การตรวจวัดแอลเลอเจนในน้ำยางธรรมชาติโดยใช้แอนติบอดีจับคู่กับอนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์

นายอาณัติ ฤทธิ์เดช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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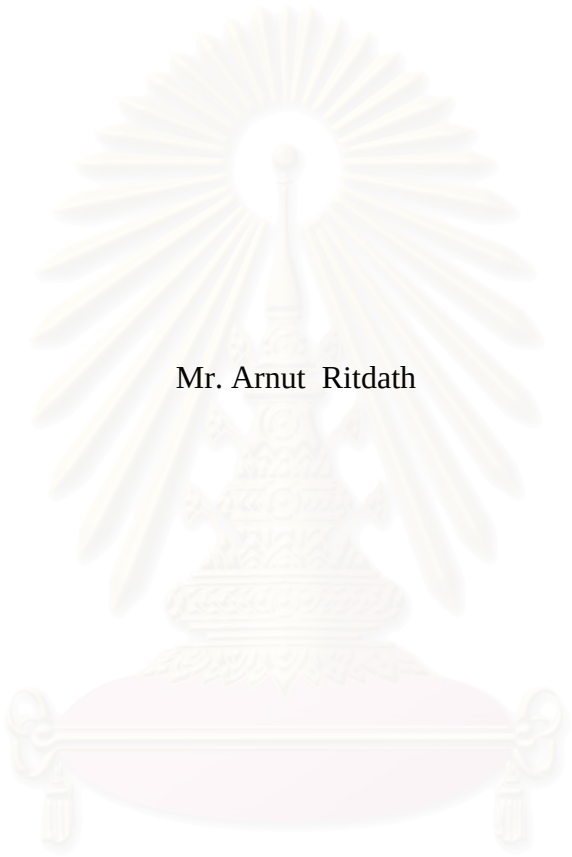
ปีการศึกษา 2549

DETECTION OF NATURAL RUBBER LATEX ALLERGENS USING ANTIBODY CONJUGATED WITH FLUORESCENT DYE-DOPED SILICA NANOPARTICLES

Mr. Arnut Ritdath



A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

Faculty of Science

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Thesis Title	Detection of natural rubber latex allergens using antibody conjugated with fluorescent dye-doped silica nanoparticles
By	Arnut Ritdath
Field of Study	Biochemistry
Thesis Advisor	Associate Professor Jariya Boonjawat, Ph. D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master's Degree.

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นายอาณัติ ฤทธิ์เดช : การตรวจวัดแอลเลอเจนในน้ำยางธรรมชาติโดยใช้แอนติบอดีจับคู่กับอนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์ (DETECTION OF NATURAL RUBBER LATEX ALLERGENS USING ANTIBODY CONJUGATED WITH FLUORESCENT DYE-DOPED SILICA NANOPARTICLES) อ.ที่ปรึกษา : รศ.ดร.จริยา บุญญวัฒน์, 106 หน้า

ในงานวิจัยนี้ ได้สังเคราะห์อนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์ [Tris (2,2'-bipyridyl dichlororuthenium (II) hexahydrate: Rubpy] ไว้ภายในโดยใช้วิธีน้ำในน้ำมัน อนุภาคที่ได้มีรูปร่างเป็นเอกภาพคือมีลักษณะกลม มีขนาดของอนุภาคโดยเฉลี่ยเท่ากับ 65 ± 5 นาโนเมตร พื้นผิวของอนุภาคถูกดัดแปลงเพื่อจับคู่กับแอนติบอดีต่อแอนติเจนของน้ำยางสกิม (น้ำหนักโมเลกุล 31 และ 39 กิโลดาลตัน) และแอนติบอดีต่อแอนติเจนของน้ำยางข้น (น้ำหนักโมเลกุล 100 กิโลดาลตัน) หลังจากจับคู่กับแอนติบอดีทั้งสองประเภทแล้ว จากการศึกษาโดยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบว่าอนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์ มีขนาดของอนุภาคเฉลี่ยเท่ากับ 50 ± 2 และ 51 ± 2 นาโนเมตร ตามลำดับ อนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์จับคู่กับแอนติบอดีต่อแอนติเจนของน้ำยางทั้งสองประเภท ถูกนำไปใช้ในการตรวจวัดโปรตีนแอนติเจนของน้ำยางและผลิตภัณฑ์จากยางธรรมชาติ ซึ่งแสดงความจำเพาะและความไวสูง โดยแสดงขีดจำกัดของการตรวจวัดถึง 0.01 นาโนกรัมต่อไมโครลิตรของโปรตีนแอนติเจนของน้ำยางสกิมและน้ำยางข้น ในขณะที่วิธีเอนไซม์ลิงค์อิมมูโนซอบเบนท์แอสเซย์ (ELISA) แสดงสัญญาณการตรวจวัดได้ต่ำสุดที่ 1 นาโนกรัมต่อไมโครลิตรของโปรตีนแอนติเจน วิธีวัดโดยใช้อนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์จับคู่กับแอนติบอดีที่พัฒนาขึ้นใหม่ มีความจำเพาะในการตรวจวัด มีความไวสูงกว่าวิธี ELISA 100 เท่า และรวดเร็วกว่าถึง 4 เท่า นอกจากนี้ ยังเป็นวิธีที่สะดวกและประหยัด เนื่องจากใช้ปริมาณตัวอย่างเพียงเล็กน้อย (5 ไมโครลิตร) และตัวอย่างเหล่านั้นไม่จำเป็นต้องทำให้เข้มข้นขึ้นหรือบริสุทธิ์ขึ้น ก็สามารถที่จะใช้อนุภาคนาโนซิลิกาที่โดปด้วยสีย้อมฟลูออเรสเซนต์จับคู่กับแอนติบอดีมาใช้เป็นตัวตรวจวัดได้

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต.....อาณัติ ฤทธิ์เดช.....

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2549

4772567123 :MAJOR BIOCHEMISTRY
KEY WORD :LATEX PROTEIN ALLERGENS / FLUORESCENT DYE-DOPED SILICA NANOPARTICLES/ DOT IMMUNOASSAY
ARNUT RITDATH: DETECTION OF NATURAL RUBBER LATEX ALLERGENS USING ANTIBODY CONJUGATED WITH FLUORESCENT DYE-DOPED SILICA NANOPARTICLES.
THESIS ADVISOR: ASSOC. PROF. JARIYA BOONJAWAT, Ph.D., 106 pp

In this research, the tris (2,2'-bipyridyl dichlororuthenium (II) hexahydrate: Rubpy)-doped silica nanoparticles (DSNP) were synthesized by using a water-in-oil microemulsion method. The DSNP are shown to be uniform spherical shape with average diameter of 65 ± 5 nm. The surfaces of DSNP were modified and conjugated with antibody against skim latex protein antigens (31 and 39 kilodalton) and antibody against concentrated latex protein antigens (100 kilodalton). After conjugation with antibody, the DSNP were apparently uniform in size, 50 ± 2 and 51 ± 2 nm respectively. Utilization of the DSNP conjugated with antibody against skim latex proteins and concentrated latex protein antigens for the detection of protein antigens in natural rubber latex and rubber products indicated that this method is specific and showed very high sensitivity with detection limit at 0.01 ng/ μL for both of skim latex antigen and concentrated latex antigen. On the contrary, the Enzyme-Linked ImmunoSorbent Assay (ELISA) exhibited signals at the detection limit only 1 ng/ μL. This newly developed method using DSNP conjugated with antibody showed 100-fold higher specificity, and 4-time more rapid than ELISA. In addition, this method is practical and economic, because sample can be used with no need for concentration or purification, the trace amount of sample is enough to be determined by using DSNP conjugated with antibody as nanoprobe.

Department.....Biochemistry.....Student's signature.....Arnut Ritdath.....
Field of study.....Biochemistry.....Advisor's signature.....Jariya Boonjawat.....
Academic year...2006.....

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ABBREVIATIONS

Ab	=	Antibody
Ag	=	Antigen
AFM	=	Atomic force microscope
CLP-Ab	=	Antibody against concentrated latex 60% protein
CLP-Ag	=	Concentrated latex 60% protein antigen
PRSV-Ab	=	Antibody against papaya ringspot virus
SLP-Ab	=	Antibody against skim latex protein
BCIP	=	5-bromo-4-chloro-3-indoyl phosphate
DSNP	=	Rubpy-doped silica nanoparticles
ELISA	=	Enzyme link immunosorbent assay
ES-EM	=	Environmental scanning electron microscope
FI	=	Fluorescent intensity
FTIR	=	Fourier Transform Infrared Spectrophotometer
IgG	=	Immunoglobulin G
kDa	=	kilodalton
NBT	=	Nitroblue tetrazolium
ng	=	nanogram
NRL	=	Natural rubber latex
PRSV	=	Papaya ringspot virus
PRSV-Ag	=	Papaya ringspot virus antigens
PRSV-IL	=	Papaya ringspot virus infected leaf
PRSV-NIL	=	Papaya ringspot virus non-infected leaf
RIAs	=	Radioimmunoassays

$[Ru(phen)_3]Cl_2$	=	Tris(1,10-phenanthroline) ruthenium(II) chloride
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLP-Ag	=	Skim latex protein antigen
SNP	=	Silica nanoparticles
TEM	=	Transmission Electron Microscope
TEOS	=	Tetraethylorthosilicate
TMTD	=	Tetramethyl thiuram disulfide
W/O	=	water-in-oil
ZnO	=	Zinc oxide

CHAPTER I

INTRODUCTION

1.1 Nanoparticle

Nanotechnology is a field of applied science and technology covering a broad range of topics. The main unifying theme is the control of matter on a scale smaller than 1 μm, normally between 1-100 nm. It is a highly multidisciplinary field (Qhobosheane *et al*., 2001). One of the most important aspects of this branch is the preparation and development of nanomaterials, such as nanoparticles.

A nanoparticle (or nanopowder) is a particle with at least one dimension less than 100 nm (Ostiguy *et al.*, 2006). Nanoparticle can be divided into two groups, the first group is natural nanoparticles, which are the result of naturally occurring phenomenon and there is no man made chemical, such as papaya ringspot virus (100 – 800 nm length and 12 nm wide) and influenza virus (H5N1: 80 – 120 nm) (Stephenson I. and Nicholson K.G., 1999). The second group is manufactured nanoparticles, which are artificially manufactured particles smaller than 100 nm in size, which have changed properties and/or functionalities (Oberdorster *et al.*, 2005). Gold nanoparticles with size ranging from 5 to 110 nm (Tapan *et al.*, 2001) and silica nanoparticle having size about 60 – 70 nm (Santra *et al.*, 2001; Lian et al., 2004) are examples of manufactured nanoparticles. Recently silica nanoparticles were used to encapsulate labeling reagents for the sensitive detection of several biological species. These nanoparticles are doped with a large number of dye molecules inside a silica matrix and provide greatly enhanced luminosity. In addition, the silica matrix protects the dye molecules from environmental oxygen, thereby providing much higher photostability (Zhao *et al.*, 2004).

1.2 Fluorescent dye-doped silica nanoparticles

Fluorescence-based techniques have generated widespread acceptance in many biochemical assays. For detecting small amount of analytes, fluorescent molecules are often exploited as efficient signal transducers. However, sample analyses in small volumes can be particularly difficult when limited amounts of signaling probes are present. As a consequence dye-doped silica nanoparticles have been used to aid in analysis (Santra *et al.*, 2001; Qhobosheane *et al.*, 2001; Lian *et al.*, 2004). Improvements in these areas are still needed to make bioassays faster, more accurate and precise, and to remove the need for time-consuming amplification protocols. Nanoparticles show great promise in this regard, largely due to their unique optical properties, high surface-to-volume ratio, and other size-dependent qualities. By using nanoparticles, researchers are able to increase sensitivity, enhance signal detection, and generate better reproducibility (Santra *et al.*, 2001).

Silica-based nanoparticles have been prepared and doped with organic and inorganic dye molecules (Santra *et al.*, 2001; Tapec *et al.*, 2002). These particles have demonstrated great promise in the fields of DNA detection, bacterial detection, and chemical sensing (Qhobosheane *et al.*, 2001; Hilliard *et al.*, 2002; Tapec *et al.*, 2003; Zhao *et al.*, 2004). As an example, when conjugated with antibody for probing target antigen, a silica nanoparticle contains thousands of dye molecules within one particle. Consequently, a significantly greater signal can be resulted from signal binding events, which leads to an increase sensitivity in comparison to single fluorophore-labeled assays (Zhao *et al*., 2004).

The bioconjugation of proteins are also allowing these particles to be used in various labeling and sensing protocols, such as *Escherichia coli* O157:H7 detection and chemical sensing (Qhobosheane *et al.*, 2001; Zhao *et al.*, 2004).

The advantages that silica nanoparticles offer in these fluorescence detection methods are largely due to their sensitivity, though the versatility of the silica substrate also plays a major role. In the development of highly effective nano-luminescent probes, silica is more appropriate shell material than polymers. Various dye – doped polymer nanoparticles have been developed (Gao *et al.*, 2006), and organic dye molecules, owing to their hydrophobic properties, are easily incorporated into the polymer matrix to form luminescent nanoparticles. Whilst polymer nanoparticles, due to their hydrophobicity and bioincompatibility, are not very well suited for bioanalysis, silica, on the other hand, has several characteristics, which make it a very attractive alternative substrate. First, it is not subject to microbial attack, and there are no swelling or porosity changes with variation in pH (Jain *et al.*, 1998). Silica is also chemically inert and optically transparent. The shell of the silica particles can act as an isolator, limiting the effect of the outside environment on the core of the particles. This is particularly important for dyes, which are sensitive to certain solvents and soluble species in the buffer solutions. Photobleaching and thermally induced degradation are primary processes that reduce the operational lifetime of a dye (Jang *et al.*, 1999). By encapsulating the dye within chemically and thermally inert silica shell, photobleaching and photodegradation of the dye can be minimized (Santra *et al.*, 2001). For bioconjugation protocols, a large amount of chemical reactions already exist for easy functionalization of silica particles to biomolecules. Consequently, the synthesis protocol for the fabrication of silica-based particles and probes is relatively simple and straightforward and requires no special conditions.

1.3 Fabrication of the fluorescent dye-doped silica nanoparticles

The reverse or water-in-oil microemulsion (w/o) is a robust and efficient method for nanoparticle fabrication which yields monodispersed particles in the nanometer size range. W/O microemulsions are isotropic and thermodynamically stable single-phase systems, consisting of three primary components: water, oil, and a surfactant (Triton X-100). Water nanodroplets are formed in the bulk oil phase, which act as a confines media, or nonoreactors, for the formation of discrete nanoparticles and dynamic produced silica nanoparticles with 60 nm diameters with minimal size distribution.

By using these fabrication methods, tetraethylorthosilicate or TEOS (Figure 1.1) is using as a precursor for synthesis of the silica nanoparticles (Figure 1.2). The versatile silica matrix and modification protocols allow them to be conjugated with various functional groups which allow for specific applications and sensing assays.

For inorganic dye-doped silica nanoparticles, Tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate (Rubpy dye) molecules (Figure 1.3) are simply placed into the reverse microemulsion system (Figure 1.4) where they are entrapped in the nanoreactor water pools (Santra *et al.*, 2001). The silica matrix then encapsulates the dye molecules and photostable luminescent particles (Figure 1.5).

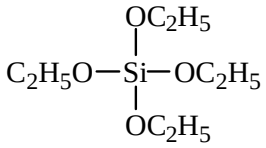


Figure 1.1 Chemical structure of tetraethyl orthosilicate (TEOS)

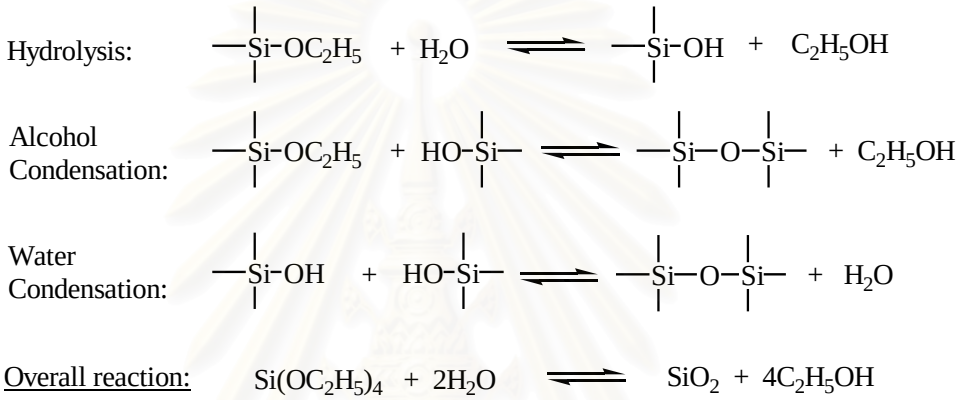


Figure 1.2 Hydrolysis and condensation reaction of TEOS to form silica

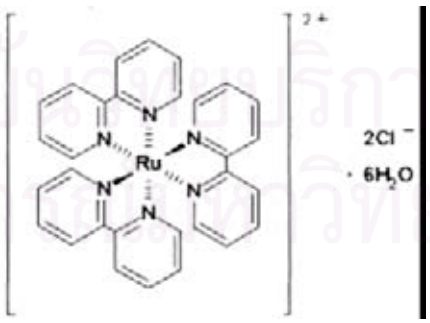


Figure 1.3 Chemical structures of tris (2,2'-bipyridyl) dicholororuthenium (II) hexahydrate (Bagwe *et al.*, 2003)

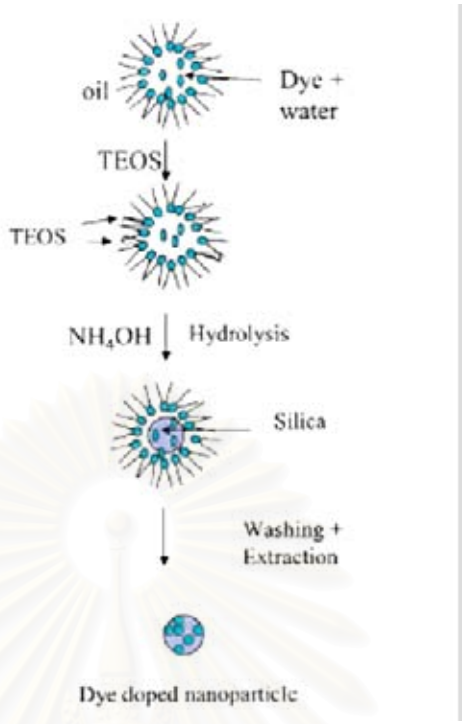


Figure 1.4 Method of preparation of dye doped silica nanoparticles using reverse micelles. (Bagwe *et al.*, 2003)

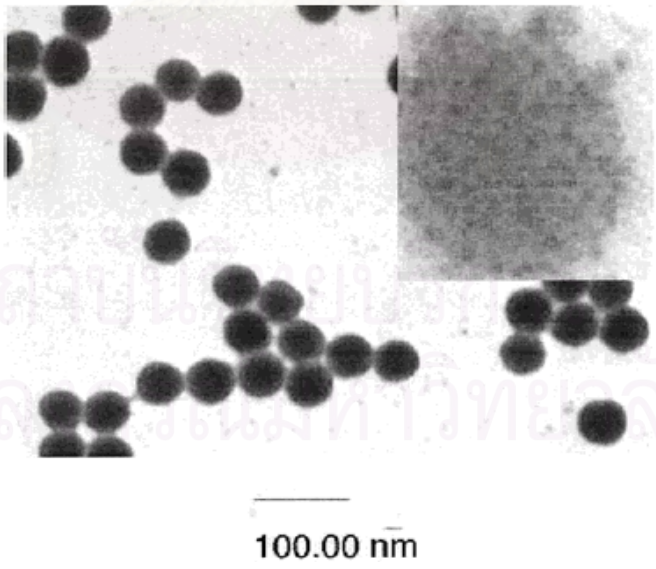


Figure 1.5 TEM image of Rubpy-doped silica nanoparticles at 70,000 magnification (inset showing a region of 184,000 magnification) (Santra *et al.*, 2001)

1.4 Biofunctionalization of silica nanoparticles

Although inorganic nanoparticles can be prepared from various materials by several methods, their coupling and functionalization with biological components are carried out with only a limit number of chemical methods. To prepare such conjugates from nanoparticles and biomolecules, the surface chemistry of the nanoparticle must be such that the ligands are fixed to the nanoparticles and thus, possess terminal functional groups that are available for biochemical coupling reactions. Binding to particle surfaces is frequently carried out via thiol group interaction (Galow *et al.*, 2000; Qhobosheane *et al*., 2001), but in most cases, a simple thiol bond to the particle surface is not sufficient to accomplish a permanent linkage. Instead, equilibrium is established with dynamic ligand exchange. To avoid this, a shell of silica is often grows on the particle itself by means of sol-gel techniques (Gerion *et al.*, 2001; Tapec, Zhao and Tan., 2003), and the linkage group pointing outwards are added as functionalized alkoxysilanes during the polycondensation process (Gerion *et al.*, 2001). The result is a relatively compact silica shell and a tight coating of the surface with coupling groups. Silica-based solid supports are effectively used for the immobilization of various biomolecules such as antibodies, enzymes, proteins, and DNA, for applications ranging from biosensors to interaction studies (Fang *et al*., 2001).

An effective bioconjugation approach for the immobilization of proteins (e.g., enzymes and antibodies) onto silica nanoparticles is to chemically modify the particle's surface with amine-reaction groups (Qhobosheane *et al.*, 2001). Subsequently, free amine groups on the surface of biomolecules can be covalently attached to the nanoparticle, leading to chemically bind silica nanoparticles conjugates. The silica nanoparticles are first silanized using trimethoxysilyl-

propyldiethylenetriamine (DETA) and then treated with succinic anhydride. The resulting carboxylate-modified particles are washed with deionized water. Two different approaches are then used to further modify the nanoparticles. The first approach uses carbodiimide hydrochloride in an activation buffer to produce *o*-acylisourea intermediate on the nanoparticle's surface. The available amine groups on the biomolecules are then bound to the modified silica nanoparticles through amine bonds by immersion of the nanoparticles in a suitable concentration of the biomolecule solution (Qhobosheane *et al*., 2001). The second approach adds a water-soluble *N*-hydroxysuccinimide compound along with carbodiimide hydrochloride to the carboxyl-modified silica nanoparticles. The active ester intermediate, which is more stable for hydrolysis, and thus, the coupling efficiency of biomolecules to the nanoparticles is increased (Zhao *et al.*, 2004). These approaches offer a more stable binding between the silica nanoparticles and biomolecules, which can last for several weeks or until the biomolecules become inactive.

1.5 Bioanalytical application for silica nanoparticles

The development of nanoparticles has shown great promise in bioanalysis due to their unique optical properties, high surface-to-volume ratio, and other size-dependent qualities. When combined with surface modifications and composition control, these properties provide probes for highly selective and ultra-sensitive bioassays due to their high quantum yield (Bui *et al.*, 2006). However, the sensitivity of methods using organic dyes is limited by a low number of recognizable components in trace amounts of biosamples, which results in an inadequate amount of detection signals. Dye molecules also typically suffer from rapid photobleaching (Gerion *et al.*, 2001), resulting in unstable and inaccurate fluorescence signals.

For effective celluler labeling techniques, biomarkers need to have excellent specificity toward biomolecules of interest, and also optically stable signal transducers. The dye-doped silica nanoparticles described offer ideal photostability and are easily conjugated through the use of the silica matrix to target-specific molecules, such as antibodies (Santra *et al.*, 2001; Zhao *et al.*, 2004).

The use of nanoparticles for biomarking has been effectively demonstrated for the labeling of leukemia cells (Qhobosheane *et al.*, 2001). A mouse anti-human CD-10 antibody has been used as the recognition element on CNBr-pretreated nanoparticles and then washed with phosphate-buffered saline to remove unbound nanoparticles. Fluorescence microscopy is then used to detect the image of the leukemia cells, whereupon the brightly fluorescent cells have been detected and correlated well to optical images (Figure 1.7).

