# CHAPTER V

# RAPID HYBRIDIZATION OF CHITOSAN-GOLD-ANTIBODY VIA METAL-FREE CLICK IN WATER-BASED SYSTEM: A MODEL APPROACH FOR NAKED-EYE DETECTABLE ANTIGEN SENSOR

## 5.1 Abstract

A Surface Plasmon Resonance (SPR) expression after hybridization of chitosan-gold nanoparticle-antibody (CS-AuNPs-Ab) based on; (i) metal-free Click chemistry and (ii) in water system as an approach for a rapid antigen sensor is proposed. Chitosan-hydroxybenzyl triazole complex enables us to carry out the conjugation of mPEG and trifluoromethylated oxanorbornadiene (OND) in water. CS-mPEG-OND further allows metal-free Click to hybridize chitosan (CS) with azido-modified gold nanoparticles (azido-AuNPs) in aqueous solution at room temperature. The CS-mPEG-OND conjugated with LipL32 antibody (Ab) not only effectively binds with LipL32 antigen (Ag) but also performs the cycloaddition with azido-AuNPs to display a change in color within 2 min. The phenomenon leads to a simple and efficient naked-eye antigen detection technique.

**Keyword**: water-soluble chitosan, oxanorbornadiene, copper-free Click Chemistry, hybridization, gold nanoparticles, rapid sensing

## 5.2 Introduction

AuNPs are accepted for its unique performances based on optical and electronic characteristics as well as the biocompatibility,(Vega *et al.*, 2010) which lead to potential biomedical applications, e. g., sensing,(Durocher *et al.*, 2009) imaging,(Cormode *et al.*, 2008) cancer treatment,(Liu *et al.*, 2008) etc. The differences in size and shape of AuNPs derive the SPR resulting in the color change which can be recognized by naked eyes. However, the point to be considered is the way to maintain dispersibility of AuNPs in water and/or buffers to meet the requirement of biomedical applications.(Kimling *et al.*, 2006) In addition, the

functionalization of AuNP surface with functional molecules is another concern since it is the way to bring in the specific molecular interaction to make AuNPs perform as an as-designed function.

Up to now, conjugation of AuNPs with a precise structure of block copolymers,(Suntivich *et al.*, 2011) biopolymers,(Prabaharan *et al.*, 2009) etc. have been reported as the way to develop novel materials with molecular recognition, responsiveness, etc. It should be noted that if we consider the material with biocompatibility,(Molinaro *et al.*, 2002) biodegradability,(Zoldners *et al.*, 2005) and nontoxicity,(Huo *et al.*, 2011) we have to accept that the naturally derived polysaccharides, such as CS, hyaluronic acid, and alginate are the good choices. Among those, CS is only the aminopolysaccharide(Dekamin *et al.*, 2013) which contains the different reactive groups in a single polymer chain, i.e. hydroxyl and amino groups. This allows us the many practical functionalizations and/or hybridizations of CS with organic molecules, polymers, inorganic nanoparticles, etc.

To our viewpoint, although the hybridization of CS with AuNPs, under non-covalent(Guo *et al.*, 2008) were successful, we realize that the hybridization via covalent bonds(Charan *et al.*, 2012) is good for stabilizing the AuNPs. In fact, CS is insoluble in water as well as most organic solvents except carboxylic acids, thus it is difficult to perform the reaction of chitosan in homogeneous reaction system. Therefore, the derivatizations of chitosan to organosoluble species such as phthaloyl-chitosan, and to water-soluble chitosan such as *N*-trimethylchitosan (TMC)(de Britto and Campana-Filho, 2004), *N*-carboxymethyl chitosan (CMC)(Aiba, 1989), are needed when we consider the hybridization via covalent bonds.

