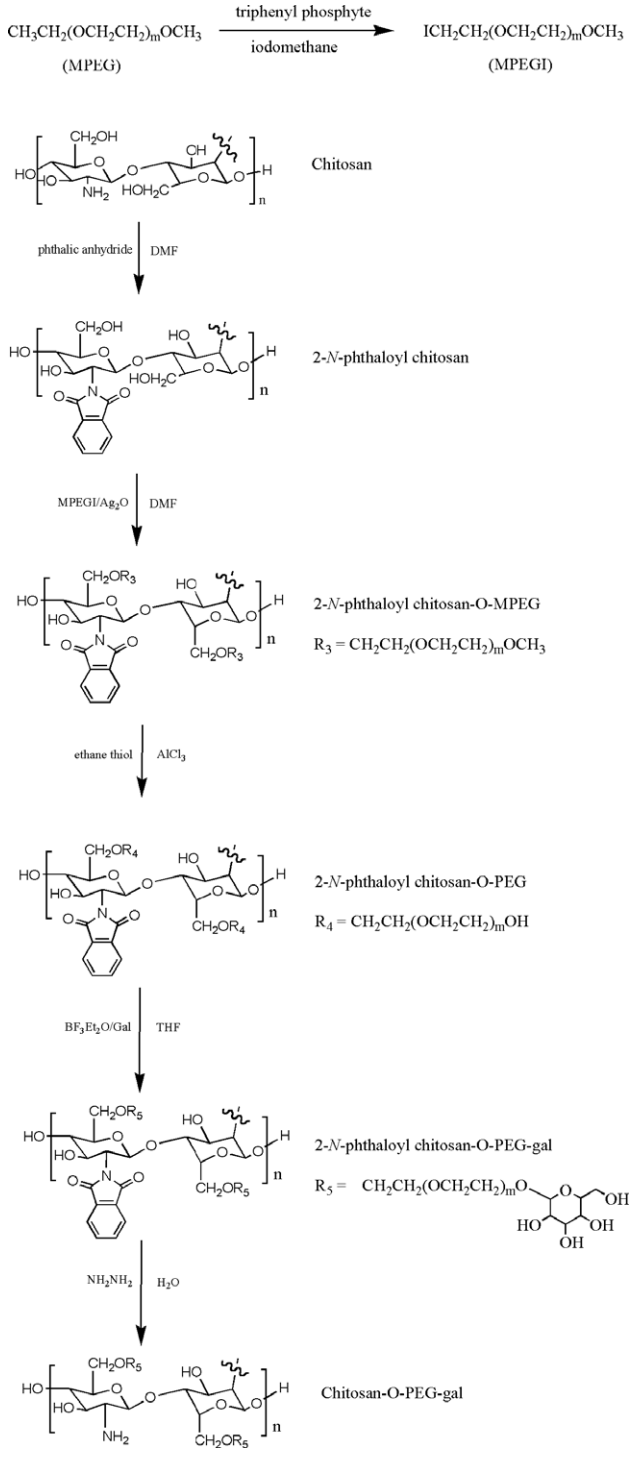
2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation 75%, low MW) and triphenyl phosphite were from Aldrich Chemical Company, Inc. (WI, USA). D(+)-Galactose (99+%) was from Acros Organics (New Jersey, USA). Poly(ethylene glycol) monomethyl ether (M_w 5000) was from Fluka Chemical Company Inc. (Buchs, Switzerland).

