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Peptide Nucleic Acid – An Opportunity for Bio-Nanotechnology

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[§]SCS-Metrohm Foundation Award for best oral presentation

Abstract: DNA is a major player in the field of bio-nanotechnology and many interesting applications have been realized using this oligonucleotide. In contrast, the use of peptide nucleic acid (PNA), which is a non-natural, neutral analogue of DNA with superior hybridization strengths compared to DNA, is still in its infancy in bio-nanotechnology. However, as demonstrated in this short review using selected studies, promising examples demonstrating the tremendous opportunities that PNA can offer for bio-nanotechnology were recently described.

Keywords: Biotechnology · Nanoparticle · Nanotechnology · Peptide nucleic acid (PNA) · Sensing

DNA bio-nanotechnology has over the last decade become an important and quite diverse field of research, including studies on the assembly of DNA strands by themselves (DNA origami),^[1] DNA modified nanoparticles,^[2] and the combination of all sorts of other nanomaterials with DNA, *e.g.* nanotubes.^[3] For applications of DNA, peptide nucleic acid (PNA), which is a non-natural analogue of DNA, has become an interesting alternative (see Fig. 1 for the comparison of the structures of PNA and DNA).^[4,5] To understand if and how PNA can impact bio-nanotechnology, the differences and consequential potential advantages of PNA over its naturally occurring analogues DNA and RNA have to be considered (Fig. 1). The nucleobases as well as their spatial arrangement are identical and complementary to each other in PNA, DNA, and RNA. Complementary strands of the different nucleic acid oligo-

mers can therefore bind to one another. Consequently, combinations of existing DNA bio-nanotechnology with PNA are possible. What distinguishes the different oligonucleotides is the backbone. While DNA and RNA possess a backbone consisting of (desoxy-)ribose sugars connected by phosphodiester, PNA relies on a *N*-(2-aminoethyl)glycine based framework, which links the subunits with amide bonds like in a peptide. This change results in a number of important differences between PNA and its natural analogues, and has sparked great interest in various fields of research.^[5] Although *N*-(2-aminoethyl)glycine has recently been found to be produced by cyanobacteria,^[6] the PNA backbone is still regarded as unnatural and as such cannot be degraded enzymatically. Most importantly, the backbone is uncharged, making PNA highly interesting for use in electronic devices. Moreover,

it eliminates the intrinsic electrostatic repulsion between individual nucleotide strands contributing to greater hybridization strengths. Partially due to this and for entropic reasons the binding strength of PNA oligomers to both complementary PNA and DNA strands is higher than of DNA complementary strands. Finally, the single base mismatch sensitivity is usually greater for PNA than for DNA.

In the past, our group has mainly been engaged in combining PNA with metal complexes,^[7] and we were wondering why PNA has only scarcely been applied in bio-nanotechnology. In this short review we highlight, on the basis of selected literature examples, how the above-mentioned properties of PNA can indeed be exploited to advance bio-nanotechnology.

Relatively much work has been done with PNA-functionalized silicon nanowires (SiNWs).^[8–11] The main reason for

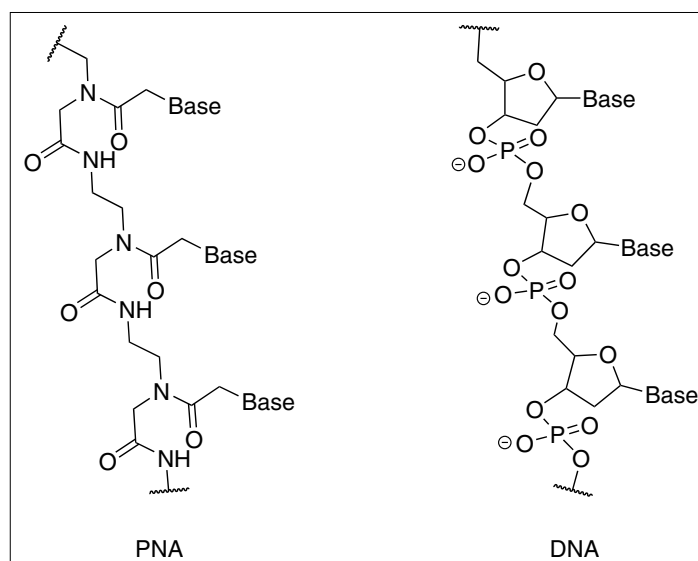


Fig. 1. Chemical structures of PNA and DNA.

