Peptide Nucleic Acids Modified Nano-Biosensor for Early Cancer Diagnosis

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ABSTRACT

A hand held electronic nanowire-device for DNA-methylation detection is described in this paper. A peptide nucleic acid (PNA) was designed as a universal capture agent for the nano-device. PNA was synthesized, and characterized by MALDI-TOF mass spectroscopy. Purified PNA was covalently linked to the nano-biosensor surface. A novel molecular signal amplification method was applied to generate methylation specific signal molecules. These signal molecules were then captured on PNA modified nano-biosensor, and measured by impedance spectra. Using electronic nano-sensor not only avoids complicated procedures in the conventional methods for DNA methylation detection, but also achieves a high sensitivity without PCR amplification.

***Keywords*:** nano-FET, impedance spectroscopy, nanowires, peptide nucleic acid, epigenetic, DNA methylation detection.

1 INTRODUCTION

Electronic biosensors used in the detection of DNA molecules have shown promising results with great features of label free and high sensitivity. However, oligonucleotides used as a capture agent on the sensing surface create a high noise background due to the negatively charged backbone. In contrast, peptide nucleic acid produces an electrically neutral advantage in the electronic detection of nucleic acids. PNA is a nucleic acid mimic, in which the sugar phosphate backbone has been replaced by a pseudo peptide-like backbone. It has unique features of thermal stability and inter-base spacing allow PNA molecules specifically and strongly binding to DNA or RNA molecules with a complementary sequence. Hybridization of PNA:DNA or PNA:RNA show low tolerance of mismatch and high resistance to nucleases. Therefore, PNA is an ideal candidate as new sensing probe of electronic biosensors. Combined with a unique signal generation process, we designed and synthesized a PNA molecule as a universal capture probe for the detection of bio-molecules. Electrochemical impedance spectroscopy (EIS) was selected as the preliminary characterization tool [3, 4] for our device, because of its wide frequency range measurement as well as sensitiveness compare to other transistor characterization tools. DNA methyaltion was chosen as the detection target, since it plays a very important role in both human development and disease, particularly in tumor initiation. A distinct set of tumor types has a high level of hypermethylation in the promoter region of tumor suppressor genes. Analyzing the methylation status in the promoter region of the tumor suppress gene will be one of the promising strategies for cancer early diagnosis. In this work, PNA synthesis and immobilization on nano-biosensor are reported. DNA methylation detection was demonstrated by using a nanowire electronic device.

2 EXPERIMENTAL

2.1 Materials and Instrumentation

The PNA monomers (Boc-T-OH) and HATU were obtained from Applied Biosystems (USA). MBHA resin and Boc-β-Ala-OH were purchased from EMD Biosciences (USA). All other chemicals and bio-chemicals used in experiments were ACS analytical grade. The PNA was synthesized and purified by Shimadzu SPD-10AVP RP-HPLC instruments, USA. The modified nano-biosensor surface images were taken on an Olympus BX60 UV microscope. Electrochemical impedance spectra were recorded using a Reference 600 from Gamry Instruments USA.

2.2 Synthesis of PNA Oligomers

We designed the experimental protocol for three simple sequences of homothymine PNAs [5, 6] table 1. The Boc-β-Ala-OH was activated with 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU)/N-ethyldiisopropylamine (DIEA) in 0.5mL NMP/Pyridine (2:1) mixture and loaded on pre swelled MBHA resin with dichloromethane (DCM) for 12 hrs. The reaction mixture was agitated with nitrogen gas at room temperature for 1 hr. Thymine PNA oligomers were synthesized manually in solid phase peptide vessel, using

standard the Boc chemistry procedure on Boc-β-Ala-(MBHA) resin with HATU/DIEA in N-methyl-2-pyrrolidone (NMP)/pyridine (2:1, v/v) as coupling reagents. PNA oligomers were cleaved from resin with trifluoromethansulphonic acid (TFMSA):trifluroacetic acid (TFA):m-cresol (2:6:1, 2 x 1 hr) and precipitated by adding cold dry ether. The crude PNA product was washed thoroughly with ether and lyophilized.

The crude PNAs were purified on RP-HPLC, using Alltech C_{18} column (5µm, 4.6 x 150 mm at 55ºC) with dual wavelength absorbance detection (260 and 220 nm) (Fig 1) and characterized by MALDI-TOF mass spectrometry. The final concentration of PNA was calculated by UV-Vis spectrophotometer at 260nm.