2.2. Synthesis of chitosan-PEG-galactose

Chitosan-*O*-PEG-galactose was synthesized as Scheme 1. First, methoxypoly(ethylene oxide) was activated by iodination and the amino groups of chitosan were protected by phthalic anhydride before pegylation. The iodo-methoxypoly(ethylene oxide) (MPEGI) attacked the hydroxyl group of chitosan and 2-*N*-phthaloyl chitosan-*O*-MPEG was obtained. Next, demethylation of 2-*N*-phthaloyl chitosan-*O*-MPEG generated hydroxyl end groups on PEG for further conjugation of galactose. Finally, the protection moieties for amino groups of chitosan were removed and chitosan-*O*-PEG-galactose was obtained. In the first step, iodo-methoxypoly(ethylene oxide) (MPEGI) was prepared (Gorochovceva & Makuška, 2004). MPEG (48.0 g), triphenyl phosphite (12.55 g), and iodomethane (2.98 mL) in the molar ratio of 1:5:5 were stirred in a reflux system at 120 °C for 12 h. The reaction mixture was left for cooling, and toluene was added to dissolve the crude product, followed by precipitating from diethyl ether. The precipitate was filtered and dried in a vacuum oven to obtain pale-yellow MPEGI. The yield of MPEGI is 82.0%. In the second step, 2-*N*-phthaloyl chitosan was prepared (Liu, Wang, Shen, & Fang, 2005; Nishimura, Kohgo, & Lurita, 1991). Chitosan (10.0 g) and phthalic anhydride (9.22 g) were heated at 130 °C in 50 mL *N*,*N*′-dimethylformamide with stirring under argon atmosphere. The suspension was filtered and washed with warm ethanol several times. The yield of 2-*N*-phthaloyl chitosan is 93.0%. In the third step, 2-*N*-phthaloyl chitosan-*O*-MPEG was synthesized (Gorochovceva & Makuška, 2004). 2-*N*-Phthaloyl chitosan (0.18 g), MPEGI (3.08 g), and Ag_2O (0.23 g) were heated at 60 °C and continuously stirred in DMF (60 mL) for 16 h. The mixture was then filtered and the solution was concentrated by rotary evaporator. The crude product was re-dissolved in dichloromethane, filtered, and concentrated again.



Scheme 1. The synthesis of chitosan-*O*-PEG-galactose.

The yield of 2-*N*-phthaloyl chitosan-*O*-MPEG is 35.5%. In the fourth step, 2-*N*-phthaloyl chitosan-*O*-PEG was prepared (Gopalakrishnan et al., 2000). 2-*N*-Phthaloyl chitosan-*O*-MPEG (3.39 g) and $AlCl_3$ (0.086 g) were continuously mixed in ethane thiol (60 mL) for 12 h at room temperature. The reaction mixture was diluted with water and then acidified with HCl aqueous (10% v/v). The precipitate was removed by filtration and then extracted with dichloromethane three times. The organic layer was collected and the solvent was removed. The yield of 2-*N*-phthaloyl chitosan-*O*-PEG is 82.0%. In the fifth step, 2-*N*-phthaloyl chitosan-*O*-PEG-galactose was synthesized (Ferrières, Bertho, & Plusquellec, 1995). 2-*N*-Phthaloyl chitosan-*O*-PEG (2.09 g), D(+)-galactose (0.084 g), and BF_3OEt_2 (0.586 mL) were dissolved in 50 mL dry THF. The reaction was stirred at 60 °C under argon atmosphere for 13 h. The solution was concentrated with rotary evaporator. The condensed viscous liquid was dissolved in dichloromethane followed by extraction with DI-water. The organic layer was collected and the solvent was removed. The yield of product is 81.8%. In the last step, chitosan-*O*-PEG-galactose was prepared (Nishimura et al., 1991). 2-*N*-Phthaloyl chitosan-*O*-PEG-galactose (1.5 g), hydrazine monohydrate (20 mL), and DI-water (40 mL) were heated at 100 °C and continuously stirred for 15 h under argon atmosphere. After cooling, the solution was diluted with water and then evaporated. This procedure was repeated for three times to remove hydrazine monohydrate. The crude product was then suspended in DI-water, filtered, and washed with ethanol and ether. The yield of chitosan-*O*-PEG-galactose is 97.9%. The synthesized intermediates and chitosan-*O*-PEG-galactose were confirmed by ^{1}H NMR (400 MHz, Bruker®) and FTIR, and the amounts of PEG and galactose conjugated with chitosan were measured by colorimetric assay using ammonium ferrothiocyanate and anthrone sulfuric acid, respectively.

