ผลของสารลดแรงตึงผิวต่อการเกิดขนาดอนุภาคระดับนาโนเมตรของไคโทซาน

นางสาวชุติมา สาราทิศ

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EFFECT OF SURFACTANTS ON THE FORMATION OFCHITOSAN NANOPARTICLES.

Ms. Chutima Saratid

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Petrochemistry and Polymer Science

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Thesis Title	Effect of Surfactants on the Formation of Chitosan Nanoparticles
By	Miss Chutima Saratid
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Piamsak Menasveta , Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Supawan Tantayanon, Ph.D.)

.....Thesis Advisor
(Associate Professor Mongkol Sukwattanasinitt, Ph.D.)

.....Member
(Assistant Professor Varawut Tangpasuthadol, Ph.D.)

.....Member
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

ชุติมา สาราทิศ : ผลของสารลดแรงตึงผิวต่อขนาดอนุภาคระดับนาโนเมตรของไคโทซาน (EFFECT OF SURFACTANTS ON THE FORMATION OF CHITOSAN NANOPARTICLES)
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ได้ทำการศึกษาผลของสารลดแรงตึงผิวที่มีต่อการเกิดอนุภาคขนาดนาโนเมตร ของไคโทซาน ในการศึกษาได้นำไคโทซานมวลโมเลกุลสูง มวลโมเลกุลปานกลางและมวลโมเลกุลต่ำ (Mv = 10^6, 1.5×10^5 และ 10^4) มาผสมกับสารลดแรงตึงผิวในระบบไมเซลล์ โดยใช้เทคนิคในการผสม 3 เทคนิค คือ เทคนิคการกวน (stirring) การโซนิเคต (sonicating) และ การวอร์เทกซ์ (vortexing) พบว่ามีเฉพาะสารลดแรงตึงผิวที่เป็นประจุลบเท่านั้น เช่นเอโอที (เอทิลเฮกซิล ซัลโฟซักซิเนต) และเอสดีเอส (โซเดียมโดเดซิลซัลเฟส) ที่สามารถทำให้ได้อนุภาคระดับนาโนเมตรกับไคโทซานในตัวกลางที่เป็นน้ำ ที่ความเข้มข้นของไคโทซานเป็น 2.5 มิลลิโมลลาร์ (mM) จะได้อนุภาคนาโนเมตร เมื่อ อัตราส่วนโดยโมลของสารลดแรงตึงผิวต่อไคโทซานมีค่ามากกว่า 14 ต่อ 1 สำหรับเอโอที และมากกว่า 20 ต่อ 1 สำหรับเอสดีเอส ซึ่งให้อนุภาคขนาด 110±10 นาโนเมตร วัดโดยเทคนิค light scattering และมีประจุที่พื้นผิวของอนุภาคเท่ากับ -47.42±5 มิลลิโวลต์ วัดโดยใช้เครื่องเซตาไซเซอร์ (zetasizer) และพบว่าอนุภาคนาโนไคโทซาน/เอโอทีสามารถดูดซับยาเททระไซคลิน (tetracycline) เข้าไปได้ 50% และอนุภาคที่ได้เก็บไว้ได้นานกว่า 1 เดือน

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา.....

4773404723 : MAJOR PROGRAM OF PETROCHEMISTRY AND POLYMER SCIENCE
KEY WORD: CHITOSAN / ANIONIC SURFACTANT / AOT / SDS / TETRACYCLINE

CHUTIMA SARATID : EFFECT OF SURFACTANTS ON THE FORMATION OF CHITOSAN NANOPARTICLES. THESIS ADVISOR : ASSOC. PROF. MONGKOL SUKWATTANASINITT, 79 pp. ISBN 974-53-2338-1.

The effects of surfactants on the formation of chitosan nanoparticles were studied. The high, medium and low molecular weight chitosan (Mv~10^6, 1.5×10^5 and 10^4) were mixed with the evaluated surfactants in a micellar system under different mixing technigues : stirring, ultrasonicating and vortexing. Only the anionic surfactants, sodium bis(ethylhexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS) allowed the formation of chitosan nanoparticles in aqueous media. At the chitosan concentration of 2.5 mM the formation of nanoparticle was observed when the mole ratio of surfactant/chitosan was higher than 14:1 and 20:1 for AOT and SDS, respectively. The size and zeta potentials of the particles determined by photo correlation spectroscopy and zetasizer were 110±10 nm and -47.42 ± 5 mV, respectively. The chitosan/AOT nanoparticles obtained can be loaded with tetracycline at AOT/chitosan/tetracycline mole raio of 20:1:1 with 50% entrapment efficiency. The suspensions of the chitosan/AOT nanoparticles were stable at ambient condition for at least 30 days.

Field of study Petrochemistry and Polymer Science Student's Chutima Saratid
Academic year 2005 Advisor's

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List of Abbreviations

nm	nanometer	CMC	carboxy methyl cellulose
ºC	degree celsius		
DI-water	deionized water	M	molar
g	gram (s)	min	minute
mV	millivolt	mL	milliliter (s)
h	hour	mM	millimolar
µm	micrometer	M_v	molecular weight
AOT	sodium bis (ethylhexyl) sulfosuccinate	sec	second
		β	beta
SDS	sodiumdodecyl sulfate	µL	microliter
TEM	transmission electron microscopy	%	percent
		%DA	percent degree of acetylation

List of Schemes

CHAPTER I

INTRODUCTION

Polymer nanoparticles have recently been widely investigated as a carrier for drug delivery. Nanoparticles have benefitial size depended properties for example the number of nanoparticles (<1 μm) crossing the intestinal epithelium is significantly greater than that of the microspheres (>1 μm). With their easy accessibility in the body, nanoparticles can be transported *via* the circulation to different body sites and the hydrophilic nanoparticles can generally circulate longer in the blood. Such systems can stay in body flouids long enough to reach specific target sites.[1] A use of colloidal carrier made of hydrophilic polysaccharides, e.g.chitosan, has arisen as a promising drug delivery system for improving the transport of macromolecules such as peptides, proteins, oligonucleotides and plasmids across biological surfaces. Surfactants, permeation enhancers, protease inhibitors, enteric coatings and bioadhesive microparticles or nanoparticles have all been used in the development towards the formation of drug delivery system.[2]

1.1 Chitosan

Chitosan is a polysaccharide containing, linearly β-(1→4)-linked 2-deoxy-*D*-aminoglucose (**Figure 1.1**).[3] Chitosan is generally obtained from the deacetylation of chitin (**Scheme 1.1**), a naturally occurring and abundantly available in marine crustaceans. Chitosan itself is also found in some microorganisms such as yeast and fungi.[4] The structure of chitin and chitosan are closely related to the most aturally abundant polymer, cellulose.

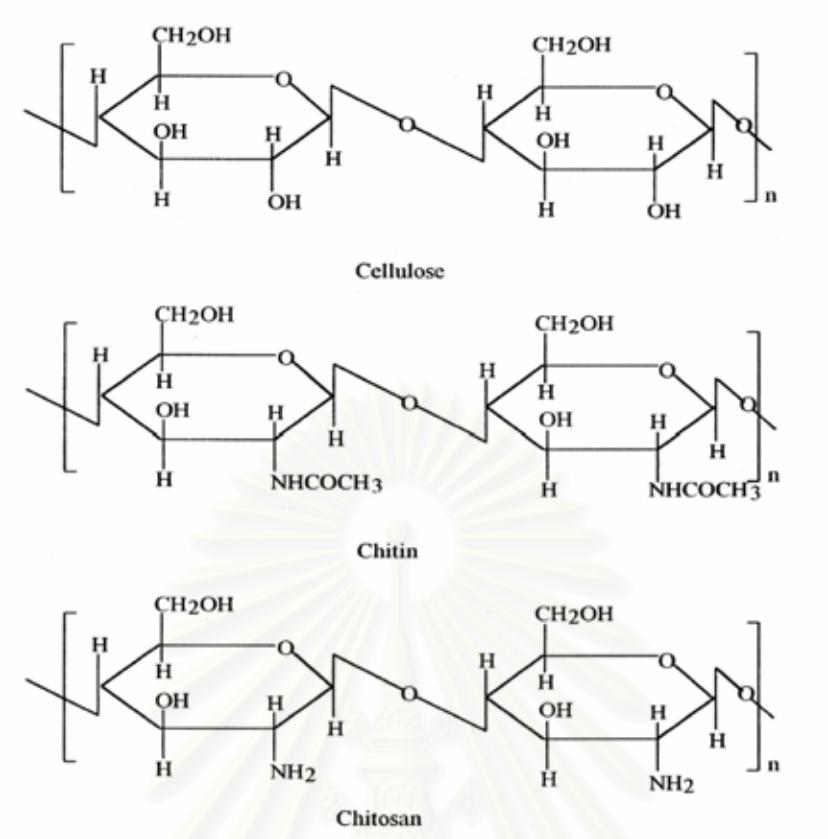
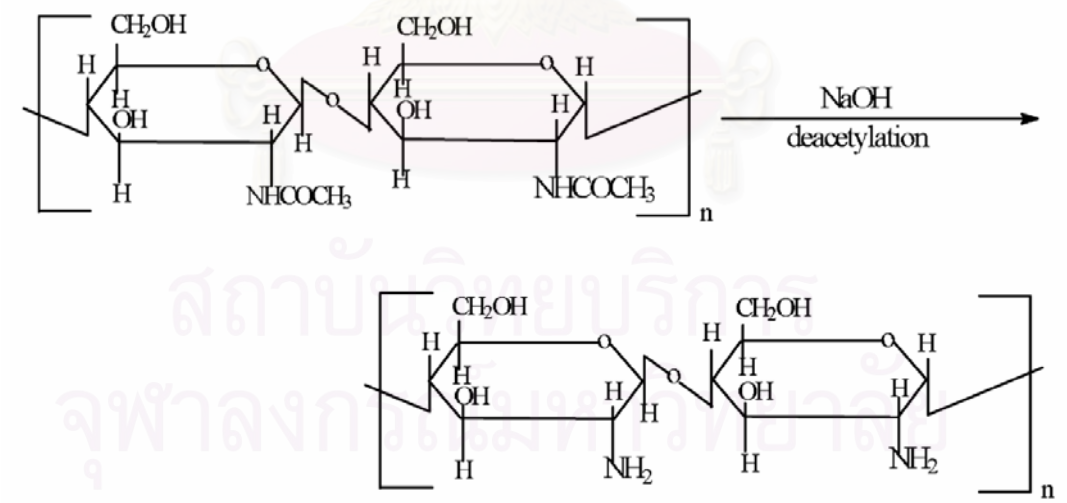


Figure 1.1 Structure of cellulose, chitin and chitosan



Scheme 1 Deacetylation of chitin to chitosan

Acetamide group of chitin can be converted into amino group to give chitosan, which is carried out by treating chitin with concentrated alkali solution. Chitin and chitosan represent long-chain polymers having molecular mass up to several million Daltons. Chitosan is relatively reactive that can be produced or fabricated into various forms such as powder, paste, film and fiber.[5-6] Chitosan is a weak base and is

therefore insoluble at alkaline and neutral pH but forms soluble salts with inorganic and organic acids such as hydrochloric acid, lactic acid, acetic acid and glutamic acid.[7] Usually 1–3% aqueous acetic acid solutions are used to solubilize chitosan, conferring to the polysaccharide with high positive charge density. Its polycationic nature allows for ionic crosslinking with multivalent anions. Solubility of chitosan depends on the distribution of free amino and *N*-acetyl groups resulted from the deacetylation process of chitin.

The primary amine groups render special properties that make chitosan very useful in pharmaceutical applications. Unlike many other natural polymers, such as dextran, pectin and arginic acid, chitosan has positive charges. Chitosan salts can bind strongly to negatively charged materials such as cell surfaces and mucus. It breaks down slowly to harmless products, amino sugars, which are completely absorbed by human body.[8] Chitosan is also degraded under the action of fermentation. It possesses antimicrobial property and absorbs toxic metals like mercury, cadmium, lead, *etc*. In addition, it has good adhesion, coagulation ability, and immunostimulating activity. If the degree of deacetylation and the molecular weight of chitosan can be controlled, then it would be a material of choice for developing biocompatible nanoparticles. Chitosan is currently extensively investigated for drug delivery systems.[9-13] Particularly, chitosan has been used in the preparation of mucoadhesive formulations, [14-17] improving the dissolution rate of the poorly soluble drugs, [18-20] drug targeting [21-22] and enhancement of peptide absorption.[23-24] Different types of chitosan-based drug delivery systems are summarized in **Table 1.1**.

Table 1.1 Chitosan-based drug delivery systems prepared by different methods for various kinds of drugs

Type of system	**Method of preparation**	**Drug**
Tablets	matrix	diclofenac sodium, pentoxyphylline, salicylic acid, Theophylline
	coating	propranolol HCl
Capsules	capsule shell	insulin, 5-amino salicylic acid
Microspheres	emulsion cross-linking	theophylline, cisplatin, pentazocine, phenobarbitone, theophylline, insulin, 5-fluorouracil, diclofenac sodium, griseofulvin, aspirin, diphtheria toxoid, pamidronate, suberoylbisphosphonate, mitoxantrone, progesterone
	coacervation/precipitation	prednisolone, interleukin-2, propranolol-HCl
	spray-drying	cimetidine, famotidine, nizatidine, vitamin D-2, diclofenac sodium, ketoprofen, metoclopramide-HCl, ovine serum albumin,ampicillin, etylpyridinium chloride, oxytetracycline, betamethasone
	ionic gelation	felodipine
Nanoparticles	emulsion-droplet coalescence	gadopentetic acid
	coacervation/precipitation	DNA, doxorubicin
	ionic gelation	insulin, ricin, bovine serum albumin, cyclosporin A
	reverse micellar method	doxorubicin
Beads	coacervation/precipitation	adriamycin, nifedipine, bovine serum albumin, salbutamol sulfate, lidocaine– HCl, riboflavin
Films	solution casting	isosorbide dinitrate, chlorhexidine gluconate, trypsin, granulocyte-macrophage colony-stimulating factor, acyclovir, riboflavine, testosterone, progesterone, beta-oestradiol
Gel	cross-linking	chlorpheniramine maleate, aspirin, theophylline, caffeine, lidocaine– HCl, hydrocortisone acetate, 5-fluorouracil

1.2 Methods for preparation of nanoparticles of chitosan

To prepare chitosan nanoparticulate systems, the strong hydrogen bonding attraction between the chitosan chains must be overcome to prevent aggregation of the particles. Currently, there are three generally known systems for the preparation of chitosan-based nanoparticles, polyanionic-cationic gelation system, reverse micelle method and structurally modified chitosan.

1.2.1 Ionic gelation

The use of complexation between negatively charged macromolecules such as DNA, tripolyphosphate (TPP) and carboxy methyl cellulose (CMC) to interact with positively charged chitosan by electrostatic forces, has attracted much attention because the process is very simple and mild **(Figure 1.2)**. In addition, reversible physical cross-linking by electrostatic interaction, instead of chemical cross-linking, has been applied to avoid the possible toxicity of reagents and other undesirable effects. Chitosan undergoes ionic gelation and precipitates to form spherical particles.[25]

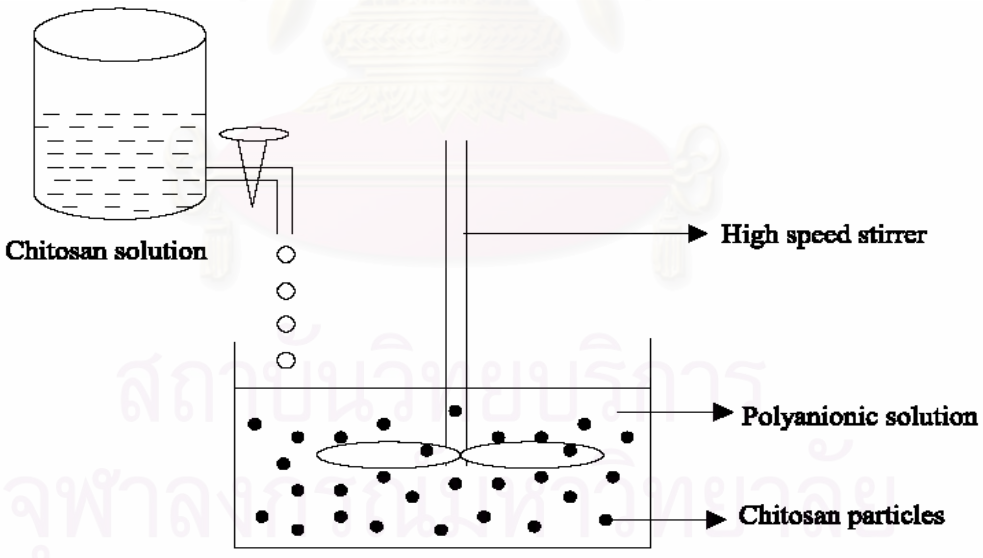


Figure 1.2 Preparation of chitosan particulate systems by ionic gelation.

1.2.2 Reverse micellar method

Reverse micelles are thermodynamically stable liquid mixtures of water, oil and surfactant. One of the most important aspects of reverse micelle hosted systems is their dynamic behavior. Preparation of ultrafine polymeric nanoparticles with narrow

size distribution could be achieved by using reverse micellar medium.[26] In this method, the surfactant is dissolved in an organic solvent to form reverse micelles. To this organic mixture, an aqueous solution of chitosan and drug is added with constant vortexing to avoid any turbidity. The aqueous phase is regulated in such a way as to keep the entire mixture in an optically transparent microemulsion phase. Additional amount of water may be added to obtain nanoparticles of larger size. A cross-linking agent may also be added to increase the stability of the particles. The maximum amount of drug that can be dissolved in reverse micelles varies from drug to drug and has to be determined by gradual increasing the amount of drug until the clear microemulsion is transformed into a translucent solution. The organic solvent is then evaporated to obtain the transparent dry mass. The material is dispersed in water and then a suitable salt is added into precipitates the surfactant out (**Figure 1.3)**.

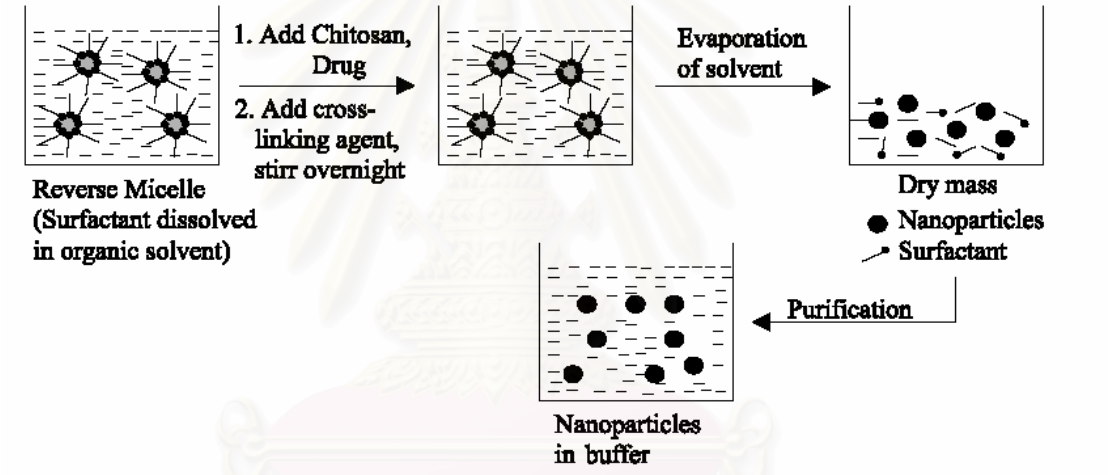


Figure 1.3 Schematic representation for preparation of chitosan particulate systems by reverse micellar.

1.2.3 Modification of chitosan molecule

Hydrophobically modified glycol chitosans (HGCs), prepared by covalent attachment of 5β-cholanic acid to glycol chitosan through amide formation, as a potential drug carrier. Depending on the degree of substitution of 5β-cholanic acid, the HGCs formed self-aggregates in an aqueous phase by intra and/or intermolecular association between hydrophobic 5β-cholanic acids.[27]

1.3 Literature reviews

In 2000, Hyroyuki, T and coworkers [28] studied the accumulation of gadolinium loaded as gadopentetic acid in chitosan nanoparticles formed by emulsion droplet. Gadopentetic acid-loaded chitosan nanoparticles were prepared for

gadolinium neutroncapture therapy. Particle size depends upon the type of chitosan, i.e., as the degree of deacetylation of chitosan decreased, particle size increased, but drug content decreased. Particles produced using 100% deacetylated chitosan had the mean particle size of 452 nm with 45% drug loading. Since gadopentetic acid is a bivalent anionic compound, it interacts electrostatically with the protonated amino groups of chitosan, which would not had occurred if a cross-linking agent was used that blocks the free amino groups of chitosan.