Click chemistry (Tornøe *et al.*, 2002) (Kolb *et al.*, 2001) is accepted as a convenient reaction since it enables the coupling between two species in mild conditions, e.g. at room temperature, in water, etc., no matter what they are polymer chains, small molecules, or inorganic nanoparticles. In the past, Click chemistry for chitosan was reported. For example, dendronized chitosan was successfully prepared by the copper-catalyzed azide-alkyne cycloaddition between propargyl functionalized poly(amidoamine) dendron and azido-chitosan.(Deng *et al.*, 2011) It should be noted that (i) the reaction has to be carry out in acetic acid which is a good solvent for chitosan, and (ii) chitosan easily forms the complex with Cu(I) which is

the catalyst for Click chemistry(Schlick, 1986), The use of acetic acid always limits the reaction condition whereas the metal ions with chitosan easily leads to a stable complex formation.

To avoid the metal complex formation, in our previous work, we demonstrated a successful metal-free Click chemistry (van Berkel *et al.*, 2007) for chitosan(Jirawutthiwongchai *et al.*, 2013) by the use of chitosan functionalized with oxanorbornadiene (CS-OND). The reaction enables us to further conjugate chitosan with various functional groups such as acid, disulfide, and silane, at room temperature. However, at that time the reaction of CS and OND was carried out in acidic solution. This limits further derivatization conditions and leads to the needs of careful purifications, especially if the goal is for biomedical applications.

Therefore, in this work, we focus on the hybridization of CS-OND with AuNPs in homogenous water reaction via metal-free Click chemistry. The work also extends to propose an Ag detection based on the SPR of AuNPs initiated from the Click chemistry between CS-OND-Ab and azido-AuNPs.

## 5.3 Experimental Section

### 5.3.1 Materials

Chitosan (CS) (deacetylation = 91 % and $M_w$ ~ 15 kDa) was provided from Chitin Research Center Chulalongkorn University, Thailand.. 1-Hydroxybenzotriazole monohydrate (HOBt·$H_2O$) and 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide)hydrochloride (EDC·HCl) were purchased from Wako Pure Chemical Industries Co., Ltd., Japan. Poly(ethylene glycol)methyl ether (mPEG, $M_n$ 5 kDa), succinic anhydride, 3-chloro-1-propanethiol, and sodium azide ($NaN_3$) were purchased from Sigma-Aldrich, Inc., USA. Oxanorbornadiene-NHS ester was prepared as reported previously. (Jirawutthiwongchai *et al.*, 2013) Gold nanoparticles, produced by laser ablation, were provided from Laser Zentrum Hannover, Germany. Dimethyl sulfoxide (DMSO) and diethyl ether was purchased from Labscan, Co., Ireland. All chemicals were used as received without further purification.

5.3.2 Synthesis of Water-soluble Chitosan-mPEG, CS-mPEG

Poly(ethylene glycol)methyl ether conjugated with carboxylic acid, mPEG-COOH, (see Appendix A for synthesis) (19.6 g, 3.9 mmol, 1.0 eq.) and EDC·HCl (2.2 g, 11.6 mmol, 3 eq.) were dissolved in water (100 mL) and cooled to 0 °C. To this solution, an aqueous solution (50 mL) containing CS (0.5 g, 3.2 mmol, 1 eq.) and HOBt (0.5 g, 3.8 mmol, 1.2 eq.), were added and stirred overnight at room temperature. The crude product obtained was purified by dialyzing against NaCl solution followed by DI water for 3 days. The product was lyophilized to obtain CS-mPEG.

5.3.3 Synthesis of Water-soluble Chitosan-mPEG-oxanorbornadiene, CS-mPEG-OND

CS-mPEG (150.0 mg, 0.05 mmol, 1 eq.) was dissolved in water (20 mL). The solution obtained was added into the THF/water (1:3 v/v, 12 mL) containing OND (18.1 mg, 0.05 mmol, 1 eq.). Diisopropylethylamine (DIPEA) (6.0 mg, 0.05 mmol, 1 eq.) was further added and the mixture was cooled to 0 °C for an hour before stirring overnight at room temperature. The solution was dialyzed against NaCl solution followed by DI water for 3 days. The product was lyophilized to obtain CS-mPEG-OND.

