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Nucleic Acid Sensing onto Peptide Nucleic Acid (PNA) Modified Solid Surfaces

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A biosensor, which is conventionally defined as "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells, to detect chemical compounds usually by electrical, thermal or optical signals" [1], comprises of at least two basic steps: first, target recognition and second, signal transduction. In nucleic acid sensing, the single-stranded DNA/RNA probes are often employed as the sensor probes, since by virtue of their capacity of binding to their complementary target nucleic acid strands with high sequence-specificity, they can almost always detect the presence of the specific target strands in the sample analyte solution, without error. Regarding transduction of the molecular recognition event into an electronic signal, mostly the optical, electrochemical, electrical, mechanical, acoustic or thermal methods are applied in the current nucleic acid sensors [2-6].

Immobilization of the nucleic acid sensor probes onto a solid substrate in the surface-based nucleic acid sensors is one of the crucial initial steps that can play a decisive role in the overall performance of the sensor. Ideally, the nucleic acid strands are to be immobilized onto the solid support in such a way that a specific recognition signal can be obtained only if they recognize their target probes *via* sequence-specific hybridization interactions. The experimental setup should be such that the various non-specific interactions, e.g. nucleobase-substrate interactions as in case of interactions between the nucleobase nitrogen and a gold substrate, are largely cancelled out or do not take place to a significant extent. Hence, experimental conditions must be adjusted for every application, and a large choice of immobilization support and methods should be considered before the most optimal arrangement can be identified. The immobilization strategy could include primarily two means, one, covalent binding *via* one end of the nucleic acid probe, e.g. binding of 5′-thiol-modified DNA oligonucleotides onto gold surface *via* gold-sulfur interactions [7], or binding of a 5′-amino-modified DNA oligonucleotide onto an epoxy-modified surface [8], and two, non-covalent binding (e.g. affinity binding based on the strong avidin biotin system, where nucleic acid is biotinylated at its 5' end and the avidin is attached directly to carbon-based surface [9].

While quite high sensitivity (femto molar to atto molar range) in target detection has been exemplified in optical/electrochemical transduction [6,10], another approach for sensitivity enhancement, i.e. by application of synthetic nucleic acid analogues like peptide nucleic acid (PNA) [11] and locked nucleic acid (LNA) [12,13] probes, which are capable of forming more stable duplexes with the DNA targets than the DNA sensor probes, may also be explored. The unique physicochemical nature of the peptidic, non-ionic backbone of PNA has promoted the use of PNA oligomers as capture probes in electrochemical, optoelectronic sensors, and microarray-based sensors [3,14,15].

PNA is an artificially synthesized polymer, invented by Peter E. Nielsen (University of Copenhagen), Michael Egholm (University of Copenhagen), Rolf H. Berg (Risø National Lab) and Ole Buchardt (University of Copenhagen) during the 1990s [11]. PNA is a DNA analog, in which a 2-aminoethyl-glycine linkage generally replaces the normal phosphodiester backbone [11,16]. A methyl carbonyl linker connects natural, as well as unusual (in some cases), nucleotide bases to this backbone at the amino nitrogens. PNA is non-ionic, achiral, and is not susceptible to hydrolytic (enzymatic) cleavage. PNAs are capable of sequence-specific binding with complementary DNA, as well as RNA obeying the Watson-Crick base paring [17,18]. Its hybrid complexes exhibit extraordinary thermal stability. PNA has high affinity to its complementary DNA or RNA molecules, mainly because of the lack of electrostatic repulsion between the uncharged PNA backbone, and that of the natural nucleic acid. Within the PNA hybrids, the order of the thermal stability of the duplexes is found to be PNA–PNA>PNA–RNA>PNA–DNA [19].

In contrast to the DNA–DNA duplexes, the stability of the PNA–DNA hybrids is not significantly affected by changes in ionic strength, except in the limit of low ionic strength, i.e. when the salt concentration is few mM, where the stability increases. The binding of PNA to a corresponding complementary DNA oligomer takes place in a sequence-specific manner, which means that the thermal stability of a hetero-duplex, where PNA is one of the components, can be considerably lowered by the presence of imperfect matches. Owing to the high sequence specificity of PNA binding to other nucleic acid strands, incorporation of any mismatch in the duplex considerably affects the T_m value of the hetero-duplex. For example, a single base mismatch could result in the lowering of the T_m value by 15°C and 11°C, in case of the 15 mer PNA–DNA and DNA–DNA duplexes, respectively [20]. This property of PNA is responsible for the remarkable discrimination between perfect matches and mismatches offered by PNA probes, and makes PNA attractive as oligonucleotide recognition elements in biosensor technologies [20].

