การพัฒนาฟังก์ชันนัลโคพอลิเมอร์บรัชสำหรับการตรึงโพรบในการประยุกต์ด้านการรับรู้เชิงชีวภาพ

นางสาวอรพรรณ เวียรชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาปิโตรเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

DEVELOPMENT OF FUNCTIONAL COPOLYMER BRUSHES FOR PROBE IMMOBILIZATION IN BIOSENSING APPLICATION

Miss Oraphan Wiarachai

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Petrochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2014

Thesis Title	DEVELOPMENT OF FUNCTIONAL COPOLYMER BRUSHES FOR PROBE IMMOBILIZATION IN BIOSENSING APPLICATION
By	Miss Oraphan Wiarachai
Field of Study	Petrochemistry
Thesis Advisor	Associate Professor Voravee Hoven, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Professor Tharapong Vitidsant, Ph.D.)

.....Thesis Advisor
(Associate Professor Voravee Hoven, Ph.D.)

.....Examiner
(Associate Professor Nuanphun Chantarasiri, Ph.D.)

.....Examiner
(Assistant Professor Yongsak Sritana-anant, Ph.D.)

.....External Examiner
(Assistant Professor Chidchanok Meechaisue, Ph.D.)

อรพรรณ เวียรชัย : การพัฒนาฟังก์ชันนัลโคพอลิเมอร์บรัชสำหรับการตรึงโพรบในการประยุกต์ด้านการรับรู้เชิงชีวภาพ (DEVELOPMENT OF FUNCTIONAL COPOLYMER BRUSHES FOR PROBE IMMOBILIZATION IN BIOSENSING APPLICATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น , 130 หน้า.

งานวิจัยนี้มีวัตถุประสงค์ที่จะพัฒนาโคพอลิเมอร์แพลตฟอร์มที่ประกอบด้วยพอลิ(2-เมทาคริโลอิลออกซีเอทิลฟอสโฟริลโคลีน) (พีเอ็มพีซี) สำหรับการประยุกต์ใช้เป็นอุปกรณ์ตรวจวัดทางชีวภาพ แพลตฟอร์มส่วนแรกเตรียมจากโคพอลิเมอร์แบบสุ่มของพอลิ(โพรพาร์จิลเมทาคริเลต) และพอลิ(2-เมทาคริโลอิลออกซีเอทิลฟอสโฟริลโคลีน) (พีพีจีเอ็มเอเอ็มพีซี) ซึ่งถูกสังเคราะห์ผ่านปฏิกิริยาพอลิเมอไรเซชันแบบ RAFT พีพีจีเอ็มเอเอ็มพีซีที่มีหมู่ปลายเป็นไทออลถูกตรึงลงบนแผ่นเซอร์เฟสพลาสมอนเรโซแนนซ์ (เอสพีอาร์) ที่เคลือบทองด้วยวิธี grafting-to ไบโอตินและเพปไทด์นิวคลีอิกแอซิด (พีเอ็นเอ) ถูกใช้เป็นโพรบต้นแบบเพื่อที่จะศึกษาการตรึงและการจับยึดอย่างจำเพาะสำหรับเซนเซอร์ในรูปแบบของแอนติเจน/แอนติบอดี และพีเอ็นเอ/ดีเอ็นเอ ตามลำดับ ทั้งนี้ทำการศึกษาผลของอัตราส่วนของโคพอลิเมอร์ต่อความหนาแน่นของตรึงโพรบ ประสิทธิภาพการตรวจวัดสารตัวอย่าง รวมถึงความสามารถในการป้องกันการดูดซับอย่างไม่จำเพาะเจาะจง แผ่นเอสพีอาร์เคลือบทองที่ถูกตรึงด้วยโคพอลิเมอร์ที่ประกอบด้วยพีจีเอ็มเอ 45 เปอร์เซ็นต์และเอ็มพีซี 55 เปอร์เซ็นต์ติดด้วยไบโอตินแสดงประสิทธิภาพที่ดีที่สุดในการตรวจวัดสเตรปตาวิดินในสารละลายพลาสมาของเลือดโดยมีค่าความเข้มข้นต่ำสุดที่ตรวจวัดได้เท่ากับ 0.95 นาโนโมลาร์ ในขณะที่เซนเซอร์แพลตฟอร์มที่ตรึงด้วยโคพอลิเมอร์ที่ประกอบด้วยพีจีเอ็มเอ 65 เปอร์เซ็นต์และเอ็มพีซี 35 เปอร์เซ็นต์ติดด้วยพีเอ็นเอสามารถตรวจวัดดีเอ็นเอที่มีลำดับเบสคู่สมได้ในปริมาณสูงที่สุดโดยมีค่าประสิทธิภาพการจับยึดเท่ากับ 71 เปอร์เซ็นต์ ในแพลตฟอร์มส่วนที่ 2 เตรียมจากโคพอลิเมอร์ของไกลโคโปรตีนที่ถูกปรับปรุงด้วยหมู่เมทาคริโลอิลและเอ็มพีซี ซึ่งพัฒนาขึ้นสำหรับการตรวจวัดฮีแม็กกลูตินินบนไวรัสอินฟลูเอนซา ไกลโคโปรตีนที่ถูกปรับปรุงด้วยหมู่เมทาคริโลอิลถูกแยกจากเซลล์เอชแอล 60 ที่มีการปรับปรุงพื้นผิวด้วยเอ็น-เมทาคริโลอิลแมนโนซามีน (แมนเอ็ม) โดยในวิธีทั้งหมดที่ได้ทำการศึกษา การตรึงไกลโคโปรตีนที่ถูกปรับปรุงด้วยหมู่เมทาคริโลอิลร่วมกับเอ็มพีซีลงไปโดยตรงบนซับสเตรทเอสพีอาร์เคลือบทองผ่านปฏิกิริยาไทออล-อีนเป็นวิธีที่มีประสิทธิภาพสูงสุด

สาขาวิชา ปิโตรเคมี ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5273870223 : MAJOR PETROCHEMISTRY
KEYWORDS: POLYMER BRUSHES, SENSOR PLATFORM, SURFACE PLASMON RESONANCE, CLICK REACTION, GRAFTING TO, RAFT POLYMERIZATION

ORAPHAN WIARACHAI: DEVELOPMENT OF FUNCTIONAL COPOLYMER BRUSHES FOR PROBE IMMOBILIZATION IN BIOSENSING APPLICATION. ADVISOR: ASSOC. PROF. VORAVEE HOVEN, Ph.D., 130 pp.

This research aims to develop copolymeric platforms containing poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) for biosensing applications. The first platform was based on copolymer of poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC) which were synthesized by RAFT polymerization. Thiol-terminated PPgMAMPC (PPgMAMPC-SH) were immobilized on gold-coated surface plasmon resonance (SPR) disk by "grafting to" approach. Biotin and peptide nucleic acid (PNA) were used as model probes to study the immobilization and specific binding for antigen/antibody and PNA/DNA biosensor, respectively. Effect of copolymer composition on probe binding density, analyte detection efficiency as well as the ability to prevent non-specific adsorption was evaluated. Gold-coated SPR chip immobilized with $PPgMA_{45}MPC_{55}$-biotin exhibited the best performance in streptavidin detection in blood plasma solution with a detection limit of 0.95 nM, while the sensor platform based on Au-$PPgMA_{65}MPC_{35}$-PNA could detect the highest amount of complementary DNA with %hybridization efficiency of 71%. The second platform was based on copolymer of methacryloyl-functionalized glycoproteins (ManM-treated glycoproteins) and MPC which was developed for influenza hemagglutinin detection. ManM-treated-glycoproteins were harvested from HL-60 cells surface-modified with *N*-methacryloyl mannosamine (ManM). Among all methods investigated, direct immobilization of ManM-treated-glycoproteins together with MPC on gold-coated SPR substrate via thiol-ene reaction was the most effective.

Field of Study: Petrochemistry Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

ACKNOWLEDGEMENTS

The accomplishment of this thesis can be attributed to the extensive support from my thesis advisor, Associate Professor Dr. Voravee P. Hoven. I am grateful for her kindly helpful suggestions, assistance, encouragement, and personal friendship throughout the course of my research. In addition, sincere appreciation is also extended to the member of thesis committee: Professor Dr. Tharapong Vitidsant, Associate Professor Dr. Nuanphun Chantarasiri, Assistant Professor Dr. Yongsak Sritana-anant and Assistant Professor Dr. Chidchanok Meechaisue for reviewing my thesis and giving valuable constructive comments and suggestions. I gratefully acknowledge Professor Dr. Yasuhiko Iwasaki for giving me valuable suggestions and an opportunity to do research in his research group at Department of Chemistry and Materials Engineering, Faculty of Chemistry, Materials and Bioengineering, Kansai University. Financial support for this work was provided by Thailand Research Fund (DBG5580003), a Distinguished Research Professor Grant, the Thailand Research Fund and Chulalongkorn University (DPG5780002), the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (RES560530126-AM), and the Thai Government Stimulus Package 2 (TKK2555), under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products and Agriculture and Chulalongkorn University Dutsadiphiphat Scholarship for academic scholarship.

Furthermore, I would like to thank all members of Organic Synthesis Research Unit (OSRU), and all my friends, for their friendliness, helpful discussions, cheerful attitude and encouragements during my thesis work. Finally, I also wish to especially thank my family members for their love, kindness and support throughout my entire study.

CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACVA	: 4,4′-Azobis(4-cyanovaleric acid)
AcSEMA	: 2-(acetylthio)ethyl methacrytale
AFM	: Atomic force microscopy
ATRP	: Atom transfer radical polymerization
BSA	: Bovine serum albumin
^{o}C	: Degree celcius
$CDCl_3$	: Deuterated chloroform
DNA	: Deoxyribonucleic acid
COOH	: Carboxyl group
COO^-	: Carboxylate group
CPD	: 4-Cyanopentanoic acid dithiobenzoate
CTA	: Chain transfer reagent
2D	: 2-Dimensional
3D	: 3-Dimensional
DMAP	: 4-(dimethylamino) pyridine
DNA	: Deoxyribonucleic acid
D_2O	: Deuterium monoxide
DP	: Degree of polymerization
DSC	: *N*′-disuccinimidyl carbonate
EDC	: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EtOH	: Ethanol
FBS	: Fetal bovine serum
FIB	: Fibrinogen
FITC	: Fluorescein isothiocyanate

FT-IR	:	Fourier transform infrared
g	:	Gram
GMA	:	Glycidyl methacrylate
GPC	:	Gel permeation chromatography
h	:	Hour
H	:	proton
HE	:	Hybridization efficiency
HL-60	:	Human promyelocytic leukemia cells
HPDS	:	Hydroxyethylpyridyl disulfide
kDa	:	Kilodalton
LODs	:	Limit of detection
LYS	:	Lysozyme
m°	:	Millidegree
MA	:	Methacrylic acid
ManM	:	*N*-methacryloylmanosamine
Mg	:	Milligram
MHz	:	Megahertz
Min	:	Minute
mL	:	Milliliter
mmol	:	Millimole
mM	:	Millimolar
$\overline{M}_n$	:	Number average molecular weight
MPC	:	2-Methacryloyloxyethyl phosphorylcholine
MW	:	Molecular weight
MWD	:	Molecular weight distribution
N_A	:	Avogadro's number

ng	: Nanogram
NHS	: *N*-Hydroxysuccinimide
nm	: Nanometer
NMR	: Nuclear magnetic resonance
NMS	: *N*-methacryloxysuccinimide
OEG	: Oligo(ethylene glycol)
PAA	: Poly(acrylic acid)
PBS	: Phosphate buffered saline
PC	: Phosphorylcholine
PDI	: Polydispersity index
PDSM	: Pyridyldisulfide ethylmethacrylate
PEG	: Poly(ethylene glycol)
PEG-N3	: Azide-terminated poly(ethylene glycol)
PEGMA	: Poly(ethylene glycol) methyl ether methacrylate
PFPA	: Pentafluorophenyl acrylate
PgMA	: Propargyl methacrylate
PHEMA	: Poly(2-hydroxyethyl methacrylate)
pI	: Isoelectric point
pKa	: Acid dissociation constant
pHEMA	: Poly(2-hydroxyethyl methacrylate)
PMA	: Poly(methacrylic acid)
PMAMPC	: Poly[(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)]
PMAMPC-SH	: Thiol-terminated poly[(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)]
pmol	: Picomole

PMPC : Poly(2-methacryloyloxyethyl phosphorylcholine)

PNA : Peptide nucleic acid

PNA-N_3 : Azide-containing peptide nucleic acid

PNMSMPC : Poly[(N-methacryloxysuccinimide)-ran-(2-methacryloyloxyethyl phosphorylcholine)]

PNMSMPC-SH : Thiol-terminated poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)]

PPgMAMPC : Poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)]

PPgMAMPC-SH : Thiol-terminated poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)]

*p*OEGMA : Poly(oligo(ethylene glycol) methacrylate)

PSGL-1 : P-selectin glycoprotein ligand-1

QCM : Quartz crystal microbalance

RAFT : Reversible addition-fragmentation chain transfer

RI : Refractive index

rms : Root mean square

SA : Streptavidin

SAM : Self-assembled monolayer

SI-ATRP : Surface-initiated atom transfer radical polymerization

SIP : Surface-initiate polymerization

S/N : Signal-to-noise ratio

SPR : Surface plasmon resonance

t : Dry film thickness

THF : Tetrahydrofuran

UV : Ultraviolet

UDDT	:	1,11-undecanedithiol
v/v	:	Volume per volume
w/v	:	Weight per volume
XPS	:	X-ray photoelectron spectroscopy
μL	:	Microliter
μmol	:	Micromole
θ_A	:	Dynamic advancing
θ_R	:	Dynamic receding
σ	:	Grafting densities
ρ	:	Mass density

CHAPTER I

INTRODUCTION

Statement of problem

Effective functionalization is certainly important for the development of materials desirable for biosensing and biomedical applications such as microarrays, microfluidic and bioactive surfaces. In order to attain specific recognition for target molecules, the covalent attachment of biomolecules acting as sensing probe, such as protein, antibody, enzyme, and DNA onto sensor surface is required. There are many strategies to prepare functionalized platform on sensor surface for immobilizing probes such as self-assembled monolayer (SAM) [1-3] of end-functionalized alkanethiol and polymeric thin film. [4-6] In particular, it has been demonstrated that the surface-grafted polymer brushes could be used to enhance sensor response by increasing the sensing probe density per area which corresponds to functional groups at the brush interface in comparison with the SAM system. [7-10] Previously we have demonstrated that surface-tethered poly(acrylic acid) (PAA) brushes prepared by surface-initiated atom transfer radical polymerization can act as a 3D precursor layer for biosensing applications. The amount of bound streptavidin (SA) on biotin immobilized on the surface-grafted PAA brushes was higher than that on biotin immobilized on self-assembled monolayer of a carboxyl-terminated alkanethiol, used as a model 2D conventional precursor layer. In addition, the PAA brushes showed very low non-specific interactions with fibrinogen and BSA protein. [7, 11, 12]

Functionality of the polymer brushes can be simply tuned by using a combination of desirable monomers in the polymerization step. In particular, the ability to resist non-specific adsorption of non-targeted analyte is extremely important for specific detection of biomolecules, especially in real biological-relevant samples in which numerous types of intererences concurrently exist. We have demonstrated earlier that non-fouling properties can be inherited in copolymer brushes containing poly (2-methacryloyloxyethyl phosphorylcholine) (PMPC), a biocompatible and

hydrophilic polymer, widely used for suppressing the adsorption of cell and plasma protein in bio-related applications. [13-16]

Functional polymer generally employed for biomolecule immobilization such as poly(oligo(ethylene glycol) methacrylate) (pOEGMA) [9, 17, 18], poly(2-hydroxyethyl methacrylate) (pHEMA) [19, 20] and poly(acrylic acid) (PAA) [7, 21-27] need to be activated with coupling agent such as *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbidiimide (EDC), *N'*-disuccinimidyl carbonate (DSC) and triethylamine, 4-(dimethylamino) pyridine (DMAP) to generate the active form of functional group before immobilization. Besides, some polymers, for example, PAA is also suffered from non-specific adsorption of positively charged components because its carboxyl groups (-COOH) can be ionized to carboxylate group ($-COO^-$), this would be problematic if the analysis is to be done in complex sample.

One of the efficient ways to reduce activation step and non-specific binding of polymer platform is the use of precursor polymer containing active functional group as probe binding units together with hydrophilic polymer as an antifouling unit. Recently, precursor layer containing alkynyl functional group have been widely used to immobilize biomolecule and polymer brushes on substrate because alkyne structure can react with azide-containing molecules via click reaction of Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition under mild condition and give high reaction yield with no side products. [28] Grafting of azide-terminated molecules to alkyne-functionalized polymer have been developed in many applications. [29, 30]

From the literatures, sensor response could be increased by using polymer brushes because of the high concentration of functional groups for probe binding. Moreover, the using of precursor polymer can reduce the activation step before probe immobilization and also suppress non-specific adsorption of non-targeted molecules. Here in this research, two functional copolymers, poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC) and poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC), were synthesized by reversible addition-fragmentation chain-transfer (RAFT) polymerization. Thiol-terminated functional copolymers was prepared and immobilized on gold-coated surface plasmon resonance (SPR) substrate by “grafting

to" method via self-assembly formation between thiol end-group of the copolymer and gold surface. Biotin and peptide nucleic acid (PNA) were used as model probes The specific detection of target molecule, streptavidin and deoxyribonucleic acid (DNA), by the developed sensing platform were determined by SPR technique to demonstrate the potential of the surface-attached PPgMAMPC for probe immobilization and subsequent detection of target molecules in biosensing application. Parameters that may affect the sensitivity and specificity in term of S/N of sensors for detecting target molecules in complex sample were investigated. These issues are likely to be important for the development of sensor platform.

Specific binding between specific receptor on the surface of the host cell and a virus is the first step of viral infection. Sialic acid on the cell surface, sialylated oligosaccharides, appear to be an essential receptor component for many animal viruses from different virus families, such as influenza A and C viruses. Recently, the modification of sialic acid by biosynthetic pathway was investigated. [22, 31-37] It has been found that mammalian cells can take up the synthetic *N*-substituted D-glucosamine and D-mannosamine derivatives and then metabolized in the sialic acid biosynthetic pathway to incorporate the structure of sialic acids with substituted *N*-acyl side chains into various glycoconjugates. [34, 36] The modification provides an opportunity to study the properties of sialic acid and cell surface modification. Therefore, this research also introduces another sensor platform prepared from methacryloyl-functionalized glycoproteins and 2-methacryloyloxyethyl phosphorylcholine (MPC) on SPR sensor chip to be used for influenza hemagglutinin detection. Sialic acid on glycoprotein acted as an active site for binding influenza hemagglutinin, whereas MPC, the hydrophilic monomeric unit enables the copolymer to suppress non-specific adsorption. Different approaches to prepare sensor platform for detecting target molecules by SPR technique were explored.

Objectives

To develop platforms based on functional copolymer brushes containing PMPC for probe immobilization and detection of target analytes to be used in biosensing applications.

Scope of investigation

The stepwise investigation was carried out as follows:

1. Literature survey for related research work.

2. Preparation and characterization of functional copolymer brushes on SPR sensor chip

3. Immobilization of sensing probe onto active functional groups containing polymer brushes.

4. Preparation and characterization of polymeric platform from methacryloyl-functionalized glycoproteins and 2-methacryloyloxyethyl phosphorylcholine on SPR sensor chip

5. Investigation on the specific interactions of sensing probe immobilized on the sensor platform with target molecule.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Biosensor

Biosensor is an analytical device consisting of two main components, bio-recognition system and transducer. The bio-recognition part provides bimolecular probes such as enzyme, protein, antibody and DNA which are recognized by target analyte. Specific binding between the target analyte and the bio-recognition part produces a signal that can be converted to a detectable signal by a transducer. Thermal, electrochemical, optical and mass are commonly detected as a signal in transducer system. The immobilization of the specific recognition molecule is particularly important aspect of the biosensors fabrication. The immobilization procedure should not only keep the biological molecule close to the surface of transducer but its biological activity should be also maintained.

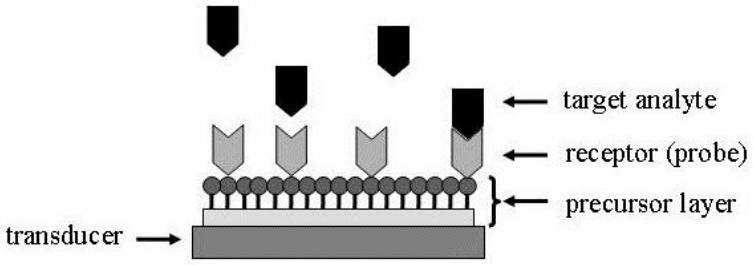


Figure 2.1 Schematic diagram of a biosensor device

The development of biosensors mainly depends on the immobilization of bioactive molecules such as protein, antibody, enzyme, and DNA [38-40] to a sensor surface or measurement platform. In principle, the sensitivity, detection limit and signal-to-noise ratio of the biosensor are affected by the density of bioactive molecules immobilized on the surface, the distance between the sensor surface and the bioactive

molecules. The resistance of non-specific adsorption of sensor platform is also an important factor that influences the sensing properties. Non-specific adsorption leads to undesirable adsorption resulting in high background noise or false positives [6, 41]. Therefore, excellent sensor platform (precursor layer) should not only allow for covalent immobilization of bioactive molecules but also resist non-specific adsorption.

There are many strategies to prepare the precursor layer on the sensor for biomolecules or probe immobilization. Self-assembled monolayer (SAM) of end-functionalized alkanethiol can be prepared especially on gold-coated substrates. However, the density of bioactive molecules cannot be enhanced because there is the two-dimentional distribution of functional compound [2, 3, 42]. The modification of surface with polymeric thin film, especially polymer brushes, has recently been used as an alternative precursor layer for the covalent immobilization of biomolecules [2, 6, 9]. It has been demonstrated previously that the surface-immobilized polymer brushes could be used for enhancement of sensor response by increasing the number of sensing probe to high concentration of functional groups at the brush interface in comparison with the SAM system. [4, 8-10]

In 2007, Yang *et al.* [10] have studied DNA hybridization, and protein–DNA interactions on streptavidin (SA) sensor chips. They have reported that the amounts of SA molecules bound on a biotin-immobilized with two dimensional (2D) carboxyl-terminated SAM were lower than that conjugated with biotin-imobilized three dimensional (3D) matrix of the carboxymethyldextran. In comparison with 2D carboxyl-terminated SAM, higher amount of biotinylated DNA was immobilized on the 3D carboxymethyldextran because of the higher reactive sites on 3D matrix. However, the limited accessibility of the DNA analyte to the immobilized DNA probes embedded inside the 3D matrix resulting in a lower DNA hybridization efficiency than that of the 2D matrix.

In 2011, Akkahat *et al.* [7] prepared surface-tethered poly(acrylic acid) (PAA) brushes by surface-initiated atom transfer radical polymerization as a 3D precursor layer for biosensing applications. The specific binding of SA on biotin-immobilized PAA brushes was higher than the binding of SA on biotin-immobilized self-assembled monolayer of a carboxyl-terminated alkanethiol, model 2D conventional precursor

layer. In addition, the PAA polymeric platform showed very low non-specific interactions with fibrinogen and BSA protein.

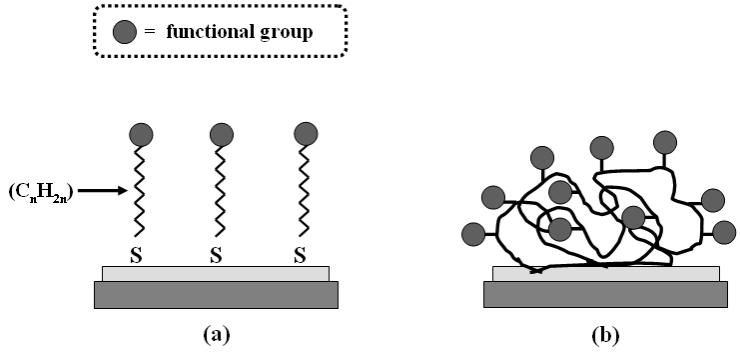


Figure 2.2 Schematic diagram of the precursor layer immobilized on the sensor surface by (a) SAM of end-functionalized alkanethiol (b) polymer

Polymer brushes exhibit a high degree of synthetic flexibility for introducing a variety of functional groups (tunable functionality). The different functional groups can be prepared by using different monomer in the polymerization step for binding the suitable probe in biosensing applications. Moreover, non-fouling properties can be introduced to the copolymer brushes to suppress non-specific adsorption resulting in increasing the efficiency of the sensors.

Poly (2-methacryloyloxyethyl phosphorylcholine) (PMPC), biocompatible and hydrophilic polymer, was widely used for reducing the adsorption of cells and plasma protein in bio-related applications. [13-16] The presence of MPC units can help in reducing the nonspecific adsorption so that the analysis can be done without the use of blocking reagents.

In 2006, Feng *et al*. [13] synthesized PMPC brushes by surface initiated atom transfer radical polymerization (ATRP). PMPC brushes with graft density of 0.06–0.39 chains/nm^2 and chain length of 5–200 monomer units were synthesized on silicon wafer surfaces. Fibrinogen was used to study protein repelling properties with the modified surface. Fibrinogen adsorption decreased significantly with increasing graft density and chain length of PMPC (graft density ≥0.29 chains/nm^2 and chain length ≥100 units).

In 1998, Ishihara *et al*. [14] have studied protein adsorption of plasma on a polymer having MPC moiety compared to protein adsorption onto poly(HEMA), poly(BMA), and BMA copolymers with acrylamide (AAm) or *N*-vinyl pyrrolidone (VPy) moieties. They found that the amount of plasma protein adsorbed on a poly(MPC) was lower than the other polymer or copolymer having a hydrophilic fraction.

Water-soluble amphiphilic phospholipid copolymer, poly[2-methacryloyloxyehtyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)], was prepared and studied by Ishihara and coworkers. [43-46] The copolymer was immobilized on gold electrode of quartz crystal microbalance (QCM) [46] to study the specific binding between active ester groups and biomolecules. They have found that the MPC moieties on the copolymer could suppress the non-specific adsorption of proteins, bovine serum albumin and γ-globulin. In addition, anti-C-reactive protein antibodies immobilized on the copolymer could be bound with the antigen without the denaturation of the antibody. This copolymer was also adsorbed on poly(L-lactic acid) nanoparticles [43] and polystyrene (PS) microtiter plate [45] to study the specific binding between antibody and antigen on the copolymer platform.

Recently, functional polymer brushes have been utilized for biomolecule immobilization, due to their versatility and robustness. Different kind of functional group of polymer brushes used for surface functionalization includes carboxylic acid (poly(acrylic acid) (PAA)) [7, 21-27] and hydroxyl group (poly(oligo(ethylene glycol) methacrylate) (pOEGMA) [9, 17, 18], poly(2-hydroxyethyl methacrylate) (pHEMA)) [19, 20]. For example:

In 2007, Lee *et al*. [9] have reported the preparation of a biocompatible and non-biofouling thin film of *p*OEGMA on gold and Si/SiO_2 substrates via the formation of SAM terminated with an initiator of atom transfer radical polymerization (ATRP) and surface-initiated ATRP. Then, terminal hydroxyl groups of side chains of *p*OEGMA were activated with *N*,*N*′-disuccinimidyl carbonate (DSC) and then reacted with (+)-biotinyl-3,6,9-trioxaundecanediamine to form biotinylated *p*OEGMA films. From surface plasmon resonance (SPR) analysis, they found that the modified films showed 10 fold signal to-noise ratio enhancement for the specific binding of

streptavidin compared with the biotinylated substrate prepared from carboxylic acid-terminated SAMs.

In 2009, Trmcic-Cvitas *et al*. [18] prepared *p*OEGMA brushes via ATRP as polymer coating for resistance of protein in complex sample such as blood, sera and plasma. The direct functionalization with SA and the coupling conditions for highest SA loading efficiency were studied. *N*,*N*′-disuccinimidyl carbonate (DSC) was the most efficient activating agent for covalent immobilization of the receptor. Then, biotinylated antibodies were immobilized on *p*OEGMA brushes to investigate the selective secondary recognition and nonspecific binding of proteins. They found that the large molecules could not infiltrate to the densely pack of polymer brushes. Moreover, the specific binding of the bulky antibody was occurred on the upper part of polymer coating.

In 2014, Vaisocherová *et al*. [20] prepared three different anti-fouling and functional polymeric platforms to study the effect of functionalization on anti-fouling platform from several types of media including blood plasma and food media. Two different polymer platform of hydroxy-functional pHEMA and carboxy-functional poly(carboxybetaine acrylamide) (pCBAA) were studied in this research in comparison with the platform immobilized with thiol-terminated OEG via self-assembly formation. The surfaces were functionalized by covalent attachment of antibodies, streptavidin, and oligonucleotides. They found that the functionalization did not affect anti-fouling properties of pCBAA, whereas pHEMA was completely lost the anti-fouling properties after hydroxyl group activation.

In 2011, Akkahat *et al*. [22] have reported the preparation of PAA brushes on gold-coated substrate as a precursor layer of the sensor. The carboxyl groups of PAA brushes was activated with *N*-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbidiimide (EDC) (**Figure 2.3**) before covalent immobilization of sensing probe such as biotin or bovine serum albumin (BSA) for detecting streptavidin (SA) or anti-BSA, respectively. As determined by SPR, the PAA brushes immobilized with biotin or BSA probes not only showed specific binding with target analytes but also maintained a high resistance to nonspecific protein adsorption.

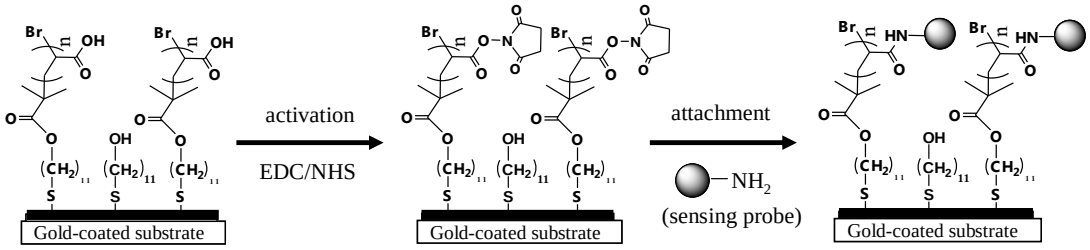


Figure 2.3 Attachment of the sensing probe on the SPR chip bearing PAA brushes [22]

From the literatures, functional polymer for biomolecule immobilization, pOEGMA, pHEMA and PAA, need to be activated with coupling agent such as *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbidiimide (EDC), *N*′-disuccinimidyl carbonate (DSC) and triethylamine, 4-(dimethylamino) pyridine (DMAP) to generate the active form of functional group before probe immobilization. Recently, polymer and copolymer containing active functional groups which are well-suited for the immobilization of biomolecules such as epoxy group (glycidylmethacrylate; GMA), *N*-hydroxysuccinimide ester group (*N*-hydroxysuccinimide; NAS) and pentafluorophenyl group (pentafluorophenyl acrylate; PFPA) were used as probe binding units together with hydrophilic polymer as an antifouling unit. For examples:

In 2008, Iwata *et al*. [47] have reported the preparation of polymer brushes and block copolymer brushes consisting of MPC and GMA by ATRP. Pyridyl disulfide groups were introduced to the polymer brushes via a reaction of epoxy groups in GMA units and Fab' fragments were immobilized onto the surfaces via a thiol-disulfide interchange reaction. Then, the specific binding of antibodies with antigens was studied. They found that the activity of the antibodies immobilized on the block copolymer brushes having biocompatible PMPC was greater than that on surfaces without PMPC in their structures.

In 2007, Park *et al*. [46] prepared the patterns of biomolecules based on polymeric self-assembled monolayers (SAM) consisting of dual functions: bio-reactive and anti-biofouling properties. The copolymers between poly(ethylene

glycol) methyl ether methacrylate (PEGMA), 3-(trimethoxysilyl)propyl methacrylate (TMSMA), and *N*-acryloxysuccinimide (NAS), poly(TMSMA-*r*-PEGMA-*r*-NAS), was synthesized by radical polymerization. After the formation of the SAM, the ability of the modified substrate to suppress non-specific adsorption of proteins was studied against bovine serum albumin. In addition, micro patterns of streptavidin were achieved using microcontact printing of NH_2-containing biotin onto the SAM of poly(TMSMA-*r*-PEGMA-*r*-NAS) on glass slides. They suggested that the other biomolecules could also be immobilized onto the modified substrate with high specificity and minimizing nonspecific adsorption.

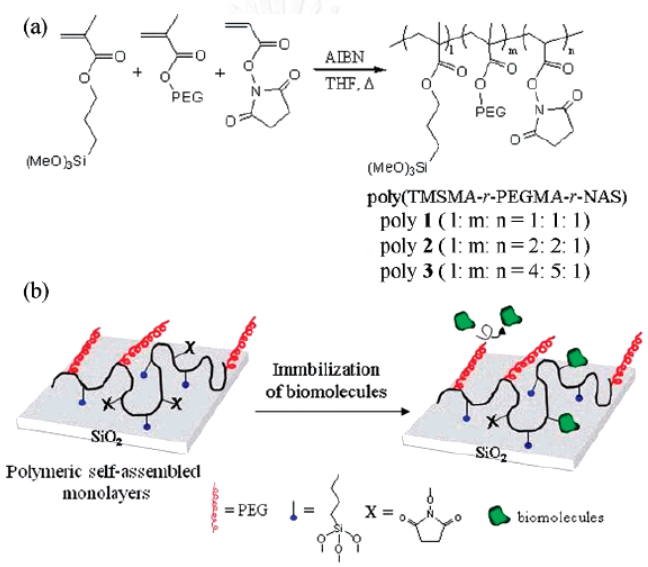


Figure 2.4 (a) Synthesis of poly(TMSMA-r-PEGMA-r-NAS) and (b) specific immobilization of biomolecules on surface immobilized with poly(TMSMA-r-PEGMA-r-NAS) [46]

In 2009, Kessler *et al*. [37] synthesized poly(methylsilsesquioxane)-poly(pentafluorophenyl acrylates) (PMSSQ-PFPA) by RAFT polymerization to prepare a material showing stable and adherent thin reactive coating on different kind of substrates: gold, polycarbonate (PC), poly(tetrafluoroethylene) (PTFE), and glass. The copolymer was immobilized on the substrate by spin coating and annealing. Then, specific binding sites of protein were immobilized on the modified substrates by dipping in a solution of a desired amine (biotin, L-thyroxine, and folic acid). The

specific binding of streptavidin, pre-albumin, and folate-binding protein were investigated by SPR, FT-IR, fluorescence spectroscopy, and AFM. The smooth homogeneous protein layers could be prepared from this method which may be useful for using in biosensor applications

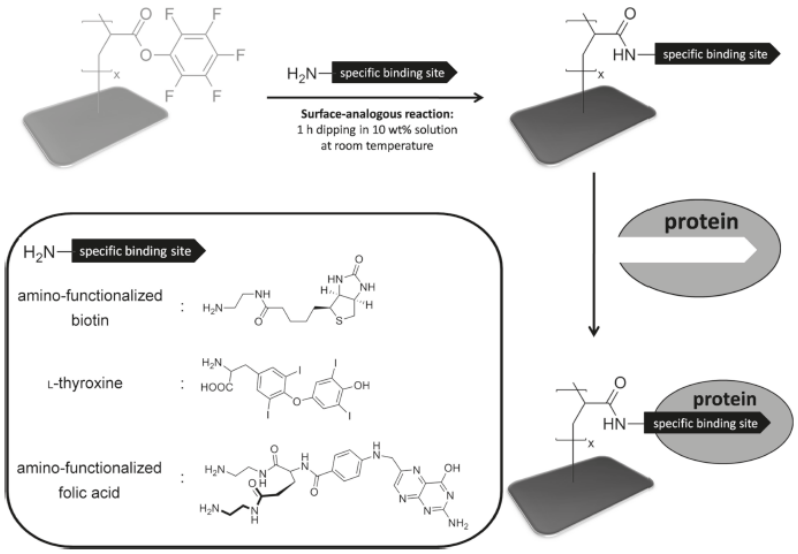


Figure 2.5 Immobilization of specific binding sites containing amino group on PMSSQ-PFPA polymers onto given substrates [37]

2.2 Click chemistry

In 2001, "Click Chemistry" was introduced by Sharpless *et al.* [48] This reaction gives high yield, and generate only harmless side products, and it can be carried out under mild reaction conditions. This concept was developed for using in the pharmaceutical, materials, and other industries. There are several well-known reactions relating with the click chemistry concept, including the hetero-Diels-Alder reaction [49], the thiol-ene reaction [50], the Staudinger ligation [51, 52], native chemical ligation [51, 53], the amidation reaction between thio acids and sulfonyl azides (sulfo-click) [54, 55] and presently the most popular and already mentioned Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition [56, 57].

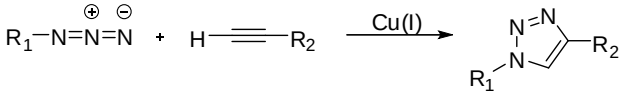


Figure 2.6 Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition.

There are a large number of the literature reviews have been published during the past years on Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition. Recently, there are a few research publications reported the use of precursor layer containing alkynyl functional groups to immobilize biomolecule or polymer brushes on substrate.

In 2009, Ostaci *et al*. [29] have reported the immoblization of azide-terminated polymer to alkyne-functionalized pseudobrushes, poly[(propargyl methacrylate)-*r*-(glycidyl methacrylate)-*r*-(methyl methacrylate)] with different monomer ratio: 27/27/46, 41/31/28, and 45/55/0. In immobilization step, alkyne-functionalized pseudobrushes were grafted by thermal ring-opening of the glycidyl groups by the silanols from the silicon substrate and azide-terminated poly(ethylene glycol)((Mw ~ 5000, 20000, and 50000 g/mol)) was immobilized on precursor layer by the Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition. The results indicated that the grafting density and thickness of the brushes on the surface depended on amount of PMA unit. The higher grafting density was observed on the substrate immobilized with copolymer having monomer ratio of 41/31/28, and 45/55/0. The obtained grafting density was 1.3, 0.3, and 0.08 chains/nm^2 for azide-terminated PEG with Mw of 5000, 20,000, and 50,000 g/mol, respectively.

In 2007, Lee *et al*. [58] prepared OEGMA polymer thin film by SAM of the ATRP initiator-containing disulfide, [$BrC(CH_3)_2COO$- $(CH_2)_{11}S]_2$ and the polymer was grown from the surface by surface-initiated ATRP. Then, azide-functionalized polymer film was prepared by the reaction between *p*OEGMA film and sodium azide. “Click” chemistry was used as a coupling reaction between azide groups of non-biofouling polymeric film and the acetylenes-containing various functional groups, such as methyl, hydroxyl, carboxylic acid, and ester groups. Biotin was also immobilized onto the *p*OEGMA film via click chemistry to study specific recognition with streptavidin by

SPR. The results showed that biotin was successfully introduced on the *p*OEGMA surface and SA could be bound to biotin at the surface.