5.3.4 Immobilization of LipL 32 Antibody on Water-soluble CS-mPEG-OND, CS-mPEG-OND-Ab

The immobilization of antibody (LipL 32: leptospiral major outer membrane protein) was carried out as reported by Arya G. et al.(Arya *et al.*, 2011) In brief, EDC (8 mg, 0.04 mmol, 3 eq.) and NHS (5 mg, 0.04 mmol, 3 eq.) were dissolved in water 3 mL at 0 °C before adding antibody (200 μL). After 30 min, an aqueous solution of CS-mPEG-OND (50 mg, 0.01 mmol, 1 eq. in water 25 mL) was added and allowed stirring at room temperature for 8 h. The product obtained was dialyzed for 3 days followed by lyophilizing to obtain the CS-mPEG-OND-Ab.

5.3.5 Metal-free Click Chemistry between CS-mPEG-OND-Ab and azido-AuNPs

The Azido-AuNPs solution (see Appendix P for synthesis) (400 µL, 1 µmol/mL consisting of 0.03 µmol/mL azido-disulfide immobilized on AuNPs) was added to CS-mPEG-OND-Ab (100 µL, 1 µmol/mL in water) solution at room temperature. After mixing, the images and absorption spectra were recorded over the reaction time.

5.3.6 Metal-free Click Chemistry between WSC-OND-Ab and Azido-AuNPs

Azido-AuNP solution (400 µL, 0.034 µmol/mL in water) was added into WSC-OND-Ab (100 µL, 0.3 µmol/mL in water). The mixture was stirred and the color of the solution was observed.

## 5.4 Results and Discussion

As our previous report, the complexation between chitosan and HOBt (CS-HOBt) enables chitosan to be soluble in water and the system is practical for conjugating reaction.(Fangkangwanwong *et al.*, 2006) Here, mPEG-COOH was further conjugated on CS-HOBt to obtain CS-mPEG. It is important to note that CS-mPEG is the water soluble product (Figure J1a). The FTIR spectrum (Figure I2c, I3c) indicates the peaks at 1730 $cm^{-1}$ (ester linkage) and 1650 $cm^{-1}$ (amide linkage) including the significant peak at 2,890 $cm^{-1}$ ($CH_2$ of mPEG). The $^1$H-NMR spectrum (Figure I1C) indicates the main chemical shifts at 3.6 ppm, 2.8 ppm, and at 3.3 ppm belonging to methylene units and methyl terminal group in mPEG chain. The conjugation of CS-mPEG with OND were carried out in water and the success of the reaction was evaluated by FTIR from the peak at 860 $cm^{-1}$ (C=C), (Figure I3c), and by $^1$H-NMR (Figure I1 D-F) from the chemical shifts at 7.2 ppm (-CH=CH-), and 5.6 ppm and 5.8 ppm (-HC-O-CH-). It is important to note that, the $^{19}$F-NMR signal showed that the chemical shift of OND at -61 ppm (Figure 5.1Ba) is shifted to -63.5 ppm (Figure 5.1Bb) indicating the covalent bond of OND on CS-mPEG. The substitution of OND on CS-mPEG was found to be in the range of ~30 % - ~ 60%

depending on the mole ratios between two components as quantitatively analyzed by $^{1}$H-NMR (Figure I1 D-F). It should be noted that although the substitution of OND was as high as 60%, the product maintained its water solubility (Figure J1b). This indicated that mPEG moieties played an important role for water solubility.