Entry	PNA	MALDI-TOF mass
1	H-βAla-T_{10}-βAlaNH$_2$	MF: $C_{116}H_{153}N_{43}O_{42}$, Calcd 2820.1; found 2821.1 $[M + H]^+$.
2	H-βAla-T_{11}-βAlaNH$_2$	MF: $C_{127}H_{167}N_{47}O_{46}$, Calcd 3086.2; found 3087.3 $[M + H]^+$.
3	H-βAla-T_{12}-βAlaNH$_2$	MF: $C_{138}H_{181}N_{51}O_{50}$, Calcd 3352.3; found, 3353.4 $[M + H]^+$.

Table1: Sequences of PNA synthesizes

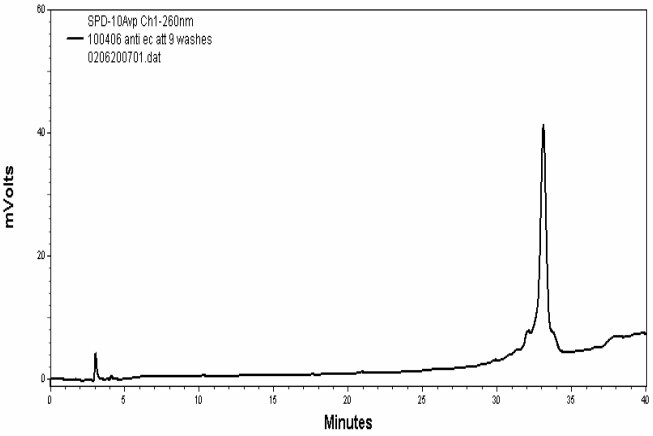

Figure 1: RP-HPLC chromatogram of synthesized PNA (H-βAla-T_{12}-βAlaNH$_2$) showing well defined purified peak around ~33 min.

2.2 Device Fabrication

A nanowire based gold nano-electronic device for DNA methylation detection was fabricated at the Cornell Nanoscale Facility (CNF), Cornell University, Ithaca NY. Low doped semiconductor nanowires (30nm-500nm) have been patterned using e-beam lithography on silicon/SiO_2 substrate. Patterned nanowires end were then connected with patterned Ti/Au (15nm/100nm) layer leaving 100nm gap between source and drain electrodes using e-beam gold deposition and standard lithography. A polyimide insulating layer was patterned on top of nano-FET using optical lithography (Fig 2).

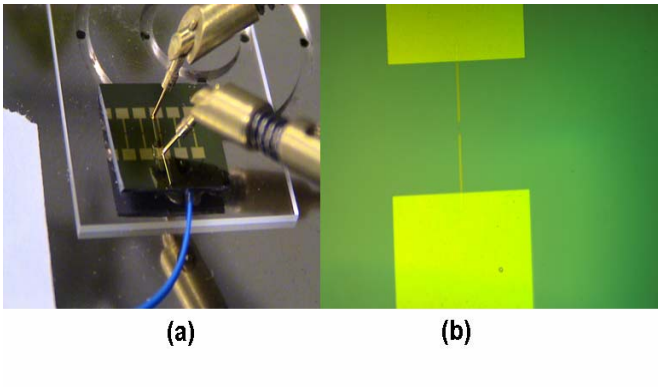

Figure 2: An outlined view of fabricated nano-FET device (a), a microscopic 20X magnification view of nano-device (b).

2.4 PNA Immobilization Method

A self assembled monolayer (SAM) of Nano-Thinker (11-mercaptoundecanoic acid, MUA) was obtained on polyimide coated nanowires surface by incubation for more than 14 hrs in a wet chamber. All MUA exposed surface was sonicated for 5 min by dipping them in 100% ethanol. After cleaning, the MUA modified chip surface was placed in an aqueous solution of 100mM 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDAC) and 25mM *N*-hydroxysuccinimide (NHS) (1:1 v/v) for 1 hr at room temperature to attach the NHS to the COOH terminal of MUA modified surface. After incubation, the surface was washed and 1 µL of 0.5 µL of 11.4 µM PNA solution was applied on the surface and incubated for 1 hr in a wet chamber in order to covalently attach these molecules. The surface was washed gently and immersed in a blocking solution of 0.01% BSA, 1mM polyethylene glycol and 1mM glycine for 1 hr. After the blocking step, the surface was washed with 1XPBS of pH 7.2 and 1 µL of targeted oligonucleotide A20 which is identical to RNA A20 signal molecule was applied and incubated for one hr and gently washed 5 times. All modified and unmodified chip surfaces were immerse in a 5µg/mL DAPI (4,6-diamidino-2-phenylindole) solution and placed on a shaker for 1 hr followed by a thorough wash with DI water and inspected under a microscope with DAPI UV filters (Fig 3).

