INCREASING AMOUNT AND ENTRAPMENT EFFICIENCY OF CHITOSAN-KETOPROFEN NANOPARTICLE USING UTRASONICATION METHOD WITH VARIED TIME AND AMPLITUDE

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ABSTRACT

Chitosan nanoparticles loaded by ketoprofen have been prepared by sonication and centrifugation methods. Preliminary tests have been carried out by conducting optimization condition of sonication including amplitude and sonication time. Turbidity data showed that the optimum condition for sonication on amplitude and sonication time at the percentage of 20% and at 60 minutes, respectively. PSA analysis indicated that decreasing of turbidity number of emulsion was also reduced particle size. PSA results showed that all formulas have a uniform particle size up to 100% with polydispersity index was below 0.3. B formula has smaller particle size and highest entrapment efficiency value than two formulae others, 222,1 nm and 87,463%, respectively. SEM and XRD analysis showed that chitosan nanoparticles loaded by ketoprofen have spherical form and semi crystalline properties, respectively.

Keywords*: chitosan, index polydispersity, entrapment efficiency, nanoparticle size.*

1. INTRODUCTION

Ketoprofen is an active component of non-steroidal anti-inflammatory drugs (NSAIDs). It is often found in commercial rheumatic drugs. Ketoprofen is not water soluble, it has low bioavailability and fast elimination time which is only 1.5–2 hours so the drug should be frequently consumed [1]. There are some way to overcome these weaknesses. One of them is by encapsulating ketoprofen with a biopolymer that acts as a drug delivery system. Pharmaceutical dosage that form nanospheres /microspheres where the drug adsorbed and or trapped in a drug carrier system is a pharmaceutical dosage form that capable of controlling drug release and accurately find the target right after oral prescription.

Chitosan is a biopolymer that has been widely investigated as a drug delivery system. Chitosan have been selected as a drug delivery system due to approval of Food and Drug monitoring agency (BPOM) with No.HK.00.05.52.6581 stated that chitosan can be used in food products. Utilization of chitosan has also received approval in other countries in the world such as the U.S which patently set chitosan as a Generally Recognised As Safe (GRAS) product by FDA. In its application, chitosan is very fragile so it is need chemical and physical modification to improve its quality. One of the chemical and physical modification that have been done in a row is to add crosslinking compound, such as sodium tripolyphosphate (STPP) and by forming a nanoparticles chitosan.

Research on chitosan as drug delivery has been widely applied. Kumar [3] have synthetized chitosan nanoparticles filled with poly (ethylene oxide) with particle sizes ranging from 200 – 1000 nm. Wu *et al.* [4] have synthesized chitosan nanoparticles as a drug containing ammonium gliserrizinat as antihepatitis agent through ionic gelation process using STTP and generate particle size in the range of 20 – 80 nm. Wahyono *et al.* [2] have synthesized chitosan nanoparticles filled with ketoprofen through ionic gelation process using STTP and oleic acid surfactat and produce a particle size ranging from 380 – 900 nm. Wahyono *et al* [2] utilized ultrasonication method combined with centrifugation. Ultrasonication conditions that being used are 130 watts power, 20 kHz frequency for 30 minutes and 20 000 rpm centrifugation for 2 hours. Although the particle produced was included into nanoparticle category, however, its polidispersity index was still larger than 0.3. It mean that the particle size is not uniform and the are micro-sized particles prescence approximately about 42%. Therefore, further research is needed to reduce polydipersity index untill it reach 0.3 in order to increase the number and the uniformity of nanoparticles so that adsorption efficiency is increased.