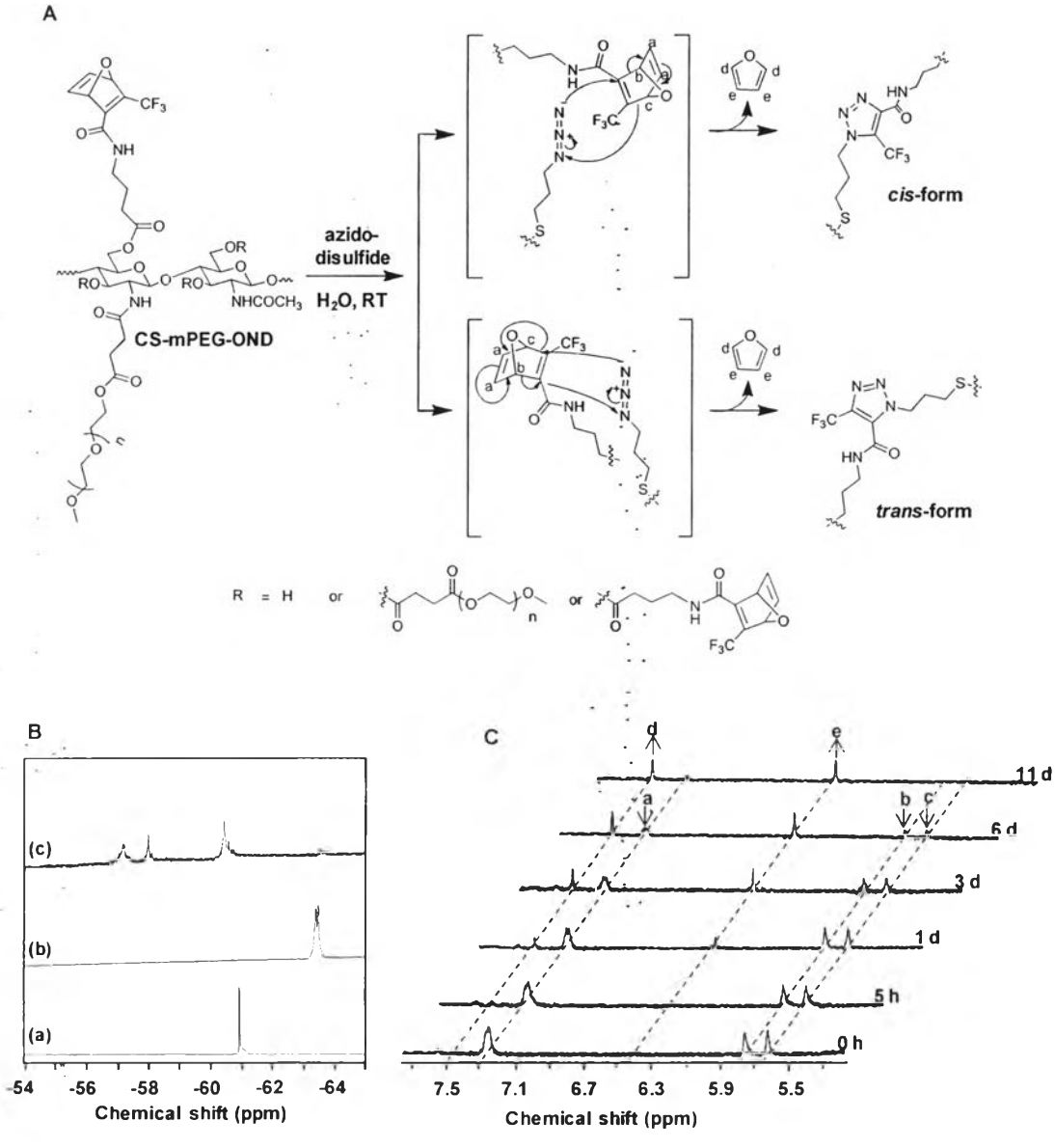


**Figure 5.1** (A) scheme of cycloaddition between CS-mPEG-OND and azido-AuNPs, (B) $^{19}$F-NMR spectra of (a) OND, (b) CS-mPEG-OND, and (c) CS-mPEG-

disulfide, and (C) $^1$H-NMR spectra of ligation progress in $D_2O$ over the reaction time between CS-mPEG-OND and azido-disulfide.

