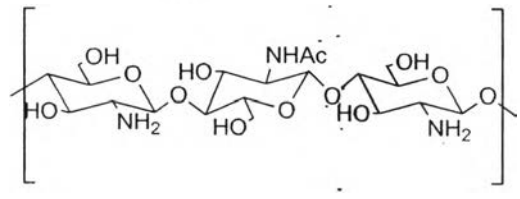
CHAPTER II
LITERATURE REVIEW

2.1 Chitosan

Chitosan (scheme 2.1) is the second-most abundant naturally occurring polysaccharide. It consists of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose and β-(1-4)-2-amino-2-deoxy-β-D-glucose (scheme 2.1). The well known properties of the chitosan are biodegradability (Zoldners *et al.*, 2005), biocompatibility (Molinaro *et al.*, 2002), and nontoxicity (Huo *et al.*, 2011). Although the chitosan gives many advantages, the limitation of dissolution in only acid aqueous solvent obstruct (scheme 2.2) to chitosan derivatization process. This leads to an attempt of changing the solvent system from acetic acid to either organic or water based system. In the past, Kurita et al. (Kurita *et al.*, 2001; Kurita *et al.*, 2007) succeeded in preparing chitosan derivative, phthaloylchitosan which is soluble in dimethylformamide (DMF), dimethyl sulfoxide (DMSO), etc.

Scheme 2.1 Structure of chitosan.

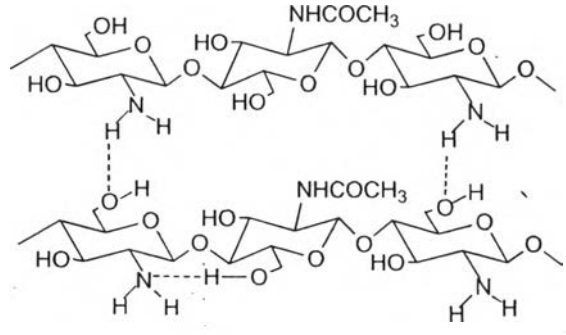


2.2 Water-soluble Chitosan

In the case of chitosan water soluble system, chitosan is normally modified as quaternized chitosan or *N,N,N*-trimethylchitosan (TMC) (de Britto and Campana-Filho, 2004), N,O-carboxymetyl chitosan (CMC) (Aiba, 1989; Muzzarelli *et al.*, 1994), and O-succinyl chitosan (Kennedy *et al.*, 1996). For the past few years, our group was firstly clarified the complexation of water-based (CS-HOBt) and proposed

this system for conjugating biomolecules (Fangkangwanwong *et al.*, 2006; Fangkangwanwong *et al.*, 2006).

Scheme 2.2 Inter- and intra- hydrogen bonding of chitosan.



2.3 Allergy

An allergy, in another word allergic disease, occurs when immune system was stimulated by an allergen. Dust-mite, cockroach, animal dander, and pollen are common allergens and are capable to stimulate a type-I hypersensitivity reaction in atopic individuals. The allergic symptoms are itchy, cough, running nose, nasal congestion, wheezing, vomiting, diarrhea, and skin rash etc. according to the part of the body contacted by the allergen. The reactions and symptoms can be explained by molecular-molecular interactions between antigen and its corresponding immunoglobulin E (IgE antibody) (Traidl-Hoffmann *et al.*, 2009). After the first exposure to the allergen, antigen presenting cell (APC) will process allergen and present it on surface of APC inducing T helper 2 cell (Th2) to be activated and to secrete some mediators i.e. interleukin-4 (IL-4) etc. The IL-4 will induce B cell to produce IgE on plasma cell, then the IgE will bind to IgE receptor on surface of mast cell (in tissue) and basophile (in circulation). If function of T helper 2 cell (Th2) is equal with T helper 1 cell (Th1), allergy and symptoms are not occurring. But when the function of Th2 is dominant, the second allergen exposure will bind with IgE on mast cell. The mast cell activation and degranulation will occur, chemicals will be released, particularly histamine resulting in allergic symptoms. At this time, there are

many treatments that have been developed for allergic diseases such as using anti-inflammatory drug, allergen immunotherapy, and allergen delivery system.

2.4 Allergen Immunotherapy: Chitosan as an Adjuvant

Allergen immunotherapy is gradually tolerated to sensitize allergen by a series of increasing administration of allergen (known as vaccine), resulting in reduction of allergic sensitization (Broos *et al.*, 2010). In general, subunit vaccines (from some part of antigen) and/or combination with adjuvant (to enhance the immune response) have been wildly developed to increase the stimulated ability in immune system which has been shown in several studies. For example, *N*-trimetyl chitosan (TMC) nanoparticles have been shown the immunogenicity increase of subunit antigen after nasal and intradermal administration by determination of IgG, IgA levels (Bal *et al.*, 2012). The alginate coated chitosan nanoparticle was identified as a subcutaneous adjuvant for hepatitis B surface antigen (HBsAg) as reported by Borges and co-worker. A high anti-HBsAg IgG content with a majority of antibodies from Th2 type was observed whereas, in cellular immune response, no significant differences were shown for the secretion IFN-γ and IL-4 (Borges *et al.*, 2008).

