การสังเคราะห์และซุปราโมเลคิวลาร์พอลิเมอไรเซชันของมอนอเมอร์ที่มีเพปไทด์นิวคลีอิก แอซิด

นางสาวรัตติยากร ดอนธงขวา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SYNTHESIS AND SUPRAMOLECULAR POLYMERIZATION OF PEPTIDE NUCLEIC ACID-CONTAINING MONOMER

Miss Ruttiyakorn Donthongkwa

A Thesis Submitted in Partial Fulfillment of the Requirements
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รัตติยากร ดอนธงขวา : การสังเคราะห์และซุปราโมเลคิวลาร์พอลิเมอไรเซชันของมอนอเมอร์ที่มีเพปไทด์นิวคลีอิกแอซิด (SYNTHESIS AND SUPRAMOLECULAR POLYMERIZATION OF PEPTIDE NUCLEIC ACID-CONTAINING MONOMER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.ธีรยุทธ วิไลวัลย์, 50 หน้า.

อันตรกริยาของนิวคลีโอเบสซึ่งเป็นหนึ่งในพันธะที่ไม่ใช่โควาเลนต์ที่เหนี่ยวนำให้เกิดการรวมตัวกันของโมเลกุลเกิดเป็นโครงสร้างที่น่าสนใจ พิร์โรลิดินิลเพปไทด์นิวคลีอิกแอซิดหรือพีเอ็นเอถูกพัฒนาขึ้นให้มีโครงสร้างเลียนแบบกรดนิวคลีอิกในธรรมชาติโดยมีโครงสร้างหลักเป็น (2′R,4′R)-prolyl/(1S,2S)-2-aminocyclopentanecarboxylic acid (acpcPNA) มีความสามารถในการเข้าคู่กับกรดนิวคลีอิกได้ตามกฎของวัตสัน-คริก นอกจากนี้ยังมีพีเอ็นเออีกชนิดหนึ่งที่ถูกพัฒนาขึ้นโดยมีความแตกต่างของสเตอริโอเคมีบนวงโพรลีนคือมีโครงสร้างหลักเป็น (2′R,4′S)-prolyl/(1S,2S)-2-aminocyclopentanecarboxylic acid (*epi*-acpcPNA) ที่มีความสามารถในการเข้าคู่กันเอง ในงานวิจัยนี้สังเคราะห์มอนอเมอร์ที่มี acpcPNA และ *epi*-acpcPNA โดยสองส่วนของ acpcPNA และ *epi*-acpcPNA ถูกเชื่อมเข้าด้วยกันผ่านมีตัวเชื่อมที่ยืดหยุ่นของพอลิเอทีลีนไกลคอลบนวัฏภาคของแข็ง ทำให้บริสุทธิ์ด้วยเทคนิคไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟีและพิสูจน์เอกลักษณ์ด้วยแมสสเปกโทรเมทรี โครงสร้างทุติยภูมิที่เกิดจากการเข้าคู่กันของมอนอเมอร์ที่มี acpcPNA และดีเอ็นเอคู่สมและโครงสร้างทุติยภูมิของมอนอเมอร์ที่มี *epi*-acpcPNA ที่เกิดการเข้าคู่กันของสาย *epi*-acpcPNA ยืนยันได้จากผลของอุณหภูมิหลอมเหลวและเทคนิคเซอร์คูลาไดครออิซึม อย่างไรก็ตามการเกิดไฮบริไดเซชันของ acpcPNA กับดีเอ็นเอคู่สมและการเข้าคู่กันของสาย *epi*-acpcPNA ไม่สามารถนำไปสู่ซุปราโมเลคิวลาร์พอลิเมอไรเซชันอย่างที่คาดไว้ซึ่งถูกพิสูจน์ด้วยเทคนิคกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดที่มีสมรรถนะสูงชนิดฟิลด์อีมิสชันและพอลิแอคริลาไมด์เจลอิเลคโตรโฟรีซีส

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RUTTIYAKORN DONTHONGKWA: SYNTHESIS AND SUPRAMOLECULAR POLYMERIZATION OF PEPTIDE NUCLEIC ACID-CONTAINING MONOMER. ADVISOR: ASSOC. PROF. VORAVEE HOVEN, Ph.D., CO-ADVISOR: PROF. TIRAYUT VILAIVAN, Ph.D., 50 pp.

Nucleobase-induced self-assembly interaction, have gained much attention as potential non-covalent driving force for generating interesting assembled structures. Pyrrolidinyl peptide nucleic acid (PNA), with (*2′R*,*4′R*)-prolyl/(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone (acpcPNA) has shown the recognition with complementary nucleic acids following Watson-Crick base rules. Another PNA system having different stereochemistry on proline ring with a backbone of (*2′R*,*4′S*)-prolyl/(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone (*epi*-acpcPNA) can undergo self-pairing. Here in this work, both acpcPNA- and *epi*-acpcPNA-functionalized monomers consisting of two PNA segments joined together by a flexible polyethylene glycol linker were prepared on solid support, purified by high performance liquid chromatography (HPLC) and characterized by MALDI-TOF mass spectrometry. The acpcPNA- and *epi*-acpcPNA-functionalized monomers showed the hybridization with complementary DNA and self-hybridization, respectively, as revealed by melting temperature and circular dichroism (CD) experiments. However, the hybridization of acpcPNA with complementary DNA and the self-hybridization of *epi*-acpcPNA did not lead to the proposed supramolecular polymerization as observed from analysis by Field emission scanning electron microscopy (FESEM) and polyacrylamide gel electrophoresis (PAGE).

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
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Ac_2O	: Acetic anhydride
acpcPNA	: Pyrrolidinyl peptide nucleic acid with (*2′R*,*4′R*)-prolyl/(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone
aegPNA	: Peptide nucleic acid with *N*-(2-aminoethyl)-glycine backbone
Calcd.	: Calculated
CD	: Circular dichroism
CuAAC	: Copper(I)-catalyzed alkyne-azide cycloaddition
DNA A16	: Deoxyribonucleic acid with base sequence of homoadenine 16 bases
DNA A8	: Deoxyribonucleic acid with base sequence of homoadenine 8 bases
DNA	: Deoxyribonucleic acid
epi-acpcPNA	:Pyrrolidinyl peptide nucleic acid with (*2′R*,*4′S*)-prolyl/(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone
FE-SEM	: Field emission scanning electron microscope
Fmoc	: Fluorenylmethyloxycarbonyl
FT-IR	: Fourier-transform infrared spectrscopy
HATU	:1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
^{1}H NMR	: Proton nuclear magnetic resonance
HPLC	: High performance liquid chromatography
MALDI-TOF	: Matrix assisted laser desorption ionization-time of flight

MeOH : Methanol

NaCl : Sodium chloride

OEG : Octa(ethylene glycol)

O-linker : Activated ethylene glycol linker

PAGE : Polyacrylamide gel electrophoresis

PEG : Polyethylene glycol

PNA : Peptide nucleic acid

SPPS : Solid phase peptide synthesis

TBE : Tris-borate-ethylenediaminetetraacetic acid

TBTA : Tris[(benzyl-1H-1,2,3-triazol-4-yl)methyl]amine

TEG : Tetra(ethylene glycol)

TEMED : Tetramethylene diamine

TFA : Trifluoroacetic acid

T_m : Melting temperature

Upy : 2-ureido-4[1H]-pyrimidinone

UV-vis : Ultraviolet–visible

Chapter I
Introduction

1.1 Introduction

The conventional polymerization is a process of tailoring monomeric units together via covalent bond to obtain a long chain polymer. On the other hand, supramolecular polymerization specially differs from the conventional polymerization in which the monomeric units are hold together by the intermolecular non-covalent interaction [1, 2] such as electrostatic, **π**-**π** stacking or hydrogen bonding interaction. These intermolecular non-covalent interactions can lead to self-assembly of molecules to obtain well-defined nanostructures with more complexity [3, 4] as can be seen from biomolecules such as secondary structures of proteins and self-assembly of nucleic acid via hydrogen bonding that can be recognized as natural supramolecular polymers. [5] Hydrogen bonding can respond to external stimuli, for instance, pH, temperature and light resulting in reversibility upon the presence and absence of external stimuli. In addition, H-bonding is moderately strong and directional so it is widely applied for preparing supramolecular polymer.[6]

In 1990, Lehn *et al.* have first defined the term "supramolecular chemistry" that was introduced to the formation of liquid crystalline supramolecular polymer comprising directional triple hydrogen bonds from recognition of diaminopyrimidine. [7] Later, Meijer's research group has reported synthesis of a quadruple hydrogen bonded molecule, called 2-ureido-4[1H]-pyrimidinone (UPy) of which structure was inspired from pyrimidine nucleobase. The architecture of UPy promotes the dimerization of itself via quadruple hydrogen bonding with a high dimerization constant. [8] In 2000, Folmer *et al.* [9] extended the chain of telechelic polymer via hydrogen bonding formed by UPy. Poly(ethylene/butylene) (PEB), molecular weight of 3.5 kDa was end-functionalized with UPy using diisocyanate molecule as a linker. The UPy-terminated PEB is literally an elastic solid meanwhile the OH-terminated PEB is a

viscous liquid (**Figure 1.1**). Dynamic oscillatory shear measurement revealed the viscoelastic behavior of UPy-terminated PEB which was not observed in OH-terminated UPy. Once UPy-terminated PEB was dissolved in chloroform, it provided a viscous solution. Upon an addition of a little amount of trifluoroacetic acid (TFA) which can break hydrogen bonding causing a substantially decrease in viscosity. This research study suggested that the UPy association can reinforce mechanical property and also provide functionalized polymer with reversibility.

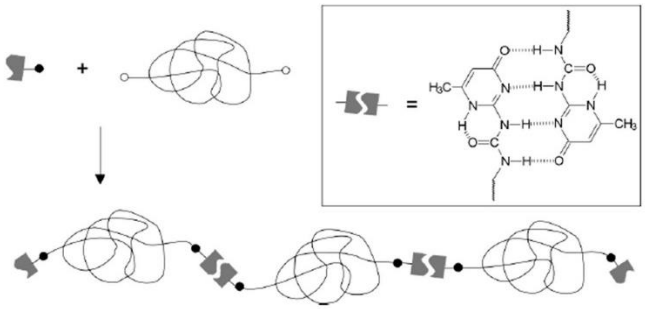

Figure 1.1. Schematic representation of supramolecular polymer formed by quadruple hydrogen-bonding of UPy-terminated PEB.[9]

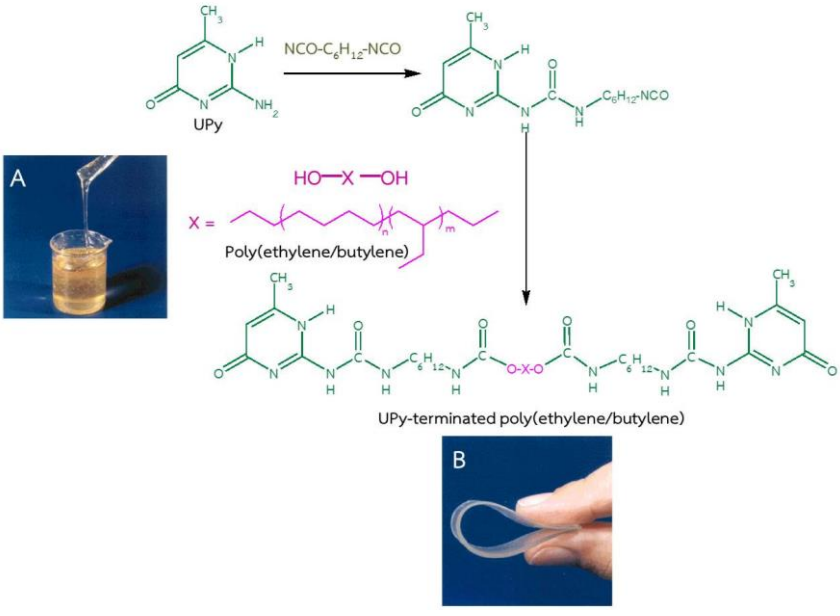

Figure 1.2 The synthetic pathway of UPy-functionalized PEB with the pictures of (A) OH-terminated PEB before functionalization, viscous liquid and (B) UPy-terminated PEB, elastic solid. [9]

The reversible dimerization of UPy groups can be utilized to prepare supramolecular pH-responsive hydrogel potentially applied in medical area. [10] In 2014, Bastings *et al.* produced the supramolecular hydrogel made from UPy units coupled with poly(ethylene glycol) via alkyl-urea spacers, molecular weight of 10 kDa [11]. This UPy hydrogel was promisingly applied in growth factor delivery for myocardial infarction treatment via catheter-injection method. The supramolecular UPy hydrogel was liquid at pH>8.5 with flow-able viscosity in vascular due to dominant enolate phenomenon of UPy group leading to disassociation of UPy groups and it could travel in a 1 m long catheter while the gelation rapidly occurred at neutral pH as can be seen from **Figure 1.3B** due to the dimerization of UPy groups. **Figure 1.3C** demonstrated that once the basic pH sample was injected into neutral pH solution, supramolecular hydrogel was formed as evidenced in red color. The rheology measurement indicated that the mechanical stiffness of UPy hydrogel (24 kPa) was close to mechanical stiffness of natural cardiac muscle (26 kPa) so this pH-responsive hydrogel is a promising material for catheter-injection.

