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นางสาวมาลินี ลี้กระจ่าง

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FILTER PAPER GRAFTED WITH PNA-CONTAINING COPOLYMER BRUSHES FOR COLORIMETRIC DNA SEQUENCE DETERMINATION

Miss Malinee Leekrajang

A Thesis Submitted in Partial Fulfillment of the Requirements
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งานวิจัยนี้มีเป้าหมายที่จะพัฒนาอุปกรณ์การตรวจวัดลำดับเบสของดีเอ็นเอบนกระดาษกรองแบบการเกิดสีโดยใช้เพปไทด์นิวคลีอิกแอซิด (พีเอ็นเอ) เป็นโพรบ เริ่มต้นจากการเตรียมกระดาษกรองที่กราฟต์ด้วยพอลิ(ไกลซิดิลเมทาคริเลต-*โค*-พอลิ(เอทิลีนไกลคอล)เมทาคริเลต (P(GMA-*co*-PEGMA)) ผ่านปฏิกิริยาพอลิเมอไรเซชันริเริ่มจากพื้นผิวด้วยกลไกแบบ reversible addition-fragmentation chain transfer จากนั้นจึงตรึงพีเอ็นเอโพรบ (Ac-GGAACCTGCGCG-LysNH_2) บนกระดาษกรองที่กราฟต์ด้วย P(GMA-*co*-PEGMA) ผ่านปฏิกิริยาการเปิดวงของหมู่อิพ็อกไซด์ในหน่วยซ้ำที่เป็นไกลซิดิลเมทาคริเลตด้วยหมู่แอมิโนในโครงสร้างของพีเอ็นเอ พิสูจน์ทราบความสำเร็จในการกราฟต์ P(GMA-*co*-PEGMA) และการตรึงพีเอ็นเอด้วยเทคนิคฟูเรียร์ทรานส์ฟอร์มอินฟราเรดสเปกโทรสโกปี จากการติดตามด้วยฟลูออเรสเซนต์ไมโครสโกปีพบว่าสภาวะที่เป็นเบสช่วยส่งเสริมการคอนจูเกตของพีเอ็นเอ การขยายสัญญาณในการตรวจวัดทำโดยอาศัยรูปแบบแซนวิชไฮบริไดเซชันที่ใช้พีเอ็นเอโพรบที่ปลายสายดัดแปรด้วยไบโอตินมีลำดับเบสเป็น b-o-o-AACACACAGACT-SerOH เป็นรีพอร์ตเตอร์โพรบร่วมกับกับเอนไซม์ฮอร์สแรดิชเปอร์ออกซิเดสที่ติดฉลากด้วยสเตรปทาวิดิน วัสดุตรวจวัดมีประสิทธิภาพในการป้องกันการยึดเกาะอย่างไม่จำเพาะเจาะจงจากดีเอ็นเอที่มีลำเบสไม่คู่สมกัน และยังสามารถแยกความแตกต่างระหว่างดีเอ็นเอที่ลำดับเบสคู่สมและดีเอ็นเอที่ลำดับเบสผิดไป 1 ตำแหน่งได้ที่ปริมาณต่ำสุดถึง 100 เฟมโตโมล นอกจากนี้ยังพบว่าการเพิ่มความเป็นไอออนของสารละลายในขั้นไฮบริไดเซชันของดีเอ็นเอโดยการเติมเกลือโซเดียมคลอไรด์ ไม่เพียงแต่ช่วยเพิ่มความเข้มของสัญญาณที่สามารถสังเกตเห็นด้วยตาเปล่า แต่ยังช่วยลดการดูดซับอย่างไม่จำเพาะเจาะจงอีกด้วย

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MALINEE LEEKRAJANG: FILTER PAPER GRAFTED WITH PNA-CONTAINING COPOLYMER BRUSHES FOR COLORIMETRIC DNA SEQUENCE DETERMINATION. ADVISOR: ASSOC. PROF.VORAVEE HOVEN, Ph.D., CO-ADVISOR: PIYAPORN NA NONGKHAI, Ph.D., 58 pp.

This work aims to develop a filter paper-based platform for colorimetric DNA detection employing peptide nucleic acid (PNA) probe. Filter paper functionalized with poly(glycidyl methacrylate-*co*-poly(ethylene glycol) methacrylate) (P(GMA-*co*-PEGMA)) was first prepared via surface-initiated reversible addition-fragmentation chain transfer (RAFT) polymerization. PNA probes (Ac-GGAACCTGCGCG-LysNH_2) were then immobilized on P(GMA-*co*-PEGMA) through ring-opening of epoxide groups in the GMA repeat units by amino groups in the PNA's structure. The success of P(GMA-*co*-PEGMA) grafting on filter paper and subsequent PNA immobilization was confirmed by Fourier transform-infrared spectroscopy. As monitored by fluorescence microscopy, the PNA conjugation can be promoted under basic condition. Signal amplification relies on sandwich-hybridization assay employing biotinylated PNA probe (b-PNA) having a sequence of b-o-o-AACACACAGACT-SerOH as reporter probe together with horseradish peroxidase-labeled streptavidin (SA-HRP). The sensing platform showed the best performance in preventing non-specific adsorption from the non-complementary DNA and discriminating between complementary and single base mismatch targets at a detection limit of at least 100 fmol. It was later found that increasing ionic strength of DNA hybridization step by NaCl addition not only can increase the signal intensity which can be visualized by naked eye, but also suppressed non-specific adsorption.

Field of Study:	Petrochemistry and Polymer Science	Student's Signature .....
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CHAPTER I

INTRODUCTION

1.1 Statement of Problem

DNA sequence determination is crucially important for clinical diagnosis, forensic identification as well as pathogen detection in food and agricultural products. Basic principle for DNA detection is generally based on the concept that DNA target is detected via specific binding to complementary nucleic acid probe, following Watson- Crick base-pairing rule. [1] Peptide nucleic acid (PNA), firstly introduced by Nielsen and co-workers in 1991 [2] is a synthetic DNA analogue having an uncharged peptide-like backbone. The suppressed electrostatic repulsion between the neutral PNA and the negatively charged DNA bring about a number of favorable DNA binding characteristics including high thermal stability, greater sequence specificity and mismatch discrimination sensitivity, and less salt-dependent affinity. Among PNA variants being developed so far, a conformationally rigid pyrrolidinyl PNA derived from D-prolyl-2-aminocyclopentane carboxylic acid (acpc) backbones (acpcPNA) developed by Vilaivan and co-workers [3] truly stands out as potential and effective nucleic acid probe for DNA biosensor mainly because the hybridized PNA·DNA duplex possess higher affinity and specificity than the original Nielsen's aminoethylglycyl PNA. The success of using acpcPNA as probe for DNA sequence determination have been continuously demonstrated by many techniques including MALDI-TOF mass spectrometry, [4] quartz crystal microbalance (QCM), [5] surface plasmon resonance (SPR) [6,7], electrochemistry [8-10], most of which require advanced instruments and have to be done in well-equipped laboratories. The development of highly sensitive and specific, yet simple and economical test kit/assays without the demand for sophisticated instruments, suitable for point-of-care usages still remains a challenge.

Inspired by research work firstly reported by Whitesides and coworkers on paper-based microfluidic devices [11] and a number of relevant publications following thereafter, paper-based DNA sensing platforms employing acpcPNA as

probes have been recently developed. The first assay is based on the concept of "Dot blot hybridization" in which filter paper grafted with quaternized poly(2-(dimethylamino)ethyl methacrylate) (QPDMAEMA) brushes was used as positively charged platform that can selectively capture PNA·DNA hybrids and prevent non-specific adsorption of non-target analytes. The hybridization event can then be visualized by an enzyme-based calorimetric assay employing horseradish peroxidase streptavidin (SA–HRP) conjugate and a chromogenic substrate. [12] Although quite an impressive detection limit (10 fmol, equivalent to 1 mL of 10 nM of DNA) can be reached by naked eye detection, the technique has a limitation for detection of DNA mixtures because the DNA was deposited on the membrane via non-specific electrostatic interactions. There should most likely be binding competition among different DNA sequences so it may not be possible to detect the desired target. To overcome the above-mentioned problem, the second platform most recently reported relies on direct immobilization of the acpcPNA probes on cellulose paper. Upon contacting with DNA analyte via capillary method, cationic dyes were introduced to electrostatically interact with negatively charged hybridized DNA to monitor the PNA-DNA binding event. A lowest detectable DNA concentration of 200 nM or 3.3 pmol per spot has been achieved. [13]

The information gained from this latest development is very fruitful in that acpcPNA covalently immobilized directly onto the paper still function efficiently in binding with the DNA target. Together with our well-established strategy to enhance active binding sites per surface area of material by using surface-grafted polymer brushes, [14] this research aims to develop another paper-based DNA sensor that can bind directly to the PNA probes. The filter paper was first grafted with poly[glycidyl methacrylate-co-poly(ethylene glycol)methacrylate] (P(GMA-co-PEGMA)) via surface-initiated reversible addition fragmentation chain transfer polymerization (SI-RAFT). [11] This copolymeric system was chosen to generate active layer for PNA binding due to the following reasons: (1) the epoxide group of the GMA unit can act as versatile active site for binding with PNA capture probe via epoxide ring opening without having to use additional coupling agent so that no by-product would be released. Hydroxyl group generated as outcome result of epoxide ring opening is well known as non-charged hydrophilic entity under neutral pH, a general DNA

hybridization condition, that should not interfere, but synergistically facilitate the detection. (2) the PEGMA unit incorporated as hydrophilic entity should prevent non-specific adsorption of non-target DNA as well as non-DNA components that may deteriorate the detection sensitivity. This latter issue is extremely important because it determines the fate of the developed platform when subjected to analysis of real biological samples. Signal amplification used in this research relies on sandwich-hybridization assay employing biotinylated acpcPNA probe (b-PNA) as reporter probe together with SA-HRP and o-phenylenediamine (OPD) substrate can be visualized by an enzyme-based colorimetric assay. The concept for this developed filter paper-based DNA sequence determination is demonstrated in **Figure 1.1**.

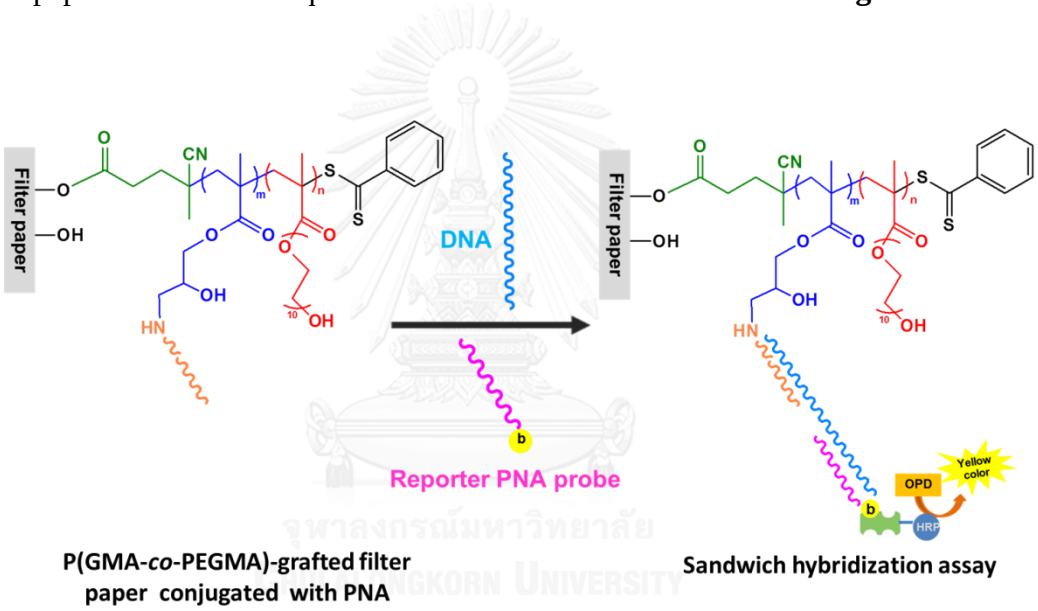


Figure 1.1 Schematic representation of enzymatic amplified colorimetric detection of DNA following sandwich-hybridization assay employing biotinylated acpcPNA probe (b-PNA) as reporter probe and filter paper-grafted P(GMA-*co*-PEGMA) brushes immobilized with acpcPNA capture probe as substrate.

1.2 Objectives

1. To prepare and characterize poly[glycidyl methacrylate-co-poly(ethylene glycol)methacrylate] (P(GMA-*co*-PEGMA)-grafted filter.
2. To determine colorimetric detection of DNA employing P(GMA-*co*-PEGMA)-grafted filter and acpcPNA probe.

1.3 Scope of Investigation

1. Literature survey for related research work.
2. Preparation and characterization of P(GMA-*co*-PEGMA)-grafted filter paper by SI-RAFT.
3. Synthesis and immobilization of modifier labeled acpcPNA
4. DNA sequence determination employing P(GMA-*co*-PEGMA)-grafted filter paper as membrane, acpcPNA as probe and colorimetric assay as the detection method using enzymatic amplification

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Peptide nucleic acid

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 structure of DNA 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 2.1**).

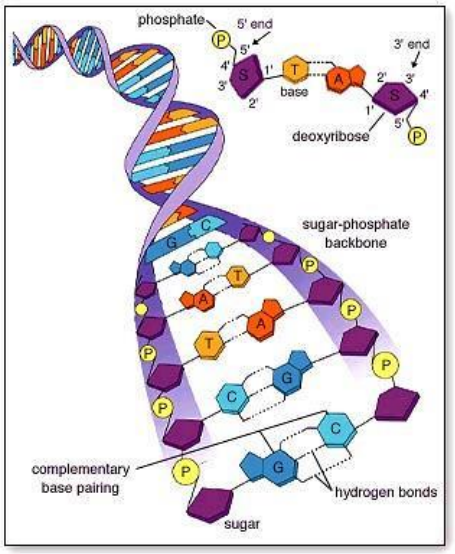


Figure 2.1 Chemical structures of DNA (http://www.mhhe.com/biosci/esp/2001_gbio/folder_structure/ge/m4/s1/)[15]

Peptide nucleic acid (PNA) is a synthetic DNA analogue first introduced by Nielsen *et al.* in 1991.[2] This PNA system called aegPNA containing repeating N-(2-aminoethyl)-glycine units having peptide linkage replacing the DNA deoxyribose–

phosphate backbone (**Figure 2.2**). The absence of negative charges of phosphate groups in the PNA backbone improves the binding affinity and promotes the high mismatch sensitivity of PNA–DNA complexes as compared with DNA–DNA complexes. [1] Recently, There are several new PNA systems with modified structures. One such sample is a conformationally rigid pyrrolidinyl PNA derived from D-prolyl-2-aminocyclopentane carboxylic acid (acpc) backbones (acpcPNA) developed by Vilaivan and co-workers. [3]

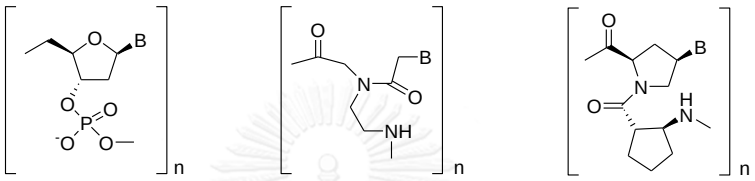


Figure 2.2 Structures of a) DNA, b) aegPNA or Nielsen's PNA and (c) (1*S*, 2*S*)-acpc PNA or Vilaivan's PNA.

