CHITOSAN-STABILIZED GOLD NANOPARTICLES FOR BIOSENSING APPLICATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
Faculty of Science
Chulalongkorn University
Academic Year 2008

อนุภาคนาโนทองคำที่ทำให้เสถียรด้วยไคโตซานสำหรับการประยุกต์ในการตรวจวัดทางชีวภาพ

นางสาวพรเพ็ญ แซ่อึ้ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

Thesis Title	CHITOSAN-STABILIZED GOLD NANOPARTICLES FOR BIOSENSING APPLICATION
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พรเพ็ญ แซ่อึ้ง: อนุภาคนาโนทองคำที่ทำให้เสถียรด้วยไคโตซานสำหรับการประยุกต์ในการตรวจวัดทางชีวภาพ (CHITOSAN-STABILIZED GOLD NANOPARTICLES FOR BIOSENSING APPLICATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.กมลวรรณ ธรรมเจริญ, 81 หน้า.

อนุภาคนาโนทองคำที่ทำให้เสถียรด้วยพอลิแซคคาไรด์ถูกสังเคราะห์โดยใช้ 2 แนวทาง แนวทางแรกใช้ไคโตซานเป็นทั้งตัวรีดิวซ์และสารทำให้เสถียร แนวทางที่ 2 ใช้โซเดียมโบโรไฮไดรด์เป็นตัวรีดิวซ์และใช้อนุพันธ์ไคโตซาน ได้แก่ เอ็น,เอ็น,เอ็น-ไตรเมทิลไคโตซาน, เอ็น-[(2-ไฮดรอกซิล-3-ไตรเมทิลแอม-โมเนียม)โพรพิล]ไคโตซานคลอไรด์ หรือเอ็น-ซักซินิลไคโตซาน เป็นสารทำให้เสถียร ผลจากการวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านพบว่า อนุภาคที่สังเคราะห์ได้เป็นทรงกลม, มีขนาดอยู่ในช่วง 7-13 นาโนเมตร และเสถียรเมื่อกระจายตัวอยู่ในน้ำอย่างน้อย 4 เดือนเนื่องจากแรงผลักระหว่างประจุบนสายโซ่พอลิเมอร์ซึ่งเคลือบเป็นชั้นนอกของอนุภาค นอกจากนี้จากการวิเคราะห์ด้วยเทคนิคโฟตอนคอร์รีเล-ชันสเปกโทรสโกปียังพบว่า ขนาด การกระจายตัวของขนาด และเสถียรภาพของอนุภาคแปรเปลี่ยนตามสัดส่วนโดยปริมาตรระหว่างสารละลายพอลิเมอร์และไฮโดรเจนเททระคลอโรออเรตน้ำหนักโมเลกุลของไคโตซาน และความเข้มข้นของสารลดแรงตึงผิวและโซเดียมโบโรไฮไดรด์ การสังเคราะห์ทางเคมีด้วยเสียงโดยใช้ไคโตซานที่ติดโบวีนซีรั่มอัลบูมินเป็นตัวรีดิวซ์และสารทำให้เสถียรทำให้ได้อนุภาคนาโนทองคำที่ทำให้เสถียรด้วยไคโตซานที่ติดโบวีนซีรั่มอัลบูมิน ซึ่งส่วนใหญ่มีลักษณะเป็นทรงกลม มีขนาดเฉลี่ย 8.2 นาโนเมตร บางส่วนมีรูปร่างเป็นสามเหลี่ยม และเสถียรเมื่อกระจายตัวอยู่ในน้ำอย่างน้อย 1 เดือน อนุภาค นาโนทองคำที่ทำให้เสถียรด้วยไคโตซานที่ติดโบวีนซีรั่มอัลบูมินรวมกลุ่มในภาวะที่มีแอนติบอดีต่อโบวีน-ซีรั่มอัลบูมิน เนื่องจากอันตรกิริยาที่จำเพาะเจาะจงของแอนติเจนและแอนติบอดี ความเข้มข้นต่ำสุดของแอนติบอดีต่อโบวีนซีรั่มอัลบูมินที่สามารถเหนี่ยวนำให้เกิดการรวมกลุ่มกันของอนุภาคซึ่งสามารถสังเกตเห็นได้ด้วยตาเปล่าคือ 20 ไมโครกรัมต่อมิลลิลิตร ผลการทดลองที่ได้แสดงให้เห็นศักยภาพในการนำอนุภาคนาโนทองคำที่ทำให้เสถียรด้วยไคโตซานที่ติดด้วยสารชีวโมเลกุลไปประยุกต์ใช้เป็นไบโอเซนเซอร์ที่อาศัยการเปลี่ยนแปลงสีหรือการดูดกลืนแสงซึ่งเกิดจากการรวมกลุ่มของอนุภาคนาโนทองคำได้

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4972400123 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: GOLD NANOPARTICLES / CHITOSAN / CHITOSAN DERIVATIVES / HOMOGENOUS IMMUNOASSAY

PORNPEN SAE-UNG: CHITOSAN-STABILIZED GOLD NANOPARTICLES FOR BIOSENSING APPLICATION. THESIS ADVISOR: ASST. PROF. VORAVEE P. HOVEN, Ph.D., THESIS CO-ADVISOR: GAMOLWAN TUMCHARERN, Ph.D., 81 pp.

Polysaccharide-stabilized gold nanoparticles (AuNPs) were synthesized by two methods. The first method employed chitosan as both reducing and stabilizing agent. The second method used sodium borohydride as a reducing agent and chitosan derivatives; *N*,*N*,*N*-trimethyl chitosan (TMC), *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), or *N*-succinyl chitosan (SCC), as a stabilizing agent. The results from transmission electron microscopy showed that the synthesized AuNPs had a spherical morphology, a size range of 7-13 nm, and are stable in aqueous solution for at least 4 months due to the repulsion between the charges on the polymer chains which act as shell coating. According to the photon correlation spectroscopy (PCS), the size, size distribution and stability of particles was found to vary with volume ratio of polymer to $HAuCl_4$, molecular weight of chitosan, concentration of surfactant, and concentration of $NaBH_4$. The synthesis based on sonochemical method using BSA-modified CS (CS-BSA) as a reducing/stabilizing agent yielded BSA-functionalized gold nanoparticles, BSA-CS-AuNPs that were mostly spherical (i.d. average=8.2 nm) with some triangular shape, and were stable in aqueous solution for at least one month. The BSA-CS-AuNPs aggregated in the presence of anti-BSA due to the antigen-antibody specific interactions. The minimum concentration of anti-BSA that can induce the aggregation of BSA-CS-AuNPs that can be visualized by naked eye was 20 µg/mL. These results demonstrated the potential of using AuNPs stabilized by biomolecule-attached chitosan as chromogenic biosensor based on the aggregation of AuNPs.

Field of study: Petrochemistry and Polymer Science Student' signature .....
Academic year: 2008 Advisor's signature .....
Co-Advisor's signature .....

ACKNOWLEDGEMENTS

First and foremost I wish to express my sincere and deep gratitude to my advisor, Assistant Professor Dr. Voravee P. Hoven and my co-advisor, Dr. Gamolwan Tumcharern for their thoughtful guidance, steady encouragement and support, and consistent generosity and consideration. Working with them has been the best course of my study. I would also like to thank all my committee members, Professor Dr. Pattarapan Prasassarakich, Assistant Professor Dr. Varawut Tangpasuthadol and Dr. Duangporn Polpanich for their valuable suggestions and time to review the thesis.

I gratefully acknowledge the financial support provided by Thailand Graduate Institute of Science and Technology (TGIST) from the National Science and Technology Development Agency (NSTDA).

I am thankful to the friendly staff of Organic Synthesis Research Unit (OSRU), and all my friends, for their friendliness, helpful discussions, cheerful attitude and encouragements during my thesis work.

Finally, I also wish to especially thank my family members for their love, kindness and support throughout my entire study.

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Anti-BSA	: Anti-bovine serum albumin
Anti-IgG	: Anti-immunoglobulin G
AFM	: Atomic force microscopy
BSA	: Bovine serum albumin
BPEI	: Branched polyethyleneimine
CMCS	: Carboxymethyl chitosan
CS	: Chitosan
CS-BSA	: Chitosan attached BSA
DD	: Degree of deacetylation
DS	: Degree of substitution
ELISA	: Enzyme-linked immunosorbent assay
FTIR	: Fourier transform infrared spectroscopy
GTMAC	: Glycidyltrimethylammonium chloride
AuNPs	: Gold nanoparticles
BSA-CS-AuNPs	: Gold nanoparticles stabilized by CS-BSA
IgG	: Immunoglobulin G
LPEI	: Linear polyethyleneimine
LSPR	: Localized surface plasmon resonance
EDC	: *N*-(3-Dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride
NHS	: *N*-Hydroxysuccinimide
HTACC	: *N*-[(2-Hydroxyl-3-trimethylammonium)propyl]

	chitosan chloride
SCC	: *N*-succinyl chitosan
TMC	: *N*,*N*,*N*-trimethyl chitosan
NMR	: Nuclear magnetic resonance spectroscopy
PBS	: Phosphate buffered saline
PCS	: Photon correlation spectroscopy
PAM	: Polyacylamide
PEO-*b*-PNiPAM	: Poly(ethylene oxide-*b*-*N*-isopropylacrylamide)
PVP	: Poly(*N*-vinyl-2-pyrrolidone)
$NaBH_4$	: Sodium borohydride
NaOH	: Sodium hydroxide
SA	: Succinic anhydride
SERS	: Surface-enhanced raman scattering
TEM	: Transmission electron microscopy
UV	: Ultraviolet
ζ-potential	: Zeta-potential

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Preparation of gold nanoparticles (AuNPs) has been extensively studied due to their unique physical and chemical properties and for potential applications in catalysis, biology and electrochemistry [1-3]. Several methods, such as the use of chemical reductants [4-5], sonochemical method [6-7], and photochemical method [8-11], can be employed for the synthesis of AuNPs. Among them, the chemical reduction is the most common method for the preparation of AuNPs. This method involves a treatment of gold salts to zero valent gold nanoparticles with a chemical reducing agent, such as citric acid, borohydride, or other organic compounds. Because AuNPs usually tend to aggregate in the medium, stabilizing agents, such as thiol compounds [12-13], surfactants [14-15], and polymers [16-18] are thus necessary in the synthesis of AuNPs. It has been reported that polymers are effectively stabilizing agent for AuNPs because they are capable of providing both electrostatic and steric stabilizations. In particular, polymers containing amino group can act as both reducing and stabilizing agents due to the fact that gold salts can be reduced by amino group in the polymer's structure. Therefore, the reduction and stabilization processes can be accomplished in one single step in the presence of amino-containing polymer.

Chitosan (CS) is a polysaccharide obtained by partial deacetylation of chitin, a natural substance found abundantly in the exoskeletons of insects, the shells of crustaceans, and fungal cell walls. A number of amino groups in the structure of chitosan is determined by the degree of deacetylation which can be ranged from more than 50% upto 100% [19]. According to recent work reported by Huang and Yang, a large number of amino groups in the CS structure has a potential to act as both reducing and stabilizing agent without any additional reducing agent [20-21]. Since CS can only be dissolved in dilute acid, such as hydrochloric acid and acetic acid, the synthesis of AuNPs has to be done under acidic condition. That somehow limits the subsequent usage especially in bio-related applications. To overcome such obstacle, a number of water soluble charged derivatives of chitosan, namely *N,N,N*-trimethyl chitosan

chloride (TMC) [22] and carboxymethylated chitosan (CMCS) [23-25], have been introduced as an alternative stabilizing agent in the synthesis of AuNPs. Both TMC and CMCS were found to be very effective stabilizing agents because of the charge repulsion along their polymeric chain in the shell layer surrounding the AuNPs.

Immunoassays based on the aggregation of AuNPs rely on specific interactions between antibody immobilized on AuNPs and the target antigen in the solution. AuNPs tend to aggregate because the dielectric constant in the vicinity of the surface is altered resulting in the change of AuNPs optical property [26]. Not only can the shifted signal reflect the degree of AuNPs aggregation, it can also be used as quantitative measure of the tested antigen. Major strategies for conjugating biomolecule to the AuNPs are physisorpion and chemisorption [27]. Although physisorption is simple, the process often suffers the chemical instability and uncontrolled orientation of antibody's active sites. In contrast, chemisorption requires more complicated procedures. The complexity is worth sacrificing considering that it can provide a better stability and site-directed immobilization of the antibody. Readily available reactive functional groups in the stabilizing agent that can be chemically bound to the desired antibody should make the synthetic process of AuNPs to be used for immunoassay simpler and more convenient. In fact, there have already been a few reports on the use of biomolecules such as cysteine, as both reducing and stabilizing agent, leucine, and asparagine, as stabilizing agent, for the synthesis of biomolecule-conjugated AuNPs [28-29].

Although there are a few literatures describing the synthesis of AuNPs using chitosan as the stabilizing/reducing agent or its charged derivative as the stabilizing agent alone, none of them have determined comparatively the size and stability of AuNPs stabilized by chitosan and its charged derivatives. Their biosensing applications in particular, have not yet been mentioned. Herein, the synthesis of AuNPs stabilized by chitosan and its charged derivatives is reported. Besides TMC which has been previously used by others [22], the investigation was also performed using other charged derivatives which could potentially act as an effective stabilizing agent; *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), and *N*-succinyl chitosan (SCC). It is anticipated that the stabilizing efficiency of the charged molecules bearing permanent charges which is independent of pH should be superior to that of chitosan.

In addition, this research is also interested in preparing AuNPs modified by biomolecules. Using bovine serum albumin (BSA) as an antigen model, two synthetic

routes based on amidation has been taken to synthesize AuNPs. One involves the BSA immobilization on the preformed AuNPs stabilized by chitosan. The other is done by using the BSA attached chitosan as a stabilizing agent for AuNPs. The resulting AuNPs are then tested for their biosensing applications with the specific antibody, anti-BSA.

1.2 Objectives

1. To synthesize and characterize AuNPs stabilized by chitosan, its charged derivatives and biomolecule-bound chitosan
2. To test biosensing application of the synthesized AuNPs

1.3 Scope of Investigation

The stepwise investigation was carried out as follows:

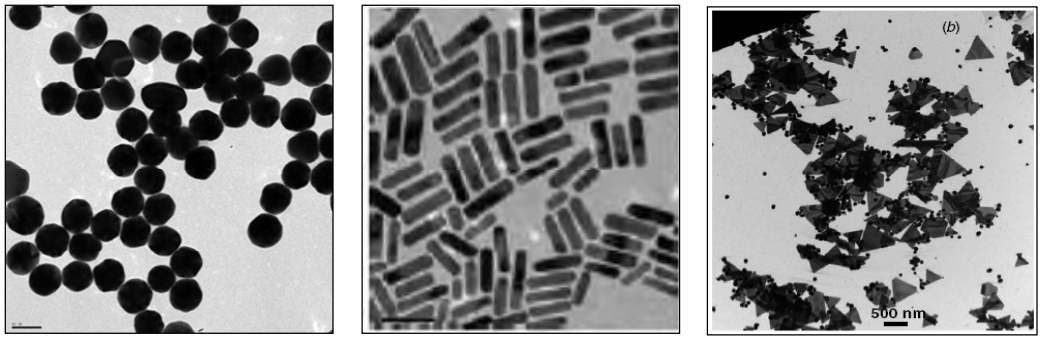
1. Literature survey for related research work
2. Synthesis of charged derivatives of chitosan
3. Synthesis of gold nanoparticles stabilized by chitosan and its derivatives via two methods.
   - 3.1 Using chitosan as both reducing and stabilizing agent
   - 3.2 Using chitosan derivative as a stabilizing agent and sodium borohydride ($NaBH_4$) as a reducing agent
4. Characterization of the synthesized gold nanoparticles
5. Preparation of AuNPs stabilized by biomolecule-bound chitosan
6. To test biosensing application of the synthesized AuNPs stabilized by biomolecule-bound chitosan

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Gold Nanoparticles and Their Optical Property

Gold nanoparticles (AuNPs) or colloidal gold is a suspension of clusters of gold atoms with sizes ranging from 1 to 100 nm [30]. The particles have a variety of shapes, sphere, rod, and triangle, as shown in Figure 2.1.



(a) (b) (c)

Figure 2.1 Different shapes of AuNPs: (a) gold nanoparticles [31], (b) gold nanorods [32], and (c) gold nanotriangles [33]

At this nanoscale, the particles have large surface to volume ratio, physical and chemical properties of them are thus unique and very different from both the bulk and the constituent atoms or molecules, most obvious example being the color change from yellow to ruby red when bulk gold is converted into nanoparticulate gold [34]. This ruby red color of gold nanoparticles is due to the presence of a plasmon absorption band. This absorption band occurs when the incident photon frequency is in resonance with the collective excitation of the conductive electrons of the particle. This effect was termed localized surface plasmon resonance (LSPR) which has an absorption band in the visible region at 530-540 nm [35]. The position of the absorption band is strongly dependent on particle size, shape, particle-particle distance, material, and environment around the particles which can be explained by calculation of extinction (absorption plus scattering) given by the Mie theory. Increasing the size of AuNPs by aggregation gives rise to a red shift in the absorption band (Figure 2.2).

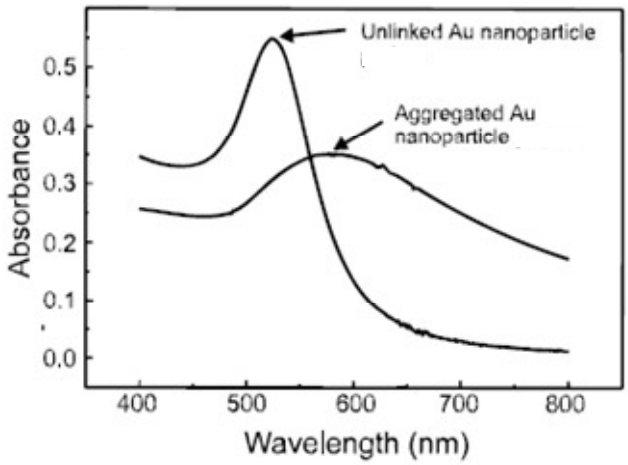


Figure 2.2 Absorption band of unlinked and aggregated AuNPs

It is well-known that an aggregation of gold nanoparticles causes the color change from red to violet or blue (Figure 2.3). The red shift in absorption band makes the gold nanoparticles attractive for many applications. Some examples include electronic and optical devices and chemical and biological sensors.

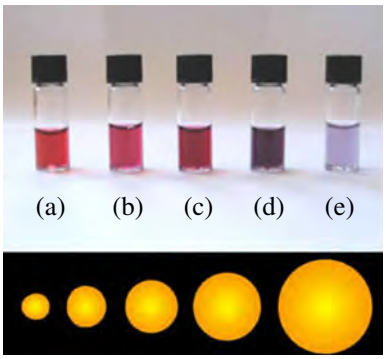


Figure 2.3 The color of AuNPs which is varied with size: (a) 18 nm, (b) 65 nm, (c) 150 nm, (d) 200 nm, and (e) 250 nm [36]

2.2 Preparation of Gold Nanoparticles

Generally, the method for the synthesis of AuNPs consists of two steps. Firstly, gold atom is produced by reduction between gold ion and reducing agent, and the produced gold atoms will come together to form gold nanoparticles. After that, stabilizing agents were coated on the surface of AuNP to prevent the particles from aggregating. There are many synthetic methods to prepare AuNPs, including chemical reduction, photolysis, radiolysis, and ultrasonic reduction, etc. Among them, the

common method is the use of chemical reductants in the synthesis of AuNPs. The methods introduced by Turkevitch et al. and Brust–Schiffrin are currently considered as the most conventional ones. The method is based on citrate reduction of $HAuCl_4$ in water. Sodium citrate firstly acts as a reducing agent. Afterwards, the negatively charged citrate ions are adsorbed onto the gold nanoparticles to prevent them from aggregation. The particles size can be manipulated during the preparation of the nanoparticles by adjusting the stoichiometric ratio of the gold nanoparticles precursor (hydrogen tetrachloroaurate) to the reducing agent (sodium citrate) [37]. This method is often used even now when a rather loose shell of ligand are required around the gold core in order to prepare a precursor to valuable AuNPs-based materials [38]. Brust–Schiffrin method published in 1994 is based on the two-phase reduction. $AuCl_4^-$ from aqueous phase is transferred to toluene phase using tetra-*n*-octylammonium bromide (TOAB) as the phase-transfer reagent and reduced by $NaBH_4$ in the presence of alkanethiols, hence subsequently generated AuNPs which are protected by a monolayer of alkanethiols. Besides, thiols are used as a classic stabilizer in the synthesis of AuNPs due to strong binding with gold.

Other stabilizing agents, such as surfactants and polymers, are also used as stabilizer to control the particle size, prevent aggregation, and introduce functionality to particle surfaces. In the case of polymers, they can usually act as stabilizing agent or as both reducing and stabilizing agent. It has been reported that polymers are effective stabilizing agent for AuNPs because they are able to combine both electrostatic and steric stabilizations.

In 2005, Hussain, *et al*. [16] reported a one-step method which led to nearly monodisperse AuNPs in the 1–4 nm size range by using highly steric polymer, alkyl thioether end-functionalized poly(methacrylic acid), as stabilizing agent. This polymer bound to the gold surface through the thioether but may also provide electrostatic stabilization due to its potential to carry a negative charge. The particle size could be controlled precisely by the molar ratio of Au to capping ligand, and the particles were readily obtainable in both aqueous and nonaqueous solutions.

In 2007, Wang, *et al.* [17] reported a one-step method for the synthesis of biocompatible gold nanoparticles stabilized by poly(*N*-vinyl-2-pyrrolidone) (PVP) by using gallic acid as reductant at room temperature. The size and shape of the synthesized AuNPs could be tuned by molar ratio of gallic acid to gold (R). For an R value of about 0.4, the prepared gold nanoparticles were the smallest in size and the

most close to spherical in shape. The incorporation of PVP effectively protected the surface of gold nanoparticles and improved their stability.

In 2008, Jeon, *et al.* [18] found that the thermoresponsive block copolymers, poly(ethylene oxide-*b*-*N*-isopropylacrylamide) (PEO-*b*-PNiPAM), were successfully used as polymeric stabilizers to prepare water-soluble AuNPs. The AuNPs stabilized by block copolymer could be obtained as the solid powder and dissolved in aqueous media. The size of water-soluble gold nanoparticles was controlled in the range of 5–30 nm under these reaction conditions.

In 2007, Perignon, *et al.* [39] prepared water stable gold nanoparticles stabilized by hyperbranched polymers chemically analogous to PAMAM dendrimers (Figure 2.4). They found that these polymers can control the growth of gold nanoparticles after reduction by sodium borohydride. Nanoparticles of ca. 4 nm were indeed obtained and stabilized in solution by the hyperbranched polymer. The average size of the nanoparticles could be easily adjusted by changing the [$HAuCl_4$]/[polymer] or [$NaBH_4$]/[$HAuCl_4$] ratios. The stability of the nanoparticles in water was mainly influenced by the molecular weight of the polymer core and the pH of the solutions.