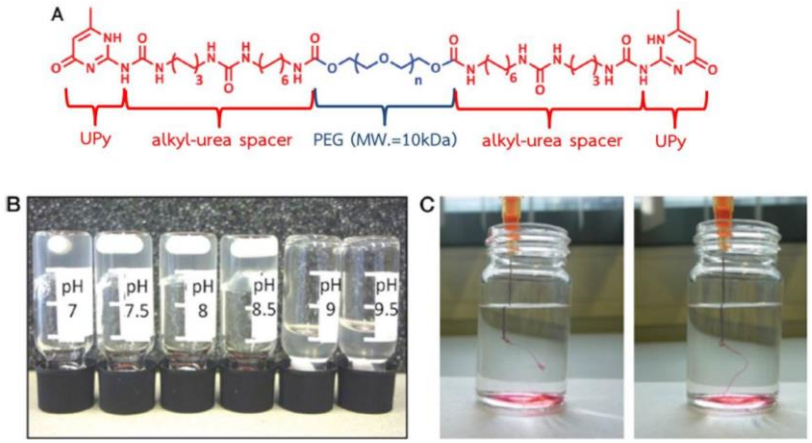

Figure 1.3 The chemical structure of UPy-functionalized poly(ethylene glycol) prepared to be used for the preparation of pH-responsive hydrogel (A), the picture of UPy-functionalized poly(ethylene glycol) in hydrogel state at pH 7-8 and liquid state at pH>8.5 (B), and the picture showing hydrogel formation when neutral pH solution of UPy-functionalized poly(ethylene glycol) being injected into basic solution.[11]

Originally, UPy unit was designed from imitating structure of natural pyrimidine nucleobase. The natural nucleobases have recognition ability following Watson-Crick rule in which adenine base (A) pairs with thymine base (T) through double hydrogen bonding and guanine base (G) pairs with cytosine base (C) through triple hydrogen bonding. [12] The recognition of nucleic acid leads the self-assembly of nucleotides resulting in various structure, for example, duplex, triplex or quadruplex structure. So the recognition of nucleotide can be used to program the desirable architecture of molecules. In 2013, Lin *et al.* investigated the assembly of uracil base-functionalized diblock copolymer, poly(**ε**-carpolactone-*block*-poly[1-(4-vinylbenzyl uracil)] (PCL-*b*-PVBU) induced by adenine-functionalized alkyl chain, 9-hexadecyladenine (AC16) [13], through the complementary interaction of uracil-adenine base pair, **Figure 1.4**. The first step, they synthesized poly(**ε**-caprolactone-*block*-poly[(4-vinylbenzyl)] (PCL-*b*-PVBA) via ring-opening polymerization of **ε**-caprolactone followed by nitroxide-mediated radical polymerization of vinylbenzyl chloride then PCL-*b*-PVBA was side chain-functionalized with uracil base through copper-catalyzed Huisgen 1,3-dipolar cycloaddition reactions. From TEM images, the pure PCL-*b*-PVBU showed lamellar structure with a long period of 35 – 45 nm resulting from **π**-**π** stacking interaction of uracil-uracil pairs. Blending AC16 with PCL-*b*-PVBU, the complementary adenine-uracil interaction was dominant over uracil-uracil pairs as can be evidenced by FTIR analysis in which the intensity of N-H stretching of free amino group decreased with elevated AC16 ratio. PCL-*b*-PVBU blended with AC16 20 wt% exhibited lamellar structure with a long period of 30 – 40 nm whereas PCL-*b*-PVBU blended with AC16 30 wt% displayed the hexagonal cylinder within lamellar structure. The different portion of AC16 affected adenine-uracil interaction and thus yielded different assembled structures.

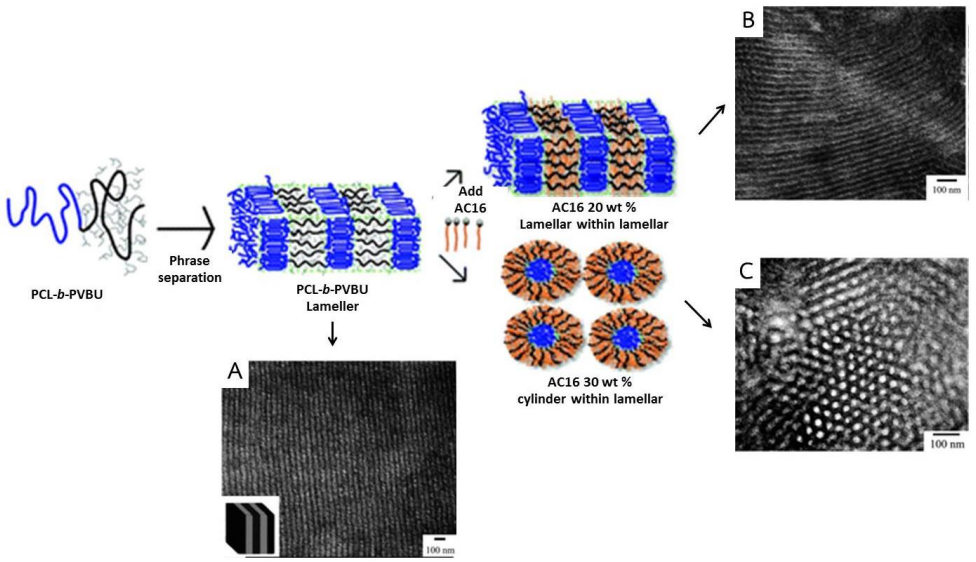

Figure 1.4 The graphic illustration presenting the assembly of PCL-b-PVBU blended with AC16 with TEM images of PCL-b-PVBU (A) before blending, (B) after blending with AC16 20 wt.%, and (C) after blending with AC16 30 wt.% .[13]

Moreover, the self-assembly of natural nucleotides guides the preparation of the impromptu hydrogel consisting of DNA and the complementary DNA. [14] The polymer chains containing DNA were cross-linked through the hybridization of DNA on polymer chain and DNA cross-linker. In 2012, Chen *et al.* fabricated polymer brushes of poly(**γ**-propargyl-L-glutamte) (PPLG) grafted by DNA (PPLG-g-DNA) prepared via copper(I) catalyzed Huisgen [3 + 2] cylcoaddition click reaction (**Figure 1.5**). [15] The formation of hydrogel was performed by adding the double stranded DNA (dsDNA) solution into PPLG-g-DNA solution. The PPLG-*g*-DNA solution suddenly changed from fluid solution to hydrogel. The oscillatory shear rheology experiment showed the storage modulus (G') was greater than the loss modulus (G") in every frequency implying the hydrogel state.

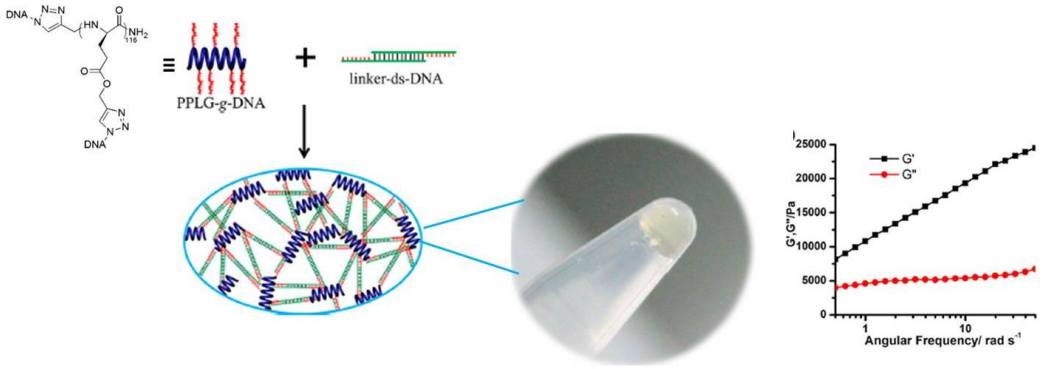

Figure 1.5 The graphic illustration of PPLG-g-DNA cross-linked by DNA cross-linker giving transparent hydrogel with the graph plotted between G', G" and angular frequency from Oscillatory rheological frequency sweep experiments indicating hydrogel state. [15]

Inspired by natural nucleotides, scientists developed the synthetic compound called peptide nucleic acid (PNA) which the backbone imitates the nucleotide analogue in which phosphate group and sugar group are replaced by pseudopeptide backbone. The first PNA named aegPNA was introduced by Nielsen and coworker in 1991. [16, 17] The aegPNA is composed of *N*-(2-aminoethyl)-glycine units repeatedly linked by peptide bonds (**Figure 1.6**). The aegPNA has a greater binding affinity with DNA since there is no effect from negatively charged repulsion of phosphate groups in the structure. [18, 19] Resulting from the polypeptide structure, aeg PNA is durable toward nuclease and proteinase. [20] Because the aeg PNA structure is flexible, it can bind with DNA both in parallel and anti-parallel direction. The synthetic PNA can also be functionalized with a variety of functional groups so that it may be applicable for desirable specific applications.

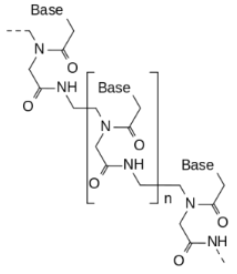

Figure 1.6 The structure of aegPNA. [16]

In 2015, Chu *et al*. fabricated a new hydrogel from copolymer conjugated with aegPNA. (M_n = 95 -110 kDa) prepared from reversible addition–fragmentation chain transfer (RAFT) polymerization of polymerizable aegPNA (having methacrylate group to be polymerized) and poly[N-(2-hydroxypropyl)methacrylamide] (polyHPMA).[21] The copolymer conjugated with aegPNA was cross-linked to form network structure via hybridization of aegPNA and complementary DNA (cross-linker). They designed the hybridization patterns into 2 types. The first type, aegPNA hybridized with complementary DNA in duplex form and the second type, aegPNA hybridized with complementary DNA in triplex form. Hydrogels could be formed from both of duplex and triplex patterns with the difference in which triplex pattern gave the faster gelation than duplex pattern owing to the stronger binding. Moreover, the speed of gelation relied on the number of aegPNA units on copolymer chain and concentration of copolymer (3 and 5 wt.%), more aegPNA units or more concentration provide faster gelation. From scanning emission microscope (SEM) images, the hydrogel from triplex hybridization has a denser structure with pore size of 2.4-2.9 μm (**Figure 1.7B**) while double helix pattern gave a structure with pore size of 85 μm. This may be explained as a result of more rigid cross-linking from triplex pattern would yield the stronger hybridization with denser gel structure. This novel aegPNA/DNA based hydrogel is promising to be applied as gene carrier for biomedical application.

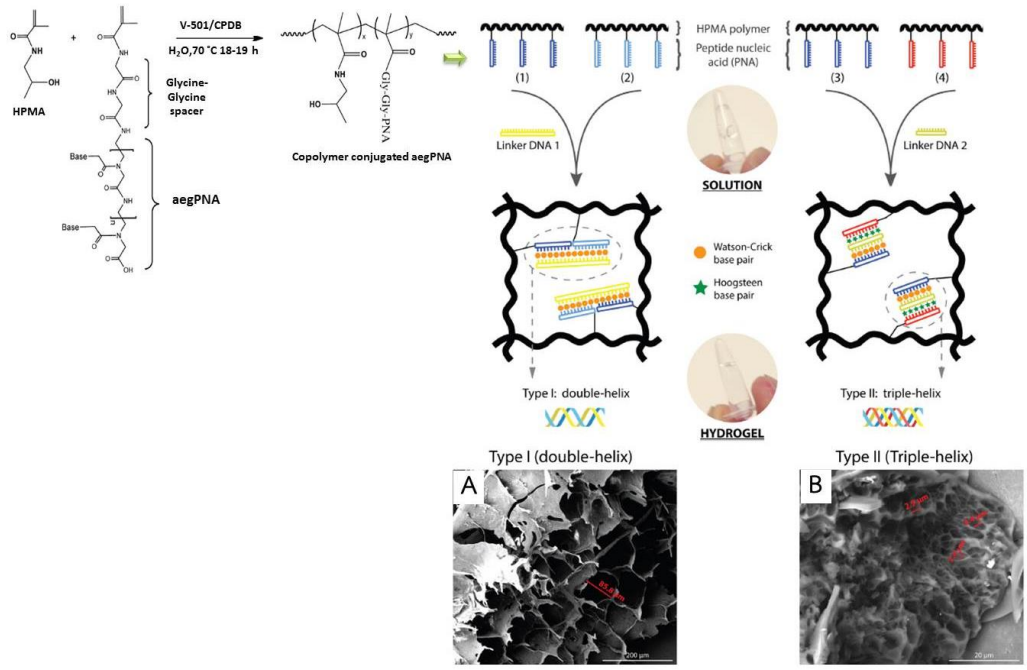

Figure 1.7 Chemical structures of copolymer conjugated with aegPNA and its hydrogel formation through PNA-DNA hybridization and SEM images of (A) hydrogel from double helix hybridization and (B) hydrogel from triplex hybridization. [21]

In 2001, Vilaivan and coworkers have developed a new pyrrolidinyl PNA [22] which has a backbone of (2'*R*,4'*R*)-nucleobase-substituted-proline alternating with (1*S*,2*S*)-2-aminocyclopentanecarboxylic acid or (2'*R*,4'*R*)-prolyl/(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone (acpcPNA). The acpcPNA has a stronger binding affinity with DNA and RNA because of its neutrally charged structure. The outstanding trait of acpcPNA over aegPNA is that acpcPNA only binds with DNA in anti-parallel direction whereas aegPNA binds with DNA in either anti-parallel or parallel direction. [23] Notably, acpcPNA has more specific binding ability because of the constrained structure allowing the arrangement of binding only in anti-parallel direction.

