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สาขาวิชาปิโตรเคมี

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

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DETERMINATION OF DNA SEQUENCES FOLLOWING DOT BLOT HYBRIDIZATION FORMAT EMPLOYING FILTER PAPER IMMOBILIZED WITH QUATERNIZED POLYMER BRUSHES AND PEPTIDE NUCLEIC ACID

Miss Praethong Laopa

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Petrochemistry

Faculty of Science

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By	Miss Praethong Laopa
Field of Study	Petrochemistry
Thesis Advisor	Associate Professor Voravee P. Hoven, Ph.D.
Thesis Co-advisor	Associate Professor Tirayut Vilaivan, Ph.D.

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

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……Examiner
(Associate Professor Nattiya Hirankarn, MD, Ph.D.)
……External Examiner
(Chaturong Suparpprom, Ph.D.)

แพรทอง เหลาภา: การตรวจหาลำดับเบสของดีเอ็นเอตามรูปแบบด็อตบล็อตไฮบริไดเซชันโดยใช้กระดาษกรองที่ตรึงด้วยควอเทอไนซ์พอลิเมอร์บรัชและเพปไทด์นิวคลีอิกแอซิด (DETERMINATION OF DNA SEQUENCES FOLLOWING DOT BLOT HYBRIDIZATION FORMAT EMPLOYING FILTER PAPER IMMOBILIZED WITH QUATERNIZED POLYMER BRUSHES AND PEPTIDE NUCLEIC ACID) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร. ธีรยุทธ วิไลวัลย์, 102 หน้า.

ในงานวิจัยนี้ได้พัฒนารูปแบบใหม่สำหรับการตรวจหาลำดับเบสของดีเอ็นเอด้วยวิธีการตรวจการเกิดสีบนกระดาษโดยใช้พิโรลิดินิลเพปไทด์นิวคลีอิกแอซิดที่มีคอนฟอร์เมชันถูกจำกัด (เอซีพีซีพีเอ็นเอ) เป็นโพรบ กระดาษกรองถูกดัดแปรพื้นผิวให้มีประจุบวกโดยการตรึงพอลิเมอร์บรัชของ QPDMAEMA เพื่อใช้จับยึดแบบจำเพาะเจาะจงกับประจุลบของดีเอ็นเอด้วยแรงระหว่างประจุแต่ไม่จับยึดกับเอซีพีซีพีเอ็นเอซึ่งเป็นกลาง พีเอ็นเอโพรบสามารถติดอยู่บนแผ่นเมมเบรนได้ในกรณีที่ดีเอ็นเอและพีเอ็นเอโพรบที่ปลายสายมีการดัดแปรมีลำดับเบสคู่สมกันเท่านั้น โมเลกุลลูกผสมของดีเอ็นเอ-พีเอ็นเอที่เกิดขึ้นสามารถตรวจวิเคราะห์ได้จากการเกิดสีผ่านการขยายสัญญาณด้วยเอ็นไซม์และปฏิกิริยาพอลิเมอไรเซชัน สำหรับวิธีการขยายสัญญาณด้วยเอ็นไซม์พบว่ามีความสามารถในการแยกความแตกต่างของดีเอ็นเอที่มีลำดับเบสผิดไปเพียง 1 ตำแหน่งได้ที่ปริมาณต่ำสุดถึง 10 เฟมโตโมล นอกจากนี้กระดาษกรองที่ตรึง QPDMAEMA ยังมีประสิทธิภาพเหนือกว่าแผ่นเมมเบรนทางการค้า เช่น ไนลอน 66 และไนโตรเซลลูโลสอีกด้วย สำหรับวิธีการขยายสัญญาณด้วยปฏิกิริยาพอลิเมอไรเซชัน พบว่าโคพอลิเมอร์ของ HEMA-Rh B และ PEGMA ซึ่งมีสีแดงสามารถเกิดขึ้นบนกระดาษเฉพาะบริเวณที่ดูดซับหมู่ริเริ่มปฏิกิริยาและปรากฏการดูดซับแบบไม่จำเพาะเจาะจงของโคพอลิเมอร์ที่ไม่ได้เกิดพันธะที่หมู่ริเริ่มปฏิกิริยาบนกระดาษกรองเพียงเล็กน้อย แต่การขยายสัญญาณของโมเลกุลลูกผสมของพีเอ็นเอ-ดีเอ็นเอในระดับพิโคโมลด้วยวิธีนี้ไม่สามารถตรวจวิเคราะห์ได้ด้วยตาเปล่า เนื่องจากปฏิกิริยาพอลิเมอไรเซชันแบบ concurrent ARGET ATRP/RAFT ต้องใช้ปริมาณของหมู่ริเริ่มปฏิกิริยาสูงมากกว่า 1 นาโนโมล

สาขาวิชา ปิโตรเคมี ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5173925023 : MAJOR PETROCHEMISTRY
KEYWORDS: POLYMER BRUSHES / PNA / DOT BLOT HYBRIDIZATION/ QPDMAEMA / ARGET ATRP

PRAETHONG LAOPA: DETERMINATION OF DNA SEQUENCES FOLLOWING DOT BLOT HYBRIDIZATION FORMAT EMPLOYING FILTER PAPER IMMOBILIZED WITH QUATERNIZED POLYMER BRUSHES AND PEPTIDE NUCLEIC ACID. ADVISOR: ASSOC. PROF. VORAVEE P. HOVEN, Ph.D., CO-ADVISOR: ASSOC. PROF. TIRAYUT VILAIVAN, Ph.D., 102 pp.

In this research, a new paper-based platform for colorimetric detection of specific DNA sequences employing a new conformationally constrained pyrrolidinyl peptide nucleic acid (acpcPNA) as a probe has been developed. The filter paper was modified to be positively charged with grafted polymer brushes of QPDMAEMA to be used for selective capturing of the negatively charged DNA, but not the neutral acpcPNA, by electrostatic interaction. Only when the sequences of the DNA and modifier labeled acpcPNA probe (m-PNA) are complementary, the probe can be immobilized through hybridization with the surface-bound DNA. The presence of DNA-PNA hybrids can be detected by colorimetric assay via either enzymatic or polymerization amplification. In enzymatic amplification mode, it is obvious that this assay was capable of discriminating a single base mismatch at a detection limit of at least 10 fmole. Moreover, the QPDMAEMA grafted filter paper exhibited a superior performance to the commercial membranes, nylon 66 and nitrocellulose. In polymerization amplification mode, it was demonstrated that the desired red copolymer of HEMA-Rh B and PEGMA grew from the initiator adsorbed on paper with minimal background from non-specific adsorption of unbound copolymer. However, the signal amplification of PNA-DNA hybridization in picomol level was not observed by naked eyes because concurrent ARGET ATRP/RAFT for this assay required initiator with a mount greater than 1 nmol.

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

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AA	: Ascorbic acid
ARGET ATRP	: Activators regenerated by electron transfer for atom transfer radical polymerization
ATRP	: Atom transfer radical polymerization
AVD	: Avidin
BSA	: Bovine serum albumin
BiB	: 2-Bromoisobutyrate
CA	: Contact angles
^{o}C	: Degree celcius
DQ	: Degree of quaternization
DNA	: Deoxyribonucleic acid
$CDCl_3$	: Deuterated chloroform
D_2O	: Deuterium oxide
DIEA	: Diisopropylethylamine
DMAEMA	: 2-(Dimethylamino)ethyl methacrylate
CTB	: Citrate buffer
EBiB	: Ethyl 2-bromoisobutyrate
EDC	: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EtOH	: Ethanol
FmocOSu	: 9-Fouorenylsuccinimidyl carbonate
fmol	: Femtomole

FT-IR	: Fourier transforms infrared spectroscopy
g	: Gram
GPC	: Gel permeation chromatography
HEMA	: 2-Hydroxyethyl methacrylate
h	: Hour
H	: Proton
KHz	: Kilohertz
LODs	: Limit of detection
Lys	: Lysine
MALDI-TOF	: Matrix-assisted laser desorption/ionization-time of flight
MeOH	: Methanol
MeI	: Methyl iodide
m°	: Millidegree
Me_6TREN	: Tris(2-(dimethylamino)ethyl)amine
Mg	: Milligram
MHz	: Megahertz
Min	: Minute
mL	: Milliliter
mmol	: Millimole
mM	: Millimolar
$\overline{M}_n$	: Number average molecular weight
MS	: Mass spectrometry
MW	: Molecular weight

MWD	: Molecular weight distribution
m/*z*	: Mass to charge ratio
ng	: Nanogram
NHS	: *N*-Hydroxysuccinimide
NMR	: Nuclear magnetic resonance spectroscopy
OPD	: *ortho*-Phenylendiamine
PNA	: Peptide nucleic acid, Polyamide nucleic acid
PBS	: Phosphate buffered saline
PDI	: Polydispersity index
pI	: Isoelectric point
pKa	: Acid dissociation constant
pmol	: Picomole
QPDMAEMA	: Quaternized poly(dimethylamino)ethyl methacrylate
QCM	: Quartz crystal microbalance
RAFT	: Reversible addition-fragmentation chain transfer
Rh B	: Rhodamine B
RX	: Organic halide
SA	: Streptavidin
Ser	: Serine
SEC	: Size exclusion chromatography
SEM	: Scanning electron microscopy
SI-ATRP	: Surface-initiated atom transfer radical polymerization
SIP	: Surface-initiate polymerization
$Sn(EH)_2$	: Tin(II) 2-ethylhexanoate

THF	: Tetrahydrofuran
TFA	: Trifluoroacetic acid
UV	: Ultraviolet
Urea-H_2O_2	: Urea-hydroperoxide
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

CHAPTER I

INTRODUCTION

1.1 DNA biosensor using pyrrolidinyl peptide nucleic acid as probe

It is well known that deoxyribonucleic acid (DNA) is a molecule that stores and transfers genetic information to next generations in all living organisms. DNA consists of repeating units of nucleotides that are connected by phosphodiester linkages. The presence of phosphate groups in backbone results in the negative charge of DNA. The core structure of DNA in all living cell forms double helix following Watson-click base paring rule; adenine (A) forms 2 hydrogen bonds with thymine (T) and cytosine (C) forms three hydrogen bonds with guanine (G) (**Figure 1.1**).

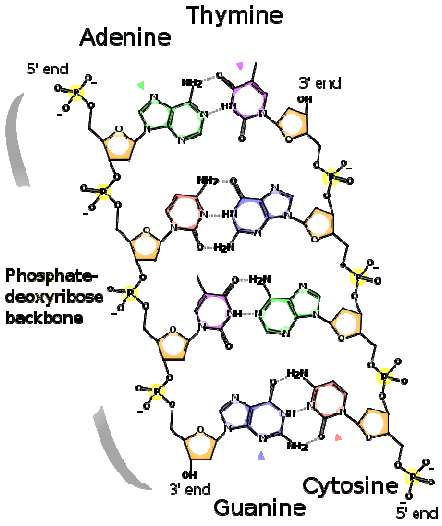
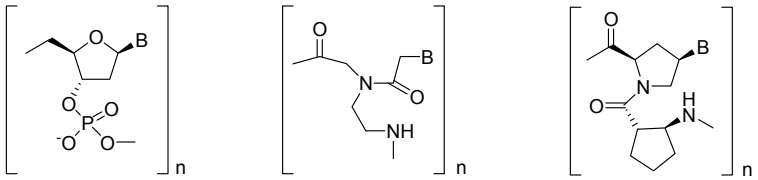


Figure 1.1 Nucleobase pair recognition by Watson-Crick hydrogen bonding and R = ribose or deoxyribose (http://en.wikibooks.org/wiki/Structural_Biochemistry/Nucleic_Acid/DNA/DNA_structure).

Since 1991, Nielsen group has introduced a unique DNA analogue called peptide nucleic acid (PNA).[1-2] This PNA system or so-called *aeg*PNA, consists of repeating *N*-(2-aminoethyl)-glycine units linked together by 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 1.2b**. The hybridization between PNA with DNA strictly follows the Watson-Crick base pairing rule 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 makes it an excellent probe for DNA sequence determination.



a **b** **c**

Figure 1.2 Structures of **a)** DNA **b)** Nielsen's PNA or aegPNA and **c)** Vilaivan's PNA or (1*S*, 2*S*)-acpc PNA.

Despite of these advantages, there are many attempts to improve the original PNA further during the past 20 years. Among several variants of PNA developed to date, the conformationally rigid pyrrolidinyl PNA derived from D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbones (acpcPNA)[3-4] introduced by Vilaivan and co-workers (**Figure 1.2c**) shows great promise because it can form PNA·DNA duplex with even higher affinity and specificity than the original Nielsen's aegPNA. Moreover, it can also form only anti-parallel hybrid with DNA because the *acpc*PNA structure contains stereochemistry (**Figure 1.3**). Therefore, this PNA system is a potential candidate for the development of a highly effective platform for DNA sequence determination.

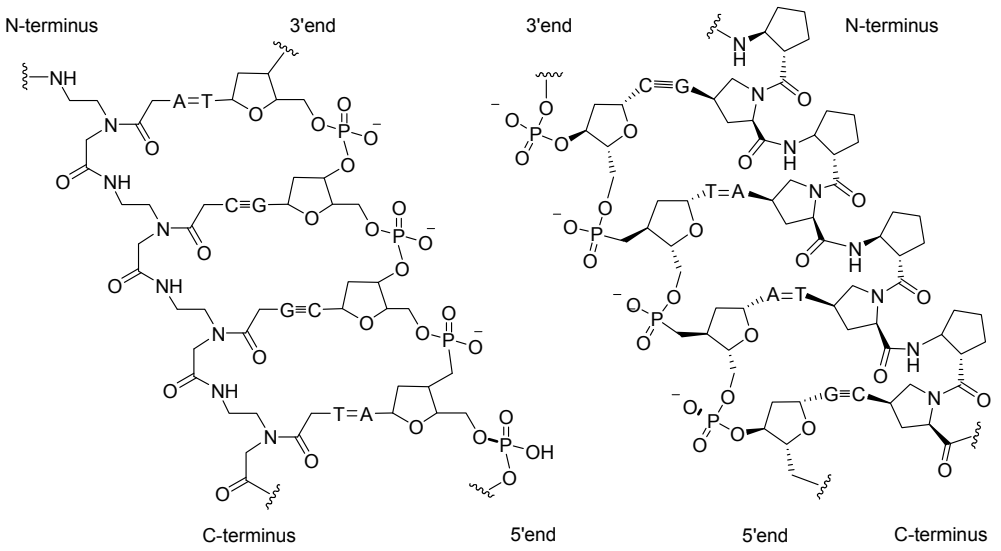


Figure 1.3 Chemical structures of a) aegPNA·DNA and b) acpcPNA·DNA duplex following Watson-Crick base paring rule.

Because of its advantages, acpcPNA has been applied as a probe to detect DNA base sequence with various techniques. In 2008, Boontha and coworkers[5] reported the high mismatch discrimination ability of the acpcPNA that led to successful applications in detection of single nucleotide polymorphisms (SNP) genotyping in combination with ion-exchange capture technique. The support-bound hybridized PNA-DNA hybrids was separated from the unhybridized probe and other impurities in the sample matrix, such as proteins and salts by simple washing (**Figure 1.4**).The presence of the hybridized PNA in the form of negatively charged PNA.DNA duplex on the positively charged ion-exchanger, Q-sepharose, was analyzed directly by MALDITOF mass spectrometry. The high specificity of the pyrrolidinyl PNA allows simultaneous multiplex SNP typing to be carried out at room temperature without the need for enzyme treatment or heating. Applications of this technique for the identification of meat species in feedstuffs and in multiplex SNP typing of the human *IL*-10 gene promoter region clearly suggested the potential of acpcPNA probes for much broader applications.

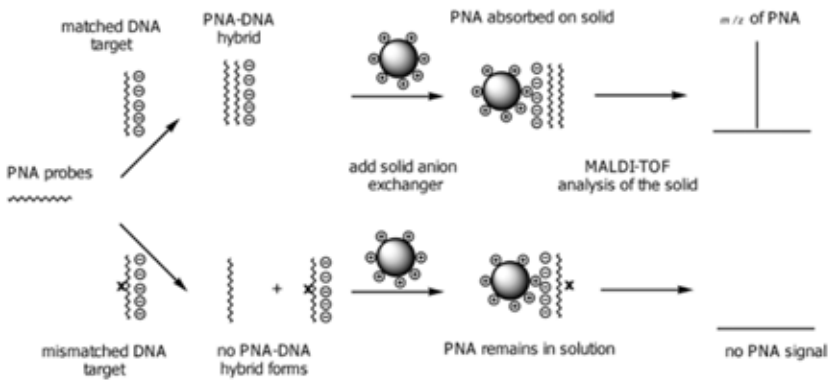


Figure 1.4 Schematic diagram showing the concept of ion-exchange capture of PNA in combination with MALDI-TOF MS in DNA sequence determination[5]

Ananthanawat *et al.* developed DNA sensors based on quartz crystal microbalance (QCM)[6] and surface plasmon resonance (SPR) techniques[7] employing acpcPNAs as probes **(Figure 1.5)**. The immobilized acpcPNA probe was shown to retain the mismatch discrimination ability. However, the direct immobilization of thiolated acpcPNA gave a relatively poor limit of detection (i.e. 0.2 μM) and low hybridization efficiency (<20%) presumably due to the too high probe density that restricts the accessibility of the target DNA. They demonstrated that the use of biotin–streptavidin–biotin chemistry for probe immobilization **(Figure 1.5)** improves the accessibility of the immobilized probes, leading to a much higher hybridization efficiency and lower limit of detection. In addition, they also used SPR technique to demonstrate the performance and hybridization properties of acpcPNA in comparison with DNA, and aegPNA in term of base-pairing specificity or ability to discriminate single base mutation, direction of binding (parallel or antiparallel) in a standard buffer and at room temperature.[8]

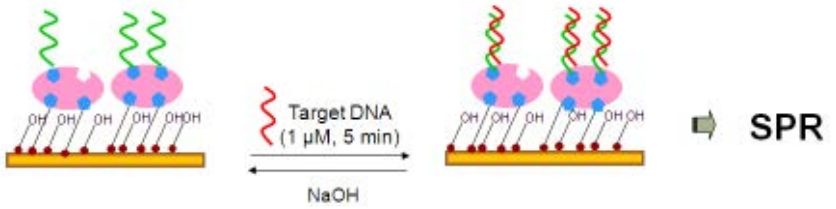


Figure 1.5 Schematic diagram showing immobilization of acpcPNA as a new sensing probe via biotin–streptavidin–biotin chemistry for detection of DNA hybridization using surface plasmon resonance (SPR).[8]

DNA-based biosensor has also been developed using fluorescence technique. In 2011, Boonlua *et.al*[9] developed a quencher-free, singly-labeled PNA beacon using a base-discriminating fluorescent (BDF) concept based on the conformationally rigid pyrrolidinyl peptide nucleic acid (acpcPNA) system **(Figure 1.6)**. A pyrene-labeled uridine (UPy) modified acpcPNA (UPy-modified acpcPNA) was synthesized. It was demonstrated that the UPy base in acpcPNA can specifically recognize adenine in DNA targets. Most importantly, the fluorescence of the UPy-modified acpcPNA increased significantly in response to the specific base pairing event between UPy and dA. As a result, the UPy-modified acpcPNA is potentially useful as a hybridization-responsive fluorescent probe for DNA sequence determination.

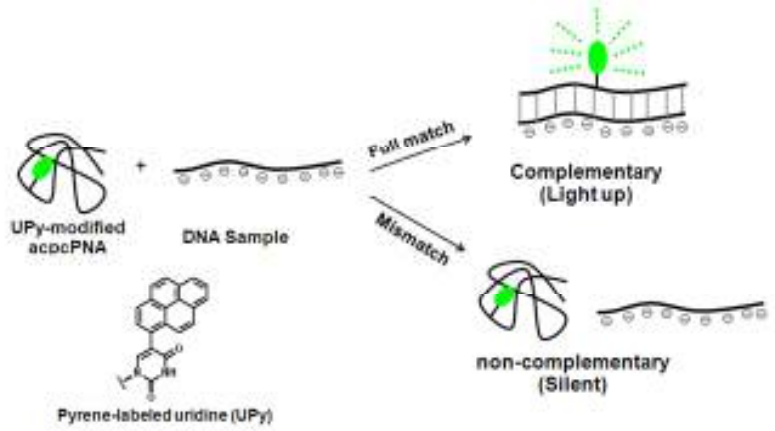


Figure 1.6 Schematic diagram showing a quencher-free, singly-labeled PNA beacon using a base-discriminating fluorescent (BDF) concept based on a pyrene-labeled uridine (UPy) modified the conformationally rigid pyrrolidinyl peptide nucleic acid (UPy-modified acpcPNA) system.[9]

In 2012, Rashatasakhon, and coworkers[10] demonstrated dendritic polycationic phenyleneethynylene fluorophores are investigated as a Förster resonance energy transfer (FRET) donor for the detection of DNA hybridization in conjunction with a fluorescein-labeled pyrrolidinyl peptide nucleic acid (Fl-acpcPNA) probe **(Figure 1.7)**. The sensing system readily detected fully complementary sequence DNA at submicromolar concentration level and distinguished it from the DNA with a single mismatch base. The cationic phenyleneethynylene dendrimer showed even higher FRET signal for detecting the target DNAs with extra hanging

nucleotide sequences that should be useful for detecting PCR-amplified or genomic DNA targets in real applications.

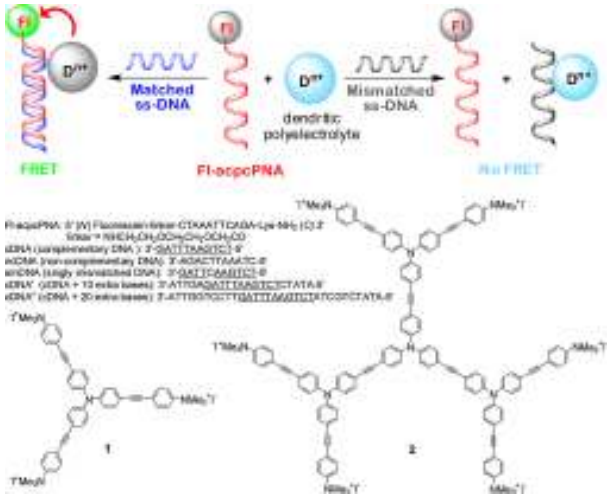


Figure 1.7 Proposed detection of DNA/PNA hybridization via FRET from polycationic dendritic fluorophores Dn+ (1 and 2) including their chemical structures and sequences of Fl-acpcPNA and DNAs used.[10]

1.2 Signal amplification for DNA biosensor

Dot blot hybridization is a technique widely used in molecular biology and genetics for detecting biomolecules. The sample containing the biological target (mostly proteins or DNA) to be detected is spotted directly on a membrane (such as nitrocellulose, nylon 66 and poly(vinylidene fluoride) (PVDF)) without prior separation. The presence or absence of a specific target can be detected by binding with a probe that can report the binding event by radioactivity, fluorescence, chemiluminescence, enzyme-based colorimetric assays or polymerization amplification

1.2.1 Radioactive assay

For many years the use of radiolabels was the method of choice for dot blot hybridization with high sensitivity. Quantification of results is possible following exposure of signal to autoradiography film or reusable storage phosphor screens in automated imaging systems. However, automated liquid handling of radioactivity is difficult due to the need for safe handling and disposal of radioactivity, and is generally limited to manual, low-throughput applications.[11]

1.2.2 Fluorescence assay

Fluorescence assay is widely used for DNA detection. Fluorescence dye is labeled directly on DNA hybridization and the signal can be easily visualized by an imaging fluorescence apparatus. Organic fluorescent dyes; fluorescein (FITC), rhodamine derivatives, oregon green, texas red, 488x and pacific blue [12-15] are largely used as labels for DNA sequence determination in the past decade. However, the limit of theses fluorescent dyes is low fluorescence intensity. In order to solve this problem, polymeric nanoparticles (frequently termed nanobeads or nanospheres)[16-17] have been developed to dope with fluorescent dyes for signal amplification. These nanospheres can contain thousands of dye molecules yielding extremely strong fluorescence. However, a major drawback for the use of polymeric nanobeads as labels is a high background. This unspecific binding to the surface occurs presumably due to their inherent tendency for agglomeration and precipitation.

1.2.3 Chemiluminescence assay

Chemiluminescence is the production of visible light (luminescence) occurring as a result of a chemical reaction. It can be exploited as a labeling method in nucleic acid hybridization. Chemiluminescence is typically about 2 orders of magnitude more sensitive than fluorescence and more than 4 orders of magnitude more sensitive than chromogenic reactions.[18-20]

1.2.4 Colorimetric assay

In recent years, colorimetric assays based on horseradish peroxidase (HRP)-catalyzed color reactions of the peroxidase substrates (3,3',5',5'-tetramethylbenzidine (TMB), o-phenylenediamine (OPD), and 2,2'-azino-di (3-ethylbenzthiazoline-6-sulfonate)(ABTS)) and H_2O_2 are most common used in DNA detection. Because of its speed and high sensitivity, colorimetric assays had been widely used for detecting target molecules. Below are literatures that describe the use of PNA probe for detecting DNA sequence and colorimetric assay for signal amplification.

In 2007, Zhang, and Appella[21] reported colorimetric sandwich-hybridization assay to detect anthrax protective antigen DNA using PNA probe. In the sandwich-hybridization strategy, one PNA was used as capture probe (PNAα) to recruit complementary DNA to a surface, and another PNA was used as a detection probe (PNAβ) to generate a signal (**Figure 1.8**). This assay was developed into a convenient

96-well plate format in which PNAα was covalently attached to a DNA-Bind plate. A biotin-labeled PNA*β*, in combination with commercially available avidin-horseradish peroxidase conjugate (HRP-avidin) and 3,3',5,5'-tetramethylbenzidine (TMB), was used to generate a signal if the target DNA is present. If a sandwich complex forms on the surface, the strong interaction between biotin and avidin will retain HRP-avidin. The HRP then catalyzed oxidation of TMB, and the enzymatic reaction was stopped by the addition of sulfuric acid, and then absorbance at 450 nm was measured. This detection system was examined the ability to detect protective antigen PA-DNA obtained from two whole cell extract of *B. anthracis*, one that has PA-DNA (Ames 35)and one that lacks PA-DNA (Ames 33). In the most sensitive detection system which 10 zmol of synthetic DNA can be detected, it was clearly able to distinguish 2.5 μg of the two cell lines, giving a colored signal visible to the naked eye.

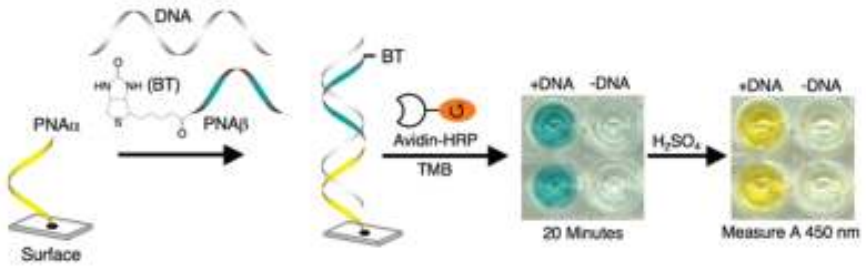


Figure 1.8 Schematic diagram of colorimetric sandwich-hybridization assays to detect anthrax protective antigen DNA using PNA probe.[21]

In 2007, Su *et al.*[22] developed an enzyme-based colorimetric assay for nucleic acids with biotinylated PNA probes immobilized on a SA-coated well plate. The hybridization of nucleic acids brings in a high density of negative charges, to which HRP can be absorbed through electrostatic interactions under a proper pH condition. The adsorbed HRP catalyzes the color reaction of TMB and H_2O_2 for colorimetric detection **(Figure 1.9).** The proposed assay has also been validated with total RNA samples extracted from two human cancer cell lines (A 549 lung cancer cell and HeLa cell) for microRNA detection in real samples. Through extensive optimizations of HRP adsorption and nucleic acid hybridization conditions, detection

limits of 0.1-0.2 nM for DNA (depending on chain length) and ~2 μg of total RNA have been achieved.

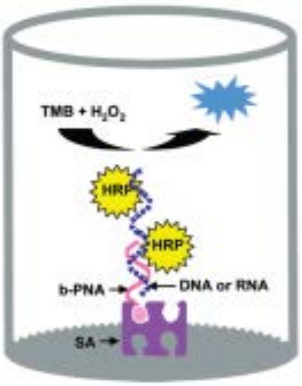


Figure 1.9 Schematic illustration of the colorimetric assay.[22]

1.2.5 Polymerization amplification

Signal amplification by polymerization has been recently emerged as a simple, rapid, sensitive, and inexpensive way to detect DNA binding. In principle, hundreds to millions of times of signals can be amplified through the chain propagation of the designated monomer from the initiator immobilized on biosensor surface under appropriate condition. The resulting polymer would thus change the optical property of the sensing spot, which later becomes visually distinguishable from the background.

The polymerization reactions have been successfully demonstrated for DNA detection including atom transfer radical polymerization (ATRP), activators generated by electron transfer for atom transfer radical polymerization (AGET ATRP), reversible addition-fragmentation chain transfer polymerization (RAFT), and photopolymerization.

In ATRP technique, the transition metal catalyst (M_t^n/ligand) reacts with an alkyl halide initiator, generating a radical and a transition metal complex by transfer of the halogen (X) to the catalyst (**Figure 1.10**). As the radical propagates by reaction with monomer (M), it is rapidly deactivated by reaction with the oxidized transition metal halide (X-M_{tn+1}/Ligand), to re-form the original catalyst and an oligomeric alkyl halide. This process repeats itself, with all chains growing in short, controlled steps, resulting in polymers with molecular weights defined by $DP_n = \Delta[M]/[I]_0$, where $[I]_0$

is the original initiator (alkyl halide) concentration, and narrow molecular weight distributions, Mw/Mn < 1.5.

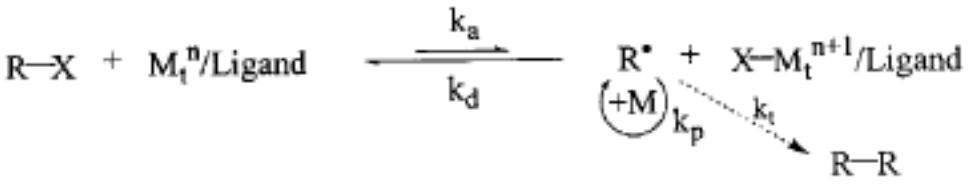


Figure 1.10 Schematic of ATRP mechanism.[23]

The application of this process for DNA sensing allowed polymer growth quantitatively at specific DNA binding site **(Figure 1.11)**.