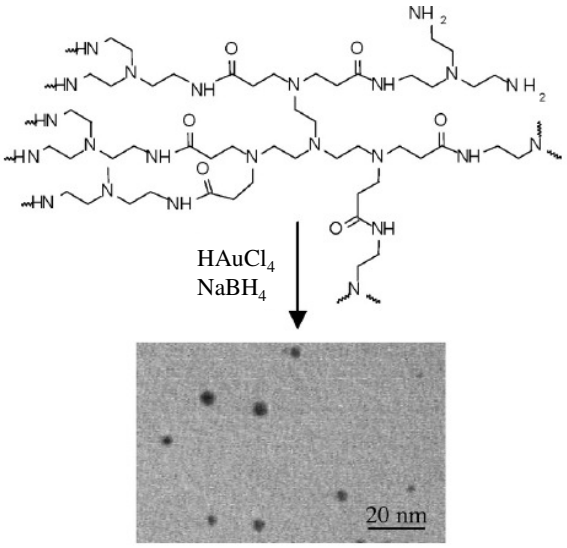


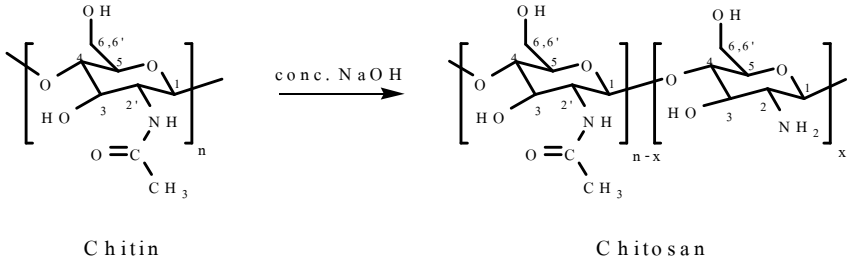
Figure 2.4 TEM image of gold nanoparticles stabilized by hyperbranched polymers chemically analogous to PAMAM dendrimers

It was also found that polymers containing amino groups can act as both reducing and stabilizing agents due to the fact that gold salts can be reduced to zerovalent gold nanoparticles by amino groups. Therefore, this method can combine the reduction and stabilization processes in one step.

In 2007, Bai, *et al.* [40] developed a facile and simple method for the preparation of polyacylamide (PAM)-stabilized AuNPs. These particles were obtained by reduction of gold salt with polyacrylamide. The AuNPs were protected by polyacrylamide immediately to produce well-dispersed AuNPs. PAM molecules can absorb on the surface of the gold nanoparticles through the formation of a coordination bond between gold atom and oxygen atom of the carbonyl groups in PAM. The synthesized AuNPs were almost spherical, and average diameter was measured to be about 15 nm.

In 2005, Sun, *et al.* [41-42] presented that polymer containing amino groups, linear polyethyleneimine (LPEI), can induce $HAuCl_4$ to spontaneously form AuNPs via heating a LPEI/$HAuCl_4$ aqueous solution, with the use of LPEI to serve as reducing agent and protective agent. The different initial molar ratio of LPEI to gold can lead to the formation of dispersed nanoparticles, quasi one-dimensional aggregates of nanoparticles or bulk metal deposits. The growth kinetics of AuNPs thus formed can be tuned by changing the initial molar ratio of LPEI to gold. Furthermore, they also reported that highly concentrated well-stable AuNPs (up to 0.16 M based on Au atom) can be prepared from branched polyethyleneimine (BPEI)-$HAuCl_4$ aqueous solution at room temperature without the additional introduction of other reducing agents and protective agents. They found that increasing molar ratio of BPEI to gold results in increasing particle size. The polymer adsorbed at the particle surface can indeed stabilize the particles due to electrosteric stabilization.

Chitosan is a linear polycationic biopolymer obtained by partial deacetylation of chitin (Scheme 2.1). From the alkali treatment in deacetylation process, acetyl groups in chitin are converted to amino groups. Thus, chitosan with many amino groups can be potentially used in the synthesis of AuNPs.



Scheme 2.1 Structures of chitin and chitosan

In 2003, Esumi, *et al.* [43] prepared gold nanoparticles in the presence of chitosan via reduction of $HAuCl_4$ with sodium borohydride and used the developed particles as antioxidant. The average particle size and catalytic activity upon elimination of hydroxyl radicals formed in an $H_2O_2/FeSO_4$ system examined by a spin-trapping method were significantly affected by the concentration of chitosan added. The size of AuNPs was ranged between 6 and 16 nm. In addition, the activity of gold nanoparticles stabilized by chitosan was 80 times higher than that of ascorbic acid, which is well known as an antioxidant.

In 2004, Huang, *et al.* [20] found that chitosan is more than a stabilizing agent and that gold salts can be reduced by amino group in its main chain without any additional reducing agent. These results illustrate that chitosan can act as both reducing and stabilizing agent for the synthesis of AuNPs. Moreover, electrostatic attractive forces between amino groups in chitosan and $AuCl^{4-}$ in solution provide an effective driving force for the formation and stabilization of the AuNPs. The morphology and size distribution of the AuNPs were varied with the concentration of both the chitosan and the precursor gold salts. The particles produced are highly stable and show no signs of aggregation after two months of storage, and the mean size was in a range of 7-20 nm.

In 2007, Yakimovich, *et al.* [44] synthesized AuNPs stabilized by chitosan under UV irradiation of chitosan solutions doped with $HAuCl_4$. They found that the polydispersity and average size of AuNPs in aqueous chitosan solutions may be governed by varying the chitosan-to-$HAuCl_4$ ratio and the molecular mass of chitosan.

In 2008, Fan, *et al.* [45] developed an efficient and rapid method for the synthesis of AuNPs which can be done within a few minutes by directly heating a reaction mixture of $HAuCl_4$ and chitosan in a microwave oven. The results showed that microwave power could effect the required time for preparing the AuNPs arising from the distinction of heating rate, and long irradiation time was favorable for complete reduction of $HAuCl_4$ when a low microwave power was applied. Besides, the spherical AuNPs, some peculiar shaped AuNPs, such as the triangular nanoplate and the gold nanorods were also observed. The average diameter of AuNPs analyzed by TEM was about 30 nm. Furthermore, this study once again confirmed that chitosan could act as both the effective reducing and stabilizing agent for the preparation of AuNPs.

. Since chitosan can only be dissolved in dilute acid, such as hydrochloric acid and acetic acid, the synthesis of AuNPs has to be done under acidic condition. That somehow limits the subsequent usage especially in bio-related applications. To overcome such obstacle, a number of water soluble charged derivatives of chitosan have been recently introduced as alternative stabilizing agent in the synthesis of AuNPs

In 2006, Ding, *et al.* [22] reported a simple method for the preparation of AuNPs stabilized by *N,N,N*-trimethyl chitosan (TMC) in aqueous solution by using $NaBH_4$ as a reducing agent. The results showed that TMC is a very effective stabilizing agent. The prepared AuNPs have a spherical morphology with diameters of about 3±0.5 nm. The special bridge structure between the stabilizing agent and the metallic nanoparticles leads to a stable shell–core structure (Figure 2.5). In a neutral aqueous solution, TMC molecules were coated around the AuNPs. The polymer shell with polycationic TMC can separate the metal cores from one another and thus prevented the aggregation of AuNPs

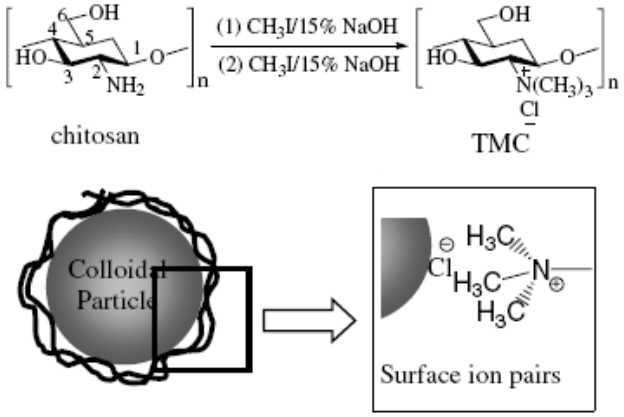


Figure 2.5 Schematic representation of the synthetic procedure for TMC and the formation of TMC-stabilized metallic nanoparticles

In 2006, Xu, *et al.* [23] successfully prepared the nanocomposite composed of carboxymethyl chitosan (CMCS) and AuNPs by using KBH_4 as a reducing agent, and used this nanocomposite with silica sol–gel method to immobilize HRP to construct a H_2O_2 electrochemical biosensor. This study found that the nanocomposite was hydrophilic even in neutral solutions, stable, and inherited the properties of the AuNPs and CMCS. For preparing the biosensor, HRP was immobilized to AuNPs stabilized by CMCS via electrostatic interaction (Figure 2.6). The resulting biosensor exhibited a fast amperometric response (5 s), a good linear response over a wide range of

concentrations from 5.0×10^{-6} to 1.4×10^{-3} M, and a low detection limit of 4.01×10^{-7} M.

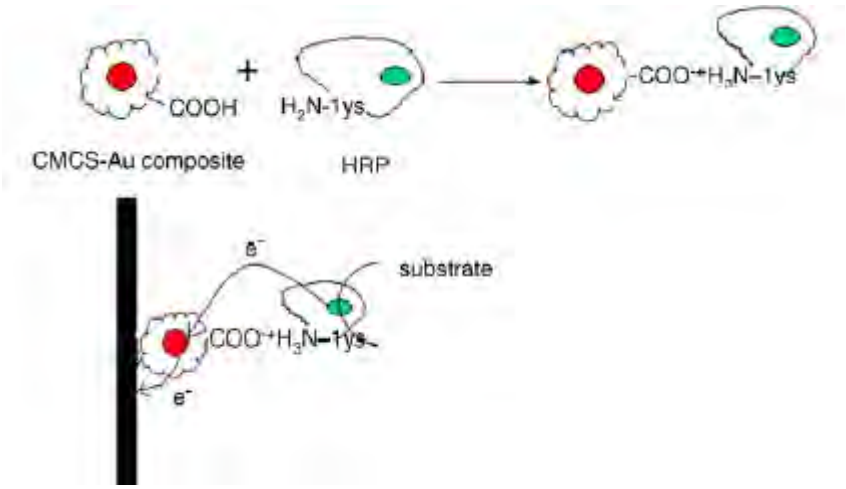


Figure 2.6 Schemes of the electrostatic interaction of CMCS–AuNPs nanocomposite and HRP and the electron transfer between the electrode and HRP

In 2007, Huang, *et al.* [24] first proposed the method to synthesize AuNPs in the alkaline CMCS solution by ultraviolet (UV) light irradiation. In the synthesis, CMCS served as both reducing agent for gold cations and stabilizing agent for AuNPs. The size, amount and morphology of AuNPs depended on the pH, concentration of $HAuCl_4$ and irradiation time of UV light. The synthesized AuNPs have a diameter size about 6.2–8.2 nm and can be stably dispersed at pH 12.4 for more than 6 months. Primary fluorescence experiments found that the fluorescence emission band of AuNPs at 400 nm varied with irradiation time, particle size, and concentrations of AuNPs and implied that the AuNPs/CMCS composites might be utilized as a new kind of fluorescence material for further constructing of biosensor.

In 2008, Laudenslager, *et al.* [25] studied the use of CMCS in the synthesis and stabilization of catalytic nanoparticles; platinum, gold, and silver nanoparticles because CMCS has a higher chelation capacity than chitosan, which has potential implications for improved catalyst formation and immobilization. This paper is the first to compare the size, morphology, and binding mechanisms of metal nanoparticles in chitosan and CMCS. The average sizes of metal nanoparticles stabilized by CMCS analyzed by TEM were 3.5±1.0, 23±11.0, and 7.7±4.2 nm for platinum, gold, and silver nanoparticles, respectively. The size and shape of gold nanoparticles varied more than the other two catalysts. In comparison between chitosan and CMCS, although CMCS has a higher chelation capacity, the mean size and size distribution of AuNPs is similar

in chitosan and CMCS and the nanoparticles were well dispersed in both solutions. Furthermore, UV-Vis absorption spectra of AuNPs did not change considerably over three weeks, indicating that gold forms particularly stable sols.

2.3 Gold Nanoparticles in Biological Applications

The unique optical property of gold nanoparticles is ensuring that gold is a candidate material for various applications as shown in Figure 2.7.

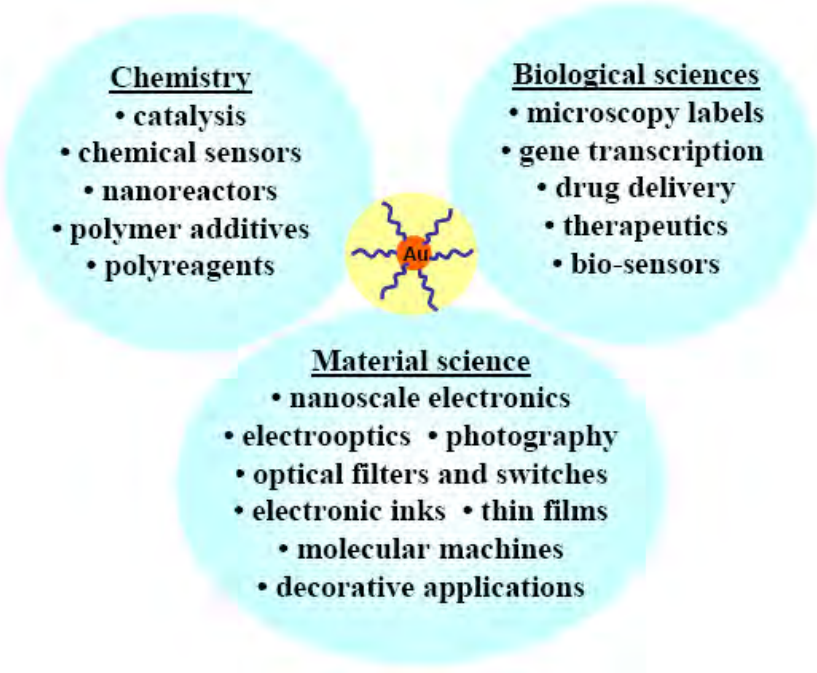


Figure 2.7 Applications of gold nanoparticles [46]

In biological applications, AuNPs are the popular materials that are used in homogeneous immunoassay. Immunoassays are tests that use antibody and antigen complexes (also called immunocomplexes) to measure the presence of a specific analyte in a sample. This property of highly specific molecular recognition of antigens by antibodies leads to high selectivity of assays which can be divided into 2 types; homogeneous and heterogenous immunoassay. Homogeneous immunoassays are faster and easier to perform than heterogeneous immunoassays because they do not require separation of unbound complexes from the bound complexes while heterogeneous immunoassays require the multiple washing and incubation steps as illustrated in Figure 2.8 [47-48].

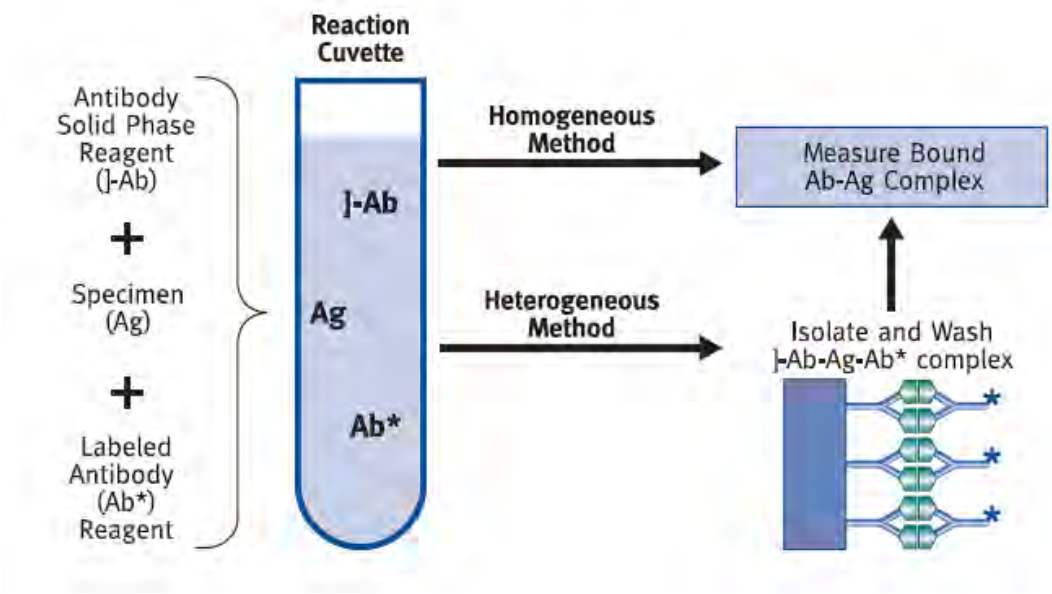


Figure 2.8 Homogeneous and heterogeneous immunoassays

In the homogeneous immunoassay, AuNPs are conjugated with antibody (or antigen) that reacts specifically with analyte by physical or chemical adsorption. Physical adsorption of protein to AuNPs is enabled by three basic phenomena: (a) charge attraction of the negative particle to the positively charged protein domains, (b) hydrophobic interaction between particle and protein, and finally (c) co-ordinate covalent binding between sulphur and noble metal. The main problems of this adsorption are that proteins gradually desorb from the particles, some molecules do not adsorb onto the particles or the adsorption of proteins is improper orientation resulting inaccessible binding site. These problems are generally overcome by covalent coupling the molecule to the particle surface.

In 1999, Weiping, *et al.* [49] proposed the convenient and efficient method to control site-directed immobilization of immunoglobulin G (IgG) antibodies onto aminopropyltriethoxylsilane (APTES) derivatized silicon wafer surfaces by using $NaIO_4$ oxidation reaction to generate aldehydes on the oligosaccharide moieties at the C-terminal IgG. The APTES derivatized surfaces were incubated with oxidized IgG to bind IgG by covalent bonds between aldehydes generated in antibody and reactive amine groups of APTES derivatized surfaces (Figure 2.9). This study found that the IgG molecule surface is stable and homogeneous, and the antigen binding capacity is increased.

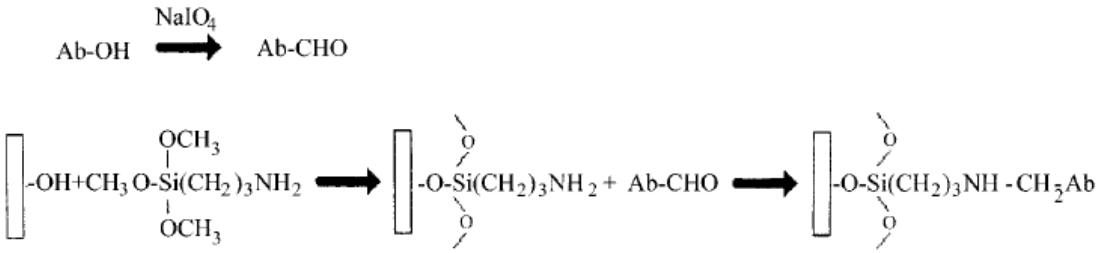


Figure 2.9 Procedure for site-directed immobilization of antibody onto APTES derivatized surface

In immunoagglutination, an antibody (or antigen) is coated on the surface of AuNPs. When a sample containing the specific antigen (or antibody) is mixed with AuNPs solution, it causes the aggregation of AuNPs because one molecule of antigen (or antibody) can bind two antibodies, and the dielectric constant around the particle is changed (Figure 2.10). Aggregation of AuNPs changes the particle size, so the optical property of AuNPs also changes because this property depends on the size of particles. The change in optical property is proportional to the analyte concentration.

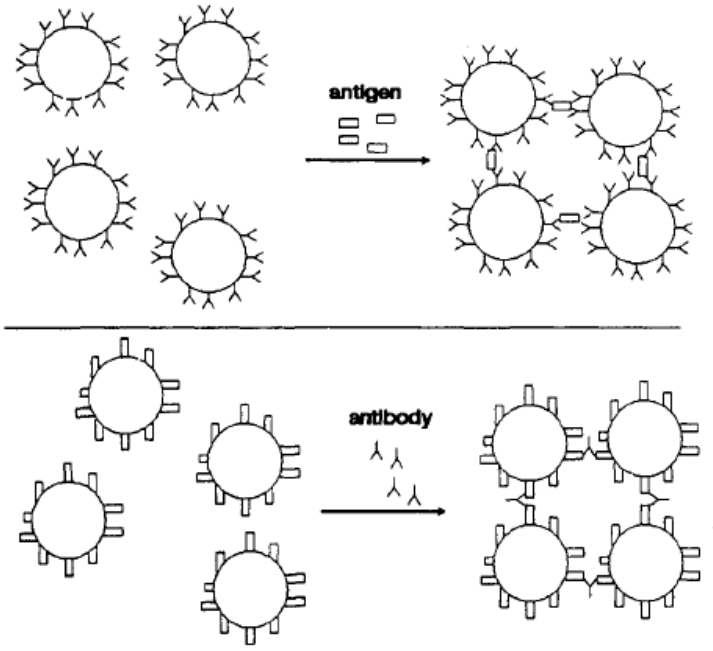


Figure 2.10 Diagram of the aggregation of antibody-coated AuNPs by antigen (upper) and of antigen-coated AuNPs by antibody (lower)

In 2008, Du, *et al.* [50] developed the homogenous noncompetitive immunoassay based on the aggregation of antibody-functionalized AuNPs by the immunoreaction coupled with light scattering technique. This study found that the light

scattering background of the suspensions of gold nanoparticles coated with antibodies is very low. The great enhancement of light scattering originated from the aggregate formation by the immunoreaction can be observed when microamounts of the antigens were directly mixed with the gold nanoparticles. The proposed immunoassay can be performed with one-step operation in homogeneous solution within 10 min and the light scattering can be easily measured by using a common spectrofluorimeter. By using human immunoglobulin G (IgG) as a model analyte, a wide dynamic range of 0.05–10 μg/mL was achieved and as low as 10 ng/mL human IgG can be detected. The proposed immunoassay has been successfully applied to the determination of human IgG in serum samples, in which the results are well consistent with those of the enzyme-linked immunosorbent assay (ELISA), indicating its high selectivity and practicality.

