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LEPTOSPIROSIS DNA DETECTION BY GOLD AND SILVER NANOPARTICLES

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ในปัจจุบัน อนุภาคทองและเงินนาโน ที่ได้รับการปรับแต่งผิวหน้า ได้ถูกประยุกต์ใช้เป็นเครื่องมือตรวจหาดีเอ็นเอด้วยการเปลี่ยนแปลงสี ประสิทธิภาพในการยึดเกาะของสารดีเอ็นเอสายเดี่ยวและดีเอ็นเอสายคู่บนผิวหน้าของอนุภาคโลหะนาโนนี้จะแตกต่างกัน โดยใช้โรคฉี่หนูเป็นแบบจำลอง ได้ออกแบบการตรวจหาดีเอ็นเอโดยอาศัยหลักการจับยึดของดีเอ็นเอ ร่วมกับสมบัติการรวมกลุ่มและการออกซิเดชันของอนุภาคทองและเงินนาโน ดีเอ็นเอสายเดี่ยวของเชื้อโรคฉี่หนูป้องกันไม่ให้อนุภาคทองและเงินนาโนรวมตัวกันจากการเหนี่ยวนำของสารละลายเกลือ ในทางตรงข้ามหลังจากการไฮบริดกับดีเอ็นเอสายทดสอบจะพบการรวมตัวกันของอนุภาคทองและเงินนาโนได้ จากการเปลี่ยนแปลงของสีของอนุภาคโลหะนาโนนี้ ในกระบวนการออกซิเดชัน ดีเอ็นเอสายคู่ระหว่างเชื้อโรคฉี่หนูกับดีเอ็นเอสายทดสอบจะป้องกันการเกิดออกซิเดชันได้ ในทางตรงข้ามพบว่าสีเหลืองของอนุภาคเงินนาโนจะถูกฟอกจางจากการออกซิไดซ์ด้วย H_2O_2 ในสารผสมของดีเอ็นเอสายคู่กับอนุภาคเงินนาโน สามารถคำนวนค่าการเปลี่ยนแปลงสีของสารละลายได้จากสเปกตรัมการตรวจวัดการดูดกลืนแสงของอนุภาคนาโน

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At present, surface modified gold and silver nanoparticles are widely applied as colorimetric DNA detectors. Binding affinity of ssDNA and dsDNA onto the metal nanoparticles are different. Using *Leptospira interogans* as a model, a novel DNA detection was designed based on the binding affinity of DNA in corporation with aggregation and oxidation properties of gold and silver nanoparticles. The ssDNA of *Leptospira interogans* prevented aggregation of silver and gold nanoparticles conducted by saline solution. On the other hand, after hybridisation with the probe DNA, aggregation of gold and silver nanoparticles can be observed as the change of colour of the metal nanoparticles. In oxidation process, dsDNA of probe and target *Leptospira interogans* DNA prevent oxidation of silver nanoparticles. In contrast, the bleaching of the yellow colour of silver nanoparticles by H_2O_2 oxidation can be observed in ssDNA/silver nanoparticles mixture. The colour changing of solutions can be calculated from light absorption spectra of nanoparticles.

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CONTENTS

Page

ABSTRACT (THAI)…… iv

ABSTRACT (ENGLISH)…… v

ACKNOWLEDGEMENTS……..… vi

CONTENTS……....…… vii

LIST OF TABLES……... x

LIST OF FIGURES…….……..... xi

LIST OF ABBREVIATIONS……..... xiii

CHAPTER I INTRODUCTION……...…….…... 1

1.1 Nanotechnology……. 1

1.2 Leptospirosis……... 1

1.3 Objectives of this research…….. 3

1.4 Scope of this research……. 4

CHAPTER II THEORY AND LITERATURE REVIEWS…….. 5

2.1 Nanotechnology……..……..... 5

2.2 Nanomaterial…..…… 7

2.2.1 Gold nanoparticles…….. 10

2.2.1.1 Synthesis of Gold nanoparticles…….. 11

2.2.1.2 Application of Gold nanoparticles…….. 12

2.2.2 Silver nanoparticles……..... 16

2.2.2.1 Synthesis of Silver nanoparticles……. 17

2.2.2.2 Application of Silver nanoparticles……. 20

2.3 Leptospirosis……..... 24

CHAPTER III MATERIALS AND METHODS……..……... 26

3.1 Chemicals ……...…. 26

3.2 Equipment and analytical instruments……. 27

3.3 Experimental procedure…… 27

3.3.1 Synthesis and characterization of metal nanoparticles….. 27

3.3.1.1 Synthesis of citrate-stabilized gold nanoparticles…….. 27

Page

3.3.1.2 Characterization of metal nanoparticles……. 28
3.3.2 Preparation of DNAs……. 28
3.3.3 Aggregation of gold nanoparticles……. 30
3.3.3.1 Effect of buffer on aggregation…….. 30
3.3.3.2 Effect of salt on aggregation……... 30
3.3.3.3 Effect of DNA on aggregation…… 31
3.3.3.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA …….. 31
3.3.4 Aggregation of silver nanoparticles……. 31
3.3.4.1 Effect of buffer on aggregation…… 31
3.3.4.2 Effect of salt on aggregation…… 32
3.3.4.3 Effect of DNA on aggregation……. 32
3.3.4.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA …….. 32
3.3.5 Oxidation of silver nanoparticles…… 33
3.3.5.1 Effect of silver nanoparticles concentration on oxidation…… 33
3.3.5.2 Effect of temperature on oxidation……. 33
3.3.5.3 Effect of hydrogen peroxide on oxidation ……. 33
3.3.5.4 Effect of DNA on oxidation …….. 34
3.3.5.5 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA ……. 34
CHAPTER IV RESULT AND DISCUSSION……...……. 35
4.1 Characterization of metal nanoparticles…… 35
4.2 Aggregation of metal nanoparticles…….. 37
4.2.1 Effect of buffer on aggregation……. 37
4.2.2 Effect of salt on aggregation……. 39
4.2.3 Effect of DNA on aggregation…….. 41

Page

4.2.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA …….. 47
4.2.5 Comparison of sensitivity between gold and silver nanoparticles……..……. 50
4.3 Oxidation of silver nanoparticles……... 51
4.3.1 Effect of silver nanoparticles concentration on oxidation.. 51
4.3.2 Effect of temperature on oxidation…… 53
4.3.3 Effect of hydrogen peroxide on oxidation …… 54
4.3.4 Effect of DNA on oxidation ……. 56
4.3.5 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA …….. 59
4.4 Comparison of sensitivity between aggregation and oxidation of silver nanoparticles…….. 60
CHAPTER V. CONCLUSION……..……...……. 61
5.1 Conclusion…… 61
5.2 Future Perspective……... 61
REFERENCES……..... 62
APPENDICES……..... 70
APPENDIX A……..... 71
APPENDIX B……..... 72
BIOGRAPHY……..... 73

LIST OF TABLES

Table		Page
2-1	Mechanisms of surface interaction between particles in liquid phase…	9
2-2	Principle of silver nanoparticles based colorimetric DNA detection….	24
3-1	Oligonucleotide sequences……..	29
3-2	Ingredient of DNA by volume……	30
4-1	CR of gold and silver nanoparticles stabilized by mixture3-5……..	51
4-2	CR of aggregation and oxidation method of silver nanoparticles……..	60

LISTS OF FIGURES

Figure		Page
1-1	Electron microscope image of Leptospira interrogans……	2
1-2	Reported cases of Leptospirosis per 100,000 populations by province, Thailand, 2008…….	2
2-1	Progress of top-down and bottom-up approaches……...	5
2-2	Example types of nanomaterials……..	7
2-3	The diameter of gold nanoparticles determines the wavelengths of light absorbed……...	10
2-4	Synthesis of AuNPs in organic solvent……	11
2-5	Synthesis of AuNPs in aqueous using Sodium 3-mercaptopropionate as stabilizer……...	12
2-6	The colour changing of AuNPs from red to blue before (A) and after (B) hybridization……..	13
2-7	Representation of Structure and Color Change of Nanoassembly in the Presence of Triplex Binder at Room Temperature…….	14
2-8	Desorption of a nonhybridized sample (a-e) and nondesorption of a hybridized sample (f-j) after addition of dextran sulfate at successive time intervals between 0 and 20 s…….....	15
2-9	The mixtures of gold nanoparticles solution and positive and negative urine samples compare with commercial test kit…….	15
2-10	The color of different size of silver colloids……	16
2-11	Atomic force microscopy (AFM) images of DNA network and synthesized nanoparticles…….	18
2-12	TEM images of the silver nanomaterial……...	19
2-13	Process of the Ag/PEG-NPs synthesis at room temperature……...	20
2-14	Antibacterial activity and mechanism of silver nanoparticles…….	21
2-15	Illustration of the reversible hybridization of two complementary DNA-silver nanoparticles due to temperature…….	22
2-16	Enzymatic reactions with CIAP (A) and PKA (B), and silver nanoparticles-based enzyme colorimetric assay (C)……	23

Figure **Page**

2-17 Microscopy of leptospiral in microscopic agglutination test……..... 25
4-1 The characterization of gold nanoparticles using spectrophotometer…….. 35
4-2 The characterization of silver nanoparticles using spectrophotometer…… 36
4-3 The characterization of metal nanoparticles using TEM……. 37
4-4 The effect of different amount of buffer to the aggregation of AuNPs..... 38
4-5 The effect of different amount of buffer to the aggregation of AgNPs..... 38
4-6 The effect of different amount of NaCl to the aggregation of AuNPs..... 40
4-7 The effect of different amount of NaCl to the aggregation of AgNPs..... 40
4-8 The effect of different amount of mixture1 to the aggregation of AuNPs... 42
4-9 The effect of different amount of mixture2 to the aggregation of AuNPs... 42
4-10 The effect of different amount of mixture1 to the aggregation of AgNPs... 43
4-11 The effect of different amount of mixture2 to the aggregation of AgNPs... 43
4-12 The effect of different amount of mixture3 to the aggregation of AuNPs... 45
4-13 The effect of different amount of mixture3 to the aggregation of AgNPs... 45
4-14 Comparison of effect of mixture1-3 on the aggregation of AuNPs……. 46
4-15 Comparison of effect of mixture1-3 on the aggregation of AgNPs……. 47
4-16 Comparison between non-complementary DNA (mixture4) and complementary DNA (mixture3) stabilized AuNPs…… 48
4-17 Comparison between non-complementary DNA (mixture4) and complementary DNA (mixture3) stabilized AgNPs…… 48
4-18 Comparison between 1-mismatched complementary DNA (mixture5) and complementary DNA (mixture3) stabilized AuNPs…… 49
4-19 Comparison between 1-mismatched complementary DNA (mixture5) and complementary DNA (mixture3) stabilized AgNPs…… 50
4-20 Colorimetric detection of oxidized AgNPs……. 53
4-21 Effect of temperature to the oxidization of AgNPs……. 54
4-22 Effect of hydrogen peroxide to the oxidization of AgNPs……..... 55
4-23 Oxidation of AgNPs stabilized by mixture1…… 56
4-24 Oxidation of AgNPs stabilized by mixture2…… 57
4-25 Oxidation of AgNPs stabilized by mixture3…… 58
4-26 Comparison of effect of mixture1-3 to the oxidation of AgNPs…….. 58
4-27 Comparison of mixture3, mixture4 and mixture5 stabilized AgNPs…….. 59

LIST OF ABBREVIATIONS

%	Percentage
ε	Molar absorptivity
λ_{max}	MaximunWavelength
°C	Degree Celsius
Δ	Delta
AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles
CH_3NO	Formamide
DNA	Deoxyrironucleic acid
dsDNA	Double stranded DNA
g	Gram
MAT	Microscopic Agglutination Test
mM	Millimolar
MNPs	Metal nanoparticles
mL	Milliliter
Mw	Molecular weight
N_A	Avogadro number
NaCl	Sodium Chloride
nm	Nanometer
PCR	Polymerase chain reaction
ppm	Parts per million
R	Radius
SSC buffer	Saline Sodium Citrate buffer
ssDNA	Single stranded DNA
µL	Microliter
Uv-vis spectroscopy	Ultraviolet and Visible spectroscopy

CHAPTER I

INTRODUCTION

1.1 Nanotechnology

Nanotechnology is the study at the atomic, molecular, and supramolecular levels on a scale of ~1–100 nm. Nanotechnology creates various new functional materials with better coatings, better fluidic flow, and a host of other improvements to everyday components due to fundamentally new properties and functions resulting from their small structure. Thus, this technology manufactures goods and systems with a vast range of applications, such as in medicine, electronics and energy production.

Nanomaterials exhibit novel properties such as unique mechanical, optical, chemical, electrical and magnetic properties (1,2) which are directly depended on their size and structure. Widely-used nanomaterial structures are nanoparticles, nanorods, nanotubes, nanowires, nanofilms, etc.

Among various types of nanomaterial, gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) particularly have been used in many applications because of their excellent photo physical and electrical properties. They also exhibit high chemical reactivity (3,4). The rich surface area of these metal nanoparticles allows surface modification with wide varieties of biomolecules, for example; DNA (5,6), protein (7), carbohydrate (8), lipids (9), enzymes (10), drugs (11), and viruses (12). Therefore, gold and silver nanoparticles can be served as promising biotechnological tools such as biosensors, drug/gene delivery devices, and contrasting agents.

1.2 Leptospirosis

Leptospirosis is an epidemic disease in human caused by a spirochete bacterium called Leptospira interrogans which have 23 serogroups and more than 200 serovars. With the size of 0.1 micron in diameter and 6 - 20 micron in length, Leptospira interrogans can be seen by dark-field or fluorescence microscope.

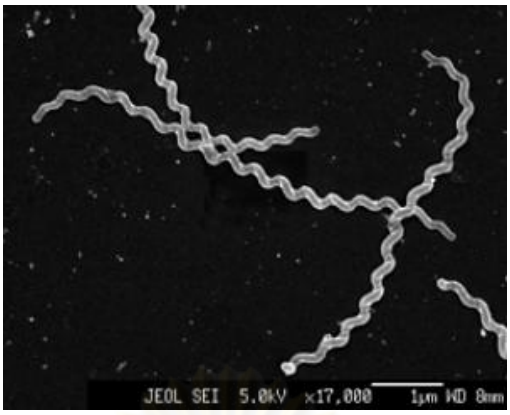


Figure 1-1 Electron microscope image of Leptospira interrogans

Leptospirosis can be infected from animals and found all over the world occasionally through out the year. It is severely spread in tropical country especially in rainy season. For Thailand, it is widespread in October and November as the rain washes germs from environment and adds up in flooded area. People or animals that have a contact with the water can be infected. During 2006-2008 more than 11,000 adults and children became infected with Leptospirosis and 195 of those infected people died.

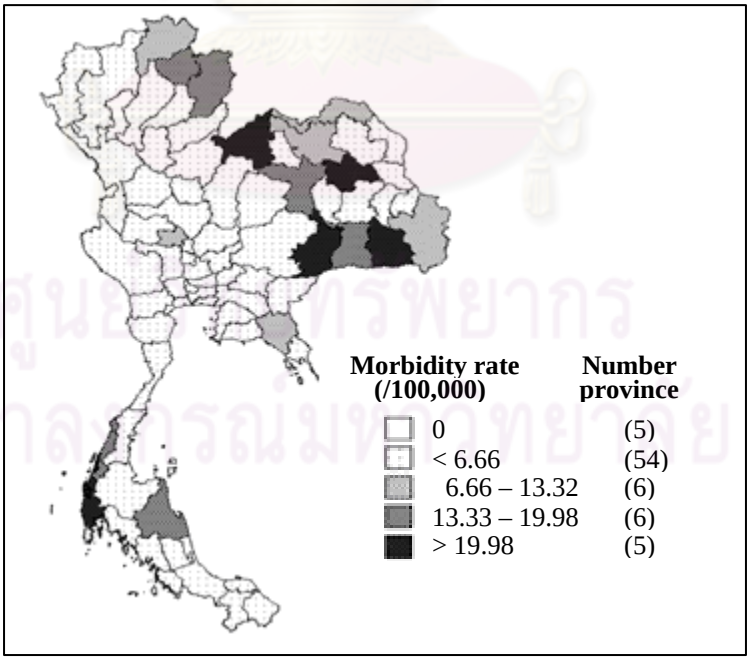


Figure 1-2 Reported cases of Leptospirosis per 100,000 populations by province, Thailand, 2008

The symptoms of Leptospirosis are similar to many other transmitted disease symptoms. It cannot be identified in a short period. The treatment should be done within 2 weeks or else the patients are risk to dead. Thus the diagnostic of Leptospirosis in laboratory is crucial. Nowadays, there are many methods for Leptospirosis detection. Each method has its advantages and drawbacks, for example, culturing this bacteria from urine samples requires a long period of infection. Whilst, and the detection of antigen is complicated, time consuming and harmful to researchers.

In present, World Health Organization (WHO) has set up a standard method for Leptospirosis detection by separating leptospira, detecting antibody by microscopic agglutination test (MAT) and detecting DNA in blood sample by polymerase chain reaction (PCR). Eventhough these method exhibit satisfied diagnostic result, but special equipments and experienced researcher are needed. Moreover, the accurate result can be detected after 5-7 days of infection in blood stream.

The quick and accurate diagnosis for the disease benefits to both patient health and economics; decrease the cost of medicine and equipments for diagnostic and treatment. Consequently, this research aims to study and develop a novel diagnosis method for Leptospirosis detection using nanotechnology. The process is to prepare short DNA probes which have a complementary base set corresponding to the infected Leptospirosis. Based on the different absorption efficiency between single-stranded and double-stranded DNA onto nanoparticles surface, nanoparticles will act like a marker which identify if there is the leptospirosis DNA or not. This technique is promising for development of fast, sensitive and economical tool for leptospirosis detection.

1.3 Objectives of this research

The objective of this research is to develop the Leptospirosis detection method using gold and silver nanoparticles.

1.4 Scope of this research

The scope of this research covers the synthesis metal nanoparticles characterized by UV-Visible spectroscopy and Transmission electron microscopy (TEM). Leptospirosis was detected by aggregation and oxidation method of metal nanoparticles. In addition, the effect of salt and DNA concentration on aggregation and oxidation method were studied.

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Nanotechnology

Nanotechnology is a multidisciplinary field which comprises of fundamental and applied sciences such as physics, chemistry, biology, medicine, and engineering. Nanotechnology involves creation and manipulation of materials in nanoscale level to create unique products which have novel properties. The study of nanotechnology is divided into 2 categories that is “top-down method”, these seek to create smaller particles in nanometer level starting from larger ones to direct their assembly and “bottom-up method” which using high technology to arrange smaller components (atoms or molecules) into more complex assemblies.