It has been reported as a PNA variant that can form a PNA·DNA duplex with even higher affinity and specificity than the original Nielsen's aegPNA. Because of its advantages, the acpcPNA has been successfully used as probe for DNA sequence determination using various techniques.

2.2 Pyrrolidinyl peptide nucleic acid -based Biosensor

In 2008, Boontha and coworkers [4] reported the detection of single nucleotide polymorphisms (SNP) genotyping in combination with ion-exchange capture technique by MALDI-TOF mass spectrometry (**Figure 2.3**). The presence of the hybridized PNA in the form of negatively charged PNA·DNA duplex on the positively charged ion-exchanger, Q-sepharose, gave high specific differentiation between complementary and single-base mismatched DNA targets. This method was carried out at room temperature without the need for biotinylation or enzymatic digestion.

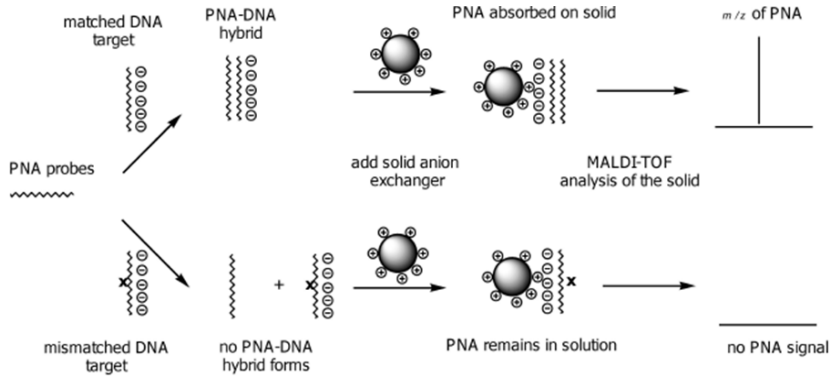


Figure 2.3 Schematic diagram showing the concept of ion-exchange capture of PNA in combination with MALDI-TOF MS in DNA sequence determination. [4]

In 2009, Ananthanawat and coworkers [5] developed DNA sensor based on quartz crystal microbalance (QCM) technique. The thiolated pyrrolidinyl peptide nucleic acids (HS-PNA) was directly immobilized on gold surface by self-assembled monolayer (SAM) formation. The step of blocking by hydroxyl-terminated thiol was found to be necessary to provide sensitive and specific platform for DNA detection. A solution of 0.5 mM phosphate buffer at pH 7.0 with no added NaCl is an appropriate binding solution that gave effective discrimination between complementary and single or multiple base mismatched DNA targets. The results from QCM technique, however, cannot satisfy levels of specificity and sensitivity for the detection of DNA hybridization. Later in the same year, they demonstrated that a similar HS-PNAs can be applied for DNA hybridization detection by using surface plasmon resonance, SPR, an alternative, non-labeling technique that should yield better detection efficiency.[6] They have found that the hybridization efficiency was improved when the length between the PNA portion and the thiol terminal was increased and/or when blocking thiol was applied. Relative high mismatch discrimination values between fully complementary DNA from one (45-54%) and two base mismatched (85-93%) DNA were achieved. Although the detection limit of this SPR-based biosensor of 0.2 μM has been improved from that of the QCM-based one (5_mM), its hybridization efficiency is still rather low (<20%). They have described this as a result of the direct HS-PNA probe immobilization yielded the densely packing of the PNA probes so that the accessibility of the target DNAs became limited. To overcome such obstacle, they

have later demonstrated that the biotinylated acpcPNA immobilized on SPR sensor chips (**Figure 2.4**) via biotin–streptavidin interactions improved the accessibility of the immobilized PNA probes to obtain a higher hybridization efficiency (59-62%) and mismatch discrimination efficiency (>90%). As opposed to aegPNA, this research also demonstrated that acpcPNA exhibited a strong preference for binding to DNA in antiparallel orientation similar to DNA. Upon using approprite regeneration conditions, the sensor can be reused with only 1.3% loss in hybridization activity per regeneration cycle.

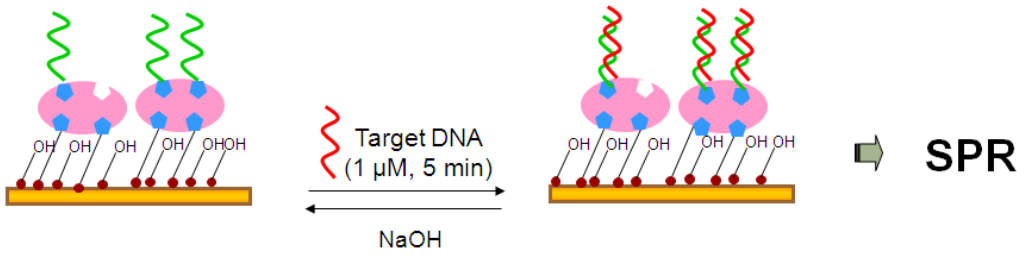


Figure 2.4 Schematic diagram showing immobilization of biotinylated acpcPNA as a new sensing probe via biotin–streptavidin–biotin chemistry using surface plasmon resonance (SPR) in DNA sequence determination. [7]

In 2012, Rashatasakhon, and coworkers [16] investigated the use of polycationic phenylene–ethynylene dendrimers as a Förster resonance energy transfer (FRET) donor and a fluorescein-labeled pyrrolidinyl peptide nucleic acid (Fl-acpcPNA) as the energy acceptor in the detection of DNA sequence (**Figure 2.5**). 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 phenylene ethynylene 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.

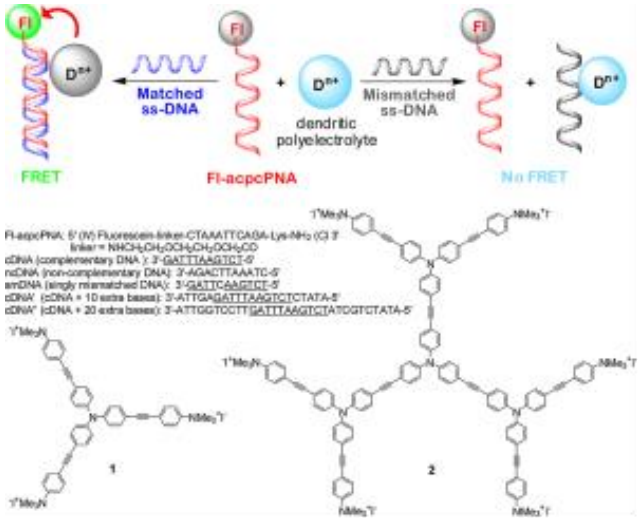


Figure 2.5 Proposed detection of DNA/PNA hybridization via FRET from polycationic dendritic fluorophores Dn+ (1 and 2). [16]

In 2013, Laopa and coworkers [12] developed a sensing platform based on quaternized poly(dimethylamino)ethyl methacrylate (QPDMAEMA) grafted on filter paper which was prepared by surface-initiated polymerization of dimethylaminoethyl methacrylate via ARGET ATRP followed by quaternization with methyl iodide. Following the concept of Dot blot hybridization, the platform was used for specific DNA sequence detection employing acpcPNA as a probe and detected by naked eye observation (**Figure 2.6**). The QPDMAEMA-grafted filter paper first captured the negatively charged DNA by electrostatic interactions. The biotinylated PNA (b-PNA) probe was then introduced through hybridization with the surface-bound DNA. Colorimetric detection was obtained via enzymatic amplification of substrate by streptavidin–horseradish peroxidase conjugate (SA–HRP) linked with b-PNA. They demonstrated that a new paper-based platform was capable of discriminating between complementary and single base mismatch targets at a detection limit of at least 10 fmol. In addition, the QPDMAEMA-grafted filter paper exhibited a superior performance to the commercial membranes, namely Nylon 66 and nitrocellulose.

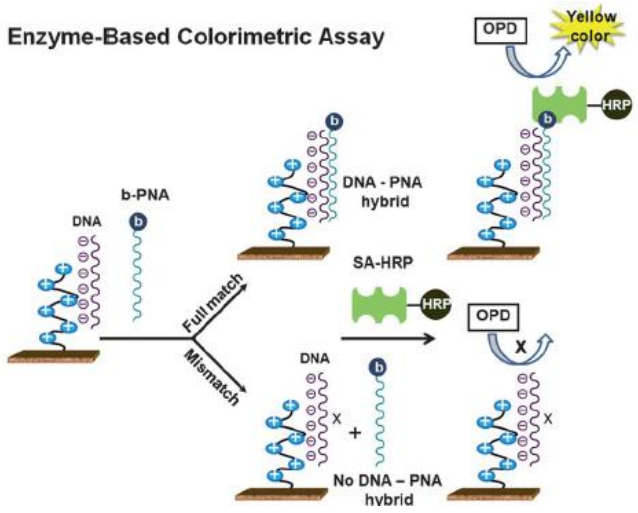


Figure 2.6 Schematic representation of enzymatic amplified colorimetric detection of DNA following Dot blot hybridization using filter paper functionalized with positively charged polymer brushes and the peptide nucleic acid probe. [12]

In 2012, Thipmanee and coworker [8] reported a new platform for a DNA biosensor based on a electrochemical capacitive transducer using pyrrolidinyl peptide nucleic acid (acpcPNA) probes. The blocking thiol with an equal length provide high binding specificity and the thiol having –OH terminating head group provide slightly better signal than that having the $–CH_3$ terminating head group. These sensors exhibited very good performances and gave low detection limit (picomolar) to response over a wide linear range (1.0×10^{-11}–1.0×10^{-8} M). The immobilized acpcPNA electrode can be reused through at least 58 cycles.

In 2013, Sankoh and coworker [9] reported a comparative study on the performances of a label-free capacitive DNA sensor using pyrrolidinyl peptide nucleic acid (acpcPNA) probe immobilized with non-conducting polymers. The electrode modified with PpPD provided highest sensitivity and lowest detection limit in the picomolar range. The sensor using the highly sensitive capacitive system can be reused for at least 68 cycles.

In 2013, Theppaleak and coworker [17] reported determination of DNA sequences in combination with an anion exchanged solid support and acpcPNA

probes by MALDI-TOF mass spectrometry (**Figure 2.7**). The positively charged magnetic nanoparticles (MNPs) coated with QPDMAEMA was used as solid support to capture negatively charged PNA·DNA duplex. This method provide high specific differentiation between full-matched and single-base mismatched target DNA sequences in two clinically relevant genes (*K-ras* and 50 methylated CpG sequence).

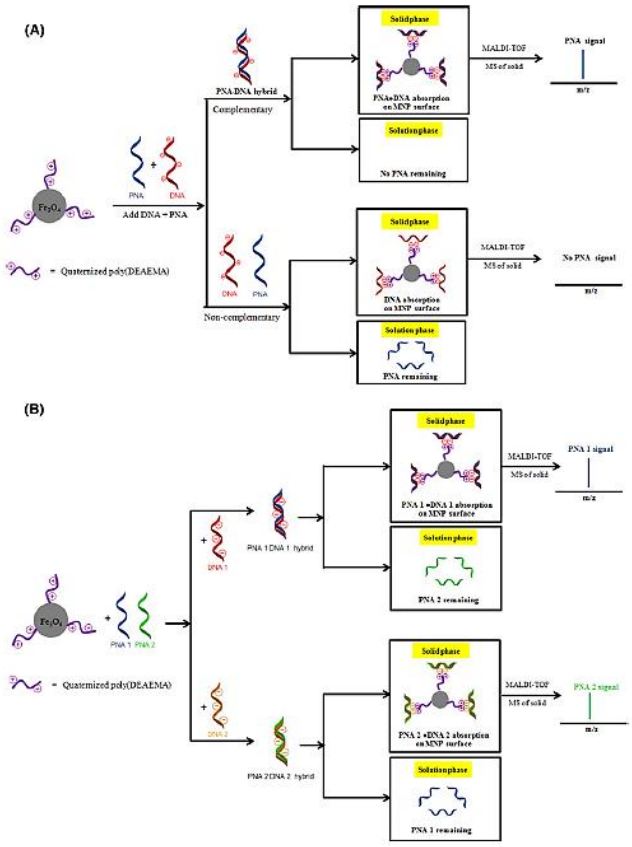


Figure 2.7 The concept of DNA sequence analysis by employing poly(DEAEMA)-grafted MNP as a nanosolid support with the use of (A) a single acpcPNA probe or (B) double PNA probes to selectively capture PNA–DNA hybrids in combination with MALDI-TOF MS. [17]

In 2014, Jampasa and coworker [10] developed a selective detection of human papillomavirus (HPV) type 16 DNA based on an immobilized anthraquinone labeled pyrrolidinyl peptide nucleic acid (PNA-AQ) probe by measuring

electrochemical signals (**Figure 2.8**). The sensor probe exhibited very high selectivity for the complementary 14 base oligonucleotide against the non-complementary 14-base oligonucleotide including HPV types 18, 31 and 33 DNA with a limit of detection and limit of quantitation of 4 and 14 nM, respectively. This DNA sensing platform was successfully developed and applied to detect the HPV type 16 DNA from a PCR amplified (240 bp fragment of the L1 gene) sample.

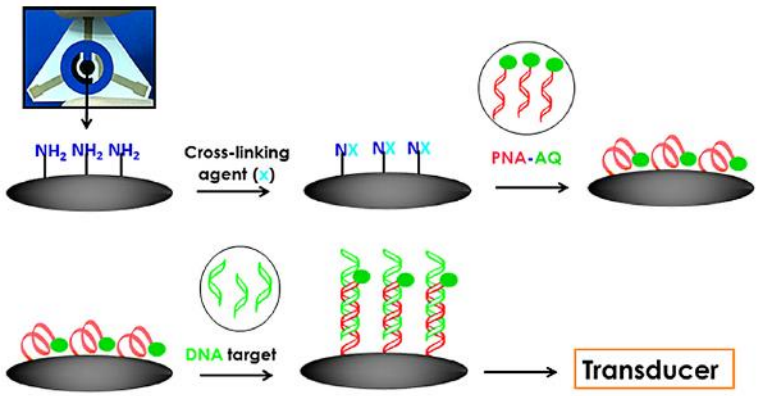


Figure 2.8 Schematic illustration of the immobilization of the PNA-AQ probe on to the CHT-SPCE and subsequent hybridization with the target DNA. [10]

In 2015, Jirakittiwut and coworkers [13] developed a cellulose-based DNA sensor which relies on direct immobilization of the acpcPNA probes. Upon contacting with DNA analyte via capillary method, cationic dyes were introduced to electrostatically interact with negatively charged hybridized DNA to monitor the PNA-DNA binding event (**Figure 2.9**). The sensor exhibited good discrimination power in the presence of nine other DNAs. A lowest detectable DNA concentration of 200 nM or 3.3 pmol per spot has been achieved.