Another approach is to use antibody-labeled in order to show great promise in the detection of single bacterium. A rapid bioassay for the precise determination of a single bacterium has been developed with clear implications in the food and clinical

industries, as well as for the identification of bioterrorism agents. Traditional methods used to detect trace amounts of bacteria tend to be laborious and time-consuming due to the complicated assay procedures (Gerion *et al.*, 2001). Rapid single bacterium detection in a large-volume sample is not yet possible, but by using dye-doped silica nanoparticle, a nanoprobe molecule has been developed to allow for the rapid, sensitive and accurate detection of single *E. coli* O157: H7 bacterium (Zhao *et al.*, 2004). Antibodies against *E. coli* O157: H7 have been conjugated to Rubpy-doped silica nanoparticles to form the nanoprobe complex, which is used to bind and label the antigen on the *E. coli* O157: H7 cell surface (Figure 1.8). The resultant fluorescence signals can then be monitored using various techniques. This bioassay takes ~20 minutes to be completed and is a convenient, highly selective method. Because the nanoparticle brings a large amount of dye molecules to the surface of the bacterium, single bacterium detection has been made possible.

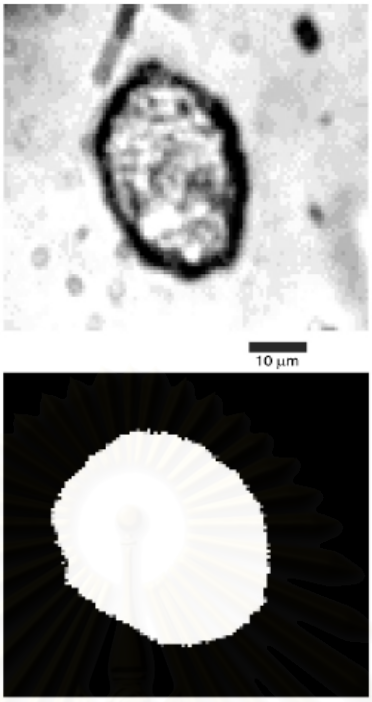


Figure 1.6 Cell staining images by optical and fluorescence microscopy

Top panel optical images of PTK2 cell. Bottom panel fluorescence image of PTK2 cell, after staining with the dye-doped nanoparticles with membrane functional group, the cells have strong fluorescence. The cell size is around 20 µm. (Qhobosheane *et al.*, 2001)

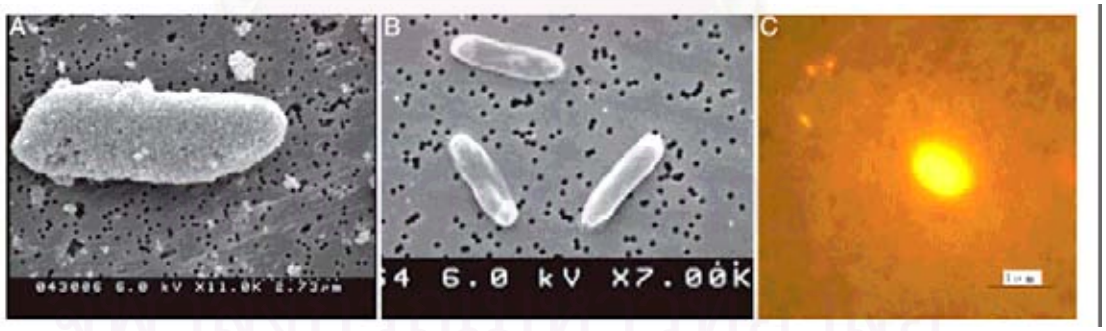


Figure 1.7 Images of bacterial cells.

(*A*) Scanning electron microscope image of *E. coli* O157:H7 cell incubated with antibody-conjugated nanoparticles. (*B*) Scanning electron microscope image of *E. coli* DH5α cell (negative control) incubated with nanoparticles conjugated with antibody for *E. coli* O157:H7. (*C*) Fluorescence image of *E. coli* O157:H7 after incubation with antibody-conjugated nanoparticles. The fluorescence intensity is strong, enabling single-bacterium cell identification in aqueous solution. (Zhao *et al.*, 2004)

1.6 Natural rubber latex proteins and latex allergy

Natural rubber is obtained from the milky secretion (latex) of various plants, but the only important commercial source of natural rubber (sometimes called Para rubber) is the tree *Hevea brasiliensis.*

Hevea Brasiliensis, the commercial rubber tree, has been introduced into Thailand about one hundred years ago. Natural rubber latex has been tapped, acid coagulated, dried, and prepared in the form of rubber smoke sheet and block, which are the raw material of tires for cars and several others “natural rubber latex (NRL) products”

Latex can also be concentrated by using a centrifuge to obtain concentrated latex 60%, according to the dry rubber content (DRC). Concentrated latex 60% is the important raw material for manufacturing thin-film products, such as gloves, condom, balloon, which are house-hold products. For health-care workers, the wide-spread hepatitis virus and AIDS have lead to the daily use of examination gloves, surgical gloves, house-hold gloves all over the world, and requirement of natural rubber concentrated latex have increased significantly. In the past two decades, latex allergies caused by frequent exposures to natural rubber products have been reported. Type I or immediate type latex allergy has been found to be caused by latex protein-allergens, and Type IV delayed hypersensitivity or chemical latex allergy has been shown to be caused by chemicals added in the vulcanisation process. Type I: latex protein allergy is systemic allergic reaction caused by circulating IgE antibodies to the proteins of natural rubber latex. Direct contact with the medical product is not needed for sensitisation to latex. Allergenic latex proteins are also absorbed on the glove powder, when latex gloves are snapped on and off, these become airborne and can be directly inhaled. Symptoms of latex protein allergy can vary from rhinitis

conjunctivitis, asthma and in severe cases anaphylaxis and death. Direct exposure to allergenic latex proteins can occur at mucosal or serosal surface by repeated operations in spina bifida patients. In 1991 the U.S Food and Drugs Administration (FDA) (Gold *et al.*, 1991) has warned of the risk of the life-threatening Type I allergy associated with NRL products. Ten to eleven percent of health care workers have already become sensitized, and over 2 percent have occupational asthma as a result of latex proteins exposure (Turjanmaa *et al.*, 1987).

The total protein content of field latex is about 1.0- 1.8 %. They are distributed in three major fractions; the rubber phase (27%), the C-serum (48%) and within the lutoid or the B-serum (25%) (Yeang H.Y., 2002). The International Union of Immunological Society (IUIS) now list 13 natural rubber latex allergens characterized at the molecular level by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) (Table 1.1).

Allergy to natural rubber latex (NRL) proteins are currently a well recognized health problem among subjects using products made of NRL, such as gloves, rubber dam and condom (Palosuo *et al.*, 2002). A major group at risk of latex allergy consists of health care workers, resulting from extended and repeated exposure to NRL proteins. The dose, the duration, and the route of exposure are the determining factors. The problems of allergic reaction to NRL proteins have raised concerns regarding the safety of the NRL products. Reducing the overall protein levels of NRL in medical devices is an initial approach, but to ensure safety, it is essential to identify and protect sensitize individuals and to prevent or minimize further sensitization by controlling exposure to allergenic proteins. This cannot be accomplished without adequate diagnostic tests and reliable methods for allergen quantitation. Although

ongoing research is direct towards quantitation of biologically relevant proteins in NRL, no standard allergen test has been developed (Tomazic-Jezic and Lucas, 2002).

Primarily, the assumption is that the total level of protein is proportional to the level of potential allergens. The Modified Lowry method, a chemical assay that measures all proteins, has therefore become the American Society for Testing Materials (ASTM) standard (D5712) since 1995. It is the first standardized and validated method with great utility for screening NRL products (Table 1.2). As a result of the availability of this standard, the Food and Drug Administration can issue a recommendation that manufacturers may state a total protein level on their products as a guide for users in product selection. With the frequent use of this method, several disadvantages become evident. As manufacturers successfully decrease the protein levels in NRL products, the assay is not sufficiently sensitive to accurately quantitate the remaining proteins. However, NRL proteins even below the detection level could induce a positive skin reaction. Furthermore, the Lowry assay is prone to the interference of chemical additives in NRL products and also includes in the measurement other proteins, which may be added during the manufacturing process. These factors are probably responsible for the generally poor correlation of the Lowry method with the biologically active protein content of the products. At present, there is an initiative to standardize amino acid analysis as another chemical method for the quantitation of NRL proteins. The analysis is based on a complete protein hydrolysis and therefore includes in the measurement of all small peptides and single amino acids that may not have any biologic relevance. However, it is a sensitive analytical method, and it may serve as a good reference method for total protein evaluation. Because of the need for expensive high performance liquid chromatography

equipment and specific technical expertise, this will be an impractical method for routine testing (Tomazic-Jezic and Lucas, 2002).

Table 1.1 Latex allergens recognized by the International Union of Immunological Societies (IUIS)

IUIS code	Identity	Molecular mass (kDa)
Hev b 1	Rubber elongation factor	58
Hev b 2	β-1,3-Glucanase	34
Hev b 3	Small rubber particle protein	24
Hev b 4	Component of microhelix complex	100
Hev b 5	Acidic protein	16
Hev b 6	Hevein precursor	20
Hev b 7	Patatin	42
Hev b 8	Profilin	14
Hev b 9	Enolase	51
Hev b 10	Superoxide dismutase (Mn)	26
Hev b 11	Chitinase	No report
Hev b 12	Lipid transfer protein	9
Hev b 13	Esterase	42

TABLE 1.2 Methods for measurement of natural rubber latex proteins, antigens, and allergens (Tomazic-Jezic and Lucas, 2002)

Method	Type	Advantages	Disadvantages
Modified Lowry method (D5712)	Chemical method, measures total proteins	Standardized, available as a kit	Insufficient sensitivity, chemical interference
Amino acid analysis (HPLC)	Chemical method, measures amino acids	Sensitive, reproducible	Expensive equipment, technical expertise
LEAP	Immunologic method, measures antigenic proteins	Sensitive, available as a kit	Assay format, not standardized
RAST inhibition assay	Immunologic method, measures antigenic or allergenic proteins	Good assay format, very sensitive	Radioactive isotope, not standardized
ELISA inhibition test	Immunologic method, measures allergenic proteins	Good assay format, colorimetric, good sensitivity	Uses human serum, cannot be standardized
ELISA inhibition test (D6499)	Immunologic method, measures antigenic proteins	Sensitive, standardized	New as a standard, unknown reproducibility of standard reagents

HPLC, High performance liquid chromatography; *LEAP*, latex ELISA for antigenic proteins.

1.7 ELISA inhibition test of natural rubber antigenic proteins

In the pursuit of a method for allergen quantitation, the next step after the chemical analyses of total protein content is an immunologic method for the measurement of antigenic proteins. The latex ELISA for antigenic proteins test, designed by Guthrie Research Institute (Beezhold, 1992), is an ELISA that uses rabbit anti-NRL protein antibodies (Table 1.2). This test has a greater sensitivity than the Modified Lowry standard test and is available as a kit. However, the test format requires binding of unknown protein samples to the assay plate, to which rabbit anti-NRL serum is then applied. The protein binding to the plate depends on the overall concentration and the molecular weight of individual proteins. Because the test samples vary in both concentration and size distribution, uniform binding of the sample proteins can be neither ensured nor validated. Two other tests used in research laboratories, the RAST inhibition and the ELISA inhibition test, both are expensive, using radioisotope (in case of RAST), consume more time and laborious, this problem has been avoided by applying a different format. The measurement of antigenic protein levels in both tests is based on the capacity of rabbit anti-NRL serum to react with the NRL protein sample. A test protein is exposed to anti-NRL serum in a separate inhibition step. After the antigen-antibody reaction is completed, the unbound antibodies are exposed to the reference antigen, and the percent of serum inhibition represents the amount of protein in the test sample. The reference antigen is a defined source of NRL protein, which is either passively attached to the assay plates or covalently bound to a solid phase. The inhibition format is both sensitive and reproducible. In addition, this format can be easily used for the quantitation of antigenic proteins with rabbit anti-NRL serum and the quantitation of specific NRL allergenic proteins with human anti-NRL serum (Palosuo *et al*., 1998). However,

neither of these methods can be developed into a standard allergen assay because the potency and specificity of human anti-NRL IgE antibodies cannot be standardized as a reference antiserum.

Although all previously described methods provide valuable approaches and information, none except the Modified Lowry method has been validated or standardized. The lack of sensitivity and specificity of the Modified Lowry method mandate the development of a good standard test. Because the knowledge about the identity of specific NRL allergens was still insufficient for development of an allergen assay, a test for quantitation of antigenic NRL proteins is the next logical step. In collaboration with Guthrie Research Institute and the National Institute for Occupational Safety and Health, a protocol for the ELISA inhibition test is recently accepted as a standard by the ASTM. The 2-step protocol includes inhibition of rabbit anti-NRL serum with a test protein sample as a separate initial step (Figure 1.9). The inhibited serum is transferred from the inhibition plates to the antigen-coated assay plates. The binding of remaining antibody to the plates is visualized by the enzyme-conjugated second antibodies and the appropriate substrate. This method measures all proteins capable of inducing an antibody binding in rabbits, and it is therefore defined as an antigenic protein assay. The inter- and intra-laboratory reproducibility and sensitivity of the test are good (Tomazic-Jezic and Lucas, 2002). However, this ELISA inhibition test is more expensive and time consuming than modified Lowry method.

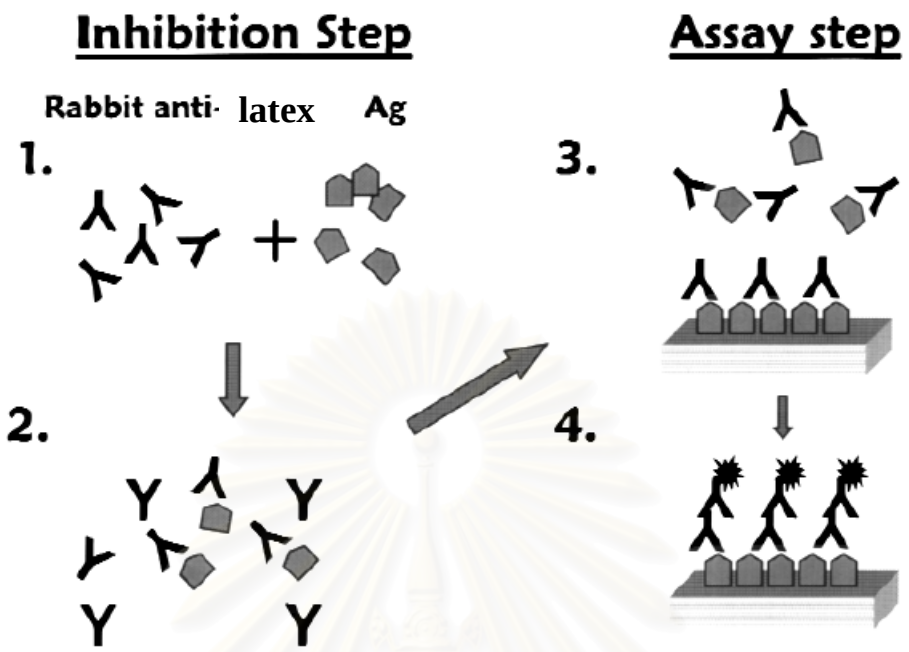


Figure 1.8 ELISA inhibition test (D6499) standard format

Latex antigen is samples of NRL. Rabbit antisera generated by immunization with standard antigen in complete (CFA) and incomplete (IFA) Freund's adjuvant. Inhibition step performed in separate plates, sera transferred in the antigen-coated assay plates. Horse radish peroxidase–conjugated secondary antibodies used to visualize reaction.

1.8 The rational and purposes of this study

Fluoroimmunoassays have been widely used in the biochemical monitoring of target antigen in complex samples, however, these traditional fluoroimmunoassays suffer from the disadvantages of inefficiency of labeling, poor photostability, and relatively low fluorescence intensities of free dyes such as fluorescein isothiocyanate (FITC) (Fang *et al.*, 2001) compared with thousand of Rubpy dye molecule inside silica network. The establishment of both simple and sensitive immunoassay for detection and quantification of trace amount of natural rubber latex proteins, using antibody conjugated with fluorescent dye-doped nanoparticles has entered the field as an extremely promising method of replacing established RIAs, ELISA and traditional fluoroimmunoassays (Cummins *et al.*, 2006).

Antibody-conjugated with fluorescent dye-doped silica nanoparticle fits well to the design of next generation immunoassays with many advantages, such as lower detection limits, lower consumption of reagents, and excellent specificity and sensitivity. In the past few years, one of the most widely studied nanoparticles for labeling was the fluorescent core-shell nanoparticles (Santra *et al.*, 2001; Bagwe *et al.*, 2004; Yang *et al.*, 2004). In comparison to conventional organic dye labels, the fluorescent core-shell nanoparticle labels offer the advantages of both higher photostability to the excitation of light and stronger fluorescent intensity resulting in an amplification factor similar to that of enzyme labels because thousands of fluorescent dye molecules are encapsulated in the matrix that also serves as a shield to protect dye from photobleaching (Santra *et al.*, 2001; Lian *et al.*, 2004; Yang *et al.*, 2004). Fluorescent core-shell nanoparticles also allow for faster analysis because one fewer reaction step is required than when enzyme labels are used. Moreover, they can be prepared easily under mild conditions with inexpensive reagents and simple

procedures. With their uniform sizes and high functionality, they can be modified easily. These advantages make them the ideal dye for various scientific research and clinical applications (Bagwe *et al.*, 2004). Their experimental results suggested that immunoassay based on dye-doped nanoparticle labels is sensitive and significantly more rapid and convenient compared to free dye labels immunoassay.

In this research, a fluoroimmunoassay for NRL protein antigens with functionalized Rubpy-encapsulated silica nanoparticles labels will therefore be developed. It is expected to be a simple and rapid method that can be used in NRL factories to screen for low protein allergen concentrated latex products. Besides, health care users can use this method to select for the NRL products with low protein antigens.

The objectives of this research are:

1. To synthesize the fluorescent dye-doped silica nanoparticles and conjugate with antibody against latex protein antigens.
2. To detect natural rubber latex protein allergens using the fluorescent dye-doped silica nanoparticles conjugated with antibody against latex protein antigens.
3. To evaluate the new method, using fluorescent dye-doped silica nanoparticles conjugated with antibody against latex protein antigens by comparison for detection limit and specificity with the indirect ELISA (ASTM D6499).

CHAPTER II

MATERIALS AND METHODS

2.1 Biological materials

2.1.1 Natural rubber latex

Three types of natural rubber latex were used in this research;

2.1.1.1 Skim latex was the serum fraction after centrifugation of latex kindly provided by Pan Asia Biotechnology Co., Ltd.

2.1.1.2 Concentrated latex was kindly provided by Pan Asia Biotechnology Co., Ltd.

2.1.1.3 Tetramethyl thiuram disulfide (TMTD) and zinc oxide (ZnO) free (TZ-free) concentrated latex Lot No. 1(24/08/05), 2(14/10/05), 3(11/11/05), 4(16/12/05), 5(08/02/06), 6(11/05/06), 6C(11/05/06), 7LG(13/07/06), 7G(13/07/06), 7C(13/07/06), 8N(22/08/06), 8O(22/08/06), 8C(22/08/06), 9A(12/10/06), 9C(12/10/06) and 10(8/11/06) from Pan Asia Biotechnology Co., Ltd.

2.1.2 Commercial rubber products

2.1.2.1 Dental dam was purchased from Hygenic Co.,Ltd.

2.1.2.2 Non-sterile examination glove Lot 06050501 was purchased from Union Rubber Glove Co.,Ltd

2.1.2.3 Disposable nitrile glove (synthetic rubber) Lot 0108207028 was purchased from Touch N Tuff Co.,Ltd

2.1.3 Antibody

2.1.3.1 Alkaline phosphatase-goat anti-rabbit IgG was purchased from Zymed Labolatories, INC.

2.1.3.2 Polyclonal rabbit IgG anti-papaya ringspot virus, was kindly provided by Dr. Srimek Chowpongpang, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University.

2.1.4 Purified viral protein antigen of papaya ringspot virus coat protein

The purified viral protein antigen of papaya ringspot virus (PRSV) was kindly provided by Dr. Srimek Chowpongpang, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University.

2.2 Chemicals

Chemicals used in this research were analytical or reagent grade.

2.3 Methods

2.3.1 Synthesis of the fluorescent dye-doped silica nanoparticles

The fluorescent dye used in this study was Tris(2,2'-bipyridyl) dichlororuthenium(II)hexahydrate (Rubpy dye), which was purchased from Sigma/Aldrich Chemical.

The silica nanoparticle and fluorescent dye-doped silica nanoparticle were performed by water-in-oil (W/O) or reverse microemulsion method according to Santra *et al.* (2001). The W/O microemulsion was prepared by mixing 1.7 mL Triton X-100, 7.14 mL cyclohexane and 1.7 mL *n*-hexanol in 50 mL plastic centrifuge tube, sonicated and added with 340 μL of deionized water. After sonication in sonicate bath

(Elma Sonic; S30H, bath size 1.5 L, 275 watt) for 10 min, 480 μL of 20 mM Rubpy dye in deionized-water (for synthesis the silica nanoparticle, the dye was not added) and 100 μL of tetraethylorthosilicate (TEOS) was added. After mixing for 20 min, 60 μL of 28- 30% NH_4OH was added to initiate the polymerization. The reaction was allowed to continue stirring for 24 h. When the polymerization was completed, an equal volume of acetone was added, and the mixture was vortexed for 1 min to break the microemulsion state. The solidified particles were collected by centrifugation at 3000 *g* for 10 min and washed 3 times with 95% ethanol. Between the washes, the particles were dispersed by vortex and sonication. The particles were air dried and weighed for yield.

2.3.2 Morphological characteristics of the silica nanoparticles

The size and morphology of silica nanoparticles and fluorescent dye-doped silica nanoparticles were characterized using JEOL JEM-2100 transmission electron microscope (TEM). The sample was prepared by sonication in absolute ethanol for 5 min, and placing a few drops of the sample on a 200 mesh copper grid. After evaporation of the solvent the particles were observed at an operating voltage of 160 kV.

2.3.3 Photostability test of the fluorescent dye-doped silica nanoparticles by spectrofluorometry

The photostability test was performed according to Bagwe *et al.* (2004) by monitor the relative fluorescent intensity of 20 mM Rubpy dye encapsulated in 1 mg of silica nanoparticle dispersing in 1 mL of deionized water in comparison with 1 mL of 20 mM free Rubpy dye in deionized water using exitation wavelength at 290 nm

and measure the emission spectra at 595 nm, in a Perkin Elmer LS55 Luminescence Spectrometer.

2.3.4 Modification of the fluorescent dye-doped silica nanoparticles surface

Surface modification of the fluorescent dye-doped silica nanoparticles was done in 2 steps according to Zhao *et al.* (2004)

2.3.4.1 Amino group linkage

The surfaces of the fluorescent dye-doped silica nanoparticles, about 20 mg particles, were chemically modified with 20 mL of 1% trimethoxysilyl-propyldiethylenetriamine in l mM acetic acid with continuous stirring for 30 min at room temperature.

2.3.4.2 Carboxyl group linkage

The modified surface of the fluorescent dye-doped silica nanoparticles were thoroughly washed 3 times in 20 mL of distilled, deionized water. After washed, the modified surface of the fluorescent dye-doped silica nanoparticles were washed with 20 mL *N*,*N*-dimethylformamide and then reacted with 20 mL of 10% succinic anhydride in *N*,*N*-dimethylformamide solution under N_2 gas for 6 h with continuous stirring. By doing so, carboxyl groups were formed onto the particle surface. The chemically modified surface of the fluorescent dye-doped silica nanoparticles was stored at 4°C in 0.1 M PBS pH 7.3 until use.

2.3.4.3 Testing of surface modification by Fourier Transform Infrared Spectrophotometer (FTIR)

Take a small amount of powder sample (about of 0.1- 2% of the KBr amount, or just enough to cover the tip of spatula) mixed with the KBr powder. Subsequently grind the mixture for 3- 5 min. When assembled the die, add the powder to the collar. Put the die together with the powder into the Qwik Handi-Press. Press the powder for 2 min to form a pellet. Disassemble the die set and take out the collar. Put the collar together with the pellet onto the sample holder and scan by FTIR (Perkin Elmer 1760X).

2.3.4.4 Characterization of surface modification by TEM

The size and the morphology of modified surface of the fluorescent dye-doped silica nanoparticles were characterized according to Method 2.3.2.

2.3.5 Preparation of polyclonal antibody to natural rubber latex protein antigens

2.3.5.1 Preparation of latex protein antigens

Two types of natural rubber latex, skim latex and concentrated latex were used for preparation of protein antigens. The 200 mL of skim latex or concentrated latex were added with 1,000 mL of cold acetone (-20 ^{0}C) and stirred. After incubation at -20 ^{0}C for 10 min and stored overnight at 10 ^{0}C the latex proteins were centrifuged at 8,000 g for 5 min. The supernatant was removed and the air-dried pellet was resuspended in 10- 20 volume of sterile distilled water. The concentration of total protein in the suspension was determined by modified Lowry Method (Lowry ASTM D 5712-99) and adjusted to 2 mg/ml.