In 2008, Chen, *et al.* [51] presented an alternative approach for aggregation-based immunoassays using Raman reporter-labeled immunogold nanoparticles as probes coupled with surface-enhanced raman scattering (SERS) detection. AuNPs were functionalized with Raman reporter and antibody successively in a two-step process before the detection of target (Figure 2.11 A). When the antigen was introduced, aggregation of AuNPs was induced by the immunoreaction between the antigen and the antibody modified on the surface of nanoparticles (Figure 2.11 B). As a result, SERS signals of the reporter on the surface of AuNPs would be greatly enhanced. Therefore, the content of human IgG could be directly determined by measuring the Raman signal of the reporter. In addition, the process of aggregation was also investigated by TEM and UV–Vis absorption spectroscopy. Utilizing human IgG as a model protein, SERS response linearly correlated with the logarithmic concentration of the target over a range from 0.1 to 15 μg/mL with a detection limit of 0.1 μg/mL.

(A) Preparation of Raman reporter-labeled immunogold nanoparticles

(B) Immunoassay protocol

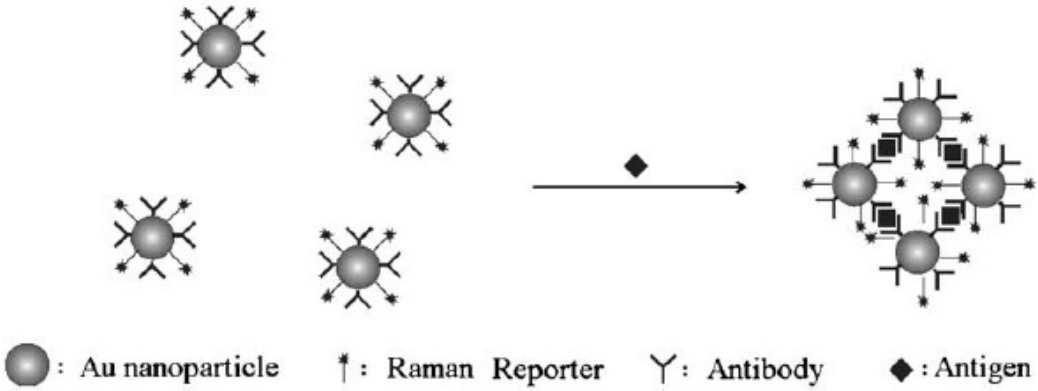


Figure 2.11 Procedure for (A) preparation of Raman reporter-labeled immunogold nanoparticles and (B) immunoassay protocol

CHAPTER III

EXPERIMENTAL

3.1 Materials

Chitosan flakes (M_V = 15 kDa, 80 kDa, and 200 kDa) with a degree of deacetylation of 90% were obtained from Seafresh Chitosan (Lab) Co., Ltd. (Thailand). Acetone, glacial acetic acid, methanol (MeOH), *N*-methylpyrrolidone (NMP), and sodium chloride (NaCl) were purchased from Merck (Germany). Anti-bovine serum albumin (anti-BSA) developed in rabbit, anti-immunoglobulin G (anti-IgG) produced in goat, bovine serum albumin (BSA), dialysis bag (cut-off molecular weight of 12,400 g/mol), phosphate buffered saline, pH 7.4 (PBS), and succinic anhydride (SA) were bought from Sigma (USA). Glycidyltrimethylammonium chloride (GTMAC), *N*-(3-dimethylaminopropyl)-*N′*-ethyl-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), sodium borohydride ($NaBH_4$), and sodium iodide (NaI) were obtained from Fluka (Switzerland). Polysorbate 80 (Tween 80) and hydrogen tetrachloroaurate ($HAuCl_4.3H_2O$) were obtained from Aldrich (USA). Centrifuge tube containing membrane with a cut-off molecular weight of 100,000 g/mol was bought from Millipore (USA). Methyl iodide (CH_3I) was purchased from Riedel-de Haen (Germany), and sodium hydroxide (NaOH) was bought from Carlo Erba (Italy). All the above chemicals were analytical grade and used as received without further purification. Moreover, all solutions were made using ultrapure distilled water that was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step (18.2 MΩ cm resistance).

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^1H NMR spectra were recorded in CF_3COOH/D_2O using Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts (δ) were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were recorded in KBr discs with a FT-IR spectrometer (Nicolet, USA), model Impact 410, with 32 scans at resolution 4 cm^{-1}. A frequency of 400-4000 cm^{-1} was collected by using TGS detector.

3.2.3 Photon Correlation Spectroscopy (PCS)

The hydrodynamic size and zeta-potential (ζ) of particles were determined using Nanosizer Nano-ZS (Malvern Instruments, UK). The solutions of AuNPs (1 mL) were sonicated for 3 min before measurement. The analysis was performed at 25°C using a scattering angle of 173°. All data are displayed as the mean ± one standard deviation and are derived from at least three independent experiments. The data were calculated using the Helmholtz-Smoluchowski equation.

3.2.4 UV-Visible Spectroscopy

UV-Vis absorption spectra of AuNPs were obtained by CARY 100 Bio UV-visible spectrophotometer (Varian Ltd., USA). The scanning wavelength was from 200 to 800 nm.

3.2.5 Transmission Electron Microscopy (TEM)

The morphology and actual size of particles were analyzed by a JEOL JEM-2010 transmission electron microscopy (Japan) operating at 200 keV. The TEM samples were prepared by dropping approximately 10 µL of AuNPs solution on the carbon-coated copper grid and dried in a dessicator before analysis. The average diameters were reported from measurements of 100 random particles for each sample using Semafore software.

3.2.6 Atomic Force Microscopy (AFM)

The presence of the polymer around the AuNPs was confirmed by a Seiko SPA 400 atomic force microscope (SII Nanotechnology Inc., Japan). The samples were prepared by dropping the AuNPs solution on a freshly cleaved mica plate and dried in a dessicator for 4 h prior to analysis. Measurements were performed in air at ambient

temperature using tapping mode and silicon tips with a resonance frequency of 115-190 kHz.

3.3 Synthesis of Charged Derivatives of Chitosan

3.3.1 *N,N,N*-trimethyl chitosan (TMC)

TMC was prepared according to a method modified from that of Sieval *et al*. [52]. Chitosan flakes (2.0 g, 0.0108 mol) were dispersed in 80 mL of NMP for 18 h at 40°C. After that, NaOH (0.87 g, 0.0216 mol), NaI (1.62 g, 0.0108 mol), and CH_3I (2.0 mL, 0.0325 mol) were added to the solution. The reaction mixture was stirred for 6 h at 40°C. The same amount of CH_3I was added and the mixture was stirred for another 18 h at the same temperature. The solution was then precipitated using acetone. The precipitate was dissolved in 6.0% (w/v) NaCl solution for 4 h in order to replace the iodide counterion with a chloride counterion. The suspension was dialyzed against deionized water in a dialysis bag at ambient temperature for 5 days to remove residual small molecules. The final product was obtained after lyophilization by a freeze dryer (model Freezeone 77520 Benchtop, Labconco, USA).

3.3.2 *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC)

HTACC was synthesized according to a method modified from that of Seong *et al*. [53]. Chitosan flakes (0.50 g, 2.708 mmol) were dissolved in 1.0 % (v/v) aqueous acetic acid (25 mL) to prepare a 2.0% (w/v) chitosan solution. GTMAC (1.6422 g, 0.0108 mol) was added to the solution and stirred for 24 h at 70°C. The solution was then dialyzed against deionized water in a dialysis bag at ambient temperature for 5 days and lyophilized to obtain a cotton-like white material. The stoichiometric ratio between GTMAC and the amino groups of chitosan was also varied in order to obtain the product with different degree of substitution (%DS).

3.3.3 *N*-succinyl chitosan (SCC)

SCC was synthesized according to a method modified from that of Aoki *et al.* [54]. Chitosan flakes (0.50 g, 2.708 mmol) were dissolved in 1.0 % (v/v) aqueous acetic acid solution (25 mL) to prepare a 2.0 % (w/v) chitosan solution. The chitosan solution

was diluted with 40 mL of MeOH. SA (1.0839 g, 0.0108 mol), dissolved in a minimum amount of acetone, was added to the chitosan solution. The mixture was vigorously stirred for 2 h at ambient temperature. The obtained viscous solution was diluted with 50 mL of water and the pH of the solution was adjusted to 10 using 2 M NaOH solution. After dialysis against deionized water in a dialysis bag at ambient temperature for 5 days, the solution was lyophilized to obtain a cotton-like white material. The stoichiometric ratio between GTMAC and the amino groups of chitosan was also varied in order to obtain the product with different degree of substitution (%DS).

3.4 Synthesis of Gold Nanoparticles (AuNPs)

All glasswares used for the synthesis of AuNPs, were cleaned with freshly prepared aqua regia solution ($HCl:HNO_3$ = 3:1, v/v) and rinsed thoroughly with distilled water prior to use.

3.4.1 Synthesis of chitosan-stabilized AuNPs (CS-AuNPs)

The stock solution of chitosan was prepared by dissolving a certain amount of chitosan in 1.0 % (v/v) aqueous acetic. The synthetic method can be summarized as follows: the chitosan solution (0.1% (w/v), varied volume) and aqueous solution of $HAuCl_4$ (2.943 mM, varied volume) in 18 mL of Milli-Q water were heated under stirring at 60°C in an oil bath for 5 min. Then Tween 80 (varied concentration), an nonionic surfactant, was added into the aqueous solution of $HAuCl_4$. Five minutes later after adding Tween 80, the chitosan solution was added into the mixture. Finally, the reaction solution was heated to 85°C under vigorous stirring until a red solution was obtained. The synthesized AuNPs was kept in the dark.

3.4.2 Synthesis of chitosan derivatives-stabilized AuNPs

Chitosan derivatives; TMC, HTACC, or SCC were dissolved in Milli-Q water. The synthesis of TMC, HTACC, or SCC stabilized-AuNPs were similar to the synthesis of chitosan-stabilized AuNPs, except that Tween 80 was not used in the synthesis and a freshly prepared and cooled $NaBH_4$ aqueous solution (29.43 mM, 1 mL of $NaBH_4$ per 1 mL of $HAuCl_4$ solution) was added in the reduction step after mixing polymer solution with aqueous solution of $HAuCl_4$.

3.5 Preparation of AuNPs Stabilized by CS-BSA (BSA-CS-AuNPs)

BSA-CS-AuNPs were synthesized via 2 steps. The first step was an attachment of BSA to amino groups on chitosan by covalent bond. The second step used chitosan attached BSA (BSA-CS) as both reducing and stabilizing agent in the synthesis of AuNPs using the optimum condition previously identified for the synthesis of CS-AuNPs.

The procedure for an attachment of BSA to chitosan consists of 3 following steps. Chitosan was first modified with SA to produce reactive sites, carboxyl groups, on chitosan molecules that could be activated with EDC and NHS. A modified chitosan was synthesized according to a method modified from that of Don *et al.* [55]. The reaction was carried out in formic acid to avoid the hydrolysis of SA in aqueous solution. 0.750 g of chitosan was dissolved in 30.0 g of formic acid and 4.06 g (10 mole equivalents) of SA was added afterwards. The solution was stirred for 24 h at ambient temperature. The solution of modified chitosan (CS-SA) then dialyzed against deionized water in a dialysis bag at ambient temperature for 5 days and lyophilized to obtain the final product.

Attachment of BSA to the carboxyl groups on chitosan was performed according to the following stepwise procedure: CS-SA (0.01 g) was dissolved in Milli-Q water (10 mL) to obtain 0.1% (w/v), EDC (3 mole equivalents) and NHS (3 mole equivalents) were added to the CS-SA solution, and the mixture was stirred at ambient temperature for 15 min. Then, BSA (1.0 mg/mL) was added to the mixture and continuously stirred at ambient temperature for 2 h to attach BSA to chitosan. To remove excess EDC, NHS, and BSA, the mixture was placed into the centrifuge tube containing membrane with a cut-off molecular weight of 100,000 g/mol (Figure 3.1) and centrifugally washed with Milli-Q water by a high speed refrigerated centrifuge (model 7780, Kubota Corporation, Japan) until excess reagents were entirely removed. It could be checked by measuring the conductance of supernatant separated after each washing cycle by a conductometer (model Orion 3 star Conductivity Benchtop, Thermo Electron Corporation, USA). The Mill-Q water was then added to the membrane centrifuge tube before sonication for 15 min. The final product (BSA-CS) was obtained after lyophilyzing the solution removed from the tube.

Figure 3.1 Centrifuge tube containing membrane with a cut-off molecular weight of 100,000 g/mol

The synthesis of chitosan attached BSA-stabilized AuNPs was similar to the synthesis of chitosan-stabilized AuNPs, except that chitosan attached BSA was used instead of chitosan.

3.6 Detection of Anti-BSA by BSA-CS-AuNPs

The solution of BSA-CS-AuNPs obtained from section 3.5 was centrifuged at 18,000 rpm for 30 min and resuspended in a phosphate-buffered saline solution (PBS). Afterwards, the solution of anti-BSA in PBS (100 µg/mL, varied volume) was added to 50 µL of the BSA-CS-AuNPs solution and the mixture was kept at ambient temperature for 2 days. Control experiments were performed by using anti-IgG and PBS.

CHAPTER IV

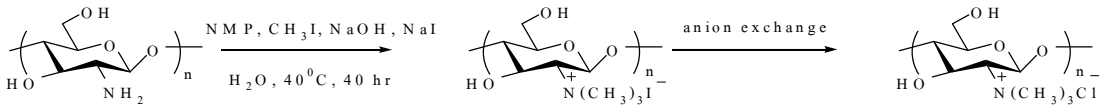
RESULTS AND DISCUSSION

This chapter was divided into 4 parts. The first part focused on the synthesis and characterization of charged derivatives of chitosan; *N,N,N*-trimethyl chitosan (TMC), *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), and *N*-succinyl chitosan (SCC). The second part concentrated on the synthesis of gold nanoparticles (AuNPs) stabilized by chitosan or its derivatives. The preparation of chitosan-stabilized AuNPs attached BSA (BSA-CS-AuNPs) was studied in the third part. And the last part, the BSA-CS-AuNPs were used to test for their biosensing applications.

4.1 Synthesis and Characterization of Charged Derivatives of Chitosan

4.1.1 *N,N,N*-trimethyl chitosan (TMC)

N,N,N-trimethyl chitosan or TMC can be synthesized via methylation of amino groups on chitosan by CH_3I (Scheme 4.1).



Scheme 4.1 Synthesis of *N,N,N*-trimethyl chitosan (TMC)

Figure 4.1 shows the ^{1}H-NMR spectra of the synthesized TMC in comparison with chitosan. The signals of methyl protons at 3.3 (b), 3.1 (c), and 2.8 (d) ppm assigned to *N,N,N*-trimethyl amino, *N,N*-dimethyl amino, and *N*-methyl amino group, respectively. Percentage of degree of trisubstitution (%DS) of methyl group on chitosan was calculated from the relative ratio between the peak integration of protons from the *N,N,N*-trimethyl amino at 3.3 ppm and the sum of integral intensities of H-2',3,4,5,6, and 6' at 3.7-4.3 ppm from chitosan (eqn. 4.1). From the data obtained, it was found that the %DS of *N,N,N*-trimethyl amino was 10.1 %.

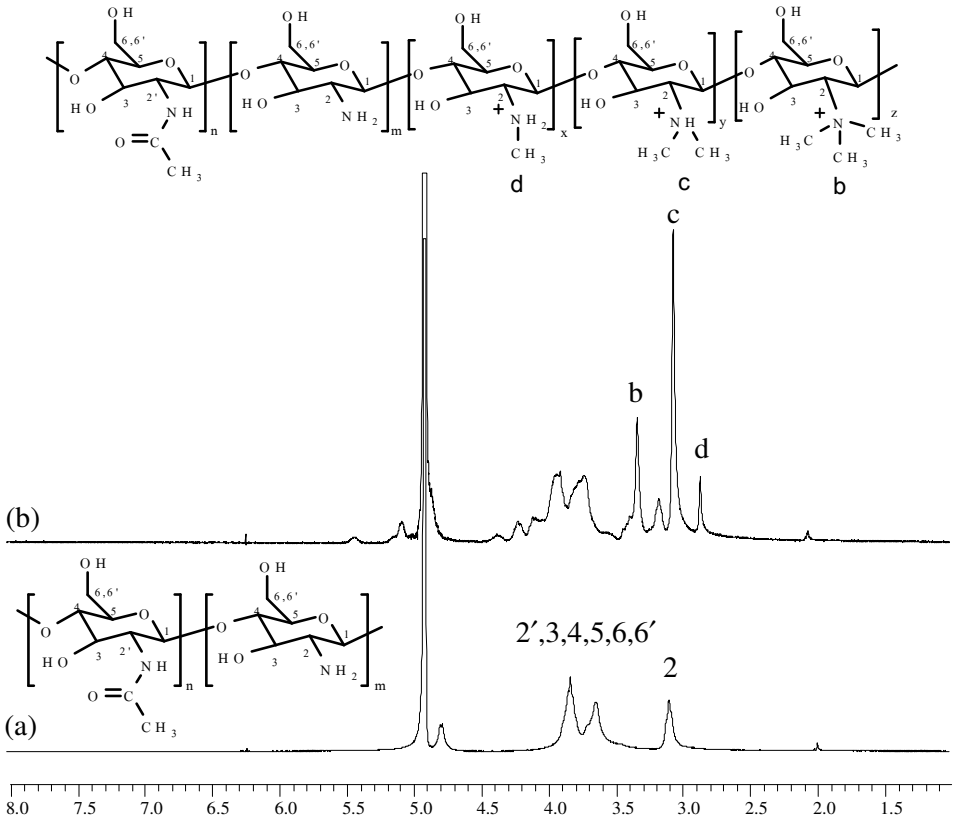


Figure 4.1 ^{1}H-NMR spectra of (a) chitosan and (b) TMC

$$\%\ DS = \left\{ \frac{\text{intergral of the } N^{+}(CH_3)_3 / 9}{\left(\frac{\text{intergral of the H - 2',3,4,5,6,6'}}{6} \right) \times \frac{90}{100}} \right\} \times 100 \qquad (4.1)$$

The structure of TMC could also be verified by FTIR analysis. The spectrum is displayed in Figure 4.2. As compared with the FTIR spectrum of chitosan, the FTIR spectrum of TMC has a new peak at 1469 cm^{-1} assigned to the C-N stretching. In addition, we found a decrement of N-H bending of primary amine in chitosan at 1596 cm^{-1}.

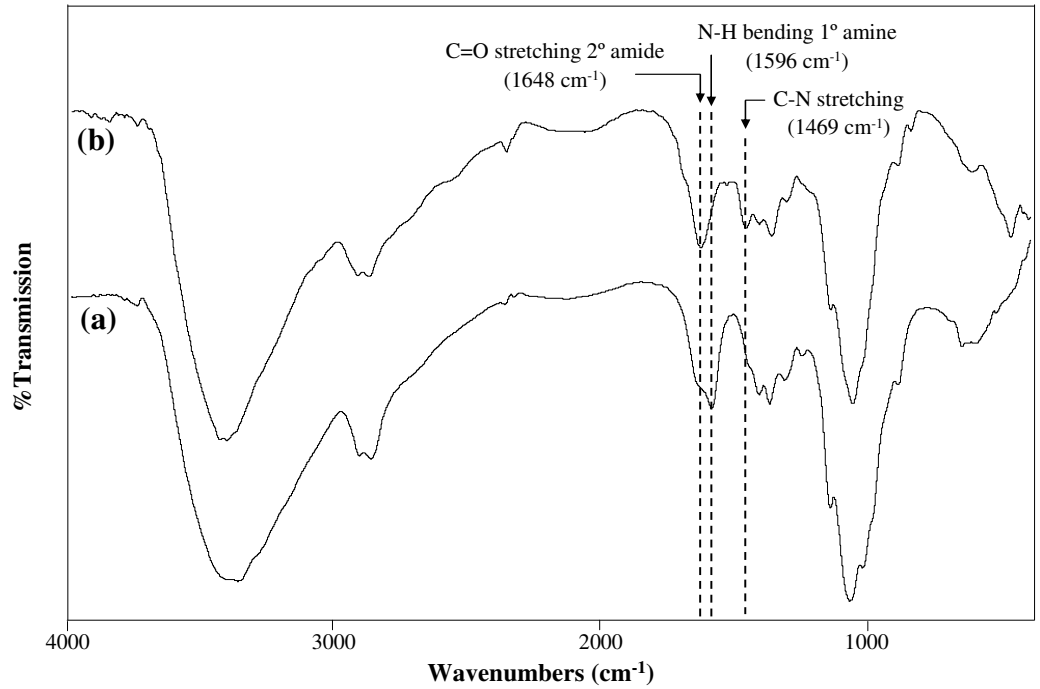
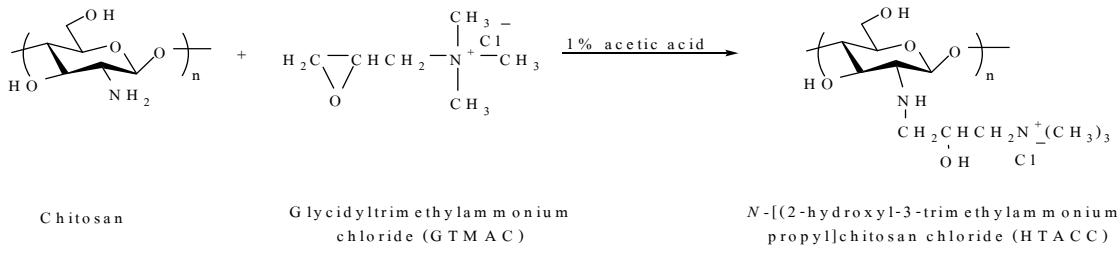


Figure 4.2 FTIR spectra of (a) chitosan and (b) TMC

4.1.2 *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC)

N-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride or HTACC was synthesized by epoxide ring opening of glycidyltrimethylammonium chloride (GTMAC) by amino groups of chitosan under acidic condition (Scheme 4.2). In theory, GTMAC mainly reacts with amino (NH_2) groups under acidic condition but preferably reacts with hydroxyl (OH) groups under neutral and alkaline conditions. The acidic condition causes protonation at the oxygen atom and makes the epoxy ring of GTMAC more reactive towards the NH_2 groups of chitosan.



Scheme 4.2 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC)

^{1}H-NMR spectra of HTACC are shown in Figure 4.3. Signals corresponding to the protons of CH_2 and CH appeared at 3.5 (c) and 4.2 (b) ppm, respectively. Furthermore, the strong peak of CH_3 at 3.2-3.4 ppm, indicating that the quaternary ammonium group of $N^{+}(CH_3)_3$ were incorporated. Percentage of the degree of substitution (%DS) of GTMAC on chitosan was calculated from the relative ratio between the peak integration of protons from the quaternary ammonium group at 3.2-3.4 ppm of GTMAC and the sum of integral intensities of H-2',3,4,5,6, and 6′ (δ 3.6-4.2 ppm) from chitosan (eqn.4.1).