The use of ultrasonic waves for the formation of nanoparticles is one of the effective methods. Ultrasonic frequency ranging from 20 kHz – 1 MHz is widely used in chemistry. This method called as sonochemistry. Sonochemical principle is closely linked to the acoustic cavitation phenomenon. It is include the formation, growth and rupture of

bubbles formed in the liquid medium [5]. Cavitation is influenced by several factors including: amplitude, temperature, pressure, concentration, and viscosity [6]. On the cavitation phenomenon, ultrasonic wave that spread in the liquid medium has the ability to generate bubble or cavity in a liquid medium. When the ultrasonic wave spred in a liquid medium, strain and density cycle is occured. Pressure decreasing resulted by a strain formed a bubble that will absorb the energy from ultrasonic waves, so the bubble can expand to a maximum size and eventually rupture [6]. The outbreak of the bubble caused an extreme condition where the local temperature rise untill 5000 K and the tpressure reaches 1000 atm with heating to cooling speed was higher than 1010K/s [5]. These extreme conditions cause the termination of the chemical bond theory called Hot Spot. Ultrasonication process with high amplitude accompanied by a time increasing will produce a high energy that used for cavitation process and will affect into gradual decreasing of particle size [7-8].

Kencana [9] produced chitosan nanoparticles sized 300 – 600 nm with ultrasonication method using 20 kHz frequency for 60 minutes. Hapsari [10] reported that the longer sonication time, the size distribution of polymeric *ferrofluid* and *polylactic acid* (PLA) tend to be smaller and more homogeneous. Ultrasonication method at frequency of 20 kHz for 20 minutes, followed by centrifugation at a speed of 15 000 rpm for 10 minutes produces chitosan nanoparticles sized around 200 – 500 nm [11], while the frequency of 20 kHz for 5 minutes, followed by centrifugation for 15 minutes at 6000 rpm produced chitosan nanoparticles sized around 39 – 300 nm [12].

This research was conducted as a follow-up study of Wahyono *et al.* [2] with the composition of the material used is based on its three best formula. To optimize the purposes of this study, the method of nanoparticle formation [2] especially in the ultrasonication modified by varying the time and amplitude. Time and amplitude variation that being used are 30, 45, 60, 75 minutes, and 20%, 25%, 30%, 35%, 40%. Modification of the method is expected to increase the number of nanoparticles and its encapsulation effeciency.

2. EXPERIMENTAL SECTION

2.1.Materials

The materials used were commercial chitosan purchased from Bratachem (DD 70.15% and 3×10^5 g/mol Molecular Weight), acetic acid 2%, oleic acid p.a., ethanol, ketoprofen is obtained from PT Kalbe Farma, STPP, and pH 7.2 Phosphate buffer solution. Nanoparticle formulation are shown in Table 1 [2].

Table 1 P, A and B formulation based on chitosan, sodium tripolyphospate (STPP), and oleic acid concentration.[2]

Formula	Chitosan	STPP	Oleic acid
	% (b/v)	(mg/ml)	(mg/ml)
P	3,00	0,84	1,5
A	2,5	0,84	0,1
B	2,5	0,84	0,8

2.2 Instrumentation

The equipment used are including Homogenyzer Ultra-Turax T8, Turbidi-meter type 2100 P, Viscometer TV-10, Ultrasonic processor Cole Parmer 130 Watt 20 kHz type probe CV 18, High Speed Himac Sentrifuse CR 21G, Particle Size Analyzer (PSA) Delsa Nano C, Buchi spray drier 190, Infrared frontier Spectrophotometer (FTIR) Perkin Elmer type Spectrum One Series, UV-Vis Spectrophotometer type UV-1700 PharmaSpec, shaker, Scanning Electron Microscopy (SEM) JEOLJSM-6360LA, pH meter Toa HM-20S, and XRD PW 1710 Philips.