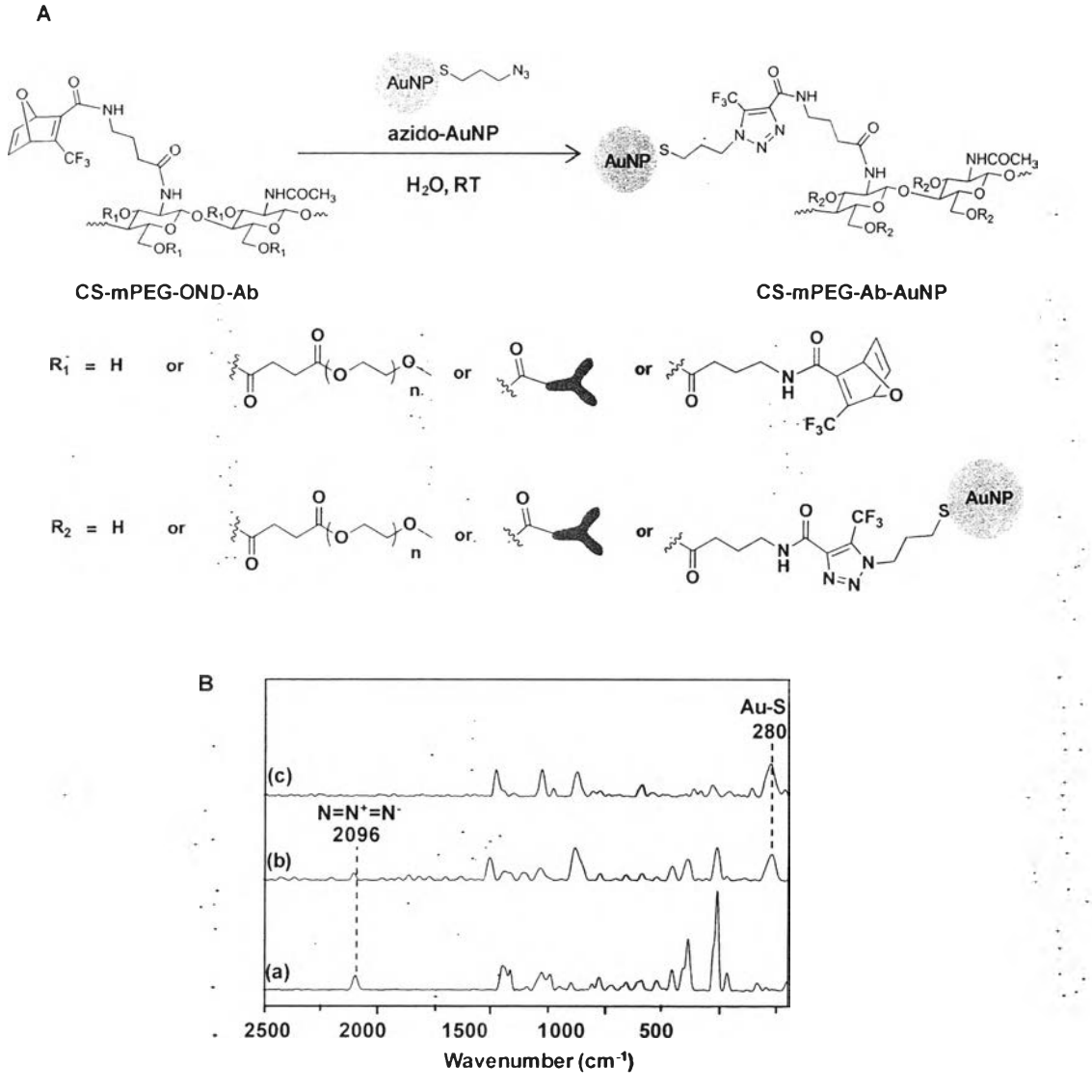


**Figure 5.2** (A) scheme of metal-free cycloaddition between CS-mPEG-OND-Ab and azido-AuNP to yield CS-mPEG-Ab-AuNP and (B) Surface-enhanced Raman spectra of (a) AuNPs, (b) azido-AuNPs, and (c) CS-mPEG-Ab-AuNPs.

