DETERMINATION OF DNA SEQUENCES USING PEPTIDE NUCLEIC ACID IN COMBINATION WITH CHITOSAN PARTICLES BY MALDI-TOF MASS SPECTROMETRY

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การหาลำดับเบสของดีเอ็นเอโดยใช้เพปไทด์นิวคลีอิกแอซิดร่วมกับอนุภาคไคโทซานด้วยมัลดิ-ทอฟแมสสเปกโทรเมตรี

นางสาวจิตติมา มีบึงพร้าว

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Thesis Title	DETERMINATION OF DNA SEQUENCES USING PEPTIDE NUCLEIC ACID IN COMBINATION WITH CHITOSAN PARTICLES BY MALDI-TOF MASS SPECTROMETRY
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จิตติมา มีบึงพร้าว: การหาลำดับเบสของดีเอ็นเอโดยใช้เพปไทด์นิวคลีอิคแอซิดร่วมกับอนุภาคไคโทซานด้วยมัลดิ-ทอฟแมสสเปกโทรเมตรี (DETERMINATION OF DNA SEQUENCES USING PEPTIDE NUCLEIC ACID IN COMBINATION WITH CHITOSAN PARTICLES BY MALDI-TOF MASS SPECTROMETRY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.ดร.สุดา เกียรติกำจรวงศ์, 87 หน้า.

การตรวจลำดับเบสของสารพันธุกรรมมีบทบาทสำคัญต่อการประยุกต์ในงานทางด้านเทคโนโลยีชีวภาพตั้งแต่ทางด้านการแพทย์ นิติวิทยาศาสตร์ เกษตรกรรม ไปจนถึงอาหาร แนวคิดของการใช้พิโรลิดินิลเพปไทด์นิวคลีอิกแอซิดที่มีคอนฟอร์เมชันถูกจำกัดชนิดใหม่ (เอซีพีซีพีเอ็นเอ) ร่วมกับตัวแลกเปลี่ยนแอนไอออนและเทคนิคมัลดิ-ทอฟแมสสเปกโทรเมตรี ได้รับการพิสูจน์เมื่อไม่นานมานี้ว่า เป็นแนวทางที่ง่ายและมีประสิทธิภาพสำหรับการตรวจลำดับเบสของดีเอ็นเอ งานวิจัยนี้เสนอการใช้อนุภาคควอเทอไนซ์ไคโทซานเป็นตัวแลกเปลี่ยนแอนไอออนเพื่อประยุกต์สำหรับการวิเคราะห์ในลักษณะเดียวกัน เตรียมอนุภาคได้โดยปฏิกิริยาควอเทอร์ไนเซชันแบบเอกพันธ์หรือวิวิธพันธ์ สามารถยืนยันการเกิดอนุภาคควอเทอร์ไนซ์ไคโทซานที่มีขนาดเล็กกว่า 1 ไมครอน ลักษณะเป็นทรงกลม และมีประจุเป็นบวก ได้ด้วยเทคนิคเอฟที-ไออาร์ โปรตอนเอ็นเอ็มอาร์ พีซีเอส และทีอีเอ็ม จากการวิเคราะห์ด้วยมัลดิ-ทอฟแมสสเปกโทรเมทรีพบว่าอนุภาคควอเทอไนซ์ไคโทซานบางชนิดเมื่อใช้ร่วมกับเอซีพีซีพีเอ็นเอสามารถตรวจสอบดีเอ็นเอที่มีลำดับเบสความยาว 9-14 เบสที่มีลำดับเบสต่างกันเพียงแค่ 1 ตำแหน่งได้ ความสำเร็จของการวิเคราะห์อย่างเลือกจำเพาะจำเป็นต้องอาศัยการล้างอนุภาคหลังจากการจับยึดกับโมเลกุลลูกผสมระหว่างพีเอ็นเอกับดีเอ็นเอด้วยสารละลาย 20 เปอร์เซ็นต์ของฟอร์มาไมด์ในสารละลายฟอสเฟตบัฟเฟอร์ นอกจากนี้ยังสามารถนำเทคนิคนี้มาประยุกต์ใช้ในการตรวจสอบลำดับเบสดีเอ็นเอสังเคราะห์ ซึ่งจำลองลำดับเบสที่ผิดปกติซึ่งเกี่ยวข้องกับโรคมะเร็งได้อย่างมีประสิทธิภาพ

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ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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JITTIMA MEEBUNGPRAW: DETERMINATION OF DNA SEQUENCES USING PEPTIDE NUCLEIC ACID IN COMBINATION WITH CHITOSAN PARTICLES BY MALDI-TOF MASS SPECTROMETRY. THESIS ADVISOR: ASST. PROF. VORAVEE P. HOVEN, Ph.D., THESIS CO-ADVISOR: PROF. SUDA KIATKAMJORNWONG, Ph.D., 87 pp.

Determination of DNA sequences is significantly important for many biotechnology-related applications ranging from medical, forensic, agriculture, and food science. The concept of using a new conformationally constrained pyrrolidinyl peptide nucleic acid (*acpc*PNA), anion-exchange captures in combination with MALDI-TOF mass spectrometry has been recently proven as a simple and effective way for DNA sequence analysis. This research has introduced chitosan quaternized chitosan particles as anion-exchange captures that may be applicable for the same purpose. The particles were prepared by either homogeneous or heterogeneous quaternization. The success of sub-micron, spherical, and positively charged quaternized chitosan particle formation was verified by FT-IR, ^{1}H NMR, PCS, and TEM analyses. Investigation by MALDI-TOF mass spectrometry suggested that some quaternized particles in combination with *acpc*PNA were capable of detecting a single mismatched base out of 9-14 base DNA sequences. The success of selective detection generally required rinsing of the particles after capturing PNA·DNA hybrid by 20% formamide in phosphate buffer solution. Potential application of this technique for a synthetic DNA of which sequence mimics mutant K-*ras* DNA, a mutated region that is associated with cancer has also been demonstrated.

Field of Study: Petrochemistry and Polymer Science Student' Signature .....
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Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

CS	: Chitosan particles
CCA	: α-cyano-4-hydroxy cinnamic acid
$CDCl_3$	: Deuterated chloroform
DEAE	: Diethylaminoethyl sepharose
DD	: Degree of deacetylation
DIEA	: Diisopropylethylamine
DNA	: Deoxyribonucleic acid
D_2O	: Deuterium oxide
DQ	: Degree of quaternization
FmocOSu	: 9-fouorenylsuccinimidyl carbonate
FT-IR	: Fourier transform infrared spectroscopy
GTMAC	: Glycidyltrimethylammonium chloride
HTACC	: *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride
Lys	: Lysine
MALDI-TOF	: Matrix-assisted laser desorption/ionization-time of flight
MBzC	: Methylated *N*-benzyl chitosan
MC	: Methylated chitosan
MeI	: Methyl iodide
MeOH	: Methanol
mg	: Milligram
MS	: Mass spectrometry
m/*z*	: Mass to charge ratio

NMR	:	Nuclear magnetic resonance spectroscopy
PCS	:	Photon correlation spectroscopy
PNA	:	Peptide nucleic acid or Polyamide nucleic acid
Ph	:	Phenyl
Q-sepharose	:	Quaternary ammonium sepharose
Ser	:	Serine
TEM	:	Transmission electron microscopy
TFA	:	Trifluoroacetic acid
TMC	:	*N*,*N*,*N*-trimethyl chitosan
TPP	:	Tripolyphosphate
μm	:	Micrometer (10^{-6} m)
nm	:	Nanometer (10^{-9} m)
ζ potential	:	Zeta potential

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Determination of the sequence of a specific region of deoxyribonucleic acid (DNA) samples has a broad range of applications ranging from medical and forensic to agriculture and food sciences. Peptide nucleic acid (PNA) which is a neutral–backbone DNA analogue firstly developed by Nielsen and co-workers has a great potential to be used as a probe for DNA biosensor [1-3]. This is mainly due to the fact that PNA can form hybrids with high affinity with complementary DNA. In addition, the hybrids are more stable than the corresponding DNA·DNA hybrid due to the absence of electrostatic repulsion between negative charge and a mismatch in a PNA·DNA hybrids [4]. In particular, a novel conformationally rigid pyrrolidinyl PNA based on D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbones synthesized by Vilaivan and co-workers exhibited desirable characteristics for development of a highly effective DNA biosensor [5,6]. This PNA system possessed at least a comparable binding affinity and sequence specificity towards DNA as that of Nielsen's PNA. On the other hand, the *acpc*PNA showed a much stronger preference for the antiparallel binding mode. The high binding affinity to complementary DNA and the powerful discrimination for single mismatched DNA, together with the high directional specificity.

A label-free technique based on the use of *acpc*PNA and anion exchanger in the step of sample preparation prior to the analysis by MALDI-TOF mass spectrometry was recently developed. The complementary PNA·DNA hybrid can be directly detected on the anion exchanger by MALDI-TOF MS after simple washing. In addition, this method could detect the hybridization event at room temperature without the need for enzyme treatment or heating [7]. The quaternary ammonium containing Q-sepharose used as the anion exchanger, however, possesses some practical limitations. It is generally commercialized in the form of liquid dispersion so the exact amount of the particles used for each experiment is unknown. The weighting of the solid Q-sepharose is not practical since the drying would destroy the swellability of the microparticles

which in turn may adversely affect the capability of DNA capturing. Therefore, this research has introduced chitosan and quaternized chitosan particles as anion-exchange captures that may be applicable for the same purpose. The ability to introduce quaternary ammonium groups to chitosan particles, whose size, charge density as well as functional group variation can be manipulated prompts us to believe that the chitosan and quaternized chitosan particles can be potentially used for the same application and perhaps overcome the problems presently encountered with the Q-sepharose.

Chitosan is a polysaccharide obtained by partial deacetylation of chitin, a natural substance found abundantly in the exoskeletons of insects, the shells of crustaceans, and fungal cell walls. Besides, it shows non-toxic in a range of toxicity tests, both in experimental animals [8] and humans [9]. Most of the amino groups in the structure of chitosan are protonated to positively charged ammonium groups in a pH range of 1–6. The binding of chitosan and DNA is based on electrostatic interactions between positively charged chitosan and negatively charged DNA. However, chitosan can bind with DNA in acidic solutions (pH 1–6) where most of the amino groups are protonated. In order to introduce permanent positively charge, chitosan can be modified by quaternization.

In this research, two routes for the preparation of quaternized particles were proposed; heterogeneous and homogeneous routes. The heterogeneous route is based on heterogeneous chemical modification of ionically crosslinked chitosan particles whereas the homogeneous route relies on the homogeneous chemical modification of chitosan followed by particle formation via ionic crosslinking. Quaternized chitosan particles namely methylated chitosan (MC) particles, methylated *N*-benzyl chitosan (MBzC) particles, *N*,*N*,*N*-trimethylchitosan (TMC) particles, and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) particles were prepared and characterized by a series of techniques which are Fourier transform-infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (^{1}H NMR), photon correlation spectroscopy (PCS), and transmission electron microscopy (TEM).

DNA sequence analysis by quaternized chitosan particles using MALDI-TOF mass spectrometry were conducted on synthetic DNA sequences using *acpc*PNA as probes. Two PNA systems were used. The first one was 9-base sequences containing a single type of base (T9 and A9). The second one was 14-base sequences containing mixed sequences. Detection limit and selectivity were also determined.

1.2 Objectives

1. To prepare and characterize quaternized chitosan particles.
2. To determine DNA sequence using *acpc*PNA as probes and quaternized chitosan particles as anion exchangers by MALDI-TOF mass spectrometry

1.3 Scope of Investigation

1. Literature survey for related research work
2. Preparation of quaternized chitosan particles by homogeneous and heterogeneous reactions.
3. Characterization of the quaternized chitosan particles
4. Determination PNA·DNA hybridization using quaternized chitosan particles by MALDI-TOF mass spectrometry

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. Peptide nucleic acid (PNA) is a DNA analogue originally introduced by Nielson *et al*. in 1991 [1]. This PNA system or so-called *aeg*PNA, consists of repeating *N*-(2-aminoethyl)-glycine units linked together by amide bonds which replaced the normal phosphodiester backbone of DNA. A methylene carbonyl linker connects natural nucleobase to this backbone at the amino nitrogen as shown in Figure 2.1.

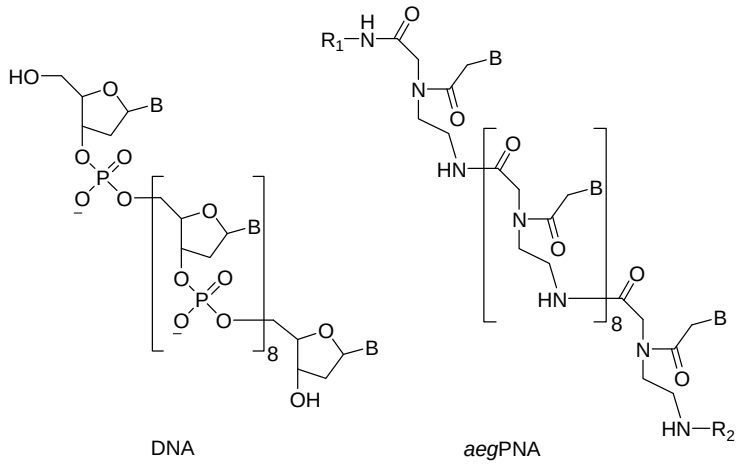
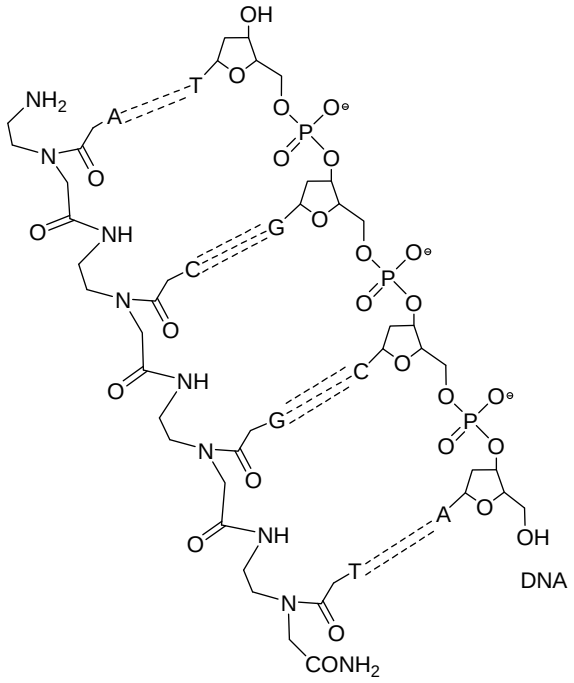


Figure 2.1 Chemical structures of DNA and *aeg*PNA

The PNA forms hybrids with high affinity with its complementary DNA. The hybrids are more stable than the corresponding DNA·DNA hybrid due to the absence of electrostatic repulsion between negative charge and a mismatch in a PNA·DNA hybrids is generally more destabilizing than a mismatch in a DNA·DNA duplex [4]. Moreover,

the hybridization between PNA with DNA strictly follows the Watson-Crick base pairing rule as in DNA hybridizations (Figure 2.2).



*aeg*PNA

Figure 2.2 Paring between complementary nucleobase of *aeg*PNA and DNA by Watson-Click base pairing

These favorable properties of PNA have attracted wide attention in medicinal researches for development of gene therapeutic (antisense and antigene) drugs [10] and in biotechnology for development of diagnostic tools [11], especially nucleic acid biosensor. A number of PNA systems have been developed but none of them had shown better properties than the original PNA. Until recently, Vilaivan and co-workers have reported synthesis of a novel conformationally rigid pyrrolidinyl PNA based on D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbones. They reported that the stereochemistry of the backbone is very important in determining the binding properties. Only the PNA containing (1*S*, 2*S*)-acpc (Figure 2.3a) can form a very stable 1:1 complex with the complementary DNA in a sequence-specific manner. In addition, thymine decamer (1*S*, 2*S*)-acpc PNA can bind to the complementary DNA with a higher affinity than Nielsen's PNA (Figure 2.3b).

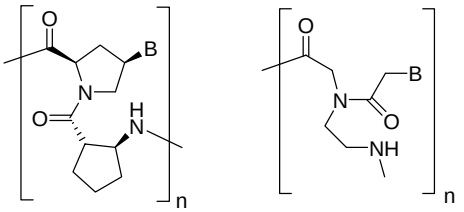


Figure 2.3 Structures of (a) (1*S*, 2*S*)-acpc PNA or Vilaivan's PNA and (b) Nielsen's PNA

2.2 Peptide Nucleic Acid-based Biosensor

The development of PNA-based biosensor has increased tremendously over the past few years as demonstrated by the large number of scientific publication. PNA offers some advantages over DNA such as higher specificity, sensitivity and accuracy in the detection of target sequence. Because of its advantages, PNA is used as a probe to detect DNA base sequence with different characterizing techniques.

The principle of Fluorescence Resonance Energy Transfer (FRET) is often used for detecting specific interactions between biological molecules. In this technique, two fluorophores including donor and acceptor fluorophores are required. The efficiency of the donor-acceptor energy transfer is reversely proportional to the sixth power of the distance between the fluorophore residues. Therefore, upon excitation at the donor absorption region and registration of the acceptor's emission, the efficiency of the energy transfer is extremely sensitive to changes in the distance between the two fluorophores. The techniques have been used in several studies of DNA hybridization.

Gaylord *et al*. [2] proposed a new method for DNA sequence using fluorescent labeled PNA with a chromophore dye (C*) (acceptor) coupled with cationic conjugated polymer (CCP), poly(9,9-bis(6 *N*,*N*,*N*-trimethylammonium)-hexyl)-fluorene phenylene) (donor). The light-harvesting properties of CCP were used to sensitize the emission of a dye on the PNA probe by FRET from CCP to the fluorphore (C*) labeled PNA. Signal transduction was controlled by binding between the PNA probe and the DNA target. The overall scheme is shown in Figure 2.4.

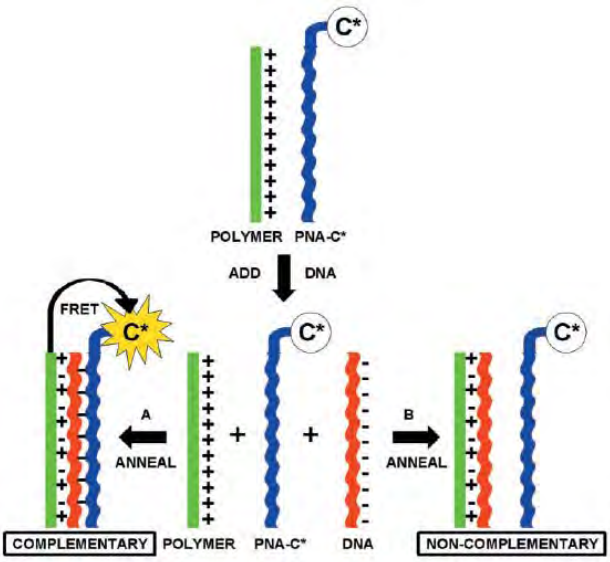


Figure 2.4 Schematic representation for the use of a water-soluble CP with a specific PNA-C* optical reporter probe to detect a complementary ssDNA sequence [2]

In 2005, Liu *et al.* [3] constructed a strand-specific DNA sensory method which is based on surface-bound peptide nucleic acids and water-soluble cationic conjugated polymers. The main transduction mechanism operates by taking advantage of the net increase in negative charge at the peptide nucleic acid surface that occurs upon single-stranded DNA hybridization. The resulting PNA·DNA complexes cause the cationic polymer to bind selectively to the surface with electrostatic. The scheme of method and structure of PNA probes attached to glass or silica surfaces are illustrated in Figure 2.5.

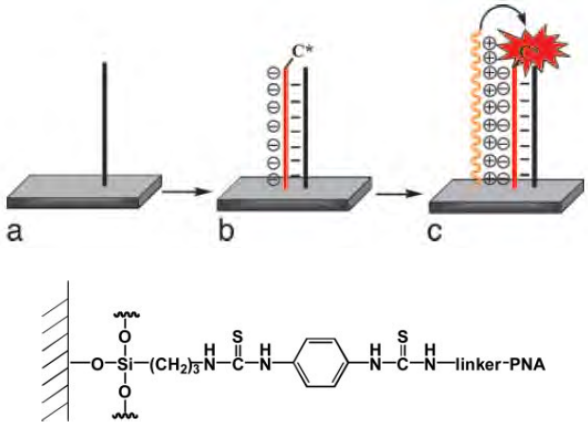


Figure 2.5 Amplification of a PNA (black) ssDNA-C* (red) solid-state sensor by polyelectrolytic deposition of the CCP (orange) and surface-bound PNA probe structure [3]

In the absence of DNA specimens, the beacon takes a closed form in which the fluorescence from a dye bound to one end of a DNA or PNA backbone is quenched by a second dye bound to another end. Upon duplex formation of the beacon with a complementary DNA specimen, these dyes are spatially separated and thus the beacon becomes fluorescent. A mismatch between the beacon and the DNA specimen is detectable because the DNA/PNA duplex is less stable and thus the beacon is less efficiently converted to the fluorescent form. A variety of applications to DNA sequence detection has been proposed using molecular beacon in combination with PNA probe.

In 2005, Ye *et al*. [12] reported use of PNA-based molecular beacons with nuclease S1 to detect genotyped SNPs. This genotyping method is based on the finding that nuclease S1 strictly recognizes a mismatch in DNA/PNA duplexes and hydrolyzes the DNA only in mismatched duplexes. When a DNA specimen has a mismatch to the PNA beacon, the DNA should be promptly digested by the enzyme, allowing the beacon to be converted to its closed form (as shown in Figure 2.6). In the absence of the mismatch, however, the DNA should be protected from the digestion, and thus the beacon retains the open form for efficient fluorescence emission.

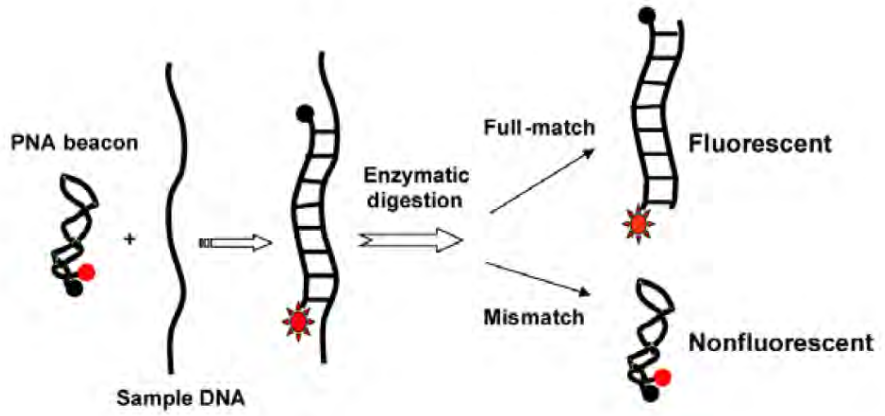


Figure 2.6 Strategy for SNP detection using the combination of PNA beacon and nuclease S1 [12]

From the variety of methods for DNA sequence analysis by using PNA as probe, some techniques showed some drawbacks. For example, in the case of enzymatic digestion method for detecting nucleic acid, it is difficult to control the condition of the reaction, especially for large-scale testing. In addition, some methods required labeled PNA probe or DNA samples that is quite complicated and expensive, yet providing

only indirect information of DNA sequence. On the other hand, matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) is interesting because it has several advantages. Firstly, this technique can be applied for a variety of compounds including protein, nucleic acids and peptide compounds. MALDI-TOF MS also takes milliseconds to complete the process of ionization, separation by size, and detection of nucleic acids. Additionally, the results are absolute, being based on the intrinsic property of mass-to-charge ratio (*m*/*z*) [13]. Because of these advantages, MALDI-TOF MS shows a great potential for high-throughput nucleic acid detection application.

Peptide nucleic acid is easily analyzed by MALDI-TOF MS, because the peptide backbone does not fragment, unlike DNA molecules, which may undergo substantial fragmentation during the MALDI process [14]; also, PNA oligomers do not tend to form adducts with metal cations, which is detrimental to MALDI-TOF mass spectrometric analysis [15], because annealing of these oligomers can be done in buffers containing low salt concentrations and also the neutral amide backbone does not have the tendency to bind to cations that may be present to the same extent as the negatively-charged backbone of DNA. There are few reports related to use of Nielsen's PNA hybridization probes with MALDI-TOF mass spectrometric method to analyze DNA sequence.

Ross and Jiang-Baucom [16,17] reported methods for SNP detection that couple the sensitivity and accuracy of MALDI-TOF MS with the specificity and stability of PNA·DNA hybridization. The methods are applied directly to polymorphisms located within human mitochondrial DNA (mtDNA) and in genomic DNA using human leukocyte antigen (HLA) DQR polymorphisms. PNA probes complementary to each of two known alleles were designed for each of the aforementioned loci. Biotin-labeled DNA is captured onto streptavidin-coated magnetic particles. Following washing, the immobilized PNA·DNA complex is then directly analyzed by MALDI-TOF MS (as shown in Figure 2.7). The PNA molecules denature from the DNA during the MALDI process, providing accurate mass spectral data. The ability to distinguish SNPs in amplified DNA consistently with high specificity is demonstrated.

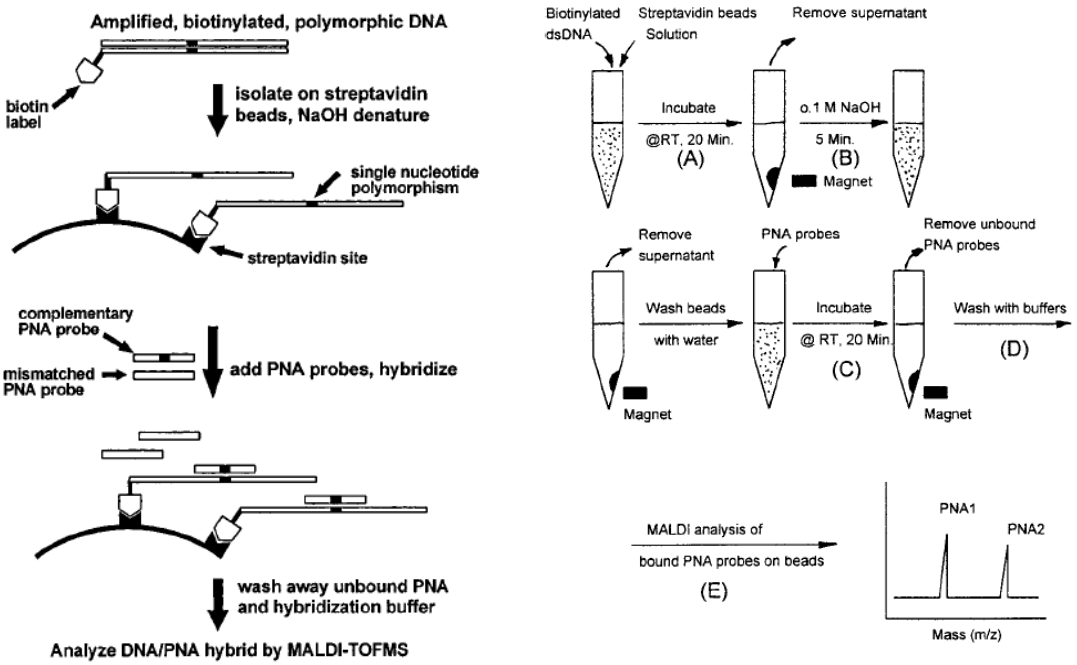


Figure 2.7 Schematic outline of detection of PNA·DNA hybridization by MALDI-TOF MS experiment [16,17]

In 2004, Ren *et al*. [18] detected single-nucleotide polymorphisms (SNPs) in double stranded DNA (dsDNA) with PNA probe by MALDI-TOF MS. In its presence, genomic dsDNA was first treated with exonuclease III and then with nuclease S1. By these one-pot reactions, single-stranded DNA fragments including the SNP sites were formed *in situ*. These fragments were directly analyzed by MALDI-TOF MS, and the identity of the DNA base at the SNP site was determined in terms of mass number. By using two or more PNA probes simultaneously, multiplex analysis was also successful.