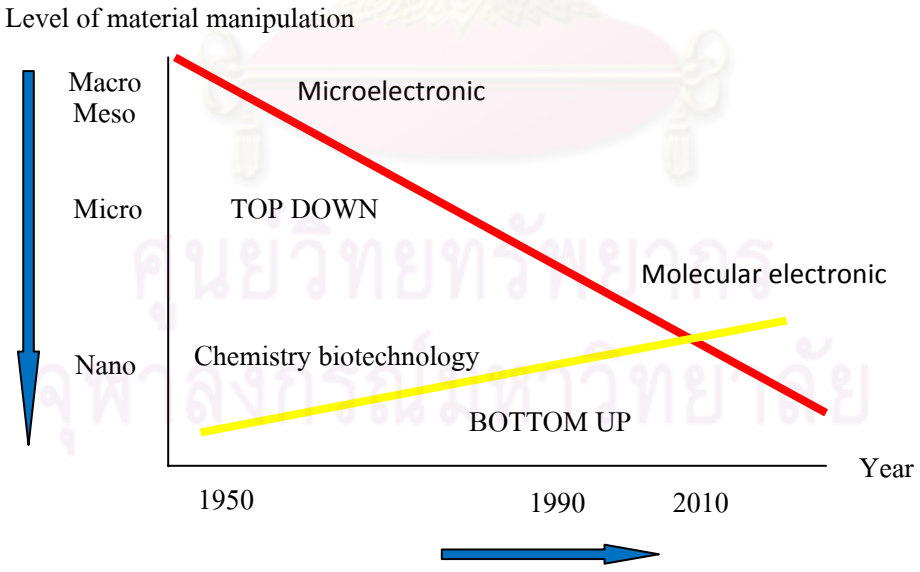


Figure 2-1 Progress of top-down and bottom-up approaches

Nowadays, nanotechnology has been applied into several applications because of their unique properties of nanomaterials. For example, nanoparticles can exhibit excellence light scattering and their light absorption is in the visible wavelength. In addition, nanomaterials which have high surface-to-volume can be applied in catalyst chemistry and nanoelectronic engineering.

In biotechnology and biomedical sciences, there is a strong dependence on having proper understanding of biochemical processes. Thus, the use of molecular self-assembly as a means to manufacture nanostructures for various nanodevices is one of the major trends, some of the nanodevices including nanoparticles, and quantum dots can be used to obtain much deeper understanding of biological processes.

Mostly applications of nanotechnology are related to health & medical areas. Typical examples include arrays (chips) for large scale DNA or protein screening. Drug delivery is another much studied example. Various self assembled peptide structures can be designed to release compounds under specific conditions. The applications of nanomaterials to biology or medicine are given below:

- Fluorescent biological labels (13)
- Drug and gene delivery (14)
- Detection of proteins (15)
- Probing of DNA structure (16)
- Tissue engineering (17)
- Biosensors (18)
- Nanobiomotors (19)
- Biomineralization (20)
- Nanorobotics (21)
- Nanocomputers (22)
- Nanorods vaccination (23)
- MRI Contrast enhancement (24)

The use of biomolecules in the energy sector has so far been rather limited. Although there are in principle possibilities for applications such as using biological light-harvesting complexes for solar energy capture, the formats are so far not very compatible. Lately there has been focused on fuel cells for using chemical redox reactions for production of electricity. Some success has been reported for such devices that include biomolecules (25) thus the method in bioelectrochemistry can be improved by nanostructured biomaterials.

2.2 Nanomaterial

Nanomaterials can be applied into metal, ceramic, polymer and composite which synthesized by controlling of matter on an atomic or molecule scale range from 1-100 nanometers. Properties of the nanomaterials are different from those of their bulk materials because of very high surface-to-volume ratio and other size-dependent properties.

Nanomaterials are classified into various groups depending on their structure and shape such as quantum dots, nanoparticles, nanowires, carbon nanotubes, nanofilms, nanocoating, nanocatalysts and nanocomposites.

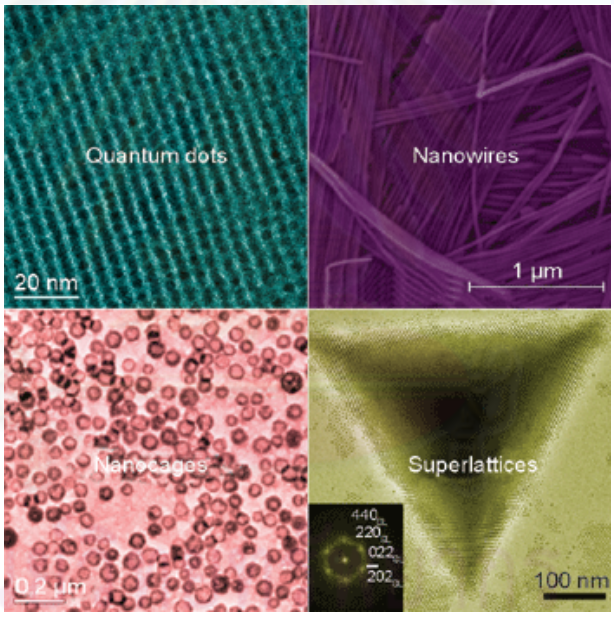


Figure 2-2 Example types of nanomaterials

One of the most nanomaterials that is widely applied in medical field is metal nanoparticles. Nanoparticles are, by definition, particles with diameters ranging from 1 to 100 nm. Metal nanoparticles are known to exist in various shapes such as spherical, triangular, cubical, pentagonal, rod-shaped, shells, and ellipsoidal. Metal nanoparticles have sizes similar to the biomolecules encountered at the cellular level. Thus, they can play great role in nanodevices that can be applied in medical and clinical applications.

When polarized light is shone onto metal nanoparticles at a specific (resonance) angel, the light will be reflected by the metal nanoparticles acting as a mirror. Photons of p-polarized light can interact with the electrons cloud of the metal nanoparticles, inducing a wave-like oscillation of the electrons cloud and thereby reducing the reflected. This phenomenon is called Surface Plasmon Resonance (SPR) (26). There are many factors that effect to wavelength of SPR phenomenon. One of the main factors is the size of nanoparticles. The wavelength can be calculated from Mie theory as shown below.

$$E(\lambda) = \frac{24\pi N_A R^3 \varepsilon^{3/2} m}{\lambda \ln 10} \left[\frac{\varepsilon_i(\lambda)}{\{\varepsilon_r(\lambda) + 2\varepsilon_m\}^2 + \varepsilon_i{}^2(\lambda)} \right]$$

When

N_A	Avogadro number
R	the radius of the spherical NP,
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

The prominent colours of many metallic nanoparticle solutions show the plasmon band in visible frequencies, unlike the bulk metals where the plasmon absorption is in the UV region which make the colour of nanoparticles different from bulk metals.

After the synthesis of nanoparticles, the dispersion and aggregation behavior of nanoparticles is generally controlled in liquid phase. The major mechanisms of surface interaction between particles in liquid phase are summarized in table 2-1.

Table 2-1 Mechanisms of surface interaction between particles in liquid phase

Surface interaction	General mechanism
van der Waals interaction	electromagnetic force between molecule and/or atom due to covalent bonds or to the electrostatic interaction of ions with one another or with neutral molecules.
Overlap of electric double layer	Electrical interaction by the overlap of electron double layer around particle in solution
Bridge force	Formation of the bridge of surfactant between particles
Hydration force	Interaction of hydrogen bond in water with hydrophilic surface on particles

The van der Waals interaction and overlapping of electric double layer is generated by the counter ions concentrated at the surface of particles by the surface charge. Low maximum value of surface potential promotes the particle aggregation. If the particles size is large, particles are able to disperse in suspension but for particle less than 100 nm, the maximum value of surface potential was too low that cause aggregation. In order to increase the maximum potential and disperse nanoparticles, anionic or cationic must be used to increase surface charge.

Thus, the colours of nanoparticles can be easily changed when nanoparticles are aroused by environment. This leads to the development of diagnosis tools particularly the modification of the surface of nanoparticles with targeted biomolecules such as DNA (27), protein (28) or carbohydrate (29).

2.2.1 Gold nanoparticles

Gold has been famous since ancient times and it was one of the main research topics for scientist since past decade. Nowadays, research paper about gold has been rapidly increased especially in nanotechnology field which concerning about gold nanoparticles (AuNPs) or gold colloid and its self-assembled monolayers, SAMs (30).

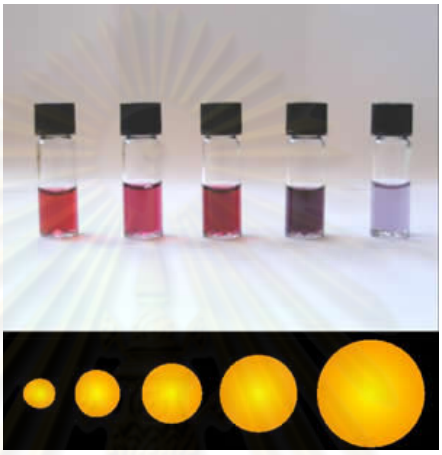


Figure 2-3 The diameter of gold nanoparticles determines the wavelengths of light absorbed.

AuNPs is one of the most stable nanoparticles among metal nanoparticles (31) and it has many interesting properties such as particles aggregation, superquenching ability in fluorescent and surface plasmon resonance. The preparations of AuNPs in both aqueous and organic solvent have been studied since 4-5 B.C. in Egypt and China. AuNPs was used for ornament or medical purpose. After that, the research about AuNPs has been done since then. In 1818, Ritchters give a reason on colour diversity in gold colloid that, it depends on the starting gold compound used for the synthesis. In 1857, Faraday presented the synthesis of AuNPs *via* a reduction process of $AuCl_4^-$ using phosphorus in CS_2 (two phase system). It has been discovered that light absorption property of prepared thin film which obtain from dehydrating AuNPs can cause reversible colour changing depends strongly on mechanic force applied (32).

2.2.1.1 Synthesis of gold nanoparticles

The synthesis of AuNPs can be operated both in aqeous and organic solution in one or two phase systems. Synthesis method requires a stabilizer which is a small organic compound or a polymer chain (33). Stabilizer will attach to AuNPs surface and prevent AuNPs from linking with each other. Thus, no aggregate or precipitate occur. These stabilizers are in equilibrium between entering and exiting from AuNPs surface (34). Consequenly, the AuNPs surface can be exchanged with other stabilizers.

In 1994, Brust reported a systhesis of AuNPs in organic solvent by the reduction of Au^{3+} with $NaBH_4$. Thiol-containing compound was used as a stabilizer. This synthesis consists of two phase system, aqeous and organic phase. The reduction of Au^{3+} went in aqeous phase, then the AuNPs was transfered to organic phase and stabilized by a thiolated organic compound. Thus, the red colour AuNPs synthesized in this method was suspended in the organic phase (35).

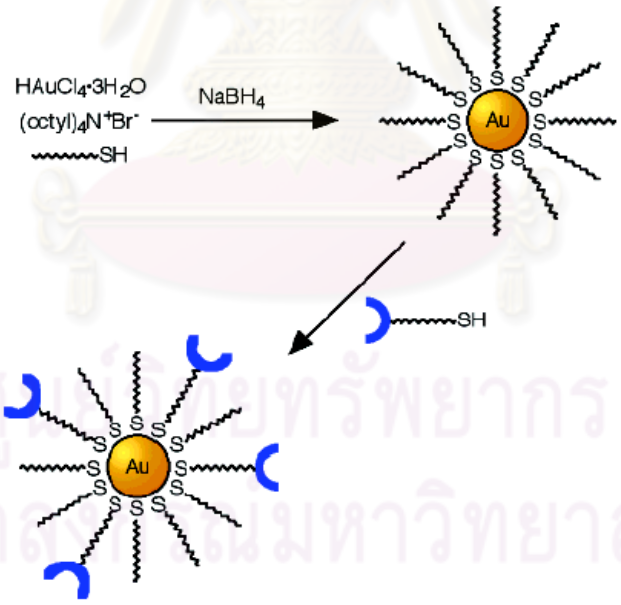


Figure 2-4 Synthesis of AuNPs in organic solvent

Synthesis of AuNPs in aqeous phase can also be done using a water soluble stabilizer. In 1999 Yonezawa and Kunitake synthesized AuNPs using sodium 3–mercaptopropionate salt as a stabilizer (36). In 1996, Graba synthesized the AuNPs using $HAuCl_4$, sodium citrate (stabilizer) and $NaBH_4$ (reducing agent) at room temperature obtaining 2.6 nm of AuNPs. When the experiment was done at 100 °C, the size of AuNPs was 60 or 125 nm depended on the concentration of $HAuCl_4$ (37). Xiao synthesized AuNPs using the same method at a temperature up to 100 °C. When $HAuCl_4$: sodium citrate ratio was 0.30, 0.50, 0.75 and 1.00, the size of AuNPs was 16±2.3 nm, 24±2.1 nm, 42±2.6 nm and 51±5.3 nm accordingly (38). The sodium citrate acts as both stabilizer and reducing agent. Moreover, the reaction condition for sodium citrate is significantly milder than that for $NaBH_4$ (39).

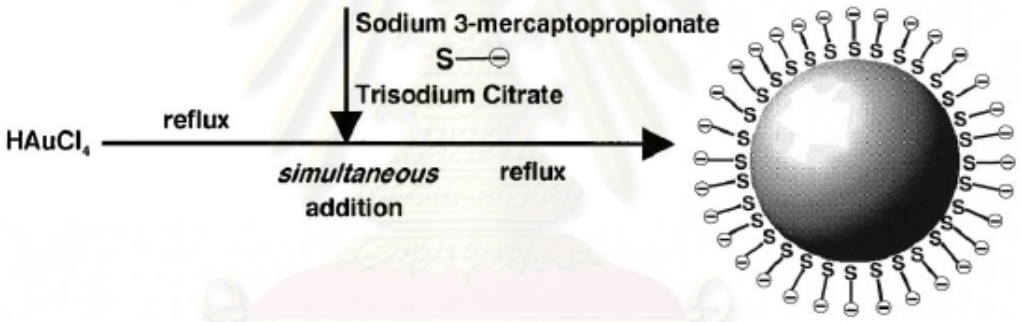


Figure 2-5 Synthesis of AuNPs in aqueous using Sodium 3-mercaptopropionate as stabilizer

2.2.1.2 Application of gold nanoparticles

AuNPs can be applied in many applications such as catalyst chemistry, Schimpf found that AuNPs can act as a catalyst in hydrogenation and oxidation reactions (40). Later, Haruta proved that the oxidation reaction of carbon monoxide can be operated at room temperature with the presence of AuNPs. (41) Moreover, gold nanoparticles have been widely used in many biomedical applications including biosensor and molecular

sensor. Himmelhaus claimed that cap-shaped AuNPs can also be used as optical biosensor. (42) Furthermore, AuNPs is applicable as ozone sensor (43) and in medical imaging (44). These applications are always associate with the surface plasmon resonance (SPR) property of AuNPs.

In 2000, Mirkin binded AuNPs with single stranded DNA constructed a novel DNA probe to detect the target DNA by hybridization process. The result showed that plasmon resonance band changed as a result of the binding between DNA probe and the target DNA. This interaction leads to changes in characteristic red colour of gold nanoparticles to a bluish-purple colour on colloidal aggregation (45).

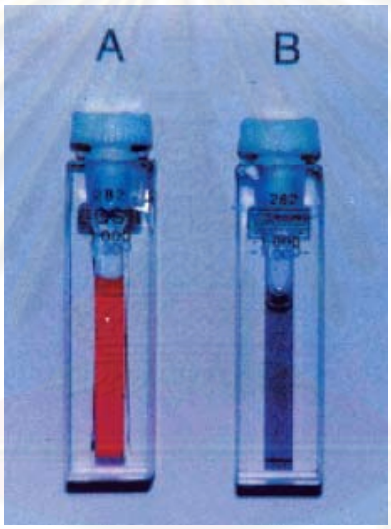


Figure 2-6 The colour changing of AuNPs from red to blue before (A) and after (B) hybridization

Besides naked eye detection, the changing of light absorption band can be detected easily by other methods. For example, detected with UV-vis spectrometer (46), electrophoretic migration (47), or by direct detection using high magnification electron microscope (48).

In 2006, Min Su used 2 different DNA probes to bind with the target single-stranded DNA in corporation with a triplex binder constructing a triplex DNA stand. This method allows dramatically improvement in discrimination between stabilizing and non-stabilizing triplex binders, thus the specificity is higher than normal hybridization (49).

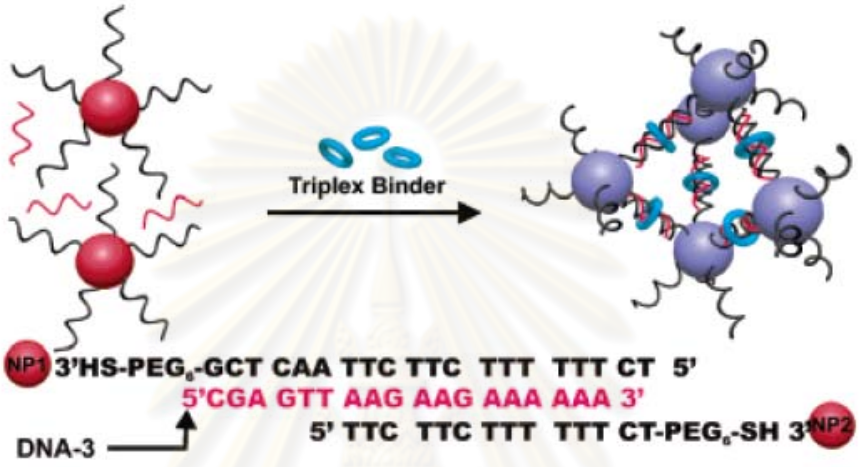


Figure 2-7 Representation of Structure and Colour Change of Nanoassembly in the Presence of Triplex Binder at Room Temperature.

For easier analyzing, Charrier bind DNA probe onto specific beads before hybridization with a target DNA. After hybridization, aggregation can be observed on the bead as two-dimensional aggregation. This aggregation caused less aggregation than three-dimensional environment due to the small amount of particles. Thus, AuNPs colour was still red. After that, dextran sulfate was added to the solution competing with the adsorbed AuNPs on the lipid layer on the bead. Therefore, it promoted desorption of AuNPs resulting in red colour of solution. In contrast, when the hybridization was carried out with target DNA, no desorption can be observed after introduction of the dextran sulfate in the solution. The adventage of this method is, as 2D supported system, it enables the design of multiplexed detection (50).

