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# Characteristics of paclitaxel-loaded chitosan oligosaccharide nanoparticles and their preparation by interfacial polyaddition in O/W miniemulsion system

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ABSTRACT

Paclitaxel-loaded chitosan oligosaccharide (CSO) nanoparticles were prepared by interfacial polyaddition between amino group of CSO and epoxy group of ethylene glycol diglycidyl ether (EGDE) in an O/W miniemulsion system. Using Span 85 and Tween 20 as the surfactants of oil phase and water phase, respectively, the stable O/W miniemulsion with about 200 nm droplet size could be obtained. When the molar ratio of EGDE to CSO was increased from 2 to 6, the average size of the obtained nanoparticles increased from 156.2 to 218.9 nm, the drug entrapment efficiency was increased from 83.68% to 92.30%, and the paclitaxel release rate was slowed. Fixing the molar ratio of EGDE to CSO at 4, the average size of prepared nanoparticles decreased from 282.1 to 140.4 nm when the total amount of CSO and EGDE was increased, while the drug entrapment efficiency increased, and the drug release rate in vitro decreased.



## 1. Introduction

Chitosan is the natural alkaline polysaccharide derived from chitin by deacetylation and consists of 2-amino-2-deoxy-(1–4*b*)-D-glucopyranose residues (D-glucosamine units) and *N*-acetyl-D-glucosamine units (Kean & Thanou, 2005), which possess distinct physicochemical and biological properties and is widely accepted as carriers for gene delivery, oral protein delivery, and controlled release systems (Gazori et al., 2009). Because of the large molecular weight, insolubility in physiological pH condition and high viscosity of chitosan (Tommeraas et al., 2002), it is necessary to degrade chitosan to prepare chitosan oligosaccharide (CSO) with low molecular weight. Particularly, chitosan oligosaccharide presents more interesting characteristics for clinical applications, which include reduced toxicity, biocompatibility, biodegradability, and good solubility in physiological condition (Duceppe & Tabrizian, 2009).

The use of biodegradable materials for nanoparticle preparation can realize sustained drug release within the target site over a period of days or even weeks. Biodegradable nanoparticles formed by PLGA and PLA have been developed for sustained drug delivery and are especially effective for drugs with an intracellular target (Panyam & Labhasetwar, 2003). Chitosan nanoparticles have been applied in potential delivery systems for vaccines (Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001; Yang et al., 2009a, 2009b), genes (Yoksan & Akashi, 2009), and anticancer agents (Li et al., 2009), which can be prepared by many methods, including solvent evaporation (Huang, Du, Yuan, & Hu, 2009), ion cross-linking (De campos, Sanchez, & Alonso, 2001; Huang, Ma, Khor, & Lim, 2002), emulsification covalent cross-linking (Lueßen et al., 1997), spray drying (Mi, Tan, Liang, & Sung, 2002), etc.

Paclitaxel (PTX) is one of the best anti-tumor drugs and exhibits a strong cytotoxic activity against a variety of solid tumors, such as breast cancer, ovarian cancer, lung cancer, and prostatic carcinoma. However, PTX has a poor solubility in water due to its hydrophobic properties, which limits its clinical applications (Zhang, Huo, Zhou, Yu, & Wu, 2009). Cremophor EL has to be used in the commercial Taxol® formulation (Cremophor EL:ethanol, 50:50, v:v), which is one of the first line formulations of paclitaxel. However, the use of Cremophor EL causes serious side effects including nephrotoxicity, neurotoxicity, and cardiotoxicity (Yang et al., 2009a, 2009b), which have limited the clinical application of Taxol. Therefore, it is important to develop a drug delivery system for paclitaxel without Cremophor EL. Many drug delivery systems for paclitaxel have been developed in the recent year, such as water soluble paclitaxel pro-drugs (Golik et al., 1996; Moosavi-Movahedi et al., 2003), liposome (Soepenberg et al., 2004; Zhang et al., 2005 and Yang et al., 2007), microsphere (Armstrong, Fleming, Markman, & Bailey, 2006; Azouz et al., 2008), emulsion (Kan, Chen, Lee, & Chu, 1999; Lundberg, Risovic, Ramaswamy, & Wasan, 2003), cyclodextrin inclusion compound (Alcaro et al., 2002; Liu et al., 2004 and

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Bouquet et al., 2009), polymeric micelle (Dabholkar, Sawant, Mongayt, Devarajan, & Torchilin, 2006; Huh et al., 2005; Lee et al., 2007) and polymeric nanoparticle.