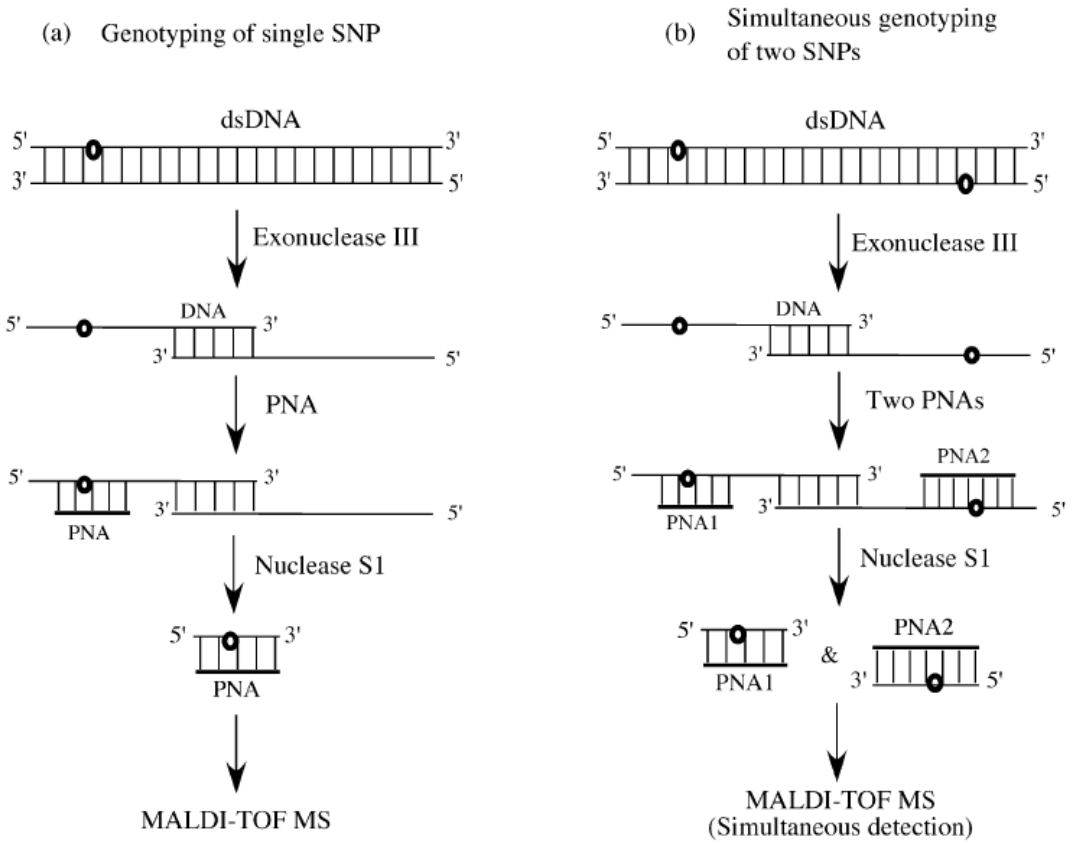


Figure 2.8 Schematic representation of the processes for SNP detection by exonuclease III/nuclease S1/PNA systems. The small circles represent the SNP sites. (a) Genotyping of one SNP by use of single PNA probe. (b) Simultaneous genotyping of two SNPs by use of two PNA probes [18]

Recently, Boontha *et al*. [7] developed a new ion-exchange capture technique introduced for label-free sample preparation in single nucleotide polymorphism (SNP) genotyping. The DNA sample is hybridized with a new pyrrolidinyl PNA probe and treated with a strong anion exchanger. The complementary PNA·DNA hybrid was selectively captured by the anion exchanger in the presence of noncomplementary or unhybridized PNA, allowing direct detection of the hybridization event on the anion exchanger by MALDI-TOF MS after simple washing. The high specificity of the pyrrolidinyl PNA allows simultaneous multiplex SNP typing to be carried out at room temperature without the need for enzyme treatment or heating. The presence of the *m/z* signal of the PNA suggested that the DNA and PNA sequences are complementary (as shown in Figure 2.9).

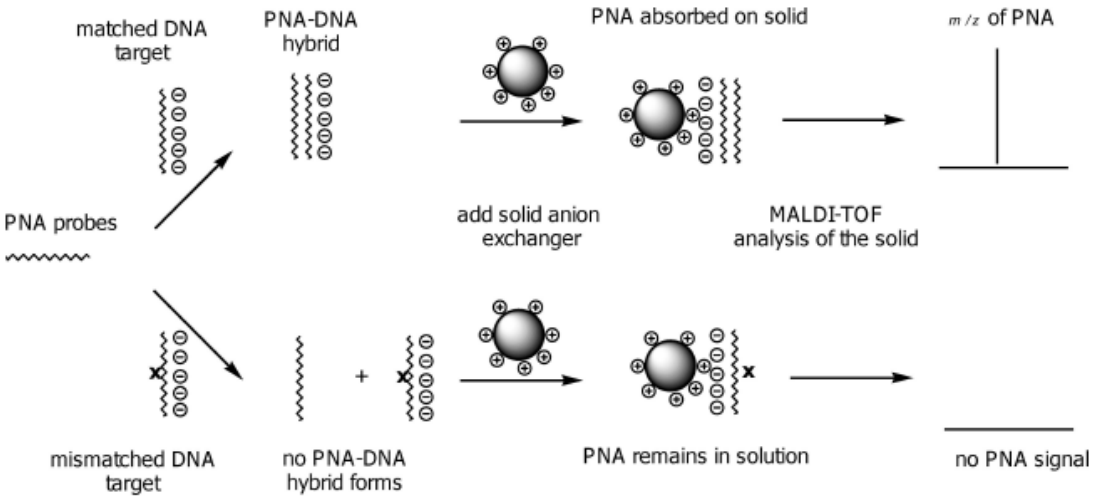


Figure 2.9 Schematic diagram showing the concept of ion-exchange capture of PNA in combination with MALDI-TOF MS in DNA sequence determination [7]

2.3 Chitosan and Derivatives

Chitin, a naturally abundant mucopolysaccharide, and the supporting material of crustaceans, insects, etc., is well known to consist of 2-acetamido-2-deoxy-β-D-glucose through a β (1→4) linkage. It is a highly insoluble material resembling cellulose in its solubility and low chemical reactivity. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. Like cellulose, it functions naturally as a structural polysaccharide. Chitin is a white, hard, inelastic, nitrogenous polysaccharide. The structures of chitin , chitosan and cellulose are shown in Figure 2.10.

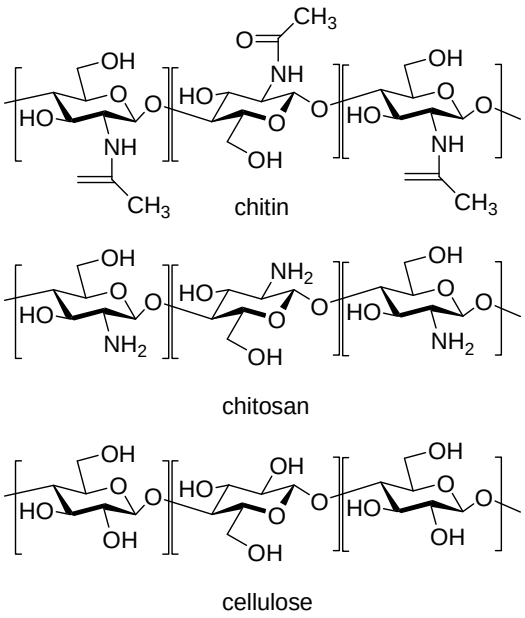
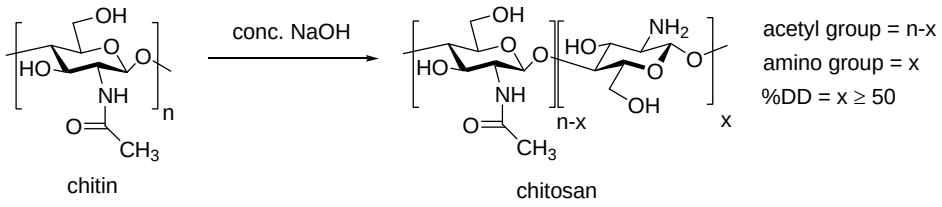


Figure 2.10 Structure of chitin, chitosan and cellulose

Chitosan are biodegradable, biocompatible, non-toxic, nonallergenic, renewable biomaterials and find applications in fields such as medicine, cosmetics, food industry and agriculture. Chitosan is prepared by deacetylation of chitin as shown in Scheme 2.1. From the alkali treatment in deacetylation process, acetyl groups in chitin are converted to amino groups. The degree of deacetylation (%DD) of chitosan ranges from 50% to 98% with an average of 80% (amino group 80% and acetyl groups 20%), depending on the crustacean species and the preparation methods [19]. Removal of the acetyl group is a harsh treatment usually performed with concentrated NaOH solution (either aqueous or alcoholic). Protection from oxygen, with a nitrogen purge or addition of sodium borohydride to the alkali solution, is necessary in order to avoid undesirable reactions such as depolymerization and generation of reactive species. The presences of 2-amino-2-deoxyglucose units in a chitosan bring the polymer into solution by salt formation. As a result, chitosan is a primary aliphatic amine that the p*K*a of chitosan amine is 6.3 so chitosan can be soluble in pH values of lower than 6.0. However, many applications of chitosan are in neutral or basic medium that chitosan is not soluble especially those in medicine, cosmetics and food.



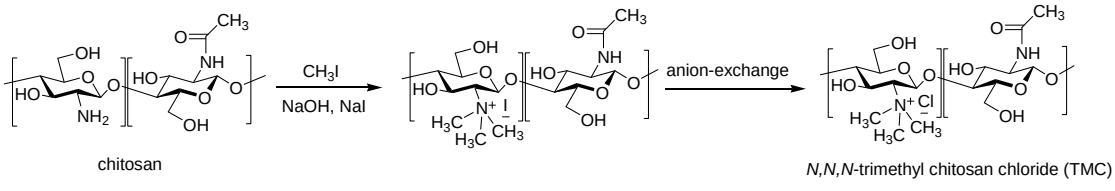
Scheme 2.1 Deacetylation of chitin

Besides, it has been shown to be non-toxic in a range of toxicity tests, both in experimental animals [8] and humans [9]. Its soft tissue compatibility has also been demonstrated by Tomihata *et al*. [20]. Chitosan has been shown to effectively bind DNA in saline or acetic acid solution and partially protect DNA from nuclease degradation [21,22].

The binding of chitosan and DNA is based on electrostatic interactions between positively charged chitosan and negatively charged DNA. However, chitosan can bind with DNA in acidic solutions (pH 1–6) where most of the amino groups are protonated. In order to introduce permanent positively charge, chitosan can be modified by quaternization. The quaternization of chitosan can be done homogeneously or heterogeneously. The following literatures describe quaternization of chitosan based on those two approaches.

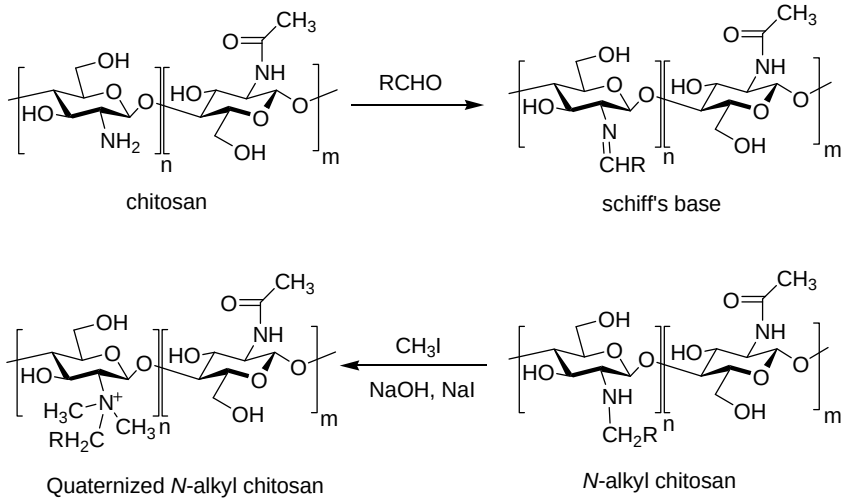
2.3.1 Homogeneous Quaternization

In 1985, Domard *et al*. [23] synthesized chitosan derivative having a quaternary ammonium salt, *N*,*N*,*N*-trimethyl chitosan chloride (TMC). The reaction was performed by reaction of a low acetyl content chitosan with methyl iodide and sodium hydroxide under controlled conditions (Scheme 2.1). The reaction yielded TMC with various degrees of quaternization. Furthermore, TMC can be soluble in water.



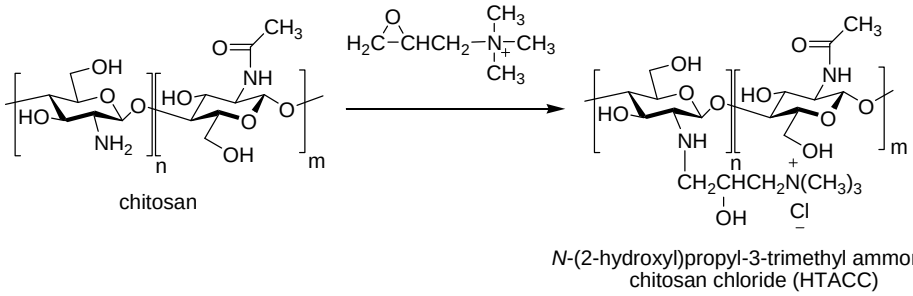
Scheme 2.2 Synthesis of *N*,*N*,*N*-trimethyl chitosan chloride (TMC)

In 2001, Jia *et al.* [24] prepared water soluble chitosan derivatives with quaternary ammonium salt; *N*,*N*,*N*-trimethyl chitosan, *N*-propyl-*N*,*N*-methyl chitosan and *N*-furfuryl-*N*,*N*-dimethyl chitosan. These derivatives were prepared by the reaction between amino groups of chitosan and appropriated aldehydes to form Schiff base intermediate and then quaternized with methyl iodide (Scheme 2.2).



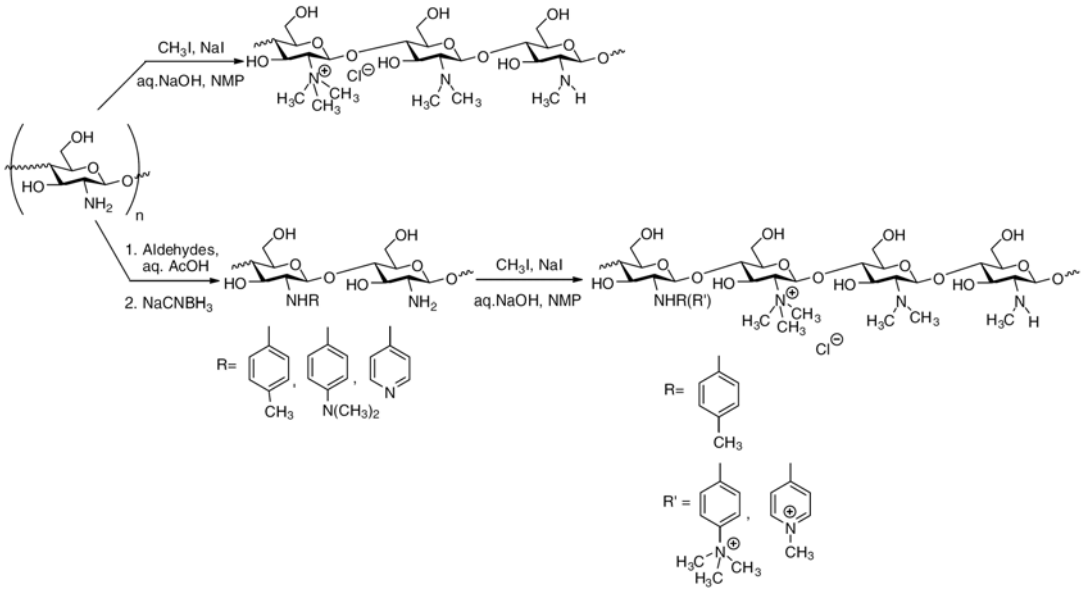
Scheme 2.3 The synthesis of quaternized *N*-alkyl chitosan

In 2003, Xu, *et al.* [25] synthesized *N*-(2-hydroxyl)propyl-3-trimethyl ammonium chitosan chloride (HTACC) which is a water-soluble derivative of chitosan (CS). It can be prepared by the reaction between glycidyltrimethylammonium chloride and CS (Scheme 2.3). HTACC nanoparticles have been formed based on ionic gelation process of HTACC and sodium tripolyphosphate (TPP).



Scheme 2.4 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium)propyl] chitosan chloride (HTACC)

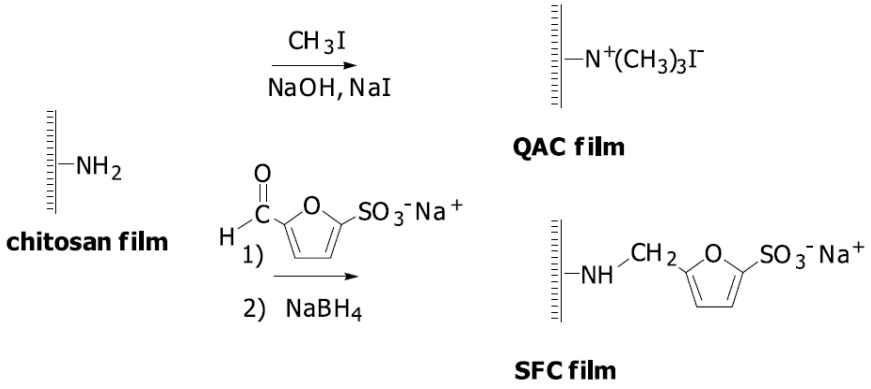
In 2008, Sajomsang *et al*. [26] reported the synthesis of *N*-arylated chitosan via Schiff bases formed by the reaction between the primary amino groups of chitosan with aromatic aldehydes followed by reduction of the Schiff base intermediates with sodium cyanoborohydride (Scheme 2.4). After that, *N*-arylated chitosan were treated with iodomethane under basic conditions. Finally, methylated *N*-alkylated chitosan was formed. The total degree of quaternization of chitosan derivatives depends on the extent of *N*-substitution and the sodium hydroxide concentration in methylation step.



Scheme 2.5 Synthesis of methylated chitosan and *N*-aryl chitosans

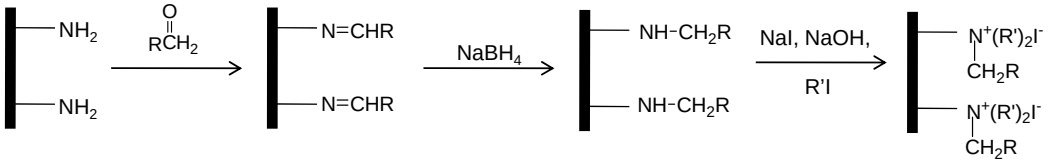
2.3.2 Heterogeneous Quaternization

In 2007, Hoven *et al*. [27] modified surface of chitosan film via methylation using methyl iodide (MeI) and reductive alkylation using 5-formyl-2-furan sulfonic acid (FFSA). The chitosan films having negative charges of *N*-sulfofurfuryl groups on their surface (SFC films) and having positive charges of quaternary ammonium groups on their surface (QAC films) exhibited selective protein adsorption against both negatively charged proteins (albumin and fibrinogen) and positively charged proteins (ribonuclease, lysozyme).



Scheme 2.6 Introduction of charged functional groups to the surface of chitosan

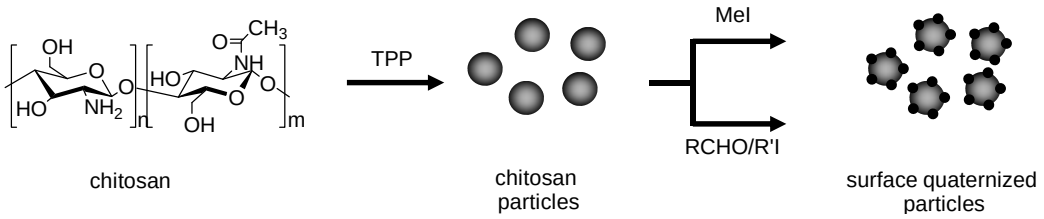
In 2006, Vallapa [28] modified chitosan surface by introducing quaternary ammonium groups via a heterogeneous two-step process: reductive alkylation using selected aldehydes followed by methylation with methyl iodide. The antibacterial activity of the surface-modified chitosan film against *Staphylococcus aureus* and *Escherichia coli* were superior to that of the virgin chitosan film. The additional positive charge and hydrophobicity introduced to the chitosan film after surface quaternization made the quaternary ammonium-containing chitosan film a more favorable substrate for interacting with the negatively-charged membrane of the bacteria.



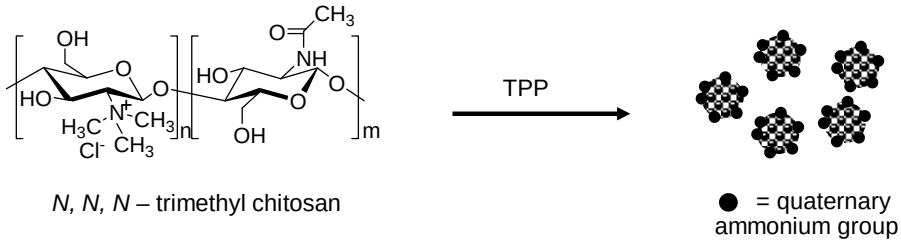
Scheme 2.7 Surface quaternization of chitosan film

In 2008, Wia-rachai [29] prepared quaternized chitosan particles using two methods. The first method was to prepare chitosan particles and their surface was then modified by heterogeneous quaternization through *N*-reductive alkylation with aldehyde followed by alkylation with alkyl iodide or direct methylation with methyl iodide (Scheme 2.7). The second method was to synthesize *N,N,N*-trimethyl chitosan by homogeneous quaternization using methyl iodide followed by particle formation (Scheme 2.8). Antibacterial activity tests against both a gram-positive bacteria, *Staphylococcus aureus*, and a gram negative bacteria, *Escherichia coli*, are performed

by turbidimetric method and viable cell counts. Minimum inhibitory concentration of some particles is also determined.



Scheme 2.8 Preparation of quaternary ammonium-containing chitosan particles by heterogeneous quaternization



Scheme 2.9 Preparation of quaternary ammonium-containing chitosan particles by homogeneous quaternization followed by particle formation

2.4 Interactions between DNA and Chitosan

In recent years, a few literatures reported the ability of the cationic form of chitosan to interact strongly with DNAs. In fact, electrostatic interaction in acid solution appears strong enough to form nanoparticles for gene delivery into cells [30]. Chitosan-DNA nanoparticles has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material for artificial skin and wound healing bandage applications [31].

In 2001, Mao *et al.* [30] prepared chitosan-DNA nanoparticles using an electrostatic interaction complex between amino groups of chitosan and phosphate groups of DNA. The chitosan-DNA nanoparticles could partially protect the encapsulated plasmid DNA from nuclease degradation as shown by electrophoretic mobility analysis. The zeta potential of these nanoparticles was in the range of +12 to +18 mV at pH lower than 6, and rapidly dropping to 0 at pH 7.2, which rendered the hydrophobicity of particles. Chloroquine could be co-encapsulated in the nanoparticles at 5.2%. This study suggested that chitosan is a versatile gene carrier with minimal

cytotoxicity. A stable and reproducible formulation has been obtained *via* surface modification of the nanoparticles.

The pK_a of the amino functional groups of chitosan makes it ideal for use in DNA extraction in a manner consistent with that described for surface charge switching. Chitosan, with pK_a of 6.3, requires a pH only in the 8.5-9.0 range to induce the DNA release state (deprotonation), delivering DNA in a PCR-ready state amenable to further processing. Recently, Cao *et al*. [32] has developed DNA purification on microchips using silica beads. A novel DNA solid-phase extraction protocol based on the pH-dependent charge of chitosan was developed specifically for low-volume DNA extraction on microchips. In this method, chitosan-coated beads could extract DNA at pH 5 and release it at pH 9. DNA extraction efficiency could be attained as high as 92%, even from complex samples such as human blood containing significant amounts of protein.

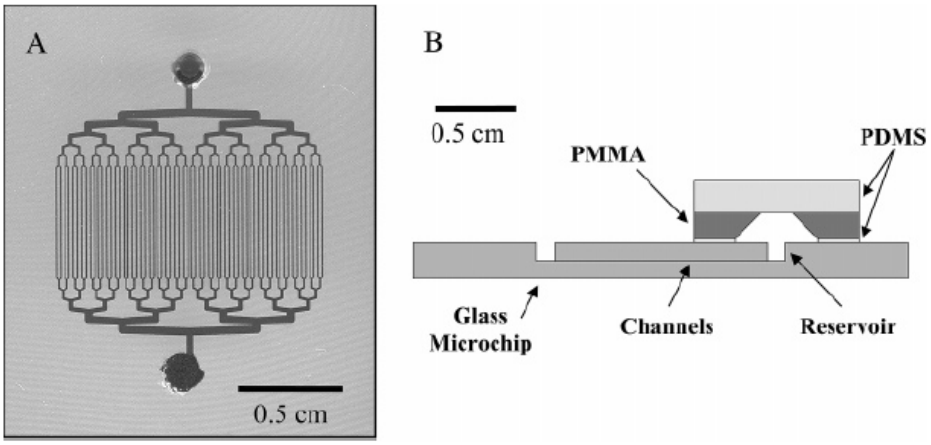


Figure 2.11 (A) Image of the high-density open channel microchip with a binary lamination design. The design contained 64 channels, each 0.5 cm. (B) Side view illustration of microchip and manual pressure device for flow generation

2.5 Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and

fragment when ionized by more conventional ionization methods. It is most similar in character to electrospray ionization both in relative softness and the ions produced (although it causes many fewer multiply charged ions). The ionization is triggered by a laser beam (normally a nitrogen laser). A matrix is used to protect the biomolecule from being destroyed by direct laser beam and to facilitate vaporization and ionization. The ionized sample and matrix fragments pass through an ion extractor which consists of a pair of electrodes. The separated ions then moved through a flight or drift tube where they will reach the detector. The peptide nucleic acid can be detected in positive ionization mode of which the protonated molecular ions ($M+H^+$) are usually the dominant species, although they can be accompanied by salt adducts, a trace of the doubly charged molecular ion at approximately half the *m/z* value, and/or a trace of a dimeric species at approximately twice the *m/z* value. The digitized data collected from the successive laser shots are summed and give out a mass spectrum based on the time-of-flight of the ions to reach the detector.

In the detection of PNA by MALDI-TOF MS, α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) was used as matrix. A solution of one of this molecule was prepared in 0.1% TFA in acetronitrile/water (1:1). The sample was dissolved in an appropriate volatile solvent at a concentration of ca. 10 pmol/µL and an aliquot (1-2 µL) of this was removed and mixed with an equal volume of a solution containing a vast excess of a matrix. The method based on MALDI-TOF MS is shown in Figure 2.12.