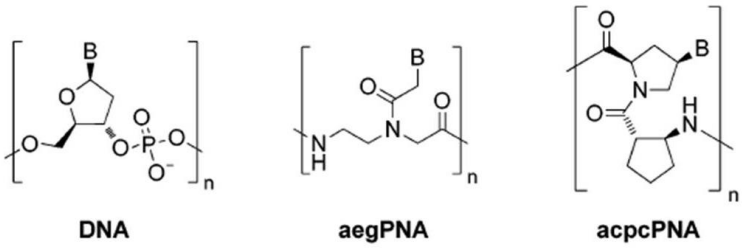

Figure 1.8 Chemical structures of DNA, aegPNA and acpcPNA. [24]

In 2006, Vilaivan and coworkers have also reported about another PNA variant of which the stereochemistry of proline ring on the original acpcPNA was changed from (*2′R,4′R*) to (*2′R,4′S*). This new PNA system was named *epi*-acpcPNA. [25] The *epi*-acpcPNA can also bind with DNA with slightly less binding affinity than acpcPNA but still stronger than natural nucleotides and more strongly bind with RNA than acpcPNA. The remarkable difference between acpcPNA and *epi*-acpcPNA is self-hybridization of *epi*-acpcPNA in anti-parallel pattern due to the proper torsional angle of the structure encouraging self-hybridization. However, *epi*-acpcPNA still prefers binding with DNA over itself.

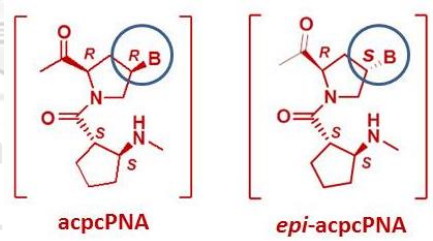

Figure 1.9 Chemical structures of acpcPNA (left) and *epi*-acpcPNA (right). [24]

Taking advantage of the powerful hybridization property of acpcPNA with DNA and self-hybridization of *epi*-acpcPNA, this research would like to explore a possibility to generate assembled structure by supramolecular polymerization of monomers functionalized by acpcPNA and *epi*-acpcPNA. Supramolecular polymerization are conducted by either hybridization of acpcPNA functionalized monomer with complementary DNA or self-hybridization of *epi*-acpcPNA functionalized monomer. It is anticipated that this developed *epi*-acpcPNA and acpcPNA- functionalized

monomers can be used as building blocks of novel self-assembled structures that can be regulated by temperature control or by the presence/absence of a DNA template.

1.2 Objective

1) To synthesize and characterize acpcPNA-functionalized and *epi*-acpcPNA-functionalized monomers

2) To determine hybridization property of acpcPNA-functionalized monomer with complementary DNA and self-hybridization of *epi*-acpcPNA-functionalized monomer

1.3 Scope of investigation

1) Synthesis and characterization of poly(ethylene glycol)-di(alkyne)

2) Synthesis and characterization of acpcPNA and *epi*-acpcPNA via solid phase peptide synthesis (SPPS) method

3) Synthesis and characterization of azide-functionalized acpcPNA and *epi*-acpcPNA

4) Conjugation of poly(ethylene glycol)-di(alkyne) with azide-acpcPNA and azide-*epi*-acpcPNA via copper(I) catalyzed alkyne-azide cycloaddition on solid support

5) Study of hybridization property of acpcPNA-functionalized monomer and complementary DNA by melting temperature determination and circular dichroism (CD) technique

6) Study of self-hybridization property of *epi*-acpcPNA-functionalized monomer by melting temperature determination and CD technique

7) Study of supramolecular polymerization of acpcPNA-functionalized monomer hybridized with complementary DNA using field emission scanning microscopy (FESEM) and polyacrylamide gel electrophoresis (PAGE)

CHAPTER II
EXPERIMENTAL

2.1 Materials

All solvents used for reactions are analytical grade and used as received, unless otherwise specified. Solutions were made with Milli-Q water purified by Millipore Milli-Q water system that involves reverse osmosis, ion exchange, and filtration steps. Tetra(ethylene glycol), octa(ethylene glycol), propargyl bromide, sodium hydride, tetrakis(acetonitrile) copper(I) hexafluorophosphate, tris[(benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), (+)-sodium-L-ascorbate were purchased from Sigma-Aldrich. Oligonucleotides were purchased from Pacific science, Ltd (Thailand). The monomers for acpcPNA and *epi*-acpcPNA synthesis were synthesized in laboratory of Prof. Tirayuth Vilaivan research group, Department of Chemistry, Chulalongkorn University, Thailand.

2.2 Characterization

Structures of tetra(ethylene glycol)-di(alkyne) (TEG-di(alkyne)) and octa(ethylene glycol)-di(alkyne) (OEG-di(alkyne)) were confirmed by ^{1}H NMR recorded on Varian NMR spectrometer (400 MHz), model Mercury-400 nuclear magnetic resonance spectrometer (USA). FT-IR spectra of TEG-di(alkyne) and OEG-di(alkyne) were recorded by FT-IR spectrometer (Perkin Elmer), model system 2000. Melting temperature (T_m) of functionalized monomers were operated on CARY 100 Bio UV-Vis spectrophotometer (Varian, Inc., USA). Circular dichroism spectra of functionalized monomers were recorded on Jasco J-815 spectropolarimeter (JASCO, Jpan). Morphological study of assembled functionalized monomers were studied on JSM-7610F Schottky Field Emission Scanning Electron Microscope (JEOL Ltd, Japan) using substrates of platinum-coated silicon wafers. Study of higher molecular weight of

functionalized monomers resulting from supramolecular polymerization was observed on OmniPAGE mini vertical systems (Cleaver Scientific Ltd, UK) equipped with nanoPAC-300 mini power supply (Cleaver Scientific Ltd, UK) and observed under UV transilluminators (Vilber Lourmat, France).

2.3 Synthesis of poly(ethylene glycol)-di(alkyne)

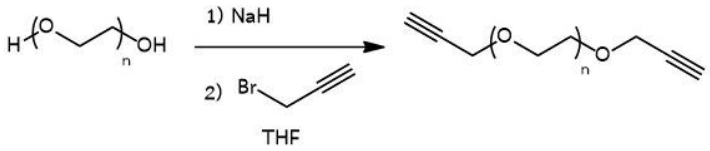

Figure 2.1 Reaction scheme for the synthesis of poly(ethylene glycol)-di(alkyne) when n = 4 is tetra(ethylene glycol) (TEG) and n = 8 is octa(ethylene glycol) (OEG).

TEG or OEG (3 mmol, 1 equiv.) and 65 % sodium hydride dispersed in mineral oil (0.42 g, 4 equiv.) were dissolved in anhydrous THF. The reaction proceeded under N_2 atmosphere at room temperature for 1 hour before was cooled down with ice bath and added with propargyl bromide (6 mmol, 2 equiv.). The reaction was stirred overnight. After that, THF was removed by evaporation. The mixture was dissolved in dichloromethane and washed three times with saturated ammonium chloride. The mixture was purified by flash column chromatography using solvent system of 3:1 (v/v) of ethylacetate:hexane. After evaporation, the yellow oily products were obtained (44% yield of TEG-di(alkyne) and 47% yield of OEG-di(alkyne)). ^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm = 2.4 (s, 1H), 3.6-3.75 (m, 4H) and 4.3 (s, 2H). FT-IR (Sodium chloride crystal window, 32 scans): wave number/cm^{-1} = 2111 (C≡C stretching) and 3256 (sp C—H stretching).

2.4 Synthesis of acpcPNA and *epi*-acpcPNA functionalized monomer

i) Synthesis of acpcPNA and *epi*-acpcPNA oligomers

In this work, acpcPNA and *epi*-acpcPNA oligomers were manually synthesized using solid-phase peptide synthesis (SPPS) strategy complied with general protocol [25] The syntheses were carried out on TenTaGel S RAM resin (Fmoc protection) in the scale of 1.5 μmol. and performed through a cycle of SPPS composed of 3 steps using 3 stock solutions below.

Stock solution#1: 20% piperidine and 2% DBU in DMF was prepared from piperidine 200 μL, DBU 20 μL, and DMF 780 μL.

Stock solution #2: 7% DIEA in DMF was prepared from DIEA 70 μL and DMF 930 μL.

Stock solution #3: 0.4 M HOAt in DMF was prepared from HOAt 5.5 mg dissolved in DMF 100 μL.

The first step of the SPPS cycle is Fmoc deprotection by using stock solution#1 (100μL). The second step is coupling with activated monomer or spacer. For the activated monomer having COOpfp group (base A, T, C and spacer), it was mixed with stock solution #2 (15μL) and stock solution#3 (15μL). For monomer having COOH group (base G, lysine), They were mixed with solution#2 (30 μL) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxide hexafluoro phosphate (HATU) (2.2 mg, 5 μmol) prior to the coupling. The third step is capping by using stock solution#2 (30μL) mixed with acetic anhydride (Ac_2O) (5 μL) to deactivate uncoupled monomers competitively reacting with the next activated monomer. Repeat the cycles until the desired acpcPNA and *epi*-acpcPNA oligomers were obtained.

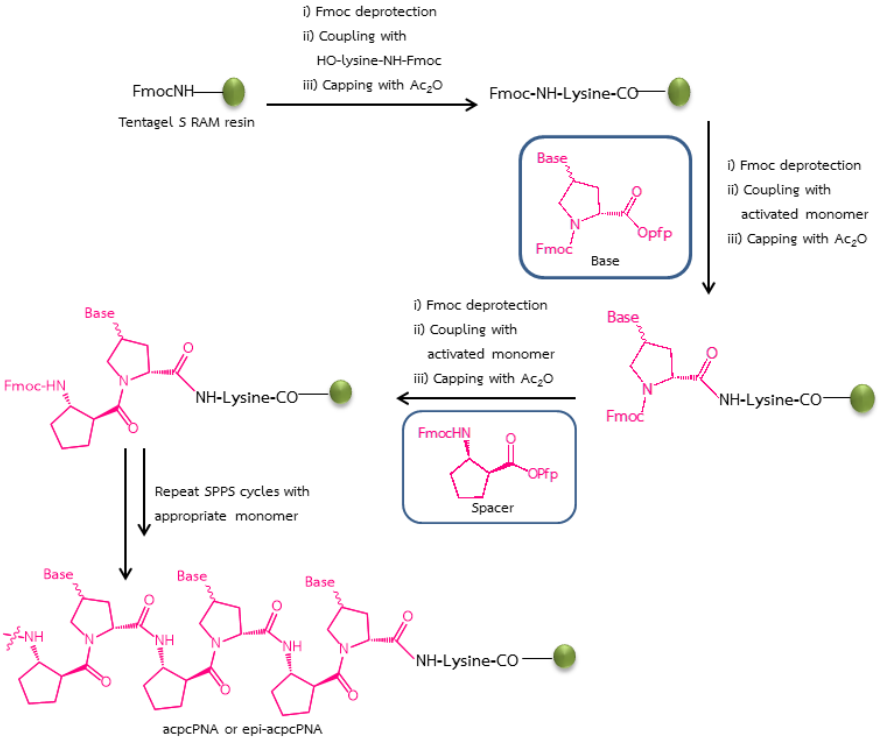

Figure 2.2 Reaction scheme for the synthesis of acpcPNA and *epi*-acpcPNA monomers.

ii) Functionalization of acpcPNA and *epi*-acpcPNA oligomers with azido groups

The acpcPNA and *epi*-acpcPNA oligomers were modified to incorporate azido groups at N-termini for subsequent CuAAC click reaction. The desired acpcPNA and *epi*-acpcPNA oligomers anchored to resin were subjected to Fmoc removal by using stock solution#1 (100 µL) then the reductive alkylation reaction was performed by immersing in the solution containing 4-azidobutanal (15 µmol, 1 equiv.), acetic acid (30 µmol, 2 equiv.) and sodium cyanoborohydride (28 µmol, 1.8 equiv.) dissolved in methanol. The reaction was kept at room temperature overnight followed by washing with MeOH (100 µL) and dried by N_2 gas flowing.