In this paper, paclitaxel-loaded chitosan oligosaccharide nanoparticles were prepared by interfacial polyaddition in O/W miniemulsion system. The effects of the time of sonication dispersion, the proportion and concentration of EGDE and chitosan oligosaccharide during preparation on several characteristics of chitosan oligosaccharide nanoparticles such as particle size, drug entrapment efficiency and drug release behavior in vitro were evaluated.

## 2. Materials and methods

### 2.1. Materials

Chitosan (95% deacetylation degree, $M_w$ = 450 kDa) was supplied by Yuhuan Marine biochemistry Co. Ltd. (Zhejiang, China). Chitosanase was purchased from Chemical Industries Co. Ltd. (Japan). Master sample of polysaccharide (Part No.: 2090-0100) was purchased from Polymer Laboratories Co. Ltd. (USA). The model drug, Paclitaxel was provided by Huzhou Zhanwang biochemical Co. Ltd., China. Ethylene glycol diglycidyl ether (EGDE) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Japan). Tween 20, Span 85 and methylene dichloride were purchased from Shanghai Chemical Reagent Co. Ltd., China. Ethanol and other chemicals were analytical reagent grade.

### 2.2. Preparation of chitosan oligosaccharide

A 3% chitosan solution was prepared by dispersing 15 g chitosan in 500 mL of distilled water. After adding 6.25 mL of 36.5% (w/v) hydrochloric acid, the temperature of the mixture was raised up to 50 °C in a bath reactor, and 1 U/mL chitosanase was added. The reaction time of hydrolysis was controlled by molecular weight measurement of chitosan, monitoring through viscosity determination. The reaction mixture was then centrifugated for 10 min at 4000 rpm. The obtained supernatant was filtered with 0.45 μm filter, and then ultrafiltered by various molecular weight cut off (NMWCO) ultrafiltration membranes (Millipore Labscale TFF system, Millipore Co. USA). The low molecular weight of chitosan, chitosan oligosaccharide (CSO) was obtained by lyophilization.

The molecular weight of the final chitosan oligosaccharide (CSO) was determined by gel permeation chromatography (GPC) with TSK-gel column (G3000SW, 7.5 mm × 30 cm I.D.) at 25 °C (Hu et al., 2006). A weighted sample of lyophilized powder of CSO was dissolved in acetate buffer solution (pH 6.0) and the final concentration was adjusted to 1.0 mg/mL. Then, 10 μL of the sample was chromatographed using acetate buffer solution (pH 6.0) as the elution buffer and a flow rate of 0.8 mL/min. Master samples of polysaccharide with different molecular weight ($M_w$ = 5.9, 11.8, 22.8, 47.3, 112, 212 K) were dissolved in acetate buffer solution (pH 6.0), and their final concentrations were set to 0.5 mg/mL. Calibration was performed by means of polysaccharide samples using the integral molecular weight distribution method.

### 2.3. Preparation of paclitaxel-loaded chitosan oligosaccharide nanoparticles

The oil in water (O/W) miniemulsion was prepared by probe-type ultrasonic treatment with methylene dichloride and CSO aqueous solution as the oil phase and aqueous phase, respectively. Paclitaxel-loaded chitosan oligosaccharide nanoparticles were prepared by polyaddition in O/W miniemulsion system at room temperature. The preparation recipes are shown in Table 1.

The aqueous phase was formulated by chitosan oligosaccharide ($M_w$ = 8000 kDa) aqueous solution and Tween 20. The oil phase, consisted of methylene chloride, EGDE, paclitaxel, and Span 85 was added into aqueous phase by stirring (DC-40, Hangzhou Electrical Engineering Instruments, China) at 400 rpm for 5 min to form the pre-emulsion. The miniemulsion was then obtained by probe-type ultrasonic treatment (500 W, 10–50 cycles with 2 s active following 3 s duration, JY92-II, Scientz Biotechnology Co. Ltd., China) of the pre-emulsion in ice-bath and then stirred at room temperature over night.

### 2.4. Determination of particle size

One milliliter miniemulsion or nanoparticles dispersion was diluted to control the concentration of droplets or nanoparticles to 0.1 mg/mL. The droplet size of the resulted miniemulsion and nanoparticles were determined using a Zetasizer (3000 HS, Malvern Instruments, UK).