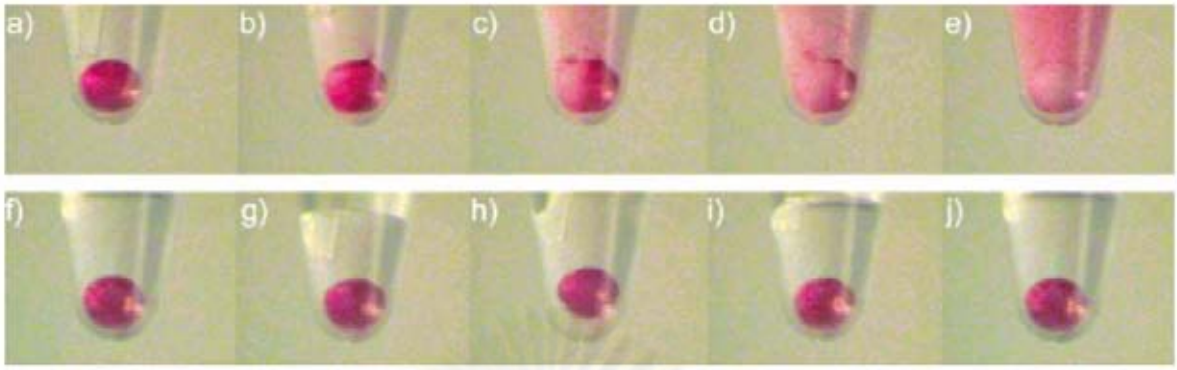


Figure 2-8 Desorption of a nonhybridized sample (a-e) and nondesorption of a hybridized sample (f-j) after addition of dextran sulfate at successive time intervals between 0 and 20 s. The solution is continuously agitated.

In 2008 Rojanathanes (51) showed the specific binding between the positive charge within the gold nanoparticle and negative charge within hCG hormone in pregnancypositive urine, while this reaction cannot be observed in pregnancy negative urine samples. As a stabilizer, hCG protects gold nanoparticles from aggregation, thus the pregnancy-positive mixture remain pink, while the pregnancy-negative mixture turned to gray.

Figure 2-9 The mixtures of gold nanoparticles solution and positive and negative urine samples compare with commercial test kit

2.2.2 Silver nanoparticles

In recent years, silver nanoparticles have received a lot of attention as discoveries in the various physical properties. These small particles maintain in hopes of use in electrical, catalytic, medical and optical fields. Like the gold nanoparticles, the extremely small size of silver nanoparticles results in the particles having a large surface area relative to their volume. This allows them to easily interact with other particles and increases their efficiency in many applications. For example, in antibiotic field, this effect can be so great that one gram of silver nanoparticles is all that is required to give antibacterial properties to hundreds of square meters of substrate material.

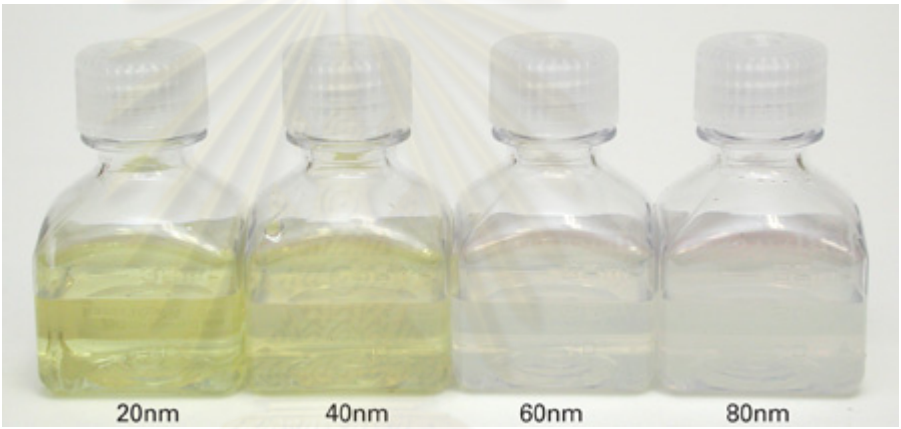


Figure 2-10 The colour of different size of silver colloids. The smaller colloids (20 and 40nm) are yellow in colour and the larger sizes (60 and 80nm) are a light gray.

Almost all the research focus has been concerned with using gold nanoparticles with very little work on other noble metal nanoparticles such as silver. This is due to the difficulty in synthesis method of silver nanoparticles which makes achieving repeatable size and optical characteristics problematic compared to gold nanoparticles synthesis. However, a silver nanoparticle also generates significant scientific and technological interests over gold nanoparticles in some applications due to the higher extinction coefficients compared with gold nanoparticles (52). Consequently, the sensitivity of the technique can be improved when using absorption spectroscopy. This benefit leads to

improved visibility due to the difference in optical brightness. In addition, silver nanoparticles provide much greater enhancements of Raman scattering than gold (53) and as such control over the aggregation of silver nanoparticles is considerable interest to those looking for optimal sensitivity using surface enhanced Raman scattering.

2.2.2.1 Synthesis of Silver Nanoparticles

Several various synthesis methods have been conducted to achieve these small particles. In silver nanoparticles synthesis, it is very important to control not only the particle size but also the particle shape and morphology as well. Previous studies also indicated that colloidal stability, particle size and morphology, and surface properties strongly depended on the specific method of preparation and the experimental conditions applied. These synthesis methods is including, but not limiting to, hydrothermal treatment (54), Tollens process (55), laser ablation in water (56), electrochemical synthesis (57), and electrochemical reduction on compact zeolite film modified electrodes (58). The silver nanoparticles are almost always either capped or stabilized with salts or organic compounds forming nanorods, nanocrystals, nanospheres, nanoprisms and particles of <10 nm diameter. Each synthesis produces different particles with varying properties depending on capping molecules.

Colloidal dispersions of silver in non-aqueous liquids, known to be difficult to prepare and to stabilize (59), have received little attention. Wet-chemical synthesis methods usually produce stable silver colloids at Ag^{+} concentrations below 0.01 M. Above this concentration, silver colloids usually become unstable or form aggregates. On the other hand, silver nanoparticles, mostly hydrosols, are perhaps most widely studied because of their easy synthesis method and important applications in catalysis (60) and photographic processes (61) and their roles in surface-enhanced Raman spectroscopy (SERS).

In 2005, Wei reported the one-step synthesis of silver nanoparticles, nanorods, and nanowires on nanoporous DNA network fabricated formed on the APTES-functionalized mica. Silver ions were first adsorbed onto the DNA network and then

reduced in sodium borohydride solution. Silver nanomaterials were formed by controlling the size of pores of the DNA network. The diameter of the silver nanoparticles and the aspect ratio of the silver nanorods and nanowires can be controlled by adjusting the DNA concentration and reduction time (62).

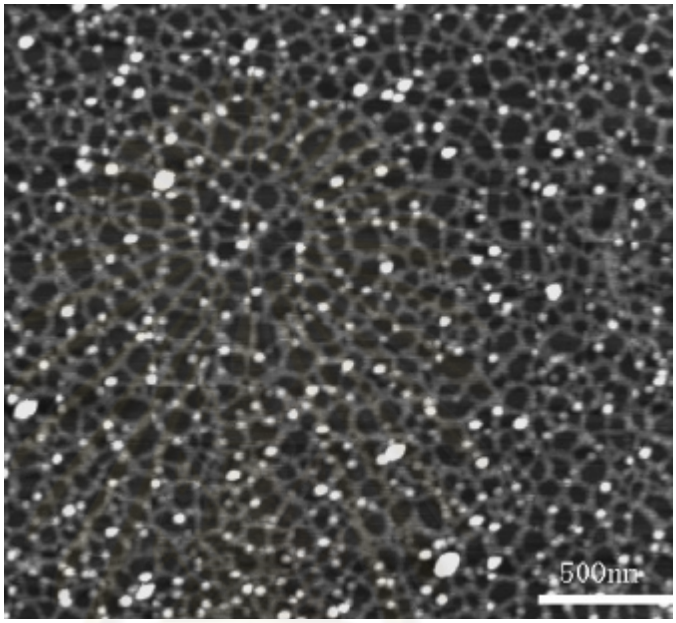


Figure 2-11 Atomic force microscopy (AFM) images of DNA network and synthesized nanoparticles

Another shape of silver nanomaterials was reported by Dong. Triangular silver nanoprisms were synthesized by stepwise reduction of silver nitrate with sodium borohydride ($NaBH_4$) and trisodium citrate. Spherical and rodlike silver nanoparticles were obtained at low concentration of $NaBH_4$ ($2x10^{-7}$ M). In the other hand, at medium concentration of $NaBH_4$ ($5x10^{-6}$ M), triangular silver nanoprisms, accompanied with some spherical silver nanoparticles, were obtained as the product. Furthermore, at high concentration of $NaBH_4$ ($5x10^{-4}$ M), the product was dominated by small spherical silver nanoparticles. Thus, the triangular nanoprism was depended on the amount of $NaBH_4$ used in the reactions (63).

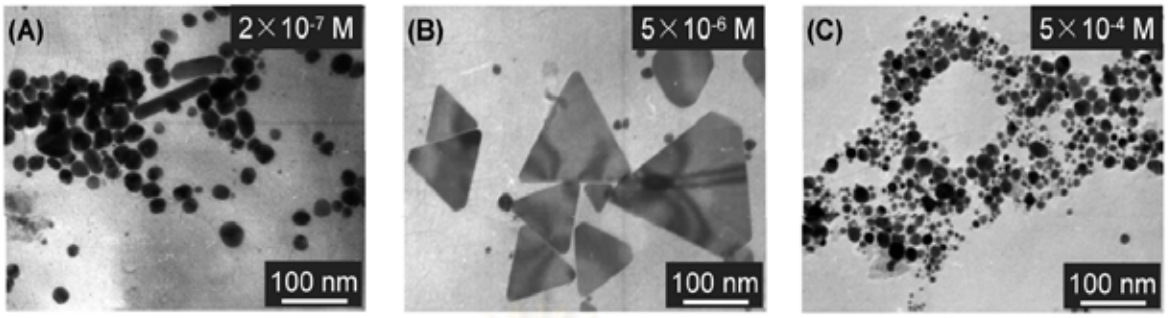


Figure 2-12 TEM images of the silver nanomaterial prepared with $2x10^{-7}$ M (A), $5x10^{-6}$ M (B) and $5x10^{-4}$ M (C) concentrations of $NaBH_4$

In 2010, Olga presented the size controllable synthesis of citrate-capped silver nanoparticles, ranging in diameter from approximately 8 to 50 nm, by a seed-mediated growth procedure. First, the silver nanoparticles seeds were synthesized by reducing $AgNO_3$ with $NaBH_4$ then synthesized larger sizes by heating a solution of silver nanoparticles seeds, $AgNO_3$, and trisodium citrate. The final diameter of the grown silver nanoparticles increased with increasing mole ratio of $AgNO_3$/Ag during the synthesis (64).

Around that time, Li reported green method to produce silver nanoparticles which particle size less than 5 nm by reduction process of nitrate with polyethylene glycol (Mw ≈ 200) without additional steps of introducing other reducing agents or stabilizers at room temperature. Therefore, the procedure required no toxic reagents, surfactant, or organic solvents involved in the whole process.

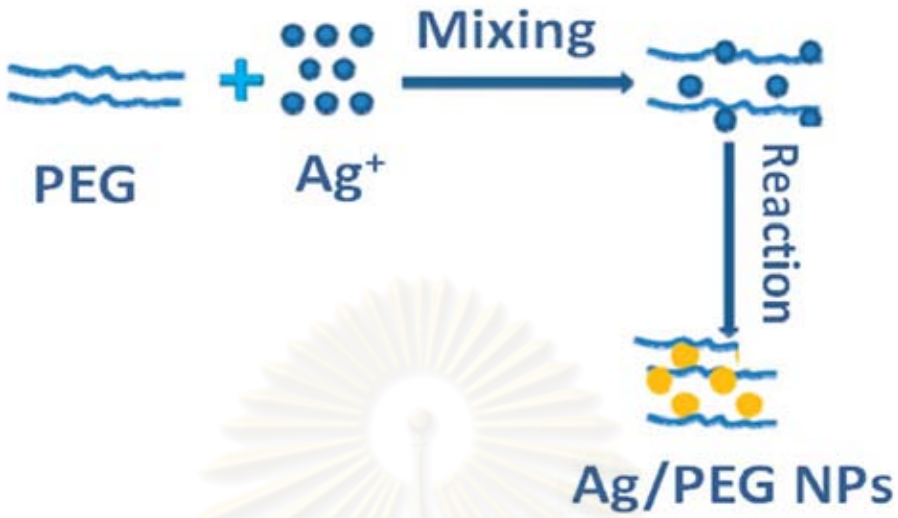


Figure 2-13 Process of the Ag/PEG-NPs synthesis at room temperature.

For synthesis of silver nanoparticles in organic solvent, Qian used a solid phase of sodium linoleate, a liquid phase of ethanol and linoleic acid, and water containing silver ions formed in the system. Ethanol in the liquid and solution phases reduced the silver ions into silver nanoparticles then absorbed on the solid phase. The product was collected at the bottom of vessel and dispersed in chloroform. Silver nanoparticles obtained from this method exhibited excellence stability which can be kept up to 4 months (65).

2.2.2.2 Application of silver nanoparticles

In nowadays silver nanoparticles application especially in antibacterial applications has become more importance due to antibiotic property of silver. For example, the coating fabric to keep it clean without washing (66), air condition containing nanoparticles in its filter (67) and the mixing of nanosilver in painting product.

Silver has been used as treatment of medical ailments for over 100 years due to its natural antibacterial and anti fungal properties. Silver is expensive metal and can be oxidized when expose to the air. As a result of these limitations, silver is become less

interesting. When nanotechnology is discovered, scientists are once again fascinated by silver nanoparticles on the reason that silver nanoparticles can diffuse though call wall of bacteria and destroy bacteria by only small amount of silver nanoparticles.

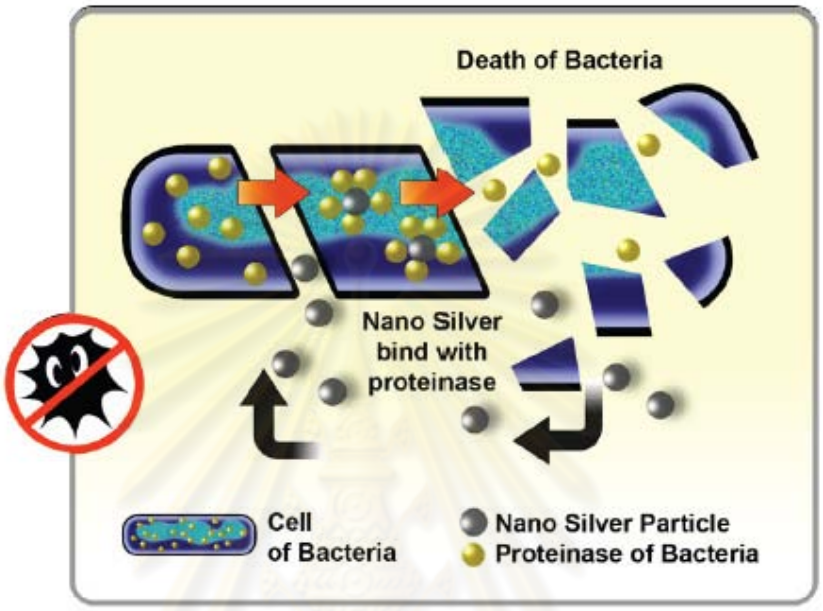


Figure 2-14 Antibacterial activity and mechanism of silver nanoparticles

One of the hypothesis about mechanism of silver nanoparticles in antibacteria process is when silver nanoparticles is in contact with bacteria and fungus, it diffuse into bacteria and fungus cell then nanoparticles which is soft acid combine with sulphydryl group (soft base) in proteinase and inhibit reactivity of this enzyme. Thus it will adversely affect cellular metabolism and inhibit cell growth.

Other potential application for silver nanoparticles is medical applications**.** It has a long history of treating wounds in the right quantities (68). Furthermore, Diagnostic biomedical optical imaging (69), Biological implants (like heart valves) (70), Dressings and bandages (71) are also application of silver nanoparticles.

In Colorimetric biosensor application, recently, Lee reported a new strategy for preparing silver nanoparticles modified oligonucleotide conjugates based upon cyclic

disulfide-anchoring groups modified DNA which shows excellence stability and can withstand NaCl concentrations up to 1.0 M. When silver nanoparticles functionalized with complementary sequences are combined, they assemble to form huge cluster. This assembly process is reversible with heating and is associated with a red shifting of the particle surface plasmon resonance (72).

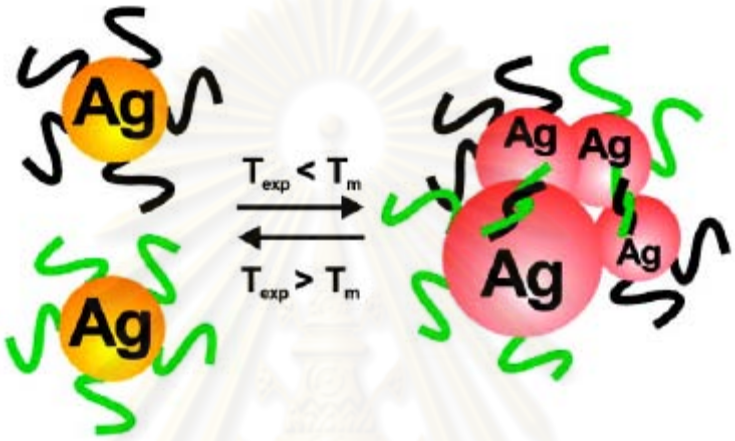


Figure 2-15 Illustration of the reversible hybridization of two complementary DNA-silver nanoparticles due to temperature.

For uses of unmodified silver nanoparticles form, Wei presented the detection of enzyme by reactions concerning adenosine triphosphate (ATP) dephosphorylation by calf intestine alkaline phosphatase (CIAP) and peptide phosphorylation by protein kinase A (PKA). The result showed that in the absence of the enzymes, unreacted ATP could protect silver nanoparticles from salt-induced aggregation, whereas in the presence of the enzymes, the reaction product of ATP (adenosine for CIAP and ADP for PKA) could not. This work provides a brilliance detection limit of 1 unit/mL for CIAP and 0.022 unit/mL for PKA (73).