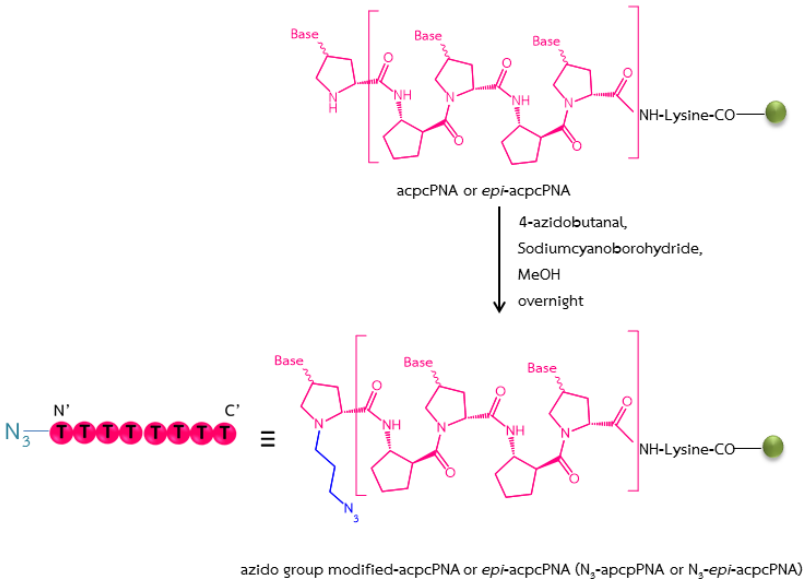

Figure 2.3 Reaction scheme for functionalization of acpcPNA and *epi*-acpcPNA oligomers with azido groups.

iii) Synthesis of acpcPNA and *epi*-acpcPNA functionalized monomer

The functionalization of monomers was performed on the solid support used for the PNA synthesis. Two types of acpcPNA-functionalized monomers having different orientation of acpcPNA were synthesized. For **type 1**, at both ends of the monomer have acpcPNA connected in the "head-to-head" direction (C---N-monomer-N---C). For **type 2**, at both ends of the monomer have acpcPNA connected in the "head-to-tail" direction (C---N-monomer-C---N). The two acpcPNA-functionalized monomers were prepared using different pathways. The **type 1** acpcPNA-functionalized monomers were prepared by Cu(I)AAC click reaction of N_3-acpcPNA on the solid support and TEG-di(alkyne) or OEG-di(alkyne) in the presence of tetrakis(acetonitrile) copper(I) hexafluorophosphate (4 μmol, 8 equiv), tris[(benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 20 μmol, 40 equiv), (+)-sodium-L-ascorbate (8 μmol, 16 equiv) in 3:1 (v/v) DMSO/t-BuOH at room temperature for 4 hours. The **type 2** acpcPNA-functionalized monomers were prepared via SPPS strategy of which the desired acpcPNA was first synthesized then the three activated monomers of ethylene glycol linker (O-linker) were coupled to the N-terminus of synthesized acpcPNA using stock solution#2 mixed

with HATU. This was followed by synthesizing the second segment of acpcPNA with the same base sequence.

For *epi*-acpcPNA functionalized monomer, the direction of *epi*-acpcPNA to monomer was “head-to-head” (C---N-monomer-N---C), there for the synthesis was the same as that of the **type 1** acpcPNA-functionalized monomer. The functionalization was formed through Cu(I)AAC click reaction of N_3-*epi*-acpcPNA and TEG-di(alkyne) under the same conditions. After the reaction was complete, the acpcPNA and *epi*-acpcPNA functionalized monomers were cleaved from the resin with trifluoroacetic acid (500 µL × 30 min × 3), dried with N_2 gas flowing and washed with diethyl ether.

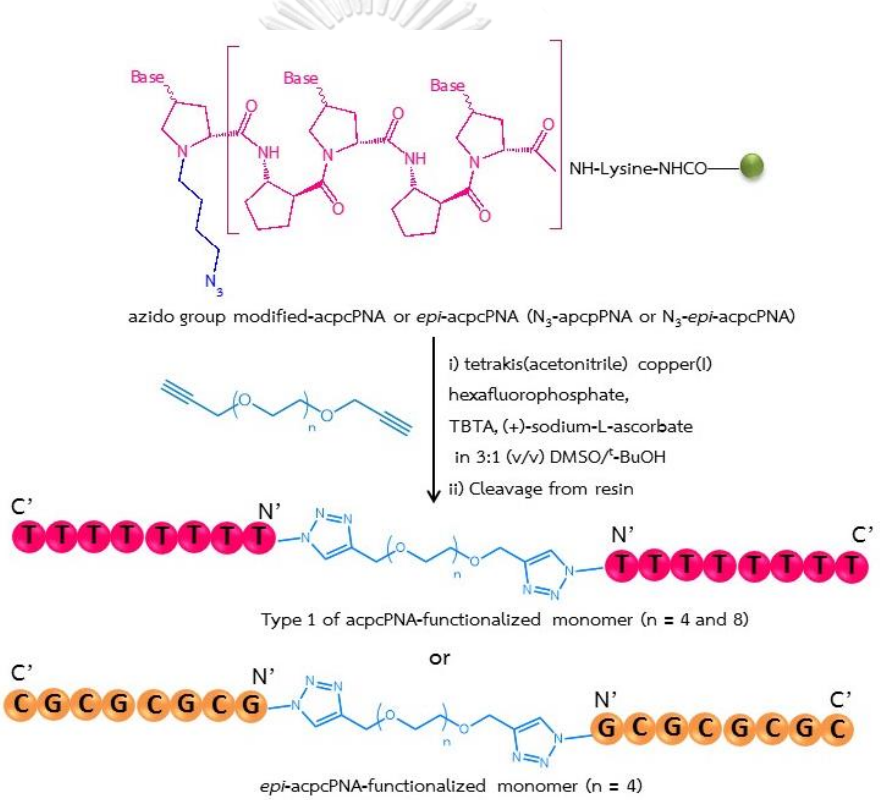

Figure 2.4. Reaction scheme for the synthesis of type 1 acpcPNA-functionalized monomer and *epi*- acpcPNA-functionalized monomer

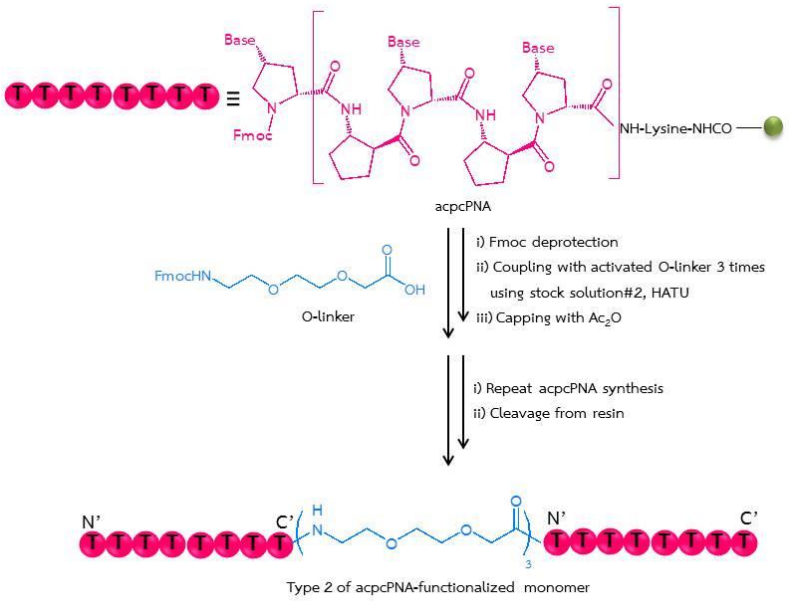

Figure 2.5. Reaction scheme for the synthesis of type 2 acpcPNA-functionalized monomer

iv) Purification and Characterization

The crude of acpcPNA or *epi*-acpcPNA functionalized monomers were dissolved in 10% methanol in Milli Q water. The crude solution was filtered through a nylon membrane filter (0.45 μm) then centrifuged for 3 times to precipitate the impure precipitants. The crude was purified by reverse phase HPLC with gradient system. There are two HPLC gradient solvent systems, solvent A (0.1% trifluoroacetic acid in acetonitrile or methanol) and solvent B (0.1% TFA in MilliQ water). The ratio of solvent A:B for eluting constantly changed from 10:90 to 90:10 over a period of 60 minutes. The fractions obtained after purification were characterized by Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The concentration of products and complementary DNA were determined by CARY 100 Bio UV-Vis spectrophotometer (Varian, USA) by determination the absorbance at wavelength of 260 nm using **ε** = 140.8 $mL.umol^{-1}.cm^{-1}$ for acpcPNA with base sequence of homothymine 16 mers and

ε = 151.2 mL.umol^{-1}.cm^{-1} for *epi*-acpcPNA with base sequence of alternating GC 16 mers obtained from http://www.chemistry.sc.chula.ac.th/pna/pna.asp.

2.5 Hybridization and self-hybridization property study of acpcPNA-functionalized monomer and *epi*-acpcPNA-functionalized monomer.

i) Melting temperature (T_m) measurement

The melting temperature (T_m) experiments of functionalized monomers were operated on CARY 100 Bio UV-Vis spectrophotometer (Varian, USA), equipped with a thermal melt system, 20 – 90 °C using a heating rate of 1 °C/min and measuring absorbance at the wavelength of 260 nm. For the hybridization of acpcPNA-functionalized monomer (**type 1** or **type 2**) and complementary DNA, the final concentration of the sample was 2 μM including 1 μM acpcPNA-functionalized monomer and 1 μM complementary DNA in the presence of 10 mM phosphate buffer and 100 mM sodium chloride. For *epi*-acpcPNA-functionalized monomer, the 2 μM *epi*-acpcPNA-functionalized monomer was used to determine the self-hybridization in the presence of 10 mM phosphate buffer and 100 mM sodium chloride. The final volume of all samples was 1 mL. The T_m was determined from the slope of melting curve plotted by KaliedaGraph 4.5 (Synergy Software) and Microsoft Excel 2010.

ii) Circular dichroism (CD) experiment

The secondary structures from self-hybridrized or DNA-hybridized functionalized monomer were investigated on Jasco J-815 spectropolarimeter (JASCO, Jpan). The experiments were performed in a wavelength range of 200 – 400 nm. For acpcPNA-functionalized monomers (**type 1** or **type 2**), the experiments were performed at 25°C under the same conditions as T_m measurement, using 1 μM acpcPNA-functionalized monomer and 1 μM complementary DNA. For *epi*-acpcPNA-functionalized monomer, the self-hybridization of 2 μM *epi*-acpcPNA-functionalized

monomer was determined from 20 to 90 °C with a heating rate of 2°C/min to observe its secondary structure when temperature was changed. The final volume of all samples was 1 mL in the presence of 10 mM phosphate buffer and 100 mM sodium chloride. The CD spectra were plotted by using Microsoft Excel 2010.

2.6 Supramolecular polymerization study of acpcPNA-functionalized monomer via hybridization with complementary DNA

i) Field Emission Scanning Electron Microscope (FESEM)

The topology of acpcPNA-functionalized monomers (**only type 1**) and complementary DNA before and after hybridization were visualized on JSM-7610F Schottky Field Emission Scanning Electron Microscope (JEOL Ltd, Japan). The solution of 10 μM acpcPNA-functionalized monomers and 10 μM complementary DNA were mixed in Milli Q water (final volume of 30 μL) in the absence of sodium phosphate buffer and sodium chloride in order to avoid salt formation during the experiments. The sample mixture was dropped on a silicon wafer (size of 1X1 cm^2) and then dried by air flowing and kept in desiccator for one night. The samples were analyzed using magnification of 5000X and 10000X.

II) Polyacrylamide Gel Electrophoresis (PAGE)

The polyacrylamide gel with a size of 4x4 $inch^2$ was prepared from 5 mL acrylamide monomer (40% w/v dissolved in deionized water), 10 mg ammonium persulfate (APS), 10 μL tetramethylethylenediamine (TEMED, a catalyst for acrylamide gel polymerization), 1 mL 1X tris-borate-EDTA (TBE) buffer and Milli Q water 3 mL. After all the components were mixed, it was loaded into the electrophoresis cell then the comb was inserted to make wells for sample loading and waited until the polyacrylamide gel harden. The samples were prepared form mixing 1 nmol acpcPNA-functionalized monomer (**type 1** or **type 2**), 1 nmol complementary DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) or 2 nmol complementary DNA A8 (ratio of acpcPNA:DNA A8

= 1:1) and 1 µL ethidium bromide (for tagging nucleotide) in 1X tris-borate-EDTA (TBE) buffer (final volume of 10 µL). The samples were then loaded into wells of polyacrylamide gel soaked in 1XTBE buffer. The samples traveled form anode electrode to cathode electrode using nanoPAC-300 mini power supply with voltage of 90 volt for 3 hours then the sample bands were observed by UV shadowing over silica gel GF254 TLC plate on the UV transilluminators at 365 nm (Vilber Lourmat, France).

CHAPTER III
RESULTS AND DISCUSSION

3.1 Synthesis of poly(ethylene glycol)-di(alkyne)

Poly(ethylene glycol) (PEG) was selected as hydrophilic linker for the synthesis of PNA-functionalized monomer because its chemistry can be easily handled in aqueous media so thus compatible with the PNA molecules. Moreover, it has been widely recognized that PEG with lower molecular weight shows good biocompatibility [26] hence it could be used in biological or medical applications. In this research, two di(alkyne)-terminated PEG derivatives having different chain length, TEG-di(alkyne) and OEG-di(alkyne) were synthesized via nucleophilic substitution of propargyl bromide with hydroxyl-terminated TEG or OEG. The products were characterized by ^{1}H NMR analysis. The ^{1}H NMR spectra (**Figure 3.1(C) and (E)**) revealed the characteristic peaks of TEG-di(alkyne) and OEG-di(alkyne) at 2.4, 3.6-3.7, and 4.3 ppm which can be assigned to $\underline{H}C{\equiv}C$—of alkyne, —$C\underline{H}_2C\underline{H}_2O$ of TEG or OEG and ${\equiv}C$—$C\underline{H}_2O$—, respectively. Additionally, The functional group of the products were confirmed by FT-IR spectra (**Figure 3.2 (A) and (B)**) which showed the peaks at 2111 and 3256 cm^{-1} representing characteristics of C≡C stretching and sp C—H stretching, respectively.