2.3. Determination of critical micelle concentration (CMC)

The critical micelle concentration of 2-*N*-phthaloyl chitosan-*O*-PEG was determined with a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) using pyrene as a fluorescence probe. The fluorescence excitation spectra of pyrene were measured at various concentrations of 2-*N*-phthaloyl chitosan-*O*-PEG (Zhao & Winnik, 1990). The concentration of pyrene was kept at 6.0×10^{-7} M. The emission wavelength was set at 390 nm and the intensities obtained from excitation wavelengths at 333 and 336 nm were recorded. The ratio of fluorescence intensities at 336 and 333 nm (I_{336}/I_{333}) was calculated and plotted against the logarithm concentrations of 2-*N*-phthaloyl chitosan-*O*-PEG. The critical micelle concentration (CMC) was defined as the midpoint of the transition region before achieved micellar region.

2.4. Determination of PEG content

The modified colorimetric method was used to determine the content of poly(ethylene glycol) in chitosan-*O*-PEG-galactose (Nag, Mitra, & Ghosh, 1996). Several concentrations of MPEG aqueous solutions were prepared. MPEG solution, ammonium ferrothiocyanate, and chloroform in the volume ratio of 1:10:10 were added into each tube, and then continuously shaken at 200 rpm for 30 min. The well-mixed solution was centrifuged at

3000 rpm for 3 min, and the chloroform layer was collected and determined by spectrophotometer at 510 nm. The calibration curve was constructed based on the various MPEG concentrations and the corresponded absorbances. Chitosan-*O*-PEG-galactose sample was prepared following the same procedure, and the concentration of PEG was re-calculated from the calibration curve according to its measured absorbance. The amount of PEG grafted onto chitosan-*O*-PEG-galactose was calculated as follows:

$$\text{PEG}(\%\ w/w) = (\text{measured PEG weight in the sample})/(\text{sample weight}) * 100\%.$$

The degree of *O*-substitution of chitosan (DS_{PEG}) by PEG was calculated as follows:

$$DS_{PEG}(\%) = (\text{PEG}(\%)/5000)/[(100 - \text{PEG}(\%))/173] * 100\%,$$

where 173 represents the average molecular weight of chitosan monomer and 5000 represents the average molecular weight of MPEG.

2.5. Determination of galactose content

The modified anthrone sulfuric acid method was used to determine the content of galactose in chitosan-*O*-PEG-galactose (Laurentin & Edwards, 2003). Several concentrations of galactose solutions were prepared and placed in a pre-cooled 96-well. The fresh anthrone sulfuric acid was prepared in an ice bath, added into the 96-well, then heated at 90 °C for 3 min. The absorbance was determined by spectrophotometer at 630 nm. The calibration curve was constructed based on several various concentrations of galactose and their corresponding absorbances. Chitosan-*O*-MPEG-galactose sample was proceeded following the same procedure, and the concentration of galactose graft onto chitosan was re-calculated from the calibration curve according to its determined absorbance. The amount of galactose grafted onto chitosan-*O*-PEG-galactose was calculated as follows:

$$\text{Gal}(\%\ w/w) = (\text{measured galactose weight in the sample})/(\text{sample weight}) * 100\%.$$

The degree of substitution of PEG (DS_g) by galactose was calculated as follows:

$$DS_g(\%) = (\text{Gal}(\%)/180)/(\text{PEG}(\%)/5000) * 100\%,$$

where 180 represents the molecular weight of galactose and 5000 represents the average molecular weight of MPEG.

3. Results and discussion

3.1. Characterization of 2-N-phthaloyl chitosan

In order to graft PEG onto the hydroxyl group of chitosan, the amino groups of chitosan is previously protected with phthalic anhydride. Fig. 1 shows the FTIR spectrum of 2-*N*-phthaloyl chitosan. The critical signals at 1777, 1712 and 721 cm^{-1} are, respectively, assigned to the carbonyl group, the tertiary amino group and the aromatic ring of phthalimido group. The peak at 3750 cm^{-1} assigned to $–NH_2$ of chitosan is shrunk and become incomparable with the –OH peak at ~3500 cm^{-1} in the spectrum. These results provide the evidence about phthaloylation of chitosan.