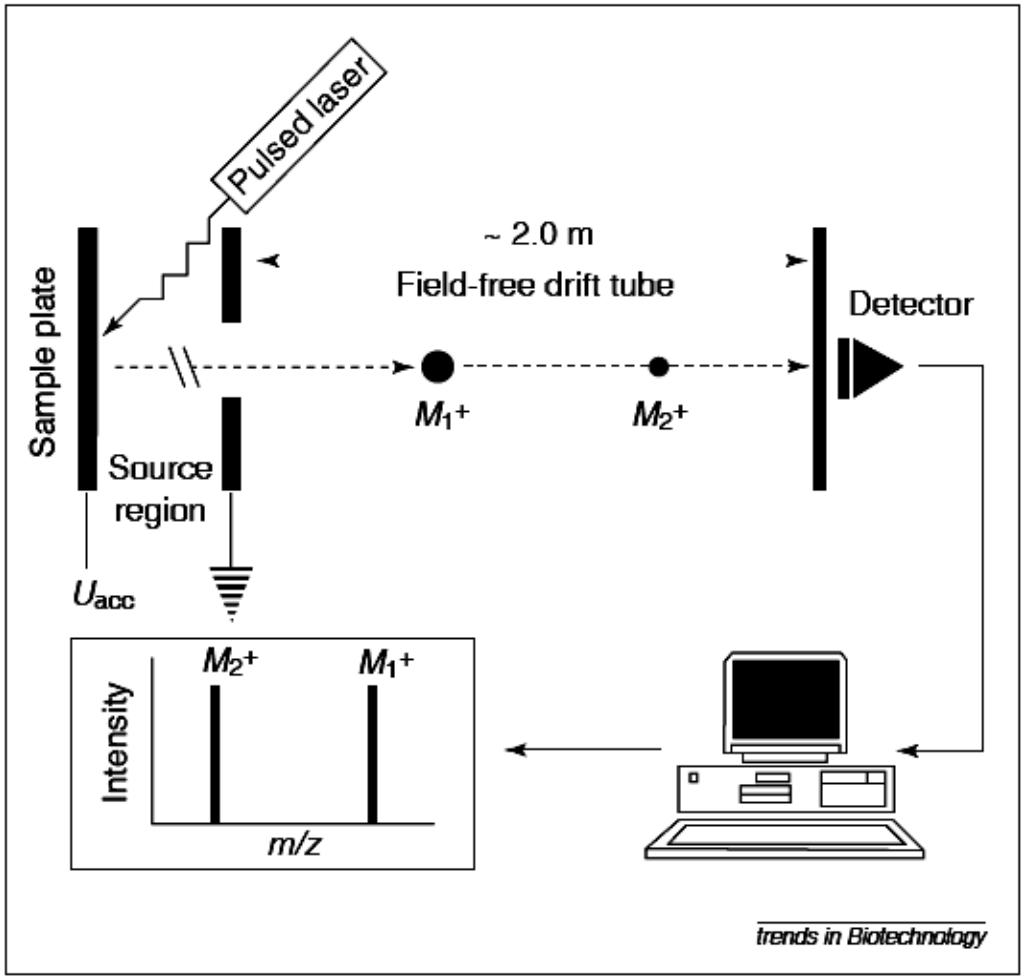


Figure 2.12 Schematic layout of the MALDI-TOF MS analysis

CHAPTER III

EXPERIMENTAL

3.1 Materials

Chitosan flake (DD 85%, M_V = 45,000 Da) was purchased from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Acetaldehyde, methyl iodide (MeI), sodium borohydride ($NaBH_4$), sodium hydroxide (NaOH), sodium iodide (NaI), and tripolyphosphate (TPP) were obtained from Fluka (Switzerland). Acetic acid, benzaldehyde, *N*-methylpyrrolidone (NMP), and methanol (MeOH) were purchased from Merck (Germany). DNA *Escherichia coli* bacteria strain B type VIII was bought from Sigma (USA). 9-flurenylsuccinimidyl carbonate were purchased from Aldrich. Oligonucleotides were purchased from Bioservice Unit, National Science and Technology Development Agency (Thailand). Hydrogen gas were obtained from Thai Industrial Gas (TIG) with high purity up to 99.5 %. All reagents and materials are analytical grade and used without further purification. 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 Equipment

3.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H NMR spectra were recorded in solution of CF_3COOH/D_2O or D_2O using a Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Fourier Transform-Infrared Spectroscopy (FT-IR)

IR spectra were collected using a Nicolet Impact 410 FT-IR spectrometer, with 32 scans at resolution 4 cm^{-1}. A frequency of 400-4000 cm^{-1} was collected by using TGS detector. All samples were prepared as KBr pellets.

3.2.3 Transmission Electron Microscopy (TEM)

The size and morphology of chitosan and quaternized chitosan particles were examined by transmission electron microscope (TEM, Model JEM-2100, Japan). The average diameter was reported from measurement of 100 random particles for each sample using Semafore software.

3.2.4 Photon Correlation Spectroscopy (PCS)

The size and ζ-potential of chitosan particles were determined using a Nanosizer Nano-ZS (Malvern Instruments, UK). The particles (~10 mg) were first dispersed in 20 mL of Milli-Q water by sonication for 3 min prior to measurement. The analysis was performed at 25°C using a scattering angle of 173°. All data are displayed as the mean ± one standard deviation and are derived from at least three independent experiments. The data were calculated using the Helmholtz-Smoluchowski equation.

3.2.5 UV-Vis Spectrophotometry

UV-Vis absorption spectra were performed by UV-Vis spectrophotometer Model FSPECGD Techna, specgene at absorbance 260 nm.

3.2.6 MALDI-TOF Mass Spectrophotometry

MALDI-TOF mass spectra of PNA were collected on Microflex MALDI-TOF mass spectrometry (Bruker Daltonics, Germany). Sample (1 μL) was mixed with 10 μL of the matrix solution consisting of α-cyano-4-hydroxycinnamic acid (CCA) in 0.1% TFA in acetonitrile/water (1:1) solution. This mixture (1 μL) was 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.

3.3 Methods

3.3.1 Preparation of Chitosan (CS) Particles

Chitosan flakes (0.2 g) were dissolved in 1% (v/v) acetic acid (100 mL) to prepare a 0.2% (w/v) chitosan solution and pH of the solution was raised to 4.6-4.8 with 10 N NaOH. CS particles were formed spontaneously upon an addition of aqueous TPP

solution (1.01 mM) 3 mL to 3 mL of chitosan solution under magnetic stirring. These formed particles were isolated by centrifugation at 12,000 rpm using a high-speed refrigerated centrifuge (Model GL21M, Yingtai Instrument, China) for 30 min interval. Supernatant was discarded and the particles were extensively rinsed with distilled water (3×). The final product was obtained after lyophilization by a freeze dryer (Labconco Corporation freeze dryer model 7753501).

3.3.2 Preparation of *N*-benzyl Chitosan Particles

An anhydrous methanol solution of benzaldehyde (10 mL) having a desired concentration was added into a flask containing chitosan particles (0.03 g). After stirring for a given time at ambient temperature, $NaBH_4$ (0.3 g, 0.8 mol) was added into the reaction mixture and the solution was stirred for 24 h at ambient temperature. After the reaction completed, the modified particles were isolated by centrifugation at 12,000 rpm for 30 min. Supernatants were discarded and the particles were extensively rinsed with methanol and dried *in vacuo*.

3.3.3 Preparation of Methylated *N*-benzyl Chitosan (MBzC) Particles

An anhydrous methanol solution of NaI (0.2 M, 10 mL) was added into a flask containing *N*-benzyl chitosan particles (0.03 g) and NaOH (0.13 g, 0.3 mol). MeI (1.25 mL) was added via syringe. Then, the reaction mixture was stirred at 50°C for 12 h. Finally, the prepared MBzC particles were isolated by centrifugation at 12,000 rpm for 30 min. Supernatant was discarded and these particles were extensively rinsed with methanol (3×) and dried *in vacuo.*

3.3.4 Preparation of Methylated Chitosan (MC) Particles

An anhydrous methanol (10 mL) was add into a flask containing chitosan particles 0.05 g. NaOH (0.13 g, 3.3 mmol) and NaI (0.28 g, 1.9 mmol) were added to the flask. Subsequently, MeI (0.4 mL, 6.4 mmol) was added every 4 h to the mixture. The reaction mixture was stirred at 50°C for 8 h. The synthesized MC particles were isolated by centrifugation at 10,000 rpm for 30 min. Supernatant was discarded and the particles were extensively rinsed with methanol and dried *in vacuo.*

3.3.5 Preparation of *N,N,N*-Trimethyl Chitosan (TMC) Particles

TMC was synthesized according to a modified method of Domard *et al.* [23]. Chitosan (2.0 g) was dispersed in 80 mL of NMP followed by adding NaOH (0.81 g, 0.02 mol) and NaI (1.51 g, 0.01 mol). MeI (4.32 g, 0.03 mol) was added every 4 h. The reaction mixture was stirred at 40°C for 8 h under magnetic stirring. The final product was precipitated using acetone and isolated by centrifugation at 12,000 rpm for 30 min. The solution was then precipitated using acetone. The precipitate was dissolved in 15.0% (w/v) NaCl solution for 4 h in order to replace the iodide counterion with a chloride counterion. The suspension was dialyzed against deionized water in a dialysis bag at ambient temperature for 5 days to remove residual small molecules. The final product was obtained after lyophilization by a freeze dryer (model Freezeone 77520 Benchtop, Labconco, USA).

To prepare TMC particles, TMC (0.2 g) was dissolved in deionized water (100 mL) to prepare a 0.2% (w/v) TMC solution. The particles were formed spontaneously upon an addition of an aqueous TPP solution of 3 mL (1.01 mM) to 3 mL of *N,N,N*-trimethyl chitosan solution under magnetic stirring. The formed particles were isolated by centrifugation at 12,000 rpm for 30 min interval. Supernatant was discarded. These particles were centrifugally washed with deionized water (3×) and then lyophilized before further use or analysis.

3.3.6 Preparation of *N*-[(2-hydroxyl-3-trimethylammonium)propyl] Chitosan Chloride (HTACC) Particles by Homogeneous Quaternization

HTACC was synthesized according to a modified method of Seong *et.al*. [33]. Chitosan (~5 g) was dissolved in 1%v/v acetic acid at ambient temperature. GTMAC (15.3 g, 4 equiv) was then added into the chitosan solution. The reaction was performed at 70°C for 24 h. After the reaction, products were dialyzed with deionized water for three days, followed by freeze-drying to obtain a cotton-like material and then dissolved in deionized water overnight. HTACC particles were formed spontaneously upon an addition of an aqueous TPP solution of 3 mL (6.06 mM) to 3 mL of HTACC solution under magnetic stirring. The obtained HTACC particles were isolated by centrifugation at 12,000 rpm for 30 min interval. Supernatant was discarded and the particles were

extensively rinsed with distilled water (3×) and then lyophilized before further use or analysis.

3.3.7 Preparation of *N*-[(2-hydroxyl-3-trimethylammonium)propyl] Chitosan Chloride (HTACC) Particles by Heterogeneous Quaternization

The chitosan particles (0.05 g) were dispersed in 10 mL deionized water. GTMAC (30.3 g, 0.2 mol) was added and the mixture was stirred for 2h. The reaction temperature was set at 80°C. The particles were then isolated by centrifugation at 12,000 rpm for a 30 min. Supernatant was discarded and the particles were extensively rinsed with methanol (3×) and dried *in vacuo.*

3.3.8 Investigation of Adsorption Efficiency of *E.coli* DNA on Particles by UV-Vis Spectroscopy

The *E.coli* DNA (0.07 mg/mL) was mixed with 800 μL sodium phosphate buffer solution (pH 7). The absorbance of the mixture was measured by UV-Vis spectroscopy at 260 nm at ambient temperature. Thereafter, the particles (1 mg) were added to DNA solution. After the mixture was incubated for 20 min, it was centrifuged in order to separate particles from the DNA solution. The absorbance of DNA solution was measured by UV-Vis spectroscopy. The adsorption efficiency can be calculated from equation 3.1.

$$[\mathrm{DNA}]^{*}_{\mathrm{particles}} = [\mathrm{DNA}]_{\mathrm{before}} - [\mathrm{DNA}]_{\mathrm{after}} \quad (3.1)$$

where, $[\mathrm{DNA}]^{*}_{\mathrm{particles}}$ is the concentration of DNA adsorbed on particles

$[\mathrm{DNA}]_{\mathrm{before}}$ is the concentration of DNA before adding particles

$[\mathrm{DNA}]_{\mathrm{after}}$ is the concentration of DNA after adding particles

The $[\mathrm{DNA}]_{\mathrm{after}}$ can be determined based on a calibration curve generated from the solution having known concentration of DNA as shown in equation 3.2.

$$Y=16.37X - 0.003 \quad (3.2)$$

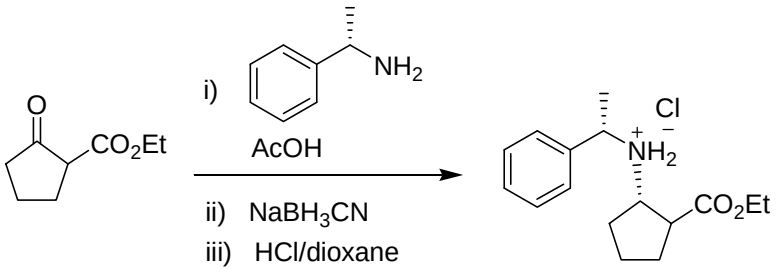
where X is DNA concentration, Y is the absorbance and $R^2 = 0.9977$

3.3.9 Synthesis of *acpc*PNA Monomer

The *acpc*PNA monomers of thymine, guanine, adenine, and cytosine were synthesized as described previously [34-36]. by Dr. Boonjira Boontha, Dr. Cheeraporn Ananthanawat, Miss. Paethong Srikaenjun, and Miss Chaloyorn Bonlua.

3.3.10 Synthesis of trans-(1S,2S)-2-aminocyclopentanecarboxylic acid (acpc) spacer

a) Ethyl (1S,2S)-2-[(1'S)-phenylethyl]-minocyclopentanecarboxylate hydrochloride (1)



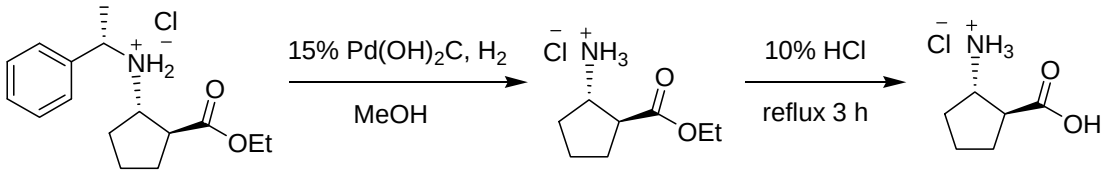
(1)

Ethyl cyclopentanone-2-carboxylate (4.0 mL, 27 mmol) was dissolved in absolute ethanol (32 mL) following by (S)-(-)-α-methylbenzylamine (3.48 mL, 30.0 mmol) and glacial acetic acid (1.54 mL, 32.0 mmol). The reaction mixture was stirred at room temperature for 2 hours to form the enamine intermediate which was monitored by TLC (7:3 hexanes/EtOAc). Next, the reaction mixture was heated to 72°C and then sodium cyanoborohydride (3.14 g, 54.0 mmol) was slowly added over a period of 5 hours. The reaction was left at room temperature overnight. When the reaction was completed as monitored by TLC (7:3 hexanes/EtOAc, $KMnO_4$), the ethanol was removed by rotary evaporation to form a white sticky residue. Water (20 mL) was added to the residue and the pH was adjusted to 7 by adding solid $NaHCO_3$. The mixture was extracted with dichloromethane (3 × 20 mL). The dichloromethane was removed by rotary evaporation to give a colorless liquid. The crude product was dissolved in 15 mL of ethyl acetate at 4°C. A freshly prepared 4N HCl in dioxane (31 mL, prepared by slowly adding 8.50 mL of acetyl chloride to a mixture of 7.02 mL ethanol and 14.58 mL dioxane) was then added. The flask was left overnight in refrigerator to allow complete precipitation of the product. After filtration, the crude product in the form of HCl salt was obtained as a white solid. This material was further

purified by repeated recrystallization from EtOH until a single isomer was obtained according to ^{1}H NMR analysis.

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.21 (3H, t, $CO_2CH_2C\underline{H}_3$), 1.59 (3H, d, $^+NH_2CHC\underline{H}_3$), 1.75 (2H, m, $CH_2C\underline{H}_2CH_2$), 1.93 (2H, m, $C\underline{H}_2CHCO$), 1.99 (2H, m, $^+NH_2CHC\underline{H}_2$), 2.33 (1H, m, $C\underline{H}CO_2Et$), 3.41 (1H, t, $^+NH_2C\underline{H}CH_2$), 3.58 (1H, q, $^+NH_2C\underline{H}CH_3$), 4.15 (2H, m, $CO_2C\underline{H}_2CH_3$), 7.41-7.63 (5H, m, phenyl). The optical rotation also corresponded to the authentic sample prepared previously in this laboratory {$[\alpha]^{25}_D$}= +17.1 (c = 1.00 g/100 mL $CDCl_3$).

b) Ethyl (1S,2S)-2-amino-cyclopentanecarboxylate hydrochloride (3)

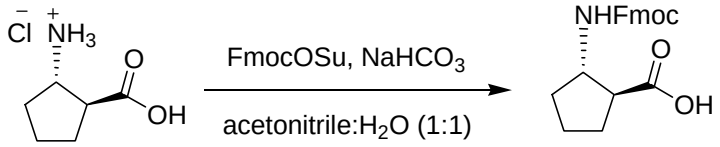


(1) (2) (3)

Ethyl (1S,2S)-2-[(1′S)-phenylethyl]-aminocyclopentane carboxylate hydrochloride (1) (0.904 g, 3.03 mmol) was dissolved in methanol (5 mL). Palladium hydroxide on charcoal (135.6 mg) was added and the reaction mixture was stirred at room temperature under H_2 atmosphere overnight. The completion of the hydrogenolysis was monitored by TLC (1:3 hexanes/EtOAc). The palladium catalyst was removed by filtration. The filtrate was evaporated by rotary evaporation to obtain compound (2) as brown oil. Without further purification, the compound (2) was refluxed with 10% HCl (10 mL) at 110 $^\circ$C for 3 hours. The mixture was allowed to cool down and the solvent was removed by rotary evaporation to obtain the title compound (3) as colorless oil, which solidified to a white solid (0.151 g) after stirring with acetone.

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.50-1.68 [m, 2H, $CH_2C\underline{H}_2CH_2$], 2.02 [m, 4H, $C\underline{H}_2$ $CH_2C\underline{H}_2$], 2.78 [q, 1H, $C\underline{H}CO_2H$], 3.75 [m, 1H, $CH_2C\underline{H}NH_3^+$].

c) (1S,2S)-2-(N-Fluoren-9-ylmethoxycarbonyl)-aminocyclopen tanecar-boxylic acid (4)

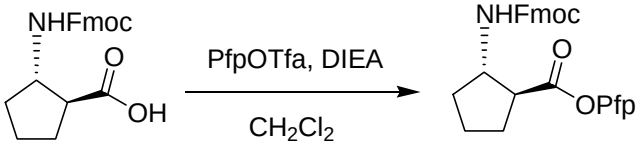


(3) (4)

The (1S,2S)-2-aminocyclopentane carboxylic acid hydrochloride (3) (0.511 g, 0.914 mmol) was dissolved in water (5 mL). $NaHCO_3$ (2.5 equiv excess) was added until the pH of the solution was ~ 8. Acetonitrile (5 mL) was added until a homogeneous solution was obtained. Next, FmocOSu (1.2 equiv) was slowly added with stirring at room temperature. The stirring was continued for 8 h while maintaining the pH at ~ 8 by addition of NaHCO3. The mixture was diluted with water (20 mL) and extracted with diethyl ether (3 × 20 mL). The aqueous phase was collected and the pH was adjusted to 2 with concentrated HCl. The white suspension was extracted with dichloromethane (3 × 20 mL). The solvent was removed by rotary evaporation and the residue was dried in vacuum to afford the title compound (4) as a white solid 0.181 g.

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 1.30-1.45 (2H, m, $CH_2C\underline{H}_2CH_2$), 1.80-2.10 (4H, m, $C\underline{H}_2CH_2C\underline{H}_2$), 2.70 (1H, q, C<u>H</u>COOH), 4.08 (1H, m, NHC<u>H</u>), 4.41-4.49 (2H, m, Fmoc aliphatic C<u>H</u> and Fmoc aliphatic $C\underline{H}_2$) and 7.22-7.79 (8H, m, Fmoc aromatic C<u>H</u>). The optical rotation also corresponded to the authentic samples prepared previously in this laboratory {$[\alpha]^{25}_D$ = + 36.4, c = 1.00g/100mL MeOH)}.

d) (1S,2S)-2-(N-fluoren-9-ylmethoxycarbonyl)-aminocyclopentane carboxylic pentafluorophenyl ester (5)



(4) (5)

To a suspension of (1S,2S)-2-(N-fluoren-9-ylmethoxycarbonyl)-aminocyclo pentanecarboxylic acid (4) and DIEA (0.71 mL) in dichloromethane (5 mL) was added PfpOTfa (0.71 mL). The resulting mixture was stirred for 2 h. The reaction was completed as indicated by TLC analysis and purified by flash column chromatography on silica gel eluting with hexanes/EtOAc (4:6) to obtain the title compound (5) as a

white solid (0.221g). The Pfp-ACPC spacer (5) was synthesized and purified (83% yield). After column chromatography and recrystallization from hexane, the Pfp-ACPC spacer (5) was obtained as a white solid

^{1}H NMR (400 MHz; $CDCl_3$): δ_H 1.68 [m, 1H; ring C<u>H</u>] 1.87 [m, 2H; ring C<u>H</u>] 2.10 [m, 1H; ring C<u>H</u>] 2.23 [m, 2H; ring C<u>H</u>] 3.11 [m, 1H; C<u>H</u>CO] 4.26 [t, ^{3}J(H,H) = 6.8 Hz, 1H; Fmoc C<u>H</u>] 4.38 [m, 1H; C<u>H</u>NH] 4.48 [d, ^{3}J(H,H) = 6.8 Hz, 2H; Fmoc C<u>H</u>$_2$] 4.98 [d ^{3}J(H,H) = 5.6 Hz, 1H; N<u>H</u>] 7.34 [m, 2H; Fmoc Ar C<u>H</u>] 7.42 [m, 2H; Fmoc Ar C<u>H</u>] 7.62 [d, ^{3}J(H,H) = 7.2 Hz, 2H; Fmoc Ar C<u>H</u>] 7.80 [d ^{3}J(H,H) = 7.2 Hz, 2H; Fmoc Ar C<u>H</u>];, $[\alpha]^{25}_{D}$ = +51.4 (c = 1.00 g/100 mL, $CHCl_3$)

3.3.11 Synthesis of acpcPNA Oligomers

In this work, three PNA oligomers (PNA_1, PNA_2, PNA(mut), and PNA(wt)) were successfully synthesized following the standard procedure previously developed for this class of PNA in our laboratory by Dr. Boonjira Boontha, Dr. Cheeraporn Ananthanawat, and Mrs. Chotima Vilaivan. The crude PNA oligomers were purified by reverse phase HPLC. After the purification, the solution of purified PNA was freeze dried and the identity of the PNA oligomers was again verified by MALDI-TOF mass spectrometry.

3.3.12 Determination of PNA·DNA Hybridization

PNA·DNA hybridization was determined based on a previously reported procedure [7]. Sequences of PNA and DNA used in this research are shown in Table 3.1. The PNA probe (10 pmol) and DNA sample (10 pmol) were mixed in 30 µL of binding buffer (10 mM sodium phosphate, pH 7) and incubated at ambient temperature for 20 min. The designated chitosan particles were then added to the PNA·DNA hybrid solution, incubated for 20 min at ambient temperature, and then centrifugally washed with 3×200 µL of Milli-Q water. Finally, 1 µL aliquots of washed particles were collected for analysis of the adsorbed PNA by MALDI-TOF mass spectrometry.

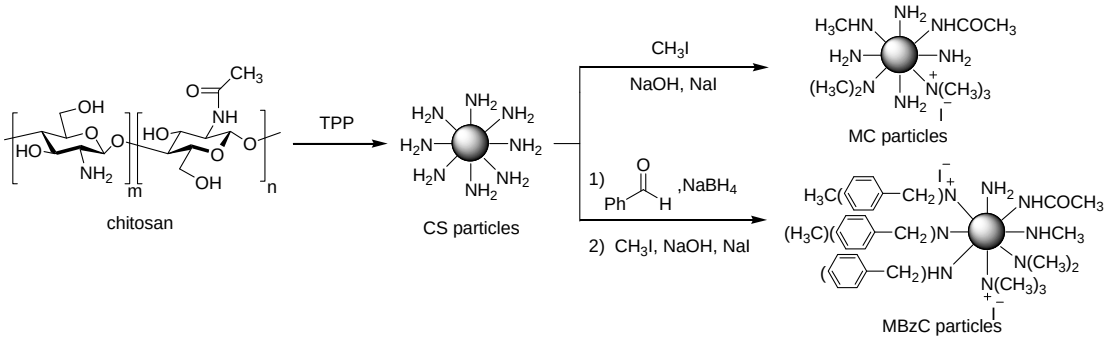
Table 3.1 PNA and DNA sequences used in this study

System	Code	Sequence	note
1	PNA_1	Ac-TTTTTTTTT-$LysNH_2$	-
	PNA_2	Ac-AAAAAAAAA-$LysNH_2$	-
	DNA_{1c}	d(5'-AAAAAAAAA-3')	complementary to PNA_1
	DNA_{1s}	d(5'-AAAACAAAA-3')	single mismatch to PNA_1
	DNA_{1f}	d(5'-AGTGATCTAC-3′)	full mismatch to PNA_1
2	PNA(mut)	Ac-Lys-O-CTACGCCAACAGCT-$SerNH_2$	-
	PNA(wt)	Ac-Lys-CTACGCCACCAGCT-$SerNH_2$	-
	DNA(mut)	d(5'-AGCTGTTGGCGTAG-3')	complementary to PNA(mut) single mismatch to PNA(wt)
	DNA(wt)	d(5'-AGCTGGTGGCGTAG-3')	complementary to PNA(wt) single mismatch to PNA(mut)
	DNA(J1)	d(5'-TAGTTGTACGTACA-3′)	full mismatch to both PNA(mut) and PNA(wt)

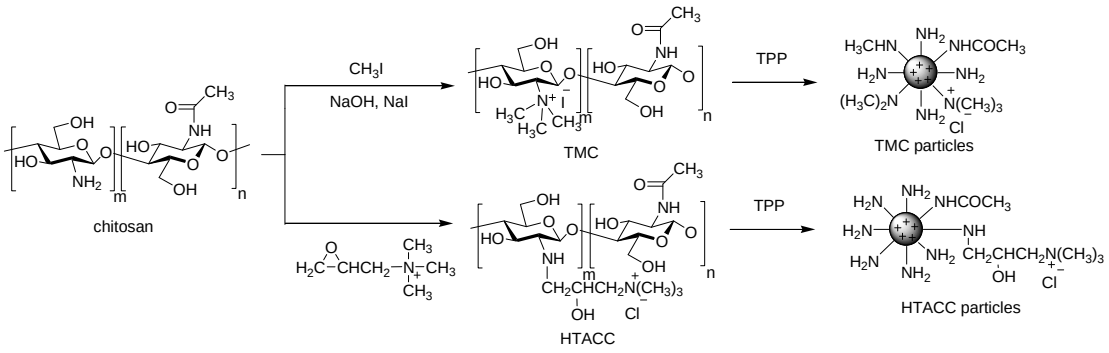
CHAPTER IV

RESULTS AND DISCUSSION

This chapter is divided into two parts. The first part focuses on the preparation and characterization of quaternized chitosan particles namely methylated chitosan (MC) particles, methylated *N*-benzyl chitosan (MBzC) particles, *N*,*N*,*N*-trimethylchitosan (TMC) particles, and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) particles. Two routes were used for particle formation; heterogeneous and homogeneous routes. The heterogeneous route (Scheme 4.1) is based on heterogeneous chemical modification of ionically crosslinked chitosan (CS) particles whereas the homogeneous route (Scheme 4.2) relies on the homogeneous chemical modification of chitosan followed by particle formation via ionic crosslinking. The second part is dedicated to sequence determination of DNA using different types of quaternized chitosan particles by MALDI-TOF mass spectrometry.



Scheme 4.1 Preparation of quaternized chitosan particles by heterogeneous route

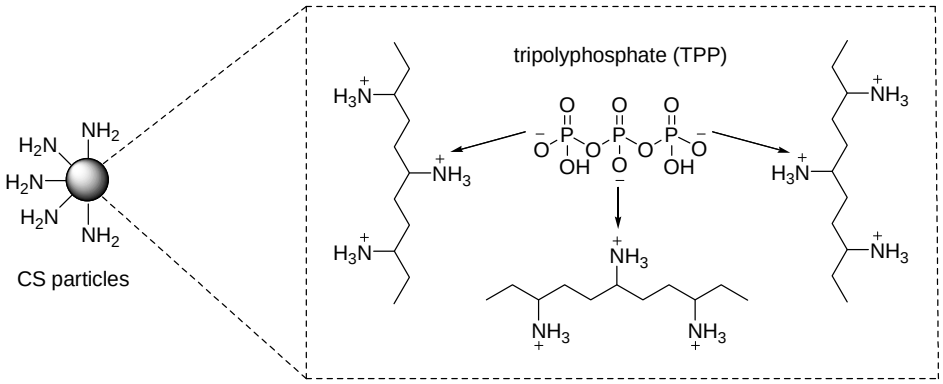


Scheme 4.2 Preparation of quaternized chitosan particles by homogeneous route

4.1 Preparation and Characterization of Quaternized Chitosan Particles

4.1.1 Quaternized Chitosan Particles Prepared by Heterogeneous Route

To prepare quaternized chitosan particles, chitosan (CS) particles were first formulated by ionic gelation between positively charged chitosan and negatively charged tripolyphosphate (TPP) at ambient temperature. Ionic crosslinking is schematically shown in Scheme 4.3. The CS particles obtained in this experiment appeared as white fine powders which are insoluble in water, dilute acidic and alkali solution.