2.3.5.2 Determination of water extractable proteins by modified Lowry method (ASTM D 5712-99)

The protein samples was carried out with 160- 200 μL of 0.1 N sodium hydroxide and 2.5- 40 μL of protein solutions, then added 75 μL of alkaline copper sulfate, Reagent A, into each well of a flat bottom, 96-well, microtiter plate, mixed and allowed to stand for 15 min at room temperature. The reaction was then added with 25 μL of dilute Folin solution, Reagent B, mixed thoroughly and allowed for 15 min at room temperature. Proteins levels were evaluated against standard protein, ovalbumin, using a microplate reader (Multiskan Ex, Labsystems) at 750 nm wavelength.

2.3.5.3 Antisera preparation

Two New Zealand white female rabbits (11–12 kilograms) were immunized with either acetone- precipitated proteins from skim latex or concentrated latex, one rabbit was immunized with skim latex proteins. The other one was immunized with concentrated latex proteins. The latex proteins were mixed with equal volume of Complete Freund's Adjuvant (CFA, Sigma, St. Louis, MO), to form a homogenous emulsion. Rabbits were injected subcutaneously at 2– 6 sites on the dorsum with 1 mL aliquots, containing 1 mg/mL of latex proteins. After a two–week rest period 3-booster injections and a two-week interval, were given using Incomplete Freund's adjuvant (IFA) instead of CFA. The total amount of latex proteins administered per rabbit was 4 mgs. Starting at week 8th, rabbits were bleeding every 2 weeks and the antibody titers were checked by an indirect enzyme-linked immunosorbent assay (indirect ELISA).

2.3.5.4 Determination of the antibody titer by Indirect Enzyme-linked Immunosorbent Assay

Indirect enzyme-linked immunosorbent assay (indirect ELISA) was used to determine the serum titer. The microtiter plate was precoated with 100 μL/ well of either acetone- precipitated proteins from skim latex or concentrated latex (final concentration 200 ng/ml) in a carbonate coating buffer pH 9.6. After incubated for 1 h at 37 ^{0}C, aspirated well and washed 3 times with 200 μL washing buffer [phosphate buffer saline (PBS) containing 0.05% Tween 20, PBST], blocked with 200 μL/ well of blocking solution (1% w/v ovalbumin in PBS) and incubated for 1 hour at 37 ^{0}C. After aspirated well, 10-fold dilutions (10^{-1}- 10^{-7}) of serum to be tested were added onto series of wells at 100 μL/ well, and used 10-fold dilutions (10^{-1}- 10^{-7}) of each normal serum, collected before immunization as negative control. After incubation for 1 h at 37 ^{0}C, aspirated well and washed 3 times with 200 μL washing buffer. After washing the plate,100 μL/well of a 1:1,000 dilution of alkaline phosphatase-goat anti-rabbit IgG was added and incubated for 1 h at 37 ^{0}C. The plate was again washed 3 times, and color reaction was started by the addition of substrate 100 μL/well of 1 mg/mL *p*-nitrophenyl phosphate, dissolved in alkaline phosphatase buffer pH 9.8 (0.1 M glycine buffer, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). After one hour, the color development was stopped by the addition of 50 μL of 3 N NaOH.

2.3.6 Conjugation between antibody and the fluorescent dye-doped silica nanoparticles

The fluorescent dye-doped silica nanoparticles were activated and conjugated with antibody according to Zhao *et al.* (2004). The carboxylated nanoparticles were activated by using 5 mL of 100 mg/mL 1-ethyl-3-3(3-dimethylaminopropyl)

carbodiimide hydrochloride and 5 mL of 100 mg/mL of *N*-hydroxy-succinimide in a *Z*-morpholinoethanesulfonic acid buffer (pH 6.8), for 25 min at room temperature with continuous stirring. The activated nanoparticles were thoroughly washed 3 times in 20 mL of distilled, deionized water. Washed nanoparticles were dispersed in 10 mL of 0.1 M PBS (pH 7.3). To covalently immobilize antibody onto the nanoparticle surfaces, 5 mL of 0.1 mg/mL nanoparticles were reacted with 5 µL antibody (1:1,000, antibody: 0.1 mg/mL nanoparticles) for 2– 4 h at room temperature with continuous stirring to form the resultant antibody-conjugated nanoparticles, followed by washing 3 times with 10 mL PBS buffer. The antibody-conjugated fluorescent dye-doped silica nanoparticles were stored at 4 ^{0}C in 0.1 M PBS (pH 7.3) for several weeks. The antibody conjugated with fluorescent dye-doped silica nanoparticles was characterized using TEM as described in Method 2.3.2, and the photostability was performed according to Method 2.3.3 by monitoring the relative fluorescent intensity of 1 mL of fluorescent dye-doped silica nanoparticles in deionized water in comparison with 1 mL of 1 mg/mL of the antibody conjugated with fluorescent dye-doped silica nanoparticles in deionized water.

2.3.7 Use of antibody conjugated with the fluorescent dye-doped silica nanoparticles to detect viral and protein antigens

2.3.7.1 Dot immunobinding assay of papaya ringspot virus (PRSV)

2.3.7.1.1 Preparation of crude extract of papaya leaf infected by PRSV

Normal papaya leaf and systemically infected leaf with PRSV were harvested. The leaves were then homogenized with 0.1 M PBS, pH 7.3 (1 g leaf/mL PBS) followed by centrifugation at 1,000 g for 10 min. The supernatant was collected for

viral detection by the antibody against PRSV conjugated fluorescent dye-doped silica nanoparticles.

2.3.7.1.2 Dot immnobinding assay of PRSV antigens by antibody against PRSV antigens conjugated with fluorescent dye-doped silica nanoparticles

The purified PRSV antigen (1mg/mL) was used as reference (positive control), crude extract of infected papaya leaf was used as unknown and crude extract of non infected leaf was used as negative control. The 3 samples of initial concentration 1 mg/ml for PRSV antigen, and 1 mg leaf/ml PBS were 10-fold diluted (10^{-1}- 10^{-7}) with PBS and the supernatants 5 μL of each sample was spotted serially into the center of each circle on a FUSION 5 nitrocellulose membrane (Whatman International Ltd.) and washed 3 times in 10 mL PBST, blocked for 30 min in 1% (w/v) albumin. After blocking, the membrane was incubated for 30 min at room temperature with 5 μL/dot of antibody against PRSV conjugated fluorescent dye-doped silica nanoparticles. After washing in PBST, the membrane was exposed under a UV light source (Vilber Lourmat UV-light) at 312 nm for 5 min and photos of fluorescent spots were recorded by a digital camera (SONY DSC-S90).

2.3.7.2 Dot immunobinding assay of natural rubber latex proteins

2.3.7.2.1 Preparation of latex proteins from TZ-free concentrated latex Lot No. 6C and 9A

TZ-free concentrated latex Lot No. 1-10 prepared from Pan Biotechnology during August 2005 to November 2006 was used to prepare natual rubber latex protein by centrifugation and collection of the clean serum as crude extract. Only the

concentrated latex lot no. 6C and 9A were devided into 2 fractions, one fraction was irradiated with gamma ray at 15 kGy. The latex from each Lot, before and after irradiation, was prepared as a rubber sheet of 20x 20 cm square, by molding in a plastic plate, then air dried at room temperature for 8-12 h. An air-dried solid rubber sheet was cut into 1 x 1 cm square size, weighed at 1 g and transferred to a 250 mL flask and added 10 mL of distilled water per gram of specimen. The flask was sealed with parafilm and extracted at 37 ^{0}C with shaking for 15 sec after adding the water and again at 120 min. The extracted solution was filtered through a filter paper (Whatman no.1) followed by centrifugation at 3,000 g for 5 min. The filtrated solution was lyophilized. The filtrated-solution was stored in a plastic tube, frozen at -80 ^{0}C, and then lyophilized for 48 h. A sample of lyophilized protein was resuspended with distilled water, determined for total protein antigen detection by dot immunoassay.

2.3.7.2.2 Dot immunobinding assay of latex protein antigens

The acetone- precipitated proteins from skim latex or concentrated, prepared according to Method 2.3.5.1, were used as reference (positive control) and latex protein antigens from method 2.3.7.2.1 were used as unknown. Each sample was diluted 10-fold (10^{-1}- 10^{-7}) in PBS, and sequentially spotted on 6 x 7 cm^{2} piece of FUSION 5 nitrocellulose membrane (Whatman International Ltd.). After blocking with 1% (w/v) albumin in PBS 4 ^{0}C overnight or 37 ^{0}C for 1 h, the membrane was incubated for 30 min at room temperature with antibody against latex proteins conjugated fluorescent dye-doped silica nanoparticles. After washing with PBST (PBS containing 0.05% Tween 20), the membrane was excited under a UV light source (Vilber Lourmat UV-light) at 312 nm for 5 min and photos of fluorescent spots were recorded by a digital camera (SONY DSC-S90).

2.3.8 Indirect Enzyme-Linked Immunosorbent Assay of viral and latex protein antigens

An indirect ELISA was used to determine the viral and latex protein antigens according to modified ASTM D 6499 by using FUSION 5 nitrocellulose membrane instead of microtiter plate. The PRSV and latex protein antigens were applied on membrane and blocked with 1% (w/v) albumin in PBS 4 ^{0}C overnight or 37 ^{0}C for 1 h. After washing with PBST, antibody against PRSV-Ag or against latex protein (1:1,000 dilution) was added, the membrane was incubated for 1 h at 37 ^{0}C. After washed the membrane 3 times with PBST, a 1:1,000 dilution of Alkaline phosphatase-goat anti-rabbit IgG (Zymed Labolatories, INC.) was added and incubated for 1 h at 37 ^{0}C. The membrane was again washed 3 times, and color reaction was developed by the addition of working solution, alkaline phosphatase buffer pH 9.5 (100 mM Tris-HCl, 100 mM NaCl and 5 mM $MgCl_2$) mixed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP). The color development was stopped by the addition of distilled water.

2.3.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of latex protein antigens

Polyacrylamide gel at 5% gel was used as stacking gel and 15% gel was used as separating gel. Tris glycine (25 mM Tris, 192 mM glycine) buffer pH 8.3 containing 0.25% w/v SDS was used as electrode buffer. Protein sample to be analyzed was dissolved in Tris buffer, containing 60 mM Tris, 2% w/v SDS, 25% v/v glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue, and boiled for 5 min prior to application to the gel. The electrophoresis was carried out at constant current of 15 mA, on a Mini-Protein (Bio-Rad) from cathode towards anode. When

the electrophoresis was completed, one part of the gel was stained with Coomassie blue R-250, whereas the other part was used for Western blotting.

2.3.10 Western Blot of natural rubber latex antigens

The transfer sandwich cassette (Bio-RAD) was used for blotting protein bands from gel. The transfer buffer (20mM Tris, 150 mM NaCl) and the cassette frame set up according to the manual (Bio-RAD Protein Blotting: A guide to transfer and detection). Proteins were transferred to a constant current of 200 mA for 3 h. Transfer may proceed overnight at a lower current. When the transfer was completed, turn off power and remove blot from apparatus. Remove SDS-gel and place in glass dish with stain. Staining was given an indication of transfer efficiency. The membrane was cut for immunostaining. Each membrane was immersed in blocking buffer, 1% (w/v) albumin or 5% (w/v) non-fat milk protein in Tris buffer saline pH 7.3 [20 mM Tris, 150 mM NaCl, TBS]. Let it blocked for 1 h at 37 ^{0}C or 4 ^{0}C overnight and then, wash the membrane in TBST for 5 min.

For labeled with indirect ELISA, the membrane was transferred into a solution of primary antibody (Rabbit anti-latex proteins) in TBS at the dilution of 1:1,000. Incubate for 1 h at 37 ^{0}C or 4 ^{0}C overnight. Washed membrane 3 times with TBST (TBS containing 0.05% Tween 20). The membrane was immersed in alkaline phosphatase-goat anti-rabbit IgG (dilution 1:1,000) and incubated at least 1 h. The membrane was washed 3 times with TBST. Make up developer solution, containing 10 mL of alkaline phosphatase buffer, 33 µL of BCIP and 66 µL of NBT. Immerse the membrane in this solution and incubated until color was developed. Stop the reaction by rinsing it with distilled water and let it be air dried.

For labeled with antibody conjugated with fluorescent dye-doped silica nanoparticles. After blocked with a solution of 1% (w/v) albumin in TBS, the membrane was incubated for 30 min at room temperature with antibody conjugated fluorescent dye-doped silica nanoparticles, and then wash it with TBST. The membrane was excited under Vilber Lourmat UV-light at 312 nm to observe the fluorescence signals.

2.3.11 Dot immunobinding assay of natural rubber latex allergen

The latex and commercial rubber samples were extracted and used to determine protein antigens.

The latex samples (Lot No. 1, 2, 3, 4, 5, 6, 6C, 7LG, 7G, 7C, 8N, 8O, 8C, 9A, 9C and 10) were centrifuged at 10,000 rpm for 30 min at 25^{o}C. The supernatant was drawn by syringe and long needle.

A piece of rubber gloves, nitrile glove and rubber dam were cut into 1x 1 cm square size, weighed and transferred to a 250 mL flask and added with 10 mL of distilled water per gram of specimen. The flask was sealed with sealing film, extraction was at 37 ^{0}C and shaken for 15 sec after with added the water again for 120 min. The extracted solution was filtered through filter paper (Whatman no.1) followed by centrifugation at 3,000 g for 5 min.

The supernatant 5 µL from each sample was spotted onto Fusion 5 nitrocellulose membrane and determined protein antigens according to Method 2.3.7.2.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Characteristics of the synthesized fluorescent dye-doped silica nanoparticles

Silica nanoparticles (SNP) were synthesized using water-in-oil (W/O) microemulsion method and characterized with transmission electron microscopy (TEM). The dried SNP are spherical shape, rather uniform in size as evident by average diameter of 54 ± 5 nm (Figure 3.1), measured from TEM micrographs (n = 100), which have the relative standard deviation in size distribution as 9.6 %. As shown in Figure 3.1a, b and d (arrows →), the SNP appears as transparent nanoparticles.

When the fluorescent dye, tris (2, 2ʹ- bipyridyl) dichlororuthenium (II) hexahydrate (Rubpy) was encapsulated in SNP. The size of dried Rubpy-doped silica nanoparticles (DSNP) have increased at 65 ± 5 nm, comparing to SNP without Rubpy dye. The DSNP are opaque inside (Figure 3.2) as a result of the presence of the Rubpy dye. Santra *et al.* (2001) also found the same phenomena.

Comparing the average particle size of DSNP (65 ± 5 nm) obtained in this research with other 7 previous reports during 2001 – 2006, Table 3.1 indicated that the DSNP prepared by either w/o microemulsion method or Stőber method resulted in approximately the same size of nanoparticles in the range of 60 – 70 nm.

From these results, the sizes of SNP and DSNP in dried form were roughly characterized by TEM. To obtain the reliable size of SNP and DSNP, further characterization should be performed by using environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM) and laser light scattering techniques.

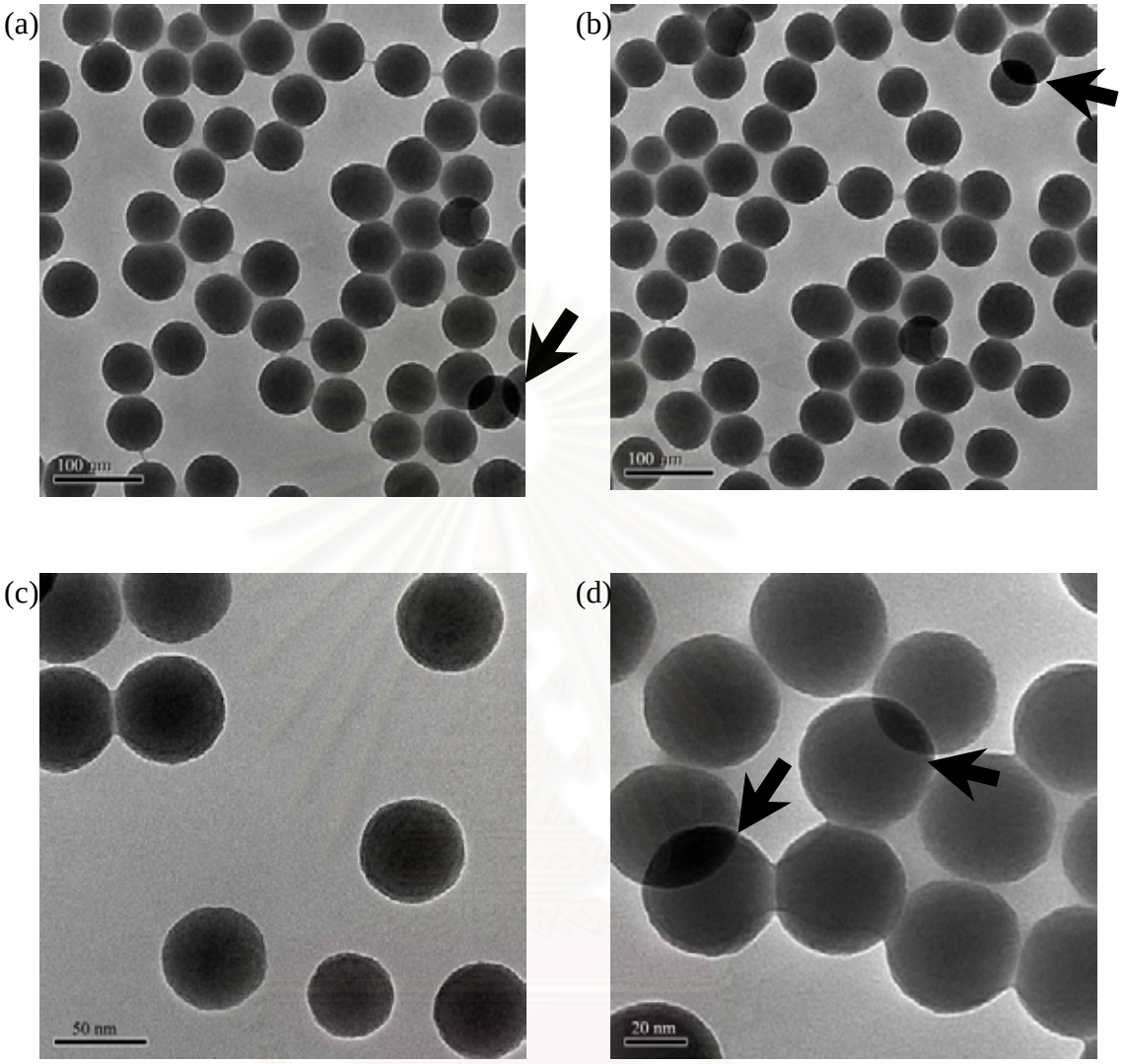


Figure 3.1 Electron micrographs of synthesized silica nanoparticles.

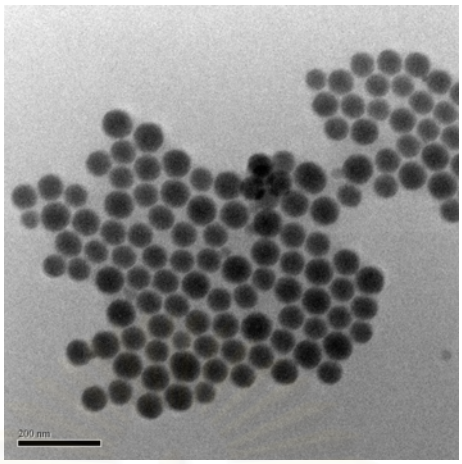
TEOS (0.439 mmole) was converted to silica nanoparticles (SNP) in water-in-oil microemulsion, harvested by precipitation with acetone, centrifuged and washed 3 times in 95 % ethanol. Air-dried particles were suspended in absolute ethanol and dropped on a copper grid for observation at 160 kV. Transparent spherical shape nanoparticles are observed in a, b and d (arrows).

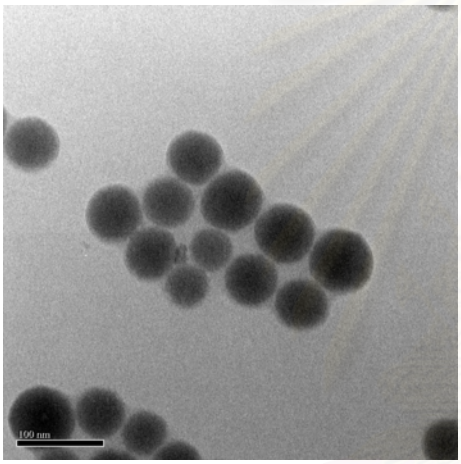
a. and b. Uniform size of SNP at 20,000 × magnification (bar = 100 nm)

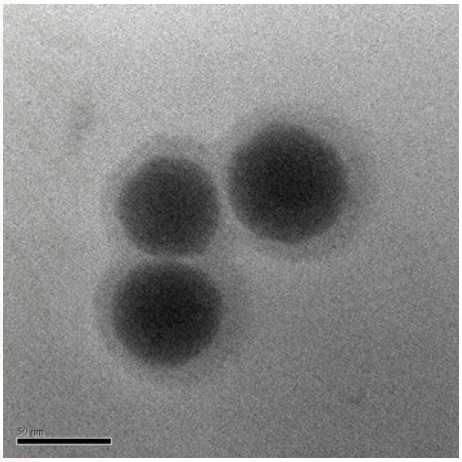
c. SNP at 40,000 × magnification (bar = 50 nm)

d. SNP at 80,000 × magnification (bar = 20 nm)

(a)







(b) (c)

Figure 3.2 Electron micrographs of Rubpy-doped silica nanoparticles

The Rubpy dye was entrapped inside silica network by using a water-in-microemulsion method to form Rubpy-doped silica nanoparticles (DSNP). The dried DSNP powder were dispersed in absolute ethanol and dropped on a copper grid for observation at 160 kV. The TEM micrographs showed that the particles are opaque, spherical and rather uniform sizewith standard deviation in size distribution of 7.7 %.

a. DSNP at 20,000 × magnification (bar = 200 nm)

b. DSNP at 40,000 × magnification (bar = 100 nm)

c. DSNP at 80,000 × magnification (bar = 50 nm)

Table 3.1 The average size of DSNP synthesized by water-in-oil (W/O) microemulsion and Stöber (S) method comparing with other reports

Size of DSNP (nm)	Methods/ dye	References
65 ± 5	W/O/ Rubpy	This research, 2007
63 ± 4	W/O/ Rubpy	Santra et al, 2001
62	W/O/ Rubpy	Qhobosheane et al., 2001
around 70	W/O Rubpy	Lian et al., 2004
60 ± 4	W/O Rubpy	Zhao et al., 2004
65 ± 8	S/ $[Ru(phen)_3]Cl_2$*	Rossi et al, 2005
60	W/O Rubpy	Wang et al., 2006
70	W/O Rubpy	Yao et al., 2006

* *Tris(1,10-phenanthroline) ruthenium(II) chloride*

3.2 The yield of the silica nanoparticles and the fluorescent dye-doped silica nanoparticles

The organo-alkoxysilane used in this work was tetraethylorthosilicate (TEOS), which was used as a precursor to create SNP $(SiO_2)_n$, and DSNP $[(SiO_2)_n$ + dye]. The fluorescent dye (Rubpy) was entrapped inside silica network. The TEOS molecule contains four ethoxy group (Figure 1.1), therefore undergoing hydrolysis and condensation to form silica (Figure 1.2). So the first step was to evaluate the conversion yield of TEOS to SNP (based on 12 lots of synthesis).

From Figure 1.2, the overall reaction demonstrated that 1 mole of TEOS can be converted to 1 mole of silica. However, in this experiment 0.439 mmole of TEOS which is equivalent to 26.37 mg of silica was used as starting material, and yielded 20.52 mg dried weight of SNP, the percent conversion yield was 78 % (w/w).

In the case of DSNP, the reaction was started with 0.439 mmole of TEOS and 9.604×10^{-3} mmole of dye all together, which is equivalent to 33.57 mg of silica and dye, and yielded 26.55 mg of dried DSNP powder. The percent conversion yield of TEOS containing Rubpy to DSNP was 79 % (w/w).

