Fluorescence Based PNA-DNA Binding Characterization on Nano-Field Effect Transistor (FET) DNA Device Surface

Shiva K. Rastogi*, Nirankar N. Mishra, Brian Filanoski, Eric Cameron, and Wusi C. Maki*

Center for Advanced Microelectronics and Biomolecular Research (CAMBR),
University of Idaho Research Park, Post Falls, ID – 83854, USA
srastogi@uidaho.edu, wmaki@cambr.uidaho.edu

ABSTRACT

A sequence of peptide nucleic acid (PNA) containing ten thymine monomers and β-alanine amino acid at both (C and N) terminal of main chain (βAla-$(T)_{10}$-βAla-NH_2) was synthesized manually using solid phase *t*Boc peptide chemistry. Synthesized PNAs were analyzed and purified using RP-HPLC and characterized by MALDI-TOF mass spectrometry. The hybridization of βAla-$(T)_{10}$-βAla-NH_2 with its single strand complementary DNA (sscDNA; A_{10}) was established in liquid phase through non denaturing gel electrophoresis and UV-thermal denaturation curve at 1 µM concentration. The T_m of hybrid was observed 40.05 °C. Further, the nano-FET devices were designed and fabricated by our group for developing DNA biosensor. The PNAs were covalently immobilized on device surface with alkyl-thiol self assembled monolayer. DAPI nucleic acid stain and fluoresceine labeling methods were used to prove the binding of PNA with A_{10} on nano FET surface from 10 nM solution, using fluorescent microscopic imaging. The model reported here has a potential capability of applying in developing PNA based biosensors for specific nucleic acid detection at room temperature.

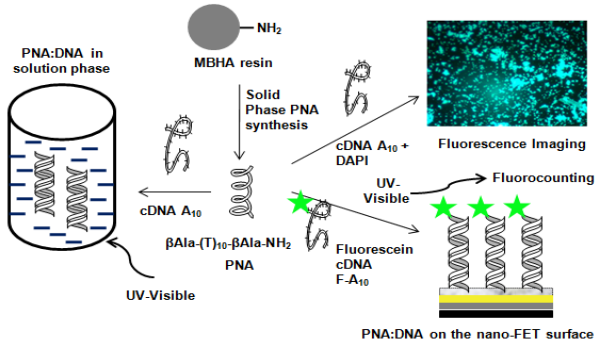
Keywords: PNA, DNA, fluorescent characterization, nano-FET device, Biosensors

1 INTRODUCTION

Peptide nucleic acid (PNA) is structural mimic of nucleic acid in which; sugar–phosphate back bone is replaced by an achiral, non-ionic pseudopeptide backbone, composed of N-(2-aminoethyl)-glycine unit [1]. Since, the backbone structure of PNA itself has no charge; there is no electrostatic repulsion between PNA and single strand complementary (sscDNA). PNA, therefore, has high sequence recognition ability and form thermally stable duplex structures with complementary single strand nucleic acid sequences via Watson-Crick base pairing [2]. Therefore, PNA has been chosen as a detection probe in the area of biomedical diagnostics [3] and food safety [4] for electronic charge detector based biosensors [5]. Leveraging on the physico- and bio-chemical properties of PNA, different electrochemical [6] and optical [7] techniques have been used to study PNA/DNA hybridization on different surface of devices for DNA detection.

Various nano field effect transistors (FET) were designed and fabricated by our group [8] with doped semiconductor nanowires (30nm-500nm) on silicon/silicon dioxide wafers coated with polyimide to develop nano FET as a DNA biosensor. In continuation of this work, we in vision, it would be a tremendous advantage for the development of nucleic acid biosensor, if complementary nucleic acid hybridization event would occur at room temperature with simple and specific length of PNA.

To address this issue, here we designed and synthesized a sequence of homothymine PNA **1**, containing β-alanine amino acid at both, the ('N' and 'C') terminal of its main chain and demonstrate its binding with sscDNA (A_{10}) in liquid and on nano-FET device surface as shown in scheme 1.



Scheme 1: Schematic diagram for synthesis of PNA **1** (βAla-$(T)_{10}$-βAla-NH_2) using solid phase resin, its binding with sscDNA in solution phase and on nano-FET device (Bottom to top layer Silicon/SiO_2/Gold/polyimide/Thiol-SAM/PNA-DNA complex).

