Poly(ethylene glycol) grafted chitosan as new copolymer material for oral delivery of insulin

Thanh Ha Ho, Thi Nu Thanh Le, Tuan Anh Nguyen and Mau Chien Dang

Laboratory for Nanotechnology, Vietnam National University in Ho Chi Minh City, Community 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam

E-mail: htha@vnuhcm.edu.vn

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Abstract
A new scheme of grafting poly (ethylene glycol) onto chitosan was proposed in this study to give new material for delivery of insulin over oral pathway. First, methoxy poly(ethylene glycol) amine (mPEGa MW 2000) were grafted onto chitosan (CS) through multiples steps to synthesize the grafting copolymer PEG-g-CS. After each synthesis step, chitosan and its derivatives were characterized by FTIR, 1H NMR Then, insulin loaded PEG-g-CS nanoparticles were prepared by cross-linking of CS with sodium tripolyphosphate (TPP). Same insulin loaded nanoparticles using unmodified chitosan were also prepared in order to compare with the modified ones. Results showed better protecting capacity of the synthesized copolymer over original CS. CS nanoparticles (10 nm of size) were gel like and high sensible to temperature as well as acidic environment while PEG-g-CS nanoparticles (200 nm of size) were rigid and more thermo and pH stable.

Keywords: chitosan, poly(ethylene glycol), copolymer, insulin, nanoparticles

Classification number: 2.05

1. Introduction

In disease medical treatment, oral route is always preferred to injection. However, it is not always possible to deliver every therapeutic agent through the oral route including vaccines, genes, proteins and peptides [1]. A common example is insulin, a peptide hormone that has to be daily administered by injection, normally before meals, to treat diabetes. If administered through gastrointestinal (GI) pathway, it will be chemically and enzymatically inactivated by the high acidity and the secreted enzymes of the stomach. Study showed that less than 0.1% of any oral insulin dose reaches the bloodstream intact [2]. Developing an efficient delivery agent that can protect insulin through the GI tract remains a persistent challenge, hence a race between big pharmaceutical companies. Chemical modification and use of mucoadhesive polymeric system, for example chitosan derivatives, seem to be promising methods [3].

Chitosan (CS) is deacetylation product from chitin, the most abundant natural polysaccharide after cellulose found in crustaceous shells. This material has clear advantages of being natural origin is that it possesses good biocompatibility and very good mucoadhesive properties [4]. For these characteristics, CS is being investigated for many applications, especially in medicine, cosmetics and nutrition [5]. One difficulty that usually comes across in exploiting CS is its insolubility in most organic solvents [6]. This makes CS a tough substance to engineer in, for example, chemical modifications or nanoparticles preparations, but at the same time gives a very interesting challenge.

Among ideas for chemically modifying CS structure, pegylation emerged to be very competent method to obtain higher soluble capacity of CS. Grafting of methoxy poly (ethylene glycol) amine (PEG) molecule onto CS backbone resulted in improving water solubility of CS [7, 8], thermal stability [9] and could be used as pH dependent drug release system for protein delivery [10–12]. In two separate studies, insulin loaded PEG-g-CS nanoparticles had 150–400 nm in size, good encapsulation capacity but unsustained release of active agents were obtained [13, 14]. Another study showed
that pegylated CS could reduce cytotoxicity of the nano-capsules hence increased cellular viability of the caco-2 cells [15].

In this study the purchased CS was first deacetylated by alkali method. Studies showed that degree of deacetylation (DD) affect CS’s physiochemical properties in such a way that high DD possessed more favorable behaviors for drug delivery [16, 17]. Pegylation of CS was performed in three steps: (1) N-phthaloylation protection, (2) O-pegylation and (3) N-phthaloylated CS deprotection (figure 1). During synthesis, solubility of the reagents was carefully examined and optimised since it was the key challenge that decided the success and yield of the outputs. The synthesis products were througly monitored by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analysis to validate their structures as well as their purity after each of the synthesis steps. Insulin loaded nanoparticles (NPs) were prepared by ionic-gelation method using tripolyphosphate (TPP) as CS cross linker and characterized by transmission electron microscopy (TEM), dialysis and UV–vis methods.