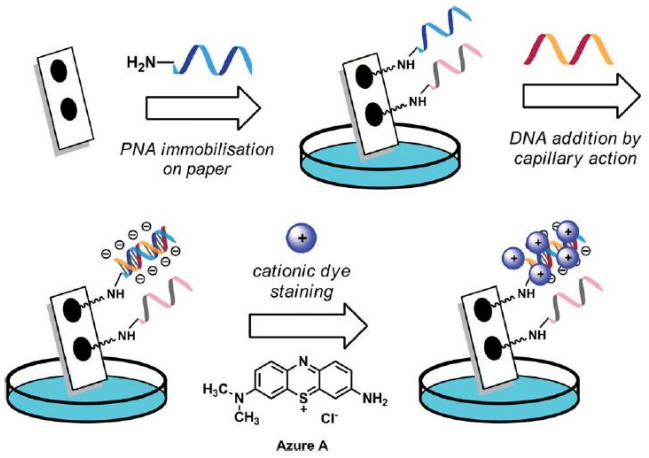


Figure 2.9 The fabrication of the new DNA sensor by covalently attaching acpcPNA onto cellulose paper, followed by cationic dye staining. [13]

2.3 Polymer brushes

Polymer brushes are polymeric chain having one end attached to a surface. Generally, polymer brushes can be prepared via two basic methods which are ‘‘grafting to’’ and ‘‘grafting from”. The “grafting to” method is done by having end-functionalized polymers reacted with a suitable substrate surface to form tethered polymer brushes. This method is simple, but the grafting density may be low because steric hindrance of polymer chains previously attached to the surface. For the “grafting from” approach, the initiator is immobilized onto the surface followed by *in situ* surface initiated polymerization to generate tethered polymers and to provide high grafting density. [18,19] Controlled radical polymerization methods that are widely used for the preparation of polymer brushes are reversible addition-fragmentation chain transfer (RAFT) polymerization and atom-transfer radical polymerization (ATRP). In this research, copolymer brushes of poly(glycidyl methacrylate-co-poly(ethylene glycol) methacrylate) (P(GMA-*co*-PEGMA)) were prepared by a “grafting from” approach via surface-initiated RAFT polymerization on filter paper. The fact that RAFT polymerization can be performed in the absence of metal catalyst unlike the ATRP truly satisfies most of bio-related applications.

Cellulose filter paper has been widely used in many applications because it is the most abundant, natural, inexpensive, biodegradable and renewable biopolymer exhibiting very good mechanical properties. Attaching polymer brushes to the surface of cellulose paper is quite convenient via chemistry with hydroxyl groups of the cellulose. [20] A number of research work have been reported on surface grafting of the polymer brushes on filter paper via both ATRP and RAFT polymerization.

In 2004, Lee and coworkers [21] reported grafting of quaternized poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) on Whatman no.1 filter paper and glass slides via ATRP to produce surface with biocidal functionality (**Figure 2.10**). The modified filter paper and glass exhibited antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*.

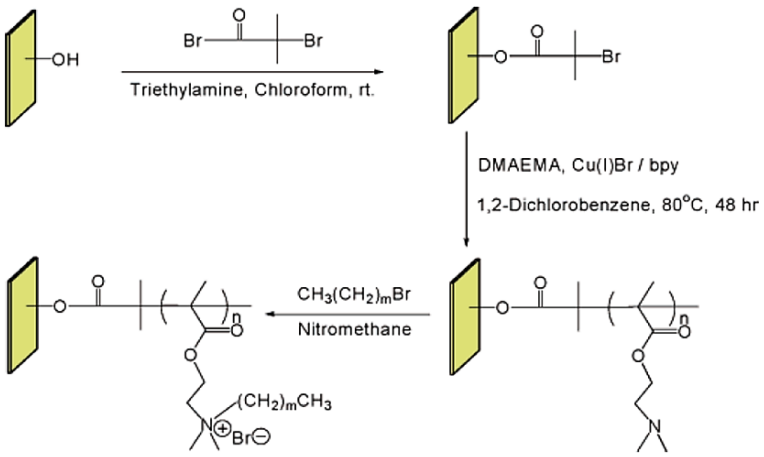


Figure 2.10 Schematic representation showing preparation of quaternized PDMAEMA grafted filter paper for antibacterial assessment. [21]

In 2008, Roy and coworkers [22] studied the grafting of PDMAEMA on a cellulose filter paper via RAFT polymerization followed by quaternization with alkyl bromides of different chain lengths (C_8-C_{16}) (**Figure 2.11**). They have found that antibacterial activity against *E.coli* of the cellulose-g-PDMAEMA quaternized with alkyl bromide having shorter alkyl chain (C8) was more effective than those quaternized with alkyl bromides having longer alkyl chain (C_{12} and C_{16}).

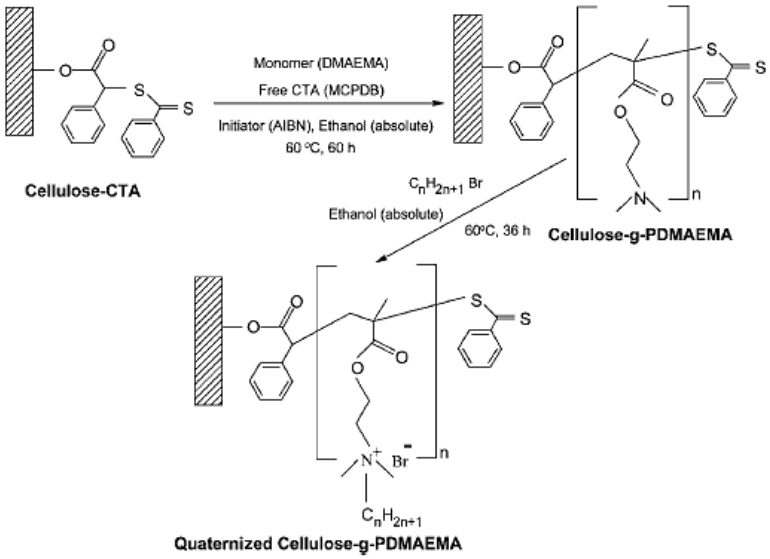


Figure 2.11 Synthesis of quaternized cellulose-g-PDMAEMA for antibacterial assessment.[22]

In 2009, Tang and coworkers [23] prepared antibacterial filter paper using silver nanoparticles as the antibacterial agent. The first step was to graft poly(*tert*-butyl acrylate) (P*t*BA) on the cellulose filter paper by surface-initiated ATRP . Upon hydrolysis, the grafted P*t*BA was transformed into poly(acrylic acid) (PAA) and yielded filter paper-g-PAA. In the presence of a reducing agent, Ag+ can be reduced *in situ* and generate silver nanoparticles deposited on the filter paper-g-PAA (**Figure 2.12**). The silver nanoparticles-decorated filter paper (filter paper-g-PAA/Ag) exhibited good antibacterial activity against *E. coli* as compared with the original filter paper and the filter paper-g-PAA.

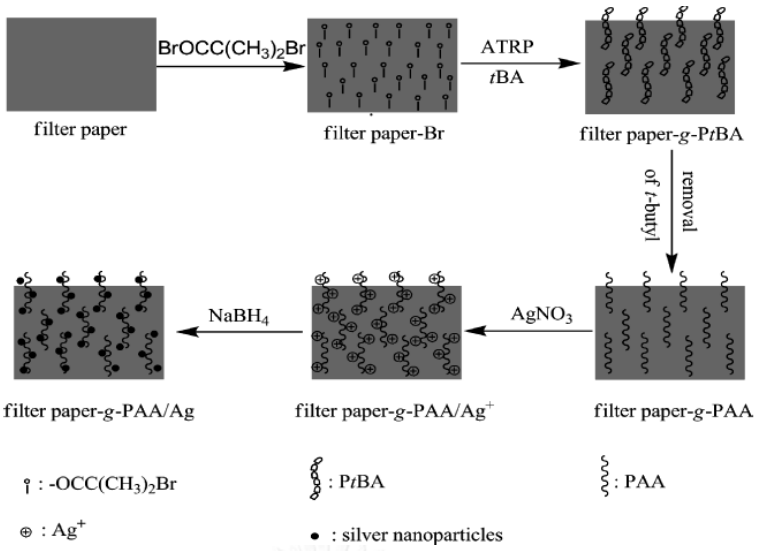


Figure 2.12 Schematic diagram illustrating the preparation of antibacterial filter paper based on surface-grafted PAA decorated with silver nanoparticles. [23]

In 2009, Hansson and coworkers [24] reported the use of activators regenerated by electron transfer-atom transfer radical polymerization (ARGET ATRP) for grafting a filter paper substrate with a range of various monomers, for example, methyl methacrylate (MMA), styrene (St), and glycidyl methacrylate (GMA) (**Figure 2.13**). ARGET ATRP is considered environmentally friendly process given that the amount of transition metal catalyst used was significantly reduced as compared with the conventional ATRP. The polymer-grafted filter papers were characterized by FT-IR. The results indicated that the amount of polymer grafted on the surface increased with increasing monomer conversion. Water contact angle (CA) measurements implied that a small amount of polymer greatly enhances the hydrophobicity of cellulose in the cases of polystyrene and poly(methyl methacrylate), in spite of low degree of polymerization.

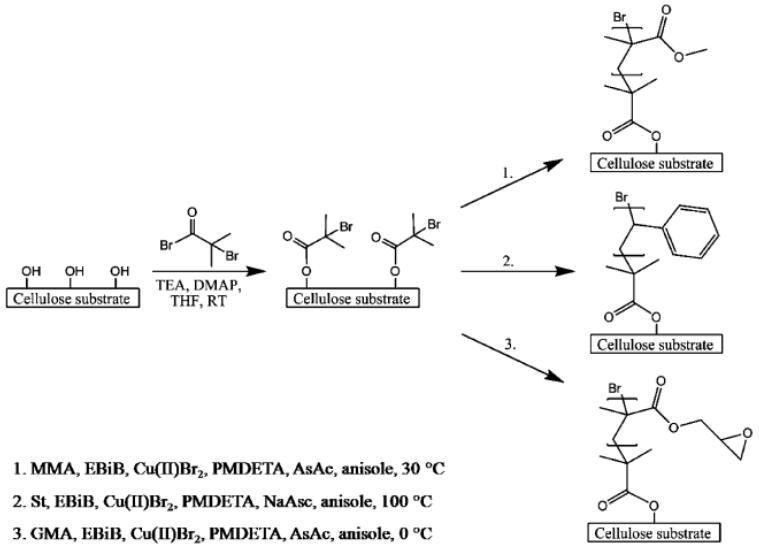


Figure 2.13 ARGET ATRP of various monomers on cellulose substrate. [24]

Poly(glycidyl methacrylate) (PGMA) is considered as one of polymer precursor that are frequently used for the preparation of functional polymers. Its repeat unit contains an epoxide ring that can readily be opened up by a variety of nucleophiles under appropriate conditions. A number of publications have reported the use of PGMA for binding with biomolecules.

In 2010, Mantel and coworkers [25] prepared PGMA thin films on gold substrates via three different pathways based on grafting onto and grafting from methods (**Figure 2.14**). The immobilization of bovine serum albumin (BSA) was done through ring-opening reaction of the epoxy groups of PGMA by amino groups of BSA. PGMA-grafted gold substrate was then tested for anti-bovine serum albumin (a-BSA) detection using sSPR technique. SPR data suggested that the substrate prepared by "grafting from" approach of which PGMA graft density was higher gave better detection efficiency than that obtained from the substrate prepared by "grafting onto" method.

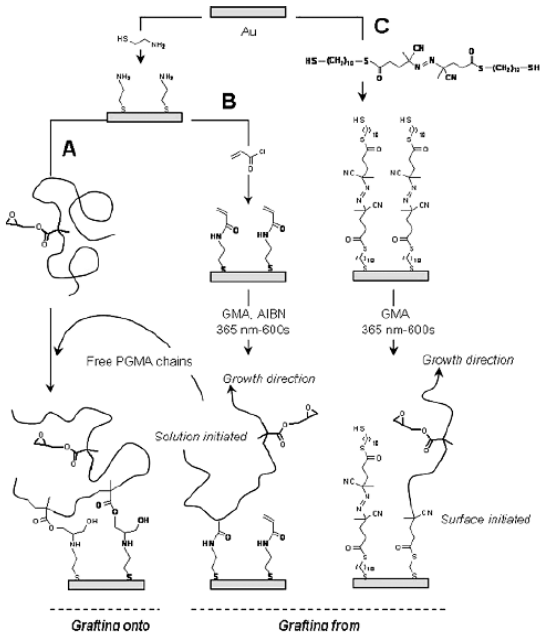


Figure 2.14 Three different pathways applied to chemically modify gold substrate with PGMA chains. (A) for the grafting-onto technique and (B and C) for the grafting-from approaches. [25]

In 2011, Lui and coworkers [11] reported about a highly sensitive microfluidic flow-through immunoassay array device that was fabricated by simply coupling the protein-arrayed P(GMA-*co*-PEGMA)-slide to a PMMA microfluidic platform (**Figure 2.15**). The multiple direct immunoassays were performed through the designed parallel flow-through microarray panels. The P(GMA-*co*-PEGMA) brush can improve larger protein loading capacity, higher antibody-antigen binding efficiency and gave lower nonspecific protein adsorption. The limit of detection (LOD) for a cancer biomarker carcinoembryonic antigen (CEA) in diluted human serum was 10 pg/mL.

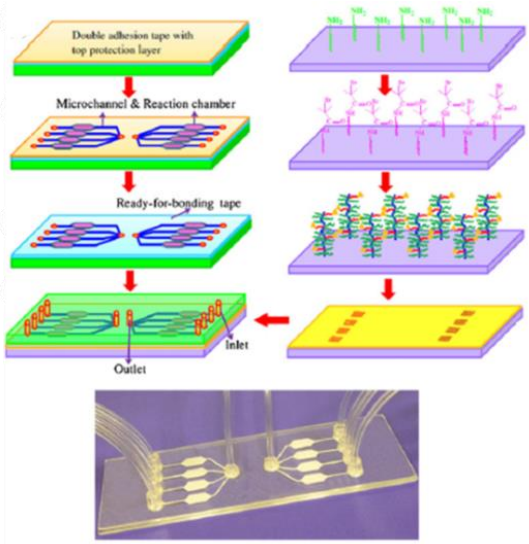


Figure 2.15 Fabrication scheme and photograph of flow-through microarray device. [11]

In 2013, Li and coworkers [26] prepared polymer brush layers based on block copolymers of poly(oligo(ethylene glycol) methacrylate) (POEGMA) and poly(glycidyl methacrylate) (PGMA) on silicon wafers by activators generated by electron transfer-atom transfer radical polymerization (AGET ATRP). These brush layers were conjugated with biomolecules via ring opening reaction of PGMA with amino groups of biomolecule, while POEGMA prevent nonspecific protein adsorption. It was shown that the conjugation of bioactive molecules to brush layer, for example, biotin with specific affinity for avidin, can be controlled by varying the thickness of the POEGMA and PGMA layers (**Figure 2.16**).