These results indicated that the synthesis of SNP and DSNP using w/o method provide average conversion yield of 78 % and 79 % (w/w) respectively. These values of conversion yields are the same as previously reported by Rossi et al. (2005), using Stőber method and $[Ru(phen)_3]Cl_2$ and obtain average conversion yield of 80 % (w/w) for DSNP.

3.3 Characteristics of surface modification of Rubpy-doped silica nanoparticles

It is well known that plentiful hydroxyl groups exist on the surface of silica. These surfaces can be easily modified with different reagents for a variety of applications. Two major modification mechanisms were used in this study; firstly by adding the amine-group and secondly by carboxyl-group. The surface modified DSNP obtained was then characterized for the presence of the added functional group by FTIR and further determined morphology by TEM.

3.3.1 Functional groups characterization by Fourier Transform Infrared Spectrophotometer (FTIR)

Before surface modification the FTIR profile of DSNP shows a strong peak at 1099 cm^{-1}, which is the characteristic assignment of ν_{as}(Si-O-Si) bond. The absorption peaks at 952, 801 and 467 cm^{-1}, which are the characteristics of ν(Si-OH), ν_s(Si-O-Si) and δ(O-Si-O) groups respectively (Figure 3.3).

After surface modification, the carbonyl stretching vibration band at 1698 cm^{-1} and the symmetric stretching vibration bands at 1420 and 1312 cm^{-1}, which are the characteristics of C=O, COO^- groups, and the absorption at 1203 cm^{-1} demonstrated the C-OH bond.

The FTIR spectra, therefore, support the presence of carboxyl group on the surface of DSNP.

The use of IR spectra to characterize (-Si-O-Si-) and (-O-Si-O-) prepared from TEOS and Fe_2O_3 nanoparticle to form Fe_2O_3-SiO_2 nanocomposite (Bruni *et al.*, 1999), have indicated the IR frequencies in the range of 1,200, 1,075, 799 cm^{-1} for (Si-O-Si) and 460 cm^{-1} for (O-Si-O).

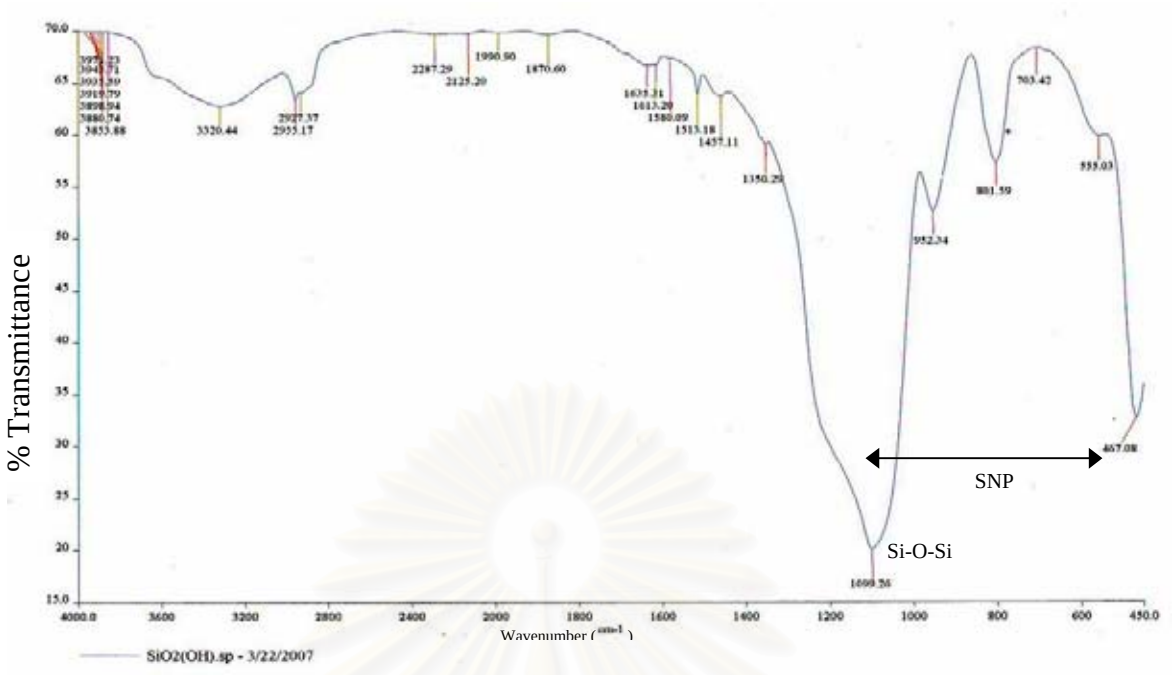


Figure 3.3 FTIR spectra of the DSNP

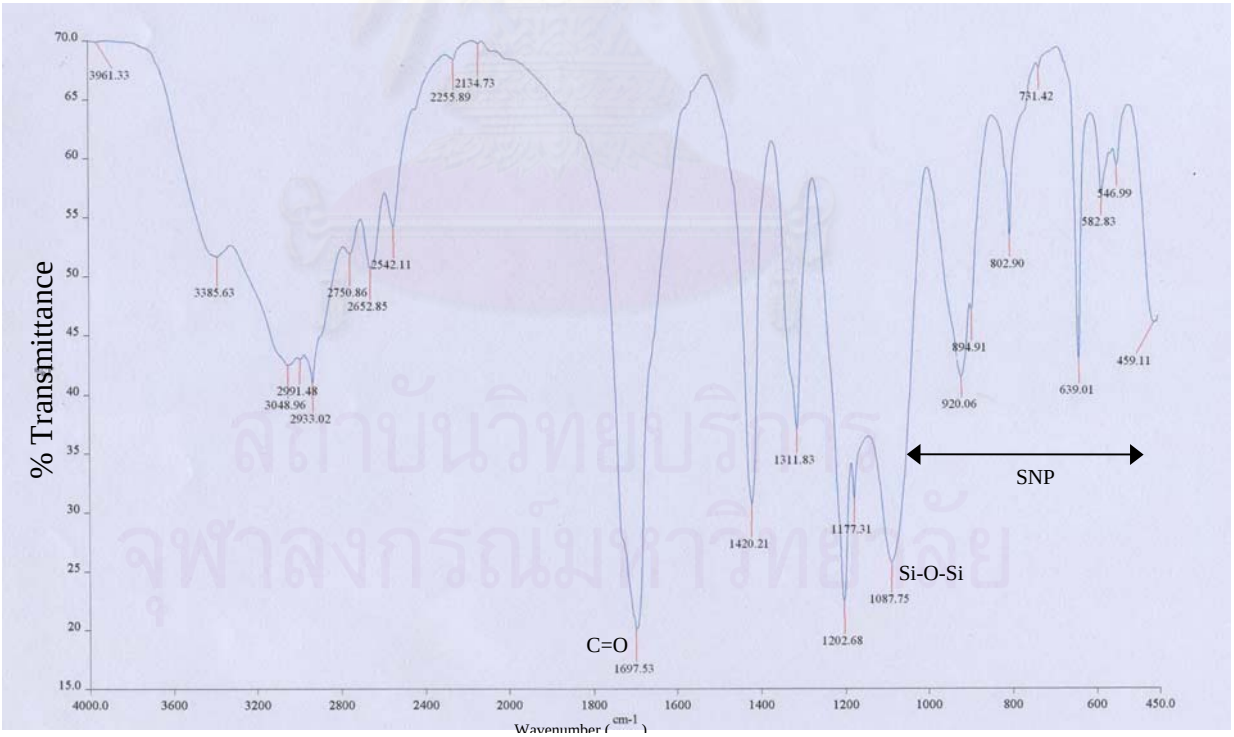


Figure 3.4 FTIR spectra of the surface modified DSNP

3.3.2 TEM

When the morphology of surface-modified DSNP was observed by TEM, the nanoparticles seem to shrink to the average diameter of 50 ± 7 nm (Figure 3.5), comparing with the DSNP (about 65 nm). The surface-modified DSNP were apparently aggregated.

Bagwe *et al.* (2006) also reported that the size of carboxylated DSNP decreased from DSNP because of strong electrostatic repulsion forces between nanoparticles. The amine groups have a positive charge, and silica groups have negative charges. The amine-modified SNP can back bond to surface silanol groups. Hence, the overall charge on the surface is very low and the particles tend to aggregate because there is no driving force on the surface of the nanoparticles to keep them apart.

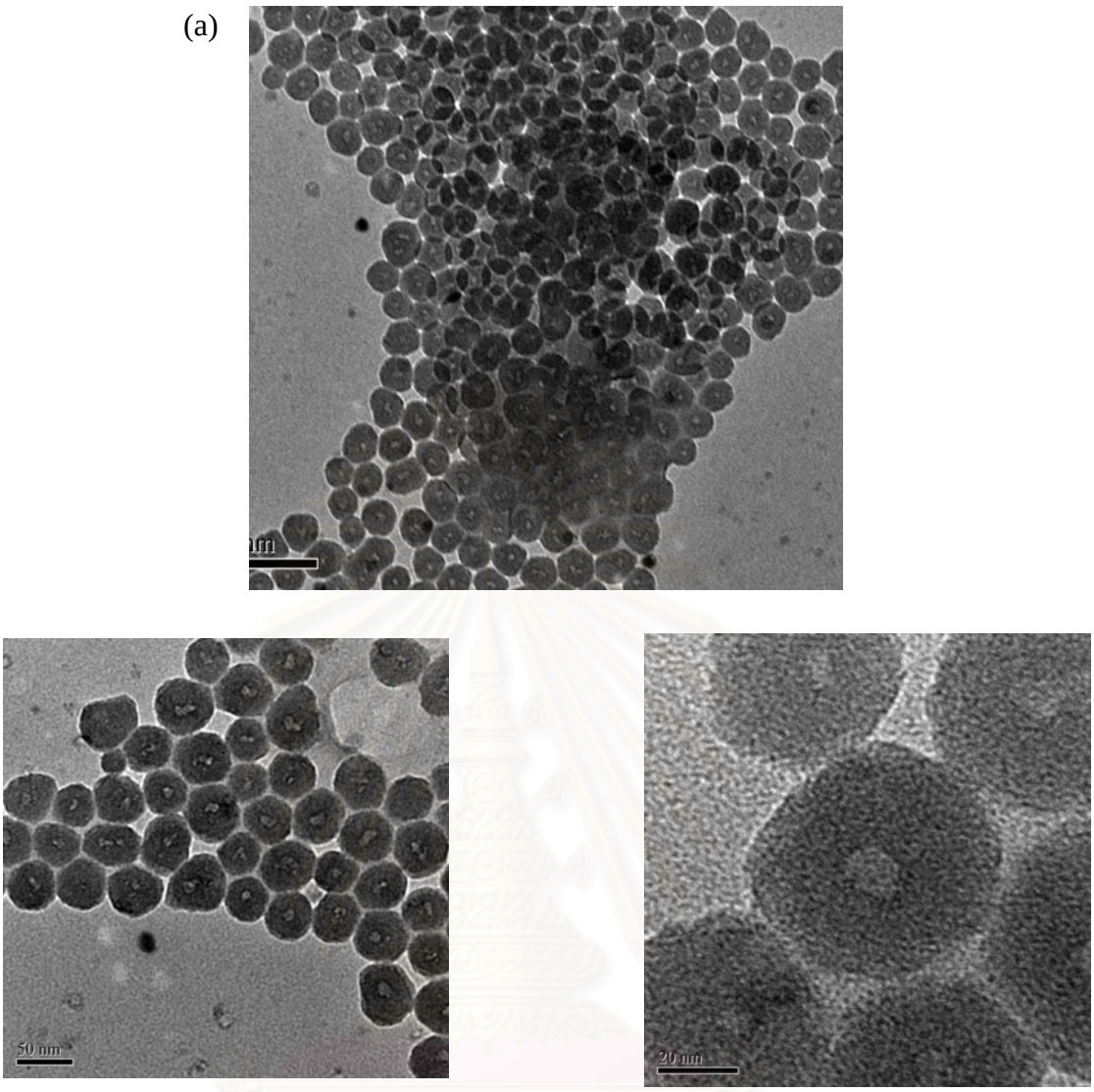


Figure 3.5 TEM micrographs of carboxylated Rubpy-doped silica nanoparticles

The linkage between DSNP was achieved through the following steps. First, the DSNP were silanized using 1% (v/v) trimethoxysilyl-propyldiethylenetriamine in 1 mM acetic acid for 30 min at room temperature. The second approach of preparing carboxylated DSNP, the silanized particles were reacted with 10% succinic anhydride in dimethylformamide under a nitrogen atmosphere and stirred for 6 h.

a. Carboxylated DSNP at 20,000 × magnification (bar = 100 nm)

b. Carboxylated DSNP at 40,000 × magnification (bar = 50 nm)

c. Carboxylated DSNP at 80,000 × magnification (bar = 20 nm)

3.4 Antibodies production and titer of rabbit IgG against latex allergens

Either antibodies (Ab) against skim latex protein antigens (SLP-Ag) or concentrated latex protein antigens (CLP-Ag) were prepared in female New Zealand White rabbits. Each animal was injected with SLP-Ag or CLP-Ag. After immunization, rabbit blood samples were collected every 2 week intervals. An indirect ELISA method was used to determine the antibody titer.

The serum antibody against skim latex proteins and concentrated latex proteins raised at the second week after the first immunization, both of SLP-Ab and CLP-Ab exhibited the titer of 1,000 (Ab concentration 10^{-3}). Later at the 4th, 6th and 8^{th} weeks, the titers of these two antibodies were raised to 10,000 (Ab concentration 10^{-4}). At this level, the serum antibodies were collected, pooled and considered high enough to conjugate with surface-modified DSNP.

3.5 Characteristics of Rubpy-doped silica nanoparticles after conjugation with antibodies

After the DSNP was modified, the carboxylated DSNP was pendented carboxylic acids at the surfaces, making them suitable for covalent coupling of proteins (antibodies).

To test the conjugation method, the crude antibodies against papaya ringspot virus (PRSV-Ab, concentration 9.5 mg/ mL) was initially used to conjugate with carboxylated DSNP, while the antibody against skim latex and concentrated latex protein antigens were under production. The DSNP-PRSV-Ab obtained exhibit the average diameter of 50 ± 2 nm, and some are in aggregated form as shown in Figure 3.6a, b and c.

From method 2.3.6, an aliquot of 5 mL (0.1 mg/ mL) DSNP was covalently attached with 5 μL (9 mg/ mL) PRSV-Ab, ratio DSNP: PRSV-Ab = 10: 1 μg (w/w), that yield 547 μg (DSNP + PRSV-Ab)/ 5005 μL, and concentration of DSNP-PRSV-Ab ≈ 100 ng/ μL.

After the SLP-Ab (6mg/ mL) and CLP-Ab (5mg/ mL) were produced, each of them was immobilized onto the particles surface. The DSNP-SLP-Ab and DSNP-CLP-Ab exhibit the same size with average diameter of 50 ± 2 nm and 51 ± 2 nm respectively (Figure 3.7 and 3.8). The DSNP after conjugation with PRSV-Abs, SLP-Abs and CLP-Abs were observed by TEM. By linking antibodies molecule to the modified DSNP surfaces, aggregated form were more dominant, whereas some single particle can be found.

In case of DSNP-SLP-Abs, ratio DSNP: SLP-Abs = 16: 1 μg (w/w) and yield of DSNP + SLP-Abs was 530 μg/ 5005 μL, which has concentration ≈ 100 ng/ μL.

For DSNP + CLP-Abs, ratio DSNP: SLP-Abs = 16: 1 μg (w/w) that yield 525 μg/ 5005 μL, which has concentration ≈ 100 ng/ μL.

These experiments demonstrated similar size of Ab-conjugated DSNP, weather conjugated with PRSV-Abs, SLP-Abs or CLP-Abs, rather uniform of about 50 nm with standard deviation less than 5 % were observed.

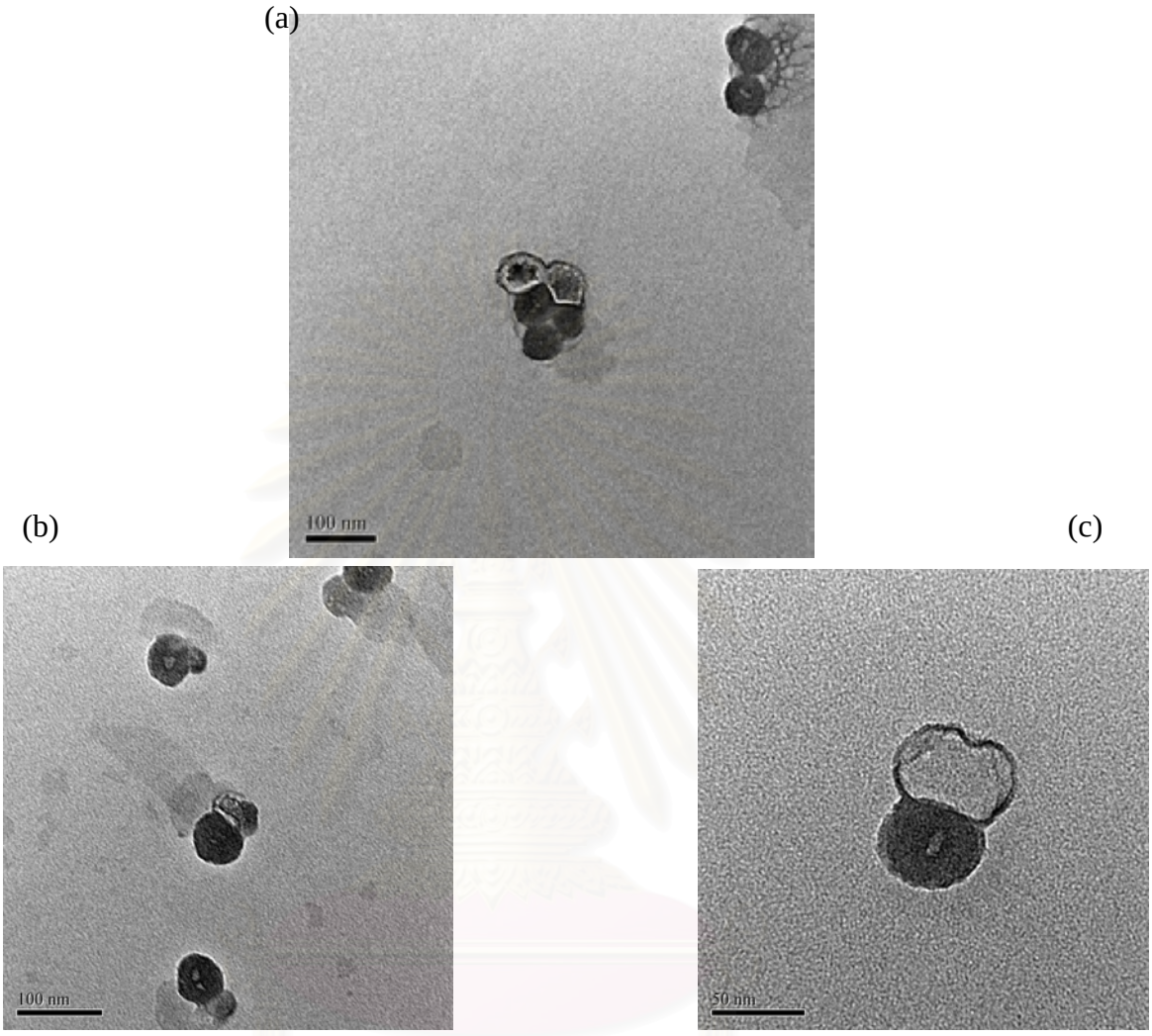


Figure 3.6 TEM micrographs of Rubpy-doped silica nanoparticles conjugated with antibody against papaya ringspot virus

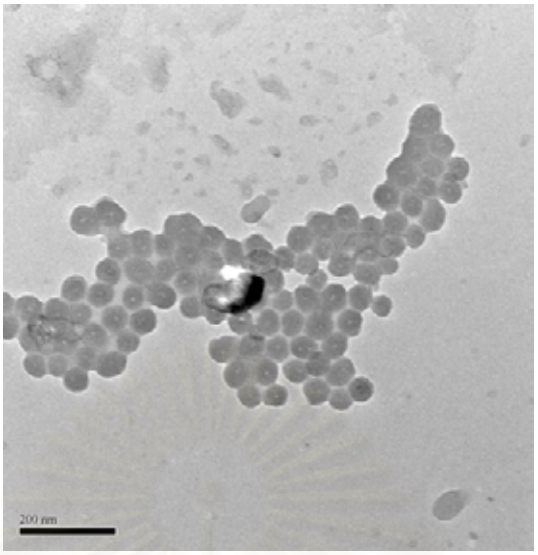
To covalently immobilize antibodies against papaya ringspot virus (PRSV-Ab) onto the DSNP surfaces, 5 mL of 0.1 mg/mL DSNP were reacted with 5 µL PRSV-Abs (1:1,000, antibody: 0.1 mg/mL DSNP) for 2– 4 h at room temperature with continuous stirring to form the resultant DSNP-PRSV-Ab, followed by washing 3 times with 10 mL PBS buffer.

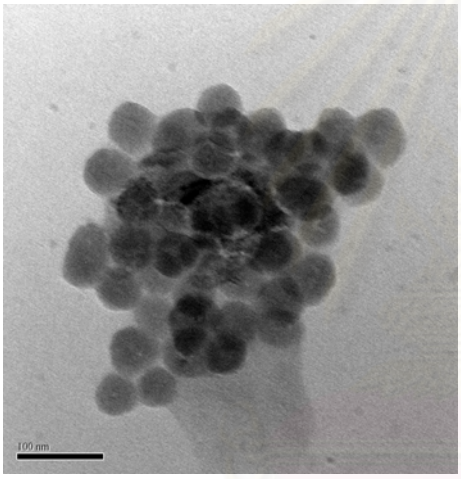
a. Carboxylated nanoparticles conjugated with PRSV-Abs at 20,000 × magnification

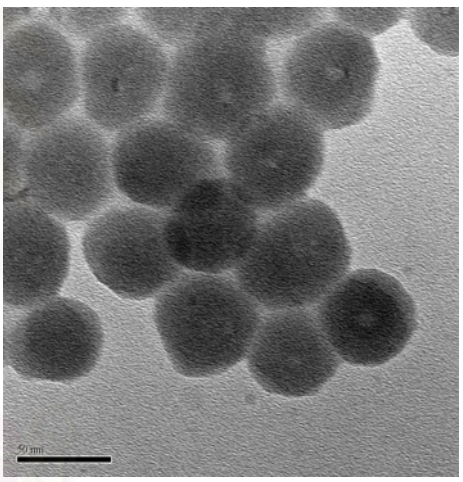
b. Carboxylated nanoparticles conjugated with PRSV-Abs at 40,000 × magnification

c. Carboxylated nanoparticles conjugated with PRSV-Abs at 80,000 × magnification

(a)







(b) (c)

Figure 3.7 TEM micrographs of Rubpy-doped silica nanoparticles conjugated with antibodies against skim latex protein antigens

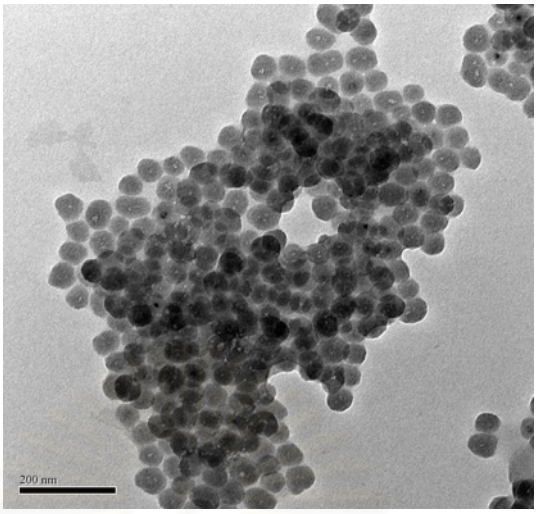
To covalently immobilize antibodies against skim latex protein antigens (SLP-Abs) onto the DSNP surfaces, 5 mL of 0.1 mg/mL DSNP were reacted with 5 µL SLP-Abs (1:1,000, antibodies: 0.1 mg/mL DSNP) for 2– 4 h at room temperature with continuous stirring to form the resultant DSNP-SLP-Abs, followed by washing 3 times with 10 mL PBS buffer.