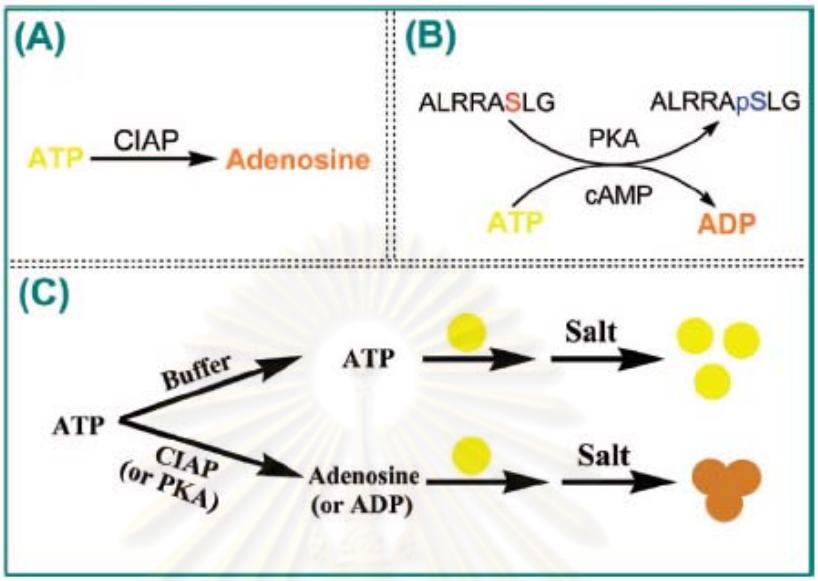
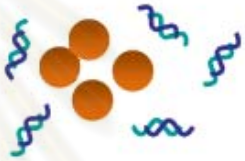


Figure 2-16 Enzymatic reactions with CIAP (A) and PKA (B), and silver nanoparticles-based enzyme colorimetric assay (C).

Therefore, silver nanoparticles can be used in both modified and unmodified form. A summary of the colorimetric DNA experimental setup and results on silver nanoparticles is provided in Table 2-2.

Table 2-2 Principle of silver nanoparticles based colorimetric DNA detection

Type I assays (using DNA- or PNA-modified nanoparticles)		*Type II assays* (using unmodified nanoparticles)	
Scheme	**Aggregation Mechanism**	**Scheme**	**Aggregation Mechanism**
Two sets of DNA conjugate 	'interparticle crosslinking aggregation through sandwich hybridization	 v.s 	Salt induced aggregation driven by London-van der Waals attractive force
Two sets of DNA conjugate 	'interparticle crosslinking aggregation through conjugate-conjugate hybridization		
One set of DNA conjugate 	'Noncrosslinking salt induced aggregation driven by London-van der Waals attractive force		
one set of PNA conjugate 			

2.3 Leptospirosis

Leptospirosis, an infectious disease that affects humans and animals, is considered one of the most common zoonosis in the world. The leptospires from infected animals will contaminate the warm lake water. They can persist in water, damp alkaline soil, vegetation, and mud with temperatures higher than 22°C for many months. Therefore, most cases of leptospirosis occur in the warm season and in rural areas.

The disease shows variable clinical manifestations so laboratory confirmation is necessary. As isolation of leptospires from clinical samples is time consuming. Thus the serology remains the main of diagnosis. The gold standard serological test is microscopic agglutination test (MAT).

In MAT test, patient sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination. The MAT is read by dark-field microscopy. Then, the titers are determined. The end point is the highest dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires compared to the control suspension. At this point, the positive result is the serum that has an antibody titer per serovar greater than or equal to 1:100

Although MAT test has high selectivity that can classify each serovar, MAT test is requires the maintenance of several leptospiral serovars in the laboratory which is expensive. It also requires the expertise personnel to read the results.

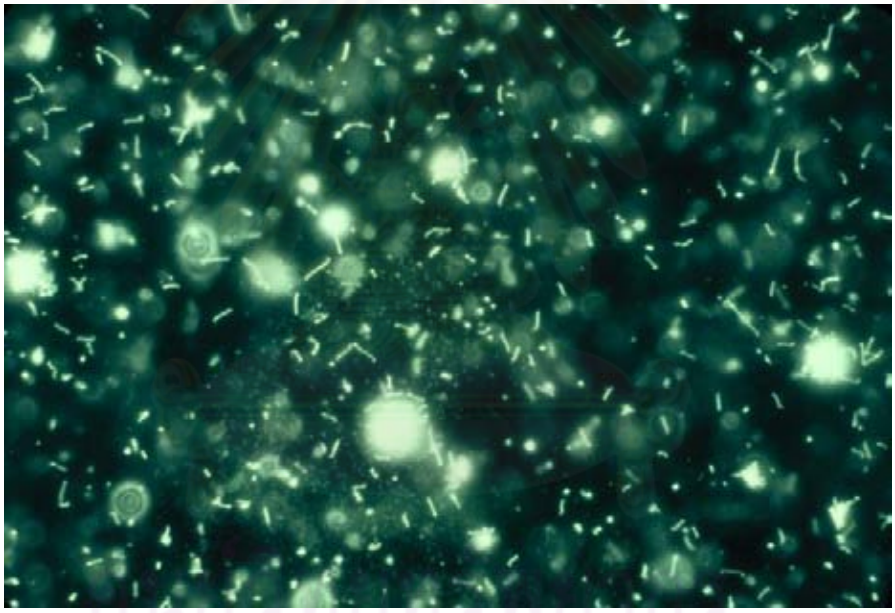


Figure 2-17 Microscopy of leptospiral in microscopic agglutination test

Besides the MAT test, there are many leptospirosis detection methods such as indirect hemagglutination test (IHA) (74), immunofluorescent assay (IFA) (75) and enzyme-linked immunosorbent assay (ELISA) (76).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

All chemicals are purchased from commercial sources and used as received, unless noted otherwise.

1. Hydrogen tetrachloroaurate (III) trihydrate : Sigma Aldrich
2. Silver nanoparticles : Department of Chemistry, Faculty of Science, Chulalongkorn University
3. Trisodium citrate dihydrate : Merk
4. Hydrogen peroxide : Thermo Fisher Scientific
5. Sodium chloride : Merk
6. Hydrochloric acid : Sigma Aldrich
7. Formamide : Sigma Aldrich
8. Dextran sulfate : Sigma Aldrich
9. Oligonucleotide : BioDesign

3.2 Equipment and analytical instuments

1. Magnetic Stirrer : Clifton
2. Micropipette : BRAND
3. Stirrer Hot Plate : BEC Thai
4. Vortex Mixer : BEC Thai
5. Spectrophotometer : Beckman Coulter
6. Transmission Electron Microscope : Hitachi High-Technologies

3.3 Experimental procedure

3.3.1 Synthesis and characterization of metal nanoparticles

3.3.1.1 Synthesis of citrate-stabilized gold nanoparticles

Citrate reduction method was first proposed by Turkevich *et al*. in 1951 (77). In a typical standard citrate reduction procedure, 24 mL of milli-Q water was heated to 80-85 °C on a strirring hot plate. With vigorously stirring, 0.5 mL of 1% tetrachloroauric acid ($HAuCl_4$) and 0.94 mL of 38.8 mM trisodium citrate were quickly added, resulting in a colour changing from yellow to grey and finally to purple. This solution was kept stirring for 2 hours. Then the solution appeared as deep red colour of gold nanoparticles. Gold nanoparticles was then cooled to room temperature. The concentration of the synthesized gold nanoparticles was 100 ppm.

3.3.1.2 Characterization of metal nanoparticles

Metal nanoparticles are commonly characterized by absorption spectroscopy and Transmission Electron Microscopy (TEM). The optical property, size and size distribution of metal nanoparticles can be investigated from their plasmon band. Generally, 1 mL of 50 ppm gold nanoparticle solution and 1 mL of 25 ppm silver nanoparticles were pipetted in a quartz cuvette and measured the absorption using UV-Vis Spectrophotometer.

A Transmission Electron Microscope (TEM) was used to view and determine the size of metal nanoparticles. TEM samples were prepared as follows: a single drop (10 μL) of metal nanoparticles solution was placed onto a copper grid. The grid was left to dry for several hours at room temperature. Then, the average size histogram of metal nanoparticles was determined by digital processing of Transmission Electron Microscopy images.

3.3.2 Preparation of DNAs

All oligonucleotides were prepared by diluting a 200 μM oligonuclotide stock solution to 20 μM. The oligonucleotide sequences used in this experiment are shown in Table 3-1.

Table 3-1 Oligonucleotide sequences

Oligonucleotides	Sequence (5’ to 3’)
Oligo1 (target leptospirosis)	TTTATTCGTCGTTACACTAC
Oligo2 (leptospirosis probe)	GTAGTGTAACGACGAATAAA
Oligo3 (non-complementary to target)	CCGAGCAATAAATCTTTACC
Oligo4 (1 mismatched complementary to target)	CTAGTGTAACGACGAATAAA

A stock DNA was prepared by adding an oligonucleotide into the hybridization buffer (0.2xSSC containing 5% formamide and 1% dextran sulfate) at room temperature for 30 min prior to use. Ratios of the oligonucleotide and the hybridization buffer are shown in table 3-2.

Table 3-2 DNA mixture composition (Oligo1 : Oligo2 : Oligo3 : Oligo4 : hybridization buffer)

DNA mixture	Oligo1 (μL)	Oligo2 (μL)	Oligo3 (μL)	Oligo4 (μL)	Hybridization buffer (μL)
mixture1 (ssDNA)	6	-	-	-	2
mixture2 (ssDNA)	-	6	-	-	2
mixture3 (dsDNA)	3	3	-	-	2
mixture4 (ssDNA)	3	-	3	-	2
mixture5 (1 mismatched dsDNA)	3	-	-	3	2

3.3.3 Aggregation of gold nanoparticles

3.3.3.1 Effect of buffer on aggregation

Into 5 eppendorf tubes contrary, 250 μl of 100 ppm AuNPs, 10, 50, 100, 150 and 200 μl of hybridization buffer were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 μl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.3.2 Effect of salt on aggregation

Into 5 eppendorf tubes contrary, 250 μl of 100 ppm AuNPs, 1, 2, 3, 4, and 5 μl of 2M NaCl were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 μl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.3.3 Effect of DNA on aggregation

Into 15 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 0.1, 0.2, 0.3, 0.4 and 0.5 µl of mixture1-3 were added. Subsequently, 4 µl of 2M NaCl was added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.3.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

Into 3 eppendorf tubes contrary, 250 µl of 100 ppm AuNPs, 0.3 µl of mixture3-5 were added. Subsequently, 4 µl of 2M NaCl was added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.4 Aggregation of silver nanoparticles

3.3.4.1 Effect of buffer on aggregation

Into 5 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 10, 50, 100, 150 and 200 µl of hybridization buffer were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.4.2 Effect of salt on aggregation

Into 5 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 1, 2, 3, 4, and 5 µl of 2M NaCl were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.4.3 Effect of DNA on aggregation

Into 15 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 1, 2, 3, 4 and 5 µl of mixture1-3 were added. Subsequently, 4 µl of 2M NaCl were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.4.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

Into 3 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 2 µl of mixture3-5 were added. Subsequently, 4 µl of 2M NaCl were added. The solutions were occasionally shaken and allowed to stand for at least 30 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.5 Oxidation of silver nanoparticles

3.3.5.1 Effect of silver nanoparticles concentration on oxidation

Into 6 eppendorf tubes contrary, 500, 250, 125, 83.33, 62.5 and 50 µl of 25, 50, 100, 150, 200 and 250 ppm AgNPs respectively, 0.8 µl of 30% hydrogen peroxide were added. The solutions were vigorous shaken immediately with vortex mixer for 5 second and allowed to stand for 15 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were diluted with milli-Q water to 500 µl and the absorbances were measured by UV-VIS spectrophotometer.

3.3.5.2 Effect of temperature on oxidation

Five eppendorf tubes containing, 250 µl of 50 ppm AgNPs were heated to 26, 30, 35, 40 and 45 °C for 30 mins. Then, 1.1 µl of 30% hydrogen peroxide were added into each tube. The solutions were vigorous shaken immediately with vortex mixer for 5 second and allowed to stand for 15 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were diluted with milli-Q water to 500 µl and the absorbances were measured by UV-VIS spectrophotometer.

3.3.5.3 Effect of hydrogen peroxide on oxidation

Into 5 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 0.5, 0.8, 1.1, 1.4 and 1.7 µl of 30% hydrogen peroxide were added. The solutions were vigorous shaken immediately with vortex mixer for 5 second and allowed to stand for 15 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.5.4 Effect of DNA on oxidation

Into 12 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 1, 2, 3 and 4 µl of mixture1-3 were added. Subsequently, 0.8 µl of 30% hydrogen peroxide were added. The solutions were vigorous shaken immediately with vortex mixer for 5 second and allowed to stand for 15 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

3.3.5.5 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

Into 3 eppendorf tubes contrary, 250 µl of 50 ppm AgNPs, 4 µl of mixture3-5 were added. Subsequently, 0.8 µl of 30% hydrogen peroxide were added. The solutions were vigorous shaken immediately with vortex mixer for 5 second and allowed to stand for 15 mins at room temperature. Then, the colours of solution mixtures were observed. Finally, the solutions were mixed with 250 µl of milli-Q water and the absorbances were measured by UV-VIS spectrophotometer.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Characterization of metal nanoparticles

The metal nanoparticles were characterized by UV-Vis spectrophotometer and Transmission Electron Microscopy (TEM). A typical absorption spectrum of metal nanoparticles was shown in Figure 4-1 (AuNPs) and Figure 4-2 (AgNPs). The plasmon wavelength maxima (λ_{max}) indicate the size of metal nanoparticles. The λ_{max} of gold and silver nanoparticles used in this research are approximately at 520 nm and 400 nm, respectively.

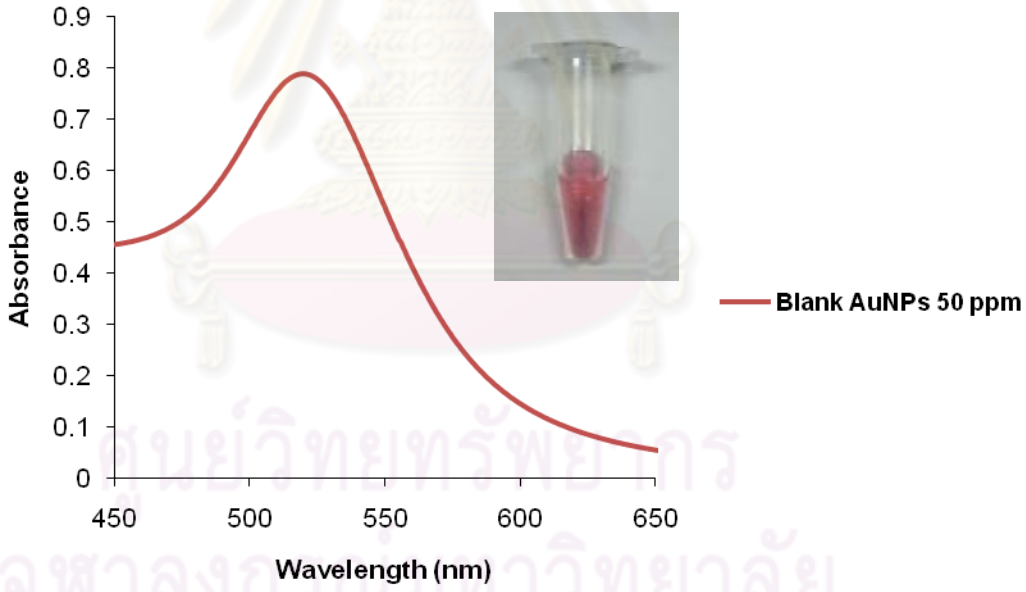


Figure 4-1 The characterization of AuNPs using spectrophotometer

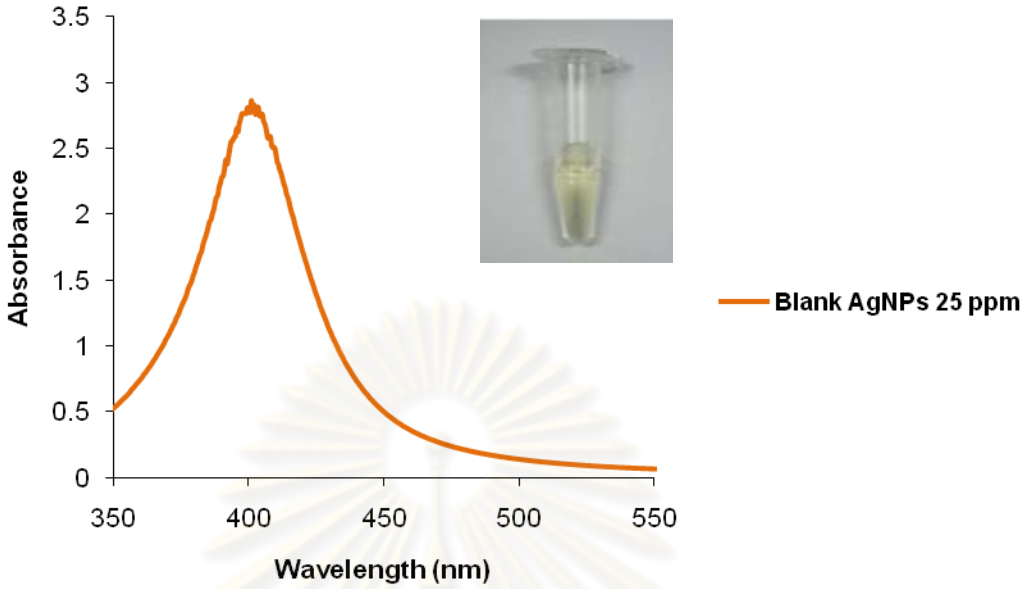


Figure 4-2 The characterization of AgNPs using spectrophotometer

Although the concentration of silver nanoparticles used in characterization is lower than gold nanoparticles, the absorbance of silver nanoparticles is higher than gold nanoparticles indicating the higher absorptivity of silver nanoparticles. Thus, this benefits in higher sensitivity of the detection of AgNPs over AuNPs.