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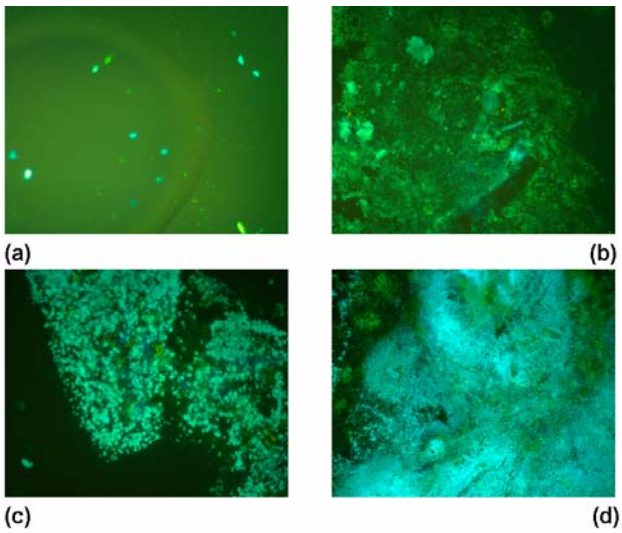

Figure 3: (a) DAPI stain image of unmodified PNA-DNA on chip (b) DAPI on modified surfaces, showing binding to modifying layer (c) DAPI images with PNA on MUA modified surface showing strong and specific binding, (d) DAPI stained DNA captured on surface showing dense DNA molecule.

2.3 Off Chip Signal Generation Model.

The off chip RNA signal molecule was generated by using the proposed model (Fig 4). A Biotin labeled probe hybridized with target DNA is captured by using streptavidin magnetic beads. A conjugate of antibody and DNA template recognize and bind to the target molecules. RNA signal molecules are generated through an in vitro transcription process. There signal molecules are captured by PNA immobilized sensing surface through sequence specific hybridization. The presence of RNA molecules on nano-biosensor are detected with respect to change electrical properties and verified by fluorescence microscopy.

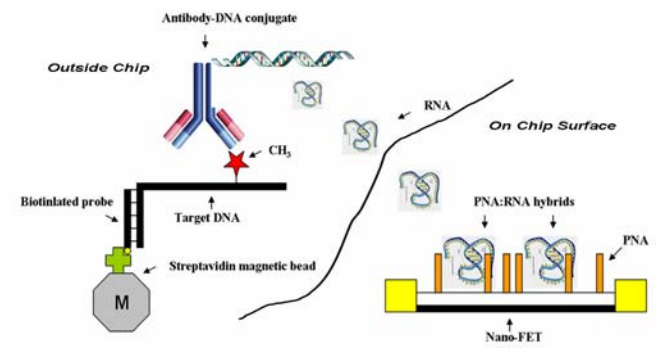

Figure 4: Schematic of signal generation model shows how the RNA signal molecule generates from methylated DNA and later capture on PNA modified nano-FET.

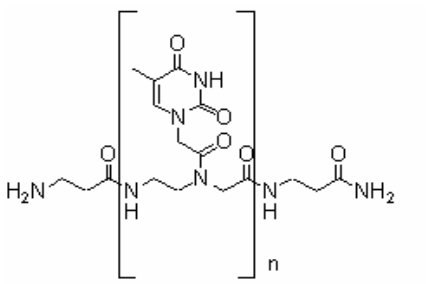

Figure 5: Structure of synthesized electrically neutral PNA compound for nanobiosensor as capturing agent for specific targeted biomolecule, where number of n varied depending on specific targeted DNA/RNA sequences.