In 2001, Jane, K.A. and coworkers [29] evaluated the potential of chitosan nanoparticles as carriers for the anthracycline drug. Doxorubicin (DOX), a cationic and hydrophilic molecule, was entrapped into nanoparticles by ionic gelation of the positively charged polysaccharide chitosan with particle size 213 nm and zeta potential +33 mV. The nanoparticles were masked from the positive charge of DOX by complexing it with the polyanion, dextran sulfate. Despite the low complexation efficiency, no dissociation of the complex was observed upon the formation of the nanoparticles. Fluorimetric analysis of the drug released in vitro showed an initial release phase, the intensity of which was dependent on the association mode, followed by a very slow release. The evaluation of the activity of DOX-loaded nanoparticles in cell cultures indicated that those containing dextran sulfate were able to maintain cytostatic activity relative to free DOX, while DOX complexed to chitosan before nanoparticle formation showed slightly decreased activity. These preliminary studies showed the feasibility of chitosan nanoparticles to entrap the basic drug such as DOX and to deliver it into the cells in its active form.

In 2001, Angela, M.D.C. and coworkers [30] studied the potential of chitosan nanoparticles as a new vehicle for the improvement of the delivery of drugs to the ocular mucosa. Cyclosporin A (CyA) was chosen as a model compound. An ionic gelation technique was conveniently modified in order to produce CyA-loaded chitosan nanoparticles. These nanoparticles had a mean size of 293 nm, a zeta potential of +37 mV. In vitro release studies, performed under sink conditions, revealed a fast release during the first hour followed by a more gradual drug release during a 24-h period.

In 2001, Cui, Z. and coworkers [31] investigated the topical application of chitosan-based nanoparticles containing plasmid DNA (pDNA) as a potential approach to genetic immunization. pDNA-condensed chitosan nanoparticles and pDNA-coated on pre-formed cationic chitosan/carboxymethylcellulose (CMC)

nanoparticles were investigated. The particle size of 180-200 nm was obtained with zeta potential of +44 mV. Both chitosan and a chitosan oligomers can complex CMC to form stable cationic nanoparticles for subsequent pDNA coating.

In 2001, Mitra, S. and coworkers [32] studied doxorubicin (DXR) commonly used in cancer therapy produces undesirable side effects such as cardiotoxicity. To minimize these size effect, attempts have been made to couple the drug with dextran (DEX) and then to encapsulate this drug conjugate in chitosan nanoparticles. The size of these nanoparticles was found to be 100 - 610 nm diameter, encapsulation of the conjugate in nanoparticles not only reduced the side effects, but also improved its therapeutic efficacy in the treatment of solid tumors.

In 2002, Yan, P.[33] prepared the insulin-loaded chitosan nanoparticles by ionotropic gelation of chitosan with TPP anions. Particle size distribution and zeta potential were determined by photon correlation spectroscopy. The ability of chitosan nanoparticles to enhance the intestinal absorption of insulin and the relative pharmacological bioavailability of insulin was investigated by monitoring the plasma glucose level of alloxan-induced diabetic rats after the oral administration of various doses of insulin-loaded chitosan nanoparticles. The positively charged, stable chitosan nanoparticles showed particle size in the range of 250–400 nm. Insulin association was up to 80%. The in vitro release experiments indicated initial burst effect, which is pH-sensitive. The chitosan nanoparticles enhanced the intestinal absorption of insulin to a greater extent than the aqueous solution of chitosan in vivo.

In 2002, Hu, Y. and coworkers [34] prepared chitosan–poly(acrylic acid) (PAA) complex nanoparticles by template polymerization of acrylic acid (AA) in chitosan solution. The physicochemical properties of nanoparticles were investigated by using size exclusion chromatography, FTIR, dynamic light scattering, transmission electron microscope and zeta potential. The molecular weight of PAA in nanoparticles increased with the increase of molecular weight of chitosan, indicating that the polymerization of acrylic acid in the chitosan solution was a template polymerization. Preparation nanoparticles carried a positive charge and showed the size in the range from 50 to 400 nm. The surface structure and zeta potential of nanoparticles was controlled by different preparation processes. The experiment of in vitro silk peptide (SP) release showed that these nanoparticles provided a continuous release of the entrapped SP for 10 days.

In 2002, Benerjee, T. and coworkers [35] studied chitosan nanoparticles cross-linked with glutaraldehyde prepared in AOT/*n*-hexane reverse micelle system. The particle size was found to vary with the amount of cross-linking. The particle size at infinite dilution is ~30 nm diameter, when 10% of the amine groups in the polymeric chains were cross-linked and it shoots up to 110 nm diameter when all the amine groups were cross-linked (100% cross-linked). TEM pictures showed that these particles were spherical in shape and remain in the form of aggregation.

In 2003, Yongmei, X. and Yumin D.[36] studied different formulations of chitosan nanoparticles produced by the ionic gelation of triphosphate (TPP) and chitosan. TEM indicated that their diameters were ranging between 20 and 200 nm with spherical shape. FTIR confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. Factors that affected the delivery of bovine serum albumin (BSA) as a model protein were studied. These included the molecular weight and the degree of deacetylation of chitosan, concentrations of chitosan and bovine serum albumin (BSA), as well as the presence of polyethylene glycol (PEG) in the encapsulation medium. Increasing molecular weight of chitosan from 10 to 210 kDa, bovine serum albumin (BSA) encapsulation efficiency was enhanced nearly twice. The total release of bovine serum albumin (BSA) in phosphate buffered saline at pH 7.4 in 8 days was reduced from 73.9% to 17.6%. Increasing deacetylation degree from 75.5% to 92% promoted the encapsulation efficiency with a decrease in release rate. Encapsulation efficiency decreased greatly by increasing the initial concentration of bovine serum albumin (BSA) and chitosan. Higher loading capacity of bovine serum albumin (BSA) enhanced the bovine serum albumin (BSA) release from nanoparticles. However, adding polyethylene glycol (PEG) hindered the bovine serum albumin (BSA) encapsulation and increased the release rate.

In 2003, Jae, H.P. and coworkers [27] prepared hydrophobically modified glycol chitosan (HGC) capable of forming nano-sized self-aggregates by the chemical conjugation of 5β-cholanic acid to the main backbone of glycol chitosan. The Arg-Gly-Asp (RGD) peptide labeled with fluoresein isothiocyanate (FITC-GRGDS) was loaded into self-aggregates at three different conditions: simple mixing, sonication, and solvent evaporation methods. It was found that the presence of FITC-labeled peptides makes the self-aggregates to be compact, possibly due to the role of both hydrophobic FITC and peptides containing carboxylic acids that allow hydrogen

bonding and electrostatic interaction with the primary amino groups in the main backbone of glycol chitosan. Overall, the self-aggregates loaded with FITC-GRGDS might be useful for monitoring or destroying the angiogenic vessels surrounding the tumor tissue.

In 2005, Yan, W. and coworkers [37] studied the ammonium glycyrrhizinate-loaded chitosan nanoparticles prepared by ionic gelation of chitosan with tripolyphosphate anions (TPP). The particle size and zeta potential of nanoparticles were determined, by dynamic light scattering (DLS) and a zeta potential analyzer, respectively. The effects, including chitosan molecular weight, chitosan concentration, ammonium glycyrrhizinate concentration and polyethylene glycol (PEG) on the physicochemical properties of the nanoparticles were studied. These nanoparticles have ammonium glycyrrhizinate loading efficiency decreased with the increase of ammonium glycyrrhizinate concentration and chitosan concentration. The introduction of PEG could significantly decrease the positive charge of particle surface. Chitosan could complex TPP to form stable cationic nanoparticles for subsequent ammonium glycyrrhizinate loading.

In 2005, Garcia-Furentes, M. and coworkers [38] studied the formation and characterization of poly(ethylene glycol) (PEG)-coated and chitosan (CS)-coated lipid nanoparticles. The goal was to study the interaction of these surface-modified lipid nanoparticles with Caco-2 cells and to evaluate the potential of the nanostructures as oral delivery systems for salmon calcitonin (sCT). In vivo studies of the response to sCT-loaded nanoparticles were performed in rats. The association of rhodamine®-loaded nanoparticles to the Caco-2 cell monolayer was independent of the surface coating of the nanoparticles (CS-coated versus PEG-coated nanoparticles). The results obtained following oral administration of sCT-loaded CS-coated nanoparticles to rats showed a significant and prolonged reduction in the serum calcium levels as compared to those obtained for control (sCT solution).

The micellation method is particularly of interest as there are variety of surfactants which may be used to produce different chitosan/surfactant nanoparticulate systems without creating new compounds with unknown toxicity. There also have not been report on the study of chitosan nanoparticles formed in a micellar system in aqueouse media. This work will describe key factors affecting the formation of chitosan/surfactant nanoparticles and molecular level interpretation for the formation of the nanoparticles.

CHAPTER II

EXPERIMENTAL SECTION

2.1 Instrument and apparatus

1. Autosizer 3000 (Melvern Instrument Ltd., UK)
2. Zetasizer (Malvern Instruments Ltd,.UK)
3. UV-Visible spectrophotometer (Varian Cary 50 Probe)
4. Refrigerator (Sharp SJ-D22L;191 L)
5. Magnetic stirrer (Coning)
6. Sonicator (Elma)
7. Pipette man (P20, P200, P1000 Gilson)
8. Syringe filter (0.2 μm PTFE, Alltech)
9. Ubbelohde viscometric tube
10. Freeze-dryer (Freezone 77520, Benchtop, Labconco)
11. Centrifuge (Beckman coulter, Avanti tm J-30I)
12. Locking dialysis membrane clamps (Membrane Filtration Product Inc.)
13. Evaporator (Rotavapor R-200 ; BUCHI)
14. Transmission electron microscopy (TEM) (CM 12 Philips, Eindhoven, Netherlands)
15. 2090 mesh copper grids
16. Floating plastic film

2.2 Chemicals

1. Chitosan (M_v~150000, 85% DD from Chemicals, Ltd, Fluka, Switzerland)
2. Chitosan (M_v ~10000, 90% DD from Laboratory preparation)
3. Chitosan (M_v ~1000000, 79% DD from Koyo Chemical, Co.,Ltd., Japan)
4. Sodium bis (ethylhexyl) sulfosuccinate (AOT, Cognis, Germany)
5. Sodium dodecyl sulfate (SDS) (Merck, Germany)
6. Benzalkonium chloride (Merck, Germany)
7. Tetracycline (Fluka, Switzerland)
8. Diclofenac (Fluka, Switzerland)
9. Nonyl phenol ethoxylate (NP-40, Cognis, Germany)
10. Span @80 (Fluka, Switzerland)
11. Glacial acetic acid, analar grade (Merck, Germany)

12. Sodium chloride, analar grade (Merck, Germany)
13. Sodium hydroxide, analar grade (Merck, Germany)
14. Hydrochloric acid, analar grade (Merck, Germany)
15. Glutaraldehyde, analar grade (Merck, Germany)
16. Tris-HCL buffer, analar grade (Merck, Germany)
17. Concentrate Ammonia, analar grade (Merck, Germany)
18. Calcium chloride, analar grade (Merck, Germany)
19. Potassium polyvinyl sulfate (PVSK), analar grade (Wako, Japan)
20. Pydidinium chloride (CPC), analar grade (Wako, Japan)
21. Toluidene blue, analar grade (Wako, Japan)

2.3 Preparation of chitosan/surfactant nanoparticles

2.3.1 Reverse micelle system

Chitosan nanoparticles were prepared by a modified reverse micelle method reported by Maitra.[35] The surfactant, AOT, was dissolved in *n*-hexane (0.04–0.1 M solution). To AOT solution (40 mL of 0.04 M), chitosan solution (400 µL of 0.621 mM) in acetic acid (6% v/v), tris–HCl buffer (176 µL;0.01%;pH 8.0), liquor ammonia (40 µL), glutaraldehyde solution (4 µL;0.01% v/v) were added, with continuous stirring at room temperature. The solution was homogenous and optically transparent. The system was left, under stirring, overnight at room temperature. The solvent was then evaporated off in a rotary evaporator and the dry mass resuspended in 20 mL of Tris–HCl buffer (pH 8.0) by sonication. $CaCl_2$ solution (4 mL : 30% w/v) was added dropwise to precipitate the surfactant as calcium salt of diethylhexylsulphosuccinate ($Ca(DEHSS)_2$). The precipitate was pelleted by centrifugation at 6000 rpm for 15 min at 25 °C. The cake of $Ca(DEHSS)_2$ was dissolved in *n*-hexane (10 mL) and the hexane solution washed two times, each time with 1 mL of Tris–HCl buffer. Total aqueous dispersion of nanoparticles was then dialyzed for ~ 2–3 h using a Spectrapore membrane dialysis bag (12 kDa cut off). The clear solution was obtained, but no particle observed by dynamic light scattering measurement.

2.3.2 Micelle system

A typical procedure for the preparation of chitosan/surfactant nanoparticles in an aqueous medium is as follows. The chitosan solution (0.5 – 5.0 mM) in an aqueous solution of acetic acid (1% v/v) was slowly added into an equal volume of aqueous surfactant solution (100 mM) under continuous stirring at room temperature over the

preparation period of 3–5 min. The preparation by other mixing methods, ultrasonication and vortexing, were also performed by using similar procedure.

2.4 Characterization of the nanoparticles

2.4.1 Determination of averaged particle size

Measurement of the averaged particle size was performed by using photon correlation spectroscopy on the Autosizer 3000 (Melvern Instruments Ltd., UK). The instrument used an air–cooled argon ion laser at 488 nm as the light source with 128 channel correlator. Based on dynamic light scattering theory, the size of the particles is related to the diffusion rate of the particles (D) according to Stoke – Einstein equation:

$$d_h = \frac{kT}{3\pi\eta D}$$

where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of medium and d_h is hydrodynamic diameter of particles. The samples were diluted prior to measuring to obtain optimal signals and thus reproducible results.

2.4.2 Study of particle shape and nanostructure

Transmission electron microscope (TEM) was used to study the particle shape and nanostructure of chitosan/surfactant particles. The nanoparticles were dispersed in millipore water to give a clear sample solution. A drop of the sample solution was allowed for air dry on a formvar coated grid. The images were taken by a TEM CM 12 (Phillips, Netherland).

2.4.3 Determination of charge and surface potential of the particles

The zeta potential of the nanoparticles was analyzed based on electrophotoretic mobility of the particles by photon correlation spectroscopy with laser doppler anemometry using Zetasizer (Melvern Instruments Ltd., UK). A sample was diluted with 0.1 mM KCl and placed in the electrophotorretic cell, where a potential of 150 mV was applied. The mobility of the particles was observed by light scattering.

2.5 Evaluation of medical encapsulation

2.5.1 Loading of drug into the nanoparticles

Tetracycline (2.5 mM) and diclofenac (1.7 mM) were loaded into the chitosan/surfactant nanoparticles at the chitosan/surfactant/drug mole ratio of 20/1/1

and 20/1/0.5, respectively. The particle size and the zeta potential was determined by photon correlation spectroscopy within one day of mixing and after being stored for 30 days. The drug loading efficiency was determined from the amount of the initial drug concentration and the concentration of the drug remained in the solution according to the following equation:

$$\text{Loading efficiency (\%)} = \frac{[\text{drug}]_{\text{initial}} - [\text{drug}]_{\text{remained in the solution}}}{[\text{drug}]_{\text{initial}}} \times 100$$

The nanoparticles were separated from the aqueous medium by ultracentrifugation at 24000 x g for 45 min. The remaining concentrations of tetracycline and diclofinac sodium salt were determined by using UV-visible spectrophotometer measuring at 357 nm and 275 nm, respectively.

2.6 Preparations and characterization of chitosan materials

2.6.1 Preparation of the low molecular weight chitosan from the medium molecular weight chitosan.

Chitosan ($M_v = 10^4$) was prepared from solution of sonication of chitosan ($M_v = 1.5\times10^5$), (12.4 mM : 1% acetic acid). The solution was sonicated for 4 h at room temperature before lyopilized by Freeze-dryer.

2.6.2 Determination of chitosan molecular weight by intrinsic viscosity

Solvent: 0.1 M acetic acid and 0.2 M NaCl in water

Dried chitosan sample (ca, 25 mg or depending on molecular weight of chitosan) was weighed accurately in a 50 mL volumetric flask, dissolved in 1 M aqueous acetic acid solution (5 mL) and water (30 mL), and stirred with a magmetic stirrer overnight. To the solution 1 M aq. NaCl solution (10 mL) was added and stirred overnight, then made up the volume to the mark with distilled water (C_1). A portion of solution (10 mL) was taken and diluted with the above solvent (0.2 M NaCl/0.1 M AcOH, 10 mL) (C_4). The temperature of a thermostatic water bath was maintained at 25°C.

Measurement of solvent viscosity: The solvent (0.2 M NaCl/0.1 M AcOH, 10 mL) was put into a Ubbelohde viscometer (**Figure 2.1**). The liquid was pushed up above the upper level (a) by a balloon pump. The time of falling between the upper (a) and lower mark (b) was measured by a stopwatch in triplicate.

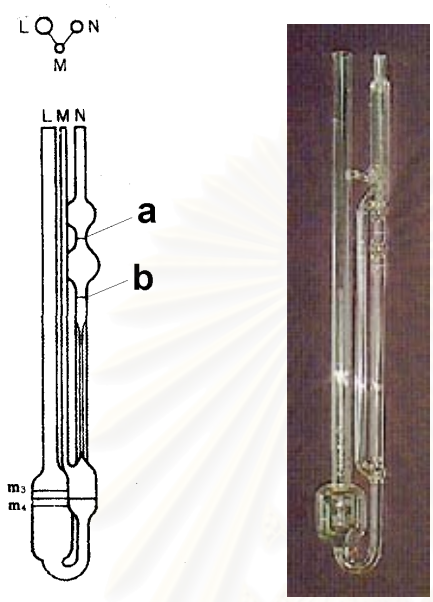


Figure 2.1 Ubbelohde viscometer

Measurement of chitosan solution viscosity: The solution (C_1, 10 mL) was put into a viscometer. The falling time was measured by a stopwatch in triplicate. The solvent (2 mL) was added through tube L and mixed well to give C_2. The falling time was measured again. The solvent (0.2 M NaCl/0.1 M AcOH, 2 mL) was added again to give C_3 and the falling time was measured. The viscometer was rinsed with the solvent (15 mL), water (15 mL) and solution C_4 (15 mL). The solution C4 (10 mL) was put into the viscometer. The falling time was measured as described aboved. The solution was diluted (C_5 and C_6) and the falling time was measured according to the procedure described for C_2 and C_3. Plot graph of η_{sp}/C and against C, and draw a straight line using the linear least square. The intrinsic viscosity [η] was obtained from the Y-intercept. Viscosity-average molecular weight can be calculated as follows:

$[\eta] = KM_v^a$ (K = 1.8 x 10^{-3}, a = 0.93 at 25 °C)

When plot graph between η_{sp} / C and C the linearity was obtained

y-intercept = $[\eta]$

$$\eta_{sp} = \frac{t - t_0}{t_0}$$

2.6.3 Determination of degree of acetylation of chitosan [39-40]

At first the solution of patassium polyvinyl sulfate solution (PVSK) must be titrated to determine the concentration. Cetyl pyridinium chloride (CPC, 12.5 mg) was weighed precisely into a 25 mL volumetric flask and the volume was made up to the mark with 0.1 M aqueous acetic acid solution. The solution (precise 5 mL) was plaed in a 25 mL beaker, three drops of 0.1% toluidine blue were added as an indicator, and the mixture was continuously stirred with a magnetic bar. The PVSK solution was added titrimetrically from a burette until the blue color of the indicator changed into reddish purple. The titration was repeated three times. In a blank titration, 0.1 M aqueous acetic acid solution was used in place of the CPC solution. The other procedures are the same as the above.

For the determination of the concentration of PVSK (N, equiv./L):

$$N = \frac{50C'}{358D}$$

When ; C' = concentration of CPC (% w/v)

D = The different between CPC and blank titration volumes (mL)

Dried chitosan (8.33 mg) was weighted precisely in a 25 mL volumetric flask and the volume was made up to the mark with 0.1 M aqueous acetic acid solution. The solution (precise 5 mL) was pipetted and placed in a 25 mL beaker and titrated as described above.

For the determination of the degree of *N*-acetylation (DA):

$$\%DA = \frac{[(50C-161ND)\ 100]}{(42ND+50C)}$$

When: N = concentration of PVSK (equiv./L)

D = different between chitosan and blank titration volumes (mL)

C = concentration of chitosan (%)

For the determination of the degree of deacetylation (DD) :

DD (%) = 100-DA (%)

The results of degree of deacetylation was shown in **Table 2.1**.