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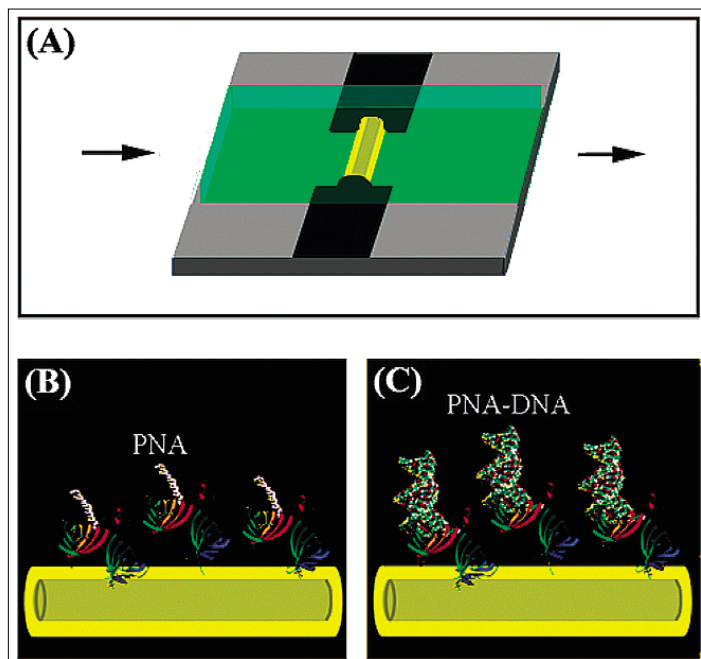


Fig. 2. (A) Schematic representation of the PNA and SiNW based sensor for DNA detection. The SiNW (yellow) connects two electrodes (black) and spans a microfluidic channel (green). The arrows indicate the direction of the flow. (B) PNA-modified SiNW before addition of complementary DNA. (C) PNA-modified SiNW after addition of complementary DNA which leads to PNA-DNA hybridization. Reprinted with permission from ref. [12]. Copyright 2004 American Chemical Society.

this is probably the uncharged nature of PNA, which is a significant advantage for SiNW-based semiconductors, as they rely on charge differences on the SiNW surface for sensing (*vide infra*). Additionally, procedures for modification of SiNWs with PNA are straight-forward and known for more than ten years.^[12] For example, PNA-functionalized SiNWs were used for label-free detection of a specific DNA sequence (Fig. 2).^[12] The SiNWs were modified with PNA as ‘receptors’ with the well-established biotin-avidin immobilization methodology. The SiNWs were used as the semiconducting element between two electrodes and were, at the same time, inside a microfluidic channel. Upon addition of the complementary DNA, sequence binding between DNA and PNA occurred, leading to a fast increase of conductance due to the electrostatic gating effect. Addition of non-complementary DNA did not substantially alter the conductance. A detection limit of 10 fM for a deletion of three bases in the gene encoding for cystic fibrosis transmembrane receptor, which leads to the corresponding disease, could be achieved due to the superior binding properties of the PNA. The sensor design and manufacturing process has since been improved, allowing preparation of highly uniform SiNW arrays.^[8] The potential advantages of using PNA compared to DNA for SiNW sensors have been pointed out by Gao and coworkers:^[8] i) increased hybridization efficiency due to stronger binding

of DNA/PNA hybrids than of DNA/DNA hybrids; ii) lower basal conductance due to the uncharged nature of PNA allowing for the use of a regime of low ionic strength, which minimizes the appearance of strong electric fields and thereby background signal; iii) the greater mismatch sensitivity of PNA compared to DNA.^[13] Indeed, PNA improved the performance of the sensor, showing greater conductance differences upon binding of the complementary DNA strand than the corresponding DNA-based SiNW sensors (see Fig. 3 for comparison between DNA- and PNA-based SiNW sensors).^[8] In another direct comparison, a SiNW sensor with PNA capture probe was four times more sensitive than the equivalent sensor with DNA-functionalization.^[9]