2.5 Chitosan Drug/Antigen Delivery System

Up to now, the treatment of allergen (or antigen) delivery system by using nanoparticle as a depot and adjuvant is a major challenge to prevent the disease (Peek *et al.*, 2008). Also in case of chitosan (Chew *et al.*, 2003; Behera *et al.*, 2011; Bal *et al.*, 2012), Vila et al. prepared the chitosan nanoparticles loaded tetanus toxoid (TT), a model antigen, via ionotropic gelation of chitosan with tripolyphosphate anions in acetic acid solution. They found that TT-loaded nanoparticles could across the nasal epithelia and could elicit an increasing and long lasting humoral immune response (indicated by IgG concentration)(Vila et al., 2004). Furthermore, the crosslinking of the antigen, ovalbumin, to TMC via disulfide bond could improve immunogenicity (Slütter *et al.*, 2010).

2.6 Nanoparticle for Delivery System

Particle size is an important property which affects the ability of delivery system. Although the suitable size range have not been seriously limited, several researchers try to produce particle size less than 1 μm which could possibly diffuse to the blood-brain barrier (Yang and Hon, 2009). Some report suggested that if the size of particles was higher than 250-300 nm, most will be filtered by the red pulp of the spleen and phagocytosed within the cells of reticuloendothelial system (Scholes *et al.*, 1993). Another report exhibited the nanoparticle size in the range of 100-200 nm which was able to enter the nasal epithelium by transcellular pathway (Csaba *et al.*, 2006). Moreover, nanoparticle size in the range of 40-50 nm has been found as a optimum range to both cell- and antibody mediated responses (Fifis et al., 2004). For nanomaterization, one of the important approaches is self assembly of hydrophobic (core) and hydrophilic (corona) structures (Yoksan *et al.*, 2003; Matsusaki *et al.*, 2006; Koo *et al.*, 2012).

2.7 Phenylalanine

In the past, phenylalanine (Phe) was grafted with chitosan (Yoksan and Akashi, 2009) and poly(amido amine) (HPAMAM) (Wang *et al.*, 2009) by conjugating/coupling reaction for complex formation with negatively charge molecule, DNA, and potential release for gene delivery. Additionally, transfection efficiency, cytotoxicity (Kono *et al.*, 2004), and transmembrane activity (translocation through phospholipids membranes) (Unterreitmeier *et al.*, 2007) were improved after modification of Phe residue. In some cases, phenylalanine was used to strengthen the hydrophobicity of the polypeptide in chitosan grafted polypeptide-based thermogelling system (Kang *et al.*, 2012).

2.8 Polyethylene Glycol

For nanomaterization, one of the important approaches is self assembly of hydrophobic (core) and hydrophilic (corona) structures (Yoksan *et al.*, 2003;

Matsusaki *et al.*, 2006; Koo *et al.*, 2012). Normally, polyethylene glycol (PEG) is a good candidate as the hydrophilic function. It provided particle stability (Prego *et al.*, 2006) and solubility (Sheng et al., 2009) in water. Moreover, PEG can prolong blood circulation time by reducing the positive charge of chitosan which can attach with negative charge of blood and lead to hemolysis (Fang *et al.*, 2006). In addition, PEGylation of chitosan would reduce its toxicity toward the nasal mucosa while maintaining its ability to open the cellular tight junctions and enhance the particle permeability (Casettari *et al.*, 2010).