Table 2.1 The molecular weight and %DD of each chitosans

Source of chitosan	**%DD**	**Molecular weight (M_v)**
Koyo chemical (Japan)	79	1000000
Fluka (Switzerland)	85	150000
Sonicating (4 h) in Laboratory	90	10000

CHAPTER III

RESULTS AND DISCUSSION

The preparation of chitosan/surfactant nanoparticles based on micellar system, involves a mixture of two aqueous solutions at room temperature. One is a solution of chitosan and the other is a solution of surfactant. The aim of this work is to investigate the effect of surfactant types, molecular weight of chitosan, concentration of chitosan solution, chitosan/surfactant mole ratio and methods for mixing chitosan and surfactant in aqueous media.

3.1 Effect of surfactant type

Three types of surfactants : cationic, anionic and nonionic, were investigated for the formation of the chitosan nanoparticulate system. There was no nanoparticle observed when cationic surfactant (benzalkonium chloride) and nonionic surfactants (NP-40 and span@80) were used. Only anionic surfactants, sodium bis(ethylhexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS) allowed the formation of nanoparticles with average diameters in submicron scale. The use of AOT gave the system in which the particle size increased gradually from 100-500 nm with increasing chitosan concentration from 0.5 mM to 5 mM. For the chitosan/SDS system, the particle size clearly moved into micron scale when the chitosan concentration was greater than 3 mM **(Figure 3.1)**. The increase in the particle size with the concentration of chitosan may be the result of decreasing surfactant/chitosan mole ratio in the solution that reduced the number of the particles formed. To confirm this hypothesis, the surfactant/chitosan mole ratio was varied by varying the concentration of AOT while fixing the chitosan concentration at 2.54 mM. The results agreed well with the hypothesis, as the size of the particles increased with the reducing surfactant/chitosan mole ratio (**Figure 3.2)**. The results also confirmed that the formation of the chitosan/SDS nanoparticles required higher surfactant/chitosan mole ratio than that in the chitosan/AOT system. The results demonstrated that AOT is more effective in preventing self-aggregation between the chitosan chains. The multi equivalents of surfactants required for the formation of nanoparticles suggested that not only the charge neutralization but also the hydrogen bond between the hydroxyl or amino groups of chitosan and the oxy-anion of the surfactant molecules are responsible for the prevention of self-aggregation. Since the molecule of SDS has

only one sulfonate group while an AOT molecule contains two more carbonyl oxygen of the ester groups, besides the sulfonate group, the stronger attraction force between chitosan and AOT can be expected. Due to greater effeency of AOT, investigations in further sections were thus focused mainly on chitosan/AOT system.

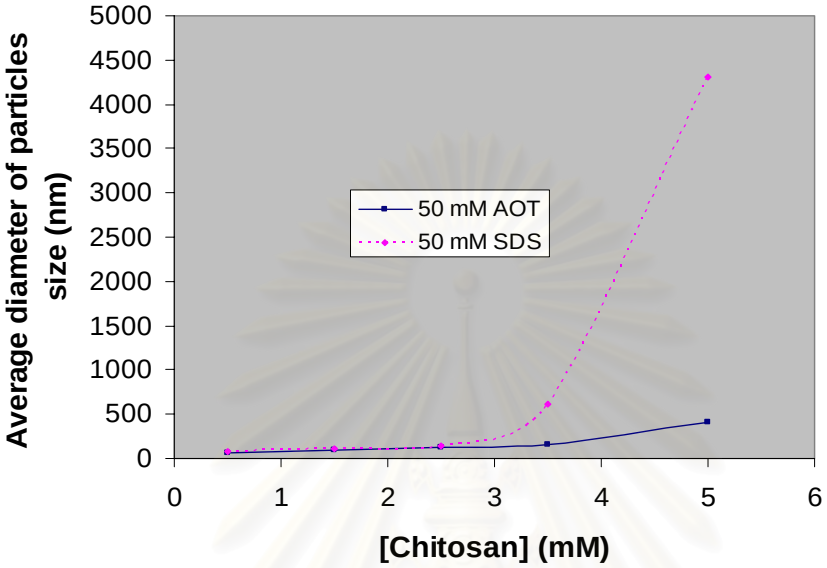


Figure 3.1 Average particle size measured by PCS in the chitosan/anionic surfactant system. (M_v of chitosan = 1.5×10^5; [anionic suractant] = 50 mM ; mixing by stirring).

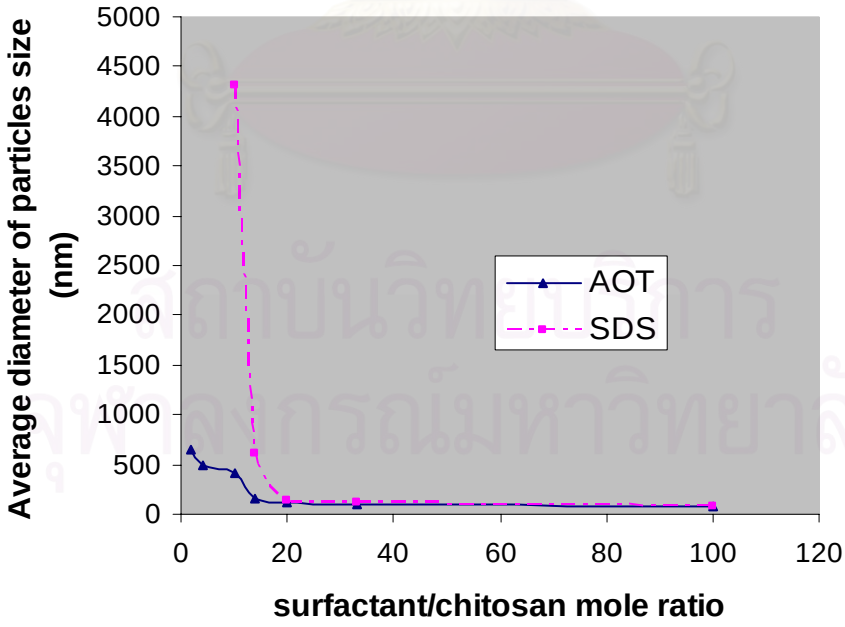


Figure 3.2 Average particle size measured by PCS in the chitosan/anionic surfactant system. With varied surfactant/chitosan mole ratio. M_v of chitosan = 1.5×10^5, [chitosan] = 2.5 mM, [anionic suractant] = 5.0-50 mM ; mixing by stirring.

3.2 Effect of molecular weight of chitosan

The effect of chitosan molecular weight on the formation of the chitosan/surfactant nanoparticles were investigated. The chitosan with three different viscosity-averaged molecular weights, M_v = 10^4, 1.5×10^5 and 10^6, were selected as representatives for high, medium and low molecular weight chitosan. When mixed with AOT, high molecular weight chitosan was precipitated out while the medium and low molecular weight chitosan allowed the formation of nanoparticles (**Table 3.1**).

Table 3.1 Size of chitosan/AOT nanoparticles formed by stirring mixtures chitosan and AOT solutions.

M_v	**Particle size (nm)**
~10^6	Precipitate
~1.5×10^5	110
~10^4	78

[chitosan] = 2.5 mM, [AOT] = 50 mM.

3.3 Effect of the mixing methods

Three methods, stirring, vortexing and ultrasonicating were used to provide roughly three different levels of mechanical energy for mixing chitosan with AOT. At higher AOT/chitosan mole ratio the size of the particles was independent to the mixing methods. When the AOT/chitosan mole ratio decreased, the size of the particles got smaller in order of stirring, vortexing and ultrasonicating, respectively (**Figure 3.3)**. The results suggested that with limited amount of surfactant (surfactant/chitosan mole ratio = 10), the mixing method providing higher energy and more uniform mechanical agitation such as ultrasonication could lead to better mixing and thus the smaller particle size.

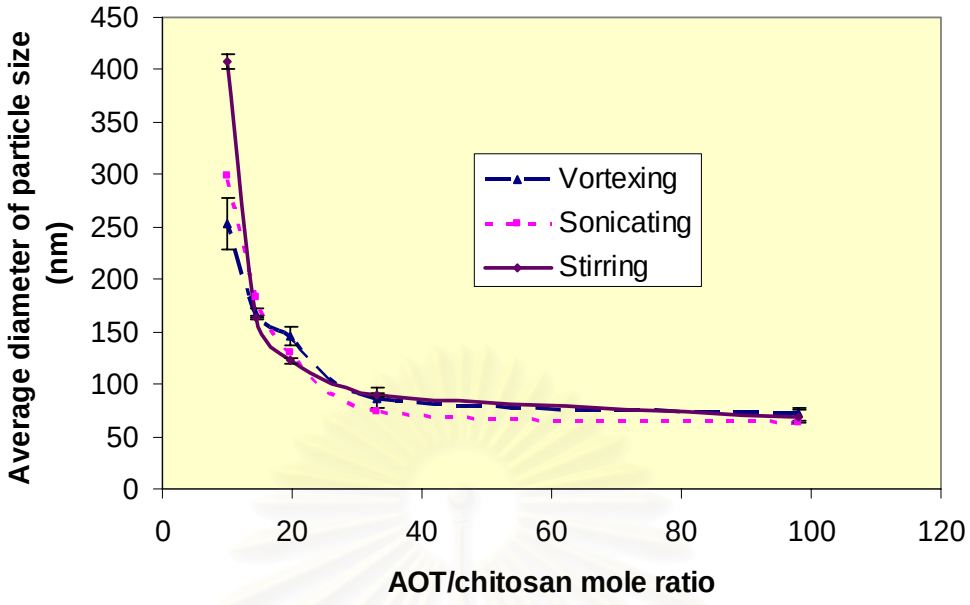


Figure 3.3 Particles sizes of chitosan/AOT nanoparticles obtained from different dispersing methods. [AOT] = 50 mM and M_v of chitosan = 1.5×10^5.

3.4 Transmission electron microscope imaging

The morphotogical examination of the chitosan/AOT nanoparticles was performed with a transmission electron microscope (TEM). TEM micrograph of a residue from the mixture of chitosan and AOT solution displayed oval solid structures with submicron sizes (**Figure 3.4 a**). It is important to note that no such structure observed in the TEM images of the residues from either chitosan or AOT solutions. The high resolution TEM image of a chitosan/AOT nanoparticles showed that there was a darker shell surrounding lighter area of the particle (**Figure 3.4 b**). To obtain more information about the structure of chitosan/AOT nanoparticles, the zeta potential of the particle was measured.

(a) **(b)**

Figure 3.4 TEM micrograph of the chitosan/AOT (a) × 25 K and (b) × 100 K.

3.5 Determination of particle surface potential (zeta potential)

The zeta potential of AOT and SDS surfactant were -79 mV and -43 mV, respectively, at pH 2.0. Upon increasing pH, the value of zeta potential of AOT and SDS were less reliable due to the highly dynamic value of AOT and SDS micelle. (**Figure 3.5** and **Figure 3.6**)

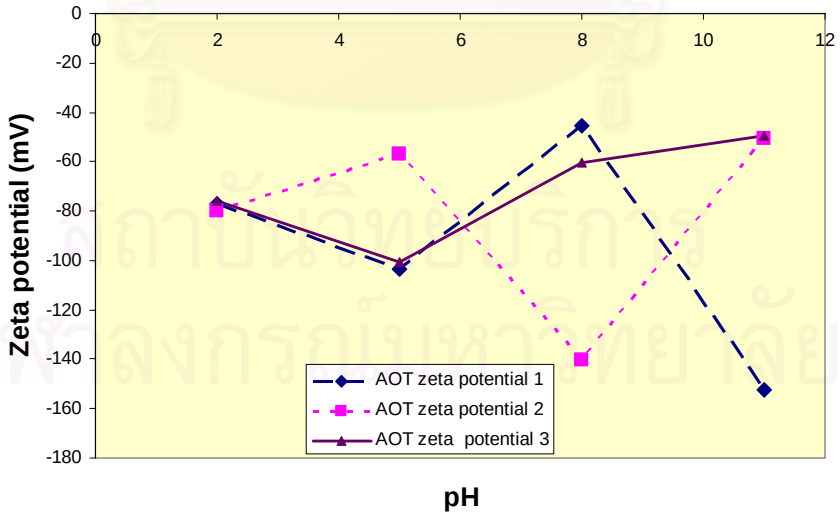


Figure 3.5 Zeta potential of AOT surfactant ; [AOT] = 100 mM

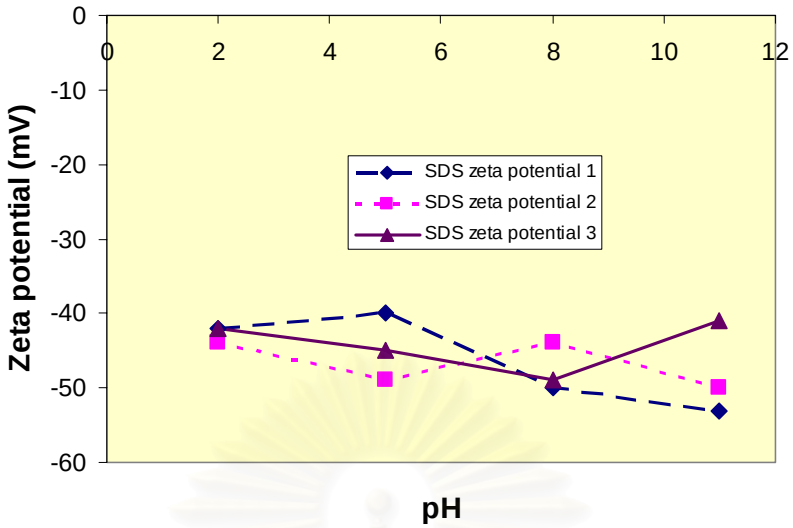
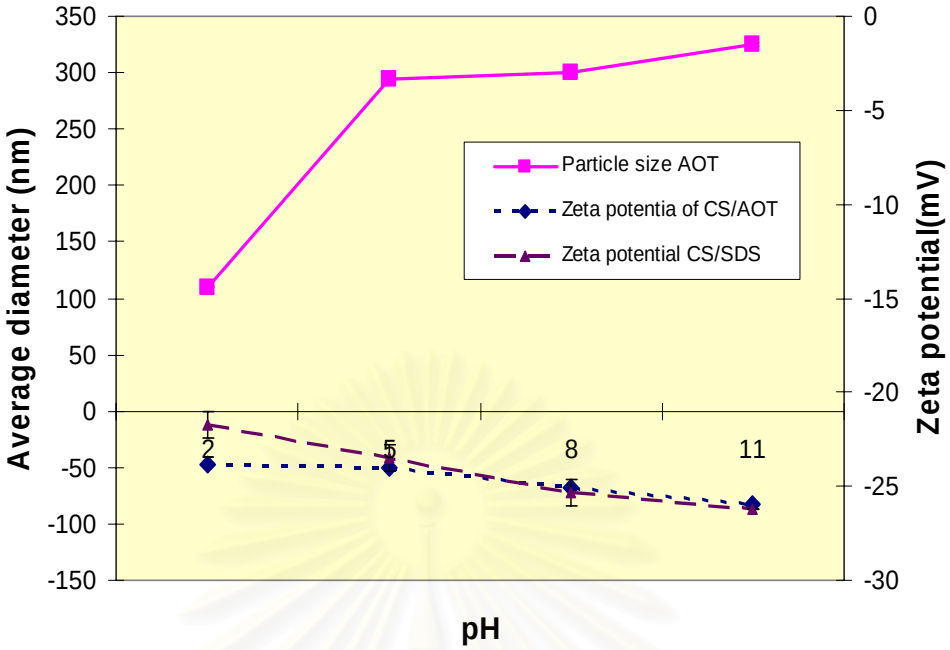


Figure 3.6 Zeta potential of SDS surfactant ; [SDS] = 100 mM

The zeta potential of chitosan/surfactant nanoparticles were slightly less negative than their corresponding surfactants. At pH 2.0, chitosan/AOT nanoparticles showed zeta potential of -79 mV and chitosan/SDS showed zeta potential of -43 mV. The zeta potential of chitosan/surfactant nanoparticles were gradually more negative with increasing pH from 2.0 to 11.0. The values of zeta potential for chitosan/surfactant nanoparticles could be readily reproduced unlike those of their corresponding surfactants. The results indicated that the surfactant molecules in/on the chitosan/surfactant nanoparticles were less dynamic (**Figure 3.7**).



. **Figure 3.7** Average particles size and zeta potential of chitosan/AOT and chitosan/SDS nanoparticles at pH 2.0 – 11.0. [chitosan] = 2.5 mM ; [AOT] = 50 mM ; [SDS] = 50 mM.

The negative potential indicated that the surface of the particle consisted mainly the anionic AOT surfactant. The structure of chitosan/AOT nanoparticles may consist of chitosan chain and AOT molecule forming a charge neutralized complex core particle surrounded by lipid bilayer of AOT. This model is consistent with the dark shell observed in the TEM image and the negative zeta potential. The lipid bilayer shell is probably attached to the charge complex of chitosan/AOT core through multi-sited hydrogen bonding.

3.6 Loading of medicine

Tetracyline and diclofinac sodium salt were selected for cationic and anionic drug models for loading into the chitosan/surfactant nanoparticles by adding a drug solution into a preformed chitosan/surfactant nanoparticle solution. The effect of the added drug on the size and storage stability of the nanoparticles were evaluated. The loading efficiencies of drugs into the nanoparticles were determined. The zeta potentials of the drug incorporated particles were measured to obtain information how the drug molecules of drugs incorporated into the particles.

3.6.1 Size and stability of chitosan/surfactant/drug nanoparticles

The size of chitosan/AOT nanoparticles were virtually unchanged after mixing with tetracycline at the chitosan/AOT/tetracycline mole ratio of 20/1/1. The average size of the particles also stayed almost the same after 30 days of storage (**Table 3.2**). Adding tetracycline into the chitosan/SDS system at the same mole ratio however increased the size of the particles slightly from 142 to 167 nm but the particles swelled further to give an average diameter of 324 nm after being kept for 15 days. Diclofenac sodium salt, on the other hand, could be mixed with chitosan/SDS system (chitosan/SDS/diclofenac sodium salt = 20/1/0.5) with slight decrease of particle size to 122 nm and it increased only slightly from 122 to 195 nm after 30 days of storing. Adding diclofenac sodium salt into the chitosan/AOT nanoparticulate system was however resulting in a precipitation of the nanoparticles due to aggregation.

It is also interesting to note that the incorporation of tetracycline also slightly increased the stability of the chitosan/AOT nanoparticles evidenced by the smaller changes in the averaged size of the particles, upon aging, comparing to the particles without the drug.

Table 3.2 Size and storage stability of chitosan/surfactant nanoparticles in the absence and the presence of drug, prepared by stirring method.

Nanoparticles	Storing (days)	Particle size (nm)
Chitosan/AOT		
Non loaded medicine	1	96
	15	98
	30	107
Tetracycline	1	95
	15	98
	30	98
Diclofenac sodium salt	1	precipitated
	15	precipitated
	30	precipitated
Chitosan/SDS		
Non loaded medicine	1	142
	15	150
	30	307
Tetracycline	1	167
	15	324
	30	precipitaed
Diclofenac sodium salt	1	122
	15	160
	30	195

[chitosan] = 1.7 mM, [surfactant] = 33.3 mM, and [drug] = 1.7 mM for tetracycline and [drug] = 0.83 mM for diclofinac sodium salt

3.6.2 Loading efficiency

The loading efficiency of drug into the nanoparticles were calculated from the following equation:

$$\text{Loading efficiency (\%)} = \frac{[\text{drug}]_{\text{initial}} - [\text{drug}]_{\text{remained in the solution}} \times 100}{[\text{drug}]_{\text{initial}}}$$

The concentrations of tetracycline and diclofenac sodium salt remained in the solution were measured from the supernatant after ultracentrifugation by UV-Vis spectrophotometer at 357 nm and 275 nm, respectively. The concentration of tetracycline in the chitosan/AOT/tetracycline was reduced from 1.32 mM to 0.66 mM and the concentration of diclofenac sodium salt in the chitosan/SDS/diclofinac sodium salt was reduced from 0.40 mM to 0.24 mM. The loading efficiency of tetracycline by chitosan/AOT nanoparticles was thus 50% and that of diclofenac sodium salt by chitosan/SDS nanoparticles was 40% (**Table 3.3**).

Table 3.3 Loading efficiency of tetracycline by chitosan/AOT nanoparticles

	Chitosan/AOT
[Tetracycline] initial (mM)	1.32
[Tetracycline] remaining (mM)	0.66
% Loading efficency	50%

[chitosan] = 2.5 mM, [AOT] = 50 mM, [tetracycline] = 1.32 mM

Table 3.4 Loading efficiency of diclofenac sodium salt by chitosan/SDS nanoparticles

	Chitosan/SDS
[diclofenac sodium salt] initial (mM)	0.40
[diclofenac sodium salt] remaining (mM)	0.24
% Loading efficency	40%

[chitosan] = 2.5 mM, [AOT] = 50 mM, [diclofenac sodium salt] = 0.40 mM.