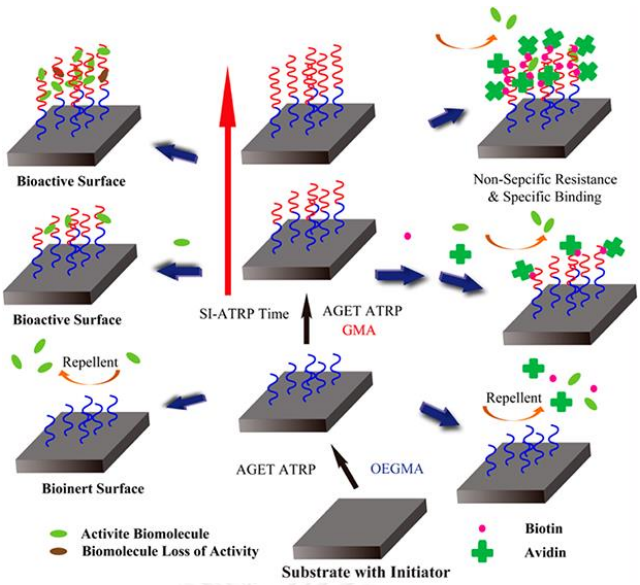


Figure 2.16 Scheme of the preparation POEGMA-b-PGMA diblock copolymer conjugated with bioactive molecules. [26]

In 2013, Li and coworkers [27] reported that patterned polymer brushes can be easily prepared by a one-step method for the immobilization of bromo-4-hydroxyacetophenone (BHAP) groups (as the active ATRP initiators) on the biaxially oriented polypropylene (BOPP) surfaces (**Figure 2.17**). The patterned BOPP-*g*-PDMAEMA and BOPP-g-PGMA films were prepared via surface initiated ATRP followed by immobilization with IgG via electrostatic interactions and covalent coupling, respectively. From fluorescence microscopic result, it was found that IgG-coupled with the patterned films can interact with target anti-IgG, namely Rhodamine-IgG and Fluorescein-IgG.

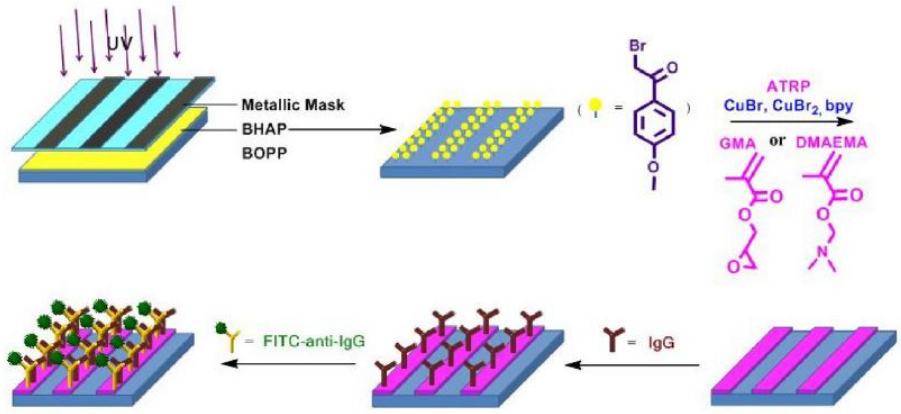


Figure 2.17 Schematic illustration of the ATRP processes of GMA and DMAEMA on BAHP-functionalized BOPP films and their resulting protein immobilization. [27]

2.4 Sandwich-hybridization assay

Sandwich hybridization approach has been successfully applied for specific detection of DNA using various mode of signal expression including colorimetric detection, electrochemical detection and microfluidic chip-based fluorescent detection. The sandwich hybridization method is based on the detection of hybridization employing two specific oligonucleotide probes. The capture probe is used to immobilize on a solid support for capturing the target nucleic acids. The other oligonucleotide probes having labeled with a detectable marker was used as the detection probe to generate a signal. [28]

Many research works have reported the use of sandwich-hybridization assay for DNA sequence determination.

In 2007, Zhang and Appella [29] developed a colorimetric sandwich-hybridization assay to detect anthrax protective antigen DNA using PNA (**Figure 2.18**). In the sandwich-hybridization strategy, one PNA is used as capture probe (PNAα) to hybridize with complementary DNA, and another PNA is used as a detection probe (PNAβ) to generate a signal. Avidin-horseradish peroxidase conjugate (HRP-avidin) and tetramethylbenzidine (TMB) were used to generate the enzymatic reaction. A clear discrimination between complementary and single-base mismatched DNA sequence was still possible at the DNA quantity of as low as 10 zmol.

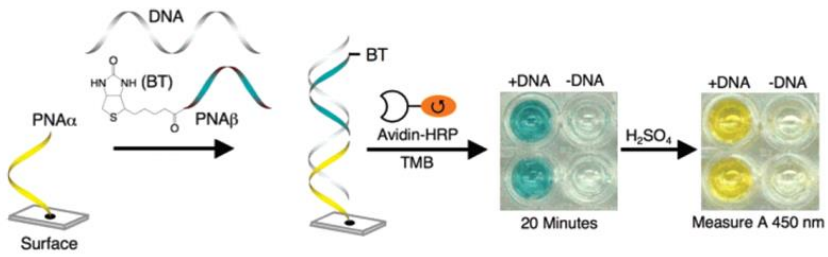


Figure 2.18 Schematic diagram of colorimetric sandwich-hybridization assays to detect anthrax protective antigen DNA using PNA probe. [29]

In 2011, Henry and coworkers [30] reported the preparation of poly(acrylamide-b-DNA) combed brushes grafted onto gold electrodes via surface initiated atom transfer radical polymerization (SI-ATRP) for the breast cancer related marker Exon16 detection using electrochemical method (**Figure 2.19**). The quantitative detection of target DNA using an enzyme-labeled reporter DNA probe in a sandwich-type format. They exhibited an excellent sensitivity of 23.5 nA nM^{-1} and a limit of detection of 2.67 nM.

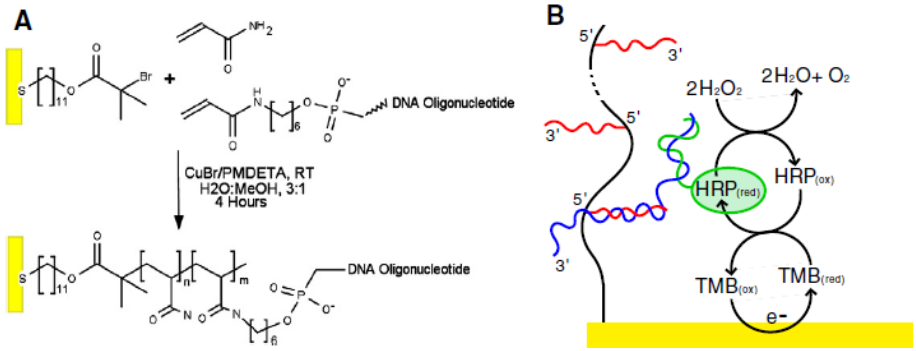


Figure 2.19 (A) SI-ATRP of acrylamide and acrylamide modified DNA oligonucleotides and (B) Schematic of the acrylamide-co-Exon16 brush and principle of the electrochemical assay. [30]

In 2013, Wang and coworkers [31] developed a microfluidic chip-based fluorescent DNA biosensor for the specific detection of single-base mismatch DNA based on the electrophoretic driving mode. The magnetic beads (MBs) were carriers in microchannel by “sandwich” hybridization strategy (**Figure 2.20**). The first probe is capture probe which was functionalized with a biotin group and then was immobilized on the surface of streptavidin-functionalized MBs. The second probe is a signal probe which was modified with a FAM (carboxyfluorescein). Target DNA can effectively hybridize with the capture probes and generated fluorescence signal. The DNA biosensor has much higher discrimination ability for the detection of single-base mismatch than biosensors previously reported.

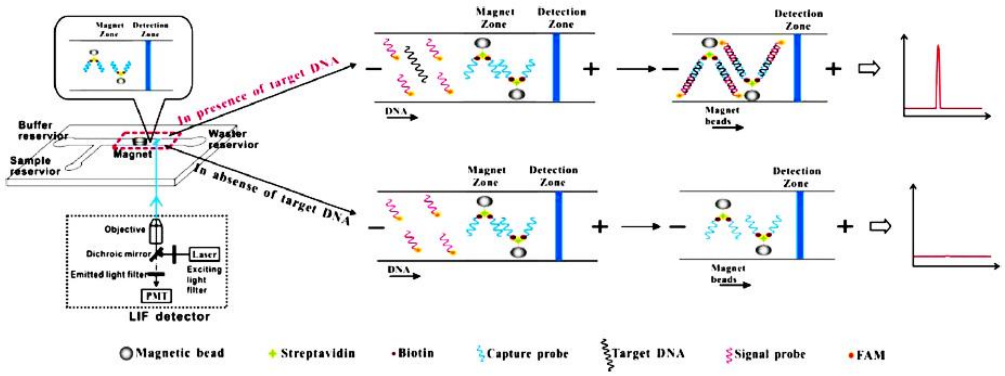


Figure 2.20 The experimental principle of the microfluidic chip-based fluorescent DNA biosensor. [31]

In 2013, Halford and coworkers [32] developed an electrochemical sensor assay to directly detect and identify bacteria based on sandwich hybridization with target DNA and ribosomal RNA (rRNA). The capture probe is anchored to the sensor surface, while the detector probe is linked with horseradish peroxidase (HRP) to obtain a signal. They demonstrated that the sensor can identify the types of bacteria (*E. coli and K. pneumonia*) in the urine of patients with urinary tract infection using recognition of bacterial species by probe pairs. The limit of detection is defined as the concentration of target required to generate a signal that is 3.29 standard deviations greater than the mean of background signals.

CHAPTER III

EXPERIMENTAL SECTION

3.1 Materials

Whatman No. 1 filter paper was used as the membrane. Polyethylene glycol methacrylate (PEGMA 98%), glycidyl methacrylate (GMA 99%), dimethyl-formamide (DMF), 4,4-azobis(4-cyanovaleic acid) (ACVA), 4-cyano-4-(phenylcarbonothio) pentanoic acid (CPD), 4-(dimethylamino) pyridine (DMAP), N,N'-Dicyclohexyl carbodiimide (DCC), *o*-phenylene- diamine (OPD), bovine serum albumin (BSA), urea–hydrogen peroxide (urea–H_2O_2) and streptavidin–horseradish peroxidase conjugate (SA–HRP) were bought from Aldrich (USA). PEGMA and GMA were purified through a column filled with basic alumina to remove the inhibitor prior to use. All reagents and materials are analytical grade and used without further purification. 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.

3.2 Equipments

3.2.1 Nuclear magnetic resonance (NMR) spectroscopy

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

3.2.2 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}.

3.2.3 Scanning electron microscopy (SEM)

The morphology of the filter paper before and after modification was examined by SEM on a JEOL (JSM-6610LV) instrument

3.2.4 X-ray photoelectron spectroscopy (XPS)

The elemental composition of the surface-modified filter paper was investigated by XPS (ESCA-3400, Shimadzu Co., Kyoto, Japan) using an Al Kα X-ray source.

3.2.5 MALDI-TOF mass spectrophotometry

MALDI-TOF mass spectra of acpcPNA were obtained on Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The samples (1 µL) were mixed with 10 µL of the matrix solution consisting of α-cyano-4-hydroxycinnamic acid (CCA) in 0.1% trifluoroacetic acid (TFA) in acetonitrile:water (1:1) solution. This mixture (1 µL) was spotted 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 processed by averaging between 20 and 30 individual laser shots.

3.2.6 High-performance liquid chromatography (HPLC)

The crude acpcPNA was purified by reversed-phase HPLC with UV detection at 260 nm. A Varian Polaris C18 analytical HPLC column (3 mm particle size 4.6 × 50 mm) was used and eluted with a gradient of 0.1% TFA in MeOH and 0.1% TFA in water.

3.2.7 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.

3.2.8 Scanner

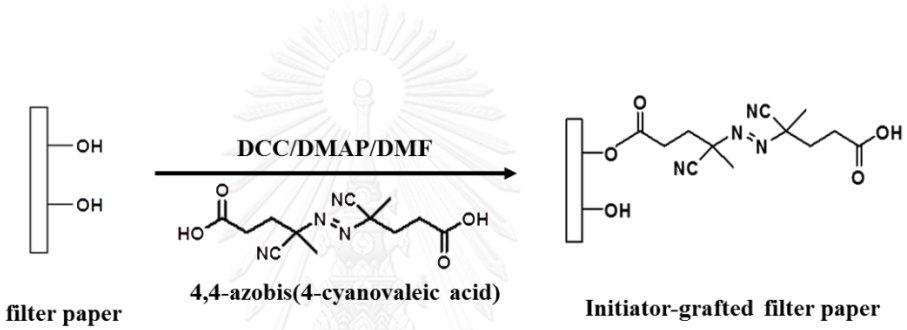
The scanned images of the tested results on filter papers were recorded on Epson Perfection V33 scanner in 24 bits Professional mode. The

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.

3.3 Experimental procedure

3.3.1 Preparation of poly(glycidyl methacrylate-co-poly(ethylene glycol) methacrylate) (GMA-*co*-PEGMA) grafted filter paper

3.3.1.1 Immobilization of initiator on filter paper



The filter paper was first immobilized with initiator. The initiator, ACVA (0.21 g, 1 mmol), DMAP (9.14 mg, 0.1 mmol) and DCC (0.19 g, 1 mmol) were dissolved in 20 mL DMF. The solution was stirred for 4 h at ambient temperature under nitrogen atmosphere before transfered to a vial containing the filter paper (4×8 cm^2). After the reaction proceeded for 20 h, the filter paper was removed from the solution and thoroughly rinsed with DMF and ethanol three times each and dried under nitrogen gas flow.

3.3.1.2 Surface-initiated reversible addition-fragmentation chain transfer (RAFT) polymerization of (GMA-*co*-PEGMA) of initiator-immobilized filter paper

The obtained initiator-immobilized filter paper was then placed in a vial containing ACVA (3.5 mg, 0.05 mmol) and CPD (14 mg, 0.0125 mmol) in 5 mL of DMF. GMA (0.928 mL, 7 mmol or 0.4 mL, 3 mmol) and PEGMA (2.28 mL, 3 mmol or 0.98 mL, 7 mmol) were then added into the mixture. The vial was then sealed with a rubber septum. The solution was deoxygenated by purging with nitrogen gas for 30 min. The SI-RAFT was then carried out at 70 °C for 4 h. The filter paper was removed from the vial and rinsed thoroughly with DMF and ethanol.