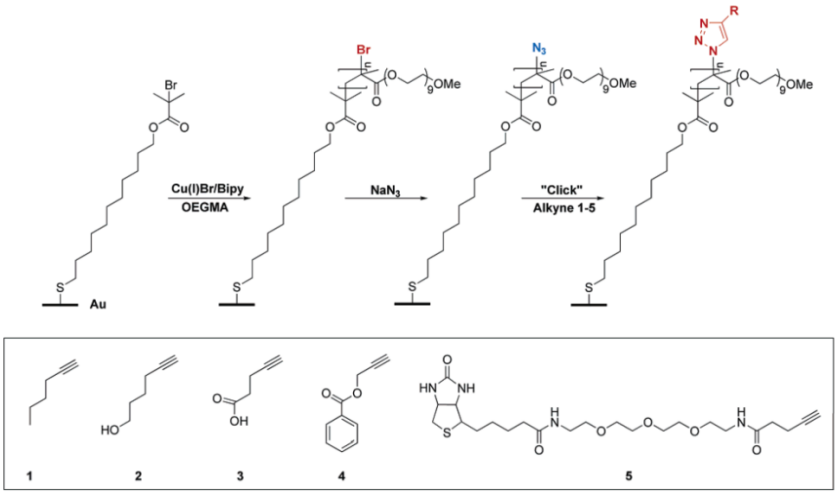


Figure 2.7 Immobilization of acetylene-containing molecules on the modified substrate: 1-hexyne (1), 5-hexyn-1-ol (2), 4-pentanoic acid (3), propargyl benzoate (4), and biotin-PEO-LC-N-pentynoate (5). [58]

In 2010, Qin *et al.* [30] have reported the preparation of microarray format by attaching the different molecules such as mannose, biotin and oligo (ethylene glycol) (OEG) having an azido tag on the monolayer of trimethylgermanyl (TMG)-alkynyl-terminated films by click reaction. It has been demonstrated that the monolayer platform functionalized with mannose and biotin showed highly specific binding with ConA and avidin, respectively **(Figure 2.8)**.

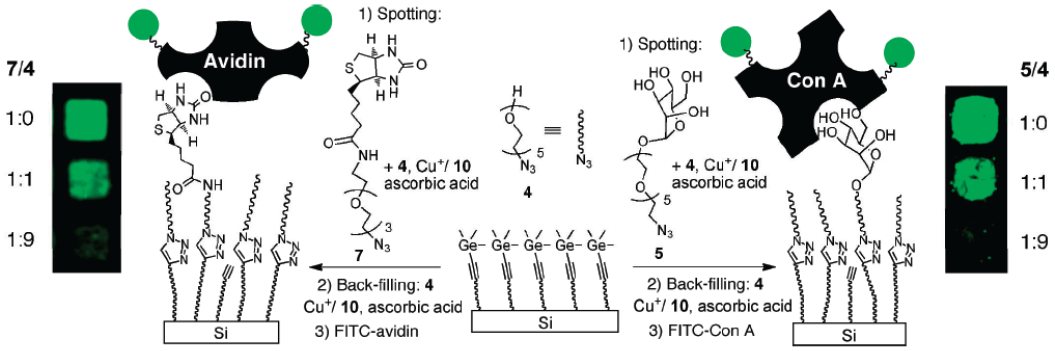


Figure 2.8 Immobilization of the biotin-N_3 and mannose-N_3 with the OEG-N_3 on the trimethylgermanyl (TMG)-alkynyl-terminated films via Cu(I)-catalyzed Huisgen

1,3-dipolar azide-alkyne cycloaddition and binding with FITC-Labeled Avidin and Con A. [30]

2.3 Reversible addition-fragmentation chain transfer (RAFT) polymerization

Recently, reversible addition-fragmentation chain transfer or RAFT polymerization, one kind of controlled radical polymerization, is attractive for development of living radical polymerization. RAFT polymerization has been used to successfully synthesize a wide range of polymers with controlled molecular weight and low polydispersity index (PDI) [59-61]. A wide variety of monomer including styrenes, acrylates, acrylamides as well as a range of other vinyl monomers are capable of undergoing RAFT polymerizing. In addition, the synthesis of variety macromolecular architectures such as gradient, block, statistical, brush, star, hyperbranched, and network copolymers can also be prepared by RAFT process.

RAFT is based on a conventional radical polymerization in the presence of a reversible chain transfer reagent (CTA). The CTA or RAFT agent such as dithioesters, thiocarbamates, and dithiocarbonates (xanthates), are used to be intermediate in the polymerization via a reversible chain-transfer process. The general structure of CTA is shown in **Figure 2.9**. The Z group serves to activate or deactivate the reactivity of the C=S bond towards addition. The R group, a homolytic leaving group, can form a stable free radical. Like other living polymerizationprocess, there is no termination step in the RAFT polymerization. There are four steps in RAFT polymerization: initiation, addition-fragmentation, re-initiation and equilibration (**Figure 2.9**).

(1) Initiation: The reaction is initiated by radical initiatorsuch as AIBN. In this step, the initiator (I) reacts with a monomer unit to produce radical species which starts an active polymerizing chain.

(2) Addition-Fragmentation: The active chain (P_n) reacts with the dithioester, which homolytically expel the the leaving group (R). This is a reversible step, with intermediate species capable of losing either the leaving group (R) or the active species (P_n).

(3) Reinitiation: The leaving group radical then reacts with another monomer species, starting another active polymer chain. This active chain (P_m) is able to go through the addition-fragmentation or equilibration steps.

(4) Equilibration: This is the fundamental step in the RAFT process which traps the majority of the active propagating species into the dormant thiocarbonyl compound. This limits the possibility of chain termination. Active polymer chains (P_m and P_n) are in an equilibrium between the active and dormant stages. While one polymer chain is in the dormant stage (bound to the thiocarbonyl compound), the other is active in polymerization.

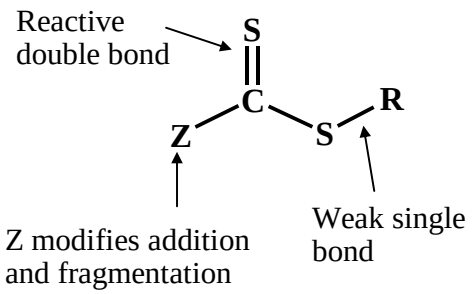


Figure 2.9 General form of RAFT agents.

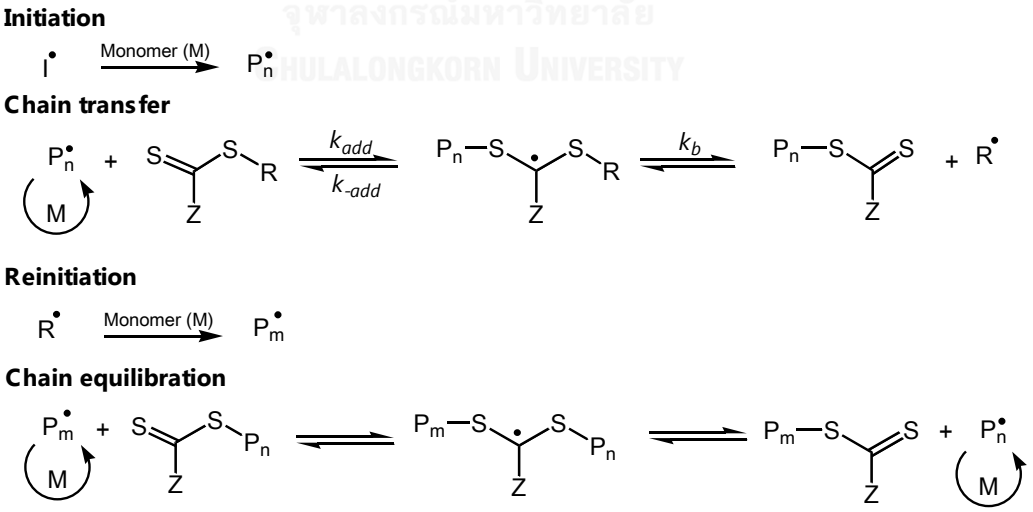


Figure 2.10 Mechanism of RAFT

The desired molecular weight with low polydispersity can be prepared by controlling the concentration of initiator and CTA. In RAFT polymerization, the concentration on the active species is kept low relative to the dormant species by controlling the amount of initiator and capping agent. This in turn will limit termination steps by radical combination and disproportionation, the polymer can continuously propagate. As a result the inertness of RAFT polymerization to protic solvents such as water, this process can potentially address the limitations that other radical polymerizations encounter in direct polymerizations of acidic monomer in aqueous media. In recent years, there are many literature have reported a well-controlled polymerization of acidic monomer such as poly(methacrylaic acid) using RAFT [62, 63].

Recently, RAFT polymerization has attracted even more attention of many researchers as versatile route to synthesize the end-functionalized polymer because after the polymerization step, the end functional group of polymer such as dithioester group can easily be converted to thiol group by aminolysis reaction of either primary or secondary amine [21, 60, 64, 65] and by $NaBH_4$ [28, 37, 66]. Consequently, thiol-terminated polymer can be readily grafted on metal surface by the interaction of metal–sulfur bonds, especially gold surfaces [21, 67, 68] that have been widely used in various bio-related applications. For example:

In 2010, Kitano *et al.* [69] prepared thiol-terminated poly(glucosylureaethyl methacrylate), Poly(GUMA-SH), by RAFT polymerization and subsequent reduction with $NaBH_4$. Then, poly(GUMA-SH) was immobilized on gold electrode and colloidal gold-immobilized glass substrate. The success of surface modification was investigated by cyclic voltammetry using hydroquinone as a probe. The localized surface plasmon resonance was used to study the adsorption of various proteins on polymer brushes. Poly(GUMA) brushes could be reduce nonspecific adsorption of proteins, such as lysozyme, bovine serum albumin, immunoglobulin G, and fibrinogen. This results imply that the synthesized polymer brush prepared in this research could be used as anti-biofouling surface in biomedical fields.

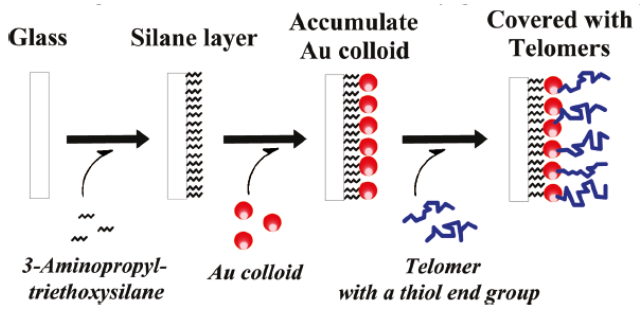


Figure 2.11 Preparation of poly(glucosylureaethyl methacrylate) brushes on Au colloid-glass chips [69]

In 2011, Gibson *et al.* [67] described a method for preparing thiol-terminated polymer for gold nanoparticles immobilization. The polymer synthesis was based on two steps of reaction. First, poly(pentafluorophenyl methacrylate) was synthesized by RAFT polymerization as a reactive precursor which can be modified by post-polymerization to prepare functional polymers with thiol end-group. Second, the modified polymers were immobilized on the surface of citrate-stabilized gold nanoparticles to produce a modified organic-inorganic hybrid particles. 75 libraries of the polymer were prepared in this research. Therefore, this method presents a powerful, accessible tool for creating model nanoparticle libraries with sensing properties.

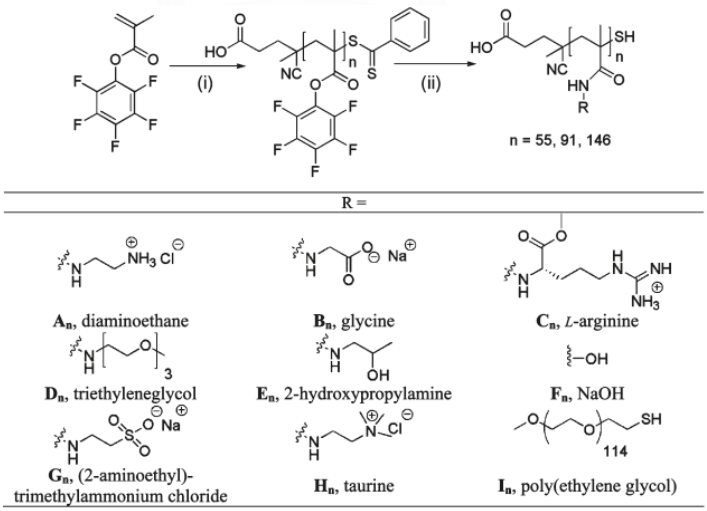


Figure 2.12 Reaction of thiol-terminated functional polymer synthesis, (i) CTA and initiator (ii) R-NH_2/NEt_3 [67]

In 2012, Akkahat *et al.* [21] synthesized thiol-terminated poly-[(methacrylic acid)-ran-(2-methacryloyloxyethyl phosphorylcholine)] (PMAMPC-SH) by RAFT polymerization and subsequent aminolysis with ethanolamine. The copolymer was immobilized on gold-coated SPR chips using the "grafting to" approach via self-assembly formation. The carboxyl groups of methacrylic acid (MA) units were attached with biotin as sensing probe for specific detection with avidin whereas the MPC units were introduced to reduce nonspecific adsorption. Copolymer brushes having 79 mol % MPC composition (molecular weight of 49.3 kDa) showed the best performance for using as platform for probe immobilization as compared with platforms based on 11-mercaptoundecanoic acid (MUA) and poly(methacrylic acid) (PMA) brushes. The detection limit for detecting AVD in blood plasma solution was 1.5 nM (equivalent to 100 ng/mL). These results imply that surface-attached PMAMPC brushe has a potential for using as sensor platform for probe immobilization and detection of target molecules in complex sample.

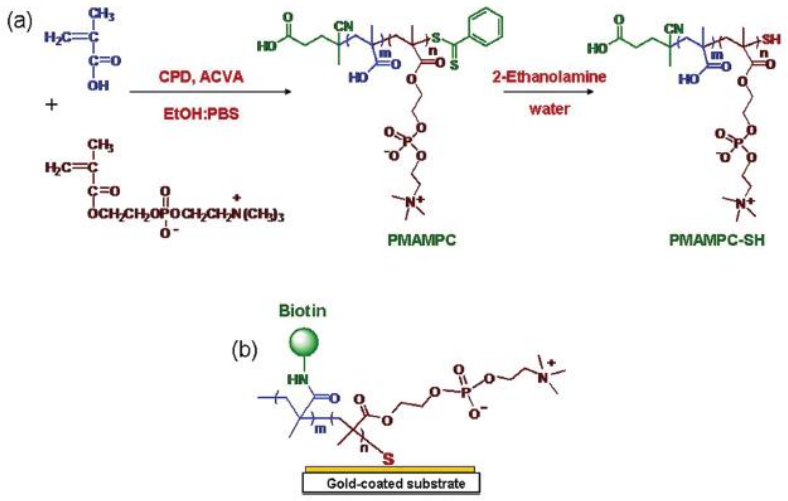
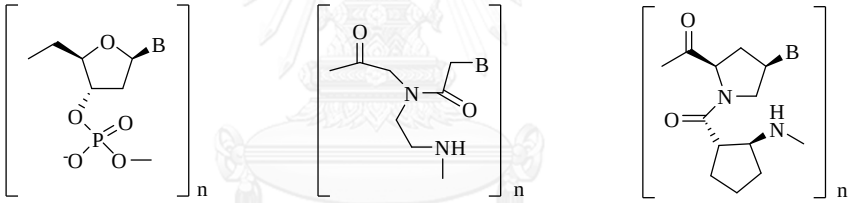


Figure 2.13 Schematic diagram: (a) synthesis of PMAMPC-SH and (b) PMAMPC-modified gold-coated SPR chip immobilized with biotin. [21]

2.4 Peptide Nucleic acid (PNA)

The well-known DNA analogue called peptide nucleic acid (PNA) was introduced by Nielsen group in 1991.[70, 71] This PNA system is *aeg*PNA, consists of repeating *N*-(2-aminoethyl)-glycine units linked together with uncharged peptide-like amide bonds which replaced the normal negatively charged phosphodiester backbone of DNA. A methylene carbonyl linker connects natural nucleobase to this backbone at the amino nitrogen as shown in **Figure 2.14 (b)**. The hybridization between PNA with DNA is based on the Watson-Crick base pairing rule as same as in DNA hybridizations. The reduced electrostatic repulsion between PNA and DNA backbones resulted in superior hybridization characteristics, e.g., higher thermal stability, stronger affinity with less dependence on salt concentration, greater sequence specificity, and higher capability of strand invasion to double-stranded DNA. The high specificity of PNA as mention above makes it an excellent probe for DNA sequence determination.



a **b** **c**

Figure 2.14 Structures of (a) DNA, (b) aegPNA and (c) (1S, 2S)-acpc PNA. [72]

From these advantages, there are many attempts to improve the original PNA further during the past 20 years. The conformationally rigid pyrrolidinyl PNA derived from D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbones (acpcPNA)[73, 74] introduced by Vilaivan and co-workers (**Figure 2.14 (b)**) shows great properties because it can form PNA·DNA duplex with even higher affinity and specificity than the original Nielsen's aegPNA (Figure 2.14 (a)). Moreover, it can also form only anti-parallel hybrid with DNA because the *acpc*PNA structure contains stereochemistry. Therefore, this PNA system is a potential substance for the development of the sensor platform for DNA detection.

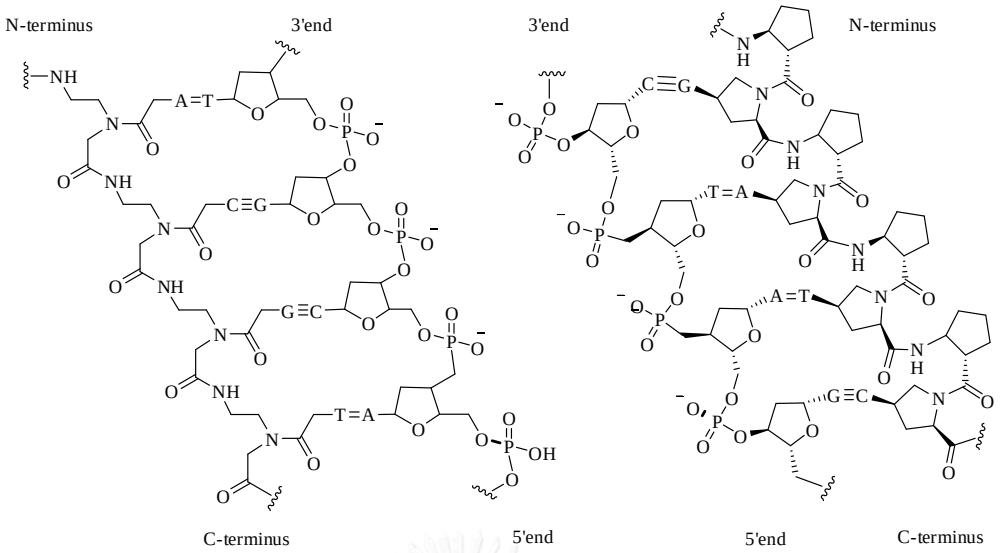


Figure 2.15 Chemical structures of (a) aegPNA·DNA and (b) acpcPNA·DNA duplex following Watson-Crick base paring rule. [72]

Because of its advantages, acpcPNA has been applied as a probe to detect DNA base sequence with various techniques including SPR technique. For example:

In 2009, Ananthanawat *et al.* [75] synthesized and immobilized thiol-terminated pyrrolidinyl peptide nucleic acids (PNA-SH) consisting d-prolyl-2-aminocyclopentanecarboxylic acid (ACPC) backbones with different lengths and types of thiol modifiers on gold-coated substrate by self-assembly formation. The hybridization efficiency of the sensor was increased with the increasing of the distance between the PNA and the thiol terminal group and blocking with thiol-containing ethylene glycol moiety after PNA-SH immobilization. The lowest DNA concentration which could be detected and discriminated between complementary and single mismatched DNA was 0.2 μM (10 pmol). This sensor platform could be bound with complementary DNA with a degree of mismatch discrimination more than 45% and hybridization efficiency of 20%.

In 2010, Ananthanawat *et al.* [76] have reported the immobilization of biotinylated acpcPNA on SPR sensor chips via biotin–streptavidin interactions. The comparison between acpcPNA, DNA and conventional peptide nucleic acid (aegPNA) probes of the same sequence was studied. The results implied that the hybridization properties of immobilized acpcPNA probe were resembled to aegPNA counterparts

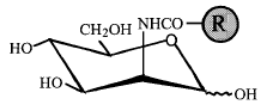
with a higher single-base mismatch sensitivity, antiparallel selectivity and low ionic strength dependence of target hybridization. The sensor can be reused for multiple cycles of hybridization with 1.3% loss in hybridization activity per regeneration cycle when using regeneration conditions (10mM NaOH, 2 min). These properties indicated the the acpcPNA can be used as sensor probes for clinical and diagnostic applications**.**

In 2011, Ananthanawat *et al.* [77] studied the binding of PNA (acpcPNA and aegPNA) to double-stranded (dsDNA) by SPR technique. SPR protocols were developed to verify the sequence rules and conditions for binding of homopyrimidine and homopurine aegPNAs to dsDNA in comparison with the information in solution phase behaviors. The results revealed that acpcPNA cannot form higher-order complexes with dsDNA through either triplex formation or duplex in vasion, while the binding behaviors of aegPNA obtained from the SPR analysis in the solid–liquid phase measurement was resembled to the literature derived from solution phase measurements.

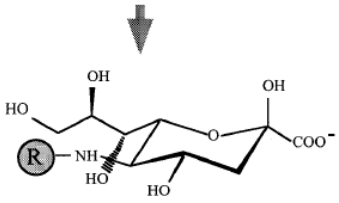
2.5 Biochemical engineering of sialic acid

Specific binding between appropriate receptor on the surface of the host cell and a virus is the first step of viral infection. Sialic acid on the cell surface, sialylated oligosaccharides, appear to be an essential receptor component for many animal viruses from different virus families, such as influenza A and C viruses (orthomyxoviruses), cardioviruses (picornaviruses), Newcastle disease virus (paramyxovirus), and primate polyomaviruses and murine [78].

For these reason, many researchers have studied the modification of sialic acid by biosynthetic pathway. From the literatures, they found that mammalian cells can take up the synthetic *N*-substituted D-glucosamine and D-mannosamine derivatives. Then, the synthetic molecules were metabolized in the sialic acid biosynthetic pathway and incorporated the structure of sialic acids with substituted *N*-acyl side chains into various glycoconjugates as shown in **Figure 2.16 and 2.17**. The biosynthetic incorporation of unphysiological sialic acids into both sialoglycoproteins and sialoglycolipids has been investigated *in vitro* and *in vivo.* [33, 36, 79-83]



N-Acyl mannosamine



N-Acyl neuraminic acid

R:	formula
- acetyl (Ac)	-CO-CH_3
- propanoyl (Prop)	-CO-CH_2-CH_3
- butanoyl (But)	-CO-CH_2-CH_2-CH_3
- pentanoyl (Pent)	-CO-CH_2-CH_2-CH_2-CH_3
- hexanoyl (Hex)	-CO-CH_2-CH_2-CH_2-CH_2-CH_3
- crotonoyl (Crot)	-CO-CH=CH-CH_3
- levulinoyl (Lev)	- CO-CH_2-CH_2-CO-CH_3
- glycolyl (Gc)	-CO-CH_2OH
- azidoacetyl (Ac-azido)	- CO-CH_2-N_3

Figure 2.16 Structure of N-acyl modified mannosamine and biochemically engineered sialic acid. [34, 36]

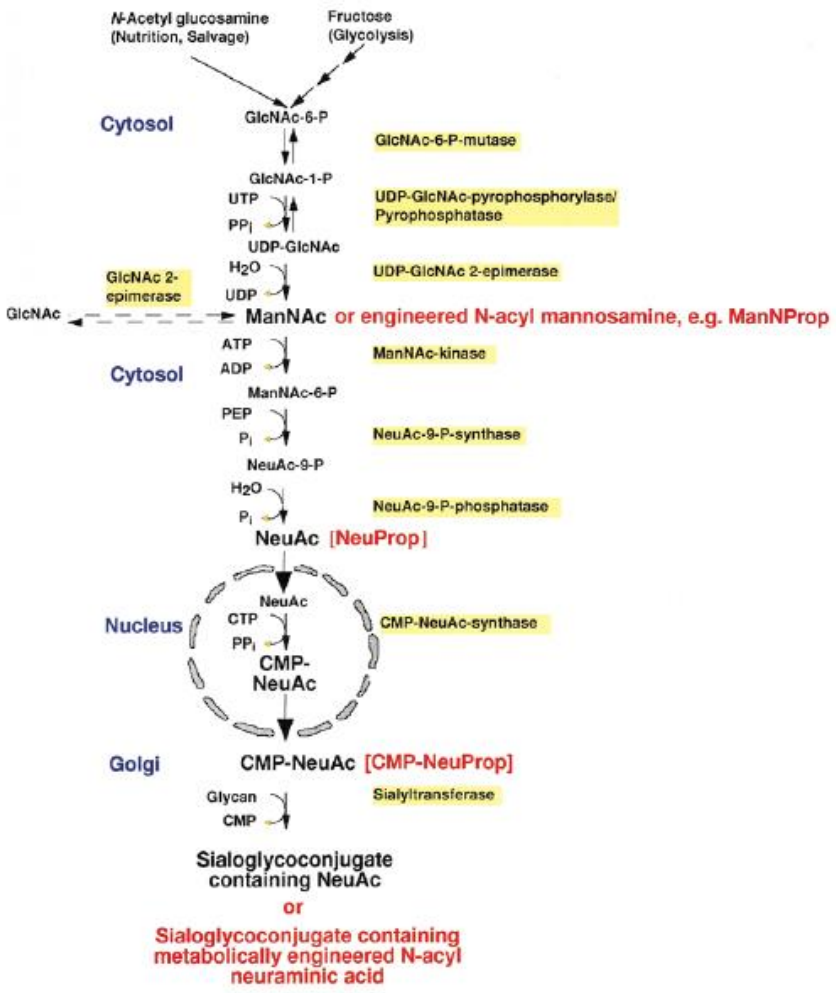


Figure 2.17 Biosynthesis pathway of sialic acid precursor [35]

The incorporation of the unnatural carbohydrates into living cells provides an opportunity to study the properties of sialic acid and cell surface modification. For example:

In 1998, Yarema *et al.* [84] reported the delivery of reactive ketone group to endogenous cell surface sialic acid residues by treating cells with the ketone-bearing metabolic precursor, *N*-levulinoylmannosamine (ManLev). The ketone was highly selective condensation reactions with specific nucleophiles such as aminooxy and hydrazide groups. Ketones-functionalized on ManLev-treated cells were then reacted with aminooxy and hydrazide-functionalized carbohydrates. The specific binding between the modified cells with lectin was determined by flow cytometry analysis. This

simple method is suitable for using in the study of carbohydrate-mediated cell surface interactions.

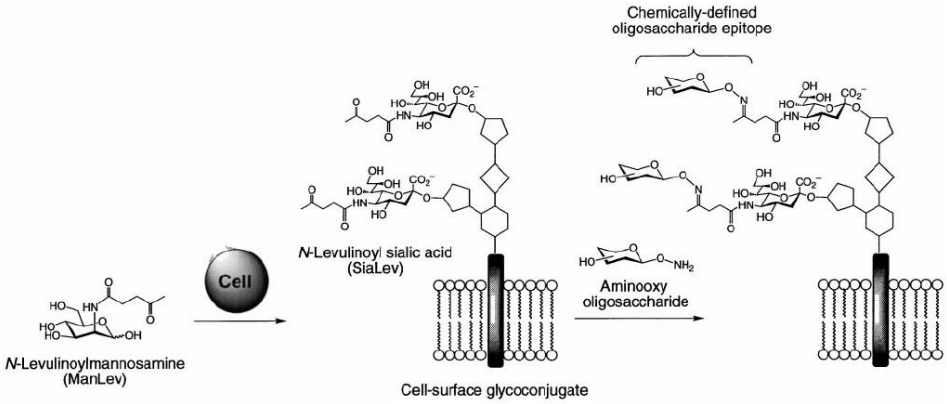


Figure 2.18 Schematic diagram showing a general approach to cell surface engineering achieved by the metabolic delivery of ketone groups to endogenous cell surface sialoglycoconjugates. [84]

In 2011, Iwasaki *et al.* [32] synthesized *N*-methacryloylmannosamine (ManMA) as a precursor for cell-surface modification at sialic-acid residues. Methacryloyl groups were firstly introduced on a living cell surface via a glycosylation pathway by treating HeLa cells with ManMA in a culture medium. After surface modification, cells were immobilized with $PEG_4$10K-SH in the presence of a photoinitiator and then exposed to UV-light (365 nm) for 10 min. The success of $PEG_4$10K-SH immobilization, PEGylation, on the cell surface was confirmed by fluorescence microscopy. Moreover, biotinylation of cell surface was also achieved by the addition of a vinyl-functionalized biotin during PEGylation. This results implies that the cell surface could be functionalized with methacryloyl group that might be useful for further surface modification.

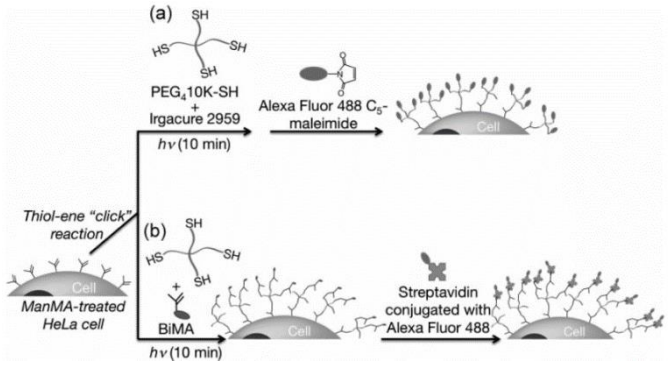


Figure 2.19 Schematic diagram of cell surface modification via methacryloyl groups delivered by glycosylation. [32]

In 2014, Iwasaki *et al*. [31] have reported the preparation of hydrogels containing methacryloyl functionalized glycoproteins of mammalian cells. Then, selectin-mediated cell adhesion on the hydrogel was investigated. HL-60 cells were cultured with the solution of ManMA and incubated for 3 days to deliver methacryloyl groups to carbohydrate residues of glycoproteins. Redox radical polymerization was used to prepare the hydrogel containing methacryloyl functionalized glycoproteins, MPC and a crosslinker. Selectin-mediated cell (HUVEC cells) adhesion on hydrogels containing glycoproteins was also investigated to study the ability of hydrogel for using in therapeutic and diagnostic applications. The results indicated that the functionalized glycoproteins could be incorporated in the synthetic hydrogels and showed the binding ability to the selectin-mediated on the surface of HUVEC cells.

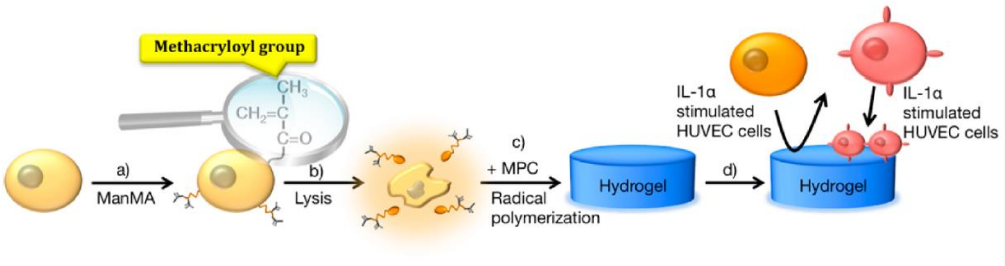


Figure 2.20 Preparation of hydrogel composed of mammalian glycoproteins: (a) metabolic oligosaccharide engineering with ManMA, (b) lysis of ManMA-treated HL-60 cells, (c) preparation of hydrogels via free radical polymerization of glycoproteins with MPC and MBA, (d) adhesion of HUVECs on hydrogels bearing glycoproteins. [31]

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

2-methacryloyloxyethyl phosphorylcholine (MPC) and thiol-terminated 4-arm poly-(ethylene glycol) $PEG_4$10K-SH were purchased from NOF Corp. (Japan). Methacrylic acid (MA) was distilled under reduced pressure to remove mono methyl ether hydroquinone, polymerization inhibitor. 4,4′-azobis (4-cyanovaleric acid) (ACVA), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB), azide-PEG_3-biotin conjugate, propargyl alcohol and coper (I) acetate were purchased from Aldrich (USA). Hydrazine monohydrate, *N,N*′-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and glycoprofile β-elimination kit was purchased from Sigma-Aldrich (USA). Deoxyribonucleic acid (DNA) was purchased from Pacific Science CO., LTD (Thailand). Milli-Q water were purified by an ultrapure water system using a Millipak-40 filter unit (0.22 μm, Millipore) and Millipore Milli-Q system that involves reverse osmosis followed by ion exchange and filtration steps (18.2 MΩ). All reagents and materials are analytical grade and used without further purification. Gold-coated SPR disk was purchased from AutoLab ESPR (Eco Chemie, The Netherlands) and Biacore T200 (GE Healthcare, Sweden). Bovine serum albumin (BSA) and lysozyme (LYZ) were purchased from Aldrich (USA). Phosphate buffered saline (PBS) pH 7.4 was purchased from Sigma (USA). Human promyelocytic leukemia (HL-60) cells were purchased from Health Science Research Resources Bank. PBS(-) [Dulbeco's PBS(-)] was purchased from Nissui (Japan). RPMI-1640 medium, fetal bolvine serum (FBS) and antibiotic-antimycotic were purchased from Gibco. RIPA lysis buffer and BCA assay kit, streptavidin and eosin Y were purchased from Thermo Fisher Scientific Inc (Japan). Alexa Fluor 488 C_5-maleimide was purchased from Invitrogen (Japan).

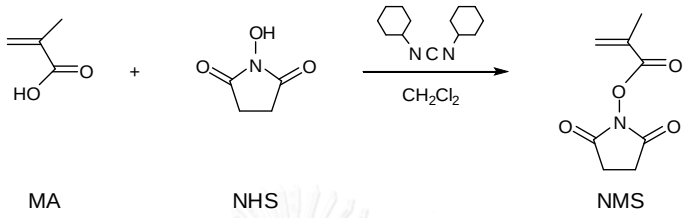
3.2 Equipments

The dynamic water contact angles were measured by a contact angle goniometer, equipped with a Gilmont syringe and a 24-gauge flattipped needle (Ramé-Hart, model 200-F1 or a First Ten Angstroms FTÅ125 goniometer, USA). All of the measurements were performed in air at ambient temperature. The measurement was taken from five different areas of the substrate and analyzed by the DROPimage standard 2.0 software. The characteristic functional groups of the copolymer brushes on the SPR sensor chip were characterized by attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy (Nicolet 6700 FT-IR spectrometer). Spectra in the infrared region (4000−650 cm^{-1}) were collected with 32 scans at a spectral resolution of 4 cm^{-1}. ^{1}H NMR spectra were recorded in methanol-d4, $CDCl_3$ or D_2O using a NMR spectrophotometer (Varian, model Mercury-400, USA or JEOL, model ECA-400, Japan) operating at 400 MHz. The disappearance of dithioester groups of copolymer was monitored on a CARY 100 Bio UV–visible spectrophotometer (Varian, Inc., USA). AFM images were recorded with Scanning Probe Microscope (NanoScope®IV, Veeco, USA). Measurements were performed in air using tapping mode with silicon nitride tip at a resonance frequency of 267–295 KHz and a spring constant 20–80 N/m were used. The copolymer immobilized on the surface was characterized by XPS (ESCA-3400, Shimadzu Co., Kyoto, Japan) using an Al Kα X-ray source. All XPS data were collected at a takeoff angle of 90°. SPR measurements were conducted using a double channel, AutoLab ESPR (Eco Chemie, The Netherlands) at Scientific and Technological Research Equipment Centre, Chulalongkorn University (Thailand) and a double channel surface plasmon resonance (SPR; Biacore T200, GE Healthcare, Sweden) sensor at Department of Chemistry and Materials Engineering, Kansai University (Japan). MALDI-TOF MS analysis was done on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CCA) as the matrix.

3.3 Experimental procedure

3.3.1 Preparation of surface-tethered poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC)

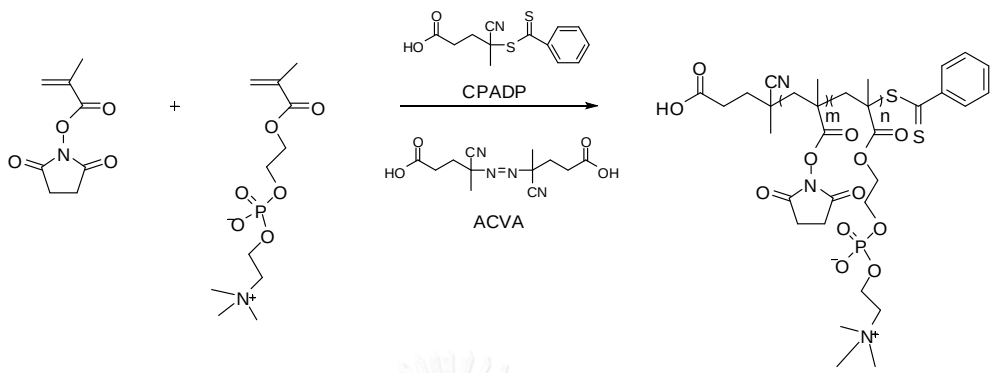
3.3.1.1 Synthesis of N-methacryloxysuccinimide (NMS) monomer



MA NHS NMS

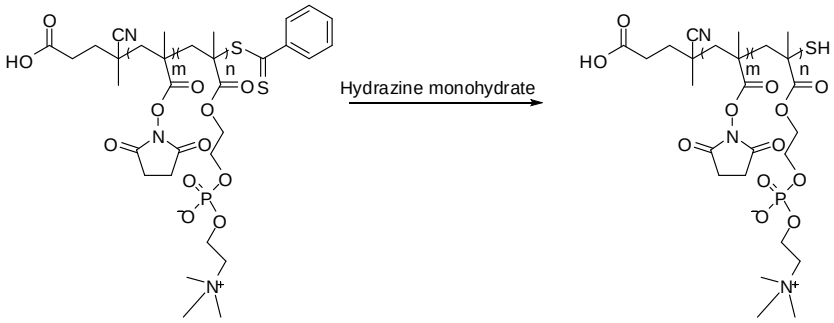
N-methacryloxysuccinimide (NMS) monomer was synthesized following the method of Percot *et al*. [85] *N*-hydroxysuccinimide (NHS) (0.84 g, 7.3 mmol) and methacrylic acid (MA) (0.5 mL, 7.3 mmol) was homogeneously dissolved with 20 mL anhydrous CH_2Cl_2 in round-bottom flask and then cooled in an ice bath. DCC (1.5 g, 7.3 mmol) dissolved in 10 mL anhydrous CH_2Cl_2 was added dropwise into the reaction flask over 1 h under magnetic stirring. The reaction mixture was stirred in an ice bath for 1 h and ambient temperature for 48 h. Insoluble dicyclohexylurea was filtrated and NMS product was purified by column chromotography using silica gel as stationary phase and mixture between hexane and ethylacetate (60:50 v/v) as eluent. The product was collected and then solvent was removed to get the desired product as white crystal. Yield: 0.64 g (48%), mp = 101-105°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm: 6.4 (s, H, H_2C=C), 5.9 (s, H, H_2C=C), 2.8 (s, 4H, O=C-H_2C-CH_2-C=O), 2.00 (s, 3H, CH_3).

3.3.1.2 Synthesis of poly[(N-methacryloxysuccinimide)-ran-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC) by RAFT polymerization



MPC monomer (0.59 g, 2 mmol), NMS monomer (0.37 g, 2 mmol), ACVA (0.7 mg, 2.5 µmol) and CPADB (5.6 mg, 20 µmol) were dissolved in 2 mL mixed solution of EtOH : THF (1:1 v/v) until the mixture solution was completely dissolved. Clear pink solution was purged with nitrogen gas for 30 min and then immersed in an oil bath at 70°C for a set reaction time. After polymerization, the reaction was terminated in an ice bath. The resulting PNMSMPC was purified by using dialysis membrane (molecular weight cut-off is 3,500 g/mol) against EtOH for 2 days, and then with DI water for 2 days. Orange cotton-like material was obtained after lyophilization. The copolymer, PNMSMPC, was characterized by ^{1}H NMR and FT-IR analysis (Section 4.1.2).