3.6.3 Zeta potential

To understand how tetracycline incorporated into the chitosan/AOT nanoparticles, the zeta potential of the chitosan/AOT/tetracycline was measured. Surprisingly, the zeta potential of the nanoparticles became even more negative with addition of tetracycline at pH 2 **(Figure 3.8)**. The result suggested that the positively charged tetracycline molecules were not on the surface of the particles but defused through the lipid bilayer shell into the particles. The increase of positive charges inside the particle demanded more negatively charged surfactant molecules to cover it. Tetracycline probably also increased the hydrogen bond interaction between the core and shell of the particle resulting in greater stability of the particles. At acidic pH, diclofenac sodium salt on the other hand is protonated into acid form which has poor solubility in water. Therefore, precipitation was observed when the chitosan/AOT was mixed with diclofenac sodium salt. In chitosan/SDS system, the acid form of diclofenac sodium salt was probably carried into the nanoparticles by the micelles of SDS.

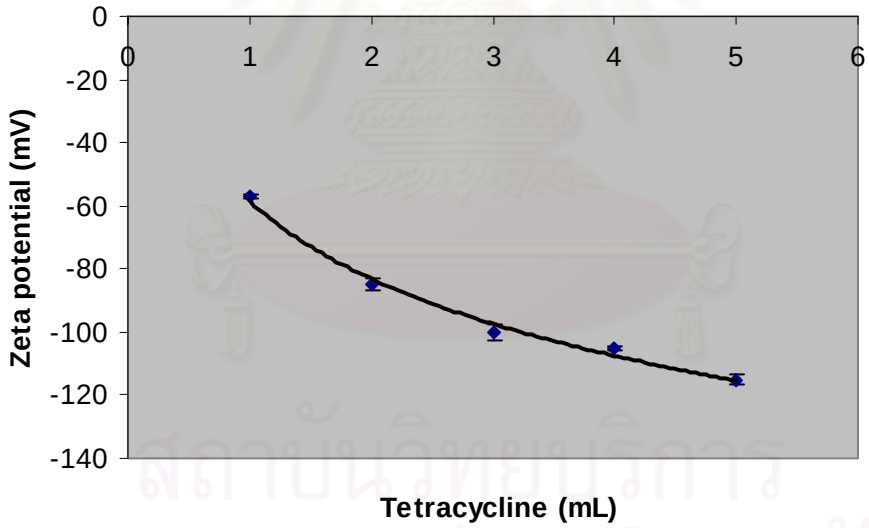


Figure 3.8 Zeta potential of chitosan/AOT/tetracycline nanoparticles after complexed with tetracycline (1.7 mM); pH = 2.0, [chitosan] = 1.7 mM, [AOT] = 33.3 mM.

CHAPTER IV

CONCLUSIONS

4.1 Conclusions

The effects of surfactants on the formation of chitosan/surfactant nanoparticles were investigated. Chitosan/surfactant nanoparticles with diameter range of 100-500 nm can be prepared in a micellar system containg an anionic surfactant such as (AOT) and (SDS). The TEM images and zeta potential suggested that the chitosan/surfactant nanoparticles consisted a dense lipid bilayer shell of AOT surrounding the chitosan-AOT hydrogel. The chitosan/AOT nanoparticles can be loaded with the tetracycline at 50% loading at [tetracycline] = 1.32 and the chitosan/SDS nanoparticles system can be loaded with diclofenac sodium salt at 40% efficiency loading at [diclofenac sodium salt] . The suspensions of the nanoparticles containing = 0.40 mM tetracycline or diclofenac were stable at ambient condition for at least 30 days.

4.2 Suggestion for future work

Releasing of drug loaded into chitosan/surfactant nanoparticles and exactly of nanostructure of chitosan/surfactant/drug nanoparticles must be more studying. It is also important to find the method for separating the nanoparticles from the solution.

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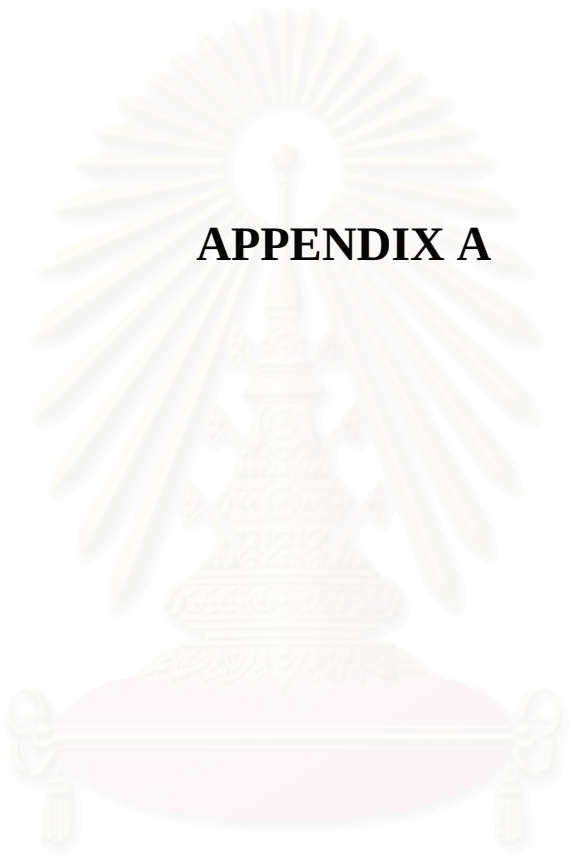
APPENDICES

APPENDIX A



Effect of surfactants on the formation of chitosan nanoparticles

Chutima Saratid[1], Monkol Sukwattansinit[2]
[1]Program of Petrochemical and Polymer science, Factory of Science, [2]Department of Chemistry, Factory of Science, Chulalongkorn University, Bangkok, Thailand.

Abstract

The effect of surfactants on the formation of chitosan nanoparticles was studied. The high, medium and low molecular weight chitosan (10^6, 1.5×10^5 and 10^4) were mixed with the evaluated surfactants in a micellar system under different mixing technigues, stirring, ultrasonicating and vortexing. Only anionic surfactants, sodium bis(ethylhexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS) allowed the formation of chitosan nanoparticles in aqueous media. The nanoparticles were presumably formed by the strong interaction between positive and negative charges of chitosan and the surfactant preventing the self aggregation of chitosan chains. The formation of the nanoparticles with diameter lower than 200 nm were observed when the mole ratio of surfactant/chitosan was higher than 14:1 and 20:1 for SDS at the chitosan concentration of 2.5 mM. The size and zeta potentials of the chitosan/AOT nanoparticles were determined by photo correlation spectroscopy 110±10 nm and -47.42 ± 5 mV, respectively. The chitosan/AOT nanoparticles obtained can be loaded with tetracycline using the mole raio of AOT/chitosan/tetracycline = 20:1:1. The suspensions of the chitosan/AOT nanoparticles/tetracycline were stable at ambient condition for at least 30 days.

Keywords : chitosan, anionic surfactant, AOT surfactant, SDS surfactant nanoparticles, tetracycline

Introduction

Polymer nanoparticles have recently been widely investigated as a carrier for drug delivery. Nanoparticles have benefitial size depended properties for example the number of nanoparticles (<1 μm) crossing the intestinal epithelium is significantly greater than that of the microspheres (>1 μm). With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites and the hydrophilic nanoparticles can generally circulate longer in the blood. Such systems can stay in body fouids long enough to reach and specific target sites.[1] A use

of colloidal carrier made of hydrophilic polysaccharides, e.g.chitosan, has arisen as a promising drug delivery system for improving the transport of macromolecules such as peptides, proteins, oligonucleotides and plasmids across biological surfaces. Surfactants, permeation enhancers, protease inhibitors, enteric coatings and bioadhesive microparticles or nanoparticles have all been used in development towards formation of drug delivery system.[2-17]

Chitosan, a polysaccharide of linearly β-(1→4)-linked, 2-deoxy-D-glucose is normally obtained from the deacetylation of chitin, a naturally occurring and abundantly available in marine crustaceans.[18] Chitosan is insoluble at alkaline and neutral pH but forms soluble as a polycationic polymer salts with inorganic and organic acids.[19] In acidic solution, the amino groups are protonated thus, making it soluble in water. Chitosan is known to be, biocompatible with living tissues since it does not cause allergic reactions and rejection.

To prepare chitosan nanoparticulate systems, the strong hydrogen bonding attraction between the chitosan chains must be over come to prevent aggregation of the particles. Currently, there are three generally known systems for preparation of chitosan-based nanoparticles, Ionic gelation system is relied on the strong electrostatic interaction of positively charged chitosan and negatively charged macromolecules.[20-26] Micellation method is relied on the favorable interaction between parts of chitosan chains with surfactant moledules to form micelles or reversed micellers.[27] Structurally modified chitosan to have less hydroxyl group and contain some hydrophobic side chains.[28-29] We are particularly interested in the micellation method as there are varieties of surfactants which may be used to produce different chitosan/surfactant nanoparticulate systems without creating new compounds with unknown toxicity. We report here is the key factors affecting the formation of chitosan/surfactant nanoparticles and our molecular interpretation for the formation of the particles.

Experiment section

High molecular weight chitosan, (M_v ~1000000), was purchased from Koyo Chemical, Co., Ltd., Japan. Medium molecular weight chitosan, (M_v ~ 150000), was purchased from Fluka, Switzerland. Low molecular weight chitosan (M_v ~ 10000), was preparated in our laboratory by sonicating a solution of chitosan the medium molecular weight for 4 hrs). AOT (Sodium bis(ethylhexyl) sulfosuccinate and nonyl

phenol ethoxylate (NP-40) were obtained from Cognis, Germany. SDS (sodium dodecyl sulfate and benzalkonium chloride were purchased from Merck, Germany. Tetracycline, diclofenac sodium salt and span@80 were purchased from Fluka, Switzerland. Other chemicals are reagent grade. All chemicals were used as received.

A typical procedure for the preparation of chitosan/surfactant nanoparticles in an aqueous medium is as follows. The chitosan solution (0.5 – 5.0 mM) in an aqueous solution of acetic acid (1% v/v) was slowly added into an equal volume of aqueous surfactant solution (100 mM) under continuous stirring at room temperature over the preparation period of 3–5 min. The preparation by other mixing methods, ultrasonication and vortexing, were also performed by using similar procedure.

Study of size distribution

Sizing of the nanoparticles was performed by photon correlation spectroscopy on the Autosizer 3000 (Melvern Instruments Ltd., UK) at 488 nm. The size of the nanoparticles was determined from the diffusion of the particles (*D*) using Stoke – Einstein equation. The samples was diluted prior to the measurement to obtain reproducible results

Determination of charge and surface potential of particles

The zeta potential of the nanoparticles was analyzed based on electrophotoretic mobility of the particles by photon correlation spectroscopy with laser doppler anemometry using Zetasizer (Melvern Instruments Ltd., UK). A sample was diluted with 0.1 mM KCl and placed in the electrophotorretic cell, where a potential of 150 mV was applied.

Study of particle shape and nanostructure

Transmission electron microscope (TEM) was used to study the particle shape and nanostructure of chitosan/surfactant particles. The nanoparticles were dispersed in millipore water to give a clear sample solution. A drop of the sample solution was allowed for air dry on a formvar coated grid. The images were taken by a TEM CM 12 (Phillips, Netherland).

Loading of drug into nanoparticles.

Tetracycline (2.5 mM) and diclofenac (1.7 mM) were loaded into the chitosan/surfactant nanoparticles at the chitosan/surfactant/drug mole ratio of 20/1/1 and 20/1/0.5, respectively. The particle size and the zeta potential was determined by photon correlation spectroscopy within one day of mixing and after being stored for

30 days. The drug loading efficiency was determined from the amount of the initial drug concentration and the concentration of the drug remained in the solution.

The nanoparticles were separated from the aqueous medium by ultracentrifugation at 24000 x g for 45 min. The remaining concentrations of tetracycline and diclofinac sodium salt were determined by using UV-visible spectrophotometer measuring at 357 nm and 275 nm, respectively.

Results and discussion

The preparation of chitosan/surfactant nanoparticles based on micellar system, involves a mixture of two aqueous solutions at room temperature. One is a solution of chitosan and the other is a solution of surfactant. The aim of this work is to investigate the effect of surfactant types, molecular weight of chitosan, concentration of chitosan solution, chitosan/surfactant mole ratio and methods for mixing chitosan and surfactant in aqueous media.

Effect of surfactant type

Three types of surfactants : cationic, anionic and nonionic, were investigated for the formation of the chitosan nanoparticulate system. There was no nanoparticle observed when cationic surfactant (benzalkonium chloride) and nonionic surfactants (NP-40 and span@80) were used. Only anionic surfactants, sodium bis(ethylhexyl) sulfosuccinate (AOT) and sodium dodecyl sulfate (SDS) allowed the formation of nanoparticles with average diameters in submicron scale. The use of AOT gave the system in which the particle size increased gradually from 100-500 nm with increasing chitosan concentration from 0.5 mM to 5 mM. For the chitosan/SDS system, the particle size clearly moved into micron scale when the chitosan concentration was greater than 3 mM **(Figure 1)**. The increase in the particle size with the concentration of chitosan may be the result of decreasing surfactant/chitosan mole ratio in the solution that reduced the number of the particles formed. To confirm this hypothesis, the surfactant/chitosan mole ratio was varied by varying the concentration of AOT while fixing the chitosan concentration at 2.54 mM. The results agreed well with the hypothesis, as the size of the particles increased with the reducing surfactant/chitosan mole ratio (**Figure 2)**. The results also confirmed that the formation of the chitosan/SDS nanoparticles required higher surfactant/chitosan mole ratio than that in the chitosan/AOT system. The results demonstrated that AOT is more effective in preventing self-aggregation between the chitosan chains. The multi

equivalents of surfactants required for the formation of nanoparticles suggested that not only the charge neutralization but also the hydrogen bond between the hydroxyl or amino groups of chitosan and the oxy-anion of the surfactant molecules are responsible for the prevention of self-aggregation. Since the molecule of SDS has only one sulfonate group while an AOT molecule contains two more carbonyl oxygen of the ester groups, besides the sulfonate group, the stronger attraction force between chitosan and AOT can be expected. Due to greater effeency of AOT, investigations in further sections were thus focused mainly on chitosan/AOT system.

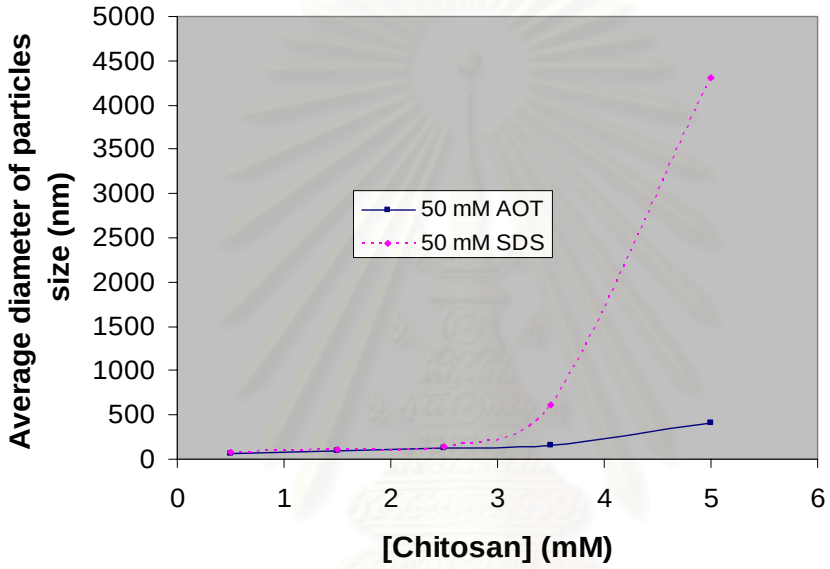


Figure 1 Average particle size measured by PCS in the chitosan/anionic surfactant system. (M_v of chitosan = 1.5×10^5; [anionic suractant] = 50 mM ; mixing by stirring).

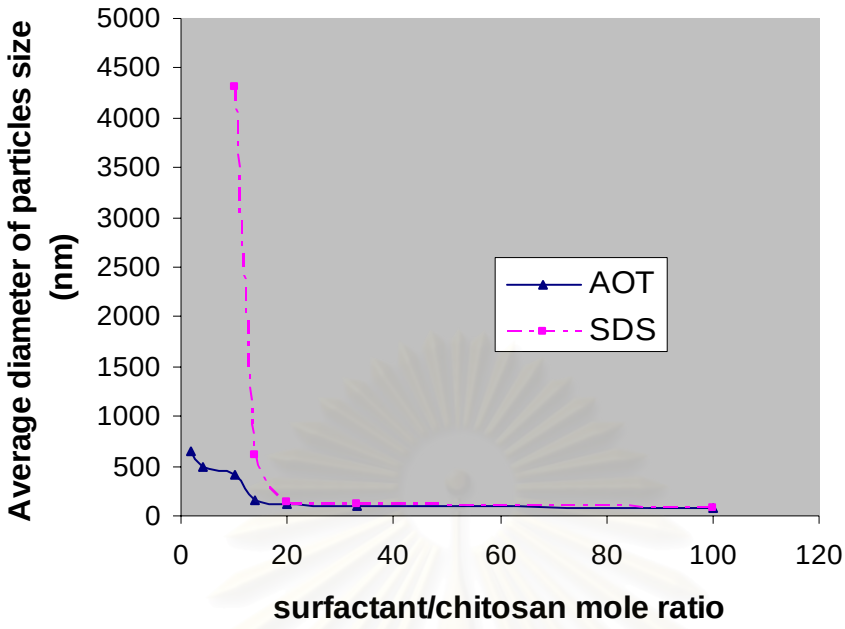


Figure 2 Average particle size measured by PCS in the chitosan/anionic surfactant system. With varied surfactant/chitosan mole ratio. M_v of chitosan = 1.5×10^5, [chitosan] = 2.5 mM, [anionic suractant] = 5.0-50 mM ; mixing by stirring.

Effect of molecular weight of chitosan

The effect of chitosan molecular weight on the formation of the chitosan/surfactant nanoparticles were investigated. The chitosan with three different viscosity-averaged molecular weights, M_v = 10^4, 1.5×10^5 and 10^6, were selected as representatives for high, medium and low molecular weight chitosan. When mixed with AOT, high molecular weight chitosan was precipitated out while the medium and low molecular weight chitosan allowed the formation of nanoparticles (**Table 1**).

Table 1 Size of chitosan/AOT nanoparticles formed by stirring mixtures chitosan and AOT solutions.

M_v	**Particle size (nm)**
~10^6	Precipitate
~1.5×10^5	110
~10^4	78

[chitosan] = 2.5 mM, [AOT] = 50 mM.

Effect of the mixing methods

Three methods, stirring, vortexing and ultrasonicating were used to provide roughly three different levels of mechanical energy for mixing chitosan with AOT. At higher AOT/chitosan mole ratio the size of the particles was independent to the mixing methods. When the AOT/chitosan mole ratio decreased, the size of the particles got smaller in order of stirring, vortexing and ultrasonicating, respectively (**Figure 3)**. The results suggested that with limited amount of surfactant (surfactant/chitosan mole ratio = 10), the mixing method providing higher energy and more uniform mechanical agitation such as ultrasonication could lead to better mixing and thus the smaller particle size.