Apart from the sensing of DNA, PNA-functionalized SiNWs have been employed to investigate an important physical property of SiNW sensors, namely the distance dependence of their field effect.^[10] The response depends on the distance between the SiNW and the charge layer, *i.e.* the charged DNA. A neutral linker between the DNA and the SiNW had therefore to be employed, and PNA fulfilled this task. The report has general implications for the future design of SiNW sensors, *e.g.* for the detection of DNA and RNA.

Single-walled carbon nanotubes (SWNTs) have also been functionalized with PNA. The attachment can be achieved covalently *via* NHS active ester chemistry^[14] or non-covalently *via* π - π -

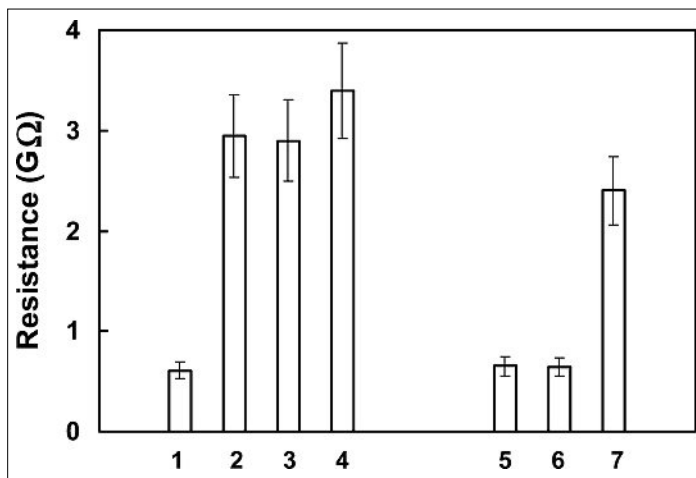


Fig. 3. Resistance of the SiNW after different treatments. (1) A blank SiNW, (2) (1)+DNA capture probes, (3) (2) after hybridized with 1.0 nM complementary PNA, (4) (2) after hybridized with 1.0 nM complementary DNA, (5) a blank SiNW, (6) (5)+PNA capture probes, and (7) (6) after hybridized with 1.0 nM complementary DNA. Same capture probe and sample sequences were used, respectively.^[8] Reprinted with permission from ref. [8]. Copyright 2007 American Chemical Society.

stacking between the PNA bases and the SWNT and electrostatic interactions between the amino groups of the PNA and the SWNT.^[15] These conjugates were capable of binding complementary DNA or RNA strands, respectively, presumably with greater strength than it would have been possible with DNA functionalized SWNTs. The absence of charges in PNA reduces non-specific electrostatic interactions with electrodes and surfaces, making them especially interesting for self-assembled nano-electronics applications.^[14] First studies into this direction have been undertaken.^[16] Interestingly, non-covalently bound DNA and PNA wrap very differently around SWNTs.^[17] This observation has, to the best of our knowledge, not been put to any use yet.

In analogy to the non-covalent binding to SWNTs, single-stranded oligonucleotides can also bind to graphene oxide, a water-soluble derivative of graphene. This interaction relies again on π - π -stacking. Because of its great π - π -stacking ability, graphene oxide is known to quench the fluorescence of dyes in close proximity.^[18] Small fluorophore-labeled PNA oligomers can be used to detect complementary DNA and RNA, as the formation of oligonucleotide duplexes can lead to their detachment from the graphene oxide. This has been applied to monitor the function of RNA polymerase. As soon as complementary RNA has been synthesized, the labeled PNA strands quantitatively bind the RNA, leading to desorption of the PNA from the graphene oxide and lighting up of the fluorophore. The advantage of PNA probes for this system lies in the stronger binding to even low concentrations of RNA, rendering the assay independent from buffer con-

centrations and external influences such as temperature.^[19]