Application of PNA as a sensor probe in nucleic acid sensor technologies holds great promise for rapid (since formation of PNA–DNA duplex is faster than formation of DNA–DNA duplex) and cost-effective detection (since lesser amount of sample is needed due to high sensitivity in PNA-based measurements) of specific DNA sequences. Usually, single-stranded PNA (ssPNA) probes are immobilized onto the transducer surface by chemical means, e.g. gold-sulfur bond formation for immobilization of thiolated ssPNA probes onto the gold surface [20]. Once the PNA sensor probes can detect the complementary (or non-complementary) target nucleic acid

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strands in the sample solution, the response from the hybridization event or a lack of it is converted into a useful electronic response by the transducer. The first report by Jensen et al. [19] in 1997 on detection of PNA–DNA and PNA–RNA hybridization using surface plasmon resonance (SPR), showed that the sensor can differentiate between a complementary and a non-complementary oligonucleotide sequence. The sensor chip used in this case was a thin gold film covered with a layer of dextran and containing streptavidin, chemically coupled to the dextran layer. Biotinylated PNA molecules were immobilized on the surface by means of strong coupling between biotin and streptavidin. The amount of the bound substance (complementary, as well as various non-complementary DNA and RNA oligonucleotides) was measured as a function of time, when a solution containing the target strands was flown over the chip surface. In this way, the association kinetics could be studied. The quartz crystal microbalance (QCM) mass measuring method can also be applied in studying hybridization of nucleic acids on solid surfaces [21-23]. The first report about the study of PNA–DNA hybridization using the QCM biosensor and PNA as the sensor probes comes from the work of Wang et al. [24], which showed that the system could differentiate between complementary and non-complementary oligonucleotides. A fast and sensitive detection of mismatched sequences was made possible by monitoring the frequency *vs.* time response of the PNA-based QCM sensor. The PNA molecules used in the above-mentioned study contained a cysteine attached to the PNA strand with the help of an ethylene glycol unit, and a PNA monolayer could be formed onto the gold-coated quartz crystal surface using this thiol-PNA construct [24]. The immobilized PNA probes exhibited remarkable sequence specificity and gave rise to rapid hybridization with the target oligonucleotides sequences. The use of PNA as recognition probe for detection of target nucleic acid strand using electrochemical means has been reported, as early as in 1998, by Wang [20]. Their method consisted of four steps: PNA probe immobilization onto the transducer surface (here, a carbon paste electrode), hybridization to target DNA strands, indicator binding and chronopotentiometric transduction. The hybridization experiment was carried out by immersing the electrode into the stirred buffer solution containing a desired target, followed by measurement of signal.

Motivated by the previous reports that PNA can potentially be a better alternative for on-surface nucleic acid detection technologies compared to DNA probes, the Mukhopadhyay group developed a sensitive and robust bio-active self assembled PNA sensor layer, which is capable of efficient and specific target detection. The formation of close association of ssPNA strands was found to be relatively straightforward, and compact self-assembled PNA films could be readily generated on solid substrates like gold (111) surface by a simple immersion method [25]. In such films, the immobilized ssPNA strands could be oriented away from the surface, as elicited from reflection absorption infrared spectroscopy (RAIRS) experiments [25], and non-specific interactions with the underlying gold substrate could be largely avoided, creating an ideal situation for the target nucleic acid strands to access the immobilized sensor PNA probes. On the contrary, the DNA films comprising the negatively charged ssDNA strands have been found to be mostly disordered/poorly ordered [26], where nonspecific DNA–surface interactions could occur through the relatively exposed nucleobases, resulting in reduced bioactivity of the film [27]. Ghosh et al. [28] have recently reported that the mismatch discrimination ability of such surface-anchored PNA layers could be successfully enhanced *via* ionic control, i.e. by varying salt concentration and the type of counter-ion. While the nature of ionic dependence of 'on-surface' behavior of PNA probes deviated significantly from the 'solution' behavior of these probes, e.g. in case of the singly mismatched duplexes, considerable similarities were also observed, e.g. in case of the fully mismatched duplexes. The single base mismatch discrimination capacity of the PNA probes could be further amplified by controlling PNA probe density using a nanoparticle-based approach, where gold (111) surface was modified with gold nanoparticles prior to PNA adsorption [29]. The simple strategy for formation of the surface-attached AuNP-PNA construct appeared to be beneficial not only because the difficulty in attaching PNA probes onto AuNPs, without AuNPs getting aggregated, could be overcome, but importantly, because this allowed an increase in the sensor probe density, and therefore, increase in the hybridization probability. Considering the need for developing sensitive, target-specific and robust high-throughput array technologies, PNA-based nucleic acid detection assays, as presented in this report, could offer practical inputs in achieving better control on on-surface DNA detection capabilities.

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