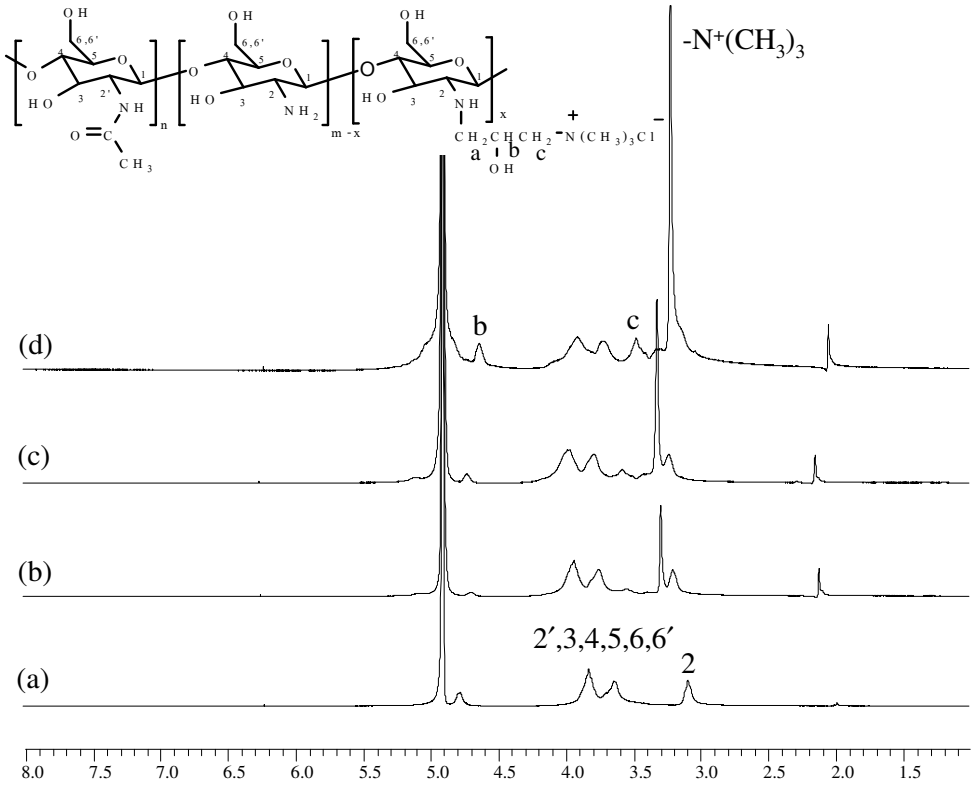


Figure 4.3 ^{1}H-NMR spectra of (a) chitosan, (b) HTACC obtained using 2 equivalents of GTMAC, reaction time 24 h, (c) HTACC obtained using 4 equivalents of GTMAC, reaction time 3 h, and (d) HTACC obtained using 4 equivalents of GTMAC, reaction time 24 h

As shown in Table 4.1, the %DS of quaternary ammonium group on chitosan was drastically increased from 20.6 to 80.0 when the GTMAC equivalent and reaction time was increased. The synthesized HTACC with 80.0 %DS was used for the synthesis of gold nanoparticles.

Table 4.1 Degree of substitution (%DS) of quaternary ammonium group on chitosan after reacting with GTMAC at 70°C in 1.0 % (v/v) acetic acid as determined by ^{1}H NMR

Equivalent of GTMAC	Reaction time (h)	%DS
2	24	20.6
4	3	35.0
4	24	80.0

The FTIR spectrum of HTACC is illustrated in Figure 4.4. The appearance of C-N stretching peak at 1478 cm^{-1} and the decrement of N-H bending of primary amine in chitosan could confirm the success of the synthesis of HTACC.

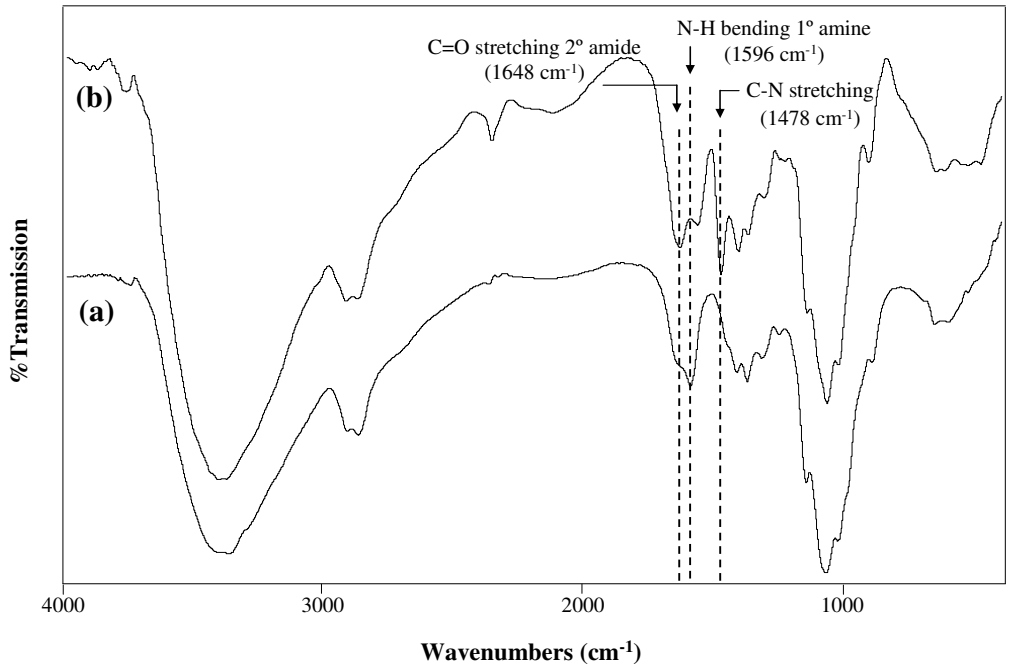
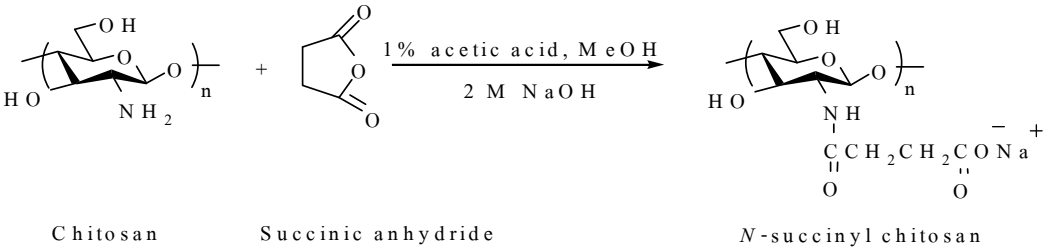


Figure 4.4 FTIR spectra of (a) chitosan and (b) HTACC

4.1.3 *N*-succinyl chitosan (SCC)

SCC was synthesized via ring opening reaction of succinic anhydride by amino groups of chitosan in aqueous methanol system at ambient temperature (Scheme 4.3).



Scheme 4.3 Synthesis of *N*-succinyl chitosan (SCC)

Figure 4.5 shows the NMR signals of the synthesized SCC. The peak at 2.6-2.8 ppm corresponded to the methylene groups (CH_2) from succinic anhydride. Moreover, %DS of SCC could also be determined from the relative ratio between the peak integration of protons from methylene groups and the sum of integral intensities of H-2', 3, 4, 5, 6 and 6' at 3.7-4.1 ppm from chitosan (eqn. 4.2).

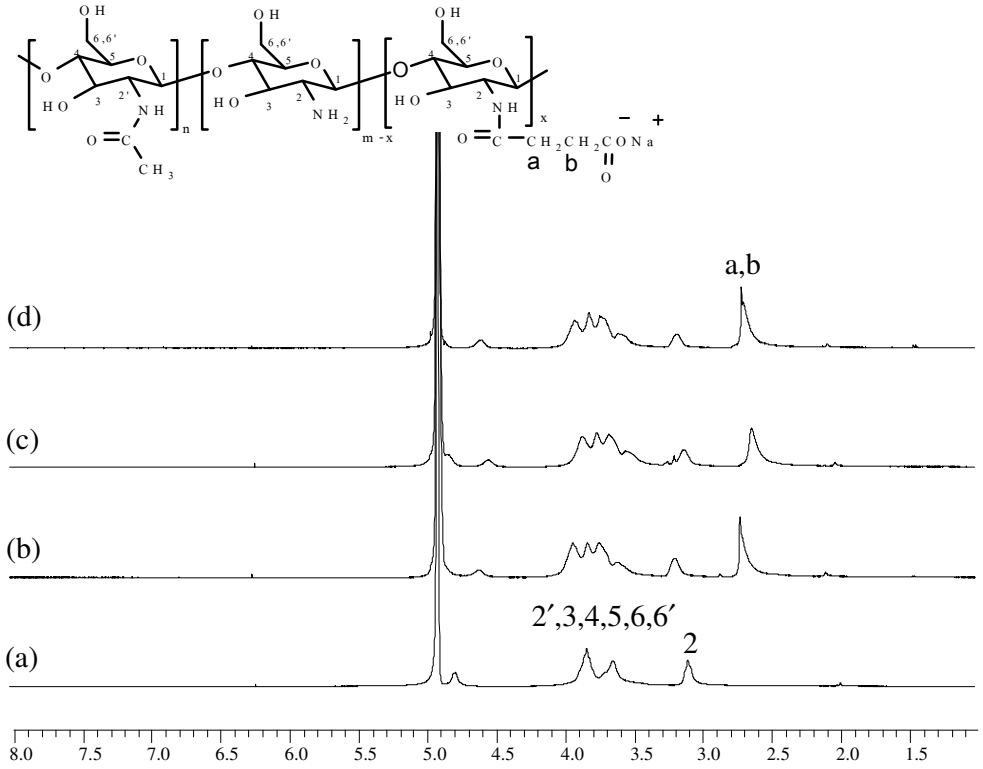


Figure 4.5 ^{1}H-NMR spectra of (a) chitosan, (b) SCC obtained using 4 equivalents of SA, reaction time 2 h, (c) SCC obtained using 8 equivalents of SA, reaction time 2 h, and (d) SCC obtained using 8 equivalents of SA, reaction time 24 h

$$\%\ DS = \left\{ \frac{\text{intergral of the } (CH_2)_2 / 4}{\left(\frac{\text{intergral of the H - 2',3,4,5,6,6'}}{6} \right) \times \frac{90}{100}} \right\} \times 100 \qquad (4.2)$$

As presented in Table 4.2, the %DS of SCC increased with the SA equivalent and reaction time. Although using 8 equivalents of SA and a reaction time of 24 h provided highest %DS, the obtained product was no longer water soluble. Therefore, the %DS of 4 equivalents of SA and a reaction time of 2 h was the optimal condition to be used for the synthesis of SCC that was later used for the synthesis of gold nanoparticles.

Table 4.2 Degree of substitution (%DS) of succinyl group on chitosan after reacting with SA at ambient temperature in 1.0 % (v/v) acetic acid as determined by ^{1}H NMR

Equivalent of SA	Reaction time (h)	%DS
4	2	57.0
8	2	55.0
8	24	67.0

The chemical structure of SCC was confirmed by FTIR analysis (Figure 4.6). There appears a new peak of carboxyl group of succinyl moiety at 1396 cm^{-1} in Figure.4.6. In addition, increment of C=O stretching and N-H bending of secondary amide at 1648 cm^{-1} and 1556 cm^{-1}, respectively and decrement of N-H bending of primary amine as compared with chitosan verified the substitution at amino groups on chitosan by groups that obtained from ring opening of succinic anhydride.

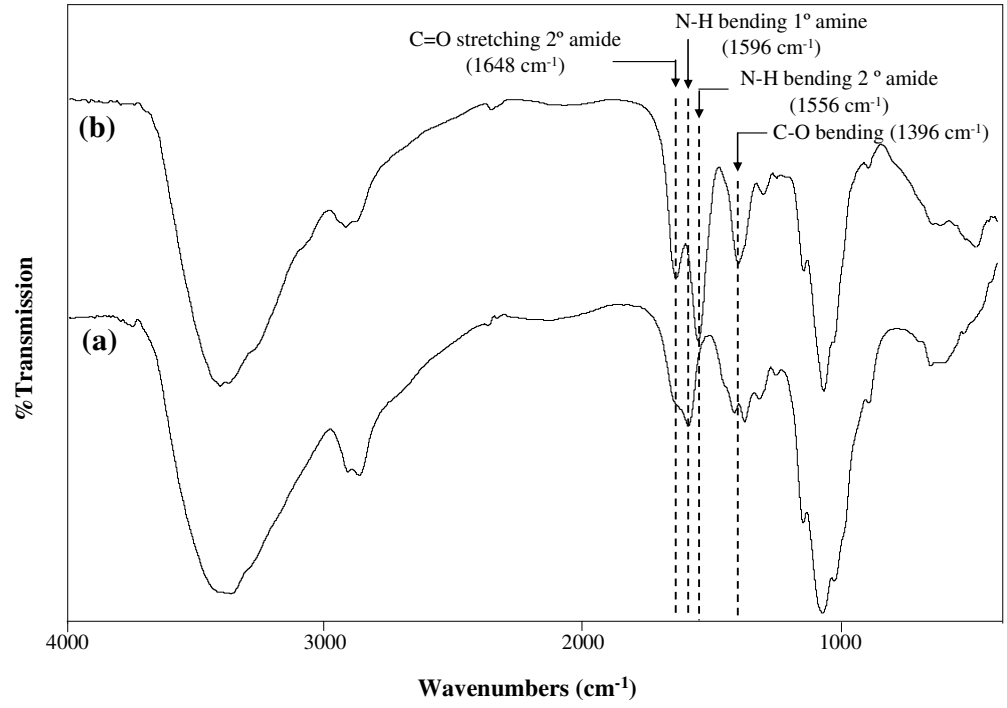


Figure 4.6 FTIR spectra of (a) chitosan and (b) SCC

4.2 Synthesis and Characterization of Gold Nanoparticles (AuNPs)

4.2.1 Gold nanoparticles stabilized by chitosan (CS-AuNPs)

AuNPs were synthesized using chitosan as both reducing and stabilizing agent. The effects of the volume ratio of chitosan to $HAuCl_4$ and molecular weight of chitosan (low molecular weight = 15 kDa, medium molecular weight = 80 kDa, and high molecular weight = 200 kDa) on the mean size, size distribution and stability of the synthesized AuNPs were studied by photon correlation spectrophotometer (PCS) which is based on a dynamic light scattering (DLS) technique. The results are shown in Figure 4.7. Apparently, the mean particle size, size distribution (expressed in term of PDI), and stability (expressed in term of ζ-potential) of AuNPs changed with the volume ratio of chitosan to $HAuCl_4$. Because the electrostatic forces between amino groups on chitosan and $AuCl_4^-$ in solution are a driving force for the formation and stabilization of the AuNPs, the effect of the volume ratio of chitosan to $HAuCl_4$ on mean particle size could be discussed by the number of adsorbing chitosan molecules on the surface of AuNPs.

When the volume ratio of chitosan to $HAuCl_4$ was low (5:5), there were only a few chitosan molecules coating on the particle, resulting in the aggregation of the particles during the synthesis, leading to the formation of large AuNPs. For the high

volume ratio of chitosan to $HAuCl_4$ (9:1), the large AuNPs might be formed due to the coating of the excess chitosan molecules on the outside layer. In this particular study, the volume ratio of 7:3 was then found to produce the smallest AuNPs. The effect of molecular weight of chitosan on mean size of CS-AuNPs agrees with a previous study [21] that the mean size of AuNPs slightly changed with the different molecular weight of chitosan. This outcome suggested that the increasing entanglement of polymeric chain as a function of chitosan MW did not affect the reducing power of amino groups as well as the ability to stabilize the synthesized AuNPs considering that the same amount of active amino groups were present in the same weight of chitosan despite its different MW. Nonetheless, this may be due to the fact that the MW variation of chitosan used in the experiment was not large enough to see such influence. (from 15 to 80 and 200 kDa). When the volume ratio of chitosan to $HAuCl_4$ of 7:3 was used for the synthesis, the ζ-potential of the AuNPs stabilized by chitosan having medium molecular weight (MCS, 80 kDa) was higher than that of the AuNPs stabilized by chitosan having low molecular weight (LCS, 15 kDa) suggesting that more stable AuNPs were formed in the former case. Also, chitosan with high molecular weight (HCS, 200 kDa) exhibited quite poor solubility. For these reasons, chitosan having medium molecular weight was then selected for further use in the synthesis of AuNPs.

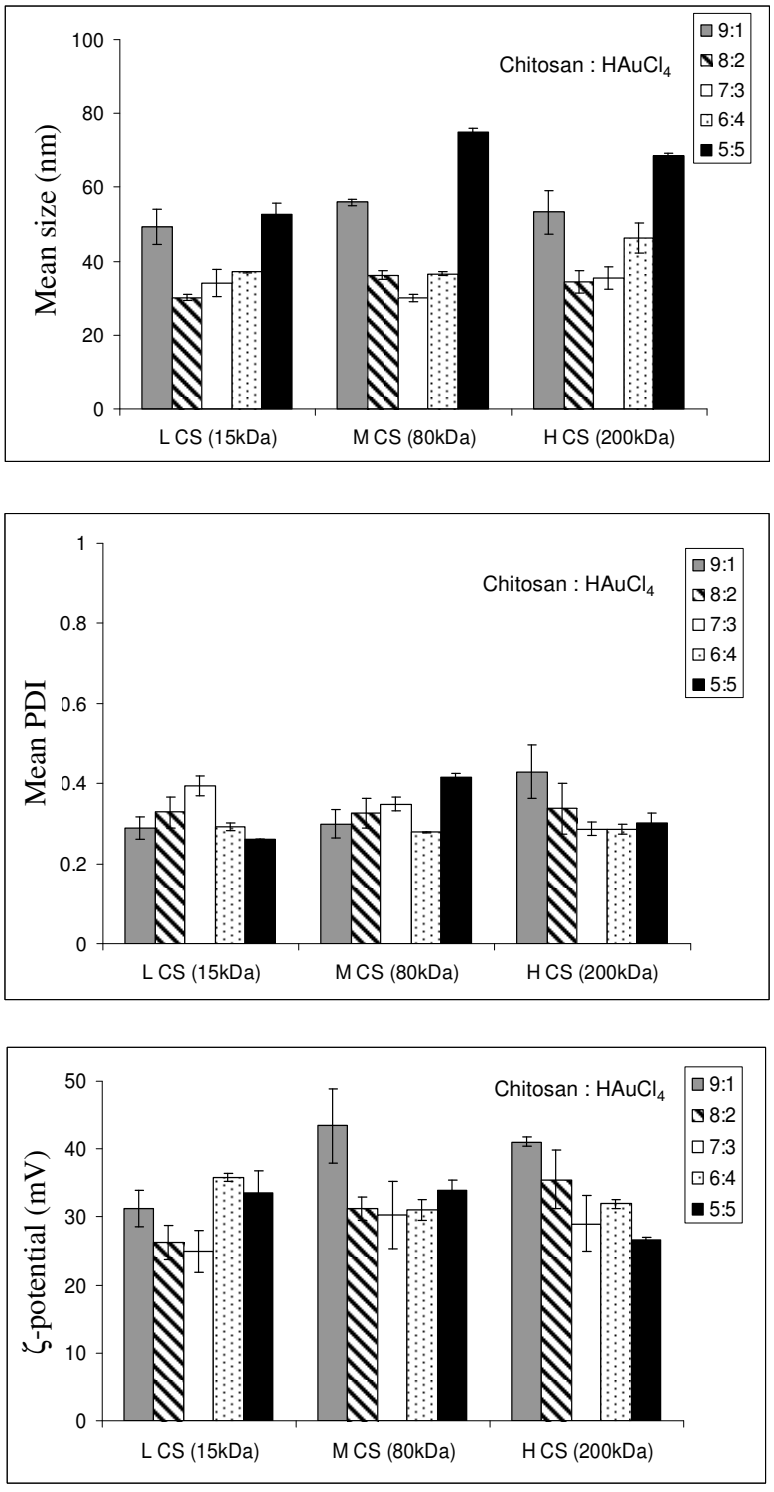


Figure 4.7 Mean size, PDI and ζ-potential of the CS-AuNPs synthesized by using varied volume ratio of chitosan to $HAuCl_4$ and molecular weight of chitosan as analyzed by PCS (independent experiment = 3)

To verify the effect of volume ratio of chitosan to $HAuCl_4$, the mean size of AuNPs stabilized by MCS analyzed by PCS was compared with that characterized by TEM (Figure 4.8). The data from TEM indicated that a relatively larger size of AuNPs was formed at the low volume ratio (5:5) implying that there was aggregation of AuNPs during the synthesis. For the high volume ratio of chitosan to $HAuCl_4$ (9:1), the mean size obtained from TEM analysis was, however, inconsistent with that obtained from PCS analysis. There was no significant size variation between the AuNPs synthesized using the volume ratios of 9:1 8:2, 7:3, and 6:4. Since the measurement is based on light scattering of particles in solution, PCS is capable of measuring hydrodynamic size of the AuNPs which combines both the gold core and the polymeric shell of the surrounding chitosan. So the excess coating of chitosan on the outside layer in the case of high volume ratio can be easily realized. Unlike PCS, TEM can only visualize the gold core of the AuNPs which exhibited greater electron density than the coated chitosan layer. Thus, the chitosan shell cannot be seen unless it is electronically denser which can be done by appropriate staining and operated with TEM having a field emission source at the accelerating voltage of 80 keV to avoid the destruction of chitosan (in this study using TEM 200 keV). This also explained why the mean sizes of AuNPs obtained from PCS were always larger than that visualized by TEM.