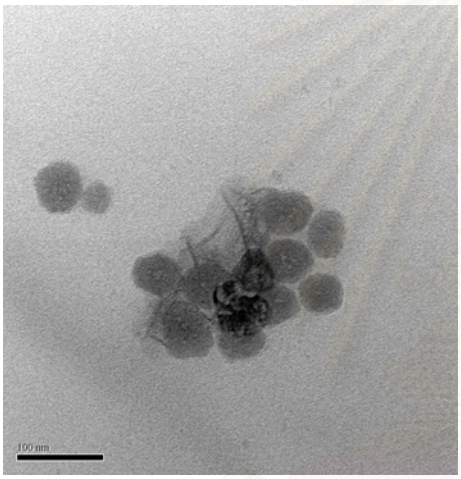
a. Carboxylated nanoparticles conjugated with SLP-Abs at 20,000 × magnification

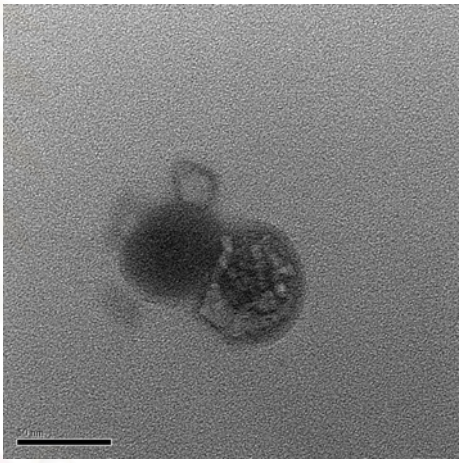
b. Carboxylated nanoparticles conjugated with SLP-Abs at 40,000 × magnification

C. Carboxylated nanoparticles conjugated with SLP-Abs at 80,000 × magnification

(a)







(b) (c)

Figure 3.8 TEM micrographs of Rubpy-doped silica nanoparticles conjugated with antibodies against concentrated latex protein antigens

To covalently immobilize antibody against concentrated latex protein antigens (CLP-Abs) onto the DSNP surfaces, 5 mL of 0.1 mg/mL nanoparticles were reacted with 5 µL CLP-Ab (1:1,000, antibodies: 0.1 mg/mL DSNP) for 2– 4 h at room temperature with continuous stirring to form the resultant DSNP-CLP-Abs, followed by washing 3 times with 10 mL PBS buffer.

a. Carboxylated nanoparticles conjugated with CLP-Abs at 20,000 × magnification

b. Carboxylated nanoparticles conjugated with CLP-Abs at 40,000 × magnification

c. Carboxylated nanoparticles conjugated with CLP-Abs at 80,000 × magnification

3.6 Photostability of the fluorescent dye-doped silica nanoparticles

In order to use Rubpy-doped silica nanoparticles as nanoprobe, the exitation and emission spectra of the DSNP (1mg/ mL) were compared with those of free Rubpy dye (20 mM), starting from 200- 800 nm at room temperature.

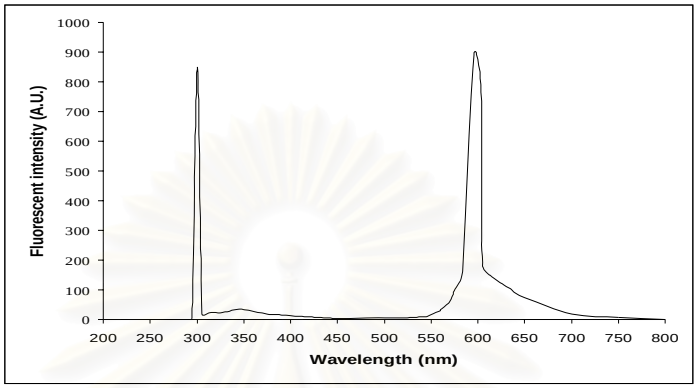
Spectrofluorometric measurements indicated that the excitation wavelength peak around 290- 310 nm, which is in the ultraviolet region and the emission spectra of either free Rubpy dye or the Rubpy in SNP showed similar emission spectra with the maximum fluorescent intensity at 595 nm (Figure 3.9a and b). There is no difference in excitation and emission wavelengths between free and entrapped Rubpy, indicating that the silica lattice did not interfere any emission spectrum.

The photostability is defined as the fluorescent intensity (FI) remained at time interval as percent relative fluorescent intensity of the FI at initial time (T = 0 h). The relative fluorescent intensity of the DSNP remained almost constant through 3 days of observation (Figure 3.10), whereas the photostability of free dye decreased to almost half during the first 12 h, and at 72 h became nearly non-detectable. The results indicate that encapsulation of Rubpy dye in silica nanoparticles enhance the stability of FI and makes them more suitable for use as nanosensor. Santra *et al.* (2001) previously reported that the relative fluorescence intensity of DSNP, when excited continuously beyond 290 nm remained constant for 1 h and the photostability of DSNP conjugated with antibodies against leukemia cell made them usable for visualizing leukemia cells under a fluorescent microscope or cell sorter. Hun and Zhang (2007) also reported that the photobleaching behavior of the DSNP was significantly less than 7.8 %, even after continuous excitation for 1 h.

It is considered that the increase of photostability of DSNP is attributed to the following factors: first, the incorporation of Rubpy dyes in silica network restraints

the movement of fluorophore molecules; second, the silica protects against the penetration of dissolved oxygen into the silica network (Yang *et al.*, 2004). Furthermore, leakage of dye molecules from the silica particles should be negligible, probably because of the strong electrostatic attractions between the positively charged inorganic dye and the negatively charged silica (Wang et al., 2006).

(a)



(b)

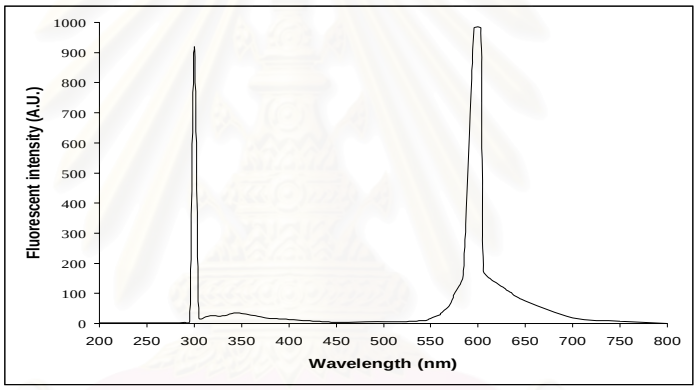


Figure 3.9 Excitation (left panel) and emission spectra (right panel) for the free Rubpy dye (a) and the Rubpy that fixed in SNP (b)

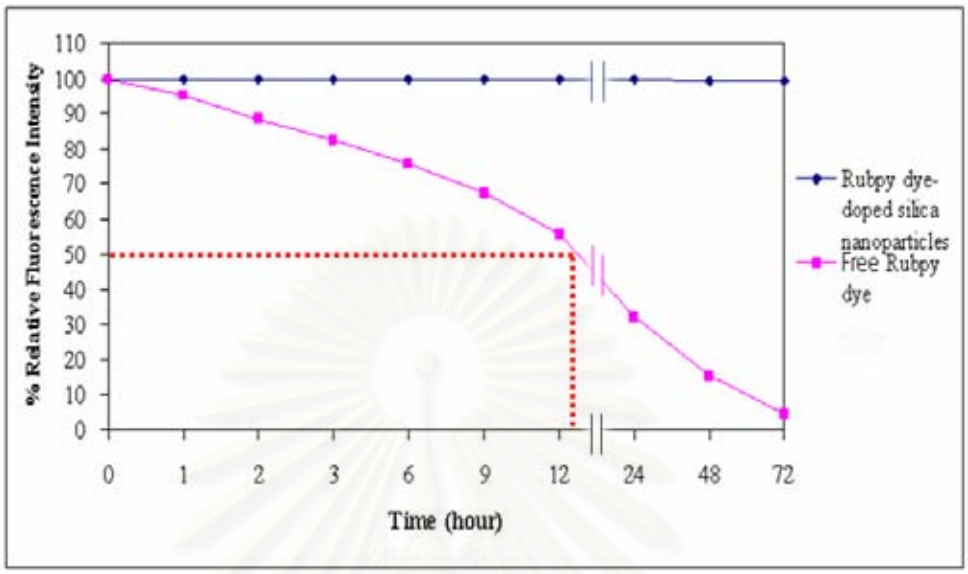


Figure 3.10 Comparison of photostability of the Rubpy-doped silica nanoparticle with the free Rubpy dye

To investigate the photostability of the Rubpy dye molecules doped inside silica nanoparticles, the time course of percent relative fluorescence intensity of dye fixed in silica nanoparticles was compared with that of free dye. Measurements were performed at its optimal excitation (290 nm) using spectrofluorophotometer. The relative photostability of Rubpy-doped silica nanoparticles suggests that the silica shell surrounding the dye molecule acts as a barrier to protect the dye from the surrounding environment.

3.7 Development of a new dot immunobinding assay using Ab-conjugate DSNP to detect protein antigen of papaya ringspot virus (PRSV)

To develop a practical method for using antibodies conjugated with DSNP (Abs-DSNP) to determine protein antigen, the DSNP linked with antibodies against papaya ringspot virus (PRSV-Ab) was used as a model to test the concept of direct dot immunobinding assay on nitrocellulose membrane instead of the 96-well microtiter plate used in ELISA method.

Based on the higher quantum yield and very high photostability of the Ab-DSNP, the amount of Ab-DSNP used in each assay can be reduced to 500 ng in 5 μL.

The purified PRSV-Ag (coat protein; 1 mg/ mL) was used as a reference (antigen concentrations varying from 10^{-1}- 10^{-7} mg/mL), and 5 μL of 100 ng/ μL was used as starting concentration. PRSV infected leaf (PRSV-IL) was used as unknown and PRSV non-infected leaf (PRSV-NIL) was used as negative control. Samples were spotted onto nitrocellulose membrane followed by incubation with DSNP conjugated PRSV-Abs. The positive fluorescent signals were observed under UV light source with a limit of detection at 0.1 ng/ μL for both of PRSV-Ag and PRSV-IL. As illustrated in Figure 3.11a the PRSV-NIL did not show any dot fluorescent signal. Besides, ELISA, was also used to detect PRSV-Ag, PRSV-IL and PRSV-NIL. Similar color-dot signals were obtained but the detection limit was at 1 ng/ μL (Figure 3.11b).

These results indicated that the sensitivity of DSNP conjugated PRSV-Abs was higher than ELISA method for 10-fold with similar specific detection.

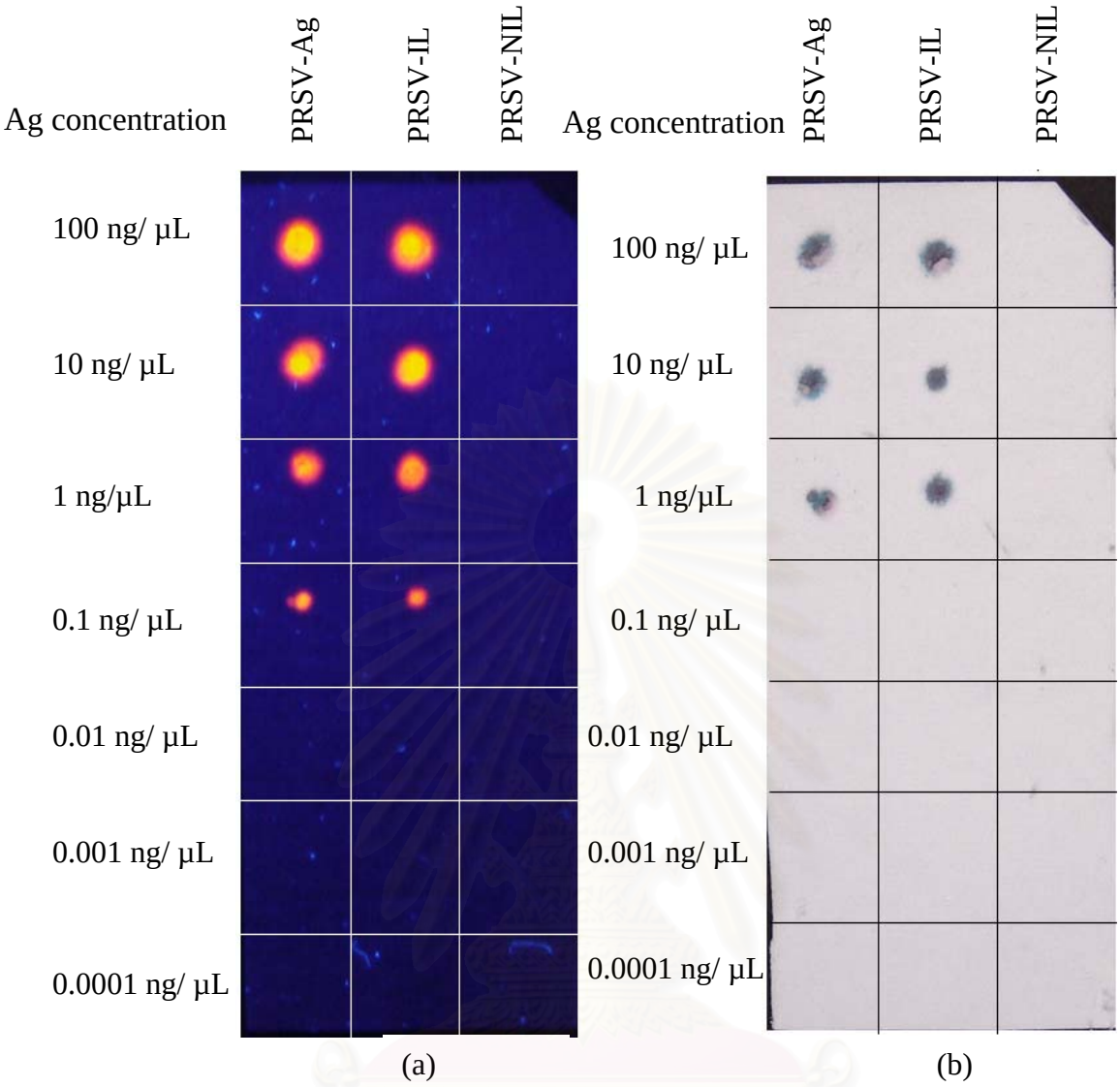


Figure 3.11 Dot immunobinding assay of PRSV-Ag using DSNP conjugated with PRSV-Abs comparing with ELISA method

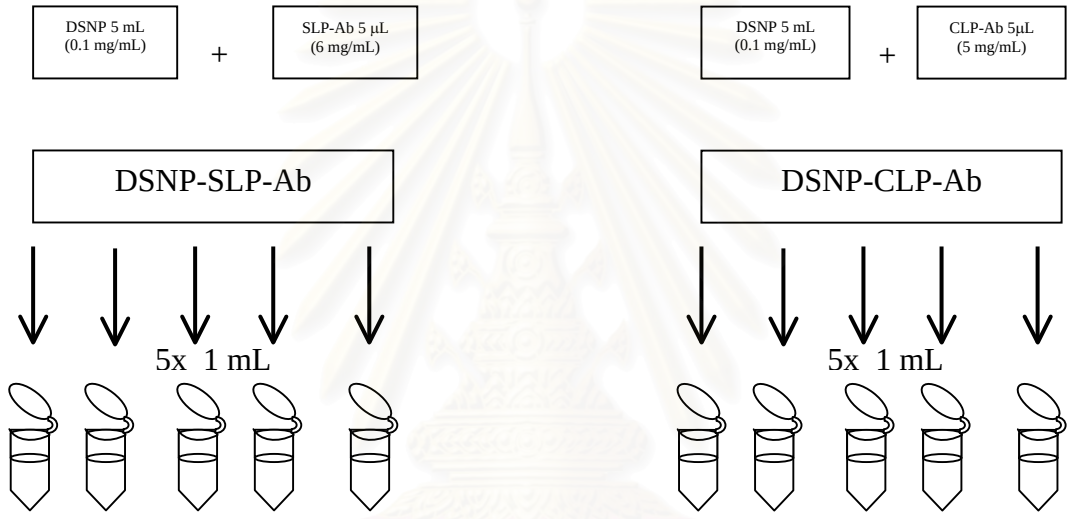
The purified PRSV coat protein antigen (1mg/mL) was used as reference (positive control), crude extract of PRSV infected papaya leaf was used as unknown, and crude extract of non infected leaf was used as negative control. All 3 samples of initial concentration 1mg/mL for PRSV antigen, and 1mg leaf/mL PBS were 10-fold diluted (10^{-1}- 10^{-7}) with PBS, and the supernatants 5 µL of each sample was spotted serially into the center of each circle on a FUSION 5 nitrocellulose membrane (Whatman International Ltd.), and washed 3 times in 10 mL PBST, blocked for 30 min in 1% (w/v) albumin. After blocking, the membrane was incubated for 30 min at room temperature with antibodies against PRSV conjugated DSNP. Then washing with PBST, the membrane was exposed under a UV light source (Vilber Lourmat UV-light) at 312 nm for 5 min and photos of fluorescent spots were captureded by a digital camera.

a. Detection with DSNP conjugated with PRSV-Ab

b. Detection by using Western blot method

3.8 Sensitivity of the Rubpy-doped silica nanoparticles conjugated with the antibodies against latex protein antigens comparing with ELISA

Since the use of DSNP conjugated with PRSV-Ab as a nanoprobe to detect viral antigens showed the limit of detection = 0.1 ng/ µL that is better than ELISA. In this experiment, the DSNP-bound antibodies against skim latex protein and concentrated latex protein antigens were optimized to the following protocol:



The diagram of dot immunoassay for 2-type of antibodies (SLP-Ab and CLP-Ab) versus 2-type of standard known concentration of latex protein antigens (SLP-Ag: Row 1 Dot 1.1-1.7 and CLP-Ag: Row 2 Dot 2.1-2.7) were shown in Figure 3.12. The water soluble protein prepared from 2 Lots of 4 rubber sheet specimens prepared from concentrated latex (6C and 9A), without gamma irradiation treatment (6C-CLP = Row 3, and 9A-CLP = Row 5) and after irradiation (Ir6C-CLP = Row 4, and Ir9A-CLP =Row 6).

When DSNP bound to SLP-Ab was used as nanoprobe, only fluorescent dots of SLP-Ag were observed with detection limit of positive fluorescent signal at 0.01 ng/ µL as evident by Row 1 Dot 1.5 (Figure 3.13a).

On the other hand, when the DSNP conjugated with CLP-Ab was used as nanoprobe, only fluorescent signal associated with CLP-Ag was observed by detecting the limit of positive fluorescent signal at 0.01 ng/ μL (Row 2 Dot 2.5).

No cross-reaction was observed between SLP-Ab versus CLP-Ag and CLP-Ab versus SLP-Ag.

As for the water extractable protein from TZ-free CLP, no matter before or after irradiation, none of them showed positive fluorescent signal with both antibodies. These results indicate the possibility that these TZ-free CLP do not contain any protein antigen specific to these antibodies.

These results demonstrated that the DSNP conjugation with both of SLP-Ab and CLP-Ab were highly specific and sensitive, which have 100-fold sensitivity higher than ELISA method (limit of detection 1 ng/ μL). In addition, the DSNP method can be done in 1 – 1.30 h, starting from blocking the membrane until the fluorescent dot were observed, while ELISA was used at least 6 h. This method is 4 times as rapid as the ELISA, compared to the total times that were used in each method.

Row	Dot No.						
1	1.1	1.2	1.3	1.4	1.5	1.6	1.7
2	2.1	2.2	2.3	2.4	2.5	2.6	2.7
3	3.1	3.2	3.3	3.4	3.5	3.6	3.7
4	4.1	4.2	4.3	4.4	4.5	4.6	4.7
5	5.1	5.2	5.3	5.4	5.5	5.6	5.7
6	6.1	6.2	6.3	6.4	6.5	6.6	6.7

Figure 3.12 Diagram of row Dot No. of referent antigen, and crude latex protein samples spotted onto membrane as a 10-fold dilution

Row 1: Dot No.1.1-1.7 = SLP (concentration of 100 to 0.0001 ng/ μL)

Row 2: Dot No.2.1-2.7 = CLP (concentration of 100 to 0.0001 ng/ μL)

Row 3: Dot No.3.1-3.7=6C-CLP (concentration of 950 to 0.00095 ng protein/g rubber)

Row 4: Dot No.4.1-4.7 = Ir6C-CLP (concentration of 1600 to 0.0016 ng protein/g rubber)

Row 5: Dot No.5.1-5.7 = 9A-CLP (concentration of 680 to 0.00068 ng protein/g rubber)

Row 6: Dot No.6.1-6.7 = Ir9A-CLP (concentration of 1700 to 0.0017 ng protein/g rubber)

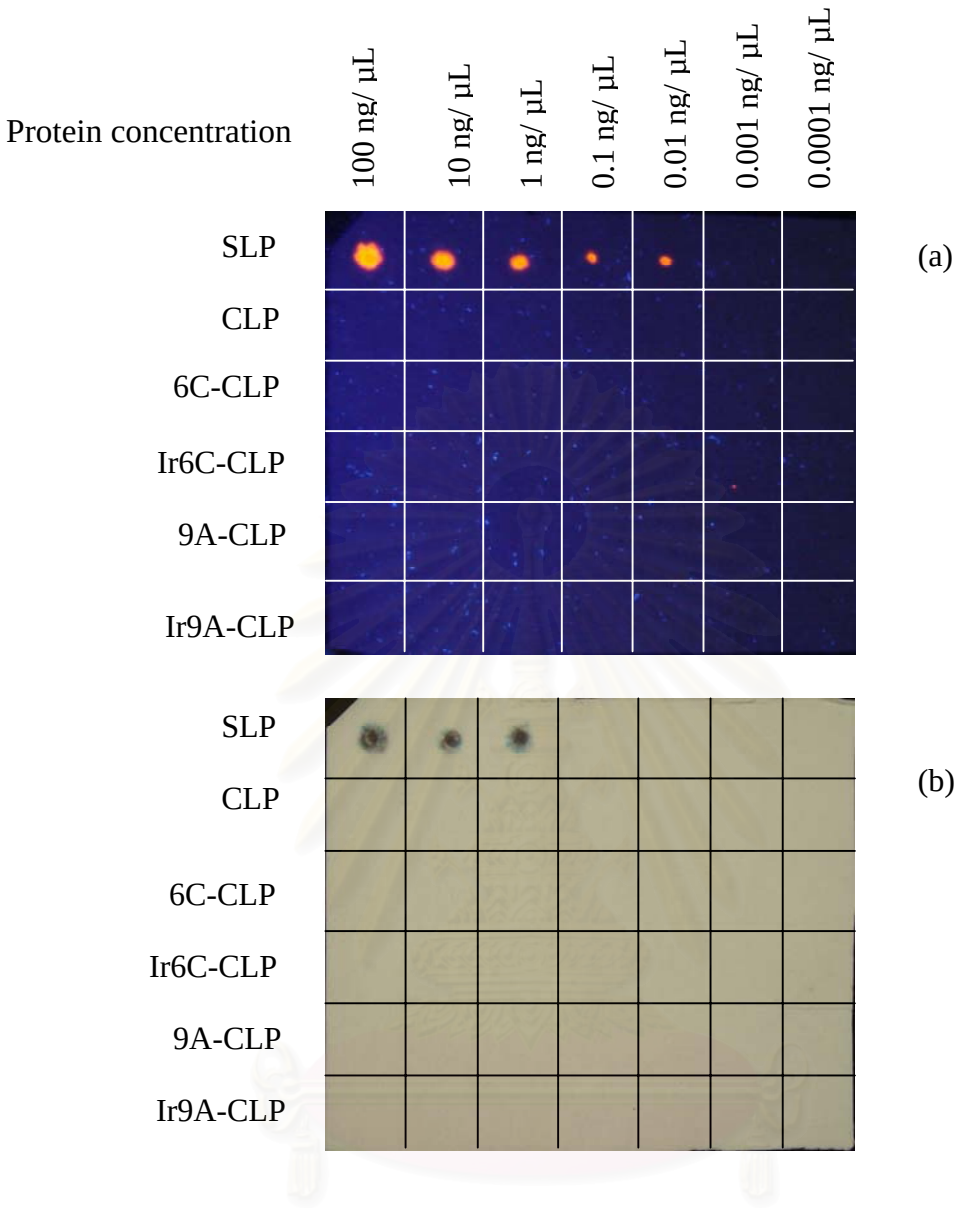


Figure 3.13 Dot immunobinding assay of latex protein by the DSNP conjugated with SLP-Ab comparing with ELISA method