3 RESULTS AND DISCUSSION

The successful synthesis of PNA produced yields in the range of 25-55%, which is a good range for longer synthetic route. The yielded PNA shows an excellent isolation from other by-product in their respective chromatogram (Fig 1). A positive ion MALDI-TOF mass spectrum is used to obtain molecular data to verify the calculated molecular weights of PNA. One of the purified PNA product (Fig 5), which we calculated molecular weight for H-βAla-T_{12}-βAla-NH_2, $C_{138}H_{181}N_{51}O_{50}$, 3352.3 and we found mass spectra at 3353.4 $[M + H]^+$ without any impurities, was used in current sensing study. All these data suggest successful synthesis of the desired PNA for the sensing device. PNA molecules were also attached on top of SAM of a polymer coated nanowires surfaces. UV microscopic images (Fig 3 a) show that without surface modification most molecules were washed away during experiment. This suggests a strong need of a stable sensing surface. On figure 2c-d DAPI images (DAPI, a blue fluorescent nucleic acid dye) are showing a dense distribution of PNA on the modified surface (Fig 2b) as well as strong capture of DNA (Fig 2d). These images also prove very specific binding on the surface due to excellent blocking reagent.

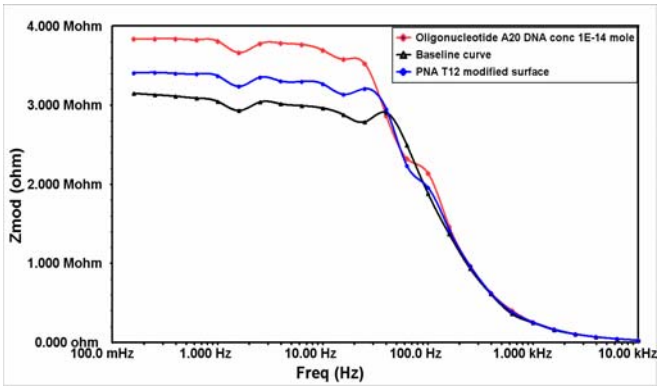

Figure 6: Electrochemical impedance spectra (Bode) PNA modified nano-FET. The spectra show a clear detection of oligo-nucleotide A20 (red line) due to attachment with PNA on the surface.

In the detection model, RNA signal molecules were generated through in vitro transcription in the presence of methylated DNA. No RNA molecules were generated in the presence of normal DNA with identical sequence. An oligonucleotide, which is an identical analog of RNA molecule was used to test detection sensitivity on the PNA modified electronic nano-device. A serial dilution of oligonucleotide was tested on PNA modified nanowire surface. A clear electronic signal was detected as shown in figure 6-7. These impedance spectra of 5 fM oligo-nucleotide showed a very good signal and noise ratio, since PNA molecules are electrically neutral.

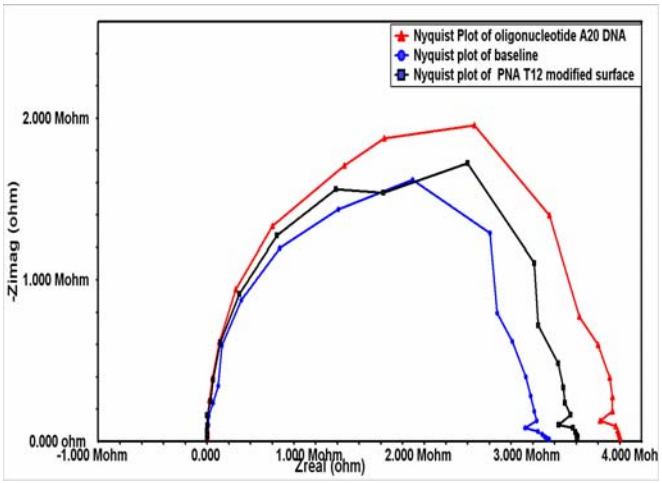

Figure 7: Electrochemical impedance spectra (Nyquist Plot) of PNA T12 modified nano-FET. The spectra show a clear detection of oligo-nucleotide A20 (red line) due to attachment with PNA on the surface.

4 CONCLUSIONS

We were successfully synthesized three homothymine based PNA and characterized by mass spectra as well as HPLC. The PNA molecule were covalently linked to the top of nanowires surface and verified by DAPI nucleic acid stain. In the detection model, significant electronic signals were detected in the presence of oligonucleotide at the fmole level. This is due to the overall impedance being influenced by molecular interaction. Electronic detection of DNA methylation shows a great potential for rapid and sensitive diagnosis in early cancer detection. Preliminary data also indicates that this device can serve as the basis for a low cost universal device for bio-molecular detection in a specific manner.

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