3.3.2 Synthesis of *acpc*PNA oligomers

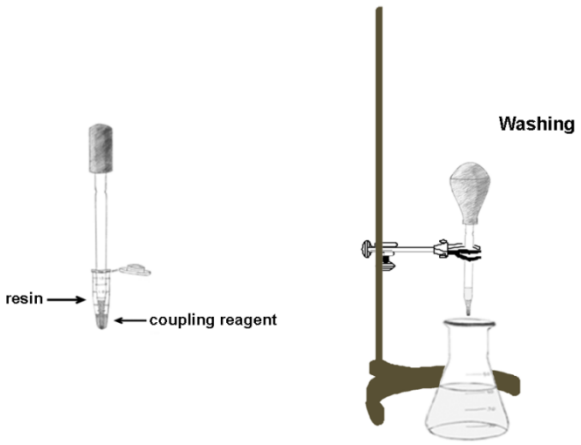


Figure 3.1 Schematic diagram synthesis of *acpc*PNA by using solid phase peptide synthesis. [33, 34]

In this work, *acpc*PNA oligomers were synthesized by using a home-made peptide synthesis. The synthesis following the standard procedure previously developed by Dr. Boonjira Boontha, Dr. Cheeraporn Ananthanawat, and Mrs. Chotima Vilaivan. The crude acpcPNA oligomers were purified by reverse phase HPLC and the identity of the PNA oligomers was verified by MALDI-TOF mass spectrometry.

3.3.2.1 Modifying the PNA oligomer

Synthesis of each labeled-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.

i) Modifying the PNA oligomer with acetic anhydride

The desired sequence of PNA capture probe on the resin was immersed with 100 μL of the reagent#1. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF. The resin was capped with 5 μL acetic acid in 30 μL of reagent#2 at room temperature for 15 min followed by washing with excess 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 protecting groups (Bz or Ibu) of PNA nucleobases (A, C, and G monomers).

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

The desired sequence of PNA reporter probe on the resin was immersed with 100 μL of the reagent#1 for 5 min. The resin was treated with a solution of the

activated ethylene glycol (egl) linker, Fmoc-egl-OPfp (1 mg) and HATU (1 mg) in 20 µL of reagent#2 for 30 min at room temperature followed by washing with excess DMF. The prepared resin was immersed with 100 µL of the reagent#1 for 5 min. After washing with excess DMF, the resin carrying free amino group was treated with the solution of activated biotin (3.92 mg, 6 µmol) in 20 µL of reagent#2 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 protecting groups (Bz or Ibu) of PNA nucleobases (A, C, and G monomers).

iii) Modifying the PNA oligomer with pentafluorobenzenesulfonyl chloride

The sequence acpcPNA (T9) oligomer on the resin was immersed with 100 µL of the reagent#1 for 5 min. The resin was capped with 5 µL pentafluorobenzenesulfonyl chloride in 30 µL of reagent#2 at room temperature for 5 days. After the specified period, the reagent was squeezed off and the reaction column was washed with excess DMF.

iv) Method for cleavage of 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 at three times with occasional agitation. After 1 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.

v) Purification and identification

The crude PNA oligomer was prepared for HPLC analysis by dissolving a mixture in 120 µL Milli-Q 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. 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% TFA 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. The PNA sequences having different modifiers used in this work are illustrated in **Table 3.1**.

Table 3.1 PNA sequences used in this study

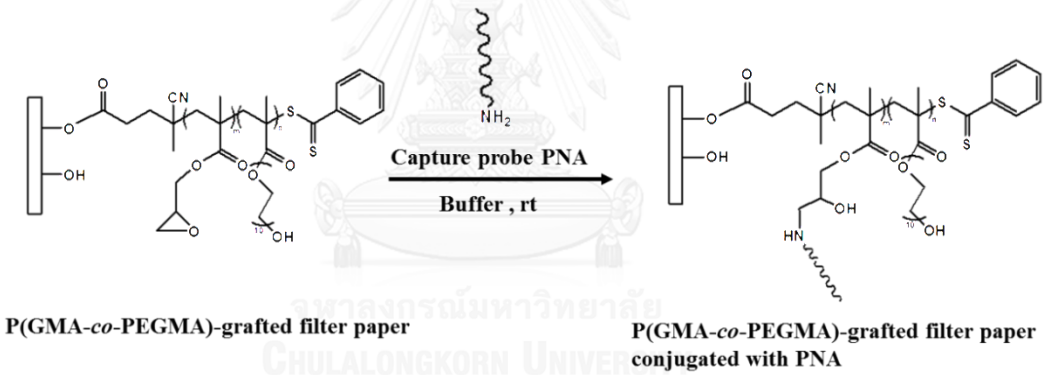
Name	Modification	Sequence	Description
PNA (Cap)	Acetic anhydride	Ac-GGAACCTGCG CG-LysNH_2	The capture probe
biotinylated PNA probe (b-PNA(Ser))	Ethylene glycol (egl) linker and biotin	b-o-o-AACACACA GACT-SerOH	The reporter probe
biotinylated PNA probe (b-PNA(Lys))	Ethylene glycol (egl) linker and biotin	b-o-o-AACACACA GACT-LysNH_2	The positive control
pentafluorobenzenes ul fonyl-labeled PNA	Pentafluorobenzenes ul fonyl chloride	penta-TTTTTTTTT-LysNH_2	Model for capture probe PNA
*fluorescence-labeled PNA	Fluorescent dye	flu-o-TTTTTTTTT-LysNH_2	Model for capture probe PNA

*This PNA was synthesized by Mrs. Chotima Vilaivan.

3.3.2.2 Melting temperature (T_m) measurement

The melting temperature (T_m) measurement of PNA and DNA were performed on a CARY 100 Bio UV-Visible spectrophotometer (Varian, Inc., USA), equipped with a thermal melt system. The final concentration of 2 µM nucleotides at a ratio of PNA : DNA = 1:1 in 10 mM phosphate buffer pH 7.0. The A_{260} of PNA·DNA was recorded in steps from of 20-90 °C in two heating and one cooling cycles (20–90–20–90) at a rate of 1.0 °C/min. The T_m was obtained from the first derivative plot after smoothing using KaliedaGraph 3.6 (Synergy Software) and Microsoft Excel 2000.

3.3.3 Immobilization of PNA capture probe on the P(GMA-*co*-PEGMA)-grafted filter paper



P(GMA-*co*-PEGMA)-grafted filter paper was incubated in an aqueous solution containing PNA(Cap) having sequence of GGAACCTGCGCG-LysNH_2 (2.7 µL, 2 nmol) in 2 mL 0.1 M phosphate buffered saline (PBS, pH 7.4) and a designated quantity of triethylamine (TEA) was added into the mixture. The solution was shaked for 24 h at room temperature and washed with 0.1 M phosphate buffer pH 7.4 and Milli-Q water, dried with nitrogen gas flow to obtain the P(GMA-*co*-PEGMA)-filter paper conjugated with PNA(Cap). Biotinylated PNA (b-o-o-AACACACAGACT-LysNH_2, b-PNA (Lys), (4.36 µL, 2 nmol) as a positive control was also immobilized on the P(GMA-*co*-PEGMA)-filer paper using the same procedure.

3.3.4 Determination of immobilization efficiency of PNA capture probe

This investigation was performed to determine the effect of triethylamine (TEA) concentration or buffer having different pH on immobilization efficiency of PNA capture probe. Firstly, the fluorescence-labeled PNA (flu-o-TTTTTTTTT-LysNH_2) was used as a model for PNA capture probe. The aqueous PNA solution having designated quantity of TEA (0.1, 0.2 and 0.3 μM) was added into 0.1 M PBS pH 7.4. 0.5 μL (200 μM) of the PNA was spotted on the marked P(GMA-*co*-PEGMA)-grafted filter paper and dried for 24 h at room temperature. The P(GMA-*co*-PEGMA)-grafted filter paper was then rinsed by acetonitrile: Milli-Q water (1:1) , 0.1 M phosphate buffer pH 7.4 and Milli-Q water three times each, then blow-dried with nitrogen gas. A similar procedure was applied for the investigation on the effect of pH of buffer (8.0, 9.0 and 10.0) by using the designated buffer to dissolve PNA without TEA addition (0.1 M phosphate buffer pH 8.0, 0.1 M carbonate-bicarbonate buffer pH 9.0, 0.1 M carbonate-bicarbonate buffer pH 10.0). The extent of fluorescence-labeled PNA immobilization was monitored under fluorescence microscope.

To determine immobilization efficiency of PNA capture probe on P(GMA-*co*-PEGMA)-grafted filter paper by XPS analysis , the pentafluorobenzenesulfonyl-labeled PNA (penta-TTTTTTTTT-LysNH_2) was employed as a probe. P(GMA-*co*-PEGMA)-grafted filer paper was incubated in an aqueous solution containing PNA (3.58 μL, 2 nmol) dissolved in 2 mL of 0.1 M PBS pH 7.4 with designated quantity of TEA (0.1 and 0.3 μM TEA) or 0.1 M carbonate-bicarbonate buffer pH 10.0.

3.3.5 Colorimetric detection for DNA sequence by sandwich-hybridization assay

All PNA and DNA sequences used in this study are displayed in **Table 3.2**. The P(GMA-*co*-PEGMA)-grafted filter paper conjugated with PNA capture probe was cut into 1×4.9 cm^2 piece and spot positions were marked with a pencil. 2 μL of the DNA sample (1 μM in 0.5 mM phosphate buffer pH 7.0) was spotted on the marked filter paper following a sequence illustrated in **Table 3.3**. The filter paper was rinsed with 0.1% Tween 20 in 0.1 M phosphate buffer pH 7.4, 0.1 M phosphate buffer pH 7.4 and Milli-Q water three times each followed by incubation in a blocking

solution (1% w/v BSA in 0.1 M PBS pH 7.4) for 30 min. A 2 µL of the biotinylated acpcPNA probe, as reporter probe, (1 µM in 0.1 M phosphate buffer pH 7.4) was spotted on filter paper at position 2, 3 and 4. After that the filter paper was rinsed with 0.1% Tween 20 in 0.1 M phosphate buffer pH 7.4, 0.1 M phosphate buffer pH 7.4 and Milli-Q water three times each and incubated in the blocking solution again for 30 min. A 2 µL of SA-HRP was spotted on filter paper at all positions followed by rinsing with 0.1 M phosphate buffer pH 7.4, Milli-Q water and 0.1 M citrate buffer (CTB, pH 5.0) three times each. 5 µL of a solution consisting of 250 µL of OPD substrate (1.6 mg/mL) and 250 µL of urea-H_2O_2 (1.6 mg/mL) was spotted on filter paper at all positions for 1 min and was finally washed with Milli-Q water.

Table 3.2 PNA and DNA sequences used in this study.

Code	Sequence	Description
PNA (Cap)	Ac-GGAACCTGCGCG-LysNH_2	Capture probe
b-PNA (Lys)	b-o-o-AACACACAGACT-LysNH_2	Positive control
b-PNA (Ser)	b-o-o-AACACACAGACT-SerOH	Reporter probe
b-DNA	d(5'-CGCGCAGGTTCC-b-3')	Positive control
DNA 1	d(5'-**CGCGCAGGTTCC**GCTAA GTCTGTGTGTT-3')	Complementary to PNA(Cap)
DNA 2	d(5'-CGCGC**T**GGTTCCGCTAAGT CTGTGTGTT-3')	Single mismatch to PNA(Cap)
DNA 3	d(5'-TATATCAAGGTTGCTACAG TGAGAGAGG-3')	Non-complementary PNA(Cap)

Table 3.3 Description of DNA and PNA sequences used for each spot on the P(GMA-*co*-PEGMA)-grafted filter paper conjugated with PNA capture probe.

Spot position	DNA	PNA	Remark[a]
1	-	b-PNA(Lys)	(+)
2	b-DNA HLA-B*1502	-	(+)
3	DNA 1 HLA-B*1502	b-PNA (Ser)	(+)
4	DNA 3	b-PNA (Ser)	(-)
5		b-PNA (Ser)	(-)
6	-	-	(-)

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

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation and characterization of P(GMA-*co*-PEGMA)-grafted filter paper

The initiator was immobilized on filter paper via the esterification of hydroxyl groups on the filter paper with ACVA using DCC as coupling agent and DMAP as catalyst. The P(GMA-*co*-PEGMA) was then grafted on filter paper via SI-RAFT polymerization to insure that the molecular weight and copolymer composition can be well manipulated. In this research, a target degree of polymerization was set at 200 and the copolymer composition was varied from GMA:PEGMA of 30:70 to 70:30. ^{1}H NMR spectra of the copolymers formed from ACVA also added in the solution are displayed in **Figure 4.1**. Equations used for the calculation of molecular weight and copolymer composition are also provided. As determined by ^{1}H NMR analysis (**Table 4.1**), the molecular weight ($\overline{M}_n$) of P(GMA-*co*-PEGMA) formed in solution closely resembled the theoretical value for the feed composition GMA:PEGMA 70:30, but was much lower than expected for the feed composition GMA:PEGMA 30:70. The copolymer composition also coincides well with the monomer feed ratio suggesting that the copolymerization was reasonably under control.

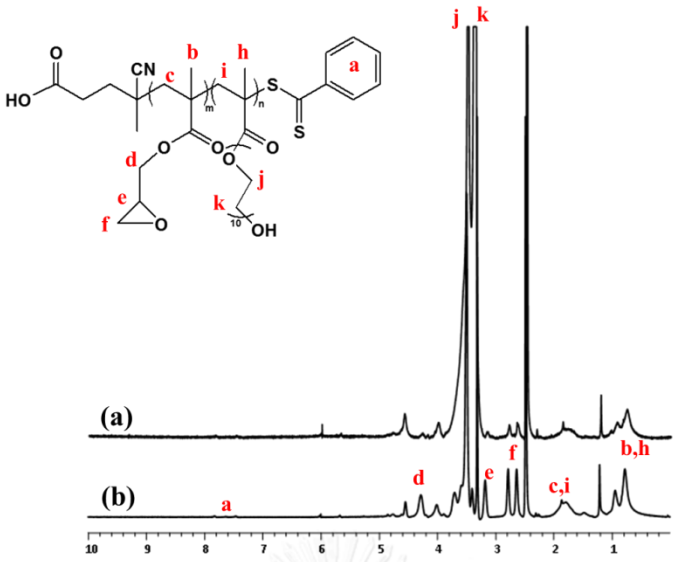


Figure 4.1 ^{1}H NMR spectra of P(GMA-*co*-PEGMA) in solution prepared by RAFT polymerization having GMA:PEGMA in the feed ratio of (a) 30:70 and (b) 70:30.

$$\text{Total unit} = \left\{ \frac{\text{intergral of the Hc,Hi,Hb and Hh/10}}{\text{intergral of the Ha/5}} \right\} \times 2$$

$$\text{GMA unit} = \left\{ \frac{\text{intergral of the Hf/2}}{\text{intergral of the Ha/5}} \right\}$$

$$\text{PEGMA unit} = \text{Total unit} - \text{GMA unit}$$

$$\text{Mn} = [\text{GMA unit} \times \text{MW of GMA (142 g/mol)} + \text{PEGMA unit} \times \text{MW of PEGMA (360 g/mol)}] \quad \textbf{(4.1)}$$

$$\text{GMA content (\%)} = \left\{ \frac{\text{GMA unit}}{\text{Total unit}} \right\} \times 100 \quad \textbf{(4.2)}$$

$$\text{PEGMA content (\%)} = \left\{ \frac{\text{PEGMA unit}}{\text{Total unit}} \right\} \times 100 \quad \textbf{(4.3)}$$

Table 4.1 Characteristics of P(GMA-*co*-PEGMA) synthesized by RAFT polymerization

Entry	GMA:PEGMA mole ratio (target)	GMA:PEGMA mole ratio (observed)	Mn (target)	Mn (observed)
1	30:70	21:79	59199	51921
2	70:30	74:26	41759	43883

The success of stepwise surface modification of filter paper was confirmed by FT-IR of which results are displayed in **Figure 4.2**. The FT-IR spectrum of the initiator-immobilized filter paper (**Figure 4.2b**) shows a band at 1738 cm^{-1} (C=O stretching) which does not appear in the unmodified filter paper (**Figure 4.2a**) suggesting that ester linkage was formed between the filter paper and carboxyl-containing initiator. The spectrum of P(GMA-*co*-PEGMA)-grafted filter paper (**Figure 4.2c,d**) exhibits a band at the same position which is also a characteristic C=O stretching of ester groups in the copolymer and a band at 910 cm^{-1} corresponding to C-O-C stretching of epoxide group of GMA. The relative peak intensity ratio between C-O-C stretching (910 cm^{-1}) and C=O stretching (1738 cm^{-1}) was found to be correspondingly increased as a function of GMA content in the copolymer. The intensity decrease of C-O-C stretching at 910 cm^{-1} found in the spectrum of P(GMA-*co*-PEGMA)-grafted filter paper after being conjugated with PNA capture probe (**Figure 4.2e**) suggested that the epoxide groups were consumed by reacting with the PNA capture probe via epoxide ring opening.