The acetone- precipitated proteins from skim latex, prepared according to Method 2.3.5.1, were used as reference (positive control) and latex protein antigens 6C-CLP, Ir6C-CLP, 9A-CLP and Ir9A-CLP were used as unknown. Each sample was diluted 10-fold (10^{-1}- 10^{-7}) in PBS, and sequentially spotted on 6 x 7 cm^2 piece of FUSION 5 nitrocellulose membrane (Whatman International Ltd.). After blocking with 1% (w/v) albumin in PBS 4 ^{0}C overnight or 37 ^{0}C for 1 h, the membrane was incubated for 30 min at room temperature with antibodies against latex proteins conjugated with fluorescent dye-doped silica nanoparticles. After washing with PBST (PBS containing 0.05% Tween 20), the membrane was excited under a UV light source (Vilber Lourmat UV-light) at 312 nm for 5 min and photos of fluorescent spots were captured by a digital camera (SONY DSC-S90).

a. Detection with DSNP conjugated with SLP-Ab

b. Detection by using ELISA method

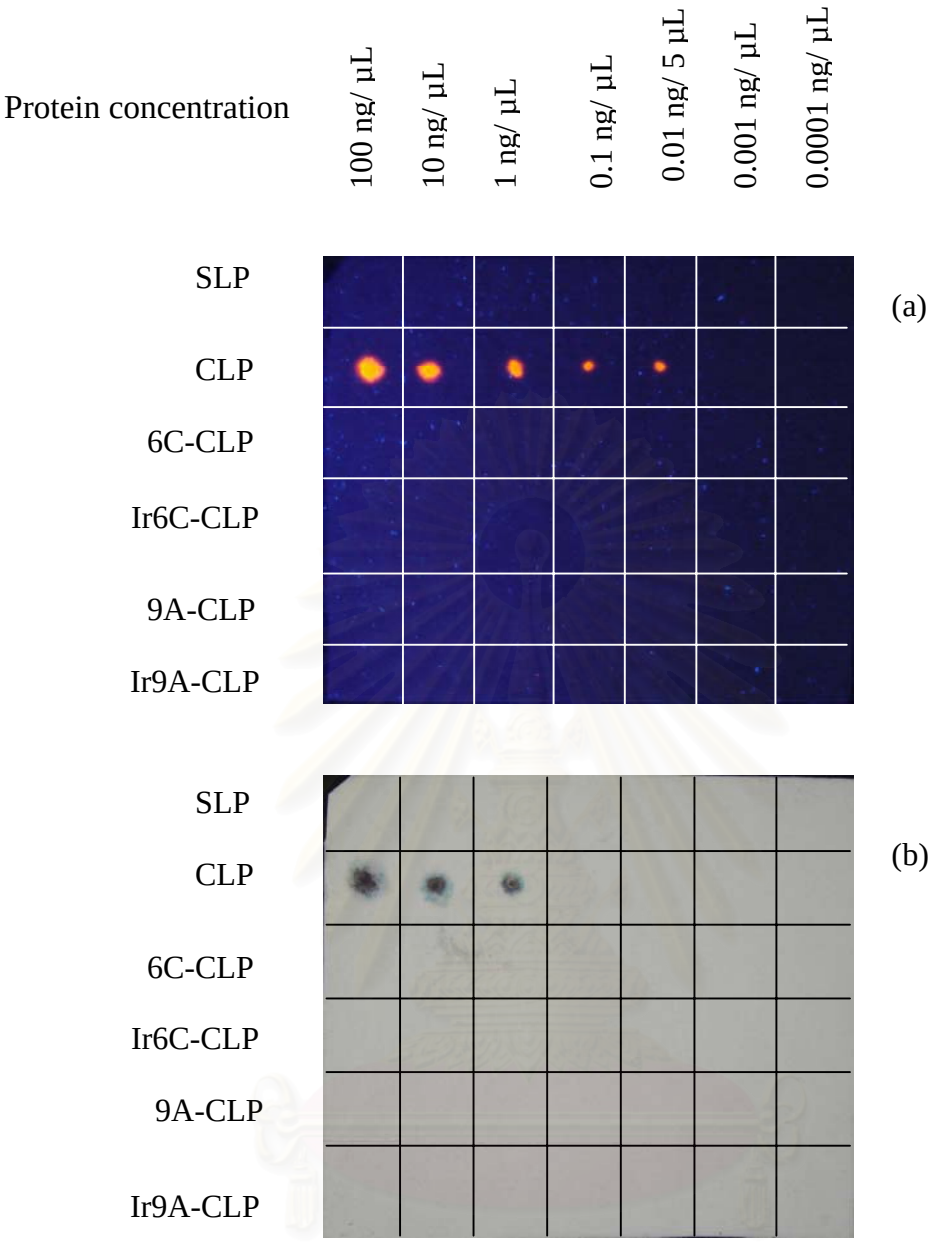


Figure 3.14 Dot immunobinding assay of latex protein by the DSNP conjugated with CLP-Ab comparing with ELISA method

The acetone- precipitated proteins from concentrated latex, prepared according to Method 2.3.5.1, was used as reference (positive control) and latex protein antigens 6C-CLP, Ir6C-CLP, 9A-CLP and Ir9A-CLP were used as unknown. Each sample was diluted 10-fold (10^{-1}- 10^{-7}) in PBS, and sequentially spotted on 6 x 7 cm^2 piece of FUSION 5 nitrocellulose membrane (Whatman International Ltd.). After blocking with 1% (w/v) albumin in PBS 4 ^{0}C overnight or 37 ^{0}C for 1 h, the membrane was incubated for 30 min at room temperature with antibody against latex proteins conjugated fluorescent dye-doped silica nanoparticles. After washing with PBST (PBS containing 0.05% Tween 20), the membrane was excited under a UV light source (Vilber Lourmat UV-light) at 312 nm for 5 min and photos of fluorescent spots were recorded by a digital camera (SONY DSC-S90).

a. Detection with DSNP conjugated CLP-Ab

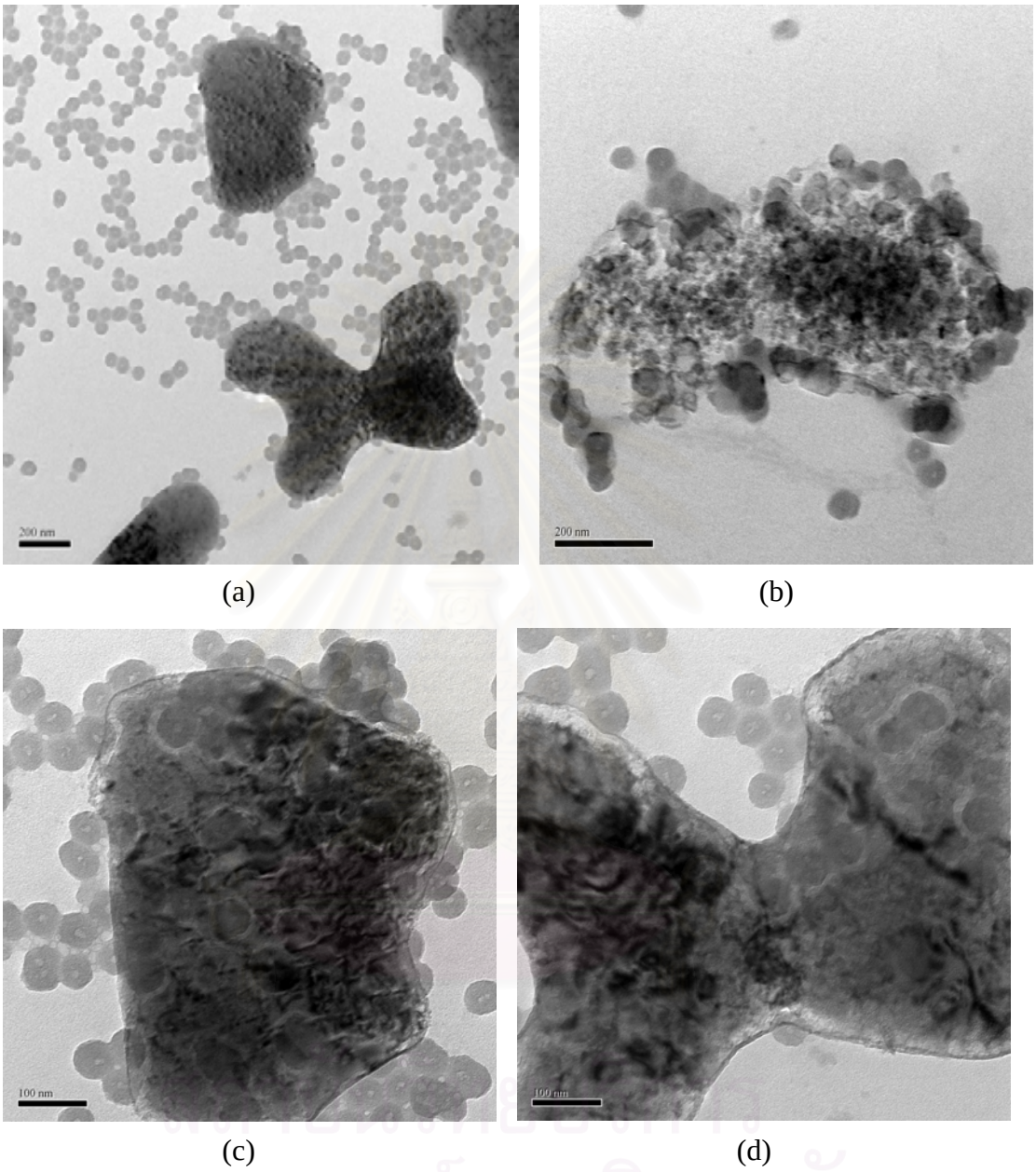
b. Detection by using ELISA method

3.9 Characteristics of Rubpy-doped silica nanoparticles conjugated with the antibodies bound to viral and protein antigen

To prove that the fluorescent signals were really emitted from DSNP bound Ab, which specifically bound to antigens. The trace amount of DSNP-Abs-PRSV-Ag (Figure 3.11), DSNP-Abs-SLP-Ag (Figure 3.13 Dot 1.1) and DSNP-Abs-CLP-Ag (Figure 3.14 Dot 2.1) was taken from the membrane and transferred to a copper grid for characterization by TEM.

Figure 3.15, 3.16 and 3.17 clearly demonstrated clusters of complexes composing of nanoparticles (DSNP-Abs) bound to respective antigens. The part that is belong to DSNP have average diameter of 51 ± 1, 50 ± 2 and 50 ± 2 nm respectively.

The results suggested the antigen size of PRSV > CLP > SLP.

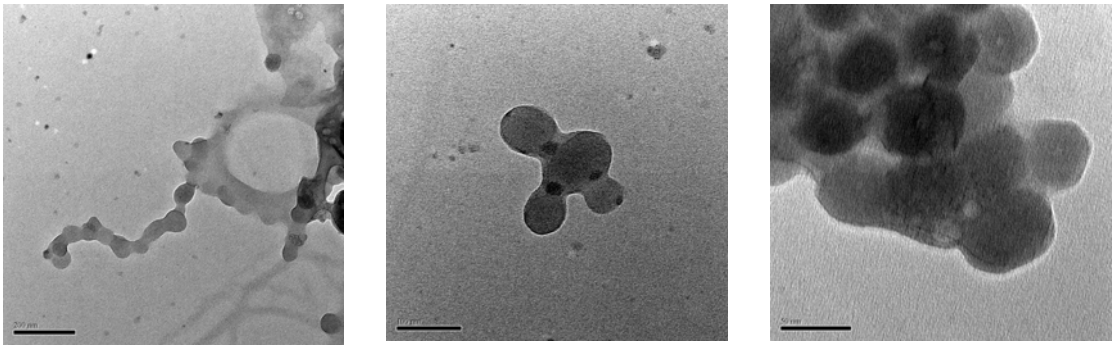


(a) (b)

(c) (d)

Figure 3.15 TEM micrographs of the DSNP conjugated PRSV-Abs binding with purified PRSV protein

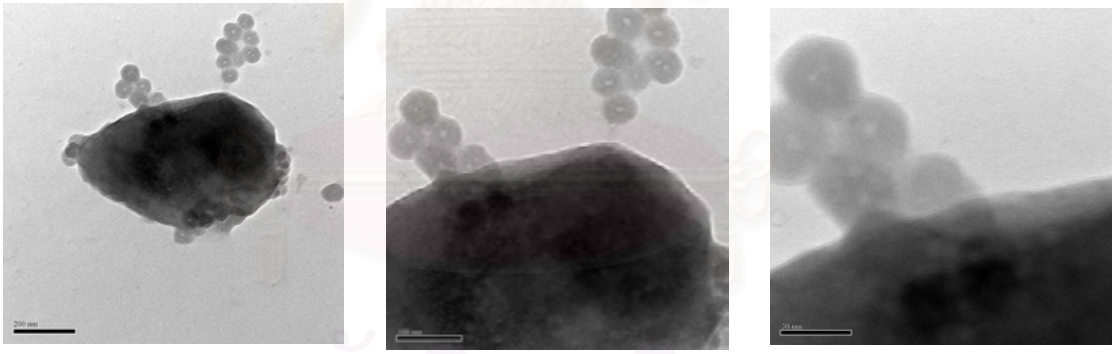
The DSNP conjugated with PRSV-Abs bound to PRSV-Ag were poked from nitrocellulose membrane fallowed by dispersed in absolute ethanol under sonication for 5 min. The sample was dropped on the copper grid and observed under TEM at (a) and (b) 20,000 x magnification and (c) and (D) 40,000 x magnification.



(a) (b) (c)

Figure 3.16 TEM image of the DSNP conjugated SLP-Abs binding with skim latex protein antigens

The DSNP conjugated with SLP-Abs binding to skim latex-Ag were poked from nitrocellulose membrane fallowed by dispersed in absolute ethanol under sonication for 5 min. The sample was dropped on the copper grid and observed under TEM at increasing magnification (a) 20,000x, (b) 40,000x and (c) 80,000x.



(a) (b) (c)

Figure 3.17 TEM micrographs of the DSNP conjugated CLP-Abs binding with concentrated latex protein antigens

The DSNP conjugated with CLP-Abs binding to concentrated latex-Ag were poked from nitrocellulose membrane fallowed by dispersed in absolute ethanol under sonication for 5 min. The sample was dropped on the copper grid and observed under TEM at increasing magnification (a) 20,000x, (b) 40,000x and (c) 80,000x.

3.10 Characterization of molecular size of skim latex protein and concentrated latex protein antigens by SDS-PAGE and immunoblotted

In this experiment, the molecular size of SLP and CLP protein antigens were characterized using SDS-PAGE followed by immunoblotting onto nitrocellulose membrane, then using DSNP conjugated with antibodies against skim latex protein antigens (DSNP-SLP-Abs) and concentrated latex protein antigens (DSNP-CLP-Abs) as nanoprobe, in comparison with conventional staining method (Coomassie blue dye-binding).

Protein bands after separation by SDS-PAGE, and after color staining with Coomassie blue show in Figure 3.18a, comparing with standard molecular weight markers (lane M). The SLP showed dominant bands with molecular weight of 31 and 39 kDa and smeared bands starting from 20.1 kDa down to lower than 14.4 kDa, while CLP showed dominant bands with molecular weight higher than 97 kDa and smeared bands of 20.1 kDa down to lower than 14.4 kDa.

After Western blotting, the SLP and CLP bands were transferred to nitrocellulose membrane. One part of membrane was detected with the DSNP-SLP-Ab, showed the specific SLP antigen bands with molecular weight of 31 and 39 kDa (Figure 3.18b). As compared with Table 1.1, both of them were identified as latex allergens as estimated from molecular mass, possibly Hev b 2: 34 kDa (β-1,3-Glucanase), Hev b 7: 42 kDa (Patatin) and Hev b 13: 42 kDa (Esterase). The other part of membrane was detected with the DSNP conjugated with CLP-Ab, exhibited latex protein antigens bands higher 97 kDa (Figure 3.18c), possibly Hev b 4: 100 kDa (component of microhelix complex).

Detection by ELISA method was also used to specify those proteins by using enzyme conjugated with goat anti-rabbit IgG and adding the color developer solution.

As shown in Figure 3.19, the SLP and CLP antigens were revealed as color bands at the same position as detected with DSNP conjugated with SLP-Ab or CLP-Ab. However, this method consumed more times at least 6 h, starting from blocking the membrane until color development, whereas the DSNP method used only 1.30 h.

When color staining by Coomassie blue for any protein was used, all of protein bands even though they were not represented as allergens are stained. In contrast, the specific latex protein antigen bands were clearly observed from both of DSNP and ELISA method. Moreover, using DSNP method was found more rapid than ELISA for 4-times as evident from total time used in each method.

On the other hand, the latex Lot No. 6C and 9A were divided into 2 fractions; one fraction was irradiated with gamma ray at 15 kGy (Ir6C and Ir9A). Each sample was used as unknown to identify the protein allergens using DSNP conjugated with antibody as nanoprobe.

Figure 3.20a showed separation of latex protein samples, Lot 6C and Ir6C- by SDS-PAGE in comparison with standard molecular weight markers (lane M). The 6C was not found any bands of protein antigen, whereas the Ir6C exhibited protein antigen bands with molecular weight of 14.4, 39, 56 kDa and a lot of slightly bands. When detecting the DSNP conjugated with CLP-Abs, there are no band were observed (Figure 3.20b).

Figure 3.21a showed separation of latex protein samples, Lot 9A and Ir9A by SDS-PAGE in comparison with standard molecular weight markers. The 9A was not found any band of protein antigen, whereas the Ir9A showed bands of proteins with molecular weight of 14.4 and 56 kDa. When detection with the DSNP conjugated with CLP-Abs, there are no any band detected on the membrane (Figure 3.21b).

These results suggested that these four latex samples did not contained latex protein antigens, Hev b 2: 34 kDa (β-1,3-Glucanase), Hev b 7: 42 kDa (Patatin), Hev b 13: 42 kDa (Esterase) and Hev b 4: 100 kDa (component of microhelix complex) which served as antigen for SLP-Abs and CLP-Abs.

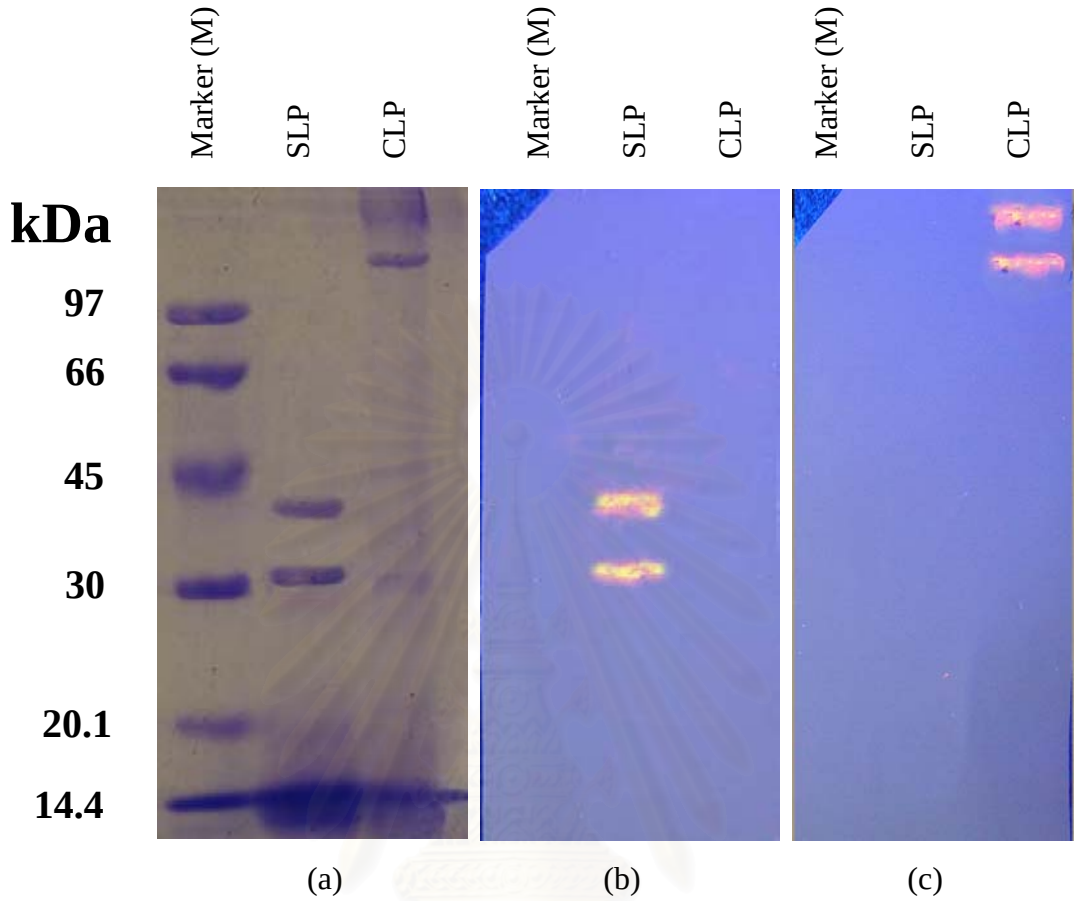


Figure 3.18 Western blot of skim and concentrated latex protein antigens when detection with DSNP conjugated with antibody against SLP or CLP

For detection with antibody conjugated DSNP. After blocked with a solution of 1% (w/v) albumin in TBS, the membrane was incubated for 30 min at room temperature with antibodies conjugated DSNP, and then washed with TBST. The membrane was excited under Vilber Lourmat UV-light at 312 nm to observe the fluorescence signals.

a. SDS-PAGE of SLP and CLP comparing with standard molecular weight marker (Phosphorylase B 97 kDa, Bovine serum albumin 66 kDa, Ovalbumin 45 kDa, Carbonic anhydrase 30 kDa, Trypsin inhibitor 20.1 kDa and α- Lactalbumin 14.4 kDa) showed SLP protein antigen bands at 31 and 39 kDa, and CLP protein antigen bands higher 97 kDa.

b. The SLP antigen bands after transferred proteins onto membrane with molecular weight of 31 and 39 kDa, when the membrane was detected by the DSNP conjugated with SLP-Abs.

c The CLP antigen bands after transferred proteins onto membrane with molecular weight higher 97 kDa, when the membrane was detected by the DSNP conjugated with CLP-Abs.

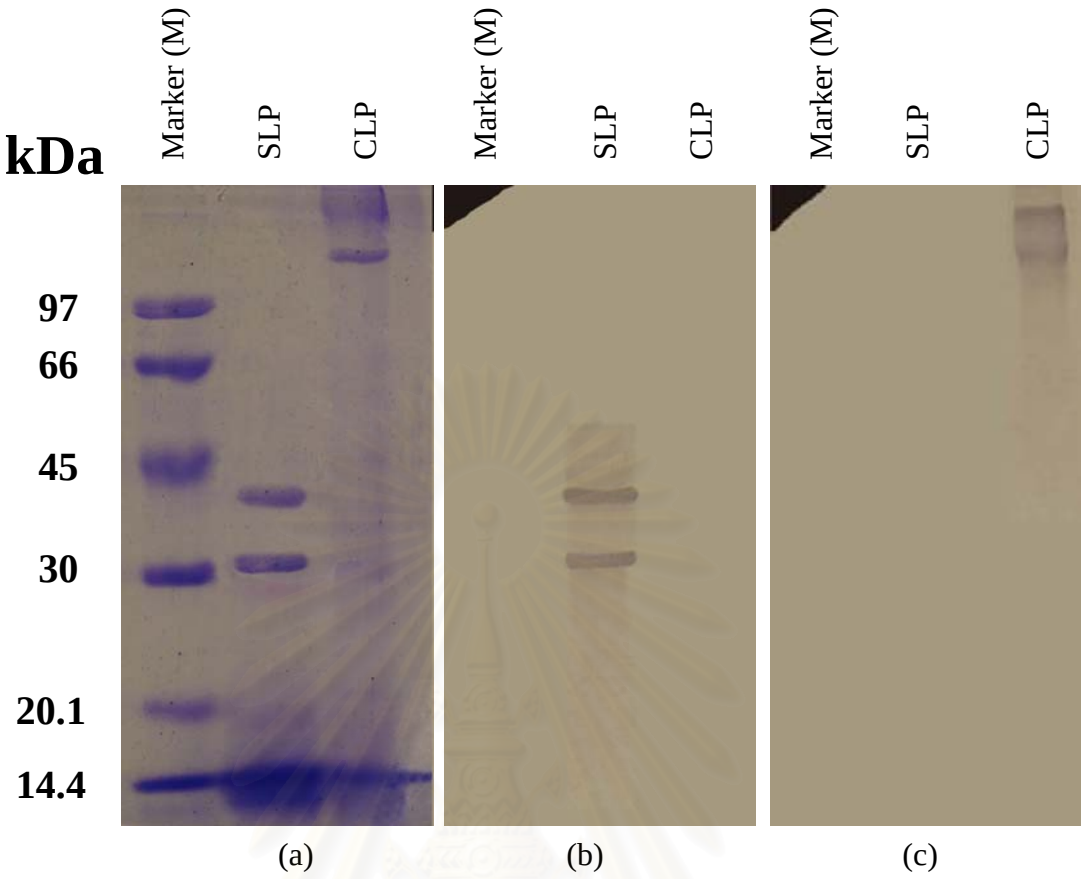


Figure 3.19 Western blot of SLP and CLP protein antigens when labeled with alkaline phosphatase-goat anti-rabbit couple with color developer solution

For detection with indirect ELISA, the membrane was transferred into a solution of primary antibody (rabbit anti-latex proteins) in TBS at the dilution of 1:1,000. Incubate for 1 h at 37 ^{0}C or 4 ^{0}C overnight. Washed membrane 3 times with TBST (TBS containing 0.05% Tween 20). The membrane was immersed in alkaline phosphatase-goat anti-rabbit IgG (dilution 1:1,000) and incubated at least 1 h. The membrane was washed 3 times with TBST. Make up developer solution, containing 10 mL of alkaline phosphatase buffer, 33 µL of BCIP and 66 µL of NBT. Immersed the membrane in this solution and incubated until color was developed. Stop reaction by rinsed with distilled water and air dried.

a. SDS-PAGE of SLP and CLP comparing with standard molecular weight marker (Phosphorylase B 97 kDa, Bovine serum albumin 66 kDa, Ovalbumin 45 kDa, Carbonic anhydrase 30 kDa, Trypsin inhibitor 20.1 kDa and α- Lactalbumin 14.4 kDa) show SLP protein antigen bands at 31 and 39 kDa, and CLP protein antigen bands higher 97 kDa.

b. The SLP antigen bands after transferred proteins onto membrane with molecular weight of 31 and 39 kDa, when detected with alkaline phosphatase-goat anti-rabbit followed by color developer solution.

c. The CLP antigen bands after transferred proteins onto membrane with molecular weight higher than 97 kDa, when detected with alkaline phosphatase-goat anti-rabbit followed by color developer solution.