Recently, the same concept for sensing RNA with a fluorophore-labeled PNA and, in this case, nano graphene oxide (NGO) has even been applied to monitor micro-RNA (miRNA) expression levels in living cells (Fig. 4).^[20] Notably, previous methods were not able to achieve this goal.^[21] NGO is negatively charged at physiological pH, and therefore uncharged PNA was expected to lead to less unspecific desorption than negatively charged DNA. Indeed, it was shown that PNA-NGO adducts are stable when treated with cell lysate, while DNA-NGO adducts disassembled leading to non-specific fluorescence. Moreover, the PNA-based system has a very low detection limit (~ 1 pM) due to the superior binding strength of PNA to miRNAs. The system could even be used to detect multiple miRNAs simultaneously by employing multiple complementary PNA strands with different dye labels.

Another area of interest for PNA application is surface-enhanced Raman spectroscopy (SERS). This technique relies on the strong amplification of Raman signals by metallic nanostructures.^[22] PNAs have already been used in this context.^[23–25] For example, Bazan and coworkers have developed a SERS sensor for single-stranded DNA (Fig. 5). Interestingly, this sensor would not work with DNA instead of PNA.^[23] More specifically, in this study, PNA was immobilized on a glass slide. Subsequently, complementary DNA was hybridized to the PNA, while non-complementary DNA was washed away. The negative charges on the backbone of the complementary DNA then lead to electrostatic attraction of positively charged silver nanoparticles (AgNPs). After addition of a SERS reporter dye, which binds to AgNPs, strongly enhanced Raman signals could be observed. If, in contrast, only non-complementary DNA was added, no enhancement was observed, as no AgNPs were deposited. The use of DNA instead of PNA would have led to non-specific deposition of the AgNPs due to the inherent negative charge of DNA.

As can be seen with the previous example, the interplay of nanoparticles with PNA is very intriguing due to the unique physico-chemical properties of nanoparticles and PNA. Indeed, PNA has also been used to functionalize nanoparticles such as silica-coated magnetic nanoparticles (γ -Fe₂O₃) immobilized on a silver substrate,^[25] gelatin nanoparticles,^[26] and others.^[27] Gold nanoparticles (AuNPs) still represent the arguably most prominent class of nanoparticles. Recently, DNA-modified AuNPs were shown to be quickly and efficiently hybridized with PNAs carrying different functionalities,