In order to demonstrate the Cu-free cycloaddition of CS-mPEG-OND and azido compound, a model reaction between CS-mPEG-OND with 30 % DS and 60 % DS of OND and mPEG, respectively, and azido-disulfide in water, instead of

using azido-AuNPs was carried out. This enables us to follow the reaction clearly by using $^{19}$F-NMR. The success of cycloaddition can be confirmed at δ ~ -57.3 and ~ -60.3 ppm for *cis* and *trans* regioisomeric forms, respectively (Figure 5.1Bc). (Krause *et al.*, 2012; Jirawutthiwongchai *et al.*, 2013) In addition, the $^{1}$H-NMR (Figure 5.1B) shows the chemical shifts at δ ~ 6.45 and ~ 7.5 ppm belonging to the methine protons of furan which is the by-product. At the same time, δs at ~ 5.7, 5.8 and 7.5 ppm belonging to OND ring are disappeared. It is important to note that the $^{1}$H-NMR also indicated that the cycloaddition started after 1 day and took about 6 days to complete the reaction. This reaction time was much shorter than that in our previous work (10 days) which the cycloaddition was carried out in 2% acetic acid solution.(Jirawutthiwongchai *et al.*, 2013)

The fact that CS-mPEG-OND is water soluble, the immobilization of LipL32 (a model Ab) can be easily done by using EDC conjugating agent and carrying out in aqueous solution at room temperature. The zeta-potential under the variation of pH (pH 2-12) to determine the isoelectricity (pI) of the compound before and after the conjugating was applied to evaluate the reaction. The point of isoelectricity (pI) of CS-mPEG-OND was changed from ~8 to ~5.5 after conjugating with Ab. This indicates the success of antibody immobilization.

The existence of azido-disulfide on the surface of AuNPs was confirmed by surface enhanced Raman spectrum (SER) at 280 cm$^{-1}$ (Figure 5.2Bb) referring to Au-S (Lu *et al.*, 2001). Here, the Au-S might be under the coordinated structure (Appendix M).

Figure 5.2Bc confirms the disappearance of azido group at 2,096 cm$^{-1}$ suggesting the formation of triazole linkage after adding azido-AuNPs to CS-mPEG-OND-Ab in water at room temperature.

The important point of the present work is that after azido-AuNPs were added to the CS-mPEG-OND-Ab aqueous solution, the color of AuNPs solution was changed from pink to violet within 2 min and last long for a day (Figure 5.3Ac). This is a rapid change and it insists the SPR change generated from the aggregation of