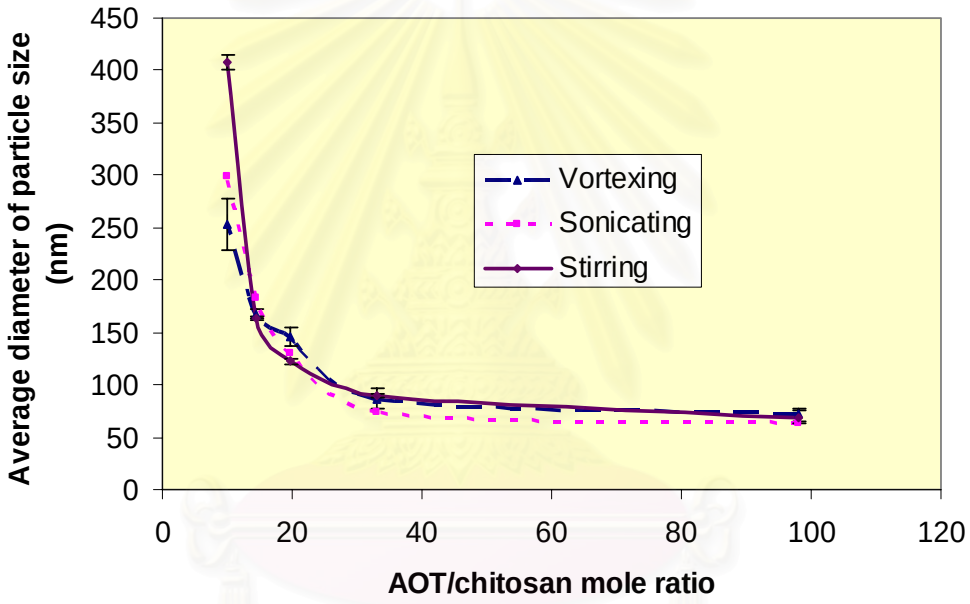
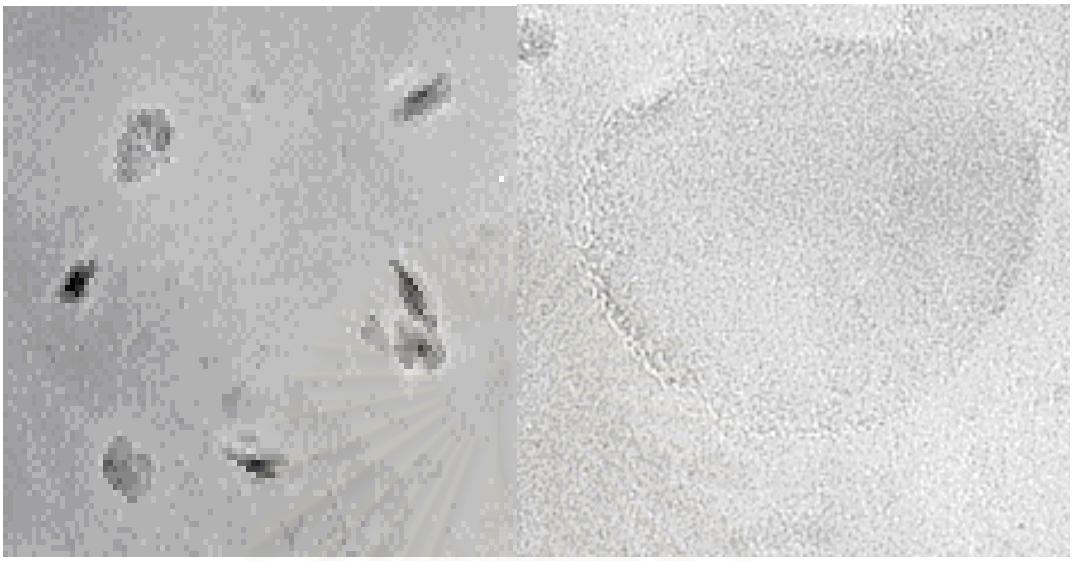


Figure 3 Particles sizes of chitosan/AOT nanoparticles obtained from different dispersing methods. [AOT] = 50 mM and M_v of chitosan = 1.5×10^5.

Transmission electron microscope imaging

The morphotogical examination of the chitosan/AOT nanoparticles was performed with a transmission electron microscope (TEM). TEM micrograph of a residue from the mixture of chitosan and AOT solution displayed oval solid structures with submicron sizes (**Figure 4 a**). It is important to note that no such structure observed in the TEM images of the residues from either chitosan or AOT solutions. The high resolution TEM image of a chitosan/AOT nanoparticles showed that there was a darker shell surrounding lighter area of the particle (**Figure 4 b**). To obtain

more information about the structure of chitosan/AOT nanoparticles, the zeta potential of the particle was measured.



(a) **(b)**

Figure 4 TEM micrograph of the chitosan/AOT (a) × 25 K and (b) × 100 K.

Determination of particle surface potential (zeta potential)

The zeta potential of AOT and SDS surfactant were -79 mV and -43 mV, respectively, at pH 2.0. Upon increasing pH, the value of zeta potential of AOT and SDS were less reliable due to the highly dynamic value of AOT and SDS micelle. (**Figure 3.5** and **Figure 3.6**)

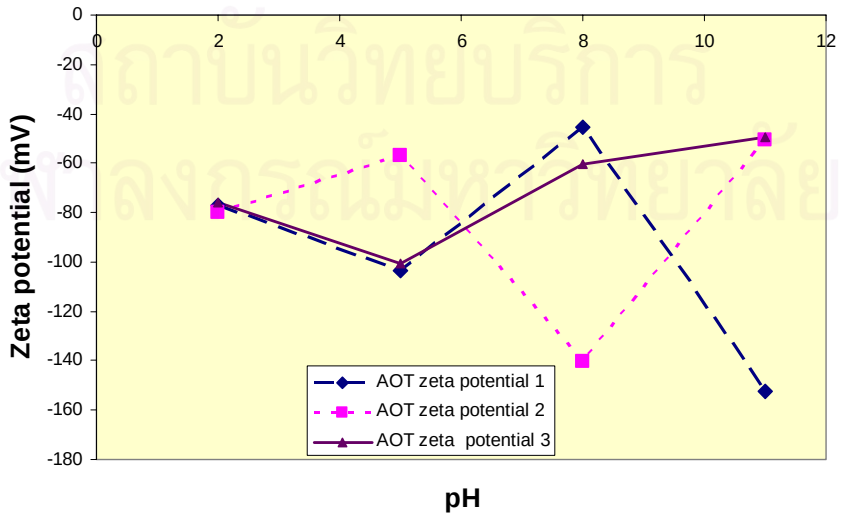


Figure 3.5 Zeta potential of AOT surfactant ; [AOT] = 100 mM

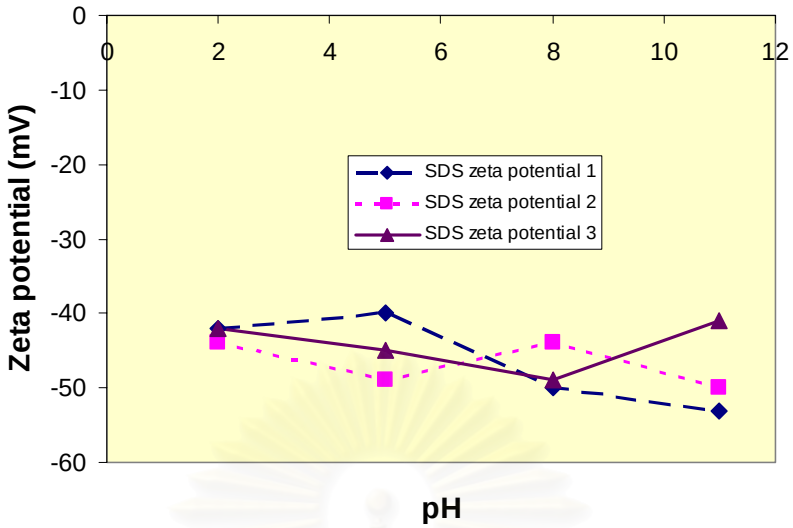


Figure 3.6 Zeta potential of SDS surfactant ; [SDS] = 100 mM

The zeta potential of chitosan/surfactant nanoparticles were slightly less negative than their corresponding surfactants. At pH 2.0, chitosan/AOT nanoparticles showed zeta potential of -79 mV and chitosan/SDS showed zeta potential of -43 mV. The zeta potential of chitosan/surfactant nanoparticles were gradually more negative with increasing pH from 2.0 to 11.0. The values of zeta potential for chitosan/surfactant nanoparticles could be readily reproduced unlike those of their corresponding surfactants. The results indicated that the surfactant molecules in/on the chitosan/surfactant nanoparticles were less dynamic (**Figure 3.7**).

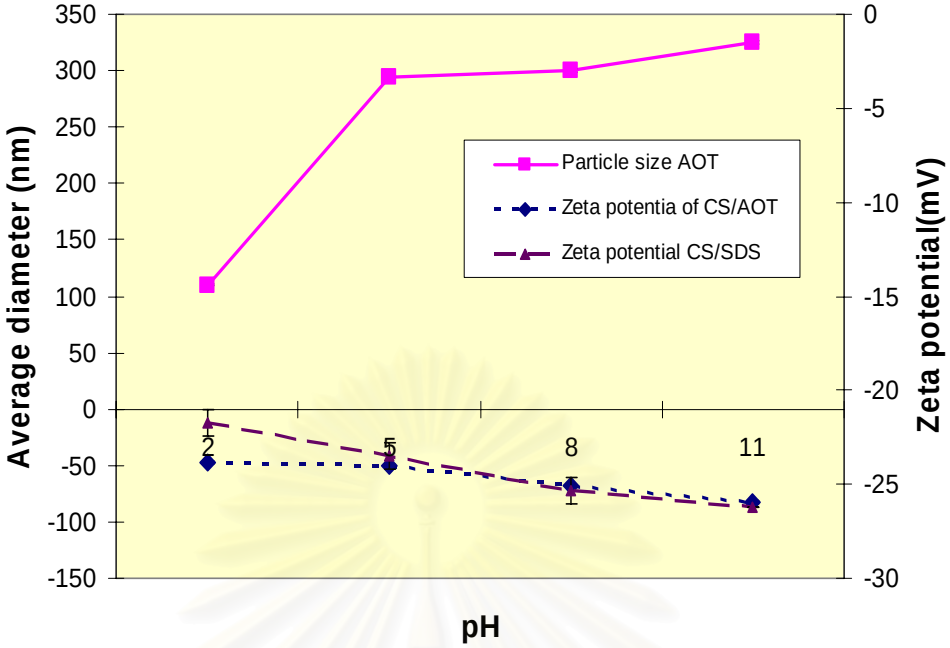


Figure 7 Average particles size and zeta potential of chitosan/AOT and chitosan/SDS nanoparticles at pH 2.0 – 11.0. [chitosan] = 2.5 mM ; [AOT] = 50 mM ; [SDS] = 50 mM.

The negative potential indicated that the surface of the particle consisted mainly the anionic AOT surfactant. The structure of chitosan/AOT nanoparticles may consist of chitosan chain and AOT molecule forming a charge neutralized complex core particle surrounded by lipid bilayer of AOT. This model is consistent with the dark shell observed in the TEM image and the negative zeta potential. The lipid bilayer shell is probably attached to the charge complex of chitosan/AOT core through multi-sited hydrogen bonding.

Loading of medicine

Tetracyline and diclofinac sodium salt were selected for cationic and anionic drug models for loading into the chitosan/surfactant nanoparticles by adding a drug solution into a preformed chitosan/surfactant nanoparticle solution. The effect of the added drug on the size and storage stability of the nanoparticles were evaluated. The loading efficiencies of drugs into the nanoparticles were determined. The zeta potentials of the drug incorporated particles were measured to obtain information how the drug molecules of drugs incorporated into the particles.

Size and stability of chitosan/surfactant/drug nanoparticles

The size of chitosan/AOT nanoparticles were virtually unchanged after mixing with tetracycline at the chitosan/AOT/tetracycline mole ratio of 20/1/1. The average

size of the particles also stayed almost the same after 30 days of storage (**Table 2**). Adding tetracycline into the chitosan/SDS system at the same mole ratio however increased the size of the particles slightly from 142 to 167 nm but the particles swelled further to give an average diameter of 324 nm after being kept for 15 days. Diclofenac sodium salt, on the other hand, could be mixed with chitosan/SDS system (chitosan/SDS/diclofenac sodium salt = 20/1/0.5) with slight decrease of particle size to 122 nm and it increased only slightly from 122 to 195 nm after 30 days of storing. Adding diclofenac sodium salt into the chitosan/AOT nanoparticulate system was however resulting in a precipitation of the nanoparticles due to aggregation.

It is also interesting to note that the incorporation of tetracycline also slightly increased the stability of the chitosan/AOT nanoparticles evidenced by the smaller changes in the averaged size of the particles, upon aging, comparing to the particles without the drug.

Table 2 Size and storage stability of chitosan/surfactant nanoparticles in the absence and the presence of drug, prepared by stirring method.

Nanoparticles	Storing (days)	Particle size (nm)
Chitosan/AOT		
Non loaded medicine	1	96
	15	98
	30	107
Tetracycline	1	95
	15	98
	30	98
Diclofenac sodium salt	1	precipitated
	15	precipitated
	30	precipitated
Chitosan/SDS		
Non loaded medicine	1	142
	15	150
	30	307
Tetracycline	1	167
	15	324
	30	precipitaed
Diclofenac sodium salt	1	122
	15	160
	30	195

[chitosan] = 1.7 mM, [surfactant] = 33.3 mM, and [drug] = 1.7 mM for tetracycline and [drug] = 0.83 mM for diclofinac sodium salt

Loading efficiency

The loading efficiency of drug into the nanoparticles were calculated from the following equation:

$$\text{Loading efficiency (\%)} = \frac{[\text{drug}]_{\text{initial}} - [\text{drug}]_{\text{remained in the solution}} \times 100}{[\text{drug}]_{\text{initial}}}$$

The concentrations of tetracycline and diclofenac sodium salt remained in the solution were measured from the supernatant after ultracentrifugation by UV-Vis spectrophotometer at 357 nm and 275 nm, respectively. The concentration of tetracycline in the chitosan/AOT/tetracycline was reduced from 1.32 mM to 0.66 mM and the concentration of diclofenac sodium salt in the chitosan/SDS/diclofinac sodium salt was reduced from 0.40 mM to 0.24 mM. The loading efficiency of tetracycline by chitosan/AOT nanoparticles was thus 50% and that of diclofenac sodium salt by chitosan/SDS nanoparticles was 40% (**Table 3**).

Table 3 Loading efficiency of tetracycline by chitosan/AOT nanoparticles

	Chitosan/AOT
[Tetracycline] initial (mM)	1.32
[Tetracycline] remaining (mM)	0.66
% Loading efficency	50%

[chitosan] = 2.5 mM, [AOT] = 50 mM, [tetracycline] = 1.32 mM

Table 4 Loading efficiency of diclofenac sodium salt by chitosan/SDS nanoparticles

	Chitosan/SDS
[diclofenac sodium salt] initial (mM)	0.40
[diclofenac sodium salt] remaining (mM)	0.24
% Loading efficency	40%

[chitosan] = 2.5 mM, [AOT] = 50 mM, [diclofenac sodium salt] = 0.40 mM.

Zeta potential

To understand how tetracycline incorporated into the chitosan/AOT nanoparticles, the zeta potential of the chitosan/AOT/tetracycline was measured. Surprisingly, the zeta potential of the nanoparticles became even more negative with

addition of tetracycline at pH 2 **(Figure 8)**. The result suggested that the positively charged tetracycline molecules were not on the surface of the particles but defused through the lipid bilayer shell into the particles. The increase of positive charges inside the particle demanded more negatively charged surfactant molecules to cover it. Tetracycline probably also increased the hydrogen bond interaction between the core and shell of the particle resulting in greater stability of the particles. At acidic pH, diclofenac sodium salt on the other hand is protonated into acid form which has poor solubility in water. Therefore, precipitation was observed when the chitosan/AOT was mixed with diclofenac sodium salt. In chitosan/SDS system, the acid form of diclofenac sodium salt was probably carried into the nanoparticles by the micelles of SDS.

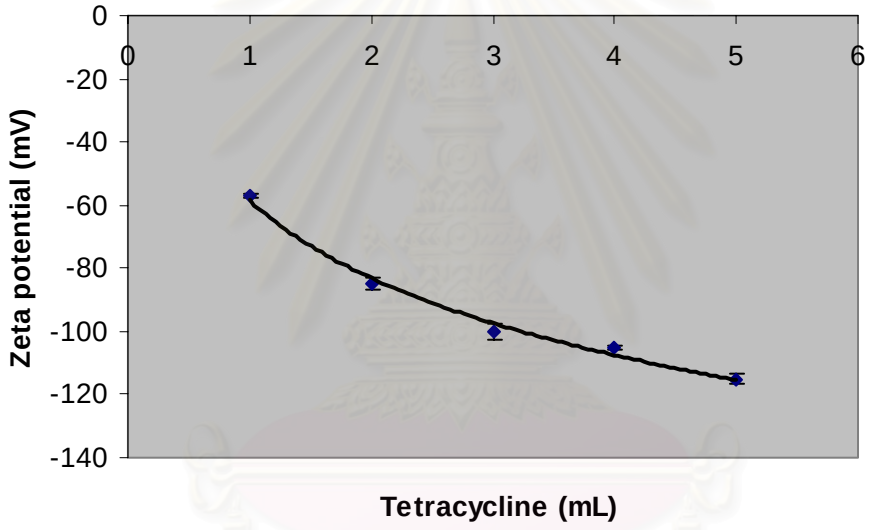


Figure 8 Zeta potential of chitosan/AOT/tetracycline nanoparticles after complexed with tetracycline (1.7 mM); pH = 2.0, [chitosan] = 1.7 mM, [AOT] = 33.3 mM.

Conclusion

The anionic surfactants such as AOT and SDS allowed the formation of nanoparticles. The size of nanoparticles could be varied from 100-500 nm. The TEM images and zeta potential suggested the structure of chitosan/AOT nanoparticle consisted of a lipid bilayer shell of AOT surrounding a charge complex core of chitosan and AOT. The positively charged medicine can be loaded to chitosan/AOT nanoparticles and diclofenac sodium salt can be loaded to chitosan/SDS nanoparticles.

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APPENDIX B

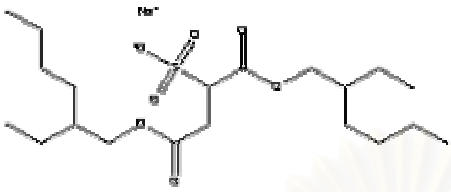


Figure B.1 Chemical structure of bis (ethylhexyl) sulfosuccinate (AOT)

Figure B.2 Chemical structure of sodium dodecyl sulfate (SDS)

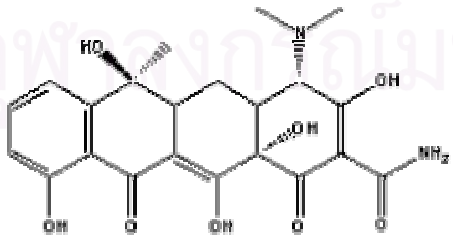


Figure B.3 Chemical structure of tetracycline

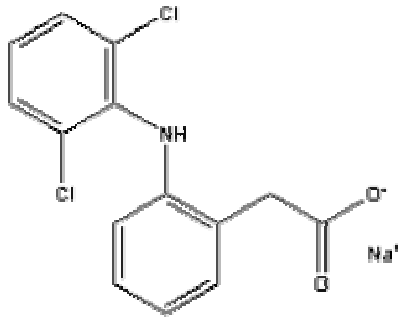


Figure B.4 Chemical structure of diclofenac sodium salt

APPENDIX C

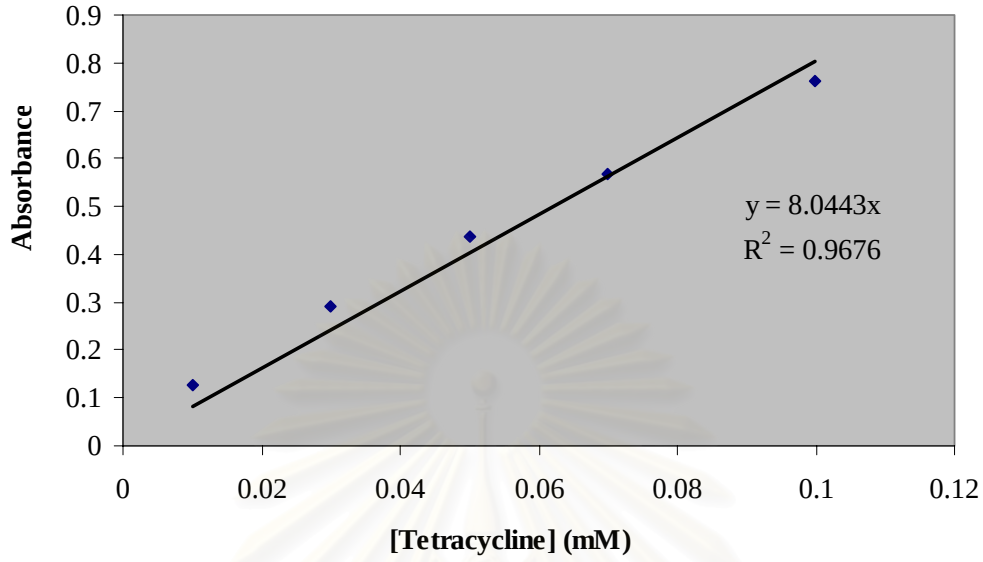


Figure C.1 Calibration curve of tetracycline in aqueous solution by UV-visible spectrophotometer at 357 nm.

