Biosensors and Bioelectronics 24 (2009) 3544–3549

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Thiolated pyrrolidinyl peptide nucleic acids for the detection of DNA hybridization using surface plasmon resonance

Cheeraporn Ananthanawat[a], Tirayut Vilaivan[b], Wanwimon Mekboonsonglarp[c], Voravee P. Hoven[b,*]

[a] Program of Macromolecular Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[c] Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 29 January 2009
Received in revised form 23 April 2009
Accepted 11 May 2009
Available online 18 May 2009

Keywords:
Peptide nucleic acid
PNA
DNA hybridization
SPR
DNA sensor

ABSTRACT

Thiolated pyrrolidinyl peptide nucleic acids (HS-PNAs) bearing D-prolyl-2-aminocyclopentanecarboxylic acid (ACPC) backbones with different lengths and types of thiol modifiers were synthesized and then characterized by MALDI–TOF mass spectrometry. These HS-PNAs were immobilized on gold-coated glass by self-assembled monolayer (SAM) formation via S atom linkage for the detection of DNA hybridization using surface plasmon resonance (SPR). The amount and the stability of the immobilized HS-PNAs, as well as the effects of spacer and blocking thiol on DNA hybridization efficiency, were determined. SPR results indicated that the hybridization efficiency was enhanced when the distance between the PNA portion and the thiol terminal was increased and/or when blocking thiol was used following the HS-PNA immobilization. The immobilized HS-PNA could discriminate between fully complementary DNA from one or two base mismatched DNA with a relatively high degree of mismatch discrimination (>45%) in PBS buffer at 25 °C. The lowest DNA concentration at which reliable discrimination between fully complementary and single mismatched DNA could still occur was at about 0.2 μM, which is equivalent to 10 pmol of DNA. This research demonstrates that using these novel thiolated PNAs in combination with the SPR technique offers a direct, rapid and non-label based method that could potentially be applied for the analysis of genomic or PCR-amplified DNA in the future.

1. Introduction

The development of DNA sensors has gained popularity over the past few years, for example because of its potential interest in applications for DNA sequencing in clinical diagnostics and in forensic sciences (Feldner et al., 2003). The basic principle of a DNA biosensor is the sequence-specific hybridization between the complementary DNA analyte(s) in solution and the single-stranded nucleic acid probe(s) tethered to solid substrates (i.e., gold). Many techniques have been developed for DNA hybridization detection, including the use of labels such as fluorescence (Ha et al., 2004) and label-free techniques such as mass spectrometry (Arlinghaus et al., 2003, 2004; Hellweg et al., 2006), piezoelectric (Duman et al., 2003; Cho et al., 2004; Skládal et al., 2004; Tombelli et al., 2005), electrochemical (Ma et al., 2006; Dharuman and Hahn, 2007; Fang et al., 2008) and optical methods (Su et al., 2005a; Niu et al., 2007; Vikholm-Lundin et al., 2007). Among the techniques, surface plasmon resonance (SPR) is a well-recognized optical-based method that relies on the measurement of changes in the refractive index, which is proportional to the mass of the bound or adsorbed species on its surface. SPR measurements are rapid, being able to be performed within minutes, and do not require specific labels (Burgener et al., 2000), which reduces not only costs and time but also reduces interference problems at the amplification stage.

Peptide nucleic acid (PNA) is a synthetic DNA analogue first introduced by Nielsen et al. in 1991 (Nielsen et al., 1991; Egholm et al., 1992). Nielsen's PNA differs from DNA in that the DNA deoxyribose–phosphate backbone is replaced by a pseudo-peptide counterpart. Nonetheless, PNA molecules retain the specific sequence dependent binding property to complementary DNA or RNA following the Watson–Crick base-pairing rules. The absence of negative charges along the PNA backbone improves the binding affinity and also promotes the high mismatch sensitivity of PNA–DNA complexes compared to DNA–DNA complexes (Hyrup and Nielsen, 1996; Arlinghaus et al., 2004). Recently, several new PNA systems with modified structures have been developed in attempts to improve the PNA's binding properties to complementary DNA or RNA sequences (Ahn et al., 2003; Govindaraju et al., 2003; Huang et al., 2004; Samuel et al., 2004; Kitamatsu et al., 2006; Merino et al., 2007).

Pyrrolidinyl PNA bearing a D-prolyl-2-aminocyclopentanecarboxylic acid (ACPC) backbone is a novel PNA system developed

* Corresponding author. Tel.: +66 2218 7627x102; fax: +66 2218 7598.
E-mail address: vipavee.p@chula.ac.th (V.P. Hoven).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.05.011

by Vilaivan et al. (Suparpprom et al., 2005; Vilaivan and Srisuwannaket, 2006). Analysis of the DNA hybridization ability of this pyrrolidinyl PNA, assessed by melting temperature and circular dichroism (CD) spectroscopy analysis, suggested that 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 pyrrolidinyl 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, render this new pyrrolidinyl PNA system a potential candidate for the development of a highly effective DNA biosensor (See Fig. S1 in the supplementary material for the structures of DNA, Nielsen's PNA and pyrrolidinyl PNA).