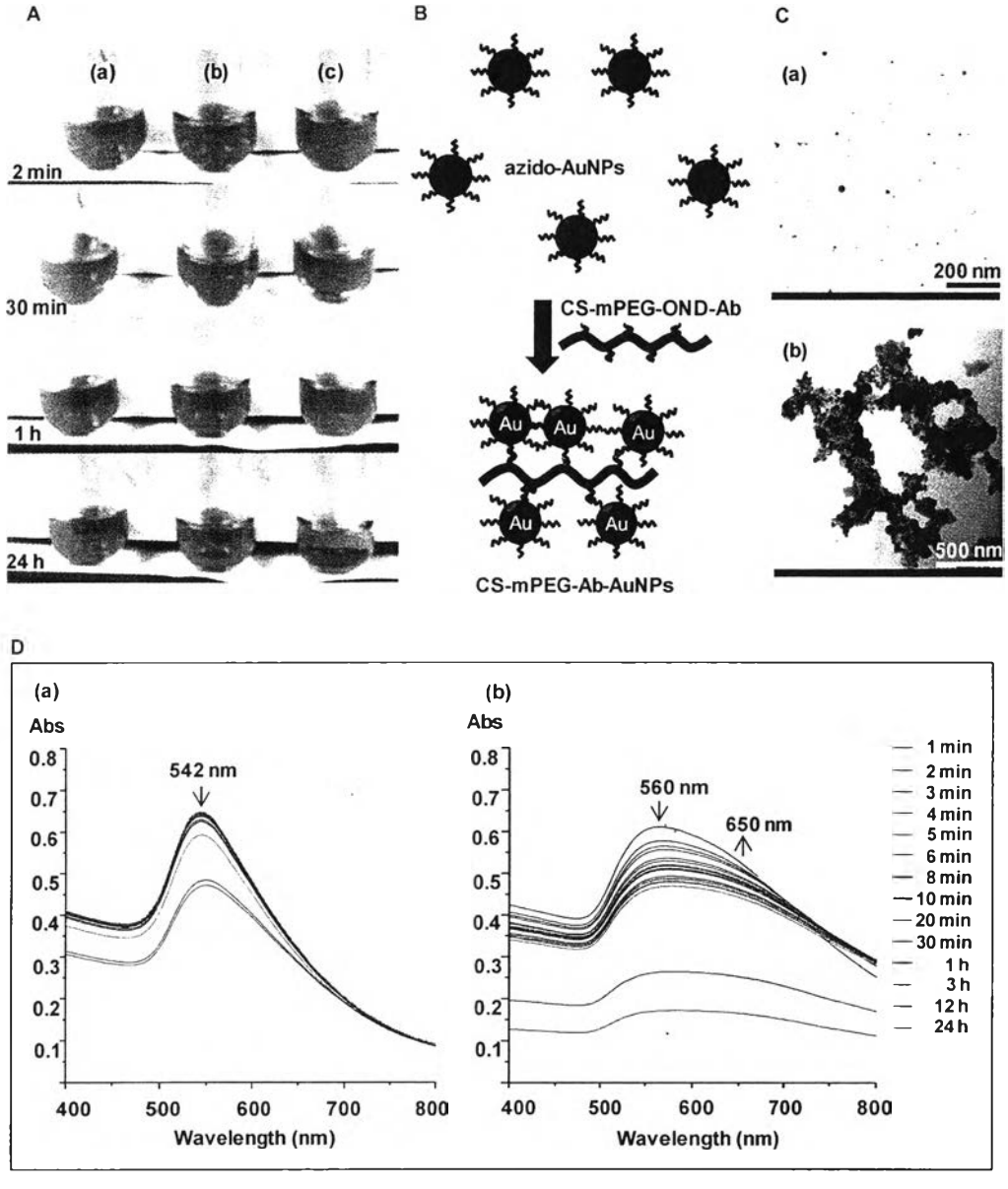


**Figure 5.3** (A) Appearances of azido-AuNPs mixing with (a) water, (b) CS-mPEG-Ab solution, and (c) CS-mPEG-OND-Ab solution after the time pass, (B) scheme showing the aggregation of azido-AuNPs after hybridizing with CS-mPEG-OND-Ab, (C) TEM micrographs of (a) azido-AuNPs, and (b) azido-AuNPs after mixing with CS-mPEG-OND-Ab for 1 day, and (D) UV-vis spectra of azido-AuNPs mixing with (a) CS-mPEG-Ab solution and (b) CS-mPEG-OND-Ab solution over the reaction time.

AuNPs under the Cu-free cycloaddition between azido-AuNPs and CS-mPEG-OND-Ab (Figure 5.3B). As seen in UV-Vis spectra (Figure M1), AuNPs solution showed a monodispersed azido-AuNPs SPR band at 542 nm. After CS-mPEG-OND-Ab was added, the peak was broaden and red-shifted to be at 560 and 650 nm (Figure 5.3D). To clarify that the aggregation of azido-AuNPs is a consequence of azido- and OND moieties, azido-AuNPs was added with CS-mPEG-Ab, which is the compound without conjugating with OND and the color was traced. As shown in Figure 5.3Ab and Figure 5.3Da, the pink color was maintained without any red-shift in UV spectrum even the time passed for 1 day.

It was also found that during the time passed, the azido-AuNPs coupled with CS-mPEG-OND-Ab continually aggregated and precipitated leading to the decrease of SPR absorbance intensity.

TEM images (Figure 5.3C) show the dark dots aggregated with gray spherical bridges within 1 day. This implies the aggregation of azido-AuNPs (dark particles) in the sizes of ~5 - 20 nm on CS-mPEG-OND-Ab (gray spheres).