Transmission Electron Microscope (TEM) was also used for determining the size and size distribution of metal nanoparticles. The size and size distribution of metal nanoparticles are shown in Figure 4-3.

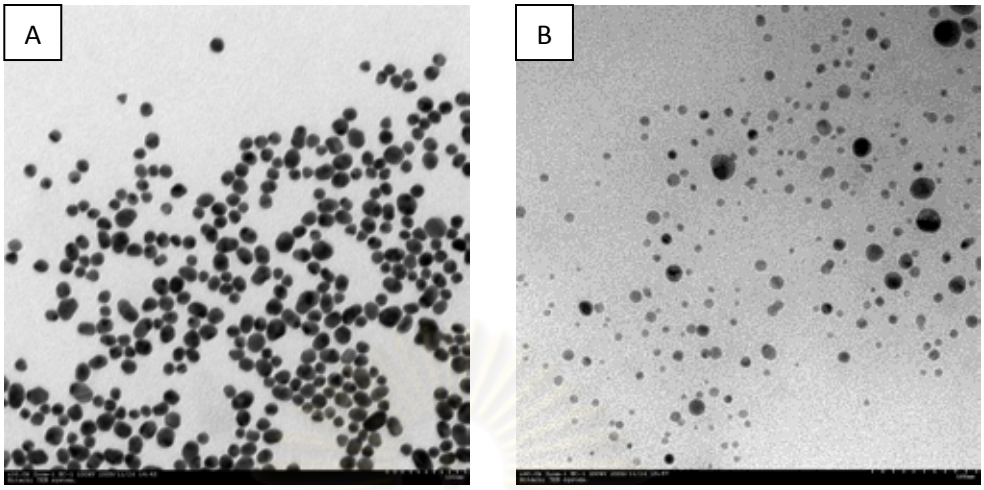


Figure 4-3 The characterization of metal nanoparticles using TEM**.** (A) Shows a spherical gold naoparticles with diameter around ≈15 nm. (B) Spherical silver nanoparticles with diameter around ≈5-20 nm.

TEM images show that both gold and silver nanoparticles are in spherical shape with different size distribution. AuNPs appeared as more uniform size distribution.

4.2 Aggregation of metal nanoparticles

4.2.1 Effect of buffer on aggregation

Due to the charge screening effect, addition of buffer or electrolyte into a solution containing metal nanoparticles resulted in a decreasing of interparticle distance and caused nanoparticles aggregation. The more electrolytes added, the more aggregation can be observed. Therefore, to make the most efficient stabilization, buffer in hybridization step should not cause aggregation of metal nanoparticles. The effect of different buffer quantity in aggregation of MNPs was shown in figure 4-4 (gold nanoparticles) and figure 4-5 (silver nanoparticles)

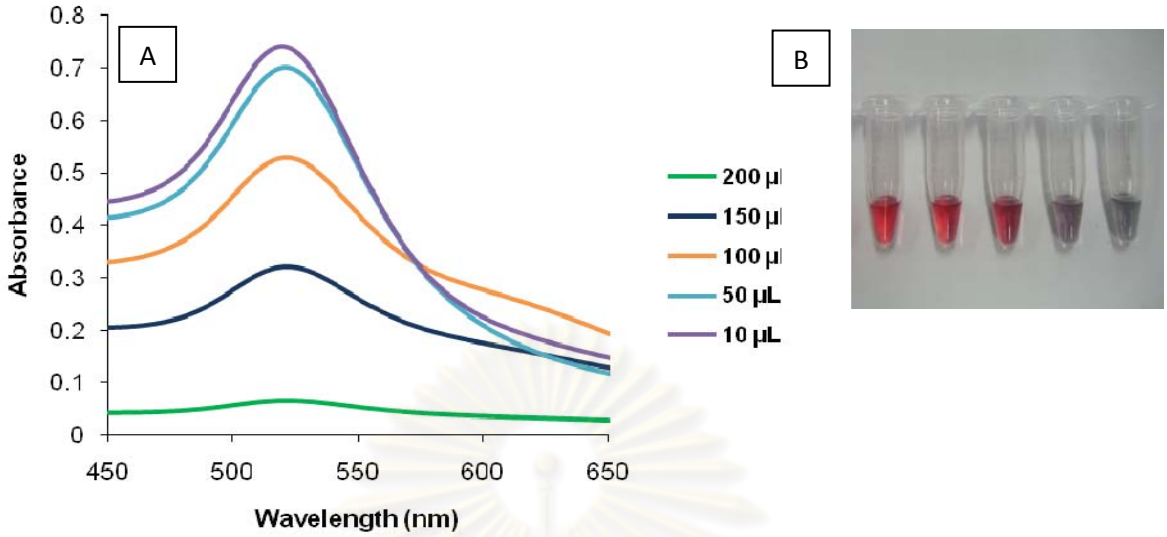


Figure 4-4 The effect of different amount of buffer to the aggregation of AuNPs. (A) UV-vis spectra of unmodified AuNPs mixing with buffer. (B) the colour changing after adding buffer, 10, 50, 100, 150 and 200 µL, from left to right, respectively.

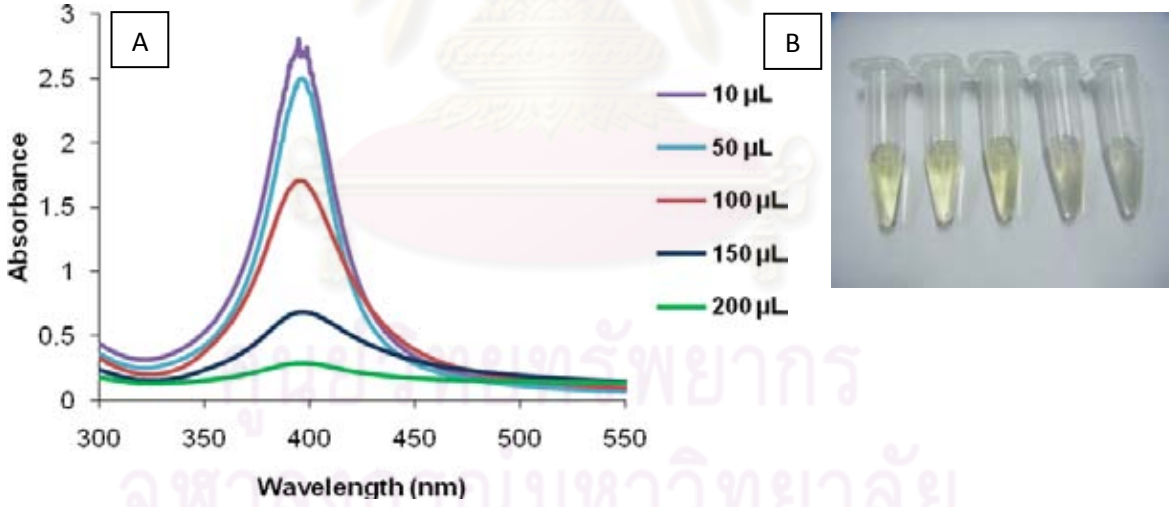


Figure 4-5 The effect of different amount of buffer to the aggregation of AgNPs. (A) UV-vis spectra of unmodified AgNPs mixing with buffer. (B) the colour changing after adding buffer, 10, 50, 100, 150 and 200 µL, from left to right, respectively.

The decreasing in absorbance at λ_{max} and a concomitant red shift in the nanoparticles plasmon band indicated an aggregation of metal nanoparticles. When high amount of buffer was added into a solution of metal nanoparticles, the nanoparticles aggregated and the solution colour turned from red to blue in AuNPs solution and yellow to pale brown in AgNPs solution.

According to the experiment, if the buffer used in hybridization was over 100 μL, the colour changing in aggregation can be observed with naked eye and the decreasing of absorbance at λ_{max} of nanoparticles cab also be detected. While in 10-50 μL of buffer, less aggregation can be observed and the colour changing of solution cannot be detected with the naked eye. Thus, the amount of buffer using in the hybridization process should not reach 50 μL.

4.2.2 Effect of salt on aggregation

The addition of NaCl into solution increased total ionic charge in solution as in the addition of buffer. Thus, the result was similar. The higher amount of salt added, the more aggregation can be observed. UV-vis spectra of mixture were shown in figure 4-6 (gold nanoparticles) and figure 4-7 (silver nanoparticles).

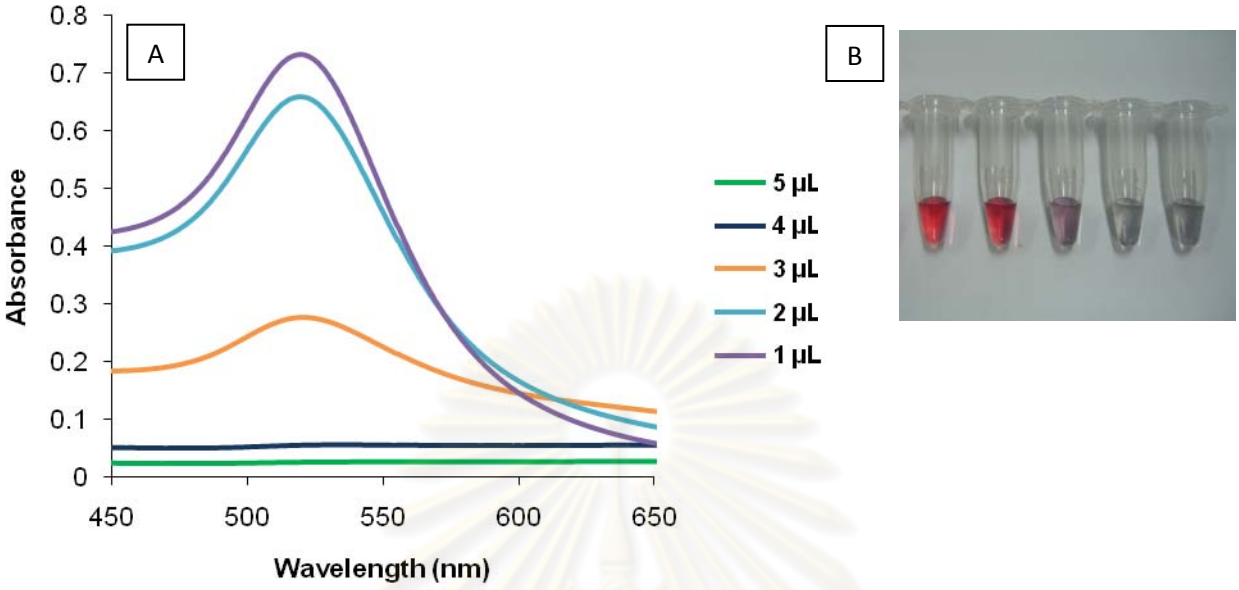


Figure 4-6 The effect of different amount of NaCl to the aggregation of AuNPs. (A) UV-vis spectra of unmodified AuNPs mixing with NaCl. (B) the colour changing after adding 2M NaCl, 1, 2, 3, 4 and 5 µL, from left to right, respectively.

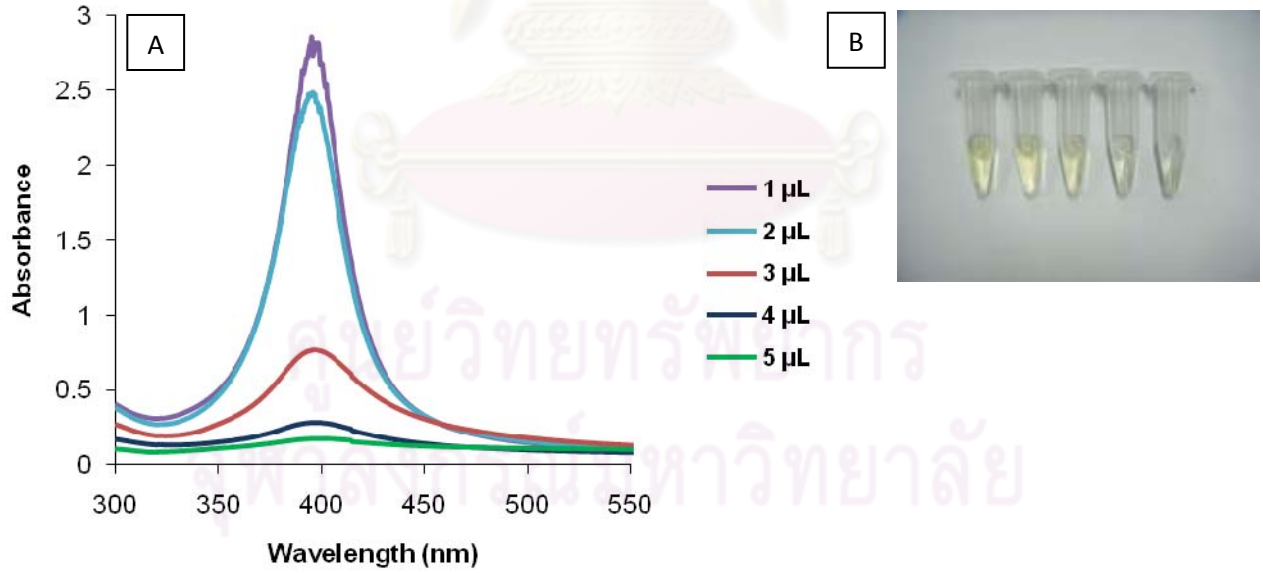


Figure 4-7 The effect of different amount of NaCl to the aggregation of AgNPs. (A) UV-vis spectra of unmodified AgNPs mixing with NaCl. (B) the colour changing after adding 2M NaCl, 1, 2, 3, 4 and 5 µL, from left to right, respectively.

UV-vis spectra showed that aggregation, which was caused by charge screening effects, suppressed the absorption intensity. This is a slow process and the size of nanoparticle gradually growing bigger. The small red shift of the λ_{max} indicated that the size of nanoparticles was slightly increased. Moreover the band width of absorption was barely changed. This indicated that size distribution remained constant.

The minimum amount of salt that caused significant changing of absorption spectra was about 3-4 μL. The following experiments used this optimum volume of salt solution.

4.2.3 Effect of DNA on aggregation

Metal nanoparticles in solution are typically stabilized by adsorbed negative ions (e.g. citrate ion) or molecule containing lone pair electron which cause negative charge on the surface. Repulsion from negative charge prevents van der Waals attraction among metal nanoparticles and thus prevents aggregation. Although the ssDNA has negative charges on the backbone causing electrostatic repulsion to the nanoparticles surface, the ssDNA is flexible and partially uncoil its structure. Under these conditions, the negative charge on the backbone is able to avert from metal nanoparticles to avoid repulsion. On the other hand, attractive van der Waals forces between the bases of ssDNA and the metal nanoparticles causes stronger ssDNA to metal interaction. The effect of DNA on AuNPs aggregation was shown in figure 4-8 (mixture1) and 4-9 (mixture2) and the effect of DNA on AgNPs aggregation was shown in figure 4-10 (mixture1) and 4-11 (mixture2).

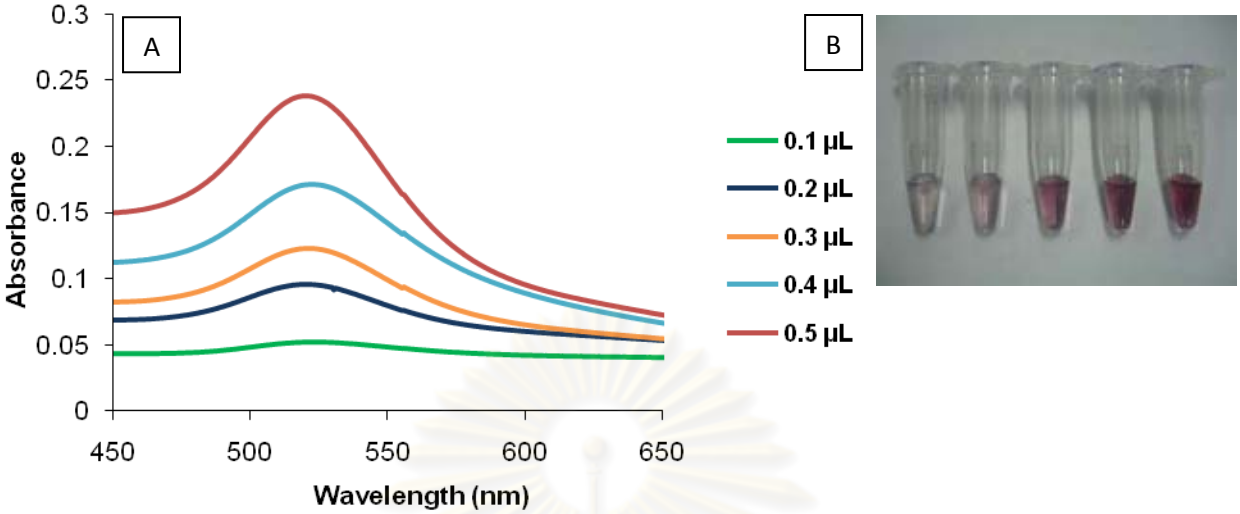


Figure 4-8 The effect of different amount of mixture1 to the aggregation of AuNPs. (A) UV-vis spectra of unmodified AuNPs stabilized by mixture1 after mixing with NaCl. (B) the colour changing of AuNPs solution stabilized by 0.1, 0.2, 0.3, 0.4 and 0.5 µL mixture1, from left to right, respectively.