Scheme 4.3 Ionic crosslinking between chitosan and TPP

FT-IR analysis of the CS particles was performed to determine their chemical functionalities in comparison with those of the pristine chitosan. As shown in Figure 4.1, there is a characteristic peak at 1650 cm^{-1} that can be assigned to C=O stretching existing in both chitosan and CS particles. The appearance of the peak at 1204 cm^{-1} assigned to P=O groups of TPP and the shifting of the N-H bending signal from 1604 cm^{-1} of chitosan to 1543 cm^{-1} of CS particles implied that the ammonium groups were crosslinked with TPP molecules.

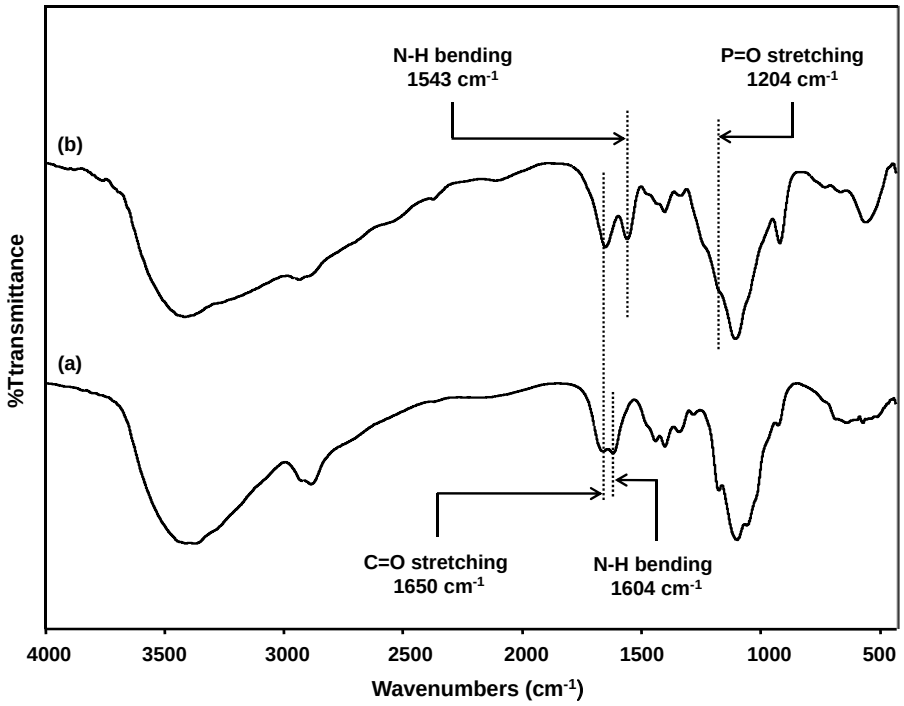
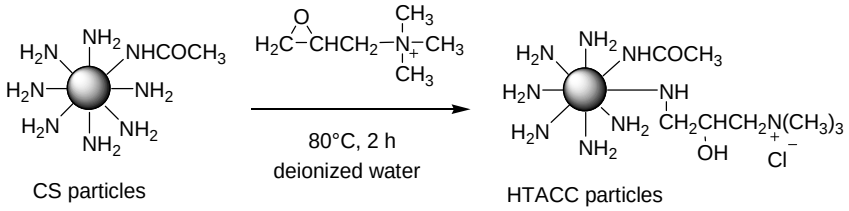


Figure 4.1 FT-IR spectra of (a) chitosan and (b) CS particles

There are three types of quaternized chitosan particles prepared via heterogeneous route. They are *N*-[(2-hydroxyl-3 trimethylammonium)propyl]chitosan chloride (HTACC) particles, methylated chitosan (MC) particles, and methylated *N*-benzyl chitosan (MBzC) particles.

HTACC particles can be prepared by epoxide ring opening of GTMAC by functional groups on the surface of chitosan particles. In principle, amino groups of chitosan are more nucleophilic than hydroxyl groups under neutral condition used in this experiment so the epoxide ring should be majorly opened by the amino groups (Scheme 4.4) [37].



Scheme 4.4 Preparation of HTACC particles by heterogeneous reaction

According to ^{1}H NMR spectra illustrated in Figure 4.2, a strong peak at 2.8 ppm indicated the presence of methyl groups in the quaternary ammonium salt groups ($N(CH_3)_3^+$) in the obtained product [38].

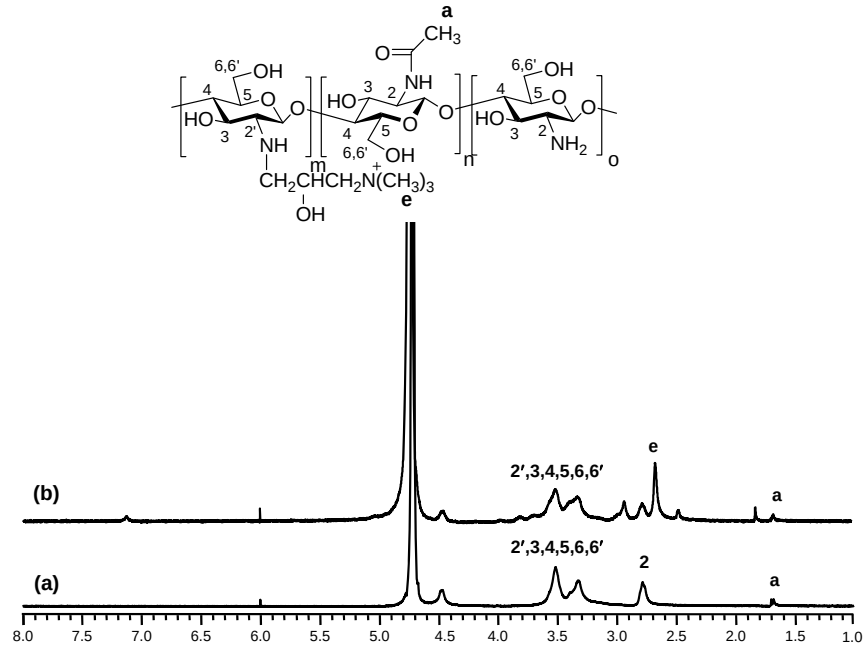
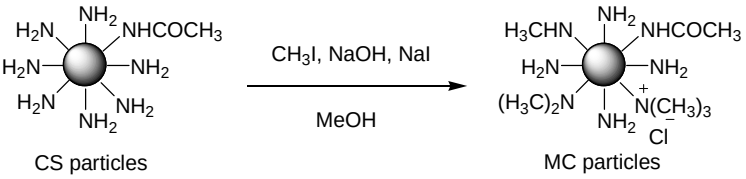
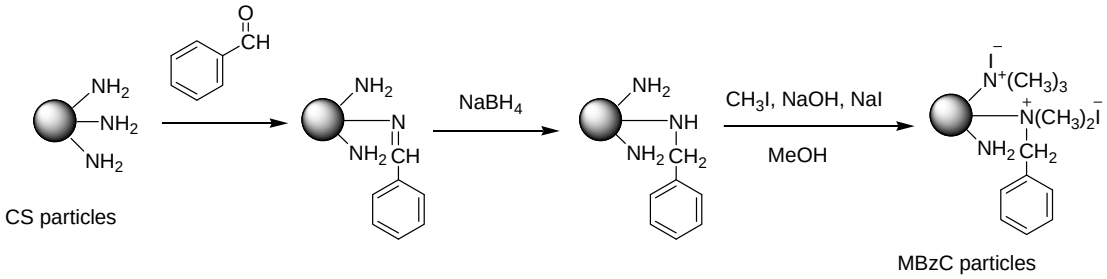


Figure 4.2 ^{1}H NMR spectra of (a) CS and (b) HTACC particles prepared by heterogeneous reaction

In the case of MC particles, quaternization was directly conducted on CS particles using methyl iodide (MeI) (Scheme 4.5). On the other hand, MBzC particles were prepared by heterogeneous reaction through 2-step process: *N*-reductive alkylation with benzaldehyde followed by methylation with MeI (Scheme 4.6). The optimized conditions for the preparation methylated *N*-benzyl chitosan (MBzC) particles were previously identified by Vallapa *et al.* [28].



Scheme 4.5 Preparation of MC particles by heterogeneous reaction



Scheme 4.6 Preparation of MBzC particles by heterogeneous reaction

FT-IR spectra of MC and MBzC particles are shown in Figure 4.3. As compared with the CS, the FT-IR spectra of both particles have a characteristic peak at 1204 cm^{-1} which can be assigned to P=O groups of TPP and the shifting of the N-H bending signal from 1604 cm^{-1} of chitosan to 1543 cm^{-1} indicating the presence of ionic crosslinking in their structure. In addition, the decrement of the N-H bending signal at 1543 cm^{-1} and the appearance of C-N stretching at 1460-1470 cm^{-1} in the spectra of MC and MBzC particles also demonstrated the success of heterogeneous quaternization of CS particles.

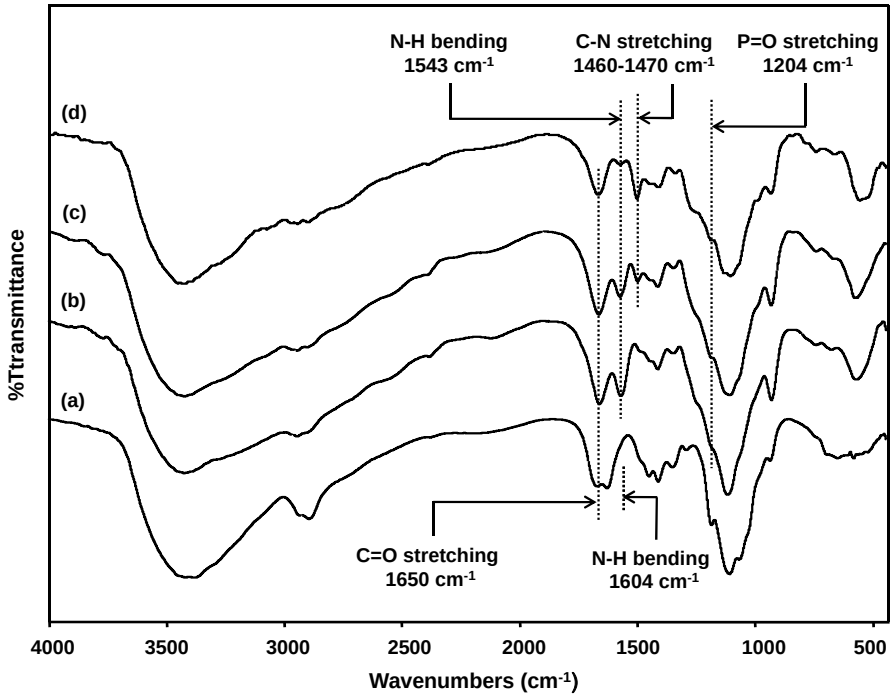


Figure 4.3 FT-IR spectra of (a) chitosan, (b) CS, (c) MC, and (d) MBzC particles

As presented in Figure 4.4, the ^{1}H NMR spectra of both MC and MBzC particles show the signal at 2.9-3.0 ppm which belongs to the methyl protons of the quaternary ammonium group while the signal at 2.7 ppm can be assigned to the methyl protons of the disubstituted amino groups. Besides, in the case of MBzC particles, the signal of aromatic proton at 7.2 ppm evidently confirms the attachment of benzyl groups at the amino moieties of chitosan.

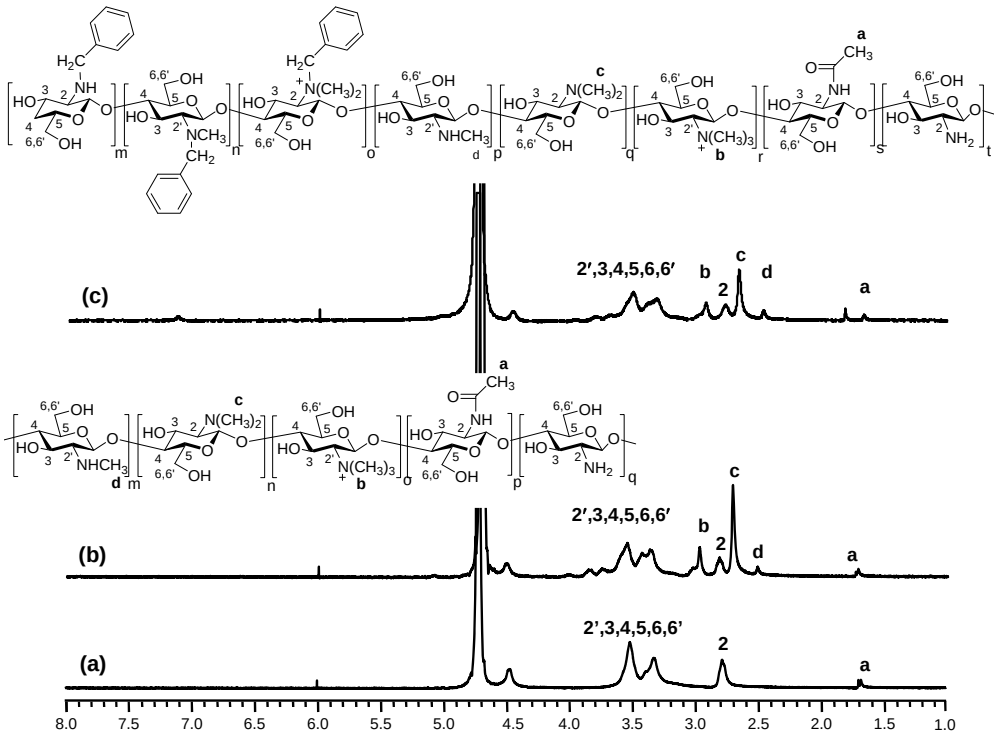


Figure 4.4 ^{1}H NMR spectra of (a) CS, (b) MC, and (c) MBzC particles

Degree of substitution of all three particles prepared by heterogeneous route can be deduced from ^{1}H NMR data. In the case of HTACC particles, the degree of quaternization (% DQ) can be determined from the relative ratio between the integration of 9 protons from 3 methyl groups ($-N^{+}(CH_3)_3$) at δ ~2.8-2.9 ppm and the peak integration of 6 protons of chitosan (δ ~3.0-3.7 ppm) following equation 4.1.

$$\text{Degree of quaternization} \quad (\%DQ_{N^{+}(CH_3)_3}) = \left[\frac{\int N^{+}(CH_3)_3 / 9}{\left(\int H-2',3,4,5,6,6' / 6\right) \times DD}\right] \times 100 \qquad (4.1)$$

For the MC particles, the degree of quaternization or degree of tri-substitution of methyl groups ($-N^{+}(CH_3)_3$) on TMC and TMC particles can be determined from the relative ratio between the integration of 9 protons from 3 methyl groups ($-N^{+}(CH_3)_3$) having signal at δ ~2.8-3.0 ppm and the peak integration of 6 protons of chitosan signal at δ ~3.2-3.8 ppm, assigned to $H_{2',3,4,5,6,6'}$ by using equation 4.2. Degree of di-substitution ($-N(CH_3)_2$) can be calculated from the relative ratio between the integration of 6 protons from 2 methyl groups having signal at δ ~2.4-2.6 ppm and 6 protons of chitosan signal at δ ~3.2-3.8 ppm, assigned to $H_{2',3,4,5,6,6'}$ following equation 4.3. Degree of mono-substitution ($-NH(CH_3)$) can be determined from the relative ratio between the integration of 3 protons from a methyl group of TMC and TMC particles signal at δ

~2.3-2.4 ppm and the peak integration of 6 protons of chitosan signal at δ ~3.2-3.8 ppm, assigned to $H_{2',3,4,5,6,6'}$ using equation 4.4.

$$\text{Degree of trimethylation} \quad (\%DS_{N^+(CH_3)_3}) = \left[\frac{\int N^+(CH_3)_3 / 9}{\left(\int H-2',3,4,5,6,6' / 6\right) \times DD}\right] \times 100 \qquad (4.2)$$

$$\text{Degree of dimethylation} \quad \left(\%DS_{N(CH_3)_2}\right) = \left[\frac{\int N(CH_3)_2 / 6}{\left(\int H-2',3,4,5,6,6' / 6\right) \times DD}\right] \times 100 \qquad (4.3)$$

$$\text{Degree of monomethylation} \quad \left(\%DS_{NHCH_3}\right) = \left[\frac{\int NHCH_3 / 3}{\left(\int H-2',3,4,5,6,6' / 6\right) \times DD}\right] \times 100 \qquad (4.4)$$

In the case of MBzC particles, the estimation of degree of substitution was simplified by assuming that the majority of quaternary ammonium groups existed in the form of $-N^+(CH_3)_3$ and presented in the form of $-NR^+(CH_3)_2$, when substituted groups (R) were benzyl groups. This assumption is based on the fact that the non-benzylated or free amino groups ($-NH_2$) left after the step of *N*-reductive benzylation are less bulky, although more electronically reactive than the benzylated amino group (-NHR) towards nucleophilic substitution of MeI. Such characteristic should favor the formation of $-N^+(CH_3)_3$ as opposed to that of the $-NR^+(CH_3)_2$. Similar assumption was also applied for mono- and di-substitution. As a result, the degree of mono-, di- and tri-substitution of MBzC particles can be calculated by using equations 4.2, 4.3, and 4.4, respectively.

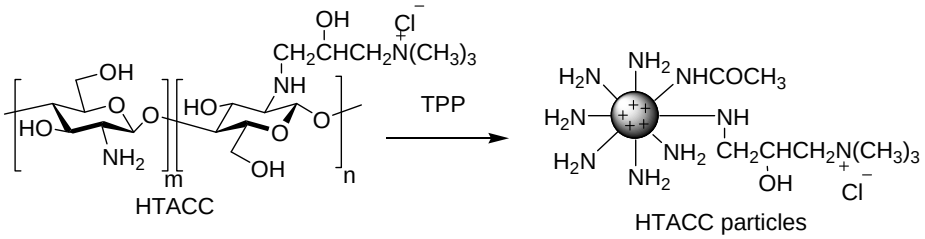
As shown the Table 4.1, the data indicate total degree of substitution ($\%DS_{total}$) was in a range of ~26-92%. The degree of di-substitution possessed higher value than those of mono- and tri-substitution. Among all particles, HTACC particles exhibited the highest degree of tri-substitution (quaternization). However, it should be emphasized that the quaternized particles were not completely dissolved in D_2O/TFA, the solvent used for ^1H NMR analysis. The data obtained from ^1H NMR analysis may not reflect the actual degree of substitution. The values can only be used as semi-quantitative measure of the extent of substitution. It should also be noted that the HTACC particles turned dark brown after quaternization. It was also later found that the heterogeneous reaction is not the appropriate route for the preparation of HTACC particles. The detail will be later discussed in the next session.

Table 4.1 Degree of substitution of quaternized chitosan particles prepared by heterogeneous route

Particles	Integration				%DS			%DS total
	H-2',3,4,5,6,6'	$-CH_3$ tri-substution	$-CH_3$ di-substution	$-CH_3$ mono-substution	tri-	di-	mono-	
HTACC	100	33.4	-	-	26.2	-	-	26.2
MC	100	17.9	63.5	2.0	14.0	74.0	4.7	92.7
MBzC	100	6.0	26.2	3.5	4.7	30.6	8.2	43.5

4.1.2 Quaternized Chitosan Particles Prepared by Homogeneous Route

The preparation of HTACC particles by homogeneous reaction is based on ionic gelation between positively charged amino groups of HTACC and negatively charged TPP at ambient temperature as shown in Scheme 4.7. The HTACC particles were obtained as white fine powder.



Scheme 4.7 Preparation of HTACC particles by homogeneous reaction

As shown in Figure 4.5, the characteristic peak of P=O groups of TPP was found at 1204 cm^{-1} in HTACC particles, whereas it was absent in chitosan and HTACC. The N-H bending peak of amino groups in chitosan at 1604 cm^{-1} shifted to 1543 cm^{-1} for both HTACC and HTACC particles indicate that most amino groups existed in the form of quaternary ammonium salt. The peaks in the range of 1460-1470 cm^{-1} observed on the spectra of HTACC and HTACC particles are the characteristic peaks of C-N stretching suggesting there are alkyl substitutions at amino positions of chitosan.

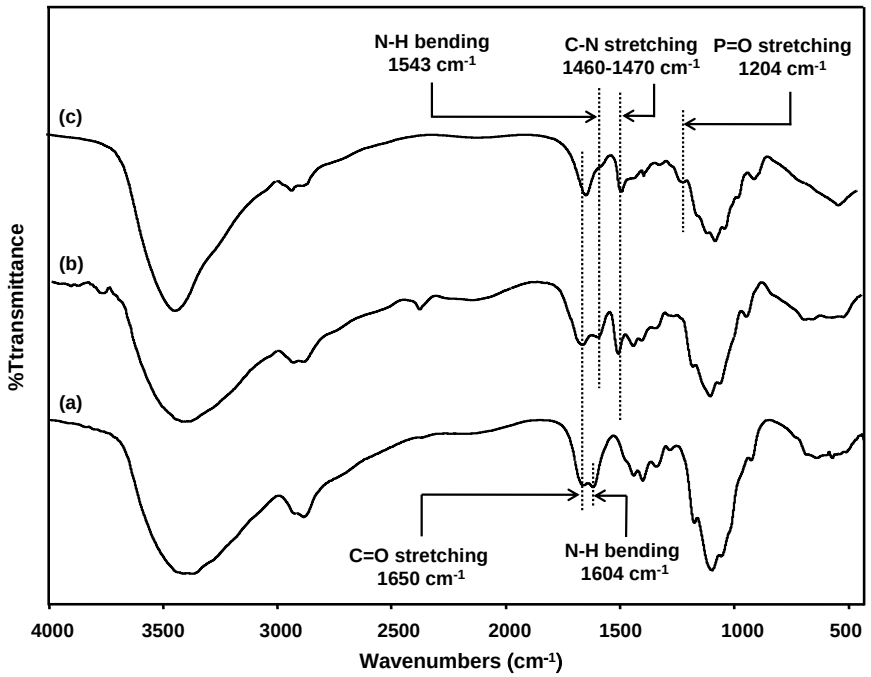


Figure 4.5 FT-IR spectra of (a) chitosan, (b) HTACC, and (c) HTACC particles

The ^{1}H NMR spectra of HTACC and HTACC particles are illustrated in Figure 4.6. The strong peak at 2.8 ppm indicated the presence of quaternary ammonium salt groups ($N^{+}(CH_3)_3$) in the obtained product.

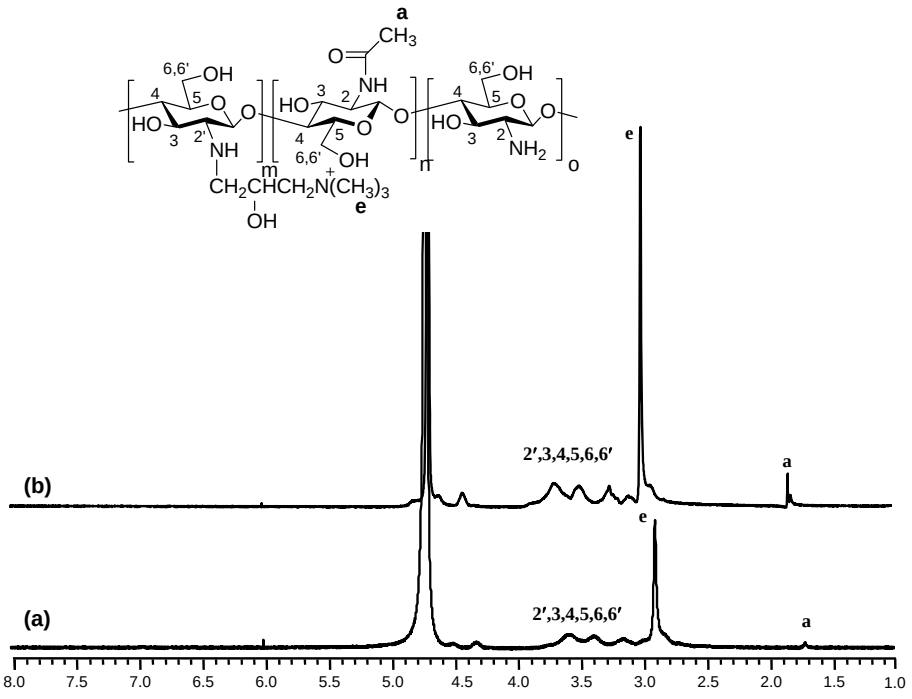
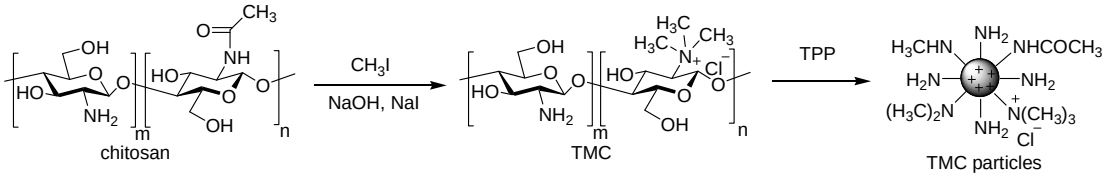


Figure 4.6 ^{1}H NMR spectra of (a) HTACC particles prepared by homogeneous reaction and (b) HTACC

In the case of TMC particles, TMC was first synthesized by direct methylation of amino groups on chitosan by MeI. After that, TMC particles were then formed by ionic gelation between positively charged groups in TMC and negatively charged TPP. Stepwise preparation is outlined in Scheme 4.8.



Scheme 4.8 Preparation of TMC particles by homogeneous reaction

FT-IR spectra of TMC and TMC particles are shown in comparison with that of chitosan in Figure 4.7, the appearance of characteristic peak at 1204 cm^{-1} corresponded to P=O groups indicated the ionic crosslinking in the TMC particles. The decreasing N-H bending peak at 1543 cm^{-1} from that of chitosan together with the emerging of C-N stretching in a wavenumber range of 1460-1470 cm^{-1} of both TMC and TMC particles suggested that methylation occurred at the amino positions.

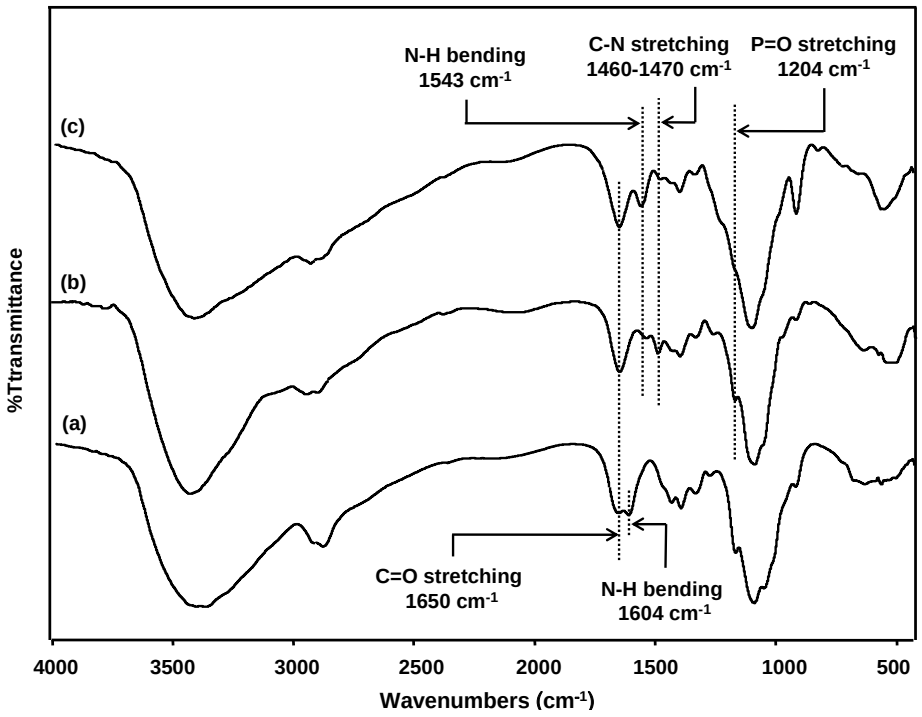


Figure 4.7 FT-IR spectra of (a) chitosan, (b) TMC, and (c) TMC particles

According to ^{1}H NMR spectra of TMC and TMC particles illustrated in Figure 4.8, the signal at 2.9 ppm indicated the methyl protons of the quaternary ammonium

groups, while the signals at 2.6 ppm and 2.5 ppm can be assigned to the methyl protons of the disubstituted and monosubstituted amino groups, respectively.