3.2. Characterization of chitosan-O-poly(ethylene glycol)

2-*N*-Phthaloyl chitosan is further modified with methoxy poly(ethylene glycol) (MPEG), where the hydroxyl group of MPEG is activated by iodination. Fig. 2 shows the FTIR spectrum of 2-*N*-phthaloyl chitosan-*O*-MPEG, and the characteristic peaks include 3500 cm^{-1} for chitosan, 1650 cm^{-1} for phthaloyl moiety, and 2900, 1500, as well as 1100 cm^{-1} for MPEG. The substitution of hydroxyl group of chitosan by MPEG results in the disappearance of the broad peak at ~3500 cm^{-1} as shown in Fig. 1.

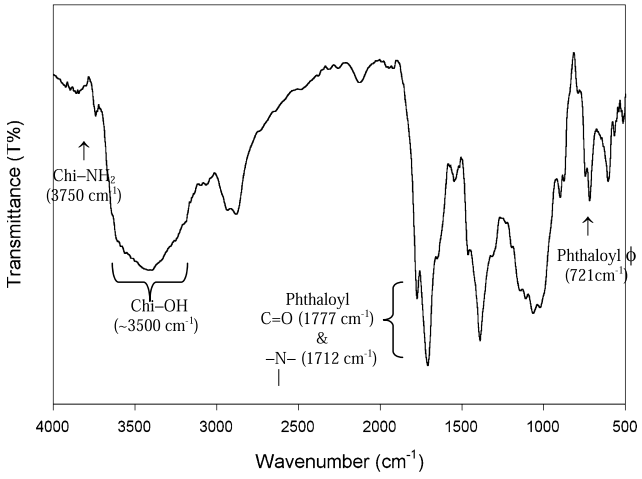


Fig. 1. FTIR spectrum of 2-*N*-phthaloyl chitosan.

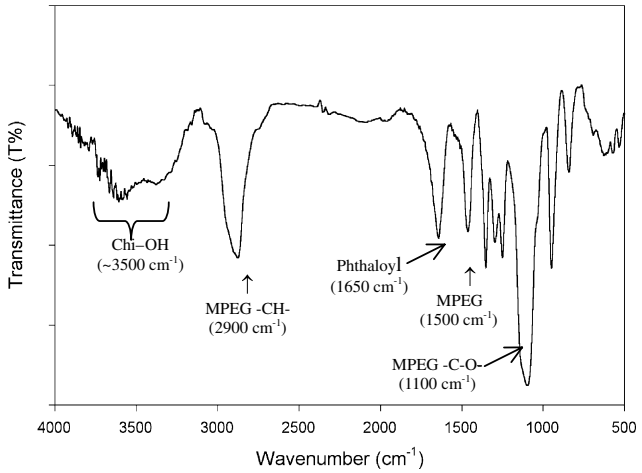


Fig. 2. FTIR spectrum of 2-*N*-phthaloyl chitosan-*O*-MPEG.

2-*N*-phthaloyl chitosan-*O*-PEG is obtained after demethylation of 2-*N*-phthaloyl chitosan-*O*-MPEG. It was proved to possess amphiphilic property and can be dissolved in both neutral aqueous (e.g., water) and organic solvents (e.g., DMF, dichloromethane). This is quite different from chitosan itself, which is only dissolved in weak acidic solution. Fig. 3 shows the ratio of fluorescence intensities at 336 and 333 nm (I_{336}/I_{333}) as a function of logarithm concentration of 2-*N*-phthaloyl chitosan-*O*-PEG. The critical micelle concentration is calculated to be 0.56 mg/mL from the midpoint of the transition region.