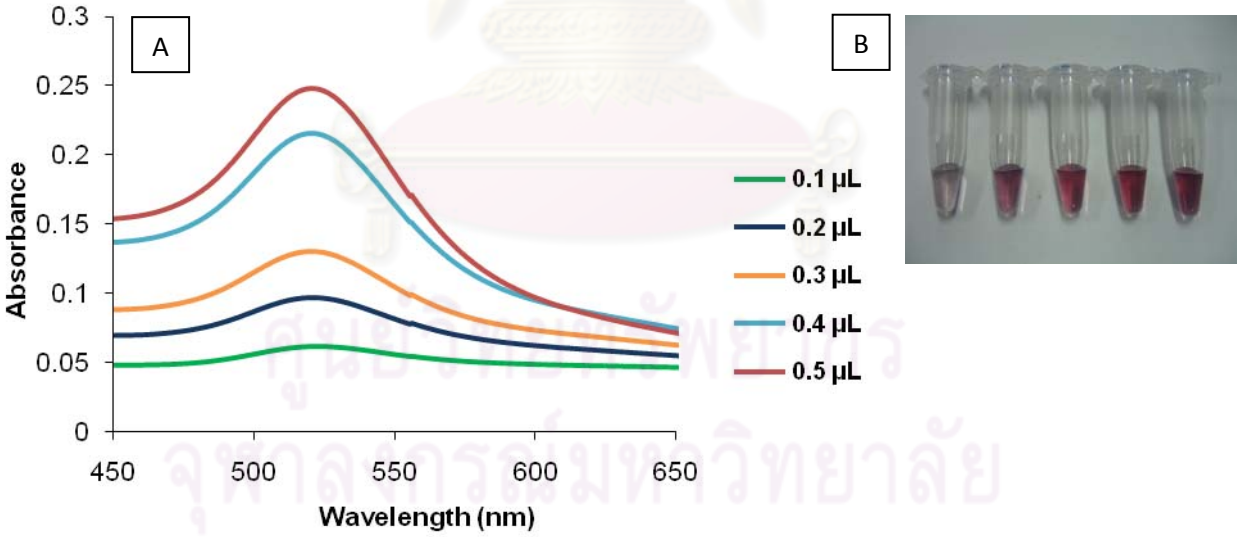


Figure 4-9 The effect of different amount of mixture2 to the aggregation of AuNPs. (A) UV-vis spectra of unmodified AuNPs stabilized by mixture2 after mixing with NaCl. (B) the colour changing of AuNPs solution stabilized by 0.1, 0.2, 0.3, 0.4 and 0.5 µL mixture2, from left to right, respectively.

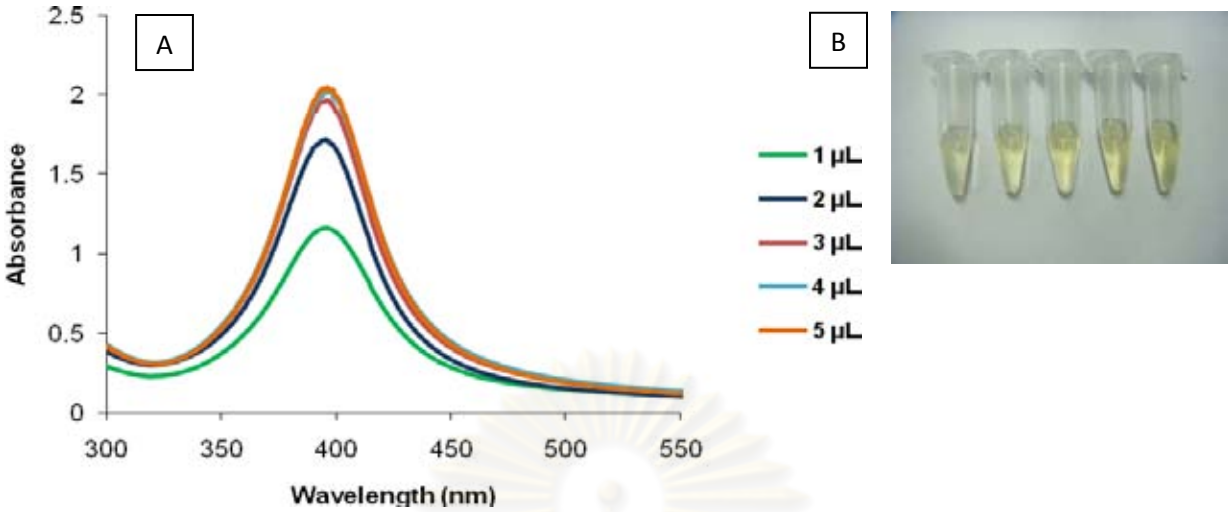


Figure 4-10 The effect of different amount of mixture1 to the aggregation of AgNPs. (A) UV-vis spectra of unmodified AgNPs stabilized by mixture1 after mixing with NaCl. (B) the colour changing of AgNPs solution stabilized by 1, 2, 3, 4 and 5 µL mixture1, from left to right, respectively.

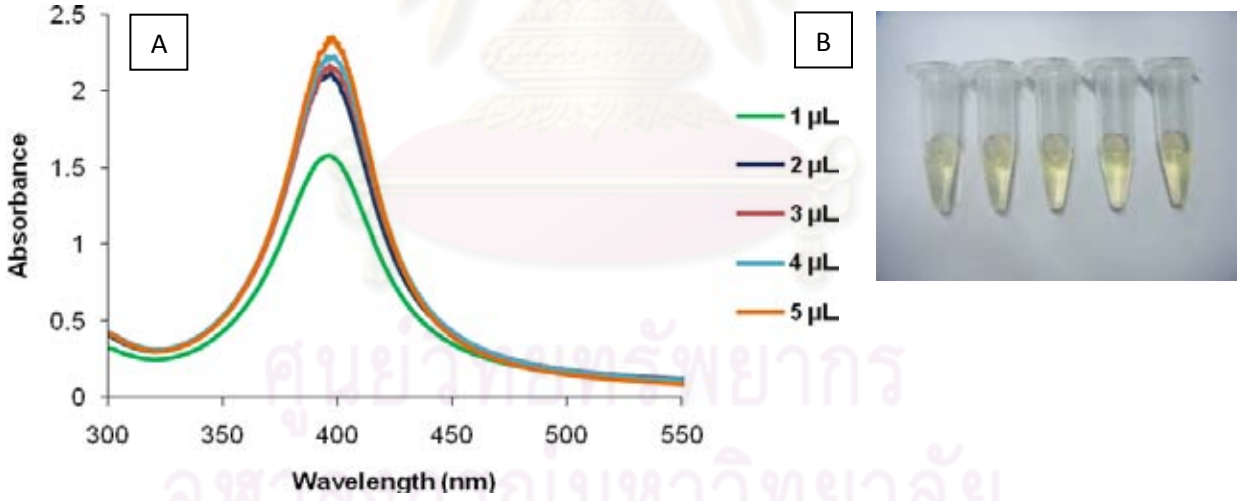


Figure 4-11 The effect of different amount of mixture2 to the aggregation of AgNPs. (A) UV-vis spectra of unmodified AgNPs stabilized by mixture2 after mixing with NaCl. (B) the colour changing of AgNPs solution stabilized by 1, 2, 3, 4 and 5 µL mixture2, from left to right, respectively.

According to the experiment, at an equal amount of mixture1 and mixture2 used to stabilize metal nanoparticles, the measured absorption intensity values were varied. The absorbance of MNPs/mixture2 mixture was higher than that of MNPs/mixture1 mixture. It indicated that the metal nanoparticles stabilization efficiency of mixture2 is higher than mixture1. Since mixture2 sequence contain more adenine base. Among four nucleobases, Adenine exhibits stronger base stacking due to its high aromaticity. Thus it promoted uncoil of DNA and caused adsorption faster and more efficiently than mixture1.

Although the colour of solution was depended on the amount of ssDNA, the addition of excess ssDNA cannot improve stability because the limited amount of nanosurface. As in AgNPs aggregation, more than 2 μL of DNA was over excess and causes insignificantly changing in colour of solution. On the other hand, solutions with only a few ssDNA had distinctly different in absorption spectra and colloid colour.

In contrast, dsDNA has different electrostatic properties due to the double-helix geometry that always turn the negatively charged phosphate backbone out. Therefore, repulsion between the charged phosphate backbone of dsDNA and the negative charge of stabilizer dominated the electrostatic interaction between the metal nanoparticles and dsDNA. As a consequence, dsDNA is not adsorbed onto the nanoparticles surface. The result of dsDNA stabilized metal nanoparticles was shown in figure 4-12 (gold nanoparticles) and figure 4-13 (silver nanoparticles)

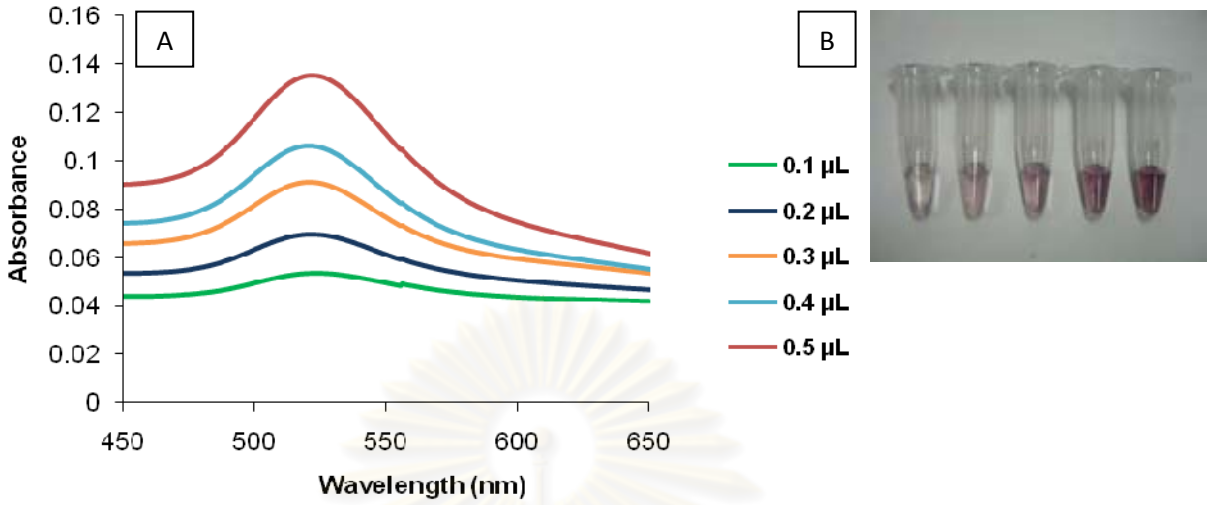


Figure 4-12 The effect of different amount of mixture3 to the aggregation of AuNPs. (A) UV-vis spectra of unmodified AuNPs stabilized by mixture3 after mixing with NaCl. (B) the colour changing of AuNPs solution stabilized by 0.1, 0.2, 0.3, 0.4 and 0.5 µL mixture3, from left to right, respectively.

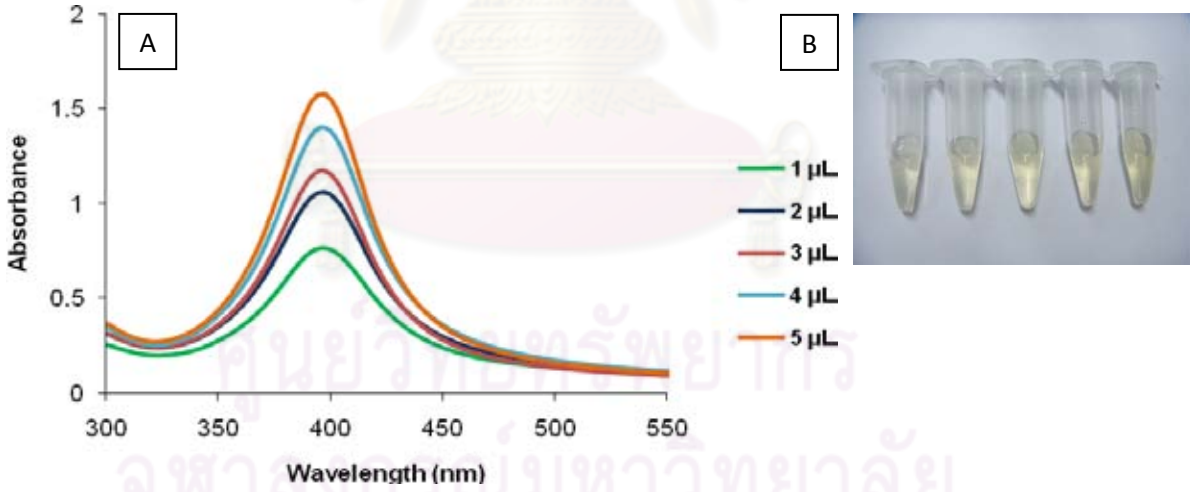


Figure 4-13 The effect of different amount of mixture3 to the aggregation of AgNPs. (A) UV-vis spectra of unmodified AgNPs stabilized by mixture3 after mixing with NaCl. (B) the colour changing of AgNPs solution stabilized by 1, 2, 3, 4 and 5 µL mixture3, from left to right, respectively.

With the increasing of mixture3, absorbance intensity was also increased though dsDNA cannot stabilize the MNPs, because hybridization was a reversible process and some of ssDNA still remained which able to stabilize metal nanoparticles.

The Experiment condition that make the aggregation of metal nanoparticles stabilized by mixture1-3 clearly detected with naked eye was shown in figure 4-14 (gold nanoparticles) and figure 4-15 (silver nanoparticles)

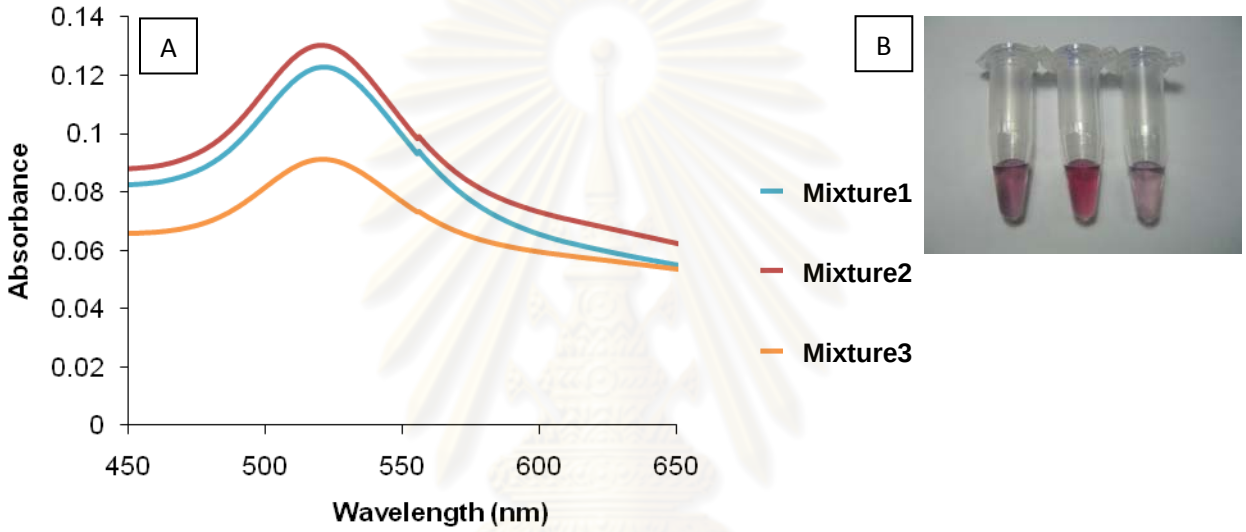


Figure 4-14 Comparison of effect of mixture1-3 on the aggregation of AuNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AuNPs stabilized by 0.3 μL mixture1-3 from left to right, respectively.

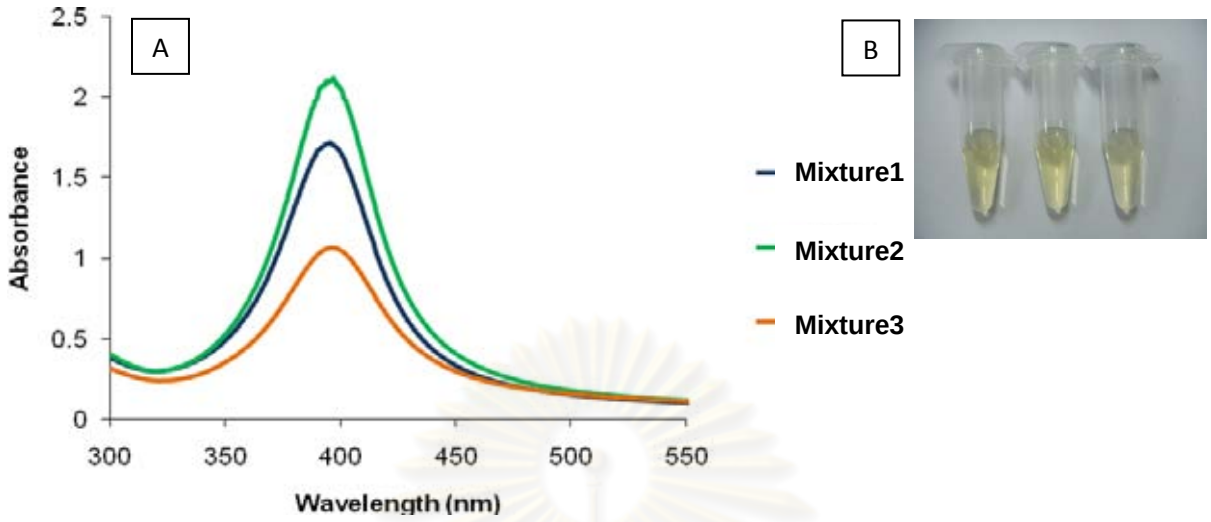


Figure 4-15 Comparison of effect of mixture1-3 on the aggregation of AgNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AgNPs stabilized by 2 μL mixture1-3 from left to right, respectively.

4.2.4 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

Non-complementary DNA is the mixture between two non-hybridizable ssDNA. It can be absorbed onto the metal nanoparticles and thus stabilizes the MNPs against the aggregation from the induction of ionic salts. Solutions with adequate quantities of non-complementary DNA prevent aggregation, whereas solutions with complementary DNA (dsDNA) do not affect the aggregation. The comparison between non-complementary DNA and complementary DNA stabilized metal nanoparticles was shown in figure 4-16 (gold nanoparticles) and figure 4-17 (silver nanoparticles).

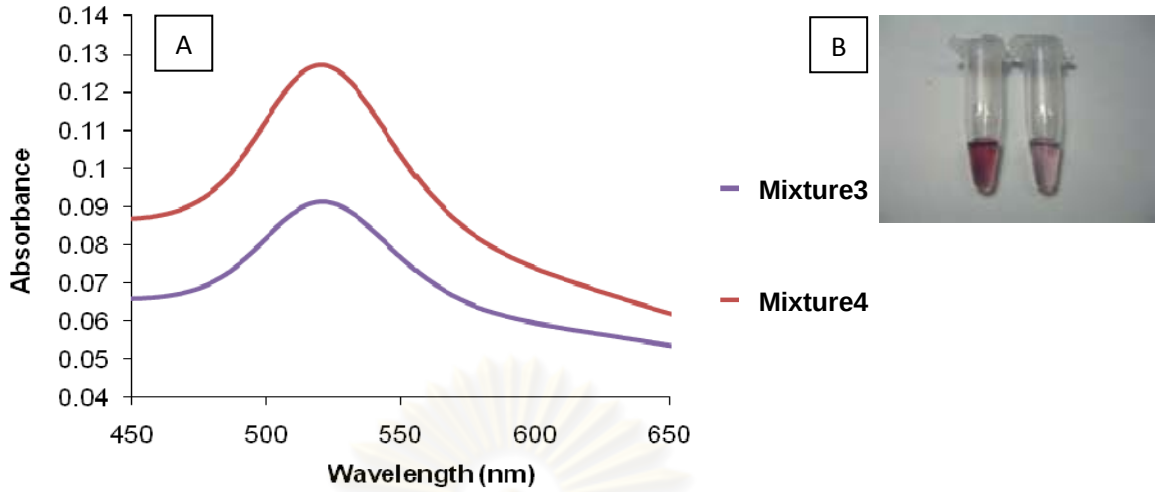


Figure 4-16 Comparison between non-complementary DNA (mixture4) and complementary DNA (mixture3) stabilized AuNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AuNPs stabilized by mixture4 (left eppendorf) and mixture3 (right eppendorf).