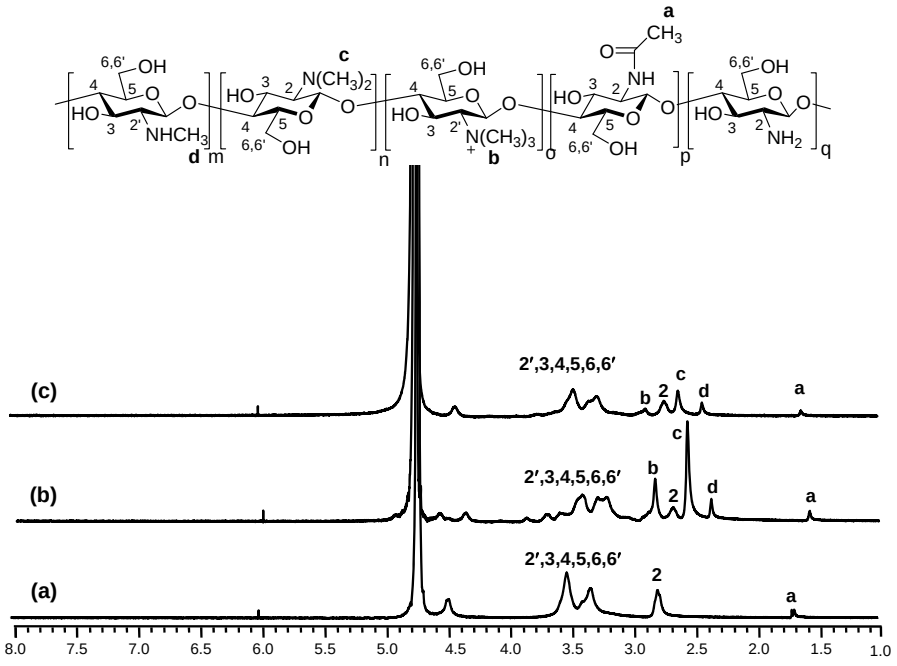


Figure 4.8 ^{1}H NMR spectra of (a) CS and (b) TMC and (c) TMC particles.

The degree of quaternization (% DQ) of HTACC and HTACC particles can be determined from ^{1}H NMR data in very much the same way as the HTACC particles prepared by heterogeneous route using equation 4.1. The estimation of degree of mono-, di-, and tri-substitution of TMC and TMC particles can be done using equation 4.2, 4.3, and 4.4, respectively. The data are outlined in Table 4.2.

Table 4.2 Degree of substitution of HTACC, TMC, HTACC and TMC particles prepared by homogeneous reaction

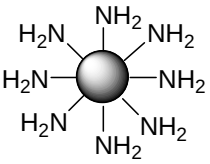
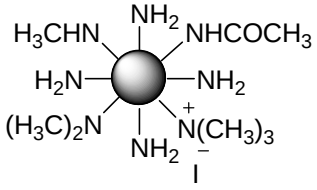
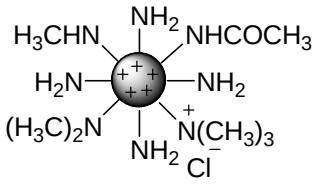
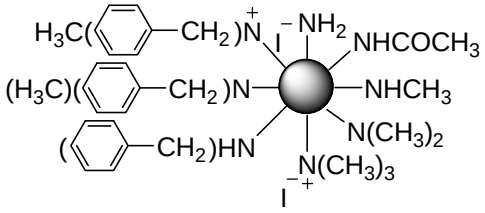
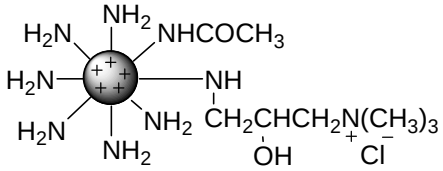
Substrate	Integration				%DS			$\%DS_{total}$
	H-2',3,4,5,6,6'	$-N^{+}(CH_3)_3$ tri-substitution	$-N(CH_3)_2$ di-substitution	$-NHCH_3$ mono-substitution	tri-	di-	mono-	
HTACC	100	123.4	-	-	96.8	-	-	96.8
HTACC particles	100	88.4	-	-	69.3	-	-	69.3
TMC	100	17.3	48.0	5.4	13.5	56.5	12.7	82.7
TMC particles	100	1.4	4.4	5.3	1.1	5.2	21.5	27.8

From the data shown in Table 4.2, it was found that the degree of quaternization (%DQ) of HTACC and TMC particles were lower than that of HTACC and TMC. This can be described by the fact that some positive charges of HTACC and TMC were ionically crosslinked with negatively charged TPP upon the particle formation. It was also observed that %DQ of HTACC particles prepared by the homogeneous route is much higher than that of the HTACC particles prepared by the heterogeneous route. Together with the fact that there is no problem with the particles turning brown in the former case, we therefore chose to use the HTACC particles prepared by the homogeneous route for further investigation. It is also interesting to note that TMC particles prepared by the homogeneous route possessed much less %DQ (~1%) in comparison with the MC particles (%DQ ~ 14%) of which functionalities should be similar. This strongly suggested that the heterogeneous approach is the more effective way to introduce trimethylated groups to chitosan particles. This is highly satisfactory considering that heterogeneous methylation is much more convenient to perform in comparison with the homogeneous methylation.

4.1.3 Characterization of Particles

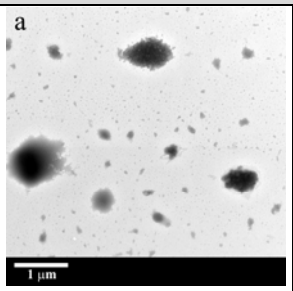
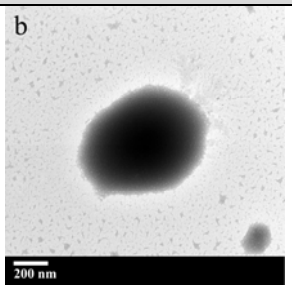
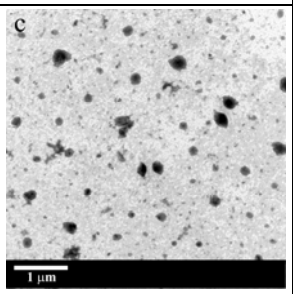
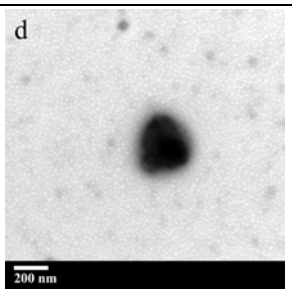
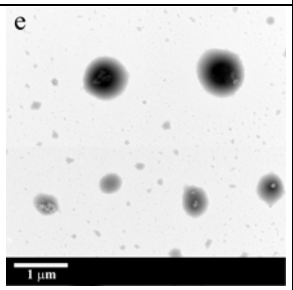
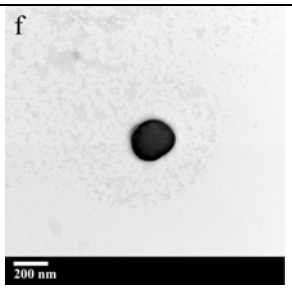
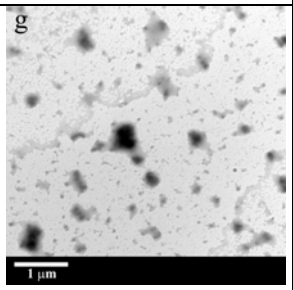
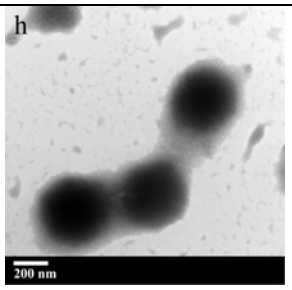
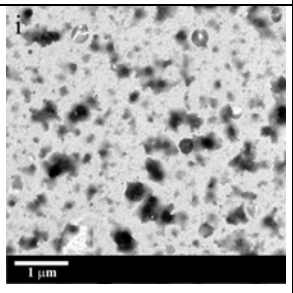
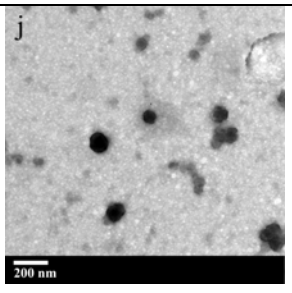
The charge characteristic of all quaternized chitosan particles expressed in term of ζ-potential was determined by Photon Correlation Spectroscopy (PCS). The data are shown in Table 4.3. All particles exhibited ζ-potential values in a positive range indicating their positively charged characteristics.

Table 4.3 ζ-potential and mean size of quaternized chitosan particles analyzed by PCS

Particles		ζ-potential (mV)	Mean size (μm)
CS particles		32.17±1.71	3.28±0.33
MC particles		30.57±1.99	1.35±0.02
TMC particles		39.77±0.21	2.57±0.33
MBzC particles		54.93±1.04	3.05±0.57
HTACC particles		24.27±0.74	0.80±0.03

The morphology and actual size of the CS, MC, TMC, MBzC and HTACC particles analyzed by TEM analysis are displayed in Table 4.4. Obviously, all particles were relatively spherical in shape. According to analysis by Semafore software, it was found that all particles have diameters in a sub-micron range (~0.13-0.40 μm). Apparently, the sizes obtained from PCS analysis were much larger than those obtained from TEM analysis. This is mainly due to the fact that the size measured by PCS is a hydrodynamic size at which the particles swell in the solution during the measurements. Whereas TEM analysis was done under reduced pressure atmosphere so the particles were in the dehydrated form.

Table 4.4 Transmission electron micrographs and mean size of (a, b) CS particles, (c, d) TMC particles, (e, f) MC particles, (g, h) MBzC particles, and (i, j) HTACC particles

Particles			**Mean size (μm)**
CS particles			0.40±0.12
MC particles			0.20±0.05
TMC particles			0.13±0.09
MBzC particles			0.16±0.05
HTACC particles			0.13±0.04

4.1.4 DNA Adsorption Efficiency of Quaternized Chitosan Particles

In this experiment, a commercially available *E.coli* DNA was used as a DNA model to determine adsorption of the negatively charged DNA on the positively charged quaternized chitosan particles by electrostatic interactions. In this study, five particles including CS, MC, TMC, MBzC, and HTACC particles were investigated. A calibration curve showing a linear relationship between DNA concentration and absorbance was established and is shown in Figure 4.9. The adsorption efficiency can be calculated following equation 3.1.

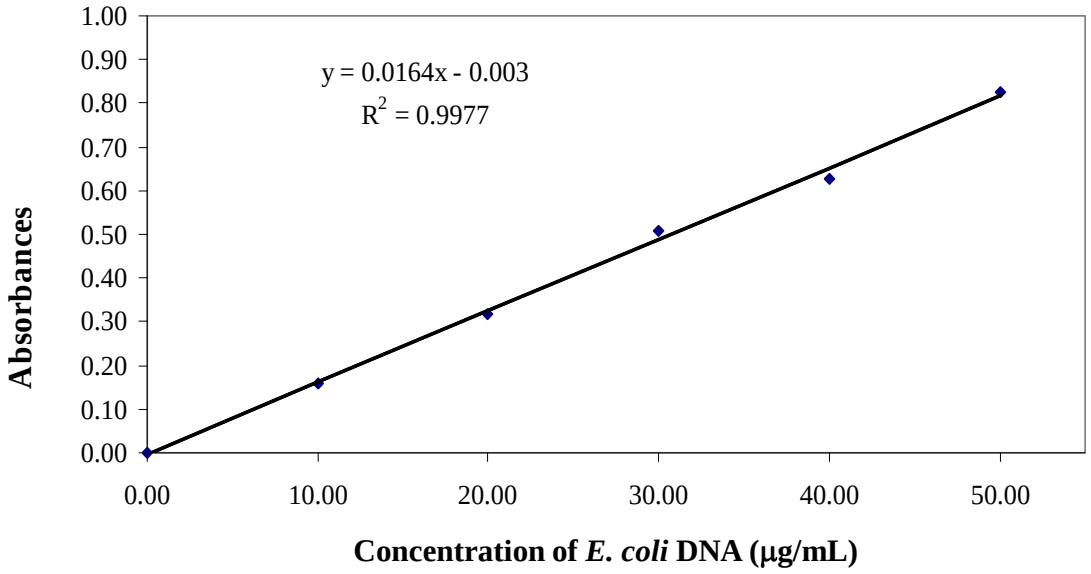


Figure 4.9 The calibration curve of *E.coli* DNA in 800 μL sodium phosphate buffer pH 7

As illustrated in Figure 4.10, it was found that the particles prepared by homogeneous reaction including HTACC and TMC particles have more adsorption efficiency than those prepared by heterogeneous reaction including MC and MBzC particles. It may be because the particles synthesized by homogeneous reaction can interact through ionic interactions between the positive charges of the quaternized chitosan particles and the negatively charged *E.coli* DNA both inside and at the surface of particles. Conversely, the particles synthesized by heterogeneous reaction can only interact on the surface of particles. The information in this section has demonstrated that all quaternized chitosan particles can interact electrostatically with DNA molecules suggesting their ability to be used for DNA sequence analysis in subsequent studies.

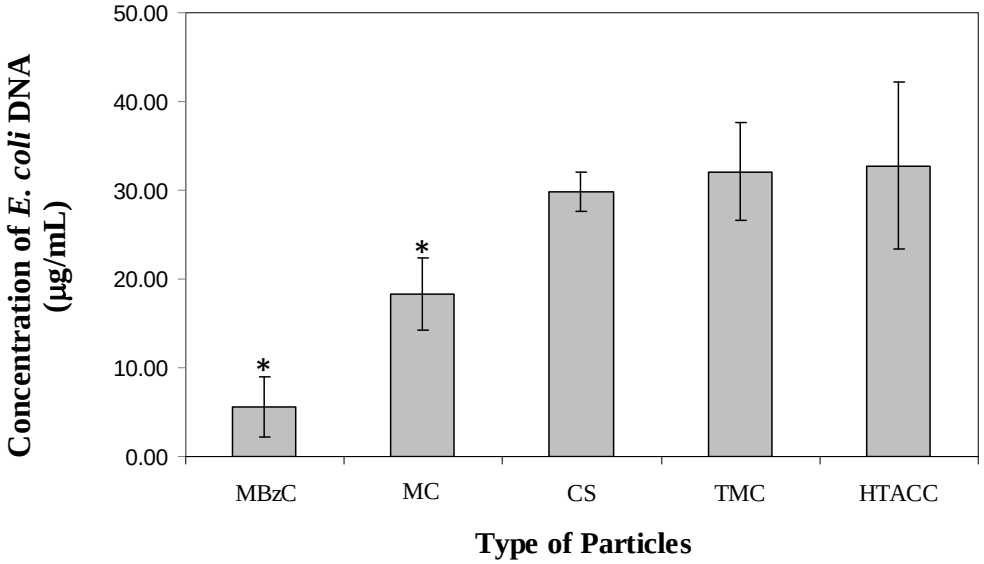


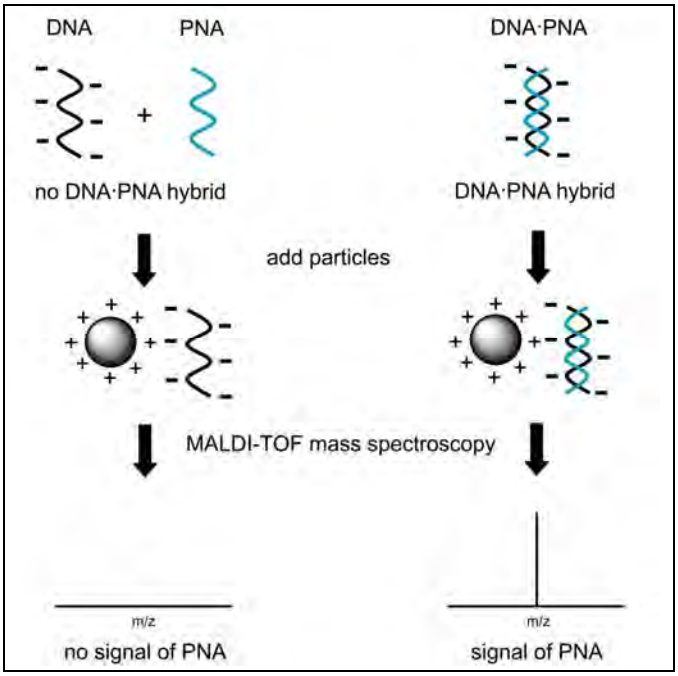
Figure 4.10 Adsorption efficiency of *E.coli* DNA on all particles. The *E.coli* DNA of 800 µL had a concentration of 50.0 µg/mL in sodium phosphate buffer pH 7 (*p = the mean difference is significant at the 0.05 level as compared with CS particles

4.2 Determination of PNA·DNA Hybridization using Quaternized Chitosan Particles by MALDI-TOF Mass Spectrometry

Analysis of DNA sequence using *acpc*PNA probes by MALDI-TOF mass spectrometry was previously identified by Boontha *et al*.[7]. This method was designed on a selective adsorption of a negatively charged PNA·DNA hybrid over unhybridized PNA onto an anion exchanger. Because PNA is an electrostatically neutral molecule, it cannot be immobilized onto the particles. On the other hand, PNA hybridized with complementary DNA target can formed a negatively charged PNA·DNA hybrid which can then be adsorbed onto the particles. The unhybridized PNA was isolated from the adsorbed PNA·DNA hybrid by simple washing. The presence of the PNA·DNA hybrid on the support can be detected by MALDI-TOF mass spectrometry. PNA is very suitable for analysis by MALDI-TOF MS technique because it is less prone to fragmentation during the MALDI process. Furthermore, due to the much better ionization of PNA than DNA under the positive ionized mode of MALDI-TOF MS analysis, interference by the mass signal of DNA is expected to be minimal [16]. The concept proposed by Boontha *et al*. [7] has been proven successful for real applications covering from the identification of meat species in feed stuffs to the multiplex SNP

typing of the human IL-10 gene promoter region. This capturing technique not only can simplify the sample preparation, but also greatly reduces the cost of analysis because anion exchangers are inexpensive and the crude DNA products obtained after PCR can be used without the demand for prior purification.

Despite the success previously described by Boontha *et al*. [7], it was found that Q-sepharose, used as the anion exchanger has some limitations. The quaternary ammonium containing Q-sepharose is generally commercialized in the form of liquid dispersion so the exact amount of the particles used for each experiment is unknown. The weighting of the solid Q-sepharose is not practical since the drying would destroy the swellability of the microparticles which in turn may adversely affect the capability of DNA capturing. The surface area of the particles can be hardly improved due to the fact the particles are only available in a micron-sized range. The variation of quaternary ammonium groups is also not possible. The ability to introduce quaternary ammonium group to the chitosan particles, whose size, charge density as well as functional group variation can be manipulated prompts us to believe that the quaternized chitosan particles can be potentially used for the same application and perhaps overcome the problems presently encountered when the Q-sepharose is used. The concept of using quaternized chitosan particles as anion exchangers is displayed in Scheme 4.9.



Scheme 4.9 Schematic diagram showing the concept of using chitosan particles as anion-exchange capture of PNA-DNA hybrid in combination with MALDI-TOF mass spectrometry for DNA sequence analysis

4.2.1 Effect of PNA concentration and Chitosan Particles on PNA·DNA Hybrid Detection

The quantity of PNA probe and DNA target that was detected by MALDI-TOF mass spectrometry using the CS particles as anion capture was optimized. PNA_1 having a T9 sequence and its complementary DNA, DNA_1 having a A9 sequence were used for this investigation. In this part, the amount of PNA was varied from 100 to 10, 1, and 0.1 pmol. From the data obtained as shown in Figure 4.11, the minimum amount of PNA probe and DNA target that can be employed for detection of DNA sequence is 10 pmol (spectrum c). No signal could be detected when the probe quantity was less than 10 pmol (spectrum d). Therefore, 10 pmol of PNA probe and DNA target was selected for the next experiments. This finding is similar to results from previous investigations by Boontha *et al*. at which Q-sepharose was used as a solid support.

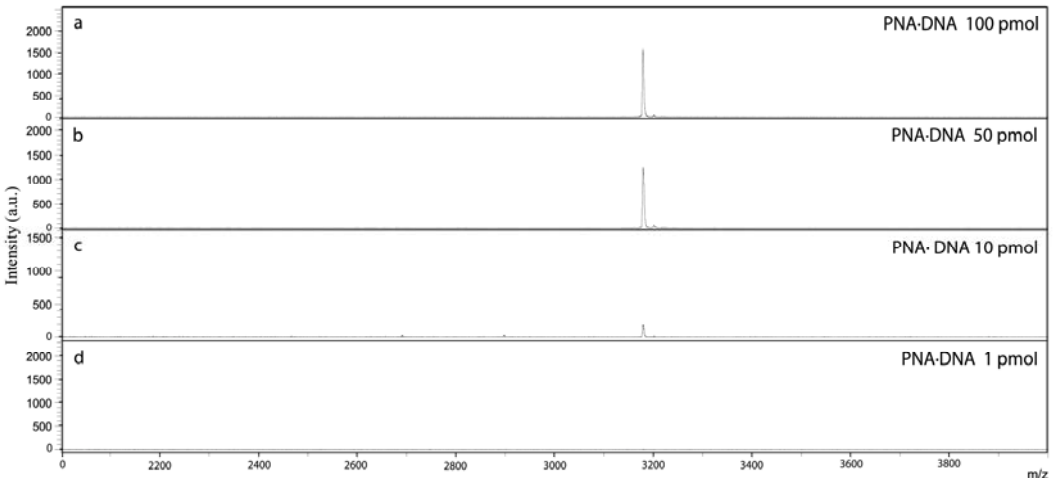


Figure 4.11 Determination of the detection limit for PNA probe (PNA_1) with DNA target (DNA_1)

To verify the proper amount of the particles, the PNA probe and DNA target was used at 10 pmol in sodium phosphate buffer pH 7 (30 μL) and the amount of particles was varied between 0.5 to 7 mg. From the mass spectra of solid (right row) shown in Figure 4.12, the PNA signals exhibited similar intensity when the particles having a weight range of 1-7 mg were used. On the other hand, the lower signal of PNA was detected when the quantity was less than 1 mg (0.5 mg). For this reason, the lowest amount of CS particles at 1 mg was selected for determination of DNA sequence.

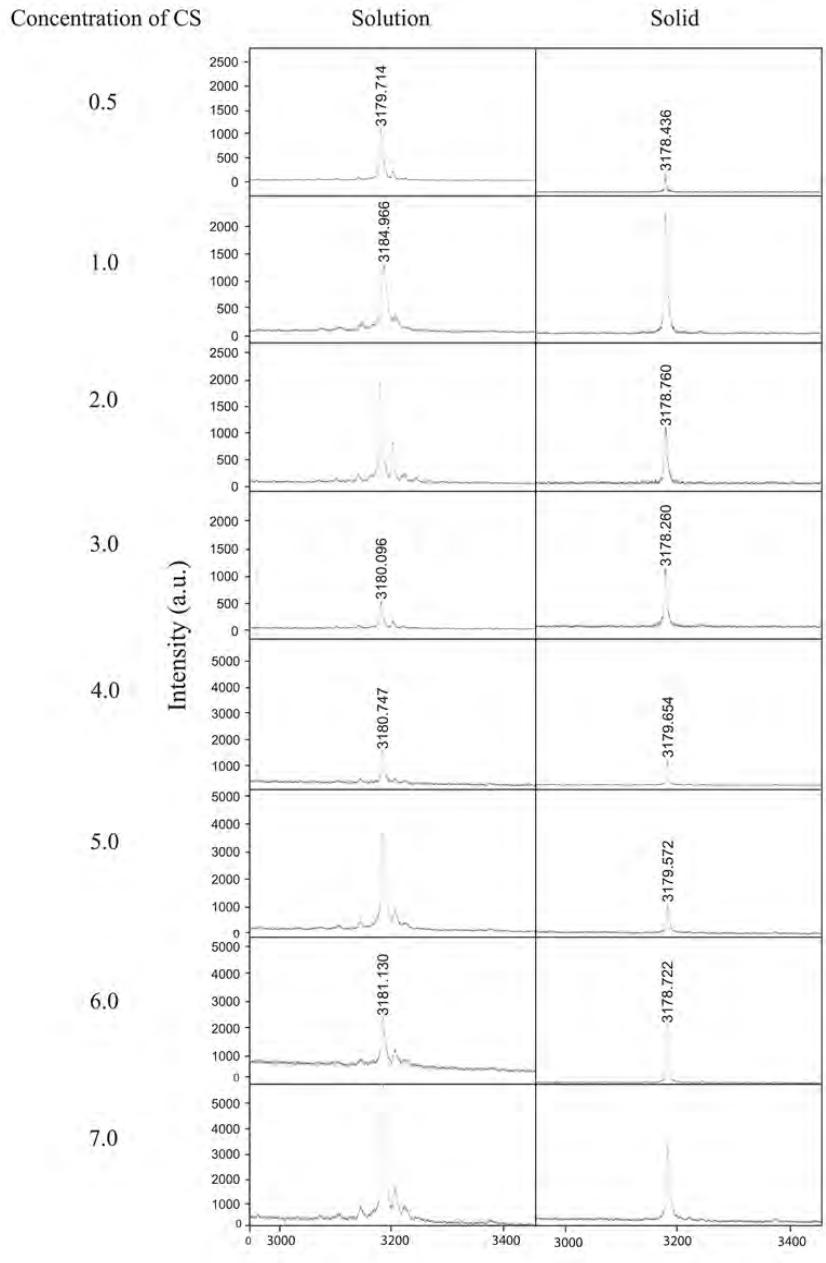


Figure 4.12 Determination of the amount of CS particles after capturing PNA·DNA hybrid (hybridization condition: chitosan particles having a weight range of 0.5-7 mg, PNA 10 pmol, DNA 10 pmol, 10 mM sodium phosphate buffer pH 7)

4.2.2 Hybridization between PNA Probe and Synthetic DNA

To test the ability of particles as anion-exchange captures of complementary PNA·DNA hybrid, PNA probe having 9 repeat units of homothymine, T9 or so-called PNA_1 was chosen for the investigation. The PNA T9 was firstly mixed in 30 µL of binding buffer and hybridized with the DNA target (listed in Table 4.5), followed by an addition of designated particles (CS, MC, TMC, MBzC and, HTACC). After that, the

particles were separated, washed with deionized water and detected by MALDI-TOF mass spectrometry. Only the signal of PNA which is complementary to the DNA target was observed. Therefore, this method is able to discriminate the specificity of hybridization.