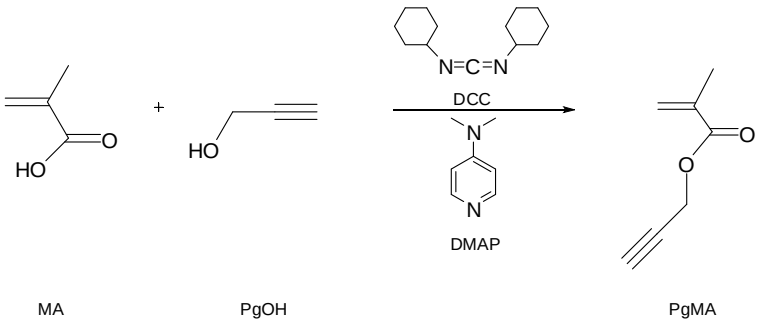
3.3.1.3 Preparation of thiol-terminated PNMSMPC (PNMSMPC-SH)



PNMSMPC-SH was prepared by aminolysis of dithioester group at chain end of the copolymer with hydrazine monohydrate. [65] 0.2 g of PNMSMPC was dissolved in mixed solution of EtOH and THF until the solution was clear. 30 mole equivalent of hydrazine monohydrate was added to the copolymer solution under magnetic stirring and stirred for 2 h at ambient temperature. Then, the mixture was added dropwise to 10 mL of 1.0 M HCl and stirred for 1 h. The obtained PNMSMPC-SH was then purified by using dialysis membrane (molecular weight cut-off = 3,500 g/mol) against HCl (aq), pH 3-4 for 2 days and with DI water for 2 days. White cotton-like product was obtained after lyophilization. The copolymer was characterized by FT-IR (Section 4.1.3).

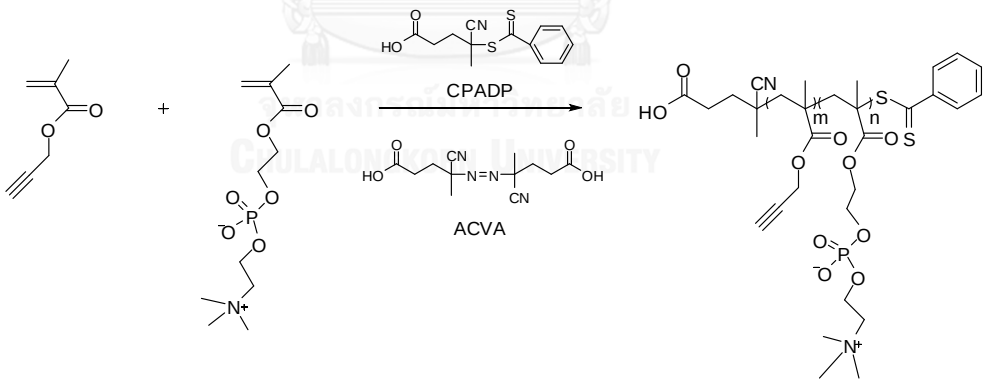
3.3.2 Preparation of surface-tethered Poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC)

3.3.2.1 Synthesis of propargyl methacrylate (PgMA) monomer



Propargyl methacrylate (PgMA) was synthesized from the following method of He *et al*. [86]. PgOH (14.5 mL, 0.25 mol), MA (21.2 mL, 0.25 mol) and DMAP (1.68 g, 13.8 mmol) was homogeneously dissolved with 200 mL anhydrous CH_2Cl_2 in round-bottom flask and then cooled in an ice bath. DCC (54.1 g, 0.26 mol) dissolved in 200 mL anhydrous CH_2Cl_2 and added dropwise into the reaction flask over 1 h under magnetic stirring. The reaction mixture was stirred in an ice bath for 1 h and then stirred at ambient temperature for 24 h. Insoluble dicyclohexylurea was filtrated and PgMA product was extracted sequentially by 1 M HCl, deionized water, 1 M NaOH, and deionized water. The solution was dried with anhydrous $MgSO_4$ and CH_2Cl_2 was removed under reduced pressure. The PgMA product was purified by vacuum distillation. Yield: 16.1 mL (51%), ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm: 6.2 (m, 1H, H_2C=C), 5.7 (m, 1H, H_2C=C), 4.8 (t, 2H, CH_2O), 2.5 (t, 1H, C≡CH), and 2.0 (m, 3H, CH_3).

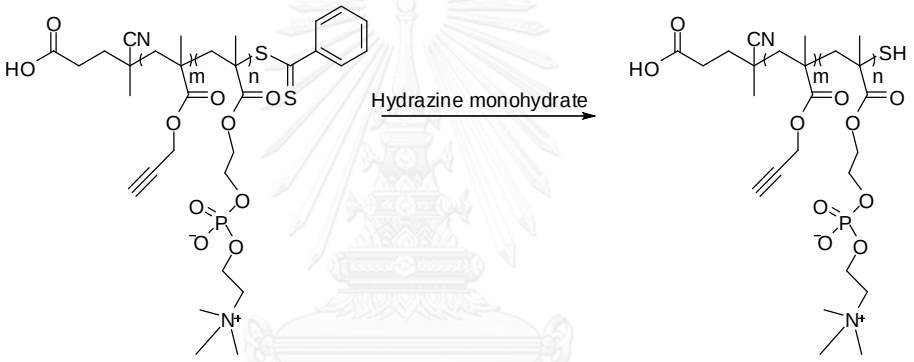
3.3.2.2 Synthesis of Poly[(propargyl methacrylate)-ran-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC) by RAFT polymerization



MPC monomer (0.59 g, 2 mmol), ACVA (0.7 mg, 2.5 µmol) and CPADB (5.6 mg, 20 µmol) were dissolved in 2 mL mixed solution of EtOH : THF (1:1 v/v) until the mixture solution was completely dissolved. Then, PgMA monomer (0.25 mL, 2 mmol) was added to the solution mixture under magnetic stirring. Clear pink solution was purged with nitrogen gas for 30 min and then immersed in an oil bath at 70°C for

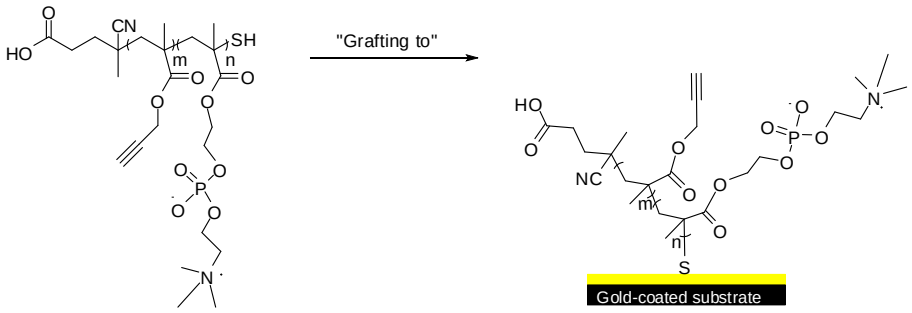
a set reaction time. After polymerization, the reaction was terminated in an ice bath. The resulting PPgMAMPC was purified by using dialysis membrane (molecular weight cut-off is 3,500 g/mol) against EtOH for 2 days, and then with DI water for 2 days. Orange cotton-like material was obtained after lyophilization. The composition of copolymer was varied by varying mole ratio of PgMA and MPC monomers in the feed. The copolymer, PPgMAMPC, was characterized by ^{1}H NMR and FT-IR analysis (Section 4.2.2).

3.3.2.3 Preparation of thiol-terminated PPgMAMPC (PPgMAMPC-SH)



PPgMAMPC-SH was prepared by aminolysis of dithioester group at chain end of the copolymer with hydrazine monohydrate. [65] 0.2 g of PPgMAMPC was dissolved in mixed solution of EtOH and THF until the solution was clear. 30 mole equivalent of hydrazine monohydrate was added to the copolymer solution under magnetic stirring and stirred for 2 h at ambient temperature. Then, the mixture was added dropwise to 10 mL of 1.0 M HCl and stirred for 1 h. The obtained PPgMAMPC-SH was then purified by using dialysis membrane (molecular weight cut-off = 3,500 g/mol) against HCl (aq), pH 3-4 for 2 days and with DI water for 2 days. White cotton-like product was obtained after lyophilization. The copolymer was characterized by ^{1}H NMR and UV-Vis spectroscopy (Section 4.2.3).

3.3.2.4 Immobilization of PPgMAMPC-SH on gold-coated SPR disk by “grafting to” method



Gold-coated SPR chip was cleaned by air plasma for 5 min, washed by Milli-Q water for 5 min, and dried under nitrogen stream. PPgMAMPC-SH was immobilized on SPR chip via self-assembly formation between thiol group of the copolymer and gold-coated surface. 0.1 mM $PPgMA_{38}MPC_{62}$-SH and $PPgMA_{45}MPC_{55}$-SH were prepared in 3 mL of EtOH, whereas $PPgMA_{65}MPC_{35}$-SH was prepared in 3 mL of mixed solution between EtOH and THF (7:3 v/v) The cleaned SPR sensor chip was immersed into the copolymer solution at ambient temperature for 48 h. The modified SPR chip was removed from the solution and rinsed by constant agitation five times for 5 min in EtOH for Au-$PPgMA_{38}MPC_{62}$ and Au-$PPgMA_{45}MPC_{55}$, in mixed solution of EtOH and THF (7:3 v/v) for Au-$PPgMA_{65}MPC_{35}$. The copolymer-immobilized SPR sensor chip was dried under nitrogen stream and was characterized by contact angle measurements, ATR-FTIR, XPS and SPR analyses (Section 4.2.4).

3.3.2.5 Immobilization of PNA probes and blocking agent [(azide-terminated poly(ethylene glycol) (PEG-N_3) and 2-azidoethanol) on the gold-coated SPR disks bearing PPgMAMPC brushes

3.3.2.5.1 Synthesis of azide-terminated PNA by solid phase peptide synthesis

a) Preparation of apparatus for solid phase peptide synthesis

Home-made peptide synthesis column equipped with a fritted glass tip was prepared in the laboratory. A new glass Pasteur pipette was plugged with glass powder and sintered on a small flame of burner. The length of sintered glass was about 3-5 mm. 7.1 mg (1.5 µmol) of Tenta Gel S RAM resin was weighed into the pipette and the pipette was equipped with a rubber teat. The resin in the pipette was swollen in DMF at least 10 min before using. For each step of synthesis, the reagent was directly suck in, eject out, hold on or agitation by manual control for the desired time. In washing step, solvent was filled from the top of pipette and eject out by squeezing the rubber teat.

b) Solid phase peptide synthesis

Thymine (T) monomer (*N*-fluoren-9-ylmethoxycarbonylamino-*cis*-4-(thymin-1-yl)-D-proline pentafluorophenyl ester, Fmoc-T-OPfp) and spacer (*N*-fluoren-9-ylmethoxycarbonyl-2-amino-cyclopentanecarboxylic acid) using in this experiment were synthesized by members in Prof. Tirayut Vilaivan's research group.

Azide-terminated PNA, N_3-TTT TTT TTT-LysNH_2, was synthesized by solid phase peptide synthesis following the procedure described earlier. [74] After removing Fmoc protecting group (i) and anchoring the first amino acid (Lys) residue (ii) on the resin, the attachment of PNA monomer was performed. For 1 cycle, the process begins with PNA monomer attatchment consisting of deprotecting (iii), coupling (iv) and end capping (v) step alternating with the attachment of spacer (deprotecting (iii), coupling (iv) and end capping (v) step**)**. The synthesis was done following this sequence: [Lys 1 cycle → T_1 1 cycle → spacer 1 cycle → T_2 1 cycle → spacer 1 cycle → T_3 1 cycle → spacer 1 cycle → T_4 1 cycle → spacer 1 cycle → T_5 1 cycle → spacer 1 cycle → T_6 1 cycle → spacer 1 cycle → T_7 1 cycle → spacer 1 cycle → T_8 1 cycle → spacer 1 cycle →

T_9 1 cycle]. Then, the *N*-terminus of PNA oligomer was modified with azide (vi) before cleaving from the resin (vii) and purification by HPLC (viii).

Three stock reagents using in the synthesis were prepared according to general protocol as follows.

● **Reagent 1#** 20% piperidine and 2% 1,8-diazabicyclo-[5,4,0]-undec-7-ene (DBU) in DMF was prepared from piperidine 200 μL, DBU 20 μL, and DMF 780 μL.

● **Reagent 2#** 7% *N*,*N*′-diisopropylethylamine (DIEA) in DMF was prepared from DIEA 70 μL and DMF 930 μL.

● **Reagent 3#** 0.4 M hydroxyl-7-azabenzotriazole (HOAt) in DMF was prepared from HOAt 5.5 mg dissolved in DMF 100 μL.

i) Removing Fmoc protecting group from the resin

The Pasteur pipette containing TantaGel S RAM Fmoc resin (6.3 mg, 1.5 μmol) was prepared as described above. The resin was treated with 100 μL of reagent#1 to deprotect Fmoc in 1.5 mL eppendorf tube for 5 min at room temperature with occasional agitation. After the specific reaction time, the reagent was squeezed off and the reaction column was washed with DMF for 3 times.

ii) Anchoring the first amino acid (Lys) residue

Fmoc-L-Lysine was first immobilized to free amino group on RAM resin employing Fmoc-L-Lys (Boc)-Opfp. Fmoc-L-Lys (Boc)-Opfp (4.8 mg, 6.8 μmol), reagent#2 and reagent#3 with occasional agitation. The prepared resin was immersed in the solution with infrequent agitation for 40 min at room temperature. After the specified reaction time, the reagent was squeezed off and the reaction column was washed with DMF for 3 times.

iii) Deprotecting Fmoc group at *N*-terminus

After the reaction (ii) was completed, the resin was treated with 100 μL of reagent#1 in 1.5 mL eppendorf tube for 15 min at room temperature with occasional agitation. After the specified reaction time, the reagent was squeezed off and the reaction column was washed with DMF for 3 times.

iv) Coupling with PNA monomer/spacer

After generating the free amino group from the deprotection step (iii), this group was further coupled with a designated PNA monomer alternately with a spacer after every PNA monomer attachment. PNA monomer or spacer (Thymine (Fmoc-T-OPfp) momomer (4 μmol), spacer (4 μmol)), 15 μL of reagent#2 and 15 μL of reagent#3 were added in 1.5 mL eppendorf tube and the prepared resin was immersed in the solution with occasional agitation for 30 min at room temperature. After the specified reaction time, the reagent was squeezed off and the reaction column was washed with DMF for 3 times.

v) End Capping

After the step of coupling, the free amino residue was capped with 5 μL acetic acid in 30 μL of reagent#2 to prevent formation of deletion sequences and facilitate purification for 5 min. After the specified reaction time, the reagent was squeezed off and the reaction column was washed with DMF for 3 times.

vi) Modifying the *N*-terminus of PNA oligomer with azide

The PNA oligomer was modified at the *N*-terminus by reductive alkylation with $N_3(CH_2)_3CHO$ following the procedure described earlier. [87] After deprotecting Fmoc grups of *N*-terminus of final T monomer, the PNA oligomer (0.5 μmol) was treated with the solution of $N_3(CH_2)_3CHO$ (15 μmol, 30 equiv) in the presence of $NaBH_3CN$ (30 μmol, 60 equiv) and acetic acid (3 μL) in MeOH (100 μL) at room temperature overnight. After the specified reaction time, the reagent was squeezed off and the reaction column was washed with MeOH for 3 times.

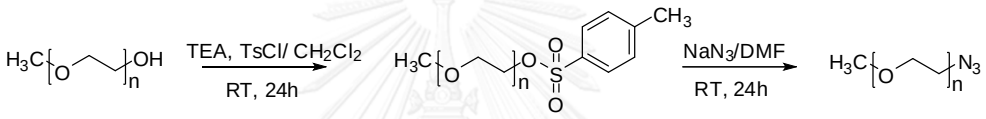
vii) Cleaving the azide-terminated PNA (PNA-N_3) oligomer from the resin

The PNA-N_3 was cleaved from the solid support by treatment with trifluoroacetic acid (TFA) (500 μL, 1h) and it was evaporated under nitrogen stream. The procedure was repeated three times. The PNA-N_3 was precipitated by an addition of diethyl ether. After washing with more diethyl ether and air-dried, the crude PNA-N_3 was obtained.

viii) Purification and characterization

The crude PNA-N_3 was dissolved in 120 μL milliQ water and purified by reverse-phase HPLC, monitoring by UV-absorbance at 260 nm. The product was eluted with a gradient system of 0.01% TFA in acetronitrile/water. Peak monitoring and data processing were performed using the Waters Empower software. Fractions from HPLC were collected manually and real-time HPLC chromatogram monitoring assisted the peak selection. The fractions containing the desired PNA-N_3 were combined and lyophilized to obtain the purified PNA-N_3, which was characterized by MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid (CCA) as the matrix.

3.3.2.5.2 Synthesis of azide-terminated poly(ethylene glycol) (PEG-N3)

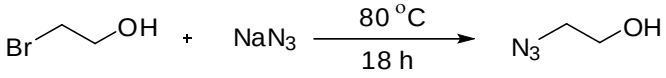


Poly(ethylene glycol) methyl ether (Mn = 550 g/mol, 10.0 mL, 20 mmol) and toluene-4-sulfonyl chloride (TsCl) (0.53g, 2.8 mmol) were completely dissolved in CH_2Cl_2 (2 mL) under nitrogen atmosphere. Triethylamine (TEA) (0.43 mL, 3.1 mmol) was dissolved in CH_2Cl_2 (2.5 mL) and then added dropwise to the above solution in an ice-water bath. The mixed solution was stirred for 24 h at room temperature and then 2.5 mL HCl (1 mol/L), 2.5 mL deionized water and 3 mL saturated NaCl were added to the reaction mixture. The mixture was extracted with CH_2Cl_2 and dried with anhydrous Na_2SO_4. Then, solvent was removed by evaporation and purified by column chromatography using the mixture between ethyl acetate and MeOH (99:1 v/v) as eluent. The monotosylated poly(ethylene glycol) (PEG-Ts), was obtained as colorless oil with 27% yield. ^{1}H NMR (400MHz, $CDCl_3$): δ/ppm: 2.4 (s, 3H, -C_6H_4-C$\underline{H}_3$), 3.0 (s, 3H, $\underline{H}_3C$-[O-CH_2-CH_2]$_n$), 3.4 (s, -O-$\underline{H}_2$C-C$\underline{H}_2$), 7.1 (d, 4H, -(C$\underline{H}_2$)$_2$-C-CH_3), 7.5 (d, 2H, -SO_3-C-(CH_2)$_2$).

To obtain PEG-N_3, sodium azide (1.05 g, 16.2 mmol) was added to a solution of the obtained PEG-Ts (3.5 mL, 5.4 mmol) in dry DMF (10 mL) under nitrogen atmosphere. The reaction mixture was stirred at 80°C for 48 h. Deionized water was added to the mixture and then the product was extracted with CH_2Cl_2. Solvent was removed by evaporation and purified by column chromatography using the mixture

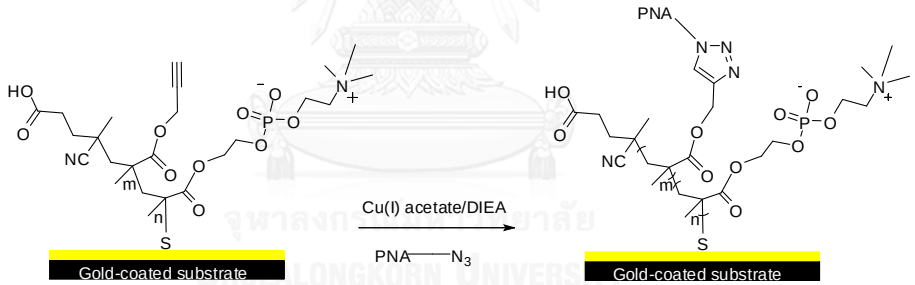
between ethyl acetate and MeOH (99:1 v/v) as eluent. As a result, PEG-N_3 was obtained as yellow liquid oil with 51% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm:2.8 (s, 3H, -CH_3), 3.3-4.0 (m, -$\underline{H}_3C$-[-O-C$\underline{H}_2$-C$\underline{H}_2$-]$_n$).

3.3.2.5.3 Synthesis of 2-azidoethanol



2-bromoethanol (2 mL, 28 mmol) and sodium azide (5.5 g, 85 mmol) were dissolved in 50 mL deionized water at 80°C for 24 h. Then, the mixed solution was extracted with diethyl ether and dried with anhydrous Na_2SO_4. After solvent evaporation, the product was obtained as colorless oil with the yield of 57% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.4 (s, 2H, N_3-CH_2-), 3.8 (s, 2H, -CH_2-OH).

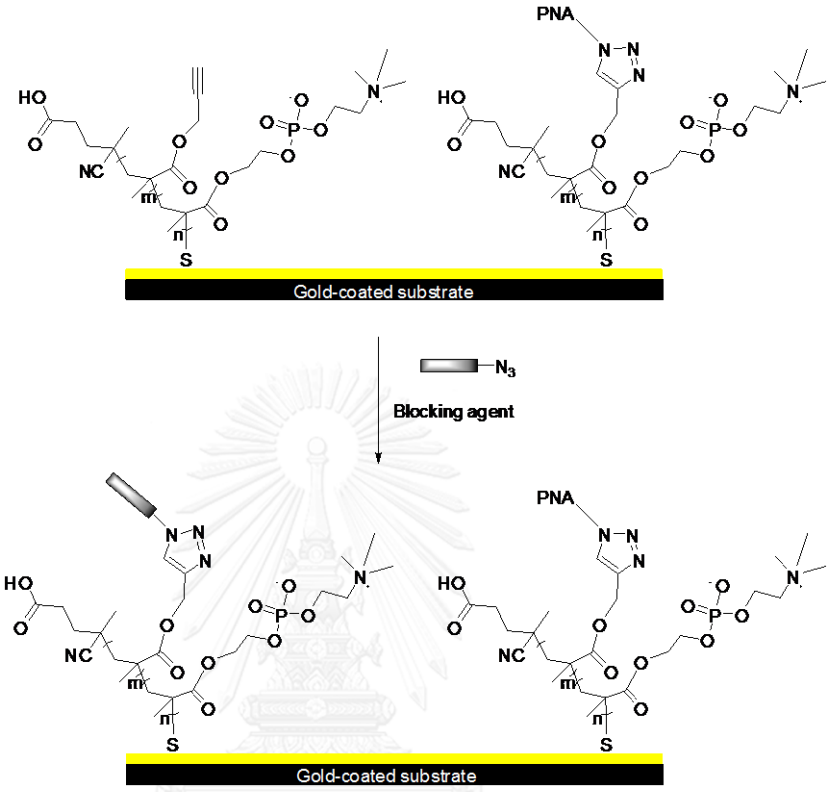
3.3.2.5.4 Immobilization of PNA probes



PNA-N_3 probe was immobilized onto the PPgMAMPC modified gold surface with the final concentration of 5 µM (10.0 nmol). Cu(I) acetate (0.1 mg, 8.2 µmol) and PNA-N_3 was dissolved in 2 mL of methanol for 10 min, then the modified SPR chip was immersed in the mixture with constant agitation for 5 min. 50 µM stock solution of DIEA (1.0 mL, 50.0 nmol), was added to the reaction mixture and then the SPR chip was immersed in this mixture for 24 h under constant agitation at ambient temperature. The modified SPR chip was removed from the solution followed by immersed and rinsed by constant agitation in 10 mM EDTA for 1 min and in methanol five times for 5 min each. The immobilized SPR sensor chip was dried

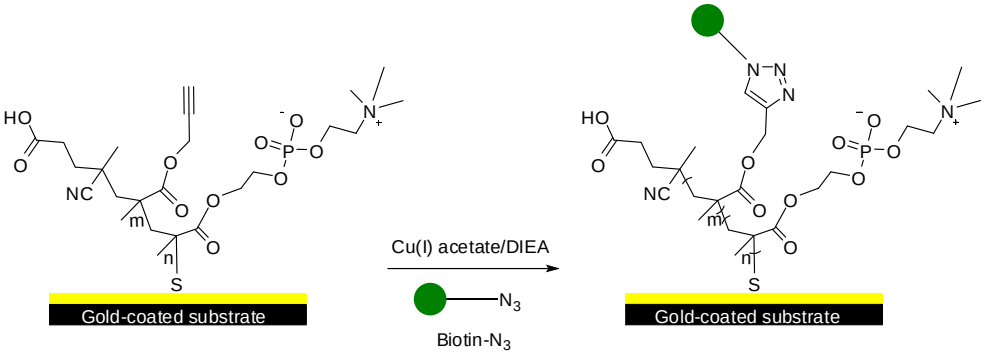
under nitrogen stream and characterized by water contact angle and SPR measurements.

3.3.2.5.5 Immobilization of blocking agent



After PNA immobilization, blocking agent (PEG-N_3 or 2-azidoethanol) was immobilized onto the remaining alkyne moieties of Au-PPgMAMPC platform with the final concentration of 1 µM. Cu(I) acetate (0.1 mg, 8.2 µmol) and blocking agent was dissolved in 2 mL of methanol for 10 min, then the modified SPR chip was immersed in the mixture with constant agitation for 5 min. 50 µM stock solution of DIEA (1.0 mL, 50.0 nmol), was added to the reaction mixture and then the SPR chip was immersed in this mixture for 24 h under constant agitation at ambient temperature. The modified SPR chip was removed from the solution followed by immersed and rinsed by constant agitation in 10 mM EDTA for 1 min and in methanol five times for 5 min each. The immobilized SPR sensor chip was dried under nitrogen stream and characterized by water contact angle and SPR measurements.

3.3.2.6 Immobilization of biotin probes on the gold-coated SPR disks bearing PPgMAMPC brushes



For immobilization of azide-PEG_3-biotin probe, Cu(I) acetate (1.23 mg, 0.01 mmol) and azide-PEG_3-biotin with the final concentration of 1 mM (0.002 mmol) were dissolved in 2 mL of 10 mM PBS for 10 min, then the modified SPR chip having PPgMAMPC copolymer was immersed in the mixture with constant agitation for 5 min. DIEA (1.71 µL, 0.01 mmol) was added to the reaction mixture and the SPR chip was immersed in the mixture for 24 h under constant agitation at ambient temperature. The modified SPR chip was removed from the solution followed by immersed and rinsed by constant agitation in 10 mM EDTA for 1 min and in 10 mM PBS 5 min each for five times.

3.3.3 SPR Measurements

The SPR measurements were conducted using a double-channel AutoLab ESPR (Eco Chemie, The Netherlands) at 25 °C, with the plane face of the prism coupled to the gold-coated glass via index matching fluid. The instrument uses a laser diode at a wavelength of 670 nm and a vibrating mirror to modulate the angle of incidence of the p-polarized light beam on the SPR substrate. An autosampler was used to inject the test solutions and the measurement of the SPR angle shift was done under non-flow liquid conditions. The SPR angle shift at the end-point of each step and after baseline subtraction (angle shift) was used to calculate the density of molecules bound onto the surface or target density, using a sensitivity factor of 120 mDegree equals to 100 ng/cm^2. This sensitivity factor is specifically calibrated for the AutoLab ESPR, which uses the N-BK 7 prism with the index of refraction of 1.518,

and the wavelength of incident light of 670 nm. Each gold-coated SPR chip bearing PPgMAMPC was first seated in a SPR cell before being stabilized with a running solution of 10 mM phosphate-buffered saline (PBS; pH 7.4) until the equilibrium SPR angle frequency value in each buffer solution was obtained, the substrate was then ready to be used.

3.3.3.1 Specific interactions between immobilized probes and target molecules on the gold-coated SPR disks bearing PAA brushes

For gold-coated SPR chip modified with PNA-N_3 probe, DNA (50 μM) in 10 mM PBS containing 100 mM NaCl was applied on the surface and left for 15 min. Unbound DNA was removed by washing with PBS for 5 min. The amount of specific binding of DNA was quantified by the shift of the SPR response angle at the end-point of the washing step and after baseline subtraction. The sensor can be regenerated by washing with 50 mM NaOH for 5 min. The hybridization efficiency (%HE) and mismatch discrimination ability (%MD) were calculated by the following equations:

$$\%HE = \frac{target\ density}{probe\ density} \times 100 \quad (1)$$

$$\%MD = \frac{\%HE\ of\ complementary\ DNA - \%HE\ of\ mismatched\ DNA}{\%HE\ of\ complementary\ DNA} \times 100 \quad (2)$$

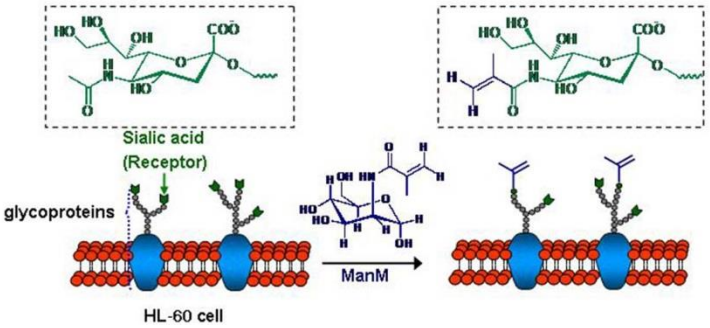
Gold-coated SPR chips bearing PPgMAMPC brushes immobilized with the desired probes were first seated in SPR cell before being rinsed with a running solution of 10 mM PBS buffer. After a baseline SPR response was stable, target molecules was applied on the modified surface. In case of surface modified with azide-PEG_3-biotin probe, streptavidin (0.1 mg/mL equivalent to 1.9 μM) in blood plasma solution (0.1 mg/mL or 0.14% in PBS buffer) was applied on the surface and left for 15 min. Unbound streptavidin was removed by washing with PBS for 5 min. The amount of specific binding of streptavidin was quantified by the shift of the SPR response angle at

the end-point of the washing step and after baseline subtraction. Non-specific binding (binding in the absence of streptavidin in blood plasma solution) was also determined in order to quantify the specific binding of streptavidin in blood plasma in terms of the signal-to-noise (S/N) ratio. The signal-to-noise (S/N) ratio was calculated by the following equation:

$$S/N = \frac{\text{SPR angle shift after exposure to blood plasma with streptavidin}}{\text{SPR angle shift after exposure to blood plasma without streptavidin}} \quad (3)$$

3.3.4 Preparation of sensing platform between methacryloyl-functionalized glycoproteins (ManM-treated HL-60 cells) and 2-methacryloyloxyethyl phosphorylcholine for influenza hemagglutinin detection

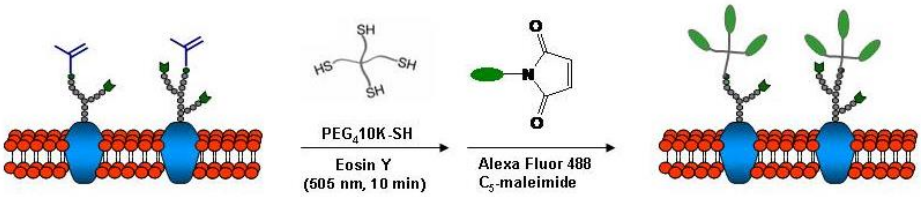
3.3.4.1 HL-60 cell surface modification with ManM



For cell surface modification, 110 μL of 50 mM ManM [M(+))] or PBS(-) [M(-)] was pipetted to each well of 24 well plate (final concentration is 5 mM). Then, 1.0 mL of HL-60 cells suspension (3.0×10^5 cells/mL) in RPMI-1640 medium supplemented with 10% FBS was added and incubated at 37 °C in a humidified atmosphere of air containing 5% CO_2 for 3 days. After 3 days, the cells were washed three times with PBS (-) to remove the remaining ManM and ManM-treated HL-60 cells were then transferred to 1.5 mL microcentrifuge tube. The cells were disrupted by ultrasonication (cycle: 40, output control: 1) 30 sec for 5 times before centrifugation at 15,000 rpm, 4°C for 1 h and the supernatant was then removed from microcentrifuge tube. 100 μL of RIPA lysis buffer was added to precipitated cells and stored at 4°C for 1 h. The cellsuspension was centrifuged at 15,000 rpm, 4°C for 1 h and then the

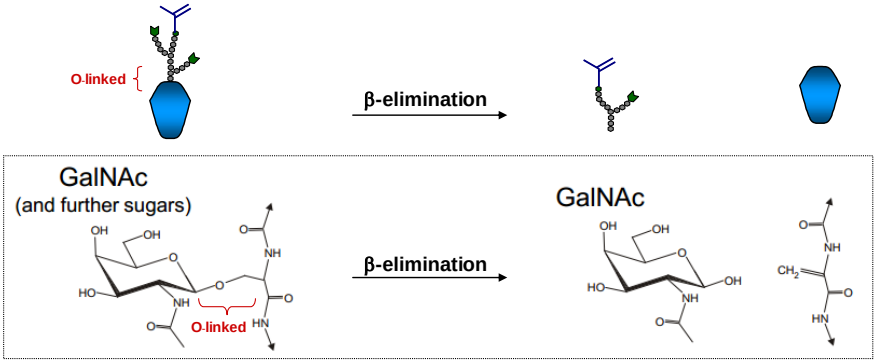
supernatant containing mixture of glycoprotein (cells lysate) was kept in new microcentrifuge tube. The concentration of glyproteins in the supernatant was measured using BCA assay kit.

3.3.4.2 Modification of ManM-treated HL-60 cell surface with $PEG_4$10K-SH

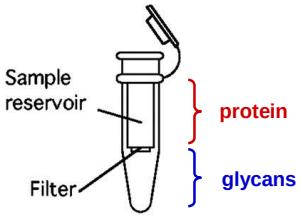


The success of carbohydrate modification on HL-60 cells with ManM was confirmed by the reaction between methacryloyl group on cell surface and thiol-terminated 4-arm poly-(ethylene glycol) ($PEG_4$10K-SH), PEGylation, via thiol-ene chemistry. After 3 days of treated cells with ManM [M(+)] in 24 well plate, the cells were washed three times with PBS (-) to remove the remaining ManM. 800 μL of RPMI-1640 medium without 10% FBS was added and 195 μL of cell solution was then transferred to 96 well plate for 4 wells. 2.5 wt% $PEG_4$10K-SH and 0.0005 wt% eosin Y was added to the cells and was exposed to visible light at 505 nm for 10 min at room temperature. The cells were washed three times with PBS (-) to remove unreacted $PEG_4$10K-SH and eosin Y. $PEG_4$10K-SH immobilized on the HL-60 cells was labeled in the dark for 10 min with 10 μg/mL Alexa Fluor 488 C_5-maleimide dissolved in the culture medium without 10% FBS. Morphology of the fluorescent-labeled cells was monitored using a confocal laser scanning microscope (LSM 5 PASCAL, Carl Zeiss Microscopy, Jena, Germany) and the fluorescence intensity on the cell surface was evaluated by flow cytometry (EPICS XL, Beckman Coulter, Brea, USA).

3.3.4.3 Separation of glycans from glycoprotein

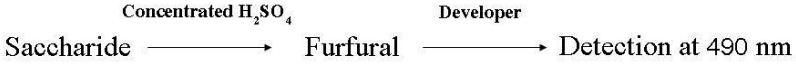


The chemical O-glycans release was performed using a Glycoprofile β-elimination kit according to the instruction. 100 µL of methacryloyl-functionalized glycoproteins was incubated with 20 µL of reagent mixture (18.8 µL of β-elimination reagent mixture and 1.2 µL of 5.0 M NaOH solution) at 4°C for 24 h. Then, neutralization of the mixture by adjusting the pH to 6-8 with 1 M HCl. O-glycans were separated from protein portion by using microcon centrifugal filter unit (YM-10 membrane, MWCO 10 kDa)



The released protein portion was eluted from microcon centrifugal filter unit by rinsing with RIPA lysis buffer. BCA assay kit was used to determine the concentration of protein in glycans and protein portion.

3.3.4.4 Determination of glycans or carbohydrate concentration by total carbohydrate calorimetric assay kit



Standard curve (calibration curve) was firstly generated by using glucose standard solution with the different concentration (0, 4, 8, 12, 16, 20 µg/well) and

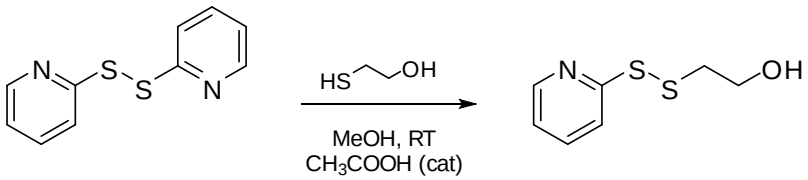
volume was adjusted to 30 µL per well. For sample preparation, 1-30 µL of sample was added to each well and the volume was adjusted to 30 µL with MilliQ water. Then, 150 µL of concentrated H_2SO_4 was added to standard and sample wells, then mix for 1 min on a shaker and incubate at 90°C for 15 min. and the plate was covered to prevent light exposure during the incubation. 30 µL of developer was added to each well and then mixed on shaker for 5 min. Absorbance of the sample was measured with microplate reader at 490 nm (A_{490}).

3.3.4.5 Immobilization of ManM-treated glycoprotein and MPC on gold-coated surface and detection of target molecules by SPR technique

In this section, ManM-treated glycoprotein and MPC were immobilized on gold-coated surface by two different methods. The first method is based on polymerization of ManM-treated glycoprotein with MPC on gold-coated surface modified with pyridyldisulfide ethylmethacrylate (PDSM) and 2-(acetylthio)ethylmethacrytale (AcSCMA). The second method is immobilization of ManM-treated glycoprotein with MPC monomer on gold-coated surface via thiol-ene chemistry.

3.3.4.5.1 Polymerization of ManM-treated glycoprotein with MPC on gold-coated surface modified with pyridyldisulfide ethylmethacrylate (PDSM)

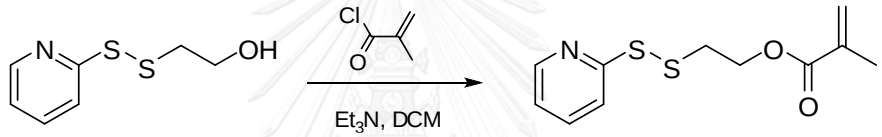
3.3.4.5.1.2 Synthesis of hydroxyethylpyridyl disulfide (HPDS)



Aldrithiol-2 (8 g, 36.3 mmol) was dissolved in 50 mL methanol and 0.8 mL of glacial acetic acid was added. Then, a solution of mercaptoethanol (1.9 g, 24.3 mmol) in 20 mL methanol was added dropwise at room temperature in 30 min under continuous stirring. After the addition was over, the reaction mixture was stirred at

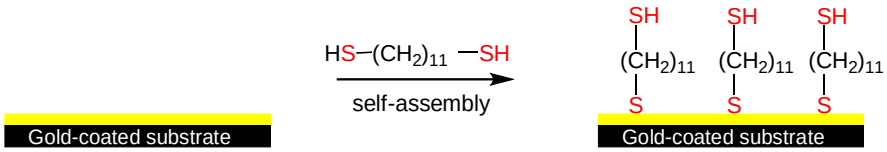
room temperature overnight. The mixture was evaporated to get the crude product as yellow oil. The crude product was then purified by column chromatography using silica gel as stationary phase and mixtures between ethyl acetate and hexane from (85:15, v/v) to (50:50, v/v) as eluents. The purification was monitored by TLC. The fraction of product with R_f = 0.30 was collected and the solvent was removed to get the desired product as yellow oil. Yield: 2.78 g (42%). ^{1}H NMR: (400 MHz, $CDCl_3$): δ/ppm: 8.5 (m, 1H, aromatic proton ortho-N), 7.6 (m, 1H, aromatic proton meta-N), 7.4 (m, 1H, aromatic proton para-N), 7.2 (m, 1H, aromatic proton, ortho-disulfide linkage), 5.8 (b, 1H, $\underline{H}OCH_2CH_2$-S-S), 3.8 (t, 2H, -S-S-C$\underline{H}_2CH_2OH$), 3.0 (t, 2H, -S-S-$CH_2C\underline{H}_2OH$).