2.3 Procedure

2.3.1 Optimation of Nanoparticle Formation [2]

This study begins with determination of optimum sonication of the P formula. Furthermore, the optimum conditions that have been reached was applied to A and B formula. Chitosan nanoparticles filled with ketoprofen was prepared by mixing chitosan, STTP and oleic acid. Chitosan was dissolved in a solution of 2% acetic acid, ketoprofen and oleic acid were dissolved in ethanol separately, and STTP was dissolved in distilled water. 50 ml of chitosan solution was added with 20 ml STTP and 20 ketoprofen 0.2 mg/L solutions. The mixture was stirred with a magnetic stirrer for 30 minutes at 400 rpm, then it added with 10 ml oleic acid. Emulsions formed was then drained with

ultrasonic waves with a frequency of 20 kHz per 25 ml for 30 minutes with an amplitude of 20%. Furthermore, each sample was sentrifuged at 19 000 rpm (RCF = 43320 g) for 2 hours. Every treatment completed was followed with turbidity measurements. Turbidity was meaured twice for both sinication and centrifugation treatment. Supernatant in the form of nanoparticles suspensions was then characterize to figure out its size, morphology and functional group. This work ws repeated by varying the time and amplitude choosen and performed with three replication, so the total formula was 96. Size measurement of ketoprofen nanoparticle conducted using PSA. While identification of its morphology and degree of crystallinity were analyzed by using SEM and XRD respectively [13].

2.3.2 Determination of Encapsulation Efficiency [14]

A total of 25 mg chitosan nanoparticles were weighed and dissolved in 50 ml pH 7.2 phosphate buffer. The mixture was shaken for 24 hours and then filtered. The absorbance of filtrate obtained is determined with a UV Vis spectrophotometer at 259.5 nm. Measured absorbance values are used to determine ketoprofen concentration with the help of standard calibration curve. Encapsulation efficiency (E) was calculated by the equation below:

$$E = \frac{\left(x \frac{mg}{l} x \frac{1L}{1000\ ml} \text{ x Extraction volume x} \frac{a\ mg}{b\ mg}\right) \text{x } 100\%}{\text{Initial ketoprofen Mass (mg)}}$$

with:

x = x value from standard calibration curve
a = total nanoparticle mass obtained
b = Nanoparticle mass that being used for determination of efficiency

3. RESULT AND DISCUSSION

3.1 Optimum Condition of Sonication in Nanoparticle Formation

The optimum condition that being observed are ultrasonication time and amplitude. The length of time and the amplitudo used in the sonication greatly affects the resulting energy, and this energy is affecting the cavitation process. Energy is directly proportiona; to the exposure time and power, it is formulated by E (J) = P (W) x t (s) [where: E = energy (joule); P = power (watt); t = time (detik)] [8]. Under this conditions, the amount of energy produced by sonicator can be calculated theoritically (Table 2.). The result shows ther is a differences between the experimental energy with and theoritical energy.This is due to the solution exposure is being done in an an open system so that the fraction of energy wasted as heat to the environment.

Table 2 Theoritical and experimental energy comparison

Time (minutes)	Experimental energy (J)		Theoritical Energy (J)*	
	20%	40%	20 %	40%
30	4188	10256	46800	93600
45	6344	14744	88920	177840
60	8185	19886	93600	187200
75	10285	24447	117000	234000

*Theoritical energy being calculated using P (power) 130 watt

Data in Table 3. Illustrates that the higher the amplitude of sonication at fixed time 30 minutes can result a greater energy. The amount of energy greatly affecting the breakdown of molecules in the emulsion. Huge energy is expected to reduce the particle size to be smaller. Turbidity measure the level of uncleareance of a solution or emulsion. The bigger the size of the particles in the solution, the higher its turbidity. Morris *et al.* [15] reported that the lower the value of turbidity in a solution, the smaller particle size will be produce. In this study, the use af any magnitude in a phase with a fixed 30 minutes ultrasonication time showing decreased value of turbidity significantly after sonication ad centrifugation tratment. Another phenomenon observed is the condition when the value of amplitude is increased, the turbidiy will incrase too, however, the change is not significant. The rising value of turbidity may be due to an increase in cavitation energy which decreases the stability of the emulsion thus might make the particle was aglomerated one and another. Rupture of stability is the main cause of increasing turbidity. The conclusion from this data is that when 20% amplitude were being used, cavitation energy has been able to reduce turbidity value that suspected to impact on particle size. Therefore, further ultrasonication conducted using 20% amplitude. This condition is then used to determine the best time of sonication to produce nanoparticles.