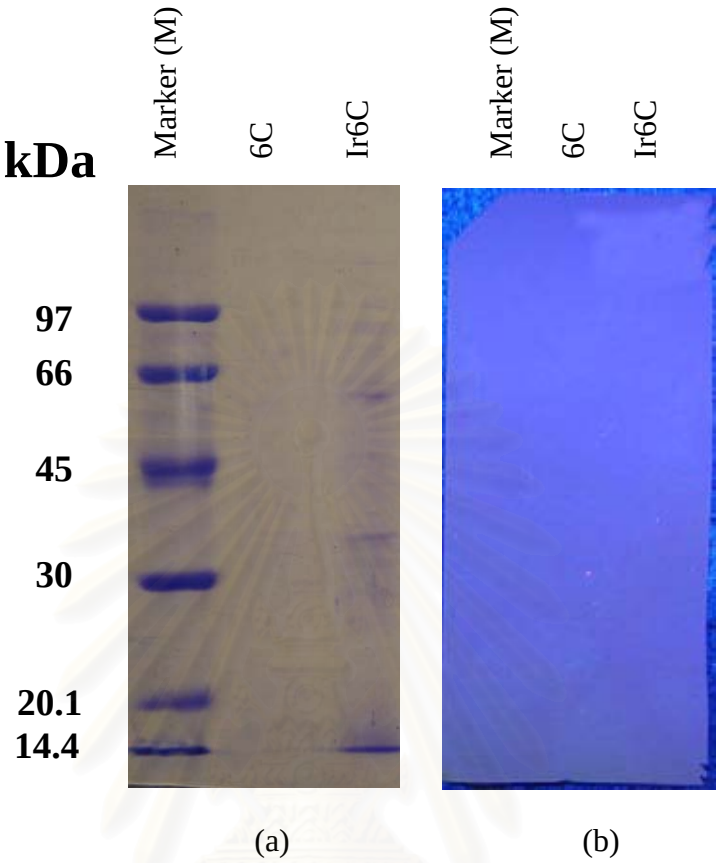


Figure 3.20 Western blot of latex protein antigens Lot 6C and Ir6C when detection with the DSNP method conjugated with CLP-Abs

a. SDS-PAGE in comparison with standard molecular weight markers (lane M). The 6C was not found any band of proteins, whereas the Ir6C showed bands of protein with molecular weight of 14.4, 39, 56 kDa and a lot of slightly bands.

b. Western blot of 6C and Ir6C when detected by the DSNP conjugated with CLP-Abs, there are no any band was detected.

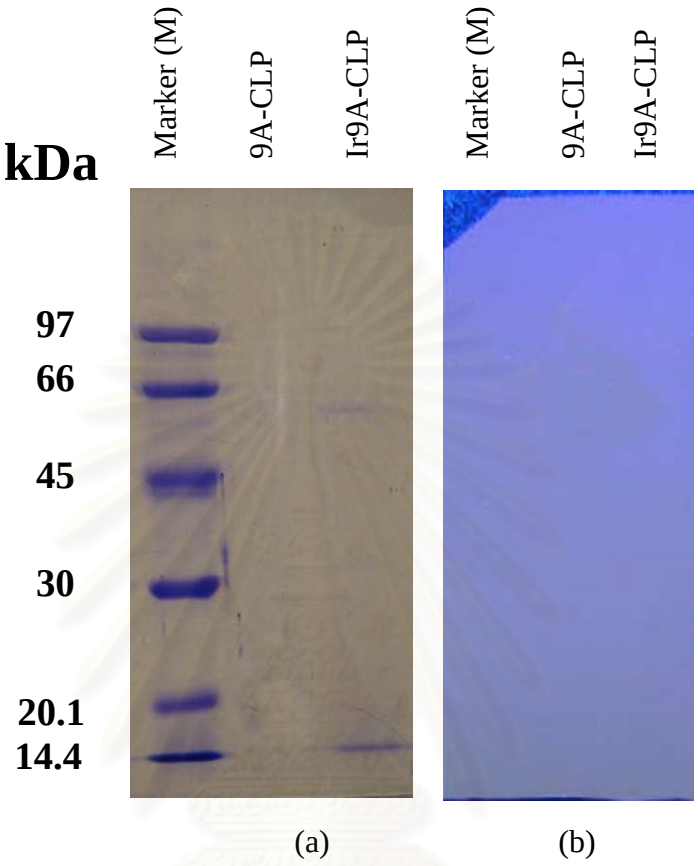


Figure 3.21 Western Blot of 9A-CLP and Ir9A-CLP when detection with the DSNP method conjugated with CLP-Abs

a. SDS-PAGE in comparison with standard molecular weight markers. The 9A was not found any band of protein, whereas the Ir9A showed bands of protein with molecular weight of 14.4 and 56 kDa.

b Western blot of 9A and Ir9A when detected with the DSNP conjugated with CLP-Abs, there are no any band was detected.

3.11 Detection of natural rubber latex antigens using DSNP conjugated with the antibodies against latex protein antigens

Apparently, this novel DSNP conjugated with SLP-Ab and CLP-Ab can be used to classify the protein antigens with high sensitivity, specificity and rapidity. In this experiment, either of the DSNP conjugated with the SLP-Ab or CLP-Ab were used as nanoprobe to quantitate the crude extracted of latex samples (Lot No. 1, 2, 3, 4, 5, 6, 6C, 7LG, 7G, 7C, 8N, 8O, 8C, 9A, 9C and 10) and crude extracted from commercial rubber products [dental dam, non-sterile examination glove and disposable nitrile glove (synthetic rubber)] that were spotted onto membrane according to Figure 3.22.

Figure 3.23a showed fluorescent dots detected by DSNP-SLP-Abs. The detection of the limit of positive fluorescent signals was at 0.01 ng/ µL, as evident by Dot No.5. Specific immunoassay was observed for all diluted crude SLP (Dot No.6-10). Only one examination glove sample (Dot No.32) shows fluorescent dot signals, which estimated the total antigens as < 0.01 ng/µL, comparing the fluorescent dot size with standard in Row 1.

Figure 3.23b, shows the DSNP-CLP-Abs was used as nanoprobe. The detection of the limit of positive fluorescent signals was at 0.01 ng/ µL as evident by Dot No.5. Specific immunobinding was observed for all diluted crude CLP (No.11-15). The latex Lot 1 (No.16), Lot 7C (No.25) and examination glove sample (No.32) were observed, when comparing with CLP standard total antigen (No.1-5). It was found that the total antigens are ≤ 0.01 ng/µL for these 3 samples.

These results suggested that latex Lot 1 and latex Lot 7C were contained with protein allergens with molecular weight of ≈ 100 kDa, which identified as Hev b 4: 100 kDa (component of microhelix complex). For examination glove, possibly

composed of multi protein allergens including molecular weight of 31, 39 and ≈ 100 kDa, which was classified as Hev b 2: 34 kDa (β-1,3-Glucanase), Hev b 7: 42 kDa (Patatin) and Hev b 13: 42 kDa (Esterase) and Hev b 4: 100 kDa (component of microhelix complex).

Each sample was determined the total protein using the modified Lowry method (Table 3.2). Crude-SLP, Crude-CLP, latex Lot 1, latex Lot 7C, and examination glove exhibited the total protein at 9 mg/ mL, 6 mg/ mL, 10 μg protein/ g rubber, 9 μg protein/ g rubber and 2.6 mg/ g rubber respectively.

These results suggested that the DSNP conjugated with antibodies can be used for both quantitative and quanlitative analysis. Because this method is very sensitive, therefore only small amount of sample can be determined without further concentration or purification in less than 2 h. This detection protocol is possibly to develop a commercial test kit for identification of rubber protein allerges with a high sensitivity and specificity.

Row					
1	1	2	3	4	5
2	6	7	8	9	10
3	11	12	13	14	15
4	16	17	18	19	20
5	21	22	23	24	25
6	26	27	28	29	30
7	31	32	33	34	35

Figure 3.22 Diagram of row Dot No. of referent antigen, and crude latex protein samples spotted onto membrane

Row 1: Dot No.1-5 = SLP-Ag or CLP-Ag (500 to 0.05 ng)

Row 2: Dot No.6-10 = Crude skim latex protein (dilution 10^{-1}-10^{-5})

Row 3: Dot No.11-15 = Crude concentrated latex protein (dilution 10^{-1}-10^{-5})

Row 4-7: Dot No.16-31 = Crude latex protein Lot 1, 2, 3, 4, 5, 6, 6C, 7LG, 7G, 7C, 8N, 8O, 8C, 9A, 9C and 10

Row 7: Dot No.32 = Examination glove

Dot No.33 = Nitrile glove (synthetic rubber)

Dot No.34-35 = Dental dam

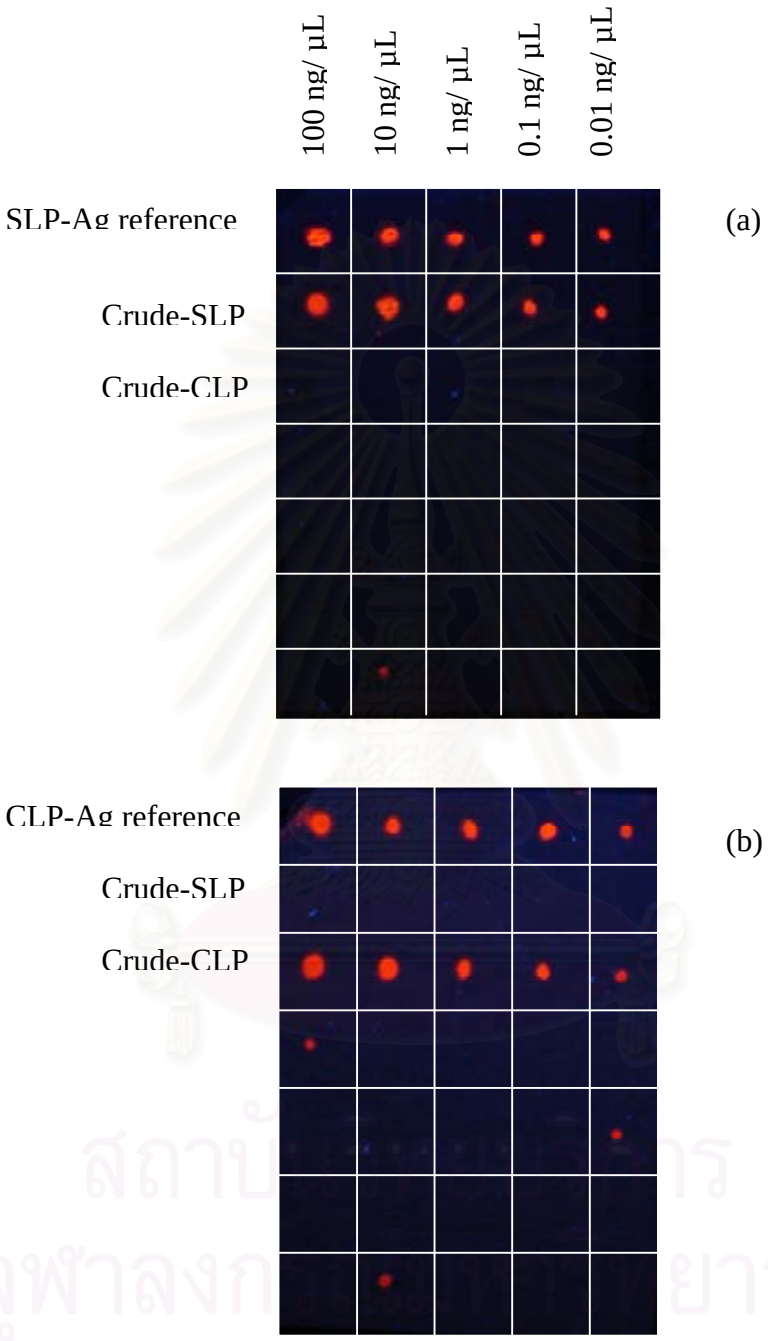


Figure 3.23 Fluoroimmunoassay of latex protein samples

a. Detection by DSNP conjugated with SLP-Abs

b. Detection by DSNP conjugated with CLP-Abs

Table 3.2 Concentration of total latex protein by Lowry method and total latex protein antigens by using DSNP conjugated with antibody against latex protein

Sample	**Total protein concentration** (modified Lowry method)	**Total antigen concentration** (detection by SLP-Ab or CLP-Ab)
Crude-SLP	9 mg/mL	100 μg/ mL (DSNP-**SLP-Ab**)
Crude-CLP	6 mg/mL	100 μg/ mL (DSNP-**CLP-Ab**)
Examination glove	2.6 mg/ g rubber	≤ 96 ng Ag/ g rubber (DSNP-**SLP**-Ab) ~ 96 ng Ag/ g rubber (DSNP-**CLP-Ab**)
latex Lot 1	10 μg protein/ g rubber	≤ 94 ng Ag/ g rubber (DSNP-**CLP**-Ab)
latex Lot 7C	9 μg protein/ g rubber	≤ 91 ng Ag/ g rubber (DSNP-**CLP-Ab**)
Other samples	4.0 – 9.9 μg protein/ g rubber	No signal was detected

3.12 Comparing for size distribution of the silica nanoparticles

In this research, the Rubpy-doped silica nanoparticles were modified to immobilize with antibodies and applied for detection viral and latex protein antigens. To elucidate that the DSNP was not changed in size. All steps that occurred with DSNP are determined as shown in Table 3.3.

From Table 3.4 indicated that the DSNP after surface modification, immobilized with antibodies and even was used in bioassay are remained in size of about 50 nm with average percent variation less than 5%. These demonstrate that the DSNP showed no swelling or porosity changes with variation in pH or no matter used in bioanalysis (Jian et al., 1998).

Table 3.3 Uniformity in size of silica nanoparticles

Size distribution of SNPs	Average* size (nm)	% Variation
Silica nanoparticle	54 ± 5	9.6
DSNP	65 ± 5	7.7
Surface modified DSNP	50 ± 7	13.6
DSNP conjugated with PRSV-Abs	50 ± 2	4.4
DSNP conjugated with SLP-Abs	50 ± 2	3.8
DSNP conjugated with CLP-Abs	51 ± 2	4.4
DSNP conjugated with PRSV-Abs binding with purified PRSV protein antigens	51 ± 1	2.3
DSNP conjugated with SLP-Abs binding with SLP	50 ± 2	4.4
DSNP conjugated with CLP-Abs binding with CLP	50 ± 2	4.5

* The average size were measured based on 12 lots of synthesized.

CHAPTER IV

CONCLUSION

1. The silica nanoparticles are transparent spherical shape with average diameter of 54 ± 5 nm and Rubpy-doped silica nanoparticles are opaque with average diameter of 65 ± 5 nm.
2. The Rubpy-doped silica nanoparticles conjugated with antibodies against skim latex antigens (31 and 39 kDa), and antibody against concentrated latex antigens (≈ 100 kDa) exhibit uniform size of 50 ± 2 nm and 51 ± 2 nm respectively.
3. The photostability of Rubpy-doped silica nanoparticles remained nearly constant for 3 days, no matter they bound with antibodies or not.
4. Utilization of Rubpy-doped silica nanoparticles conjugated with antibodies for detection of latex allergens indicated that specific and higher sensitivity of 0.01 ng/ μL antigen for both of skim latex protein antigens and concentrated latex protein antigens.
5. The very high sensitivity of the newly developed Rubpy-doped silica nanoparticles are 100-fold higher, 4-time rapid and similar specificity when comparing with ELISA.
6. The Rubpy-doped silica nanoparticles has potential to classify protein antigen by molecular weight when use in SDS-PAGE.
7. Simple extract from crude sample can be determined by Rubpy-doped silica nanoparticles conjugated with antibodies, no need for further concentrated or purified.

FUTURE RESEARCH

From this research, clearly demonstrated the potential of the fluorescent dye doped silica nanoparticles in bioassay, which have highly sensitivity specificity and rapid. Nowadays, many viral and other harmful biological agents such as H5N1, SARS and HIV can cause pandemic to population health. The rapid bioassay with highly sensitive and specific are needed. Bird flu is one of the most dangerous for animals and human. It can be distributed in the air and can lead the living things to death when infected by inhaling. To protect and assay with immediately, the DSNP conjugated with antibody against H5N1 should be developed for serving at the important tool to monitor the bird flu viral (H5N1) as fast as we can.

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APPENDICES

Appendix 1

Size of silica nanoparticles

1. Size of silica nanoparticles

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	56.25	26	54.17	51	56.25	76	54.17
2	56.25	27	45.83	52	56.25	77	45.83
3	52.08	28	60.42	53	52.08	78	60.42
4	43.75	29	58.33	54	43.75	79	58.33
5	58.33	30	56.25	55	58.33	80	56.25
6	54.17	31	56.25	56	54.17	81	56.25
7	45.83	32	56.25	57	45.83	82	56.25
8	60.42	33	52.08	58	60.42	83	52.08
9	58.33	34	43.75	59	58.33	84	43.75
10	56.25	35	58.33	60	56.25	85	58.33
11	56.25	36	54.17	61	56.25	86	54.17
12	56.25	37	45.83	62	56.25	87	45.83
13	52.08	38	60.42	63	52.08	88	60.42
14	43.75	39	58.33	64	43.75	89	58.33
15	58.33	40	56.25	65	58.33	90	56.25
16	54.17	41	56.25	66	54.17	91	56.25
17	45.83	42	56.25	67	45.83	92	56.25
18	60.42	43	52.08	68	60.42	93	52.08
19	58.33	44	43.75	69	58.33	94	43.75
20	56.25	45	58.33	70	56.25	95	58.33
21	56.25	46	54.17	71	56.25	96	54.17
22	56.25	47	45.83	72	56.25	97	45.83
23	52.08	48	60.42	73	52.08	98	60.42
24	43.75	49	58.33	74	43.75	99	58.33
25	58.33	50	56.25	75	58.33	100	56.25

AV	54.17
STDEV	5.21

2. Size of fluorescent dye-doped silica nanoparticles

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	64.15	26	64.15	51	64.15	76	64.15
2	67.92	27	67.92	52	67.92	77	67.92
3	71.70	28	64.15	53	71.70	78	64.15
4	52.83	29	67.92	54	52.83	79	67.92
5	60.38	30	67.92	55	60.38	80	67.92
6	64.15	31	64.15	56	64.15	81	64.15
7	67.92	32	67.92	57	67.92	82	67.92
8	64.15	33	71.70	58	64.15	83	71.70
9	67.92	34	52.83	59	67.92	84	52.83
10	67.92	35	60.38	60	67.92	85	60.38
11	64.15	36	64.15	61	64.15	86	64.15
12	67.92	37	67.92	62	67.92	87	67.92
13	71.70	38	64.15	63	71.70	88	64.15
14	52.83	39	67.92	64	52.83	89	67.92
15	60.38	40	67.92	65	60.38	90	67.92
16	64.15	41	64.15	66	64.15	91	64.15
17	67.92	42	67.92	67	67.92	92	67.92
18	64.15	43	71.70	68	64.15	93	71.70
19	67.92	44	52.83	69	67.92	94	52.83
20	67.92	45	60.38	70	67.92	95	60.38
21	64.15	46	64.15	71	64.15	96	64.15
22	67.92	47	67.92	72	67.92	97	67.92
23	71.70	48	64.15	73	71.70	98	64.15
24	52.83	49	67.92	74	52.83	99	67.92
25	60.38	50	67.92	75	60.38	100	67.92

AV 64.91
STDEV 5.03

3. Size of surface modification of the DSNP

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	58.06	26	54.84	51	58.06	76	54.84
2	51.61	27	48.39	52	51.61	77	48.39
3	38.71	28	54.84	53	38.71	78	54.84
4	54.84	29	41.94	54	54.84	79	41.94
5	41.94	30	58.06	55	41.94	80	58.06
6	54.84	31	58.06	56	54.84	81	58.06
7	48.39	32	51.61	57	48.39	82	51.61
8	54.84	33	38.71	58	54.84	83	38.71
9	41.94	34	54.84	59	41.94	84	54.84
10	58.06	35	41.94	60	58.06	85	41.94
11	58.06	36	54.84	61	58.06	86	54.84
12	51.61	37	48.39	62	51.61	87	48.39
13	38.71	38	54.84	63	38.71	88	54.84
14	54.84	39	41.94	64	54.84	89	41.94
15	41.94	40	58.06	65	41.94	90	58.06
16	54.84	41	58.06	66	54.84	91	58.06
17	48.39	42	51.61	67	48.39	92	51.61
18	54.84	43	38.71	68	54.84	93	38.71
19	41.94	44	54.84	69	41.94	94	54.84
20	58.06	45	41.94	70	58.06	95	41.94
21	58.06	46	54.84	71	58.06	96	54.84
22	51.61	47	48.39	72	51.61	97	48.39
23	38.71	48	54.84	73	38.71	98	54.84
24	54.84	49	41.94	74	54.84	99	41.94
25	41.94	50	58.06	75	41.94	100	58.06

AV	50.32
STDEV	6.83

4. Size of the DSNP conjugated with the antibody against papaya ringspot virus

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	50.00	26	48.00	51	48.00	76	50.00
2	50.00	27	50.00	52	46.00	77	52.00
3	48.00	28	50.00	53	48.00	78	54.00
4	46.00	29	50.00	54	48.00	79	52.00
5	48.00	30	50.00	55	50.00	80	54.00
6	48.00	31	48.00	56	50.00	81	50.00
7	50.00	32	46.00	57	52.00	82	50.00
8	50.00	33	48.00	58	54.00	83	48.00
9	50.00	34	48.00	59	52.00	84	46.00
10	50.00	35	50.00	60	54.00	85	48.00
11	48.00	36	50.00	61	50.00	86	48.00
12	46.00	37	52.00	62	50.00	87	50.00
13	48.00	38	54.00	63	48.00	88	50.00
14	48.00	39	52.00	64	46.00	89	50.00
15	50.00	40	54.00	65	48.00	90	50.00
16	50.00	41	50.00	66	48.00	91	48.00
17	52.00	42	50.00	67	50.00	92	46.00
18	54.00	43	48.00	68	50.00	93	48.00
19	52.00	44	46.00	69	50.00	94	48.00
20	54.00	45	48.00	70	50.00	95	50.00
21	50.00	46	48.00	71	48.00	96	50.00
22	50.00	47	50.00	72	46.00	97	52.00
23	48.00	48	50.00	73	48.00	98	54.00
24	46.00	49	50.00	74	48.00	99	52.00
25	48.00	50	50.00	75	50.00	100	54.00