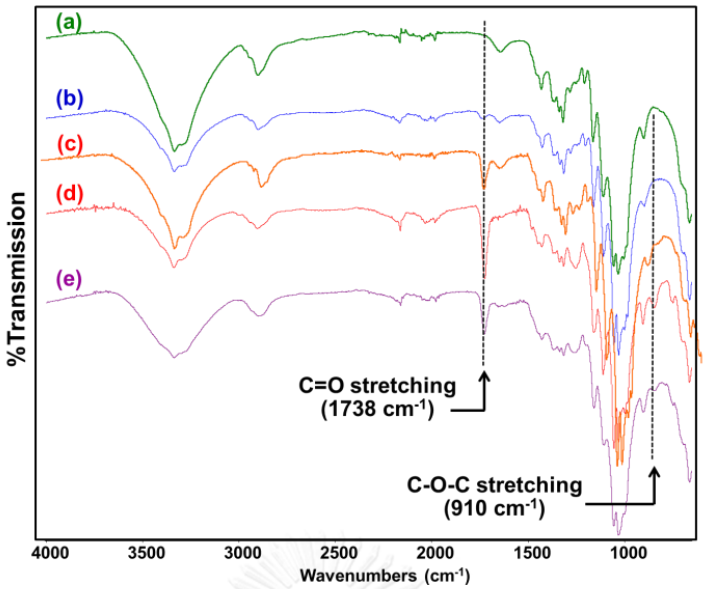


Figure 4.2 FT-IR spectra of the filter paper: (a) unmodified, (b) immobilized with initiator, grafted with P(GMA-*co*-PEGMA) having GMA:PEGMA of (c) 30:70 and (d) 70:30, and (e) conjugated with PNA capture probe.

As illustrated in **Figure 4.3**, there were no noticeable microscopic changes in morphology of the filter paper undergoing initiator immobilization and copolymer grafting suggesting that the coated polymer layer is relatively thin. Nevertheless, the filter paper grafted with P(GMA-*co*-PEGMA) exhibits better tearing resistance as opposed to the unmodified filter paper ,especially after repetitive exposure to variety of solutions during PNA probe conjugation and DNA hybridization studies.

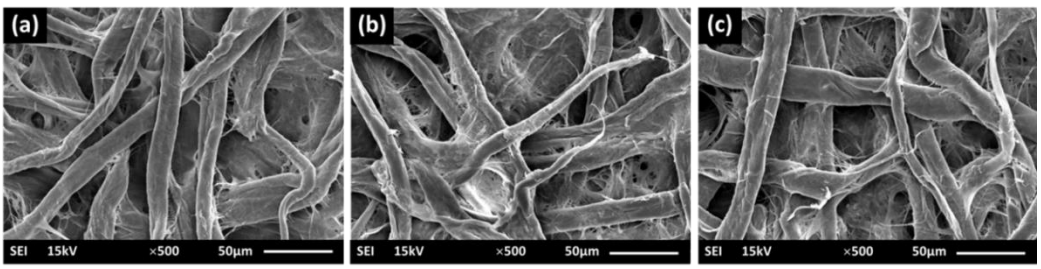
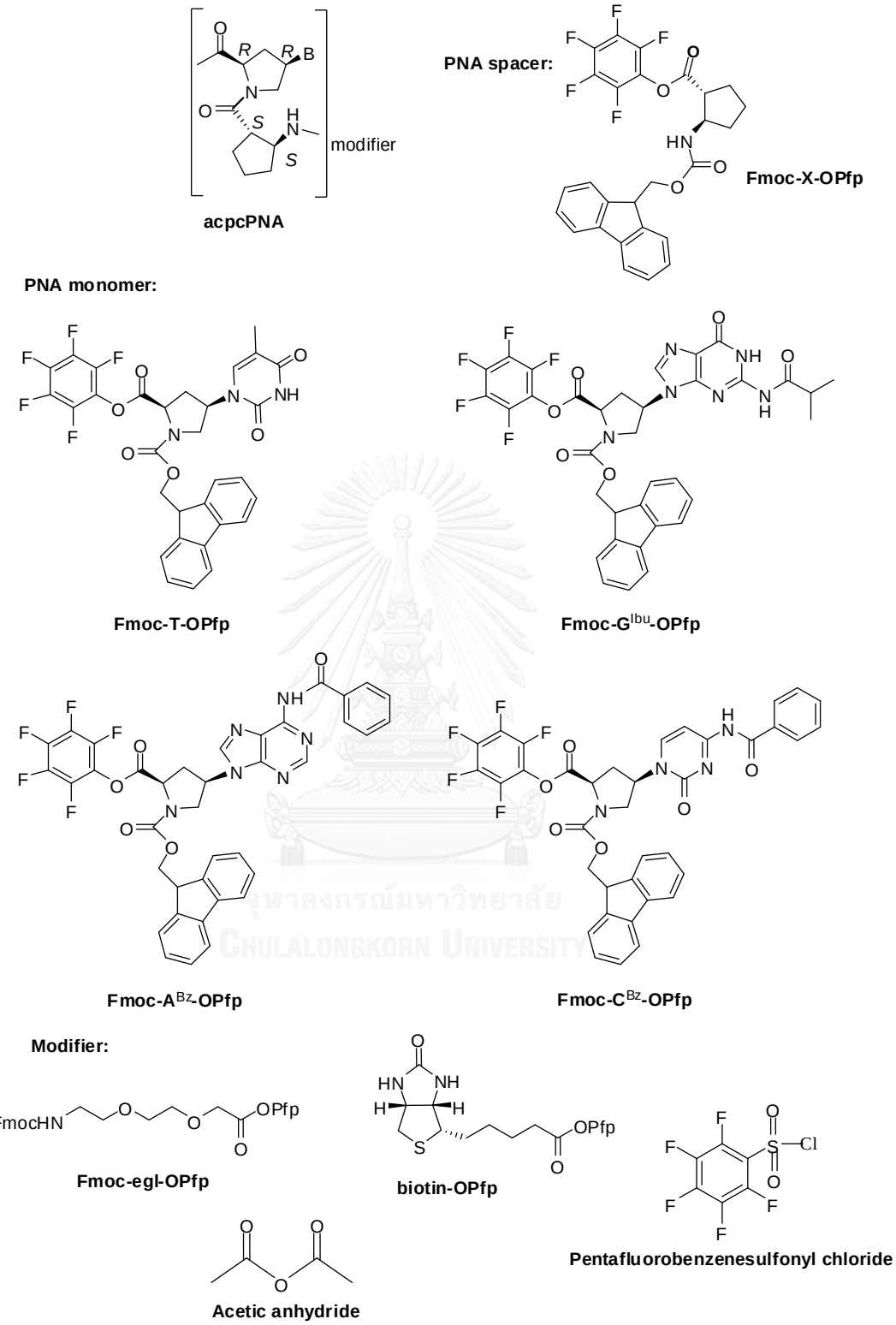


Figure 4.3 SEM images of the filter paper: (a) unmodified, (b) immobilized with initiator, (c) grafted with P(GMA-*co*-PEGMA) (GMA:PEGMA of 70:30).

4.2 Synthesis of *acpc*PNA oligomers

acpcPNA Probes were synthesized using solid phase peptide synthesis according to procedures previously reported in literatures. [33, 34] The activated Fmoc-protected acpcPNA monomers, spacer, activated ethylene glycol (egl) linker and modifier such as biotin, acetic anhydride, pentafluorobenzenesulfonyl chloride were used in solid phase peptide synthesis (**Scheme 4.1**). The PNA probe were usually produced in a stepwise fashion starting from C (carboxyl) terminus to N (amino) terminus in a series of coupling cycles. The technique involves growing of a peptide, the first amino acid (lysine, serine) was attached to the free amino group on the RAM resin. The next step of the synthetic cycle, the PNA monomer and spacer were coupled alternately until the desired PNA sequence was obtained. Nucleobase protecting groups (Bz or Ibu) in the PNA chain were removed by a treatment with 1:1 concentrated aqueous ammonia:dioxane 1:1 at 65 ^{o}C for 15 h. After nucleobase side-chain deprotection and cleavage from the solid support, the crude acpcPNA was purified by reversed-phase HPLC. After purification, the solution of PNA was lyophilized and the identity of the acpcPNA oligomers was verified by MALDI-TOF mass spectrometry and thermal denaturation experiments (after hybridization with complementary DNA sequences) (**Table 4.2**).



Scheme 4.1 Structure of activated PNA monomers, spacer, activated ethylene glycol (egl) linker and modifier for solid phase peptide synthesis.

Table 4.2 Characteristics of *acpc*PNA oligomers synthesized by solid phase method.

Name	sequence	m/z (target)	m/z (observed)	T_m (°C)
PNA (Cap)	*N*-Ac-GGAACCTGCGCG-$LysNH_2$-*C*	4259.55	4259.38	77.6
b-PNA (Ser)	*N*-biotin-$(egl)_2$-AACACACAG ACT- SerOH -*C*	4630.02	4630.99	62.2
b-PNA (Lys)	*N*-biotin-$(egl)_2$-AACACACAG ACT- $LysNH_2$ -*C*	4670.12	4669.21	not measured
penta-PNA(T9)	*N*-penta-TTTTTTTTT$LysNH_2$-*C*	3368.47	3368.63	not measured
PNA (T9) [a]	*N*-flu-(egl)-TTTTTTTTT-$LysNH_2$-*C*	-	-	-

[a]This PNA was synthesized by Mrs. Chotima Vilaivan.

4.3 Determination of immobilization efficiency of PNA capture probe

In principle, the epoxide ring opening can be promoted under basic condition. To be able to monitor the effect of a base (TEA in this particular case) addition or pH of buffer used in PNA conjugation step on immobilization efficiency by fluorescence microscopy, a fluorescence-labeled PNA (flu-o-PNA(T9)-$LysNH_2$) was used as a model for PNA capture probe. Firstly, different TEA concentration (0.1, 0.2 and 0.3 μM) was introduced to PNA solution dissolved in 0.1 M phosphate buffer saline (PBS, pH 7.4). According to the fluorescence images shown in **Figure 4.4**, an intensity of green illumination of the spots became stronger as a function of TEA concentration indicating that the PNA conjugation was quantitatively improved upon base addition. It should be noted that pH of the PNA solution was not altered after TEA addition. Alternatively, the enhancement of PNA conjugation was much more pronounced once buffer solution was used instead of the 0.1 M PBS pH 7.4 (**Figure 4.5**). The strongest fluorescence intensity which indicated the maximum immobilization quantity, was achieved for the conjugation in buffer solution having

pH 10.0 (0.1 M $NaCO_3$-$NaHCO_3$ buffer). Notably, low background illumination was also found on most of the controlled unmodified filter paper implying a certain level of non-specific adsorption of the flu-o-PNA(T9)-LysNH_2.

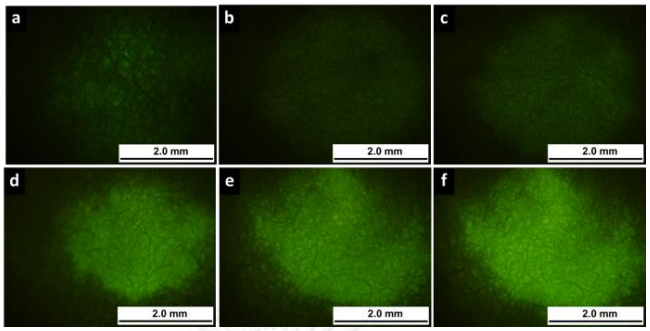


Figure 4.4 Fluorescence images of filter paper after flu-o-PNA(T9)-LysNH_2 conjugation in 0.1 M PBS pH 7.4 with added TEA of (a,d) 0.1, (b,e) 0.2, and (c,f) 0.3 µM : (a-c) unmodified filter paper; (d-f) P(GMA-*co*-PEGMA)-grafted filter paper (GMA:PEGMA ,70:30).

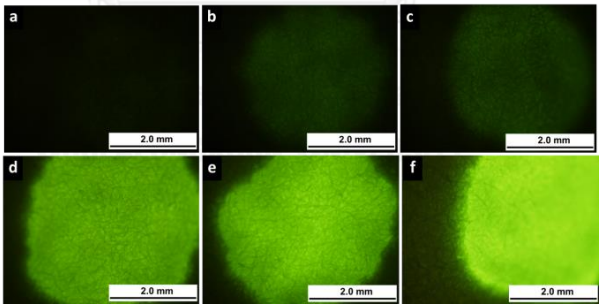


Figure 4.5 Fluorescence images of filter paper after flu-o-PNA(T9)-LysNH_2 conjugation in buffer solution having pH of (a,d) 8.0, (b,e) 9.0, and (c,f) 10.0: (a-c) unmodified filter paper; (d-f) P(GMA-*co*-PEGMA)-grafted filter paper (GMA:PEGMA ,70:30).

To further chemically identify whether the PNA capture probe can be bound onto the P(GMA-*co*-PEGMA)-grafted filter paper, XPS analysis of the P(GMA-*co*-

PEGMA)-grafted filter paper after conjugation with another model of PNA capture probe, the pentafluorobenzenesulfonyl-labeled PNA (penta-PNA(T9)-LysNH_2) using the selected solutions previously employed for fluorescence studies: 0.1 M PBS pH 7.4 with added 0.1 and 0.3 µM TEA and buffer solution of pH 10 was performed. This fluorine-tagged PNA provides additional fluorine atoms in its structure enabling a convenient identification of its existence from fluorine composition. From the XPS atomic composition data presented in **Table 4.3**, the fluorine content of ~0.6 %. was detected on all substrates after conjugation verifying that the top most surface (< 5 nm, the sampling depth of XPS technique) of the P(GMA-*co*-PEGMA)-grafted filter paper were saturately covered with relatively same amount of penta-PNA(T9)-LysNH_2, independent of conjugation condition. From application viewpoint, fluorescence technique is therefore more appropriate to probe the extent of PNA conjugation than the highly surface sensitive technique like XPS, given that the detection relies on not only solution adsorption on top of the surface but also absorption to a certain depth within the paper-based platform.