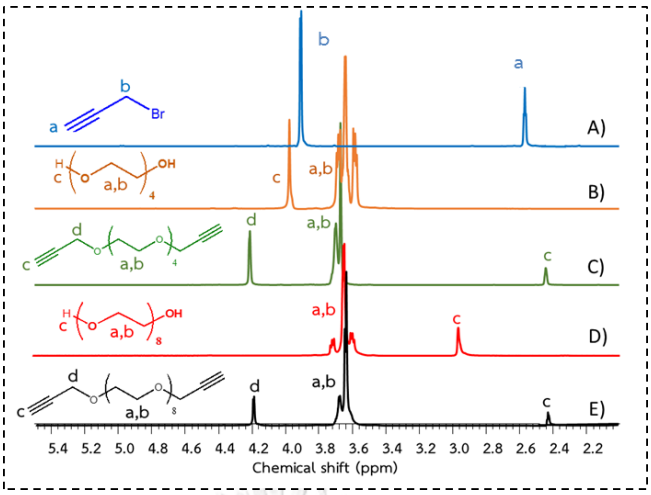

Figure 3.1 ^{1}H NMR spectra of (A) propargyl bromide, (B) tetra(ethylene glycol), (C) tetra(ethylene glycol)-di(alkyne), (D) octa(ethylene glycol), and (E) octa(ethylene glycol)-di(alkyne).

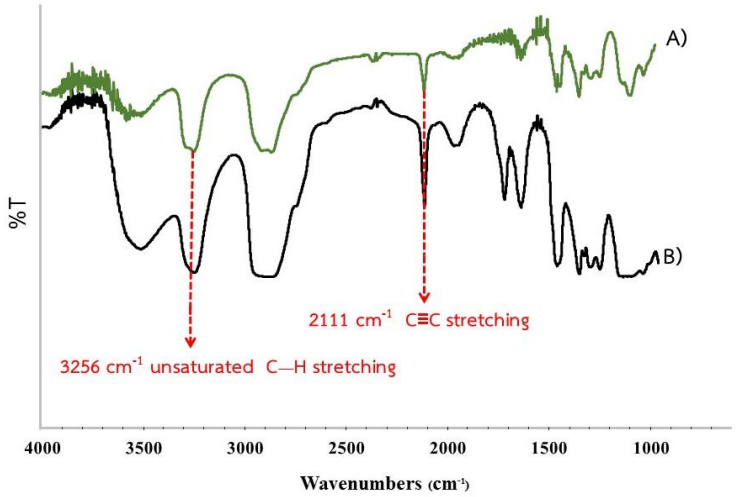

Figure 3.2 FT-IR spectra of (A) tetra(ethylene glycol)-di(alkyne) and (B) octa(ethylene glycol)-di(alkyne).

3.2 Synthesis and characterization of acpcPNA and *epi*-acpcPNA functionalized monomers

The acpcPNA with a sequence of homothymine 8 mers (N-TTT TTT TT-C) and *epi*-acpcPNA with sequence of alternating guanine and cytosine 8 mers (N-GCG CGC GC-C), a sequence which can form a self-hybridization, were manually synthesized via

solid phase peptide synthesis (SPPS) which is a general method for synthesizing peptides. The acpcPNA and *epi*-acpcPNA were synthesized from C terminus to N terminus by coupling the monomers in alternation with the 2-aminocyclopentanecarboxylic acid spacer. FmocLysine(Mtt)OH was firstly attached to the resins to improve aqueous solubility of the PNA. Other activated monomers and spacers were subsequently added with respect to the desired base sequence. The successful coupling of each activated monomer can be primarily checked from stock solution#1 after Fmoc deprotection since Fmoc group contains fluorenyl group with strong UV adsorption so its presence can be monitored by thin layer chromatography. When the desired acpcPNA and *epi*-acpcPNA were obtained, the side chain protecting groups (Ibu or Bz) were cleaved by immersing in 1:1 dioxane:concentrated ammonia at 65 °C for 15 hours. After that, acpcPNA or *epi*-acpcPNA were functionalized with azido group at N-termini via reductive alkylation with 4-azidobutanal in the presence of $NaBH_3CN$ as reducing agent.

The type 1 acpcPNA-functionalized monomer and *epi*-acpcPNA-functionalized monomer which have a head-to-head direction of the two PNA strands as C---N-monomer-N---C were prepared on Tentagel S RAM resin via CuAAC click reaction. This is a cycloaddition of azide and alkyne compounds selectively forming 1,4-disubstituted triazole groups. Typically, the CuAAC click reaction of symmetric di(alkyne) compounds and azide compounds bound to resins cannot be done at one time. In other words, one alkyne end has to be connected first followed by the other alkyne. In contrast to this belief, in this research, we found that two alkyne end groups can be simultaneously connected. The type 2 acpcPNA-functionalized monomer was synthesized using different procedure. Three molecules of O-linker considered as units of poly(ethylene glycol) were continually coupled to N-terminus of acpcPNA by performing the amide coupling on RAM resin then followed by synthesizing acpcPNA with the same sequence. According to this procedure, the direction of two acpcPNA parts is the same chain (C---N-monomer-C---N). All functionalized monomers were purified by HPLC and characterized by MALDI-TOF mass spectrometry. The observed masses of both precursor molecules (**Table 3.1**) and functionalized monomers (**Table 3.2**) were close to the calculated masses confirming the identity of the expected

products were obtained. The HPLC traces and mass spectra of the functionalized monomers are shown in **Figure 3.3** and **3.4**, respectively.

Table 3.1 Molecular weight of poly(ethylene glycol)-di(alkyne), acpcPNA and *epi*-acpcPNA

Sample	Sequence (N to C)	m/z (calculated)	m/z (found)
TEG-di(alkyne)	-	270.0	270.0
OEG-di(alkyne)	-	446.0	446.0
acpcPNA	TTT TTT TT	2693.9	2691.3
epi-acpcPNA	GCG CGC GC	2733.9	2729.8
N_3- acpcPNA	TTT TTT TT	2791.9	2791.0
N_3- *epi*-acpcPNA	GCG CGC GC	2831.9	2831.9

Table 3.2 Molecular weight of type 1 and type 2 acpcPNA-functionalized monomers and *epi*-acpcPNA functionalized monomer

Functionalized monomer	m/z (calculated)	m/z (found)
Type 1 acpcPNA-functionalized TEG	5853.8	5853.5
Type 1 acpcPNA-functionalized OEG	6029.8	6033.9
Type 2 acpcPNA-functionalized monomer	6069.5	6072.4
epi-acpcPNA-functionalized TEG	5933.8	5491.2

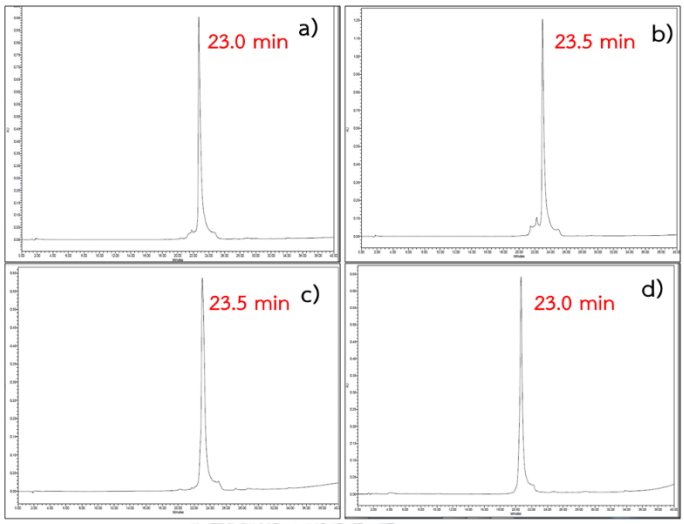

Figure 3.3 Reverse phase HLPC spectra of (a) type 1 acpcPNA-functionalized-TEG, (b) type 1 acpcPNA-functionalized-OEG, (c) type 2 acpcPNA-functionalized monomer, and (d) *epi*-acpcPNA-functionalized monomer.

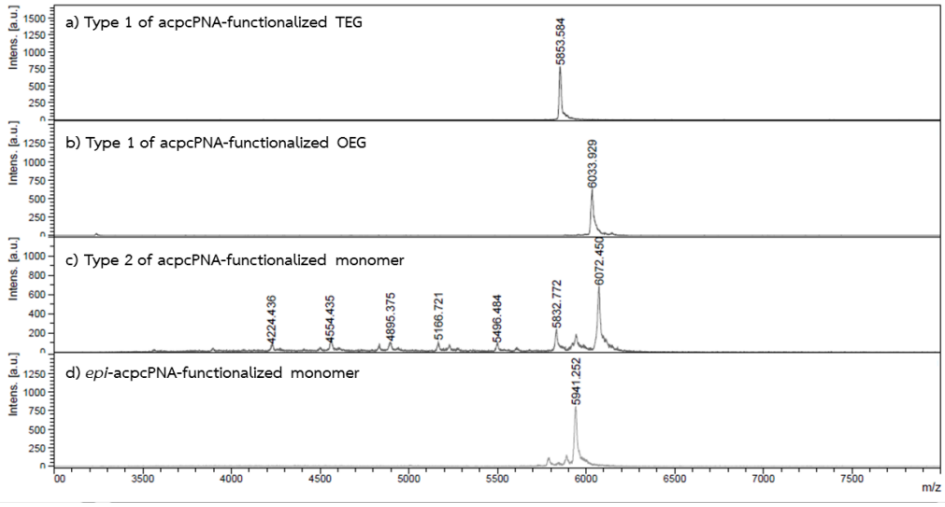

Figure 3.4 Mass spectra of (a) type 1 acpcPNA-functionalized-TEG, (b) type 1 acpcPNA-functionalized-OEG, (c) type 2 acpcPNA-functionalized monomer, and (d) *epi*-acpcPNA-functionalized monomer.

Most functionalized monomers gave the expected mass (MALDI-TOF) as a single peak except type 2 acpcPNA-functionalized monomer (**Figure 3.4**) which showed some by-products left which were incompletely coupled PNA chains even after second HPLC purification. Nevertheless, it could be still acceptable to be further used since the

expected peak was the major one. All functionalized monomers were lyophilized then dissolved in Miili Q water to be kept as stock solutions. Their concentrations were determined via UV-vis spectrophotometer by measuring the absorbance at wavelength of 260 nm in which oligonucleotides absorb UV light. The concentration of functionalized monomers can be calculated from optical density (OD) found using the equation below.

$$OD = Absorbance\ at\ 260\ nm\ \times dilution\ factor \qquad \textbf{Eq. 3.1}$$

Where dilution factor = total volume ÷ volume of PNA added

$$Concentration = \frac{OD \times 1000}{\varepsilon} \qquad \textbf{Eq. 3.2}$$

Where ε of acpcPNA with base sequence of homothymine 16 mers = 140.8

ε of *epi*-acpcPNA with base sequence of alternating GC 16 mers = 151.2

3.3 Hybridization of acpcPNA-functionalized monomer and self-hybridization of *epi*-acpcPNA-functionalized monomer

It is anticipated that hybridization of the type 1 acpcPNA-functionalized monomers with DNA A16 (ratio of acpcPNA:DNA A16 = 2:1 or acpcPNA:DNA A8 = 1:1) and self-hybridization of *epi*-acpcPNA-functionalized monomer. Type 1 and type 2 acpcPNA-functionalized monomers would polymerize in the presence of complementary DNA to form linear polymers as shown in **Figure 3.5a** and **Figure 3.5b**, respectively. The *epi*-acpcPNA-functionalized monomer would form another linear supramolecular polymer by self-hybridization as shown in **Figure 3.5c**.

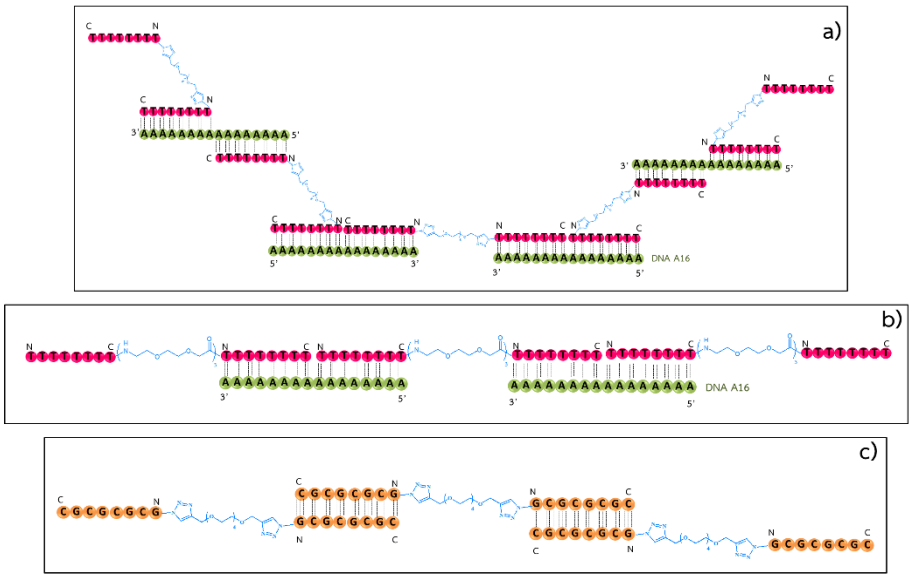

Figure 3.5 Schematic representation of proposed format of hybridization between (a) type 1 acpcPNA-functionalized monomer and DNA A16 (b) type 2 acpcPNA-functionalized monomer and DNA A16 and (c) self-hybridization of *epi*-acpcPNA-functionalized monomer.