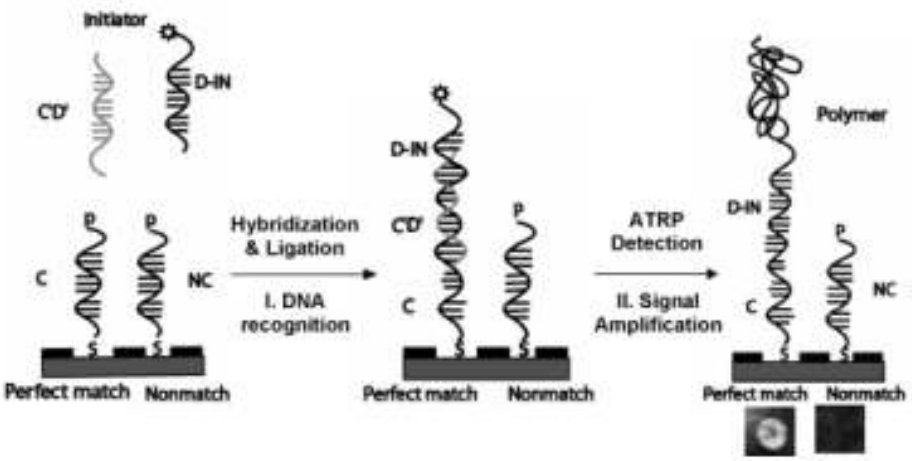


Figure 1.11 Schematic drawing of ATRP-assisted DNA detection.[24]

However, in all cases, the polymerization-based amplification step has been conducted in an oxygen-free environment because oxygen quenches propagating radicals. To solve this problem, AGET ATRP has been developed by using reducing agent to bring the Cu (II) complexes back to the corresponding ATRP-active, lower oxidative state of catalytic complexes. During this redox cycling process, oxygen is consumed prior to the onset of polymer growth. Therefore, all agents can be thoroughly mixed in the presence of air, and the reducing agent can be added at a controlled rate. In 2009, Qian and He[25] reported the employment of AGET ATRP to achieve signal amplification upon DNA hybridization. The detection limit was estimated at ~0.2 pmol, with better sensitivity achievable by extending the reaction time. Detection of single point mutation using AGET ATRP was highly sequence

specific. Together, AGET ATRP was demonstrated as a promising solution for qualitative recognition of specific DNA sequences with better reproducibility, simpler assay procedure, and faster assay turn-around than previously reported method, which gaves the way for future development of portable DNA sensors for point-of-need applications (**Figure 1.12**).

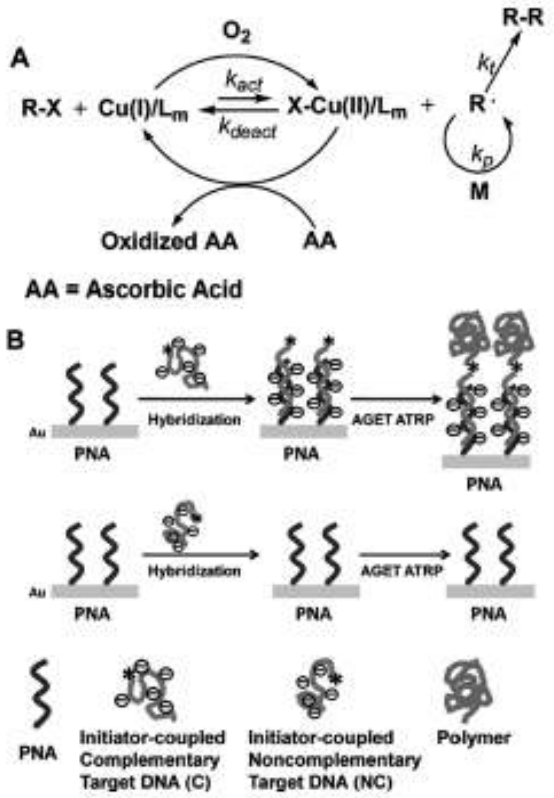


Figure 1.12 AGET ATRP-assisted DNA detection.[25]

RAFT polymerization operates on the principle of a reversible chain transfer process, facilitated by chain transfer agents (CTAs, containing thiocarbonylthio groups) attached on DNA molecules. The initial radicals formed using conventional thermal, photochemical, redox, or γ-irradiation methods attack the monomers and form P• that start chain growth. P• interacts with CTA preferably and CTA-containing Px•, Py•, or Pz• are formed in solution and on the surface simultaneously (**Figure 1.13**). The oscillating nature of CTA plays a critical role in RAFT that enables the "controlled" growth of polymer chains without compromising the growth rate. Through this technique, as low as 2000 copies of DNA can be visualized.[26]

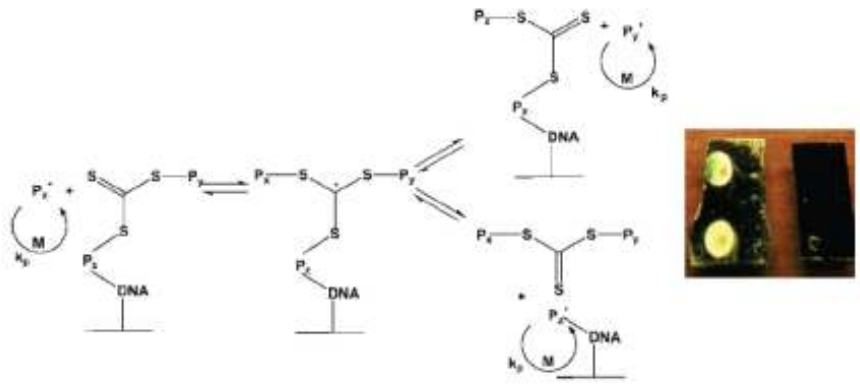


Figure 1.13 Mechanism of RAFT polymerization on DNA covered surface. The image (right side) illustrates the polymer growth from 1 fM target DNA concentration applied on Au surface.[26]

In the application of photoinitiated free radical polymerization in DNA sensing, rapid polymerization starts after photo irradiation of polymer mixture. In 2008, Hansen *et al.* developed a non-enzymatic method for quantifying surface concentrations of labeled DNA targets by coupling regulated amounts of polymer growth to complementary biomolecular binding on array-based biochip **(Figure 1.14)**. Polymer film thickness measurements in the 20–220 nm range vary logarithmically with labeled DNA surface concentrations over two orders of magnitude with a lower limit of quantitation at 60 molecules/μm^2 ($\sim 10^6$ target molecules). In an effort to develop this amplification method towards compatibility with fluorescence-based methods of characterization, incorporation of fluorescent nanoparticles into the polymer films was also evaluated. The resulting gains in fluorescent signal enable quantification using detection instrumentation amenable to point-of-care settings.[27]

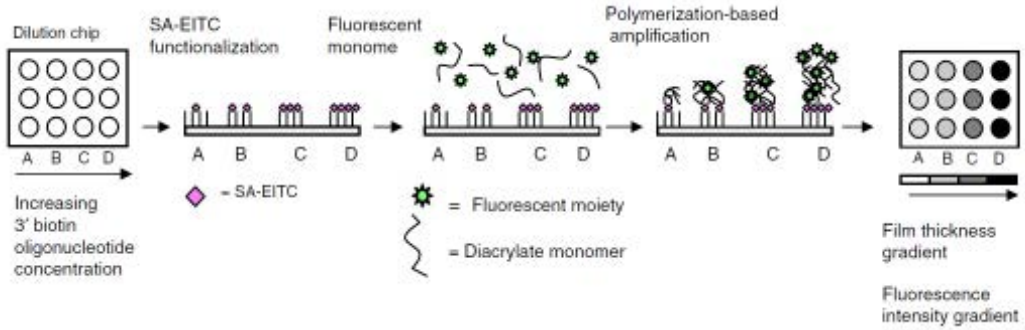


Figure 1.14 Schematic diagram of photopolymerization amplification for DNA detection.[27]

1.3 Cellulose grafted with polymer brushes

Polymer brushes are ultrathin polymer coatings consisting of end-tethered (grafted, anchored) polymer chains stretched away from the substrate or the interface. In the past decade, the polymer brushes have been widely used to modify surface to give new materials for desirable application.[28-33] Surface-initiated polymerization (SIP), such as atom transfer radical polymerization (ATRP) is one technique commonly used in preparation of polymer brushes with predetermined degrees of polymerization and narrow polydispersities. In addition, ATRP is especially attractive due to its mild reaction conditions (room temperature in many cases); use of readily available catalysts, initiators and monomers; and tolerance to impurities.[34-37]

However, one of the disadvantages of ATRP, which limits its widespread industrial utilization, is that the transition metal complexes have to be removed from the reaction mixture and preferably recycled. To reduce the residual transition metal in the final product, activators regenerated by electron transfer or ARGET ATRP has been developed by Matyjaszewski and coworkers.[36,38] In ARGET ATRP, an inactive Cu (II) salt, which is added from the beginning of the polymerization, is rapidly reduced to an active Cu (I) species by a reducing agent. These methods allow ATRP to be conducted with significantly lower concentrations of catalyst in a few ppm levels (**Figure 1.15**).

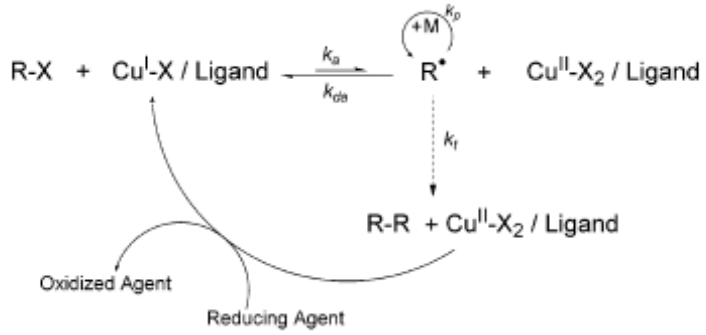


Figure 1.15 Proposed mechanism for activators regenerated by electron transfer for atom transfer radical polymerization (ARGET ATRP).[38]

Among all solid substrates used for grafting polymer chains, celluloses has been widely popular because cellulose is an abundant, inexpensive, biodegradable and renewable biopolymer exhibiting very good mechanical properties. In addition, the hydroxyl groups on the cellulose can easily react with initiator groups.

In 2002, Carlmark and Malmström[39] firstly reported cellulose fibers, in the form of a conventional filter paper, have been successfully grafted with poly(methyl acrylate) (PMA) using ATRP, mediated by Me_6-TREN and Cu(I)Br. The initially hydrophobic cellulose was first modified by reacting the hydroxyl groups with 2-bromoisobutyryl bromide where upon methyl acrylate was grafted from the surface. The resulting polymer grafted papers were extremely hydrophobic, ($\theta_a = 133°$). FT-IR analysis indicates that the grafted amount can be controlled.

Later, the same group of authors[40] reported the grafting of PMA and block copolymer of PMA and PHEMA on cellulose fibers using ATRP method and characterize the materials by gravimetric analysis, FT-IR, ESCA, and AFM. To verify that the polymerization initiated from the surface was “living” and the chain end of the first layer of PMA was still active, a second layer hydrophilic poly(2-hydroxyethyl methacrylate) (HEMA), was grafted on the PMA layer, creating a block copolymer grafting on the fibers. After the layer of PHEMA had been attached, FT-IR revealed a large increase in the carbonyl content and the former hydrophobic paper having PMA became hydrophilic. This suggests that the hydrophilic/hydrophobic behavior of a cellulose surface can be tailored by surface-initiated polymerization via ATRP.

In 2004, Lee and coworkers[31] reported the use of ATRP for growing long chain, low polydispersity polymers on Whatman no.1 filter paper and on amino glass slides using DMAEMA as a monomer (**Figure 1.16**). The tertiary amino group of the DMAEMA, which was pendant to the main chain of the polymer, was then easily quaternized by ethyl bromide to provide an effective biocidal functionality. The permanence of the antimicrobial activity was demonstrated through incubating the modified materials with either *Escherichia coli* or *Bacillus subtilis*. Quaternary ammonium group are believed to cause cell death by disrupting cell membranes allowing release of the intracellular contents. Atomic force microscopic imaging of cells on modified glass surfaces supports this hypothesis.

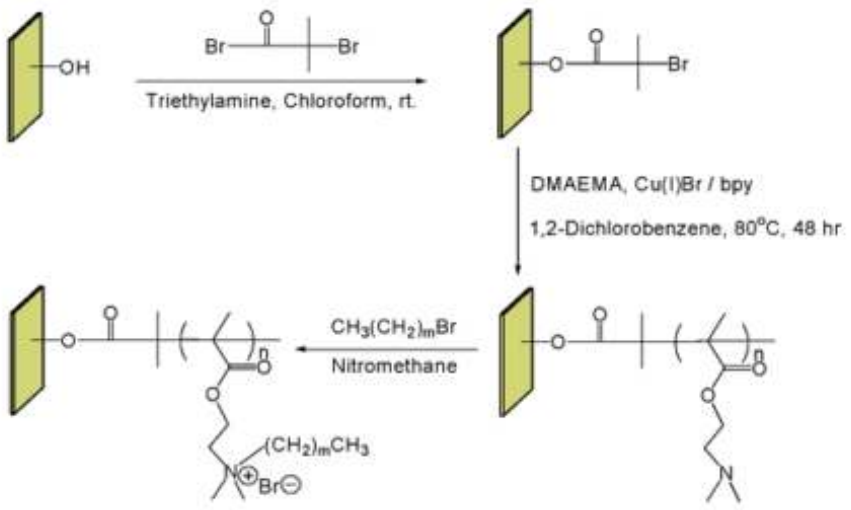


Figure 1.16 Schematic representation showing preparation of quaternized PDMAEMA grafted filter paper for antibacterial assessment.[31]

In 2009, Hansson *et al*.[27] applies ARGET ATRP for grafting methyl methacrylate (MMA), styrene (St), and glycidyl methacrylate (GMA) on cellulose. The grafted filter papers were evaluated by FT-IR. The results suggested that the amount of polymer on the surface increased with increasing monomer conversion. Water contact angle (CA) measurements implied that covering layers of PMMA and PS were formed on the cellulose substrate, making the surface hydrophobic, in spite of low degree of polymerization. The CA of the PGMA-grafted filter papers revealed that, by utilizing either aprotic or protic solvents when washing the substrates, it was possible to either preserve or hydrolyze the epoxy groups. Independent of the solvent

used, all grafted filter papers were essentially colorless after the washing procedure because of the low amount of copper required when performing ARGET ATRP. Nevertheless, surface modification of cellulose via ARGET ATRP truly facilitates the manufacturing since no thorough freeze-thaw degassing procedures are required.

Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) has attracted a significant attention in recent years as a pH and temperature responsive polymer[41] for an increasing number of applications in drug delivery, bioseparation, and microfluidic areas. In addition, quaternized PDMAEMA (PQDMAEMA) is a good candidate for development of cationic antimicrobial surfaces as described above.[31] Recently, PQDMAEMA brushes covalently attached to a range of solid surfaces have demonstrated high levels of antibacterial activity.[31] Well-defined PDMAEMA (co)polymers, block copolymers, and surface tethered brushes have been successfully prepared via ATRP due to its tolerance to a variety of functional groups on the monomers.[33,42]

Because of limitation of ATRP as previous described in above, Dong and Matyjaszewski[43] developed an ARGET ATRP for the preparation of well-defined PDMAEMA homopolymer with and without the addition of external reducing agents in the presence of limited amounts of air. The DMAEMA monomer, containing tertiary amine group, can serve as an internal reducing agent in ARGET ATRP, but can also compensate for the presence of limited amounts of oxygen impurities **(Figure 1.17)**. This technique can be potentially applied to other monomers containing functional reducing moieties, such as amines or phenols. In addition, ARGET ATRP was successfully applied to the synthesis of PDMAEMA brushes from the surfaces of silicon wafers with grafting density ~0.3 chains/nm^2.

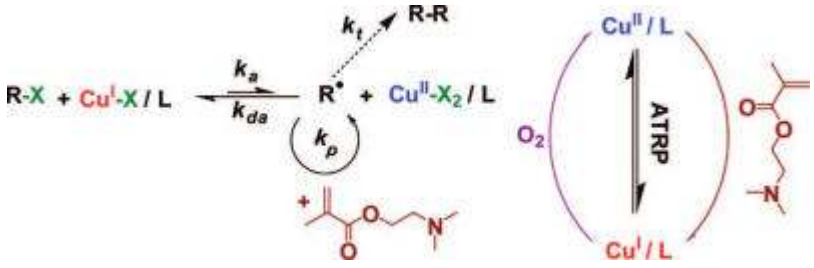


Figure 1.17 Schematic diagram of ARGET ATRP of DMAEMA with internal reducing agent in the presence of air.[43]

1.4 Statement of Problem

As described above, acpcPNA possess a high potential to be applied as probe to detect DNA base sequence with various techniques including QCM[6], SPR[7-8], fluorescence[9-10], and MALDI-TOF mass spectrometry.[5] However, these techniques require advanced instruments that are suitable for only in well-equipped laboratories and hence cannot be used on-site in remote or rural areas having limited clinical experts and medical facilities.

Therefore, this research is interested to apply acpcPNA for DNA sequence determination following "Dot blot hybridization" because this technique can analyze multiple samples inexpensively with high accuracy and in a high-throughput fashion. In development of dot blot hybridization, three major components must be considered including the membrane, the probe and the detection method. Commercial membranes; nitrocellulose[45-46] PVDF[47] and nylon 66[48] are generally hydrophobic due to undesirable non-specific adsorption between membrane and PNA. To reduce this non specific interaction, strong buffer solution containing metal ion ($MgCl_2$)[49], organic solvent (acetonitrile, formamide)[5,45,48-49], anionic detergent (sodium dodecyl sulfate)[5,45,48-49] or combinations there of must be employed, which may negatively affect the PNA·DNA hybridization. In order to solve this problem, this research aims to develop a hydrophilic and positively charged membrane from inexpensive filter papers to selectively capture DNA-PNA, including the ability to prevent non specific adsorption of other non-DNA components.

To prepare a positively charged membrane, the filter paper was surface-modified by grafted polymer brushes of quarternized poly(2-(dimethylamino)ethyl methacrylate (QPDMAEMA)[39-40,50-51] via surface-initiated activator regenerated by electron transfer atom transfer radical polymerization (ARGET ATRP).[43,52-53] ARGET ATRP is a robust and versatile technique that requires only low concentrations of transition metal catalysts and can accurately control the density of the charge which depends on the length of the polymer.

The QPDMAEMA grafted filter paper should readily capture the negatively charged DNA, but not the neutral acpcPNA. Only when the sequences of the DNA and the modifier labeled acpcPNA probe (m-PNA) are complementary that the probe will be immobilized, through hybridization with the surface-bound DNA. The non-

complementary as well as the excess PNA probes may be removed by simple washing (**Figure 1.18**).

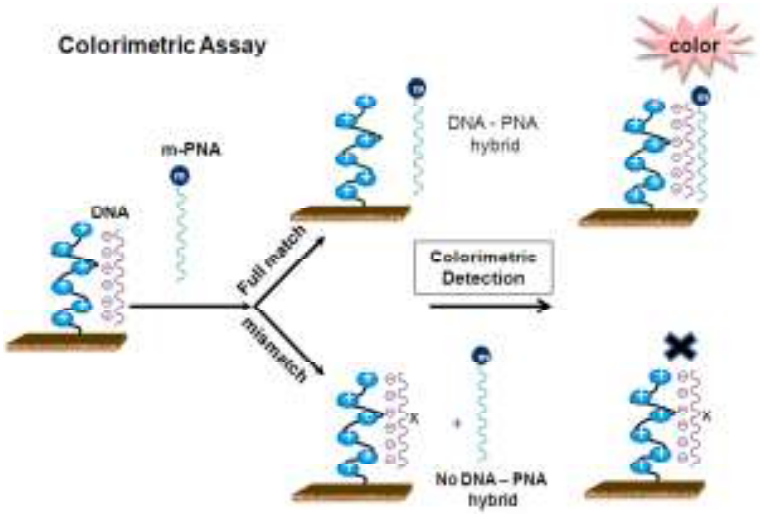


Figure 1.18 Schematic representation of concept of DNA sequence determination following "Dot blot hybridization" using filter paper functionalized with positively charged polymer brushes and peptide nucleic acid probe.

The presence of the m-PNA·DNA hybrid on the membrane can be visualized by a colorimetric assay employing either enzymatic amplification and polymerization amplification (**Figure 1.19**). In enzymatic amplification mode, acpcPNA end-functionalized with biotin was used as a probe. The complementary b-PNA-DNA hybridization can be visualized by naked eye observation of the yellow product generated by the enzymatic amplification employing horseradishes peroxides (HRP) labeled streptavidin (SA-HRP) and a chromogenic substrate such as *o*-phenylenediamine (OPD).[21-22,45-46,49,54-55] In polymerization amplification mode, acpcPNA end-capped with ARGET ATRP initiator (I-PNA) was used as a probe while rhodamine B-functionalized 2-hydroxyethylmethacrylate (HEMA-Rh B) and poly(ethylene glycol)methacrylate (PEGMA) were used as monomers. The signal of I-PNA-DNA hybridization can be amplified through the chain propagation of these monomers from the initiator end caped-PNA to obtain red color of copolymer on QPDMAEMA grafted filter paper.

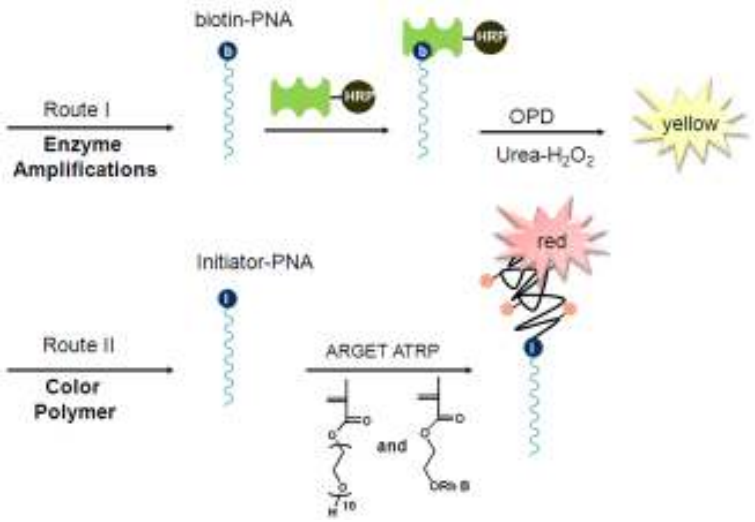


Figure 1.19 Schematic of signal amplification for DNA·PNA hybridization determination.

1.5 Objectives

1. To prepare and characterize quaternized poly(2-(dimethylamino)ethyl methacrylate (QPDMAEMA) grafted filter paper (QPDMAEMA grafted filter paper)
2. To determine DNA sequence following "Dot blot hybridization" employing QPDMAEMA grafted filter paper as membrane, acpcPNA as probe and colorimetric assay as the detection method.

1.6 Scope of investigation

1. Literature survey for related research work
2. Preparation and characterization of QPDMAEMA grafted filter paper by SIP via ARGET ATRP
3. Synthesis of biotin labeled acpcPNA and initiator end-capped acpcPNA
4. DNA sequence determination following "Dot blot hybridization" employing QPDMAEMA grafted filter paper as membrane, acpcPNA as probe and colorimetric assay as the detection method using 2 routes for signal amplification:
   - 4.1 Enzymatic amplification
   - 4.2 Polymerization amplification

CHAPTER II

METHODS AND MATERIALS

2.1 Materials

A filter paper, Whatman No.1, was used as membrane. 2-(Dimethylamino)ethyl methacrylate (DMAEMA) (Aldrich, 98%), polyethylene glycol methacrylate (PEGMA) (Aldrich, 98%), 2-hydroxyethyl methacrylate (HEMA) polyethylene glycol methacrylate (PEGMA) (Aldrich, 98%), was passed through a column filled with basic alumina to remove the inhibitor. The ligand, tris[2-(dimethylamino)ethyl]amine (Me_6TREN) was synthesized according to literature procedures from tris(2-aminoethyl)amine (98%, Aldrich).[56] Rhodamine B (Aldrich, 98%), Ethyl 2-bromoisobutyrate (EBiB) (Fluka, >97%), 2-bromoisobutyryl bromide (Aldrich, 98%), 2-bromoisobutyric acid (Sigma-Aldrich, ≥ 98%), copper (II) bromide (Fluka, 99%), tin(II) 2-ethylhexanoate ($Sn(EH)_2$) (Aldrich, 95%), L-ascorbic acid (AA) (Sigma-Aldrich, > 99%), 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid (Sigma-Aldrich, > 97%), 4-*N,N*-dimethyl aminopyridine (DMAP) (Fluka, 95%), methyl iodide (Aldrich, >99%), streptavidin-horseradish peroxidase enzyme (SA-HRP) complexes (Sigma-Aldrich, Protein, ≥80%, 80-150units/mg protein), o-phenylendiamine (OPD) (Aldrich, 98%), 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich, ≥99%), urea-peroxide (Urea-H_2O_2) substrate kit (Merk, for analysis 35%), BSA (Sigma-Aldrich, >96%), Blotting-Nylon 66 membranes, type B, positive sheet (Fluka, 0.45 μm pore size, surface (quaternary ammonium groups) 100%), Hybond ECL Nitrocellulose Membrane or Amersham Hybond™ ECL™ (GE Healthcare Life Sciences, 0.45 μm pore size, Unsupported, 100% pure nitrocellulose membrane) were used as received.

All used solvents were purchased from Fluka (Switzerland), Merck (Germany), Aldrich Chemical Co., Ltd. (USA). Solvents for reactions are reagent grade and used without purification, otherwise specified. Dichloromethane was dried over CaH_2 under reflux and nitrogen atmosphere. Tetrahydrofuran (THF) was dried over sodium and benzophenone under reflux and nitrogen atmosphere. Nitrogen gas was obtained from

TIG. Oligonucleotides were purchased from Bioservice Unit, National Science and Technology Development Agency (Thailand). Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step.

2.2 Equipment

2.2.1 Nuclear magnetic resonance (NMR) spectroscopy

^{1}H and ^{13}C NMR spectra were recorded in solution of $CDCl_3$ or DMSO-d_6 on a Varian Mercury-400 nuclear magnetic resonance spectrometer operating at 400 MHz. Chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (TMS).

2.2.3 Fourier transform-infrared spectroscopy (FT-IR)

Infrared spectra of materials scraped from the surface-modified filter paper and prepared as KBr pellets were collected on a Nicolet Impact 6700 FT-IR spectrometer with 32 scans at a resolution of 4 cm^{-1} in a frequency range of 400-4000 cm^{-1}. IR spectra of copolymers of Rh B-HEMA-co-PEGMA on QPDMAEMA grafted filter paper were obtained using ATR FT-IR mode.

2.2.3 Scanning electron microscopy (SEM)

The surface morphology of the filter paper before and after stepwise modification was observed by SEM on a JEOL (JSM-6480LV, Japan) instrument.

2.2.4 Contact angle measurements

The water contact angles were measured in air at ambient temperature using a contact angle goniometer, model 200-F1, equipped with a Gilmont syringe and a 24-gauge flat-tipped needle (Ramé-Hart, USA). The data for each sample was taken from five different areas of the substrate and analyzed by DROPimage standard 2.0, after which they were expressed as the arithmetic mean value ± standard deviation (SD).

2.2.5 Size exclusion chromatography (SEC)

The molecular weight and polydispersity index of the free poly(2-(dimethylamino)ethyl methacrylate (PDMAEMA) formed in solution were measured by a Waters GPC system (USA), performed at 35 °C using THF (1.0 $mL.min^{-1}$) as the mobile phase and a Waters E600 column connected to the RI detector. Narrow polystyrene standards were used for generating a calibration curve.

2.2.6 X-ray photoelectron spectroscopy (XPS)

The surface composition of the surface-modified filter paper was characterized by XPS on a Physical Electronics Quantum 2000, using monochromatic Al k-alpha X-Rays, and standard dual ion-electron neutralization.

2.2.7 MALDI-TOF mass spectrophotometry

The mass spectra of acpcPNA were recorded on a Microflex MALDI-TOF mass spectrometry (Bruker Daltonics, Germany). Sample (1 μL) was mixed with 10 μL of the matrix solution consisting of α-cyano-4-hydroxycinnamic acid (CCA) in 0.1% TFA in acetonitrile/water (1:1) solution. This mixture (1 μL) was deposited onto the target, allowed to dry, and analyzed in positive ion linear time-of-fight mode with an accelerating voltage +20 kV. All spectra were performed by averaging between 20 and 30 individual laser shots.

2.2.8 UV-Vis spectrophotometer

Melting temperature (T_m) measurements of PNA·DNA complex was performed on a CARY 100 Bio UV-Visible spectrophotometer (Varian, Inc., USA) equipped with a thermal melt system.

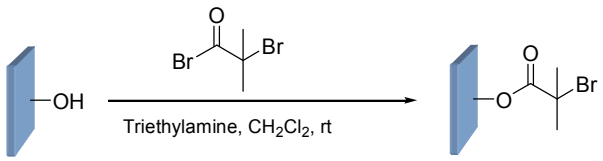
2.2.9 Scanner

The scanned images of the tested results on filter papers were recorded on XEROX WorkCentre 3119 scanner in 24 bits RGB mode. The brightness/contrast/resolution were set to 128/128/300. The images were saved as TIFF-files. The intensity of each spot was determined using Scion Image software by first converting to gray scale at 300 dpi. Intensity measurements were carried out using the Line tool to select area for analysis to obtain profile images.

2.3 Experimental procedure

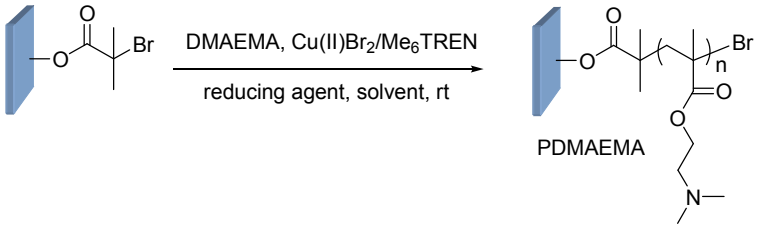
2.3.1 Preparation of quaternized poly(dimethylamino)ethyl methacrylate (QPDMAEMA) grafted filter paper.

2.3.1.1 Preparation of initiator immobilized filter paper



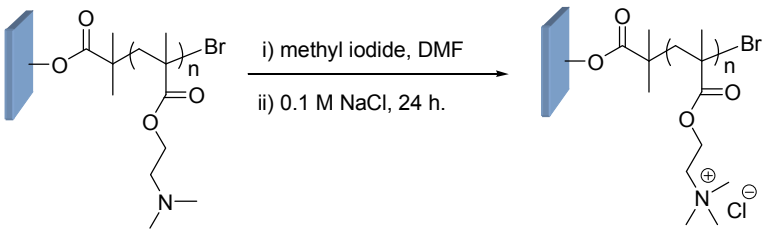
Initiator immobilized filter paper was prepared by a modification of Carlmark's protocol. The filter paper (3.5 x 8 cm^2) was washed with dichloromethane and ultrasonicated for two min prior to immerse in a solution mixture of triethylamine (2.40 mL, 17.0 mmol) and a catalytic amount of 2-dimethyl aminopyridine (DMAP) (39 mg, 0.3 mmol) in 25 mL dichloromethane under stirring 10 min. Then, 2-bromoisobutyrylbromide (0.60 mL, 1.90 mmol) was slowly dropped to the solution. The reaction was performed for 16 h at ambient temperature with gentle agitation. After the reaction was complete, the filter paper was thoroughly rinsed with dichloromethane following with methanol and then air-dried.