### 2.5. Determination of drug encapsulation efficiency

The content of paclitaxel was determined by high performance liquid chromatograph (HPLC). A Hypersil C18 column (150 mm × 3.9 mm) was used. The mobile phase consisted of acetonitrile and water (45:55, v/v) with a flow rate of 1 ml/min. The wavelength was set at 240 nm and the column temperature was 35 °C. The separated drug-loaded nanoparticles by centrifugation were re-dispersed into 30 ml of PBS (pH 7.4) with 2 M sodium salicylate and surged by vortexing (XW-80A, Instruments Factory of Shanghai Medical University, China) for 3 min to dissolve the unloaded drug. Then the dispersions were centrifuged at 20,000 rpm for 15 min (3K30, Sigma, Germany) and the supernatant was filtrated with 0.22 μm filter. The drug content in the obtained supernatant was measured by HPLC as described above. The drug entrapment efficiency (EE) was calculated from

$$EE = (W - W_0)/W \times 100\% \quad (1)$$

where $W$ is the weight of the drug added in the system, $W_0$ is the analyzed weight of the drug in the supernatant after centrifugation.

### 2.6. In vitro release studies

The precipitate of drug-loaded nanoparticles were re-dispersed in 30 ml PBS (pH 7.4) containing 2 M sodium salicylate and surged by vortexing (XW-80A, Instruments Factory of Shanghai Medical University) for 3 min, and then shaken horizontally (SHEL-LAB1227-2E, SHELLAB, USA) at 37 °C and 60 strokes/min. One milliliter of the dispersion was withdrawn from the system at definite time intervals. The dispersions were centrifuged at 20,000 rpm for 15 min (3K30, Sigma, Germany) and the supernatant was filtered through a 0.22 μm filter. The paclitaxel content in the filtrate was determined by HPLC method as described above. The drug content at 0 time was considered as the content of unloaded drug.

## 3. Results and discussions

### 3.1. Preparation of stable O/W miniemulsion

The droplet diameter of the emulsion prepared by mechanical stirring usually ranges from several to tens of microns, while nano-sized miniemulsion with narrow size distribution can be obtained via applying probe-type ultrasonic treatment. In this paper, the miniemulsion was prepared by probe-type ultrasonic treatment with methylene chloride and chitosan oligosaccharide solution as the oil phase and aqueous phase, respectively. The

**Table 1**
Preparation recipe of paclitaxel-loaded chitosan oligosaccharide (CSO) nanoparticles.

| Recipe | Recipe 1 | Recipe 2 | Recipe 3 | Recipe 4 | Recipe 5 | Recipe 6 | Recipe 7 |
|---|---|---|---|---|---|---|---|
| Paclitaxel (mg) | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Methylene dichloride (mL) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| EGDE (mmol) | 0.8 | 0.8 | 0.4 | 1.2 | 0.4 | 1.2 | 0.2 |
| Span 85 (g) | / | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Tween 20 (g) | 0.05 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| $H_2O$ (mL) | 9.5 | 9.5 | 9.5 | 9.5 | 9.5 | 9.5 | 9.5 |
| CSO (mmol) | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.3 | 0.05 |

miniemulsions prepared by recipe 1 in which Tween 20 was the only water soluble surfactant. The droplet size became bigger after 5 min, and the separation of the oil phase and water phase occurred after a few hours. However, the miniemulsion prepared by recipe 2 was completely stable for several hours because of the use of both Tween 20 and the lipophilic surfactant Span 85. Fig. 1 showed the droplet diameter of the miniemulsion prepared by recipe 2 as a function of the cycles of ultrasonic treatment. As shown in Fig. 1, the size of miniemulsion droplet reduced from 293.6 to 201.0 nm with the cycles of ultrasonic treatment increasing from 10 to 20. However, the cycles of ultrasonic treatment would not have a significant effect on the sizes of miniemulsion droplet when it exceeded 20. The droplet size of miniemulsion almost kept in 200 nm when the cycles of ultrasonic treatment was increased from 20 to 50. Consequently, 30 cycles of ultrasonic treatment in the preparation process of O/W miniemulsion was employed for the preparation of PTX-loaded CSO nanoparticles in the following studies.