Both type 1 and type 2 acpcPNA-functionalized monomers were studied for their hybridization properties with complementary homoadenine 16 mers DNA (DNA A16) through the melting temperature experiment using UV-vis spectrophotometry. The melting temperature (T_m) is the temperature at which 50% of double stranded DNA becomes unhybridized [27] therefore the T_m can provide evidence for the hybridization of acpcPNA-functionalized monomer and DNA A16. The base sequence of complementary DNA has to be two-fold longer than the functionalized acpcPNA monomer so that the DNA can link two functionalized monomers together. Increasing temperature causes separating the base stacking of acpcPNA hybridized with DNA resulting in the absorbance increase. The measurement operated from 20 to 90 °C yielded the melting curves of which the maximum slope of melting curves give T_m values.

As shown in **Figure 3.6**, The T_m of type 1 and type 2 acpcPNA-functionalized monomers hybridized with DNA A16 in the presence of salt were 80 and 81 °C, respectively. Both types of acpcPNA-functionalized monomer gave approximately the

same T_m implying that the direction of base sequence does not affect the acpcPNA and DNA hybridization. The fact that both acpcPNA-functionalized TEG and acpcPNA-functionalized OEG gave similar T_m also suggested that a change in the number of ethylene oxide repeat unit from 4 of TEG to 8 of OEG did not affect the hybridization. Notably, the T_m values of the acpcPNA-functionalized monomers hybridized with DNA A16 in the absence of added salt shown in **Figure 3.7** were higher than 85 °C suggesting that in the presence of salt, the binding between the acpcPNA and complementary DNA was weaker [22].

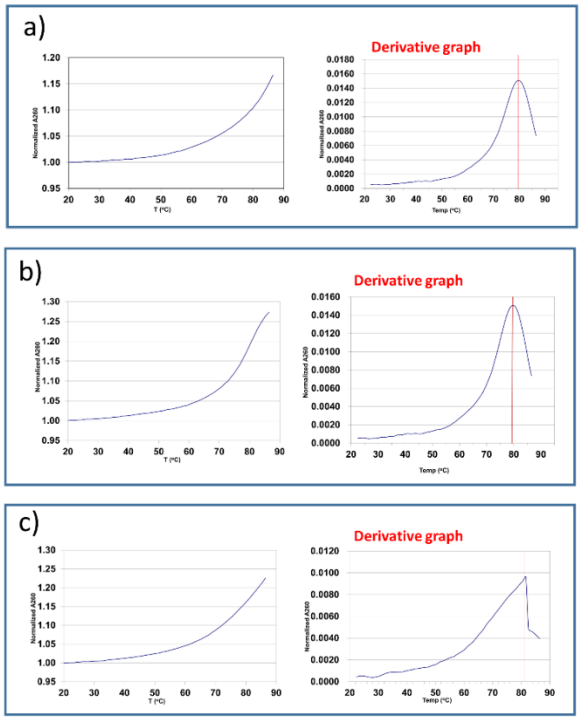

Figure 3.6 Melting curves and derivative plots of a) 1 μM type 1 acpcPNA-functionalized TEG + 1 μM DNA A16, b) 1 μM type 2 acpcPNA-functionalized OEG + 1 μM DNA A16, and c) 1 μM type 2 acpcPNA-functionalized monomer + 1 μM DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) under a condition with 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

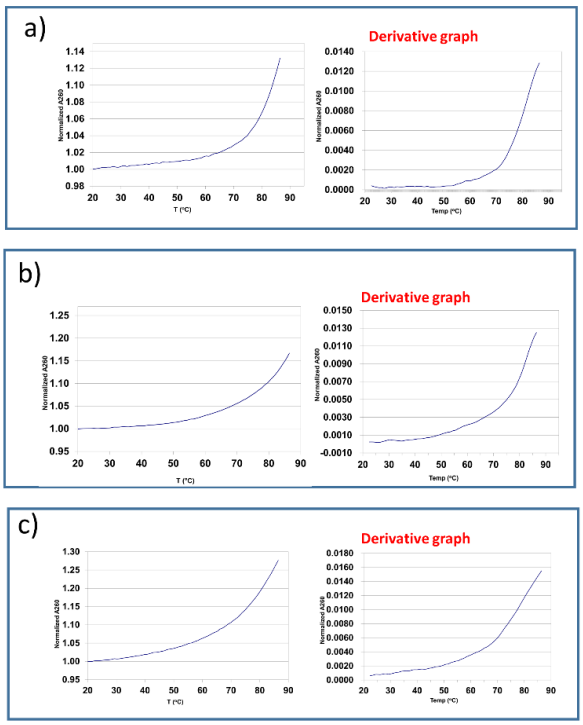

Figure 3.7 Melting curves and derivative plots of a) 1 μM type 1 acpcPNA-functionalized TEG + 1 μM DNA A16, b) 1 μM type 2 acpcPNA-functionalized OEG + 1 μM DNA A16, and c) 1 μM type 2 acpcPNA-functionalized monomer + 1 μM DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) under a condition without 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

The acpcPNA-functionalized monomers (both type 1 and type 2) were hybridized with complementary homoadenine 8 mer DNA (DNA A8) which has the base sequence matching to that of the acpcPNA functionalized monomers as a reference condition whereby the supramolecular polymerization could not form. As depicted in **Figure 3.8**, the type 1 and type 2 acpcPNA-functionalized monomers hybridized with DNA A8 in the presence of salt exhibited T_m of 64 and 61 °C, respectively. By definition, T_m is the temperature at which the acpcPNA.DNA hybrid is 50% separated. The lower T_m of the hybrid between acpcPNA-functionalized monomer and DNA A8 (T_m ≈ 61-64 °C) than the hybrid between acpcPNA-functionalized monomer and DNA 16 (T_m ≈ 80 °C) may be described as a consequence of the ease of separating 4 base pairs from

the acpcPNA and DNA A8 hybrid as opposed to the separating of 8 base pairs from the acpcPNA.DNA A16 hybrid.

On the other hand, in the absence of salt, there were 2 melting transitions shown in **Figure 3.9**, T_m at 36 and 80°C for type 1 acpcPNA functionalized monomer and 39 and 75 °C for type 2 acpcPNA functionalized monomer suggesting the formation of a triplex [28], possibly by a folding of acpcPNA **(Figure 3.10)**. The T_m value of approximately 40 °C was a transition changing from triplex to duplex structure. The T_m of approximately 80 °C was a transition changing from duplex structure to single stranded structure [29] which exactly matched the T_m of the unmodified acpcPNA with same base sequence when hybridized with DNA A8. (80°C). Information of all melting transition is concluded in **Table 3.3**.

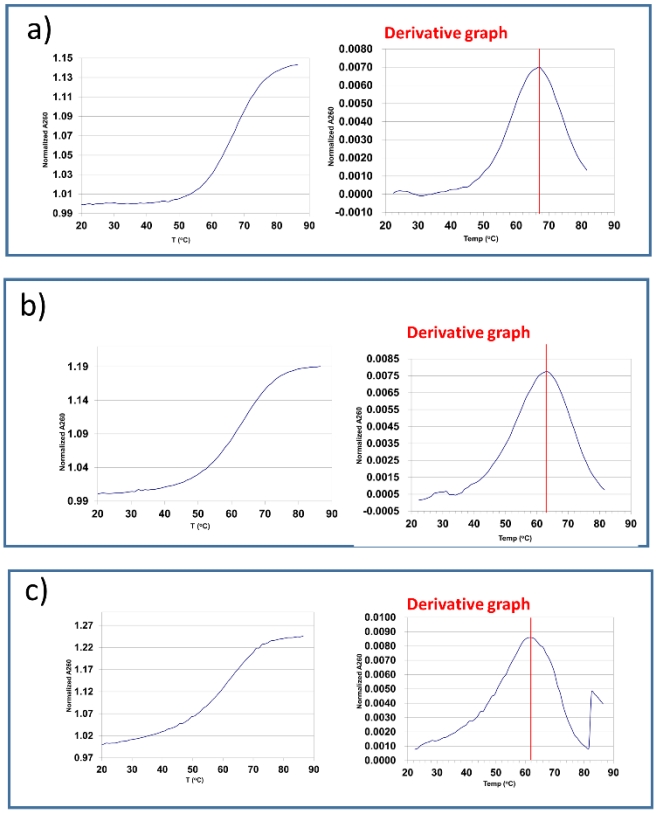

Figure 3.8 Melting curves and derivative plots of a) 1 μM type 1 acpcPNA-functionalized TEG + 2 μM DNA A8, b) 1 μM type 2 acpcPNA-functionalized OEG + 2 μM DNA A8, and c) 1 μM type 2 acpcPNA-functionalized monomer + 2 μM DNA A8 (ratio of acpcPNA:DNA = 1:1) under a condition with 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

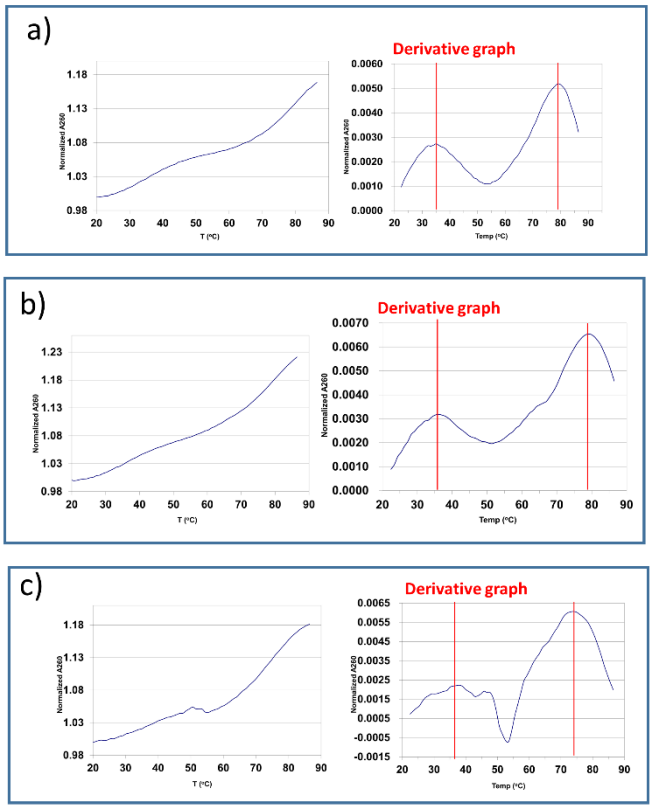

Figure 3.9 Melting curves and derivative plots of a) 1 μM type 1 acpcPNA-functionalized TEG + 2 μM DNA A8, b) 1 μM type 2 acpcPNA-functionalized OEG + 1 μM DNA A8, and c) 1 μM type 2 acpcPNA-functionalized monomer + 2 μM DNA A8 (ratio of acpcPNA:DNA = 1:1) under a condition without 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

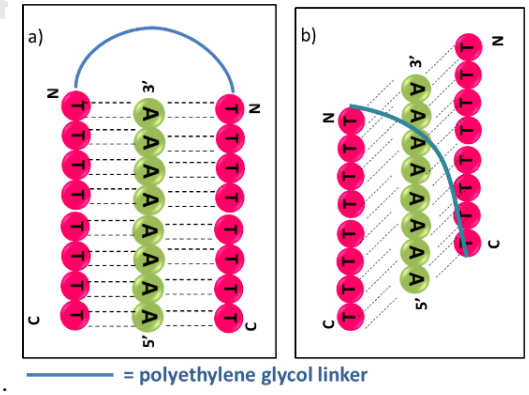

Figure 3.10 The suggested formation of a triplex from a) type 1 acpcPNA-functionalized monomer + DNA A8 and b) type 2 acpcPNA-functionalized monomer + DNA A8 under condition without 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

Table 3.3 Melting temperature (T_m) of homothymine acpcPNA-functionalized monomers in the presence of DNA.