2 EXPERIMENTAL

2.1 Reagents and Instrumentation

Unless otherwise noted, starting materials and other chemicals were obtained from commercial sources and used without any further purification. All the glass wears were rinsed with deionized water (Water Pro PS, Labconco, 18.2 Ω). Positive ion MALDI-TOF mass spectra were obtained using an Applied Biosystem (4800 TOF-TOFMALDI) mass spectrometer. Matrices used for MALDI-TOF analysis was: a saturated sinapinic acid solution (in 01% TFA in water). HPLC purification and analysis were performed on Shimadzu, 10 Avp and Prevail C18 5µ, 150 mm x 4.6 mm column from Alltech-Associates, Inc. for analytical separation and C18 Monomeric, 120A from GRACE VYDAC for semi-preparative separation. UV/Vis

spectra were recorded on UV-Pharma Spec – 1700, spectrometer, Shimadzu. PNA/DNA hybridization in solution phase was performed in Techne-Techgene thermal cycler. Gel spots were visualized at 254 nm UV-C, using 3UV™ transilluminator from UVP, upland, CA. 4′,6-diamidino-2-phenylindole DAPI images were taken from Olympus BX60 optical microscope, diagnostics model # 2.2.1.

2.2 Synthesis of PNA

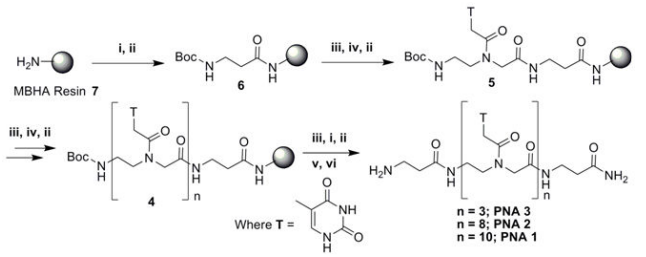
General Method for synthesis of PNA 1

Synthesis of PNA **1** was performed manually in 10 ml solid phase peptide vessel, using *t*Boc chemistry Ørum et al. [9] as elutriated in scheme 2. Boc-β-Ala-OH (23 mg, 0.118 meq) was activated with HATU (43.3 mg/0.112 meq)/DIEA (25 µL, 0.147 meq) in 500 µL NMP/Pyridine (2:1) mixture and loaded on pre swelled MBHA resin (50 mg, 0.0295 mmol) with DCM at rt for overnight. The reaction mixture was agitated with nitrogen gas at rt for 3 h. This coupling was repeated twice. Boc group of Boc-β-Ala-(MBHA) resin and all other intermediates were depredated with TFA:m-cresol (95:5, v/v) 3 min twice followed by resin was washed with DCM (x3), DMF (x3), 5% DIEA/DCM (x1), DMF (x3) and DCM (x3). PNA monomer, Boc-T-OH was coupled with resin though HATU/DIEA (0.112 meq/0.147 meq) in NMP/pyridine (v/v 2:1; 500 µL) as coupling reagents under nitrogen agitation gas at rt for 1 h. and washed with DCM (x3), DMF (x3), 5% DIEA/DCM (x1), DMF (x3) and DCM (x3). After every coupling of amino acid and monomers, free amines of unreacted oligomer resin were capped with NMP:Pyridine:Ac_2O (v/v/v, 2:2:1; 3 mL) for 15 min twice at rt . After that resin was washed with DCM (x3), DMF (x3), 10% pip/NMP (x1), DMF (x3) and DCM (x3).

PNA oligomers were cleaved from resin using "low-high TFA-TFMSA" procedure Berg and coworkers [10], (i) TFA:m-cresol (95:5; v/v) 3 min twice and (ii) TFMSA:TFA:m-cresol (200 µL, 2:6:1) for 1.5 h. and precipitated by adding cold anhydrous diethyl ether and lyophilized with water:acetonitrile mixture (4:1; v/v).The crude PNAs were analyzed and purified on RP-HPLC, using Alltech C_{18} column at 55°C with a flow rate of 1 mL/min by running two gradient methods with buffer A: Water (0.1% TFA) and buffer B: acetonitrile (0.1% TFA) with dual wavelength absorbance detection (260 and 220nm). Purified PNAs were characterized by MALDI-TOF mass spectrometry. The overall yields of PNAs were 25-55%. All purified PNA **1**, 2, and **3** were dissolved in pure DI water aliquot in portions and stored at – 20°C. Synthesis was monitored with Kaiser Test [11] and at two intermediate PNA oligomers **2** and **3** stage. The yield of purified PNA **1** was obtained in the range of 55% with more than 98% of purity.