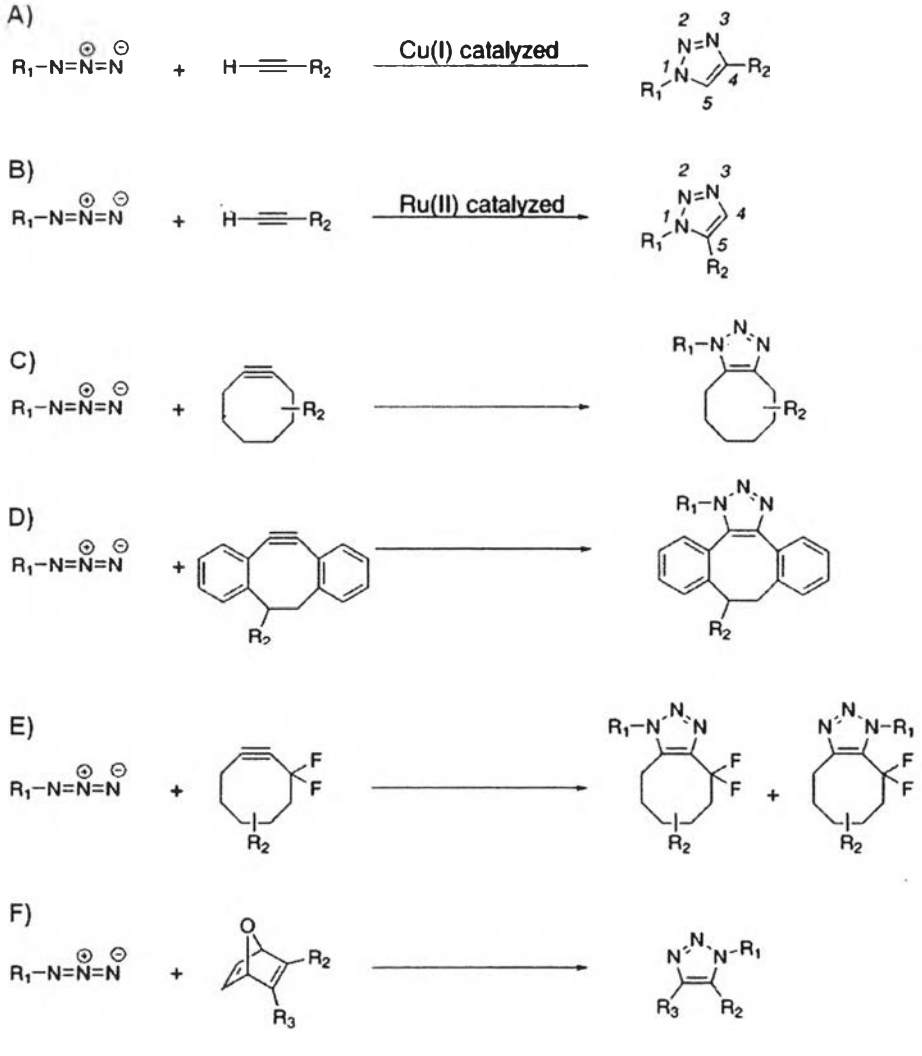
2.9 Click Chemistry

In 2001, Sharpless (Kolb *et al.*, 2001; Kolb *et al.*, 2001) and Medal(Tornøe *et al.*, 2002) independently proposed the concept of "click chemistry" which provided nearly perfect reactions by the Cu(I)-catalyzed 1,3-dipolar cycloaddition. In order to exhibit many advantages, e. g. (i) high yield, harmless side products, mild condition, the click reaction has been wildly applied in many synthesis fields, especially biological field with macromolecules. There are several well-known reactions that merge with the click chemistry concept, including the Diels-Alder reaction, the thiol-ene coupling, Staudinger ligation, native chemical ligation, the amidation reation between thio acids and sulfonyl azides (sulfo-click), and copper(I) catalyzed alkyne-azide cycloaddition (CuAAC).

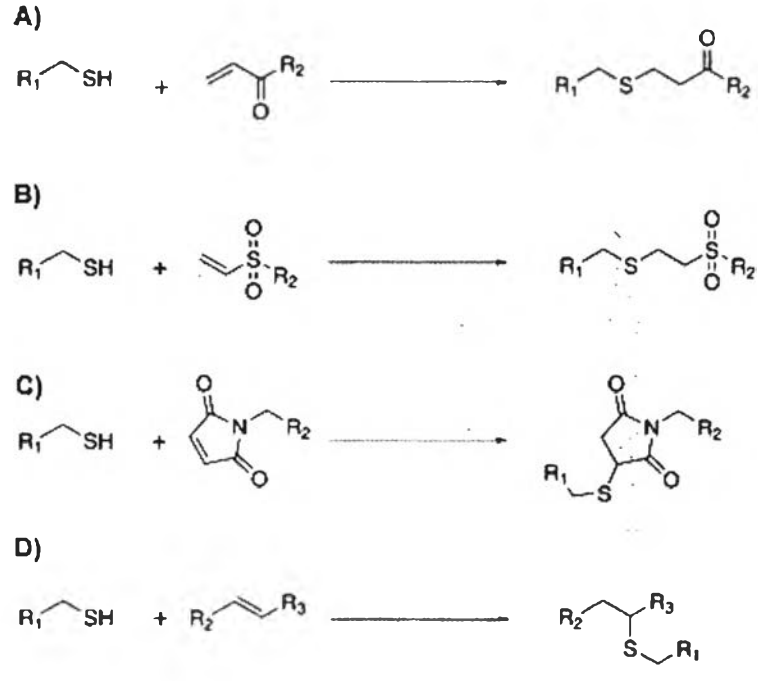
The CuAAC is presently popular due to the fast reaction even in ambient reaction condition, and very selective reaction. The cycloaddition is similar to the classical Huisgen cycloaddition reaction except the faster reaction and main forming of 1, 4-dipolar cycloaddition. However, this still shows the drawback of Cu contamination which is difficult to remove and is a problem to apply in biomedical field. Thus, the ring strain is used instead of alkyne. Bertozzi et al. (Agard *et al.*, 2004) demonstrated the [3+2] cycloaddition of azides and cyclooctyne derivatives but it has lower reactivity toward azides and slower rate reaction comparing to the CuAAC. Then, cyclooctyne was introduced by several 4-dibenzocyclooctynols to improve the reactivity. However, the cyclooctyne derivative was insoluble in water. This was developed again by Bertozzi and co-workers. They synthesized a second