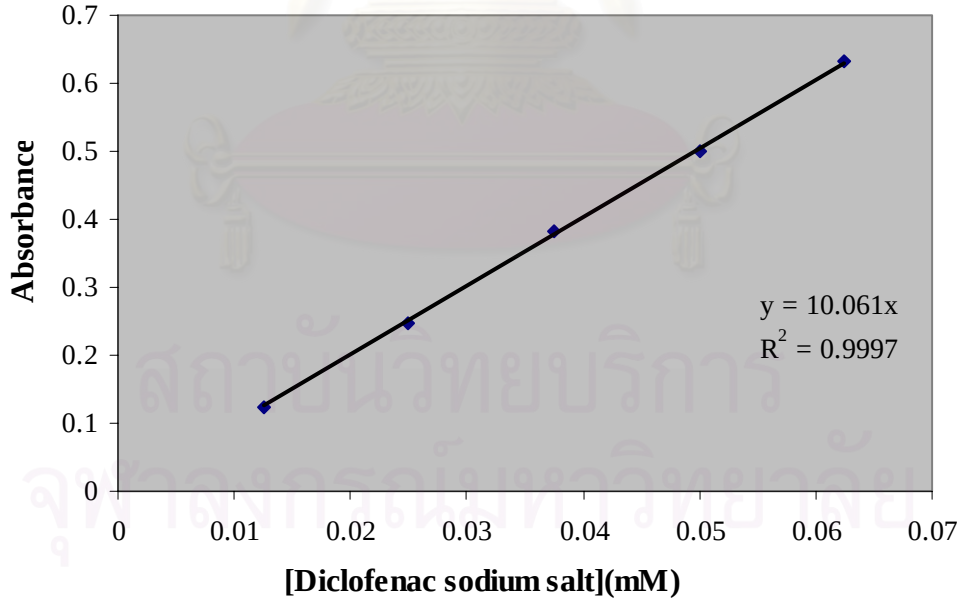
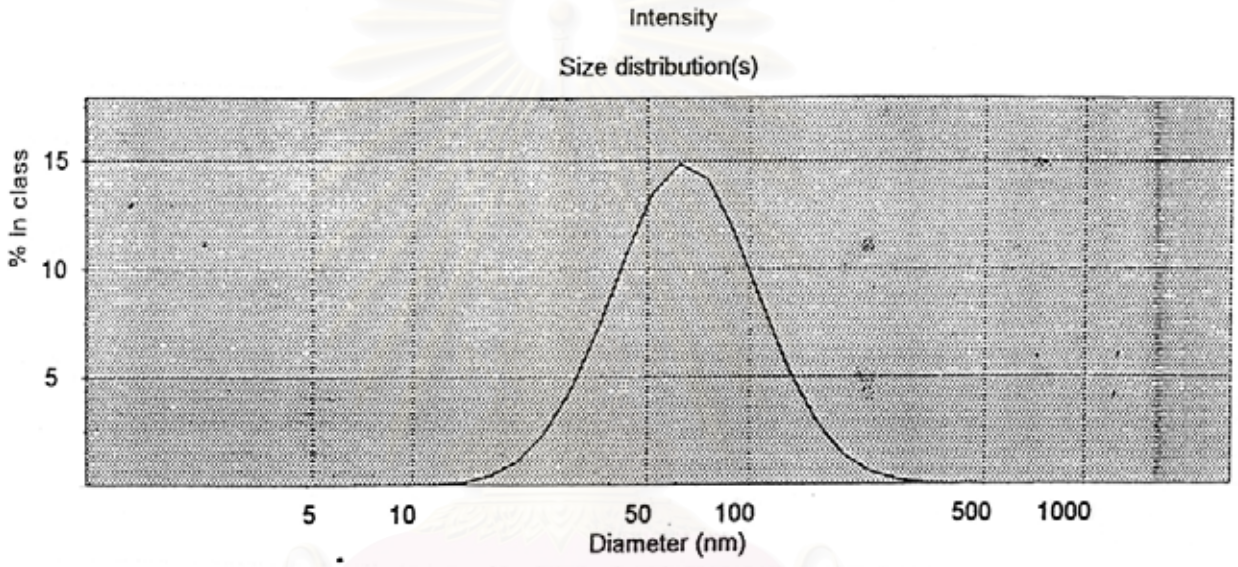


Figure C.2 Calibration curve of diclofenac sodium salt in aqueous solution by UV-visible spectrophotometer at 275 nm.

APPENDIX D

Result

Quality Factor:	Pass	Intensity Mean (nm):	72.6
Z Average Mean(nm):	64.2		
Polydispersity:	0.25	Analysis Mode:	Monomodal

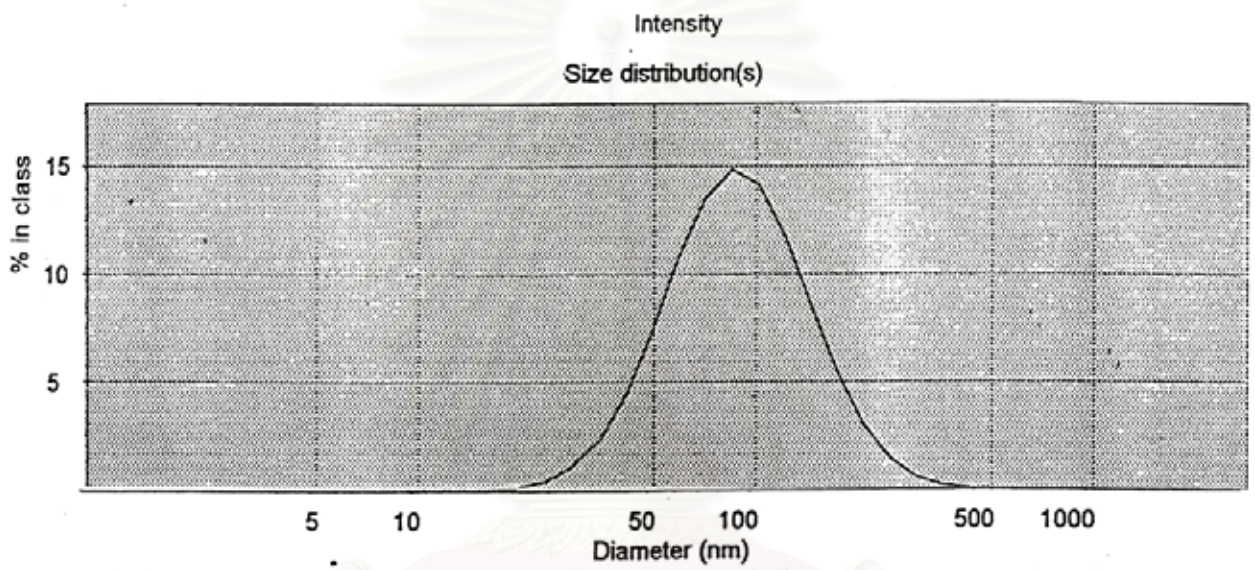


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
6.8	0.0	29.7	4.5	130.8	5.3
8.1	0.0	35.8	7.4	157.4	2.9
9.8	0.0	43.0	10.8	189.4	1.4
11.8	0.0	51.8	13.5	228.0	0.6
14.2	0.1	62.3	14.8	274.4	0.2
17.0	0.4	75.0	14.2	330.2	0.1
20.5	1.1	90.3	11.8	397.4	0.0
24.7	2.3	108.7	8.5	478.3	0.0

Figure D.1 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 0.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	98.2
Z Average Mean(nm):	87.4		
Polydispersity:	0.23	Analysis Mode:	Monomodal

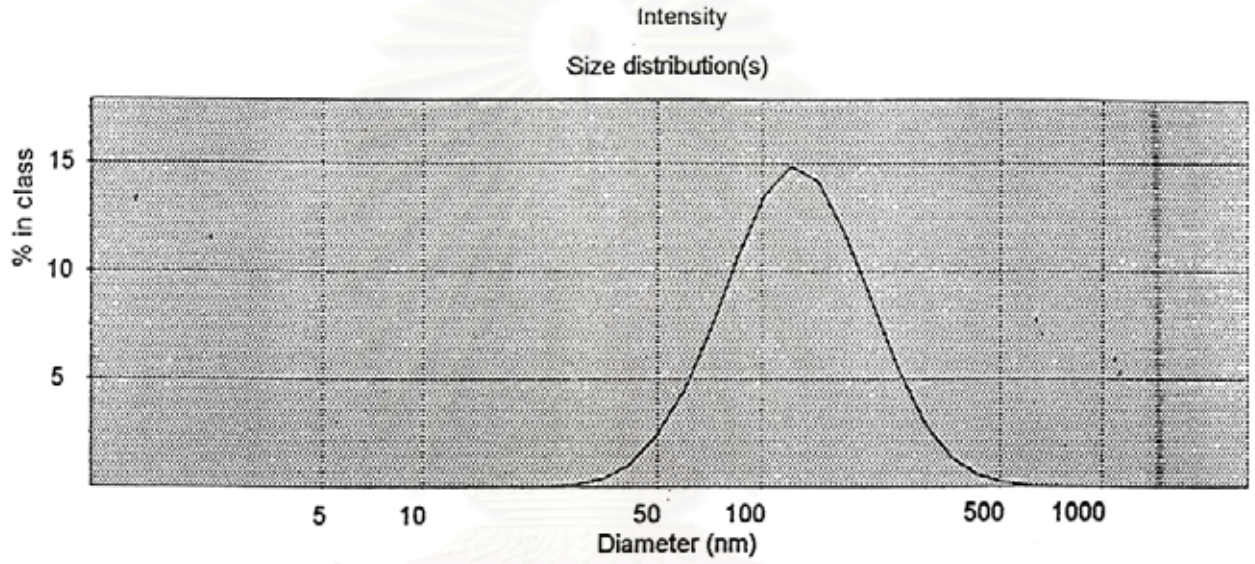


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
9.8	0.0	41.3	4.5	174.5	5.4
11.7	0.0	49.4	7.4	209.0	2.9
14.0	0.0	59.2	10.7	250.3	1.4
16.8	0.0	70.9	13.5	299.7	0.6
20.1	0.1	84.9	14.8	358.9	0.2
24.0	0.4	101.6	14.2	429.8	0.1
28.8	1.1	121.7	11.8	514.7	0.0
34.5	2.3	145.7	8.5	616.4	0.0

Figure D.2 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 1.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	141.3
Z Average Mean(nm):	125.7		
Polydispersity:	0.23	Analysis Mode:	Monomodal

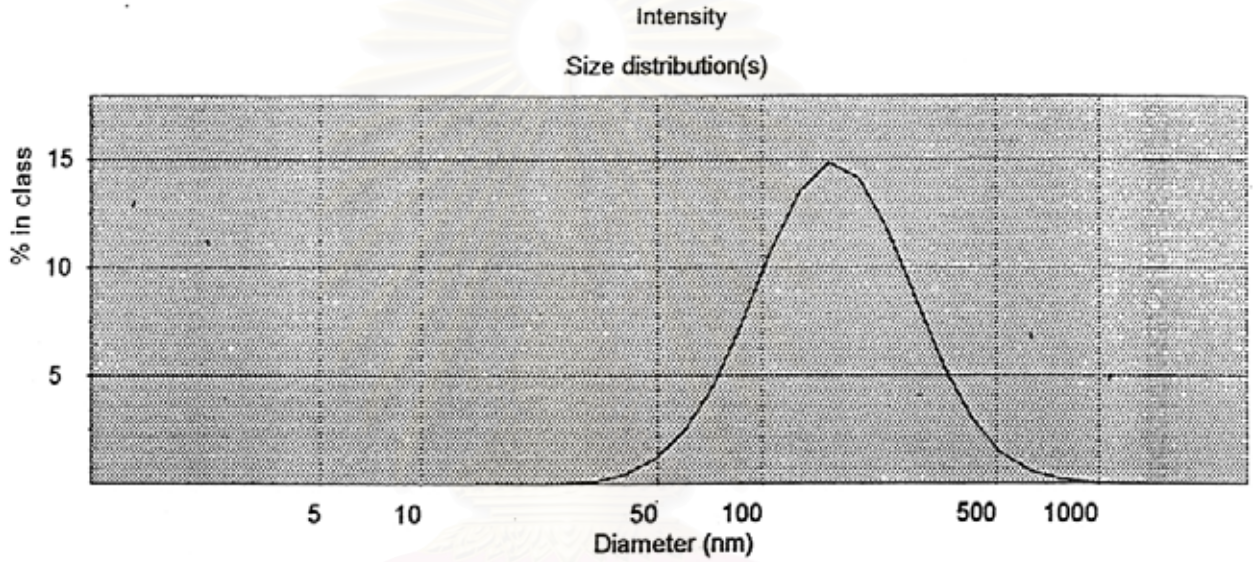


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
14.1	0.0	59.4	4.5	250.7	5.4
16.9	0.0	71.1	7.4	300.2	2.9
20.2	0.0	85.2	10.7	359.3	1.4
24.2	0.0	102.0	13.5	430.2	0.6
28.9	0.1	122.1	14.8	515.0	0.2
34.6	0.4	146.1	14.2	616.5	0.1
41.5	1.1	174.9	11.8	738.1	0.0
49.6	2.3	209.4	8.5	883.6	0.0

Figure D.3 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 2.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	187.9
Z Average Mean(nm):	163.3		
Polydispersity:	0.28	Analysis Mode:	Monomodal

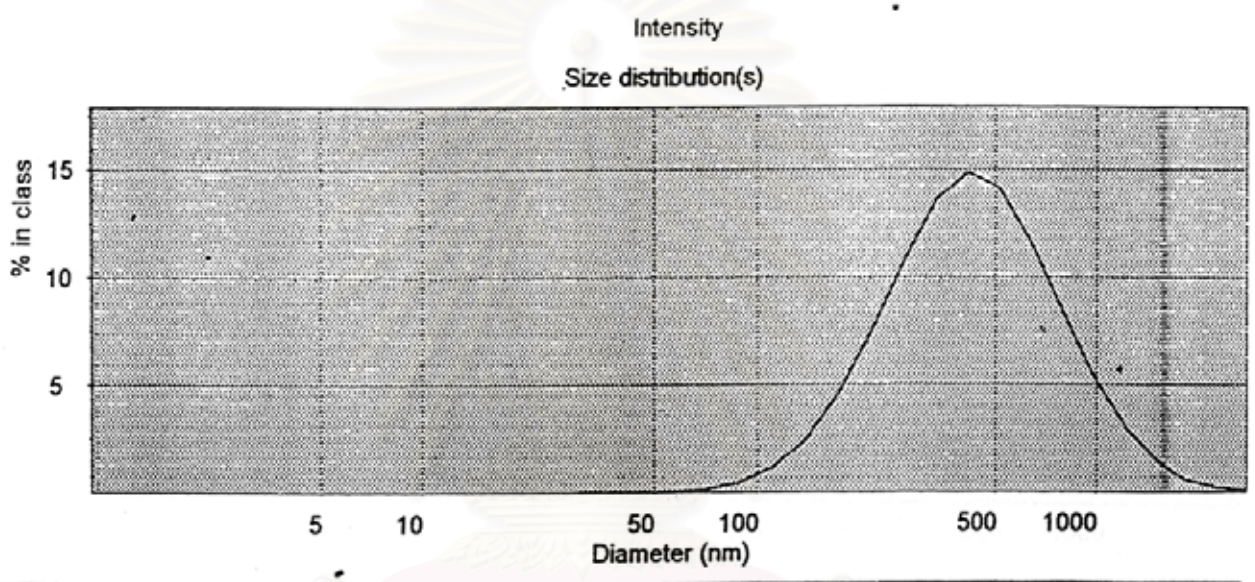


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
14.8	0.0	71.8	4.5	349.7	5.3
18.0	0.0	87.6	7.5	426.2	2.9
21.9	0.0	106.7	10.8	519.5	1.4
26.7	0.0	130.1	13.6	633.1	0.6
32.6	0.1	158.5	14.8	771.6	0.2
39.7	0.4	193.2	14.1	940.4	0.1
48.4	1.1	235.4	11.7	1146.1	0.0
58.9	2.4	286.9	8.5	1396.8	0.0

Figure D.4 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 3.0 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	507.7
Z Average Mean(nm):	426.4		
Polydispersity:	0.35	Analysis Mode:	Monomodal

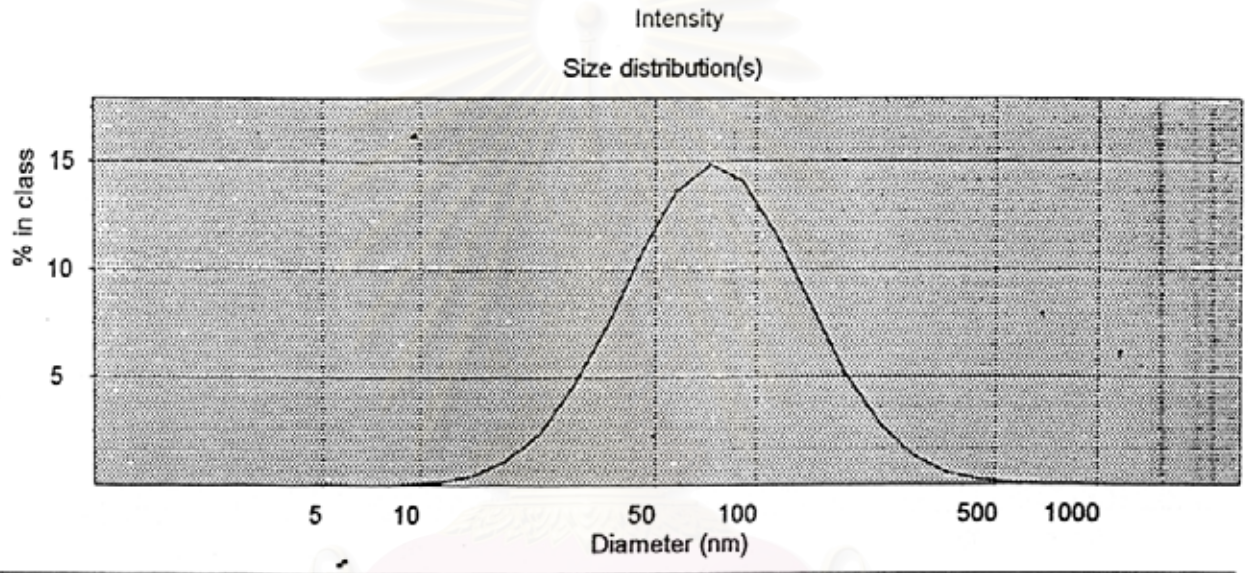


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
29.4	0.0	171.5	4.5	999.3	5.3
36.7	0.0	213.8	7.5	1245.5	2.9
45.7	0.0	266.5	10.8	1552.5	1.4
57.0	0.0	332.1	13.6	1935.1	0.6
71.0	0.2	414.0	14.8	2412.1	0.2
88.6	0.4	516.0	14.1	3006.5	0.1
110.4	1.1	643.2	11.7	3747.5	0.0
137.6	2.4	801.7	8.4	4671.1	0.0

Figure D.5 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 5.0 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	90.0
Z Average Mean(nm):	74.6		
Polydispersity:	0.38	Analysis Mode:	Monomodal

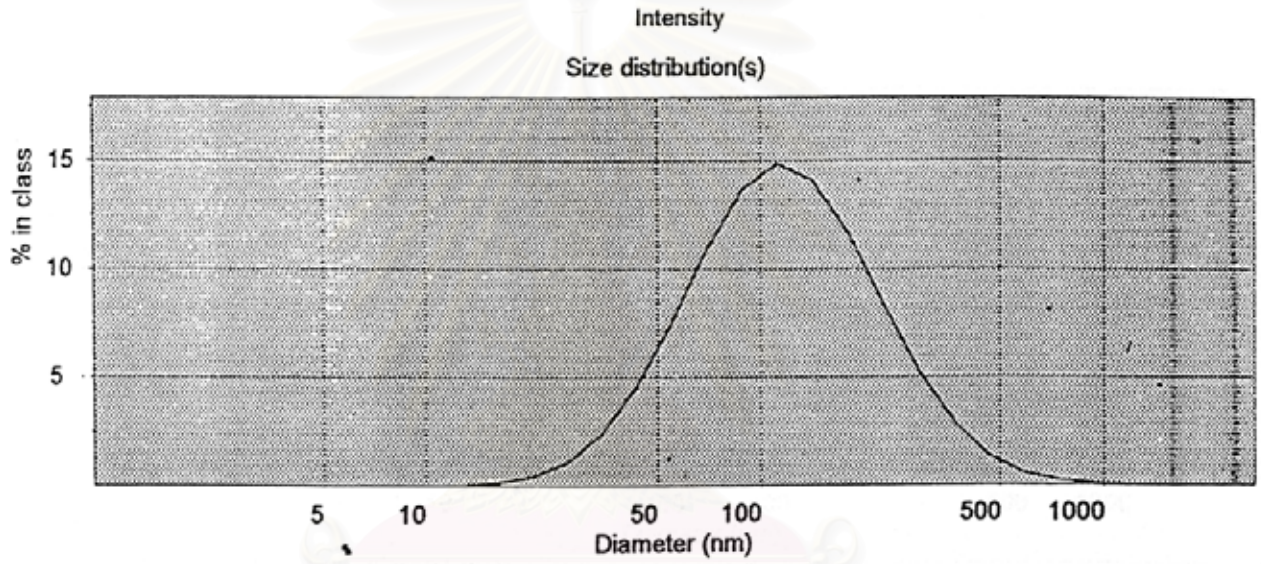


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
4.6	0.0	29.0	4.6	180.9	5.3
5.8	0.0	36.4	7.5	227.4	2.9
7.3	0.0	45.8	10.9	285.9	1.4
9.2	0.0	57.6	13.6	359.5	0.6
11.6	0.2	72.4	14.9	452.0	0.2
14.6	0.4	91.0	14.1	568.3	0.1
18.3	1.1	114.4	11.7	714.5	0.0
23.0	2.4	143.9	8.4	898.3	0.0

