Synthesis and Electrochemical Characterization of Metallocene–PNA Oligomers

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The synthesis of ferrocene- and cobaltocenium–PNA conjugates with *C*-terminal cysteine residues by solid phase methods is described. Melting points with complementary DNA reveal that both metallocenes stabilize the duplex, although to a different extent. The effect is more pronounced when there is an overhang of the DNA at the PNA amino terminus. The conjugates were studied by electrochemical methods in solution. They were also immobilized on Au microelectrodes in self-assembled monolayers (SAMs) and further characterized electrochemically on the surface.

Introduction

Peptide Nucleic Acid (PNA) oligomers are synthetic DNA analogues which have gained considerable attention because of their favourable properties, such as high stability in biological media and sequence-selective binding to RNA and DNA.[1–3] In addition to medicinal applications as a lead structure for antisense agents, analytical applications in molecular biology have been suggested for PNA.[4] In the sequence-specific detection of RNA or ss-DNA, PNA oligomers are superior to ss-DNA because of their higher binding affinity which allows for detection of shorter oligomer sequences.[5,6] At the same time, mismatch sensitivity is improved for PNA·DNA duplexes compared to homologous ds-DNA. In addition, the stability of PNA·DNA duplexes is far less dependent on factors such as salt concentration and impurities than a purely DNA-based detection system would be.[7,8]

Electrochemical systems for DNA detection are potentially cheaper and more reliable than the conventional fluorescence spectroscopy. Ferrocene and its derivatives are often used in such devices because of their favourable electrochemical properties.[9,10] The electrochemically active metal complex is covalently linked to a DNA oligomer which is readily immobilized on an electrode surface,[11,12] making this scheme also suitable for DNA arrays.[13,14] Because of the advantageous properties of PNA, it seems ideal to construct electrochemical DNA sensors using metal-PNA probes. Wang and coworkers have explored PNA-based DNA sensors.[15,16] These workers used freely diffusing metal complexes or so-called reagentless detection. No PNA oligomers with covalently bound electrochemically active metal complexes were known at the time. Our group has subsequently reported the first organometallic PNA monomers and oligomers,[17–19,10] followed by other groups.[20,21] No attempt has been made so far to exploit the favourable combination of properties in metallocene–PNA conjugates for electrochemical DNA detection. In fact, no metallocene conjugates with PNA oligomers were comprehensively characterized so far.

In this paper, we report the synthesis of metallocene–PNA oligomers with thiol groups for immobilization on Au microelectrodes. Ferrocene PNA oligomers are compared to the isostructural but positively charged cobaltocenium derivatives to avoid solubility problems associated with the lipophilic ferrocene group. The metallocene–PNA oligomers are characterized in their interaction with complementary DNA. They were deposited in self-assembled monolayers (SAMs) on Au microelectrodes. The two metallocenes are compared in terms of their suitability for an electrochemical DNA detection system.

Results and Discussion

For this study, a 10-mer PNA sequence was synthesized by standard solid phase techniques on TentaGel R PHB-Cys(Trt)-Fmoc resin. After synthesis of the PNA oligomer, the last Fmoc group was cleaved, and activated ferrocene carboxylic acid (**1**) or cobaltocenium carboxylic acid (**2**) was added. After cleavage from the resin by concentrated TFA, precipitation from cold ether and lyophilization, two PNA oligomers were obtained with sequences $CpFeC_5H_4$-

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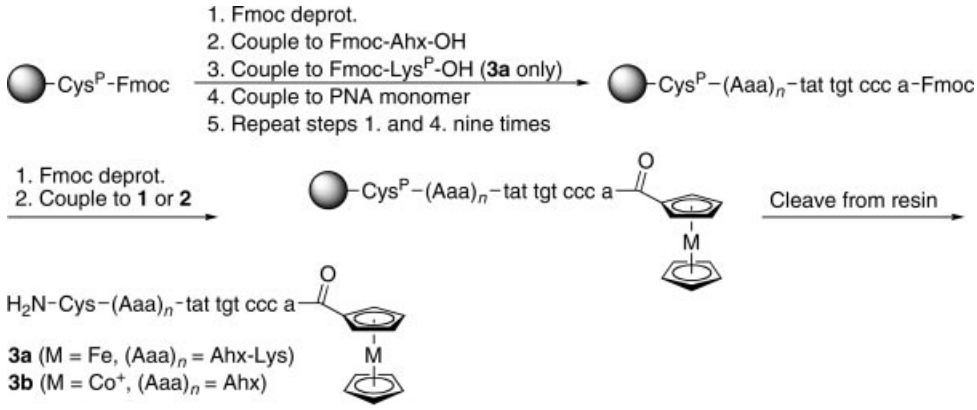

Scheme 1. Synthesis of metallocene–PNA oligomers **3**. See Supporting Information for details.