Table 4.5 PNA and DNA sequences used in section 4.2.2

Code	Sequence	Note	*m/z*
PNA_1	Ac-TTTTTTTTT-$LysNH_2$	-	3176.9
DNA_{1c}	d(5'-AAAAAAAAA-3')	complementary to PNA_1	-
DNA_{1s}	d(5'-AAAACAAAA-3')	single mismatch to PNA_1	-
DNA_{1f}	d(5'-AGTGATCTAC-3′)	full mismatch to PNA_1	-
PNA_2	Ac-AAAAAAAAA-$LysNH_2$	-	3260.2

MALDI-TOF analysis of all particles after being washed by deionized water as displayed in Figure 4.13 revealed mainly the signal of PNA probe, only when the complementary DNA (A9) was employed. There were no signals detected in the cases of both single-mismatched (DNA_{1s}) and full-mismatched (DNA_{1f}) DNA targets suggesting the specificity of the detection. The success of analysis was further demonstrated by the fact that there was no appearance of PNA signal in the absence of the hybridized DNA implying that there were no non-specific interactions between the positively-charged particles and the neutral and uncharged PNA. And that the PNA can only be linked to the particles through the hybridization with the complementary DNA that was later attracted electrostatically to the particles. However, the mismatch discrimination cannot be realized in the case of the MBzC particles. The signals of PNA probe were seen both in the PNA solution and on the particles regardless of the mismatching of the DNA target. This non-specific interaction is believed to stem from the presence of hydrophobic benzyl moieties introduced to the MBzC particles. It should also be noted that there was no DNA signal appearing in the mass spectra although it exist concurrently with the PNA in the PNA·DNA hybrid that was captured by the particles. This can be explained as a consequence of the more effective ionization of PNA than DNA in the positive ion mode.

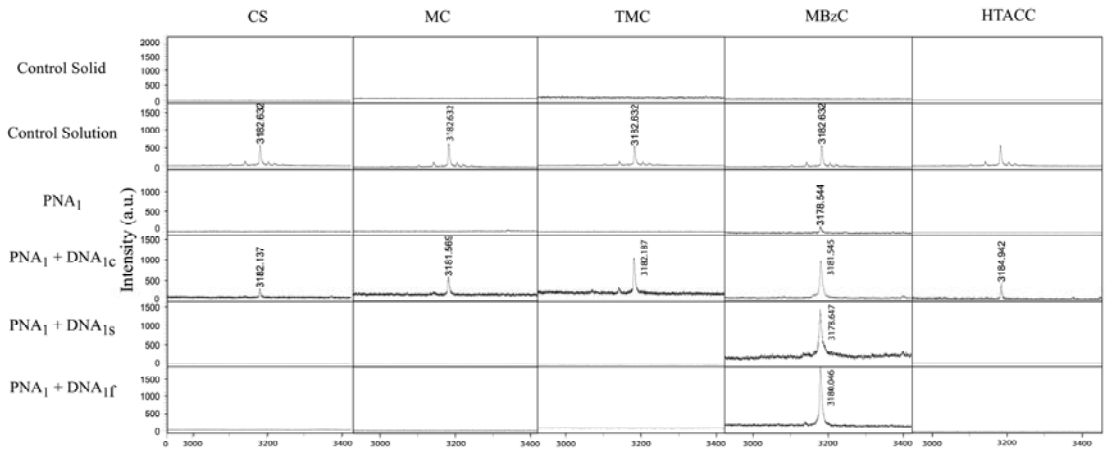


Figure 4.13 MALDI-TOF mass spectra of particles after capturing the PNA·DNA hybrid using PNA_1 as a probe

4.2.3 Application in Synthetic Mutant K-*ras* DNA Analysis

Analysis of real DNA samples usually requires a probe having longer than 9 bases. In order to investigate the applicability of quaternized chitosan particles for DNA sequence analysis, PNA probe having 14 bases, of which sequence mimics mutant K-*ras* DNA (DNA mut) and wild-type K-*ras* DNA (DNA wt), a mutated region that is associated with cancer were used. The sequences of the PNA probes and DNA targets used in this section are illustrated in Table 4.6.

Table 4.6 PNA and DNA sequences used in section 4.2.3

Code	Sequence	Note	*m/z*
PNA(mut)	Ac-Lys-O-CTACGCCAACAGCT-$SerNH_2$	-	5071.9
PNA(wt)	Ac-Lys-CTACGCCACCAGCT-$SerNH_2$		4902.8
DNA(mut)	d(5'-AGCTGTTGGCGTAG-3')	complementary to PNA(mut) single mismatch to PNA(wt)	-
DNA(wt)	d(5'-AGCTGGTGGCGTAG-3')	complementary to PNA(wt) single mismatch to PNA(mut)	-
DNA_{3f} (J1)	d(5'-TAGTTGTACGTACA-3')	full mismatch to both PNA(mut) and PNA(wt)	-

The data shown in Figure 4.14 reveal that only HTACC particles exhibited satisfactory behavior by showing the signal of PNA probe when the complementary DNA (DNA(mut)) was employed. And there were no signals detected in the cases of both single-mismatched (DNA(wt)) and full-mismatched (DNA(J1)) DNA targets suggesting the specificity of the detection. It should be noted that the MALDI-TOF analysis was carried out after simple washing the particles 3 times with Mill-Q water. This truly implies that HTACC can prevent non-specific adsorption of the non-complementary targets without having to use specific washing treatment. However, the detected signal was relatively low in the complementary case in comparison with other particles. MC, TMC, and MBzC particles can only discriminate the complementary DNA target from the full-mismatched DNA target, but not from the single-mismatched DNA target. On the other hand, CS particles cannot at all distinguish the complementary DNA target from the non-complementary ones suggesting that a high degree of non-specific interactions occurred even without the DNA target.

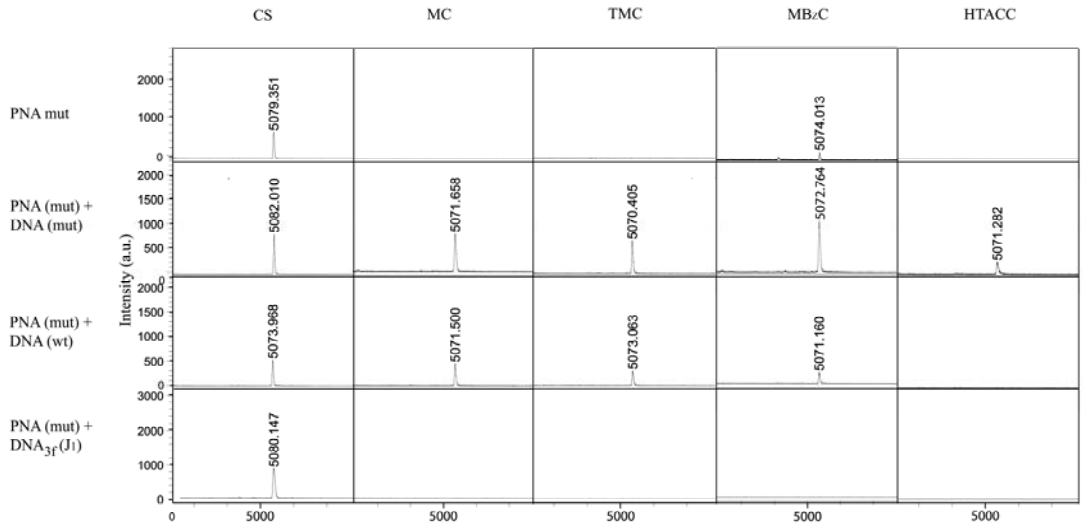


Figure 4.14 MALDI-TOF mass spectra of particles after capturing the PNA·DNA hybrid using PNA(mut) as a probe

Similar findings were also observed in the case when PNA(wt) was used as a probe (Figure 4.15) except that the discrimination cannot be realized by the MBzC particles.

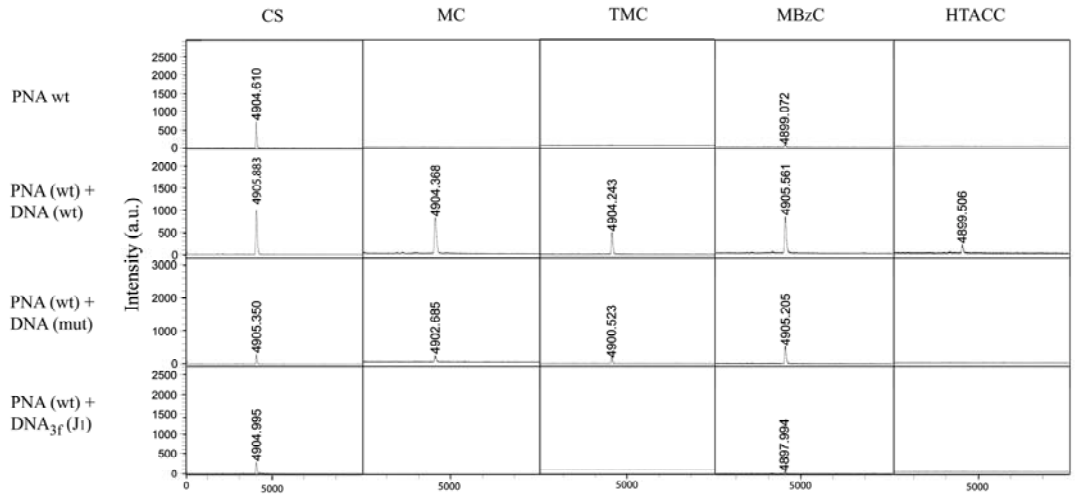


Figure 4.15 MALDI-TOF mass spectra of particles after capturing the PNA·DNA hybrid using PNA(wt) as a probe

To get rid of the non-specific interactions, formamide was added to the binding buffer that was used for rinsing the particles after capturing the PNA.DNA hybrid and washing with MilliQ water. This method has been proven successful by Ross *et.al.* [16]. They have reported that formamide can reduce the hydrophobic interactions between PNA and solid support (streptavidin magnetic bead in their case) and destabilize the non-complementary DNA·PNA hybrids. MALDI-TOF mass spectra obtained from the particles rinsed by 20% formamide in binding buffer are shown in Figure 4.16. Apparently, the nonspecific absorption of PNA·single-mismatched DNA hybrid on all particles except CS particles could be eliminated both in the cases when PNA(mut) and PNA(wt) were used as probes. The results illustrated in Figure 4.17 have demonstrated that the rinsing by 20% formamide solution did not destroy the PNA·complementary DNA hybrid both in the cases when PNA(mut) and PNA(wt) were used as probes. This characteristic is a desirable feature considering that the capturing of the designated hybrid was still effective.

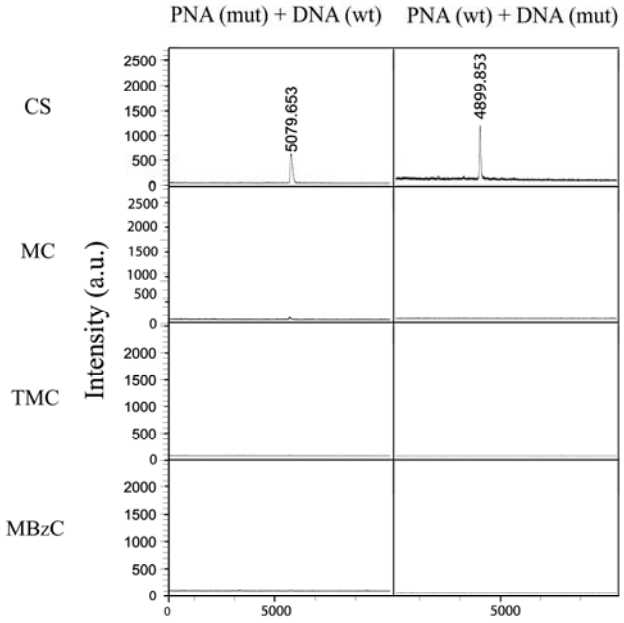


Figure 4.16 MALDI-TOF mass spectra of CS, MC, TMC, and MBzC particles after capturing the PNA·single-mismatched DNA hybrid after washing with MilliQ water and 20% formamide in binding buffer using PNA(mut) or PNA(wt) as a probe

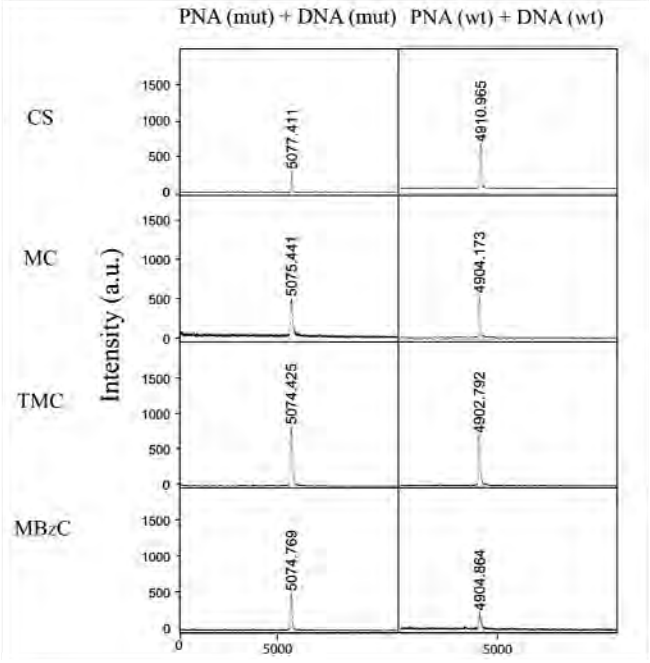


Figure 4.17 MALDI-TOF mass spectra of CS, MC, TMC, and MBzC particles after capturing the PNA·complementary DNA hybrid after washing with MilliQ water and 20% formamide in binding buffer using PNA(mut) or PNA(wt) as a probe

4.2.4 Selectivity of Synthetic PNA·DNA Hybridization

To verify the use of quaternized chitosan particles as anion-exchange captures in practice, two different PNA probes were mixed in a 30 μL binding buffer and hybridized with the DNA target followed by an addition of designated particles. The system of PNA_1 and PNA_2 of which sequence is T9 and A9, respectively was tested first. The concept of the selectivity test is outlined in Figure 4.18.

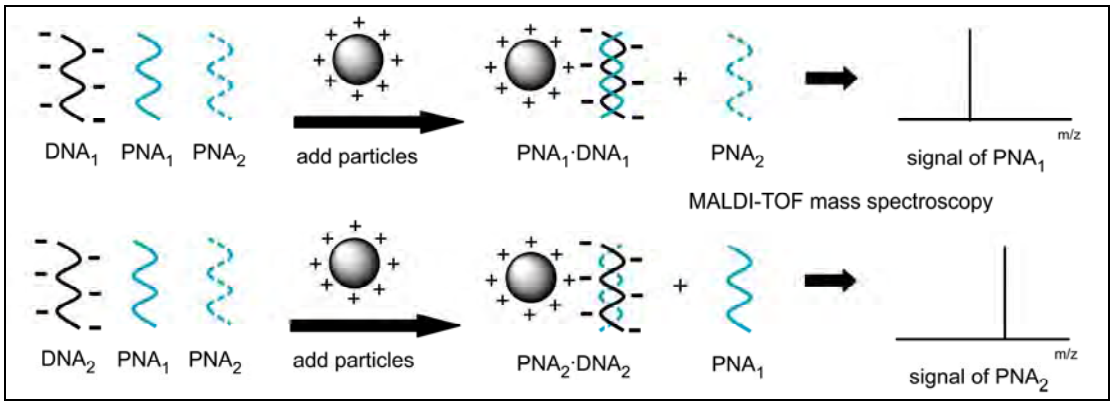


Figure 4.18 The concept of DNA sequence determination by employing quaternized chitosan particles to selectively capture complementary PNA·DNA hybrids followed by subsequent MALDI-TOF analysis

Typically, the signal of PNA_1 and PNA_2 as analyzed by MALDI-TOF MS would appear at m/z 3176.9 and 3260.2, respectively. The signals should appear at the same positions if the two PNAs exist together. As analyzed by MALDI-TOF MS, there was non-specific adsorption of PNA_1 only on MBzC particles and PNA_2 on all particles except HTACC particles.

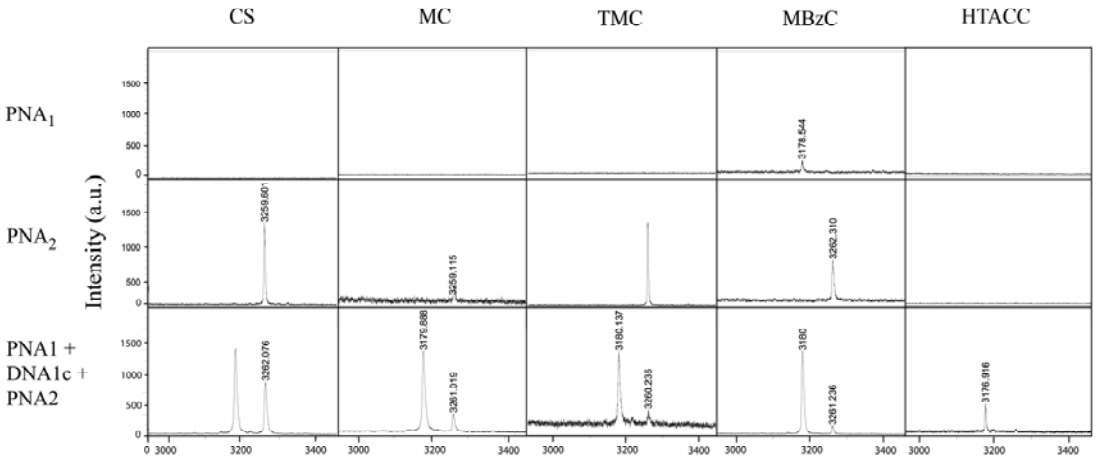


Figure 4.19 MALDI-TOF mass spectra of particles after exposure to PNA_1 or PNA_2 alone or the mixture of PNA_1 and PNA_2 that was previously hybridized with the DNA_1 and rinsed with MillQ water

In order to seek for a method to suppress the nonspecific adsorption of the non-complementary probe, PNA_2 in this particular case, a series of rinsing have been attempted. Among all reagents tested, 20% formamide seemed to be the most effective one in removing non-specific adsorption of PNA_2 from all particles except the CS particles (Figure 4.20). However, the signal from PNA_1 became very low implying that some of the PNA·complementary DNA hybrid (PNA_1·DNA_1) might be destroyed. We suspected that this might be a consequence of hydrogen bonding between adenine base in PNA_2 sequence and the un-quaternized free amino groups of chitosan. This is the reason why the non-specific adsorption was not dominated in the case of HTACC particles of which %DQ was the highest (69.3%) in comparison with other particles (See Table 4.1 and 4.2 for %DQ).

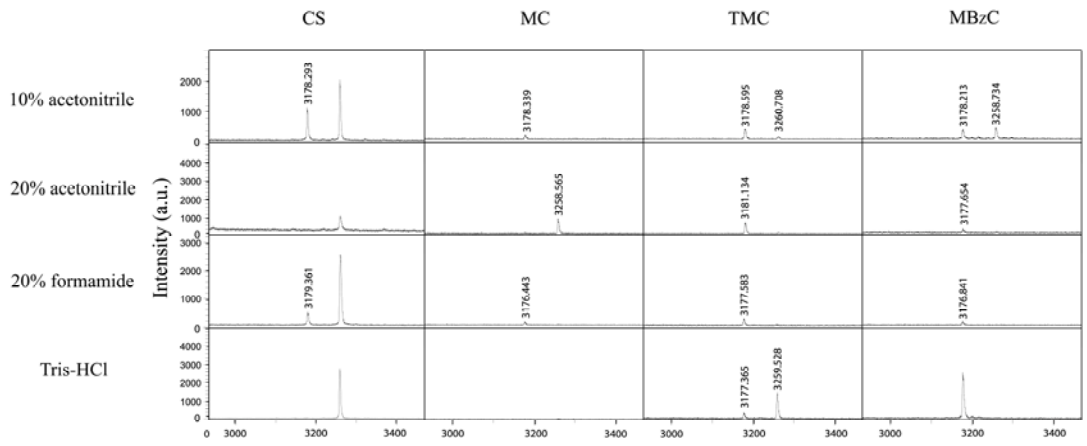


Figure 4.20 MALDI-TOF mass spectra of CS, MC, TMC, and MBzC particles after exposure to the mixture of PNA_1 and PNA_2 that was previously hybridized with the DNA_1 after rinsing with selected reagents

Selectivity test was also conducted on the system of PNA(mut) and PNA(wt) of which sequences contain 14 mixed bases. Typically, the signal of PNA(mut) and PNA(wt) as analyzed by MALDI-TOF MS would appear at m/z 5071.9 and 4902.8, respectively. According to Figure 4.21, all particles showed no non-specific adsorption of either PNA(mut) and PNA(wt). Upon using 20%formamide as the rinsing reagent after hybridization, MC, TMC, and HTACC particles possessed good selectivity by showing only the signal of PNA that was hybridized with the complementary DNA target. Among these three particles, MC gave the best performance due to the following characteristics. The PNA signal was high (higher than that of the HTACC particles) with very low non-specific adsorption of the non-complementary probe. Despite their comparable performance, the method to prepare the MC particles which is based on the heterogeneous route is less time-consuming and less complicated than that was used for the preparation of the TMC particles which is based on the homogeneous method.

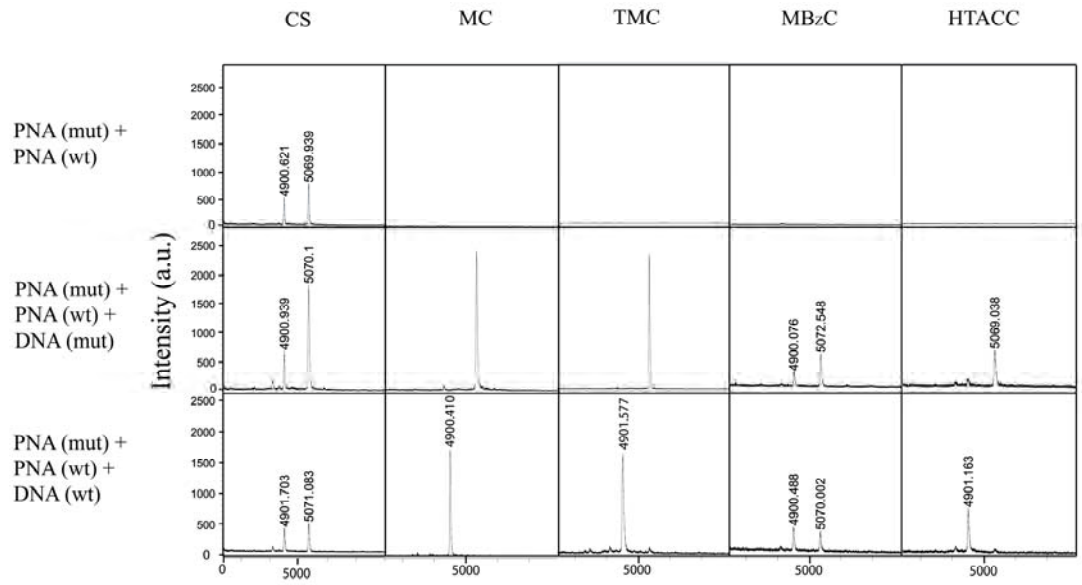


Figure 4.21 MALDI-TOF mass spectra of particles after exposure to the mixture of PNA(mut) and PNA(wt) alone or the mixture of PNA(mut) and PNA(wt) that was previously hybridized with selected DNA target (PNA(mut) or PNA(wt)) after rinsing with 20%formamide

CHAPTER V

CONCLUSION AND SUGGESTIONS

A series of quaternized chitosan particles having different alkyl substituents were prepared. Methylated chitosan (MC) particles and methylated *N*-benzyl chitosan (MBzC) particles were obtained by heterogeneous chemical modification of ionically crosslinked chitosan (CS) particles whereas *N,N,N*-trimethylchitosan (TMC) particles and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) particles were obtained by homogeneous chemical modification of chitosan followed by particle formation via ionic crosslinking. The success of particle formation was verified by ^{1}H NMR and FT-IR analyses. The highest degree of quaternization (%DQ) of 69.3% was obtained in the case of HTACC particles. Charge characteristic of the particles as determined by PCS suggested that all particles carried positive charges with ζ-potential ranging from +24.29 to +59.43. The results from TEM analysis indicated that all particles were relatively spherical and had diameters in a range of 0.13-0.39 μm. All particles were capable of binding electrostatically with a model DNA, *E.coli* DNA suggesting their ability to be used for DNA sequence analysis.

The concept of using quaternized chitosan particles as anion exchange captures in combination with pyrrolidinyl PNA (*acpc*PNA) and MALDI-TOF mass spectrometry for determination of DNA sequence has been demonstrated. It is obvious that the anion capturing ability of some chitosan particles are comparable to that of the commercial anion exchanger, Q-sepharose. The minimal detectable amount of DNA target is 10 pmol. The specific detection of hybridization event can also be realized and is strongly affected by the variation of functionality introduced to the chitosan particles in the step of quaternization. Among all particles, HTACC is the most effective anion exchangers to be used for synthetic DNAs having T9 and A9 sequences. On the other hand, MC particles gave the best performance for detection of synthetic 14-mer, mixed base DNAs of which sequence mimics mutant K-*ras* DNA, a mutated region that is associated with cancer. Rinsing by 20% formamide solution in PBS buffer was required to achieve selective DNA sequence determination in both cases.

From practical point of view, the most effective quaternized chitosan particles should also be tested with real DNA samples.

REFERENCES

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

[2] Gaylord, B. S.; Heeger, A. J.; Bazan, G. C., DNA detection using water-soluble conjugated polymers and peptide nucleic acid probes. *Proceedings of the National Academy of Sciences of the United States of America* 99 (17) (2002): 10954-10957.

[3] Liu, B.; Bazan, G. C., Methods for strand-specific DNA detection with cationic conjugated polymers suitable for incorporation into DNA chips and microarrays. *Proceedings of the National Academy of Sciences of the United States of America* 102 (3) (2005): 589-593.

[4] Hyrup, B.; Egholm, M.; Nielsen, P. E.; Wittung, P.; Norden, B.; Buchardt, O., Structure-activity studies of the binding of modified peptide nucleic acids (PNAs) to DNA. *Journal of the American Chemical Society* 116 (18) (1994): 7964-7970.

[5] Vilaivan, T.; Lowe, G., A novel pyrrolidinyl PNA showing high sequence specificity and preferential binding to DNA over RNA. *Journal of the American Chemical Society* 124 (32) (2002): 9326-9327.

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

[7] 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* 80 (21) (2008): 8178-8186.

[8] Rao, S. B.; Sharma, C. P., Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential. *Journal of Biomedical Materials Research* 34 (1) (1997): 21-28.

[9] Aspden, T. J.; Masons, J. D. T.; Jones, N. S.; Lowe, J.; Skaugrud, W.; Illum, L., Chitosan as a nasal delivery system: The effect of chitosan solutions on *in vitro* and *in vivo* mucociliary transport rates in human turbinates and volunteers. *Journal of Pharmaceutical Sciences* 86 (4) (1997): 509-513.

[10] Hamilton, S. E.; Simmons, C. G.; Kathiriya, I. S.; Corey, D. R., Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chemistry & Biology* 6 (6) (1999): 343-351.

[11] Nielsen, P. E., Peptide nucleic acid: A versatile tool in genetic diagnostics and molecular biology. *Current Opinion in Biotechnology* 12 (1) (2001): 16-20.

[12] Ye, S.; Miyajima, Y.; Ohnishi, T.; Yamamoto, Y.; Komiyama, M., Combination of peptide nucleic acid beacon and nuclease S1 for clear-cut genotyping of single nucleotide polymorphisms. *Analytical Biochemistry* 363 (2) (2007): 300-302.

[13] Griffin, T. J.; Tang, W.; Smith, L. M., Genetic analysis by peptide nucleic acid affinity MALDI-TOF mass spectrometry. *Nature Biotechnology* 15 (13) (1997): 1368-1372.

[14] Zhu, L.; Parr, G. R.; Fitzgerald, M. C.; Nelson, C. M.; Smith, L. M., Oligodeoxynucleotide fragmentation in MALDI/TOF mass spectrometry using 355-nm radiation. *Journal of the American Chemical Society* 117 (22) (1995): 6048-6056.

[15] Shaler, T. A.; Wickham, J. N.; Sannes, K. A.; Wu, K. J.; Becker, C. H., Effect of impurities on the matrix-assisted laser desorption mass spectra of single-stranded oligodeoxynucleotides. *Analytical Chemistry* 68 (3) (1996): 576-579.

[16] Ross, P. L.; Lee, K.; Belgrader, P., Discrimination of single-nucleotide polymorphisms in human DNA using peptide nucleic acid probes detected by MALDI-TOF mass spectrometry. *Analytical Chemistry* 69 (20) (1997): 4197-4202.