3.3.4.5.1.2 Synthesis of pyridyldisulfide ethylmethacrylate (PDSM)



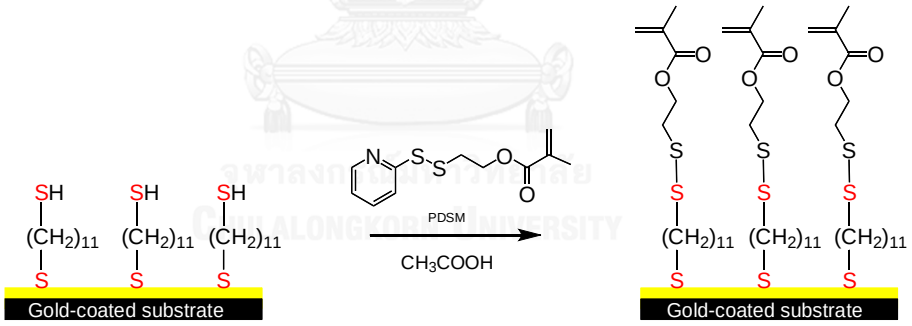
HPDS (2.43 g, 13.0 mmol) in 10.0 mL of dry dichloromethane was added 1.58 g (15.6 mmol) of triethylamine and the mixture was cooled in an ice bath. Then, a solution of methacryloyl chloride (1.36 g, 13.0 mmol) was added dropwise with continuous stirring. The mixture was stirred in an ice bath for 3 h and then stirred at room temperature for 3 h. The stirring was stopped and the solid was removed by filtration. The filtrate was washed with brine and then 3×20 mL distilled water. The organic layer was collected, dried over anhydrous $MgSO_4$ and concentrated by rotary evaporation (T< 30°C) to get crude product as brown oil. The product was purified by column chromatography using silica gel as stationary phase and mixture between hexane and ethylacetate (80:20 (%v/v)) was used as eluent. The fraction of product with R_f = 0.50 was collected and the solvent was removed to get the desired product as colorless oil. Yield: 2.55 g (77%). ^{1}H NMR: (400 MHz, CDCl3): δ/ppm: 7.9 (m, 1H, aromatic proton ortho-N), 7.7 (m, 2H, aromatic proton meta-N and para-N), 7.3 (m, 1H, aromatic proton, orthodisulfide linkage), 6.3 (d, 1H, vinylic proton, cis-ester), 5.8 (d, 1H, vinylic proton, trans-ester) 4.6 (t, 2H, -S-S-$CH_2C\underline{H}_2O$-), 3.3 (t, 2H, -S-S-C$\underline{H}_2CH_2O$-), 2.1 (s, 3H, methyl proton of the methacryloyl group).

3.3.4.5.1.3 Immobilization of 1,11-undecanedithiol (UDDT) on gold-coated surface



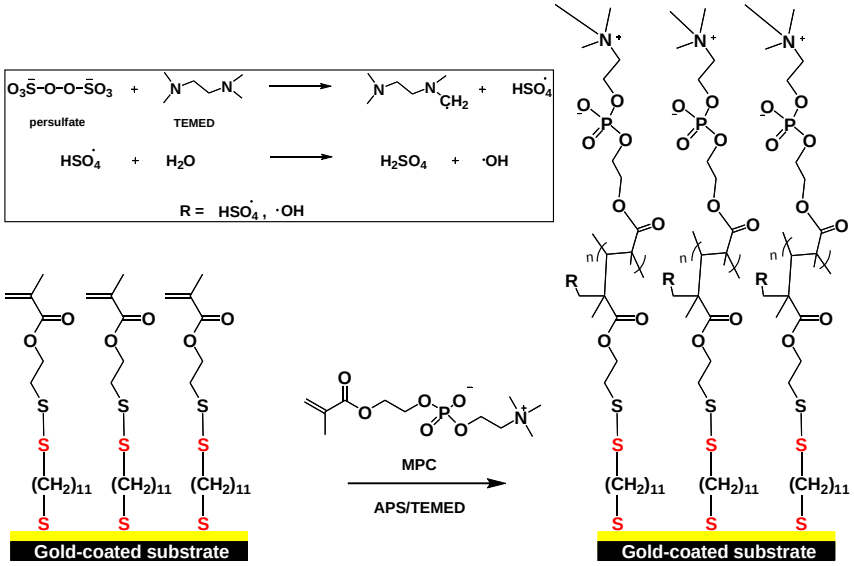
1×1 cm^2 of gold-coated surface was cleaned by oxygen plasma for 2 min, and then immersed in MilliQ water immediately. After drying with nitrogen stream, gold-coated substrate was immersed in 1.0 mM of UDDT in ethanol for 24 h at ambient temperature. The substrate was removed from the solution, then rinsed with ethanol with constant agitation 5 min each for 4 times and rinsed with THF for 1 min. Contact angle measurements were used to characterize the changing of wettability on the modified surface.

3.3.4.5.1.4 Immobilization of PDSM on UDDT-modified surface



UDDT-modified gold surface was immersed in solution of PDSM (1.5 mmol/L, 1.0 mL) with 0.1 µL glacial acetic acid for 24 h. The substrate was removed from the solution, and then rinsed with ethanol with constant agitation 5 min each for 5 times. Contact angle measurements were used to characterize the changing of wettability on the modified surface.

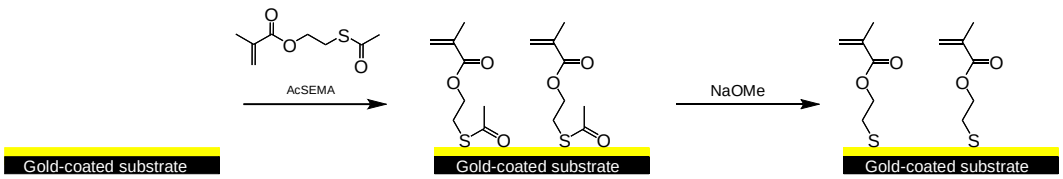
3.3.4.5.1.5 Polymerization of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on gold-coated surface modified with PDSM



A solution of MPC in RIPA lysis buffer (1.0 mol/L, 60 µL) was mixed with APS in PBS (0.22 mol/L, 9 µL) and 3 µL TMEDA by vortex. After 3 min of mixing, the solution was coated on PDSM-modified surface by spin coating (step1: 500 rpm for 5 sec, step 2: 5000 rpm for 20 sec) and was then incubated in incubator at 25°C for 18 h. The substrate was removed from the solution, and rinsed with MilliQ water with constant agitation 5 min each for 5 times. Contact angle measurements were used to characterize the changing of wettability on the modified surface.

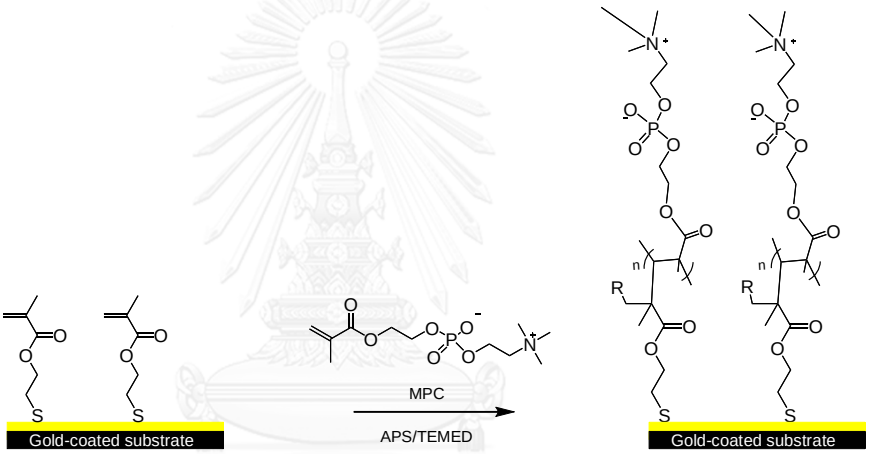
3.3.4.5.2 Polymerization of ManM-treated-glycoprotein with MPC on gold-coated surface modified with 2-(acetylthio)ethylmethacrytale (AcSCMA)

3.3.4.5.2.1 Immobilization of AcSEMA on UDDT-modified surface



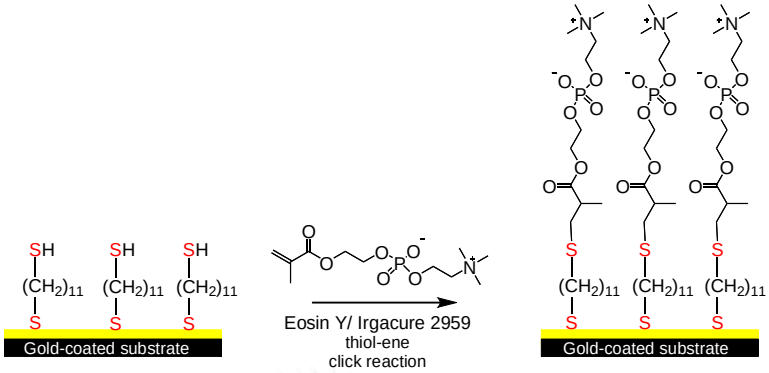
Gold-coated surface was immersed in solution of AcSEMA (1.0 mmol/L, 1.0 mL) for a set reaction time. The substrate was removed from the solution, and then rinsed with ethanol with constant agitation 5 min each for 5 times. Then, the modified surface was immersed in the solution of 1 mM sodium methoxide (NaOMe) in ethanol for 24 h and rinsed with ethanol with constant agitation 5 min each for 5 times. Contact angle measurements were used to characterize the changing of wettability on the modified surface.

3.3.4.5.2.2 Polymerization of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on gold-coated surface modified with AcSEMA



A solution of MPC in RIPA lysis buffer (1.0 mol/L, 60 µL) was mixed with APS in PBS (0.22 mol/L, 9 µL) and 3 µL TMEDA by vortex. After 3 min of mixing, the solution was coated on AcSEMA-modified surface by spin coating (step1: 500 rpm for 5 sec, step 2: 5000 rpm for 20 sec) and was then incubated in incubator at 25°C for 18 h. The substrate was removed from the solution, and then rinsed with MilliQ water with constant agitation 5 min each for 5 times. Contact angle measurements were used to characterize the changing of wettability on the modified surface.

3.3.4.5.3 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) on modified gold-coated surface via thiol-ene click reaction



Eosin Y (8.1 mg, 12.5 μmol) and MPC (50 mmol/L, 500 μL) were dissolved in 500 μL of 10% RIPA lysis buffer in MilliQ water. UDDT-modified surface was immersed in 500 μL of the solution containing eosin Y and MPC. Then, the substrate was irradiated with visible light at 505 nm (diatance between lamp and surface was 2 cm, power output was 5.1 mW/cm^2) for a set reaction time. The solution containing only eosin Y was used as the control experiment. In the case of using Irgacure 2959 as catalyst, Irgacure 2959 (2.8 mg, 12.5 μmol) and MPC (50 mmol/L, 500 μL) were dissolved in 500 μL of 10% RIPA lysis buffer in MilliQ water. UDDT-modified surface was immersed in 500 μL of the solution containing Irgacure 2959 and MPC. Then the substrate was irradiated with UV light at 365 nm (distance between lamp and surface was 2 cm, power output was 1.6 mW/cm^2) for a set reaction time. The solution containing only Irgacure 2959 was used as the control experiment. After a set reaction time, the substrate was removed from the solution and rinsed with MilliQ water by constant agitation 5 min each for 5 times. The substrate was dried with stream of nitrogen and then characterized by water contact angle measurements.

3.3.4.5.4 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ManM-treated-glycoprotein on modified gold-coated surface via thiol-ene click reaction

MPC monomer (2.5 mol/L, 300 µL), Irgacure 2959 (1.68 mg, 7.5 µmol) and glycoprotein (final concentration was 1000 µg/mL) were dissolved in 10% RIPA lysis buffer in MilliQ water. Then, the solution was covered with aluminium foil and mixed by vortex for 10 min and then transfer to 24 well plates. Thiol-modified SPR chip was immersed in the solution and irradiated with UV light at 365 nm for 15 min. Solution containing only Irgacure 2959 was used for the control experiment. The substrate was removed from the solution, immersed in PBS and stored at 4°C for 18 h to remove the unbound polymer and then rinsed by constant agitation in PBS (-) 5 min each for 5 times. The substrate was dried with stream of nitrogen and characterized by water contact angle measurements.

3.3.4.5.5 Determination of PSGL-1 antibody (N-16) binding to PSGL-1 glycoprotein on the modified surface by SPR

The SPR measurements were conducted using Biacore T200 (GE Healthcare) at 25 °C. An autosampler was used to inject the test solutions. The SPR angle shift at the end-point of each step and after baseline subtraction (“angle shift”) was used to calculate the density of molecules bound onto the surface or target density, using a sensitivity factor of 1 response unit (RU) equals 1 pg/mm^2. Each gold-coated SPR chip bearing either MPC and glycoproteins were first seated in a SPR chip holder before being stabilized with a running solution of 10 mM PBS(-) or 10 mM PBS(-) with 0.05% P20 until the equilibrium SPR angle frequency value in each buffer solution was obtained, the substrate was then ready to be used. PSGL-1 antibody (10 µg/mL) was used as target molecules to confirm the immobilized PSGL-1 glycoprotein on the surface before using hemagglutinating virus of Japan envelope (HVJ-E).

3.3.4.5.6 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ManM-treated-HL-60 cells on modified gold-coated surface via thiol-ene click reaction

110 μL of 50 mM ManM [M(+))] or PBS(-) [M(-)] was pipetted to each well of 24 well plate (final concentration is 5 mM) for 12 wells. Then, 1.0 mL of HL-60 cells suspension (3.0×10^5 cells/mL) in RPMI-1640 medium supplemented with 10% FBS was added and incubated at 37 °C in a humidified atmosphere of air containing 5% CO_2 for 3 days. After 3 days, the cells were washed three times with PBS(-) to remove the remaining ManM and ManM-treated HL-60 cells were then transferred to 1.5 mL microcentrifuge tube. 200 μL of RIPA lysis buffer was added to precipitated cells and stored at 4°C for 1 h. MPC monomer in RIPA lysis buffer (0.2 mol/L, 300 μL) was mixed with Irgacure 2959 (30 mg, 13.8 μmol). Then, the 500 μL of solution was covered with aluminium foil and mixed by vortex for 10 min and then transfer to 24 well plates. Thiol-modified gold-coated surface was immersed in the solution and irradiated with UV light at 365 nm for 15 min. The substrate was removed from the solution, rinsed by constant agitation in PBS(-) 5 min for 5 times. The substrate was dried with stream of nitrogen and characterized by water contact angle and SPR measurements.

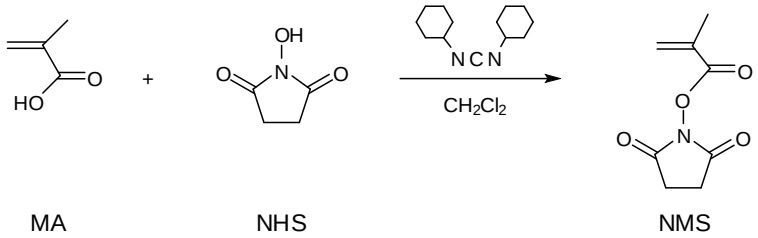
CHAPTER IV

RESULTS AND DISCUSSION

This part is divided into 2 sections. The first section reveals the synthesis and biosensing applications of two functional copolymer: poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC) and poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC). The immobilization of the copolymer brushes was performed by "grafting to" approach via self-assembly formation of thiol-terminated polymer chain on gold-coated SPR disk. Effects of copolymer composition and molecular weight on the ability to prevent non-specific adsorption and to detect analyte were evaluated. Biotin and peptide nucleic acid (PNA) were used as model probe to study the immobilization and specific binding in antigen/antibody and PNA/DNA biosensor, respectively. The second section explains the preparation of sensing platform based on methacryloyl-functionalized glycoproteins (ManM-treated glycoproteins) and 2-methacryloyloxyethyl phosphorylcholine (MPC) for influenza hemagglutinin detection. Surface modification of HL-60 cells with *N*-methacryloyl mannosamine (ManM) and the separation of ManM-treated-glycoproteins from HL-60 cells were investigated. The method to immobilize ManM-treated-glycoproteins and MPC on gold-coated substrate and the ability to detect antibody PSGL-1 as model target analyte was studied.

4.1 Preparation of surface-tethered poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC)

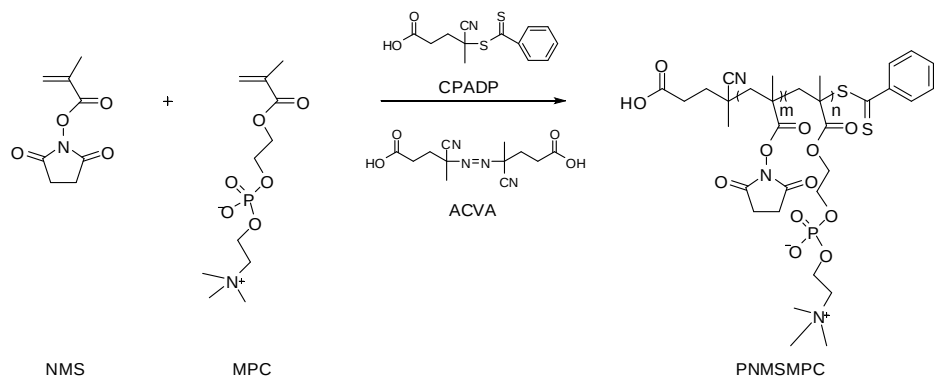
4.1.1 Synthesis of *N*-methacryloxysuccinimide (NMS) monomer



Scheme 4.1 Synthesis of *N*-methacryloxysuccinimide (NMS) monomer

N-methacryloxysuccinimide (NMS) monomer was synthesized by esterification between hydroxyl groups of *N*-hydroxysuccinimide (NHS) and carboxyl groups of methacrylic acid MA by using DCC as coupling agent in dichloromethane (Scheme 4.1). The product was obtained in 45% yield as white solid crystal. The structure of NMS monomer was confirmed by ^{1}H NMR and FT-IR analysis. The spectra are shown in Figure A-1 and Figure A-2 (Appendix A).

4.1.2 Synthesis of poly[(N-methacryloxysuccinimide)-ran-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC) by RAFT polymerization



Scheme 4. 2 Synthesis of PNMSMPC by RAFT polymerization

Reversible addition-fragmentation chain transfer (RAFT) is a controlled free radical polymerization that can produce a well-defined (co)polymer with narrow polydispersity index. The synthesis of poly[(*N*-methacryloxysuccinimide)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PNMSMPC) copolymer using RAFT polymerization was carried in the mixed solvent of EtOH and THF (1:1 v/v) in the presence of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) and 4,4'-azobis(4-cyanovaleric acid) (ACVA) as a chain transfer agent (CTA) and radical initiator, respectively. A ratio of CTA/initiator, ratio of monomer/CTA, and polymerization time were fixed at 8, 200 and 8h, respectively. Moreover, the copolymer ratio between NMS and MPC was fixed at 50:50 to study polymerization condition and aminolysis reaction before preparation of this functional copolymer as a precursor layer.

After polymerization for 8 h, PNMSMPC copolymer was characterized by ^{1}H NMR and FT-IR as shown in **Figure 4.1 and 4.2**, respectively. From ^{1}H NMR analysis, the characteristic peaks of NMS unit (-CO-C$\underline{H}_2$-C$\underline{H}_2$-CO = 2.6-2.7 ppm) and MPC unit ((-N(C$\underline{H}_3$)$_3$ = 3.1 ppm, -C$\underline{H}_2$N-CH$_2$-O- =3.5 ppm, -POC$\underline{H}_2$C$\underline{H}_2$N-COOC$\underline{H}_2$, –C$\underline{H}_2$OP = 3.8-4.3 ppm) were clearly observed. The peak intensity at 3.8-4.3 ppm attributed to the -POC$\underline{H}_2$C$\underline{H}_2$N- COOC$\underline{H}_2$ and –C$\underline{H}_2$OP proton of the MPC unit and at 0.6-2.3 ppm attributed to CH_2 and CH_3 proton of both MPC and NMS units were used to calculate the copolymer composition. The aromatic protons attributed to the dithiobenzoate group at the chain end of PNMSMPC were also observed around 7.4-7.9 ppm. From FT-IR analysis, the characteristic absorption peaks of MPC unit, C=O stretching (ester) at 1730 cm^{-1}, O=P−O−$_{asym}$ stretching at 1240 cm^{-1}, O=P−O−$_{sym}$ stretching at 1087 cm^{-1} and −N^{+}(CH$_3$)$_3$ stretching at 960 cm^{-1}, were observed, whereas the characteristic absorption peaks of succinimidyl groups (C=O stretching) of NMS unit was observed at 1805 and 1725 cm^{-1}, respectively.

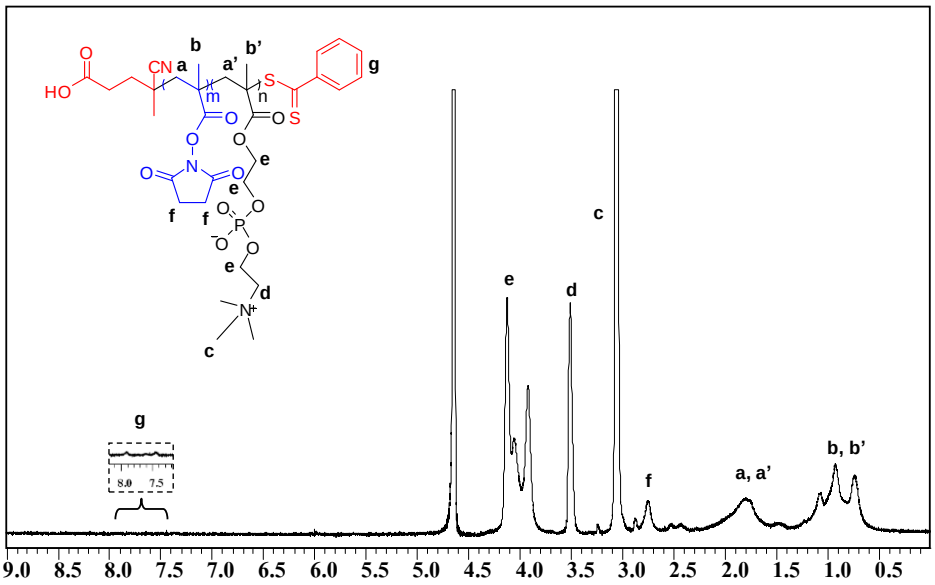


Figure 4.1 ^{1}H NMR spectrum of $PNMS_{36}MPC_{64}$

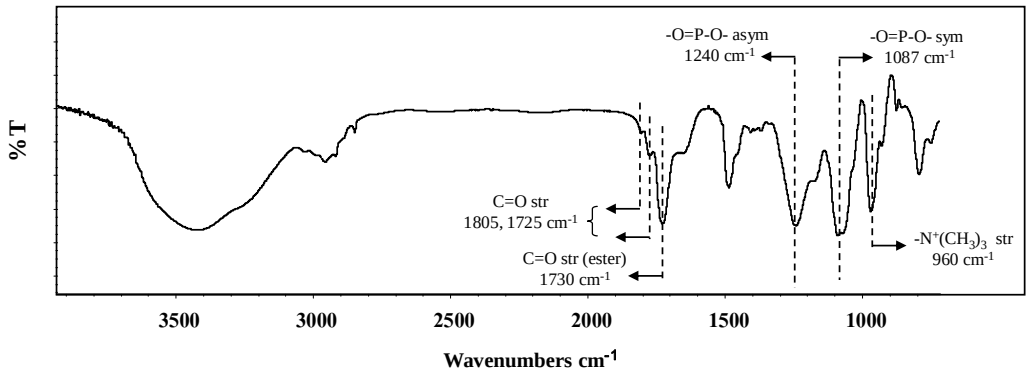


Figure 4.2 FT-IR spectrum of $PNMS_{36}MPC_{64}$

The copolymer composition and molecular weight ($\overline{M}_n$) determined by ^{1}H NMR. All monomer content (degree of polymerization) was calculated from relative ratio between integral of the CH_3 protons of both MPC and NMS units (position a, a', b, b') and integral of the aromatic protons of dithiobenzoate group at the chain end of PNMSMPC using equation (4). MPC composition was calculated from a relative ratio between integral of protons at position e and integral of aromatic protons of dithiobenzoate group. Then, the composition of NMS and molecular weight of the copolymer could be calculated using equation (6) and (7), respectively.

After calculation, the copolymer composition of NMS:MPC was 36:64 ($\overline{M}_n$ = 26.5 kDa). The obtained copolymer composition was not resembled the copolymer ratio in the feed (NMS:MPC was 50:50). This result is the same as previously report by Akkahat *et al* [21] in case of poly[(methacrylic acid)-*ran*-poly(2-methacryloyloxyethyl phosphorylcholine)] (PMAMPC) copolymer synthesis (composition in feed (%) of PMA:MPC=50:50). This might be the reason of higher reactivity of MPC monomer than NMS monomer in polymerization process.

$$All\ monomer\ content\ (unit) = \left\{\frac{\frac{integral\ of\ (position\ a,a',b,b')}{10}}{\frac{integral\ of\ (position\ g)}{5}}\right\} \quad (4)$$

$$MPC\ content\ (unit) = \left\{\frac{\frac{integral\ of\ (position\ e)}{6}}{\frac{integral\ of\ (position\ g)}{5}}\right\} \quad (5)$$

$$NMS\ content\ (unit) = All\ monomer\ content\ (unit) - MPC\ content\ (unit) \quad (6)$$

Molecular weight = [MPC content (unit) × M_n of MPC] + [NMS content (unit) × M_n of NMS] + M_n of RAFT agent (7)

4.1.3 Preparation of thiol-terminated PNMSMPC (PNMSMPC-SH)

The dithiobenzoate group at the chain end of PNMSMPC prepared by RAFT polymerization can be converted to a thiol group by aminolysis with hydrazine [65]. The $PNMS_{36}MPC_{64}$, before and after hydrolysis was characterized by FT-IR as illustrated in **Figure 4.3**. After aminolysis, the appearance of a characteristic peak at 1639 cm^{-1} of C=O stretching (amide), 1543 cm^{-1} of N-H bending, and 1703 cm^{-1} of C=O stretching (carboxylic acid) were observed. This result implies that the chemical structure of $PNMS_{36}MPC_{64}$ polymer backbone was changed after aminolysis. As a result of highly reactive functional groups on the copolymer, hydrazine can act as

nucleophile to attack at both dithioester and succinimidyl groups on the copolymer (**Figure 4.3 (b-II)**). In addition, these active functional groups can react with moisture in air as can be evidenced in **Figure 4.3 (b-I)**. For this reason, PNMSMPC copolymer was not suitable to be further used as sensor platform because the functional groups on the copolymer are very reactive to react with non-target molecules resulting in decreasing the active site for binding the specific probe.

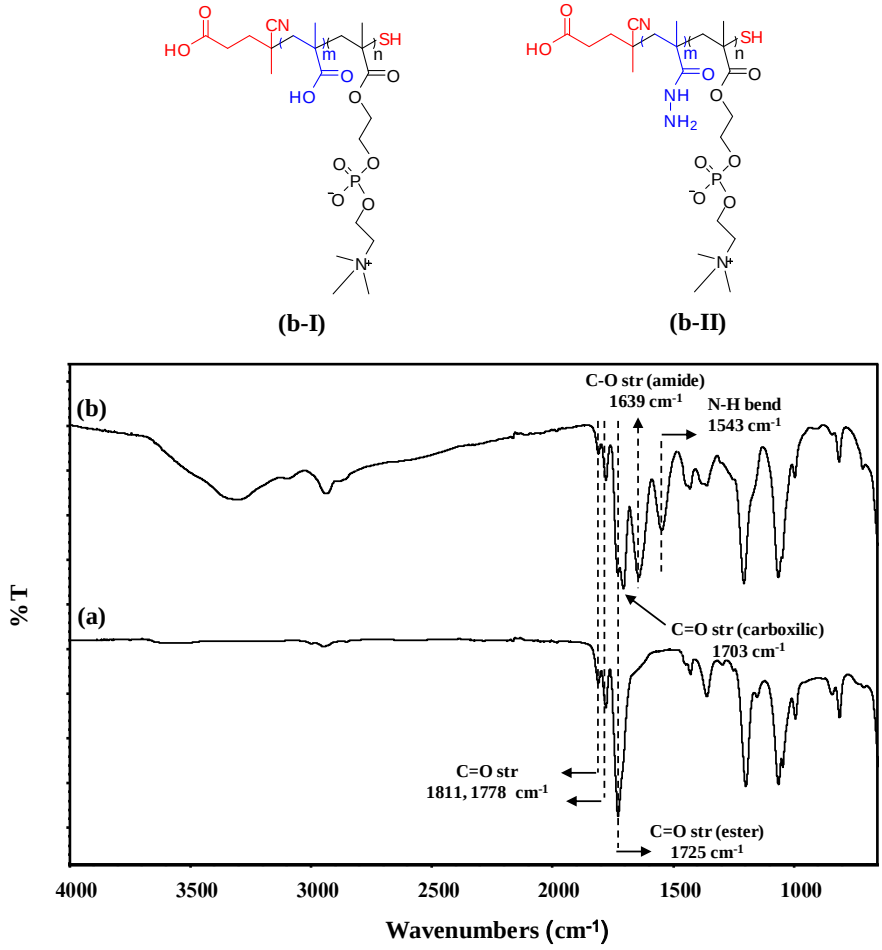
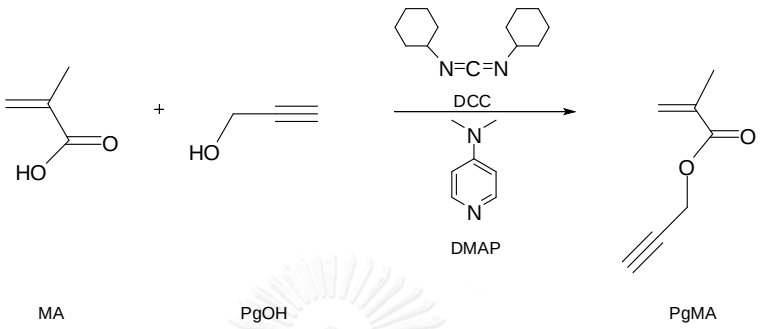


Figure 4.3 FT-IR spectra of (a) $PNMS_{36}MPC_{64}$ and (b) $PNMS_{36}MPC_{64}$ after animolysis

4.2 Preparation of surface-tethered poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC)

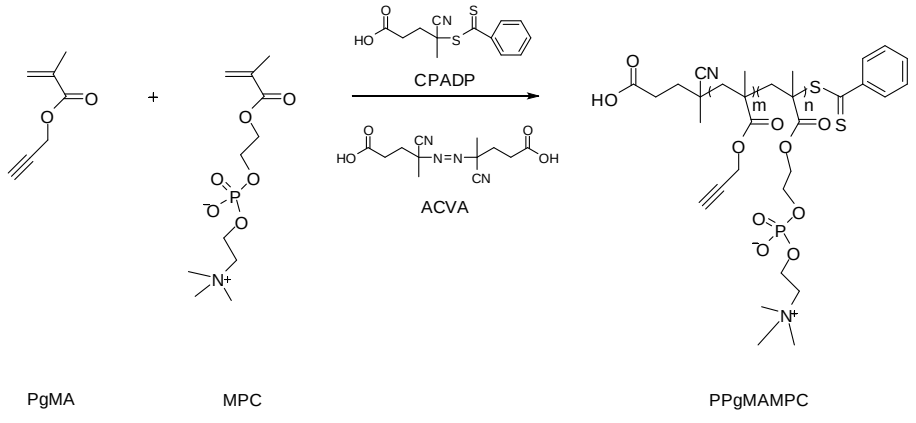
4.2.1 Synthesis of propargyl methacrylate (PgMA) monomer



Scheme 4.3 Synthesis of propargyl methacrylate (PgMA) monomer

Propargyl methacrylate (PgMA) monomer was synthesized by esterification between hydroxyl groups of propargyl alcohol (PgOH) and carboxyl groups of methacrylic acid MA by using DCC and DMAP as coupling agent in dichloromethane **(Scheme 4.3)**. The product was obtained in 51% yield as colorless oil. The structure of NMS monomer was confirmed by ^{1}H NMR and FT-IR analysis. The spectra are shown in **Figure A-3** and **Figure A-4** (Appendix A).

4.2.2 Synthesis of poly[(propargyl methacrylate)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PPgMAMPC)



Scheme 4.4 Synthesis of PPgMAMPC by RAFT polymerization

PPgMAMPC copolymer was synthesized via RAFT polymerization by using CPADP and ACVA as chain transfer agent (CTA) and radical initiator (In), respectively. The composition of PgMA and MPC unit in the copolymer ($PPgMA_mMPC_n$) is assigned as m and n, respectively. The data shown in **Table 4.1** suggested that an optimal ratio of [CTA]/[In] that yielded a copolymer with well-controlled molecular weight characteristic was 8. The control over the copolymerization process was also demonstrated for the targeted degree of polymerization (DP) of 200 from the linear first-order relationship between ln[Mo]/[M] and polymerization time (**Figure 4.4**) suggesting that polymeric radical concentration remained unaltered throughout the period of polymerization. As determined by ^{1}H NMR. **Figure 4.5** shows ^{1}H NMR spectra of PPgMAMPC copolymer with the different copolymer composition ($PPgMA_{65}MPC_{35}$, $PPgMA_{45}MPC_{55}$, $PPgMA_{38}MPC_{62}$), the characteristic peak of PgMA unit (-C≡C<u>H</u> = 2.40 ppm (position g), -O-C<u>H$_2$</u>-C≡CH = 4.55-4.80 ppm (position f)) and MPC unit ((-N(C<u>H$_3$</u>)$_3$ = 3.25 ppm (position c), -C<u>H$_2$</u>N-CH$_2$-O- =3.75ppm (position d), -POC<u>H$_2$</u>C<u>H$_2$</u>N- , -COOC<u>H$_2$</u> –C<u>H$_2$</u>OP = 4.0-4.4 ppm (position e)) were clearly observed. Furthermore, the signal at 7.40-7.95 ppm can be assigned to aromatic proton in the copolymer. The signal at position f increased when the copolymer having high PgMA monomer ratio.

The copolymer composition was correspondingly varied as a function of monomer ratio in feed (**Table 4.2**). Molecular weight of the copolymers also closely resembled the expected target (35,375-46,327 kDa). The copolymer composition and molecular weight ($\overline{M}_n$) determined by ^{1}H NMR. All monomer content (degree of polymerization) was calculated from relative ratio between integral of the CH_3 protons of both MPC and PgMA units (position a, a', b, b') and integral of the aromatic protons of dithiobenzoate group at the chain end of PPgMAMPC using equation (7). MPC composition was calculated from a relative ratio between integral of protons at position e and integral of aromatic protons of dithiobenzoate group. Then, the composition of NMS and molecular weight of the copolymer could be calculated using equation (9) and (10) respectively.

$$All\ monomer\ content\ (unit) = \left\{\frac{\frac{integral\ of\ (position\ a,a',b,b')}{10}}{\frac{integral\ of\ (position\ h)}{5}}\right\} \quad (7)$$

$$MPC\ content\ (unit) = \left\{\frac{\frac{integral\ of\ (position\ e)}{6}}{\frac{integral\ of\ (position\ h)}{5}}\right\} \quad (8)$$

$$PgMA\ content\ (unit) = All\ monomer\ content\ (unit) - MPC\ content(unit) \quad (9)$$

Molecular weight = [MPC content (unit) × M_n of MPC] + [PgMA content (unit) × M_n of PgMA] + M_n of RAFT agent (10)

Functional group identity of the synthesized copolymer was verified by FT-IR analysis as shown in **Figure 4.6**. Appearance of characteristic peaks: C=O (ester) stretching at 1725 cm^{-1}, asymmetric O=P−O− stretching at 1240 cm^{-1}, symmetric O=P−O− stretching at 1087 cm^{-1}, and $-N^+(CH_3)_3$ at 960 $cm^{-1}$of the MPC units and -C≡C- stretching at 2127 cm^{-1} of which intensity proportionally increased with the PgMA content truly confirmed the successful formation of copolymers.

Table 4.1 Optimal ratio of [CTA]/[In] of PPgMAMPC (targeted DP of 100) synthesized by RAFT polymerization.

Monomer composition in feed (%)		Monomer composition in copolymer (%)[a]		[CTA]/[In]	Target $\overline{M}_n$ (kDa)[a]	$\overline{M}_n$ (kDa)[a]	Copolymer abbreviation
PgMA	MPC	PgMA	MPC				
20	80	15	85	4	26.3	66.8	$PPgMA_{15}MPC_{85}$
20	80	15	85	8	26.3	20.1	$PPgMA_{15}MPC_{85}$

- [a] calculated from ^1H NMR data
- Polymerization time = 24 h

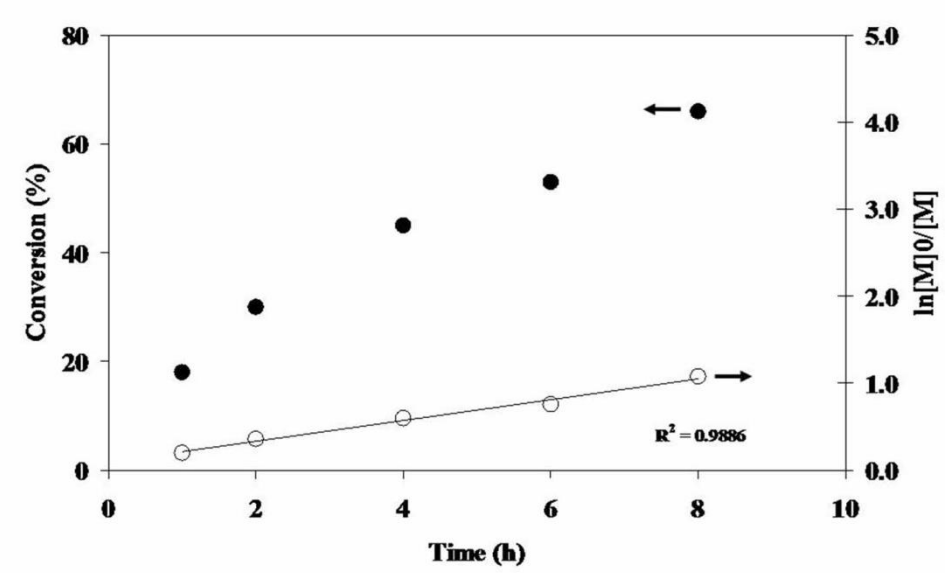


Figure 4.4 Percentage of conversion (●) and semilogarithmic plot of MPC conversion (○) as a function of time for $PPgMA_{50}MPC_{50}$ with targeted DP of 200.