generation of difluorinated cyclooctynes which showed similar reaction kinetics as CuAAC. Unfortunately, these molecules are difficult to prepare. Lastly, Rutjes et al. used a [3+2] cycloaddition-retro-Diels-Alder ligation method in which trifluoromethyl-substituted oxanorbornadiene derivatives react with azido-molecule to form a triazole linkage (scheme 2.3).

Scheme 2.3 (A) Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction, (B) ruthenium(II) –catalyzed cycleaddition, (C) ring strain promoted cycloaddition, (D) ring strain promoted cycloaddition with 4-dibenzocyclooctynol, (E) second generation of ring strain promoted cycloadditon, (F) the tandem [3+2] cycloaddition-retro-Diels-Alder ligation method. (van Dijk *et al.*, 2009).



Scheme 2.4 (A) Michael addition between thiols and acrylates, (B) thiols and vinyl sulfones, (C) thiols and maleimides, (D) thiols and alkene (R_2 and R_3 have to be electron withdrawing groups) (Dondoni, 2008).



Another reaction is also called as Click reaction which is the Michael addition (Thiol-ene or Thiol-Click) (Gress *et al.*, 2007) between thiols and acrylates, acrylamide, vinyl sulfones. This reaction gives a high efficiency and orthogonality to provide various types of functional groups. However, it has a limitation of sensitivity of the free thiol toward oxidation into a disulfide (scheme 2.4).

2.10 Oxanorbornadiene for Click Chemistry

In 2007, oxanorbornadiene was firstly synthesized to use instead of cyclooctyne in Cu-free click chemistry. Rutjes et al. produced this compound by the combination of ring strain and eletrondeficiency (at double bond ring) occurring from oxa-bridged bicyclic systems (van Berkel *et al.*, 2007). It was found that the reaction of oxanorbornadiene was approximately fivefold higher than those of the

aliphatic alkynes. These oxanorbornadiene have successfully attached on macromolecules, i.e. polyethylene glycol, polysialic acid, and alginate. Oxanorbornadiene functionalized with short polyethylene glycol was further conjugated with azido-cyclo-(Arg-Gly-Asp-D-Phe-Orn) and showed favourable hydrophilicity to apply in pharmaceutical application (van Berkel *et al.*, 2008). Another report showed the 25 % oxanorbornadiene derivative derivatization on polysialic acid by conjugating reaction and using oxanorbornadine derivative as a nucleophilic molecule (Su *et al.*, 2010). The lasted work by Krause et al. reported the modified polysaccharide alginate with RGD-pentapeptides by this Cu-free click. The oxanorbornadiene-alginate was synthesized prior to form cycloaddition with azido-based RGD-pentapeptides (Krause *et al.*, 2012).

2.11 Derivatization of Chitosan/Polymer via Cu-free Click Chemistry

The use of Cu-free click chemistry mostly is in the biomedical/biological field by using bioorthogonal strain-promoted azide-alkyne cycloaddition (SPAAC). Lallana et al. reported the efficiency of SPAAC for functionalizing biomolecules under physiological conditions. Azide-substituted chitosan-g-PEG nanostructure was coupled with cyclooctyne-functionalized immunoglobulin G (IgG) to obtain immune nano particle (Lallana *et al.*, 2009). Another report showed a new in vivo active targeting strategy for nanoparticles in living cell. First, azido- unnatural sialic acid which is targetable glycans, were artificially generated in target cancer cells by an intratumoral injection of the precursor, tetraacetylated N-azidoacetyl-d-manosamine (Ac_4ManNAz). Second,. dibenzyl cyclooctyne was introduced on nano-sized PEGylated liposomes then it was injected into-bearing mice to bind with azido-unnatural sialic acid on the target cancer cell surface via Cu-free click chemistry (Koo *et al.*, 2012).

2.12 Gold Nanoparticles

In 1857, Faraday who is the first one has successfully synthesized gold nanoparticles in colloidal solution and described the relations of gold to light. This

discovered phenomenon inspired the motivated researches over the world now. In general, gold nanoparticles are in the form of solution with various sizes and colors.