This investigation suggested that the attachment of PNA probe is apparently more efficient under basic condition and that the conditions with added 0.1 µM TEA in 0.1 PBS pH 7.4 and in buffer solution of pH 10 were selected for the step of PNA capture probe immobilization on P(GMA-*co*-PEGMA)-grafted filter paper to be further analyzed for DNA detection.

Table 4.3 Atomic composition of surface-modified filter paper as determined by XPS analysis

Sample	Atomic composition (%)				
	C	**O**	**N**	**F**	**S**
Immobilized with initiator	53.41	43.45	3.15	-	-
Grafted with P(GMA-*co*-PEGMA)	57.00	41.15	1.58	-	0.27
Immobilized with penta-PNA(T9)-$LysNH_2$ in 0.1 M PBS pH 7.4	57.20	39.98	1.95	0.60	0.28
Immobilized with penta-PNA(T9)-$LysNH_2$ in 0.1 M PBS pH 7.4 with 0.1 µM TEA	61.14	36.41	1.53	0.59	0.34
Immobilized with penta-PNA(T9)-$LysNH_2$ in 0.1 M PBS pH 7.4 with 0.3 µM TEA	64.09	33.73	1.23	0.65	0.29
Immobilized with penta-PNA(T9)-$LysNH_2$ in 0.1 M $NaCO_3$-$NaHCO_3$ pH 10.0	61.84	35.72	1.51	0.62	0.31

4.4 Colorimetric detection for DNA sequence by sandwich-hybridization assay

The P(GMA-*co*-PEGMA)-grafted filter paper was tested for its efficiency as a sensing platform for DNA sequence analysis by sandwich-hybridization assay employing two PNA probes (**Figure 4.6**) of which sequences are designed in response to DNA sequences in human leukocyte antigen (HLA) alleles. In particular, this research focuses on HLA-B*1502 alleles having strong correlation with a severe skin disorder called Stevens-Johnson syndrome in response to carbamazepine (a drug used to treat seizures).[35] PNA(Cap) of a 12-base sequence of

Ac-GGAACCTGCGCG-LysNH_2 was used as the capture probe to be immobilized on the surface of P(GMA-*co*-PEGMA)-grafted filter paper for capturing the DNA target. b-PNA (Ser), a biotinylated PNA probe having a sequence of b-o-o-AACACACAGACT-SerOH was used as a reporter probe to generate a signal upon enzymatic amplification using the optimal condition (in terms of substrate/enzyme concentration and activation time) previously identified by Laopa *et al*. [12] Two formats of the test results are displayed: scanned images (column A) and profile images as analyzed by Scion Image (column B). The profile image provides the semi-quantitative intensity of the colorimetric readout. Details of DNA and PNA sequences and the description of samples/probes applied in each spot (1–6) are showed in Tables 3.1 and 3.2, respectively. A biotinylated PNA (b-PNA(Lys)) having the same sequence as the reporter probe (b-PNA(Ser)) was immobilized on the position 1 as a positive control to illustrate the maximum signal generated by the enzymatic reaction. A biotinylated DNA (b-DNA) was spotted on the position 2 as another positive control to demonstrate that enzymatic signal amplification via hybridization can be achieved once the PNA is complementary with the DNA.

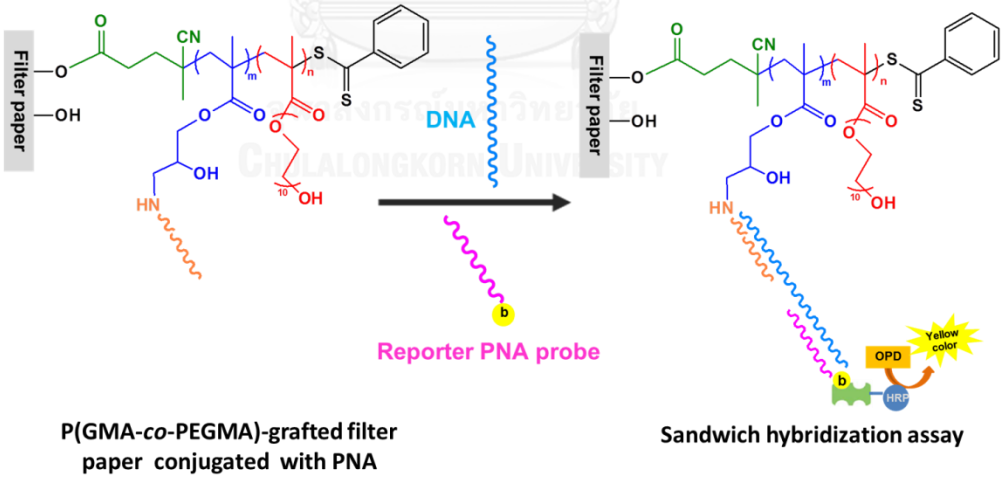


Figure 4.6 Schematic representation of enzymatic amplified colorimetric detection of DNA following sandwich-hybridization assay employing biotinylated acpcPNA probe (b-PNA) as reporter probe and filter paper-grafted (P(GMA-*co*-PEGMA) brushes conjugated with acpcPNA capture probe as substrate.

From the results shown in **Figure 4.7**, the position 3 with DNA 1 which is complementary to the PNA capture probe (PNA(Cap)) shows yellow spots visible by the naked eyes with similar intensity to that of the positive control on the position 2 but with much stronger intensity than that of the position 4 with the non-complementary DNA 2. Apparently, the filter paper grafted with the copolymer with greater composition of PEGMA (GMA:PEGMA = 30:70) shows a much better signal readout implying that the ability to suppress non-specific adsorption by hydrophilic PEGMA entities are very important for the detection efficiency. The fact that low non-specific adsorption of b-PNA(Ser) and SA–HRP with PNA-P(GMA-*co*-PEGMA)-grafted filter paper appearing on the position 5 and 6, respectively still existed suggesting that further investigation to seek for optimized rinsing condition is still required.

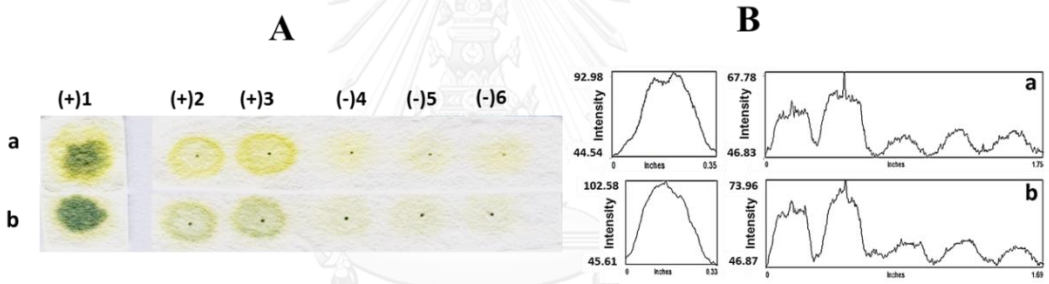


Figure 4.7 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating the copolymer composition of GMA:PEGMA (a) 30:70, (b) 70:30.

Another test was conducted on the P(GMA-*co*-PEGMA)-grafted filter paper conjugated with PNA(Cap) in 0.1 PBS pH 7.4 containing 0.1 μM TEA to determine an impact of ionic strength of DNA binding solution on DNA hybridization efficiency. As revealed in **Figure 4.8**, very strong signal readout on position 1 and 2 which were spotted with b-PNA(Lys) as a control for reporter probe indicated that the signal amplification through biotin-SA-HRP conjugation function efficiently. A relatively strong signal appearing at position 3 spotted with the complementary DNA sequence preliminarily signify the specificity of the test. Upon increasing ionic

strength of DNA hybridization step by NaCl addition from 10 to 100 mM not only can increase the signal intensity, but also suppressed non-specific adsorption from non-complementary DNA sequence [(-4)], reporter probe [(-5)], and SA-HRP [(-6)], allowing more effective discrimination. It was found that 0.5 mM phosphate buffer pH 7.0 with added 100 mM NaCl (row C, **Figure 4.8**) gave highest signal. For this reason, it was chosen as the hybridization buffer for all subsequent experiments.

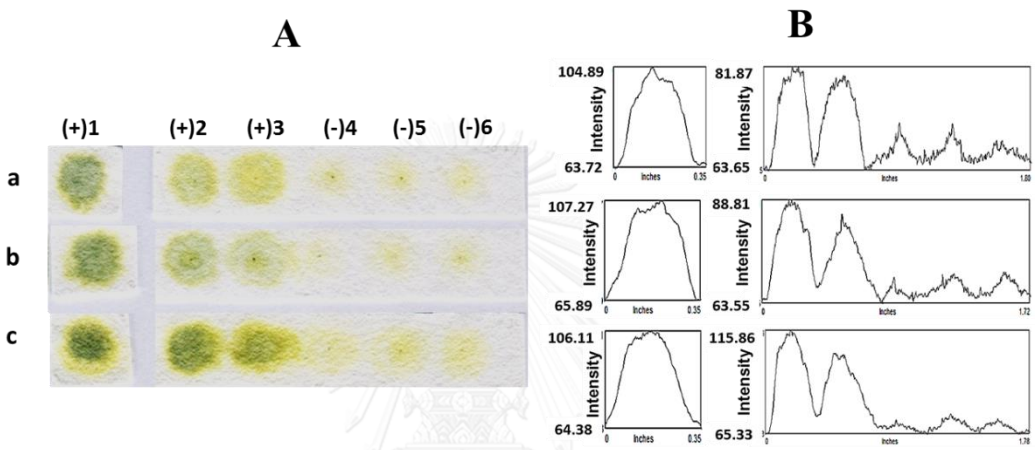


Figure 4.8 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating the effect of salt addition to the hybridization buffer (0.5 mM phosphate buffer pH 7.0): (a) 10 mM NaCl, (b) 50 mM NaCl, and (c) 100 mM NaCl.

To compare the effect of PNA capture probe quantity and binding efficiency, three conditions for PNA(Cap) conjugation on the P(GMA-*co*-PEGMA)-grafted filter paper were employed: in 0.1 PBS pH 7.4 with added 0.1 or 0.3 µM TEA and in buffer solution of pH 10,which supposedly yielded varied quantity of bound PNA(Cap) from low to high as previously identified by fluorescence microscope. The results shown in **Figure 4.9** demonstrate that the detection efficiency which can be judged from the ability to provide high intensity signal to the complementary DNA1[(+3)] as opposed to the non-complementary DNA3 [(-4)] and simultaneously suppress non-specific adsorption of b-PNA(Ser) [(-5)] and SA–HRP [(-6)] seems to be inversely

proportional to the immobilized PNA(Cap) quantity. This unexpected outcome may be described as a result of the high probe density that restricts the accessibility of the target DNA. Therefore, the quantity of PNA(Cap) immobilized on the P(GMA-*co*-PEGMA)-grafted filter paper which was obtained from the immobilization in 0.1 M PBS pH 7.4 containing 0.1 µM TEA was optimal for DNA hybridization

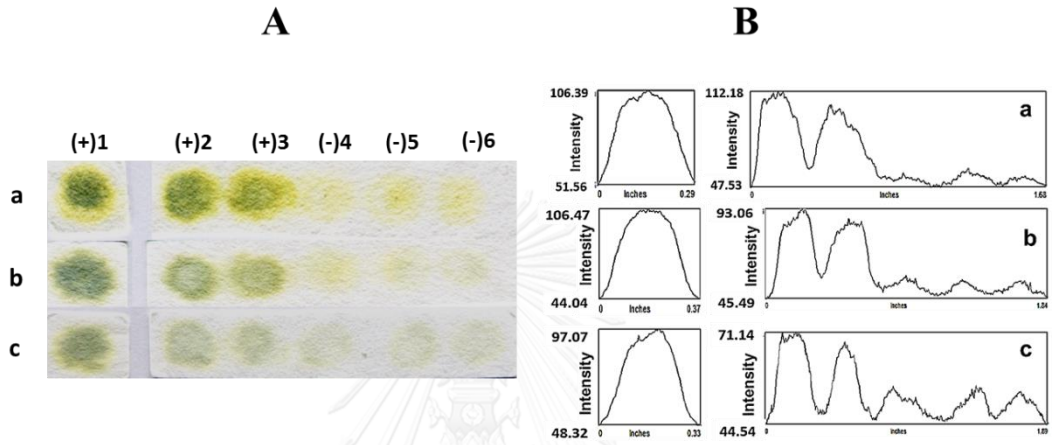


Figure 4.9 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating the effect of PNA(Cap) immobilization condition: in 0.1 M PBS pH 7.4 with added TEA of (a) 0.1 and (b) 0.3 µM, and (c) in buffer solution pH 10.

DNA 2, another DNA sequence having one base mismatch from the complementary DNA, DNA1 was also subjected to the test. Detail of DNA and PNA sequences and description of samples/probes applied in each spot (1-7) of this particular test are illustrated in **Table 4.4**. From the results shown in **Figure 4.10**, the DNA1 [(+3)] which is complementary to the PNA(cap) shows green spots visible by the naked eyes with similar intensity to that of the positive control b-PNA(Lys) [(+1)] and b-DNA [(+2)]. This illustrated an excellent specificity to distinguish single base mismatch [(-4)] and non-complementary [(-5)] in the DNA targets.

Table 4.4 Description of DNA and PNA sequences used for each spot of the test results shown in Figure 4.10

Sequence	Spot position						
	1	**2**	**3**	**4**	**5**	**6**	**7**
DNA	-	b-DNA	DNA 1	DNA 2	DNA 3	-	-
PNA	b-PNA (Lys)	-	b-PNA (Ser)	b-PNA (Ser)	b-PNA (Ser)	b-PNA (Ser)	-
[a]Remark	(+)	(+)	(+)	(-)	(-)	(-)	(-)

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

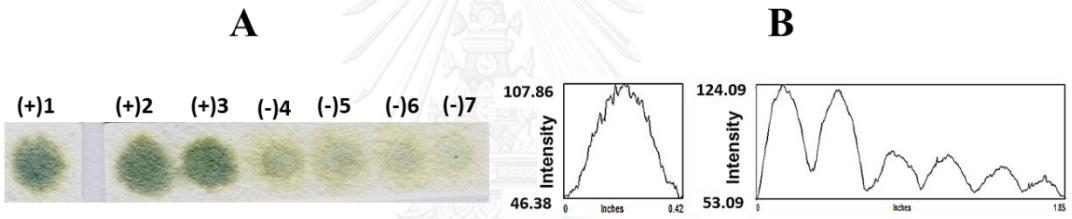


Figure 4.10 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating the specificity and efficiency of PNA(Cap) and b-PNA(Ser) to distinguish complementary, single mismatched and non-complementary DNA targets. The positive results are shown at b-PNA(Lys) [(+1)], b-DNA [(+2)] and DNA 1 [(+)3].