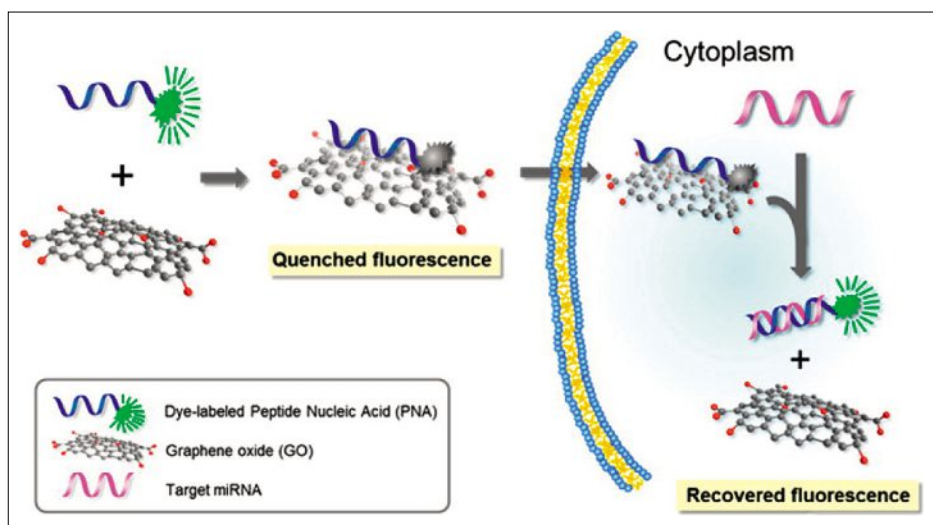


Fig. 4. Schematic representation of the working principle of intracellular miRNA sensing using NGO and PNA. Extracellularly, fluorophore labeled PNA binds to NGO, leading to quenching of the fluorophore. After crossing of the cellular membrane, the PNA-NGO complex stays intact until a complementary miRNA strand is in proximity. A PNA-RNA duplex is then formed leading to desorption of the construct from NGO. Thereby, the fluorophore is not quenched anymore and can be detected. Adapted with permission from ref. [20]. Copyright 2013 American Chemical Society.

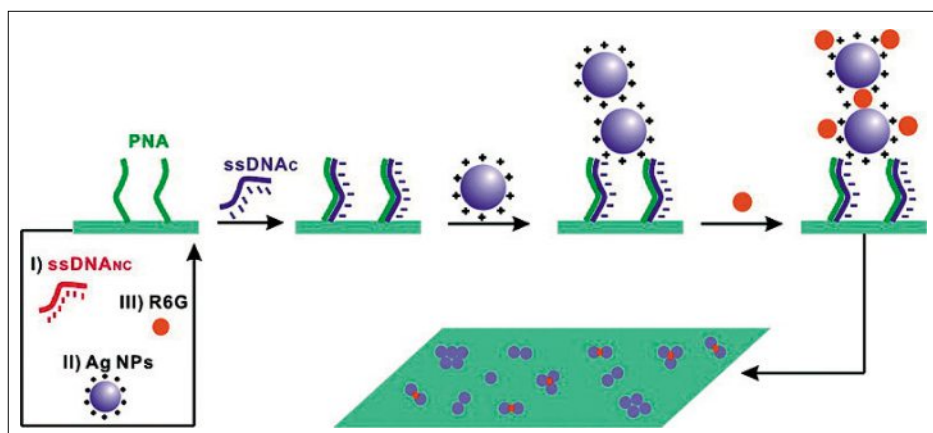


Fig. 5. PNA is deposited on a glass slide. Addition of non-complementary DNA with AgNPs and a SERS reporter (Rhodamine 6 G, R6G) leads to no assemblies. Complementary DNA strands bind to the PNA, thereby immobilizing negative charges. Positively charged AgNPs are electrostatically bound to the hybrids, R6G can bind to the AgNPs, and finally SERS can be observed. Reprinted with permission from ref. [23]. Copyright 2007 American Chemical Society.

ranging from small molecules like fluorophores and drugs, to differently sized biomolecules like siRNA, aptamers and antibodies.^[28] Their *in vitro* use as imaging agent has been demonstrated.

However, there are, generally, only very few reports about PNA-modified AuNPs, especially about directly PNA-modified AuNPs, *i.e.* with attachment of the PNA to the gold surface of the NP.^[29–31] There is a straight-forward explanation: Addition of PNAs to unmodified AuNPs lead to their agglomeration and subsequent precipitation from solution (Fig. 6). This effect is thought to be mainly due to the adsorption of PNA bases to gold surfaces, which is stronger than for negatively charged DNA.^[32] Thus, the initial stabilization of the AuNPs is lost and the general tendency of the NPs to aggregate induces the destabilization.

The precipitation is permanent, and the non-specific binding of PNA to AuNPs is therefore not reversible, in contrast to its binding to graphene oxide (*vide supra*). It has been shown that this aggregation tendency is even strong enough to be used for sequence-selective quantification of DNA^[33] and RNA.^[34]

The problem can be circumvented by immobilizing the AuNPs *e.g.* on an Au(1,1,1) surface *via* thiol/amine linkers before modification with thiol-linked PNA.^[35] The pre-disposition of the AuNPs obviously prevents aggregation upon addition of PNA. It was found that the single-mismatch sensitivity of PNA/DNA hybrids is already improved on unmodified Au(1,1,1) surfaces, compared to PNA/DNA hybrids in solution.^[31] Immobilization of AuNPs on the Au(1,1,1)