Table 4.2 Reaction conditions, copolymer composition and molecular weight of PPgMAMPC synthesized by RAFT polymerization

composition in feed (%)		Monomer: CTA (mole ratio)	composition in copolymer (%) *a		$\overline{M}_n$ ($\times 10^3$) *a (Da)	abbreviation
PgMA	MPC		PgMA	MPC		
70	30	100	64	36	18.9	$PPgMA_{64}MPC_{36}$
70	30	200	65	35	38.4	$PPgMA_{65}MPC_{35}$
70	30	300	69	31	52.9	$PPgMA_{69}MPC_{31}$
50	50	200	45	55	33.1	$PPgMA_{45}MPC_{55}$
30	70	200	38	62	40.0	$PPgMA_{38}MPC_{62}$

● polymerization time = 6 h

*a = calculated from ^{1}H NMR

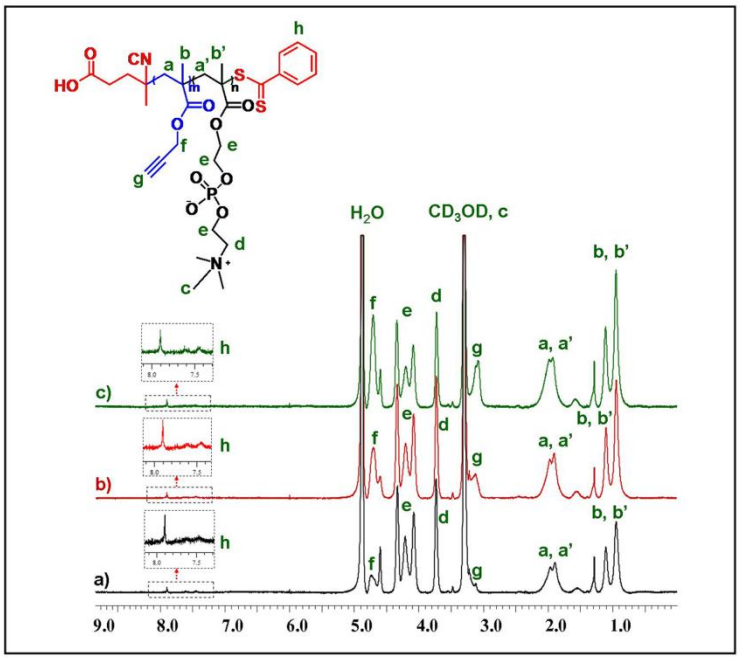


Figure 4.5 ^{1}H NMR spectra (a) $PPgMA_{38}MPC_{62}$ (40.0 kDa), (b) $PPgMA_{45}MPC_{55}$ (33.1 kDa) and (c) $PPgMA_{65}MPC_{35}$ (38.4 kDa) copolymer

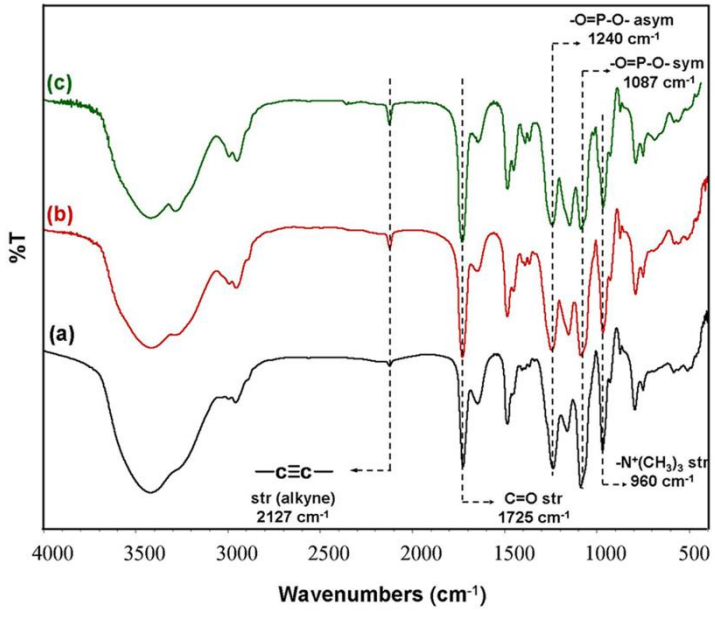
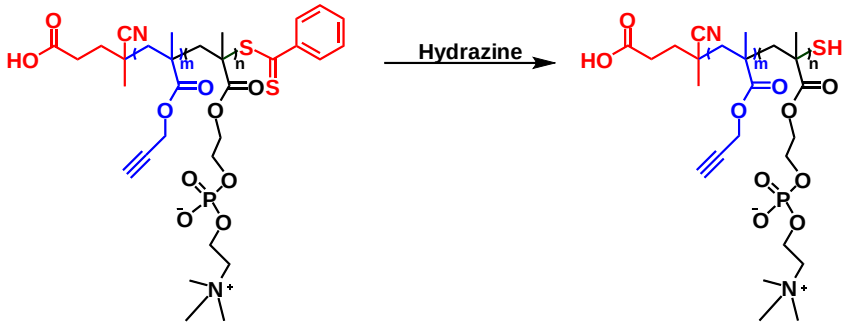


Figure 4.6 FT-IR spectra of (a) $PPgMA_{38}MPC_{62}$ (40.0 kDa), (b) $PPgMA_{45}MPC_{55}$ (33.1 kDa) and (c) $PPgMA_{65}MPC_{35}$ (38.4 kDa) copolymer

4.2.3 Preparation of thiol-terminated PPgMAMPC (PPgMAMPC-SH)



Scheme 4.5 Preparation of PPgMAMPC-SH

Upon aminolysis by hydrazine, the dithiobenzoate group at the chain end of PPgMAMPC copolymer was transformed into thiol groups and then yielded PPgMAMPC-SH . [65] As can be realized from the disappearance of aromatic proton signal at 7.40-7.95 ppm in ^{1}H NMR spectra and UV-visible absorption peak at 305 nm after aminolysis (**Figure 4.7 and 4.8**).

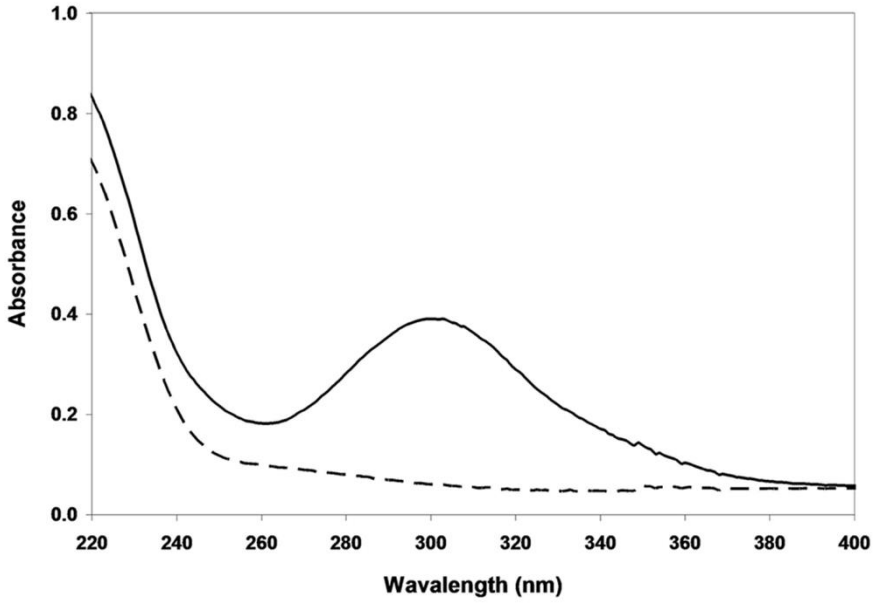


Figure 4.7 UV-vis absorption spectra for $PPgMA_{65}MPC_{35}$ (38.4 kDa) before (—) and after (---) aminolysis

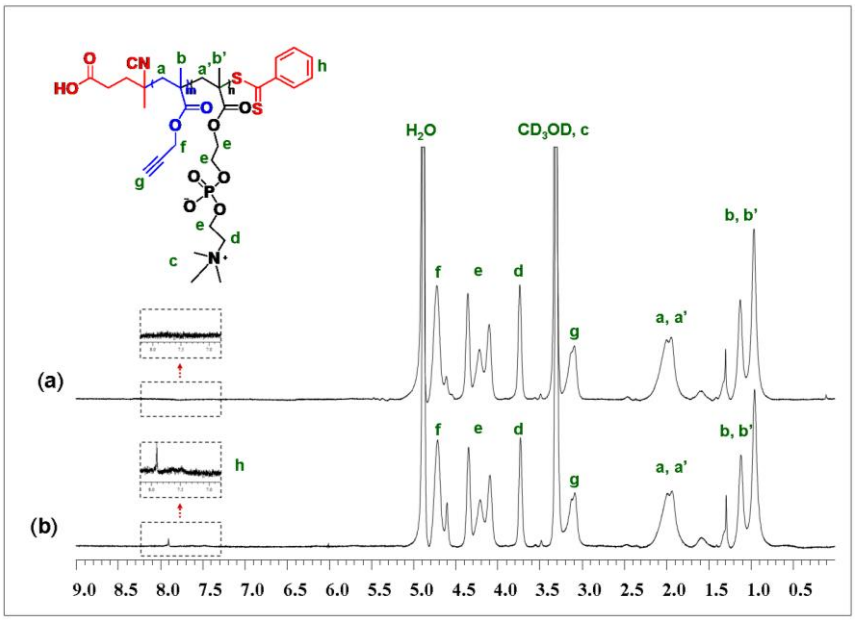
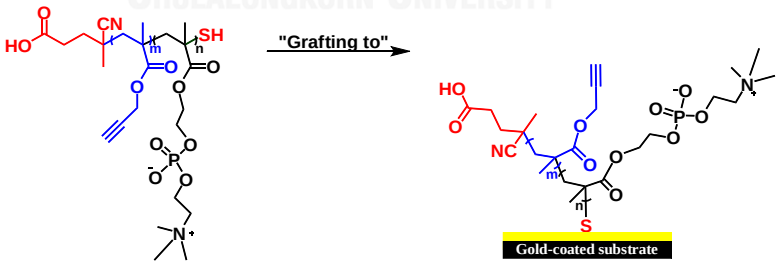


Figure 4.8 ^{1}H NMR spectra for $PMA_{65}MPC_{35}$ (38.4 kDa) copolymer (a) before and (b) after aminolysis

4.2.4 Immobilization of PPgMAMPC-SH on gold-coated SPR chip



Scheme 4.6 Immobilization of PPgMAMPC-SH on gold-coated SPR chip

Unlike, $PPgMA_{38}MPC_{62}$-SH and $PPgMA_{45}MPC_{55}$-SH, $PPgMA_{65}MPC_{35}$-SH having higher composition of the more hydrophobic PgMA was not soluble in EtOH but can dissolve in(in the mixed solution of EtOH and THF (7:3 v/v). Au-S bonds between the gold layer onSPR chip and the thiol-terminated copolymer via “grafting

to" approach upon immersion of the freshly cleaned gold-coated SPR chip in the copolymer solution at ambient temperature for 48 h. The existence of PPgMAMPC copolymer brushes on SPR chip was confirmed by water contact angle measurement, SPR, ATR-FTIR, and XPS analyses.

As shown in **Table 4.3**, the advancing (θ_A) water contact angles of SPR chip was dropped from 71.9 ± 1.3 ° to 44.6 ± 0.9° and 46.6 ± 3.5° upon immobilization of $PPgMA_{38}MPC_{62}$-SH and $PPgMA_{45}MPC_{55}$-SH, respectively indicating their highly hydrophilic character. The copolymer with higher hydrophobic content of PgMA, $PPgMA_{65}MPC_{35}$-SH yielded the gold surface with even higher contact angle (87.9 ± 4.2°) than the pristine gold surface. The chemisorption of the copolymer brushes on the gold-coated SPR chip was also monitored by SPR technique. SPR angle shift upon PPgMAMPC-SH immobilization was used to calculate the amount of the copolymer bound onto the surface, using a sensitivity factor of 120 mDegrees equals to 100 ng/cm^2 (**Table 4.3**).

Table 4.3 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization and subsequent biotin attachment.

Sample	Water contact angle (°)	SPR data	
		Angle shift (mDegree)	Amount of adsorbed copolymer (ng/cm^2)
Au	71.9 ± 1.3	-	-
Au-$PPgMA_{38}MPC_{62}$	44.6 ± 0.9	1024.8 ± 81.8	854.0 ± 68.1
Au-$PPgMA_{45}MPC_{55}$	46.6 ± 3.5	882.1 ± 166.6	735 ± 138.9
Au-$PPgMA_{65}MPC_{35}$	87.9 ± 4.2	1323.1 ± 56.1	1102 ± 46.8
Au-$PPgMA_{38}MPC_{62}$-biotin	32.7 ± 2.7	15.2 ± 7.4	12.7 ± 6.1
Au-$PPgMA_{45}MPC_{55}$-biotin	41.6 ± 1.3	229.5 ± 50.4	191.2 ± 42.0
Au-$PPgMA_{65}MPC_{35}$-biotin	62.2 ± 1.7	209.5 ± 6.7	174.5 ± 5.6

Figure 4.9 shows XPS spectra of the gold-coated substrate immobilized with $PPgMA_{45}MPC_{55}$-SH as compared with the substrate before immobilization (bare gold). Determined at a takeoff angle of 90°, phosphorus (P_{2p}) and nitrogen (N_{1s}) signals attributed to the phosphorylcholine group of the MPC units were observed on the substrate indicating that the copolymer can be bound to the gold-coated substrate. The signal from S_{2p} of bound thiol at a binding energy of 163 eV was also detected implied that the copolymer was strongly adsorbed on the substrate by the interaction between thiol groups of the copolymer and gold surface. From ATR-FTIR analysis, the characteristic absorption peaks of MPC unit, (C=O stretching (ester) at 1725 cm^{-1}, O=P−O−$_{asym}$ stretching at 1264 cm^{-1}, O=P−O−$_{sym}$ stretching at 1091 cm^{-1} and $-N^{+}(CH_3)_3$ stretching at 968 cm^{-1}, were observed as shown in **Figure 4.10**, whereas the characteristic absorption peaks of PgMA unit, -C≡C- stretching at 2127 cm^{-1}, was not clearly observed due to the appearance of absorption peak at the same range of bare gold.

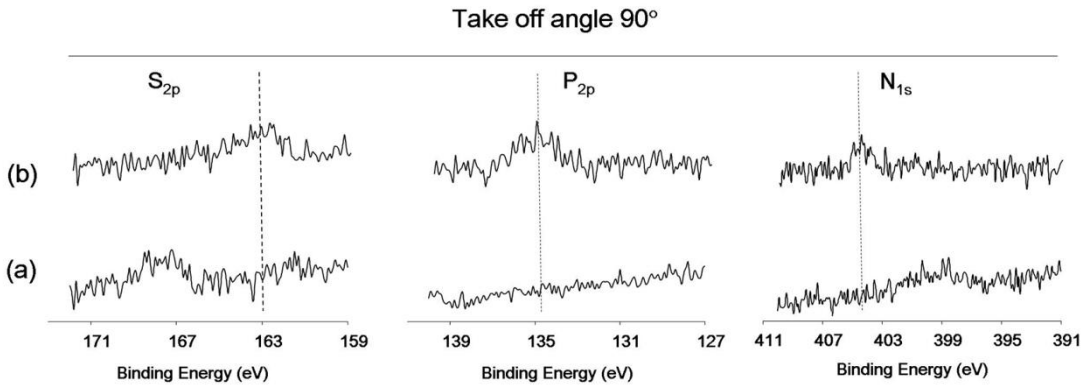


Figure 4.9 XPS spectra of gold-coated substrate (a) bare gold and after being adsorbed with (b) $PPgMA_{45}MPC_{55}$-SH.

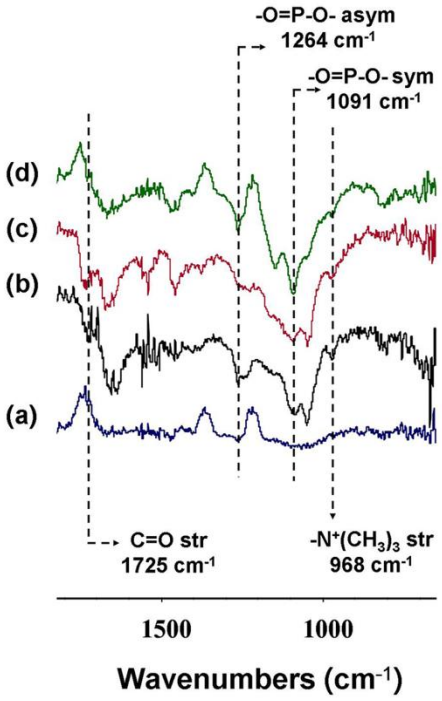


Figure 4.10 ATR-FTIR spectra of gold-coated SPR chips: (a) bare gold and after immobilization with (b) $PPgMA_{38}MPC_{62}$-SH (40.0 kDa), (c) $PPgMA_{45}MPC_{55}$-SH (33.1 kDa), and (d) $PPgMA_{65}MPC_{35}$-SH (38.4 kDa).

4.2.5 Synthesis of azide-terminated PNA (PNA-N_3) by solid phase peptide synthesis

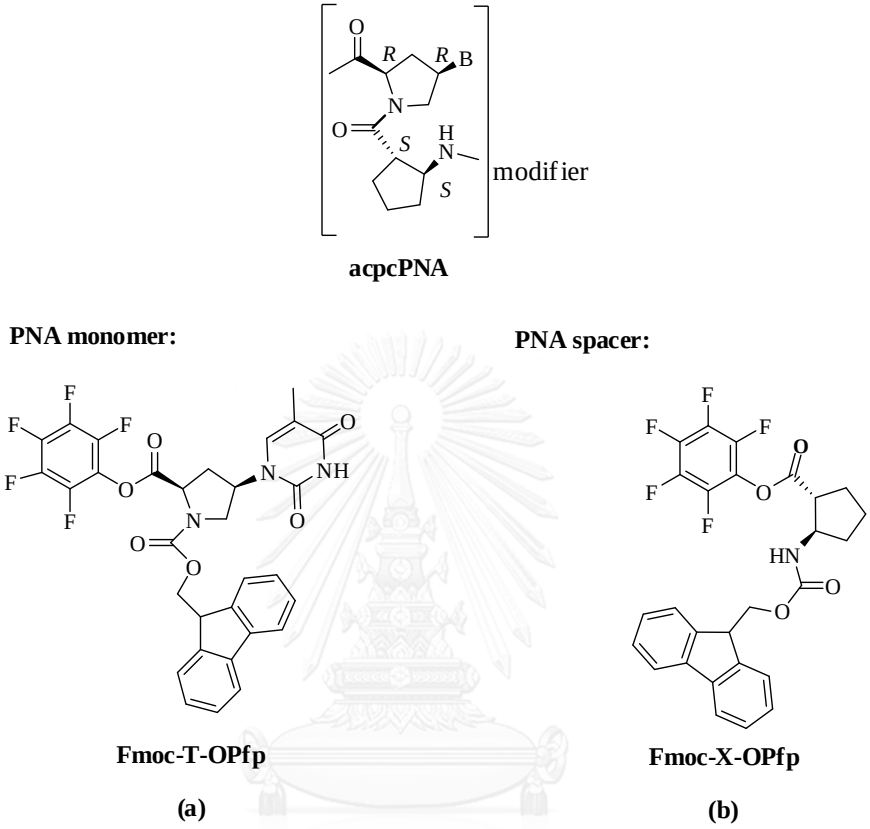


Figure 4.11 Structure of activated PNA monomers (a) and spacer (b) for solid phase peptide synthesis.

The conformationally rigid pyrrolidinyl PNA derived from D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbones (acpcPNA) [73, 74] introduced by Vilaivan and co-workers shows great properties because it can form PNA·DNA duplex with even higher affinity and specificity. Therefore, this PNA system is a candidate for the development of a highly effective sensor platform for DNA sequence detection.

The PNA-N_3, N_3-TTT TTT TTT-LysNH_2, was prepared for using as model probe to study the immobilization and specific binding in PNA/DNA biosensor. The activated Fmoc-protected acpcPNA monomers, spacer, and modifier (N_3-$(CH_2)_3$-CHO) were used in solid phase peptide synthesis shown in **Figure 4.11**. Solid phase peptide

synthesis of PNA was synthesized following the standard protocol previously developed by Vilaivan and co-workers.[88, 89] The peptide chain is grown from amino acid building blocks on solid support, TantaGel S RAM Fmoc resin. The peptides synthesis is usually extended from C (carboxyl) terminus to N (amino) terminus through a series of coupling cycles. An Fmoc-protected amino acid with polar side chain (Lys) was first coupled to the resin. Then, the PNA monomer (Fmoc-T-OPfp) and spacer (Fmoc-X-OPfp) were coupled alternately until the desired PNA sequence was obtained. Azide-terminated PNA was synthesis following the procedure described earlier. [74] The deprotected *N*-terminus of the last PNA monomer (Fmoc-T-OPfp) was coupled with N_3-$(CH_2)_3$-CHO by reductive alkylation for overnight. Finally, the PNA oligomer was cleaved from the resin by treatment with TFA. The crude PNA was alternatively washed with ether and air-dried for three times to give a white precipitate of the crude PNA.

The crude PNA oligomers were purified by reverse phase HPLC. The gradient system for azide-terminated PNA oligomer is 0.01% TFA in acetronitrile/water. After the purification, the solution of the purified PNA was lyophilized and characterized the structure of the PNA oligomers by MALDI-TOF mass spectrometry. The *m/z* data obtained from MALDI-TOF analysis for PNA are shown in **Figure 4.12,** and HPLC chromatogram is illustrated in **Figure 4.13**. A single sharp peak of azide terminated PNA was obtained after purification by reverse phase HPLC. The mass detected from MALDI-TOF mass spectrometry observed **(***m/z* = 3,122.9) closely resembled the calculated values (*m/z* = 3,124.0). This result confirmed the success of PNA-N_3 synthesis.

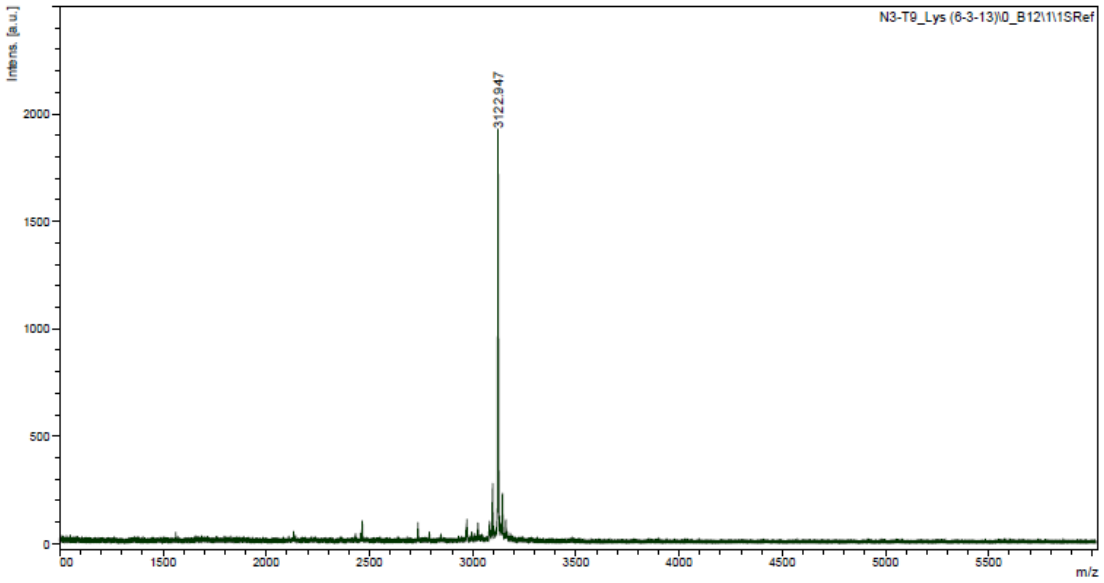


Figure 4.12 MALDI-TOF MS spectrum of the PNA-N_3 (N_3-TTT TTT TTT-$LysNH_2$)

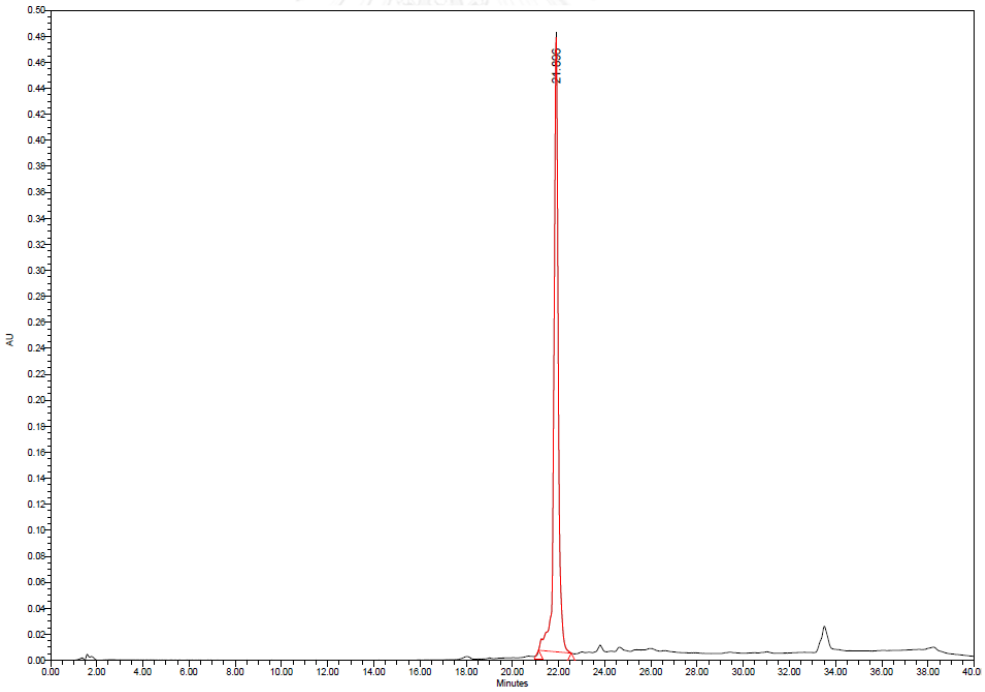
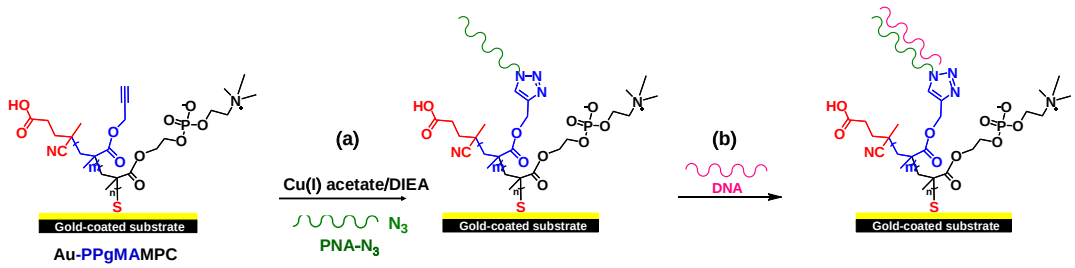


Figure 4.13 Analytical HPLC chromatogram (260 nm) of PNA-N3 (N3-TTT TTT TTT-LysNH2)

4.2.6 Specific interaction of the PNA probes immobilized on the gold coated SPR disks bearing PPgMAMPC brushes with target DNA

The specific binding of sensing probes with target molecules was determined by SPR measurement. The PNA was immobilized on the alkynyl group of PgMA unit in the PPgMAMPC copolymer brushes outside the SPR instrument based on Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition. In this research, PNA probe was firstly used as sensing probe to study the specific detection with complementary and single-mismatched DNA **(Table 4.4)**. In order to determine the ability of sensor platform to detect DNA due to the Watson–Crick base pairing rules, 50 μM DNA sample and 10 mM PBS buffer (running buffer) were used in this experiment to study the specific binding with PNA probes. The change of SPR angle shift is proportional to the amount of target molecules bound with the specific probe immobilized on the sensor platform. The efficiency of this sensor platform was investigated in order to quantify the specific binding in term of hybridization efficiency (%HE) and mismatch discrimination ability (%MD) (equation 1 and 2).



Scheme 4.7 Schematic diagram showing (a) Immobilization of PNA probe on PPPgMAMPC-modified SPR chip (b) specific binding between DNA and probe

$$\%HE = \frac{target\ density}{probe\ density} \times 100 \qquad (1)$$

$$\%MD = \frac{\%HE\ of\ complementary\ DNA - \%HE\ of\ mismatched\ DNA}{\%HE\ of\ complementary\ DNA} \times 100 \qquad (2)$$

Table 4.4 Synthesized azide-terminated PNA probe and the DNA sequence used in this study.

Description	Sequence
PNA-N_3	N_3-TTT TTT TTT-LysNH_2
Complementary DNA	5'-AAA AAA AAA-3'
Single mismatch DNA	5'-AAA A<u>T</u>A AAA-3'

4.2.6.1 Effect of copolymer concentration and ionic strength

The effect of copolymer concentration and ionic strength to the hybridization efficiency was investigated in this experiment. From the research previously reported in our laboratory [21], the copolymer concentration using in the preparation of PMAMPC sensor platform is 0.2 mM. To optimize the hybridization condition, the copolymer concentration of $PPgMA_{65}MPC_{35}$-SH (38.4 kDa) was varied in the immobilization step via self-assembly formation between thiol-terminated PPgMAMPC copolymer and gold-coated substrate. To study the effect of ionic strength, 100 mM NaCl was added to the hybridization solution and compared the results with the condition without 100 mM NaCl. The hybridization time, PNA and DNA concentration were kept constant at 5 min, 5 μM and 50 μM, respectively.

As shown in **Table 4.5**, the advancing (θ_A) water contact angles of the $PPgMA_{65}MPC_{35}$-modified SPR chip correspondingly decreased after PNA attachment indicating that the hydrophobic alkyne moieties from the PgMA repeat units were bound with PNA. The data in **Table 4.6** show that the PNA probe density on Au-0.2 mM $PPgMA_{65}MPC_{35}$ was slightly higher than that on Au-0.1 mM $PPgMA_{65}MPC_{35}$. However, the higher $\%HE_{com}$ was observed on the sensor platform immobilized with 0.1 mM $PPgMA_{65}MPC_{35}$. It is obvious that the DNA molecules may have limited accessibility to the densely immobilized PNA probes on the copolymer which has limited swellability due its hydrophobic nature. Therefore, the copolymer concentration of 0.1 mM was selected as the optimized copolymer concentration for subsequent experiments.

As shown in **Table 4.6**, in the condition with and without 100 mM NaCl, %HE was increased from 42% to 71%, while %MD was nearly the same because not only the SPR signal of complementary DNA was increased but the signal of single mismatch DNA were also increased. This may be explained as a result of intermolecular electrostatic repulsion between the strands of DNA hybridized on the modified gold-coated substrate being weaker in high ionic strength condition. The charged on DNA strands are shielded by the ion of salt, so the ability of DNA hybridization is improved [90]. Therefore, 100 mM NaCl was applied in the later part of experiment.

Table 4.5 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization and subsequent PNA attachment.

Sample	Advancing water contact angle (°)
Au	71.9 ± 1.3
Au-0.1 mM PPgMA$_{65}$MPC$_{35}$	79.4 ± 5.4
Au-0.2 mM PPgMA$_{65}$MPC$_{35}$	85.3 ± 10.5
Au-0.1 mM PPgMA$_{65}$MPC$_{35}$-PNA	65.2 ± 1.5
Au-0.2 mM PPgMA$_{65}$MPC$_{35}$-PNA	59.2 ± 3.9

Table 4.6 PNA probe density, hybridization efficiency of complementary DNA (%HE_{com}) and mismatch discrimination ability (%MD) of the modified gold-coated SPR chips

Sample	PNA probe density ($\times 10^3$ molecules/cm^2)	%HE_{com}	%MD
Au-0.1 mM $PPgMA_{65}MPC_{35}$-PNA	2.1 ± 1.1	42%	54%
Au-0.2 mM $PPgMA_{65}MPC_{35}$-PNA	2.8 ± 0.1	30%	58%
Au-0.1 mM $PPgMA_{65}MPC_{35}$-PNA with 100 mM NaCl	2.1 ± 1.1	71%	58%

4.2.6.2 Effect of copolymer chain length

The number of active functional groups of PgMA unit can be increased when increasing the copolymer chain length, so the effect of copolymer chain length to the hybridization efficiency was studied in this part. The copolymer chain length was varied by varying the molecular weight of the copolymer ($PPgMA_{64}MPC_{36}$ (18.9 kDa), $PPgMA_{65}MPC_{35}$ (38.4 kDa), $PPgMA_{69}MPC_{31}$ (52.9 kDa)). The hybridization time, PNA and DNA concentration were kept constant at 5 min, 5 μM and 50 μM, respectively. Moreover, copolymer concentration of 0.1 mM and the addition of 100 mM NaCl were also used in this experiment.

As shown in **Table 4.7**, the advancing (θ_A) water contact angles of the PPgMAMPC-modified SPR chip slighly decreased after PNA attachment indicating that the hydrophobic alkyne moieties from the PgMA repeat units were bound with PNA. The data in **Figure 4.14 and Table 4.8** indicate that the gold-coated substrate immobilized with $PPgMA_{65}MPC_{35}$-SH (38.4 kDa) showed the highest %HE and %MD. The hybridization efficiency was decreased when molecular weight of copolymer was increased to 52.9 kDa. These results indicate that the increase of polymer chain length to 38.4 kDa could be used to promote the specific binding to the sensor platform but the longer polymer chain length (52.9 kDa) may have limited accessability of copolymer immobilized onto the substrate via grafting to method

resulting in low amount of probe density and hybridization efficiency of the sensor platform **(Table 4.8)**. Therefore, the molecular weight of 38.4 kDa was selected as optimal molecular weight for using in later part of experiment.

Table 4.7 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization with different molecular weight and subsequent PNA attachment

Sample	Advancing water contact angle (°)
Au	71.9 ± 1.3
Au-PPgMA$_{64}$MPC$_{36}$ (18.9 kDa)	87.9 ± 1.9
Au-PPgMA$_{65}$MPC$_{35}$ (38.4 kDa)	87.9 ± 4.2
Au-PPgMA$_{69}$MPC$_{31}$ (52.9 kDa)	85.3 ± 10.5
Au-PPgMA$_{64}$MPC$_{36}$ (18.9 kDa)-PNA	79.9 ± 3.6
Au-PPgMA$_{65}$MPC$_{35}$ (38.4 kDa)-PNA	74.5 ± 2.2
Au-PPgMA$_{69}$MPC$_{31}$ (52.9 kDa)-PNA	59.2 ± 3.9

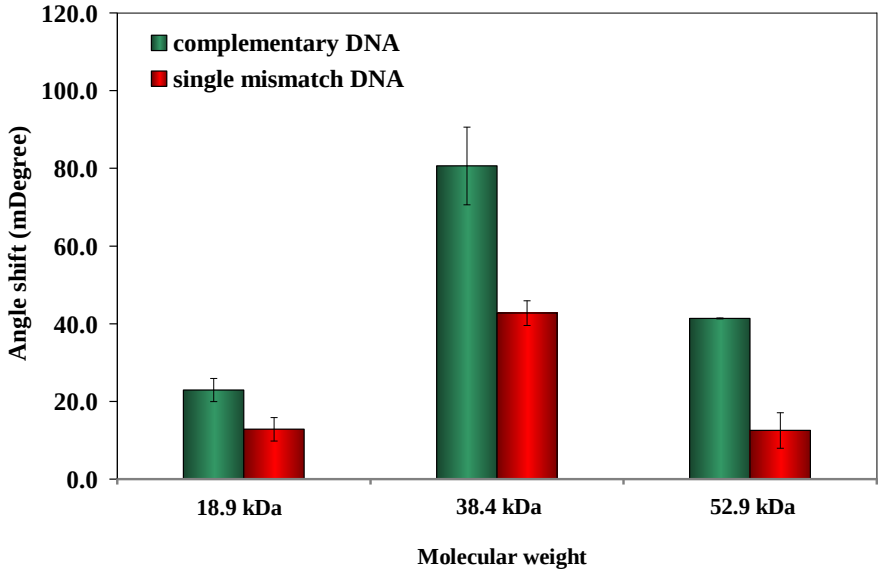


Figure 4.14 SPR angle shift corresponding to the binding of complementary DNA (5'-AAA AAA AAA-3') and single mismatch DNA (5'-AAA ATA AAA-3') on the surface modified with 0.1 mM $PPgMA_{64}MPC_{36}$ (18.9 kDa), $PPgMA_{65}MPC_{35}$ (38.4 kDa), and $PPgMA_{69}MPC_{31}$ (52.9 kDa) and immobilized with PNA.

Table 4.8 PNA probe density, hybridization efficiency of complementary DNA ($\%HE_{com}$) and mismatch discrimination ability (%MD) of the modified gold-coated SPR chips with different molecular weight

Sample	PNA probe density ($\times 10^3$ molecules/cm^2)	$\%HE_{com}$	%MD
Au-$PPgMA_{64}MPC_{36}$ (18.9 kDa)-PNA	2.5 ± 0.2	17%	44%
Au-$PPgMA_{65}MPC_{35}$ (38.4 kDa)-PNA	2.1 ± 1.1	71%	58%
Au-$PPgMA_{69}MPC_{31}$ (52.9 kDa)-PNA	1.3 ± 0.1	67%	54%

4.2.6.3 Effect of copolymer composition

The effect of copolymer composition to the hybridization efficiency was studied in this part. The different ratio between the active site for probe binding and the hydrophilic monomeric unit to suppress non-specific adsorption was varied by varying

the copolymer composition of PPgMAMPC ($PPgMA_{38}MPC_{62}$-SH (40.0 kDa), $PPgMA_{45}MPC_{55}$-SH (33.1 kDa), and $PPgMA_{65}MPC_{35}$-SH (38.4 kDa). The hybridization time, PNA and DNA concentration were kept constant at 5 min, 5 μM and 50 μM, respectively. Moreover, copolymer concentration of 0.1 mM and the addition of 100 mM NaCl were also used in this experiment.

Table 4.9 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization with different copolymer composition and subsequent PNA attachment.

Sample	Advancing water contact angle (°)
Au	71.9 ± 1.3
Au-$PPgMA_{38}MPC_{62}$	44.6 ± 0.9
Au-$PPgMA_{45}MPC_{55}$	46.6 ± 3.5
Au-$PPgMA_{65}MPC_{35}$	87.9 ± 4.2
Au-$PPgMA_{38}MPC_{62}$-PNA	66.5 ± 2.8
Au-$PPgMA_{45}MPC_{55}$-PNA	60.6 ± 2.2
Au-$PPgMA_{65}MPC_{35}$-PNA	74.5 ± 2.2

As shown in **Table 4.9**, the advancing (θ_A) water contact angles of SPR chip was increased from 44.6 ± 0.9° and 46.6 ± 3.5° to 66.5 ± 2.8° and 60.6 ± 2.2° upon immobilization of PNA probe on Au-$PPgMA_{38}MPC_{62}$-SH and Au-$PPgMA_{45}MPC_{55}$-SH, respectively because of the immobilization of hydrophobic PNA molecules on the surface. The copolymer with higher hydrophobic content of PgMA, $PPgMA_{65}MPC_{35}$-SH, the contact angle (87.9 ± 4.2°) was decreased to 74.5 ± 2.2° after PNA immobilization. These results confirm the immobilization of PNA probes on the copolymer-modified substrate.

The sensing platform based on Au-PPgMA$_{65}$MPC$_{35}$-PNA having highest amount of PgMA unit can detect the highest amount of complementary DNA (85.5 ± 10.9 mDegree) with %HE of 71% that was higher than %HE of sensing platform based on Au-S-PNA[75] and Au-biotin-PNA[76] as shown in **Figure 4.15 and Table 4.10**. However, this copolymeric platform still exhibited high non-specific interactions towards single mismatch DNA because of the remaining alkyne moieties, hydrophobic part, on the polymeric platform. That is why %MD was still not high when compared with the Au-biotin-PNA platform.