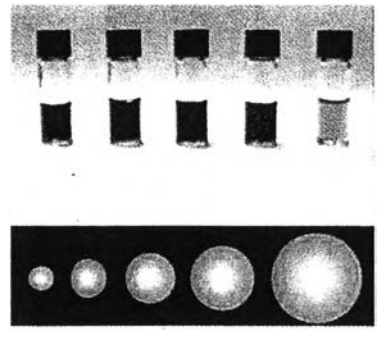


Figure 2.1 Solution of gold nanoparticles at various sizes.

2.13 Synthesis of Gold Nanoparticles

Various types of AuNPs preparations have been reported. Mainly, the preparation can be categorized in two main types. First type is "bottom-up" (chemical transformation) and another type is "top-down" (physical manipulation) (Saha *et al.*, 2012).

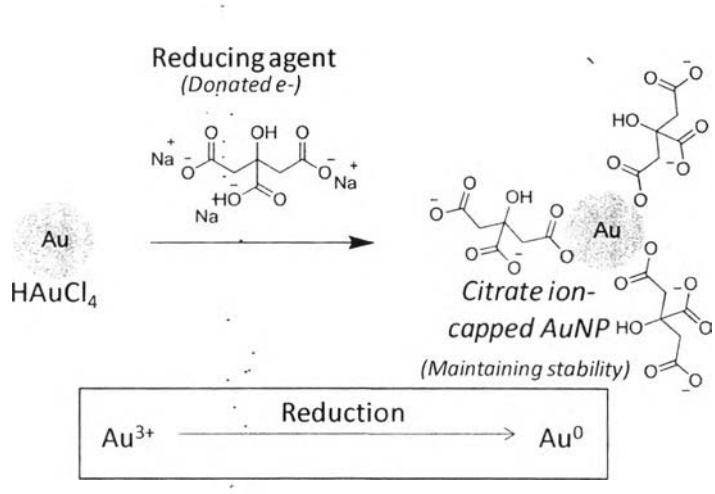
2.13.1 Chemical Transformation

One of the most popular approaches is using citrate reduction of $HAuCl_4$ in water firstly reported by Turkevich in 1951. In his work, citric acid can act as reducing and stabilizing agent to provide AuNPs with the diameter of 20 nm. In Further studies, the sodium citrate was used instead of citric acid enabling to control AuNP size by varying feed ratio of $HAuCl_4$ to sodium citrate and the kinetic of AuNPs was studied to the reason of AuNPs clustered formation (Kimling *et al.*, 2006).

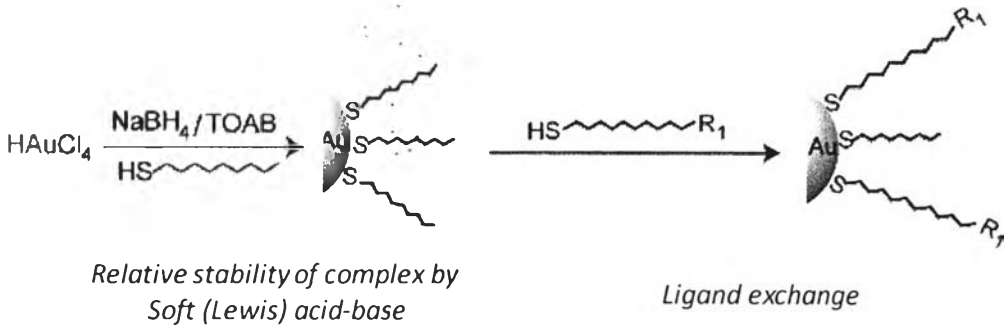
Another well-known approach is Brust-Schiffrin Method for thiol-protected AuNP which was reported by Brust and Schiffrin in 1994 (Brust *et al.*, 1994). They proposed the strong thiol-gold interactions produced by two-phase synthesis. The $AuCl_4$ was transferred from aqueous phase to toluene using the

surfactant tetraoctylammonium bromide (TOAB) and reduced by sodium borohydride ($NaBH_4$) in the presence of dodecanethiol. The thiol-protected AuNPs showed the high stability with controlled diameters in the range 1.5-5 nm. This AuNPs can be further functionalized by ligand exchange (Scheme 2.6) of different types of thiol ligands (Ingram *et al.*, 1997).

Scheme 2.5 Synthesis of gold nanoparticles by citrate-capped AuNP.



Scheme 2.6 Synthesis of gold nanoparticles by Au-S coordination.



2.13.2 Physical Manipulation

The physical methods for manipulating gold particles, mostly, are thermolysis, digestive ripening, laser irradiation of colloids and laser ablation of solid in liquid.