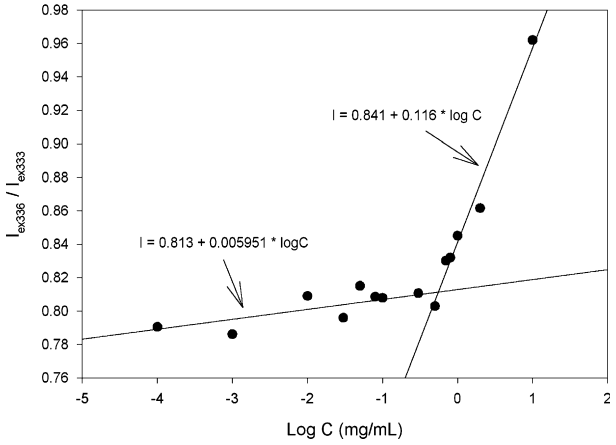


Fig. 3. CMC of 2-*N*-phthaloyl chitosan-*O*-PEG.

This result suggests that pegylated chitosan is capable of forming micelles spontaneously as concentration above critical micelle concentration. The size of micelles is measured to be 294.5 ± 2.3 nm, and the polydispersity is 0.123 ± 0.021. The similar aggregation phenomenon has been observed for chitosan-2-*N*-PEG (Ochi, Nishizawa, & Ohya, 1998). The corresponding particle sizes of chitosan-2-*N*-PEG aggregates are in the range of 70–120 nm dependent of the amount of PEG. More compact aggregates are formed with low PEG, however, the aggregates become looser with larger size by increasing conjugated PEG, which even form an unimolecular micelle in aqueous solution. Ouchi *et al.* further demonstrated the chitosan-2-*N*-PEG aggregates possess a pH-dependent character to manipulate compound release. Where the hydrophobic substance is taken up into the chitosan-2-*N*-PEG aggregates in a neutral aqueous solution, and then released by changing pH to an acidic condition.

3.3. Characterization of chitosan-O-PEG-galactose

Chitosan-6-*O*-PEG-galactose is prepared by galactosylation of 2-*N*-phthaloyl chitosan-*O*-PEG following by removal of phthaloyl moiety. The ^{1}H NMR spectra of 2-*N*-phthaloyl chitosan-*O*-PEG, 2-*N*-phthaloyl chitosan-*O*-PEG-galactose, and chitosan-*O*-PEG-galactose are shown in Fig. 4. The chemical shift at δ 8.05 ppm referred to the aromatic protons of phthaloyl moiety appears in the