It should be noted that although the cycloadditon between the CS-mPEG-OND and azido-disulfide components as traced by $^{1}$H-NMR was ~ 1 day, when it comes to the solution system, the color of gold nanoparticle solution was found to change from pink to violet only after 2 minutes.

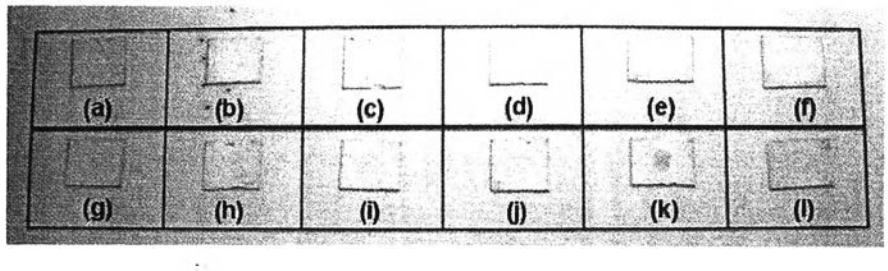


**Figure 5.4** Appearances of nitrocellulose membranes after soaking with azido-AuNPs by first spotting with (g) - (l) and without (a)-(f) Ag followed by dropping with: (a), (g) PBS buffer; (b), (h) CS-mPEG; (c), (i) CS-mPEG-Ab; (d), (j) CS-mPEG-OND; (e), (k) CS-mPEG-OND-Ab; and (f), (l) Ab solution.

In order to see the possibility to apply the system in detecting Ag, the simple method adapting from Dot blot Elisa was applied (Appendix Q for procedure). After coating Ag and CS-mPEG-OND-Ab on nitrocellulose membrane, the membrane was washed thoroughly to remove the unbound Ab . The membrane obtained was rinsed with azido-AuNPs. It should be noted that the violet spot was observed in a sudden (Figure 5.4k). In addition, a series of comparative studies were carried out as follows. The spots belonging to the samples without Ag (Figure 5.4a-f), without Ab (Figure 5.4j) and without OND (Figure 5.4h, i) do not exhibit the color. Figure 5.4 confirms the performance of CS-mPEG-OND-Ab in terms of (i) providing Ag-Ab specific interaction, (ii) allowing Click chemistry with azido-AuNPs, and (iii) expressing the rapid SPR as the Ag detection by the naked eye.

## 5.5 Conclusions

CS-HOBt was a good system for the conjugation of mPEG and OND. The substitutions of mPEG and OND depended on the molar ratios of the reactants. By simply mixing CS-mPEG-OND-Ab and azido-AuNPs in the solution, metal-free Click Chemistry occurred, resulting in a SPR shift within 2 minutes. When this approach was applied on the nitrocellulose membrane which was firstly coated with LibL 32 Ag, then a second coating with CS-mPEG-OND-Ab was selectively bound with Ag, followed by rinsing with azido-AuNPs. This process allowed us to detect Ag by the appearance of the violet spot. This phenomenon produced a novel type of Ag sensor with rapid response and naked-eye detectability in a water-based system.

## 5.6 Acknowledgments

The authors would like to thank Dr. Csaba Laszlo Sajti and Annette Barchanski, Nanotechnology Department, Nano-Materials Group, Laser Zentrum Hannover for gold nanoparticles, Dr. Gerald Draeger and Dr. Andreas Krause, Institute of Organic Chemistry and Center of Biomolecular Drug Research, University of Hannover, Germany for the suggestion. Appreciation is also expressed for the Dusadee Pipat Fund of Chulalongkorn University for the Ph.D. program

scholarship, Center of Innovative Nanomaterial (CIN), Chulalongkorn University, Intergrated Innovation Academic Center (IIAC), Centenary Academic Development Project, Chulalongkorn University, and the Research, Development and Engineering (RD&E) fund through the National Nanotechnology Center (NANOTEC), Thailand, for research funds.

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