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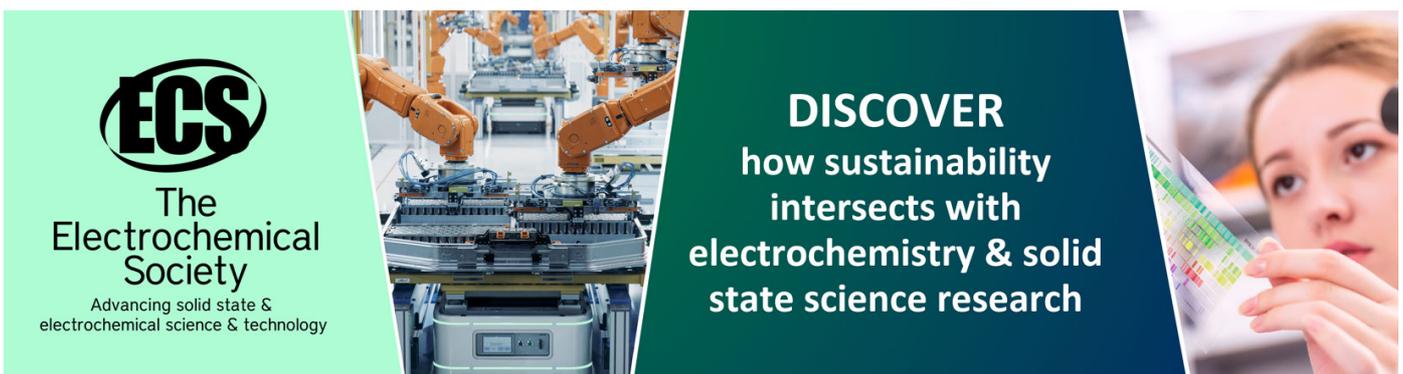
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## Effect of tween 80 on nanoparticle preparation of modified chitosan for targeted delivery of combination doxorubicin and curcumin analogue

Anita Sukmawati<sup>1</sup>, Wahyu Utami<sup>1</sup>, Ratna Yuliani<sup>1</sup>, Muhammad Da'i<sup>1</sup> and Akhmad Nafarin<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Pabelan, Surakarta, 57162, Indonesia

anita.sukmawati@ums.ac.id

**Abstract.** Delivery of anticancer is facing several problems including unspecific delivery of active substance to the targeted cell. The conjugation between chitosan and folate (chitosan-FA) was used for nanoparticle preparation containing combination of doxorubicin (DOX) and curcumin analogue, *2,5-bis-(4-hydroxy,3,5-dimethyl)-benzylidincyclopentanone*, as active substances. The purpose of this research is investigating formulation aspect for chitosan-FA nanoparticle by addition various tween 80 to achieve desired nano-size particle. The ionic gelation method was used for nanoparticle preparation using 0.05% w/v chitosan-FA with addition of 0.1 and 0.5% v/v of tween 80. The result showed that the high concentration of tween 80 during nanoparticle preparation lead to formation of smaller size particle. The 111.8 ±4.11 nm particle size was revealed by addition of 0.5% v/v tween 80 during chitosan-FA nanoparticle preparation loaded with active substances.

### 1. Introduction

Delivery of active agent for cancer therapy has several challenging aspect including how to specifically bring the agents to the targeted cell, particularly when the cancer cell spread to the body, an anticancer therapy is needed for the patient [1]. However, the common problem related to anticancer therapy is several drawbacks including gastrointestinal and immune system problem causing by unspecific delivery of anticancer agents. Thus, cancer therapy using specific targeted therapy should be developed. Nanoparticle can be used for delivering an anticancer since the ability of the system for protecting the active substance and the nano-size particle would improving the penetration across the mucosal epithelium [2,3]. Moreover, the nano-size particle usually provide a larger surface area for attachment with the cell and shorter path for drug diffusion to the target cell [4].

One of potential polymer for drug delivery is chitosan, a deacetylated chitin based material. This material offers several advantages including low toxicity, biodegradable and has immuno-stimulating effect [2]. Moreover, the amine primer groups on chitosan can be chemically modified by attaching with specific ligand for targeted delivery. Drug incorporation into nano-polymeric system also can be used for controlled release of drug over the extended period and functionalized with appropriate ligand for targeted delivery of active substance [5].