Figure D.6 Size distributions of chitosan/SDS nanoparticles ; [SDS] = 50 mM ; [chitosan] = 0.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	141.4
Z Average Mean(nm):	115.3		
Polydispersity:	0.41	Analysis Mode:	Monomodal

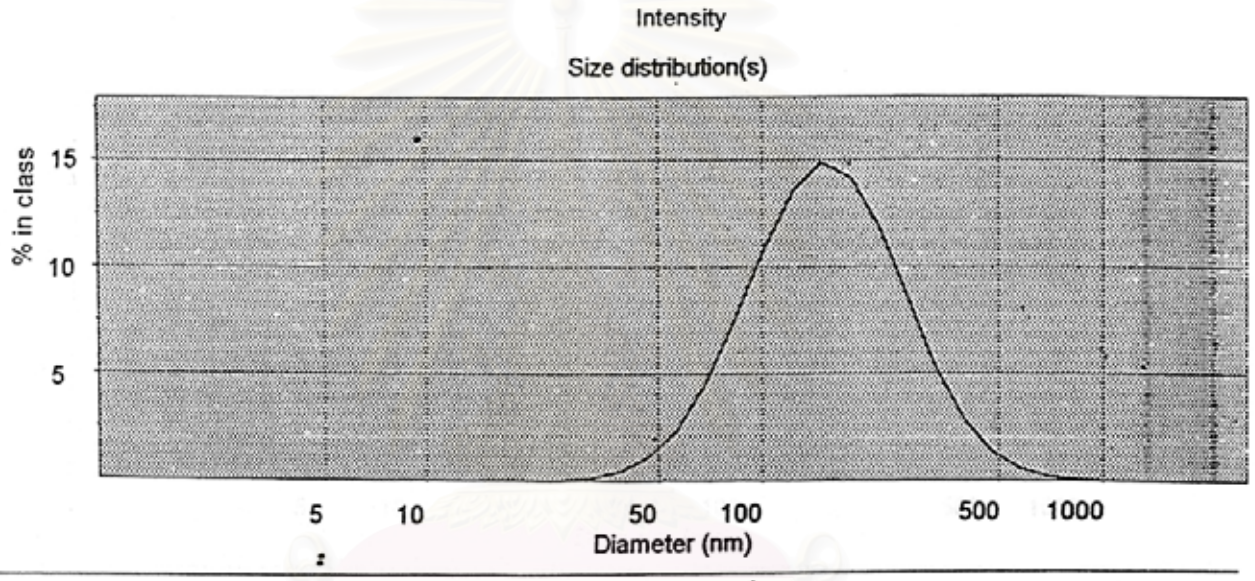


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
6.4	0.0	43.2	4.6	290.3	5.2
8.2	0.0	54.8	7.5	368.4	2.9
10.3	0.0	69.5	10.9	467.4	1.4
13.1	0.0	88.2	13.6	593.1	0.6
16.7	0.2	112.0	14.9	752.6	0.2
21.1	0.4	142.1	14.1	955.0	0.1
26.8	1.1	180.3	11.6	1211.8	0.0
34.0	2.4	228.8	8.4	1537.7	0.0

Figure D.7 Size distributions of chitosan/SDS nanoparticles ; [SDS] = 50 mM ; [chitosan] = 1.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	175.6
Z Average Mean(nm):	153.3		
Polydispersity:	0.27	Analysis Mode:	Monomodal

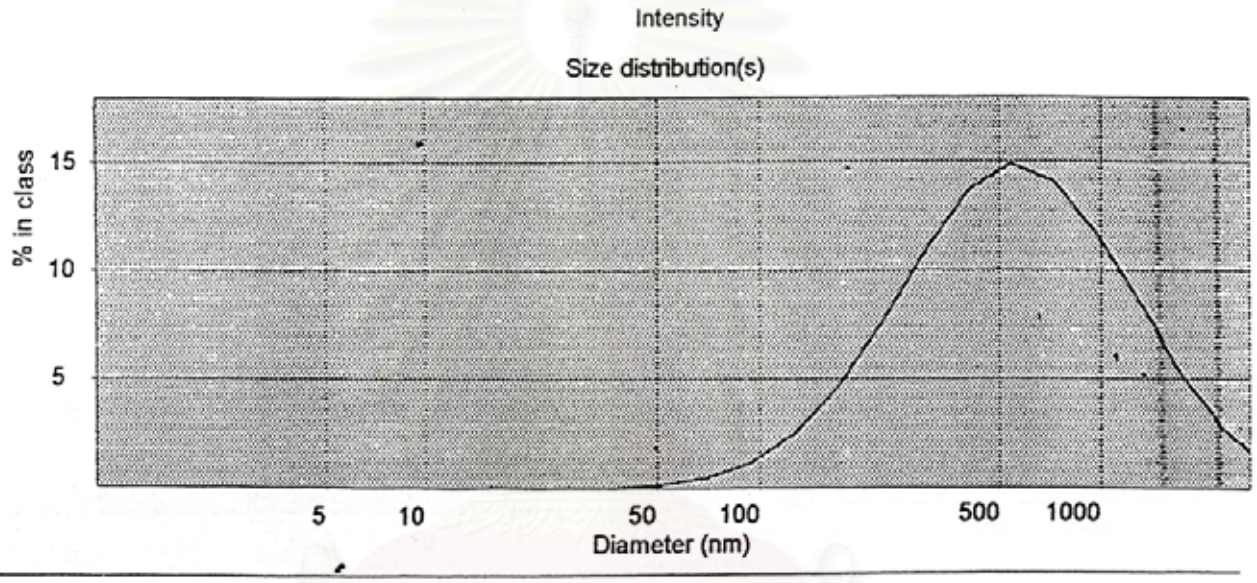


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
14.4	0.0	68.4	4.5	324.0	5.3
17.5	0.0	83.0	7.5	393.6	2.9
21.3	0.0	100.9	10.8	478.1	1.4
25.8	0.0	122.5	13.6	580.7	0.6
31.4	0.1	148.8	14.8	705.4	0.2
38.1	0.4	180.8	14.1	856.8	0.1
46.3	1.1	219.6	11.7	1040.8	0.0
56.3	2.4	266.7	8.5	1264.2	0.0

Figure D.8 Size distributions of chitosan/SDS nanoparticles ; [SDS] = 50 mM ; [chitosan] = 2.5 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	772.9
Z Average Mean(nm):	564.1		
Polydispersity:	0.63	Analysis Mode:	Monomodal

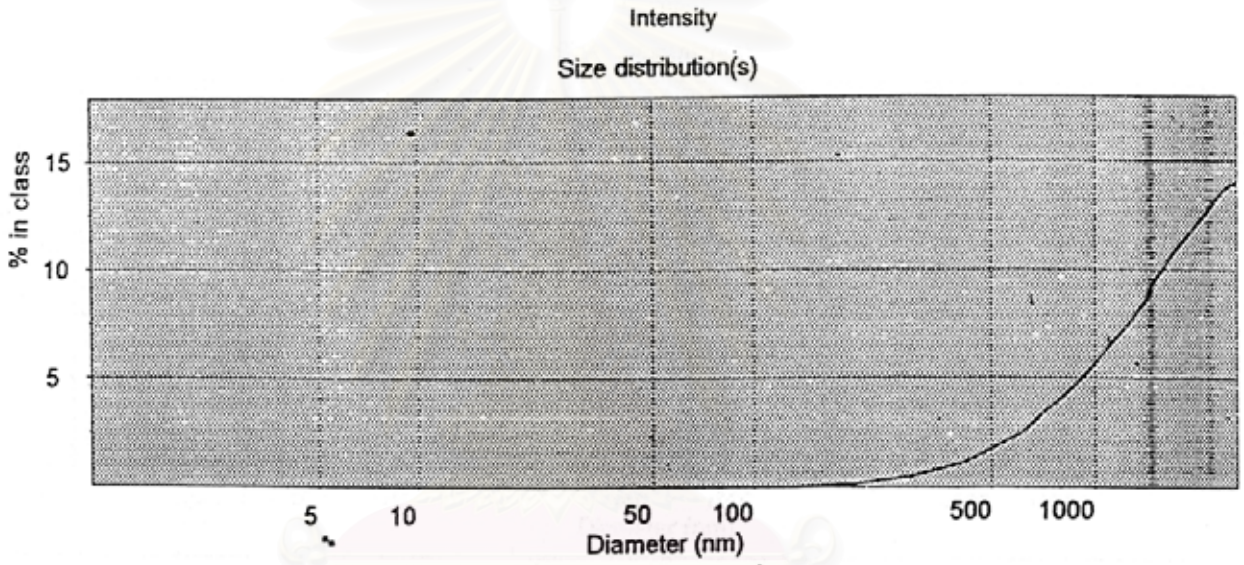


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
15.7	0.0	167.7	4.6	1788.6	5.2
21.1	0.0	225.4	7.6	2404.5	2.8
28.4	0.0	303.1	10.9	3232.3	1.3
38.2	0.0	407.4	13.7	4345.2	0.5
51.4	0.2	547.7	14.9	5841.2	0.2
69.0	0.4	736.3	14.1	7852.4	0.1
92.8	1.1	989.8	11.6	10555.9	0.0
124.8	2.4	1330.5	8.3	14190.3	0.0

Figure D.9 Size distributions of chitosan/SDS nanoparticles ; [SDS] = 50 mM ; [chitosan] = 3.0 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Fail	Intensity Mean (nm):	6701.4
Z Average Mean(nm):	4066.7		
Polydispersity:	1.00	Analysis Mode:	Monomodal

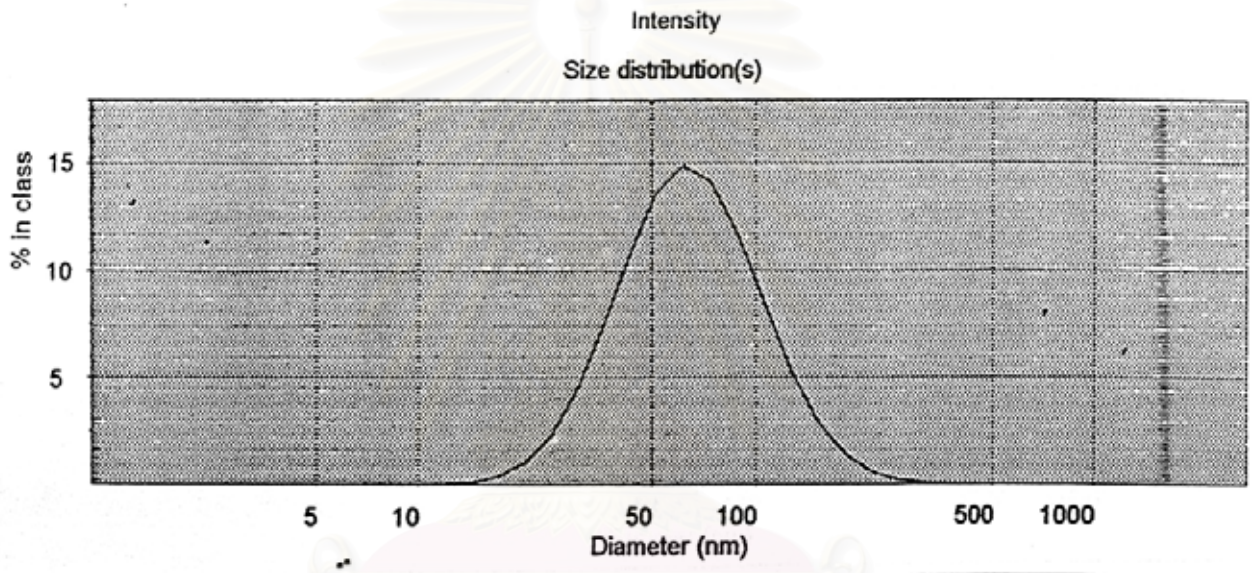


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
45.1	0.0	889.2	4.7	17531.3	5.1
65.5	0.0	1290.8	7.7	25448.8	2.8
95.0	0.0	1873.7	11.0	36941.8	1.3
138.0	0.0	2719.9	13.7	53625.4	0.5
200.3	0.2	3948.3	14.9	77843.5	0.2
290.7	0.5	5731.4	14.0	112999.0	0.1
422.0	1.1	8319.8	11.5	164031.2	0.0
612.6	2.5	12077.1	8.2	238110.5	0.0

Figure D.10 Size distributions of chitosan/SDS nanoparticles ; [SDS] = 50 mM ; [chitosan] = 5.0 mM ; stirring method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	71.4
Z AverageMean(nm):	63.4		
Polydispersity:	0.24	Analysis Mode:	Monomodal

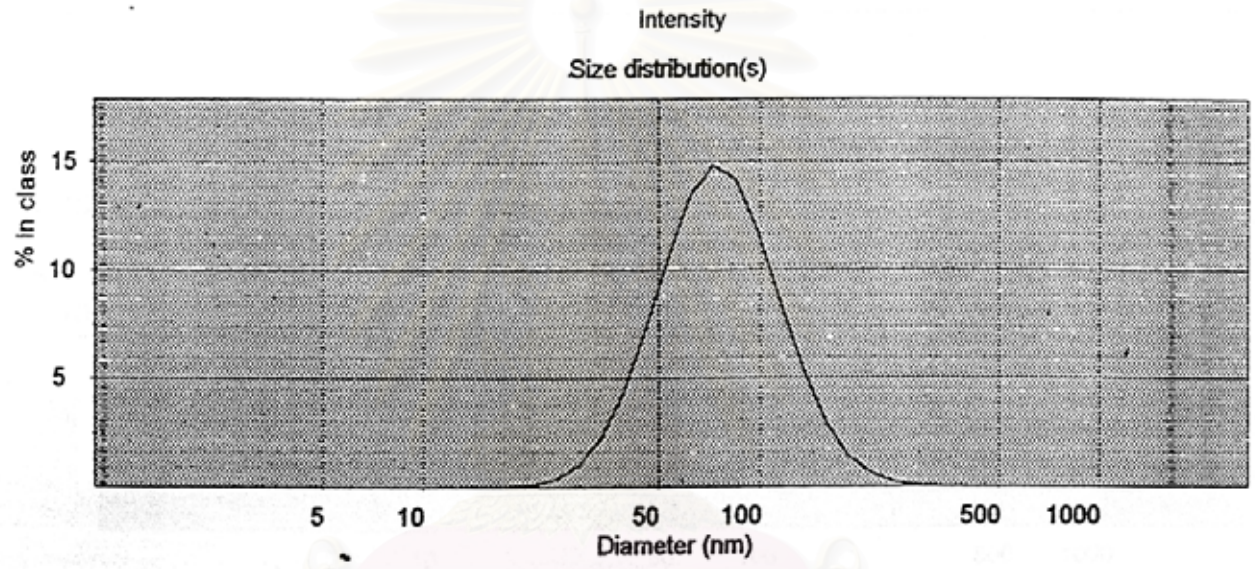


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
6.9	0.0	29.7	4.5	127.4	5.3
8.3	0.0	35.7	7.4	152.8	2.9
10.0	0.0	42.8	10.7	183.3	1.4
12.0	0.0	51.3	13.5	219.9	0.6
14.4	0.1	61.5	14.8	263.8	0.2
17.2	0.4	73.8	14.2	316.5	0.1
20.7	1.1	88.6	11.8	379.6	0.0
24.8	2.3	106.2	8.5	455.4	0.0

Figure D.11 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 0.5 mM ; sonicating method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	81.2
Z Average Mean(nm):	74.6		
Polydispersity:	0.17	Analysis Mode:	Monomodal

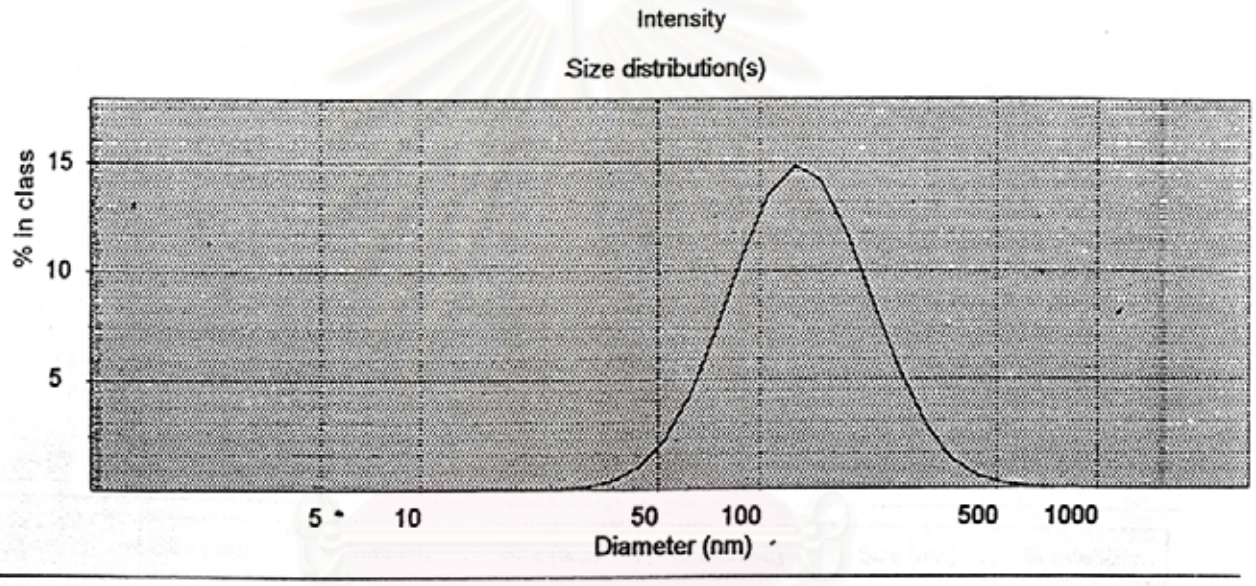


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
11.6	0.0	39.3	4.4	133.6	5.4
13.5	0.0	45.8	7.3	155.6	3.0
15.7	0.0	53.3	10.6	181.3	1.4
18.3	0.0	62.2	13.5	211.3	0.6
21.3	0.1	72.4	14.8	246.2	0.2
24.8	0.4	84.4	14.2	286.9	0.1
28.9	1.0	98.4	11.9	334.3	0.0
33.7	2.3	114.6	8.6	389.6	0.0

Figure D.12 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 1.5 mM ; sonicating method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	146.5
Z Average Mean(nm):	130.9		
Polydispersity:	0.22	Analysis Mode:	Monomodal