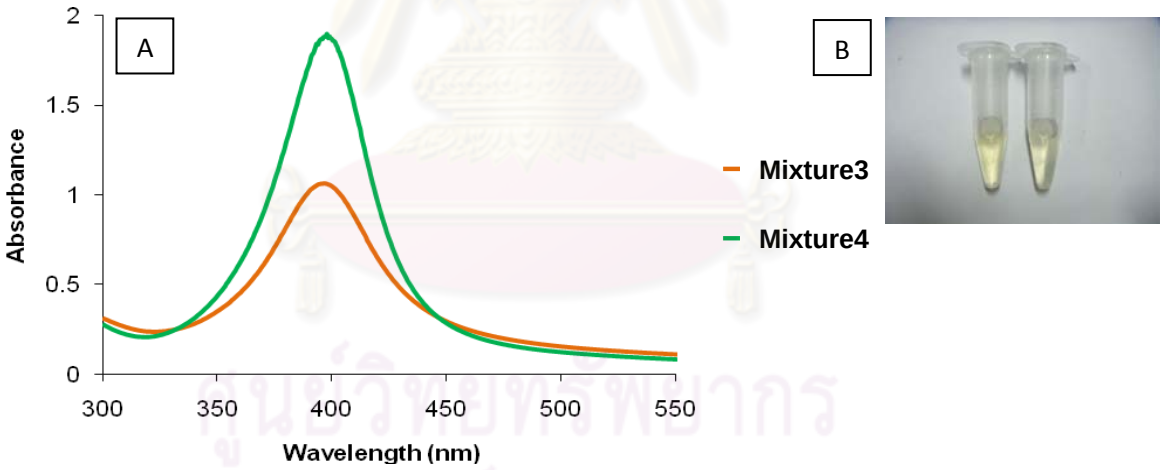


Figure 4-17 Comparison between non-complementary DNA (mixture4) and complementary DNA (mixture3) stabilized AgNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AgNPs stabilized by mixture4 (left eppendorf) and mixture3 (right eppendorf).

Typically, 1-mismatched complementary DNA containing a few base pairs always has lower melting temperature than a perfect complementary DNA for 6-7 °C. Thus, a solution of 1-mismatched complementary DNA contains higher number of ssDNA than those in a perfect matched DNA. As a consequence, 1-mismatched complementary DNA is a better stabilizer for metal nanoparticles than a perfect matched DNA but not as a non- complementary DNA. The comparison between 1-mismatched complementary DNA and complementary DNA stabilized metal nanoparticles was shown in figure 4-18 (gold nanoparticles) and figure 4-19 (silver nanoparticles)

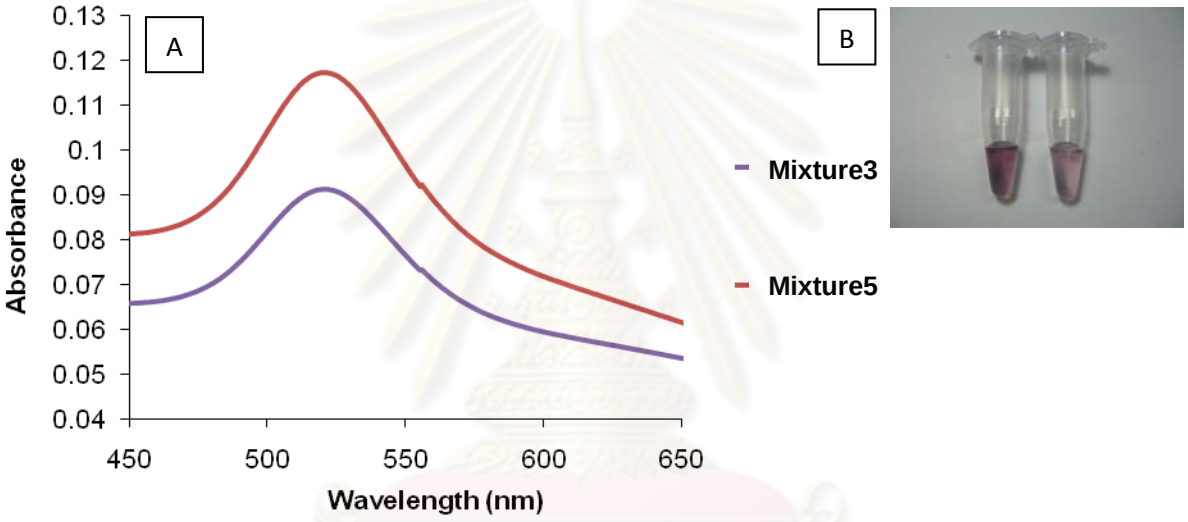


Figure 4-18 Comparison between 1-mismatched complementary DNA (mixture5) and complementary DNA (mixture3) stabilized AuNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AuNPs stabilized by mixture5 (left eppendorf) and mixture3 (right eppendorf).

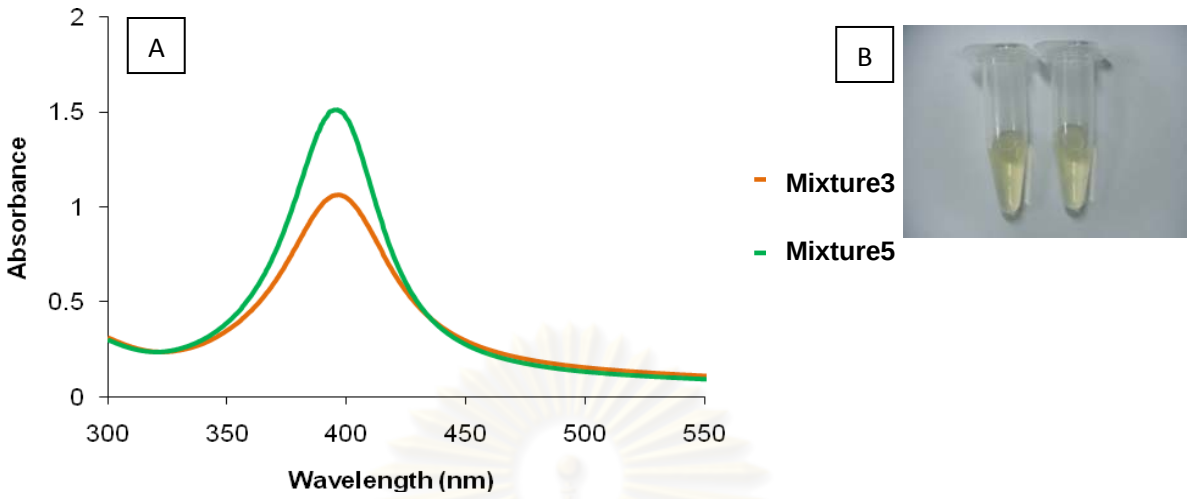


Figure 4-19 Comparison between 1-mismatched complementary DNA (mixture5) and complementary DNA (mixture3) stabilized AgNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AgNPs stabilized by mixture5 (left eppendorf) and mixture3 (right eppendorf).

4.2.5 Comparison of sensitivity between gold and silver nanoparticles

A quantitative expression of sensitivity of the detection can be exhibited by colorimetric response (CR) (78) value as shown in table 4-1.

Table 4-1 CR of gold and silver nanoparticles stabilized by mixture3-5

Metal nanoparticles	Type of DNA	CR	ΔCR (comparing with mixture3)
AuNPs	mixture3	0.29	0
	mixture4	0.22	0.07
	mixture5	0.23	0.05
AgNPs	mixture3	0.13	0
	mixture4	0.04	0.09
	mixture5	0.08	0.05

The CR values describe the colour changing comparing with a blank solution. Higher CR value indicates stronger colour changing by aggregation of metal nanoparticles.

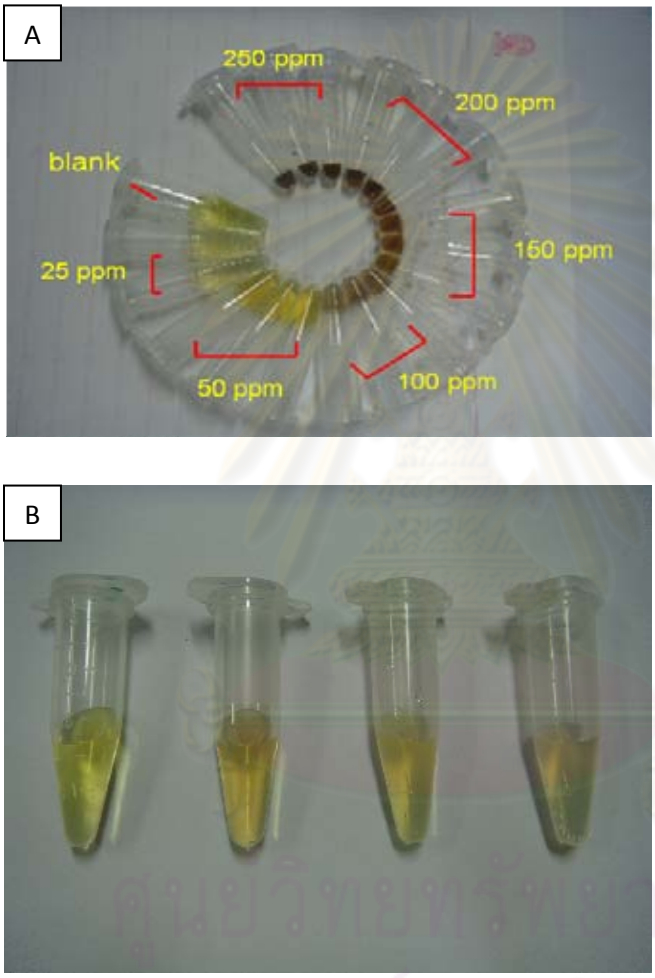
Sensitivity of this experiment was measured by ΔCR value. The difference in ΔCR of AuNPs and AgNPs considered statistically insignificant. Furthermore, ΔCR of both AuNPs and AgNPs in mixture4 and mixture5 were low. This indicated that the DNA detection by aggregation of metal nanoparticles lacks of sensitivity.

4.3 Oxidation of silver nanoparticles

4.3.1 Effect of silver nanoparticles concentration on oxidation

Direct evidence of the interaction between AgNPs and hydrogen peroxide was illustrated in Figure 4-20. In oxidation process of AgNPs, AgNPs was surrounded by silver ions; they induced aggregation of AgNPs. In higher concentration, the aggregation was clearly detected as the colour changing to deep brown shade. When AgNPs 100 ppm was oxidized, the colour changed from yellow to orange-brown and the absorbance was dropped down, while in 50 ppm AgNPs solution, the colour changing was hardly

observed. Because higher particle population of 100 ppm AgNPs, they leaded to aggregation of silver ion. Comparing between AgNPs 25 ppm and 50 ppm, absorbance of 25 ppm AgNPs was weaker because of the faster oxidation in lower AgNPs concentration. According to the high E^0 of the bigger particles, the aggregation process predominated in higher concentration of AgNPs.



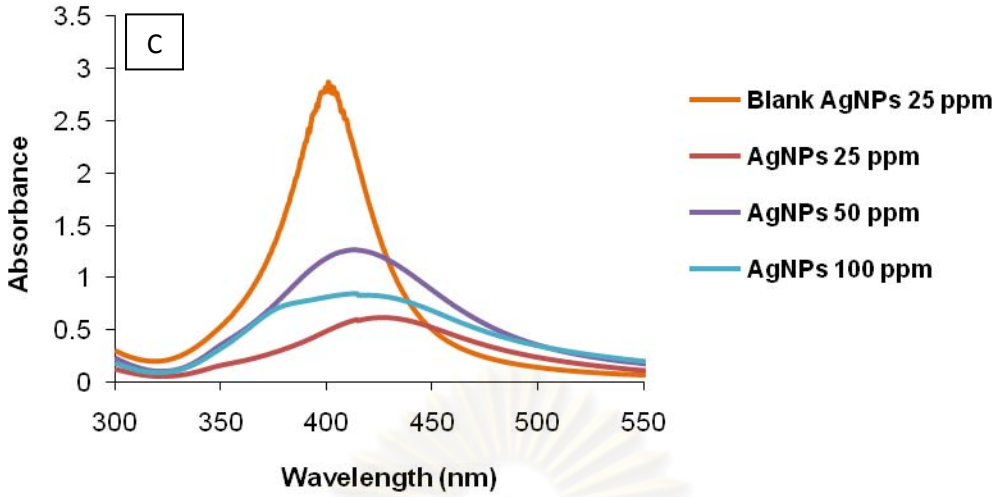


Figure 4-20 Colorimetric detection of oxidized AgNPs. (A) The colour changing of AgNPs before diluting with milli-Q water. (B) The colour changing of AgNPs after diluting with milli-Q water, on the last left is the blank 25 ppm AgNPs solution. The others are oxidized 100, 50, and 25 ppm AgNPs, from left to right, respectively. (C) Absorption spectra of blank AgNPs and oxidized AgNPs in different concentration.

4.3.2 Effect of temperature on oxidation

Generally, hydrogen peroxide decomposes into water and oxygen gas over time but under normal conditions, the decomposition is so slow as to be imperceptible. The rate of decomposition is dependent on the temperature and concentration of the peroxide. At high temperature, the rate of decomposition is higher than the rate of oxidation of AgNPs. As a consequence, the efficiently of oxidation of AgNPs is reduced. The effect of temperature to the oxidation of AgNPs was shown in figure 4-21.

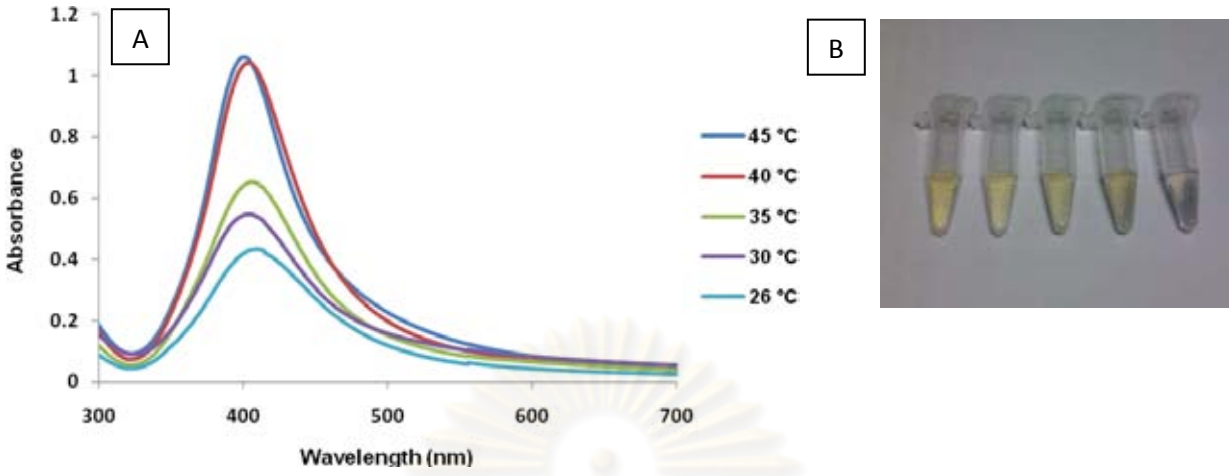


Figure 4-21 Effect of temperature to the oxidation of AgNPs. The UV-vis spectra (A) and the colour changing (B) of AgNPs oxidized at 45, 40, 35, 30 and 26 °C, from left to right, respectively.

4.3.3 Effect of hydrogen peroxide on oxidation

The effect of hydrogen peroxide was shown in figure 4-22. The higher concentration of peroxide used, the more oxidation was observed. The hydrogen peroxide used is over excess for oxidation of AgNPs. Furthermore, is not stable under direct sunlight, freshly prepared hydrogen peroxide was recommended. The oxidation completed within 15 minutes after that the excess amount of hydrogen peroxide gradually decomposed.