2. Materials and methods

2.1. Materials

Chitosan was obtained from HiMedia (India); methoxy poly (ethylene glycol) amine (PEG) (MW 2000), insulin recombinant human (≥27.5 IU/mg), phthalic anhydride (PhA), N, N-dimethylformamide (DMF), triethylamine (Et₃N), p-toluenesulfonyl chloride (TsCl), dichloromethane (DCM) and acetic acid glacial (AA) were purchased from Sigma Aldrich® Chemical Co., and were used as received. Sodium tripolyphosphate (TPP) was purchased from Acros OrganicTM, Dialysis tubing (MW cut off 12 000–14 000) was purchased from Carolina®. All other reagents were chemical grade and used as received.

2.2. Deacetylation of chitosan

Chitosan (CS) powder was refluxed at 120 °C in 50% KOH solution of 1:20 (w/v) ratio under nitrogen atmosphere for 6 h. After the reaction, the slurries was filtered and washed with distilled water until pH neutral then dried at 40 °C and stored under vacuum. Deacetylation degree (DD) of CS was measured by reserve acid-base titration and FTIR methods as fully explained by Renata et al [18] expressed by following formulae

\[ DD(\%) = \frac{V_{\text{HCl}} - V_{\text{NaOH}}}{0.0042(V_{\text{HCl}} - V_{\text{NaOH}}) + m_{\text{CS}}} \times 100 \]

\[ DD(\%) = 100 - 115 \frac{A_{1655}}{A_{3450}} \]

where \( m_{\text{CS}} \) is the weight in g of CS, \( V \) stands for volume in ml of (0.1 N) solutions used for titration, \( A_{1655} \) and \( A_{3450} \) are values of FTIR absorbance from baseline.

2.3. Grafting of PEG onto chitosan

2.3.1. N-phthaloylation of chitosan. Deacetylated CS (5.00 g, DD=90% so 25.0 mmol of glucosamine units) and PhA (12.1 g, 81.8 mmol) in DMF:H₂O = 95:5 (100 ml) was refluxed at 120 °C under nitrogen atmosphere. After 5–6 h of reaction, the cooled mixture was precipitated in ice-water, collected by filtration, washed overnight with MeOH, and air dried to obtain a pale tan powdery material N-phthaloylchitosan (PhACS) (yield: 81%). IR (KBr): \( \nu \) 3450 (O–H), 2902 (C–H, pyranose), 1773 and 1716 (C=O, imide), 1394 (C=C, PhA), 1150–1010 (C–O, pyranose), and 721 cm⁻¹ (arom, phthaloyl) (figure 2).

2.3.2. O-pegylation of chitosan. PhACS (1 g, 3.19 mmol) was dispersed in 30 ml of DCM:DMSO 50:50. The mixture was cooled to 0 °C then TsCl (1.5 eq) and Et₃N (1.5 eq) were gradually added. The reaction was kept at 0 °C for 1 h then 15 °C for 5 h and room temperature overnight. The mixture was then precipitated in ice-water, filtered, washed with MeOH and air dried. The powder was then dispersed in DCM together with PEG and the mixture was gently stirred at room temperature for 5 days. The slurries were then washed with EtOH and air dried. IR (KBr): \( \nu \) 3440 (O–H), 2905 (C–H, pyranose), 1654 (amide I, N-PEG), 721 cm⁻¹.

2.3.3. N-phthaloylated chitosan deprotection. PEG-PhACS from previous (3.0 mmol of PhACS units), NaBH₄ (5 eq) 

Figure 1. Scheme of PEG-g-CS synthesis and nanoparticles preparation steps: (1) N-phthaloylation of CS, (2) pegylation, (3) deprotection of N-pegylated PhACS and (4) nanoparticles preparation by crosslinking with TPP.
were dissolved in 30 ml of 2-propanol:H₂O 6:1 and stirred overnight. Acetic acid (18 eq) was then added and the mixture was air closed and heated to 80 °C in 2 h. After the reaction, the slurries was filtered and washed with EtOH, air dried to obtain the final product PEG-g-CS dried powder. IR (KBr): 3432 (broad, OH, NH and NH₂), 2900 (C–H, pyranose), 1100 cm⁻¹ (C–O, pyranose). ¹H NMR: δ 5–5.2 (1H, br, H-1 of GlcN), 3.6–3.9 (m, H-3, H-4, H-5, H-6, H-6’, N–CH₂b– of N-alkylated PEG), 3.1–3.4 (singlet of –OCH₂– of PEG), 3.5 (~OCH₃), 3.4–3.5 (br s, H-2).