In order to minimize the toxicity of drug, combination of potent anticancer can be used since it will reduce the required dose of each drug. In this research, doxorubicin (DOX), a potent anticancer, is combined with a curcumin analogue, *2,5-bis-(4-hydroxy,3,5-dimethyl)-benzylidincyclopentanone* or pentagamavuno-1 (PGV-1) for inducing apoptosis in cancer cell as described in previous research by Hermawan and Meiyanto [6,7]. Nano particulate system using chitosan is proposed for delivering these agents since the chitosan nanoparticle (NP) can be selectively delivered anticancer agents to the tumour cell and has enhance permeation and retention (EPR) effect inside the cell [1,8]. However, retention and permeation of the particle inside the cell



strongly influenced by tumour type, location and vascular density of tumour and further affect the effectivity to the cancer cell [10]. As consequences, the modification of chitosan nanoparticle is needed to achieve specific delivery to cancer cell.

Folic acid (FA) is proposed to use as a ligand for specific delivery of nanoparticle since it enhancing endocytosis of particle through folate receptor [11]. Additionally, FA is a relative stable ligand, has lower immunogenic activity and has high affinity with folate receptor. Nanoparticle conjugate with folic acid entered the cancer cell via folate receptor-mediated endocytosis and transferred through cell's organelles by vesicular trafficking and then releases the active substances into cell cytoplasm [11]. The conjugation of FA into chitosan polymer can be performed through selective amidation of primary amine of chitosan as described by Yang *et al* [11].

The physical characteristics of particle including the particle size, is strongly influenced by nanoparticle preparation [13,14]. Further consequences, it will affect the activity of the nanoparticle inside the cancer cell. This research will explore the preparation and characterisation of chitosan modified folic acid nanoparticle. The conjugation of FA into chitosan polymer would be characterised using spectrophotometer UV. Modified chitosan nanoparticle was prepared using ionic gelation method using 0.1% of sodium tripolyphosphate (Na TPP). Tween 80 as surfactant was added during the nanoparticle preparation in order to achieve the desired size of particle. The effect of various concentration of tween 80 towards particle size was investigated.

## 2. Material and Methods

### 2.1 Material.

Chitosan (95% deacetylation, 150 kDa, pharmaceutical grade) purchased from CV. ChiMultiguna, Indonesia, 2,5-bis-(4-hydroxy, 3,5-dimethyl)-benzylidene-cyclopentanone (pentagamavunon-1, PGV-1) purchased from Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia, sodium tripolyphosphate (Na TPP), sodium hydroxide (NaOH) and acetic acid were purchased from Bratachem, Indonesia. Folic acid (FA), *N*-(*e*-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC hydrochloride), dimethyl sulfoxide (DMSO), doxorubicin hydrochloride (DOX-HCl) and phosphate buffer saline (PBS) tablet were purchased from Sigma-Aldrich.

### 2.2 Preparation of chitosan modified folic acid.

Conjugation of folic acid (FA) to chitosan was prepared using the method as described in Yang, *et al* (2010) [11]. The FA was attached to chitosan with 0.2 mol ratio to 1 mol chitosan. A 250 mg of chitosan (1.55 mmol) was dissolved in 50 ml of 1M acetic acid. The 20 mL DMSO solution containing 136.83 mg (0.31 mmol) of FA and 59.43 mg (0.31 mmol) of EDC hydrochloride then added to chitosan solution and continuously stirred using magnetic stirrer for 18 hour in the dark. After 18 hour, 1 M sodium hydroxide was dripped into the solution to bring the pH to 9. The mixture then centrifuged at 2500 rpm to precipitate the chitosan modified folic acid. Chitosan modified folic acid (chitosan-FA) then dissolved in 50 ml water and dialyzed against phosphate buffer saline (PBS) for 3 days then continuing dialyzed against water for 4 days. The solution containing chitosan-FA then freeze dried to get the sponge mass of chitosan-FA and stored at 4°C.

### 2.3 Confirmation of chitosan modified folic acid.

A 10 mg of chitosan-FA was dissolved in 10 ml of 1M acetic acid. The solution then diluted to 100 µg/ml using water and scanned with spectrophotometer UV-VIS (Genesys 10S) at 200-300 nm. The measured absorbance spectrum was compared with chitosan solution in acetic acid, folic acid solution in water and EDC hydrochloride in water.