To determine the minimal quantity of DNA targets that can still allow a clear differentiation between fully complementary and single-mismatched DNA, the immobilized PNA(Cap) probe was hybridized with DNA1 and DNA2 at different concentrations ranging from 2 pmol to 10 fmol and detected using 2 pmol of b-PNA(Ser) probe. As presented in **Figure 4.11**, the detection limit of this colorimetric method is at least down to 100 fmol (equivalent to 1 µL of 100 nM DNA). Although this platform is 5 times less sensitive than the previously developed platform based on

filter paper grafted with QPDMAEMA brushes (10 fmol detection limit), the inferior performance can be forgiven considering that the limitation of detection of DNA mixtures can be overcome. Besides, the fact that the discrimination power was not sacrificed via sandwich-hybridization assay of which the use of two PNA probes was required truly highlights the specificity of this acpcPNA system. It should be emphasized that the assay developed in this research does not at all require stringent washing condition (i.e. 20% acetonitrile in PBS buffer) to remove non-specific binding. This outcome suggested that the grafting of copolymer having ability to resist non-specific adsorption is also another key to the success of this developed assay.

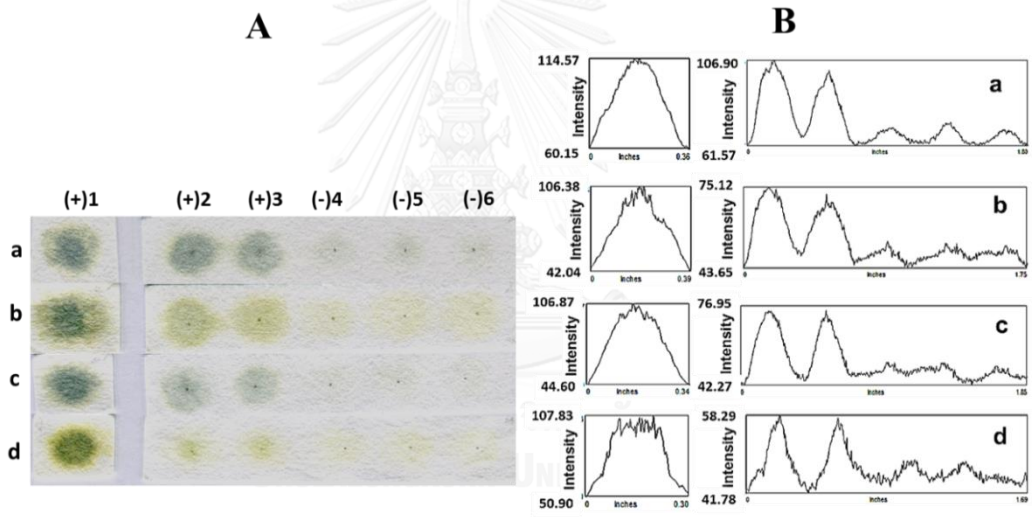


Figure 4.11 Representative images (column A: scanned image and column B: profile image) of the test results demonstrating detection limit of complementary DNA 1 and single mismatched DNA 2 using 2 µL of 1 µM b-PNA(Ser) as probe: (a) 2 pmol (2 µL of 1 µM), (b) 1 pmol (1 µL of 1 µM), (c) 100 fmol (1 µL of 100 nM), and (d) 50 fmol (0.5 µL of 100 nM).

CHAPTER IV

CONCLUSION AND SUGGESTIONS

In this work, it has been demonstrated that the P(GMA-*co*-PEGMA)-grafted filter paper can be prepared by SI-RAFT. Molecular weight and composition of the copolymers determined from ^{1}H NMR data suggested that the polymerization process was reasonably under control. The stepwise surface modification of the filter paper can be verified by FTIR analysis. The intensity decrease of C-O-C stretching at 910 cm^{-1} of the P(GMA-*co*-PEGMA)-grafted filter paper after conjugating with PNA capture probe implied that the epoxide groups of the grafted copolymer were consumed and PNA capture probe has been bound to the surface. Using flu-o-PNA(T9)-LysNH_2 as a model for capture probe and monitored by fluorescence microscopy, it was found that the PNA conjugation via epoxide ring opening can be promoted under basic condition either by the addition of TEA (0.1-0.3 µM) to 0.1 M PBS pH 7.4 or the use of buffer solution having pH of 8.0-10.0.

Upon PNA capture probe immobilization, the developed paper-based platform can be used for DNA sequence determination using sandwich-hybridization assay. Both PNA capture probe and PNA reporter probe were designed in response to DNA sequences in HLA-B*1502 alleles, the human leukocyte antigen alleles having strong correlation with a severe skin disorder called Stevens-Johnson syndrome. The P(GMA-co-PEGMA)-grafted filter having GMA:PEGMA of 30:70 was chosen as appropriate platform for the tests. Immobilization of the PNA capture probe in 0.1 M PBS pH 7.4 with added 0.1 µM TEA was the optimal condition. The best hybridization efficiency could be achieved at in 0.5 mM phosphate buffer, pH 7.0 with an addition of 100 mM NaCl. The enzymatic signal amplification by SA–HRP and OPD can discriminate complementary from single- mismatched and non-complementary DNA targets at a detection limit of at least 100 fmol which can be realized by naked eye. It is anticipated that this P(GMA-co-PEGMA)-grafted filter paper can potentially be developed into an alternative paper-based platform for DNA sequence determination in a high throughput format.

To be able to apply this platform for analysis of real biological samples, it is necessary to perform tests on DNA having longer sequences of which their complementary regions with both PNA capture probe and PNA reporter probe do not necessarily locate at the end of the DNA chains. This is, therefore, a subject of our future investigation.

REFERENCES

1. Hyrup, B.; Nielsen, P. E., Peptide nucleic acids (PNA): Synthesis, properties and potential applications. *Bioorganic & Medicinal Chemistry* **1996,** *4* (1), 5-23.
2. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O., Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **1991,** *254*, 1497-1500.
3. Vilaivan, T.; Srisuwannaket, C., Hybridization of pyrrolidinyl peptide nucleic acids and DNA: Selectivity, base-pairing specificity, and direction of binding. *Organic Letters* **2006,** *8* (9), 1897-1900.
4. Boontha, B.; Nakkuntod, J.; Hirankarn, N.; Chaumpluk, P.; Vilaivan, T., Multiplex mass spectrometric genotyping of single nucleotide polymorphisms employing pyrrolidinyl peptide nucleic acid in combination with ion-exchange capture. *Analytical Chemistry* **2008,** *80* (21), 8178-8186.
5. Ananthanawat, C.; Vilaivan, T.; Hoven, V. P., Synthesis and immobilization of thiolated pyrrolidinyl peptide nucleic acids on gold-coated piezoelectric quartz crystals for the detection of DNA hybridization. *Sensors and Actuators B-Chemical* **2009,** *137* (1), 215-221.
6. Ananthanawat, C.; Vilaivan, T.; Mekboonsonglarp, W.; Hoven, V. P., Thiolated pyrrolidinyl peptide nucleic acids for the detection of DNA hybridization using surface plasmon resonance. *Biosensors and Bioelectronics* **2009,** *24* (12), 3544-3549.
7. Ananthanawat, C.; Vilaivan, T.; Hoven, V. P.; Su, X., Comparison of DNA, aminoethylglycyl PNA and pyrrolidinyl PNA as probes for detection of DNA hybridization using surface plasmon resonance technique. *Biosensors and Bioelectronics* **2010,** *25* (5), 1064-1069.
8. Thipmanee, O.; Samanman, S.; Sankoh, S.; Numnuam, A.; Limbut, W.; Kanatharana, P.; Vilaivan, T.; Thavarungkul, P., Label-free capacitive DNA sensor using immobilized pyrrolidinyl PNA probe: effect of the length and terminating head group of the blocking thiols. *Biosensor and Bioelectronics* **2012,** *38* (1), 430-435.

9. Sankoh, S.; Samanman, S.; Thipmanee, O.; Numnuam, A.; Limbut, W.; Kanatharana, P.; Vilaivan, T.; Thavarungkul, P., A comparative study of a label-free DNA capacitive sensor using a pyrrolidinyl peptide nucleic acid probe immobilized through polyphenylenediamine and polytyramine non-conducting polymers. *Sensors and Actuators B: Sensors and Actuators B: Chemical* **2013,** *177*, 543-554.

10. Jampasa, S.; Wonsawat, W.; Rodthongkum, N.; Siangproh, W.; Yanatatsaneejit, P.; Vilaivan, T.; Chailapakul, O., Electrochemical detection of human papillomavirus DNA type 16 using a pyrrolidinyl peptide nucleic acid probe immobilized on screen-printed carbon electrodes. *Biosensors and Bioelectronics* **2014,** *54*, 428-434.

11. Liu, Y.; Wang, W.; Hu, W.; Lu, Z.; Zhou, X.; Li, C. M., Highly sensitive poly[glycidyl methacrylate-co-poly(ethylene glycol) methacrylate] brush-based flow-through microarray immunoassay device. *Biomedical Microdevices* **2011,** *13* (4), 769-777.

12. Laopa, P.S.; Vilaivan, T.; Hoven, V. P., Positively charged polymer brush-functionalized filter paper for DNA sequence determination following Dot blot hybridization employing a pyrrolidinyl peptide nucleic acid probe. *Analyst* **2013,** *138* (1), 269-277.

13. Jirakittiwut, N.; Panyain, N.; Nuanyai, T.; Vilaivan, T.; Praneenararat, T., Pyrrolidinyl peptide nucleic acids immobilised on cellulose paper as a DNA sensor. *RSC Advances* **2015,** *5* (31), 24110-24114.

14. Akkahat, P.; Hoven, V. P., Introducing surface-tethered poly(acrylic acid) brushes as 3D functional thin film for biosensing applications. *Colloids and Surfaces. B, Biointerfaces* **2011,** *86* (1), 198-205.

15. *DNA Structure*. http://www.mhhe.com/biosci/esp/2001_gbio/folder_structure/ge/m4/s1/ 2015 [cited 2015 21 June].

16. Rashatasakhon, P.; Vongnam, K.; Siripornnoppakhun, W.; Vilaivan, T.; Sukwattanasinitt, M., FRET detection of DNA sequence via electrostatic interaction of polycationic phenyleneethynylene dendrimer with DNA/PNA hybrid. *Talanta* **2012,** *88*, 593-598.

17. Theppaleak, T.; Rutnakornpituk, B.; Wichai, U.; Vilaivan, T.; Rutnakornpituk, M., Magnetite nanoparticle with positively charged surface for immobilization of peptide nucleic acid and deoxyribonucleic acid. *Journal of Biomedical Nanotechnology* **2013,** *9* (9), 1509-1520.

18. Zhao, B.; Brittain, W. J., Polymer brushes: Surface-immobilized macromolecules. *Progress in Polymer Science* **2000,** *25* (5), 677-710.

19. Gurbuz, N.; Demirci, S.; Yavuz, S.; Caykara, T., Synthesis of cationic N-[3-(dimethylamino)propyl]methacrylamide brushes on silicon wafer via surface-initiated RAFT polymerization. *Journal of Polymer Science Part A: Polymer Chemistry* **2011,** *49* (2), 423-431.

20. Lindqvist, J.; Malmström, E., Surface modification of natural substrates by atom transfer radical polymerization. *Journal of Applied Polymer Science* **2006,** *100* (5), 4155-4162.

21. Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell, A. J., Permanent, nonleaching antibacterial surfaces. 1. Synthesis by atom transfer radical polymerization. *Biomacromolecules* **2004,** *5* (3), 877-882.

22. Roy, D.; Knapp, J. S.; Guthrie, J. T.; Perrier, S., Antibacterial cellulose fiber via RAFT surface graft polymerization. *Biomacromolecules* **2008,** *9* (1), 91-99.

23. Tang, F.; Zhang, L.; Zhang, Z.; Cheng, Z.; Zhu, X., Cellulose filter paper with antibacterial activity from surface-initiated ATRP. *Journal of Macromolecular Science, Part A* **2009,** *46* (10), 989-996.

24. Hansson, S.; Östmark, E.; Carlmark, A.; Malmström, E., ARGET ATRP for versatile grafting of cellulose using various monomers. *ACS Applied Materials & Interfaces* **2009,** *1* (11), 2651-2659.

25. Bernand-Mantel, D.; Chehimi, M. M.; Millot, M. C.; Carbonnier, B., Protein-functionalized ultrathin glycidyl methacrylate polymer grafts on gold for the development of optical biosensors : An SPR investigation. *Surface and Interface Analysis* **2010,** *42* (6-7), 1035-1040.

26. Li, X.; Wang, M.; Wang, L.; Shi, X.; Xu, Y.; Song, B.; Chen, H., Block copolymer modified surfaces for conjugation of biomacromolecules with control of quantity and activity. *Langmuir* **2013,** *29* (4), 1122-1128.

27. Li, C. Y.; Xu, F. J.; Yang W. T., Simple strategy to functionalize polymeric substrates via surface-initiated ATRP for biomedical applications. *Langmuir* **2013,** *29* (5), 1541-1550.

28. Rautio, J.; Barken, B. K.; Lahdenperä, J.; Breitenstein, A.; Molin, S.; Neubauer, P., Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates. *Microbial Cell Factories* **2003,** *2* (1), 1-9.

29. Zhang, N.; Appella, D. H., Colorimetric detection of Anthrax DNA with a peptide nucleic acid sandwich-hybridization assay. *Journal of the American Chemical Society* **2007,** *129* (27), 8424-8425.

30. Henry, O. Y. F.; Kirwan, S.; Debela, A. M.; O'Sullivan, C. K., Electrochemical genosensor based on three-dimensional DNA polymer brushes monolayers. *Electrochemistry Communications* **2011,** *13* (11), 1155-1158.

31. Wang, Z.; Fan, Y.; Chen, J.; Guo, Y.; Wu, W.; He, Y.; Xu, L.; Fu, F., A microfluidic chip-based fluorescent biosensor for the sensitive and specific detection of label-free single-base mismatch via magnetic beads-based "sandwich" hybridization strategy. *Electrophoresis* **2013,** *34* (15), 2177-2184.

32. Halford, C.; Gau, V.; Churchill, B. M.; Haake, D. A., Bacterial detection & identification using electrochemical sensors. *Journal of Visualized Experiments* **2013,** (74), e4282, 1-8.

33. Suparpprom, C., Synthesis and nucleic acid binding properties of novel peptide nucleic acids carrying beta amino acid spacer. Doctoral disertation, Department of Chemistry, Faculty of Science, Chulalongkorn University, 2006.

34. Ananthawat, C., Development of DNA sensor based on peptide nucleic acid and surface plasmon resonance. Doctoral disertation, Program in Macromolecular Science, Faculty of Science, Chulalongkorn University, 2009.

35. Ferrell, P. B. Jr.; McLeod, H. L., Carbamazepine, HLA-B*1502 and risk of Stevens-Johnson syndrome and toxic epidermal necrolysis: US FDA recommendations. *Pharmacogenomics* **2008,** *9* (10), 1543-1546.

APPENDIX

VITA

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