Table 3. Turbidity value after sonication and centrifugation for 30 minutes using different amplitude for P formula

Formula	Sonication		Turbidity (NTU)		
	A (%)	E (Joule)	Initial	Sonication	centrifugation
P1	20	4188	224,00	141,80	121,60
P2	25	5645	224,00	143,60	123,60
P3	30	7195	224,00	145,00	124,60
P4	35	8635	224,00	148,60	126,40
P5	40	9710	224,00	149,20	127,80

It is also seen in Table 4. that the longer sonication time with fixed 20% amplitude produce grater energy. The length of ultrasonication time tends to make the solution homogeneous and lower the particle size distribution [10]. Data in Table 4. showed that the use of a fixed value of the amplitude range of the higher sonication time can reduce the value of a good emulsion turbidity after sonication and centrifugation treatment. However, another phenomenon was observed when using 75 minutes even though the changes are not significant. With time increasing, the cavitation energy is increased too and risng temperature affected the emulsion. High temperature rise is the evidenced by the reduction in volume of 3 – 4 ml solution. High temperature rise causes the solvent reached the boiling point and produce larger cavitation bubbles, which inhibits the activity of sound transmission process and negate the effect of energy that allows particle aglomerated back [8]. From the result of this study, it is concluded that the optimum conditions for the formation of nanoparticles chitosan-STPP contains ketoprofen using ultrasonication is at 20% amplitude with duration of 60 minutes.

Table 4. Turbidity value after sonication and centrifugation using 20% amplitude with different time length for P formula

Formula	Sonication Time (minutes)	Energy (Joule)	Initial turbidity (NTU)	After Sonication (NTU)	After Centrifugation (NTU)
P0	30	4190	224,0	141,8	121,6
P1	45	6344	229,0	176,4	95,0
P2	60	8885	229,0	168,0	88,4
P3	75	10101	229,0	181,0	96,4

3.2 Nanoparticle Size and adsorption efficiency

Determination of ketoprofen nanoparticle size performed using PSA. Characterization was carried out at 25^{o}C, using water as solvent with refractive index 1.333 and degrees of viscosity 0.8878 cP. Measurement of PSA has three basic distributions which are intensity, volume, and number distribution. Each distribution shows the range of sizes and the percentage, the average particle size and polydispersity index. Polydispersity index as an indicator of uniformity of size and uniform particle size is when the polydispersity index is less than 0.3. In this research, the basic distribution used is the number distribution because it has been known that the majority of ketoprofen nanoparticles are spherical. Spherical shape is determined using SEM. The data in Table 5. showed that all the formulas in the ultrasonication treatment with amplitude of 20% fro 60 minutes produced below 1000 nm sized particles close to 100% and the third formula has uniform size because it polydispersity index was less than 0.3. From the third formula, the average size of the nanoparticles is B formula is 222.1 nm which is the smallest, followed by P and A formula with respectively have 366.4 and 381.2 nm ofe average size.

Table 5. Turbidity, size, polydispersity, number, and adsorption efficiency relation of P, A, and B nanoparticle at 60 minutes sonication using 20% amplitude

Formula	concentration*		Turbidity (NTU)	Size Average (nm)	IP	Number of nanoparticle (%)	EP (%)
	C (%b/v)	Oleic Acid (mg/mL)					
P	3,0	1,5	88,4	366,4	0,259	99,99	81,856
A	2,5	0,1	36,6	381,2	0,225	99,77	70,662
B	2,5	0,8	70,6	222,1	0,275	99,99	87,364

Note: C = Chitosan, IP = Polydispersity Index dan EP = Entrapment Efficiency.