Functionalized monomer	**Complementary DNA**	**Ratio of acpcPNA:DNA**	**Solution condition**	**1st T_m (°C)**	**2nd T_m (°C)**
Type 1 acpcPNA-functionalized TEG	A16	2:1	Phosphate buffer pH 7.4, NaCl	80	-
Type 1 acpcPNA-functionalized OEG	A16	2:1	Phosphate buffer pH 7.4, NaCl	80	-
Type 1 acpcPNA-functionalized TEG	A16	2:1	Milli Q	>85	-
Type 1 acpcPNA-functionalized OEG	A16	2:1	Milli Q	>85	-
Type 2 acpcPNA-functionalized monomer	A16	2:1	Phosphate buffer pH 7.4, NaCl	81	-
Type 2 acpcPNA-functionalized monomer	A16	2:1	Milli Q	85	-

Type 1 acpcPNA-functionalized TEG	A8	1:1	Phosphate buffer pH 7.4, NaCl	64	-
Type 1 acpcPNA-functionalized OEG	A8	1:1	Phosphate buffer pH 7.4, NaCl	64	
Type 1 acpcPNA-functionalized TEG	A8	1:1	Milli Q	35	80
Type 1 acpcPNA-functionalized OEG	A8	1:1	Milli Q	37	79
Type 2 acpcPNA-functionalized monomer	A8	1:1	Phosphate buffer pH 7.4, NaCl	61	-
Type 2 acpcPNA-functionalized monomer	A8	1:1	Milli Q	39	75
Unmodified acpcPNA	A8	1:1	Milli Q	80	-

Circular dichroism (CD) spectroscopy is a technique that can be used for evaluating secondary structure of polynucleotides and proteins by differential absorption of left- and right-handed circularly polarized light [30]. The absorption

bands can be observed at the wavelength where the absorption takes place. Similar conformational change was observed in both type 1 and type 2 acpcPNA-functionalized monomers when hybridized with DNA A16 (**Figure 3.11 and 12**) which revealed negative absorption bands at 208 and 250 nm. The increased intensity in CD signal of the acpcPNA functionalized monomers when hybridized with DNA A16 as compared with the CD signal of the DNA A16 alone indicated that there was the interaction between acpcPNA functionalized monomers and DNA A16. Besides, the fact that the magnitude of the hybridized CD signals was greater than the summation of CD signals of the acpcPNA functionalized monomers and DNA A16 can also be used as another supporting evidence of the interactions. Such trend was observed for both type 1 and type 2 acpcPNA-functionalized monomers and both in the presence and absence of salt. The overall shapes of the CD spectra in all cases are in good agreement with simple pT_n – dA_n hybrid reported [22].

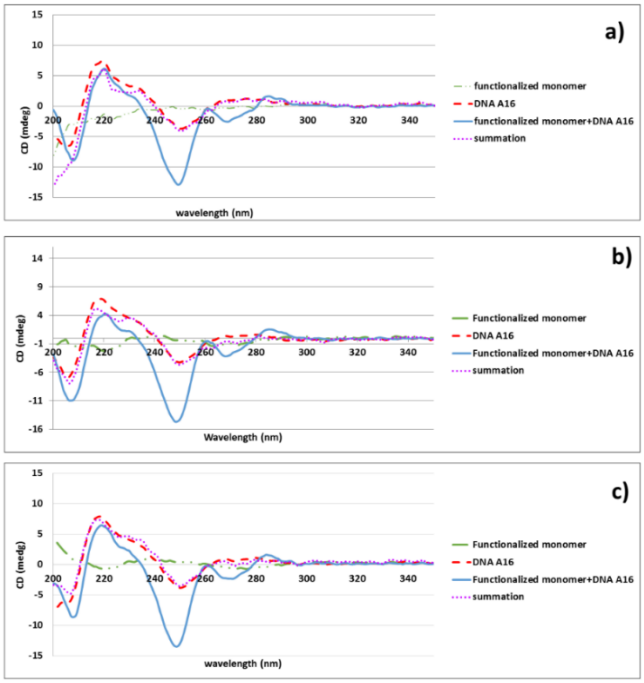

Figure 3.11 CD spectra of 1 μM type 1 acpcPNA-functionalized TEG (a), 1 μM type 1 acpcPNA-functionalized OEG (b) and 1 μM type 2 acpcPNA-functionalized monomer (c) hybridized with 1 μM DNA A16 under condition with 10 mM phosphate buffer, 100 mM NaCl.

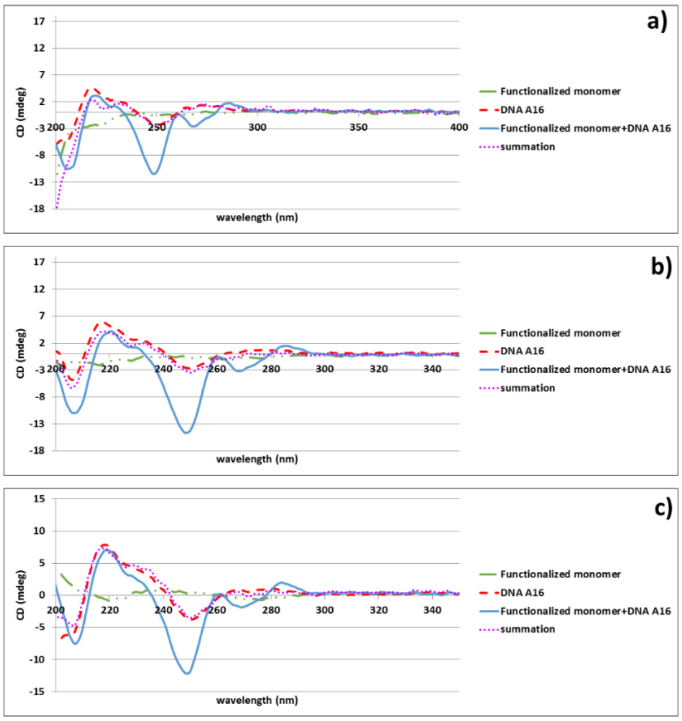

Figure 3.12 CD spectra of type 1 acpcPNA-functionalized TEG (a), type 1 acpcPNA-functionalized OEG (b) and type 2 acpcPNA-functionalized monomer (c) hybridized with DNA A16 under condition without 10 mM phosphate buffer, 100 mM NaCl.

DNA-, RNA- and self-pairing properties of *epi*-acpcPNA was reported by Taechalertpaisarn *et al*. [24]. *epi*-acpcPNA shows the ability to bind with DNA and RNA as well as to itself. There was no evidence of hybrid formed from the complementary acpcPNA strands [29] whereas two strands of self-complementary *epi*-acpcPNA provided an antiparallel self-hybrid as shown by melting curve and stronger CD signals compared to acpcPNA. Self-hybridization study of the 2 μM *epi*-acpcPNA functionalized monomer was also performed in the presence of 10 mM phosphate buffer and 100 mM NaCl. From **Figure 3.13**, the melting transition cannot be identified in the first round of heating. However a single melting transition with T_m of 64 °C was detected in the second round of heating, supporting the function of self-hybrids. Upon heating for the third round, a slightly lower T_m was found at 58 °C implying that there might be more than one self-hybridization pattern given that the *epi*-acpcPNA functionalized monomer consisting of alternating cytosine and guanine base sequence.

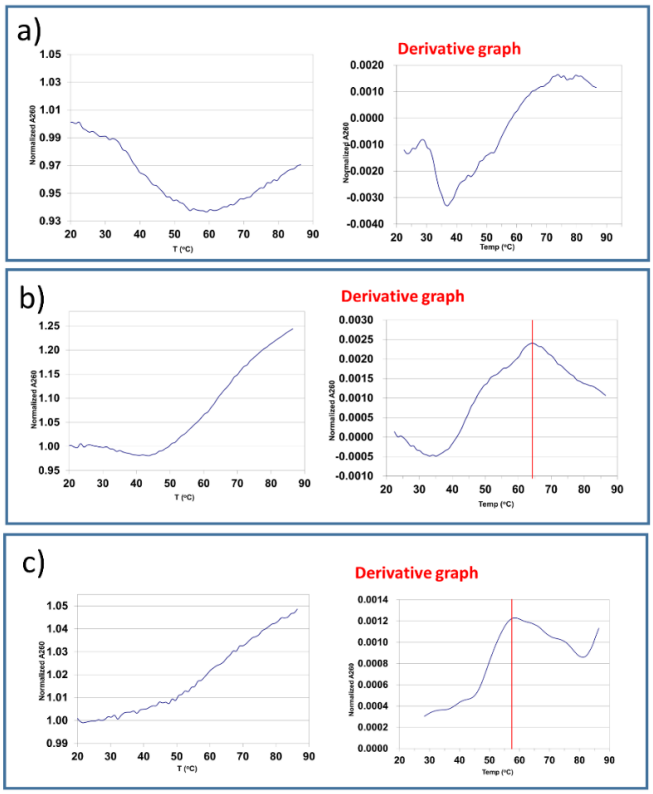

Figure 3.13 Melting curve and derivative graph of *epi*- acpcPNA-functionalized monomer from the 1st experiment (a), the 2nd experiment (b) and the 3rd experiment (c) under the condition with 10 mM phosphate buffer, 100 mM NaCl.

The CD measurements of *epi*-acpcPNA functionalized monomer were performed by varying temperature from 20 to 90 °C to study the self-hybridization of *epi*-acpcPNA functionalized monomer (**Figure 3.14**). At the temperature under T_m of the functionalized monomer (20-50 °C), the helical and structural pattern were observed from negative band at wavelength of 215 and 255 nm. When temperature rose from 50 to 90 °C, the negative peak at 255 nm became broader with lower intensity suggesting the unstacking of the nucleobase.

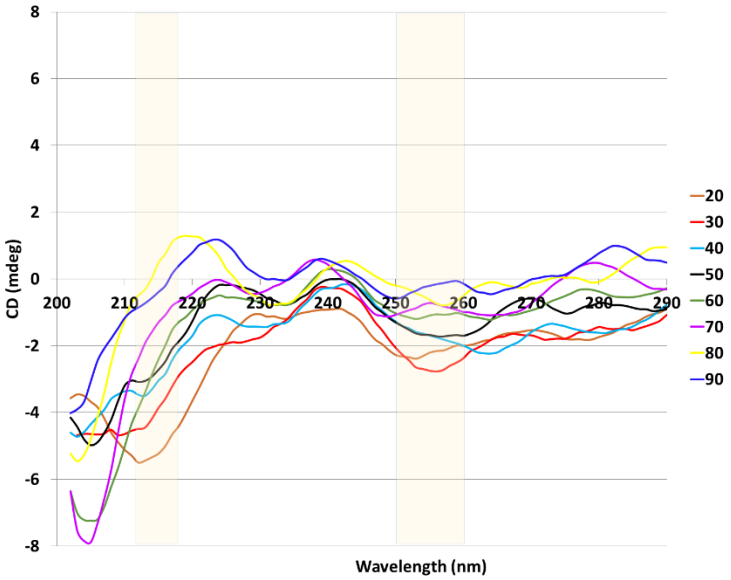

Figure 3.14 CD spectra at wavelength of 200-300 nm of *epi*-acpcPNA-functionalized monomer operated in a temperature range of 20-90 °C

The graph of CD signal at 215 nm and 255 nm was plotted between temperature range of 20-90 °C and CD intensity **(Figure 3.15)** to investigate the intensity change as a function of time. This graph showed the gradual reduction of CD intensity when temperature rose which is consistent with the unpairing of the self-duplex derived from *epi*-acpcPNA-funcionalized monomer. It can be concluded that the *epi*-acpcPNA functionalized monomer can form self-hybrids at the temperature under its T_m, although there may be several species of the self-hybrid as a mixture present together according to the observed several possible transitions.

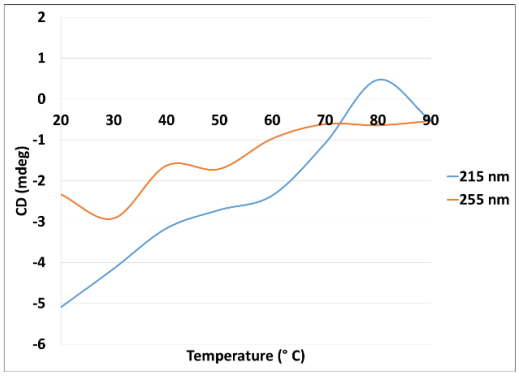

Figure 3.15 A plot between temperature and CD intensity of *epi*-acpcPNA-functionalized monomer at wavelength of 215 and 255 nm.

3.4 Morphological study of acpcPNA-functionalized monomer upon hybridization with complementary DNA

The type 1 acpcPNA-functionalized monomer before and after DNA addition was subjected to morphological analysis via Field Emission Scanning Electron Microscope (FESEM) which is a technique that can be used to monitor the formation of assembled micro/nanostructures on surface. It is assumed that the result may be used as a preliminary evidence of supramolecular polymerization. We expected to see changes either in size or shape of assembled structures upon hybridization between the acpcPNA-functionalized monomer and DNA. The preparation of samples for FESEM analysis was different from that previously employed for hybridization study, in which 100 mM NaCl stabilizing negative charges of DNA was added in 10 mM phosphate buffer in order to adjust pH to neutral. Here in this experiment, no buffer was used and the pH of the type 1 acpcPNA-functionalized monomer was adjusted to be neutral by adding concentrated ammonia solution. And the sample solution was dissolved in filtered Milli Q water in the absence of NaCl to avoid the salt crystal formation upon drying. As depicted in **Figure 3.16**, the FESEM micrographs of the acpcPNA-functionalized TEG (**Figure 3.16(a)**) and acpcPNA-functionalized OEG (**Figure 3.16(e)**) showed the ellipsoidal-like structures having size in a range of 0.2 – 0.4 μm. The free complementary DNA A16 (**Figure 3.16(b)**) appeared in spindle shapes having size around 1 μm. After the hybridization, the FESEM micrographs show assembled

structures of spindle shapes (**Figure 3.16(d) and (f)**) in a size range of 1 – 1.4 μm, very much similar to that observed for DNA A16 alone except in the case of the acpcPNA-functionalized TEG hybridized with DNA A16 of which the assembled structure was twice longer (2.6 nm) (**Figure 3.16(c)**). Overall, the results from FESEM analysis cannot conclusively verify the supramolecular polymerization.