2.4. Preparation and characterization of PEG-g-CS nanoparticles loaded with insulin

Aqueous PEG-g-CS solutions (from 0.1 to 0.5 mg ml⁻¹) were first prepared in AA (from 0.1 to 2%) solution. Aqueous TPP (1.0 mg ml⁻¹) was then slowly added with a rate of 0.2 ml min⁻¹. The reaction mixture was kept vigorously stirred for about 1 h then gently stirred for another 1 h. NPs’ size and shape were determined by TEM visualization.

Insulin encapsulation was evaluated by dialysis method and UV–vis measurement. Dialysis tubing was first pre-conditioned by hot water in 1 h. Then, the NPs mixture from preparation step was poured directly into the tube, closed the two ends and the filled tube was soaked into distilled water for 2 h for the unloaded insulin molecules to across the membrane and then evaluated by UV–vis at 276 nm.
3. Results and discussion

3.1. Deacetylation of chitosan

Deacetylation degree (DD) was measured before and after deacetylation (DA) reaction and by both methods to determine the effectiveness of the deacetylation process. The idea behind using two different methods was that to increase the precise of results while DD determination by NMR method was temporally unavailable. After deacetylation, DD was increased and reached over 90% (table 1).

Figure 3 shows absorbance values interpretation at 3450 and 1655 cm$^{-1}$ for the DD calculations. It can be seen that after deacetylation reaction, the band at 1655 cm$^{-1}$ (C=O, acetyl groups) decreases as compared to the band at

Figure 5. $^1$H NMR spectrums of (a) chitosan, (b) PEG and (c) PEG-g-CS.

Figure 6. TEM visualization of (a) chitosan nanoparticles (CS NPs), (b) insulin loaded CS NPs, (c) insulin loaded PEG-g-CS aggregates and (d) PEG-g-CS NPs.
3450 cm\(^{-1}\) (O–H groups). Following the formula (2), this would result in increasing deacetylation degree value which was confirmed in table 1 results.

### 3.2. Grafting of PEG onto chitosan

Chemical modifications of CS were carried out under heterogeneous conditions. Special care must be paid to various problems during synthesis such as the limit choice of reaction, low level of reagents’ solubility, control of regioselective substitution, purification of the products. Besides, because CS is thermo instable, all heat assist reactions had to be taken place under nitrogen atmosphere or air sealed to avoid CS molecules degradation.

In figure 4 we see that the appearance of stronger IR absorptions at 2882 (CH\(_2\)) and 1171 cm\(^{-1}\) (C–O–C) showed the existence of PEG chains. All of the obtained PEG-g-CS was soluble in DMSO as a sign of solubility improving of grafting copolymers over the origin CS.

The final purified product PEG-g-CS was analyzed by \(^1\)H NMR (figure 5). Peaks spectrum in the range of 3.0–4.0 ppm were multiples and not well separated due to the overlapping of peak of PEG methylene group and peaks of saccharine units of CS. Methyl group of PEG was clearly observed at 3.3 ppm.

### 3.3. Nanoparticles preparation

Nanoparticles preparation with CS and PEG-g-CS were realized using TPP as crosslinking agent. Results in TEM visualization (figure 6) showed that NPs prepared from CS were more gel-like. They were also thermo instable and hard to centrifuge. NPs prepared from PEG-g-CS were larger in size but much more rigid and stable. Loading tests showed less insulin was encapsulated by PEG-g-CS (5.2% compared to 60% of CS NPs).

### 4. Conclusion

PEG-g-CS copolymers were prepared through a new protection–graft–deprotection route with phthaloylchitosan as an intermediate. PEG branches were grafted regioselectively at the hydroxyl groups of phthaloylchitosan, and then phthaloyl groups were deprotected to regenerate the free amino groups. The graft copolymers obtained presented improved thermo stability and less gel-like.

However, more investigation was needed to carry out to improve the synthesis yield and productivities and the insulin loading capacity.

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