2.3.1.2 Surface-initiated polymerization of (dimethylamino)ethyl methacrylate (DMAEMA) on initiator immobilized filter paper by ARGET ATRP



The initiator immobilized filter paper was placed in a 32 mL scintillation vial containing DMAEMA (16.8 mL, 100 mmol), $CuBr_2$ (1.2 mg, 0.005 mmol), Me_6TREN (57.0 mg (0.25 mmol), and EBiB (74 μL, 0.5 mmol) dissolved in 4.5 mL acetone. After stirring for 10 min, a solution of $Sn(EH)_2$ (490 mg, 1.2 mmol) in 3 mL acetone was then added toto the mixture. The vial was then sealed with a rubber septum. The volume of free space above the solution was 7.7 mL. The reaction was left stirring for a set reaction time at ambient temperature. The filter paper was taken out from the vial, successively washed with THF and MeOH to remove the residual monomer and the catalyst and was finally air-dried to yield PDMAEMA grafted filter paper.

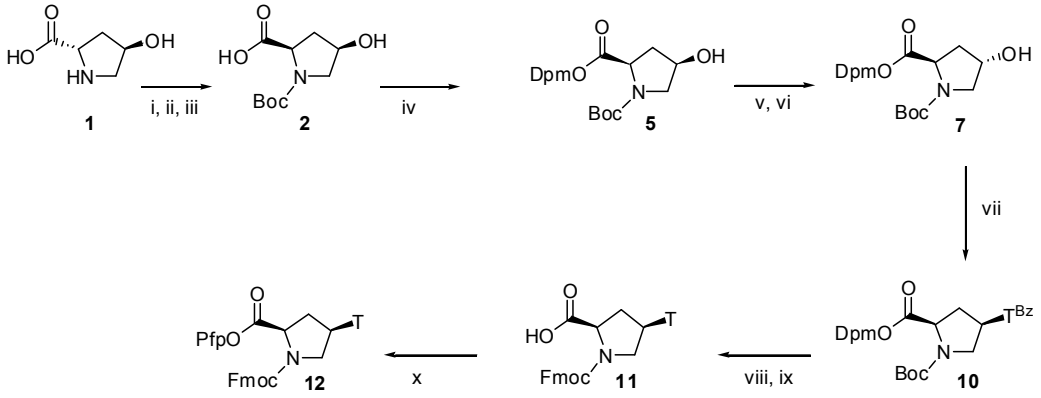
2.3.1.3 Preparation of quaternized poly(dimethylamino)ethyl methacrylate (QPDMAEMA) grafted filter paper



The PDMAEMA grafted filter paper was placed in 25 mL DMF containing excess amount of methyl iodide (100 μL). After stirring for 20 h, the filter paper was removed from the solution and washed three times using MeOH followed by THF under sonication and then air-dried. The filter paper was immersed in 100 mL of 0.1M NaCl solution for 24 h, soaked in 500 mL of deionized water for 48 h and was finally air-dried to obtain QPDMAEMA grafted filter paper.

2.3.2 Synthesis of intermediate and activated acpcPNA monomers

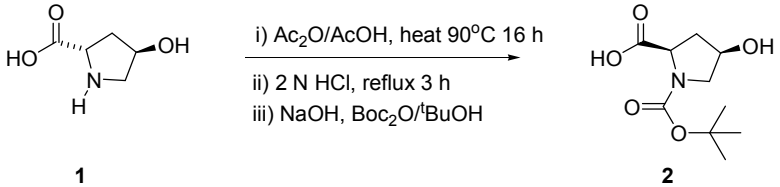
Fmoc-T-OPfp (12)



Scheme 2.1 Synthesis of intermediate and activated acpcPNA monomers Fmoc-T-OPfp **(12)**; i) Ac_2O/AcOH, heat 90 ^oC 16 h; ii) 2 M HCl, reflux 5 h; iii) Boc_2O, ^{t}BuOH, NaOH (aq), overnight; iv) Ph_2CN_2, EtOAc, overnight; v) HCO_2H, Ph_3P, DIAD, THF, overnight; vi) NH_3, MeOH, 2 h; vii) N^3-T^{Bz}, Ph_3P, DIAD, THF, overnight; viii) TFA, anisole; ix) FmocOSu, $NaHCO_3$, H_2O , MeCN; x) Pfp-OTfa, DIEA, CH_2Cl_2

2.3.2.1 Synthesis of intermediate for pyrrolidinyl monomers

cis-4-hydroxy-D-proline (2)

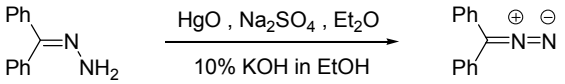


cis-4-Hydroxy-L-proline **(2)** was synthesized from *trans*-4-hydroxy-L-proline **(1)** (29.46 g, 224.3 mmol), acetic anhydride (80 mL) and acetic acid (80 mL) at 90 °C under purged nitrogen for 16 h. After reaction was complete, the reaction mixture was allowed to cool at ambient temperature. Water (50 mL) was added and then the mixture was removed by rotary evaporator at 60 °C to give dark thick oil. The crude product was dissolved in 2 M hydrochloric acid (100 mL) and then refluxed for another 2 h.

After solvent removal, the mixtures of *cis*-D and *trans*-L hydrochloride as dark thick oil were obtained. These were redissolved in solution of NaOH (19.52 g, 488 mmol) in water (100 mL) at 0 °C and then the solution of Boc_2O (48.89 g, 224.0 mmol) in *tert*-butanol (100 mL) was slowly added for 30 min. The reaction was allowed to stir at ambient temperature for 8 h and a pale brown solid was formed. The solvent was removed under reduced pressure to give a crude product, which was dissolved in water (100 mL) and then was extracted with diethyl ether (3 × 20 mL). The collected liquid layer was adjust to pH ~6-7 by adding concentrated HCl then was adjusted to pH ~ 2-3 by adding $NaHSO_4$. The mixture was extracted by ethyl acetate (3 × 50 mL) and then the collected organic layer was removed to give brown oil. This residue was recrystallized by ethyl acetate to give the desired product **(2)** as white solid (13.60 g, 28 % yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.32 [s, 9H, C<u>H</u>$_3$ Boc] 1.80 [m, 1H, C<u>H</u>$_2$(3′)] 2.30 [m, 1H, C<u>H</u>$_2$(3′)] 3.09 [m, 1H, C<u>H</u>$_2$(5′)] 3.46 [m, 1H, C<u>H</u>$_2$(5′)] 4.07, 4.12 [m, 2H, C<u>H</u>(4′) and C<u>H</u>(2′)].

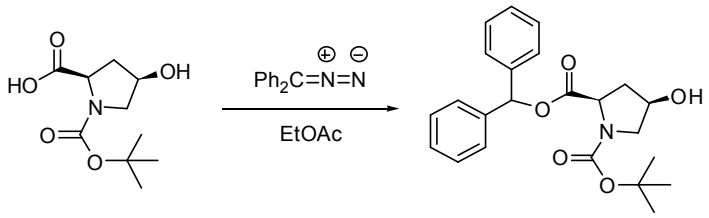
2.3.2.2 Synthesis of diphenyldiazomethane (4)



3 **4**

The 10% potassium hydroxide in ethanol (15 mL) was added dropwise in the mixture of benzophenone hydrazone **(3)** (13.74 g, 70.0 mmol), mercuric oxide (yellow) (15.16 g, 70.0 mmol), anhydrous sodium sulfate (9.94 g, 70.0 mmol) and diethyl ether (150 mL) until the solution turned to purple color under stirring in the dark for 6 h. The solution mixture was filtered to remove the used mercuric oxide and sodium sulfate mixture and washed with diethyl ether. The collected organic solution was removed by rotary evaporation without heating to obtain product **(4)** (9.30 g, 47.9 mmol) as a purple liquid, which was used for the next step without further purification.

2.3.2.3 Synthesis of *N-tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester (5)

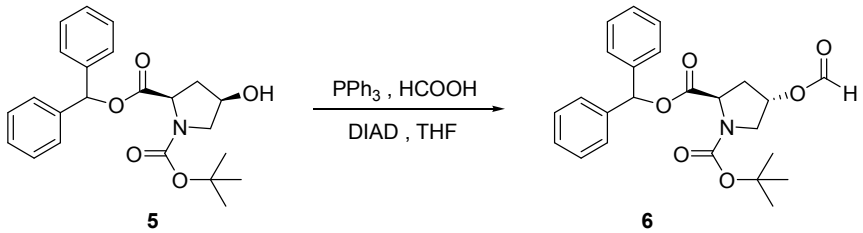


2 **5**

Freshly prepared diphenyldiazomethane **(4)** (9.30 g, 47.9 mmol) diluting with ethyl acetate (30 mL) was added dropwise to the solution of *N*-Boc-*cis*-4-hydroxy-D-proline **(2)** (4.00 g, 17.3 mmol) in ethyl acetate (5 mL) until the purple color persists under stirring in the dark at 0 ^{o}C. The reaction was allowed to stir at ambient temperature for 8 h. If the solution became colorless, more diphenyldiazomethane reagent was added to the reaction mixture until the purple color persists again. The solvent was evaporated and the crude mixture was dissolved in a small amount of ethyl acetate. This solution was added dropwise to a flask containing vigorously stirred hexane (200 mL). White solid precipitated **(5)** was collected by filtration and air-dried (5.43 g, 79 % yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.17, 1.39 [2×s, 9H, C$\underline{H}_3$ Boc rotamers] 1.98 [t, J = 8.4 Hz, 1H, 1×C$\underline{H}_2$(3′)] 2.16-2.33 [br m, 1H, 1×C$\underline{H}_2$(3′)] 3.47-3.55 [m, 2H, C$\underline{H}_2$(5′)] 4.24 [m, 1H, C$\underline{H}$(4′)] 4.34, 4.40 [dd, J = 23.6, 6.2 Hz, 1H, C$\underline{H}$(2′)] 6.81, 6.88 [2×s, 1H, C$\underline{H}$Ph$_2$ rotamers] 7.25 [m, 10H, phenyl C$\underline{H}$].

2.3.2.4 Synthesis of *N*-tert-butoxycarbonyl-trans-4-formyl-D-proline diphenylmethyl ester (6)



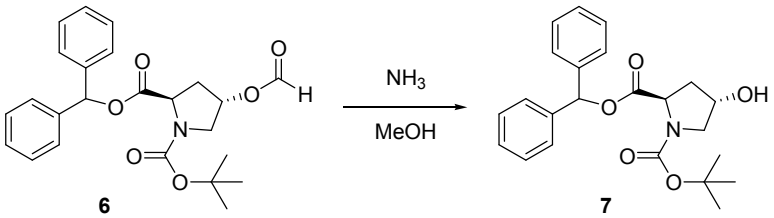
5 **6**

Diisopropyl azodicarboxylate (DIAD) (3.6 mL, 17.8 mmol) was added dropwise via syringe within 15 min to a mixture of *N-tert*-butoxycarbonyl-*cis*-4-hydroxy-D-proline diphenylmethyl ester **(5)** (5.00 g, 12.6 mmol), triphenylphosphine (4.10 g, 15.6 mmol) and formic acid (60 μL, 15.9 mmol) in dry THF (50 mL) under

nitrogen atmosphere at 0 ^{o}C. The reaction was allowed to stir at room temperature for 8 h. The solvent was evaporated and the residue was purified by column chromatography on silica gel using hexane: ethyl acetate (3:1) as eluent to give clear oil. The desired product was recrystallized with ethyl acetate and hexane and obtained as white solid **(6)** (4.06 g, 76 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.27, 1.49 [2×s, 9H, C$\underline{H}_3$ Boc] 2.18 [m br, 1H, 1×C$\underline{H}_2$(3′)] 2.45 [m br, 1H, C$\underline{H}_2$(3′)] 3.64, 3.76 [2×m br, 2H, C$\underline{H}_2$(5′)] 4.53, 4.61 [2×m br, 2H, C$\underline{H}$(4′)] 5.37 [2×m br, 1H, C$\underline{H}$(2′)] 6.93, 6.97 [2×s, 1H, C$\underline{H}$Ph$_2$ rotamers] 7.27-7.36 [m, 10H, phenyl C$\underline{H}$] 7.98 [C$\underline{H}$O].

2.3.2.5 Synthesis of *N*-tert-butoxycarbonyl-trans-4-hydroxy-D-proline diphenylmethyl ester (7)

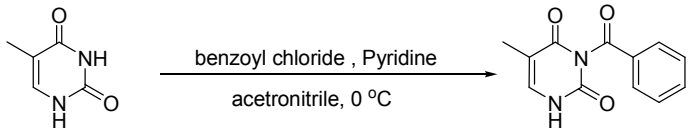


6 **7**

Concentrated aqueous ammonia solution (3 mL) was added to a solution mixture of *trans*-4-formate ester **(6)** and methanol (20 mL) under stirring at ambient temperature. After 1 h, the reaction was completed as monitored by TLC analysis using hexane: ethyl acetate (3:1) as eluent. The solvent was removed under reduced pressure and the oily residue was recrystallized with ethyl acetate and hexane (1: 5) to give white solid **(7)** (3.52 g, 70% yield 2 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.25, 1.48 [2×s, 9H, C$\underline{H}_3$ Boc rotamers] 2.03 [br m, 1H, 1×C$\underline{H}_2$(3′)] 2.25, 2.35 [br 2×m, 1H, 1×C$\underline{H}_2$(3′)] 3.62 [m br, 2H, C$\underline{H}_2$(5′)] 4.44 [br m, 1H, C$\underline{H}$(4′)] 4.56-4.63 [dt, J = 26.8, 7.6 Hz, 1H, C$\underline{H}$(2′)] 6.91, 6.96 [2×s, 1H, C$\underline{H}$Ph$_2$ rotamers] 7.35 [m, 10H, phenyl C$\underline{H}$].

2.3.2.6 Synthesis of N^3-benzoylthymine

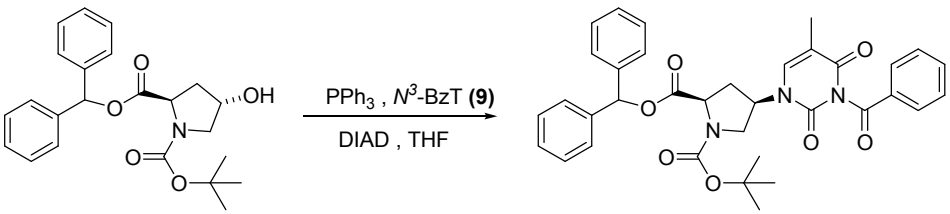


8 **9**

Pyridine (100 mL) was slowly added to the solution of thymine **(8)** (6.00 g, 47.6 mmol) in acetronitrile (150 mL) at 0 °C under stirring. Benzoyl chloride (18.9 mL, 150.0 mmol) was then slowly added and the reaction was allowed to stir at ambient temperature for 8 h. The reaction was monitored by TLC analysis using ethyl acetate: hexane (1:1) as eluent. After reaction completed, water (50 mL) was added to the reaction mixture and the solvent was removed using a rotary evaporator. The residue was recrystallized with methanol and yielded white solid **(9)** (6.00 g, 55% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ_H 1.80 [s, 3H, C<u>H</u>$_3$] 7.51 [s, 1H, C<u>H</u>] 7.59 [t, J = 7.9 Hz, 2H, C<u>H</u>(3,5) Ar] 7.75 [t, J = 7.4 Hz, 1H, C<u>H</u>(4) Ar] 7.92 [d, J = 7.3 Hz, 2H, C<u>H</u>(2,6) Ar].

2.3.2.7 Synthesis of *N*-*tert*-butoxycarbonyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline diphenylmethyl ester (10)

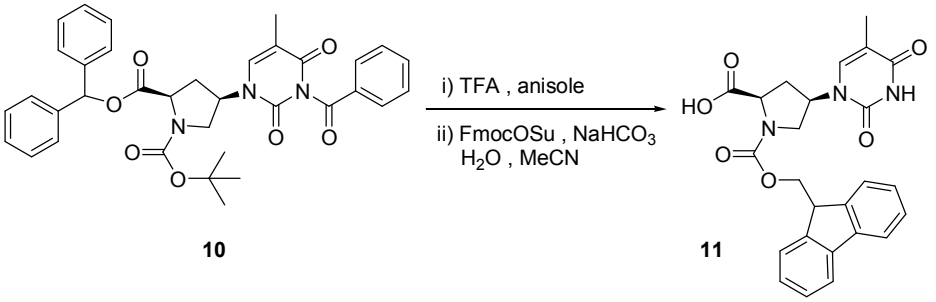


7 10

DIAD (1.2 mL, 6.10 mmol) was slowly added via syringe within 15 min in the stirring mixture of the protected *trans*-4-hydroxy-D-proline **(7)** (1.00 g, 2.52 mmol), triphenylphosphine (4.96 g, 5.57 mmol), N^3-benzoylthymine **(9)** (0.70 g, 3.02 mmol) and dry THF (30 mL) under nitrogen at 0 °C. The reaction mixture was allowed to stir at ambient temperature for 8 h. After the reaction completed (monitered by TLC using hexane: ethyl acetate (2:1) with ammonium molybdate reagent), the solvent was evaporated and the residue was recrystallized by methanol to give the product **(10)** as white solid (0.69 g, 44 % yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.25, 1.29 [s, 9H, C<u>H</u>$_3$ Boc rotamers] 1.78, 1.81 [s, 3H, C<u>H</u>$_3$ Thymine] 2.05 [m, 1H, C<u>H</u>$_2$(3′) Proline] 2.84 [m, 1H, C<u>H</u>$_2$(3′) Proline] 3.54-3.68 [m, 1H, C<u>H</u>$_2$(5′) Proline] 4.00 [m, 1H, C<u>H</u>$_2$(5′) Proline] 4.53, 4.64 [m, 1H, C<u>H</u>(4′) Proline] 5.26 [m, 1H, C<u>H</u>(2′) Proline] 6.92 [s, 1H, C<u>H</u>Ph$_2$] 7.36 [s, 1H, C6<u>H</u> Thymine] 7.35 [m, 10H, Phenyl Dpm] 7.52 [t, J = 7.9 Hz, 2H, C<u>H</u>(3,5) Ar Bz] 7.63 [t, J = 7.4 Hz, 1H, C<u>H</u>(4) Ar Bz] 7.92 [d, J = 7.4 Hz, 2H, C<u>H</u>(2,6) Ar Bz]

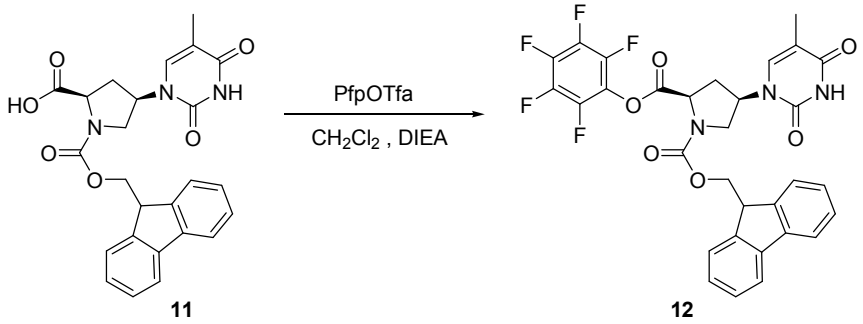
2.3.2.8 Synthesis of (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline (11)



Trifluoroacetic acid (2 mL) was added to the mixture of (*N*-*tert*-butyloxycarbonyl)-*cis*-4-(thymin-1-yl)-D-proline diphenylmethyl ester **(10)** (0.5 g, 0.82 mmol) and anisole (1 mL) under stirring at room temperature for 8 h. The TFA was removed by a gentle stream of nitrogen and then diethyl ether was added to the residue. White solid obtained was collected by filtration, then was immediately dissolved in 1:1, water (H_2O): acetonitrile (MeCN) (5 mL/mmol) and treated with solid $NaHCO_3$ until pH 8 (pH paper). FmocOSu (0.33 g, 0.98 mmol) was added to small portions with stirring at ambient temperature for 8 h and the solvent was removed by rotary evaporation. The residue was diluted with water (10 mL) and extracted with diethyl ether (3 × 20 mL). After purging the extracted aqueous layer to remove the dissolved ether with a gentle stream of N_2, concentrated HCl was added dropwise to adjust pH to 2. The product **(11)** as white solid (0.28 g, 72 % yield) was obtained after filtration, washed with water, diethyl ether and dried under vacuum.

^{1}H NMR (400 MHz; DMSO-d_6): δ_H 1.76 [s, 3H, C$\underline{H}_3$ Thymine] 2.14, 2.22 [2×m, 1H, 1×C$\underline{H}_2$(3′) rotamers] 2.60, 2.70 [2×m, 1H, 1×C$\underline{H}_2$(3′) rotamers] 3.47 [m, 1H, 1×C$\underline{H}_2$(5′) rotamers] 3.86 [m, 1H, 1×C$\underline{H}_2$(5′) rotamers] 4.26 [m, 1H, C$\underline{H}$ Fmoc] 4.30 [m, 2H, C$\underline{H}_2$ Fmoc] 4.44 [m, 1H, C$\underline{H}$(2′) rotamers] 4.97, 5.31 [2×m, 1H, C$\underline{H}$(4′) rotamers] 7.15 [s, 1H, $\underline{H}$6 Thymine] 7.31 [m, 2H, C$\underline{H}$ Fmoc] 7.39 [m, 2H, C$\underline{H}$ Fmoc] 7.59 [d, ^3J(H,H) = 7.0 Hz, 2H, C$\underline{H}$ Fmoc] 7.85 [d, ^3J(H,H) = 7.0 Hz, 2H, C$\underline{H}$ Fmoc] 11.30 [s, 1H, N$\underline{H}$ Thymine].

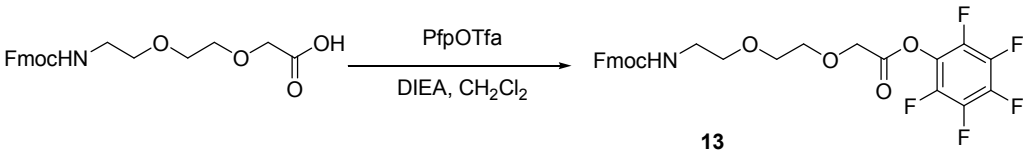
2.3.2.9 Synthesis of (*N*-fluoren-9-ylmethoxycarbonyl)-*cis*-4-(thymin-1-yl)-L-proline pentafluorophenyl ester (12)



Diisopropylehtylamine (DIEA) (167 μL, 0.97 mmol) and pentafluorophenyl trifluoromethyl acetate (PfpOTfa) (168 μL, 0.95 mmol) were added to solution of (*N*-fluoren-9-ylmethoxycarbonyl)-*trans*-4-(thymin-1-yl)-D-proline **(10)** (0.30 g, 0.64 mmol) in dichloromethane (5 mL) under stirring at ambient temperature for 0.5 h. After reaction completed as monitored by TLC using hexane: ethyl acetate (2:1), the solution mixture was diluted with dichloromethane and extracted with sat. $NaHCO_3$ (3x10 mL) following with water (3x10 mL) and 10% v/v hydrochloric acid (HCl) (3x10 mL). The collected organic layer was dried with Na_2SO_4 and then was filtered off. After solvent removal, the oily residue that was dissolved in a small amount of dichloromethane was added dropwise to a flask containing vigorously stirred hexane (20 mL). The product **(11)** as white solid (0.32 g, 83 %yield) was collected by filtered off and dried under vacuum.

^{1}H NMR (400 MHz; $CDCl_3$): δ_H 1.93 [s, 3H; C$\underline{H}_3$ Thymine] 2.33, 2.39 [2×m, 1H; 1×C$\underline{H}_2$(3′) rotamers] 2.97 [m, 1H; 1×C$\underline{H}_2$(3′) rotamers] 3.60, 3.75 [2×m, 1H; 1×C$\underline{H}_2$(5′) rotamers] 3.95, 4.04 [2×m, 1H; 1×C$\underline{H}_2$(5′) rotamers] 4.23 [m, 1H; Fmoc C$\underline{H}$] 4.52 [m, 2H; Fmoc C$\underline{H}_2$] 4.68, 4.76 [2×m, 1H; C$\underline{H}$(2′) rotamers] 5.22, 5.31 [2×m, 1H; C$\underline{H}$(4′) rotamers] 7.15 [s, 1H; T $\underline{H}$6] 7.30 [m, 2H; C$\underline{H}$ Fmoc] 7.39 [m, 2H; C$\underline{H}$ Fmoc] 7.56 [d, ^3J(H,H) = 7.0 Hz, 2H; C$\underline{H}$ Fmoc] 7.76 [d, ^3J(H, H) = 7.0 Hz, 2H; C$\underline{H}$ Fmoc] 10.16 [s, 1H; N$\underline{H}$ Thymine].

2.3.3 Synthesis of activated ethylene glycol (egl) linker, 2-[2-(Fmoc-amino)ethoxy]ethoxyacetic acid pentafluorophenyl ester or Fmoc-egl-OPfp (13)



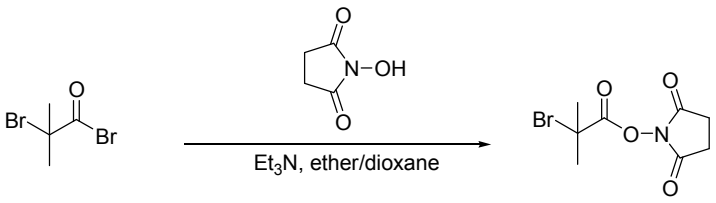
13

The titled compound **(13)** was synthesized according to the same procedure used for compound **12,** except 2-[2-(Fmoc-amino)ethoxy]ethoxyacetic acid (0.10 g, 0.25 mmol) was used in place of (*N*-fluoren-9-ylmethoxycarbonyl)-*trans*-4-(thymin-1-yl)-D-proline **(11)**. The desired compound (**13**) was obtained as white powder (0.180 g, 79 %).

^{1}H NMR (400 MHz; $CDCl_3$): δ_H 3.42 [t, 2H, O-C$\underline{H}_2$-CH_2-N], 3.59 [t, 2H, O-CH_2-C$\underline{H}_2$-O], 3.70 [t, 2H, O-C$\underline{H}_2$-CH_2-O], 3.81 [t, 2H, O-CH_2-C$\underline{H}_2$-O], 4.20 [t, 2H, O-CH_2-C$\underline{H}$-Ar, J = 6.4 Hz], 4.39 [d, 2H, CH2-C$\underline{H}_2$-N, J = 6.4 Hz], 4.51[s, 2H, O-C$\underline{H}_2$-CO2], 5.25 [s, 1H, N$\underline{H}$], 7.29 [t, 2H, Ar$\underline{H}$], 7.39 [t, 2H, Ar$\underline{H}$, J = 7.5 Hz], 7.58 [d, 2H, Ar$\underline{H}$, J = 7.7 Hz], 7.76 [d, 2H, Ar$\underline{H}$, J = 7.7 Hz].

2.3.4 Synthesis of modifier

2.3.4.1 Synthesis of 2-bromoisobutyric acid *N*-hydroxysuccinimide (14)



14

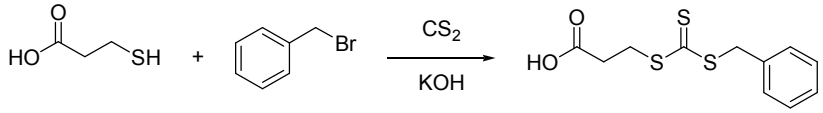
The mixture of 2-hydroxysuccinimide (0.10 g, 0.87 mmol) and triethylamine (121 µL, 0.87 mmol) in dioxane (2 mL) was slowly added to the solution of 2-bromoisobutyryl bromide (118 µL, 0.73 mmol) in diethyl ether (3 mL) under stirring at 0 ºC. The reaction was allowed to stir at ambient temperature for 8 h. After solvent removal under reduced pressure, the crude product was dissolved in diethyl ether and then extracted with sat.$NaHCO_3$. The organic phase was collected and then dried with Na_2SO_4. After filtration, the collected organic solvent was evaporated to give the

product **(14)** as white solid (0.18 g, 95%). The product was used without further purification.

^{1}H NMR (400 MHz; $CDCl_3$): δ_H 2.18 [s, 6H, C<u>H</u>$_3$-C], 2.89 [t, 4H, 2xC<u>H</u>$_2$ of NHS].

2.3.4.2 Synthesis of 3-benzylsulfanylthiocarbonylsufanyl propionic acid *N*-hydroxysuccinimide ester (16)

i) Synthesis of 3-benzylsulfanylthiocarbonylsufanyl propionic acid (15)



15

3-Mercaptopropionic acid (5 mL, 47.0 mmol) was added to a solution of potassium hydroxide (6.50 g, 115.0 mmol) in water (60 mL). After the dropwise addition of carbon disulfide (7.5 mL), the orange solution was allowed to stir at room temperature for a further 5 h. The benzyl bromide (9.9 g, 57.0 mmol) was added and the mixture solution was heated for 12 h at 80 ºC. Dichloromethane (100 mL) was added to the cooled reaction mixture and then hydrochloric acid was slowly added until the organic layer turned yellow. The liquid layer was extracted with dichloromethane (2 x 100 mL). The collected organic layers were dried over anhydrous magnesium sulfate. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel with a mobile phase of hexane: ethyl acetate (3: 1) to obtained the product **(15)** as yellow powder (0.40 g, 3%).

^{1}H-NMR (400 MHz; $CDCl_3$): δ_H 2.84 [t, 2H, CH_2-C<u>H</u>$_2$COOH, J = 7.17 Hz], 3.62 [t, 2H, C<u>H</u>$_2$-S, J =7.17 Hz], 4.61 [s, 2H, ArC<u>H</u>$_2$], 7.27 [m, 5H, Ar<u>H</u>], 8.85 [br, 1H, COO<u>H</u>].

ii) Synthesis of 3-benzylsulfanylthiocarbonylsufanyl propionic acid *N*-hydroxysuccinimide ester (16)

15 **16**

A mixture of EDC.HCl and *N*-hydroxysuccinimide (NHS) (51 mg, 0.44 mmol) in dioxane was slowly added drop wise to the solution of the compound **(15)** (0.10 g, 0.37 mmol) in dichloromethane under stirring at 0 ºC. The solution was allowed to

warm up to room temperature and the reaction was monitored by TLC with a mobile phase of hexane: ethyl acetate (4: 1). After reaction completed (8 h), the solvent was removed under reduced pressure and the product was extracted with dichloromethane and sat.$NaHCO_3$. After drying of the organic phase (Na_2SO_4) and evaporation of the solvent, the product **(16)** was obtained as yellow powder which was later used without further purification (0.122 g, 90 %yield).