### 2.4 Preparation of chitosan-FA nanoparticle.

The nanoparticle of chitosan-FA was prepared using a method as described in Zhang *et al* [15] with modification. A 100 mg of chitosan-FA was dissolved in 200 ml of 0.5% acetic acid solution containing 0.1 and 0.5% v/v of tween 80 to give polymer concentration 0.05% w/v. The mixture was stirred for 3 minutes at 1500 rpm using magnetic stirrer (IKA). Afterwards, the pH was measured and adjusted to 4 using 1N sodium hydroxide. A 20 ml of 0.1% Na TPP then added by slow dripping under magnetic stirrer to form nanoparticle. The mixture then continued to stir for 4 hours and isolated by centrifugation at 12,500 rpm. The precipitated mass then freeze dried to give dry particle and keep in the dark at 4°C.

The chitosan-FA nanoparticle loading with drugs was prepared using the method described above with addition of 1 ml DOX (2 mg/mL) and PGV-1(10 mg/ml) to give the final concentration of 2% w/w and 10%

w/w drugs to polymer, respectively. Chitosan nanoparticle also prepared using the same method in preliminary experiment. The variables applied for the nanoparticle formulation is given in **Table 1**.

Table 1. Formulation of chitosan and chitosan-FA nanoparticles

Polymer	Tween 80 (%v/v)	Active substances
Chitosan	-	DOX-PGV-1
Chitosan-FA	-	-
Chitosan-FA	0.1	-
Chitosan-FA	0.5	-
Chitosan-FA	0.5	DOX-PGV-1

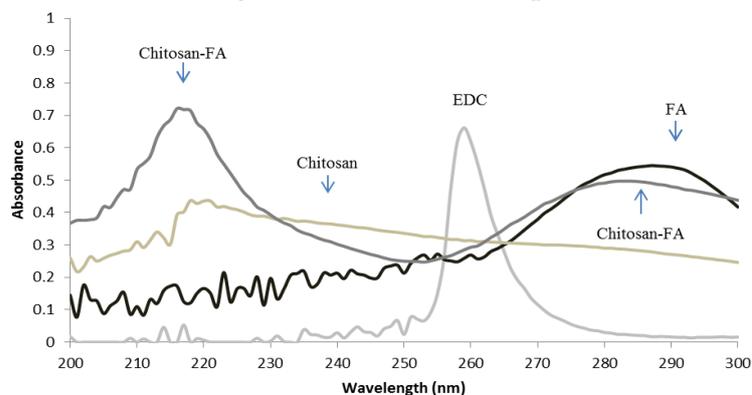
**Evaluation of particle size.** Particle size and polydispersity index of nanoparticles were evaluated by laser diffraction analysis using particle size analysis (PSA, Horiba SZ100) at  $25 \pm 0.5^\circ\text{C}$ . Ten milligram of nanoparticle was dispersed in aqua pro injection and directly measured using PSA to obtained particle size and polydispersity index. All measurements were performed triplicate.

### 3. Result and Discussion

#### 3.1 Preparation and identification of chitosan modified folic acid.

The chitosan modified folic acid (chitosan-FA) was successfully synthesized with  $53.2 \pm 0.02\%$  of yield. Sequential process involved during chitosan-FA preparation including purification by dialysis might reduce the yield of chitosan-FA. During dialysis process, the unbound folic acid (FA) and soluble EDC hydrochloride diffused outside of the dialysis membrane and removed from chitosan-FA.

Conjugation of chitosan FA as well as purification process was confirmed via UV absorption spectra. The UV absorption spectra of chitosan, FA, EDC hydrochloride are shown in **Fig. 1**.



**Fig. 1.** Confirmation of chitosan-FA conjugation and purification by UV absorption spectroscopy. Chitosan (2.5 mg/ml) and chitosan-FA (100  $\mu\text{g}/\text{ml}$ ) were measured in 1 M acetic acid solution while folic acid (2  $\mu\text{g}/\text{ml}$ ) and EDC hydrochloride (10  $\mu\text{g}/\text{ml}$ ) were measured in water.