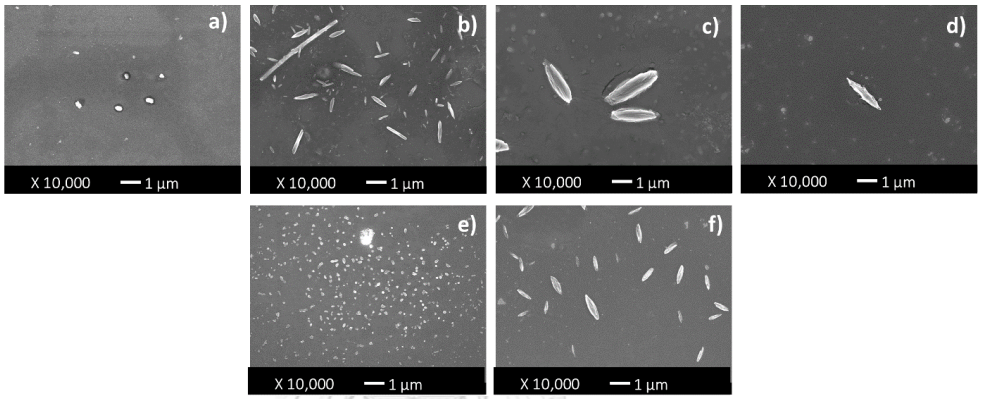

Figure 3.16 FESEM micrographs of acpcPNA-functionalized TEG (a), DNA A16 (b), acpcPNA-functionalized TEG hybridized with DNA A16 (c-d), acpcPNA-functionalized OEG (e), and acpcPNA-functionalized OEG hybridized with DNA A16 (f).

Polyacrylamide gel electrophoresis (PAGE) was chosen as an alternative tool to determine whether the hybridization of the acpcPNA-functionalized monomer (both type 1 and type 2) and complementary DNA A16 can lead to supramolecular polymerization. In principle, PAGE is a method for separating charged molecules such as DNA by monitoring the band movement on a cross-linked polyacrylamide gel prepared from acrylamide, ammonium persulfate (initiator) and TEMED (catalyst) in 1X TBE buffer. Molecules with lower molecular weight and/or higher charge would travel faster than molecules with higher molecular weight. PAGE takes a role as molecular sieve which will let the preferable DNA go through the pores affecting DNA mobility. The migration of the double-stranded DNAs are driven by electricity owing to negatively charged phosphate groups on DNAs and the band could be observed by

appropriate DNA stains or by UV light. Here in this experiment, it is anticipated that if there was hybridization between the acpcPNA functionalized monomer and DNA induced by nucleobase recognition, the bands should stay on the top of polyacrylamide gel due to the high molecular weight of the polymer. In addition, the bands would be broaden as a result of heterogeneous nature of the polymer. Both type 1 and type 2 acpcPNA-functionalized monomers after hybridized with complementary DNA A16 showed the new bands with slower mobility (**Figure 3.17-3.18**) than the DNA A16 alone indicating the hybridization of acpcPNA functionalized monomer and DNA A16. The acpcPNA-functionalized monomer alone would not move due to the absence of negative charge. However, the fact that the bands of acpcPNA-functionalized monomers hybridized with complementary DNA A8 also showed almost the same mobility as those of acpcPNA-functionalized monomers hybridized with complementary DNA A16 indicated that molecular weight of the A8 (ratio of acpcPNA:DNA A8 = 1:1) and DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) hybrids were similar. This leads to the conclusion that the proposed supramolecular polymer was not formed.

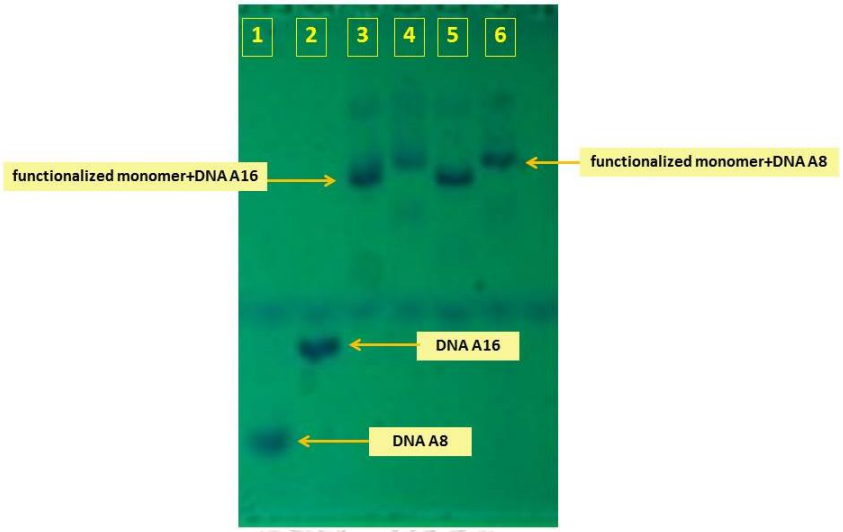

Figure 3.17 Image of PAGE represents DNA A8 (lane 1), DNA A16 (lane 2), type 1 acpcPNA-functionalized TEG hybridized with DNA A16 (lane 3), type 1 acpcPNA-functionalized TEG hybridized with DNA A8 (lane 4), type 1 acpcPNA-functionalized OEG hybridized with DNA A16 (lane 5) and type 1 acpcPNA-functionalized OEG hybridized with DNA A8 (lane 6).

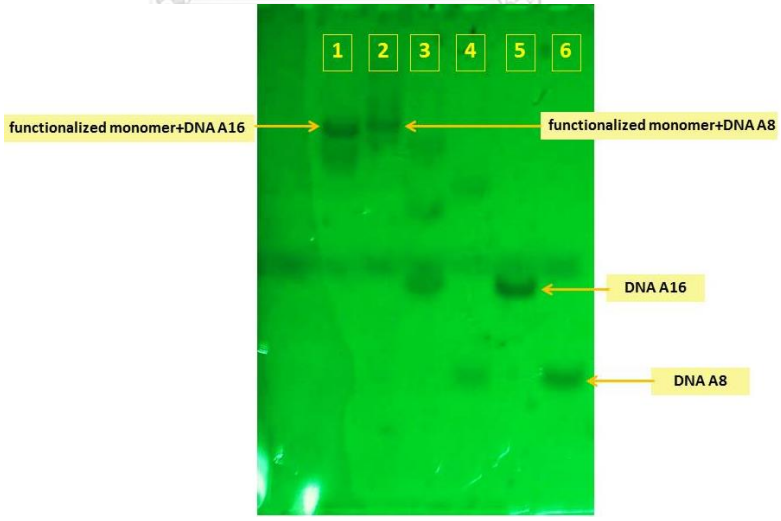

Figure 3.18 Image of PAGE represents type 2 acpcPNA-functionalized monomer hybridized with DNA A16 (lane 1), type 2 acpcPNA-functionalized monomer hybridized with DNA A8 (lane 2), unmodified acpcPNA hybridized with DNA A16 (lane 3),

unmodified acpcPNA hybridized with DNA A8 (lane 4), DNA A16 (lane 5) and DNA A8 (lane 6).

Herein we have proposed thepossible pattern of hybridization between acpcPNA-functionalized monomers (type 1 and type 2) and DNA A16 as deduced from T_m results. We proposed that one chain of acpcPNA-functionalized monomers (type 1 and 2) consisting of two homothymine segments at both ends of the linker would hybridize with one strand of DNA A16 **(Figure 3.19)** based on the same T_m value between the acpcPNA-functionalized monomers and DNA A16 hybrids (T_m = 80-81 °C) and the free acpcPNA with homothymine base 8 mers and DNA A16 hybrids with the ratio of acpcPNA:DNA A16 = 2:1 (T_m = 80°C) **(Figure 3.20).**

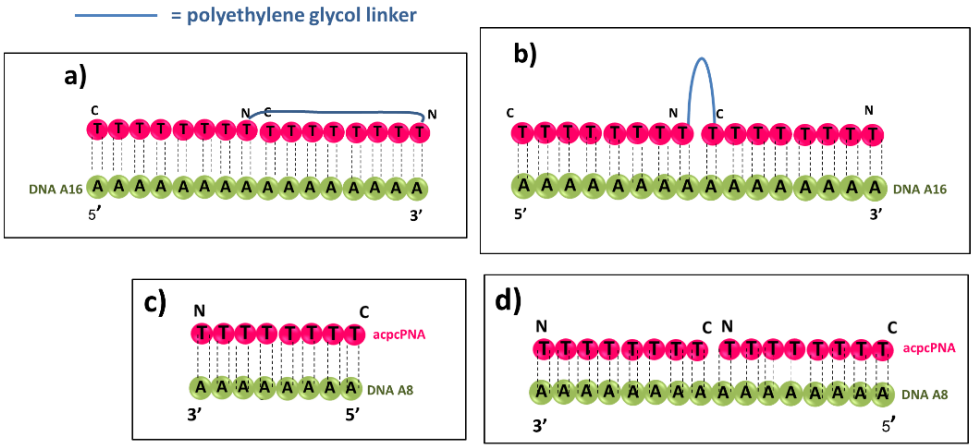

Figure 3.19 The proposed hybridizaion pattern of type 1 acpcPNA-functionalized monomer a), type 2 acpcPNA-functionalized monomer b), hybridized with DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) as compared with free homothymine acpcPNA + DNA A8 (ratio of acpcPNA:DNA A8 = 1:1) c) and free homothymine acpcPNA + DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) d).

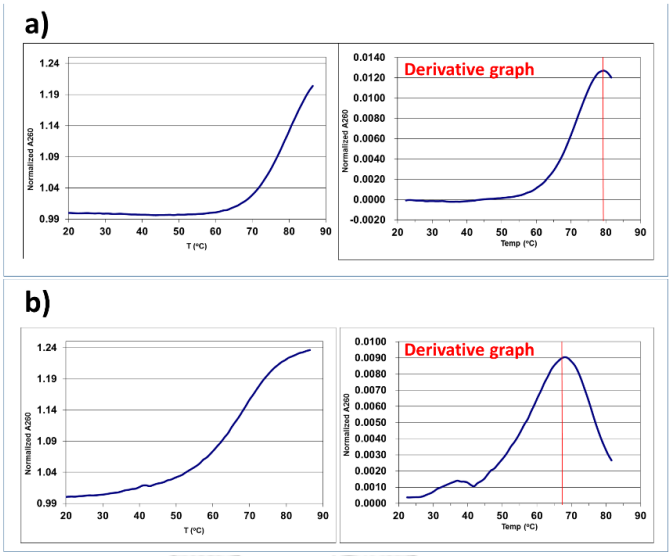

Figure 3.20 Melting curves and derivative plots of a) 2 μM free homothymine 8 mers + 1 μM DNA A16 (ratio of acpcPNA:DNA A16 = 2:1) and B) 1 μM free homothymine 8 mers + 1 μM DNA A8 (ratio of acpcPNA:DNA A8 = 1:1) under a condition with 10 mM phosphate buffer pH 7.4, 100 mM NaCl.

From previous works [32, 33], they studied the change of viscosity after the assembly of polypeptide-functionalized polymers via the examination of microrheology [34]. That might be an alternative way to investigate the supramolecular polymerization of acpcPNA- and *epi*-acpcPNA-functionalized monomers in the future.

CHAPTER VI
CONCLUSION AND SUGGESTIONS

Herein, we attempted to study supramolecular polymerization via utilizing non-covalent bond which is hydrogen bond formed by hybridization of acpcPNA with complementary DNA, or self-hybridization of *epi*-acpcPNA inspired from self-assembly of natural nucleic acid. We have successfully prepared acpcPNA-functionalized monomers and *epi*-acpcPNA-functionalized monomer which were purified by HPLC and characterized by MALDI-TOF. The acpcPNA functionalized monomers consist of two short acpcPNA or *epi*-acpcPNA segments linked together via linker made of polyethylene glycol (repeating unit of 4 or 8). Two types of acpcPNA-functionalized monomers having different relative direction of the two acpcPNA segments were made to linker. **Type 1** has the "head-to-head" orientation of acpcPNA to monomer (C---N-linker-N---C) which was prepared by CuAAC click reaction of tetra(ethylene glycol)-di(alkyne) or octa(ethylene glycol)-di(alkyne) and azide modified-acpcPNA. **Type 2** has the "head-to-tail" direction of acpcPNA to monomer (C---N-linker-C---N), which was prepared by SPPS strategy. For *epi*-acpcPNA functionalized monomer, the direction of *epi*-acpcPNA to linker was "head-to-head" so it was synthesized via the same method as **type 1** acpcPNA using CuAAC click reaction of tetra(ethylene glycol)-di(alkyne) and azide modified-*epi*-acpcPNA. Both of acpcPNA- and *epi*-acpcPNA-functionalized monomers showed the hybridization with complementary DNA and self-hybridization, respectively, as revealed by melting temperature and circular dichroism (CD) studies. However, the hybridization of acpcPNA and the self-hybridization of *epi*-acpcPNA did not lead to supramolecular polymerization as suggested by FESEM and PAGE techniques. Nevertheless, it is believed that acpcPNA-functionalized monomers may serve as useful precursor that can efficiently hybridize with DNA be used in applications of DNA and gene delivery or detection.

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