^{1}H-NMR (400 MHz; $CDCl_3$): δ_H 2.25 [t, 4H, 2x$C\underline{H}_2$ of NHS], 3.09 [t, 2H, CH_2-$C\underline{H}_2CO$, J = 6.8 Hz], 3.70 [t, 2H, $C\underline{H}_2$-S, J = 6.8 Hz], 4.61 [s, 2H, Ar$C\underline{H}_2$], 7.29 [m, 5H, Ar$\underline{H}$].

2.3.5 Synthesis of acpcPNA oligomers

All peptide oligomers were synthesized by using a home-made peptide synthesis column of which set-up is shown in **Figure 2.1**. The resin was weighed accurately into the pipette. The resin in the pipette was then immersed in DMF for at least 10 min before use. For each reaction, the reagent was directly sucked in, ejected out by manual control for the specified period. After the reaction was complete, the reaction was washed by DMF solvent for three times to eliminate the excess reagents.

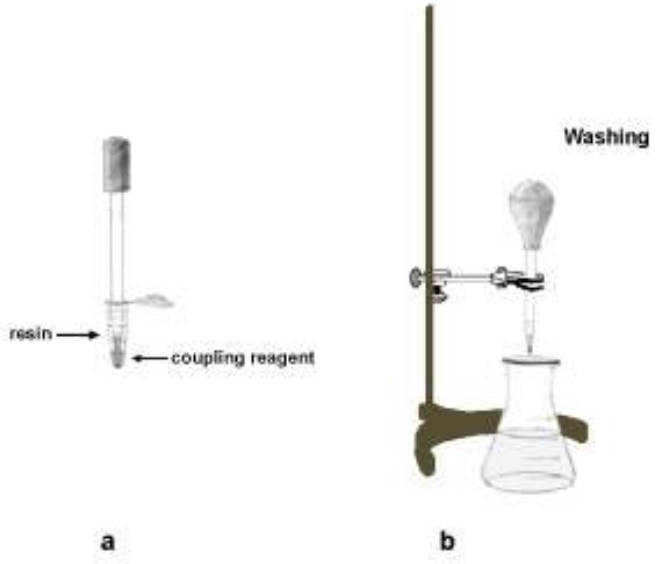


Figure 2.1 Schematic diagram of the manual technique for solid phase peptide synthesis; **a)** coupling, deprotection and cleaving process; **b)** washing process. [57-58]

Synthesis of each biotin and initiator-modified PNA was carried out on 1.5 μmol scale from three stock reagents that were prepared according to general protocol as follows.

- **Reagent#1** (20% piperidine and 2% DBU in DMF) was prepared from piperidine 200 μL, DBU 20 μL, and DMF 780 μL.
- **Reagent#2** (7% DIEA in DMF) was prepared from DIEA 70 μL and DMF 930 μL.
- **Reagent#3** (0.4 M HOAt in DMF) was prepared from HOAt 5.5 mg dissolved in DMF 100 μL.

The general synthesis protocol was divided into steps as follows.

i) Removing Fmoc protecting group form the resin

The reaction pipette containing TentaGel S RAM Fmoc resin (6.3 mg, 1.5 μmol) was prepared as described above. The Fmoc group on resin was deprotected with 100 μL of reagent#1 in a 1.5 mL eppendorf tube for 5 min at room temperature with occasional agitation. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF for three times. The free amino group on the RAM resin was obtained.

ii) Anchoring of the first amino acid residue

Lysine was first attached to the free amino group on the RAM resin. The prepared resin was immersed in a mixture solution of Fmoc-L-Lys (Boc)-OPfp (9.52 mg, 15 μmol), reagent#2 and reagent#3 with occasional agitation for 1 h at room temperature. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF.

iii) Deprotection of the Fmoc protection group at N-terminal

After the amino acid coupling (**ii)** was completed, the resin was treated with 100 μL of the reagent#1 for 5 min at room temperature with occasional agitation. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF. The used deprotecting reagent can be used to determine the coupling efficiency by diluting with an appropriate volume of methanol and then the UV-absorbance of dibenzofulvene-piperidine adduct at 264 nm measured. The first UV-absorbance of the adduct, released from the preloaded Fmoc-L-Lys (Boc)-resin, was

assumed to be 100%. Such determination of coupling efficiency was advantageous in terms of determining how the solid phase reaction progress. The efficiency should be >95 % for each step in order to give acceptable yield of the 9-15 mer PNA oligomer.

iv) Coupling with pyrrolidinyl monomer and spacer

The free amino group which was obtained from the deprotection step (iii) was further coupled with a designated PNA monomer, alternately with a spacer after every acpcPNA monomer attachment. The resin in the reaction pipette was treated with the solution mixture of PNA monomer (6.0 μmol) or spacer (6.0 μmol) in 15 μL of reagent#2 and 15 μL of reagent#3 for 30 min at room temperature with occasional agitation. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF.

v) End capping

After coupling step (iv), 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 period, the reagent was squeezed off and the reaction column was washed with excess DMF.

vi) Modifying the PNA oligomer with ethylene glycol (egl) linker and biotin

After the final deprotecting Fmoc group of spacer, the desired sequence acpcPNA oligomer on the resin was treated with a solution of the activated ethylene glycol (egl) linker, Fmoc-egl-OPfp **(13)** (3.30 mg, 6.0 μmol) in 30 μL anhydrous DMF for 1 h at room temperature followed by washing with excess DMF. The resin was then treated with 100 μL of the reagent#1. After washing with excess DMF, the resin carrying free amino group was treated with the solution of activated biotin **(17)** (3.92 mg, 6 μmol) in 30 μL anhydrous DMF for 1 h at room temperature followed by washing with DMF. Finally, the resin in the reaction pipette was treated with 1:1 concentrated aqueous ammonia:dioxane 1:1 at 65 ^{o}C for 15 h in a screwed cap test tube in order to remove nucleobase protecting groups (Bz, Ibu) of PNA nucleobases (A, C, and G monomers).

vii) Modifying the PNA oligomer with ethylene glycol (egl) linker and initiator

The desired sequence acpcPNA oligomer on the resin was treated with 1:1 concentrated aqueous ammonia:dioxane 1:1 at 65 ^{o}C for 15 h in a screwed cap test tube in order to remove nucleobase protecting groups (Bz, Ibu) of PNA nucleobases (A, C, and G monomers). After the specified period of time, the reagent was squeezed off and the reaction column was washed exhaustively with DMF. Then, the free amino group on resin was coupled with activated ethylene glycol (egl) linker, Fmoc-egl-OPfp **(13)** (3.30 mg, 6.0 μmol) in 30 μl anhydrous DMF for 1 h at room temperature followed by washing with DMF. The resin was immersed in 100 μL of reagent#1 for 5 min followed by washing with excess DMF. After that, the resin in the pipette was treated with the solution of 2-bromoisobutyric acid *N*-hydroxysuccinimide ester **(14)** (3.96 mg, 15.0 μmol) or 3-benzylsulfanylthiocarbonylsufanylpropionic acid *N*-hydroxysuccinimide ester **(16)** (5.54 mg, 15.0 μmol) in 30 μL of anhydrous DMF for 2 h at ambient temperature with occasional agitation. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF.

viii) Method for cleavage PNA oligomer from the resin

The cleavage of PNA oligomer from the resin was done by treatment with trifluoroacetic acid or TFA at room temperature for 1 h with occasional agitation. After one hour, the trifluoroacetic acid in the cleavage solution was removed by a nitrogen stream in fume hood. The resin was treated with another portion of TFA to ensure a complete cleavage of the peptide from the resin. The sticky residue was treated with diethyl ether to precipitate the crude PNA. Finally, the crude peptide was air dried at room temperature and stored dried at -20 °C until used.

ix) Purification and identification

The crude PNA oligomer was prepared for HPLC analysis by dissolving a mixture in 120 μL deionized water. The solution was filtered through a nylon membrane filter (0.45 μm). Analysis and purification was performed by reverse phase HPLC, monitoring by UV-absorbance at 260 nm and eluting with a gradient system of 0.1% TFA in acetonitrile/water for initiator end-capped PNA and 0.1% TFA in methanol/water for biotin labeled PNA. The HPLC gradient system used two solvent systems which are solvent A (0.1 % trifluoroacetic acid in acetonitrile or methanol) and

solvent B (0. 1% trifluoroacetic acid in MilliQ water). The elution began with A:B (10:90) for 5 min followed by a linear gradient up to A:B (90:10) over a period of 60 min, then hold for 5 min before reverted back to A:B (10:90). After freeze-drying, the identity of the PNA oligomer was verified by MALDI-TOF mass spectrometry.

x) T_m experiments: DNA hybridization studies of PNAs in solution phase

The sample for T_m measurement was prepared by mixing calculated amounts of stock DNA and PNA solutions to give a final concentration of 2 μM nucleotides at a ratio of PNA (or DNA): DNA = 1:1 in 10 mM sodium phosphate buffer (PBS, pH 7.0). The Abs_{260} was recorded in steps from 20 to 90 ^oC (block temperature) in two heating and one cooling cycles (20-90-20-90) with a temperature increment of 1^oC/min. The results were normalized by dividing the absorbance at each temperature by the initial absorbance. The temperature was corrected by applying the equation derived from measuring the actual temp (T_{actual}) in the cuvette using a temperature probe, against the set temperature (T_{block}) over the range of 20–90 ^oC. A linear relationship $T_{actual} = 0.9696T_{block}–0.8396$ was obtained with $r^2 > 0.99$ (linear regression analysis). The T_m was obtained from the first derivative plot after smoothing using Kalieda Graph 3.6 (Synergy Software) and Microsoft Excel XP (Microsoft Corp.).[5]

2.3.6 Method of DNA-PNA detection following "Dot blot hybridization"

2.3.6.1 Enzymatic amplification

The QPDMAEMA grafted filter paper was cut into 1.0 × 7.0 cm^2 and the spot positions were marked with a pencil. The designated DNA and b-PNA sequences from a list in **Table 2.1** were spotted on the filter paper following a sequence indicated in **Table 2.2**. A 2 μL of the DNA sample (1 μM in the solution of 0.4 M NaOH containing 10 mM EDTA) was spotted on the filter paper using a micropipette. The filter paper was air-dried and 2 μL of the biotinylated acpcPNA probe (1 μM in the solution of 0.1 M sodium phosphate buffer (PBS) pH 7.4) was subsequently introduced. The filter paper was rinsed immediately three times with phosphate buffer solution and Milli-Q water followed by incubation in a blocking solution (1% BSA, w/v) for 30 min. A 2 μL of SA-HRP (10 μg/mL) was then spotted onto the filter paper at the position that the DNA sample was immobilized. The filter paper was then rinsed three times with PBS,

Milli-Q water, and citrate buffer (CTB, 0.1 M, pH 5). Two concentration of PBS solutions (0.1 M, 50 mM) with or without added 0.1 M NaCl used in the step of washing and blocking were tested in order to find the optimal condition that yields the most efficient detection without drying, the filter paper was immersed in a solution consisting of 250 μL of 1.6 mg/mL OPD substrate (freshly prepared prior to use) and 250 μL of 1.6 mg/mL urea-H_2O_2 for 1 min and was finally washed with deionized water.

Table 2. 1 PNA and DNA sequences used in this study

Code	Sequence	Description
b-DNA SLE2	d(5′-b-TTGGGAAGGGGAA-3′)	Positive control
DNA SLE1	d(5′-TTGGGAGGGGGAA-3′)	Complementary to b-PNA SLE1 Single mismatch to b-PNA SLE2
DNA SLE2	d(5′-TTGGGAAGGGGAA-3′)	Complementary to b-PNA SLE2 Single mismatch to b-PNA SLE1
DNA (AG)	d(5′-TGTGGATAGTGA-3′)	Non complementary to b-PNA (TG)
DNA (AC)	d(5′-GTCATAGCATCA-3′)	Complementary to b-PNA (TG)
b-PNA SLE1	b-$(egl)_2$-TTCCCCCTCCCAA-Lys	-
b-PNA SLE2	b-$(egl)_2$-TTCCCCTTCCCAA-Lys	-
b-PNA (TG)	b-$(egl)_2$-TGATGCTATGAC-Lys	-

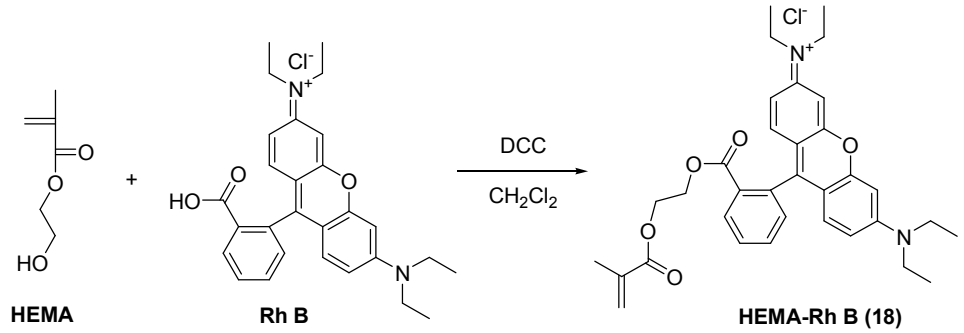
Table 2.2 Description of DNA and PNA sequences used for each spot of the test

Spot position	DNA	PNA	Remark[a]
1	b-DNA SLE2	-	(+)
2	DNA SLE1	b-PNA SLE2	(-)
3	DNA SLE2	-	(-)
4	DNA SLE2	b-PNA SLE2	(+)
5	-	b-PNA SLE2	(-)
6	-	-	(-)

[a](+) = positive result, (-) = negative result

2.3.6.2 Polymerization amplification

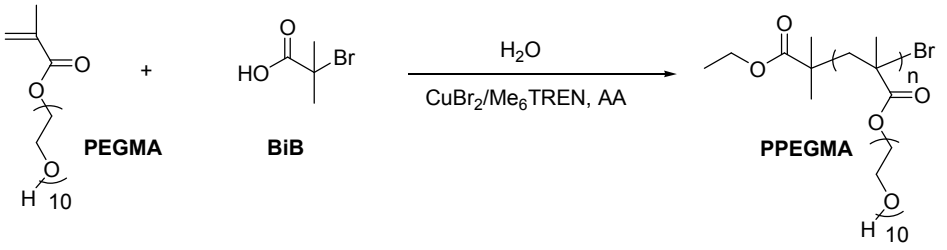
i) Synthesis of rhodamine B functionalized 2-hydroxyethyl methacrylate (Rh B-HEMA) (18)



Rhodamine B was attached to hydroxyl group of 2-hydroxyethyl methacrylate (HEMA) using standard DCC coupling reagent. A solution of DCC (1.59 g, 7.71 mmol) in dichloromethane was slowly added dropwise to the reaction mixture of rhodamine B (3.00 g, 6.04 mmol) and HEMA (1.48 mL, 12.19 mmol) in dichloromethane under stirring at 0 ºC. The mixture was allowed to stir at ambient temperature for 3 days. The solid was removed by filtration and the solution was concentrated by rotary evaporator. The product **(18)** was purified by column chromatography on silica gel with a mobile phase of acetone: dichloromethane (1: 4, v/v) and yielded a red powder (0.104 g, 3%). ^{1}H-NMR (400 MHz; D_2O): δ_H 1.15 [t, 24H, C<u>H</u>$_3$-CH$_2$], 1.73 [s, 3H, C<u>H</u>$_3$-C], 3.03 [t, 4H, NC<u>H</u>$_2$-CH$_3$, *J* = 7.6 Hz], 3.25 [t, 4H, N$^+$C<u>H</u>$_2$-CH$_3$, *J* = 7.17 Hz], 4.22 [t, 2H, COOCH$_2$-C<u>H</u>$_2$-O], 4.55 [t, 2H, COOC<u>H</u>$_2$-CH$_2$-O], 5.53, 5.80 [s, 2H, C<u>H</u>$_2$=C], 6.60 [s, 1H, Ar<u>H</u>, *J* = 7.6 Hz], 6.72 [d, 2H, Ar<u>H</u>, *J* = 7.6 Hz], 7.39 [d, 1H, Ar<u>H</u>, *J* = 11.20 Hz], 7.59 [m, 4H, Ar<u>H</u>].

ii) Solution polymerization of Rh B-HEMA and PEGMA

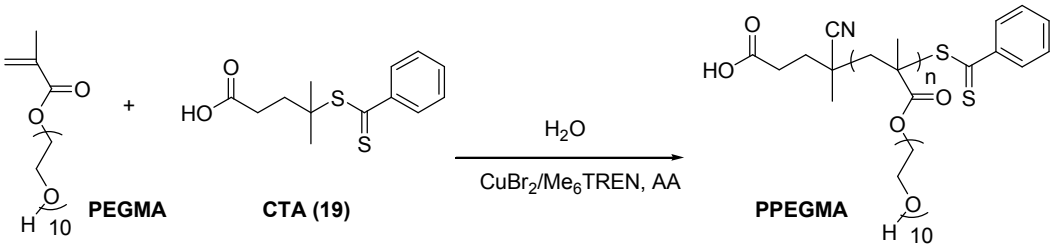
a) Synthesis of poly(2-hydroxyethyl methacrylate) (PPEGMA) by ARGET ATRP



α-bromoisobutyric acid (BiB) (0.51 mg, 3.05 μmol), water (1 mL), PEGMA (0.2 mL, 0.61 mmol), $CuBr_2$ (1 mg, 4.48 μmol) and Me_6TREN (7.4 mg, 1.44 mmol) were added to a 10 mL scintillation vial. The solution of ascorbic acid (AA) (53 mg, 0.30 mmol) in 1 mL deionized water was then added to the reaction mixture and the vial was immediately sealed with rubber septum. The volume of free space above the solution was 8.0 mL. The polymerization was carried out at ambient temperature under stirring for varied period of time. After specified reaction time, the polymerization mixture was subjected to dialysis (MWCO 1200) for 3 days against DMSO to remove the unreacted monomer and other small molecules. After that, polymer solution was lyophilized and the identity of the PPEGMA was confirmed by ^1H NMR in D_2O.

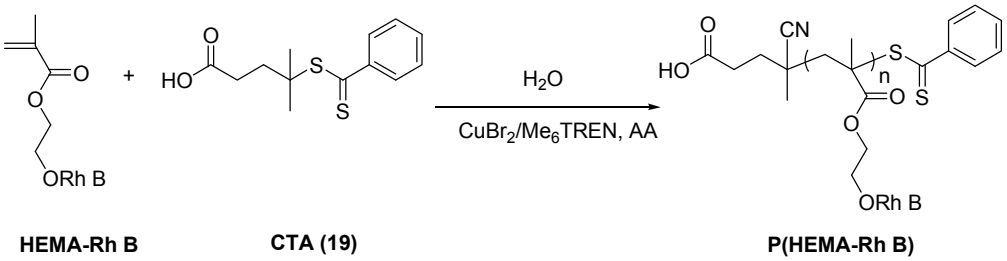
^1H-NMR (400 MHz; D_2O): δ_H 0.80-2.20 [m, 5H, (-C<u>H</u>$_2$C(C<u>H</u>$_3$))], 3.60 [t, 2H, COOCH$_2$-C<u>H</u>$_2$O], 4.15 [t, 2H, COOC<u>H</u>$_2$-CH$_2$O].

b) Synthesis of PPEGMA by concurrent ARGET ATRP/RAFT



The polymerization procedure was similar to the method previously described for ARGET ATRP, except 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid, CTA **(19)** (0.85 mg, 3.05 μmol) or 3-benzylsulfanylthiocarbonylsufanylpropionic acid, BPA **(16)** (0.83 mg, 3.05 μmol) was used in place of α-bromoisobutyric acid.

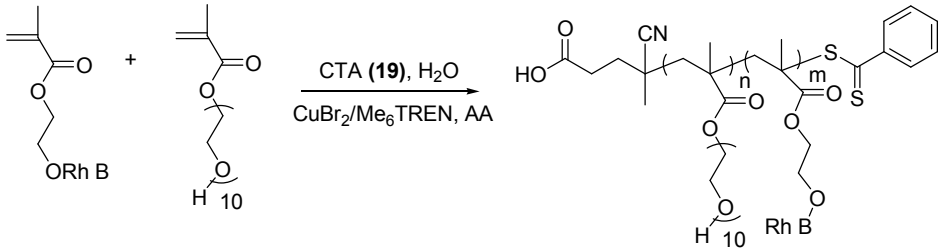
c) Synthesis of poly(rhodamine B functionalized 2-hydroxyethyl methacrylate) (P(Rh B-HEMA)) by concurrent ARGET ATRP/RAFT



4-Cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid, CTA **(19)** (0.85 mg, 3.05 μmol) was added to the mixture of monomer Rh B-HEMA (10 mg, 21 μmol), $CuBr_2$ (1 mg, 4.48 μmol) and Me_6TREN (7.4 mg, 1.44 mmol) in acetone (2 mL). The solution of AA (53 mg, 0.30 mmol) in 1 mL deionized water was then added to the reaction mixture and the vial was immediately sealed with rubber septum. The volume of free space above the solution was 8.0 mL. The polymerization was carried out at ambient temperature under stirring for 30 min and then the polymerization mixture was dialyzed for 3 days. After that, polymer solution was lyophilized and the identity of the P(Rh B-HEMA) was confirmed by ^{1}H NMR in deuterated dimethyl sulfoxide (DMSO-d_6).

^{1}H-NMR (400 MHz; DMSO-d_6): δ_H 0.80-1.21 [m, 5H, (-C<u>H</u>$_2$C(C<u>H</u>$_3$))], 3.33 [t, 2H, COOCH$_2$-C<u>H</u>$_2$O], 3.65 [t, 2H, COOC<u>H</u>$_2$-CH$_2$O], 6.91-7.98 [10H, Ar<u>H</u> of Rh B].

d) Synthesis of PPEGMA-co-P(Rh B-HEMA) by concurrent ARGET ATRP/RAFT



HEMA-Rh B **PEGMA** **PPEGMA-co-P(HEMA-Rh B)**

4-Cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid **(19)** (0.85 mg, 3.05 μmol), water (1 mL), PEGMA (0.2 mL, 0.61 mmol), Rh B-HEMA (10 mg, 21 μmol), $CuBr_2$ (1 mg, 4.48 μmol) and Me_6TREN (7.4 mg, 32 μmol) were added to a 10 mL scintillation vial. The reaction vial was then sealed immediately with rubber septum after an addition of AA (53 mg, 0.30 mmol). The volume of free space above the solution was 8.0 mL. The polymerization was carried out at ambient temperature under stirring for 30 min and stopped by exposure to air. The polymerization mixture was dialyzed and lyophilized according to the method described in **(a)** to give PPEGMA-co-P(Rh B-HEMA).

^{1}H-NMR (400 MHz; D_2O): δ_H 0.80-2.21 [b, 5H, (-C<u>H</u>$_2$C(C<u>H</u>$_3$))], 3.60 [t, 2H, COOCH$_2$-C<u>H</u>$_2$O], 4.15 [t, 2H, COOC<u>H</u>$_2$-CH$_2$O], 7.4-8.10[10H, Ar<u>H</u> of Rh B].

iii) Surface-initiated polymerization of PPEGMA-co-P(Rh B-HEMA) on QPDMAEMA grafted filter paper by ARGET ATRP or concurrent ARGET ATRP/RAFT

The reaction initiator, 3-benzylsulfanylthiocarbonylsufanyl propionic acid **(15)**) was spotted on the QPDMAEMA grafted filter paper size 1x2.5 cm^2. After air-dried, the paper was immersed in the solution mixture of PEGMA (0.2 mL, 0.61 mmol), Rh B-HEMA (10 mg, 21 μmol), $CuBr_2$ (1 mg, 4.48 μmol), Me_6TREN (7.4 mg, 32 μmol) and 1 mL deionized water in a 10 mL scintillation vial. The solution of AA (53 mg, 0.30 mmol) in DI water (1 mL) was added to the reaction mixture and the vial was immediately sealed with rubber septum. After a specified period of time, the filter paper was immediately rinsed thoroughly with excess amount of water.

iv) General protocol of polymerization amplification for DNA sequence determination

A 2 μL of DNA SLE2 was first spot on the QPDMAEMA grafted filter paper and followed by I-PNA SLE2 which was synthesized in section 2.3.5 (see **Table 2.3**). The filter paper was immediately rinsed three times with 0.1 M PBS having 0.1 M NaCl and Milli-Q water. The filter paper was then immersed in a 10 mL scintillation vial containing a solution mixture of PEGMA (0.2 mL, 0.61 mmol), Rh B-HEMA (10 mg, 21 μmol), $CuBr_2$ (1 mg, 4.48 μmol), Me_6TREN (7.4 mg, 32 μmol) and 1 mL deionized water. After the solution of AA (53 mg, 0.30 mmol) in DI water (1 mL) was added, the vial was immediately sealed with rubber septum and the polymerization was performed at ambient temperature under stirring for 30 min. After the polymerization was ended by exposure to air, the filter paper rinsed thoroughly with excess amount of water.

Table 2.3 Description of DNA and PNA sequence used for each spot of the test

Spot position	DNA	PNA	Remark[a]
1	DNA SLE2	I-PNA SLE2	(+)
2	-	I-PNA SLE2	(-)
3	-	-	(-)

[a](+) = positive result, (-) = negative result

CHAPTER III

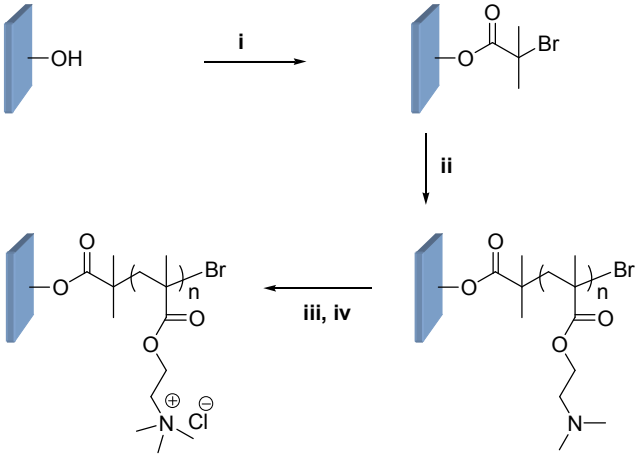
RESULTS AND DISCUSSION

This chapter is divided into three parts. The first part focuses on the preparation and characterization of quaternized polymer brush grafted filter paper namely quarternized poly(2-(dimethylamino)ethyl methacrylate (QPDMAEMA). The second part is synthesis of acpcPNA oligomers labeled with biotin or initiator. The final part is dedicated to sequence determination of DNA following "Dot blot hybridization" using QPDMAEMA grafted filter paper and acpcPNA probes. The signal amplification to detect DNA-PNA duplex on QPDMAEMA grafted filter paper was accomplished by colorimetric methods via enzymatic and polymerization amplification.

3.1 Preparation and characterization of QPDMAEMA grafted filter paper

3.1.1 Preparation of QPDMAEMA grafted filter paper

QPDMAEMA grafted filter paper was prepared by surface-initiated polymerization (SIP) of 2-(dimethylamino)ethyl methacrylate (DMAEMA) on filter paper followed by quaternization with methyl iodide (**Scheme 3.1**).



Scheme 3.1 The modification of filter paper; i) 2-bromoisobutyryl bromide, triethylamine, DMAP, CH_2Cl_2; ii) DMAEMA, $CuBr_2/Me_6TREN$, $Sn(EH)_2$, acetone; iii) methyl iodide, DMF; iv) 0.1 M NaCl, 24 h.

The initiator immobilized filter paper was prepared by the reaction of hydroxyl groups on the filter paper with 2-bromoisobutyryl bromide in the presence of triethylamine and *N,N*-dimethylaminopyridine (DMAP) in dichloromethane. According to the data shown in **Table 3.1**, the filter paper became increasingly hydrophobic after the reaction. The maximum coverage of 2-bromoisobutyryl group on the filter paper was reached upon esterification for at least 16 h.

Table 3.1 Water contact angle of filter paper surface as a function of esterification time.

Esterification time (h)	**Advancing Water Contact Angle (degree)**
0	~0
2	118.50 ± 5.04
5	120.74 ± 7.77
8	123.52 ± 5.88
16	125.50 ± 1.00
24	125.70 ± 0.50

The filter paper having 2-bromoisobutyrate was then subjected to SIP of DMAEMA monomer via a recently developed living radical polymerization process based on activators regenerated by electron transfer for atom transfer radical polymerization (ARGET ATRP). The fact that polymerization can be performed under a limited amount of air using a ppm level of a catalyst in the presence of reducing agent without a need for rigorous deoxygenation truly adds simplicity to the method. The reactions can also be carried out at ambient temperature (33-39 ºC). Monomer consumption was determined by ^{1}H NMR analysis using $CDCl_3$ as solvent and % conversion can be calculated from the relative ratio between the integration of 2 protons from methylene groups (CH_2) of polymer at δ ~4.2-4.3 ppm and the peak integration of 2 protons of methylene groups of monomer (δ ~3.9-4.1 ppm) (see Appendixes **Figure A-1**) following equation **3.1**.

$$\%conversion = \left[\frac{\int CH_2 polymer/2}{\int CH_2 polymer/2 + \int CH_2 monomer/2}\right] \times 100 \quad \textbf{(3.1)}$$

To directly determine the molecular weight of PDMAEMA on filter paper is not practically possible because the amount of attached polymer brushes than can be removed was be too low to be characterized by GPC. Previously, a sacrificial initiator, ethyl 2-bromoisobutyrate (EBiB), has been used to monitor the polymerization process and assuming that the ratio between the free initiator and monomer determines the degree of polymerization.[39-40] This is a common method to control the length of the grafted polymer from surface confined initiators. Moreover, the controlled growth of the polymer in solution was found to be independent of the surface initiator density.[31] Therefore, this study used this method and assumed that the molecular weights (M_n) and the molecular weight distribution of the polymers in solution were essentially equal to polymer growing from the filter paper.

Table 3.2 presents PDMAEMA characteristics synthesized via ARGET ATRP of DMAEMA initiated by EBiB in the presence of $CuBr_2$/Me_6TREN complex. For the target degree of polymerization (DP) at 200 ($[DMAEMA]_0/[EBiB]_0$ = 200:1), it was found that the $[Sn(EH)_2]_0/[CuBr_2]_0$ of 400 was the optimal ratio that gave the polymer with well-controlled characteristic (M_n (exp) = 28,495 being close to M_n (theo) of 28,260 and PDI = 1.28) **(Table 3.2, entry 5)**. Increasing the ratio to 667 does not provide a positive impact of the polymerization **(Table 3.2, entry 6)**. On the other hand, the PDI tended to be higher at the ratio of 250. Ascorbic acid (AA) was also tested as an alternative reducing agent. Due to its stronger reducing power than Sn(II) species , the polymerization using AA as the reducing agent proceeded faster than that using $Sn(EH)_2$ as the reducing agent. The polymerization process was less controllable and the resulting PDMAEMA possessed $\boldsymbol{M_n}$ (exp) deviated from the M_n (theo) (**Table 3.2**, **entries 1 and 2**). It should be emphasized that acetone is a better solvent for polymerization of DMAEMA using this catalytic system than anisole **(Table 3.2, entry 3)**, the solvent used for the synthesis of PDMAEMA via ARGET ATRP using $CuCl_2$/tris[(2-pyridyl)methyl]amine (TPMA) complex as the catalyst, previously reported by others.[43] This is quite desirable given that acetone is a non-toxic organic

solvent having low boiling point so it can be easily removed from the filter paper after SIP process by air drying without the need for heating.