[17] Jiang-Baucom, P.; Girard, J. E.; Butler, J.; Belgrader, P., DNA typing of human leukocyte antigen sequence polymorphisms by peptide nucleic acid probes and MALDI-TOF mass spectrometry. *Analytical Chemistry* 69 (23) (1997): 4894-4898.

[18] Ren, B.; Zhou, J. M.; Komiyama, M., Straightforward detection of SNPs in double-stranded DNA by using exonuclease III/nuclease S1/PNA system. *Nucleic Acids Research* 32 (4) (2004): e42.

[19] Ilium, L., Chitosan and Its Use as a Pharmaceutical Excipient. *Pharmaceutical Research* 15 (9) (1998): 1326-1331.

[20] Tomihata, K.; Ikada, Y., *In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives. *Biomaterials* 18 (7) (1997): 567-575.

[21] MacLaughlin, F. C.; Mumper, R. J.; Wang, J.; Tagliaferri, J. M.; Gill, I.; Hinchcliffe, M.; Rolland, A. P., Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *Journal of Controlled Release* 56 (1-3) (1998): 259-272.

[22] Richardson, S. C. W.; Kolbe, H. V. J.; Duncan, R., Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics* 178 (2) (1999): 231-243.

[23] Domard, A.; Rinaudo, M.; Terrassin, C., New method for the quaternization of chitosan. *International Journal of Biological Macromolecules* 8 (2) (1986): 105-107.

[24] Jia, Z.; shen, D.; Xu, W., Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research* 333 (1) (2001): 1-6.

[25] Xu, Y.; Du, Y.; Huang, R.; Gao, L., Preparation and modification of *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a protein carrier. *Biomaterials* 24 (27) (2003): 5015-5022.

[26] Sajomsang, W.; Tantayanon, S.; Tangpasuthadol, V.; Daly, W. H., Synthesis of methylated chitosan containing aromatic moieties: Chemoselectivity and effect on molecular weight. *Carbohydrate Polymers* 72 (4) (2008): 740-750.

[27] Hoven, V. P.; Tangpasuthadol, V.; Angkitpaiboon, Y.; Vallapa, N.; Kiatkamjornwong, S., Surface-charged chitosan: Preparation and protein adsorption. *Carbohydrate Polymers* 68 (1) (2007): 44-53.

[28] Vallapa, N. Preparation and antibacterial activity of quaternary ammonium-containing chitosan surface. Chulalongkorn University, 2006.

[29] Wia-rachai, O. Antibactrerial activity of quaternary ammonium-contraining chitosan particles. Chulalongkorn University, 2008.

[30] Mao, H.-Q.; Roy, K.; Troung-Le, V. L.; Janes, K. A.; Lin, K. Y.; Wang, Y.; August, J. T.; Leong, K. W., Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *Journal of Controlled Release* 70 (3) (2001): 399-421.

[31] Dodane, V.; Vilivalam, V. D., Pharmaceutical applications of chitosan. *Pharmaceutical Science & Technology Today* 1 (6) (1998): 246-253.

[32] Cao, W.; Easley, C. J.; Ferrance, J. P.; Landers, J. P., Chitosan as a polymer for pH-induced DNA capture in a totally aqueous system. *Analytical Chemistry* 78 (20) (2006): 7222-7228.

[33] Seong, H.-S.; Whang, H. S.; Ko, S.-W., Synthesis of a quaternary ammonium derivative of chito-oligosaccharide as antimicrobial agent for cellulosic fibers. *Journal of Applied Polymer Science* 76 (14) (2000): 2009-2015.

[34] Lowe, G.; Vilaivan, T., Amino acid bearing nucleobases for the synthesis of novel peptide nucleic acid. *Journal of the Chemical Society, Perkin Transactions 1* (4) (1997a): 539−546.

[35] Lowe, G.; Vilaivan, T., Dipeptides bearing nucleobases for the synthesis of novel peptide nucleic acids *Journal of the Chemical Society, Perkin Transactions 1* (4) (1997b): 547−554.

[36] Lowe, G.; Vilaivan, T., Solid phase synthesis of novel peptide nucleic acid. *Journal of the Chemical Society, Perkin Transactions 1* (4) (1997c): 555−560.

[37] Wu, J.; Wei, W.; Wang, L. Y.; Su, Z. G.; Ma, G. H., A thermosensitive hydrogel based on quaternized chitosan and poly(ethylene glycol) for nasal drug delivery system. *Biomaterials* 28 (13) (2007): 2220-2232.

[38] Lim, S. H.; Hudson, S. M., Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group. *Carbohydrate Research* 339 (2) (2004): 313-319.

APPENDICES

APPENDIX A

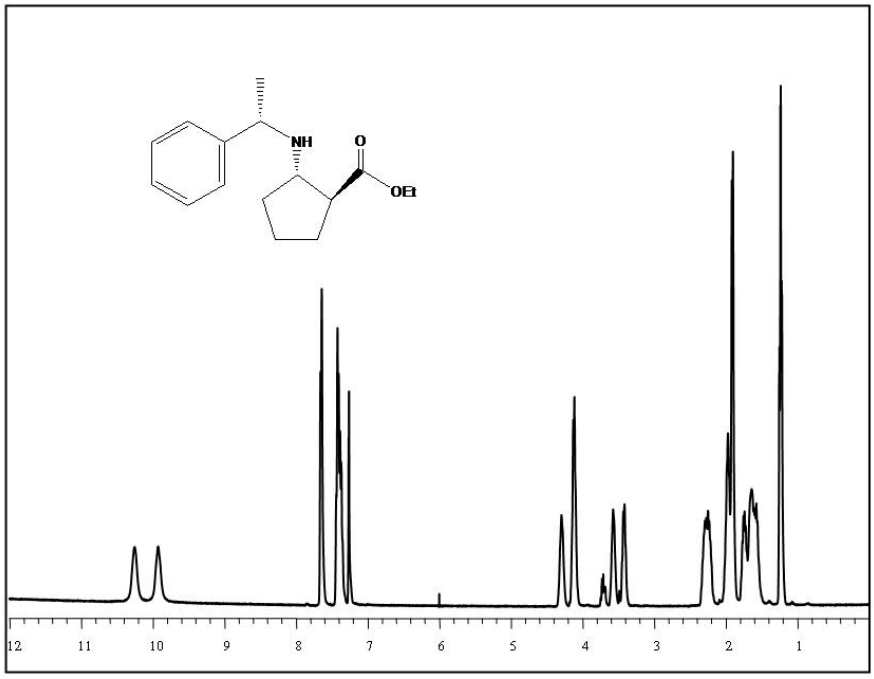


Figure A.1 ^{1}H-NMR spectrum of ethyl (1*S*,2*S*)-2-[(1'*S*)-phenylethyl]-aminocyclo-pentane carboxylate hydrochloride (1)

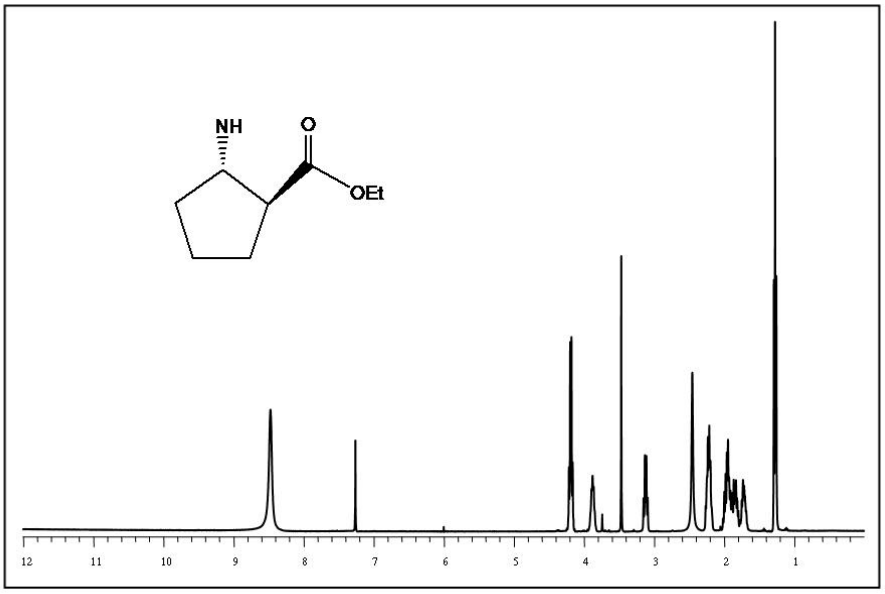


Figure A.2 ^{1}H-NMR spectrum of Ethyl (1*S*,2*S*)-2-aminocyclopentane carboxylate

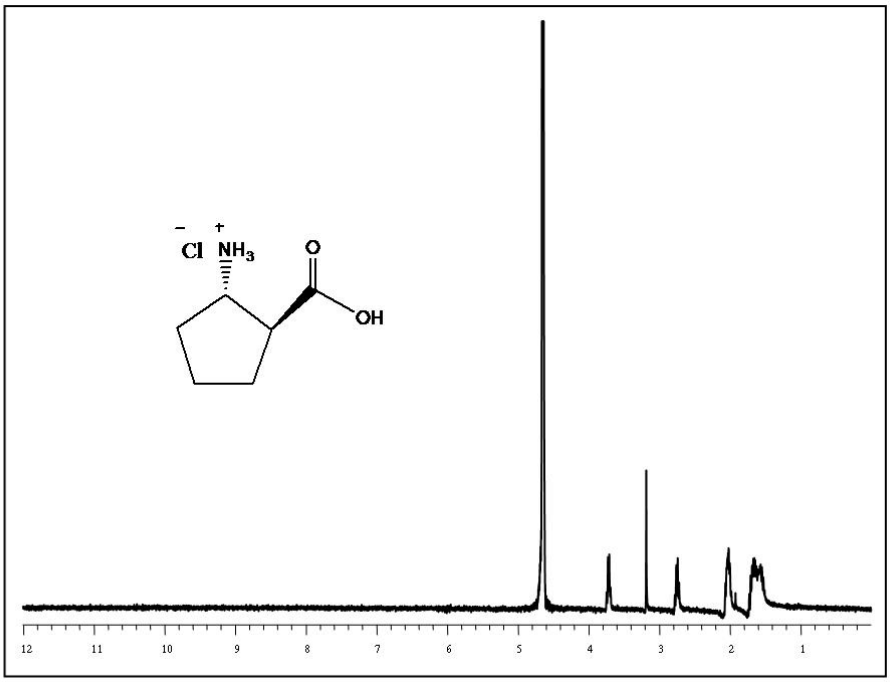


Figure A.3 ^{1}H-NMR spectrum of Ethyl (1*S*,2*S*)-2-amino-cyclopentanecarboxylate hydrochloride (3)

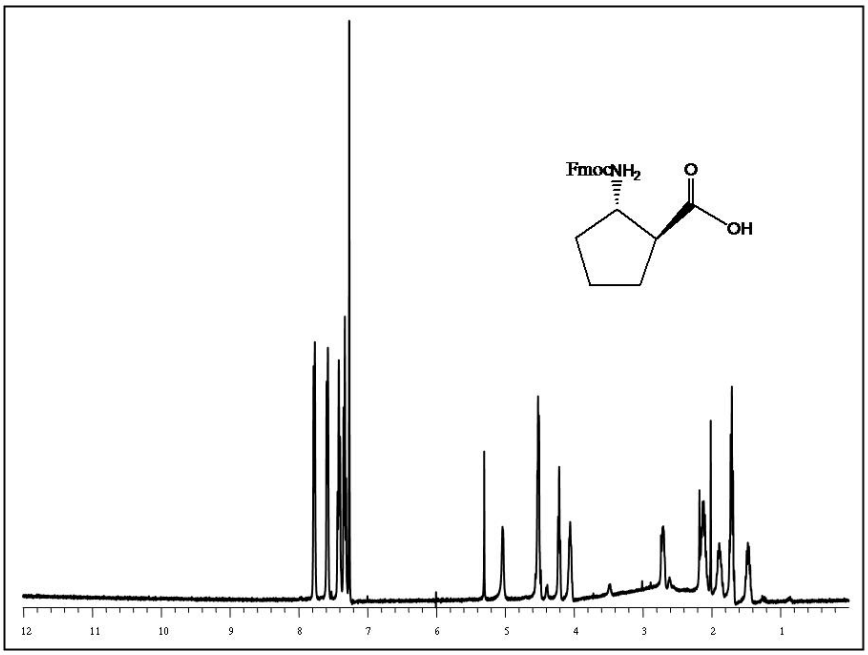


Figure A.4 ^{1}H-NMR spectrum of (1*S*,2*S*)-2-(*N*-Fluoren-9-ylmethoxycarbonyl)-aminocyclopentanecarboxylic acid (4)

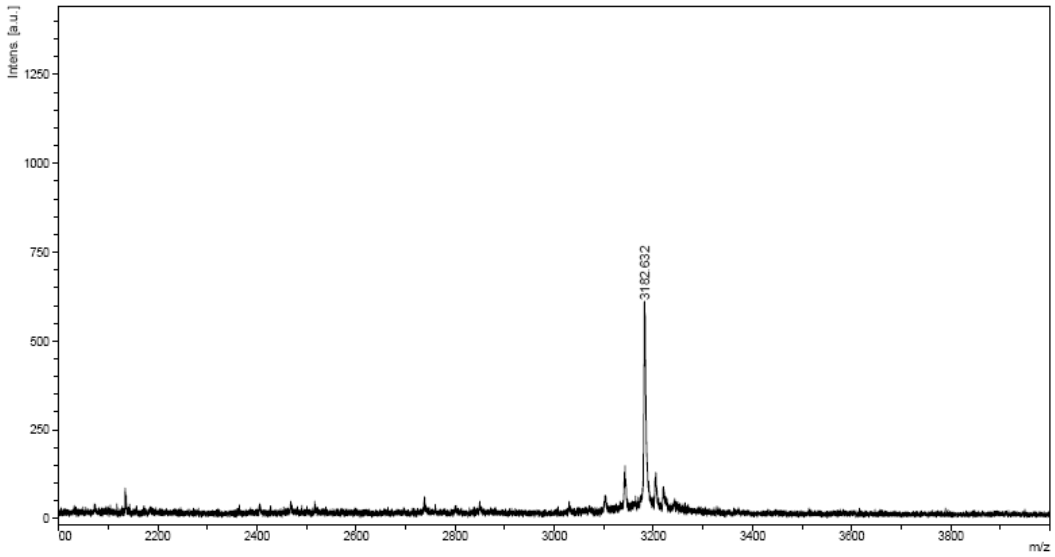


Figure A.5 MALDI-TOF mass spectrum of Ac-TTTTTTTTT-LysNH$_2$ (PNA$_1$)

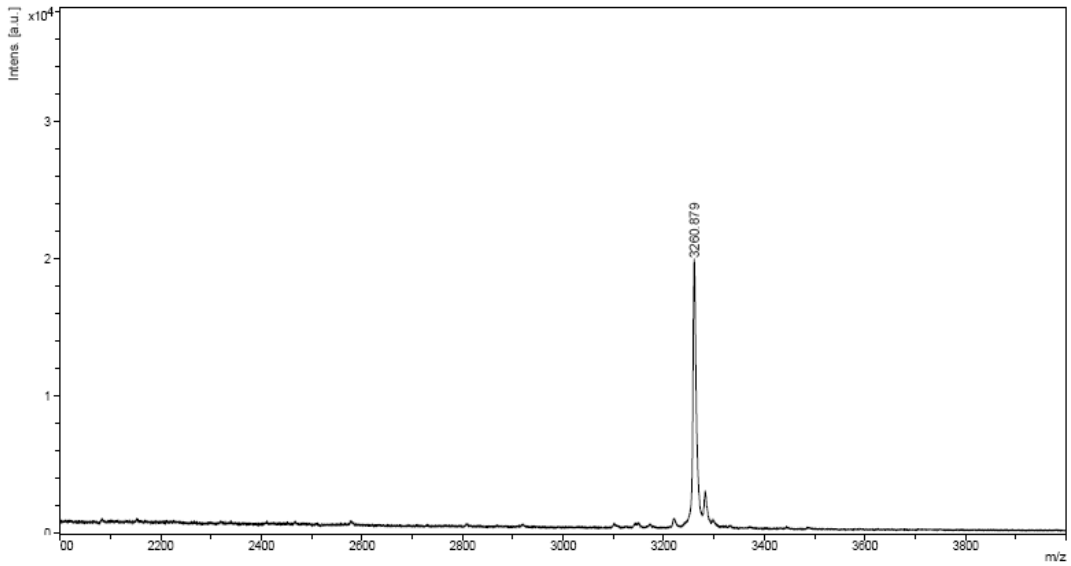


Figure A.6 MALDI-TOF mass spectrum of Ac-AAAAAAAAA-LysNH$_2$ (PNA$_2$)

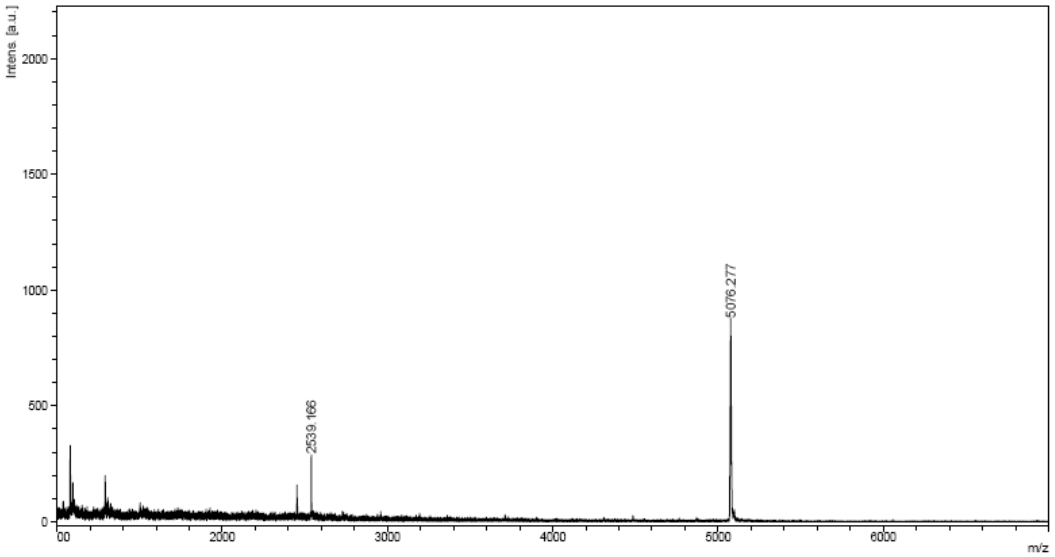


Figure A.7 MALDI-TOF mass spectrum of Ac-Lys-O-CTACGCCAACAGCT-SerNH_2 (PNA(mut))

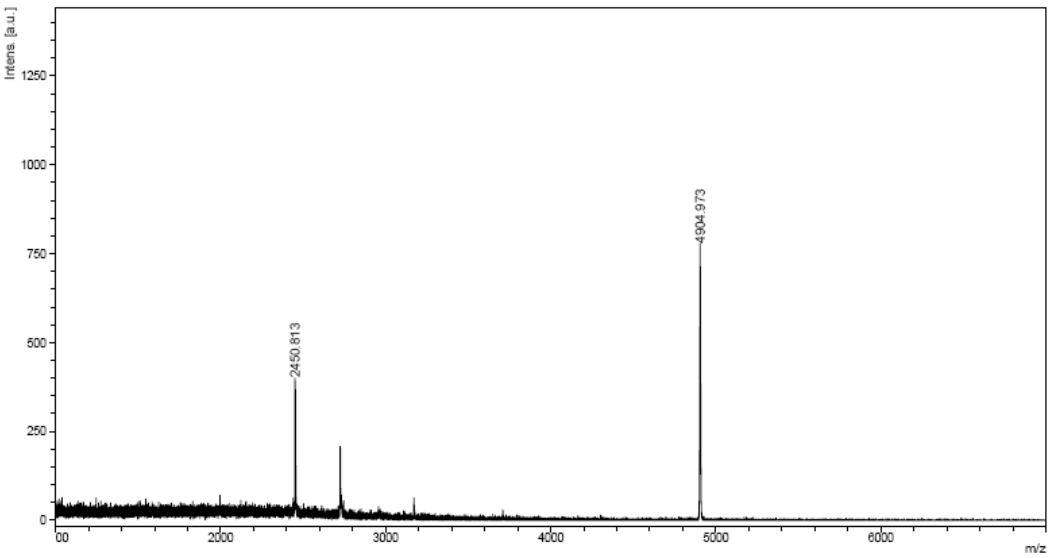


Figure A.8 MALDI-TOF mass spectrum of Ac-Lys-CTACGCCACCAGCT-SerNH_2 (PNA(wt))

Table A.1 Adsorption efficiency of *E.coli* DNA on all particles

particles	Concentration of *E. coli* DNA on particles			
	no.1	no.2	no.3	average
CS	27.71	29.55	32.06	29.77 ± 2.18
MC	21.09	13.54	20.29	18.31 ± 4.14
TMC	25.99	33.53	36.66	32.06 ± 5.48
MBzC	2.14	5.76	8.89	5.59 ± 3.37
HTACC	22.25	40.34	35.62	32.74 ± 9.38

APPENDIX B

B.1 Pure and Applied Chemistry International Conference 2009, Faculty of Science, Naresuan University, Phitsanulok, Thailand.
The Outstanding Poster Presentation Award

B.2 The 2nd Polymer Graduate Conference of Thailand, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

B.3 The 11th Pacific Polymer Conference, Cairns, Australia.

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Abstracts

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ABSTRACTS

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S7-PO-36

Detection of DNA-PNA Hybridization Using Quaternized Chitosan Particles in Combination with MALDI-TOF Mass Spectrometry

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Determination of DNA sequences are significantly important for many biotechnology-related applications ranging from medical, forensic, agriculture and food science. The concept of using a new conformationally rigid pyrrolidinyl peptide nucleic acid (PNA), strong anion-exchange captures such as commercially available Q-Sepharose in combination with MALDI-TOF mass spectrometry has been proven as a simple and effective way for single nucleotide polymorphism genotyping. This research has introduced quaternized chitosan particles as alternative anion-exchange captures that may be applicable for the same purpose. The particles were prepared by homogeneous and heterogeneous reactions between chitosan and methyl iodide. Because the particles are positively charged, they can only bind ionically with the negatively charged DNA. PNA is a neutral molecule so it cannot be adsorbed by quaternized chitosan particles unless it is hybridized with the negatively charged complementary DNA to form a negatively charged DNA-PNA hybrid. The facts that the complementary PNA-DNA hybrid can be selectively captured by the positively charged quaternized chitosan particles and the particles can be easily separated from non-complementary and unhybridized PNA allow direct detection of the PNA-DNA hybridization by MALDI-TOF mass spectrometry after washing without the use of enzyme treatment or heating.

REFERENCES

1. Boontha, B.; Nakkuntod, J.; Hirankarn, N.; Chumpluk, P.; Vilaivan, T. *Anal. Chem.* **2008**
2. Vilaivan, T.; Srisuwannaket, C. *Org. Lett.* **2006**, 8, 1897–1900.
3. Hoven, V. P.; Tangpasuthadol, V.; Angkitpaiboon, Y.; Vallapa, N.; Kiatkamjornwong, S. *Carbohydr. Polym.* **2007**, 68, 44-53

S7-PO-37

Structures and Mechanisms of the Mukaiyama Aldol Reactions between Metal Organic Frameworks-505 Encapsulated Formaldehyde and Silyl Enol Ether: An ONIOM Study

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The reaction mechanism of the Mukaiyama-aldol reaction of encapsulated formaldehyde and silyl enol ether catalyzed by metal organic frameworks (MOF-505) is systemically investigated by the ONIOM (our-Own-N-layer Integrated molecular Orbital + Molecular Mechanics) approach utilizing two-layer ONIOM schemes (B3LYP/6-31G(d,p) : UFF). For comparison, the Mukaiyama-aldol reaction of silyl enol ether and formaldehyde was also studied with the bare Cu^+ cation as an excessive charged catalyst. Three model systems were utilized to study this reaction: (1) a non-promoted uncatalyzed model: $O{=}CH_2/H_3SiOHC{=}CH_2$, (2) a formaldehyde in MOF-505: $O{=}CH_2$@MOF-505/$H_3SiOHC{=}CH_2$, and (3) a naked Cu^+ cation as a catalyst: $Cu(I)/O{=}CH_2/H_3SiOHC{=}CH_2$. The Mukaiyama aldol reaction of silyl enol ether with formaldehyde takes place in single concerted reaction step. It was found that metal-organic frameworks lead to an energy barrier for the reaction ΔE_{act} of 12.0 kcal/mol. This compares to values of ΔE_{act} of 13.7 kcal/mol for the uncatalyzed system and 6.9 kcal/mol for the reaction on the naked Cu^+ cation. In order to promote the Mukaiyama-aldol reaction, the metal-organic framework not only moderately reduces the reaction barrier but it also prevents a polymerization of the formaldehyde reagent.

REFERENCES

1. Choomwattana, S.; Maihom, T.; Khongpracha, P.; Probst, M.; Limtrakul, J. *J. Phys. Chem.* **2008**, 112, 10855–10861.
2. Sangthong, W.; Probst, M.; Limtrakul, J. *J. Mol. Struct.* **2005**, 748, 119-127.
3. Maihom, T.; Namuangruk, S.; Nanok, T.; Limtrakul, J. *J. Phys. Chem. C* **2008**, 112, 12914–12920.
4. Limtrakul, J.; Nanok, T.; Jungsuttiwong, S.; Khongpracha, P.; Truong, T. N. *Chem. Phys. Lett.* **2001**, 349, 161-166.