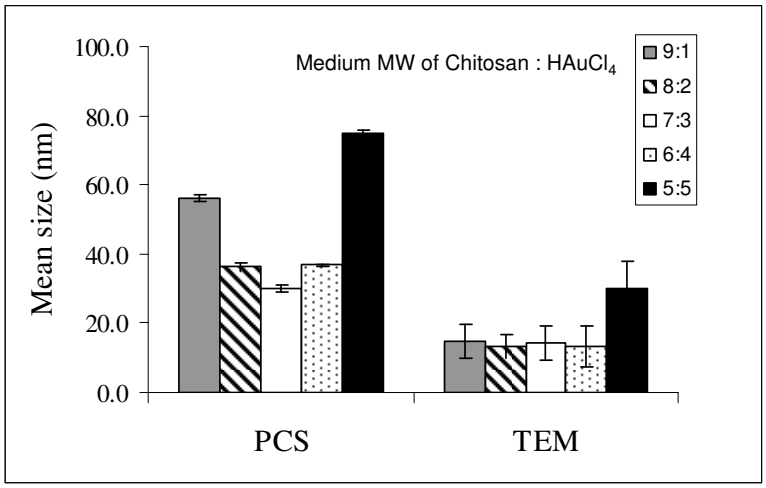


Figure 4.8 Mean size of the AuNPs stabilized by MCS as analyzed by PCS and TEM

In general, there are two types of repulsive forces that help stabilizing the AuNPs, electrostatic and steric repulsion. In our case, the CS-AuNPs cannot be re-

dispersed in neutral aqueous solution after the purification step due to the lack of permanent charge in the chitosan molecules at pH above its pKa (~6.5) that should provide electrostatic stabilization. To be able to re-disperse the CS-AuNPs, Tween 80 was then added to the reaction solution to introduce the steric stabilization to the particles. Table 4.3 presents the effect of Tween 80 concentration on the mean size, PDI and ζ-potential of the synthesized CS-AuNPs. In this study, it was found that 1% (v/v) was the lowest Tween 80 concentration that yielded re-dispersable AuNPs (See the data after re-dispersion). As compared with the CS-AuNPs synthesized in the absence of Tween 80, the ζ-potentials of CS-AuNPs made in the presence of 0.25, 0.50, and 1.0% (v/v) of Tween 80 dramatically decreased (See the data after re-dispersion). This is somewhat expected because the AuNPs were shielded by Tween 80. Upon raising the Tween 80 concentration to 2.0% (v/v), the ζ-potential also increased to 10.1 mV. This may be due to the extensive formation of micelles at the concentration a lot higher than the critical micelle concentration (CMC) of Tween 80 which is 0.012 mM. It should be noted that lowest concentration used in this study (0.25% (v/v)) which is equivalent to 2.0 mM exceeded the CMC value.

Table 4.3 Mean size, PDI and ζ-potential of the CS-AuNPs using MCS: $HAuCl_4$ = 7:3 and varied Tween 80 concentration as analyzed by PCS (independent experiment = 3)

[Tween 80] (%(v/v))	Size (nm)		PDI		ζ-potential (mV)	
	As synthesized	After re-dispersion	As synthesized	After re-dispersion	As synthesized	After re-dispersion
-	30.0	N/A	0.349	N/A	30.2	N/A
0.25	62.6	68.6	0.460	0.386	12.5	3.4
0.50	64.3	72.3	0.386	0.407	13.4	3.6
1.00	27.3	45.6	0.461	0.394	15.3	3.5
2.00	24.8	39.3	0.462	0.276	10.0	10.1

N/A: the data is not available because AuNPs aggregated during the purification step.

4.2.2 Gold nanoparticles stabilized by chitosan derivatives

AuNPs were prepared using sodium borohydride ($NaBH_4$) as a reducing agent and chitosan derivatives; TMC, HTACC, or SCC, as a stabilizing agent. The effects of

the volume ratio of chitosan derivatives to $HAuCl_4$ and the concentration of $NaBH_4$ on the formation of AuNPs were investigated by PCS. The mean size, PDI and ζ-potential of the chitosan derivative-stabilized AuNPs were displayed in Figure 4.9. The particle sizes of AuNPs stabilized by HTACC and SCC were smaller than those of AuNPs stabilized by CS. The sizes of TMC-stabilized AuNPs were, however, larger than others. TMC with its lower degree of substitution (10.1 %DS) than HTACC (80.0 %DS) and SCC (57.0 %DS) should yield lower charge density and degree of charge repulsion among the AuNPs at neutral pH so they tended to aggregate and form larger AuNPs. The sizes of the synthesized TMC-AuNPs in this experiment were larger than those previously reported by Ding, et al. [22] of which %DS of the TMC used for the synthesis was relatively higher (51.1%). It should be noted that the chitosan was dissolved in 1% acetic acid in the synthesis of AuNPs while TMC, HTACC, and SCC were dissolved in Milli-Q water resulting the sizes of TMC-stabilized AuNPs were larger than the AuNPs stabilized by CS.

Although HTACC is positively charged in nature, the synthesized AuNPs stabilized by HTACC showed ζ-potential in a negative range. This was presumably caused by the chloride counterion being in close vicinity to the positively charged quaternary groups of HTACC located in the exterior of AuNPs. It is believed that HTACC and SCC were effective stabilizing agents due to their high permanent charge densities. The optimal condition that yielded AuNPs with small size, high ζ-potential and narrow size distribution (low PDI) was the ratio of 8:2 for the AuNPs stabilized by TMC and SCC and 6:4 for the AuNPs stabilized by HTACC. It should be noted that the data for the AuNPs stabilized by TMC using the ratios of 5:5 and 6:4 were not presented in Figure 4.9 because the prepared AuNPs readily aggregated and precipitated during the synthesis. Also, the characteristic of AuNPs stabilized by HTACC using the volume ratio of 9:1 could not be measured by PCS because the concentration of the as-prepared AuNPs was too low.

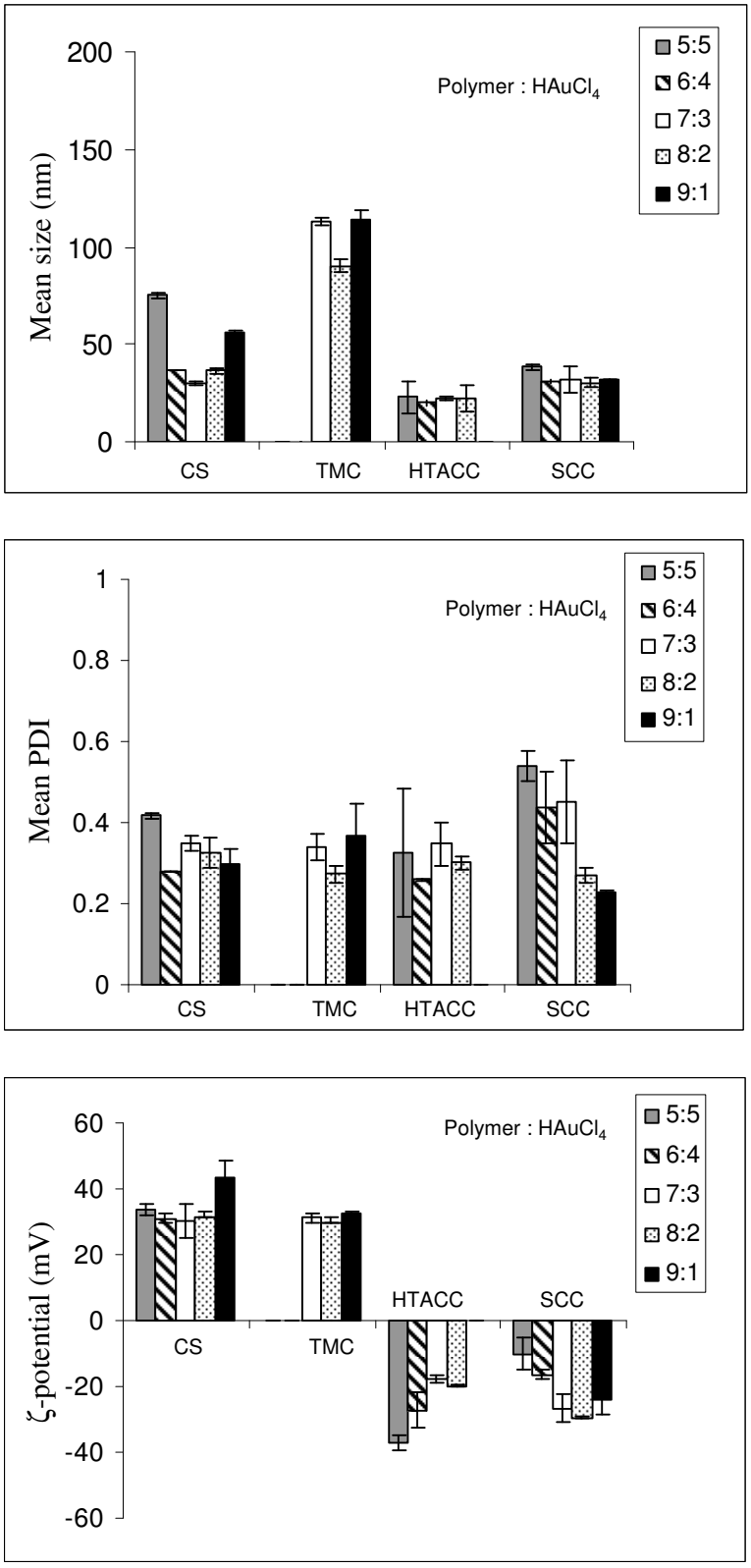


Figure 4.9 Mean size, PDI and ζ-potential of the chitosan derivative-stabilized AuNPs using varied volume ratio of polymer to $HAuCl_4$ as analyzed by PCS

To determine the effect of $NaBH_4$ concentration on the characteristic of AuNPs, the series HTACC-AuNPs was synthesized. The $NaBH_4$ concentration was changed from 0.02943 M to 0.05886 M, 0.04414 M, and 0.01472 M which corresponded to 2, 1.5, and 0.5 times of 0.02943 M, respectively. The AuNPs apparently tended to aggregate during the synthesis when the $NaBH_4$ concentration was too low (0.05886 M) and too high (0.01472 M). Although the $NaBH_4$ concentration of 0.04414 M yielded reasonably stable AuNPs, the resulting AuNPs were larger in size and broader in size distribution as can be realized from the results shown in Table 4.4. The $NaBH_4$ concentration of 0.02943 M was then considered as the optimal concentration for the synthesis of AuNPs.

Table 4.4 Mean size, PDI and ζ-potential of the HTACC-AuNPs synthesized using volume ratio of HTACC:$HAuCl_4$ of 7:3, varied concentration of $NaBH_4$ as analyzed by PCS

Concentration of $NaBH_4$ (M)	Size (nm)	PDI	ζ-potential (mV)
0.02943	22.1	0.347	-17.6
0.04414	44.1	0.776	-32.4

4.2.3 TEM analysis of the synthesized AuNPs

The morphology and actual size of CS-AuNPs, TMC-AuNPs, HTACC-AuNPs, and SCC-AuNPs synthesized using the volume ratio of MCS:$HAuCl_4$ = 7:3 were analyzed by TEM analysis, as displayed in Figure 4.10. Obviously, the particles were spherical in shape and the mean size of CS-AuNPs, TMC-AuNPs, HTACC-AuNPs, and SCC-AuNPs was 13.9±5.8, 8.0±2.2, 7.6±3.2, and 12.2±4.9 nm (n=100), respectively. It was also found that the size of AuNPs stabilized by chitosan derivatives were smaller than the CS-AuNPs which were consistent with the hydrodynamic size analyzed by PCS, except the TMC-AuNPs. The reason was discussed earlier in section 4.2.1. TEM images showed some degree of AuNPs aggregation. In addition, the size of TMC-AuNPs from TEM at the ratio of 8:2 and 9:1 was 22.6±9.6 and 32.5±8.9 nm, respectively which was consistent with the PCS analysis.

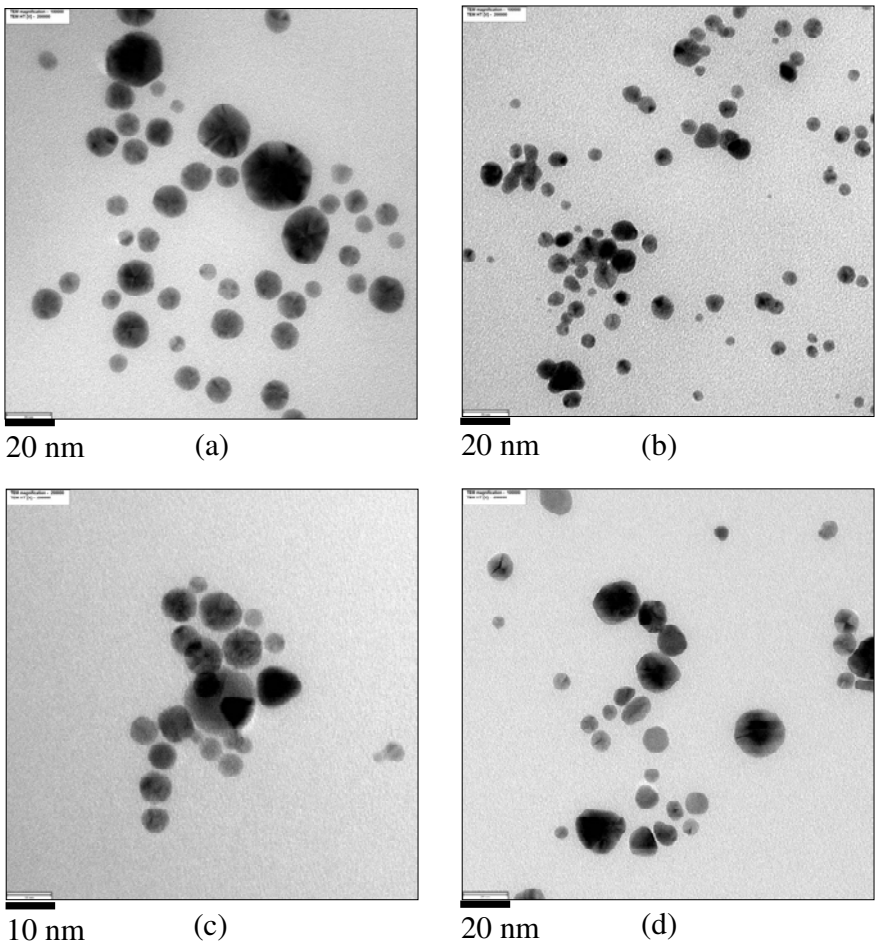


Figure 4.10 TEM images of AuNPs stabilized by (a) CS, (b) TMC, (c) HTACC, and (d) SCC

The optical property of the AuNPs was characterized by UV-vis spectroscopy (Figure 4.11). All spectra exhibited an absorption band around 520 nm, which is a typical plasmon resonance band for AuNPs. According to Table 4.5, the absorption bands of the CS-AuNPs, HTACC-AuNPs, and SCC-AuNPs being stored more than 4 months were only slightly shifted from the as-synthesized ones suggesting the high stability of the synthesized AuNPs. Due to its largest size, TMC-AuNPs were not stable and aggregated upon storage.

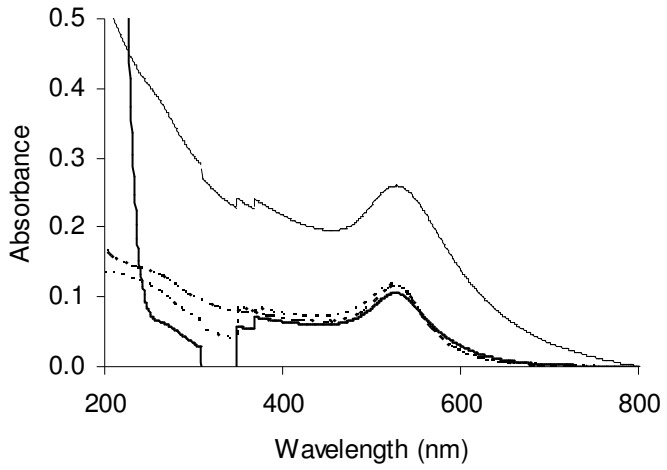


Figure 4.11 UV-Vis absorption spectra of the AuNPs stabilized by (a) CS (solid line), (b) TMC (dashed line), (c) HTACC (dotted line), and (d) SCC (dash-dotted line)

Table 4.5 UV-Vis absorption band of the as-synthesized AuNPs and the AuNPs after storing for more than 4 months

Type of AuNPs	Absorption band (nm)	
	As-synthesized	After storing
CS-AuNPs	527	523
TMC-AuNPs	529	N/A
HTACC-AuNPs	524	528
SCC-AuNPs	526	530

N/A: the data is not available because TMC-AuNPs aggregated during the storage.

To confirm the presence of polymers around the AuNPs and identify the possible interaction between the polymers and AuNPs, FTIR spectra of all AuNPs were recorded. All spectra in Figure 4.12 showed a characteristic IR feature of chitosan and its derivatives around 1626 cm^{-1}, which can be assigned as an absorption band of C=O stretching of secondary amide of acetamide in chitosan. Furthermore, the disappearance of the N-H bending vibration of primary amine at 1596 cm^{-1} implied that amino groups may locate close to the AuNPs, causing an impediment in IR vibration.

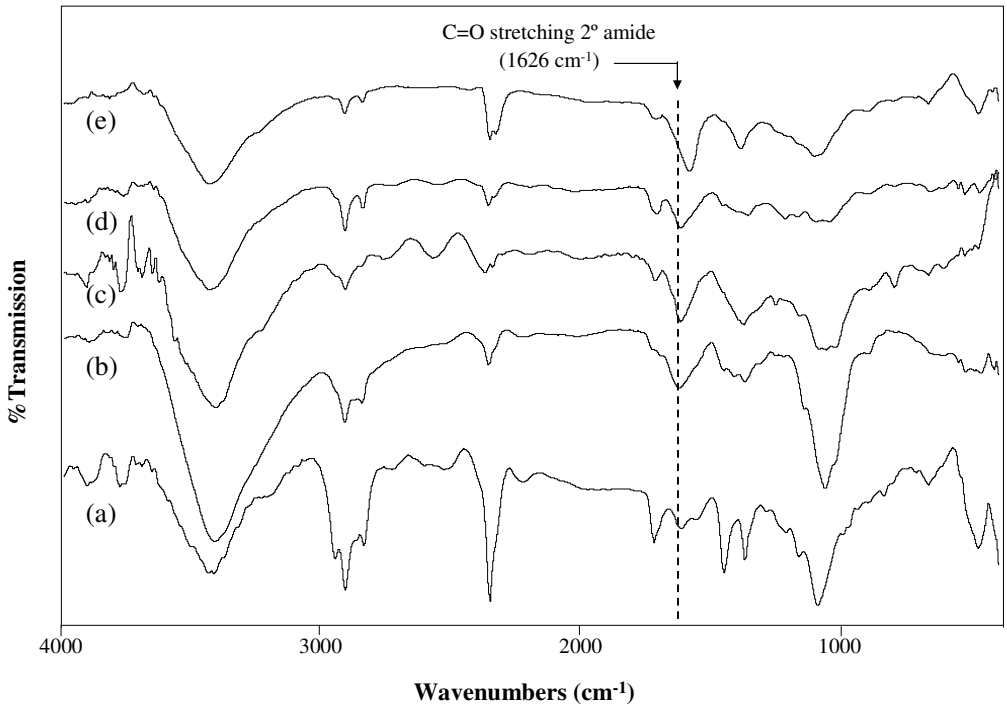


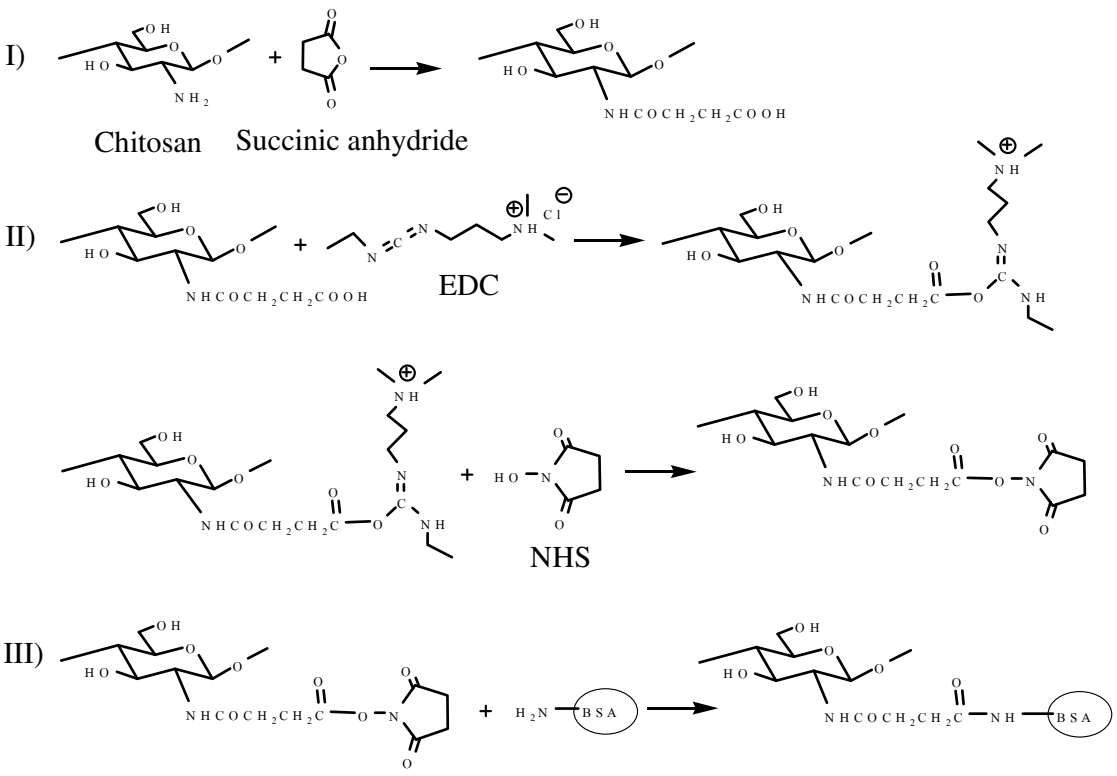
Figure 4.12 FTIR spectra of AuNPs stabilized by (a) CS, (b) TMC, (c) HTACC, (d) SCC, and (e) trisodium citrate

4.3 Preparation of AuNPs Stabilized by CS-BSA (BSA-CS-AuNPs)

In the preparation of biosensor, BSA was selected as a model of antigen. BSA-CS-AuNPs were synthesized via 2 steps. Firstly, BSA was bound to amino groups of chitosan by covalent coupling. Later on, chitosan attached BSA (CS-BSA) was used to synthesize AuNPs with the optimum condition previously identified for the CS-AuNPs in section 4.2.1.

4.3.1 Attachment of BSA to amino groups of chitosan (CS-BSA)

Attachment of biomolecule to the surface of the particles by covalent linkage provides many advantages in comparison with the conventional physisorption. Covalent binding can overcome a problem due to desorption of biomolecule from the particles and has an ability to provide site-directed immobilization. A proper pathway for attachment BSA to chitosan consists of the following 3 steps as shown in Scheme 4.4.



Scheme 4.4 Attachment of BSA to chitosan

In the first step, chitosan was modified with succinic anhydride (SA) to introduce carboxyl groups to chitosan molecule. The reaction was carried out in formic acid to avoid the hydrolysis of SA in aqueous solution. Figure 4.13 presents the FTIR spectra of chitosan before and after chemical modification by SA. As compared with the virgin chitosan, an increment of C=O stretching and N-H bending of secondary amide at 1665 cm^{-1} and 1578 cm^{-1}, respectively and a decrement of N-H bending of primary amine in the modified chitosan (CS-SA) could be used as evidences to confirm the amidation between amino groups of chitosan and SA.