### 3.2. Preparation of chitosan oligosaccharide nanoparticles

The chitosan oligosaccharide nanoparticles were then prepared by interfacial polyaddition between the epoxy group of EGDE and the amino group of CSO at room temperature in the O/W miniemulsion system. The process of polyaddition and a possible structure of the polymer derived from this reaction are described in Fig. 2. A CSO with 8000 Da weight average molecular weight was used. The drug and EGDE were dissolved in the oil phase in O/W miniemulsion, and the CSO was dissolved in the water phase. It was designed that the CSO and EGDE could react via polyaddition between epoxy group of EGDE and amino group of CSO on the surface of oil droplet, to form a cross-linked polymeric matrix for the encapsulation of hydrophobic drug–paclitaxel. After the remaining methylene chloride was completely volatilized, the paclitaxel-loaded CSO nanoparticles aqueous dispersion was obtained.

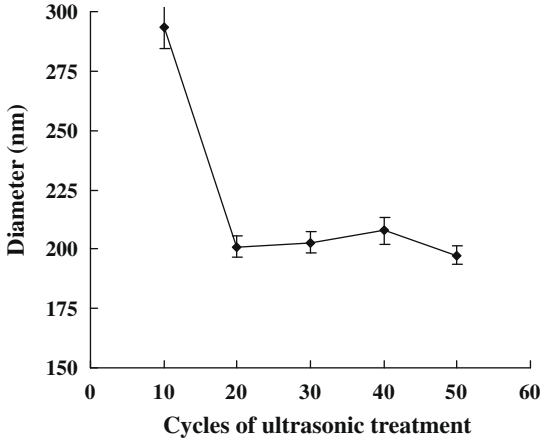


**Fig. 1.** Effect of cycles of ultrasonic treatment on the droplet diameter of O/W miniemulsion.

### 3.3. Effects of molar ratio of EGDE to CSO on the characteristics of nanoparticles

The CSO with 8000 Da average molecular weight had about 45 primary amino groups per CSO molecule, and EGDE had two epoxy groups per EGDE molecule. It was considered that the molar ratio of EGDE to CSO would affect the properties of the resultant paclitaxel-loaded nanoparticles prepared by interfacial polyaddition of EGDE and CSO in an O/W miniemulsion system. The paclitaxel-loaded CSO nanoparticles were then prepared by polyaddition between epoxy group of EGDE and amino group of CSO, using different molar ratios of EGDE to CSO. The molar ratio of EGDE to CSO varied from 2 to 6.

The particle sizes and drug entrapment efficiencies of the resultant paclitaxel-loaded CSO nanoparticles are shown in Fig. 3. It can be seen that the particle diameter increased from 156.2, 202.3 to 218.9 nm as the molar ratio of EGDE to CSO increased from 2, 4 to 6, while the drug entrapment efficiency of the nanoparticles increased from 83.68%, 88.45% to 92.30%. The increase of molar ratio of EGDE to CSO would enhance the reaction amount of CSO in water phase with the EGDE in oil phase, and the polymer content in nanoparticles. As a result, the particle size increased with increasing molar ratio of EGDE to CSO. Moreover, the increased polymer content in nanoparticles and the increased cross-link degree of CSO caused by increasing the molar ratio of EGDE to CSO, could also improve the efficiency of drug entrapment.

Fig. 4 shows the in vitro drug release behaviors of paclitaxel-loaded CSO nanoparticles prepared using different molar ratios of EGDE to CSO. It was found that all of the drug releases in vitro were prolonged up to 72 h, although the CSO were of nano size. The in vitro drug release was decreased with increasing molar ratio of EGDE to CSO. The accumulative percentage of released drug in 72 h was decreased from 89.5%, 78.0% to 69.4%, when the molar ratio of EGDE to CSO in the nanoparticles increased from 2, 4 to 6. The reduced drug release rate may originate from the increasing particle size, polymer content in nanoparticles and the increased cross-link degree of CSO on increasing the molar ratio of EGDE to CSO.

### 3.4. Effects of total amount of EGDE and CSO on the characteristics of the nanoparticles

The total amount of EGDE and CSO in the preparation of paclitaxel-loaded chitosan oligosaccharide nanoparticles by polyaddition of CSO and EGDE was also investigated. The total molar number of EGDE and CSO was varied from 0.25, 0.5, 1.0 to 1.5 mmol, while the molar ratio of EGDE to CSO was fixed at 4. The particle sizes and drug entrapment efficiencies of resulting paclitaxel-loaded CSO nanoparticles are shown in Fig. 5. As shown in Fig. 5, it is clear that the particle diameter was kept about 280 nm when the total molar number of CSO and EGDE increased