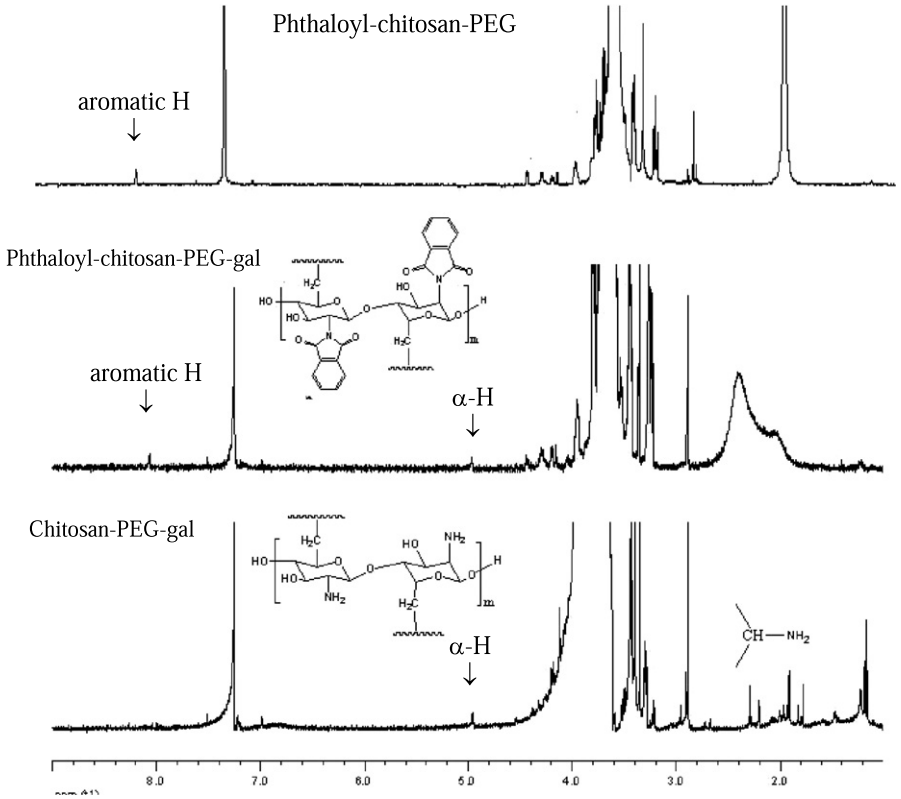


Fig. 4. ^{1}H NMR spectrum of 2-*N*-phthaloyl chitosan-*O*-PEG, 2-*N*-phthaloyl chitosan-*O*-PEG-galactose, and chitosan-*O*-PEG-galactose.

spectra of 2-*N*-phthaloyl chitosan-*O*-PEG and 2-*N*-phthaloyl chitosan-*O*-PEG-galactose, while disappears in the spectrum of chitosan-*O*-PEG-galactose. Meanwhile, the coupling of C2-methin group (δ 2.3 ppm) with $-NH_2$ group (δ 1.95 ppm) is clearly observed after removal of phthaloyl moiety in the final product. On the other hand, the critical peak at δ 5.0 ppm representing the α-H of galactose is clearly appeared after galactosylation. Fig. 5 shows the FTIR spectrum of final product chitosan-*O*-PEG-galactose. The prominent change is the growing of the peak at $\sim$3800 cm^{-1} corresponding to the reforming of $-NH_2$ group of chitosan after removal of phthaloyl moiety. The contents of PEG and galactose conjugated with chitosan are measured to be $98.09 \pm 4.63\%$ w/w and $3.06 \pm 0.54\%$ w/w, respectively. In other words, the degree of *O*-substitution of chitosan by PEG (DS_{PEG}) and the degree of substitution of PEG by galactose (DS_g) are 177.69% and 86.7%, respectively. There are two hydroxyl groups, C6–OH and C3–OH, in each monosaccharide residue of chitosan. Exclusively high DS_{PEG} indicates both hydroxyl groups are substituted by PEG polymer chains. It has been reported that the feed molar ratio of chitosan and MPEGI plays an important role on determining chitosan's pegylation extent (Gorochovceva & Makuška, 2004). Poly(ethylene glycol) is a biocompatible material and widely applied in pharmaceutics. The flexibility of hydrophilic PEG chain not only prevents plasma protein adsorption, but also avoids delivery carriers taken up by reticular endothelial system and prolongs circulation time in blood (Stolnik, Illum, & Davis, 1995). It is therefore proposed that the chitosan-*O*-PEG-galactose aggregates form a core-shell architecture, where chitosan is compacted in the core due to intermolecular hydrogen bonding and hydrated PEG chain with high mobility is swept out and covered the outer shell of the aggregates. On the other hand, further prominent attachment of galactose onto hydroxyl end group of PEG allows chitosan-*O*-PEG-galactose possessing sufficient quantity of targeting moieties for asialoglycoprotein receptor on hepatocytes. However, the targeting efficiency of synthesized chitosan-*O*-PEG-galactose onto hepatocytes needs further investigated.

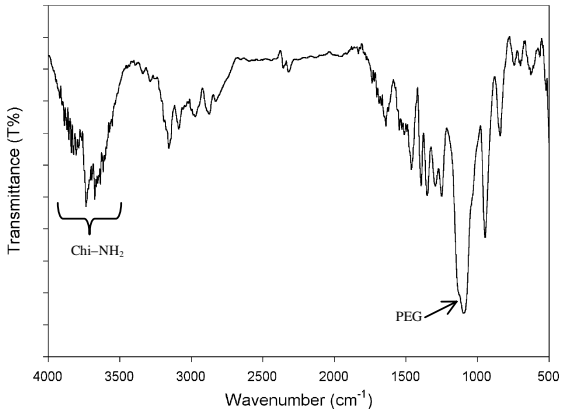


Fig. 5. FTIR spectrum of chitosan-*O*-PEG-galactose.