Table 3.2 Characteristic of PDMAEMA synthesized by ARGET ATRP

Entry	$[Sn(EH)_2]_0/[CuBr_2]_0$[a]	solvent	time (h)	% conv.[c]	M_n(theo)[d]	M_n(exp)[e]	PDI[e]
1	125[b]	anisole	19	99	31,086	49,377	1.39
2	125[b]	acetone	24	91	28,260	21,670	1.38
3	250	anisole	19	99	31,086	53,488	1.30
4	250	acetone	24	72	22,608	21,670	1.38
5	400	acetone	24	90	28,260	28,495	1.28
6	667	acetone	24	90	28,260	23,983	1.35

[a]DMAEMA/EBiB/$CuBr_2$/Me_6TREN = 200:1:0.006:0.25. $[DMAEMA]_0$ = 4.12 M, The polymerization was conducted in a scintillation vial with volume of free space = 7.7 mL. [b]Ascorbic acid was used as reducing agent instead of $Sn(EH)_2$. [c]Determined by ^{1}H NMR in $CDCl_3$. [d]M_n(theo) = ($[DMAEMA]_0/[EBiB]_0$) × conversion. [e]Determined by GPC in THF based on polystyrene standards.

A relatively linear increase in monomer consumption as a function of polymerization time (**Figure 3.1**) together with a linear increase of molar mass with %conversion (**Figure 3.2**) suggested that the polymerization is living in character. A slight deviation from a straight-line kinetic plot (**Figure 3.1**) indicated that the numbers of radicals present in the system changed, to some extent, during the reaction, but control was still maintained. As revealed in **Figure 3.2**, PDI was lower at relatively high monomer conversion (> 60%) but became high again (PDI = 1.32) when the monomer was totally consumed (~100 %conversion), suggesting that diffusion controlled radical-radical coupling at high conversion cannot be totally suppressed.

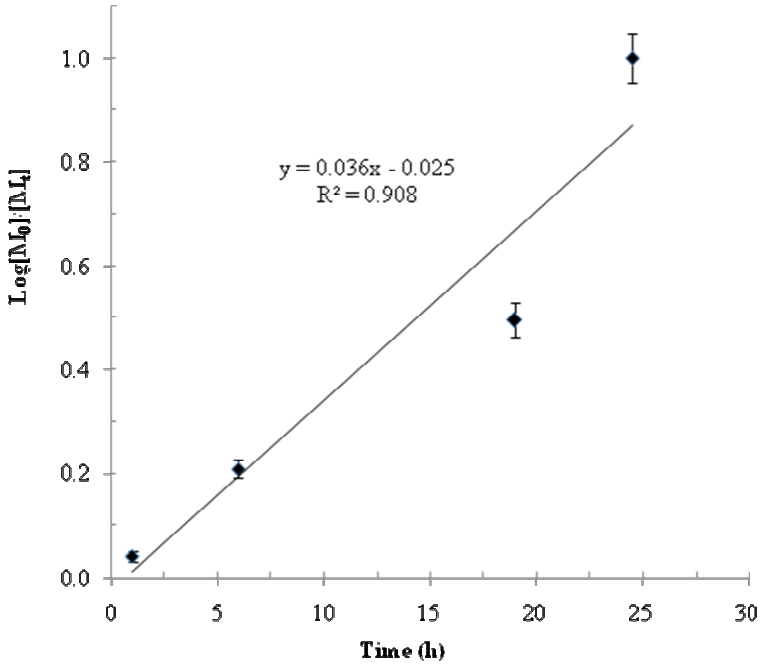


Figure 3.1 Kinetic plot of monomer consumption as a function of time during ARGET ATRP of DMAEMA with ratio $[Sn(EH)_2]_0/[CuBr_2]_0 = 400$ (Table 3.2, entry 5).

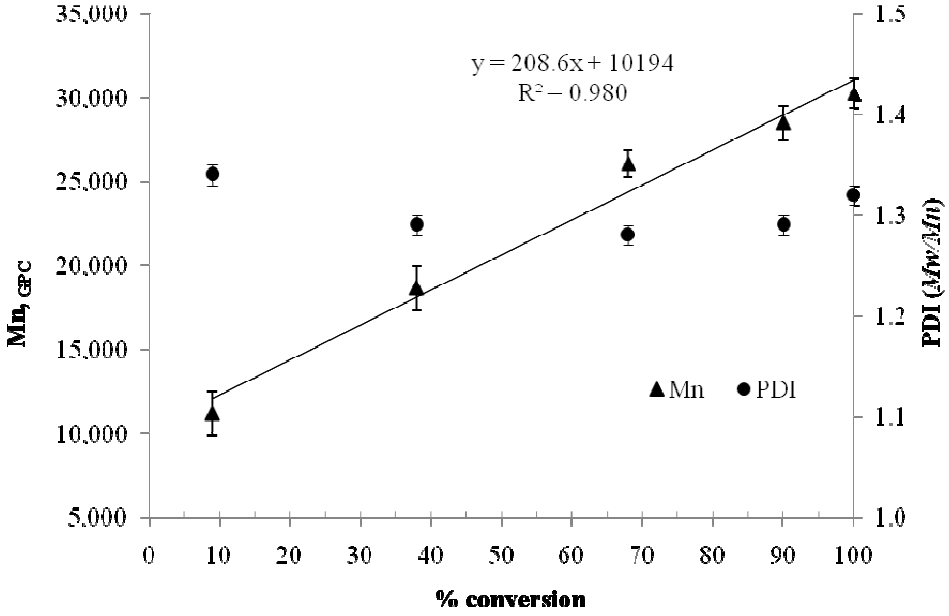


Figure 3.2 Plots of M_n(exp) and PDI as a function of % conversion of DMAEMA in ARGET ATRP using $[Sn(EH)_2]_0/[CuBr_2]_0 = 400$ (Table 3.2, entry 5).

The ability to control molecular weight of PDMAEMA is demonstrated in **Table 3.3**. Apparently, the molecular weight of PDMAEMA correspondingly increases as a function of $([DMAEMA]_0/[EBiB]_0$ from 100:1, 200:1 to 400:1 or target DP of 100, 200, and 400, respectively. The PDI values approaching 1.0 suggest that the polymerization can be well controlled and gave PDMAEMA with narrow molecular weight distribution.

Table 3.3 Characteristic of PDMAEMA synthesized by ARGET ATRP having varied target DP

Entry	Target DP[a]	% conv.[b]	M_n(theo)[c]	M_n(exp)[d]	PDI[d]
1	100	85	13,345	21,010	1.24
2	200	90	28,260	28,495	1.28
3	400	69	43,332	42,650	1.18

[a]EBiB/$CuBr_2$/Me_6TREN = 1:0.006:0.25. The polymerization was conducted in a scintillation vial with volume of free space = 7.7 mL. [b]Determined by ^{1}H NMR in $CDCl_3$. [c]M_n(theo) = $([DMAEMA]_0/[EBiB]_0)$ × conversion. [d]Determined by GPC in THF based on polystyrene standards.

Quaternization of PDMAEMA-grafted filter paper was accomplished by methylation of the amino groups of PDMAEMA on filter paper with methyl iodide at ambient temperature as shown in **Scheme 3.1**. The iodide counter ion must be exchanged for chloride by treatment with 0.1 M NaCl. This step is mandatory because it was found that iodide ion can be oxidized into iodine which makes the paper turn slightly yellowish upon storage. After air-dried, the QPDMAEMA grafted filter paper having chloride as the counter ion was obtained. The filter paper became extremely hydrophilic with unmeasurable water contact angle (~ 0^o) after being grafted with PDMAEMA and its wetting behavior remained unaltered after quaternization.

3.1.2 Characterization of QPDMAEMA grafted filter paper

The functional group identity of each surface-modified filter paper was determined by FT-IR as shown in **Figure 3.3**. The FT-IR spectra **(Figure 3.3b)** of the initiator immobilized filter paper shows a band at ~1728 cm^{-1} (C=O stretching of 2-bromoisobutyryl ester) which is not present in the virgin filter paper **(Figure 3.3a)**.

After being grafted with PDMAEMA, the absorption peak for the carbonyl group appears at the same position with greater intensity **(Figure 3.3c)**, which is characteristic of the C=O stretching of PDMAEMA.[31,59] No significant change in the IR bands was observed after quaternization **(Figure. 3.3d)**.

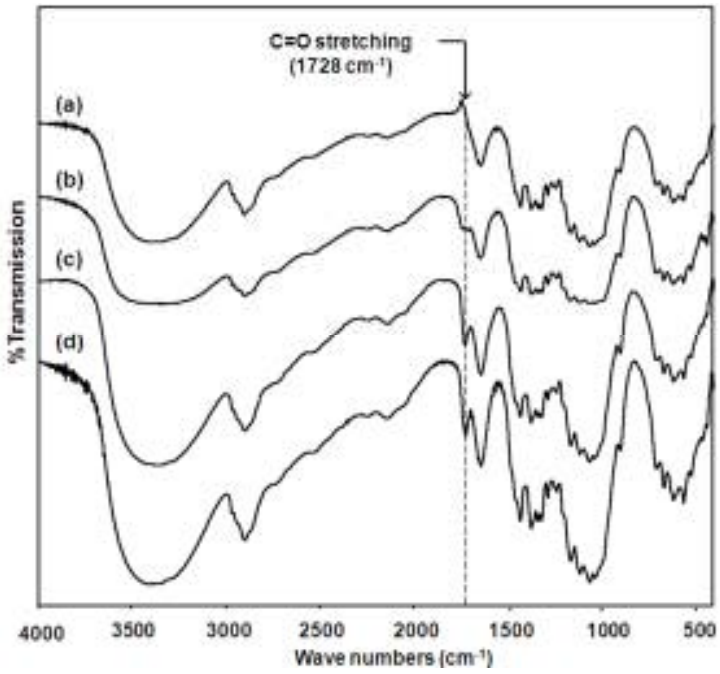


Figure 3.3 FT-IR spectra of a) unmodified filter paper, b) initiator immobilized, c) PDMAEMA grafted filter paper and d) QPDMAEMA grafted filter paper filter paper.

The morphology of all the surface-modified filter paper was examined by SEM. As demonstrated in **Figure 3.4**, there was no visible changes of surface morphology of the filter paper particularly after being grafted with PDMAEMA having target DP of 200 (both before and after quaternization) implying that the layer of coated polymer is relatively thin. This observation coincides with the fact that the physical strength and resistance to bending of the filter paper was not affected by polymer grafting. On the other hand, the filter paper became tougher and plastic-like upon grafting with PDMAEMA having higher target DP of 400 implying that the thicker polymer layer was deposited on the filter paper.

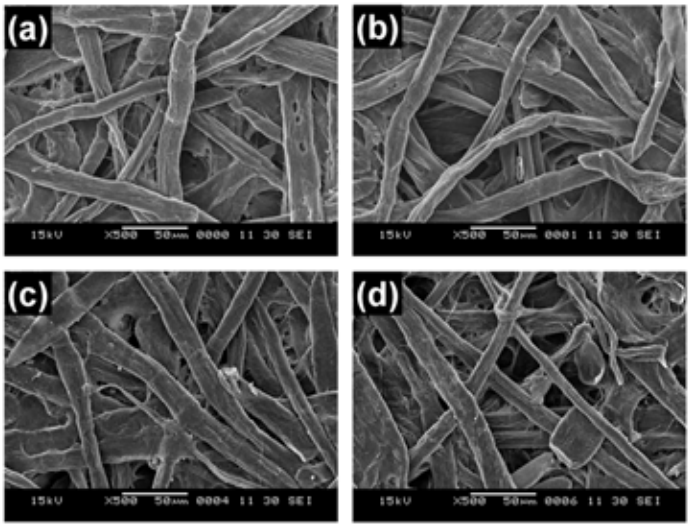


Figure 3.4 SEM images of filter paper: a) unmodified, b) immobilized with initiator, c) grafted with PDMAEMA (target DP =200), and d) grafted with QPDMAEMA (target DP =200).

The elemental compositions of all surface-modified filter paper were evaluated by XPS. From the XPS atomic composition data illustrated in **Table 3.4**, the detection of 0.8% bromine were on the initiator immobilized filter paper suggests that there are bromoester groups available for initiating polymerization from the surface of filter paper. A trace amount of bromine (~0.2%) on the PDMAEMA and QPDMAEMA grafted filter papers. indicated that there are still living polymer chain ends in the form of dormant bromoester species. The presence of atomic signal of nitrogen on the PDMAEMA and QPDMAEMA grafted filter papers, confirms the successful polymer attachment via SIP process. The C/N ratio of 8.1 **(Table 3.4)** correspond quite well with the theoretical value of PDMAEMA **(8)** (C/N ratio, 8/1). An increase in C/N ratio to 11.9 together with the emergence of the N_{1s} peak at 402.0 eV on the QPDMAEMA grafted filter paper **(Figure 3.5)** strongly indicate that additional carbon atoms has been introduced upon quaternization and that most nitrogen existed in the less electronegative form of quaternary ammonium groups.[33] The extent of surface quaternization estimated from the relative ratio between the area of the N_{1s} from the quaternary ammonium entities and that of the regular, non-quaternized one appearing at 398.6 eV was about 80%. This figure is relatively high suggesting that the QPDMAEMA grafted filter paper should be positively charged

and readily available to electrostatically absorb DNA. To test such adsorption ability **(Table 3.4)**, a synthetic 13mer DNA corresponding to partial sequence of human *IL-10* promoter gene **(DNA SLE2)** was used as a model. A 1 µL of 10 µM DNA in 0.4 M NaOH containing 10 mM EDTA was spotted on the QPDMAEMA grafted filter paper. XPS analysis of the paper after extensive washing to remove unbound DNA indicated that the phosphorus content of 1.2 %, a characteristic signal of the phosphate group on DNA was detected implying that DNA molecules being adsorbed on the QPDMAEMA grafted filter paper.

Table 3.4 Atomic composition of surface-modified filter paper determined by XPS analysis.

Sample	**Atomic composition (%)**						**C/N**
	C	**O**	**Br**	**N**	**Cl**	**P**	
Unmodified	58.2	41.7	N/D	N/D	N/D	N/D	-
Immobilized with initiator	57.7	41.5	0.8	N/D	N/D	N/D	-
Grafted with PDMAEMA	65.0	26.8	0.2	8.0	N/D	N/D	8.1
Grafted with QPDMAEMA	69.2	24.9	0.2	5.8	<0.1	N/D	11.9
Grafted with QPDMAEMA after DNA adsorption	60.4	33.6	0.1	4.6	0.15	1.2	13.1

N/D = Not detected

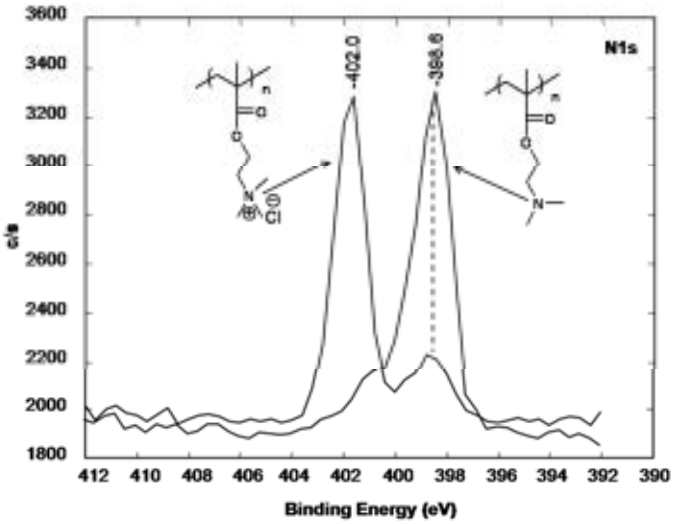
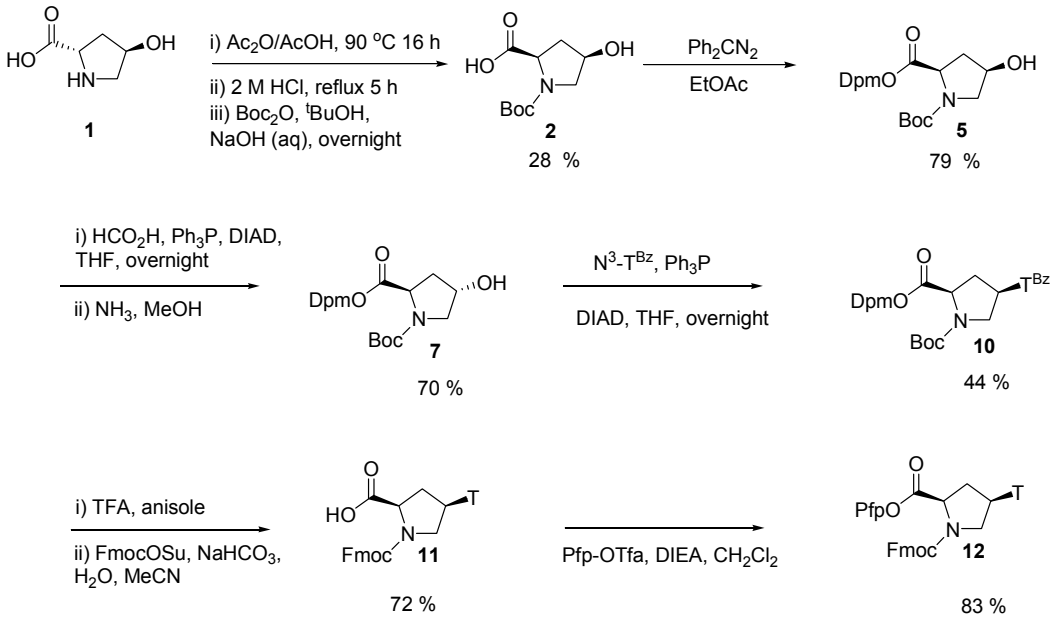


Figure 3.5 N_{1s} XPS spectra of PDMAEMA (right) and QPDMAEMA (left) grafted filter papers.

3.2 Synthesis of acpcPNA oligomers

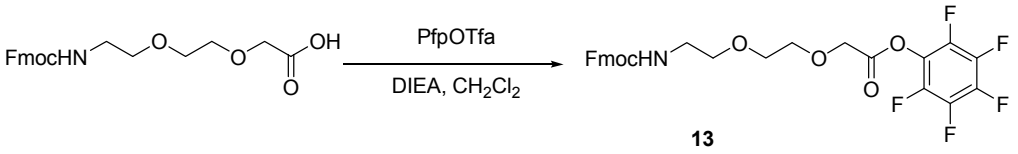
3.2.1 Synthesis of intermediate and activated acpcPNA monomers Fmoc-T-OPfp (12)



Scheme 3.2 Synthesis of intermediate and activated acpcPNA monomers Fmoc-T-OPfp (**12**).

The PNA monomer, Fmoc-T-OPfp **(12)**, was synthesized according to stepwise procedure shown in **Scheme 3.2**. The synthesized compound was identified by ^{1}H NMR and compared to standard spectra in the literature before usage.[58,60]

3.2.2 Synthesis of activated ethylene glycol (egl) linker (13)



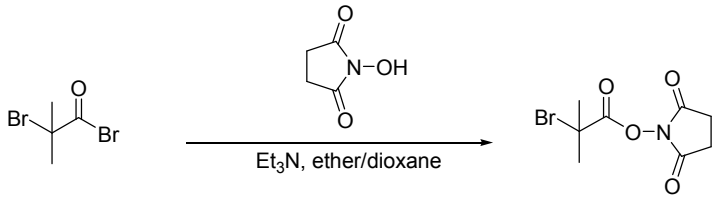
13

Scheme 3.3 Synthesis of activated ethylene glycol (egl) linker **(13)**

The Fmoc-egl-OPfp **(13)** was synthesized by reacting 2-[2-(Fmoc-amino)ethoxy]ethoxyacetic acid with PfpOTfa and DIEA as described previously for the PNA monomers **(Scheme 3.3)**. [4-5,57] The product (**13**) was obtained in 79 % yield as a white solid.

3.2.3 Synthesis of modifier

3.2.3.1 Synthesis of 2-bromoisobutyric acid *N* -hydroxysuccinimide (14)

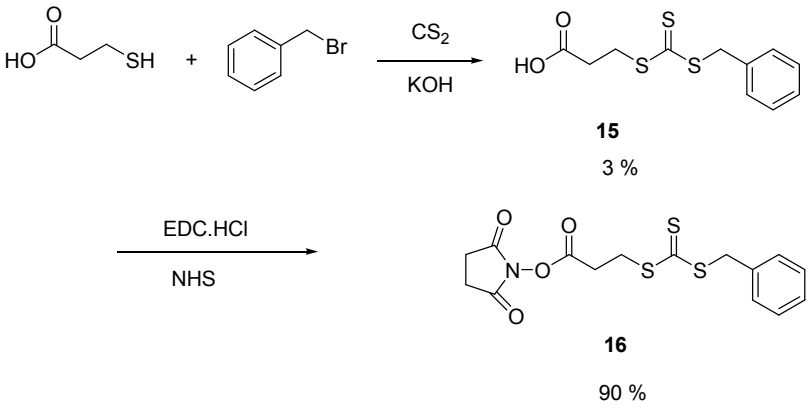


14

Scheme 3.4 Synthesis of 2-bromoisobutyric acid *N* -hydroxysuccinimide **(14)**.

Initiator **(14)** was synthesized from 2-bromoisobutyryl bromide, 2-hydroxysuccinimide, and triethylamine in ether/dioxane (1:1) (**Scheme 3.4**). The product (**14**) was obtained in 95 %yield as white solid. The structure of product was confirmed by ^{1}H NMR and its spectrum is shown in **Figure B-1** (Appendix B).

3.2.3.2 Synthesis of 3-benzylsulfanylthiocarbonylsufanyl propionic acid *N*-hydroxysuccinimide ester (16)



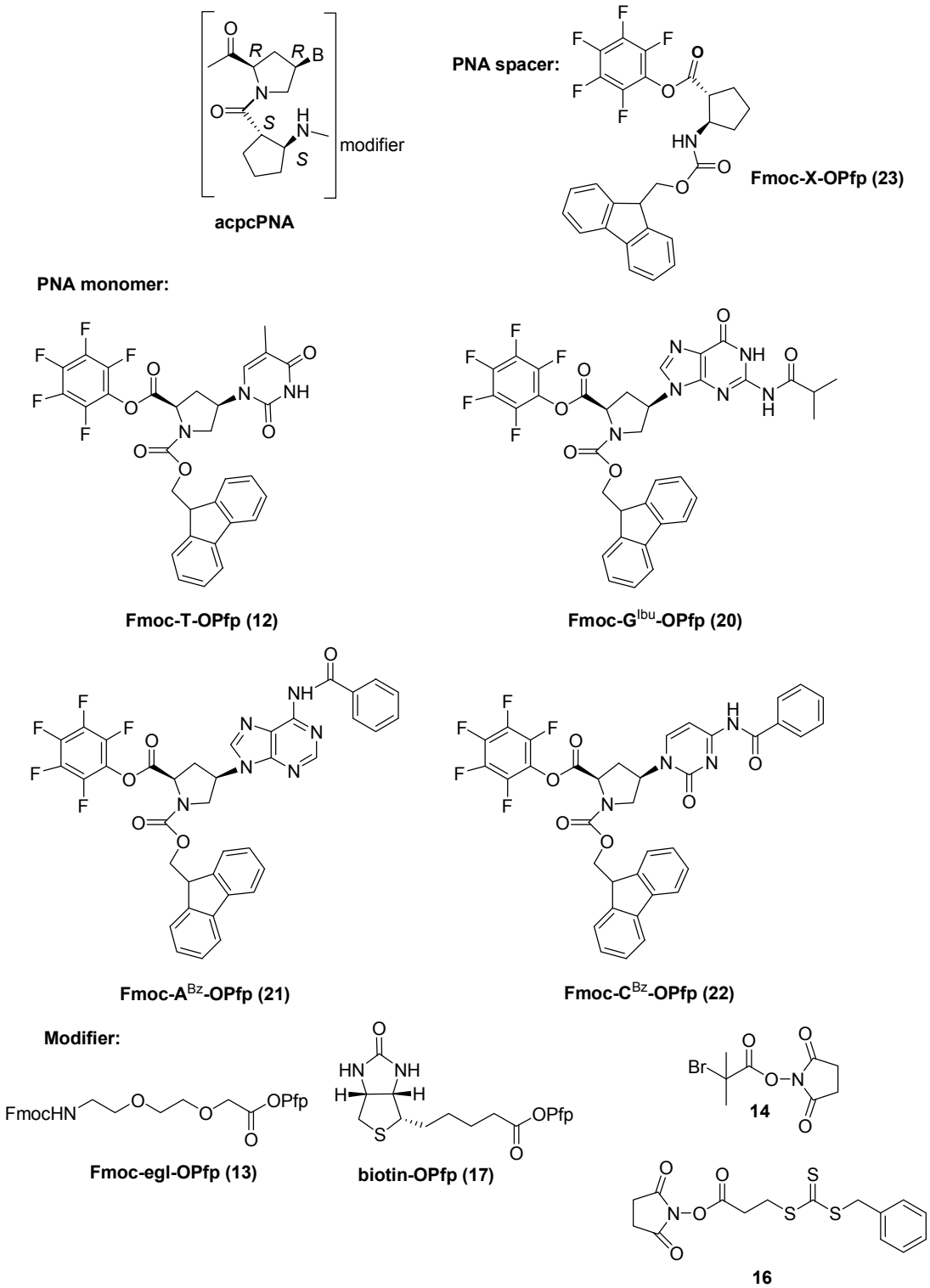
Scheme 3.5 Synthesis of 3-benzylsulfanylthiocarbonylsufanyl propionic acid *N*-hydroxysuccinimide ester (**16**).

Initiator **(15**) was synthesized from 3-mercaptopropionic acid, carbon disulfide, and benzyl bromide in aqueous KOH followed the procedure previously reported by Stenzel.[61] The product (**16**) was obtained in 3 %yield as yellow solid and was further reacted with PfpOTfa and DIEA. The active ester (**16**) was obtained in 90 % yield as a yellow solid (**Scheme 3.5**). The structure of product was confirmed by ^{1}H NMR and its spectrum is shown in **Figure B-3** (Appendix B).

3.2.4 Solid phase synthesis

The activated Fmoc-protected acpcPNA monomers **(12, 20-22)**, spacer **(23)**, activated ethylene glycol (egl) linker **(13)** and modifier **(14, 16 and 17)** were used in solid phase peptide synthesis (**Scheme 3.6**). The three monomers, Fmoc-G^{Ibu}-OPfp **(20),** Fmoc-A^{Bz}-OPfp **(21)** and Fmoc-A^{Bz}-OPfp **(22)**, were synthesized according to the reported procedure by Dr. Woraluk Munsawat, Miss Chalotorn Boonlua, and Miss Boonsong Ditmangklo as previously described.[58,60]

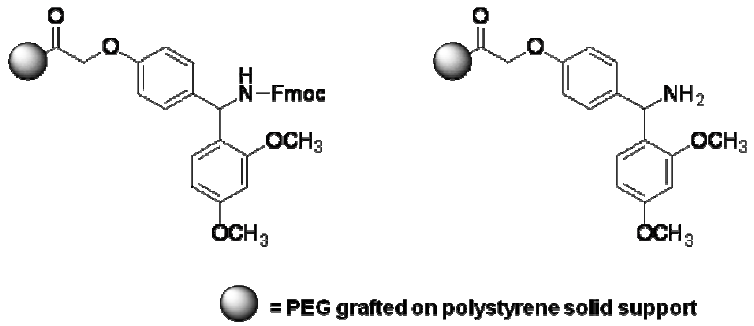
The PNA spacer, Fmoc-X-OPfp **(23)**, was synthesized following the method reported by Miss Chalotorn Boonlua and Miss Jittima Meebungpraw.[58, 60]



Scheme 3.6 Structure of activated PNA monomers **(12, 20−22)**, spacer **(23)**, activated ethylene glycol (egl) linker **(13)** and modifier **(14, 16, and 17)** for solid phase peptide synthesis.

The biotin-OPfp **(17)** was synthesized by Dr. Cheeraporn Ananthanawat. The identity of the product **(17)** were confirmed by ^{1}H NMR and compared to standard spectra in the literature before usage.[4-5,57]

Solid phase peptide synthesis (SPPS) of PNA was performed according to the standard protocol previously developed in our laboratory.[58,60] The technique involves growing of a peptide chain from amino acid building blocks on solid support such as TentaGel[63] resin containing a polyethylene glycol (PEG) grafted on polystyrene resin (**Scheme 3.7**). The synthetic peptides are usually extended from C (carboxyl) terminus to N (amino) terminus through a series of coupling cycles. An appropriate Fmoc-protected amino acid with polar side chain (Ser, Asp, Lys) was first coupled to the resin in order to prevent the self aggregation of the peptide chain and to increase the solubility of the peptide in aqueous medium due to the repulsion of the positively charged side chain.[1-2] The next step of the synthetic cycle, the PNA monomer and spacer were coupled alternately until the desired PNA sequence was obtained. For synthetic biotin labeled PNA, the deprotected N-terminus of the last spacer (Fmoc-X-OPfp **(23)**) was coupled with the activated ethylene glycol (egl) linker, Fmoc-egl-OPfp **(13)**, to increase the solubility and the distance between the PNA. After Fmoc deprotection of egl linker, the activated biotin **(17)** was then coupled to obtain the biotin labeled PNA. For PNA containing A, C or G nucleobases, the nucleobase protecting groups (Bz, Ibu) remained on the PNA chain were removed by a treatment with 1:1 aqueous ammonia:dioxane at 60 ^{o}C for 6 h. For synthetic initiator labeled PNA, this step could be done before the modification with a hydrophilic diethyleneglycol ("egl") linker step. The initiator groups ((**14**) and (**16**)) were then coupled after Fmoc cleavage to give the initiator-end capped PNA. Finally, the PNA oligomer was cleaved from the resin by treatment with TFA. A sticky residue was obtained after TFA removal with a stream of nitrogen gas in the fume hood. The crude PNA was washed with ether and air-dried to give a white precipitate of the crude PNA.



Scheme 3.7 TentaGel S RAM Fmoc resin and TentaGel S RAM resin.

3.2.5 Purification and identification of the crude PNA oligomers

The crude PNA oligomers were purified by reverse phase HPLC. The gradient system for biotin **(17)** labeled PNA oligomers and initiator (**14**) end-capped PNA oligomer is 0.1% TFA in methanol/water. The initiator (**16**) labeled PNA oligomer was successfully purified with a gradient system of 0.1% TFA in acetonitrile/water since the initiator group (**16**) is sensitive to methanolysis. After the purification, the solution of the purified PNA was lyophilized and the identity of the PNA oligomers was verified by MALDI-TOF mass spectrometry. The *m/z* data obtained from MALDI-TOF analysis for each PNA are illustrated in **Table 3.5,** and the mass spectra are shown in Appendix A.