CO-a-ccc-tgt-tat-Lys-Ahx-Cys-NH_2 (**3a**) and [$CpCoC_5H_4$-CO-a-ccc-tgt-tat-Ahx-Cys-NH_2]$^+$ (**3b**, Ahx denotes ω-aminohexanoic acid). An *N*-acetylated oligomer Ac-a-ccc-tgt-tat-Lys-NH_2 (**3c**) was prepared for comparison (Scheme 1).

Purification and characterization of the PNAs was performed by RP-HPLC and MALDI-TOF mass spectrometry, respectively. The MS for **3a** shows a signal at *m*/*z* = 3233.8, which matches exactly the calculated value of 3233.3 for [M + H]$^+$. Spectra of similar quality were obtained for **3b** and **3c** (see Supporting Information). It should be noted that **3b** carries a positive charge already. The presence of the covalently attached metallocene group is also immediately apparent from the orange-brown or yellow colour of the resin for **3a** or **3b**, respectively, in comparison to white **3c**. A similar colour change was observed for a Mo organometallic complex attached to the pentapeptide enkephalin.[22] We recently found that attaching a cobaltocenium group to peptides significantly enhances the cellular uptake and nuclear localization of such conjugates.[23]

The metal–PNA conjugates were hybridized to complementary DNA in buffered aqueous solution in order to study the influence of the metal complexes on duplex stability. The melting curves for **3**·DNA were recorded in a temperature range of 20–80 °C (see Figure 1 and Table 1), using exactly matching DNA (**I**) and a complementary DNA strand which has three bases overhang at the metallocene PNA end (**II**). Several conclusions can be drawn from the melting temperature data: (1) the uncharged ferrocene influences duplex stability even if the PNA and DNA match exactly [Δ*T* (**3a·I**–**3c·I**) = 4.3 °C], (2) overhanging DNA stabilizes a PNA·DNA duplex [Δ*T* (**3a·II**–**3a·I**) = 1.5 °C], and (3) a positively charged metallocene further stabilizes the PNA·DNA duplex only if the DNA, which is formally a polyanion, overlaps the PNA to make an interaction with the metallocenium cation possible. In effect, the increase in melting temperatures is 5.1 °C in going from **3b·I** to **3b·II**, whereas it is only 1.5 °C in the ferrocene system. The second observation is in agreement with previous results.[5] The third observation has been suggested in work with coordination compounds but could not be proven independently of other factors.[24]

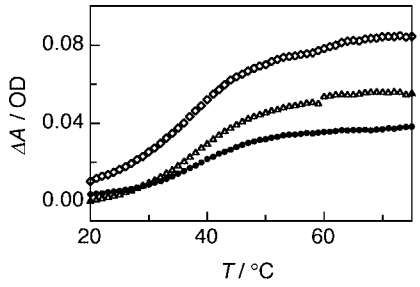

Figure 1. Melting profiles of **3a·I** (open triangles), **3b·I** (open squares), and **3c·I** (filled circles). See Supporting Information for experimental details.

Table 1. PNA·DNA UV melting temperatures (°C). DNA sequences are 5′-ATA-ACA-GGG-T-3′ (**I**) and 5′-ATA-ACA-GGG-TAA-T-3′ (**II**). See Supporting Information for experimental details.

PNA/DNA	I	II
3a	42.8 ± 0.5	44.3 ± 0.5
3b	37.6 ± 0.5	42.6 ± 0.5
3c	38.5 ± 0.5	n. d.

Solution electrochemistry of the metallocene PNAs **3a** and **3b** alone shows a quasireversible wave at E^0 = 0.20 V (**3a**, see Figure 2) and E^0 = –1.51 V (**3b**) vs. Fc/Fc^+ in acetonitrile/H_2O (1:1) solution for the one-electron transitions of the metallocenes. Electron-transfer properties of a ferrocene–PNA monomer and dimer were studied by Baldoli, Maiorana and coworkers.[25] The metal-free conjugate **3c** does not show any signal at comparable concentration. En route to the design of a device for electrochemical DNA analysis, we investigated the electrochemistry of ferrocene–PNA conjugates immobilized on Au electrode surfaces.