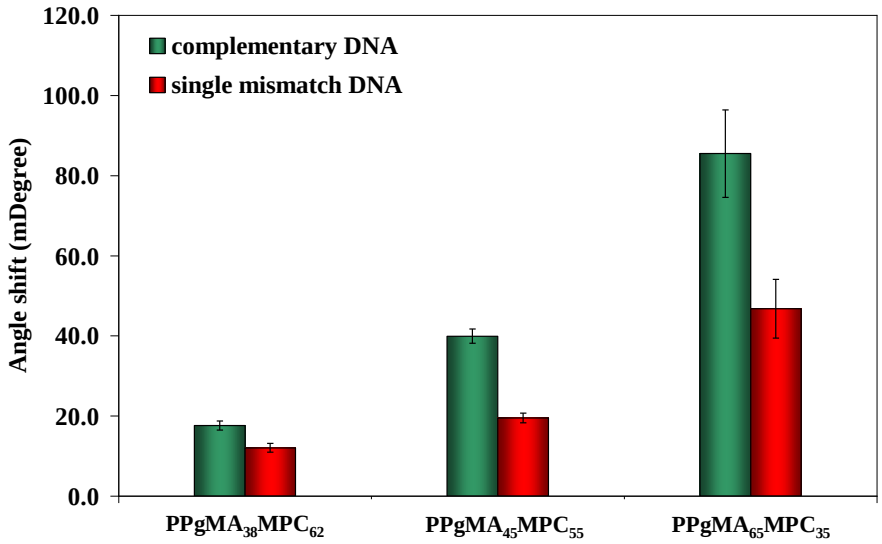


Figure 4.15 SPR angle shift corresponding to the binding of complementary DNA (5'-AAA AAA AAA-3') and single mismatch DNA (5'-AAA ATA AAA-3') on the surface modified with 0.1 mM PPgMA$_{38}$MPC$_{62}$-SH (40.0 kDa), PPgMA$_{45}$MPC$_{55}$-SH (33.1 kDa), and PPgMA$_{65}$MPC$_{35}$-SH (38.4 kDa) and immobilized with PNA.

Table 4.10 PNA probe density, hybridization efficiency of complementary DNA (%HE_{com}) and mismatch discrimination ability (%MD) of the modified gold-coated SPR chips with different copolymer composition

Sample	PNA probe density ($\times 10^3$ molecules/cm^2)	%HE_{com}	%MD
Au-PPgMA$_{38}$MPC$_{62}$-PNA	1.6 ± 0.0	20%	32%
Au-PPgMA$_{45}$MPC$_{55}$-PNA	4.9 ± 0.1	61%	52%
Au-PPgMA$_{65}$MPC$_{35}$-PNA	2.1 ± 1.1	71%	58%
Au-S-PNA [75]	4.5 ± 0.5	20%	54%
Au-biotin-PNA [76]	0.8 ± 0.1	58%	>90%

4.2.6.4 Effect of blocking agent

4.2.6.4.1 Effect of azide-terminated poly(ethylene glycol) (PEG-N_3) as blocking agent

As a result of high non-specific interactions towards single mismatch DNA on the sensor platform (Au-PPgMA$_{65}$MPC$_{35}$-PNA) in **4.2.6.3 section**, it was anticipated that the use of hydrophilic blocking agent for suppressing non-specific adsorption may improve the efficiency of the sensor. In this section, the effect of azide-terminated poly(ethylene glycol) (PEG-N_3) as blocking agent was investigated. The hybridization time, PNA and DNA concentration were kept constant at 5 min, 5 μM and 50 μM, respectively. Copolymer concentration of 0.1 mM and the addition of 100 mM NaCl were used in this experiment.

After PNA probe immobilization onto the SPR chip modified with PPgMAMPC-SH, blocking agent, PEG-N_3, was then immobilized on remaining alkyne moieties of the modified surface by Cu-catalyzed azide/alkyne cycloaddition as shown in **Figure 4.16**.

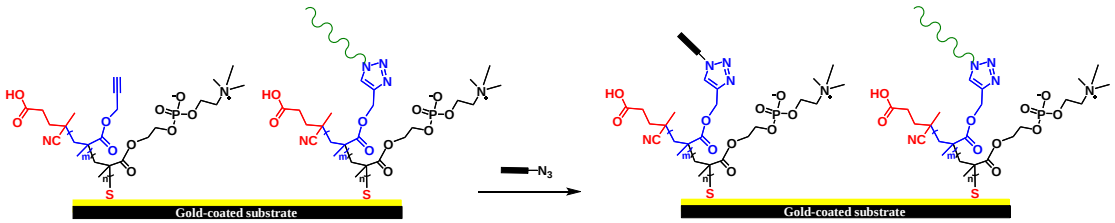


Figure 4.16 Immobilization of PEG-N_3 as blocking agent

From The data shown in **Table 4.11**, the advancing (θ_A) water contact angles of SPR chip was decreased from 74.5 ± 2.2° to 49.9 ± 1.3° indicating the success of immobilization of hydrophilic PEG-N_3. However, the SPR angle shift and %HE of the polymeric platform for complementary and single-mismatch DNA was dropped as shown in **Figure 4.17** and **Table 4.12**. These might be the reason of immobilization of high molecular weight hydrophilic PEG ($\overline{M}_n$ = 550 g/mol) on the polymeric platform-immobilized with PNA probes ($\overline{M}_n$ = 3,122.9 g/mol). The target DNA having hydrophobic backbone of polynucleotide might be suppressed from highly hydrophilic sensor platform.

Table 4.11 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization subsequent PNA attachment and blocking with PEG-N_3

Sample	Advancing water contact angle (°)
Au	71.9 ± 1.3
Au-PPgMA$_{65}$MPC$_{35}$	87.9 ± 4.2
Au-PPgMA$_{65}$MPC$_{35}$-PNA	74.5 ± 2.2
Au-PPgMA$_{65}$MPC$_{35}$-PNA-PEG	49.9 ± 1.3

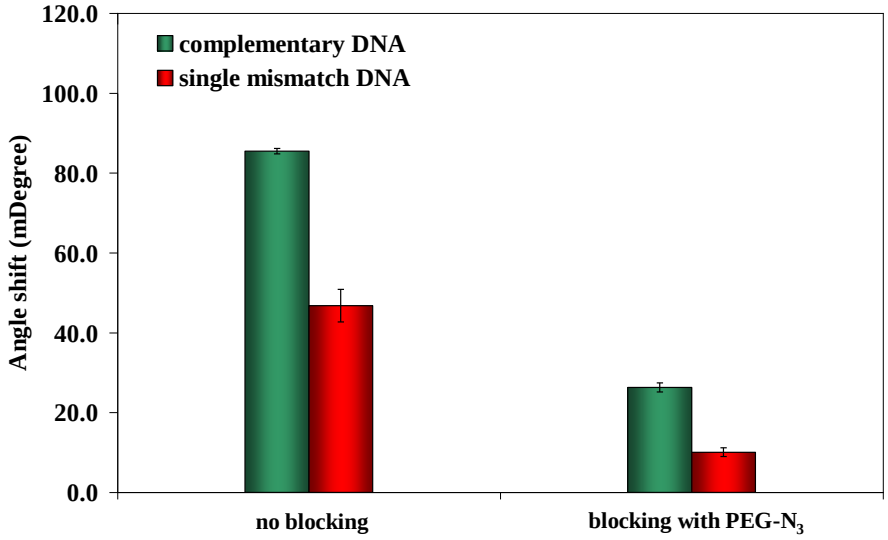


Figure 4.17 SPR angle shift corresponding to the binding of complementary DNA (5'-AAA AAA AAA-3') and single mismatch DNA (5'-AAA ATA AAA-3') on the surface modified with 0.1 mM $PPgMA_{65}MPC_{35}$ (38.4 kDa) and immobilized with PNA, no blocking and blocking with PEG-N_3

Table 4.12 PNA probe density, hybridization efficiency of complementary DNA ($\%HE_{com}$) and mismatch discrimination ability (%MD) of the modified gold-coated SPR chips blocking with PEG-N_3 after PNA probe immobilization

Sample	PNA probe density ($\times 10^3$ molecules/cm^2)	$\%HE_{com}$	%MD
Au-$PPgMA_{65}MPC_{35}$-PNA	2.1 ± 1.1	71%	58%
Au-$PPgMA_{65}MPC_{35}$-PNA-PEG	2.1 ±1.1	23%	62%

4.2.6.4.2 Effect of 2-azidoethanol and mercaptoethanol as blocking agent

In this section, low molecular weight azide-containing hydrophilic molecule, 2-azidoethanol, was used as blocking agent to react with the remaining alkyne moieties of sensor platform. Moreover, mercaptoethanol was also used as blocking substance immobilized on gold-coated surface via self-assembly formation between thiol and gold to improve the efficiency of the sensor (**Figure 4.18**). The hybridization time, PNA and DNA concentration were kept constant at 5 min, 5 μM and 50 μM, respectively. Copolymer concentration of 0.1 mM and the addition of 100 mM NaCl were used in this experiment.

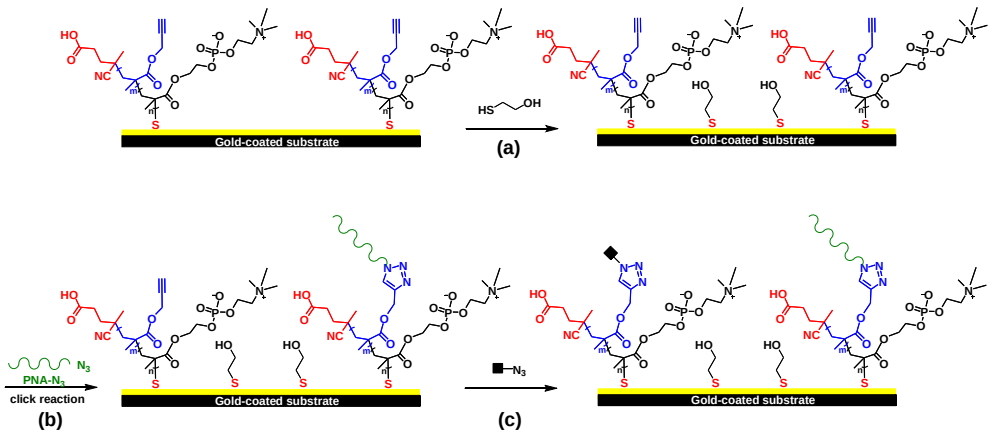


Figure 4.18 Schematic of immobilization of (a) mercaptoetanol, (b) azide-containing PNA and (c) 2-azidoethanol on the surface-tethered 0.1 mM $PPgMA_{69}MPC_{31}$ (52.9 kDa).

In the case of blocking the polymeric platform with only 2-azidoethanol or mercaptoethanol together with 2-azidoethanol, the advancing (θ_A) water contact angles of SPR chip was decreased in every step of immobilization as shown in **Table 4.13**. These results indicate the success of immobilization of PNA probes and blocking agent. The SPR signal of the polymeric platform for complementary and single-mismatched DNA was dropped as shown in **Figure 4.19**. Although %MD of Au_2-$PPgMA_{69}MPC_{31}$–(mercaptoethanol)-PNA-(2-azido ethanol) was slightly

increased in comparison with Au_1-$PPgMA_{69}MPC_{31}$-PNA and Au_1-$PPgMA_{69}MPC_{31}$-PNA-(2-azido ethanol) substrate, %HE of the sensor platform was decreased when increased the step of surface blocking **(Table 4.14).** As a result of DNA consisting of hydrophobic backbone of polynucleotide, the sensor platform having highly hydrophilic properties can not only reduce non-specific adsorption of non-targeted molecules but it also suppresses the binding of target DNA. Therefore, the using of hydrophilic blocking agent could not improve hybridization efficiency of this sensor platform.

Table 4.13 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization and subsequent PNA attachment using variation of blocking methods.

Sample	Water contact angle (°)
Bare Au	74.8 ± 3.5
Au-$PPgMA_{69}MPC_{31}$	90.4 ± 3.8
Au_1-$PPgMA_{69}MPC_{31}$-PNA	74.5 ± 2.2
Au_1-$PPgMA_{69}MPC_{31}$-PNA-(2-azido ethanol)	64.5 ± 1.8
Au_2-$PPgMA_{69}MPC_{31}$-(mercaptoethanol)	80.2 ± 2.7
Au_2-$PPgMA_{69}MPC_{31}$–(mercaptoethanol)-PNA-(2-azido ethanol)	44.5 ± 7.8

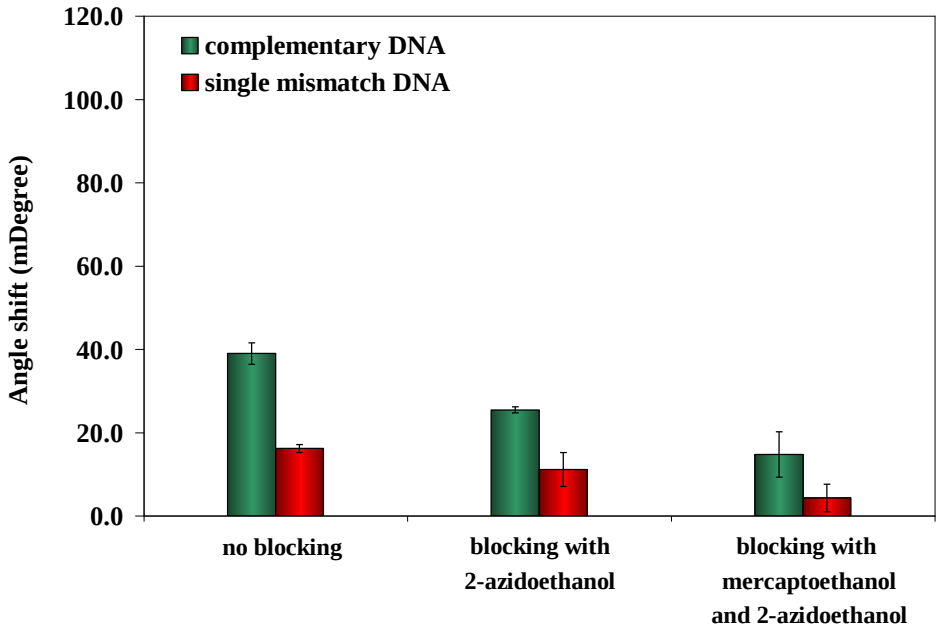
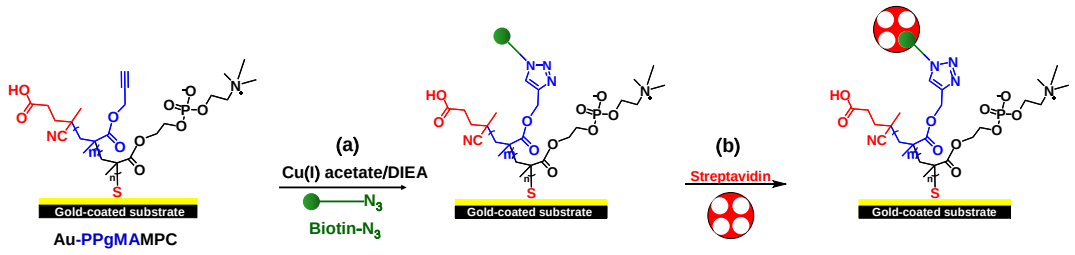


Figure 4.19 SPR angle shift corresponding to the binding of complementary DNA (5'-AAA AAA AAA-3') and single mismatch DNA (5'-AAA ATA AAA-3') on the surface-modified with 0.1 mM $PPgMA_{69}MPC_{31}$ (52.9 kDa) and immobilized with PNA using variation of blocking methods.

Table 4.14 PNA probe density, hybridization efficiency of complementary DNA ($\%HE_{com}$) and mismatch discrimination ability (%MD) of the modified gold-coated SPR chips using variation of blocking methods

Sample	PNA probe density ($\times 10^3$ molecules/cm^2)	$\%HE_{com}$	%MD
Au-$PPgMA_{69}MPC_{31}$-PNA	1.3 ± 0.1	67%	54%
Au_1-$PPgMA_{69}MPC_{31}$-PNA-(2-azido ethanol)	2.1 ± 0.1	26%	56%
Au_2-$PPgMA_{69}MPC_{31}$–(mercaptoethanol)-PNA-(2-azido ethanol)	2.6 ± 0.2	10%	65%

4.2.7 Specific interaction of the biotin probes immobilized on the gold coated SPR disks bearing PPgMAMPC brushes with streptavidin



Scheme 4.8 Schematic diagram showing (a) Immobilization of biotin probe on PPPgMAMPC-modified SPR chip (b) specific binding between streptavidin and probe

Biotin was used as the first sensing probe model due to its high binding affinity with streptavidin (dissociation constant (K_D) ≈ 10^{-4} M). [91] Azide-PEG_3-biotin were immobilized onto the SPR chip modified with PPgMAMPC-SH copolymer outside the SPR instrument by Cu-catalyzed azide/alkyne cycloaddition [57, 92] between azide-PEG_3-biotin and alkyne moieties of the PgMA units in the copolymer. As shown in **Table 4.15**, the water contact angles of the PPgMAMPC-modified SPR chip correspondingly decreased after biotin attachment indicating that the hydrophobic alkyne moieties from the PgMA repeat units were consumed and bound with biotin having 3 repeat units of hydrophilic PEG spacer. The amounts of immobilized biotin were significantly increased from 12.7 ± 6.1 to 191.2 ± 42.0 ng/cm^2 when the PgMA composition in the copolymer was increased from 38% to 45%.However, further increase in the PgMA composition to 65% yielded inferior quantity of probe binding implying that biotin may have limited accessability to alkyne moieties of the copolymer with high PgMA content of which swellability may be suppressed due to its inherent hydrophobicity. Its water contact angle (87.9 ± 4.2°) was much higher than those of the PPgMAMPC-modified SPR chip having PgMA content of 38 and 45% (44.6 ± 0.9° and 46.6 ± 3.5°, respectively).

Table 4.15 Water contact angle data and SPR angle shift of the gold-coated SPR chips upon copolymer immobilization and subsequent biotin attachment.

Sample	Water contact angle (°)	SPR	
		Angle shift (mDegree)	ng/cm^2
Au	71.9 ± 1.3	-	-
Au-PPgMA$_{38}$MPC$_{62}$	44.6 ± 0.9	1024.8 ± 81.8	854.0 ± 68.1
Au-PPgMA$_{45}$MPC$_{55}$	46.6 ± 3.5	882.1 ± 166.6	735 ± 138.9
Au-PPgMA$_{65}$MPC$_{35}$	87.9 ± 4.2	1323.1 ± 56.1	1102 ± 46.8
Au-PPgMA$_{38}$MPC$_{62}$-biotin	32.7 ± 2.7	15.2 ± 7.4	12.7 ± 6.1
Au-PPgMA$_{45}$MPC$_{55}$-biotin	41.6 ± 1.3	229.5 ± 50.4	191.2 ± 42.0
Au-PPgMA$_{65}$MPC$_{35}$-biotin	62.2 ± 1.7	209.5 ± 6.7	174.5 ± 5.6

Non-specific adsorption of biotin-immobilized PPgMAMPC copolymer platform having different copolymer composition on gold-coated SPR chip was investigated against non-targeted proteins, bovine serum albumin (BSA; 69 kDa, pI = 4.8), lysozyme (LYS; 14 kDa, pI = 12) and 0.14% blood plasma (equivalent to 0.1 mg/mL of protein in PBS buffer pH 7.4) in comparison with the targeted analyte, streptavidin (SA; 60 kDa, pI = 5). In PBS buffer solution (pH 7.4), BSA and LYS were selected as model proteins that exhibit negative and positive charge, respectively at pH 7.4 in PBS buffer solution. Blood plasma represents a complex biological-mimic media comprising about 7% (70 mg of proteins per milliliter of plasma) of different proteins, for example,fibrinogen, albumin, and globulin and other components such as water, inorganic ions, and organic compound.[93] As anticipated, the biotin-immobilized PPgMAMPC platform was specifically bound to the targeted SA (**Figure 4.20**). The bound content of SA apparently increased with the PgMA in the copolymer and biotin composition. Non-specific adsorption of the non-targeted proteins (BSA, LYZ, 0.14% blood plasma) is highly suppressed on the biotin-

immobilized PPgMAMPC platform as opposed to the bare gold surface. These results indicated that MPC units present in the PPgMAMPC copolymer are of paramount importance in suppressing non-specific protein adsorption. This evidently agree well with the work previously reported on other MPC–containing (co)polymers. [14, 21, 94, 95] Although the copolymeric platform having the highest PgMA content of 65% exhibited the greatest bound SA content, it exhibited highest non-specific adsorption of blood plasma.

In principle, the binding ratio between biotin and SA should be four if all of biotin molecules could be bound to SA. However, the biotin/SA binding ratio listed above the bar graphs in **Table 4.16** was found to be much higher than the theoretical value indicating that not all of the immobilized biotin was conjugated to the SA. The biotin/SA binding ratio of the sensing platform immobilized with $PPgMA_{45}MPC_{55}$-SH copolymer was highest when compared with the other copolymer composition. This may be explained as a result of the limited immobilization of the biotin probe to the PgMA unit that was embedded inside the inner layer of the polymer brushes as a consequence of low biotin/SA binding ratio of the sensor platform immobilized with higher composition of PgMA ($PPgMA_{65}MPC_{35}$-SH).

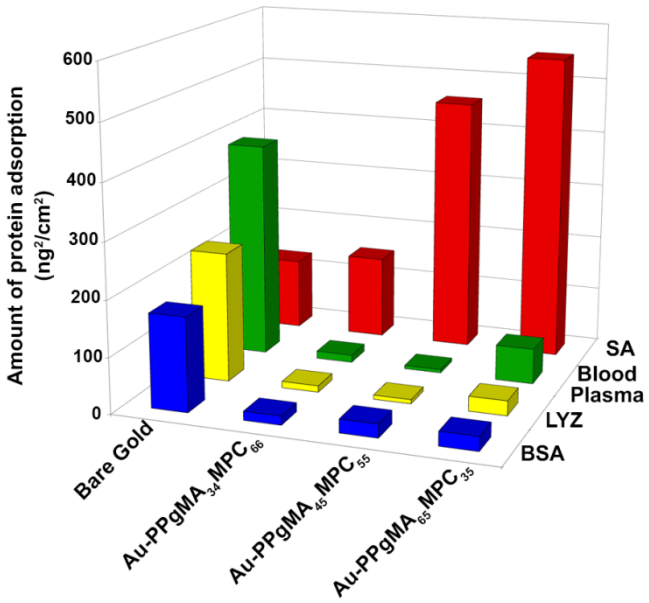


Figure 4.20 Protein adsorption on SPR chip modified with PPgMAMPC- having different copolymer composition

Table 4.16 SPR angle shift and amount of adsorption corresponding to the steps of biotin immobilization and subsequent SA (0.1 mg/mL) binding in PBS solution (10 mM, pH 7.4) on SPR chip modified with PPgMAMPC- having different copolymer composition

sample	Immobilized biotin (mDegree)	bound SA (mDegree)	immobilized biotin (pmol/cm^2)	bound SA (pmol/cm^2)	mole of [immobilized biotin]/[bound SA]
$PPgMA_{38}MPC_{62}$	15.2±7.4	179.8±14.8	56.5±27.3	2.5±0.2	23
$PPgMA_{45}MPC_{55}$	229.5±50.4	549.4±34.3	852.4±187.3	7.6±0.5	112
$PPgMA_{65}MPC_{35}$	209.5±6.7	652.0±18.0	778.1±25.0	9.2±0.2	85

The biotin-immobilized PPgMAMPC platform with different copolymer composition was further investigated to determine the lowest SA concentration detectable in complex protein sample (blood plasma). Concentrations of SA were varied in a range of 1.9 µM - 0.019 nM (equivalent to 0.1 mg/mL – 0.01 µg/mL) in 0.14% blood plasma (0.1 mg/mL). Moreover, non-specific binding with blood plasma (in the absence of SA) was also measure to evaluate the sensor efficiency in terms of the signal-to-noise (S/N) ratio as shown in **Figure 4.21**. S/N ratio can be calculated from equation 1. [9, 21] The SPR angle shift obtained from complex sample having SA is considered as signal (S) of detection, whereas that obtained from the sample without SA, target molecules, is considered as background or noise (N) of the detection. The results illustrated in **Figure 4.21** suggest that SPR chip modified with copolymer platform having 65% of PgMA composition ($PPgMA_{65}MPC_{35}$-SH) showed highest non-specific binding with blood plasma in control experiment (absence of SA in 0.14% blood plasma). This might be due to the non-specific adsorption between the remaining alkyne moieties on the copolymer platform and non-targeted protein in blood plasma. Limits of detection (LODs) of sensor platform that can discriminate between the target and non-targeted binding signal (S/N $\geq$ 2) of $PMA_{38}MPC_{62}$-SH, $PMA_{45}MPC_{55}$-SH and $PMA_{65}MPC_{35}$-SH were found to be 1.9 nM (0.1 µg/mL), 0.95 nM (0.05 µg/mL) and 0.19 µM (10 µg/mL), respectively. These results suggested that the copolymer platform based on 45% PgMA composition showed the best efficiency in detecting SA in blood

plasma solution. The detection limit of this PPgMA$_{45}$MPC$_{55}$ platform (0.95 nM equivalent to 50 ng/mL) was even lower than that (1.5 nM equivalent to 100 ng/mL) obtained from the platform based on PMAMPC, previously developed in our group [21] The fact that PPgMAMPC sensor platform can be directly immobilized with azide-containing biotin probe via click reaction without the step of functional group activation as required for PMAMPC platform make it attractive as copolymer platform for probe binding in biosensing applications.

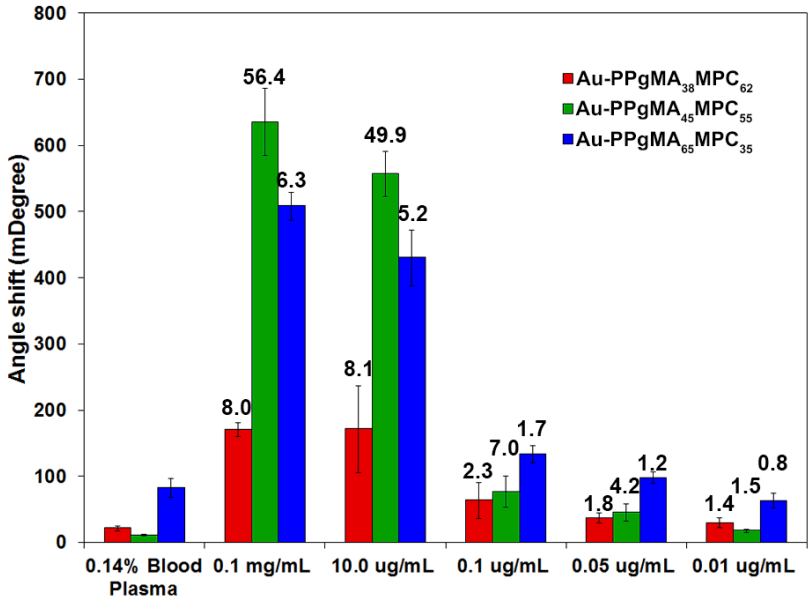
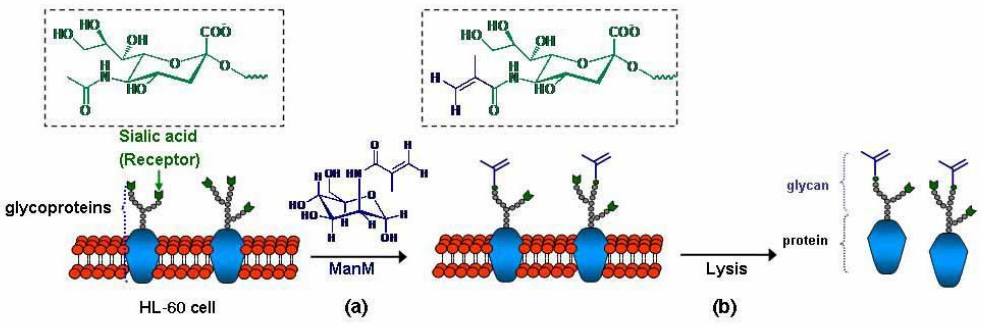


Figure 4.21 SPR angle shift corresponding to the binding of components from 0.14% blood plasma and SA having different concentrations in 0.14% blood plasma and S/N ratio of SA binding in blood plasma on sensor platform (labeled on bar chart) based on gold after immobilization with PPgMA$_{38}$MPC$_{62}$-SH, PPgMA$_{45}$MPC$_{55}$-SH, and PPgMA$_{65}$MPC$_{35}$-SH (38.4kDa).

4.3 Preparation of sensing platform between methacryloyl-functionalized glycoproteins and 2-methacryloyloxyethyl phosphorylcholine for influenza hemagglutinin detection

Influenza hemagglutinin is a glycoprotein found on the surface of the influenza viruses. This part is responsible for binding the virus to cells with sialic acid on the membranes, such as cells in the upper respiratory tract or erythrocytes resulting in an infectious illness [96, 97]. In this section, methacryloyl-functionalized glycoproteins and 2-methacryloyloxyethyl phosphorylcholine (MPC) were immobilized on gold-coated surface to prepare the sensing platform. Sialic acid on glycoprotein should serve as an active site for binding influenza hemagglutinin, whereas MPC, the hydrophilic monomeric unit enables the copolymer to suppress non-specific adsorption. The specific detection of target molecules by the developed sensing platform were determined by SPR technique to demonstrate the potential of the surface-attached copolymer for detection of target molecules in biosensing application.

4.3.1 HL-60 cell surface modification with *N*-methacryloyl mannosamine (ManM)



Scheme 4.9 Schematic of (a) HL-60 cell surface modification with ManM and (b) lysis of glycoprotein from HL-60 cells

Methacryloyl group was delivered to oligosaccharide or glycan of P-selectin glycoprotein ligand-1 (PSGL-1) on human promyelocytic leukemia (HL-60) cells by biosynthesis of sialic acid which is the terminal residues of oligosaccharide covering the mammalian cell surface. The biosynthesis process begins with the synthesis of uridine diphosphate (UPD)-*N*-actyl glucosamine (UPD-GlcNAc) in cytoplasm, then UPD-GlcNAc is catalyzed by UPD-GlcNAc 2-epimerase to produce *N*-acetyl manosamine (ManNAc). ManNAc derivatives are processed in the sialic acid biosynthesis and the non-natural functional groups are transferred to the terminus of oligosaccharide by sialyltransferase [98, 99].

The preparation of methacryloyl-functionalized glycoproteins started from treating HL-60 cells (3.0×10^5 cells/mL) with ManM, M (+), at a final concentration of 5 mM for 3 days. After 3 days, the cells were washed with PBS (-), to remove the remaining ManM. Then, the cells were disrupted by ultrasonication and centrifuge to separate glycoprotein. RIPA lysis buffer was used to dissolve mixture of glycoprotein (cells lysate) from the precipitated cells. The concentration of glycoproteins was measured by using BCA assay kit.

From phase contrast micrographs shown in **Figure 4.22**, cell viability and proliferation of HL-60 cells treating with ManM were not measurably different fromthe cells treated with PBS(-), M(-), as control experiments. This result indicates that the using of 5 mM ManM in this experiment did not have any impact on behavior of HL-60 cells. Concentrations of glycoprotein M(+) and M(-) were measured byBCA assay [100]. BCA assay consists of two steps of reactions. First, Cu^{2+} ions (copper(II) sulfate) in the assay are reduced to Cu^{+} ions by peptide bonds in protein structure. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution and then two molecules of bicinchoninic acid chelate with each Cu^{+} ion, yielding a purple-colored solution that can strongly absorb light at 570 nm. The concentration of glycoprotein M(+) and M(-) were found to be 682 μg/mL and 839 μg/mL, respectively.

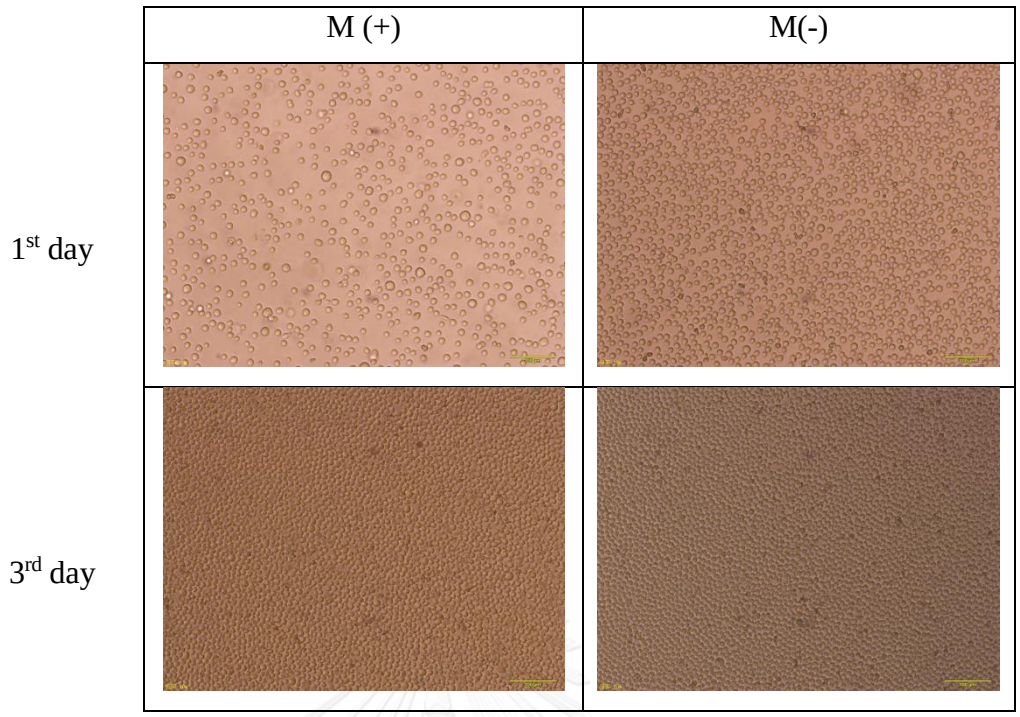
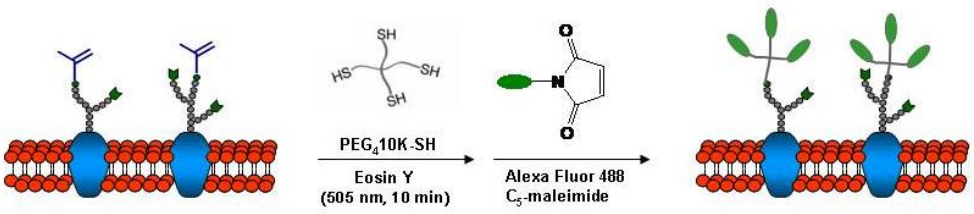


Figure 4.22 Phase contrast micrographs of HL-60 cells after treating with ManM (M+) and PBS(-) (M-) for 3 days

4.3.2 Modification of ManM-treated HL-60 cell surfaces with thiol-terminated 4-arm poly(ethylene glycol) ($PEG_4$10K-SH)



Scheme 4.10 ManM-treated HL-60 cell surface immobilized with thiol-terminated 4- $PEG_4$10K-SH and Alexa Fluor 488

The immobilized methacryloyl group on HL-60 cell surface was confirmed by thiol-ene reaction. After treating cells with 5 mM ManM for 3 days, thiol-terminated 4-arm poly(ethylene glycol) ($PEG_4$10K-SH) was immobilized to methacryloyl groups on

the cell surface by exposure to the visible light at 505 nm for 15 min in culture media containing ManM-treated HL-60 cells, $PEG_4$10K-SH and eosin Y (catalyst). Then, the remaining thiol groups of $PEG_4$10K-SH on the cells surfaces can react with Alexa Fluor 488 C_5-maleimide via thiol-maleimide click chemistry. As expected, fluorescence signal should only be detected on ManM-treated HL-60 cells, whereas the non-treated cells should not give signal. From **Figure 4.23 (a,b),** the results from confocol laser scaning microscope were similar to the results from flow cytometry technique. Fluorescence intensity of ManM-treated HL-60 cells was higher than non-treated HL-60 cells due to the interaction between $PEG_4$10K-SH and fluorescence probe. However, non-treated HL-60 cells showed lower fluorescence intensity because the non-specific interaction between cell membrane and Alexa Fluor 488 C_5-maleimide. Fluorescence signal was not observed in native HL-60 cells. These results indicate the success of methacryloyl group immobilization on HL-60 cells surfaces.

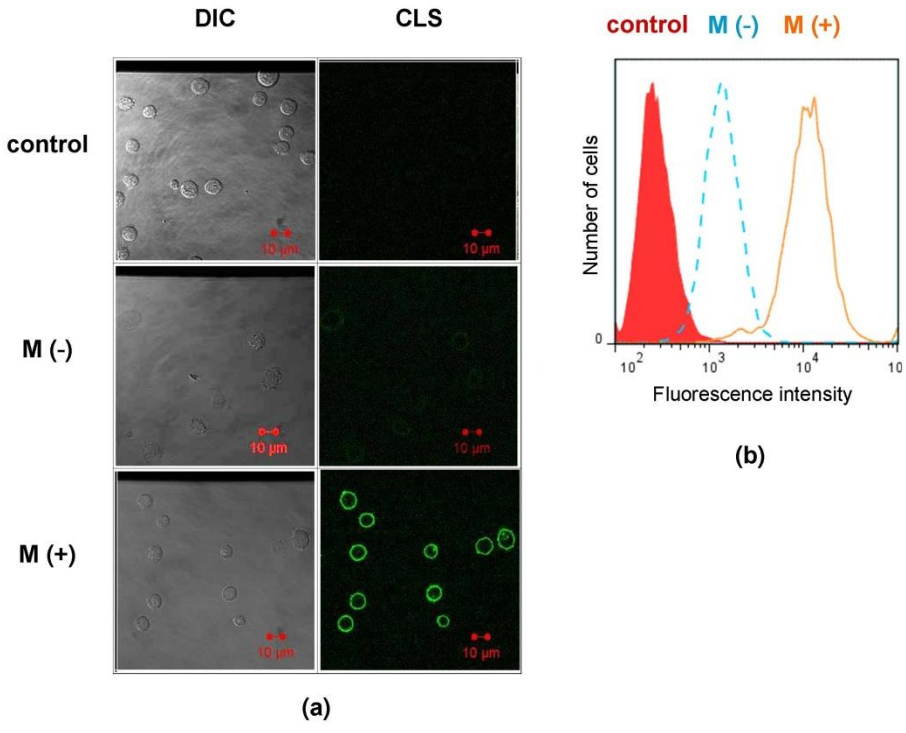
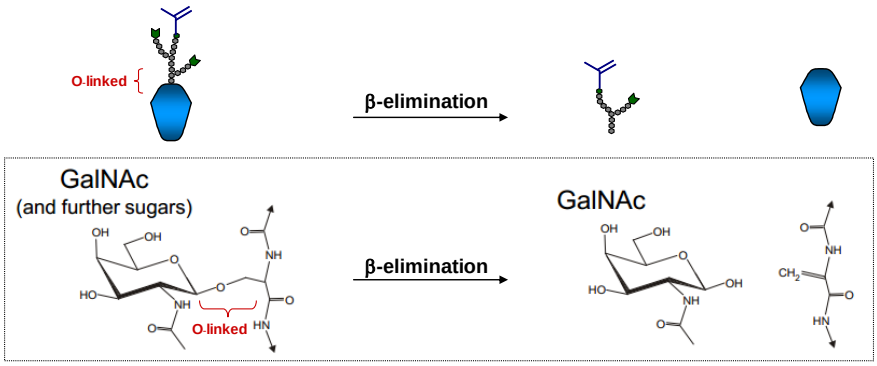


Figure 4.23 (a) Differential interference contrast micrograph (DIC) and confocol laser scanning micrograph of ManM-treated HL-60 cells (M(+)), non-treated HL-60 cells (M(-)) and native HL-60 cells (control experiment), (b) Fluorescence intensity determined by flow cytometry.