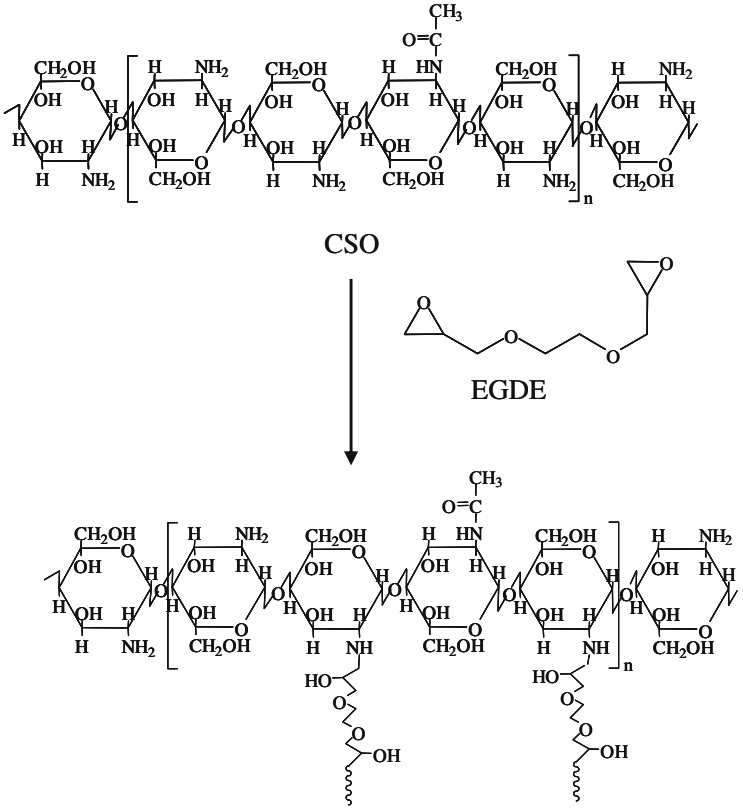


**Fig. 2.** Reaction scheme of polyaddition of CSO and EGDE and a possible structure of the copolymer.

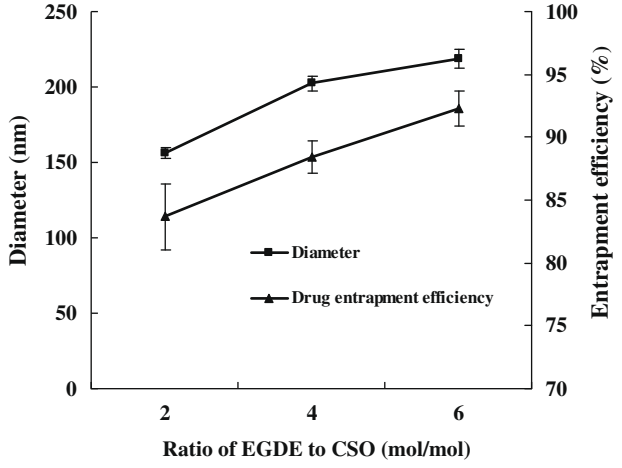


**Fig. 3.** Effect of molar ratio of EGDE to CSO on the diameter of nanoparticles and drug entrapment efficiency.

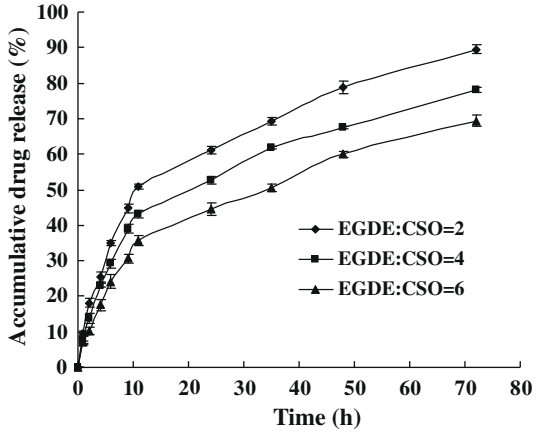


**Fig. 4.** *In vitro* drug release behaviors of paclitaxel loaded CSO nanoparticles prepared using different molar ratio of EGDE to CSO.

from 0.25 to 0.5 mmol. However, the particle size decreased from 280 to about 140 nm as the molar number of EGDE and CSO further increased from 0.5 to 1.5 mmol. On the other hand, the drug entrapment efficiency of resulting CSO nanoparticles improved from 77.25%, 83.87% to about 87% when the molar number of EGDE and CSO increased from 0.25, 0.5 to 1.0 mmol. On further increase from 1.0 to 1.5 mmol, the drug entrapment efficiency did not change much. The increase of total molar number of EGDE and CSO could improve the reaction efficiency between CSO and EGDE, which favored the formation of a larger amount of CSO nanoparticles. As a result, the particle size decreased, and the drug entrapment efficiency was improved. Also it was apparent that the CSO nanoparticle had about 280 nm size when the total molar number of EGDE and CSO was 0.25 mmol, which was bigger than that of the original oil droplet in the miniemulsion. This result implied the coagulation between the oil droplets or nanoparticles by the reaction might have happened, which led the bigger particle size and poorer drug entrapment efficiency.