S7-PO-38

Conducting Polymer Composites of Polystyrene and Poly(3,4-ethylenedioxythiophene) Prepared by Heat-Activated Polymerization of 2,5-Dibromo-3,4-ethylenedioxythiophene

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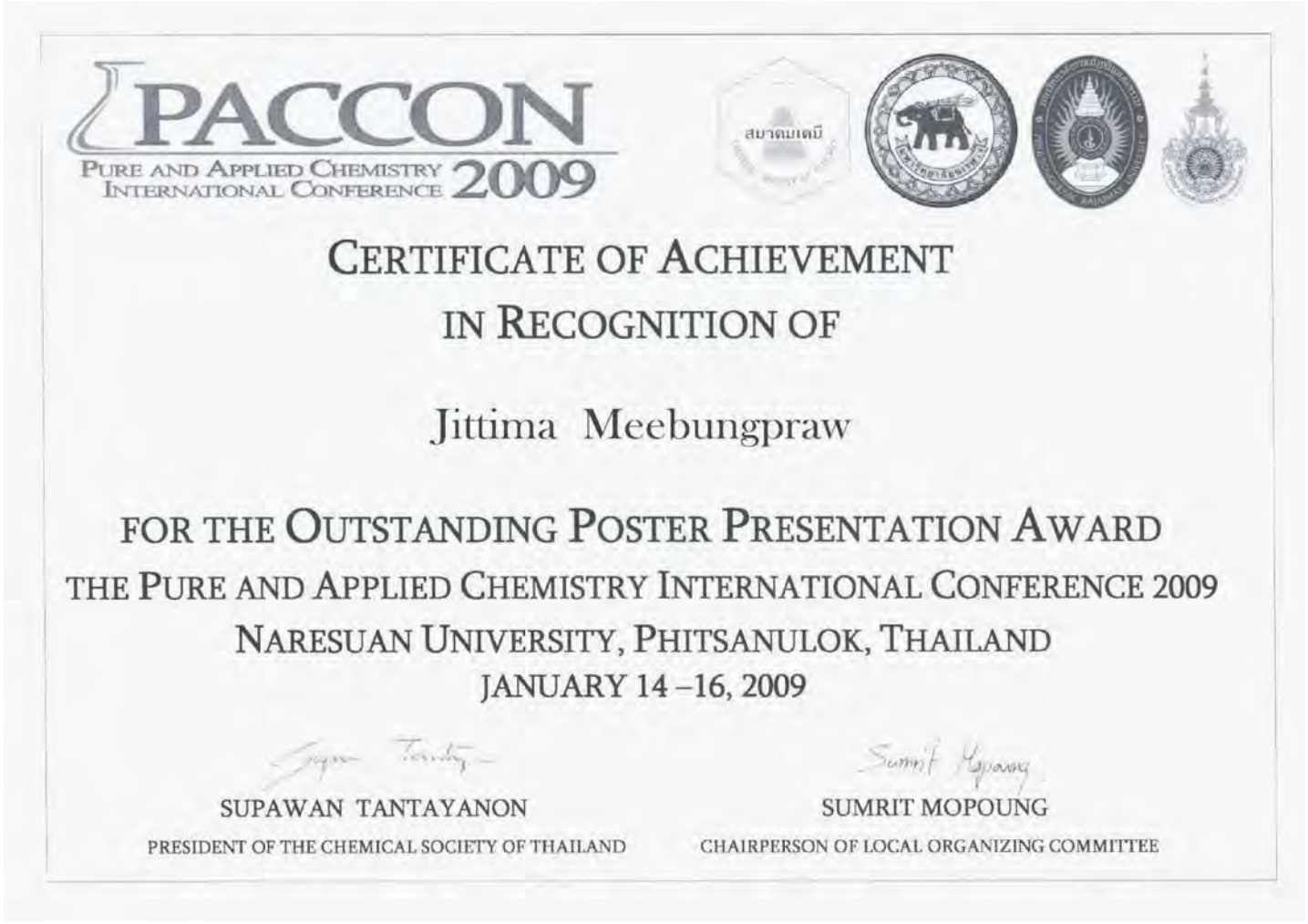
Conducting polymer composites containing poly(3,4-ethylenedioxythiophene) (PEDOT) was prepared by heat-activated polymerization of 2,5-dibromo-3,4-ethylenedioxythiophene (DBEDOT) in the presence of polystyrene (PS). A thin film was first fabricated by drop casting or electrospinning a solution mixture of PS and DBEDOT on solid substrates. After the solvent was removed, the solid state polymerization of the DBEDOT crystals embedded in the PS matrix was then



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CERTIFICATE OF ACHIEVEMENT
IN RECOGNITION OF

Jittima Meebungpraw

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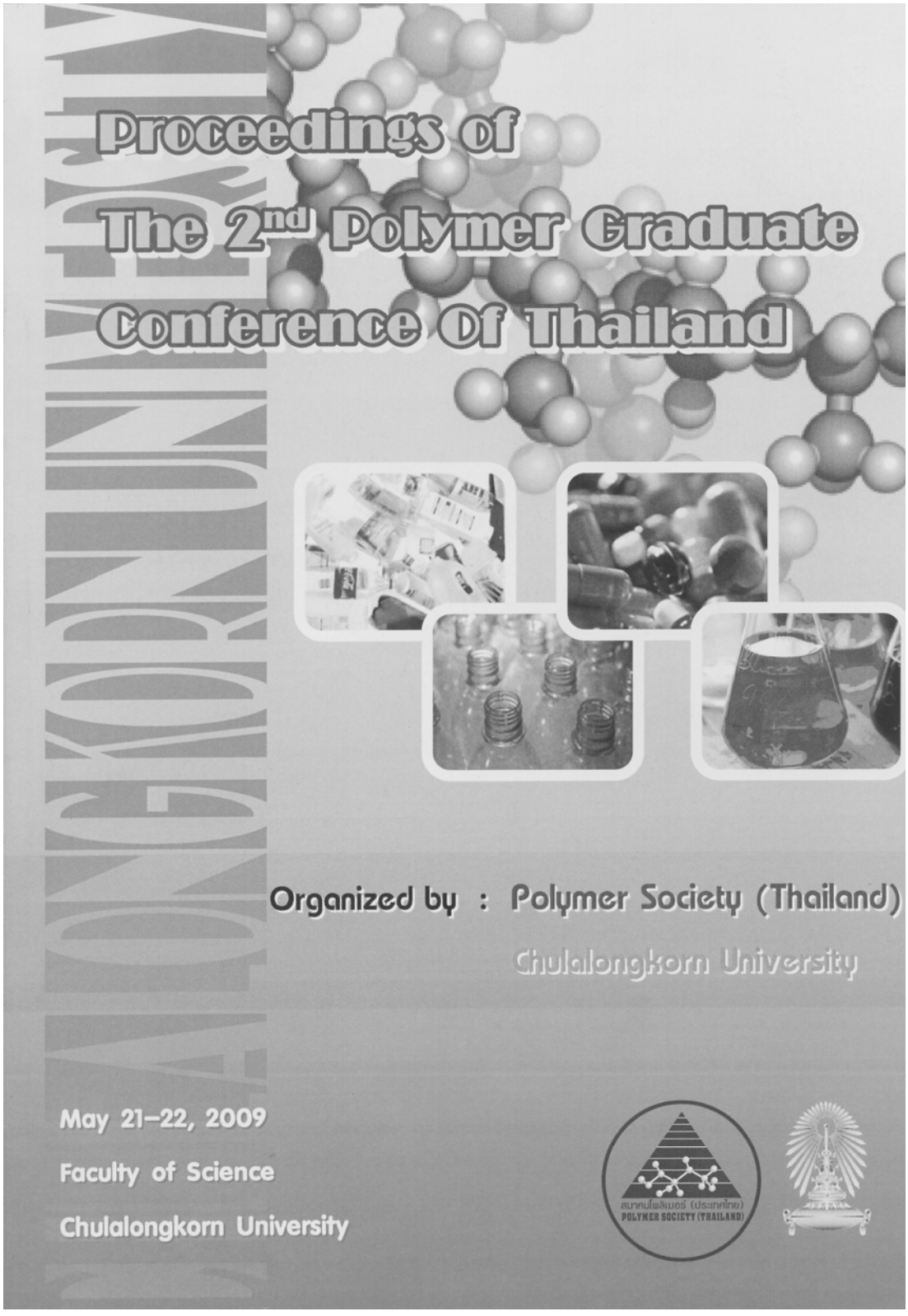
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Determination of DNA Sequences using Peptide Nucleic Acid in Combination with Chitosan Particles by MALDI-TOF Mass Spectrometry

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Abstract

The concept of using a new conformationally rigid pyrrolidinyl peptide nucleic acid (PNA), strong anion-exchange captures in combination with MALDI-TOF mass spectrometry has been recently proven as a simple and effective way for DNA sequence analysis. This research has introduced chitosan and quaternized chitosan particles as anion-exchange captures that may be applicable for the same purpose. The success of particle formation was verified by FT-TR, ^{1}H-NMR analyses. Investigation by MALDI-TOF mass spectrometry suggested that chitosan and some quaternized particles in combination with PNA (T9) are capable of distinguishing the complementary DNA sequence (A9) from the non-complementary (single and full mismatched) DNA sequences.

Keywords: Chitosan; Quaternized chitosan particles; Solid support; Complementary DNA

1. Introduction

Determination of DNA sequences are significantly important for many biotechnology-related applications ranging from medical, forensic, agriculture and food science. A recently developed label-free technique based on the use of peptide nucleic acid (PNA), a neutral-backbone analogue of DNA and anion exchanger was used in the step of sample preparation prior to the analysis by MALDI-TOF Mass Spectrometry [1]. The quaternary ammonium containing Q-sepharose used as the anion exchanger, however, possesses some practical limitations. It is generally commercialized in the form of liquid dispersion so the exact amount of the particles used for each experiment is unknown. The weighting of the solid Q-sepharose is not practical since the drying would destroy the swellability of the microparticles which in turn may adversely affect the capability of DNA capturing. The ability to introduce quaternary ammonium groups to the surface of chitosan particles, whose size, charge density as well as functional group variation can be manipulated prompts us to believe that the chitosan and quaternized chitosan particles can be potentially used for the same application and perhaps overcome the problems presently encountered with the Q-sepharose. The concept of using chitosan particles as anion exchangers is displayed in Figure 1.

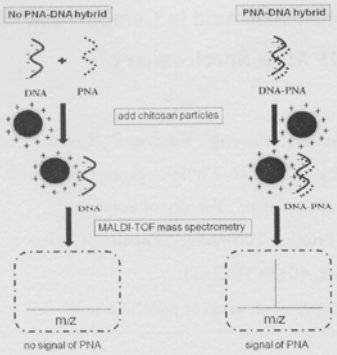

Figure 1. Schematic diagram showing the concept of using chitosan particles as anion-exchange capture of PNA-DNA hybrid in combination with MALDI-TOF mass spectrometry in DNA sequence analysis.

Hypothetically, all particles especially those are quaternized should be positively charged at pH 7 so they can only bind ionically with the negatively charged DNA. PNA is a neutral molecule so it cannot be adsorbed by the particles unless it is hybridized with the negatively charged complementary DNA to form a negatively charged DNA-PNA hybrid. The facts that the complementary PNA-DNA hybrid can be selectively captured by the positively charged chitosan and quaternized chitosan particles and the particles can be easily separated from non-complementary and unhybridized PNA should allow direct detection of the PNA-DNA hybridization by MALDI-TOF mass spectrometry after washing.

2. Experimental

2.1 Preparation of Chitosan and Quaternized Chitosan Particles

Chitosan particles formed spontaneously upon an addition of 3 mL of an aqueous tripolyphosphate (TPP) solution to a 3 mL of chitosan solution dissolved in 1%v/v acetic acid under magnetic stirring. The particles were obtained after rinsing and isolation by centrifugation at 10,000 rpm and lyophilization.

Methylated chitosan particles were prepared by two methods. The first method was based on heterogeneous methylation of chitosan particles using NaOH, NaI and MeI in methanol at 50°C for 8 h [2]. The second method involved particle formation by crosslinking *N,N,N*-trimethyl chitosan (TMC) by TPP. The detail of TMC synthesis is available elsewhere [3]. The particles prepared by the first and second method are defined as QAC and TMC particles, respectively.

Another quaternized chitosan particles, methylated *N*-benzyl chitosan particles (MBzC) were prepared by heterogeneous reaction through 2-step process: *N*-reductive alkylation with benzaldehyde followed by methylation with MeI [4]. In brief, an anhydrous methanol solution of benzaldehyde (1 M) was added into a flask containing chitosan particles. After stirring for 4 h at ambient temperature. $NaBH_4$ was then added into the reaction mixture and the solution was stirred for 24 h. After that, the particles were subjected to heterogeneous methylation using NaOH, NaI and MeI in methanol at 50°C for 12 h. The MBzC particles were obtained after isolation by the same methods described for the chitosan particles.

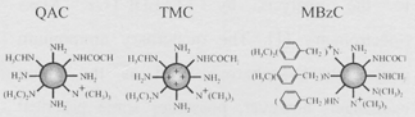

Figure 2. Chemical structures of quaternized chitosan particles.

2.2 Determination of PNA-DNA Hybridization

A PNA probe (10 pmol) and DNA sample (10 pmol) were mixed in 50 µL of binding buffer (10 mM sodium phosphate, pH 7) and incubated at ambient temperature for 20 min. The quaternized chitosan particles were then added to the PNA-DNA hybrid solution, incubated for 20 min at ambient temperature, and then centrifugally washed with 3×200 µL of Milli-Q water at ambient temperature. Finally, 1 µL aliquots of washed particles was re-moved and mixed with 10 µL of the matrix solution consisting of α-cyano-4-hydroxycinnamic acid in 0.1% TFA in acetonitrile/water (1:2) solution. The sample mixture (100 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 (on Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). All spectra were processed by averaging between 20 and 30 individual laser shots.

3. Results and discussion

From ^{1}H NMR spectra of quaternized chitosan particles illustrated in Figure 3, the signal at 2.9-3.0, 2.7, and 2.5 ppm can be assigned to the methyl protons of the trisubstituted, disubstituted, and monosubstituted amino groups, respectively. In the case of MBzC particles, the signals of aromatic protons at 7.2 ppm additionally confirmed the attachment of benzyl groups to the amino moieties of chitosan.

The FT-IR spectra of quaternized chitosan particles are shown in Figure 4. The appearance of characteristic peak at 1204 cm^{-1} assigned to P=O groups of TPP and the shifting of the N-H bending signal from 1604 cm^{-1} of chitosan to 1547 cm^{-1}of all particles (CS, TMC, QAC, and MBzC) indicated that the amino groups of chitosan and TMC were ionically crosslinked by TPP. As compared with the CS particles, the decrement of the N-H bending signal at 1547 cm^{-1} and the appearance of C-N stretching at 1460-1470 cm^{-1} in the spectra of QAC, TMC, and MBzC particles also demonstrated the success of quaternized particle formation.

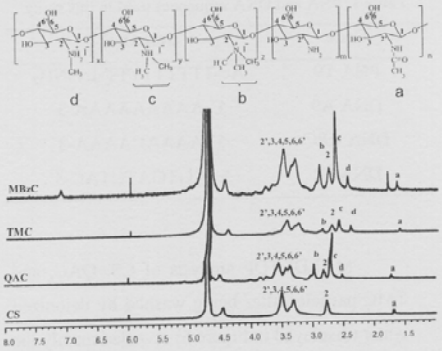

Figure 3. ^{1}H NMR spectra of CS, QAC, TMC and MBzC particles.

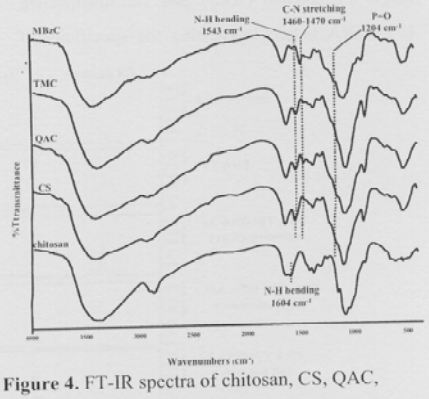

Figure 4. FT-IR spectra of chitosan, CS, QAC, TMC and MBzC particles.

To test the ability of particles as anion-exchange captures of complementary PNA-DNA hybrid, PNA probe having 9 repeat units of homothymine, T9 was chosen for the investigation. The T9 was firstly mixed in 100 µL of binding buffer and hybridized with the DNA target (listed in Table 1), followed by an addition of designated particles (CS, QAC, TMC, and MBzC).

Table 1. PNA and DNA sequences used in this study

Code	Sequence
PNA T9	Ac-TTTTTTTTT-LysNH$_2$
DNA A9	5′-AAAAAAAAA-3′
DNA A8C	5′-AAAACAAAA-3′
DNA F2	5′-AGTGATCTAC-3′

MALDI-TOF analysis of CS, QAC, and TMC particles after being washed by deionized water (displayed in Figure 5) revealed mainly the signal of PNA probe, only when the complementary DNA (A9) was employed. There were no signals detected in the cases of both single-mismatched (A8C) and full-mismatched (F2) DNA targets suggesting the specificity of the detection. The success of analysis was further demonstrated by the fact that there was no appearance of PNA signal in the absence of the hybridized DNA implying that there were no non-specific interactions between the positively-charged particles and the neutral and uncharged PNA. And that the PNA can only be linked to the particles through the hybridization with the complementary DNA that was later attracted ionically to the particles. However, the mismatch discrimination cannot be realized in the case of the MBzC particles. The signals of PNA probe were seen both in the PNA solution and on the particles regardless of the mismatching of the DNA target. This non-specific interaction is believed to stem from the presence of hydrophobic benzyl moieties introduced to the MBzC particles. It should also be emphasized that there was no DNA signal appearing in the mass spectra although it exist concurrently with the PNA in the PNA-DNA hybrid that was captured by the particles. This can be explained as a consequence of the more effective ionization of PNA than DNA in the positive ion mode.

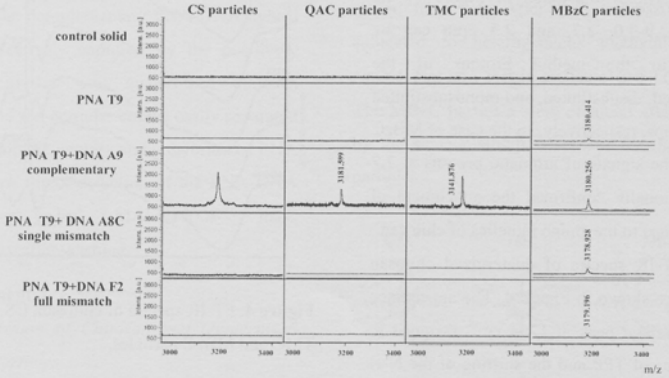

Figure 5. MALDI-TOF mass spectra of particles before (row 1) and after incubation with PNA-probe in the presence of different DNA target (row 3-5) in comparison with the PNA probe in solution (row 2).

4. Conclusion

The concept of using chitosan and quaternized chitosan particles as anion exchange captures in combination with pyrrolidinyl PNA and MALDI-TOF mass spectrometry for determination of DNA sequence has been validated. It is obvious that the anion capturing ability of some chitosan particles are comparable to that of the commercial anion exchanger, Q-sepharose. The specific detection of hybridization event can also be realized and is strongly affected by the variation of functionality introduced to the chitosan particles in the step of quaternization.

Acknowledgements

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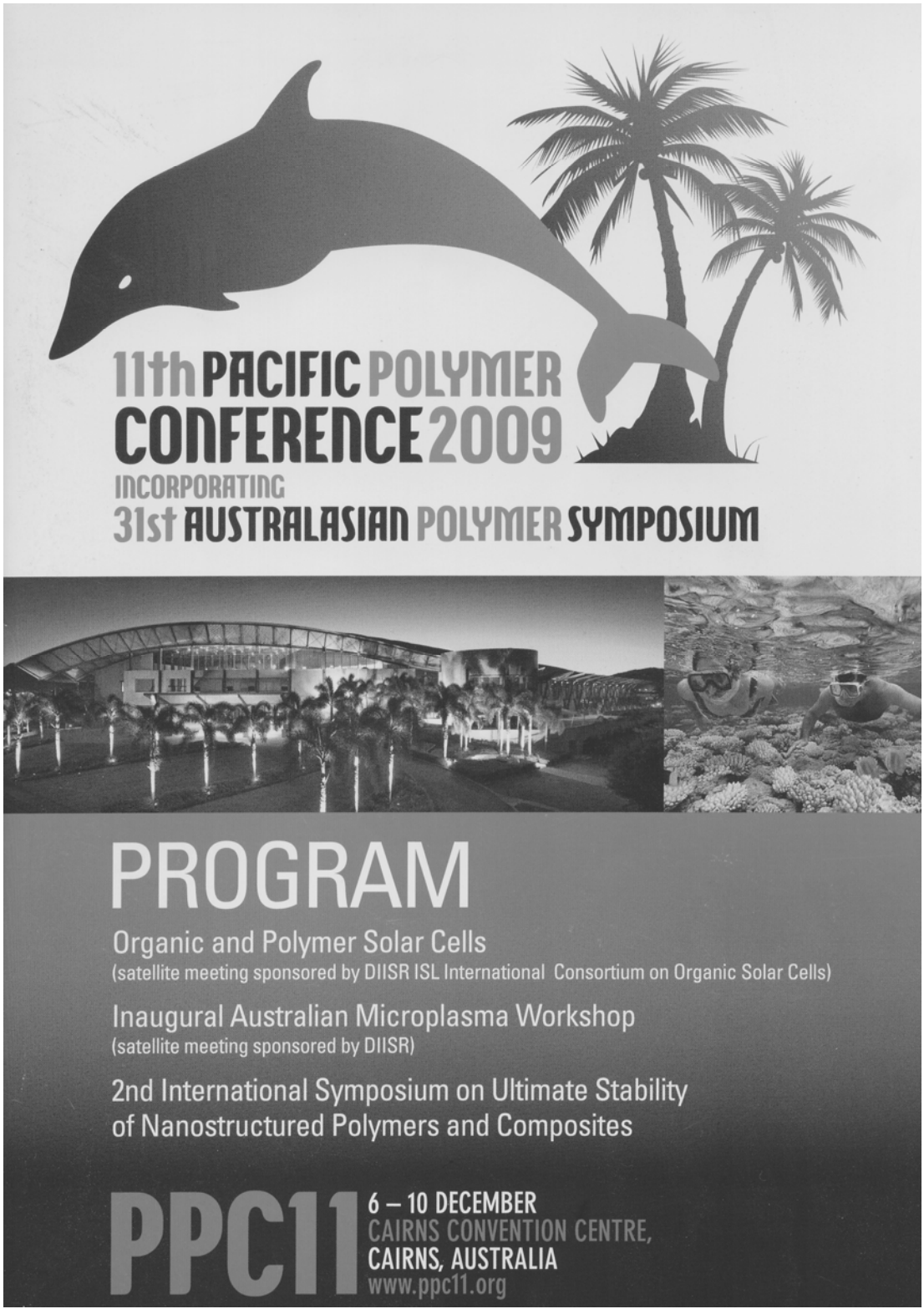
References

[1] Boontha, B., Nakkuntod, J., Hirankarn, N., Chumpluk, P and Vilaivan, T. Anal. Chem., **80**, 8178-8186, (2008).

[2] Hoven, V. P., Tangpasuthadol, V. Angkitpaiboon, Y., Vallapa, N. and Kiatkamjornwong, S. Carbohydr. Polym., **68(1)**, 44-53, (2007).

[3] Sieval, A. B., Thanou, M.,Kotze, A. F., Verhoef, J. C., Brussee, J. and Junginger, H. E. Carbohydr. Polym., **36(2-3)**, 157-165, (1998).

[4] Vallapa, N. Preparation and antibacterial activity of quaternary ammonium-containing chitosan surface, Master's thesis, Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University., **2006.**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Determination of DNA Sequences using Peptide Nucleic Acid in combination with Chitosan Particles by MALDI-TOF Mass Spectrometry

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Determination of DNA sequences are significantly important for many biotechnology-related applications ranging from medical, forensic, agriculture and food science. The concept of using a new conformationally rigid pyrrolidinyl peptide nucleic acid (PNA), strong anion-exchange captures such as commercially available Q-Sepharose in combination with MALDI-TOF mass spectrometry has been proven as a simple and effective way for single nucleotide polymorphism genotyping.[1] This research has introduced chitosan particles having different moieties as alternative anion-exchange captures that may be applicable for the same purpose. The strategy is schematically outlined in Figure 1.

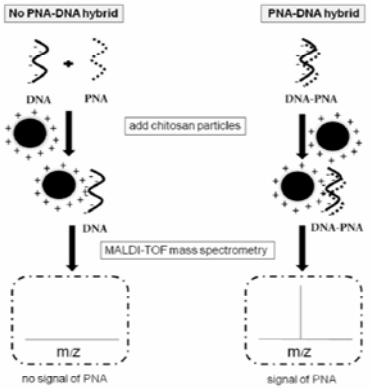

***Figure* 1:** Schematic diagram showing the concept of using chitosan particles as anion-exchange capture of PNA-DNA hybrid in combination with MALDI-TOF mass spectrometry in DNA sequence analysis

In principle, the positively charged chitosan particles can only bind ionically with the negatively charged DNA. The neutral PNA cannot be adsorbed by the particles unless it is hybridized with the negatively charged complementary DNA to form a negatively charged DNA-PNA hybrid. The facts that the complementary PNA-DNA hybrid can be selectively captured by the positively charged chitosan particles which can be easily separated from non-complementary and unhybridized PNA should allow direct detection of the PNA-DNA hybridization by MALDI-TOF mass spectrometry after washing. To test the ability of all chitosan particles as anion-exchange captures of complementary PNA-DNA hybrid, PNA1 probe, T9 was first hybridized with the DNA target (listed in Table 1), followed by an addition of the designated chitosan particles. MALDI-TOF analysis of all particles (except DMBz) after being washed by deionized water, shown in Figure 2, revealed mainly the signal of PNA1, only when the complementary DNA1 (D9) was employed. There were no signals detected in the cases of both single-mismatched (A8C) and full-mismatched (F2) DNA targets suggesting the specificity of the detection. The success of analysis was further

demonstrated by the fact that there was no appearance of PNA1 signal in the absence of the hybridized DNA implying that there were no non-specific interactions between the positively-charged particles and the neutral and uncharged PNA1. And that the PNA1 can only be linked to the particles through the hybridization with the complementary DNA that was later attracted ionically to the particles. However, the mismatch discrimination cannot be realized in the case of the DMBz particles. The signals of PNA1 probe were seen on the particles regardless of the mismatching of the DNA target. This non-specific interactions are believed to stem from the presence of hydrophobic benzyl moieties introduced to the DMBz particles. Among all particles tested, HTACC particles seem to give the best performance considering that they did not exhibit non-specific interactions with PNA2 probe, so that the detection of PNA1 was possible even in the presence of PNA2. It should be noted that the signal of PNA1 and PNA2, typically appear at m/z 3180 and 3260, respectively. That was not the case for CS, QAC, and DMBz particles. The hydrophilic hydroxyl groups which is a part of quaternary ammonium groups render the HTACC particles superior anion-exchange captures.

Table 1 PNA and DNA sequences used in this study

Code	Sequence	Note
PNA1 (T9)	Ac-TTTTTTTTT-LysNH_2	-
DNA (A9)	d(5'-AAAAAAAAA-3')	complementary to PNA1
DNA (A8C)	d(5'-AAAACAAAA-3')	single-mismatch to PNA1
DNA (F2)	d(5'-AGTGATCTAC-3′)	full-mismatch to PNA1
PNA2 (A9)	Ac-AAAAAAAAA-LysNH_2	-

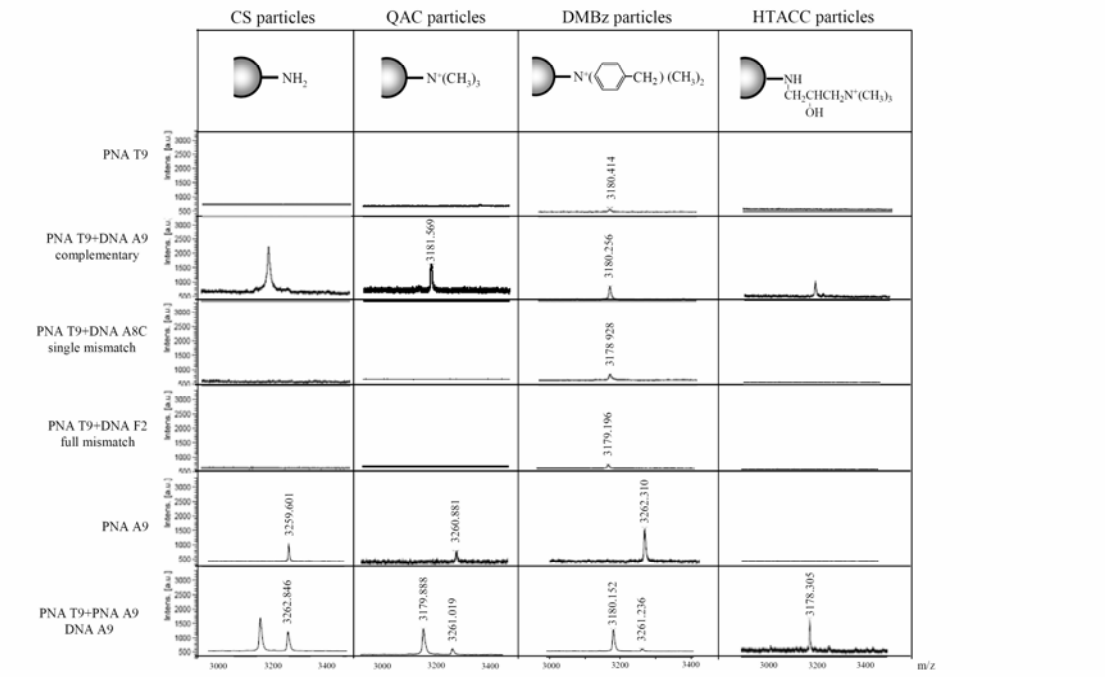

Figure 2: MALDI-TOF mass spectra of particles after capturing the PNA-DNA hybrid

References

[1] Boontha, B.; Nakkuntod, J.; Hirankarn, N.; Chumpluk, P.; Vilaivan, T. *Anal. Chem.* **2008,** *80*, 8178-8186.

[2] Hoven, V. P.; Tangpasuthadol, V.; Angkitpaiboon, Y.; Vallapa, N.; Kiatkamjornwong, S. *Carbohydr. Polym.* **2007,** 68(1), 44-53.

VITAE

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