2.4 Electrophoretic Gel Mobility Shift Assay.

The PNA **1** and A_{10} and two single strands-



Scheme 2. Synthetic route for solid phase synthesis of PNA **1** (βAla-$(T)_{10}$-βAla-NH_2); (i) MBHA resin, Boc-βAla-OH, HATU/DIEA, NMP/Pyridine, 3h, rt; (ii) NMP/Pyridine/Ac_2O, 15 min x 2, rt; (iii) TFA:m-Cresol, 3 min. x 2, rt; (iii) Boc-T-OH, HATU/DIEA, NMP/Pyridine, 3h, rt. (iv) TFA/m-Cresol, 3 min x 2, rt; (v) TFMSA/TFA/m-Cresol 1.5 h rt.

noncomplementary ss-non-cDNA (**1**: 5'/5AmMC6/TAA AGT TAA TGG TAA ATG AA3' and **2**: 5'/5 ThioMC6-D/CCG TCT GAA ACT GAT3') were mixed individually at 1:1 µM concentrations ratio in 10 µL water and suspended in 20 µL TE buffer (10 mM Tris-HCl and 1.0 mM EDTA). The samples were denatured at 85°C for 5 min followed by slow cooling to 22°C and holding the PNA-DNA complex at same temperature for 60 min followed by refrigeration at 4°C for 10 min. The samples were loaded on the gel bromophenol blue (BPB) was used as the tracer dye separately in an adjacent well. Gel electrophoresis was performed on a 15 % nondenaturing polyacrylamide gel (acrylamide/bisacrylamide 19:1) at constant power supply of 200 V and 10 mA in 40% sucrose in TBE buffer pH 8.0, until the BPB migrated to three-fourths of the gel length. During electrophoresis, the temperature was maintained at 5°C with ice bath. The spots were visualized at 254 nm, through UV shadowing by illuminating the gel placed on a transilluminator.

2.5. UV-T_m Measurment

The concentrations of PNAs and DNAs were calculated using the generally applied extinction coefficients (OD_{260}/µmol) of the corresponding nucleotides: A, 15.4 and T, 8.8. 1 µM of each strand (PNA and DNA) were mixed into TE (Tris/EDTA) buffer, pH 7.5, containing TrisCl (10 mM) and EDTA (1 mM), and annealed by keeping the samples at 90 °C for 5 min followed by slow cooling to 20 °C. Thermal denaturation profiles (A_{260} vs. T) for duplexes were recorded on a UV-Vis Cary 100 spectrophotometer using quartz optical cells with a path length of 1.0 cm, by varying the temperature from 20 °C to 80 °C at a ramp of 1.0 °C/min. Data were processed using Cary WinUV version 3.00 software, and thermal denaturation temperatures (T_m-values) were obtained using the derivative method. Reported thermal denaturation temperatures are an average of three measurements within ±1.0 °C.

2.6. Protocol for PNA/DNA binding on device surface.

The chip surfaces were place in 100% ethanol and sonicated for 10 min, thoroughly washed with 100% ethanol and DI three times. Then the gold surface was dried through nitrogen gas current and placed in argon plasma cleaner for cleaning 20 min. The formation of carboxylic group terminated alkane thiol SAM on the gold surface was accomplished by overnight immersing in 5 mM 11-merceptoundecanoic acid and 25 mM, 3-merceptopropionic acid (1:10 v/v) in ethanol solutions. After that sonicate in 100% ethanol for 5 min and washed with DI water 5 times, allow to dry 10 min in air. 2 µl of an aqueous solution of 100 mM EDAC and 25 mM N-hydroxysuccinimide (NHS) was droped on the gold surface and allow to incubate for 1 h at rt to activate free end of carbolylic acid group of SAM and then washed with water 5 times. PNA **1** (50 mM) was heated at 50°C for 10 min then allow to cool in ice bath for 2 min, 1.5 µl of this PNA was dropped on the activated SAM of gold surface and incubate 24 h at rt in a humid chamber, washed with water 5 times and allowed to air dry for 10 min. The unactivated surface was blocked with 3 µL of blocking buffer (1X PBS+0.01% BSA+1mM+PEG+1mM Glycine) for 3h at rt then washed with PBS and water three times each. Binding of A_{10} or F-A_{10} as accompanied with SAM immobilized PNA **1** on gold surface at rt. DNA (1.5 µl) in TE buffer (10 mM+EDTA 1mM, pH 8.0) was dropped over PNA **1** spot on surface and allow to incubate at rt. in humid chamber followed by washing with TE and water three times each and allow to air dry at rt for 10 min. Each surface was immersed in 1 µg aqueous solution of DAPI and gently shaked for 1 h at rt, followed by aqueous washing 3 times, allow to stain each spot of gold surface. The various surfaces were then analyzed using confocal fluorescent microscope under UV-visible light.