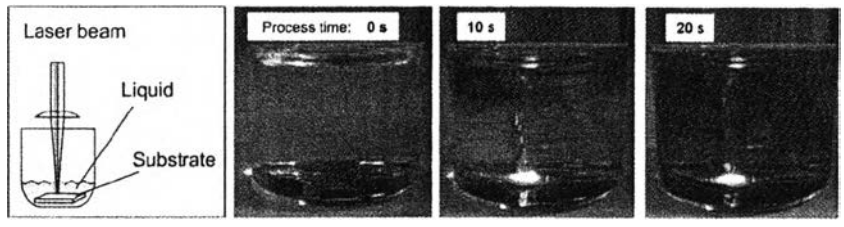
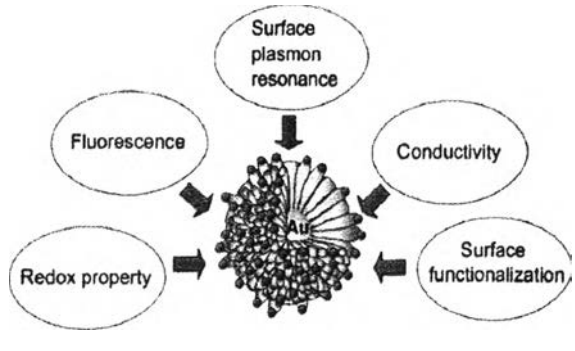


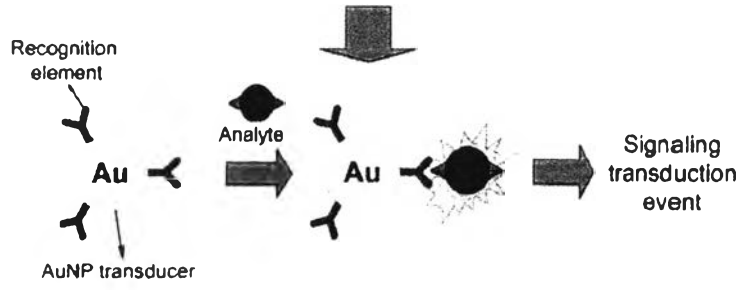
Figure 2.2 Gold nanoparticles by laser ablation.

In case of Laser ablation, recently, it has been shown the higher advantages compared to the conventional synthesis, e.g., (i) the higher possibility of biomolecule conjugation on the surface of gold, ligand-free gold nanoparticle surface, (ii) decrease the amount and cost of the reducing and stabilizing agents, (iii) lower noise of characterization, i.e., surface-enhanced Raman and (iv) ablation in polymer solution allows the easiness of embedding on the surface of gold (Barcikowski and Mafuné, 2011). For the detail of AuNP surface, it was demonstrated that the nanoparticles were partially oxidized by the oxygen present in solution. The hydroxylation of the Au-O compounds, followed by a proton loss to give surface Au-O^-, resulted in the negative charging of the nanoparticles leading to enhance the stability (Sylvestre *et al.*, 2004).

2.14 Physical Properties of Gold Nanoparticles

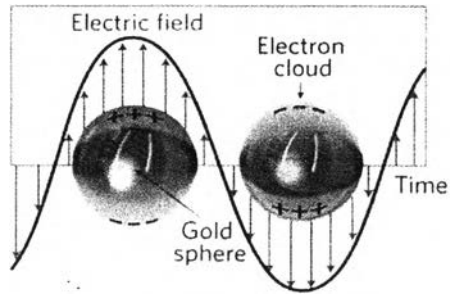
Scheme 2.7 Physical properties of AuNPS and the concept of AuNP-based detection system (Saha *et al.*, 2012).





2.14.1 Theory of Surface Plasmon Resonance (SPR)

Scheme 2.8 Resonance of gold nanoparticles and electric field of light.



When the polarized light from the electric field which is perpendicular to wave's direction (magnetic field was not considered) is exposed to the surface of gold nanoparticles at a specific angel, the light will be reflected by the gold nanoparticles. Photons (energy or electromagnetic force of light) of p-polarized light can interact with the electrons cloud of the AuNPs resulting in inducing a wave-like oscillation of the electron cloud and reducing the reflection. This phenomenon is called Surface Plasmon Resonance (SPR). The main factor related to the wavelength changing of SPR is the size of particles as shown in the Mie theory equation below.

$$E(\lambda) = \frac{24\pi N_A R^3 \varepsilon^{3/2} m}{\lambda ln10} a_0 \left[\frac{\varepsilon_i(\lambda)}{\{\varepsilon_r(\lambda) + \varepsilon_m\}^2 + \varepsilon_i^2(\lambda)}\right]$$

Where, N_A Avogadro number

R The radius of the spherical nanoparticle

ε_m Dielectric constant of the surrounding medium

λ The wavelength of the light

ε_r The real parts of the dielectric function of the metallic

ε_i The imaginary parts of the dielectric function of the metallic

2.15 Visual Detection

Visible indication based on the aggregation of gold nanoparticles is highly advantageous for rapid detection of biological entities, which even untrained persons can perform without specialized instrumentation (Lim *et al.*, 2012).