AV	49.60
STDEV	2.16

5. Size of the DSNP conjugated with the antibody against skim latex protein

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	50.00	26	52.00	51	50.00	76	52.00
2	52.00	27	52.00	52	52.00	77	52.00
3	46.00	28	50.00	53	46.00	78	50.00
4	48.00	29	50.00	54	48.00	79	50.00
5	50.00	30	52.00	55	50.00	80	52.00
6	52.00	31	50.00	56	52.00	81	50.00
7	52.00	32	52.00	57	52.00	82	52.00
8	50.00	33	46.00	58	50.00	83	46.00
9	50.00	34	48.00	59	50.00	84	48.00
10	52.00	35	50.00	60	52.00	85	50.00
11	50.00	36	52.00	61	50.00	86	52.00
12	52.00	37	52.00	62	52.00	87	52.00
13	46.00	38	50.00	63	46.00	88	50.00
14	48.00	39	50.00	64	48.00	89	50.00
15	50.00	40	52.00	65	50.00	90	52.00
16	52.00	41	50.00	66	52.00	91	50.00
17	52.00	42	52.00	67	52.00	92	52.00
18	50.00	43	46.00	68	50.00	93	46.00
19	50.00	44	48.00	69	50.00	94	48.00
20	52.00	45	50.00	70	52.00	95	50.00
21	50.00	46	52.00	71	50.00	96	52.00
22	52.00	47	52.00	72	52.00	97	52.00
23	46.00	48	50.00	73	46.00	98	50.00
24	48.00	49	50.00	74	48.00	99	50.00
25	50.00	50	52.00	75	50.00	100	52.00

AV	50.20
STDEV	1.90

6. Size of the DSNP conjugated with the antibody against concentrated latex protein

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	48.00	26	50.00	51	48.00	76	50.00
2	50.00	27	48.00	52	50.00	77	48.00
3	48.00	28	52.00	53	48.00	78	52.00
4	52.00	29	50.00	54	52.00	79	50.00
5	54.00	30	54.00	55	54.00	80	54.00
6	50.00	31	48.00	56	50.00	81	48.00
7	48.00	32	50.00	57	48.00	82	50.00
8	52.00	33	48.00	58	52.00	83	48.00
9	50.00	34	52.00	59	50.00	84	52.00
10	54.00	35	54.00	60	54.00	85	54.00
11	48.00	36	50.00	61	48.00	86	50.00
12	50.00	37	48.00	62	50.00	87	48.00
13	48.00	38	52.00	63	48.00	88	52.00
14	52.00	39	50.00	64	52.00	89	50.00
15	54.00	40	54.00	65	54.00	90	54.00
16	50.00	41	48.00	66	50.00	91	48.00
17	48.00	42	50.00	67	48.00	92	50.00
18	52.00	43	48.00	68	52.00	93	48.00
19	50.00	44	52.00	69	50.00	94	52.00
20	54.00	45	54.00	70	54.00	95	54.00
21	48.00	46	50.00	71	48.00	96	50.00
22	50.00	47	48.00	72	50.00	97	48.00
23	48.00	48	52.00	73	48.00	98	52.00
24	52.00	49	50.00	74	52.00	99	50.00
25	54.00	50	54.00	75	54.00	100	54.00

AV	50.60
STDEV	2.21

7. Size of the DSNP conjugated with the antibody against PRSV protein binding with purified PRSV proteins

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	52.78	26	51.67	51	52.78	76	51.67
2	48.33	27	50.00	52	48.33	77	50.00
3	50.56	28	49.44	53	50.56	78	49.44
4	51.11	29	50.56	54	51.11	79	50.56
5	51.11	30	50.56	55	51.11	80	50.56
6	51.67	31	52.78	56	51.67	81	52.78
7	50.00	32	48.33	57	50.00	82	48.33
8	49.44	33	50.56	58	49.44	83	50.56
9	50.56	34	51.11	59	50.56	84	51.11
10	50.56	35	51.11	60	50.56	85	51.11
11	52.78	36	51.67	61	52.78	86	51.67
12	48.33	37	50.00	62	48.33	87	50.00
13	50.56	38	49.44	63	50.56	88	49.44
14	51.11	39	50.56	64	51.11	89	50.56
15	51.11	40	50.56	65	51.11	90	50.56
16	51.67	41	52.78	66	51.67	91	52.78
17	50.00	42	48.33	67	50.00	92	48.33
18	49.44	43	50.56	68	49.44	93	50.56
19	50.56	44	51.11	69	50.56	94	51.11
20	50.56	45	51.11	70	50.56	95	51.11
21	52.78	46	51.67	71	52.78	96	51.67
22	48.33	47	50.00	72	48.33	97	50.00
23	50.56	48	49.44	73	50.56	98	49.44
24	51.11	49	50.56	74	51.11	99	50.56
25	51.11	50	50.56	75	51.11	100	50.56

AV	50.61
STDEV	1.16

8. Size of the DSNP conjugated with the antibody against skim latex protein binding with skim latex protein

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	48.00	26	50.00	51	48.00	76	50.00
2	52.00	27	50.00	52	52.00	77	50.00
3	54.00	28	48.00	53	54.00	78	48.00
4	46.00	29	52.00	54	46.00	79	52.00
5	50.00	30	50.00	55	50.00	80	50.00
6	50.00	31	48.00	56	50.00	81	48.00
7	50.00	32	52.00	57	50.00	82	52.00
8	48.00	33	54.00	58	48.00	83	54.00
9	52.00	34	46.00	59	52.00	84	46.00
10	50.00	35	50.00	60	50.00	85	50.00
11	48.00	36	50.00	61	48.00	86	50.00
12	52.00	37	50.00	62	52.00	87	50.00
13	54.00	38	48.00	63	54.00	88	48.00
14	46.00	39	52.00	64	46.00	89	52.00
15	50.00	40	50.00	65	50.00	90	50.00
16	50.00	41	48.00	66	50.00	91	48.00
17	50.00	42	52.00	67	50.00	92	52.00
18	48.00	43	54.00	68	48.00	93	54.00
19	52.00	44	46.00	69	52.00	94	46.00
20	50.00	45	50.00	70	50.00	95	50.00
21	48.00	46	50.00	71	48.00	96	50.00
22	52.00	47	50.00	72	52.00	97	50.00
23	54.00	48	48.00	73	54.00	98	48.00
24	46.00	49	52.00	74	46.00	99	52.00
25	50.00	50	50.00	75	50.00	100	50.00

AV	50.00
STDEV	2.20

9. Size of the DSNP conjugated with the antibody against concentrated latex protein binding with concentrated latex protein

No.	Size (nm)	No.	Size (nm)	No.	Size (nm)	No.	Size (nm)
1	52.00	26	50.00	51	52.00	76	50.00
2	50.00	27	50.00	52	50.00	77	50.00
3	48.00	28	54.00	53	48.00	78	54.00
4	46.00	29	52.00	54	46.00	79	52.00
5	52.00	30	48.00	55	52.00	80	48.00
6	50.00	31	52.00	56	50.00	81	52.00
7	50.00	32	50.00	57	50.00	82	50.00
8	54.00	33	48.00	58	54.00	83	48.00
9	52.00	34	46.00	59	52.00	84	46.00
10	48.00	35	52.00	60	48.00	85	52.00
11	52.00	36	50.00	61	52.00	86	50.00
12	50.00	37	50.00	62	50.00	87	50.00
13	48.00	38	54.00	63	48.00	88	54.00
14	46.00	39	52.00	64	46.00	89	52.00
15	52.00	40	48.00	65	52.00	90	48.00
16	50.00	41	52.00	66	50.00	91	52.00
17	50.00	42	50.00	67	50.00	92	50.00
18	54.00	43	48.00	68	54.00	93	48.00
19	52.00	44	46.00	69	52.00	94	46.00
20	48.00	45	52.00	70	48.00	95	52.00
21	52.00	46	50.00	71	52.00	96	50.00
22	50.00	47	50.00	72	50.00	97	50.00
23	48.00	48	54.00	73	48.00	98	54.00
24	46.00	49	52.00	74	46.00	99	52.00
25	52.00	50	48.00	75	52.00	100	48.00

AV	50.20
STDEV	2.28

Appendix 2

Yields of the nanoparticles

1. Yield of the silica nanoparticles

Lot	Weight (mg)
1	21.67
2	20.54
3	20.15
4	20.23
5	20.73
6	20.41
7	20.53
8	20.28
9	20.20
10	20.96
11	20.25
12	20.32
AV	20.52
SD	0.43

2. Yield of the fluorescent dye-doped silica nanoparticles

Lot	Weight (mg)
1	27.16
2	26.45
3	26.32
4	26.51
5	26.69
6	26.82
7	26.44
8	26.74
9	26.35
10	26.41
11	26.38
12	26.37
AV	26.55
SD	0.25

Appendix 3

Fluorescent intensity of the free dye and the DSNP

1. Fluorescent intensity of the free Rubpy dye

Time(hour)	Fluorescence Intensity(A.U.)	%Relative Fluorescence Intensity
0	985.77	100
1	935.79	94.93
2	873.24	88.58
3	812.08	82.38
6	748.57	75.94
9	665.73	67.53
12	548.79	55.67
24	315.28	31.98
48	150.34	15.25
72	46.38	4.70

2. Fluorescent intensity of the DSNP

Time(hour)	Fluorescence Intensity(A.U.)	%Relative Fluorescence Intensity
0	999.99	100
1	999.99	100
2	999.99	100
3	999.67	99.97
6	999.33	99.93
9	998.97	99.90
12	998.61	99.86
24	997.55	99.76
48	994.98	99.50
72	991.23	99.12

Appendix 4

Water extractable protein determination by Lowry method (ASTM D 5712-99)

1. Extraction of water extractable proteins from concentrated latex 60% and commercial rubber dam (ASTM D 5712-99)

The concentrated latex 60% was poured onto the 20x 20 cm square plate then air dried at room temperature for 8-12 hours. A solid rubber sheet was cut with 1x 1 cm square size, weighed and transferred to a 250 ml flask and added 10 ml of distilled water per gram of specimen. The flask was sealed with sealing film; extraction was at 37 ^{0}C and shook for 15 seconds after adding the water and again at 120 minutes. The extracted solution was filtered through filter paper (Whatman no.1) followed by centrifugation at 3,000 g for 5 minutes. The filtrated solution was lyophilized.

2. Concentration of latex proteins by lyophilization

The filtrated-solution was aliquoted in plastic tube, frozen at -80 ^{0}C, then lyophilized for 48 hours. Random sampling of lyophilized protein was resuspended with distilled water and assayed for water extractable protein.

3. Determination of water extractable proteins by modified Lowry method (ASTM D 5712-99)

3.1 Solution for modified Lowry method

Solution C: 6% w/v of sodium carbonate

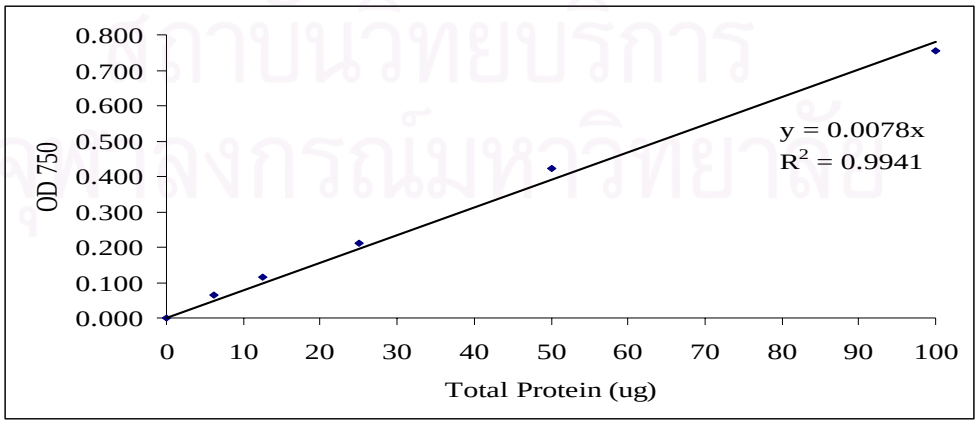
Solution D: 1.5% w/v of copper sulfate in 3% w/v of sodium citrate

Reagent A: Alkaline copper sulfate (10 parts of C: 0.2 part of D)

Reagent B: Folin Reagent

3.2 Measurement water extractable protein by modified Lowry method

The lyophilized protein samples were resuspended with distilled water. The reaction was carried out with 160- 200 µl of 0.1 N sodium hydroxide and 2.5- 40 µl of protein solutions, then added 75 µl of alkaline copper sulfate, Reagent A, into each well of a flat bottom, 96-well, microtiter plate, mixed and allowed to stand for 15 minutes at room temperature. The reaction was then added with 25 µl of dilute Folin solution, Reagent B, mixed thoroughly and allowed for 15 minutes at room temperature. Proteins levels were evaluated against standard protein, ovalbumin, using a microplate reader (Multiskan Ex, Labsystems) at 750 nm wavelength.



Standard curve of ovalbumin measured by modified Lowry method.

Water extractable protein of dry rubber sheet compared between before and after irradiation vulcanization (RV) by gamma ray at 15 kGy.

Water extractable protein from latex 60% Lot No.	Protein content (µg protein / g rubber)	Protein content (µg protein / g RV rubber)
1(24/08/48)	10.0	27
2(14/10/48)	6.8	21
3(11/11/48)	4.8	17
4C(16/12/48)	6.9	34
5(08/02/49)	7.9	24
6A(11/05/49)	4.2	9
6C(11/05/49)	9.5	16
7LG(13/07/49)	5.2	24
7G(13/07/49)	9.5	24
7C(13/07/49)	9.3	43
8O(22/08/49)	4.7	23
8C(22/08/49)	9.9	33
9A(12/10/49)	6.8	17
Com. Dental Dam	29	29

Appendix 5

Preparation of polyclonal antibody to natural rubber latex protein (NRL) antigens

1. Preparation of protein antigens

Two types of NRL, skim latex and concentrated latex 60%, were used for preparation of protein antigens. The 200- ml skim latex or concentrated latex 60% were added with 1,000 ml of cold acetone (-20 ^{0}C) under stirring. After incubation at -20 ^{0}C for 10 minutes and store overnight at 10 ^{0}C the latex proteins were centrifuged at 8,000 g for 5 minutes. The supernatant was removed and air-dried pellet was resuspended in 10- 20 volume of buffer solution or distilled water.

2. Antisera preparation

New Zealand white female rabbits (11–12 kilograms) were immunized with either acetone- precipitated proteins from skim latex and concentrated latex 60%, using Complete Freund's Adjuvant (CFA, Sigma, St. Louis, MO). For rabbits immunized with CFA, an equal volume of antigen was mixed with CFA to form a homogenous emulsion. Rabbits were injected subcutaneously at 2– 6 sites on the dorsum with 1 ml aliquots, containing total of 1 mg/ml of protein. After a rest period, about 2 weeks, 3 booster injections were given using Incomplete Freund's adjuvant (IFA). The total amount of antigen administered per rabbit was 4 mg/ml. Starting at week 8, rabbits were bleed every 2 weeks and the antibody titers were checked by an indirect ELISA.

3. Determination of the antibody titer by ELISA

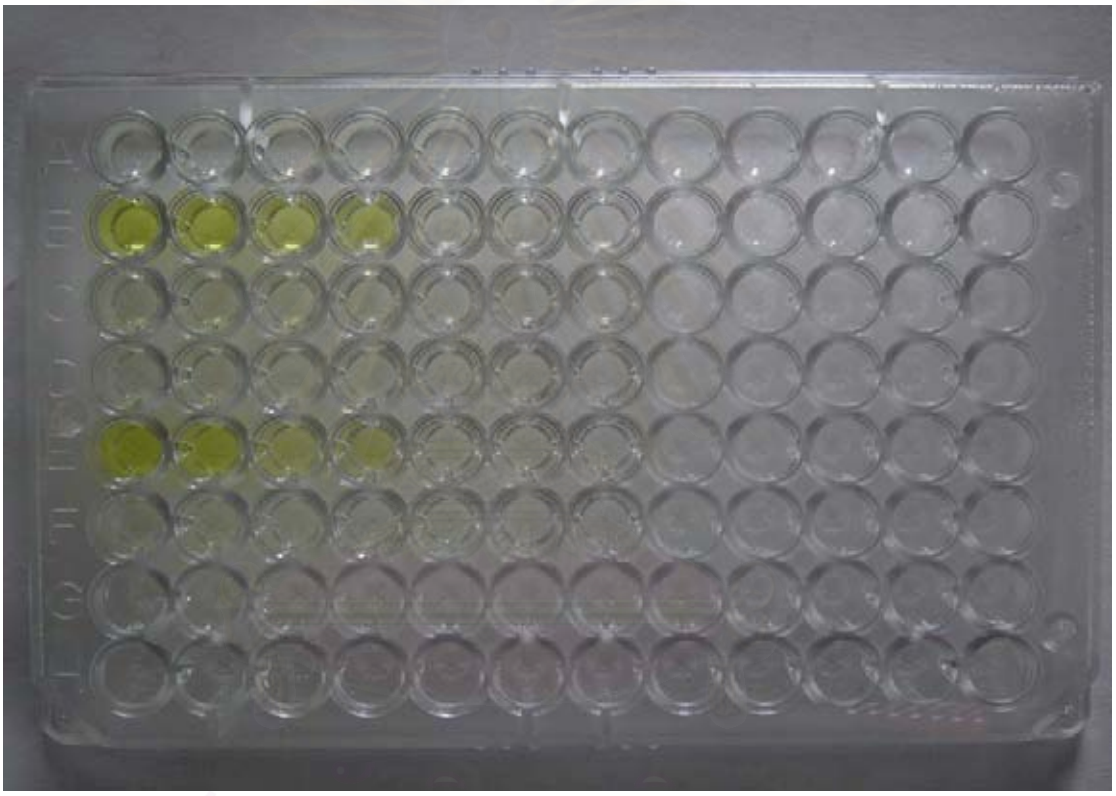
The microtiter plate was coated by using 100 μl/ well of latex protein (final concentration 200 ng/ml) in a carbonate coating buffer pH 9.6. After incubated for 1 hour at 37 ^{0}C, aspirate well and washed 3 times with 200 μl washing buffer [phosphate buffer saline (PBS)] containing 0.05% Tween 20, PBST, blocked with 200 μl/well of blocking solution (1% ovalbumin/PBS) and incubated for 1 hour at 37 ^{0}C. After aspirate well, seven 10-fold (10^{-1}- 10^{-7}) dilutions of serum to be tested were added onto series of wells at 100 μl/well, using seven 10-fold (10^{-1}- 10^{-7}) dilutions of each normal serum as negative control. After incubation for 1 hour at 37 ^{0}C, aspirate well and washed 3 times with 200 μl washing buffer. After washing the plates, a 1/1000 dilution of Alkaline phosphatase-goat anti-rabbit IgG was added and incubated for 1 hour at 37 ^{0}C. The plates were again washed 3 times, and color reaction was started by the addition of substrate 1 mg/ml *p*-nitrophenyl phosphate (pNPP), dissolved in buffer pH 9.8 (0.1 M glycine buffer, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). The color development was stopped by the addition of 50 μl of 3 N NaOH.

4. Antibody titer

An indirect ELISA method was used to determine the antibody titer, SLP and CLP, and titer 4 was observed for day 52, 66 and 90 after immunization that antisera.

The crosslink between each antibody was checked. The microtiter plate was coated with skim latex proteins in row A, B, C and concentrated latex 60% proteins in row D, E and F, and determined by indirect ELISA method. For row A and D the normal serum seven ten-fold dilutions (10^{-1}- 10^{-7}) was used as 1st antibodies (negative control), row B and E the antibody against skim latex proteins and concentrated latex 60% proteins were used as 1st antibodies respectively. On row C and F the

concentrated latex 60% proteins and skim latex proteins were used as 1st antibodies respectively. All of the antiserum including normal serum (seven ten-fold dilutions) was added to each well from column 1 to 7. The results from lanes C and F (Figure 3.2) demonstrated that no crosslink occur between antibody against skim latex proteins and concentrated latex 60% proteins, only lane B and E have a yellow color and shown the titer value as 4 for both antibody.



The microtiter plate for inhibition ELISA assay for titer checked and antibodies cross-linking test.

Appendix 6

Solution for SDS-PAGE

1. Tris glycine electrode buffer

(25 mM Tris, 192 mM glycine)

Tris	3.0	g
Glycine	14.4	g
SDS	1.0	g
H_2O	1	L

2. Tris-HCl stock solution pH 8.8

(2 M Tris)

Tris	24.2	g
H_2O	100	ml

(adjust pH to 8.8 with $HCl_{conc.}$ Or 0.1 M NaOH)

3. Tris-HCl stock solution pH 6.8

(1 M Tris)

Tris	12.2	g
H_2O	100	ml

(adjust pH to 6.8 with $HCl_{conc.}$ Or 0.1 M NaOH)

4. Sample buffer

Tris-HCl stock solution pH 6.8	0.6	ml
10 % SDS	2	ml
2-mercaptoethanol	0.5	ml
1 % bromophenol blue	1	ml
H_2O	0.9	ml

5. Acrylamide stock

Acrylamide	29.2	g
Bis	0.8	g
H_2O	100	ml

6. Ammonium persulfate 0.1 g/ml

7. 15 % Seperating gel

Stock gel (30 %)	10	ml
Stock buffer pH 8.8	5	ml
H_2O	5	ml
Ammonium persulfate	100	µl
TEMED	10	µl

8. Stacking gel

Stock gel (30 %)	1.34	ml
Stock buffer pH 6.8	2.0	ml
H_2O	4.6	ml
Ammonium persulfate	60	µl
TEMED	10	µl

9. Staining solution

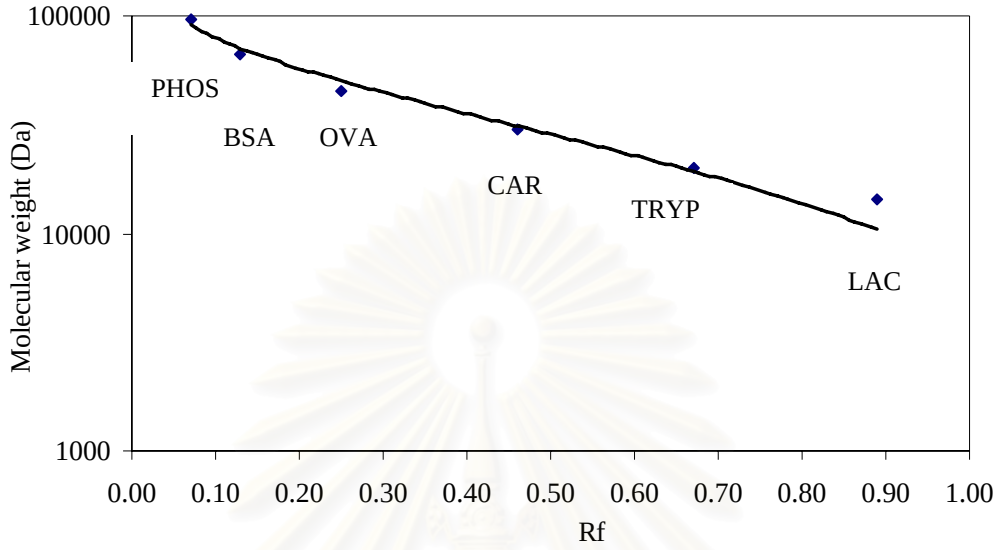
Coomassie Blue R-250	1.0	g
Methanol	450	ml
Glacial acetic acid	100	ml
H_2O	450	ml

10. Destain solution

Methanol	100	ml
Glacial acetic acid	100	ml
H_2O	800	ml

Appendix 7

Standard curve of standard protein for SDS-PAGE



Molecular weight markers calibration curve of SDS-PAGE

PHOS:	Phosphorylase B	97	kDa
BSA:	Bovine serum albumin	66	kDa
OVA:	Ovalbumin	45	kDa
CAR:	Carbonic anhydrase	30	kDa
TRYP	Trypsin inhibitor	20.1	kDa
LAC	α- Lactalbumin	14.4	kDa

BIOGRAPHY

Mr. Arnut Ritdath was born on August 3, 1982. He graduated with a Bachelor in Science majoring in General Science from Kasetsart University in 2004. He continued his study in the Master Program of Biochemistry, Faculty of Science at Chulalongkorn University, since 2004.