3 RESULT AND DISCUSSION

The study of simple sequence of thymine PNA was investigated, which include the design, synthesis, and binding with sscDNA in liquid phase and on gold surface. The main aim was to evaluate the binding of PNA/DNA on gold surface on room temperature for developing PNA based DNA biosensor device.

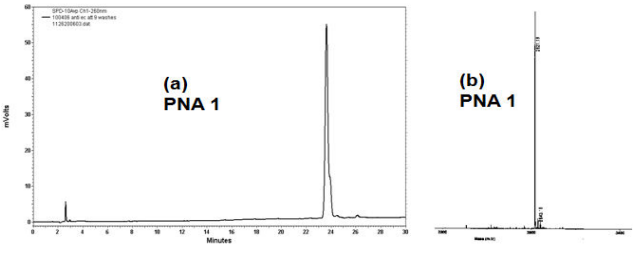


Figure 1a. RP-HPLC Chromatogram of pure βAla-$(T)_{10}$-βAla-NH_2; method: 0 - 10 min, 95 - 89% A, 10 – 20 min, 89% A, 20 – 42 min, 89 – 84% A, 42 – 43 min, 84 – 95% where A = (0.1% TFA in water) and B = (0.1% TFA in acetonitrile) **(b)** Positive ion MALDI-TOF mass of PNA **1**; calcd $[M+H]^+$ = 2821.12 and found = 2821.18

Since, the pyrimidine (T) PNA oligonucleotides binds sequence specifically to homopurines (A) regions of DNA by triple helix formation through T-A-T triplets [1], this property PNA motivate us for selecting PNA **1** for our study. We incorporated the β-alanine amino acid at N-terminus that would be used for covalent immobilization with self assembled monolayer (SAM) on gold surface and at C-terminus for easy elongation of PNA on resin. The RP-HPLC chromatogram in fig. 1a, shows the synthesize PNA 1 was obtained in very high purify > 98% and fig 1b shows the observed molecular weight of PNA **1** from MALDI-TOF mass spectroscopy which confirm the presence of PNA **1**.

In solution phase PNA **1** and A_{10} DNA hybridization was first characterized by using non denaturing electrophoretic gel shift assay. The two ss-non-cDNA strands (**1** and **2**) were randomly selected as negative control, their mixture PNA **1** were also run in gelelctrophoresis to check the non specific binding. All, the bands visualized on a fluorescent TLC background are shown in figure 2a. The two concentration ratio of PNA **1**/ DNA A_{10} were used for gelelectrophoresis. In case of 1:1 µM ratio there was very poor shift was observed between the bands (data not shown here), whereas at 2:1 µM ratio gives better shifting, which indicates that PNA **1** would be triplicating with A_{10} DNA.

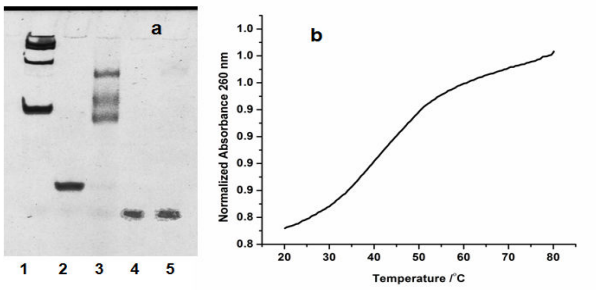


Figure 2. (a) 15% nondenaturing gel electrophoresis mobility shift of PNA **1** – A_{10} complexes: line 1 = marker; line 2 = A_{10}; line 3 = (βAla-$(T)_{10}$-βAla-NH_2) : A_{10} (2:1 µM) at rt.; line 4 = PNA **1**: ss-non-cDNA 1; line 5 = ss-non-cDNA **2**, (**b**) Thermal denaturation profile (A_{260} vs. T) of 1:1 mixture of βA-$(T)_{10}$-βA-NH_2 – A_{10} (1µM each strand) in TE buffer (10 mM Tris-HCl and 1.0 mM EDTA) at pH = 7.0. Temperature was raised from 20 to 80 ºC at a ramp of 1.0 ºC/min.

On the other hand the two negative controls did not show any significant shifting on the gel, it means the PNA 1 is not binding with ss-non-cDNA. Further, thermal characteristic graph of PNA **1**/A_{10} mixture is shown in figure 2b. This is shows the temperature dependence of UV-visible absorption curve at 260 nm of PNA **1**/A_{10} DNA complex at 1:1 µM mixture. The melting temperature was observed to be 40.05 °C from derivative method, which clearly suggests that PNA **1** is forming a complex and can be used for binding below its melting temperature.