We have recently reported the success of employing immobilized thiolated pyrrolidinyl PNA (HS-PNA) for the detection of DNA hybridization using a quartz crystal microbalance (QCM). However, the combination of pyrrolidinyl PNA with the QCM technique did not yield satisfactory levels of specificity and sensitivity (Ananthanawat et al., 2009). This research aims to further explore the applicability of similar HS-PNAs for DNA hybridization detection by using SPR as an alternative, non-labeling technique. It is anticipated that SPR can deliver a better detection efficiency compared to that achieved by QCM. Parameters that may affect the sensitivity and specificity of DNA sensors, including the length and the type of thiol modifier that link the pyrrolidinyl PNA portion and the thiol end, the HS-PNA immobilization process, detection limit, specificity and stability of sensor, were also evaluated.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Fluka (Switzerland), Merck (Germany) or Aldrich (USA), and were purified as appropriate. The solid support for peptide synthesis (TentaGel S RAM resin) and Fmoc-L-Lys(Boc)-OPfp were obtained from Fluka and Calbiochem Novabiochem (USA), respectively. The four pyrrolidinyl PNA monomers and spacer were synthesized as described previously (Vilaivan and Srisuwannaket, 2006). Oligonucleotides were purchased from the BioService Unit, National Science and Technology Development Agency and BioDesign Co., Ltd. (Thailand). Solutions were made with MilliQ water, obtained by purification with a Millipak® 40 filter unit (0.22 μm, Millipore, USA). Phosphate buffered saline (PBS) pH 7.4 was purchased from Sigma (USA).

2.2. SPR equipment

SPR measurements were conducted using a double channel, AutoLab ESPR (Eco Chemie, The Netherlands) at 25 °C. An autosampler was used to inject the test solutions and the measurement of the SPR angle shift was done under non-flow liquid conditions. The measured SPR angle shifts were converted into mass uptakes, using a sensitivity factor of 120 mDegrees per 100 ng/cm^2 PNA and DNA (see Section 3.4.1), for the calculation of the amounts of immobilized PNA and hybridized DNA to PNA.

2.3. Methods

2.3.1. Synthesis of HS-PNA oligomers and melting temperature (T_m) measurements

The HS-PNAs were synthesized manually by solid phase peptide synthesis on TentaGel S RAM resin (0.24 mmol/g substitution) preloaded with Fmoc-L-Lys(Boc)-OPfp. A detailed description of the synthesis has been presented elsewhere (Ananthanawat et al., 2009). After the attachment of the last nucleobase, the PNAs were modified at the N-terminus with different thiol modifiers. Cleavage of the PNA oligomers from the resin was done concurrently with the removal of the *S*-protecting group by treatment with 10% (v/v) anisole in trifluoroacetic acid (TFA). The TFA was removed by a stream of nitrogen and the residue was washed with diethyl ether. The identity of each HS-PNAs was verified by MALDI–TOF mass spectrometry (Bruker Daltonik GmbH, Germany). The sequences of HS-PNA oligomers synthesized, together with their complementary and mismatched DNA targets used in this investigation, are listed in Table 1.

Table 1
Summary of the synthesized HS-PNA probes and the target DNAs used in this study.

Name	Description	Sequence
9 mers		
P9C2	PNA1	$HS(CH_2)_2CO$-TTT TTT TTT-$LysNH_2$
P9C10	PNA1	$HS(CH_2)_{10}CO$-TTT TTT TTT-$LysNH_2$
P9C2-egl	PNA1	$HS(CH_2)_2CO$-egl-TTT TTT TTT-$LysNH_2$
D9comp	Complementary DNA to PNA1	5′-AAA AAA AAA-3′
D9M1T	Single-mismatched (T) DNA to PNA1	5′-AAA ATA AAA-3′
D9M1C	Single-mismatched (C) DNA to PNA1	5′-AAA ACA AAA-3′
D9M1G	Single-mismatched (G) DNA to PNA1	5′-AAA AGA AAA-3′
D9M2T	Double-mismatched (T) DNA to PNA1	5′-AAA TAT AAA-3′
D9M2C	Double-mismatched (C) DNA to PNA1	5′-AAA CAC AAA-3′
D9M2G	Double-mismatched (G) DNA to PNA1	5′-AAA GAG AAA-3′
15 mers		
P15C2-egl	PNA2	$HS(CH_2)_2CO$-egl-TGT ACG TCA CAA CTA-$LysNH_2$
D15comp	Complementary DNA to PNA2	5′-TAG TTG TGA CGT ACA-3′
D15M1	Single-mismatched DNA to PNA2	5′-TAG TTG GGA CGT ACA-3′
D15M2	Double-mismatched DNA to PNA2	5′-TAG TTG AGT CGT ACA-3′

Melting temperature (T_m) measurements were performed to determine the stability of HS-PNA·DNA hybrids in solution under the same conditions previously reported (Ananthanawat et al., 2009), and the results are summarized in Table S1 (supplementary material).

2.3.2. Immobilization of HS-PNA on gold-coated SPR disk

Before immobilization, the bare gold-coated SPR disk was cleaned with piranha solution (3:1 H_2SO_4: 30% (v/v) H_2O_2), and then rinsed with MilliQ water and dried under a gentle stream of nitrogen gas.

For the immobilization step, 500 μL of the 1.0 μM HS-PNA aqueous solution was dropped on top of the cleaned disk. The disk was incubated at ambient temperature (31 °C) for 24 h before rinsing with MilliQ water and dried with a light stream of nitrogen gas. Following the HS-PNA immobilization, the disk was treated with blocking solution (1 mM) at ambient temperature (31 °C) for 1 h. 2-Mercaptoethanol (MEL) and 11-mercaptoundecanol (MUL) were used as blocking reagents corresponding to the thiol modifier with a length of C2 and C10, respectively. After additional rinsing and drying, the disk was seated in the SPR cell and was ready to be used for detection of DNA hybridization. Before DNA hybridization measurements, the disk was rinsed with a running buffer of 5× PBS (0.05 M phosphate buffer, 0.69 M NaCl, 0.0135 M KCl, pH 7.4). The difference of angles between before and after HS-PNA immobilization was used to quantify the amount of the immobilized HS-PNA on the gold surface or probe density. The angle shift was calculated