2.15.1 Development of Analyte Detection

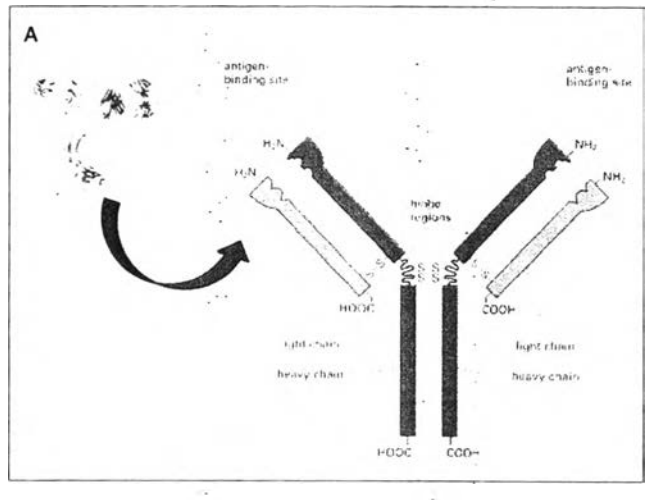
Nowadays, the development of detection is focused on the requirements of (i) fast detection, (ii) ease of use, (iii) high sensitivity, and (iv) economical cost. The crosslink is a one of the important strategies to induce the aggregation of gold lead to fast signal to detect. Especially, if the cross linker is designed from the analytes. Zhenyu *et al.* reported the visual detection of copper(II) based molecule on the aggregation of gold nanoparticles by click chemisty. They coated the 1-azidoundecan-11-thiol and alkyne-tagged molecules on the surface of gold. Once the Cu analyte was added, the triazole from the part of azido- and alkano-moieties is formed resulting in the color change from aggregation (Lin *et al.*, 2012).

Whereas the another way round is also focused on the method of crosslinked cleavage. In order to detect the thiol-based molecules, i.e., cysteine, glutathione, and cysteinylglycine which can break the disulfide bond, the gold nanoparticles were firstly prepared in the state of aggregation by using glutathione disulfide as a crosslinker before. When the analytes were exposed in the presence of modified gold nanoparticles, the individual gold nanoparticles would reversely form again. Then the color change from purple to red pink is detected (Durocher *et al.*, 2009).

2.15.2 Antigen Detection by Antibody-conjugated AuNP

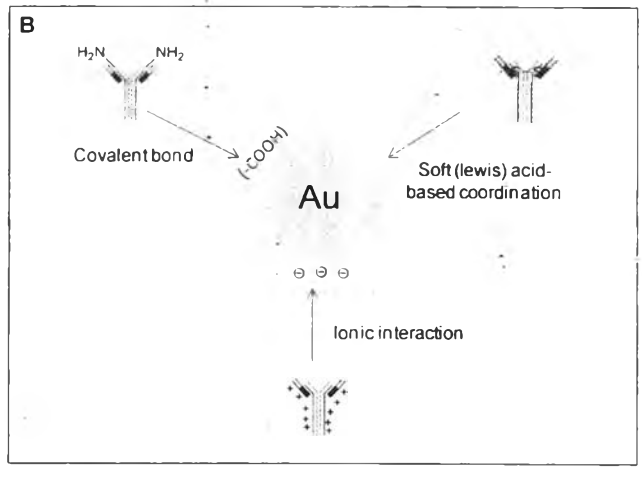
Before applying the gold nanoparticles to the detection, the particles have been prepared the surface firstly. The bifunctional ligands of the type X-R-SH (X= -COOH, -OH, $-NH_2$) were mostly exposed to the surface depending on the binded further molecules as shown in scheme 2.9.

Scheme 2.9 (A) Structure of antibody consisting of various types of functional groups and (B) interactions between AuNP and antibody.

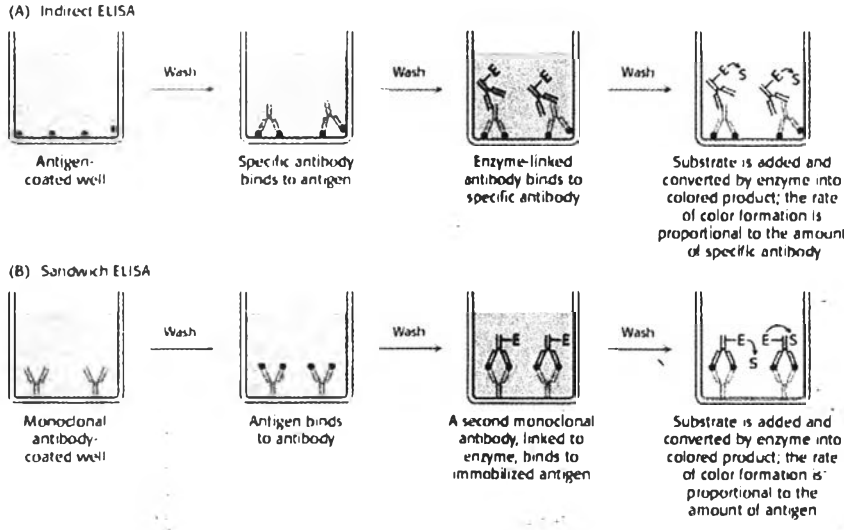


http://nfs.unipv.it/nfs/minf/dispense/immunology/lectures/files/structure_abs_tcr.html

http://captain-nitrogen.tumblr.com/post/17850004239/the-story-of-the-immune-system-warfare-at-the