Gold microelectrodes were prepared from Au wire (50 μm diameter) sealed into glass capillaries as described before.[26] After polishing on alumina and electrochemical preparation, the thiolated PNAs were immobilized on the Au microelectrodes as a duplex with DNA **II**. For those electrodes, an electrochemical signal can be recorded at *E* = 0.44 V for the ferrocene–PNA–DNA duplex (**3a·II**, see Figure 2) vs. Ag/AgCl in aqueous KCl (3 M) in cyclic vol-

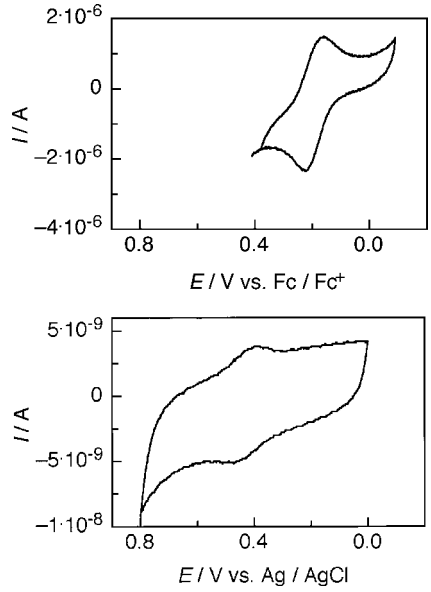

Figure 2. Cyclic voltammograms of **3a** in solution (top, CH_3CN/H_2O solution with 0.1 M $NaClO_4$, ca. 1 mM, platinum electrode with 2 mm diameter, vs. Fc/Fc^+, scan rate 0.5 Vs^{-1}) and of **3a·II** immobilized on the Au surface (bottom, aq. phosphate buffer at pH = 7, Au microelectrode, vs. Ag/AgCl, scan rate 2 Vs^{-1}). See Supporting Information for details.

tammetry (CV) as well as square wave voltammetry (SWV). Under identical conditions, no signal could be observed for the cobaltocenium–PNA conjugate **3b·II**. Ferrocene–DNA conjugates on Au electrodes are known to stand aligned at an angle around 45°. We suspected that the charged cobaltocenium–PNA conjugate might flip to the surface because of the flexible Ahx linker. Indeed, we were able to record a SWV for a cobaltocenium–PNA·DNA duplex that had no Ahx linker and was therefore more rigid (H_2N-Cys-tat-tgt-ccc-a-Cc·5′-ATA-ACA-GGG-T-3′; Cc: $CpCoC_5H_4$-CO-). In this case, a signal at $E = -0.92$ V was observed in the SWV.

From the electrochemical experiment, the surface concentration Γ_0* of **3a·I** was calculated from the oxidative peak current, the known scan rate and the surface area to be $\Gamma_0^* = 4\times10^{-11}$ mol cm^{-2}. This corresponds to roughly 7.3×10^8 molecules on an effective electrode surface of 2.8×10^{-5} cm^{-2}. For this calculation, the roughness factor for the surface area was experimentally determined by Cu-UPD to be 1.43. Under the assumption of complete electrode coverage by a self-assembled monolayer (SAM) this results in a footprint of 384 Å^2 per molecule, which is in agreement with previous measurements on Au-immobilized DNA and simple molecular geometry considerations.

Conclusions

In conclusion, we have presented the synthesis of metallocene PNA conjugates with thiol linkers for immobilization on Au microelectrodes. The synthesis of the first comprehensively characterized metallocene–PNA oligomers by solid phase peptide synthesis (SPPS) is described. Given the ubiquitous use of ferrocene in electrochemical sensors, the easy accessibility of the ferrocene–PNA system along with its favourable properties are worth mentioning. The use of a cobaltocenium–PNA conjugate is reported for the first time. Because ferrocene and cobaltocenium groups are almost isostructural but differ in charge as well as redox properties, subtle effects can be studied in detail as exemplified for the thermal stability of the metallocene bioconjugates in interaction with complementary DNA. The cobaltocenium–PNA conjugate shows excellent chemical stability upon storage and in solution. On the other hand, only the ferrocene–PNA shows clean electrochemical behaviour in the surface-immobilized duplex with complementary DNA. Taking together the ease of synthesis, chemical stability of the conjugates and favourable electrochemical properties, the metallocene–PNA system seems ideally suited for the development of electrochemical DNA arrays. Such devices are currently being developed in our groups.

Supporting Information is available online for this manuscript, including all experimental details as well as HPLC and MS data for PNA oligomers **3a**–**c**. Standard abbreviations are used for DNA bases (capital letters). The same small letters are used to denote PNA bases. Cys^P and Lys^P denotes protected cysteine (S-Trt) and lysine residue (N_ε-Boc-Lys).

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