Fig. 6. Evolution of aggregation and precipitation of AuNPs upon addition of PNA. Time frame from first to last picture: less than one minute.

surfaces further increased the mismatch discrimination, mainly due to the greater melting temperatures (T_m) of the fully complementary hybrids.^[35] This effect was greater for PNA than for DNA probes. The study therefore shows the potential of combining AuNPs with PNA. Notably, the T_m of the PNA/DNA hybrid was dependent on the size of the AuNPs, leading to higher T_m with smaller NP diameter. This was attributed to the greater PNA probe density on smaller particles. This last point shows that the ‘nanoscopic’ environment has a noticeable impact on the molecular level. Consequently, it can be expected that particles that are not immobilized on a surface have distinct properties. Apart from that, having the particles in solution allows for additional applications, e.g. they can be added to setups with differently functionalized surfaces.

Recently, in collaboration with the Bach group at Monash University (Australia), we have reported a new method for generating such directly PNA modified AuNPs in solution.^[29] To overcome the above-mentioned stability issues, we proposed a two-step process for the synthesis (Fig. 7). First, the AuNPs were modified with a thiolated surfactant which provides steric and electrostatic stabilization. In a second step, the particles were treated with thiol-linked PNA without inducing agglomeration of the AuNPs. The PNA-modified AuNPs were tested in comparison to analogous DNA-modified AuNPs by assaying the self-assembly properties towards PNA modified gold surfaces. The test was carried out in the absence of ions,

where DNA–DNA assembly would not be possible due to electrostatic repulsion between the charged oligonucleotide backbones. The PNA-modified AuNPs showed superior binding affinity towards the complementary PNA strands on the surface. Notably, the nature of the thiol linker (short, long, monothiol, trithiol) did not have an influence on the outcome of the self-assembly experiment. The methodology therefore seems to be flexible and the linker of the PNA can, in future studies, be chosen to best fit the application, without running into problems with nanoparticle stability again. These new PNA-modified AuNPs will likely lead to new innovations, as many of the great applications of DNA-modified AuNPs can potentially be improved by using PNA-modified AuNPs instead.

In summary, DNA-based bio-nanotechnology can be greatly advanced by the use of PNA. Worthy of note, there are many more examples than the ones described above, e.g. miRNA sensing with PNA in nanogaps^[36] or self-assembled PNA/DNA nanostructures.^[37] Nevertheless, it has to be pointed out that the field is still in its infancy, and studying PNA for bio-nanotechnology will still hold many obstacles. There are, for example, also reports where the use of PNA instead of DNA brings basically no advantages.^[38] Incompatibility issues like those of ‘naked’ AuNPs towards PNA could be encountered for other systems as well. Nevertheless, the promise this technology holds is great and further investigations are likely to lead to innovative discoveries.

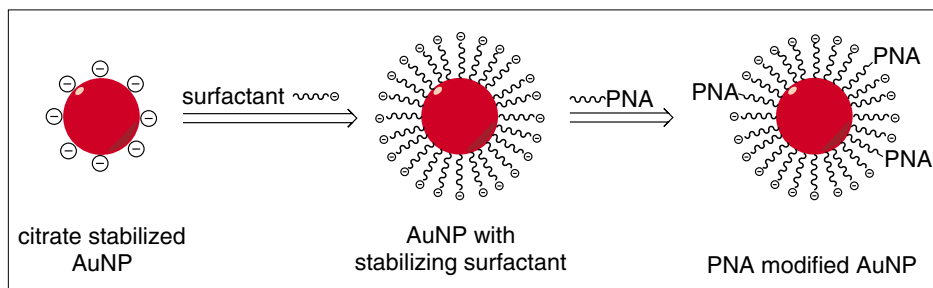


Fig. 7. Overview of the two-step process for functionalization of AuNPs with a stabilizing surfactant and thiol-linked PNA. Adopted with permission from ref. [29]. Copyright 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

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