Fig. 6 showed the in vitro drug release behaviors of paclitaxel-loaded CSO nanoparticles prepared using different molar number of EGDE and CSO. From Fig. 6, it was clear that all the drug releases in vitro were also prolonged for 72 h. The in vitro drug release rate of CSO nanoparticles prepared by 1.0 and 1.5 mmol molar numbers

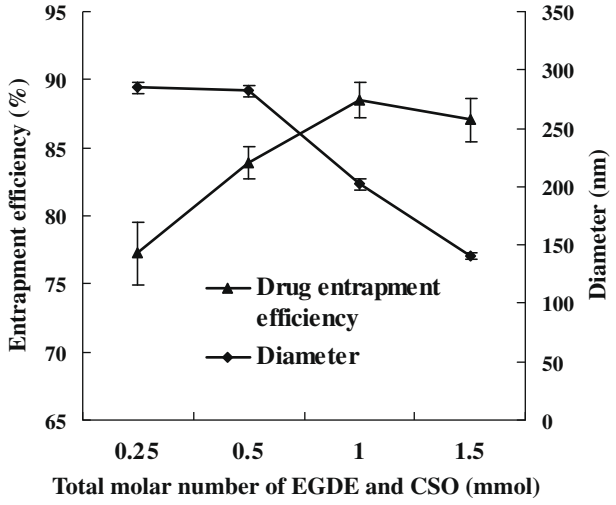


**Fig. 5.** Effect of total molar number of EGDE and CSO on the the diameter of nanoparticles and drug entrapment efficiency.

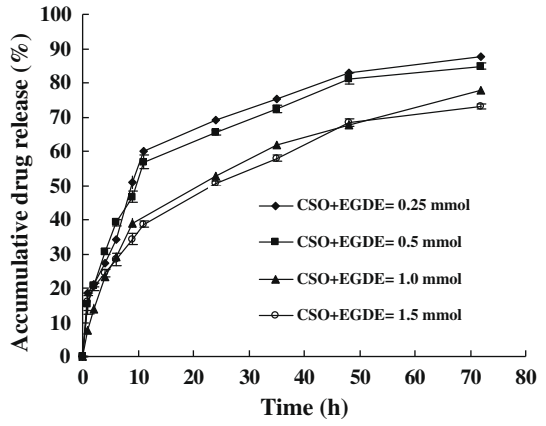


**Fig. 6.** *In vitro* drug release behaviors of paclitaxel-loaded CSO nanoparticles prepared using different total molar number of EGDE and CSO, in which the molar ratio of EGDE to CSO was fixed at 4.

of EGDE and CSO was slower than that of the nanoparticles prepared by 0.25 or 0.5 mmol molar number of EGDE and CSO, although the CSO nanoparticles prepared using 1.0 and 1.5 mmol molar numbers of EGDE and CSO had a smaller particles size. As mentioned above, the increase of total molar number of EGDE and CSO could improve the reaction efficiency between CSO and EGDE, promoting the polyaddition conducted on the surface of oil droplet of miniemulsion, and consequently, efficiently encapsulating the drug inside CSO nanoparticles. It is noteworthy that the burst drug release in the first 12 h slowed down from about 60% below to 40% as the molar number of EGDE and CSO increased to 1.0 or 1.5 mmol.

## 4. Conclusions

The paclitaxel-loaded chitosan oligosaccharide nanoparticles could be simply and easily prepared by interfacial polyaddition in O/W miniemulsion system over a certain range. The size of nanoparticles and the drug entrapment efficiency linearly with the amount of cross-linker (EGDE). Following a burst drug release from nanoparticles at the initial stage, which could be moderated by increasing the amount of EGDE there was a steady release at the latter stages. Moreover, the nanoparticles with relatively higher charged molar ratio of EGDE had a slower release rate. Not only did the drug entrapment efficiency improve significantly with increasing amounts of carrier matrix, but also the drug release rate of nanoparticles slowed.

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