4. Conclusion

Chitosan-*O*-PEG-galactose was synthesized through hydroxyl groups of chitosan, which followed several steps including protection of amino group of chitosan, pegylation of chitosan, galactosylation of pegylated chitosan, and final removal of protection to obtain chitosan-*O*-PEG-galactose. The synthesized intermediates and final product were characterized and confirmed by ^1H NMR and FTIR. The pegylated chitosan possesses amphiphilic property in terms of soluble in both neutral aqueous (e.g., water) and organic solvents (e.g., DMF, dichloromethane). The corresponding critical micelle concentration is measured to be 0.56 mg/mL, and the size of micelles is 294.5 ± 2.3 nm with polydispersity 0.123 ± 0.021. The contents of PEG and galactose conjugated in the final product, chitosan-*O*-PEG-galactose, are $98.09 \pm 4.63\%$ w/w and $3.06 \pm 0.54\%$ w/w, respectively. In terms of the degree of *O*-substitution of chitosan by PEG (DS_{PEG}) and the degree of substitution of PEG by galactose (DS_g) are 177.69% and 86.7%, respectively. Exclusively high DS_{PEG} indicates both C6–OH and C3–OH of chitosan are conjugated with PEG polymer chains. Further prominent attachment of galactose onto hydroxyl end group of PEG allows chitosan-*O*-PEG-galactose to possess sufficient quantity of targeting moieties for asialoglycoprotein receptor on hepatocytes.

Acknowledgement

This work was supported by National Science Council in Taiwan (NSC 94-2320-B-002-079).

References

Choi, E. J., Kim, C. H., & Park, J. K. (1999). Synthesis and characterization of starch-g-polycaprolactone copolymer. *Macromolecules, 32*, 7402–7408.

Ferrières, V., Bertho, J. N., & Plusquellec, D. (1995). A new synthesis of *O*-glycosides from totally *O*-unprotected glycosyl donors. *Tetrahedron Letters, 36*, 2749–2752.

Gopalakrishnan, G., Kasinath, V., Pradeep Singh, N. D., Thirumurugan, R., Shanmuga Sundara Raj, S., & Shanmugam, G. (2000). A new synthetic route to dihydrobenzopyran via tandem demethylation cyclisation. *Molecules, 5*, 880–885.

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

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.

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., & Lee, H. G. (2003). Design of a microporous controlled delivery system for theophylline tablets. *Journal of Controlled Release, 89*, 179–187.

Lin, W. J., Lee, H. G., & Wang, D. M. (2004). The influence of plasticizers on the release of theophylline from microporous controlled tablets. *Journal of Controlled Release, 99*, 415–421.

Lin, W. J., Wang, C. L., & Chen, Y. C. (2005). Comparison of two pegylated copolymeric micelles and their potential as drug carriers. *Drug Delivery, 12*, 223–227.

Liu, L., Wang, Y., Shen, X., & Fang, Y. (2005). Preparation of chitosan-g-polycaprolactone copolymers through ring-opening polymerization of ε-caprolactone onto phthaloyl-protected chitosan. *Biopolymers, 78*, 163–170.

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

Nishimura, S. I., Kohgo, O., & Lurita, K. (1991). Chemospecific manipulations of a rigid polysaccharide: syntheses of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications. *Macromolecules, 24*, 4745–4748.

Ochi, T., Nishizawa, H., & Ohya, Y. (1998). Aggregation phenomenon of PEG-grafted chitosan in aqueous solution. *Polymer, 39*, 5171–5175.

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.

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

Stolnik, S., Illum, L., & Davis, S. S. (1995). Long circulating microparticulate drug carriers. *Advanced Drug Delivery Reviews, 16*, 195–214.

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

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

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

Zhao, C. L., & Winnik, M. A. (1990). Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers. *Langmuir, 6*, 514–516.