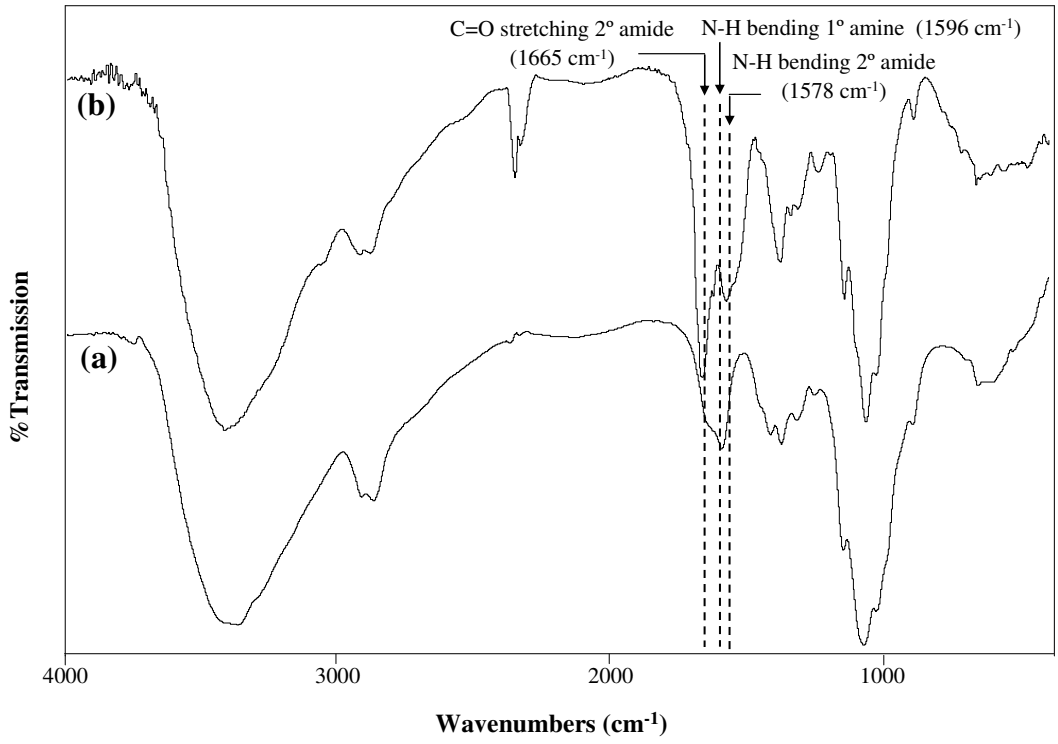


Figure 4.13 FTIR spectra of (a) CS and (b) CS-SA

After chitosan was modified, carboxyl groups in chitosan were bound with BSA through EDC/NHS activation. After the reaction was completed, the excess reagents; EDC, NHS, and BSA, were washed with Milli-Q water by membrane centrifugation. The purification efficiency was monitored by measuring a conductance of supernatant obtained after each washing cycle. As displayed in Table 4.6, the excess reagents should be entirely removed after 7 cycles of centrifugational wash because the conductance dramatically decreased. The conductance of MilliQ-water is 1.1 μS/cm.

Table 4.6 Conductance of supernatant after membrane centrifugation

Centrifugation (cycle)	1	2	3	4	5	6	7
Conductance (μS/cm)	2625.0	1091.0	214.9	67.3	20.5	14.1	10.9

The success of BSA attachment onto chitosan can be confirmed by the absorption band of phenyl groups in tryptophan and tyrosine, amino acids in BSA, at

around 275 nm in UV-Vis spectra (Figure 4.14) [56]. Such signal in the spectrum of the SA-modified chitosan after reacting with BSA (CS-SA-BSA) appeared at the same position that was observed in the pure BSA and can be easily distinguished from those of the SA-modified chitosan (CS-SA).

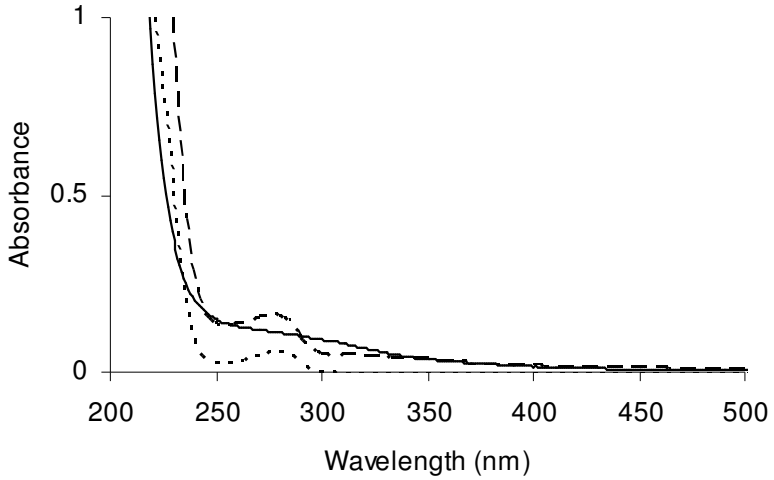


Figure 4.14 UV-Vis absorption spectra of (a) CS-SA (solid line), (b) CS-SA-BSA (dashed line), and (c) BSA (dotted line)

4.3.2 Synthesis of AuNPs stabilized by CS-BSA (BSA-CS-AuNPs)

The synthesis of AuNPs stabilized by CS-BSA was similar to the synthesis of AuNPs stabilized by pure chitosan, except that the CS-BSA was used instead of pure chitosan. Because BSA can be denatured at the temperature above 62 ºC, BSA-CS-AuNPs cannot be synthesized by heating as done in previous experiments. In this research, sonochemical synthesis was introduced as an alternative method in order to avoid the problem due to BSA denaturation. From the results shown in Table 4.7, it was found that the size of CS-AuNPs prepared by sonication was similar to that of the CS-AuNPs prepared by heating suggesting that sonochemical synthesis could be used for the synthesis of AuNPs with the same optimum condition previously identified for the synthesis based on thermal treatment.

Table 4.7 Mean size, PDI and ζ-potential of the CS-AuNPs prepared by heating and sonication as analyzed by PCS using MCS: $HAuCl_4$ = 7:3 (v/v), 1.00 % Tween 80, and independent experiment = 3

Method	Size (nm)	PDI	ζ-potential (mV)
Heating	45.6	0.394	3.5
Sonication	56.6	0.393	3.4

This result was further supported by TEM analysis as illustrated in Figure 4.15. The CS-AuNPs prepared by heating and sonication were spherical in shape, and the mean size was 13.9±5.8 and 9.1±2.3 nm (n=100), respectively. This finding agrees with the data obtained by PCS that there was no significant difference between the size of AuNPs prepared by heating and sonication. Therefore, sonochemical process could be used for the synthesis of BSA-CS-AuNPs.

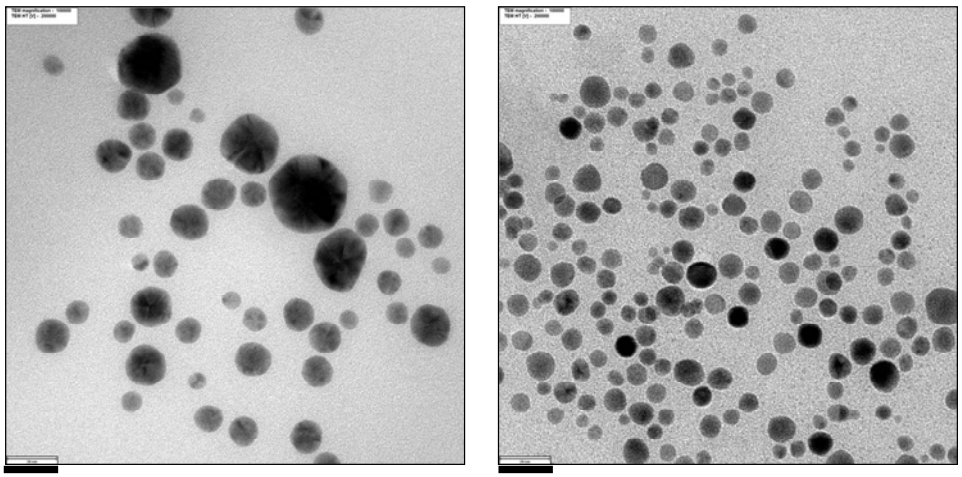


Figure 4.15 TEM images of CS-AuNPs prepared by (a) heating and (b) sonication

Upon using the sonication method, the synthesized AuNPs stabilized by CS-BSA had a mean particle size, PDI, and ζ-potential of 286 nm, 0.436, and +19.9 mV, respectively according to PCS analysis. In addition to the commonly observed spherical morphology, the AuNPs stabilized by BSA-CS (BSA-CS-AuNPs) also appeared in the form of triangle as can be seen in Figure 4.16. In fact, it has been formerly reported that BSA can function as a structure directing agent to produce gold nanoparticles with

triangular morphology under mild condition [33]. For the spherical morphology, the core size of the particles was 8.2±2.8 nm (n=100) (Table 4.8). This dimension was apparently similar to that of the CS-AuNPs synthesized by the same method. The enormous difference between the size of the core gold analyzed by TEM and the hydrodynamic size determined by PCS truly suggested that the BSA-CS-AuNPs had a very thick shell layer. This is quite reasonable considering that chitosan was immobilized by such a large molecule of BSA.

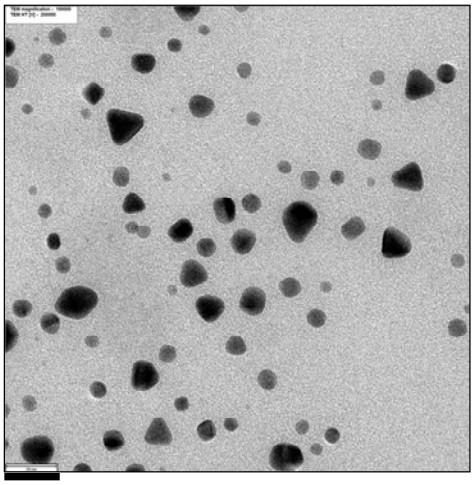


Figure 4.16 TEM images of BSA-CS-AuNPs prepared by sonication

Table 4.8 Mean size of the AuNPs prepared by sonication using MCS: $HAuCl_4$ = 7:3 (v/v) and 1.00 % of Tween 80)

Technique	Size (nm)	
	CS-AuNPs	BSA-CS-AuNPs
PCS	56.6	286
TEM	9.1	8.2

The presence of the polymeric shell of CS-BSA around the AuNPs can also be realized by AFM analysis. This can be done based on the fact that the organic layer of the CS-BSA is less denser and softer than the inorganic core of the gold nanoparticles. Figure 4.17 can clearly demonstrate the presence of CS-BSA surrounding the AuNPs. As can be seen from the 3D image on the right side, the core gold appears as protrusions with a greater height than the polymeric shell. It should be noted that the

relative dimension of the core gold and polymeric shell may not correspond with the actual size measured by TEM and the hydrodynamic size analyzed by PCS. This may be explained by 2 reasons: (1) AFM analysis was performed under semi-dried condition, (2) there was force applied during the measurement.

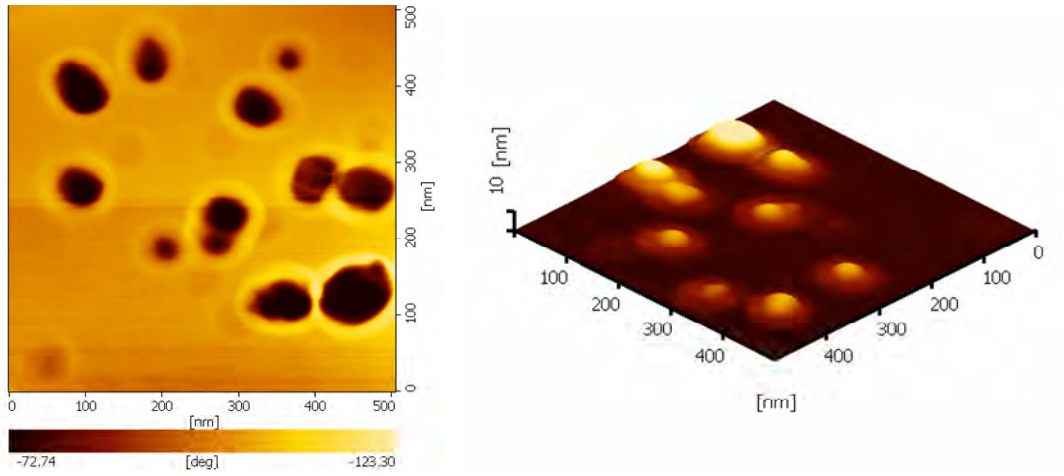


Figure 4.17 AFM images of BSA-CS-AuNPs

In addition, the AuNPs stabilized by CS-BSA were also characterized by UV-Vis spectroscopy to determine the optical property and stability of the particles. As shown in Figure 4.18, the spectrum of the as-synthesized AuNPs shows an absorption band at 531 nm which is a typical plasmon resonance band of AuNPs. Upon storing for one month, the absorption band slightly shifted to 537 nm. This evidently suggested that the BSA-CS-AuNPs were quite stable.

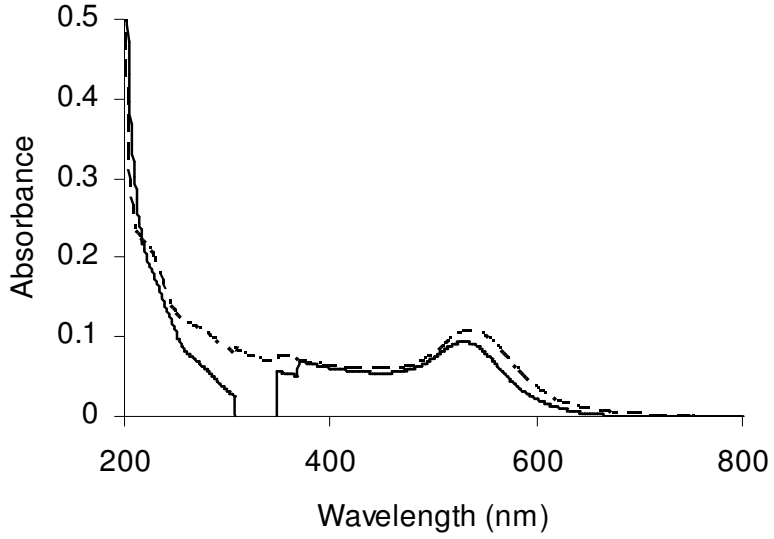


Figure 4.18 UV-Vis absorption spectra of CS-BSA AuNPs: (a) as-synthesized (solid line) and (b) after one month storage (dashed line)

4.3.3 Attempt to attach BSA on AuNPs stabilized by chitosan

We also attempted to synthesize the BSA-CS-AuNPs by binding BSA on the previously formed CS-AuNPs through a same stepwise process as shown in Scheme 4.4 except that CS-AuNPs were used as substrates instead of chitosan. Although there was no change of UV-Vis absorption spectra of the CS-AuNPs after the reaction with succinic anhydride (SA) (Figure 4.19) which implied that the chemical modification by SA was successful and did not induce the aggregation of CS-AuNPs, the conditions and reagents used in the second and third steps somehow caused CS-AuNPs aggregation. This can presumably be explained as a consequence of self-crosslinking between the introduced carboxyl groups and the remaining unreacted amino groups of chitosan in the presence of EDC/NHS.

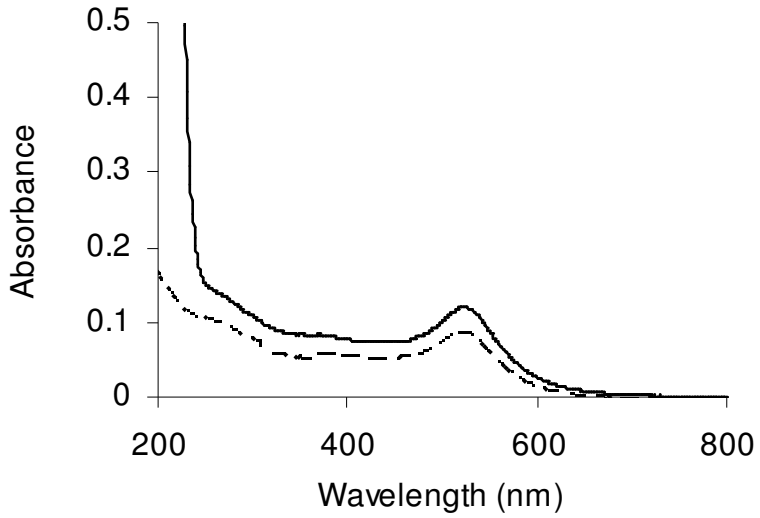


Figure 4.19 UV-Vis absorption spectra of CS-AuNPs (a) before (solid line) and (b) after (dashed line) reacting with succinic anhydride

4.4 Detection of Anti-BSA by BSA-CS-AuNPs

In this section, anti-BSA was used as a target molecule to test for biosensing application of BSA-CS-AuNPs prepared in section 4.3.2. When the specific interactions between BSA attached on the BSA-CS-AuNPs and anti-BSA in the solution occur, the AuNPs should aggregate because two BSA molecules can typically bind one anti-BSA molecule [57], and the binding of antigen-antibody should also change the dielectric constant around the particles. Aggregation of AuNPs changes the optical property because this property depends on the size of particles; also, the color of AuNPs changes from red to violet. Figure 4.20 shows the appearance of the BSA-CS-AuNPs solution after an addition of anti-BSA having varied concentration. The blank solution is a BSA-CS-AuNPs in PBS solution without anti-BSA. From the data obtained, it was found that the minimum concentration of anti-BSA that can induce the aggregation of BSA-CS-AuNPs and can be observed by naked eye was 20 μg/mL. In addition, we also found that the precipitates showed the red color instead of the violet color. It may be because the anti-BSA can induce the aggregation of the BSA-CS-AuNPs, but the vicinity between the AuNPs is not adequate to change the plasmon absorption band due to the fact that the synthesized AuNPs have a very thick shell layer. Therefore, the AuNPs did not change in the optical property.

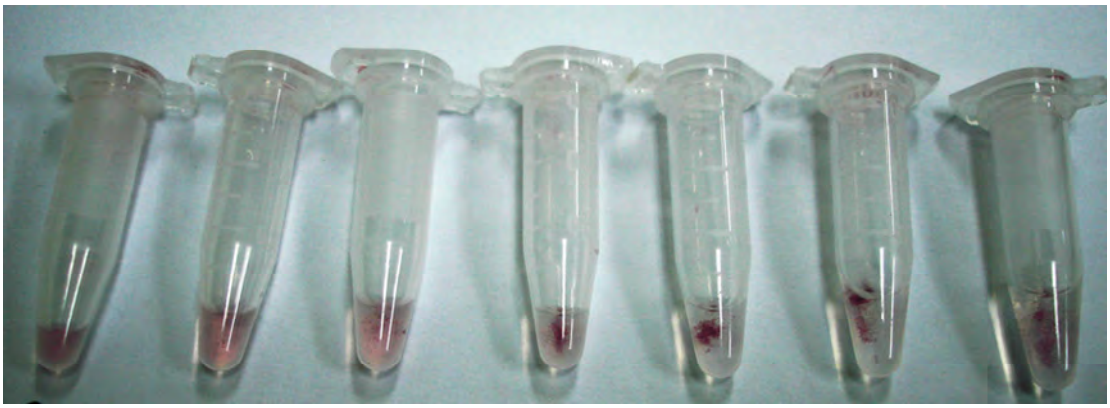
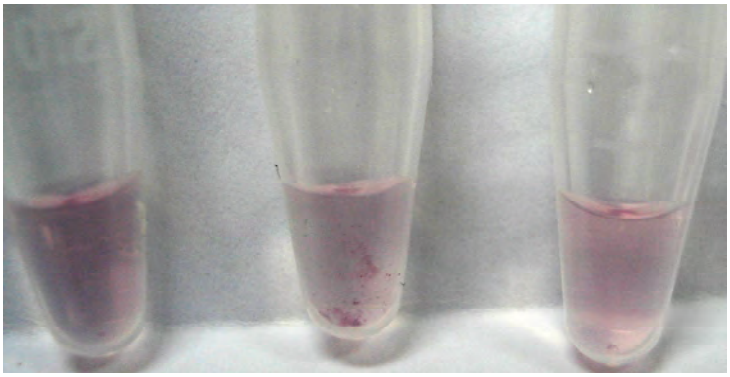


Figure 4.20 Appearance of BSA-CS-AuNPs solution after an addition of anti-BSA having varied concentration

To confirm that the interaction between BSA-CS-AuNPs and anti-BSA did not arise from nonspecific interactions, anti-IgG was also tested as a non-specific target molecule in comparison with the anti-BSA at the concentration of 30 µg/mL. Figure 4.21 illustrates the appearance of the BSA-CS-AuNPs solutions after the addition of anti-BSA and anti-IgG for 2 days. The control in this experiment was the BSA-CS-AuNPs that has been added with PBS solution having the same volume as the anti-BSA and anti-IgG solutions which were also dissolved the PBS. Apparently, there was no aggregation of the BSA-CS-AuNPs upon an addition of the anti-IgG implying that the binding of anti-BSA to the BSA-CS-AuNPs should take place through specific antigen-antibody interactions. These results strongly indicate the potential of the synthesized AuNPs for chromogenic biosensor based on the aggregation of AuNPs.



PBS | Anti-BSA 30 µg/mL | Anti-IgG 30 µg/mL

Figure 4.21 Appearance of BSA-CS-AuNPs solution after an addition of PBS (control), anti-BSA and anti-IgG

CHAPTER V

CONCLUSION AND SUGGESTIONS

This investigation has demonstrated that chitosan can act as both reducing and stabilizing agents for the synthesis of AuNPs due to the fact that gold salts can be reduced to zerovalent gold nanoparticles by amino groups in chitosan's structure. Moreover, chitosan derivatives; TMC, HTACC, and SCC, were effective stabilizing agents due to the repulsion between the charged polymers which act as shell coating for the AuNPs. Gold nanoparticles with different size, size distribution and stability could be obtained by using a different volume ratio of polymer to $HAuCl_4$, molecular weight of chitosan, concentration of Tween 80, and concentration of $NaBH_4$. The synthesized AuNPs have a spherical morphology and a size range of 7-13 nm. AuNPs are stable in the aqueous solution up to 4 months as indicated by no significant changes in UV-Vis absorption band.