Scheme 2.10 Type and procedure of ELISA.



http://exploreable.wordpress.com

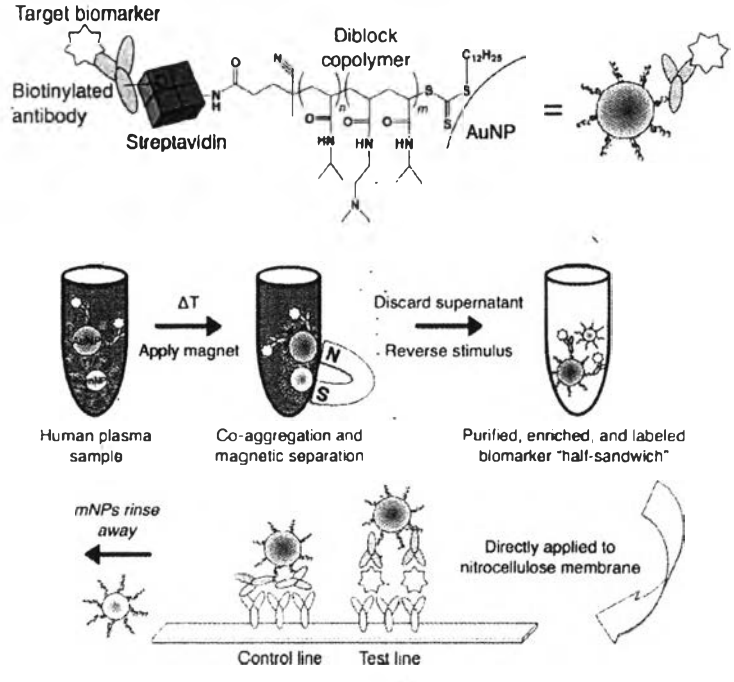
ELISA (Enzyme Linked Immunosorbent Assay) is widely used and is available in the market for antigen detection/diagnosis (scheme 2.10). However, the accuracy, rapid detection, and high sensitivity are still developed.. One of developments is increase the amplification of optical signal by applying gold nanoparticle which used as carriers of the signaling antibody anti-CA15-3-HRP (horseradish peroxidase) (Ambrosi *et al.*, 2009).

2.15.3 Polymer and/or Chitosan for Visual Detection

In recent year, not only the citrate based molecule but also polymeric-based compounds, e.g., sialic based polymer, chitosan derivative, gelatine, alginate, and polyethylenimine (PEI) have been reported as the stabilizers. For multi-proposed application, many researchers have an attempt to apply the polymers to functionalize with AuNP.

Sialic based polymer was coated on the surface of gold and showed the stabilized gold nanoparticles ~ 20 nm. It also showed the recognized property of influenzavirus (Lee *et al.*, 2013). The report of Huang et al. showed the properties of chitosan which could stabilized AuNP (same as that of glucose) and exhibited the various shape as polygonal and size of AuNP (Huang and Yang, 2004).

Scheme 2.11 Gold nanoparitcle modified with responsive polymer linked with biotin-lated antibody and the strategy of developed lateral flow immunoassay (Nash *et al.*, 2012).



The Integration of magnetite, gold nanoparticle, and polymer functions could provide the advance biomarker detection. Nash *et al.* (Nash *et al.*, 2012) proposed the strategy of rapid diagnosis and antigen purification as shown in scheme 2.11. A linear diblock copolymer with a thermally responsive poly(N-isopropylacrylamide) (pNIPAm) segment and a gold-binding block composed of NIPAm-co-N,N-dimethylaminoethyl acrylamide was prepared by reversible addition fragmentation chain transfer (RAFT) polymerization. The diblock copolymer was used to functionalize gold nanoparticles (AuNPs), with subsequent bioconjugation to yield thermal responsive polymer-AuNPs which were co-decorated with streptavidin. These AuNPs could complex with biotinylated capture antibodys which were bound to plasmodium falciparum histidine- rich protein. The gold-labeled biomarker was then purified and mixed with magnetic nanoparticles which were decorated with pNIPAAm. When a thermal stimulus was applied at the presence of magnetic field,

coaggregation of the AuNP half-sandwiches with the polymer coated iron oxide nanoparticles was created and separated from bulk serum.