Chitosan has maximum absorbance at  $\lambda$  218 nm, related to chromophore groups from N-acetyl glucosamine and glucosamine [16]. However, the absorption peak was weak compared to chitosan-FA. Chitosan-FA absorbance reached maximum at  $\lambda$  216 nm and 282 nm with high intensity related to  $n \rightarrow \pi^*$  and  $\pi \rightarrow \pi^*$  transition corresponding to amide bond formation and C=C bond transition in chitosan-FA [17]. FA itself has the maximum absorbance at  $\lambda$  287 nm related to  $\pi \rightarrow \pi^*$  of aromatic ring in folic acid. The unrepresented of EDC hydrochloride peak in chitosan-FA revealed that the dialysis method can be used for chitosan-FA purification.

#### 3.2 Preparation and particle size determination of nanoparticles.

Ionic gelation method was used for nanoparticle preparation by interaction of positive charge of chitosan and negative charge of Na TPP. In this research, the effect of tween 80 on particle size was investigated. For nanoparticle delivery purposes, the size of particle may vary from 10 nm-1000 nm. However, the smaller size of particle will be easier to uptake by the cell and has a larger surface area. As consequences, a smaller particle will be related to faster drug release [18].

In order to obtain desired nano-size particle, at the beginning of experiment, the chitosan-FA nanoparticle was prepared without loaded with active substances. It can be seen at Table 2. that the chitosan-FA NP without tween 80 lead to the formation of micro-size particle and high polydispersity index (PI). However, preliminary studies using the same method showed that the method can be used to produce nano-size particle with polydispersity index under 0.7 indicating homogeneity of particle. This diverse result might relate to folate conjugation into chitosan molecule. Addition of folate molecule into chitosan backbone affected chitosan molecular properties. Chitosan-FA had higher molecular size than chitosan itself. High molecular size of polymer revealed to produce bigger particle size as evaluated in earlier experiment by Caetano *et al* and Sarwar *et al* [19, 20].

Table 2. Particle size and polydispersity index of chitosan and chitosan-FA nanoparticles

Polymer	Tween 80 [%v/v]	Active substances	Particle size [nm] $\pm$ SD	Polydispersity index [PI] $\pm$ SD
Chitosan	-	DOX-PGV-1	812.8 $\pm$ 11.94	0.50 $\pm$ 0.21
Chitosan-FA	-	-	5581.4 $\pm$ 33.67	4.25 $\pm$ 1.92
Chitosan-FA	0.1	-	554.2 $\pm$ 40.46	0.44 $\pm$ 0.12
Chitosan-FA	0.5	-	476.6 $\pm$ 11.94	0.49 $\pm$ 0.06
Chitosan-FA	0.5	DOX-PGV-1	111.8 $\pm$ 4.11	0.50 $\pm$ 0.21

Tween 80 addition during nanoparticle preparation improved the particle size of chitosan FA into nano-scale (Table 2). Nevertheless, the addition of 0.5% v/v of tween 80 leading to the formation of smaller particle size compared to 0.1% v/v tween 80. The result indicated that tween 80 might act as stabilizing agent during nanoparticle formation and reducing the surface energy leading to inhibition of crystal growth [21]. Conversely, the experiment by Asasutjarit *et al* reported that increased tween 80 concentration ranged from 0 to 0.03% v/v acted as amphiphilic molecule and can be deposited at the surface of particle resulting in rising of particle size [22]. It can be determined that different range of tween concentration applied during nanoparticle preparation could be altered the size of particle by diverse effect.

The 0.5% v/v tween 80 then continue to use for preparing chitosan-FA loaded with active substances, DOX and PGV-1. Evaluation on particle size showed that the size of particle was 111.8  $\pm$ 4.11 nm with the addition of active substances. This size was lower than unloaded chitosan-FA prepared using the same method. Generally, the size of particle will be bigger with the presence of active substance although the increasing the particle size are not significant [23, 24]. This unexpected result might be related to the addition of active substances, mainly DOX, since DOX added to the formula in was the form of doxorubicin hydrochloride. The presence of hydrochloride salt from DOX in nanoparticle preparation might contribute to solubility of the chitosan-FA in acetic acid, hence, the fine particles were formed. However, the other aspect related to nanoparticle morphology, encapsulation efficiency of active substances and drug release should be investigated.

#### 4. Conclusion

This study present that chitosan-FA was successfully conjugated and purified as confirmed by UV absorbance. Moreover, this research present that addition tween 80 affected the size of chitosan-FA nanoparticle. The higher concentration tween 80 applied during formulation decreased the size of nanoparticle. The desired nanosize particle was achieved using 0.5% v/v tween 80 and need further identification using scanning electron microscope.

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