For the preparation of biosensor, BSA-CS-AuNPs can be synthesized via 2 steps. Firstly, BSA was bound to amino groups of chitosan by covalent coupling. Later on, CS-BSA was used to synthesize AuNPs with the optimum condition identified for the CS-AuNPs. The sonochemical synthesis was used as the alternative method in the synthesis of BSA-CS-AuNPs to avoid BSA denaturation. BSA-CS-AuNPs were mostly spherical with a diameter of 8.2 nm. Some of them were triangles. They were stable in aqueous solution for more than one month. The synthesized BSA-CS-AuNPs tended to aggregate upon an addition of anti-BSA caused by the specific interactions between the BSA attached on the BSA-CS-AuNPs and the anti-BSA in the solution. The minimum concentration of anti-BSA that could induce the aggregation of BSA-CS-AuNPs that can be visualized by naked eye was 20 µg/mL. Interactions that could lead to detectable aggregation of BSA-CS-AuNPs in the presence of non-specific antigen, anti-IgG was observed.

Further work on testing the specificity and sensitivity of the BSA-CS-AuNPs against other non-specific species or proteins is necessary to determine their efficiency in comparison with the AuNPs-BSA which are prepared by the conventional method based on physisorption. Using the similar strategy of binding the designated antibody to

the molecule of chitosan, which can act as both reducing and stabilizing agent prior to the synthesis of AuNPs, biosensing applications based on other pairs of antigen-antibody should also be possible.

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APPENDICES

APPENDIX A

Table A.1 Mean size, PDI and ζ-potential of the CS-AuNPs synthesized by using varied volume ratio of chitosan to $HAuCl_4$ and molecular weight of chitosan as analyzed by PCS (independent experiment = 3)

$CS:Au^{3+}$	Mean size (nm)		
(mL)	LCS	MCS	HCS
9 :1	49.3±4.8	56.0±0.9	53.3±6.0
8 :2	30.1±0.8	36.3±1.1	34.5±3.1
7 :3	34.0±3.8	30.0±1.0	35.4±3.1
6 :4	37.1±0.1	36.7±0.4	46.3±4.1
5 :5	52.8±2.9	74.9±1.2	68.6±0.6

$CS:Au^{3+}$	PDI		
(mL)	LCS	MCS	HCS
9 :1	0.289±0.029	0.299±0.036	0.430±0.067
8 :2	0.328±0.038	0.326±0.038	0.338±0.064
7 :3	0.394±0.025	0.349±0.018	0.287±0.017
6 :4	0.292±0.010	0.279±0.001	0.286±0.012
5 :5	0.262±0.001	0.417±0.008	0.302±0.024

$CS:Au^{3+}$	ζ-potential (mV)		
(mL)	LCS	MCS	HCS
9 :1	31.2±2.8	43.4±5.4	41.1±0.6
8 :2	26.3±2.4	31.2±1.8	35.5±4.3
7 :3	24.9±3.1	30.2±5.0	29.0±4.1
6 :4	35.8±0.6	31.1±1.6	31.9±0.7
5 :5	33.5±3.4	33.8±1.7	26.6±0.3

Table A.2 Mean size of the AuNPs stabilized by MCS synthesized by using varied volume ratio of polymer to $HAuCl_4$ as analyzed by PCS and TEM

MCS:Au^{3+} (mL)	Mean size (nm)	
	PCS	TEM
9:1	56.0±0.9	14.7±5.0
8:2	36.3±1.1	13.3±3.5
7:3	30.3±1.0	14.2±4.8
6:4	36.7±0.4	13.4±5.8
5:5	74.9±1.2	30.0±7.8

Table A.3 Mean size, PDI and ζ-potential of the chitosan derivative-stabilized AuNPs synthesized by using varied volume ratio of polymer to $HAuCl_4$ as analyzed by PCS (independent experiment = 3)

polymer:Au^{3+}	Mean size (nm)		
(mL)	TMC	HTACC	SCC
9 :1	113.7±4.7	N/A	31.7±0.2
8 :2	90.0±3.3	22.2±6.6	30.4±2.1
7 :3	113.2±1.8	22.1±0.7	32.0±6.6
6 :4	N/A	19.9±1.4	30.7±1.1
5 :5	N/A	22.9±8.3	38.4±1.6

polymer:Au^{3+}	PDI		
(mL)	TMC	HTACC	SCC
9 :1	0.369±0.080	N/A	0.228±0.004
8 :2	0.273±0.022	0.302±0.016	0.270±0.017
7 :3	0.338±0.032	0.347±0.054	0.451±0.104
6 :4	N/A	0.259±0.001	0.437±0.087
5 :5	N/A	0.325±0.158	0.541±0.038

polymer:Au^{3+}	ζ-potential (mV)		
(mL)	TMC	HTACC	SCC
9 :1	32.6±0.6	N/A	-24.1±4.4
8 :2	30.0±1.5	-20.2±0.6	-29.5±0.1
7 :3	31.3±1.3	-17.6±1.0	-26.6±4.5
6 :4	N/A	-27.2±5.4	-16.3±1.6
5 :5	N/A	-37.3±2.2	-10.0±5.1

N/A= is not available because of prepared AuNPs readily aggregated and precipitated during the synthesis.

APPENDIX B

B.1: The 16th Science Forum, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

B.2: The 5th National Chitin-Chitosan Conference, Center for Chitin-Chitosan Biomaterials (CCB), Metallurgy and Materials Science Research Institute (MMRI), Chulalongkorn University, Bangkok, Thailand.

B.3: The 5th Thailand Materials Science and Technology Conference, Miracle Grand Convention Hotel, Bangkok, Thailand. Best Poster Award for Student in Biomedical Materials & Devices Session

B.4: Pure and Applied Chemistry International Conference 2009, Faculty of Science, Naresuan University, Phitsanulok, Thailand. The Outstanding Poster Presentation Award


The Science Forum 2008
การประชุมวิชาการ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ครั้งที่ 16 ประจำปี 2551
วันที่ 13 - 14 มีนาคม 2551
FACULTY OF SCIENCE
CHULALONGKORN UNIVERSITY
ISBN 978-974-03-2086-9

POSTERS

Development of antibacterial fillers from quaternized chitosan particles

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This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical devices, health, food, textile, and personal hygiene industries. Two routes have been proposed to introduce quaternary ammonium groups in order to enhance the antibacterial activity of chitosan particles. Route 1 is to prepare chitosan particles by ionic gelation and modify the particle surface by heterogeneous quaternization using methyl iodide (MeI). Route 2 involves the synthesis of *N,N,N*-trimethyl chitosan by homogeneous quaternization using MeI followed by particle formation using ionic gelation. Results from FT-IR and ^{1}H-NMR analyses confirmed the success of the quaternary ammonium-containing particle formation. The antibacterial activity, tested against *Staphylococcus aureus* (a gram positive bacteria) by viable cell counting, indicated that the additional positive charges introduced to the chitosan particles provide the quaternary ammonium-containing chitosan particles with a superior antibacterial activity compared to the native chitosan particles in a neutral pH range.

Keywords: chitosan, antibacterial activity, quaternary ammonium group

Gold nanoparticles stabilized by chitosan and its derivatives

Pornpen Sae-ung[1], Gamolwan Tumcharern[2] and Voravee P. Hoven[3]*

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A new series of polysaccharide-stabilized gold nanoparticles (AuNPs) were synthesized by two methods. The first method employs chitosan as both a reducing and a stabilizing agent. The second method uses sodium borohydride as a reducing agent and chitosan derivatives; *N,N,N*-trimethyl chitosan (TMC), *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), or *N*-succinyl chitosan (SCC), as a stabilizing agent. The morphology, size and stability of AuNPs were evaluated by UV-vis spectroscopy, photon correlation spectroscopy (PCS), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR). The AuNPs have a spherical morphology and a size range of 5-14 nm. The size, size distribution and stability of the particles varied with the molar ratio of polymer to $HAuCl_4$. It is anticipated that the chitosan and its derivatives surrounding the AuNPs will allow shell functionalization, biomolecule conjugation and the formation of AuNPs-labeled biomolecules that can be applied for biosensing applications.

Keywords: gold nanoparticles, chitosan, chitosan derivatives

เอกสารประกอบการประชุมวิชาการ ไคติน-ไคโตซานแห่งประเทศไทย ครั้งที่ 5

24-25 กรกฎาคม 2551

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จัดโดย ศูนย์วัสดุชีวภาพไคติน-ไคโตซาน
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สนับสนุนโดย ศูนย์เทคโนโลยีโลหะและวัสดุแห่งชาติ
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

Proceedings of the Fifth National Chitin - Chitosan Conference

July 24-25, 2008
Bangkok, Thailand

Organized by:

Center for Chitin-Chitosan Biomaterials(CCB),
Metallurgy and Materials Science Research Institute,
Chulalongkorn University

Supported by: National Metal and Materials Technology Center
National Science and Technology Development Agency

อนุภาคนาโนทองคำที่ทำให้เสถียรโดยไคโตซานและอนุพันธ์ที่มีประจุของไคโตซาน

Gold nanoparticles stabilized by chitosan and its charged derivatives

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บทคัดย่อ: อนุภาคนาโนทองคำที่ทำให้เสถียรด้วยพอลิแซคคาไรด์ถูกสังเคราะห์โดยใช้ 2 แนวทาง แนวทางที่ 1 ใช้ไคโตซานเป็นทั้งตัวรีดิวซ์และสารทำให้เสถียร แนวทางที่ 2 ใช้โซเดียมโบโรไฮไดรด์เป็นตัวรีดิวซ์และใช้อนุพันธ์ไคโตซาน ได้แก่ เอ็น,เอ็น,เอ็น-ไตรเมทิลไคโตซาน, เอ็น-[(2-ไฮดรอกซิล-3-ไตรเมทิลแอมโมเนียม)โพรพิล]ไคโตซานคลอไรด์ หรือเอ็น-ซักซินิลไคโตซาน เป็นสารทำให้เสถียร ผลจากการวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านพบว่า อนุภาคที่สังเคราะห์ได้เป็นทรงกลม, มีขนาดอยู่ในช่วง 5-14 นาโนเมตร ความเสถียรเมื่อกระจายตัวอยู่ในน้ำเกิดขึ้นเนื่องจากแรงผลักระหว่างประจุบนสายโซ่พอลิเมอร์ซึ่งเคลือบเป็นชั้นนอกของอนุภาค นอกจากนี้จากการวิเคราะห์ด้วยเทคนิคโฟตอนคอร์รีเลชันสเปกโทรสโกปียังพบว่าขนาด, การกระจายตัวของขนาด และเสถียรภาพของอนุภาค แปรเปลี่ยนตามสัดส่วนโดยปริมาตรระหว่างสารละลายพอลิเมอร์และไฮโดรเจนเททระคลอโรออเรต โดยคาดว่าไคโตซานและอนุพันธ์ที่ล้อมรอบอนุภาคนาโนทองคำจะสามารถใช้ในการเชื่อมต่อกับสารชีวโมเลกุลและนำไปประยุกต์ใช้เป็นไบโอเซนเซอร์ได้

Abstract: Polysaccharide-stabilized gold nanoparticles (AuNPs) were synthesized by two methods. The first method employed chitosan as both reducing and stabilizing agent. The second method used sodium borohydride as a reducing agent and chitosan derivatives; *N,N,N*-trimethyl chitosan (TMC), *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), or *N*-succinyl chitosan (SCC), as a stabilizing agent. The result from transmission electron microscopy showed that the synthesized AuNPs had a spherical morphology and a size range of 7-14 nm. They are stable in aqueous solution due to the repulsion between the charges on the polymer chains which act as shell coating. Moreover, from the photon correlation spectroscopy (PCS), it was found that the size, size distribution and stability of particles varied with volume ratio of polymer solution to $HAuCl_4$. It is anticipated that the chitosan and its derivatives surrounding the AuNPs will allow biomolecule conjugation that can be applied for biosensing applications.

Introduction

Preparation of gold nanoparticles (AuNPs) has been extensively studied due to their unique physical and chemical properties. They hold great potential for applications in catalysis, biological nanosensor and optoelectronic nanodevice. Chemical reduction is the most common method for the preparation of AuNPs. This method involves a treatment with a chemical reducing agent, such as citrate acid, borohydride, or other organic compounds to convert gold salts to zerovalent gold nanoparticles Because AuNPs usually tend to aggregate in the medium, stabilizing agents are thus necessary in the synthesis of AuNPs. It has been reported that some polymers can act as stabilizing agent for the synthesis of AuNPs. The stabilization generally occurs through charge or steric repulsion [1]. Chitosan (CS) is a polysaccharide obtained by partial deacetylation of chitin, a natural substance found abundantly in the exoskeletons of insects, the shells of crustaceans, and fungal cell walls. Chitosan has been used as both reducing and stabilizing agent due to the fact that gold salts can be reduced by amino group in its main chain without any additional reducing agent [2]. Taking advantage of its ability to act as both reducing and stabilizing agent for the synthesis of AuNPs, together with the fact that chitosan consists of reactive functional groups such as amino and hydroxyl group that can be further functionalized. This research aims to use chitosan and its charge derivatives as stabilizers in the synthesis of AuNPs. It is anticipated that the chitosan derivatives-stabilized AuNPs are more stable than the chitosan-stabilized AuNPs due to the presence of permanent charges of chitosan derivatives. In addition, the chitosan and its derivatives surrounding the AuNPs should also allow further shell functionalization, especially binding to bioactive molecules.

Materials and Methods

$HAuCl_4$ and succinic anhydride (SA) were purchased from Aldrich. Chitosan flake (degree of deacetylation 90.0% and molecular weight 80 kDa) was obtained from Seafresh Chitosan (Lab) Co., Ltd. (Thailand). Sodium borohydride ($NaBH_4$), methyl iodide (MeI), sodium hydroxide (NaOH), sodium iodide (NaI), and Glycidyltrimethylammonium chloride (GTMAC) were purchased from Fluka (Switzerland). Glacial acetic acid and methanol (MeOH) were purchased from Merck (Germany). All reagents and materials are analytical grade and used without further purification. All solutions were made using ultrahigh purity water purified using a Milli-Q Plus system (Millipore Co.).

Synthesis of chitosan-stabilized AuNPs: All glasswares used were cleaned in a bath of freshly prepared aqua regia solution ($HCl:HNO_3$ 3:1) and then rinsed thoroughly with H_2O prior to use. Before the preparation of AuNPs, the stock solutions of chitosan were prepared by dissolving a certain amount of chitosan in 1% acetic acid solution. The synthetic method can be summarized as follows: the chitosan solution (0.1%w/v) and aqueous solution of $HAuCl_4$ (2.943 mM) in 18 mL of Milli Q water were separately heated at 60°C in an oil bath for 5 min. Then, the chitosan solution was added into the aqueous solution of $HAuCl_4$. The mixture was heated to 85°C under vigorous stirring until a red solution was obtained. The synthesized AuNPs was kept in the dark.

Synthesis of chitosan derivatives-stabilized AuNPs: Chitosan derivatives; TMC, HTACC, and SCC, were prepared according to Hamman et al. [3], Seong et al. [4], Sashiwa et al.[5], respectively. They were dissolved in Milli Q water. The synthesis of AuNPs stabilized by TMC, HTACC and SCC were similar to the synthesis of chitosan-stabilized AuNPs, except 10 mole equivalents of $NaBH_4$ (29.43 mM) was added in the reduction step after mixing the polymer solution with aqueous solution of $HAuCl_4$.

Results and Discussion

The effects of the volume ratio of polymer to $HAuCl_4$ on the mean size, size distribution and stability of the synthesized AuNPs were studied using PCS which is based on a dynamic light scattering (DLS) technique. The results are shown in Fig.1. Apparently, the mean particle size, size distribution, and stability of AuNPs changed with the volume ratio of polymer to $HAuCl_4$. In addition, the particle size of AuNPs stabilized by HTACC and SCC were smaller than that of AuNPs stabilized by CS. The size of TMC-stabilized AuNPs were, however, larger than others because of its lower degree of substitution (23%DS) in comparison with HTACC (70 %DS) and SCC (40 %DS). The lower charge density at pH ~ 7 of TMC led to the lower degree of charge repulsion between TMC-stabilized AuNPs so they tended to aggregate and form larger particles. The optimal condition that yielded AuNPs with small size, high zeta potential and narrow size distribution was the ratio of 7:3.

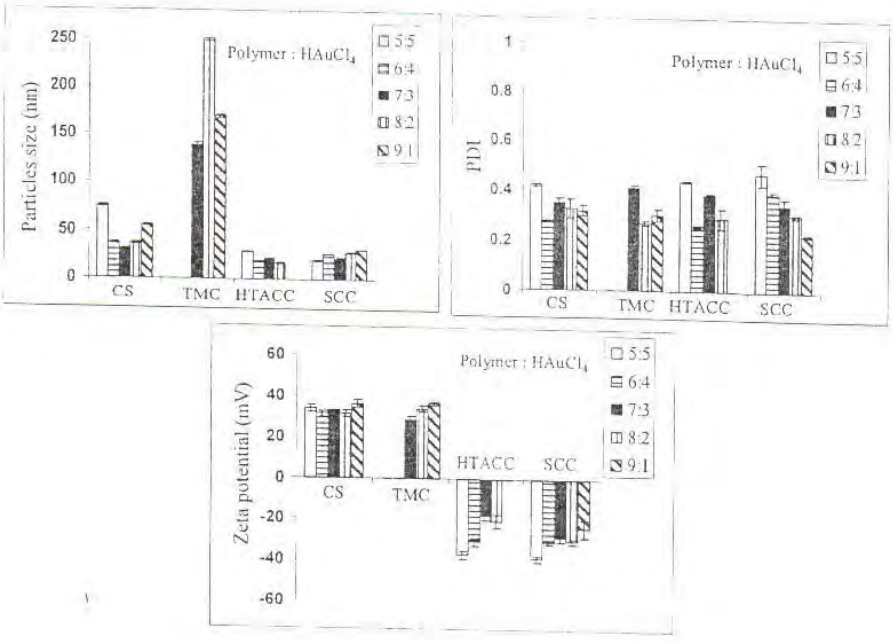

Fig. 1: Charcteristics of AuNPs stabilized by chitosan (CS) and its charged derivatives

The morphology and actual size of AuNPs (polymer:$HAuCl_4$ = 7:3) were determined by TEM analysis, as displayed in Fig.2. Obviously, the particles were spherical in shape and have a relatively narrow size

distribution ranging from 7 to 14 nm. Moreover, the apparent particle size visualized by TEM analysis is larger than the hydrodynamic size characterized by PCS.

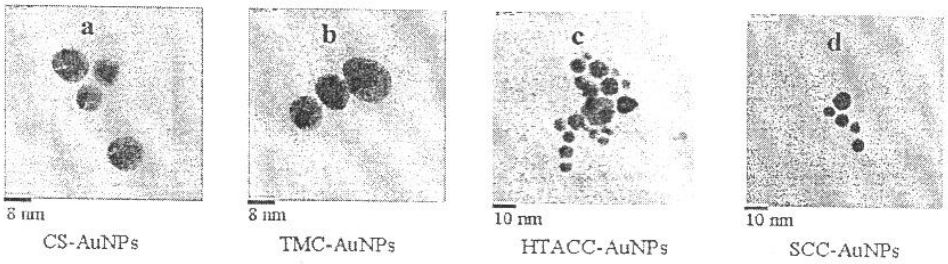

Fig. 2: TEM images of AuNPs stabililzed by CS (a), TMC (b), HTACC (c), and SCC (d)

The optical properties of the AuNPs were characterized using UV-vis spectroscopy (Fig.3). All spectra exhibit an absorption band around 520 nm, which is a typical plasmon resonance band for AuNPs, indicating the formation of AuNPs. To confirm the presence of the polymers around the AuNPs, FTIR spectra were measured. All spectra in Fig.4 show a characteristic IR feature of chitosan and its derivatives around 1630 cm^{-1}, which is an absorption band of C=O stretching (Amide I) of acetamide in chitosan. Furthermore, the disappearance of the N-H bending vibration (Amide II) at 1598 cm^{-1} implied that amino groups may locate close to the AuNPs, causing an impediment in IR vibration.

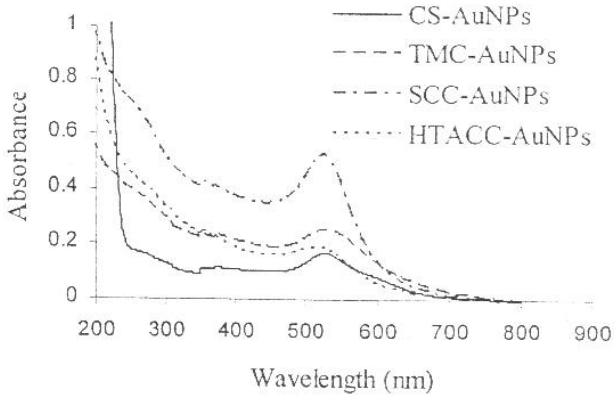

Fig. 3: UV-vis absorption spectra of AuNPs stabililzed by CS and its charged derivatives

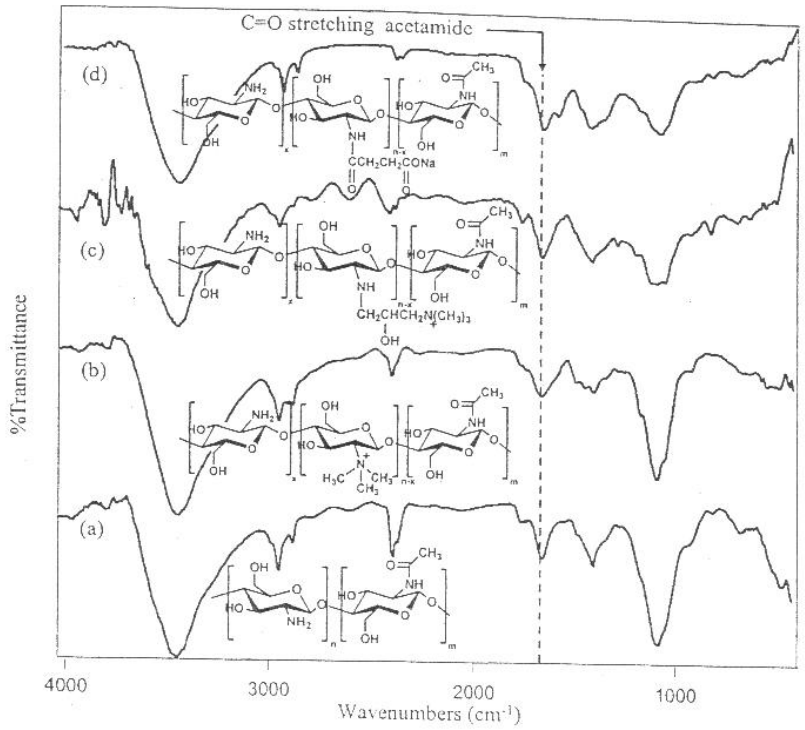

Fig. 4: FTIR spectra of AuNPs stabilized by CS (a), TMC (b), HTACC (c), and SCC (d)

Conclusion

This investigation has demonstrated that chitosan can act as both reducing and stabilizing agents for the synthesis of AuNPs due to the fact that gold salts can be reduced to zerovalent gold nanoparticles by amino group in chitosan's structure. Moreover, chitosan derivatives: TMC, HTACC, and SCC, were effective stabilizing agents due to the repulsion between the charged polymers which act as shell coating for the AuNPs. Gold nanoparticles with different size, size distribution and stability could be obtained by using a different volume ratio of polymer to $HAuCl_4$. The synthesized AuNPs have a spherical morphology and a size range of 7-14 nm. Currently, we are investigating the attachment of biomolecules to the active moieties of chitosan and its derivatives (NH_2, COOH) surrounding the AuNPs in order to determine their potential in biosensing applications.