The difference in the average size of each formula is due to differences in the composition of its nanoparticles constitutent [16]. P formula has grater an average particle size than B formula but smaller than A formula. P formula used higher chitosan concentration than two other formulas, so the emulsion viscosity was higher so the solving process becomes les effective. Meanwhile, the A formula has the largest average size between the other two formulas, this is due to the low concentration of oleic acid which only 0.1 mg/ml thus might not be able to stabilize the particles making the particle aglomerated back. Role of oleic acid is to lower the interfacial energy of solution so it can prevent the onset of surface aggregates. Size range of the third formula was 10 – 1000 nm [17–18].

Nakorn *et al.* [11] produce chitosan nanoparticles sized 215–420 nm by dissolving 20 mg of chitosan into 40 ml acetic acid 2.0% (v/v) wrer added 20 ml STPP 0.75 mg/ml and then sonicated for 5 min and centrifuged for 15 min at 6000 rpm. Wahyono *et al.* [2] has resulted in chitosan nanoparticles with average size of 380 – 900 nm by using chitosan concentration of 3% (w/v), STPP 0.84 mg/ml, and oleic acid 1 frequency of 20 kHz in 30 minutes and cenrifuged at 20.000 rpm within 2 hours. Saha *et al*. [12] chitosan nanoparticles with size of 200 – 500 nm with a polydispersity index below 0.3. He mixed the STPP to chitosan solution (not specified concentration) were sonicated for 20 minutes, then centrifuged at 15.000 rpm for 10 minutes. This study was able to produce almost 100% nanoparticles when compared with previous research, which only reach 58.08% [2], as well as its nanoparticle size.

Adsorption efficiency can describe how much ketoprofen adsorbed in chitosan nanoparticles. The data in table 5 showed that the efficiency of ketoprofen adsorption for formula P, A, and B respectively are 81.856%, 70.663%, ad 87.364%. From the data in table 5 we can see the relationship between average particle size with adsorption efficiency. The smaller the average particle size, the greater the adsorption eficiency. B formula has the highest ketoprofen adsorption efficiency compare to two other formulas. This is because the B formula has the smallest average particle size which is 222.1 nm. The smaller the particle, the grater is its surface area. With the growing amount of surface area, adsorption ability of chitosan nanoparticle is increased. This adsorption efficiency value is higher than those previous study with adsorption efficiency value of hydrazin with chitosan-STPP nanoparticle is 15.8% [19] *amonium glycyrrhizinate* with Chitosan-STTP modified with polyetylene glycol is 63% [4], and ketoprofen with chitosan-STTP nanoparticle is 79,79% [2]. According to Cho *et al.* [19], there are to ways of hydralazin entry into chitosan nanoparticles, the first is only adsorb at the surface and the other is when the hydralazin absorbed into the cavity of chitosan nanoparticles (Figure 1). If the surface area is wide then there is higher possibility that the ketoprofen are being adsorb at the surface so it can go out. On the orther hand, the trapped ketoprofen will need extra time to be released. Those thing is the reason behind the differences of adsorption efficiency value. Ketoprofen nanoparticle characterization is then using B formula ketoprofen.

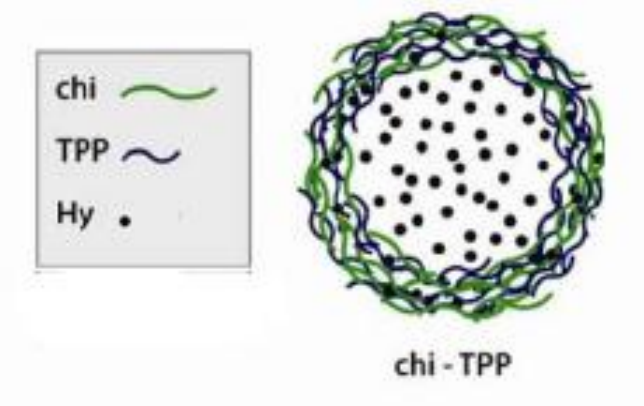

Figure 1. Hydralazin coating in chitosan-TPP nanoparticle [19]

3.3 Morphological and crystalinity degree characterization

Morphological characterization was conducted to identify the shape and uniformity of size of the nanoparticle. Characterization is conducted using SEM analysis. SEM photo of formula B chitosan nanoparticle (Figure 2.) showed that ketoprofen loaded chitosan nanoparticles have a spherical shape intact and separate from one another. The agnitude of the particle size looks more uniform and is supported by data from PSA which gives its index polydispersity value less than 0.3.