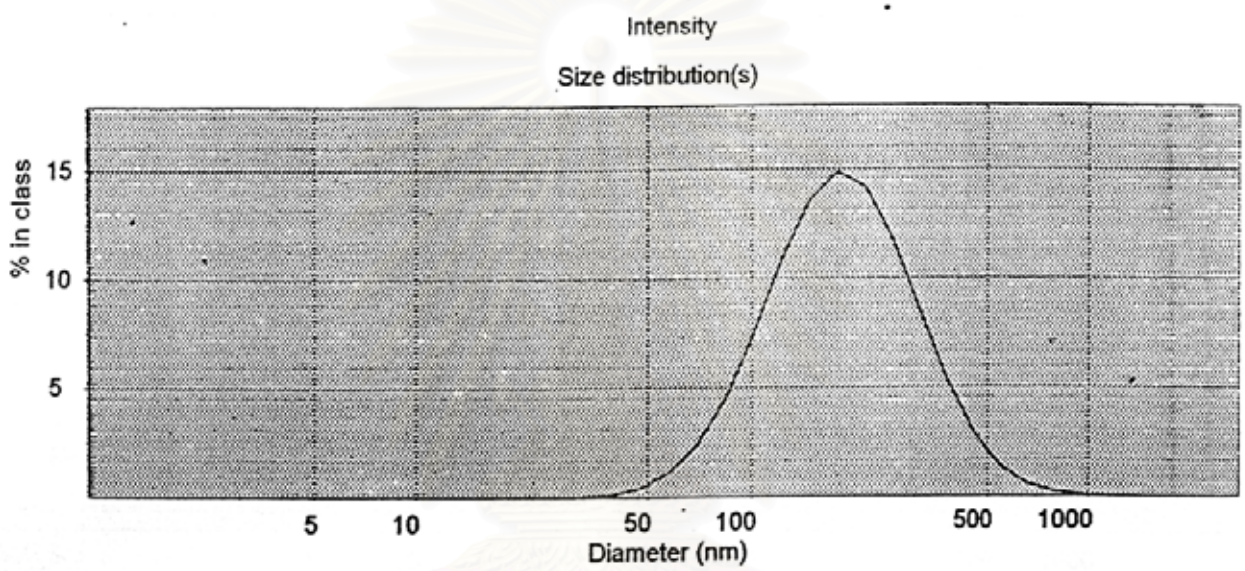


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
15.3	0.0	62.7	4.5	257.6	5.4
18.2	0.0	74.9	7.4	307.3	2.9
21.8	0.0	89.3	10.7	366.7	1.4
26.0	0.0	106.6	13.5	437.5	0.6
31.0	0.1	127.1	14.8	522.0	0.2
36.9	0.4	151.7	14.2	622.8	0.1
44.1	1.1	181.0	11.8	743.0	0.0
52.6	2.3	215.9	8.5	886.5	0.0

Figure D.13 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 2.5 mM ; sonicating method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	207.5
Z Average Mean(nm):	182.7		
Polydispersity:	0.26	Analysis Mode:	Monomodal

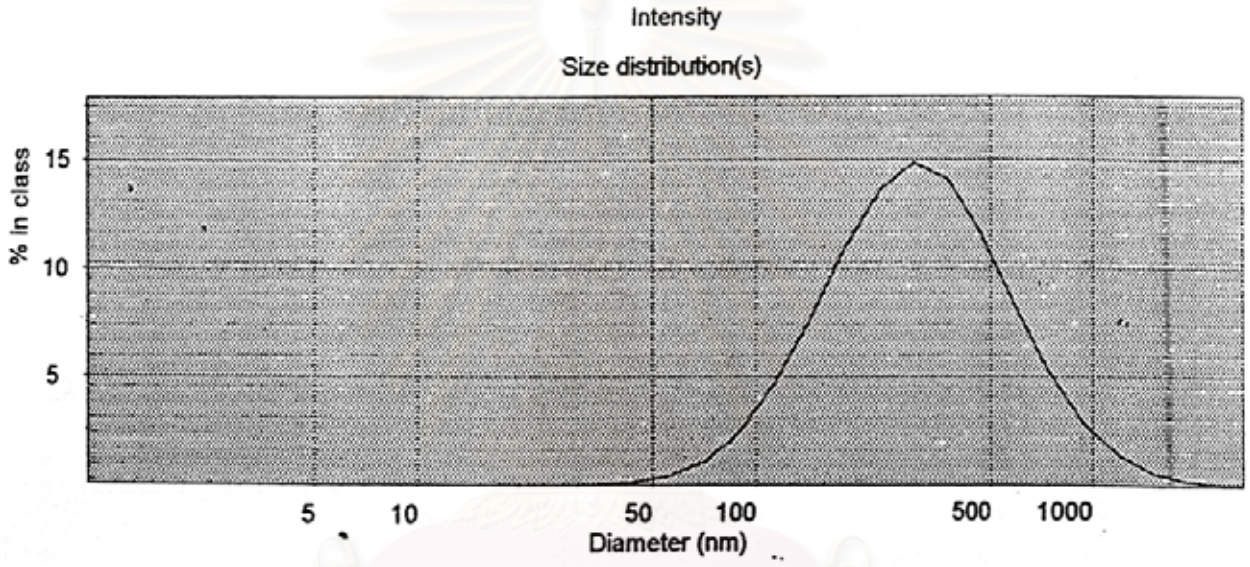


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
18.5	0.0	83.5	4.5	376.6	5.3
22.4	0.0	100.8	7.4	454.7	2.9
27.0	0.0	121.7	10.8	548.9	1.4
32.6	0.0	146.9	13.5	662.5	0.6
39.3	0.1	177.4	14.8	799.8	0.2
47.5	0.4	214.1	14.2	965.5	0.1
57.3	1.1	258.5	11.7	1165.4	0.0
69.2	2.3	312.0	8.5	1406.9	0.0

Figure D.14 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 3.0 mM ; sonicating method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	365.8
Z Average Mean(nm):	299.3		
Polydispersity:	0.40	Analysis Mode:	Monomodal

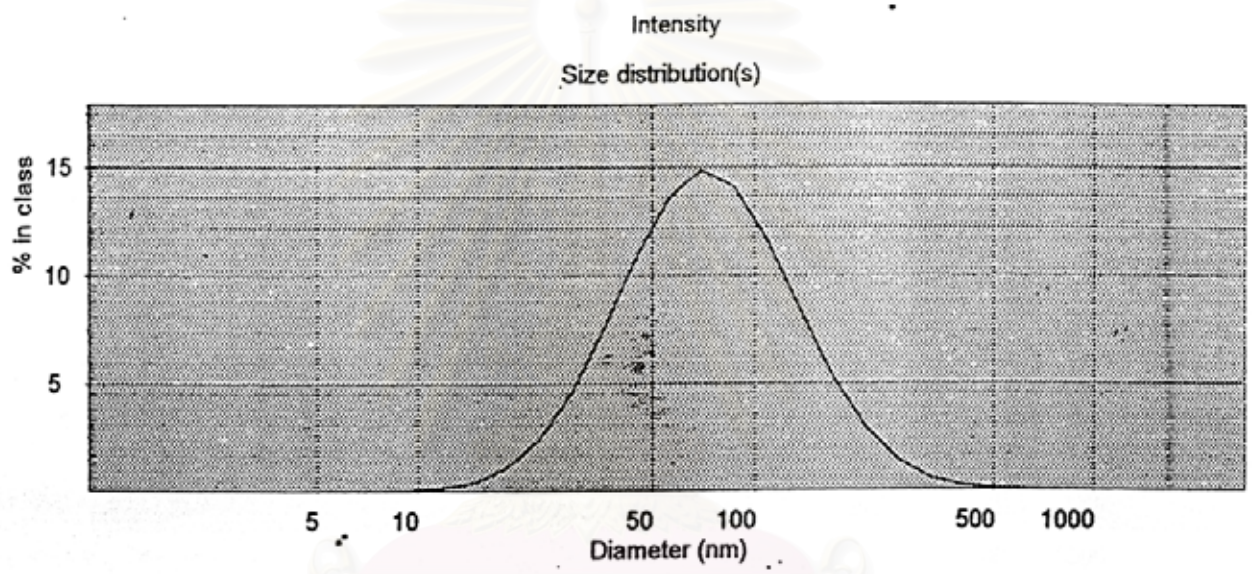


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
17.1	0.0	112.9	4.6	747.5	5.2
21.6	0.0	143.0	7.5	946.6	2.9
27.4	0.0	181.2	10.9	1198.9	1.4
34.7	0.0	229.4	13.6	1518.4	0.6
43.9	0.2	290.6	14.9	1922.9	0.2
55.6	0.4	368.0	14.1	2435.3	0.1
70.4	1.1	466.0	11.6	3084.3	0.0
89.2	2.4	590.2	8.4	3906.1	0.0

Figure D.15 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 5.0 mM ; sonicating method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	85.5
Z Average Mean(nm):	71.8		
Polydispersity:	0.35	Analysis Mode:	Monomodal

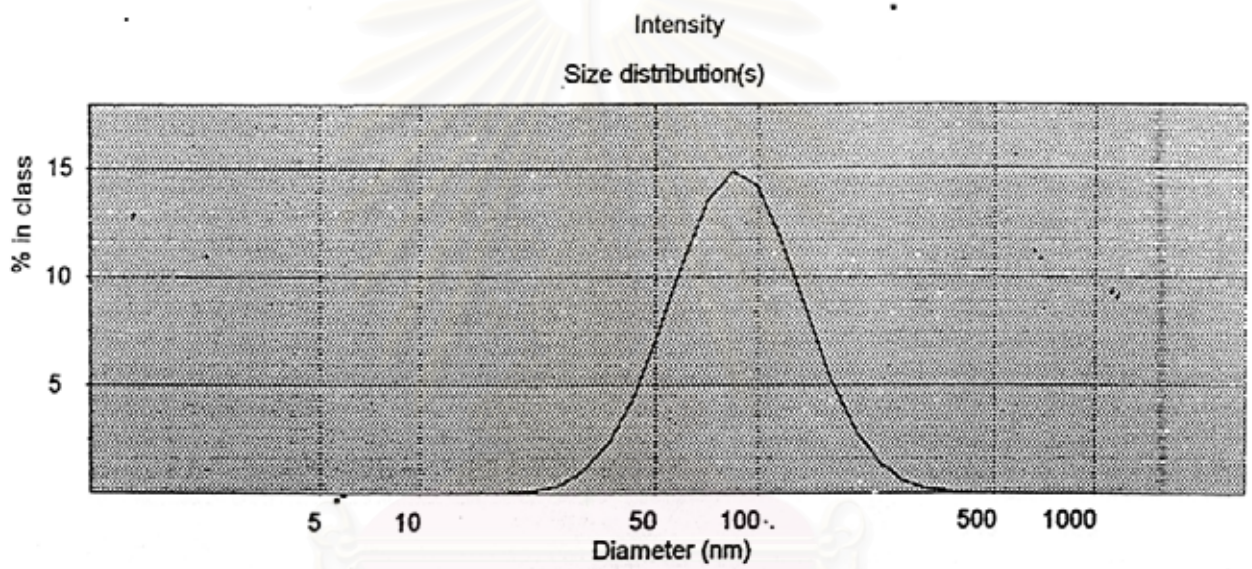


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
4.9	0.0	28.8	4.5	168.4	5.3
6.2	0.0	36.0	7.5	209.9	2.9
7.7	0.0	44.8	10.8	261.8	1.4
9.6	0.0	55.9	13.6	326.3	0.6
11.9	0.2	69.7	14.8	406.9	0.2
14.9	0.4	86.9	14.1	507.3	0.1
18.6	1.1	108.3	11.7	632.4	0.0
23.1	2.4	135.1	8.4	788.5	0.0

Figure D.16 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 0.5 mM ; vortexting method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	95.3
Z Average Mean(nm):	86.3		
Polydispersity:	0.20	Analysis Mode:	Monomodal

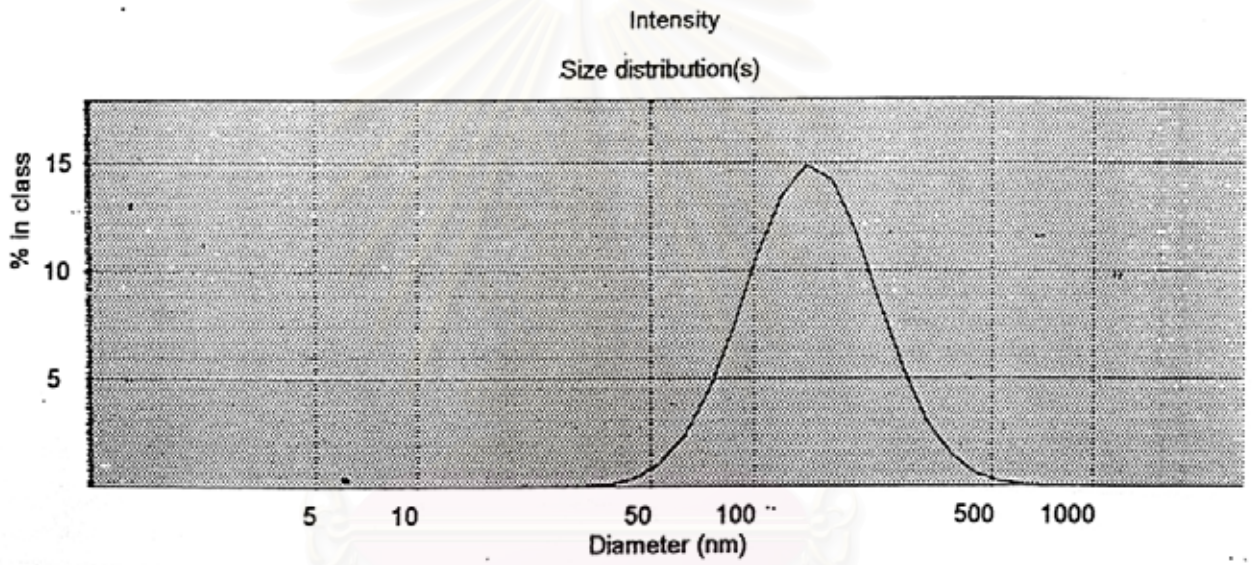


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
11.4	0.0	43.1	4.4	162.8	5.4
13.5	0.0	50.9	7.4	192.3	3.0
15.9	0.0	60.1	10.7	227.0	1.4
18.8	0.0	70.9	13.5	268.1	0.6
22.2	0.1	83.8	14.8	316.6	0.2
26.2	0.4	98.9	14.2	373.8	0.1
30.9	1.0	116.8	11.8	441.4	0.0
36.5	2.3	137.9	8.6	521.2	0.0

Figure D.17 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 1.5 mM ; vortexting method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	161.2
Z Average Mean(nm):	146.3		
Polydispersity:	0.19	Analysis Mode:	Monomodal

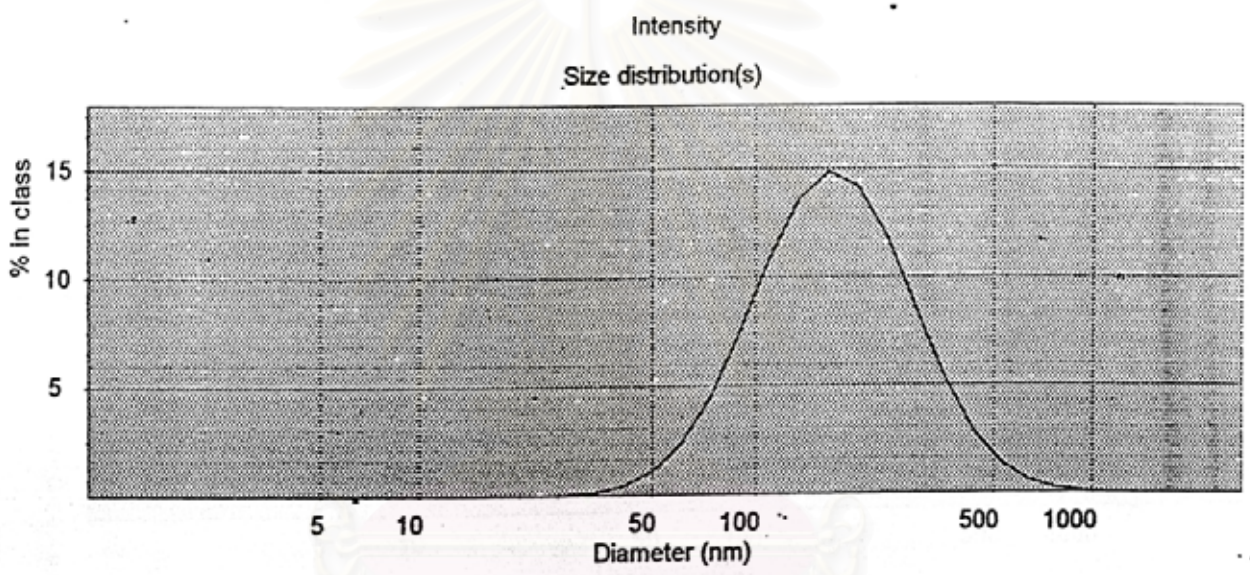


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
19.7	0.0	73.5	4.4	274.3	5.4
23.2	0.0	86.7	7.4	323.3	3.0
27.4	0.0	102.2	10.7	381.1	1.4
32.3	0.0	120.5	13.5	449.3	0.6
38.1	0.1	142.0	14.8	529.7	0.2
44.9	0.4	167.4	14.2	624.4	0.1
52.9	1.0	197.3	11.8	736.1	0.0
62.4	2.3	232.6	8.6	867.8	0.0

Figure D.18 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 2.5 mM ; vortexting method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	191.8
Z Average Mean(nm):	167.0		
Polydispersity:	0.28	Analysis Mode:	Monomodal

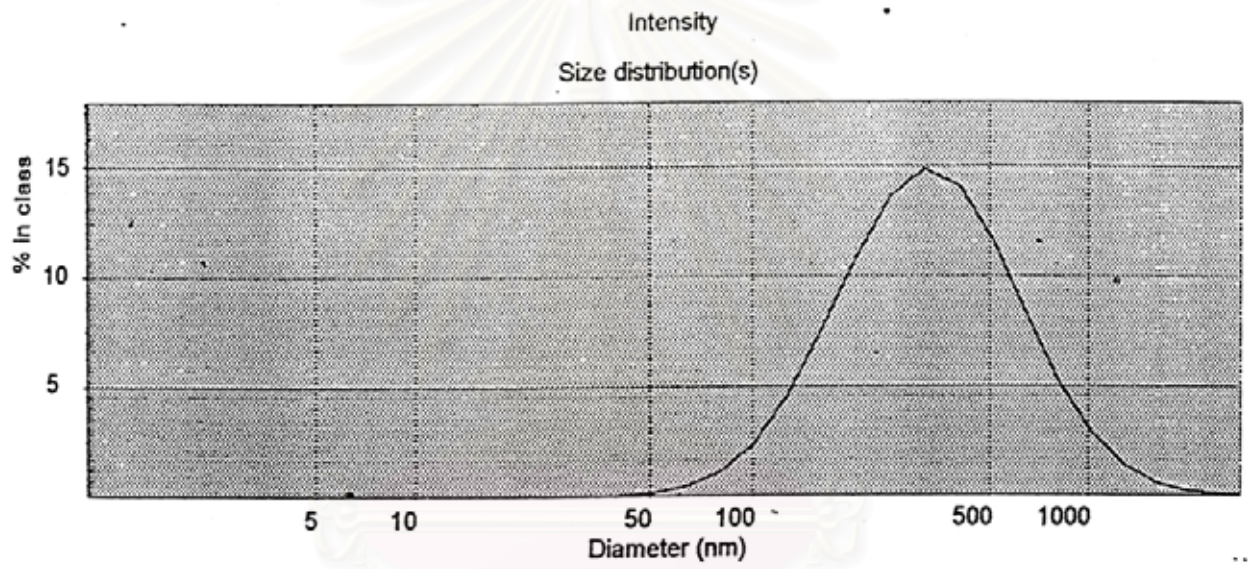


Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
15.4	0.0	74.0	4.5	355.4	5.3
18.7	0.0	90.0	7.5	432.4	2.9
22.8	0.0	109.5	10.8	526.2	1.4
27.8	0.0	133.3	13.6	640.2	0.6
33.8	0.1	162.2	14.8	778.9	0.2
41.1	0.4	197.3	14.1	947.7	0.1
50.0	1.1	240.1	11.7	1153.1	0.0
60.8	2.4	292.1	8.5	1403.0	0.0

Figure D.19 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 3.0 mM ; vortexting method by light scattering measurement.

Result

Quality Factor:	Pass	Intensity Mean (nm):	404.0
Z Average Mean(nm):	332.4		
Polydispersity:	0.39	Analysis Mode:	Monomodal



Size (nm)	% Intensity	Size (nm)	% Intensity	Size (nm)	% Intensity
19.8	0.0	127.2	4.6	818.9	5.2
24.9	0.0	160.5	7.5	1033.6	2.9
31.5	0.0	202.6	10.9	1304.5	1.4
39.7	0.0	255.7	13.6	1646.5	0.6
50.1	0.2	322.7	14.9	2078.0	0.2
63.3	0.4	407.3	14.1	2622.7	0.1
79.8	1.1	514.1	11.7	3310.2	0.0
100.8	2.4	648.9	8.4	4177.8	0.0

Figure D.20 Size distributions of chitosan/AOT nanoparticles ; [AOT] = 50 mM ; [chitosan] = 5.0 mM ; vortexting method by light scattering measurement.

VITAE

Miss Chutima Saratid was born on August 25th, 1978 in Lopburi, Thailand. She received a Bachelor Degree of Science, majoring in Chemistry from Thammasat University, in 1999. Since 2004, she has been a graduate student studying Petrochemical and Polymer Science as her major course at Chulalongkorn University. During his studies towards the Master's Degree, he was awarded a teaching assistant scholarship by the Faculty of Science.

Her present address is Priwan mansion II, Soi 52/2, Rhamkamheung Road, Haumark, Bangkapi, Bangkok, Thailand 10240.