Acknowledgements

This research is financially supported by Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA).

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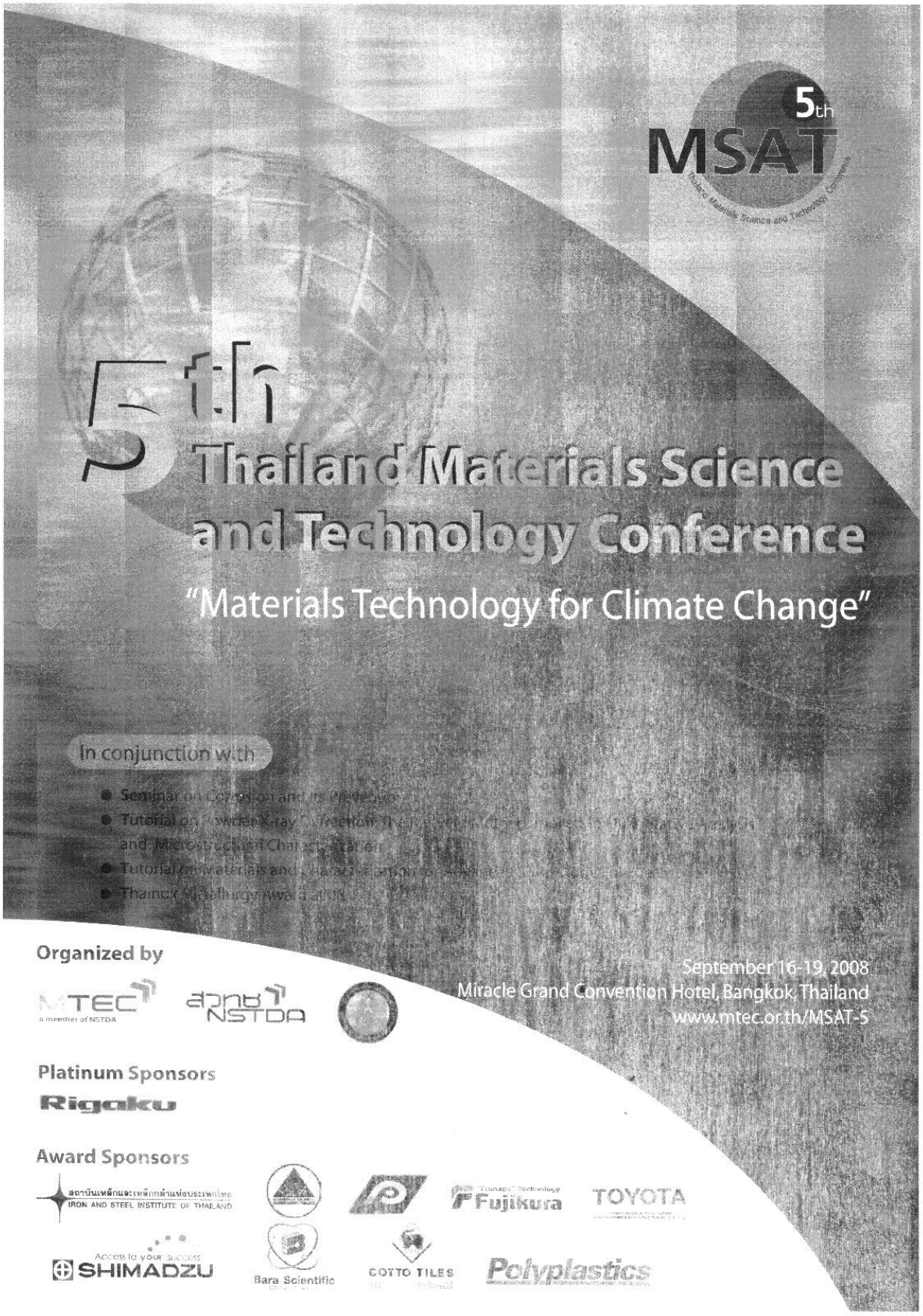
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Chitosan-Stabilized Gold Nanoparticles for Biosensing Application

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Abstract

Polysaccharide-stabilized gold nanoparticles (AuNPs) were synthesized by employing chitosan as both reducing and stabilizing agent. The morphology, size and stability of AuNPs were evaluated by UV-vis spectroscopy, dynamic light scattering (DLS) technique, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). The results showed that the AuNPs have a spherical morphology and a size range of 8-12 nm. They are stable in aqueous solution due to the repulsion between the charged polymers which act as shell coating. It was also found that the size, size distribution and stability of particles varied with volume ratio of chitosan to $HAuCl_4$, concentration of surfactant, and molecular weight of chitosan. The reactivity of amino groups of the chitosan surrounding the AuNPs was tested against phenylalanine, an amino acid that can represent peptide-based biomolecules.

1. Introduction

Preparation of gold nanoparticles (AuNPs) has been extensively studied due to their unique physical and chemical properties and for potential applications in catalyst, biological nanosensor and optoelectronic nanodevice [1]. Chemical reduction is the most common method for the preparation of AuNPs. This method involves a treatment with a chemical reducing agent, such as citrate acid, borohydride, or other organic compounds to convert gold salts to zerovalent gold nanoparticles. Because AuNPs usually tend to aggregate in the medium, stabilizing agents are thus necessary in the synthesis of AuNPs. It has been reported that some polymers can act as stabilizing agent for the synthesis of AuNPs. The stabilization generally occurs through charge or steric repulsion [2].

Chitosan is a polysaccharide obtained by partial deacetylation of chitin, a natural substance found abundantly in the exoskeletons of insects, the shells of crustaceans, and fungal cell walls. Chitosan has been used as both reducing and stabilizing agent due to the fact that gold salts can be reduced by amino group in its main chain without any additional reducing agent [3].

Taking advantage of its ability to act as both reducing and stabilizing agent for the synthesis of AuNPs, together with the fact that chitosan consists of reactive functional groups such as amino and hydroxyl group, this research aims to synthesize chitosan-stabilized AuNPs and to test the reactivity of the amino groups of chitosan towards covalent binding with biomolecules. It is anticipated that the biomolecule-bound chitosan-stabilized AuNPs will be useful for the development of chromogenic biosensor based on the agglomeration of AuNPs.

2. Experimental

2.1 Synthesis of chitosan-stabilized AuNPs

All glasswares used were cleaned in a bath of freshly prepared aqua regia solution ($HCl:HNO_3$ 3:1) and then rinsed thoroughly with H_2O prior to use. Before the preparation of AuNPs, the stock solutions of chitosan were prepared by dissolving a certain amount of chitosan in 1% acetic acid solution. The synthetic method can be summarized as follows: the chitosan solution (0.1%w/v) and aqueous solution of $HAuCl_4$ (2.943 mM) in 18 mL of Milli Q water were separately heated at 60°C in an oil

bath for 5 min. Then, Tween 80 and the chitosan solution were sequentially added into the aqueous solution of $HAuCl_4$. The mixture was heated to 85°C under vigorous stirring until a red solution was obtained. The synthetic AuNPs was kept in the dark.

2.2 Attachment of biomolecule to amino group of chitosan

Phenylalanine was conjugated with chitosan molecules by EDC/NHS activation. Conjugation was started by adding phenylalanine 5 mole equivalent followed by the ECD/NHS 3 mole equivalent and stirred at room temperature for 2 days. After the reaction was completed, the solution was dialyzed against Milli Q water in a dialysis bag to remove residual small molecules. After 5 days of dialysis, the solution was freeze-dried to obtain a cotton-like white material.

3. Results and Discussion

3.1 Synthesis of chitosan-stabilized AuNPs

The effects of the molar ratio of chitosan to $HAuCl_4$ and molecular weight of chitosan on the mean size, size distribution and stability of the synthetic AuNPs were studied using a zetasizer which is based on a dynamic light scattering (DLS) technique. The results are shown in Figure 1. Apparently, the mean particle size, size distribution, and stability of AuNPs changed with the volume ratio of chitosan to $HAuCl_4$ and molecular weight of chitosan. The conditions that yield AuNPs with small size, high zeta potential and narrow size distribution are the ratio of 7:3 and medium molecular weight of chitosan (low molecular weight = 15kDa and medium molecular weight = 80kDa).

The morphology and actual size of AuNPs (medium molecular weight chitosan:$HAuCl_4$ = 7:3) were determined by TEM analysis, as displayed in Figure 2. Obviously, the particles were spherical in shape and have a relatively narrow size distribution ranging from 8 to 12 nm. Moreover, the apparent particle size visualized by TEM analysis is larger than the hydrodynamic size characterized by DLS.

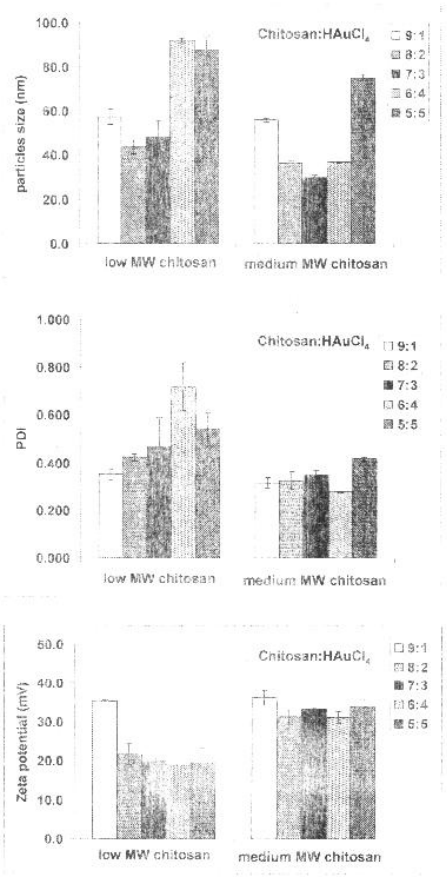

Figure 1 DLS analysis of chitosan-stabilized AuNPs

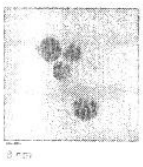

Figure 2 TEM image of AuNPs stabilized by medium MW chitosan (chitosan:$HAuCl_4$ = 7:3)

In addition, Tween 80, as a nonionic surfactant, was used to prevent particle aggregation during purification. Table 1 illustrates the effect of the concentration of Tween 80 on the mean size, size distribution and stability of synthetic AuNPs. From the results, the zeta potential was decreased due to the fact that the AuNPs was shielded by Tween 80. The proper condition was 1%v/v of Tween 80.

Table 1 DLS analysis of medium MW chitosan-stabilized AuNPs + Tween 80

Concentration of Tween 80	Mean size (nm)	Mean PDI	Mean zeta potential (mV)
0.25%v/v	44.3	0.433	19.4
0.50%v/v	48.6	0.477	9.9
1.00%v/v	30.0	0.494	11.5
2.00%v/v	41.4	0.506	13.2

3.2 Synthesis of biomolecule-attached chitosan-stabilized AuNPs

The attachment of phenylalanine on amino group in chitosan's structure was confirmed by ^{1}H-NMR. From ^{1}H-NMR spectrum (Figure 3), the signal at 7.2-7.4 ppm can be assigned to the protons of the aromatic group in phenylalanine's structure. Evidences from the NMR analysis implied that the attachment of phenylalanine to the chitosan backbone was successful. The degree of substitution (%DS) of phenylalanine of 13.4% was obtained. This value was calculated based on the relative ratio between the integration of 5 protons from aromatic groups of phenylalanine and the peak integration of 6 protons (H-2', 3, 4, 5, 6, 2) of chitosan (δ2.9-3.8 ppm).

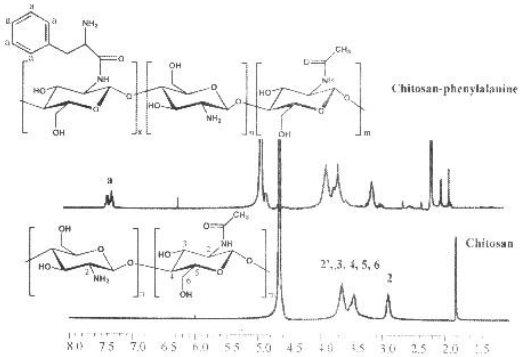

Figure 3 ^{1}H-NMR spectra of chitosan and phenylalanine-attached chitosan

Using the optimized condition previously identified (medium MW chitosan:$HAuCl_4$= 7:3, 1.0%v/v Tween 80), AuNPs was synthesized using phenylalanine-attached chitosan as both reducing and stabilizing agent. The mean size, mean PDI, and mean zeta potential of the AuNPs stabilized by phenylalanine- attached chitosan were 44.8 nm, 0.351, and 10.8 mV, respectively. These characteristics closely resembled those of the AuNPs stabilized by chitosan.

4. Conclusions

This study showed that chitosan can act as both reducing and stabilizing agents for the synthesis of AuNPs due to the fact that gold salts can be reduced to zerovalent gold nanoparticles by amino group in chitosan's structure. Gold nanoparticles with different size, size distribution and stability could be obtained by using a different volume ratio of chitosan to $HAuCl_4$, concentration of Tween 80, and molecular weight of chitosan. The synthetic AuNPs have a spherical morphology and a size range of 8-12 nm. Phenylalanine-attached chitosan can be used in the synthesis of AuNPs. This strongly indicates the possibility of using other biomolecule-attached chitosan for the synthesis of AuNPs which should be applicable for biosensing applications.

Acknowledgement

This research is financially supported by Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA).

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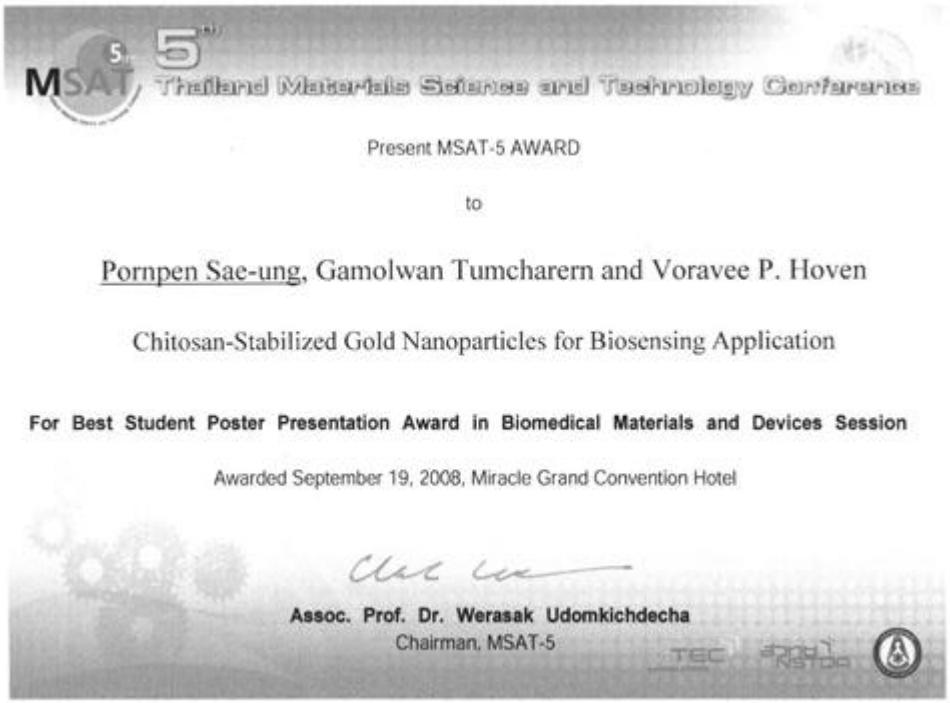

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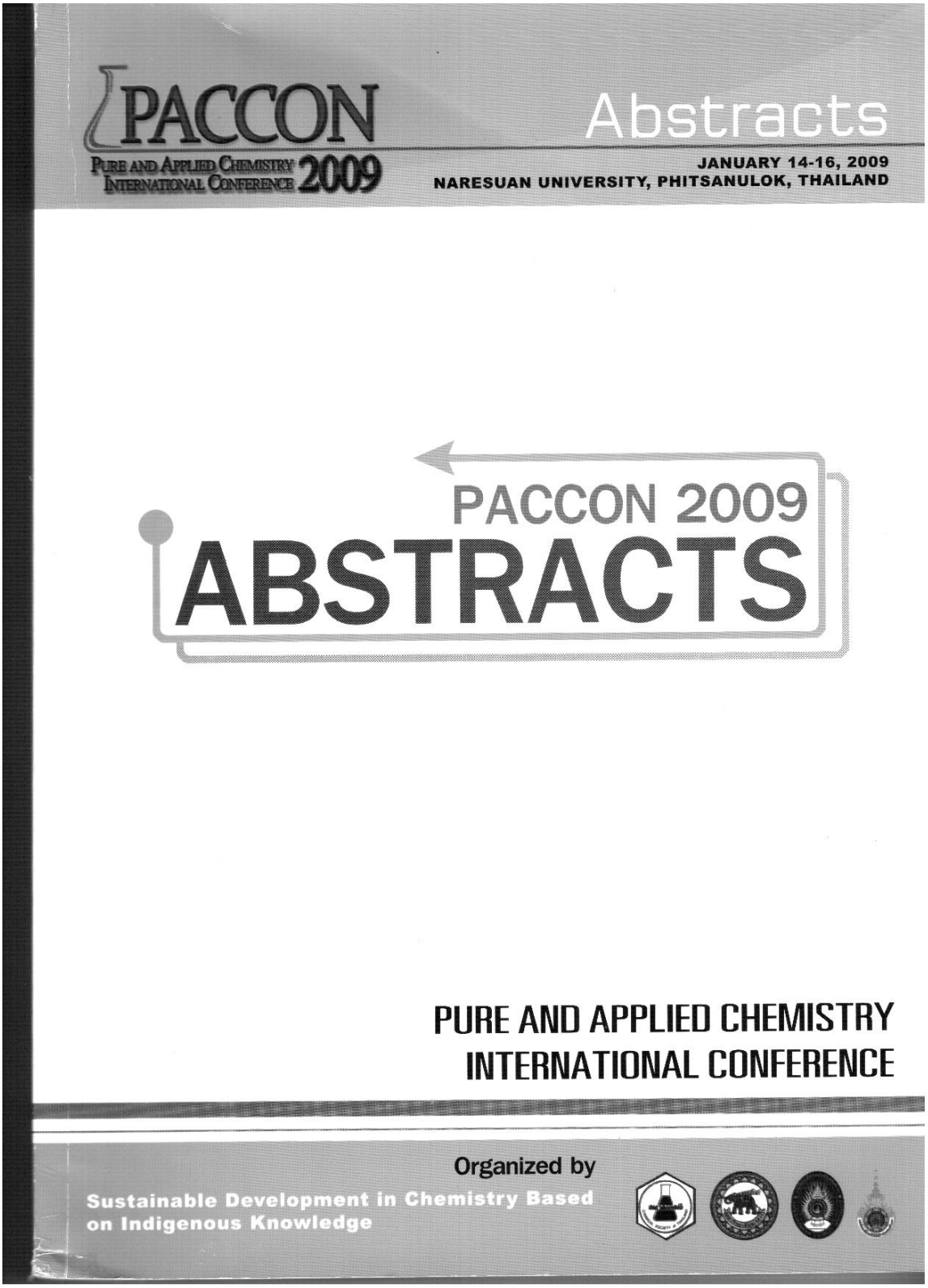
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S7-PO-27

Synthesis and Characterization of Biofunctionalized Gold Nanoparticles Stabilized by Chemically Modified Chitosan

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Gold nanoparticles (AuNPs) were first synthesized by employing chitosan as both reducing and stabilizing agent. Gold salts can be reduced to zerovalent gold nanoparticles by amino groups in the main chains of chitosan. The morphology, size and stability of AuNPs were evaluated by UV-vis spectroscopy, dynamic light scattering, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FT-IR). The results showed that the AuNPs have spherical morphology and a size range of 11.5±3.5 nm. They are stable in aqueous solution due to the repulsion between the charged chitosan which acts as shell coating. It was also found that the size, size distribution and stability of the AuNPs varied with volume ratio of chitosan to $HAuCl_4$, concentration of surfactant, and molecular weight of chitosan. As analyzed by UV-vis spectroscopy, bovine serum albumin (BSA), a model of antigen, can successfully be attached to the amino groups of chitosan. Moreover, a one-step synthesis of AuNPs can be achieved using BSA-modified chitosan as both reducing and stabilizing agent. These results strongly indicate the possibility of using the synthesized AuNPs for chromogenic biosensor based on the agglomeration of AuNPs.

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S7-PO-28

Quaternary Ammonium-Containing Chitosan Particles: Preparation and Antibacterial Activity

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This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical device, health, food, textile, and personal hygiene industries. Two routes have been proposed to introduce quaternary ammonium groups in order to enhance the antibacterial activity of chitosan particles. Route 1 is to firstly prepare chitosan particles by ionic gelation and then modify the surface of the particles by heterogeneous quaternization using selected aldehydes and alkyl iodides. Route 2 involves the synthesis of *N,N,N*-trimethyl chitosan by homogeneous quaternization using MeI. The particles were then formed by ionic gelation. Results from FT-IR and ^{1}H-NMR analyses confirmed the success of the quaternary ammonium-containing particle formation. The antibacterial activity tested against *S.aureus* (gram positive bacteria) and *E.coli* (gram negative bacteria) by optical density (OD_{600}) and viable cell counting methods indicated that the additional positive charges introduced to the chitosan particles rendered the quaternary ammonium-containing chitosan particles superior antibacterial activity to the native chitosan particles in a neutral pH range. Furthermore, the particles prepared by heterogeneous route exhibited higher antibacterial activity than those prepared by homogeneous route.

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Miss Pornpen Sae-ung was born in Bangkok, Thailand, on May 13th, 1984. She received a Bachelor Degree of Science in Chemistry from the Faculty of Science, Chulalongkorn University, Bangkok in 2006. In the same year, she started as a Master Degree student with a major in Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University and completed the program in May, 2009.

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September 2008	The 5th Thailand Materials Science and Technology Conference, Miracle Grand Convention Hotel, Bangkok, Thailand. Best Poster Award for Student in Biomedical Materials & Devices Session
January 2009	Pure and Applied Chemistry International Conference 2009, Faculty of Science, Naresuan University, Phitsanulok, Thailand. The Outstanding Poster Presentation Award