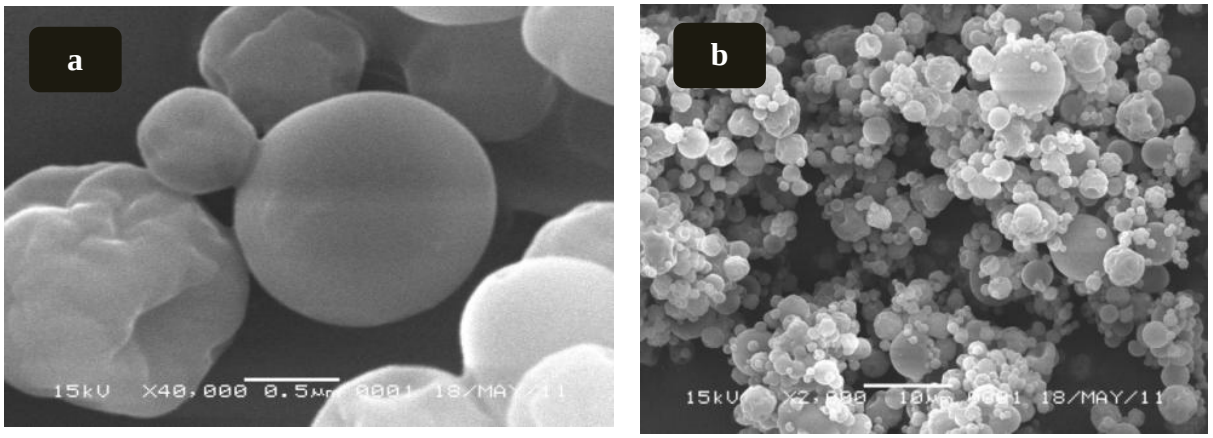

Figure 2. SEM photograph of B Formula at 40.000x (a), and 2000x (b) magnification.

XRD analysis performed to determine the phase that forms on the sample and its degree of crystallinity. Pure chitosan diffractogram patterns indicate different angles with high intensity at 2θ 10,186^0; 19,958^0 and 22,048^0. Meanwhile, diffractogram pattern of ketoprofen loaded chitosan nanoparticles showed a few peaks at 2θ 20,7046^0; 23,4860^0; 44,0646^0; 77,5456^0; 64,4362^0. 2θ pure chitosan at 19,958^0 and 22,048^0 indicated shifted into 20,7046^0 and 23,4860^0 and its intensity become lower, this is due to chitosan was crosslinked with STPP. The degree of crystallinity is a quantity that specifies the number of crystals content in a material by comparing the area of the crystalline and amorphous curve. Higher degree of crystallinity of a material make that material become more crystalline. The lower degree of crystallinity make them become more amorf. Among the amorf and crystalline phases, there is a semi crystalline phases. From diffractogram analysis, B formula has 48% degree of crystallinity, those value is higher than pure chitosan with only 37% degree of crystallinity. Ketoprofen encapsulation process is able to increase the degree of crystallinity and change the chitosan which amorf at the begining to become semi crytalline. The increase of this degree of crystallinity is also indicated that chitosan matrix is filled with ketoprofen.

4. CONCLUSIONS

Optimum condition of chitosan nanoparticle synthesis loaded with ketoprofen was when using sonication methode at 20% amplitude for 60 minutes. SEM and XRD analysis shows that chitosan nanoparticle loaded with ketoprofen

has spherical form and is semi-crystalline, respectively, while the PSA analysis shows that all three formula sized less than 400 nm with polydispersity less than 0.3. B formula was the best formula for having the average particle size of 222,1 nm with adsorption efficiency reached 87,364%.

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