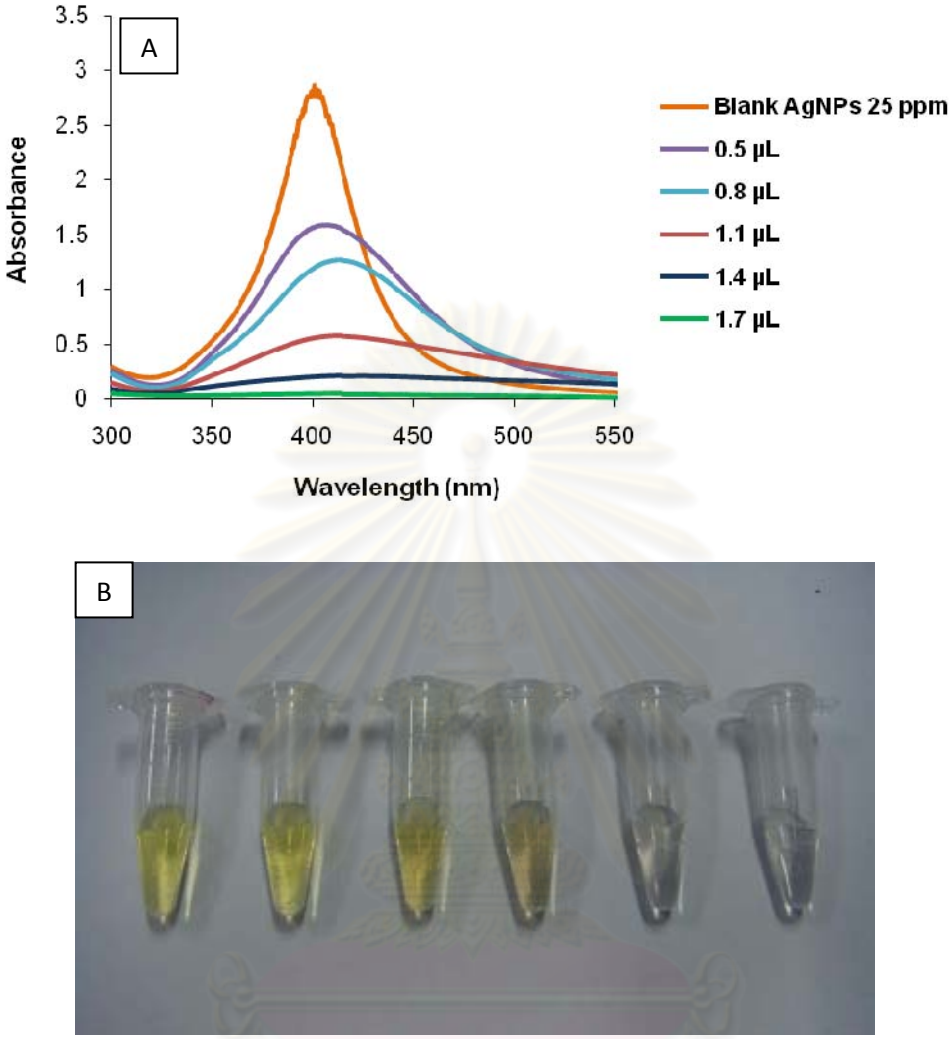


Figure 4-22 Effect of hydrogen peroxide to the oxidation of AgNPs. (A) The UV-vis spectra of AgNPs oxidized by different volume of hydrogen peroxide. (B) The colour changing of AgNPs after oxidizing, the first one from the left vial is blank 25 ppm AgNPs solution. The others are AgNPs oxidized with 0.5, 0.8, 1.1, 1.4, and 1.7 µL of hydrogen peroxide, from left to right, respectively.

Silver ion was not only increased the charge screening effects in the solution but also acted like a catalyst that bound AgNPs together resulting in faster aggregation. The smaller size of non-aggregated AgNPs and aggregated AgNPs was oxidized in different

rate. Thus, the λ_{max} obtained from oxidation process was much weaker than the one obtained from aggregation process.

4.3.4 Effect of DNA on oxidation

Addition of ssDNA into AgNPs solution before oxidation caused the stabilization of AgNPs by ssDNA and thus prevented silver ion deposition onto AgNPs. As a consequence, the oxidation process is faster than the aggregated AgNPs. Therefore, adding more ssDNA promote the oxidation. From this experiment, the addition of 4 μL of ssDNA, the complete oxidation of AgNPs to silver ion was observed from the colourless solution as shown in figure 4-23 (mixture1) and figure 4-24 (mixture2).

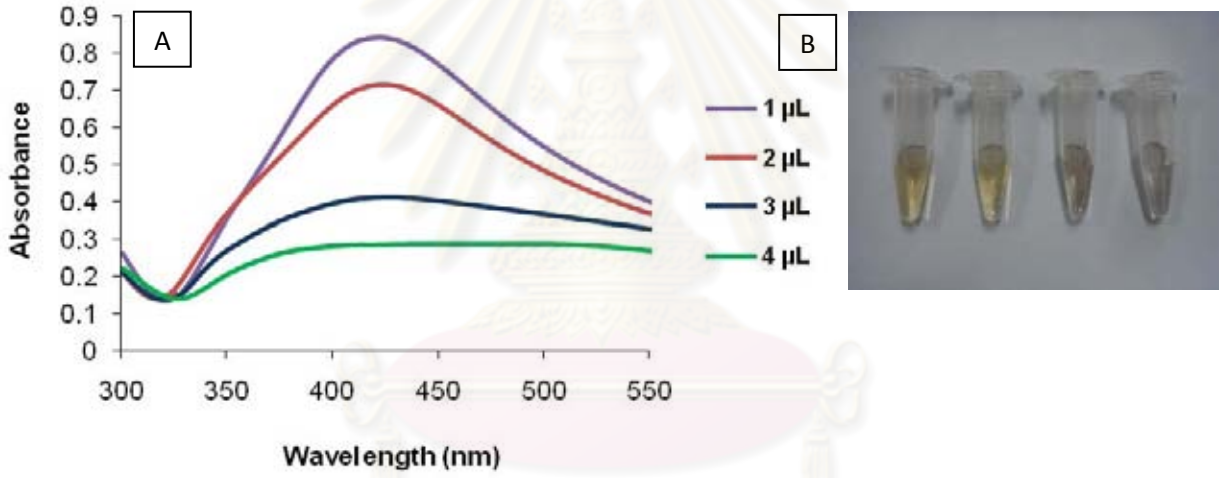


Figure 4-23 Oxidation of AgNPs stabilized by mixture1. (A) The UV-vis spectra of oxidized AgNPs. (B) The colour changing of AgNPs containing 1, 2, 3 and 4 μL mixture1, from left to right, respectively.

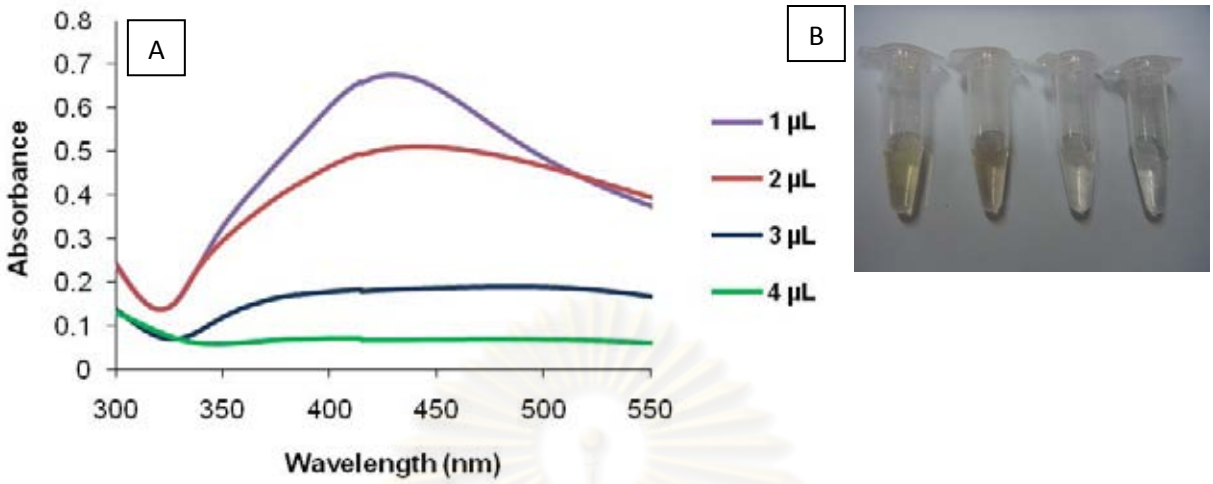


Figure 4-24 Oxidation of AgNPs stabilized by mixture2. (A) The UV-vis spectra of oxidized AgNPs. (B) The colour changing of AgNPs containing 1, 2, 3 and 4 µL mixture2, from left to right, respectively.

Single and double standed DNA have different binding affinity onto AgNPs surface due to their electrostatic properties. In figure 4-25, AgNPs solution containing dsDNA was not absorbed onto the particles. As a consequence, the silver ion from oxidized AgNPs bound onto the remaining AgNPs in solution. As the aggregation proceeded, the oxidation with hydrogen peroxide was then prevented.

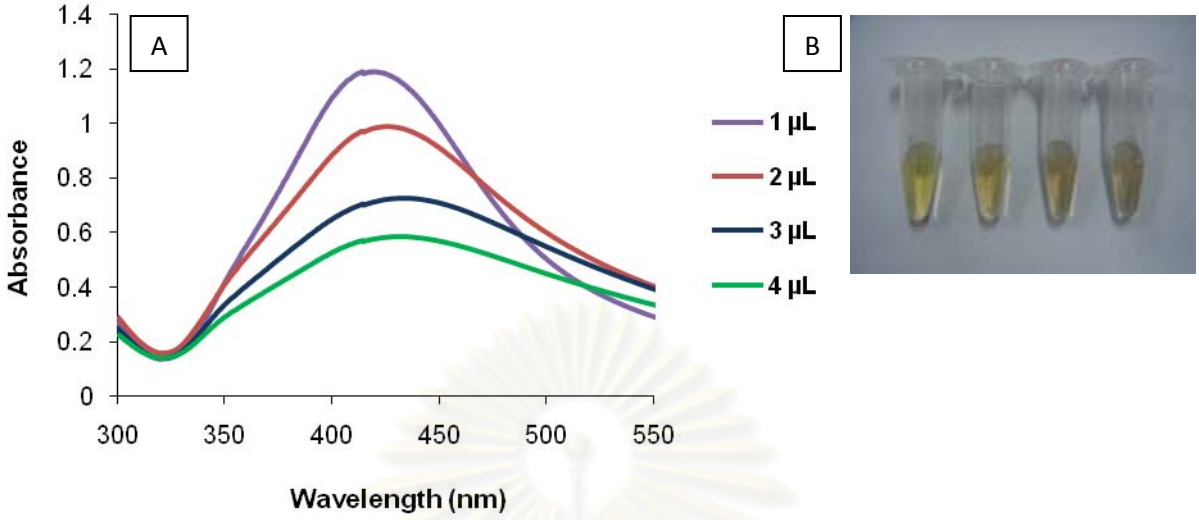


Figure 4-25 Oxidation of AgNPs stabilized by mixture3. (A) The UV-vis spectra of oxidized AgNPs. (B) The colour changing of AgNPs containing 1, 2, 3 and 4 µL mixture3, from left to right, respectively.

The Experiment condition that made the oxidation of silver nanoparticles stabilized by mixture1-3 clearly detected with naked eye was shown in figure 4-26

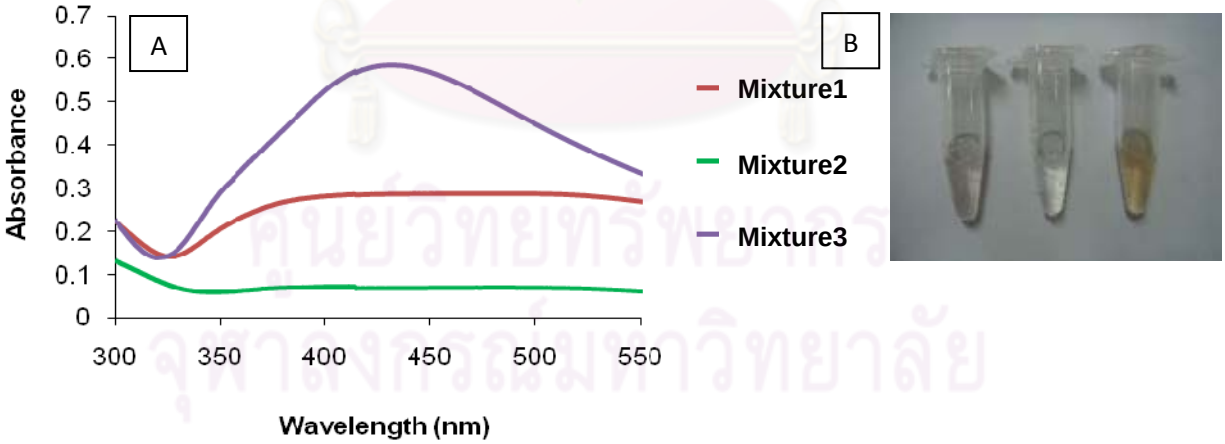


Figure 4-26 Comparison of effect of mixture1-3 to the oxidation of AgNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AgNPs stabilized by 4 µL mixture1-3 from left to right, respectively.

4.3.5 Colorimetric detection of non-complementary DNA, complementary DNA and 1-mismatched complementary DNA

As in aggregation experiment, the affinity of DNA onto AgNPs surface was ranked from mixture4, mixture5 and mixture3. The oxidation efficiency was cooperative with the stabilization efficiency of DNA. Therefore, the colour changing in mixture5 stabilized silver nanoparticles was the easiest to be detected with the naked eye and mixture4 and mixture3 is the second and the third, respectively. The comparison between mixture4, mixture5 and mixture3 stabilized metal nanoparticles was shown in figure 4-27

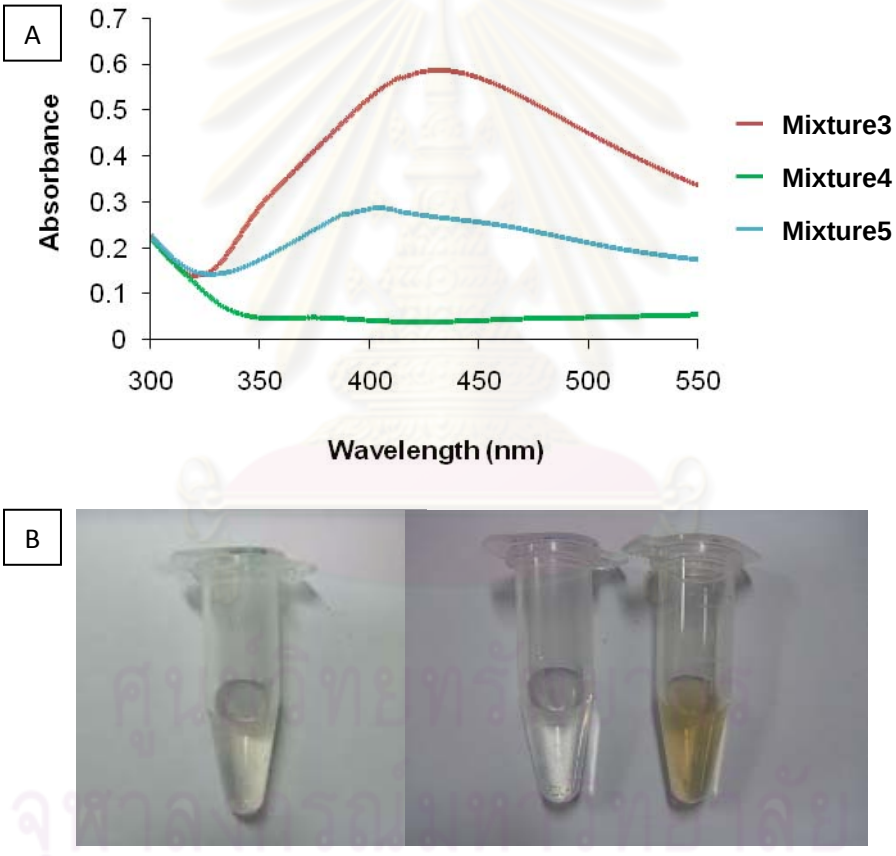


Figure 4-27 Comparison of mixture3, mixture4 and mixture5 stabilized AgNPs. (A) The UV-vis spectra and (B) the colour changing of unmodified AgNPs stabilized by, from left to right, mixture5, mixture4 and mixture3, respectively.

4.4 Comparison of sensitivity between aggregation and oxidation of silver nanoparticles

The colorimetric response (CR) of aggregation and oxidation method is defined in table 4-2.

Table 4-2 CR of aggregation and oxidation method of silver nanoparticles

Method	Type of DNA	CR	ΔCR (comparing with mixture3)
Oxidation	mixture3	0.15	0
	mixture4	0.73	0.58
	mixture5	0.30	0.15
Aggregation	mixture3	0.13	0
	mixture4	0.04	0.09
	mixture5	0.08	0.05

Obviously, ΔCR of oxidation was higher than ΔCR of aggragation both in non-complementary DNA and 1-mismatched complementary DNA. The ΔCR of non-complementary DNA was 6 times higher but for the 1-mismatched complementary DNA was 3 times higher. This difference in ΔCR of oxidation and aggregation method considered statistically significant.

CHAPTER IV

CONCLUSION

5.1 Conclusion

We have developed a novel metal nanoparticle-based colorimetric *Leptospira*'s DNA detection method, using the aggregation of unmodified gold and silver nanoparticles and the oxidation behavior of unmodified AgNPs as the sensing element. Besides the common advantages of colorimetric assay such as simplicity, high sensitivity, and low cost, our method using unmodified AgNPs could further simplify the experimentation since it is a label-free nanoparticle. Sensitivity of the aggregation of gold and silver nanoparticles considered statistically insignificant. This indicated the less effectiveness of aggregation of metal nanoparticles. Comparing with the aggregation method, the oxidation method exhibits 6 times and 3 times stronger colour alterations. Thus, this novel oxidation procedure can be used as a new venue of research exploiting the advantages of the oxidation of AgNPs.

5.2 Future Perspective

- Optimizing the experimental condition to meet the most effective DNA detection
- Use genomic DNA
- Use PNA to increase the sensitivity of the detection

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APPENDICES

APPENDIX A

REAGENTS AND PREPARATIONS

1. Stock 1% hydrogen tetrachloroaurate (100 mL)

$HAuCl_4 \cdot 3H_2O$	1	g
Milli-Q water	100	mL

2. Stock 38.8 mM sodium citrate (100 mL)

$C_6H_5Na_3O_7 \cdot 2H_2O$	1.1411	g
Milli-Q water	100	mL

3. 2x Saline Sodium Citrate buffer (2x SSC)

$C_6H_5Na_3O_7 \cdot 2H_2O$	0.0882	g
NaCl	0.1753	g
Milli-Q water	100	mL

pH was adjusted to 7.0 with HCl

4. Stock Hybridization buffer (10 mL)

CH_3NO	0.5	mL
Dextran sulfate	0.1	g
2x SSC	1	mL
Milli-Q water	8.5	mL

APPENDIX B

COLORIMETRIC RESPONSE CALCULATION

Equation

$$CR = (PB_0 - PB_I)/PB_0$$

$$PB = A_{colour}/(A_{colour} + A_{baseline})$$

Where

A is the absorbance at the maximum absorption of the yellow band (400 nm) for AgNPs or red band (520 nm) for AuNPs and at the baseline (700 and 800 nm for AgNPs and AuNPs, respectively) in a UV-vis spectrum

PB_0 is the baseline/colour ratio of the control sample (before induction of a colour changes; blank solution), while PB_I is the value obtained after the colorimetric aggregation or oxidation

Biography

Mr. Apiratt Thitimon was born on November 8, 1983 in Bangkok, Thailand. He graduated with the Bachelor Degree of Chemistry in Faculty of Science at Chulalongkorn University, Bangkok in 2006; he enrolled in the Master Degree program of Chemistry, Faculty of Science at Chulalongkorn University, Bangkok in 2006 and completed the program in 2009