4.3.3 Separation of glycans from glycoproteins



Scheme 4.11 Separation of glycans from glycoprotein by using Glycoprofile β-elimination kit.

In this experiment, sialic acid, terminal residues of oligosaccharide, on methacryloyl-functionalized PSGL-1 glycoproteins was used as an active site of the sensor platform for detecting influenza hemagglutinin. However,non-specific adsorption withprotein residues of glycoproteins may be possible. Therefore, oligosaccharide residues or glycans should be separated from glycoproteins structure by using Glycoprofile β-elimination kit. By using this kit, the *O*-glycan linkage between glycans and proteins was hydrolyzed by dilute alkaline solution under mild condition. Then, glycans were separated from the mixture by centrifugation in centrifugal filter unit. The success of glycans separation from glycoprotein was confirmed by using BCA assay kit to analyze concentration of protein in glycans portion. From **Table 4.17**, concentration of protein was not detected in glycans portion of M(+) and M(-) suggesting the success of glycans separation.

Concentration of glycans was analyzed by total carbohydrate calorimetric assay kit. The assay relies on the phenol-sulfuric acid method. The oligosaccharides were hydrolyzed to monomers and converted to furfural by mixing with concentrated H_2SO_4 Then, furfural reacted with the developer to form a chromogen that can be quantified by measuring the absorbance at 490 nm. Concentrations of glycans M(+) and glycans M(-) were found to be 239 and 192 μg/mL, respectively.

Table 4.17 Concentration of protein in glycan and protein portion of M(+) and M(-) sample after separation with Glycoprofile β-elimination kit

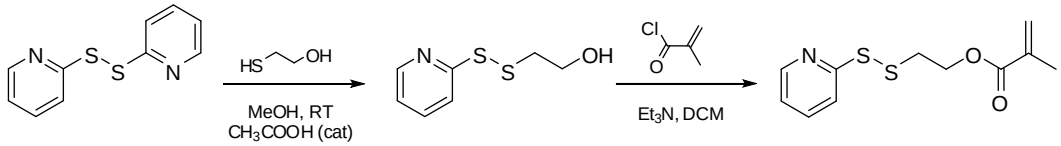
Protein concentration (μg/mL)	M(+)	M(-)
Glycans portion	0	0
Protein portion	165	145

4.3.4 Immobilization of ManM-treated glycoprotein and MPC on gold-coated surface and detection of target molecules by SPR technique

In this section, ManM-treated glycoprotein and MPC were immobilized on gold-coated surface by two different methods. The first method is based on polymerization of ManM-treated glycoprotein with MPC on gold-coated surface modified with pyridyldisulfide ethylmethacrylate (PDSM) and 2-(acetylthio)ethylmethacrytale (AcSEMA). The second method is immobilization of ManM-treated glycoprotein with MPC monomer on gold-coated surface via thiol-ene chemistry.

4.3.4.1 Preparation of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on gold-coated sustrate modified with pyridyldisulfide ethylmethacrylate (PDSM)

4.3.4.1.1 Synthesis of pyridyldisulfide ethylmethacrylate (PDSM)



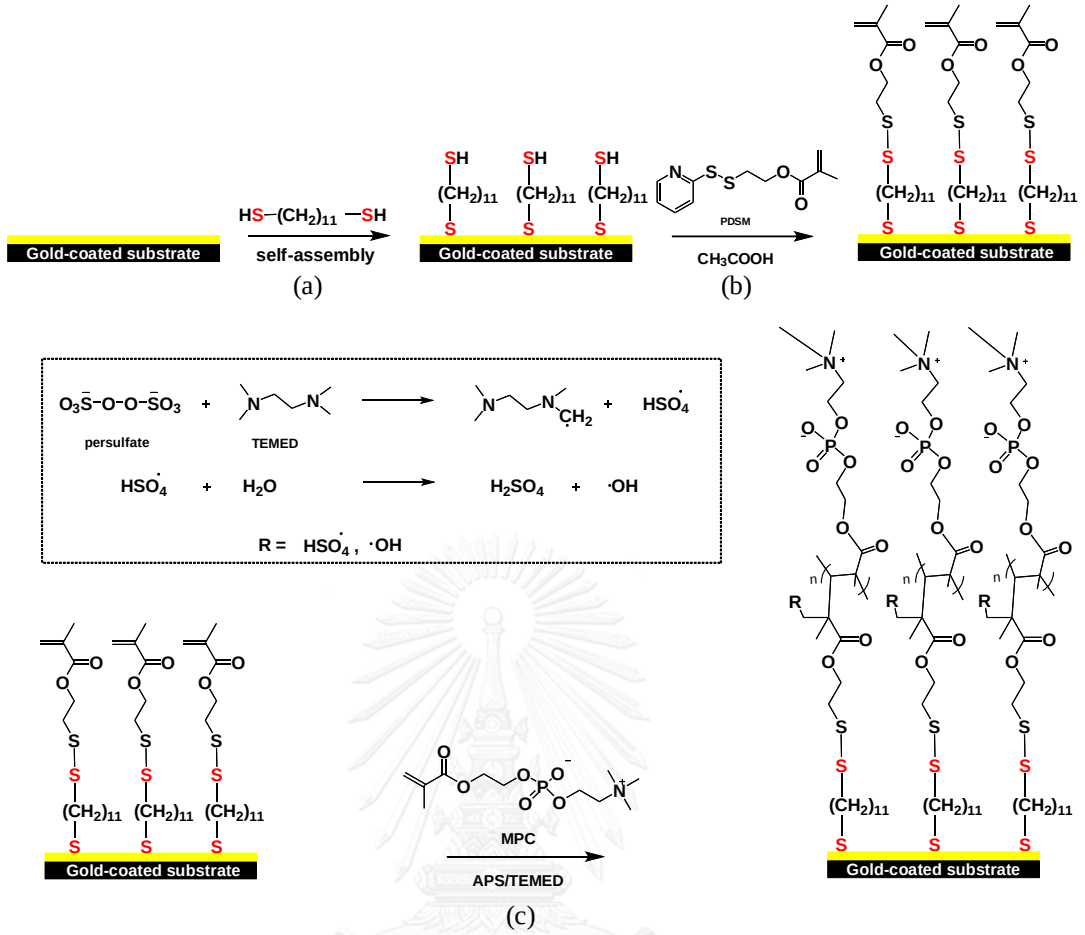
Scheme 4.12 Synthesis of PDSM

Pyridyldisulfide ethylmethacrylate (PDSM) monomer containing reactive pyridyl disulfide groups was synthesized by 2-step reaction **(Scheme 4.12)** following the procedure reported in literature [101]. Firstly, hydroxyethylpyridyl disulfide

(HPDS) was synthesized by the disulfide exchange reaction between aldrithiol-2 and mercaptoethanol (yield = 42%). Secondly, PDSM was reacted with methacryloyl by nucleophilic substitution. The product was obtained in 77% yield as colorless oil. The structure of PDSM monomer was confirmed by ^{1}H NMR analysis. The spectra are shown in **Figure A-7 and A-8 (Appendix A)**.

4.3.4.1.2 Preparation of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on gold-coated sustrate modified with pyridyldisulfide ethylmethacrylate (PDSM)

To optimize the condition for immobilization of ManM-treated glycoprotein and MPC on gold-coated surface via redox polymerization, PMPC was firstly immobilized on the substrate. Gold-coated substrate was firstly cleaned by oxygen plasma for 2 min to remove an organic layer, and then immobilized with UDDT to introduce thiol-terminated functional groups on the surface. Water contact angle data from **Figure 4.24** shows the decreasing of contact angle after cleaning with oxygen plasma and the increasing of contact angle was observed after immobilization with UDDT. This might be the reason of the attachment of dithiol molecules having long chain hydrocarbon on the surface. From the results, there was no significant change of contact angle after HPDS immobilization on the surface. However, the contact angle was decrease*n* from 89.63°/62.07° to 56.36°/25.42° (Δ =33.27°/36.65°) after polymerization of PMPC on the modified surface. These results implied that PMPC were successfully immobilized on the substrate via redox radical polymerization. As a result, the wettability of the surface was decreased after immobilization of hydrophilic polymer. Although PMPC was successfully immobilized on PDSM-modified surface, this substrate is not appropriate for using in biosensing applications. Pyridyldisulfide functional groups are reversible disulfide conjugate which make them reactive with thiol containing biorelated molecules such as protein in complex sample. Therefore, the polymer layer can be removed when using this substrate as sensor platform for detecting biomolecules.



Scheme 4.13 Preparation of PMPC on gold-coated substrate: (a) immobilization of undecanedithiol (UDDT) on gold-coated surface, (b) immobilization PDSM on thiol-terminated surface and (c) preparation of PMPC by redox-initiated free radical polymerization

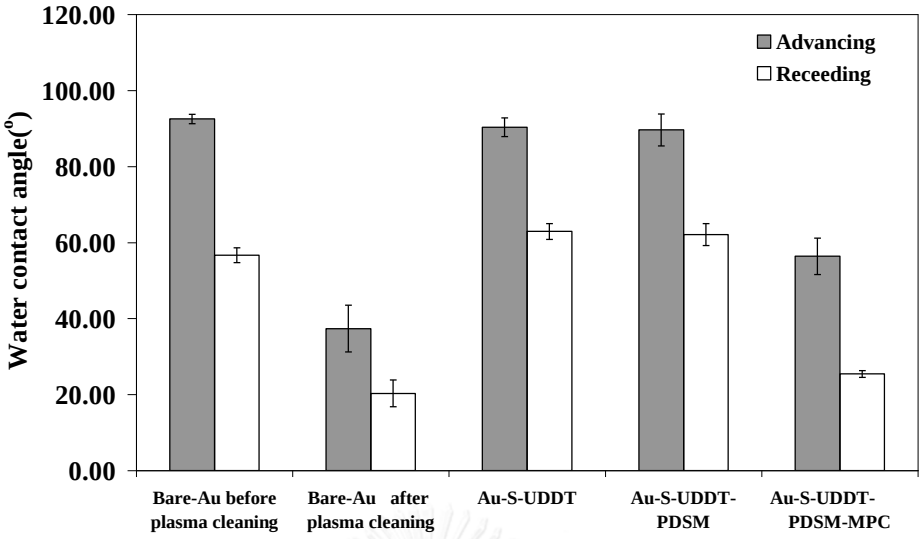
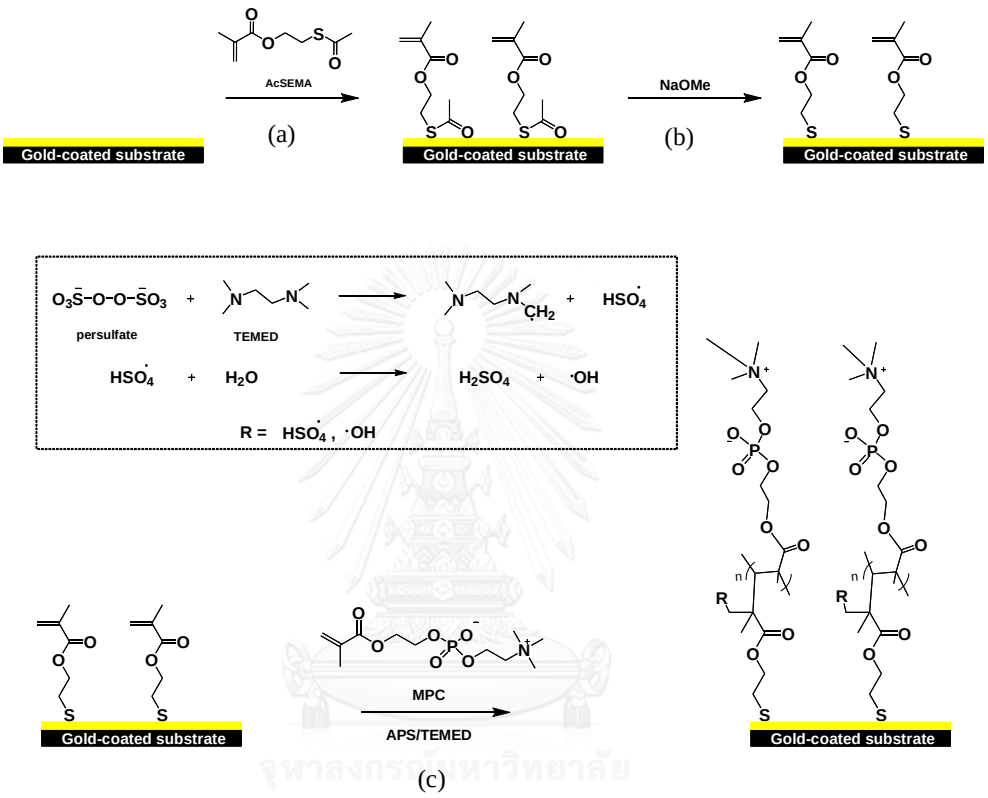


Figure 4.24 Water contact angle (θ_A/θ_R) of gold-coated substrate after immobilization of undecanedithiol (UDDT), PDSM and preparation of PMPC by redox-initiated free radical polymerization

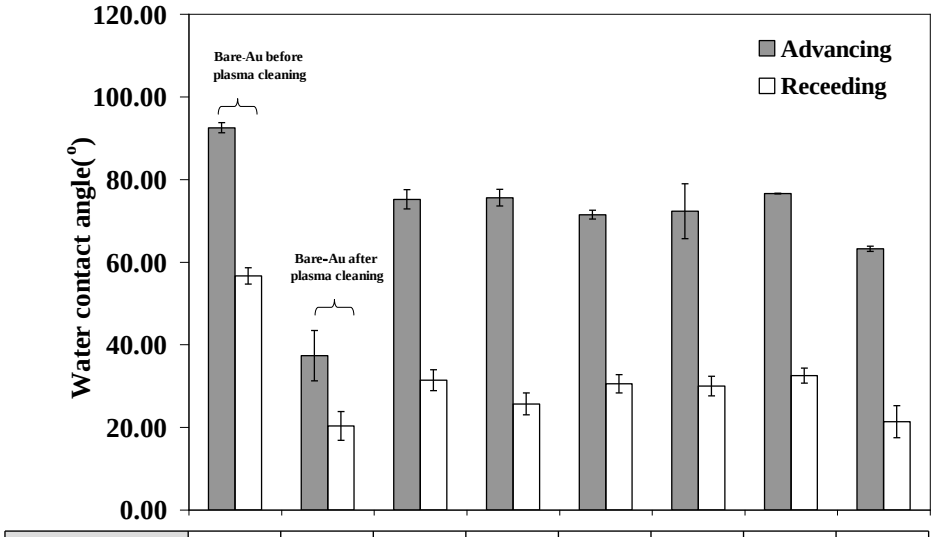
4.3.4.2 Preparation of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) on gold-coated sustrate modified with 2-(acetylthio)ethylmethacrytale (AcSEMA)

In this experiment, AcSEMA monomer containing thioester functional groups was attatched on gold-coated surface and then thioester grouop was hydrolyzed by sodium methoxide (NaOMe) to introduce thiol group as strong binding site on gold surface. PMPC polymer was immobilized on the modified surface by redox polymerization as shown in **Scheme 4.14**. Concentrations of AcSEMA and time for immobilization were varied as shown in **Figure 4.25**. Water contact angles of the substrate (θ_A/θ_R) were increased after immobilization of AcSEMA in comparison with bare-Au after plasma cleaning. Contact angle of the substrate immobilized with 1 and 10 mM AcSEMA for 24 and 48 h, respectively were not different. Therefore, immobilization of 1.0 mM AcSEMA for 24 h was selected as the optimal condition for surface modification. After treating the modified substrate with NaOMe, hydrolysis, at 24 h, water contact angle was the same as before hydrolysis. The wettability of the

surface was slightly decreased after polymerization of PMPC on the AcSEMA-modified surface. This result indicates that AcSEMA-immobilized gold-coated substrate was not appropriate to immobilize PMPC via redox polymerization. It might be the reason of low stability of AcSEMA on the gold-coated surface.



Scheme 4.14 Preparation of PMPC on gold-coated substrate: (a) immobilization of AcSEMA on gold-coated substrate, (b) hydrolysis by sodium methoxide (NaOMe) and (c) preparation of PMPC by redox-initiated free radical polymerization



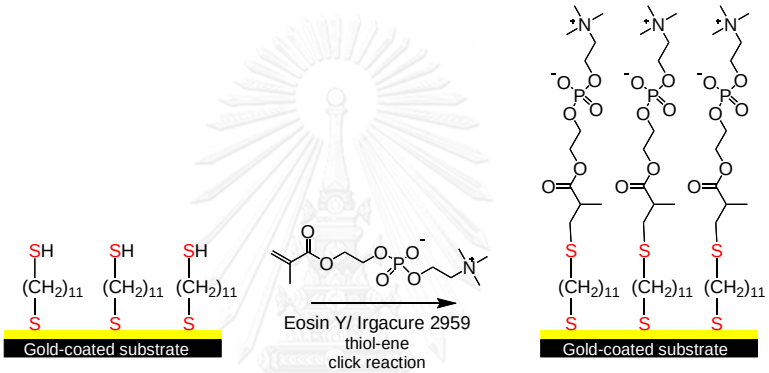
AcSEMA (a)	-	-	1 mM 24 h	10 mM 24 h	1 mM 48h	10 mM 48 h	1 mM 24 h	1 mM 24 h
NaOMe (b)	-	-	-	-	-	-	24 h	24 h
Redox polymerization (PMPC) (c)	-	-	-	-	-	-	-	18 h

Figure 4.25 Water contact angle (θA/θR) of gold-coated substrate and surface-modified: (a) AcSEMA, (b) NaOME and (c) PMPC with different concentration and reaction time

4.3.4.3 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) on modified gold-coated surface via thiol-ene click reaction

From **Figure 4.26**, to optimize the condition of surface modification, only MPC monomer was immobilized on gold-coated surface via thiol-ene click reaction with the interaction between methacryloyl group of MPC and thiol-modified gold-coated (Au-UDDT) substrate by using eosin Y as photoinitiator. Irradiation time was varied from 30-60 min, while the concentration of MPC monomer was fixed at 50 mM in 10% RIPA lysis buffer. The reaction containing only eosin Y was measured as control experiment. After irradiation for 30 and 60 min, water contact angle (θ_A/θ_R) was not different from the control experiment and Au-UDDT substrate. This result implies that MPC monomers could not be effectively immobilized on gold-coated substrate via thiol-ene click reaction by using eosin Y as photoinitiator. Therefore, Irgacure 2959 was used as photoinitiator instead of eosin Y in this reaction. As shown in **Table 4.18**,

irradiation time, concentration and volume of MPC solution using in the reaction were varied to optimize the condition for thiol-ene reaction of MPC on the substrate. MPC could be immobilized on thiol-modified surface via thiol-ene reaction by using Irgacure 2959 as photoinitiator because water contact angle (θ_A/θ_R) of the modified gold-coated substrate was dramatically decreased at 15 min of irradiation when treating with 500 µL of MPC solution. From the results, 300 µL of 2.5 M MPC was chosen as the optimal condition for thiol-ene reaction because the using of low-volume of total solution can reduce the amount of glycoprotein using in next experiment.



Scheme 4.15 Immobilization of MPC on modified gold-coated surface via thiol-ene click reaction by using eosin Y or Irgacure 2959 as photoinitiator

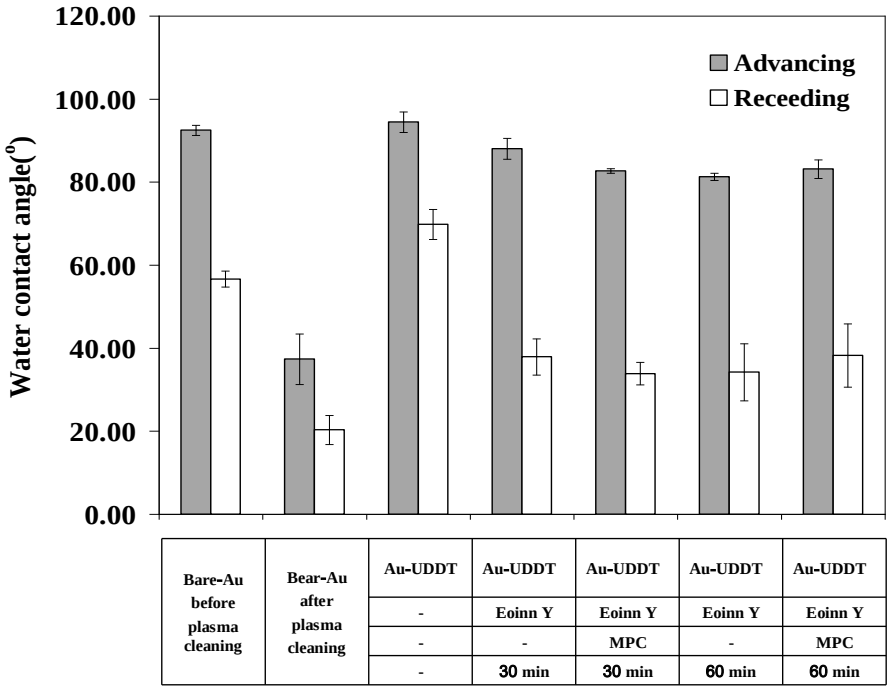


Figure 4.26 Water contact angle (θ_A/θ_R) of gold-coated substrate modified with 50 mM MPC by using eosin Y as photoinitiator

Table 4.18 Water contact angle (θ_A/θ_R) of gold-coated substrate modified with MPC by varying irradiation time, MPC concentration and volume of solution using Irgacure 2959 as photoinitiator

time (min)	MPC concentraton (M)	volume (µL)	WCA (°)	
			θ_A	θ_R
15	1.0	60	83.88	54.31
		300	81.71	43.00
		500	18.48	14.06
	2.5	60	81.09	47.82
		300	28.50	13.28
		500	23.03	17.66
30	1.0	500	48.79	18.26
60	1.0	500	60.61	21.58

4.3.4.4 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ManM-treated-glycoprotein on modified gold-coated surface via thiol-ene click reaction and determination of PSGL-1 antibody (N-16) binding to PSGL-1 glycoprotein on the modified surface by SPR

As shown in **Table 4.19**, water contact angle (θ_A/θ_R) of modified SPR chip with 2.5 M MPC and 1000 μg/mL ManM-treated glycoprotein M(+) amd M(-) were 37.16°/22.03° and 32.59°/14.67° respectively. This result suggests the success of surface immobilization. In this experiment, antibody PSGL-1 was used as model of target molecules binding with ManM-treated PSGL-1 glycoprotein M(+) on the sensor platform. 0.05% P20 in PBS(-) was used as running buffer in this experiment. From SPR sensorgram shown in in **Figure 4.27**, there was no changing of SPR response unit (RU) of surface immobilized ManM-treated glycoprotein M(-) and ManM-treated glycoprotein M(+). These results indicate that PSGL-1 antibody was suppressed from both modified substrate implying that only MPC was immobilized on the substrate. This is because MPC (Molecular weight = 295.27 Da) shows higher reactivity to immobilize on the thiol-terminated surface than ManM-treated glycoprotein M(+) (Molecular weight > 120 kDa) [31]. For next experiment, concentration of MPC monomer was decreased to 0.2 M for increasing a chance of surface binding between methacryloyl-functionalized glycoproteins M(+) and thiol-modified surface.

Table 4.19 Water contact angle (θ_A/θ_R) of gold-coated substrate immobilized with different MPC concentration and 1000 μg/mL ManM-treated glycoprotein M(+), M(-) for 15 min by using Irgacure 2959 as photoinitiator

sample	MPC concentration (M)	glycoprotein (μg/mL)	volume (μL)	WCA (°)	
				θ_A	θ_R
Au-UDDT	-	-	-	87.26	62.83
Au-UDDT-MPC-glycoprotein (M+)	2.5	1000	300	37.16	22.03
				32.59	14.67
Au-UDDT-MPC-glycoprotein M(-)	0.2		500	64.87	22.44
				49.30	31.02

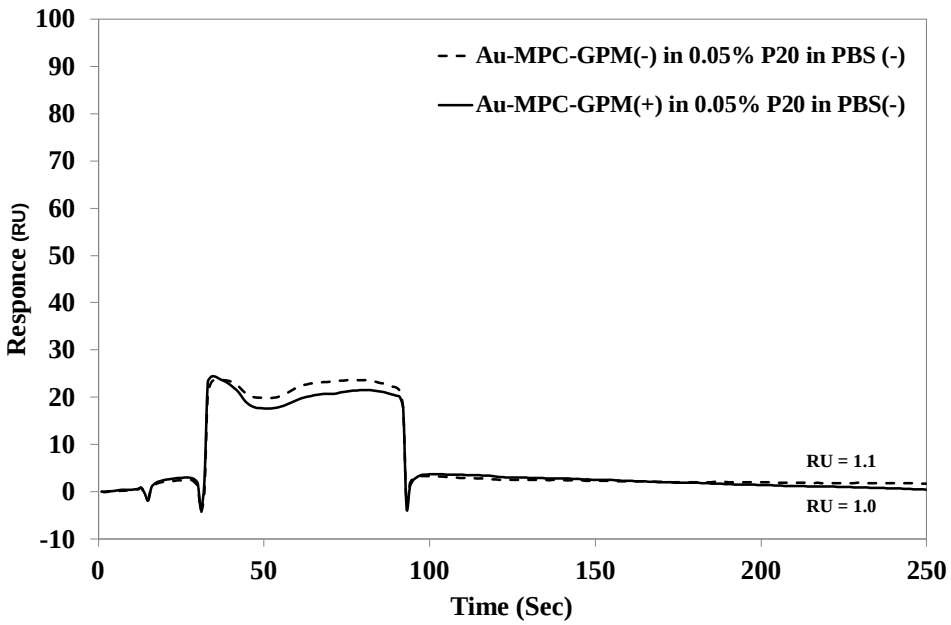


Figure 4.27 SPR sensorgrams of the gold-coated SPR chip bearing MPC and ManM-treated glycoprotein detecting 10 μg/mL PSGL-1 antibody as target molecules

The results from **Table 4.19**, water contact angles (θ_A/θ_R) of modified SPR chip with 0.2 M MPC and 1000 μg/mL ManM-treated glycoprotein M(+) amd M(-) were 64.87°/22.44° and 49.30°/31.02° respectively. This result suggests the immobilization of MPC and ManM-treated glycoprotein on the surface. For surface modification, 500

μL of 0.2 M MPC in 10% RIPA lysis buffer, 1000 μg/mL glycoprotein and Irgacure 2959 was used in this study. The specific binding between substrate modified ManM-treated PSGL-1 glycoprotein M(+) and antibody PSGL-1 was studied by SPR technique. From **Figure 4.28**, PSGL-1 antibody can be bound on the surface modified ManM-treated glycoprotein M(-) (74.1 RU) more than on ManM-treated glycoprotein M(+) (24.6 RU). These results implied that ManM-treated glycoprotein M(+) might not be immobilized on the surface via the interaction between thiol and gold but it may attach by non-specific interaction resulting in non-specific adsorption of the antibody on the surface. This might be the reason of the decreasing of methacryloyl group on MamM-treated glycoprotein from the effect of ultrasonication in glycoprotein separation step. Therefore, the amount of ManM-treated glycoprotein M(+) immobilized on the modified surface was low. To protect methacryloyl group of the glycoprotein, direct immobilization of ManM-treated HL-60 cells on thiol-modified surface was studied in next experiment.

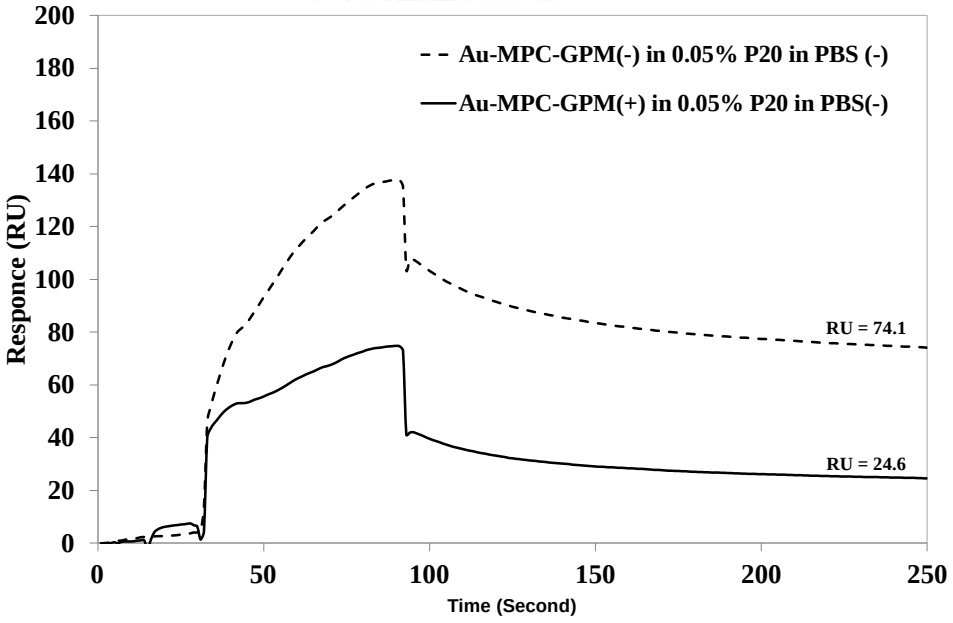


Figure 4.28 SPR sensorgrams of the gold-coated SPR chip bearing MPC and ManM-treated glycoprotein detecting 10 μg/mL PSGL-1 antibody as target molecules.

4.3.4.5 Immobilization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ManM-treated-HL-60 cells on modified gold-coated surface via thiol-ene click reaction

After 3 days of treating HL-60 cell with ManM, the cells were washed three times with PBS(-) to remove the remaining ManM and then ManM-treated HL-60 cells M(+) or M(-) were directly immobilized on thiol-modified gold-coated via thiol-ene reaction substrate to protect methacryloyl groups from the effect of sonication in the glycoprotein separation step.

As shown in **Figure 4.29**, water contact angle (θ_A/θ_R) of the substrate immobilized with ManM-treated HL-60 cells (M+) and M(-) was decreased to 17.86°/14.68° and 22.20°/19.21°, respectively, when compared to Au-UDDT substrate. The specific binding between modified substrate and antibody PSGL-1 was studied by SPR technique. In **Figure 4.30**, amount of detected PSGL-1 antibody on ManM-treated HL-60 cells (M+) (5.6 RU) was insignificantly higher than on ManM-treated HL-60 cells (M-) (2.2 RU). It might be the reason of the high reactivity of low molecular weight MPC that can attach on the surface more than ManM-treated HL-60. For this reason, the direct immobilization of MPC and ManM-treated HL-60/ManM-treat glycoprotein on the substrate via thiol-ene reaction is not suitable to prepare the sensor platform for binding with target molecules.

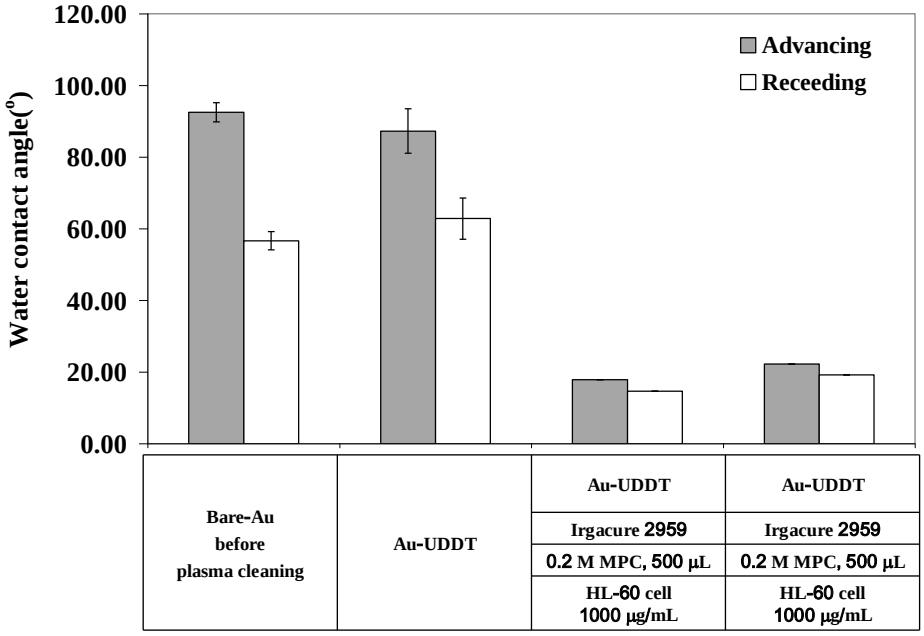


Figure 4.29 Water contact angle (θ_A/θ_R) of gold-coated substrate immobilized with 0.2 M MPC and ManM-treated HL-60 M(+), M(-) for 15 min by using Irgacure 2959 as photoinitiator

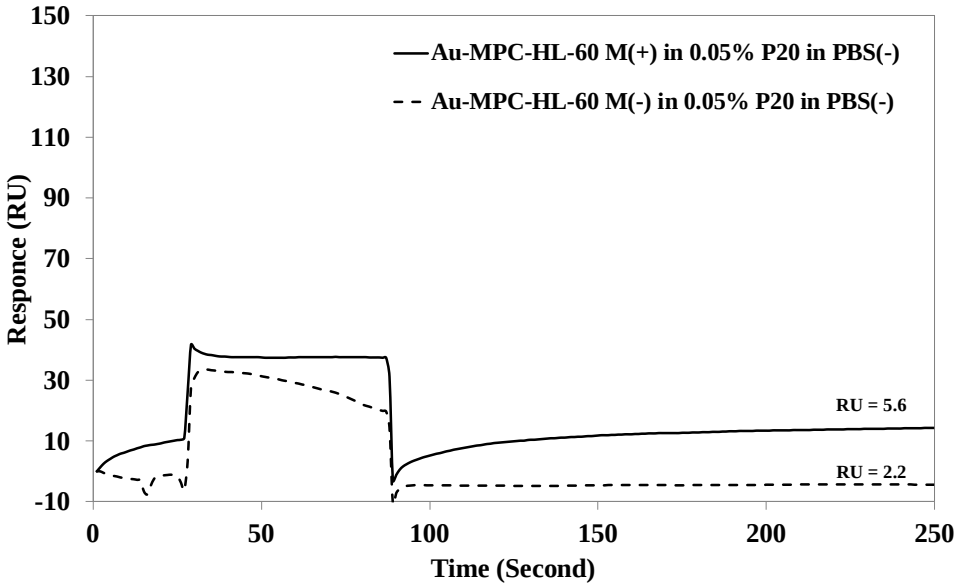


Figure 4.30 SPR sensorgrams of the gold-coated SPR chip bearing MPC and ManM-treated glycoprotein detecting 10 µg/mL PSGL-1 antibody as target molecules

CHAPTER V

CONCLUSIONS

Functional copolymer brushes of PNMSMPC and PPgMAMPC and their corresponding thiol-terminated derivatives, PNMSMPC-SH and PPgMAMPC-SH with well-controlled molecular weight and copolymer composition were successfully synthesized via RAFT polymerization as confirmed by ^{1}H NMR and FT-IR analysis. It was found that PNMSMPC was not a suitable polymeric platform for probe binding. Their succimidyl groups are hydrolytically unstable. Besides, they were partially removed during aminolysis by hydrazine, the necessary step used for dithioester conversion to thiol.

The success of PPgMAMPC-SH grafting on gold-coated SPR chip via "grafting to" approach was verified by ATR-IR, XPS, and water contact angle measurements. Alkyne moieties of PgMA units in the copolymers were capable of binding with azide-containing molecules via click reaction based on Cu-catalyzed azide/alkyne cycloaddition for biotin-immobilized platform, specific detection of streptavidin (SA) in complex sample, 0.14% blood plasma, was determined by SPR technique. The platform based on Au-PPgMA$_{45}$MPC$_{55}$ exhibited the best performance in terms of biotin immobilization efficiency, subsequent SA detection as well as the ability to suppress non-specific adsorption of non-target analytes and yielded high S/N ratio. In addition, the detection limit for detecting SA in blood plasma solution was found to be 0.95 nM (equivalent to 0.05 µg/mL) which was lower than the platform based on poly[(methacrylic acid)-*ran*-(2-methacryloyloxyethyl phosphorylcholine)] (PMAMPC) developed by Akkahat *et al*. [21] (detection limited for detecting avidin in blood plasma was 1.5 nM (0.10 µg/mL)). To explore the potential of the surface-attached PPgMAMPC as sensing layer in DNA biosensing application, azide-containing PNA probe was also immobilized on the platform. The sensor platform based on Au-PPgMA$_{65}$MPC$_{35}$-PNA could detect the highest amount of complementary DNA (85.5 ± 10.9 mDegree) with %HE of 71% which was higher than the sensor platform developed in our research group based on thiolated

pyrrolidinyl peptide nucleic acids (PNA-SH)[75] (%HE = 20%) and biotinylated PNA immobilized on SPR sensor chips via biotin–streptavidin (%HE = 58%).[76] These results suggested that the substrate immobilized with PPgMAMPC brushes could be a new potential copolymer platform for azide-containing probe immobilization for detecting target molecules in biosensing applications.

To prepare the sensor platform from methacryloyl-functionalized glycoproteins and MPC, methacryloyl-functionalized glycoprotein (ManM-treated glycoprotein) was prepared by biosynthetic pathway in human promyelocytic leukemia (HL-60) cells and used as monomer for surface immobilization via redox polymerization or direct immobilization by thiol-ene click reaction. The success of ManM delivery to the surface of HL-60 cells was confirmed by click reaction with fluorescent dye on cell surface. The polymeric platform, PMPC, was not successfully prepared from redox polymerization on gold-coated surface modified with pyridyldisulfide ethylmethacrylate (PDSM) and 2-(acetylthio)ethylmethacrytale (AcSEMA) as determined by water contact angle measurement. In contrast, the immobilization of MPC monomer on thiol-terminated gold-coated surface via thiol-ene click chemistry was successfully prepared by using Irgacure 2959 as photoinitiator at 365 nm. It was found that the optimize condition for immobilization of MPC and ManM-treated glycoprotein was 0.2 mM MPC in 10% RIPA lysis buffer, irradiation time of 15 min and 500 µL total volume of solution, respectively. In addition, 1000 µg/mL of ManM-treated glycoprotein was used for immobilization with MPC on thiol-modified substrate. Specific binding between PSGL-1 glycoprotein and antibody PSGL-1 was determined as model system. The result suggests that thiol-ene click chemistrty was not suitable for immobilization of ManM-treated glycoprotein and MPC on the substrate because the reactivity of MPC immobilization is higher than ManM-treated glycoprotein.

To improve the immobilization of ManM-treated glycoproteins on gold-coated SPR chip, future work will focus on the immobilization via copolymer of PMPC containing thiol groups. In the first step, PMPC containing thiol groups will be immobilized on surface via grafting to approach. ManM-treated glycoproteins will be later immobilized to thiol groups in PMPC copolymer via thiol-ene reaction. It is

anticipated that this method will provide high binding of ManM-treated glycoproteins on gold-coated SPR surface.