Table 3.5 Characteristics of *acpc*PNA oligomers synthesized by solid phase method.

Name	**sequence**	***m/z*** **(observed)**	**T_m(°C)**
b-PNA (T9)	*N*-biotin-$(egl)_2$-TTTTTTTTT-egl-LysNH_2-*C*	3798.22	77.6
b-PNA(SLE1)	*N*-biotin-$(egl)_2$-TTCCCCCTCCCAA-LysNH_2-*C*	4879.92	59.3
b-PNA(SLE2)	*N* -biotin-$(egl)_2$-TTCCCCTTCCCAA-LysNH_2-*C*	4898.64	65.5
b-PNA(AG)	*N* -biotin-$(egl)_2$-TGATGCTATGAC-LysNH_2-*C*	4724.46	-[a,b]
I-PNA(T9)	*N* -Initiator(**14**)-$(egl)_2$-TTTTTTTTT-egl-LysNH_2-*C*	3720.84	77.6
I-PNA(SLE2)	*N* -Initiator(**16**)-egl-TTCCCCTTCCCAA-LysNH_2-*C*	4778.10	-[b]

[a]This PNA was synthesized by Mrs. Chotima Vilaivan. [b]T_m was not determined.

3.2.6 T_m experiments of modified PNA: DNA hybrids in solution

The melting temperature (T_m) is the temperature at which one-half of a particular DNA duplex will dissociate and become single strand DNA. This process can be monitored by spectrophotometer at 260 nm. Unstacked bases (random coil single stranded DNA) absorb more light than neatly stacked (double helix) base pairs (**Figure 3.6**).

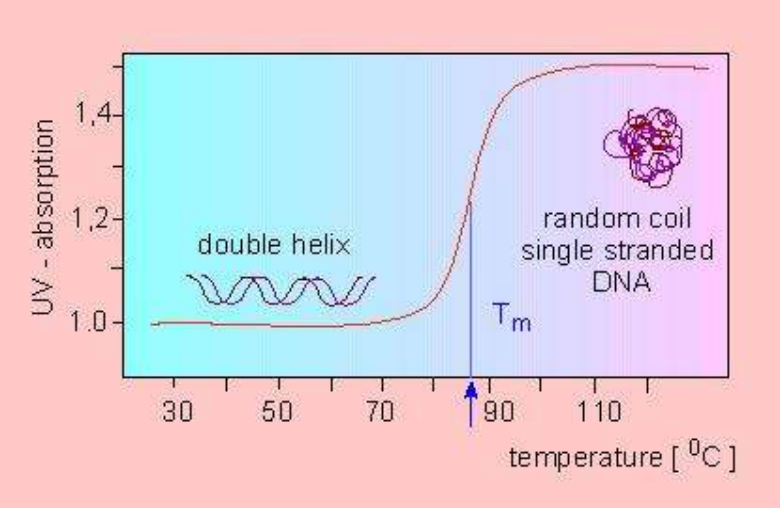


Figure 3.6 Melting curve of DNA (thermal denaturation) (http://www.biologie.uni-hamburg.de/b-online/e21/14.htm).

Investigation of the binding properties of each synthesized PNA oligomer with its complementary DNA and mismatched DNAs in solution was investigated by T_m measurement at 260 nm. **Figure 3.7** shows an example of melting curve of biotin-egl-TTTTTTTTT-egl-Lys (b-PNA (T9)) with its complementary DNA (5'-AAAAAAAAA-3') and non-complementary DNA (5'-TTTTTTTTT-3'). The T_m values of these PNA·DNA duplexes and others are summarized in **Table 3.5**.

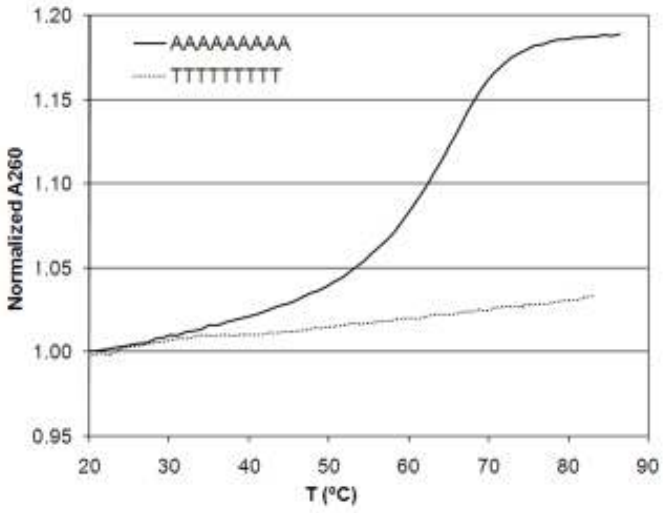


Figure 3.7 Representative melting curve of biotin-$(egl)_2$-TTTTTTTTT-egl-Lys (b-PNA (T9)) with its complementary DNA (5'-AAAAAAAAA-3') and full mismatched DNA (5'-TTTTTTTTT-3').

3.3 Determination of PNA·DNA hybridization following "Dot blot hybridization" using QPDMAEMA grafted filter paper

3.3.1 Enzymatic amplification

The concept of DNA sequence determination following "Dot blot hybridization" using enzymatic amplification is illustrated in **Figure 3.8**. After dot blotting DNA on membrane, a DNA target was captured via electrostatic interactions between the positive charges of the QPDMAEMA brushes and negative charges of the phosphate backbone of DNA. Upon hybridization with biotin labeled acpcPNA probe (b-PNA), the PNA·DNA hybridization can be visualized by naked eye observation of the yellow product generated by the enzymatic amplification employing horseradish peroxides (HRP) labeled streptavidin (SA-HRP). HRP is catalyst for the oxidation of amine and phenol derivatives, using H_2O_2 as the oxidant. o-Phenylenediamine (OPD) was used as a chromogenic substrate for the colorimetric reaction for HRP and H_2O_2 (**Scheme 3.8**). The absorbance of the colored product of OPD, 2,3-diaminophenylenazine (DAP), produced can be measured spectrophotometrically at a wavelength of 450 nm (pH 5) or 492 nm (pH 1).

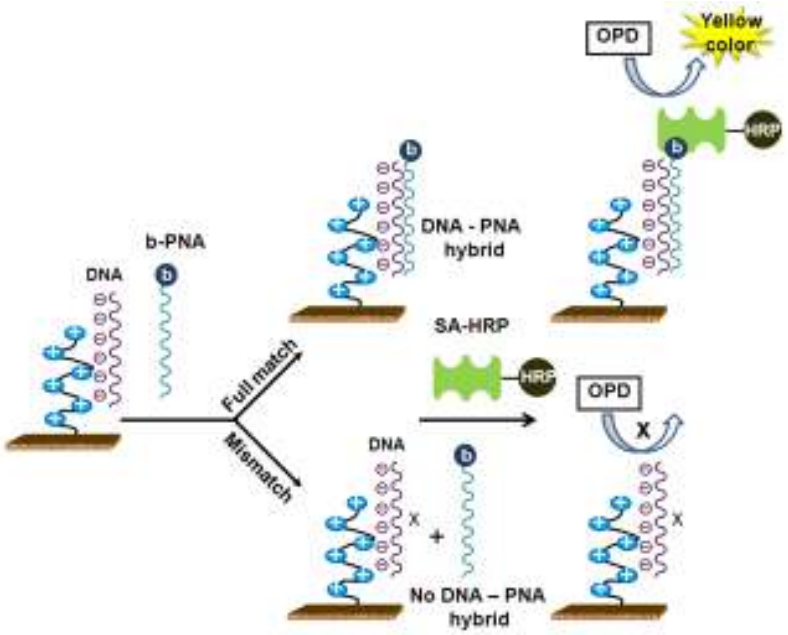
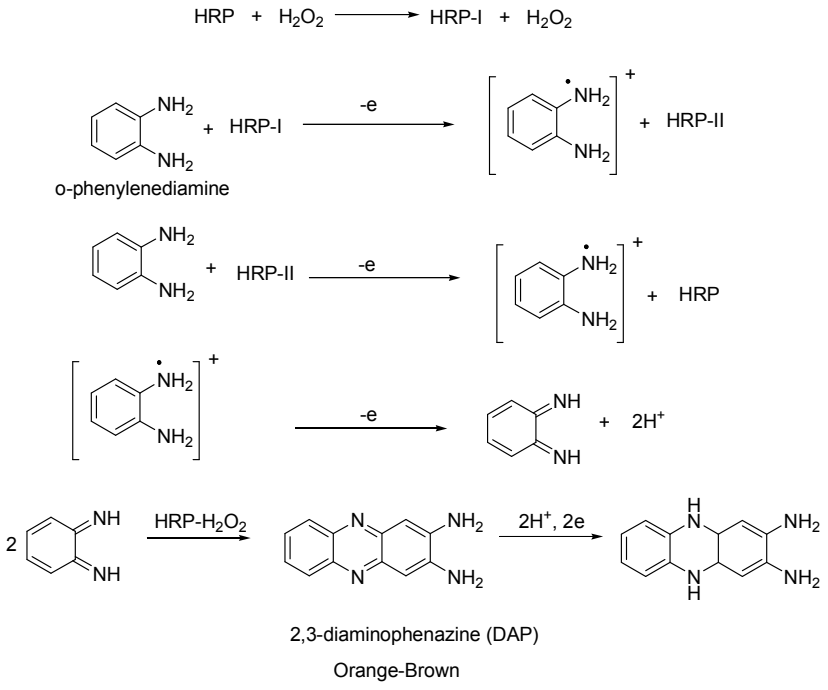


Figure 3.8 Schematic diagrams showing the concept of DNA sequence determination hybridization following "Dot blot hybridization" using enzymatic amplification.



Scheme 3.8 Schematic diagram illustrates mechanism of HRP catalyzed reaction of OPD and H_2O_2

It should be emphasized that most of the tests were performed on the QPDMAEMA grafted filter paper prepared using target DP of 200 because polymer

layer of this target DP is relatively thin which did not affected to the physical strength and resistance to bending of the filter paper. The results are displayed in two formats: scanned images (column A) and profile images as analyzed by Scion Image (column B). The profile image should provide semi-quantitative intensity of the colorimetric readout and therefore reflecting the testing efficiency and facilitating background and signal differentiation. Preliminary optimization was first carried out with 1 pmol of b-DNA SLE2 as a positive control by varying the enzyme, substrate, concentration and reaction time.

3.3.1.1 Effect of amount and type of HRP enzyme

The results shown in **Figure 3.9** suggest that SA-HRP was superior to avidin-HRP, and the optimal amount was 20 ng/spot since this condition provided high signal and minimal background due to non-specific adsorptions (**Figure 3.9d**).

3.3.1.2 Effect of concentration and type of substrate and activation time

According to **Figure 3.10**, the optimum concentration of substrate mixture is 250 μL of 1.6 mg/mL OPD and 250 μL of 1.6 mg/mL urea-H_2O_2 and the optimum activation time is 1 min because this condition gave high signal to background ratio (**Figure 3.10c**). Increasing concentration of substrate mixtures and/or reaction time gave stronger signal, but so as the non-specific adsorption (background). When TMB [21-22] was used as substrate in place of OPD, a stronger color was obtained, but also with higher background (**Figure 3.10a**). In addition, the blue color disappeared after storage at room temperature suggesting that the complex formed was not stable and that TMB was not the appropriate substrate.

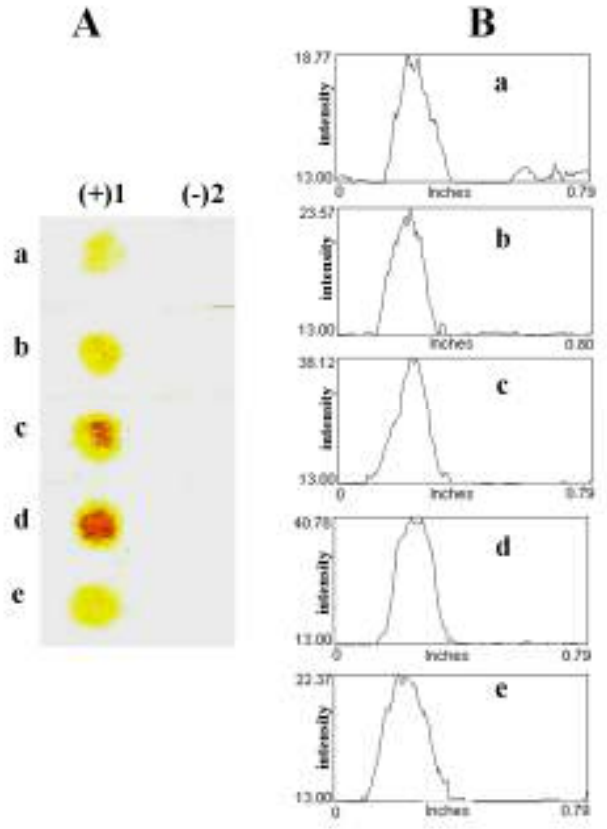


Figure 3.9 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the effect of amount and type of HRP enzyme ((+)1= w/ b-DNA SLE2 and (-)2 = w/o b-DNA SLE2): a) 20 ng avidin-HRP (2 μL of 10 μg/mL), b) 50 ng avidin-HRP (2 μL of 25 μg/mL), c) 30 ng SA-HRP (3 μL of 10 μg/mL), d) 20 ng SA-HRP (2 μL of 10 μg/mL), and e) 10 ng SA-HRP (1 μL of 10 μg/mL).

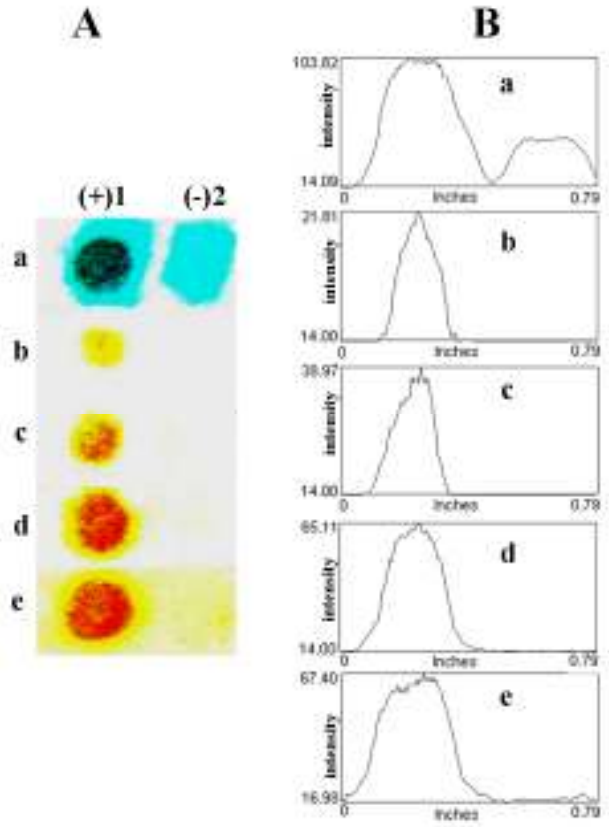


Figure 3.10 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the effect of type and concentration of substrate and reaction time used in the step of enzymatic signal amplification ((+)1= w/ b-DNA and (-)2 = w/o b-DNA) using 20 ng SA-HRP (2 μL of 10 μg/mL): a) 250 μL of 1.6 mg/mL of TMB in DMF/ H_2O (1/9) and 250 μL of 1.6 mg/mL of urea-H_2O_2 for 1 min b) 250 μL of 0.8 mg/mL OPD and 250 μL of 0.8 mg/mL urea-H_2O_2 for 1 min, c-e) 250 μL of 1.6 mg/mL OPD and 250 μL of 1.6 mg/mL urea-H_2O_2 for 1, 2 and 5 min, respectively.

3.3.1.3 Effect of blocking solution before SA-HRP addition

The use of a blocking solution (especially 1% BSA) has been found to be extremely important to prevent nonspecific interaction between the SA-HRP and QPDMAEMA grafted filter paper as demonstrated in **Figure 3.11**. As seen on **Figure 3.11b**, both expected specific hybridization and unwanted non-specific adsorption were totally suppressed after blocking with a common blocking reagent for bioassay, 1%

skim milk suggesting that it is not a proper blocking reagent. Therefore, 1% BSA was selected as blocking solution for all experiments.

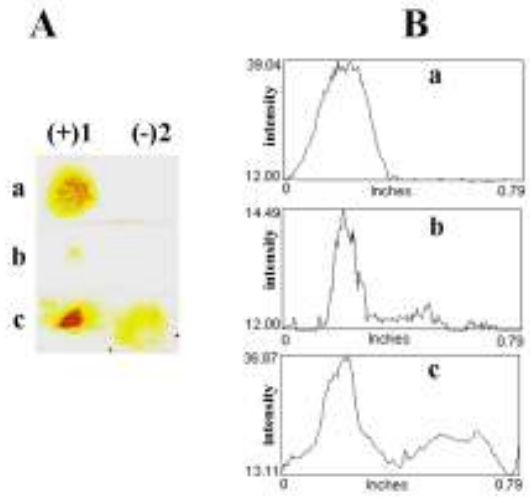


Figure 3.11 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the effect of blocking solution before SA-HRP addition ((+)1 = w/ b-DNA and (-)2 = w/o b-DNA): a) 1% BSA in 0.1 M PBS pH 7.4 containing 0.1 M NaCl, b) 1% skim milk in 0.1 M PBS pH 7.4 containing 0.1 M NaCl, c) w/o blocking. The tests were performed using the mixture of 250 μL of 1.6 mg/ mg/mL OPD and 250 μL of 1.6 mg/mL urea-H_2O_2 as substrate mixture to activate color for 1 min.

3.3.1.4 Effect of washing solution after SA-HRP addition

In this experiment, the optimal washing solution after SA-HRP hybridization was identified. The results illustrated in **Figure 3.12** have demonstrated that the yellow color on the paper was not observed by naked eyes when 0.1 M PBS pH 7.4 containing 0.1 M NaCl and Milli-Q water were used as washing solution after SA-HRP addition (**Figure 3.12a**). In contrast, the yellow color was visualized when 0.1 M CTB pH 5 and Milli-Q water were used as washing solution (**Figure 3.12b**). The rinsing by both 0.1 M PBS pH 7.4 containing 0.1 M NaCl and 0.1 M CTB pH 5 and Milli-Q water provided even better result than the rinsing by 0.1 M CTB pH 5 and Milli-Q water alone. This can be explained by the fact that the hybridization between biotin and SA-HRP is most effective under neutral condition when 0.1 M PBS pH 7.4 containing 0.1 M NaCl was

used as washing solution and the performance of HRP catalyzed oxidation reaction of OPD and urea-H_2O_2 is most favorable at lower pH when 0.1 M CTB pH 5 was used as washing solution. Combining those two washing solutions, therefore, gave better detection results.

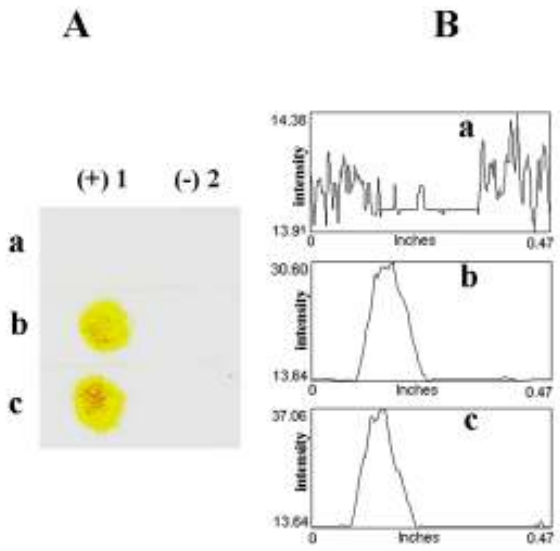


Figure 3.12 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating effect of washing solution after SA-HRP addition ((+)1= w/b-DNA and (-)2 = w/o b-DNA): a) 0.1 M PBS pH 7.4, containing 0.1 M NaCl and Milli-Q water, b) 0.1 M CTB pH 5 and Milli-Q water, c) 0.1 M PBS pH 7.4, containing 0.1 M NaCl, then Milli-Q water and 0.1 M CTB pH 5.

3.3.1.5 Effect of washing solution after PNA hybridization and SA-HRP addition

To test the ability of QPDMAEMA grafted filter paper as membrane for DNA sequence determination following "Dot blot hybridization", two closely related synthetic 13mer oligodeoxynucleotide (DNA SLE1 and DNA SLE2) differing by just one base were spotted onto the membrane and the sequences detected by a b-PNA SLE2 probe. Detail of DNA and PNA sequences and description of DNA and PNA sequences used for each spot of the test (spot 1-6) are displayed in **Table 3.6** and **3.7**, respectively. A biotinylated DNA (b-DNA SLE2) was included as a positive control to show a maximum signal that can be generated. The effects of washing and blocking solutions after PNA hybridization and SA-HRP addition were examined using the

optimal conditions mentioned above (See results in **Figure. 3.9d, 3.10c, 3.11a,** and **3.12c**).

Table 3.6 PNA and DNA sequences used in this study**.**

Code	Sequence	Description
b-DNA SLE2	d(5′-b-TTGGGAAGGGGAA-3′)	Positive control
DNA SLE1	d(5′-TTGGGAGGGGGAA-3′)	Complementary to b-PNA SLE1 Single mismatch to b-PNA SLE2
DNA SLE2	d(5′-TTGGGAAGGGGAA-3′)	Complementary to b-PNA SLE2 Single mismatch to b-PNA SLE1
DNA (AG)	d(5′-TGTGGATAGTGA-3′)	Non-complementary to b-PNA (TG)
DNA (AC)	d(5′-GTCATAGCATCA-3′)	Complementary to b-PNA (TG)
b-PNA SLE1	b-$(egl)_2$-TTCCCCCTCCCAA-Lys	-
b-PNA SLE2	b-$(egl)_2$-TTCCCCTTCCCAA-Lys	-
b-PNA (TG)	b-$(egl)_2$-TGATGCTATGAC-Lys	-

Table 3.7 Description of DNA and PNA sequences used for each spot of the test results shown in **Figure 3.14, and 3.16-3.19.**

Spot	DNA	PNA	Remark
1	b-DNA (SLE2)	-	Positive control (+)
2	DNA (SLE1)	b-PNA (SLE2)	Negative control (-)
3	DNA (SLE2)	-	Negative control (-)
4	DNA (SLE2)	b-PNA (SLE2)	Positive result (+)
5	-	b-PNA (SLE2)	Negative control (-)
6	-	-	Negative control (-)

Only the signal of b-DNA SLE2 [(+)1)] and DNA SLE2/b-PNA SLE2 [(+)4] **(Table 3.6)** gave yellow spots visible by naked eyes **(Figure 3.13)**. Apparently, the signal at the positive control position [(+)1)] is more intense when 0.1 M NaCl was added to washing and blocking solutions **(Figure 3.13a)** than that observed in the case when salt was not included **(Figure 3.13b)** suggesting salt dependency of DNA·DNA hybridization. That was not the case for the tested position [(+)4] of which intensity readout in both cases **(Figure 3.13a-b)** was not affected by salt addition. Lowering the PBS concentration down from 0.1 M to 50 mM in the absence of 0.1 M NaCl **(Figure 3.13d)** gave inferior detection efficiency with slight yellowish tint observed on the two negative controls [(-)5) and (-)6)] Such non-specific adsorption disappeared and a good detection result was obtained (comparable to the result in **Figure 3.13b**) upon the addition of 0.1 M NaCl **(Figure 3.13c)**. The reason that 50 mM PBS in the absence of 0.1 M NaCl was reduced ionic strength results in reduce ability to remove non-specific interaction. Therefore, 0.1 M PBS without salt addition **(Figure 3.13b)** was chosen as washing and blocking buffer for all subsequent experiments.

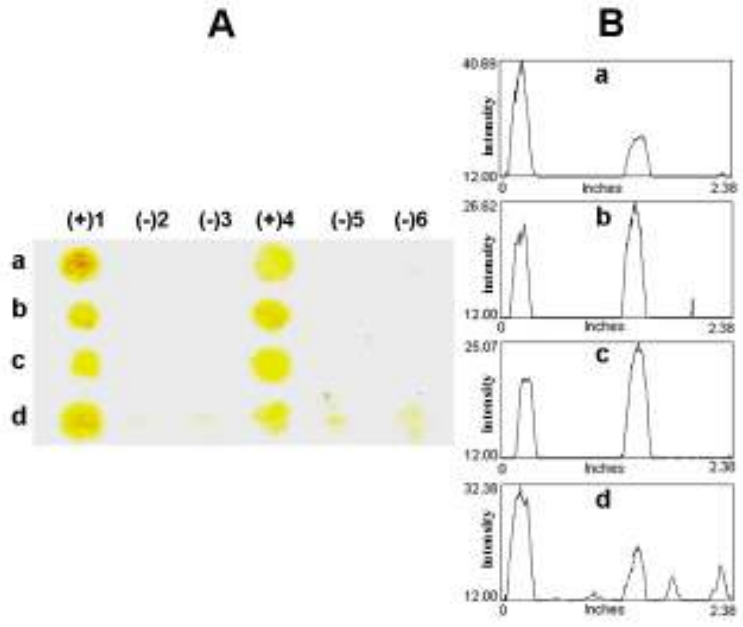


Figure 3.13 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the effect of PBS concentration and salt addition in washing and blocking solutions: a) 0.1 M PBS with 0.1 M NaCl, b) 0.1 M PBS without 0.1 M NaCl, c) 50 mM PBS with 0.1 M NaCl, and d) 50 mM PBS without 0.1 M NaCl.

3.3.1.6 Specificity of PNA·DNA hybridization

Three acpcPNA probes: b-PNA SLE2 and b-PNA SLE1, which are differing by only one base, and an unrelated b-PNA (TG) were chosen for specificity determination. Detail of DNA and PNA sequences and description of samples/probes applied in each spot (1-6) are illustrated in **Table 3.6** and **3.8**, respectively. The data presented in **Figure 3.14a** and **b** reveal that the Dot blot hybridization using the QPDMAEMA-grafted filter paper and acpcPNA probes offers an excellent specificity to distinguish single base mismatches in the DNA target consisting a partial sequence of human *IL-10* promoter gene (SLE1 and SLE2) by showing only the signal of b-PNA that was hybridized with the complementary DNA target. In addition to these two sequences, the same technique employing b-PNA (TG) as a probe can also successfully distinguish between DNA between complementary and non-complementary DNA targets since the yellow spot was only observed at the position of the complementary DNA target (AC) (**Figure 3.14c**). This illustrated the general applicability of the membrane for detection of various DNA sequences.

Table 3.8 Description of DNA and PNA sequences used for each spot of the test results shown in **Figure 3.15**

Entry	Sequence	Spot position					
		1	**2**	**3**	**4**	**5**	**6**
a	DNA	b-DNA SLE 2	DNA SLE 1	DNA SLE 2	DNA SLE2	-	-
	PNA	-	b-PNA SLE2	-	b-PNA SLE2	b-PNA SLE2	-
	[a]Remark	(+)	(-)	(-)	(+)	(-)	(-)
b	DNA	b-DNA SLE 2	DNA SLE 1	DNA SLE 2	DNA SLE2	-	-
	PNA	-	b-PNA SLE1	-	b-PNA SLE1	b-PNA SLE1	-
	[a]Remark	(+)	(+)	(-)	(-)	(-)	(-)
c	DNA	b-DNA SLE 2	DNA (AG)	DNA (AC)	DNA (AC)	-	-
	PNA	-	b-PNA (TG)	-	b-PNA (TG)	b-PNA (TG)	-
	[a]Remark	(+)	(-)	(-)	(+)	(-)	(-)

[a](+) = positive result, (-) = negative result

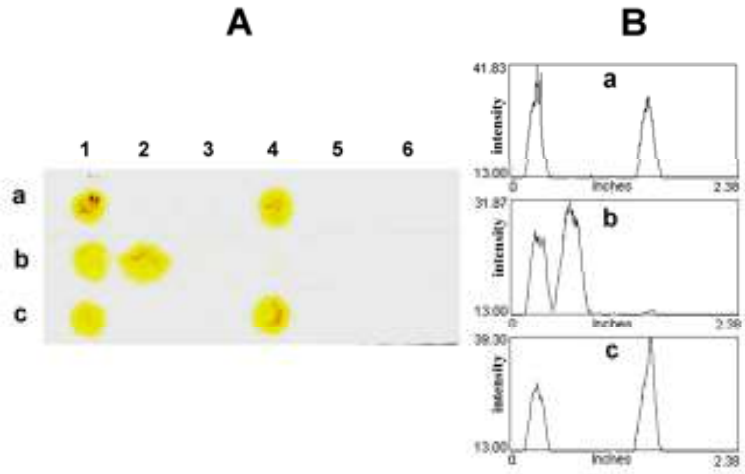


Figure 3.14 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the specificity and efficiency of a) b-PNA SLE2, b) b-PNA SLE1 to distinguish complementary and single mismatched DNA (SLE2 and SLE1) targets or c) complementary DNA (AC) and non-complementary DNA (AG) using b-PNA (TG). The positive results are shown by the yellow spots at position 4 for the tests in entries a and b, and 2 for entry b.

3.3.1.7 Detection efficiency in comparison with commercial membranes

To compare the performance of the modified filter paper described in this work with commercial membranes, the detection of DNA SLE2 with b-PNA SLE2 was selected for the investigation. The detail of DNA and PNA sequences and description of DNA and PNA sequences used for each spot of the test (spot 1-6) are displayed in **Table 3.6** and **3.7**, respectively. The results present in **Figure 3.15** that only the QPDMAEMA grafted filter paper provided a clear-cut discrimination between complementary and single mismatched DNA **(Figure 3.15d)**. On the other hand, the unquaternized PDMAEMA grafted filter paper gave low signals even with the positive control **(Figure 3.15c)**, indicating that the quaternary ammonium groups of PDMAEMA are essential for efficient capture of DNA. In addition, this quaternary ammonium groups are important to their ability to be used for DNA sequence analysis because the amino group of unquaternized PDMAEMA grafted filter paper could be protonated led to changed pH and decreased efficiency of DNA-PNA hybridization.

The commercial nitrocellulose and nylon 66 membranes showed substantial nonspecific interactions as shown by the presence of dark spots with the non-complementary DNA target. In the case of nitrocellulose **(Figure 3.15a),** the appearance of b-PNA signal in the absence of the hybridized DNA suggested that there were non-specific interactions between the surface of membrane and the b-PNA probe. With nylon 66 **(Figure 3.15b)**, the colored spots were observed in all six positions implying that there were non-specific interactions between the membrane surface and SA-HRP as well.