PNA/DNA binding characterization on the surface was carried out by covalently immobilizing PNA **1** onto the nano FET surface. The fluorescent images of different surface are shown in fig. 3. The DAPI stain (dark greenish-blue spots) on different surfaces (as a negative control) were showing not more than ca. 5% DAPI, but the dense DAPI stain can be seen on the surface having immobilized PNA **1** and A_{10} complex. This shows the presence of target sscDNA on the immobilized PNA **1** with surface.

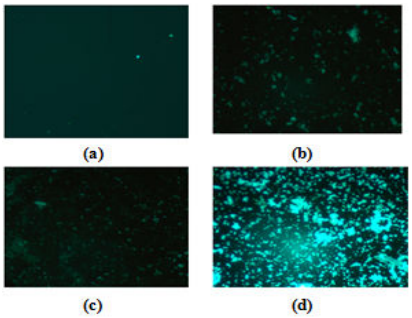


Figure 3. Confocal microscopic images of DAPI fluorescent stain on nano FET surface (3 x 3 mm) under UV-Visible light. PNA **1** – A_{10} complex were stained with DAPI as shown in figure **d**: (**a**) blank surface; (**b**) surface + thiol SAM; (**c**) surface + thiol SAM + βAla-$(T)_{10}$-βAla-NH_2; (**d**) surface + thiol SAM + βAla-$(T)_{10}$-βAla-NH_2/A_{10}. All images were taken through reflected light on a bright background.

PNA **1** - sscDNA binding was further investigated on nano FET surface using eight different concentrations of F-A_{10} DNA. The fluorocounting of F-A_{10} are shown in the graph of fig. 4a. This indicates that the fluorocount reading of less than 10 nM concentrations of F-A_{10} shows nearly equivalent to the blank reading and above 100 nM concentrations reach the saturation point and fluorocount readings become constant.

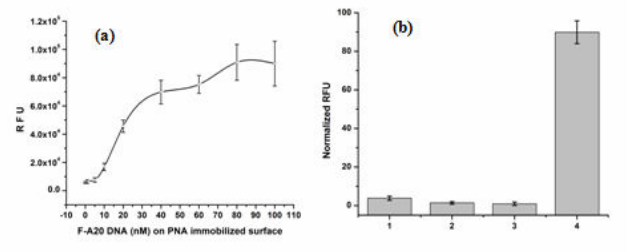


Figure 4 a. Complexes of immobilized PNA **1** on nano FET surface with fluoresceine labeled cDNA (F-A_{10}) were characterized through a microplate fluorescence reader using excitation wavelength of 485±20 and emission at 528±20 λ; (**b**) relative fluorescent unit of: bar **1** = blank surface; bar **2**: surface + F-A_{10}; bar **3**: surface + thiol SAM + F-A_{10}; bar **4**: surface + thiol SAM + βAla-$(T)_{10}$-βAla-NH_2 : F-A_{10}. 10 nM F-A_{10} DNA were used on each surface to characterize PNA **1** - sscDNA binding.

The fluorocounting of F-A_{10} are shown in the graph of fig. 4a. This indicates that the fluorocount reading of F-A_{10} DNA at less than 10 nM concentrations shows nearly equivalent to the blank reading and above 100 nM concentrations, it reaches to the saturation point and then fluorocount readings become constant. The bar graph of figure 3b showes significant fluorocount readings of F-A_{10} at 10 nM concentration on the PNA immobilized surface compared to blank and control surface. Each experiment was repeated three times to test the reproducibility. All data reported here with mean signal and with ±standard deviation (S.D.). The study of binding of PNA 1 with mismatch DNA is currently being investigated on nano FET surface using electrochemical method and will be reported soon.

4 CONCLUSION

In summary, in this report, we have been demonstrated the successful synthesis of PNA βAla-$(T)_{10}$-βAla-NH_2 and its ability to bind with sscDNA with A_{10} and F-A_{10} in liquid phase and onto the nano FET surface at rt. The model reported here has a potential capability of applying in developing PNA based biosensors for nucleic acid detection.

5 ACKNOWLEDGEMENTS

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[1]Shiva K. Rastogi and Wusi Maki, University of Idaho, CAMBR, 721 Lochsa St, Ste. 8, Post Falls, ID 83854, USA
Tel: 208-262-2019; 208 262 2006; Fax: 208 262 2001
srastogi@uidaho.edu wmaki@cambr.uidaho.edu