REFERENCES

1. Nakamura, F.; Ito, E.; Hayashi, T.; Hara, M., Fabrication of COOH-terminated self-assembled monolayers for DNA sensors. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2006,** *284-285*, 495-498.
2. Senaratne, W.; Andruzzi, L.; Ober, C. K., Self-assembled monolayers and polymer brushes in biotechnology: Current applications and future perspectives. *Biomacromolecules* **2005,** *6* (5), 2427-48.
3. Su, X. L.; Li, Y., A self-assembled monolayer-based piezoelectric immunosensor for rapid detection of *Escherichia coli* O157:H7. *Biosensors and Bioelectronics* **2004,** *19* (6), 563-74.
4. Feng, C. L.; Zhang, Z.; Forch, R.; Knoll, W.; Vancso, G. J.; Schnherr, H., Reactive thin polymer films as platforms for the immobilization of biomolecules. *Biomacromolecules* **2005,** *6* (6), 3243-3251.
5. Lee, B. S.; Lee, J. K.; Kim, W. J.; Jung, Y. H.; Sim, S. J.; Lee, J.; Choi, I. S., Surface-initiated, atom transfer radical polymerization of oligo(ethylene glycol) methyl ether methacrylate and subsequent click chemistry for bioconjugation. *Biomacromolecules* **2007,** *8* (2), 744-749.
6. Masson, J. F.; Battaglia, T. M.; Kim, Y. C.; Prakash, A.; Beaudoin, S.; Booksh, K. S., Preparation of analyte-sensitive polymeric supports for biochemical sensors. *Talanta* **2004,** *64* (3), 716-25.
7. Akkahat, P.; Hoven, V. P., Introducing surface-tethered poly(acrylic acid) brushes as 3D functional thin film for biosensing applications. *Colloids and Surfaces B: Biointerfaces* **2011,** *86* (1), 198-205.
8. Kurosawa, S.; Aizawa, H.; Talib, Z. A.; Atthoff, B.; Hilborn, J., Synthesis of tethered-polymer brush by atom transfer radical polymerization from a plasma-polymerized-film-coated quartz crystal microbalance and its application for immunosensors. *Biosensors and Bioelectronics* **2004,** *20* (6), 1165-1176.
9. Lee, B. S.; Chi, Y. S.; Lee, K.-B.; Kim, Y.-G.; Choi, I. S., Functionalization of poly(oligo(ethylene glycol) methacrylate) films on gold and Si/SiO_2 for

immobilization of proteins and cells: SPR and QCM studies. *Biomacromolecules* **2007,** *8* (12), 3922-3929.

10. Yang, N.; Su, X.; Tjong, V.; Knoll, W., Evaluation of two- and three-dimensional streptavidin binding platforms for surface plasmon resonance spectroscopy studies of DNA hybridization and protein-DNA binding. *Biosensors and Bioelectronics* **2007,** *22* (11), 2700-2706.
11. Riccardi, C. M.; Cole, K. S.; Benson, K. R.; Ward, J. R.; Bassett, K. M.; Zhang, Y.; Zore, O. V.; Stromer, B.; Kasi, R. M.; Kumar, C. V., Toward "stable-on-the-Table" enzymes: improving key properties of catalase by covalent conjugation with poly(acrylic acid). *Bioconjugate Chemistry* **2014,** *25* (8), 1501-1510.
12. Wang, Y.-M.; Cui, Y.; Cheng, Z.-Q.; Song, L.-S.; Wang, Z.-Y.; Han, B.-H.; Zhu, J.-S., Poly(acrylic acid) brushes pattern as a 3D functional biosensor surface for microchips. *Applied Surface Science* **2013,** *266*, 313-318.
13. Feng, W.; Brash, J. L.; Zhu, S., Non-biofouling materials prepared by atom transfer radical polymerization grafting of 2-methacryloloxyethyl phosphorylcholine: Separate effects of graft density and chain length on protein repulsion. *Biomaterials* **2006,** *27* (6), 847-855.
14. Ishihara, K.; Nomura, H.; Mihara, T.; Kurita, K.; Iwasaki, Y.; Nakabayashi, N., Why do phospholipid polymers reduce protein adsorption? *Journal of Biomedical Materials Research* **1998,** *39* (2), 323-330.
15. Lewis, A. L., Phosphorylcholine-based polymers and their use in the prevention of biofouling. *Colloids and Surfaces B: Biointerfaces* **2000,** *18* (34), 261-275.
16. Yoshimoto, K.; Hirase, T.; Madsen, J.; Armes, S. P.; Nagasaki, Y., Non-fouling character of poly[2-(methacryloyloxy)ethyl phosphorylcholine]-modified gold surfaces fabricated by the ‘Grafting to’ method: comparison of its protein resistance with poly(ethylene glycol)-modified gold surfaces. *Macromolecular Rapid Communications* **2009,** *30* (24), 2136-2140.
17. Jeong, S. P.; Lee, B. S.; Kang, S. M.; Ko, S.; Choi, I. S.; Lee, J. K., Binding behaviors of protein on spatially controlled poly[oligo(ethylene glycol)

methacrylate] brushes grafted from mixed self-assembled monolayers on gold. *Chemical Communications* **2014,** *50* (40), 5291-5293.

18. Trmcic-Cvitas, J.; Hasan, E.; Ramstedt, M.; Li, X.; Cooper, M. A.; Abell, C.; Huck, W. T. S.; Gautrot, J. E., Biofunctionalized protein resistant oligo(ethylene glycol)-derived polymer brushes as selective immobilization and sensing platforms. *Biomacromolecules* **2009,** *10* (10), 2885-2894.
19. Gam-Derouich, S.; Lamouri, A.; Redeuilh, C.; Decorse, P.; Maurel, F.; Carbonnier, B.; Beyazit, S.; Yilmaz, G.; Yagci, Y.; Chehimi, M. M., Diazonium salt-derived 4-(dimethylamino)phenyl groups as hydrogen donors in surface-confined radical photopolymerization for bioactive poly(2-hydroxyethyl methacrylate) grafts. *Langmuir* **2012,** *28* (21), 8035-8045.
20. Vaisocherova, H.; Sevcu, V.; Adam, P.; Spackova, B.; Hegnerova, K.; de los Santos Pereira, A.; Rodriguez-Emmenegger, C.; Riedel, T.; Houska, M.; Brynda, E.; Homola, J., Functionalized ultra-low fouling carboxy- and hydroxy-functional surface platforms: functionalization capacity, biorecognition capability and resistance to fouling from undiluted biological media. *Biosensors and Bioelectronics* **2014,** *51*, 150-157.
21. Akkahat, P.; Kiatkamjornwong, S.; Yusa, S.-I.; Hoven, V. P.; Iwasaki, Y., Development of a novel antifouling platform for biosensing probe immobilization from methacryloyloxyethyl phosphorylcholine-containing copolymer brushes. *Langmuir* **2012,** *28* (13), 5872-5881.
22. Akkahat, P.; Mekboonsonglarp, W.; Kiatkamjornwong, S.; Hoven, V. P., Surface-grafted poly(acrylic acid) brushes as a precursor layer for biosensing applications: effect of graft density and swellability on the detection efficiency. *Langmuir* **2012,** *28* (11), 5302-5311.
23. Audouin, F.; Larragy, R.; Fox, M.; O'Connor, B.; Heise, A., Protein immobilization onto poly(acrylic acid) functional macroporous polyHIPE obtained by surface-initiated ARGET ATRP. *Biomacromolecules* **2012,** *13* (11), 3787-3794.
24. Cullen, S. P.; Liu, X.; Mandel, I. C.; Himpsel, F. J.; Gopalan, P., Polymeric brushes as functional templates for immobilizing ribonuclease A: Study of binding kinetics and activity. *Langmuir* **2007,** *24* (3), 913-920.

25. Dai, J. H.; Bao, Z. Y.; Sun, L.; Hong, S. U.; Baker, G. L.; Bruening, M. L., High-capacity binding of proteins by poly(acrylic acid) brushes and their derivatives. *Langmuir* **2006,** *22* (9), 4274-4281.

26. Qu, Z.; Chen, K.; Gu, H.; Xu, H., Covalent immobilization of proteins on 3D poly(acrylic acid) brushes: mechanism study and a more effective and controllable process. *Bioconjugate Chemistry* **2014,** *25* (2), 370-378.

27. Thilakarathne, V.; Briand, V. A.; Zhou, Y.; Kasi, R. M.; Kumar, C. V., Protein polymer conjugates: improving the stability of hemoglobin with poly(acrylic acid). *Langmuir* **2011,** *27* (12), 7663-7671.

28. van Dijk, M.; Rijkers, D. T. S.; Liskamp, R. M. J.; van Nostrum, C. F.; Hennink, W. E., Synthesis and applications of biomedical and pharmaceutical polymers via click chemistry methodologies. *Bioconjugate Chemistry* **2009,** *20* (11), 2001-2016.

29. Ostaci, R. V.; Damiron, D.; Grohens, Y.; Leger, L.; Drockenmuller, E., Click chemistry grafting of poly(ethylene glycol) brushes to alkyne-functionalized pseudobrushes. *Langmuir* **2010,** *26* (2), 1304-1310.

30. Qin, G.; Santos, C.; Zhang, W.; Li, Y.; Kumar, A.; Erasquin, U. J.; Liu, K.; Muradov, P.; Trautner, B. W.; Cai, C., Biofunctionalization on alkylated silicon substrate surfaces via "click" chemistry. *Journal of the American Chemical Society* **2010,** *132* (46), 16432-16441.

31. Iwasaki, Y.; Matsunaga, A.; Fujii, S., Preparation of biointeractive glycoprotein-conjugated hydrogels through metabolic oligosacchalide engineering. *Bioconjugate Chemistry* **2014,** *25* (9), 1626-1631.

32. Iwasaki, Y.; Matsuno, H., Metabolic delivery of methacryloyl groups on living cells and cell surface modification via thiol-ene “Click” reaction. *Macromolecular Bioscience* **2011,** *11* (11), 1478-1483.

33. Kayser, H.; Ats, C.; Lehmann, J.; Reutter, W., New amino sugar analogues are incorporated at different rates into glycoproteins of mouse organs. *Experientia* **1993,** *49* (10), 885-7.

34. Kayser, H.; Zeitler, R.; Kannicht, C.; Grunow, D.; Nuck, R.; Reutter, W., Biosynthesis of a nonphysiological sialic acid in different rat organs, using

N-propanoyl-D-hexosamines as precursors. *Journal of Biological Chemistry* **1992,** *267* (24), 16934-8.

35. Keppler, O. T.; Horstkorte, R.; Pawlita, M.; Schmidt, C.; Reutter, W., Biochemical engineering of the *N*-acyl side chain of sialic acid: biological implications. *Glycobiology* **2001,** *11* (2), 11R-18R.
36. Keppler, O. T.; Stehling, P.; Herrmann, M.; Kayser, H.; Grunow, D.; Reutter, W.; Pawlita, M., Biosynthetic modulation of sialic acid-dependent virus-receptor interactions of two primate polyoma viruses. *Journal of Biological Chemistry* **1995,** *270* (3), 1308-1314.
37. Kessler, D.; Roth, P. J.; Theato, P., Reactive surface coatings based on polysilsesquioxanes: controlled functionalization for specific protein immobilization. *Langmuir* **2009,** *25* (17), 10068-10076.
38. Rusmini, F.; Zhong, Z.; Feijen, J., Protein immobilization strategies for protein biochips. *Biomacromolecules* **2007,** *8* (6), 1775-1789.
39. Senaratne, W.; Andruzzi, L.; Ober, C. K., Self-assembled monolayers and polymer brushes in biotechnology: Current applications and future perspectives. *Biomacromolecules* **2005,** *6* (5), 2427-2448.
40. Xu, F. J.; Neoh, K. G.; Kang, E. T., Bioactive surfaces and biomaterials via atom transfer radical polymerization. *Progress in Polymer Science* **2009,** *34* (8), 719-761.
41. Masson, J.-F.; Battaglia, T. M.; Davidson, M. J.; Kim, Y.-C.; Prakash, A. M. C.; Beaudoin, S.; Booksh, K. S., Biocompatible polymers for antibody support on gold surfaces. *Talanta* **2005,** *67* (5), 918-925.
42. Nakamura, F.; Ito, E.; Hayashi, T.; Hara, M., Fabrication of COOH-terminated self-assembled monolayers for DNA sensors. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2006,** *284-285*, 495-498.
43. Goto, Y.; Matsuno, R.; Konno, T.; Takai, M.; Ishihara, K., Polymer nanoparticles covered with phosphorylcholine groups and immobilized with antibody for high-affinity separation of proteins. *Biomacromolecules* **2008,** *9* (3), 828-833.
44. Mark, S. S.; Nishizawa, K.; Takai, M.; Ishihara, K., A bioconjugated phospholipid polymer biointerface with nanometer-scaled structure for highly

sensitive immunoassays. In *Bioconjugation Protocols*, Humana Press: 2011; Vol. 751, pp 491-502.

45. Nishizawa, K.; Konno, T.; Takai, M.; Ishihara, K., Bioconjugated phospholipid polymer biointerface for enzyme-linked immunosorbent assay. *Biomacromolecules* **2008,** *9* (1), 403-407.
46. Park, J.; Kurosawa, S.; Takai, M.; Ishihara, K., Antibody immobilization to phospholipid polymer layer on gold substrate of quartz crystal microbalance immunosensor. *Colloids and Surfaces B: Biointerfaces* **2007,** *55* (2), 164-172.
47. Iwata, R.; Satoh, R.; Iwasaki, Y.; Akiyoshi, K., Covalent immobilization of antibody fragments on well-defined polymer brushes via site-directed method. *Colloids and Surfaces B-Biointerfaces* **2008,** *62* (2), 288-298.
48. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click chemistry: diverse chemical function from a few good reactions. *Angewandte Chemie International Edition* **2001,** *40* (11), 2004-2021.
49. Gacal, B.; Durmaz, H.; Tasdelen, M. A.; Hizal, G.; Tunca, U.; Yagci, Y.; Demirel, A. L., Anthracene-maleimide based Diels-Alder "Click Chemistry" as a novel route to graft copolymers. *Macromolecules* **2006,** *39* (16), 5330-5336.
50. Lowe, A. B., Thiol-ene "click" reactions and recent applications in polymer and materials synthesis. *Polymer Chemistry* **2010,** *1* (1), 17-36.
51. Nilsson, B. L.; Kiessling, L. L.; Raines, R. T., Staudinger Ligation: A peptide from a thioester and azide. *Organic Letters* **2000,** *2* (13), 1939-1941.
52. Saxon, E.; Armstrong, J. I.; Bertozzi, C. R., A "Traceless" Staudinger ligation for the chemoselective synthesis of amide bonds. *Organic Letters* **2000,** *2* (14), 2141-2143.
53. Ruttekolk, I. R.; Duchardt, F.; Fischer, R.; Wiesmuller, K.-H.; Rademann, J.; Brock, R., HPMA as a scaffold for the modular assembly of functional peptide polymers by native chemical ligation. *Bioconjugate Chemistry* **2008,** *19* (10), 2081-2087.
54. Merkx, R.; Brouwer, A. J.; Rijkers, D. T. S.; Liskamp, R. M. J., Highly efficient coupling of B-substituted aminoethane sulfonyl azides with thio

acids, toward a new chemical ligation reaction. *Organic Letters* **2005,** *7* (6), 1125-1128.

55. Shangguan, N.; Katukojvala, S.; Greenberg, R.; Williams, L. J., The reaction of thio acids with azides: A new mechanism and new synthetic applications. *Journal of the American Chemical Society* **2003,** *125* (26), 7754-7755.

56. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B., A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angewandte Chemie International Edition* **2002,** *41* (14), 2596-2599.

57. Tornoe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *The Journal of Organic Chemistry* **2002,** *67* (9), 3057-3064.

58. Lee, B. S.; Lee, J. K.; Kim, W.-J.; Jung, Y. H.; Sim, S. J.; Lee, J.; Choi, I. S., Surface-initiated, atom transfer radical polymerization of oligo(ethylene glycol) methyl ether methacrylate and subsequent click chemistry for bioconjugation. *Biomacromolecules* **2007,** *8* (2), 744-749.

59. Moad, G.; Chong, Y. K.; Postma, A.; Rizzardo, E.; Thang, S. H., Advances in RAFT polymerization: the synthesis of polymers with defined end-groups. *Polymer* **2005,** *46* (19), 8458-8468.

60. Sumerlin, B. S.; Lowe, A. B.; Stroud, P. A.; Zhang, P.; Urban, M. W.; McCormick, C. L., Modification of gold surfaces with water-soluble (co)polymers prepared via aqueous reversible addition-fragmentation chain transfer (RAFT) polymerization. *Langmuir* **2003,** *19* (14), 5559-5562.

61. Willcock, H.; O'Reilly, R. K., End group removal and modification of RAFT polymers. *Polymer Chemistry* **2010,** *1* (2), 149-157.

62. Rinaldi, D.; Hamaide, T.; Graillat, C.; D'Agosto, F.; Spitz, R.; Georges, S.; Mosquet, M.; Maitrasse, P., RAFT copolymerization of methacrylic acid and poly(ethylene glycol) methyl ether methacrylate in the presence of a hydrophobic chain transfer agent in organic solution and in water. *Journal of Polymer Science Part A: Polymer Chemistry* **2009,** *47* (12), 3045-3055.

63. Weber, C.; Remzi Becer, C.; Guenther, W.; Hoogenboom, R.; Schubert, U. S., Dual responsive methacrylic acid and oligo(2-ethyl-2-oxazoline) containing graft copolymers. *Macromolecules* **2010,** *43* (1), 160-167.

64. Shan, J.; Nuopponen, M.; Jiang, H.; Kauppinen, E.; Tenhu, H., Preparation of poly(*N*-isopropylacrylamide)-monolayer-protected gold clusters: Synthesis methods, core size, and thickness of monolayer. *Macromolecules* **2003,** *36* (12), 4526-4533.

65. Shen, W.; Qiu, Q.; Wang, Y.; Miao, M.; Li, B.; Zhang, T.; Cao, A.; An, Z., Hydrazine as a nucleophile and antioxidant for fast aminolysis of RAFT polymers in air. *Macromolecular Rapid Communications* **2010,** *31* (16), 1444-1448.

66. Lowe, A. B.; Sumerlin, B. S.; Donovan, M. S.; McCormick, C. L., Facile preparation of transition metal nanoparticles stabilized by well-defined (co)polymers synthesized via aqueous reversible addition-fragmentation chain transfer polymerization. *Journal of the American Chemical Society* **2002,** *124* (39), 11562-11563.

67. Gibson, M. I.; Danial, M.; Klok, H.-A., Sequentially modified, polymer-stabilized gold nanoparticle libraries: Convergent synthesis and aggregation behavior. *ACS Combinatorial Science* **2011,** *13* (3), 286-297.

68. Nelson, K. E.; Gamble, L.; Jung, L. S.; Boeckl, M. S.; Naeemi, E.; Golledge, S. L.; Sasaki, T.; Castner, D. G.; Campbell, C. T.; Stayton, P. S., Surface characterization of mixed self-assembled monolayers designed for streptavidin immobilization. *Langmuir* **2001,** *17* (9), 2807-2816.

69. Kitano, H.; Hayashi, A.; Takakura, H.; Suzuki, H.; Kanayama, N.; Saruwatari, Y., Anti-biofouling properties of a telomer brush with pendent glucosylurea groups. *Langmuir* **2009,** *25* (16), 9361-9368.

70. Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E., PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **1993,** *365* (6446), 566-568.

71. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O., Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **1991,** *254* (5037), 1497-1500.

72. Laopa, P. Determination of DNA sequences following dot blot hybridization format employing filter paper immobilized with quaternized polymer brushes and peptide nucleic acid. Doctoral dissertation, Program in Petrochemistry, Faculty of Science. Chulalongkorn Univesity, Bangkok, Thailand, 2011.

73. Suparpprom, C.; Srisuwannaket, C.; Sangvanich, P.; Vilaivan, T., Synthesis and oligodeoxynucleotide binding properties of pyrrolidinyl peptide nucleic acids bearing prolyl-2-aminocyclopentanecarboxylic acid (ACPC) backbones. *Tetrahedron Letters* **2005,** *46* (16), 2833-2837.

74. Vilaivan, T.; Srisuwannaket, C., Hybridization of pyrrolidinyl peptide nucleic acids and DNA: Selectivity, base-pairing specificity, and direction of binding. *Organic Letters* **2006,** *8* (9), 1897-1900.

75. Ananthanawat, C.; Vilaivan, T.; Mekboonsonglarp, W.; Hoven, V. P., Thiolated pyrrolidinyl peptide nucleic acids for the detection of DNA hybridization using surface plasmon resonance. *Biosensors and Bioelectronics* **2009,** *24* (12), 3544-3549.

76. Ananthanawat, C.; Vilaivan, T.; Hoven, V. P.; Su, X., Comparison of DNA, aminoethylglycyl PNA and pyrrolidinyl PNA as probes for detection of DNA hybridization using surface plasmon resonance technique. *Biosensors and Bioelectronics* **2010,** *25* (5), 1064-1069.

77. Ananthanawat, C.; Hoven, V. P.; Vilaivan, T.; Su, X., Surface plasmon resonance study of PNA interactions with double-stranded DNA. *Biosensors and Bioelectronics* **2011,** *26* (5), 1918-1923.

78. Burness, A. T. H., *Virus receptors (Part II)*. Chapman and Hall: London, 1981; p 65–84.

79. Collins, B. E.; Fralich, T. J.; Itonori, S.; Ichikawa, Y.; Schnaar, R. L., Conversion of cellular sialic acid expression from *N*-acetyl- to *N*-glycolylneuraminic acid using a synthetic precursor, *N*-glycolylmannosamine pentaacetate: inhibition of myelin-associated glycoprotein binding to neural cells. *Glycobiology* **2000,** *10* (1), 11-20.

80. Herrmann, M.; von der Lieth, C. W.; Stehling, P.; Reutter, W.; Pawlita, M., Consequences of a subtle sialic acid modification on the murine polyomavirus receptor. *Journal of Virology* **1997,** *71* (8), 5922-31.

81. Schmidt, C.; Stehling, P.; Schnitzer, J.; Reutter, W.; Horstkorte, R., Biochemical engineering of neural cell surfaces by the synthetic N-propanoyl-substituted neuraminic acid precursor. *Journal of Biological Chemistry* **1998,** *273* (30), 19146-19152.

82. Stehling, P.; Grams, S.; Nuck, R.; Grunow, D.; Reutter, W.; Gohlke, M., In *vivo* modulation of the acidic *N*-glycans from rat liver dipeptidyl peptidase IV by *N*-propanoyl-d-mannosamine. *Biochemical and Biophysical Research Communications* **1999,** *263* (1), 76-80.

83. Wieser, J. R.; Heisner, A.; Stehling, P.; Oesch, F.; Reutter, W., In vivo modulated *N*-acyl side chain of *N*-acetylneuraminic acid modulates the cell contact-dependent inhibition of growth. *FEBS Letters* **1996,** *395* (2-3), 170-173.

84. Yarema, K. J.; Mahal, L. K.; Bruehl, R. E.; Rodriguez, E. C.; Bertozzi, C. R., Metabolic delivery of ketone groups to sialic acid residues: application to cell surface glycoform engineering. *Journal of Biological Chemistry* **1998,** *273* (47), 31168-31179.

85. Percot, A.; Lafleur, M.; Zhu, X. X., New hydrogels based on *N*-isopropylacrylamide copolymers crosslinked with polylysine: membrane immobilization systems. *Polymer* **2000,** *41* (19), 7231-7239.

86. He, H.; Zhang, Y.; Gao, C.; Wu, J., 'Clicked' magnetic nanohybrids with a soft polymer interlayer. *Chemical Communications* **2009,** (13), 1655-1657.

87. Ditmangklo, B.; Boonlua, C.; Suparpprom, C.; Vilaivan, T., Reductive alkylation and sequential reductive alkylation-click chemistry for on-solid-support modification of pyrrolidinyl peptide nucleic acid. *Bioconjugate Chemistry 24* (4), 614-625.

88. Srisuwannaket, C. Synthesis and DNA-binding properties of pyrrolidinyl peptide nucleic acids bearing (1S, 2S)-2-aminocyclopentane carboxylic acids

spacer. Doctoral dissertation, Department of Chemistry, Faculty of Science. Chulalongkorn Univesity, Bangkok, Thailand, 2005.

89. Suparpprom, C. Synthesis and nucleic acid binding properties of novel peptide nucleic acids carrying beta amino acid spacer. Doctoral dissertation, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, 2006.
90. Herne, T. M.; Tarlov, M. J., Characterization of DNA probes immobilized on gold surfaces. *Journal of the American Chemical Society* **1997,** *119* (38), 8916-8920.
91. Green, N. M., Avidin. *Advances in Protein Chemistry* **1975,** *29*, 85-133.
92. Huisgen, R., 1,3-Dipolar cycloadditions. Past and future. *Angewandte Chemie International Edition in English* **1963,** *2* (10), 565-598.
93. Hansen, J. T., *Netter's Clinical Anatomy*. 3rd edition ed.; Elsevier Inc: Philadelphia, PA, 2014.
94. Ishihara, K.; Oshida, H.; Endo, Y.; Ueda, T.; Watanabe, A.; Nakabayashi, N., Hemocompatibility of human whole blood on polymers with a phospholipid polar group and its mechanism. *Journal of Biomedical Materials Research* **1992,** *26* (12), 1543-1552.
95. Kim, G.; Park, S.; Jung, J.; Heo, K.; Yoon, J.; Kim, H.; Kim, I. J.; Kim, J. R.; Lee, J. I.; Ree, M., Novel brush polymers with phosphorylcholine bristle ends: synthesis, structure, properties, and biocompatibility. *Advanced Functional Materials* **2009,** *19* (10), 1631-1644.
96. Olofsson, S.; Kumlin, U.; Dimock, K.; Arnberg, N., Avian Influenza and sialic acid receptors: More than meets the eye? *The Lancet Infectious Diseases* **2005,** *5* (3), 184-8.
97. Skehel, J. J.; Wiley, D. C., Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual Review of Biochemistry* **2000,** *69*, 531-69.
98. Grunholz, H.-J.; Harms, E.; Opetz, M.; Reutter, W.; Cerny, M., Inhibition of in vitro biosynthesis of *N*-acetylneuraminic acid by *N*-acyl- and *N*-alkyl-2-amino-2-deoxyhexoses. *Carbohydrate Research* **1981,** *96* (2), 259-270.

99. Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R., Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. *Science* **1997,** *276* (5315), 1125-1128.

100. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C., Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **1985,** *150* (1), 76-85.

101. Wong, L.; Boyer, C.; Jia, Z.; Zareie, H. M.; Davis, T. P.; Bulmus, V., Synthesis of versatile thiol-reactive polymer scaffolds via RAFT polymerization. *Biomacromolecules* **2008,** *9* (7), 1934-1944.

APPENDIX

APPENDIX A

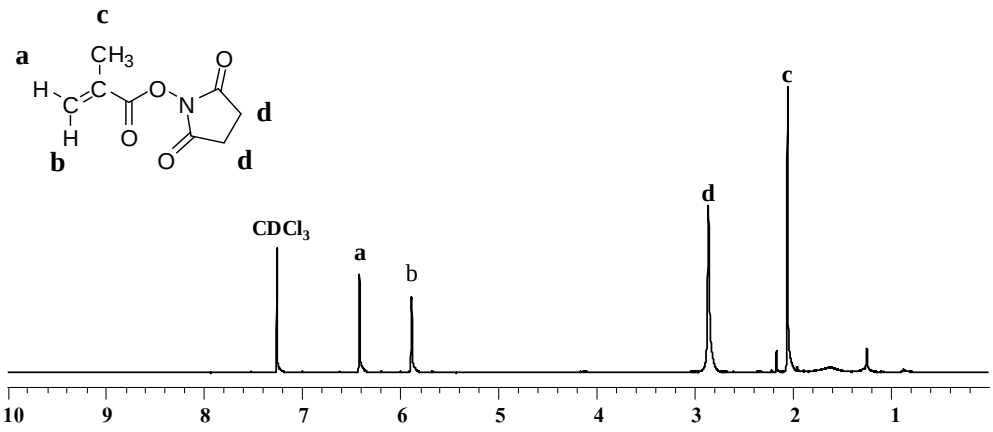


Figure A- 1 ^{1}H-NMR spectrum of NMS monomer in $CDCl_3$

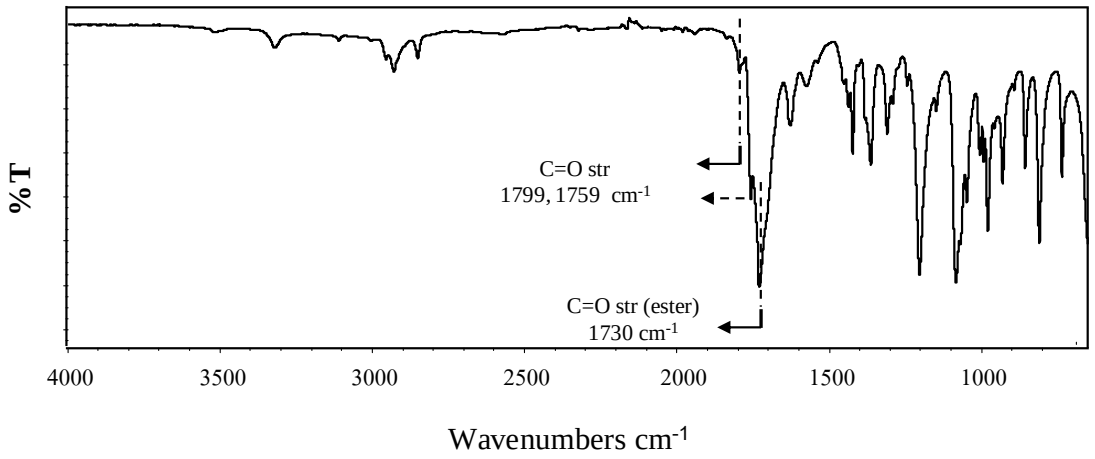


Figure A- 2 FT-IR spectrum of NMS monomer

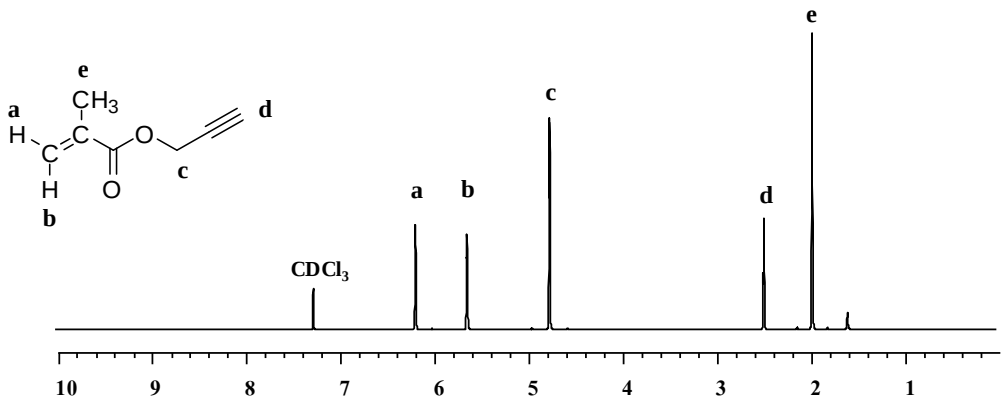


Figure A- 3 ^{1}H-NMR spectrum of PgMA monomer in $CDCl_3$

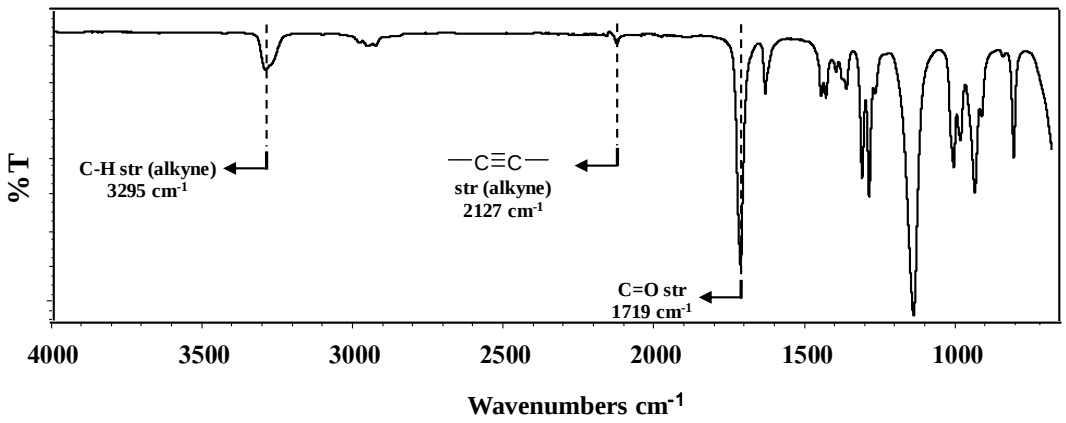


Figure A- 4 FT-IR spectrum of PgMA monomer

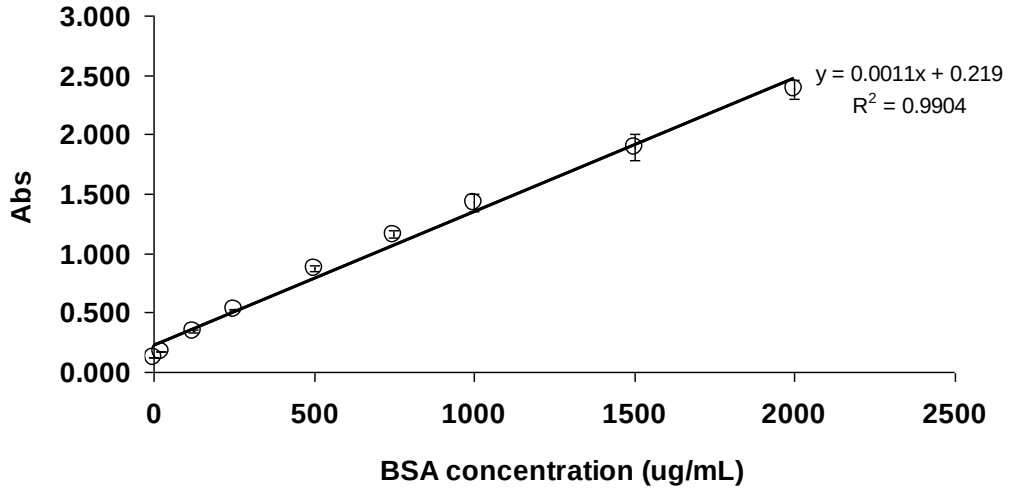


Figure A- 5 Calibration curve of BCA assay

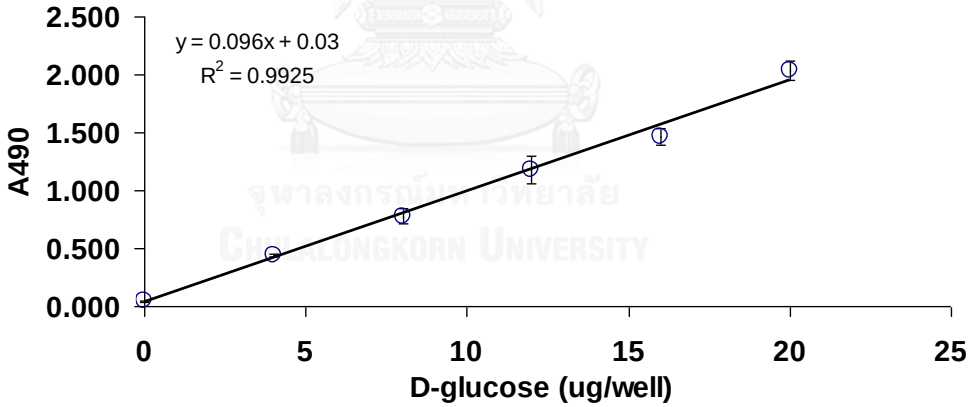


Figure A- 6 Calibration curve of total carbohydrate calorimetric assay

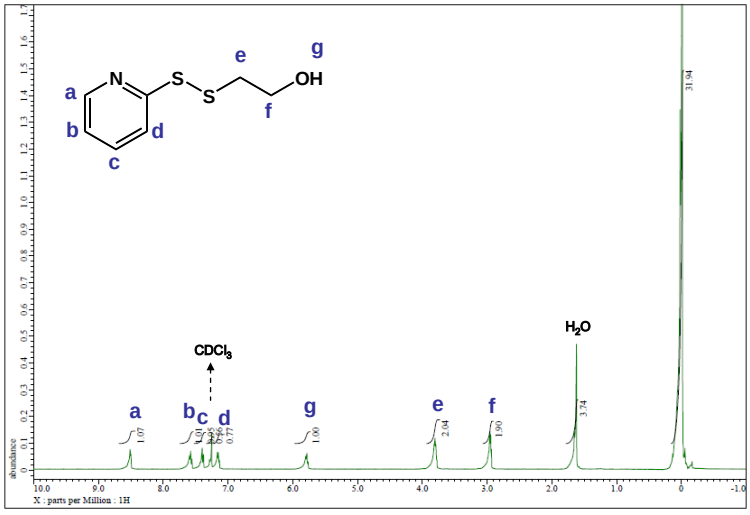


Figure A- 7 ^{1}H-NMR spectrum of HPDS in $CDCl_3$

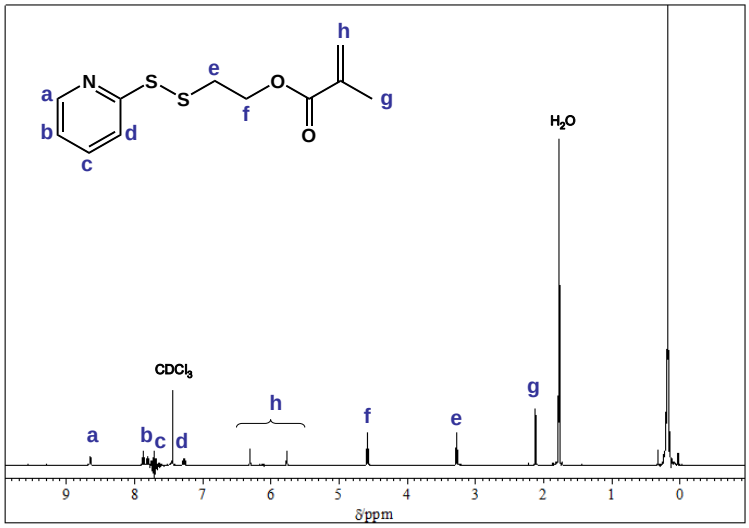


Figure A- 8 ^{1}H NMR spectrum of PDSM in $CDCl_3$

VITA

Miss Oraphan Wiarachai was born in Nakhon Phanom, Thailand, on February 19th, 1984. She received a Bachelor Degree of Science (Chemistry) from the Department of Chemistry, Faculty of Science, Chulalongkorn University in 2006 and graduated with the Master Degree of Science, majoring in Petrochemistry and Polymer Science from Faculty of Science, Chulalongkorn University in 2009. She began her PhD study in Program of Petrochemistry, Faculty of Science, Chulalongkorn University in the academic year of 2009 and graduated in the academic year of 2014.

Awards:

2013 Poster Presentation Award in the 8th International Symposium in Science and Technology at Kansai University

2008 Best Poster Presentation Award in Symposium: Nanomaterials in the 16th Science Forum, Faculty of Science, Chulalongkorn University

Presentation in Conference:

2014 "Clickable and Anti-fouling Copolymer Platform of Poly[(propargyl methacrylate)-ran-(2-ethacryloyloxyethyl phosphorylcholine)] for Biosensing Applications" Macro 2014, Chiang Mai, Thailand

2013 "Clickable and Anti-fouling Copolymer of Poly[(propargyl methacrylate)-ran-(2-methacryloyloxyethyl phosphorylcholine)] for Biosensing Applications" The 8th International Symposium in Science and Technology at Kansai University, Japan

2013 "Synthesis and Characterization of Clickable and Anti-fouling Copolymer for Bio-related Applications" The 3rd Polymer Conference of Thailand, Bangkok, Thailand