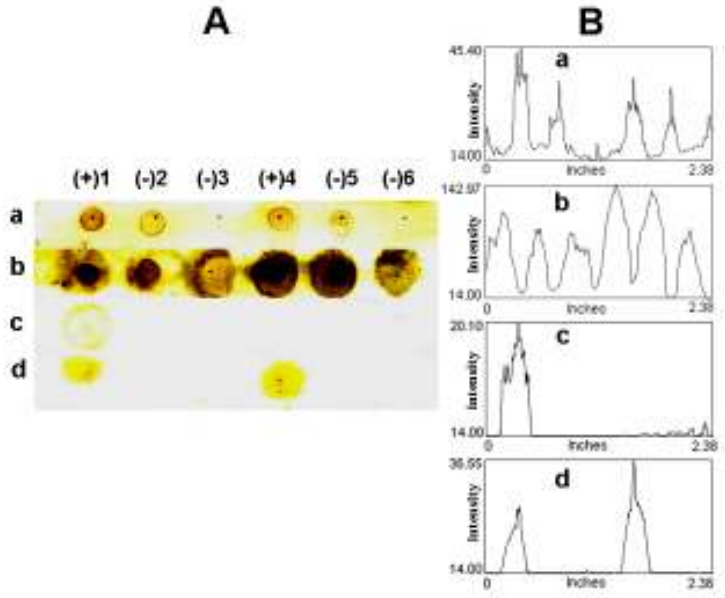


Figure 3.15 Representative images (column A: scanned image and column B: profile image) of the test result demonstrating the performance of the QPDMAEMA grafted filter paper in comparison with commercial membranes: a) nitrocellulose, b) Nylon 66, c) PDMAEMA, and d) QPDMAEMA grafted filter paper.

3.3.1.8 Stability of QPDMAEMA grafted filter paper

To test the stability of the QPDMAEMA functionalized filter paper upon storage, the Dot blot experiments were carried out using a membrane that has been stored at room temperature (27-33 ºC) for 1 month in comparison with the freshly prepared membranes. The results shown in **Figure 3.16** demonstrate that both membranes provided comparable results in distinguishing complementary and mismatched DNA targets suggesting that the modified filter paper is stable and the detection efficiency is not affected upon storage for at least one month. This outcome

is quite desirable from practical perspective.

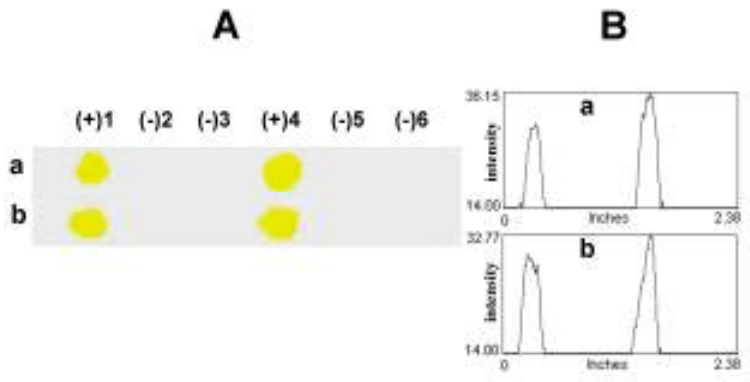


Figure 3.16 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating sequence determination of DNA SLE2 sample using b-PNA SLE2 probe on the QPDMAEMA-grafted filter paper: a) freshly prepared and b) stored at room temperature (27-33 ºC) for 1 month.

3.3.1.9 Limit of detection (LODs)

The lowest amount of DNA that can still allow a clear discrimination between fully complementary (DNA SLE2) and single-mismatched DNA (DNA SLE1) was evaluated by spotting different quantities of the DNA targets (2 pmol to 10 fmol) and detecting using 2 pmol of b-PNA SLE2 probe. The results in **Figure 3.17** show that unambiguous discrimination, which can be quoted as the detection limit of this colorimetric method, is at least down to 10 fmol (equivalent to 1 µL of 10 nM DNA).

Although this detection limit is somewhat higher than some other reports [21-22,48-49] (see **Table 3.9**), our main objective to develop positively charged filter paper-based membrane for simple naked eyes detection of DNA using PNA probe and rapid analysis of multiple DNA samples with a short reaction time (less than 1 hour from DNA spotting to color readout) has been fulfilled. We have previously demonstrated that this level of sensitivity is more than sufficient to detect DNA samples from standard PCR products [5] and, if necessary, it should be possible to improve the detection limit further using polybiotin tags[13,21] or polymeric enzymes.[21]

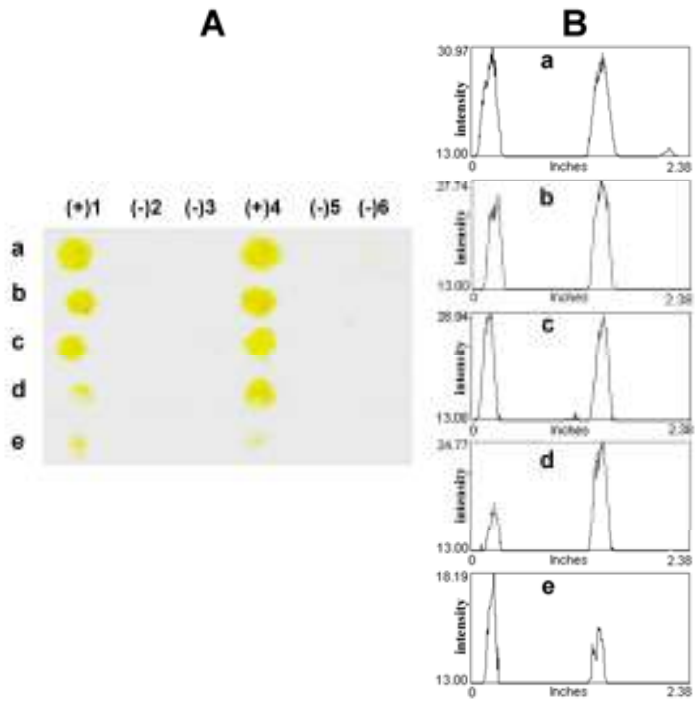


Figure 3.17 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating detection limit of complementary DNA SLE2 and single mismatched DNA SLE1 using 2 μL of 1 μM b-PNA SLE2 as probe: a) 2 pmol (2 μL of 1 μM), b) 1 pmol (1 μL of 1 μM), c) 100 fmol (1 μL of 100 nM), d) 50 fmol (0.5 μL of 100 nM),and e) 10 fmol (1 μL of 10 nM).

Table 3.9 Comparison of detection limit with other reports

Author	Detection limit	Probe	Enzyme	Substrates	surface	Time (h)
Reed *et al.*[48]	0.8 µg real DNA	Radio labeled DNA	-	-	Nylon	16
LEARY *et al.*[49]	1-2 pg (0.5 fmol) real DNA	b-DNA	Apoly(BAP) complex	NBT/BCIP	nitrocellulose	4.75
Zhang *et al*.[21]	10 zmol (5 aM, synthetic DNA) 2.5 µg (real DNA)	b-PNA (2 biotin)	Avidin-polyHRP	TMB/H_2O_2	DNA-BIND®96 well plate	4
Su *et a*l.[22]	10 fmol (0.2 nM, synthetic DNA) 2 µg (real RNA)	b-PNA	HRP	TMB/H_2O_2	streptavidin (SA)-coated polystyrene 96-well plates	3
Boontha *et al.*[5]	1-10 pmol (1 µM, synthetic DNA) 25 ng (real DNA)	acpcPNA	-	-	Q-sepharose	~1
Our research	10 fmol (10 nM, synthetic DNA)	b-acpcPNA	SA-HRP	OPD/urea-H_2O_2	QPDMAEMA grafted filter paper	<1

3.3.1.10 Applications in detection of PCR Samples

Since this detection system successfully detected single-stranded (synthetic) DNA as described in above, the ability of this technique to detect double-stranded DNA derived from PCR amplification was further investigated. The preliminary study revealed that this technique could be detected PCR product enriches target sequences but the high concentration of sample was required. This resulted in high detection limit for double-stranded DNA, which was necessary to improve in the future. In addition, PCR product was long double helix DNA molecule, which is necessary denatured by heating at 95 ºC, becomes two single-strand DNA chains before hybridization step. PNA probe are short oligoneucleotide so the nature of interaction of acpcPNA with PCR product should be further investigated.

3.3.2 Polymerization amplification

The concept of DNA sequence determination following "Dot blot hybridization" using polymerization for signal amplification is displayed in **Figure 3.18**. After dot blotting DNA on membrane, a DNA target was immobilized via electrostatic interactions between the positive charges of the QPDMAEMA brushes and negative charges of the phosphate backbone of DNA. Upon hybridization with initiator end-capped acpcPNA probe (I-PNA), the PNA·DNA hybridization can be detected by naked eye observation of the colored copolymer product generated by the ARGET ATRP polymerization. In this research, two monomers have been used, poly(ethylene glycol)methacrylate (PEGMA) and rhodamine B-functionalized 2-hydroxyethyl methacrylate (Rh B-HEMA). Rhodamine B is a hydrophilic fluorescence dye containing carboxylic group that can react with hydroxyl groups of HEMA and yielded Rh B-HEMA monomer. PEGMA was used as the comonomer to render the copolymer water solubility so that the copolymer formed in the solution (free polymer) can be easily removed with a mild condition by simple aqueous washing which should not interfere the stability of the PNA·DNA hybrid. The copolymer formed should remain intact only when it grew from the I-PNA hybridized with its complementary DNA that was bound electrostatically to the QPDMAEMA grafted filter paper.

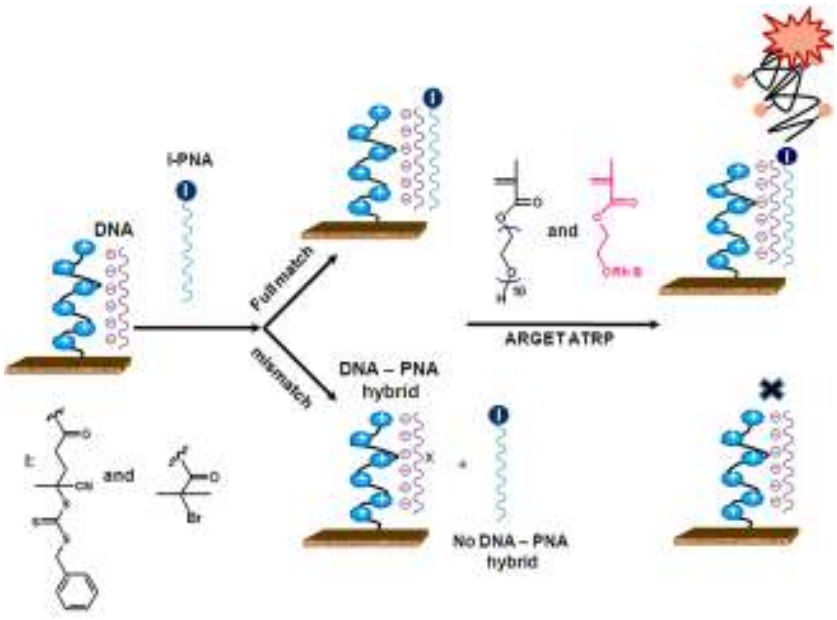
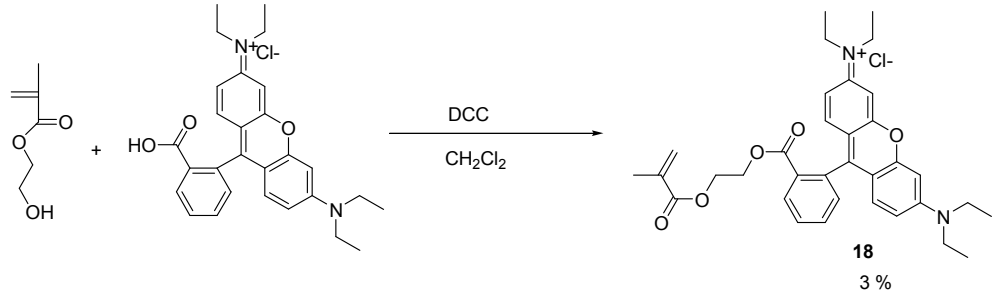


Figure 3.18 Schematic diagram showing the concept of DNA sequence following "Dot blot hybridization" using polymerization amplification

3.3.2.1 Synthesis and characterization of rhodamine B-functionalized 2-hydroxyethyl methacrylate (Rh B-HEMA) (18)

Rh B-HEMA was synthesized from rhodamine B (Rh B) and 2-hydroxyethylmethacrylate (HEMA) using DCC as coupling reagent in dichloromethane for 48 h (**Scheme 3.9**). The product was obtained in low yield (3%) as red solid. The structure of product was confirmed by ^{1}H NMR and its spectrum is shown in **Figure C-1 (**Appendix C).

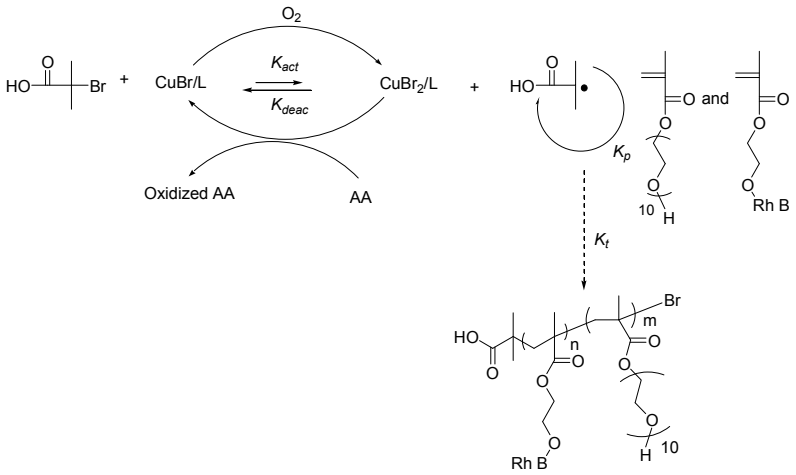


Scheme 3.9 Synthesis of rhodamine B-functionalized 2-hydroxyethyl methacrylate (Rh B-HEMA).

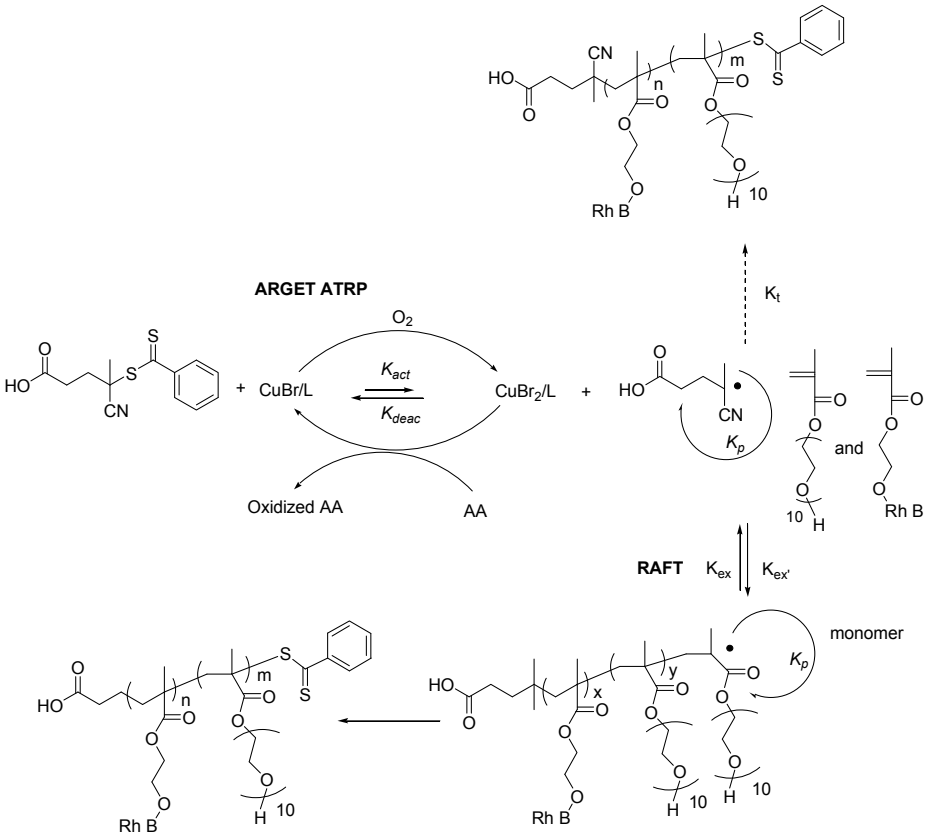
3.3.2.2 Solution copolymerization of Rh B-HEMA and PEGMA by ARGET ATRP

Copolymerization of Rh B-HEMA and PEGMA in aqueous solution was tested via 2 routes; ARGET ATRP and concurrent ARGET ATRP/RAFT. ARGET ATRP used 2-bromobutyric acid as initiator and mechanism of this polymerization was shown in **Scheme 3.10.** Concurrent ARGET ATRP/RAFT which is the living polymerization process under control two equilibrium both metal complex redox equilibrium and chain transfer equilibrium used 4-cyano-4-(thiobenzoylthio)pentanoic acid as initiator and chain transfer agent (see **Scheme 3.11**). The catalyst mixture of $CuBr_2$/Me_6TREN was dissolved in water and added to an aqueous solution of Rh B-HEMA/PEGMA (1:35 by mole) derived from the optimal ratio of copolymer of Methyl red modified-HEMA and PEGMA via ARGET ATRP from previous report.[62] Ascorbic acid (AA) was selected as the reducing agent because it is water soluble and environmentally friendly. The concentration of Cu(II) in the system is

2.24 mM. Cu (II)/AA with the ratio of 1:67 was employed not only to reduce Cu (II), but also to consume the oxygen in the free space above the solution (8 mL) of the reaction vial. Polymerization progress and percentage of monomer conversion were monitored by ^{1}H NMR. From the NMR spectrum shown in **Figure 3.19a**, the signal at 0.8-2.2 and 4.15 ppm can be assigned to the methylene in methacrylate part (-C$\underline{H}_2$C(CH$_3$)) and methylene group in ethylene glycol part (O$\underline{CH}_2$CH$_2$O) of homopolymer of PEGMA (PPEGMA), respectively. In the case of homopolymer of Rh B-HEMA (P(Rh B-HEMA), the signal at ~6.9-7.8 ppm indicated the presence of aromatic moiety in the obtained product (**Figure 3.19b**). In addition, the presence of signals at ~6.9-7.8 ppm assigned to aromatic moiety and at 4.15 ppm assigned to methylene group of ethylene glycol part in **Figure 3.19c** confirm that the copolymerization via both ARGET ATRP (**Figure C-2** (Appendix C)) and concurrent ARGET ATRP/RAFT mechanisms was successful. This outcome provides important information that can be further applied for polymerization amplification.



Scheme 3.10 Copolymerization of Rh B-HEMA and PEGMA via ARGET ATRP mechanism.



Scheme 3.11 Copolymerization of Rh B-HEMA and PEGMA via concurrent ARGET ATRP/RAFT mechanism.

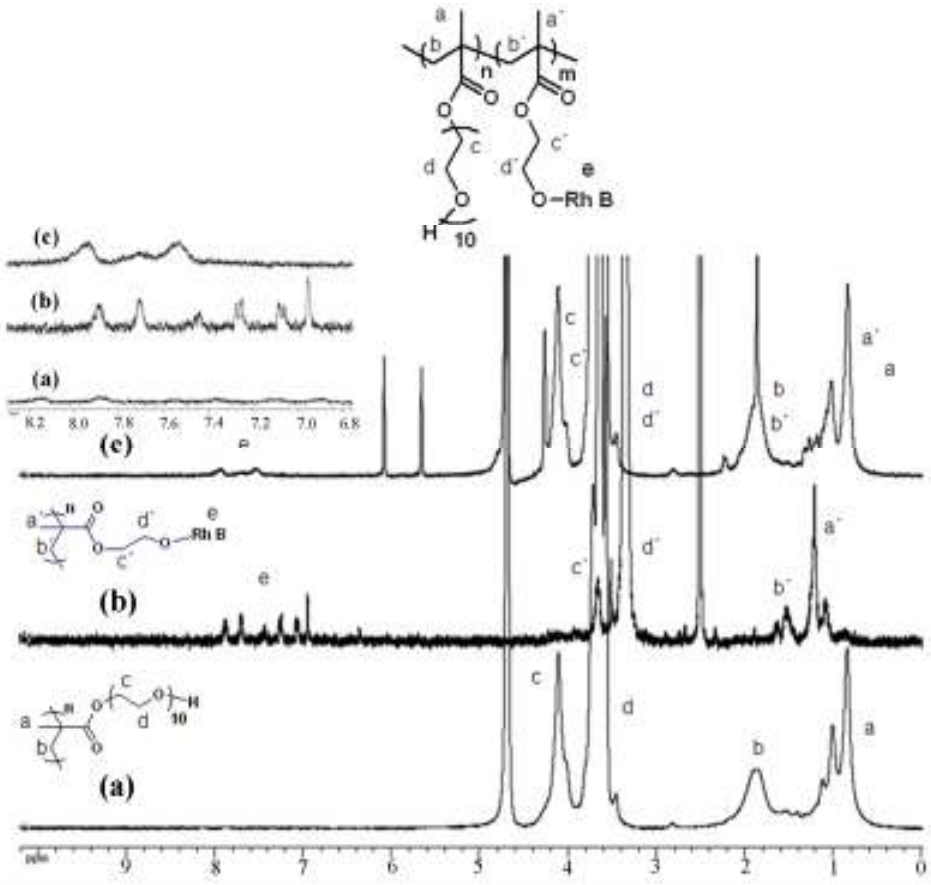
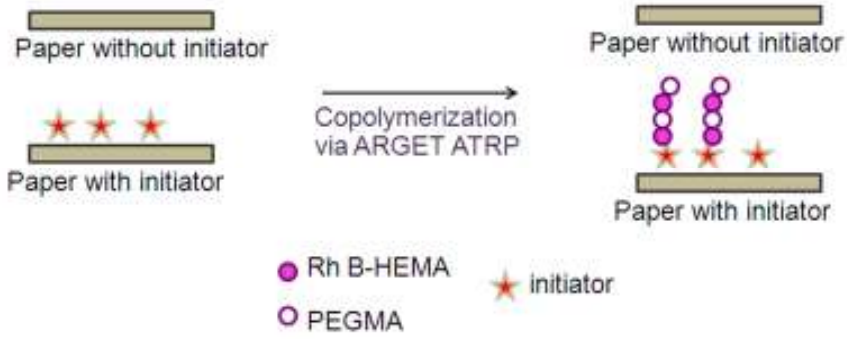


Figure 3.19 ^{1}H NMR spectra after dialysis and lyophilization of a) P(PEGMA), b) P(Rh B-HEMA), and c) P(PEGMA)-co-P(Rh B-HEMA) synthesized by concurrent ARGET ATRP/RAFT using 4-cyano-4-(thiobenzoylthio)pentanoic acid as initiator.

3.3.2.3 Synthesis and characterization of P(PEGMA)-co-P(Rh B-HEMA) brushes grown on QPDMAEMA grafted filter paper

Using the information of the successful copolymerization of PEGMA and Rh B-HEMA described in the previous section, the surface initiated copolymerization of the two monomers on the QPDMAEMA grafted filter paper was tested (**Scheme 3.12)**. After the selected initiator was spotted on the QPDMAEMA grafted filter paper, the substrate was immersed in a mixture containing monomers and catalyst to allow the copolymer to grow specifically from the initiator. After a specified period of time, the paper was rinsed excessively with DI water.



Scheme 3.12 Copolymerization of Rh B-HEMA and PEGMA on QPDMAEMA grafted filter paper.

For the test, 72 nmol (1 μL taking from 72 mM solution) of each initiator was employed. α-Bromoisobutyric acid (BiB) was tested for copolymerization via ARGET ATRP whereas 4-cyano-4-(thiobenzoyl)sulfanyl)pentanoic acid (CTA, **19**), and 3-benzylsulfanylthiocarbonylsufanyl propionic acid (BPA, **15**) were tested for copolymerization via concurrent RAFT/ARGET ATRP. The results shown in **Figure 3.20** demonstrated that the growth of copolymer on the paper surface was not observed when BiB was used as the initiator (**Route A**). In contrast, copolymerization via concurrent RAFT/ARGET ATRP was favorable as can be realized from the strong pink stain on the paper physically adsorbed with CTA (**Route B**) and BPA (**Route C**). The absence of background signal strongly suggested that aqueous washing was sufficient to remove the unbound free copolymer formed in the solution. Despite its effectiveness in initiating the copolymerization on the paper, CTA **(19)** cannot be further used for signal amplification because it was found that acpcPNA end functionalized with CTA was not stable upon cleavage by TFA, the reagent used to take off the modified PNA from solid phase. Therefore, only BPA-end capped acpcPNA was subjected to further investigation.

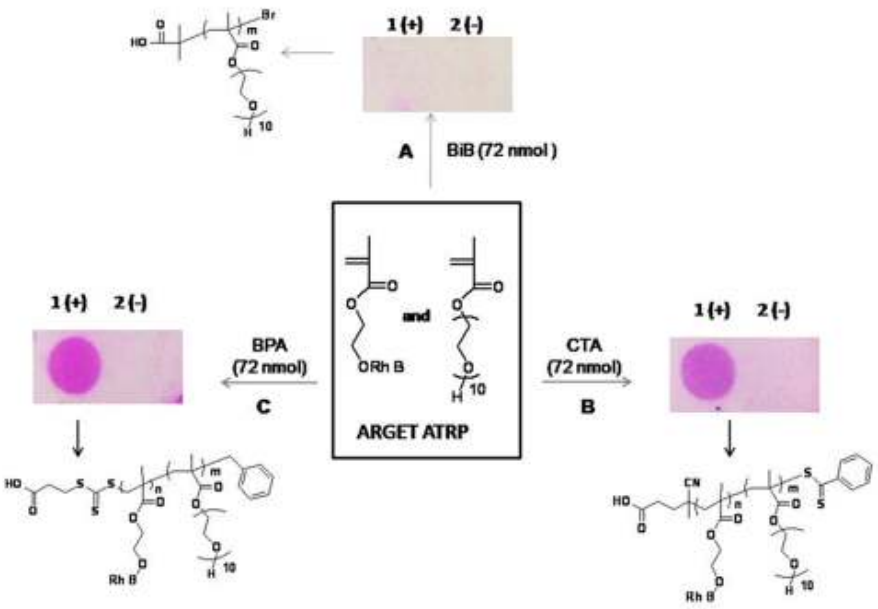


Figure 3.20 Test result demonstrating the effect of type of initiator used for surface-initiated copolymerization of PEGMA and Rh B HEMA from QPDMAEMA grafted filter paper with (1(+)) and without initiator (2(-)).

To better understand why the copolymer did not grow on the paper when BiB was used as the initiator, a kinetic study was performed to examine the effect of initiator for polymerization of PEGMA (target DP = 200) in solution. At a desired time interval, the solution was taken out and analyzed by ^{1}H NMR. The PEGMA conversion was determined based on the integration of proton signals of vinyl group in PEGMA (δ6.0, **Figure 3.19**) and methylene group (δ0.5-1.0, **Figure 3.19**) in the polymer repeat unit. The kinetic plots shown in **Figure 3.21** suggested that the conversion increased linearly with time. The rate of polymerization initiated by BiB was faster than that initiated by CTA. The gelation took place within 30 min of polymerization initiated by BiB. For this reason, the homopolymerization of PEGMA was so fact that it became difficult for the more sluggish monomer, Rh B-HEMA to be incorporated into the copolymer by ARGET ATRP mechanism. In contrast, the slower kinetic of ARGET ATRP/RAFT allowed the copolymerization to take place. Without added initiator, self-polymerization of PEGMA via ARGET ATRP was also possible but presumably under an uncontrolled manner.

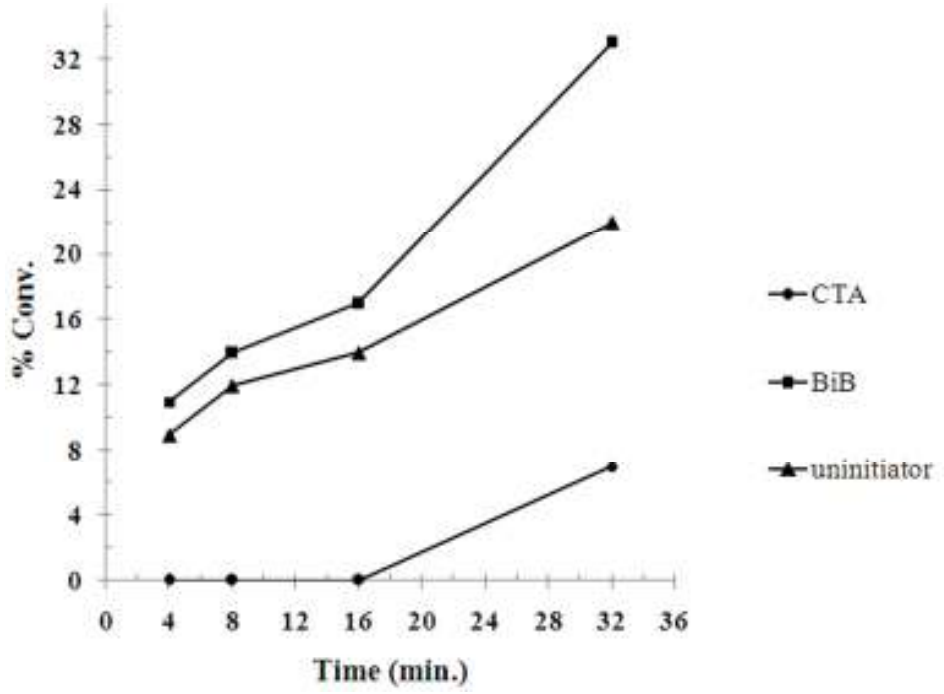


Figure 3.21 Kinetic plots of % conversion versus time in ARGET ATRP of PEGMA. Experimental condition; initiator: PEGMA = 1:200, $CuBr_2$: Me_6TREN (1:50), $CuBr_2$: AA (1:67), the volume of free space above solution was 8.0 mL.

To confirm the growth of the copolymer from the surface, the QPDMAEMA grafted filter paper without initiator (**Figure 3.20**, Route C, spot position 2(-)), and with initiator (**Figure 3.20**, Route C, spot position 1(+)) were characterized by ATR FT-IR (**Figure 3.22**). Apparently, the intensity of characteristic C=O stretching peak at 1723 cm^{-1} significantly increased from that of the QPDMAEMA grafted filter paper without initiator (**Figure 3.22c**) to that of the QPDMAEMA grafted filter paper with initiator (**Figure 3.22d**) suggesting that the additional intensity came from the copolymer grown from the initiator bound to the paper. The emergence of signal due to C=C stretching of aromatic moiety at 1543 cm^{-1} on the QPDMAEMA grafted filter paper with initiator truly verified that Rh B-HEMA was incorporated into the copolymer (**Figure 3.22d**).

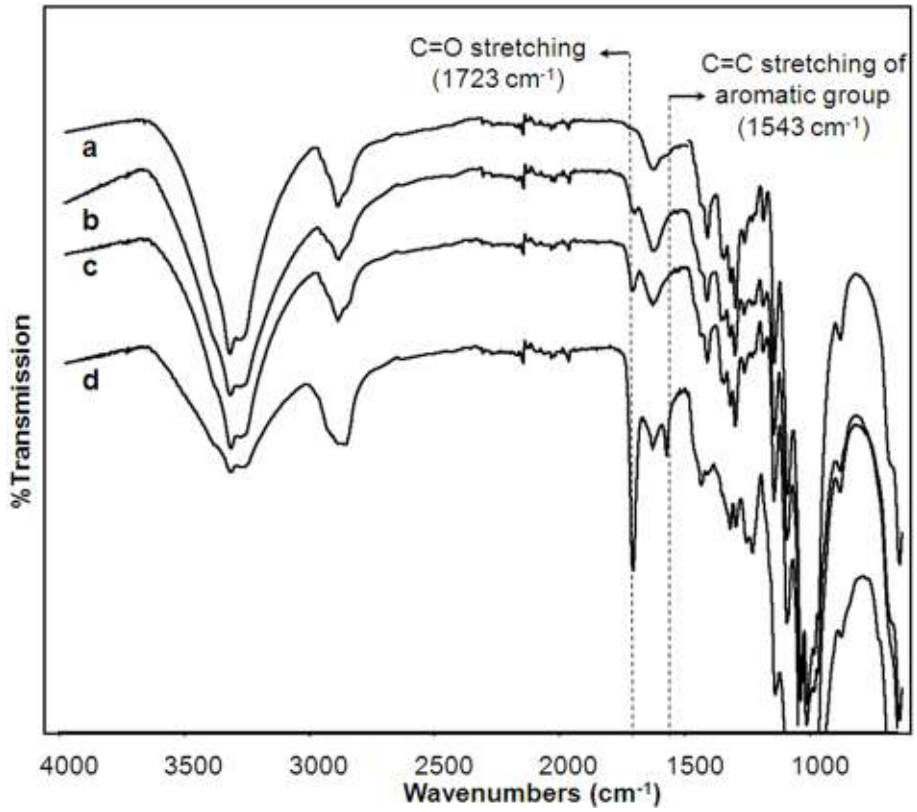


Figure 3.22 ATR-FT IR spectra of a) virgin filter paper, b) QPDMAEMA grafted filter paper, c) QPDMAEMA grafted filter paper without initiator (Figure 3.20, Route C, spot position 2(-)), and d) QPDMAEMA grafted filter paper with initiator (Figure 3.20, Route C, spot position 1(+)) after subjected to copolymerization of PEGMA and Rh B HEMA.

3.3.2.1 DNA sequence determination using concurrent ARGET ATRP/ RAFT for signal amplification

DNA sequence determination following Dot blot hybridization employing concurrent ARGET ATRP/RAFT polymerization of PEGMA and Rh B HEMA for signal amplification was tested using 2 pmol of DNA-SLE2 and 2 pmol of I-PNA SLE2 having end functionalized BPA. After DNA SLE2 was applied on the QPDMAEMA grafted filter paper, I-PNA SLE2 was then hybridized and the filter paper was washed immediately three times each with 0.1 M PBS and Milli-Q water to remove excess and unhybridized I-PNA SLE2. Description of DNA and PNA sequences used for each spot of the test (spot 1-3) is displayed in **Table 3.9**. After

that, the filter paper was then immersed in the solution containing monomers and catalysts as mentioned above (See section **3.3.2.3**).

Table 3.9 Description of DNA and PNA sequences used for each spot of the test results shown in **Figure 3.22**

Entry	Sequence	Spot position		
		1	**2**	**3**
a	DNA	DNA SLE 2	-	-
	PNA	I-PNA SLE2 (2 pmol)	I-PNA SLE2 (2 pmol)	-
	[a]Remark	(+)	(-)	(-)
b	Initiator (BPA)	72 nmol	1 nmol	100 pmol
	PNA	-	-	-
	[a]Remark	(+)	(+)	(+)

[a](+) = positive result, (-) = negative result

The test results shown in **Figure 3.23, entry a** indicated that the signal of DNA SLE2/I-PNA SLE2 [(+)1] was not observed implying that the amount of I-PNA SLE2 might be too low to generate enough quantity of the colored copolymer that can be visualized by naked eye. In order to seek for the minimum I-PNA SLE2 required for effective signal amplification, we conducted a set of experiments by spotting directly the BPA initiator with various quantity from 100 pmol to 1 nmol and 72 nmol replace amount of I-PNA SLE2 on the QPDMAEMA grafted filter paper before emerging the spotted paper in the polymerization solution. As seen in **Figure 3.23, entry b**, only the position with 72 nmol initiator [(+)1] gave red spots visible by naked eyes. These results indicated that the effective signal amplification requires initiator group of greater than 1 nmol. This requirement is not practical given that the I-PNA amount to begin with was 1.5 μmol (the quantity obtained from each solid phase synthesis cycle) and only part of that, the amount of which cannot be estimated, would be hybridized with the immobilized DNA. Therefore, it was assumed that the

polymerization amplification was thus considered much less efficient compared to the enzymatic amplification of which LOD has reached as low as 10 fmol.

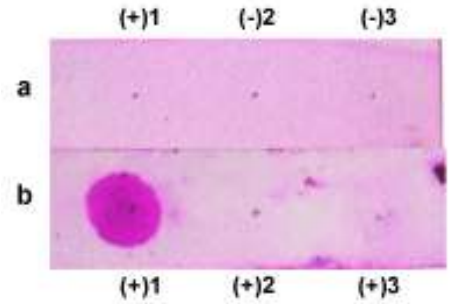


Figure 3.23 Representative image of the test result demonstrating the effect of I-PNA SLE2 quantity and on signal amplification by polymerization: a) 2 pmol of I-PNA SLE2 and b) 72 nmol [(+)1], 1 nmol [(+)2], and 100 [(+)3] pmol of initiator (BPA).

CHAPTER IV

CONCLUSION AND SUGGESTIONS

In this research, the QPDMAEMA grafted on the filter paper was synthesized by surface-initiated polymerization of DMAEMA via ARGET ATRP followed by quaternization with methyl iodide. The molecular weight and polydispersity index of the polymer can be controlled by adjusting the ratio of $Sn(EH)_2$/initiator and reaction time. The surface-modified filter paper was characterized by FT-IR, SEM and XPS. The FT-IR spectra indicated that the PDMAEMA was successfully grafted on filter paper. No significant change in the IR bands was observed after quaternization. From SEM, the surface morphology of all surface-modified filter paper exhibited no observable changes upon polymer grafting suggesting that the layer of coated polymer was relatively thin. As characterized by XPS, the detection of atomic signal of N_{1s} peak at 398.6 eV on the PDMAEMA and N_{1s} peak at 402.0 eV on the QPDMAEMA grafted filter papers confirmed the successful polymer attachment via SIP process. In addition, the extent of surface quaternization was about 80% and the QPDMAEMA grafted filter paper were capable of binding electrostatically with a model DNA, DNA SLE2 by detecting 1.2 % of phosphorus, a characteristic signal of the phosphate group on DNA suggesting their ability to be used for DNA sequence analysis.

Biotin labeled acpcPNA and initiator end capped acpcPNA were synthesized by solid phase peptide synthesis. The crude acpcPNA probes having biotin and initiator **(14)** were purified by reverse phase HPLC with a gradient of 0.1% TFA in MeOH and 0.1% TFA in water. However, the initiator **(16)** labeled PNA oligomer was purified with a gradient system of 0.1% TFA in acetronitrile and 0.1% TFA in water since it is reactive to aminolysis and alcoholysis.

The concept of using QPDMAEMA grafted filter paper as membrane for DNA sequence determination following “Dot blot hybridization” employing modifier labelled acpcPNA as probe and colorimetric assay for signal amplification has been

demonstrated. In enzyme amplification mode, it is obvious that the QPDMAEMA grafted filter paper showed better performance for DNA sequence detection than commercial membranes, nylon 66 and nitrocellulose when biotinylated acpcPNA probe was used. The low non-specific interaction of the modified filter paper, together with the high specificity of the PNA probe allows single mismatch discrimination down to 10 fmol of DNA target to be detected.

In polymerization amplification, rhodamine B-functionalized 2-hydroxyethylmethacrylate (HEMA-Rh B) monomer was synthesized and characterized by ^{1}H NMR. The copolymerization of HEMA-Rh B and PEGMA in solution and on QPDMAEMA grafted filter paper via concurrent RAFT/ARGET ATRP was also investigated. However, the signal amplification for DNA sequence determination via RAFT/ARGET ATRP copolymerization of HEMA-Rh B and PEGMA required the amount of initiator greater than 1 nmol to form enough colored copolymer to be detected by naked eyes. Therefore, the polymerization amplification was thus considered much less efficient compared to the enzymatic amplification.

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APPENDICES

APPENDIX A

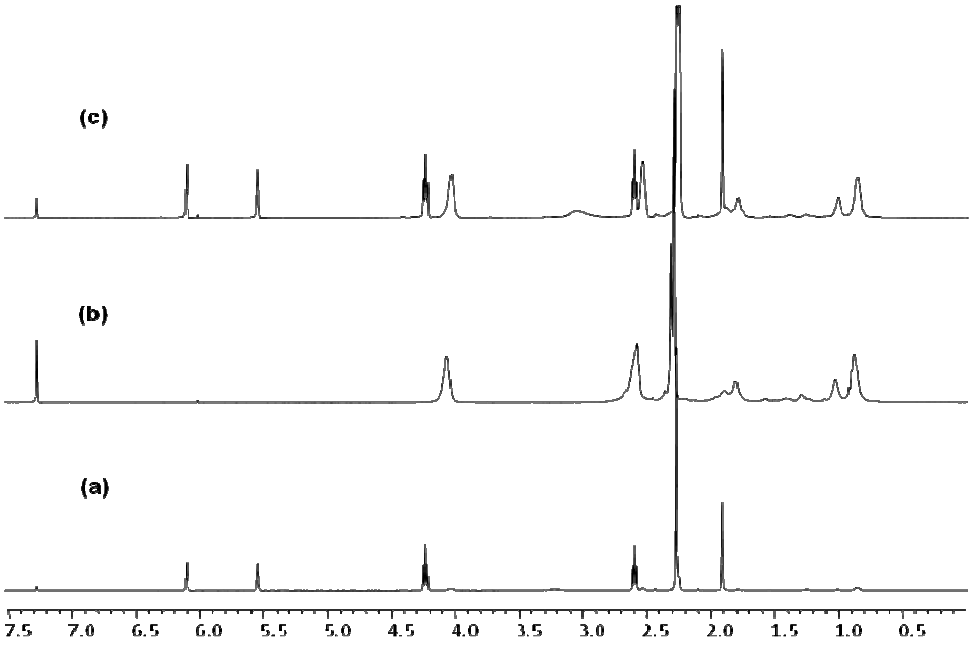

Figure A-1 Schematic of ^{1}H NMR spectra of a) DMAEMA monomer, b) PDMAEMA, and c) crude reaction of PDMAEMA via ARGET ATRP

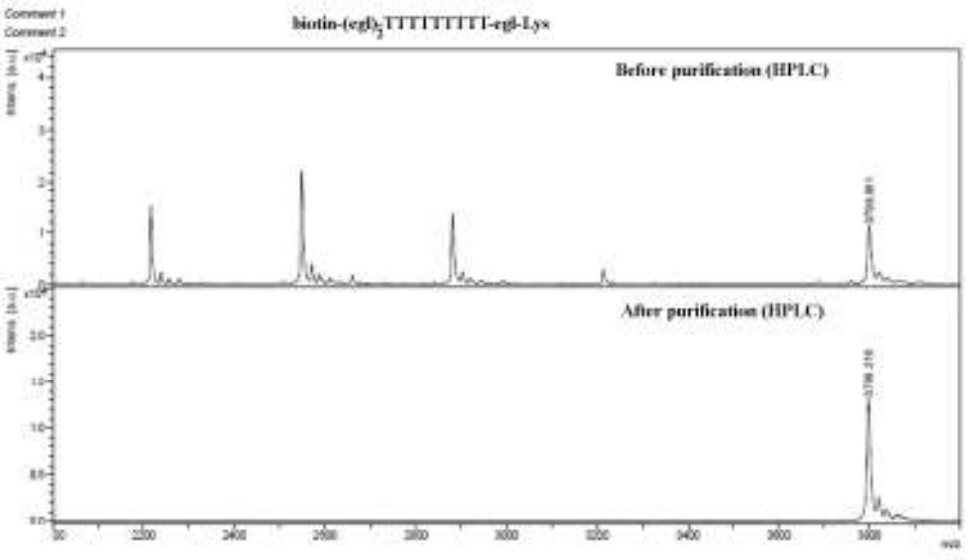

Figure A-2 Mass spectra of biotin-$(egl)_2$-TTTTTTTT-egl-Lys before and after purification by HPLC.

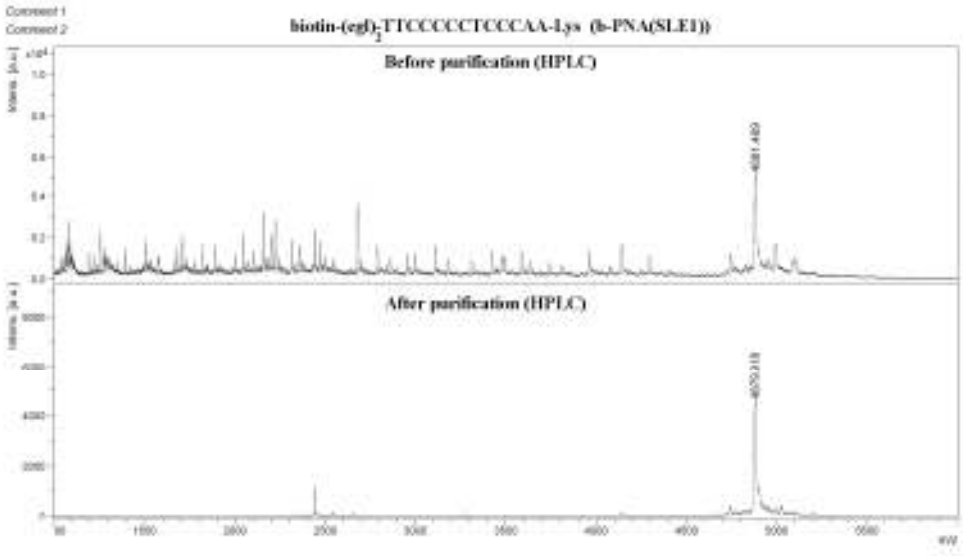

Figure A-3 Mass spectra of biotin-$(egl)_2$-TTCCCCCTCCCAA-Lys (b-PNA(SLE2)) before and after purification by HPLC.

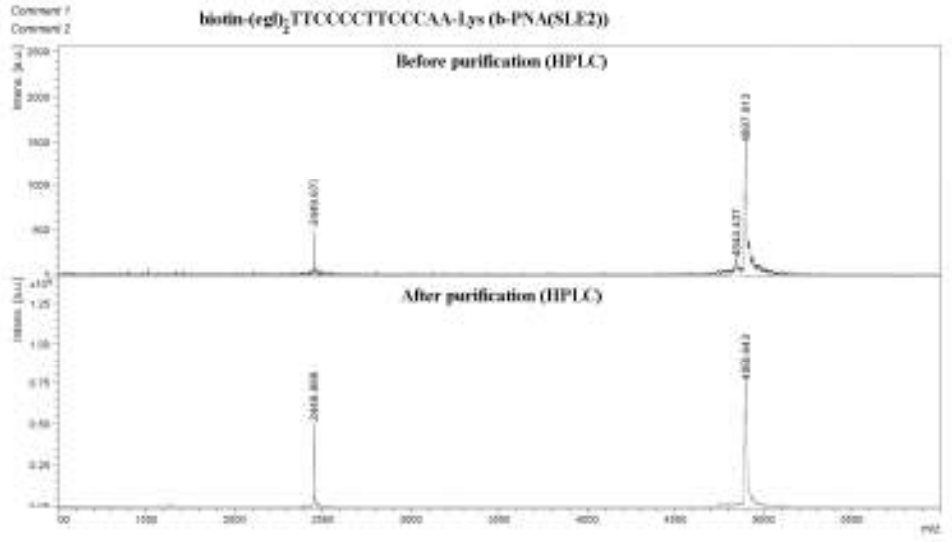

Figure A-4 Mass spectra of biotin-$(egl)_2$-TTCCCCTTCCCAA-Lys (b-PNA(SLE2)) before and after purification by HPLC.

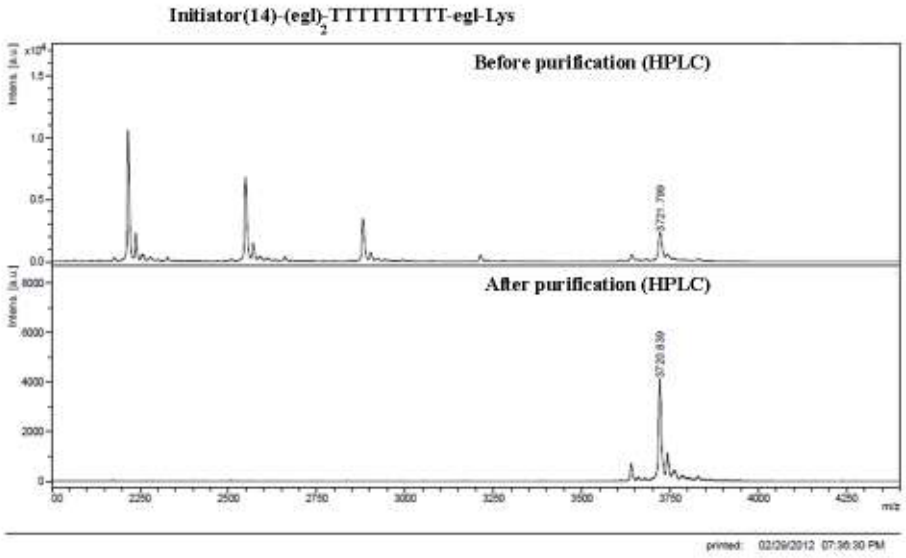

Figure A-5 Mass spectra of initiator (**14**)-(egl)$_2$-TTTTTTTTT-egl-Lys (I-PNA(T9)) before and after purification by HPLC.

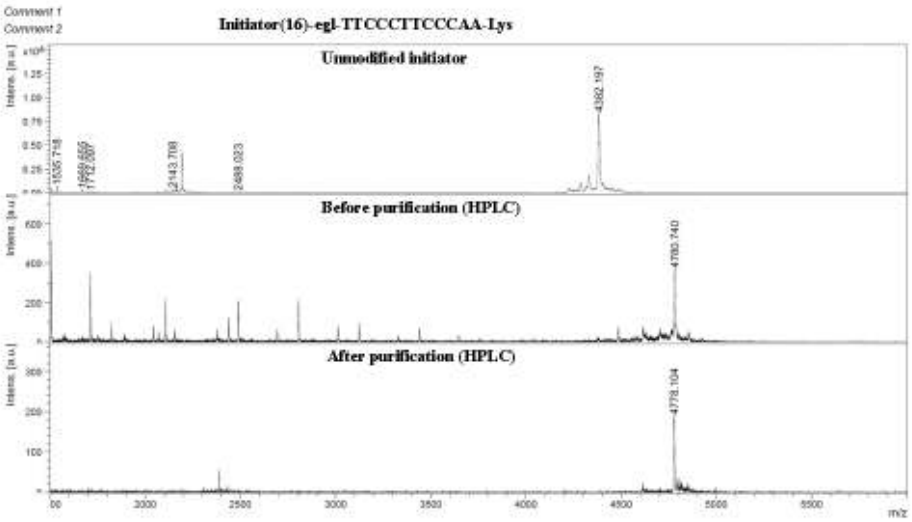

Figure A-6 Mass spectra of initiator (**16**)-egl-TTCCCCTTCCCAA-Lys (I-PNA (SLE2)) before and after purification by HPLC.

APPENDIX B

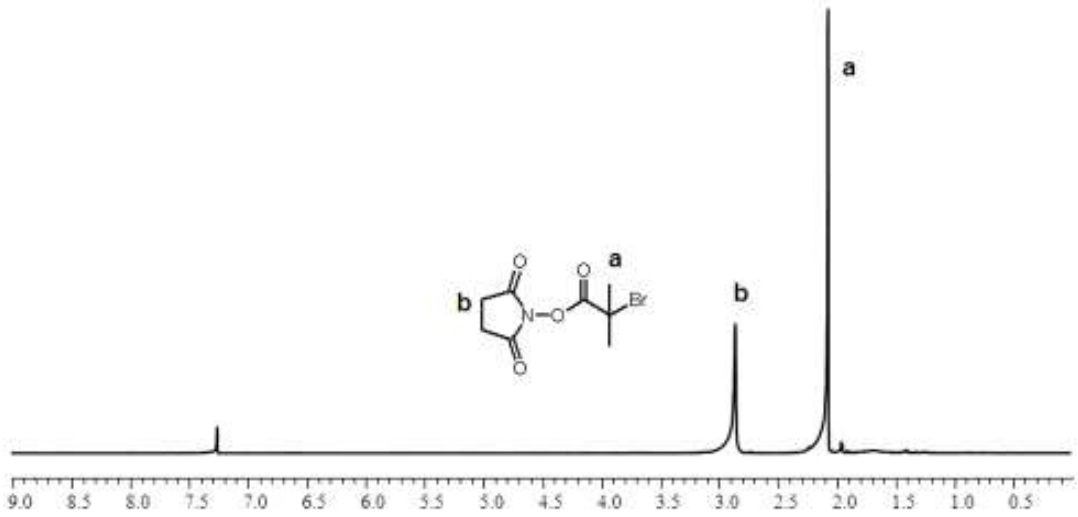

Figure B-1 ^{1}H NMR spectrum of 2-bromoisobutyric acid *N*-hydroxysuccinimide (**14**).

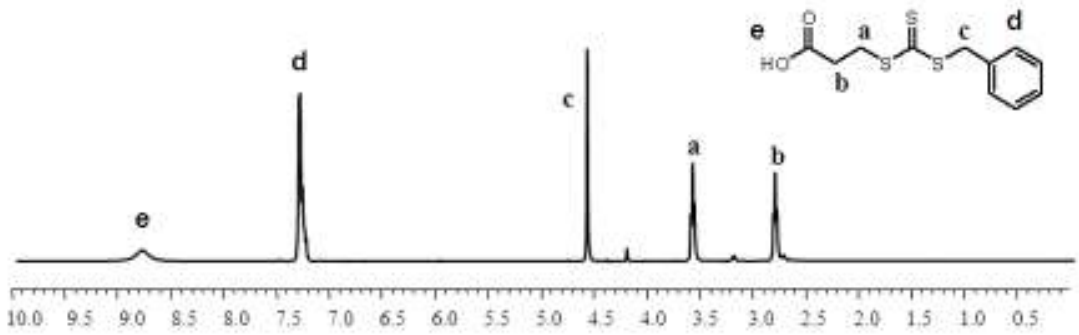

Figure B-2 Sc ^{1}H NMR spectrum of 3-benzylsulfanylthiocarbonylsufanyl propionic acid (**15**).

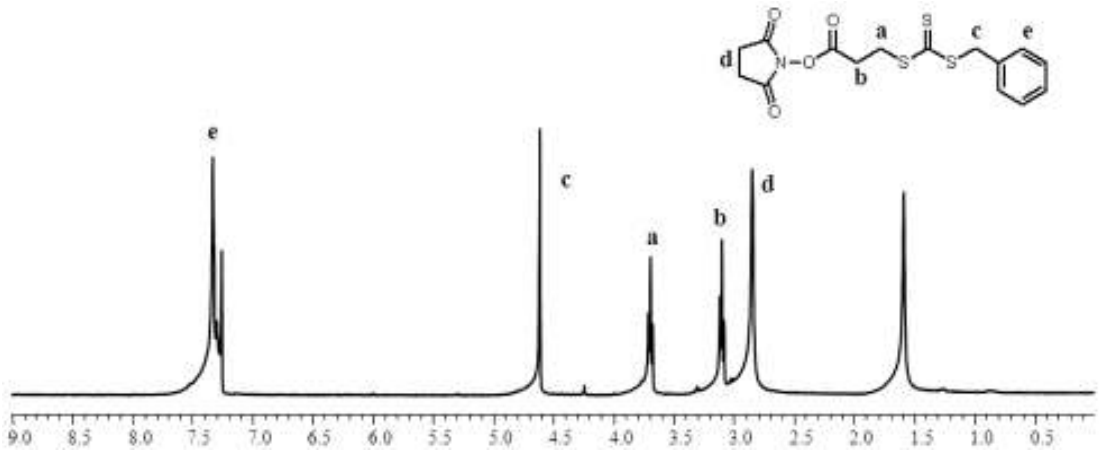

Figure B-3 ^{1}H NMR spectrum of 3-benzylsulfanylthiocarbonylsufanyl propionic acid *N*-hydroxysuccinimide ester (**16**).

APPENDIX C

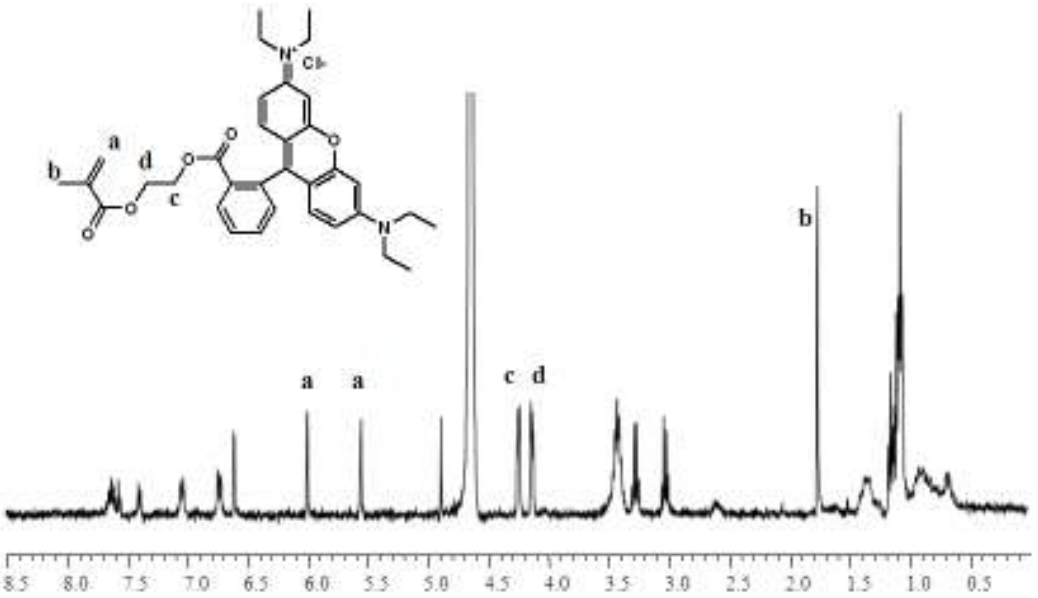

Figure C-1 ^{1}H NMR spectrum of rhodamine B-functionalized 2-hydroxyethylmethacrylate (Rh B-HEMA).

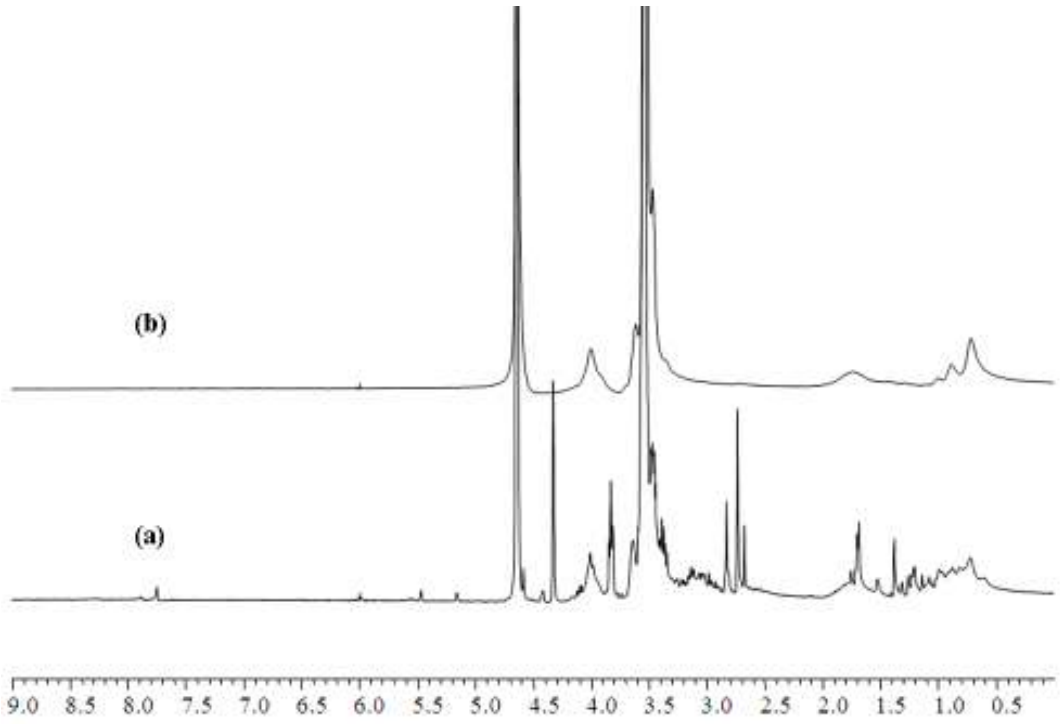

Figure C-2 Schematic of ^{1}H NMR spectra of copolymer of Rh B-HEMA and PEGMA via ARGET ATRP **(a)** before purification **(b)** after purification

VITAE

Miss Praethong Laopa was born on May 12nd, 1979 in Roi-ed province, Thailand. She received the Bachelor Degree of Science, majoring in Chemistry from Khonkaen University in 2002 and graduated with the Master Degree of Science, majoring in Chemistry from Faculty of Science, Chulalongkorn University in 2005. She has been worked at Kasetsart University, Chalermphrakiat Sakon Nakhon Province Campus in 2006 to present. She began PhD studying in Program in Petrochemistry, Faculty of Science, Chulalongkorn University in the academic year of 2008 and graduated in the academic year of 2011.

Presentation in Conference:

May 2010	The 11th World Congress on Biosensors (Biosensor 2010), **"**Enzymatic amplified colorimetric detection of DNA following "Dot Blot" format employing filter paper functionalized with quaternized polymer brushes and peptide nucleic acid probe", Glasgow, United Kingdom, *Poster Presentation*
September 2011	The 14th Asian Chemical Congress 2011 (14 ACC), "Detection of DNA following "Dot Blot" format employing filter paper functionalized with quaternized polymer brushes and peptide nucleic acid probe", Bangkok,Thailand, *Oral Presentation*
January, 2012	Pure and Applied Chemistry International Conference (PACCON 2012), "Development of signal amplification method assisted by polymerization for DNA detection", Chiang Mai, Thailand, *Poster Presentation*