A highly sensitive electrochemical genosensor based on Co-porphyrin labelled DNA

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We report the use of Co porphyrins as electrochemical tags for highly sensitive and selective genosensor. Avian Influenza Virus based DNA sequences characteristic for H5N were detected at femtomolar levels from competing non-complementary sequences through hybridisation of the labeled DNA.

The development of a global community by improved travelling and globalisation brings along comfort in live that is unprecedented in history. Through the rapid movement of people and goods, together with a steady improvement of medical treatment, both the availability of products (in particular animals and food products) from far-away corners of the world as well as extended lifespans can be enjoyed. As a consequence, this brings about new challenges for society, and particularly the fast and broad spread of local infection diseases facilitates the development of an epidemic. The development of novel, very sensitive and fast analytical techniques for medical diagnostics, food control and environmental screening is therefore a high priority. Among the variety of available analytical techniques[1] which are currently applied in those areas, the use of electrochemical genosensors is very promising as it can be applied using relatively simple instrumentation and shows quick and sensitive response to the analyte.

Generally, in electrochemical genosensors the formation of the DNA duplex (hybridisation to an immobilised ssDNA) is monitored *via* change of current or electrical potential value, either

using label-free or labelled systems. Label-free genosensors can be based on the changes of oxidation/reduction peak current of electroactive oligoducleotides (ODNs) themselves,[2] on the use of ion-channels in amperometric sensors,[3] or include an electroactive intercalator;[4] the latter was shown to show efficiencies in terms of electron transfer rates in the range of 1.5 to 40 s-1 and allows detecting sequence mismatches.[5] For the former systems, changes in response are a consequence of electrode surface changes after hybridisation inducing steric hindrance for marker ions to reach the surface of the electrode.[6] Redox labels are generally attached to the unbound end of the attached DNA (E-DNA), where ferrocene[7] (Fc) or methylene blue[8] are most commonly used markers. In the "signal-off" architecture the electrochemical response is greatly diminished going from the flexible ssDNA to the rigid dsDNA,[9] whereas "signal-on" systems show the opposite effect.[10] The selectivity and sensitivity of the E-DNA sensors arises from a combination of a conformational change upon hybridisation, together with the redox labels being active at potentials far from those of most electroactive biomolecules typical for clinical and environmental samples, thus being resistant to interfering contaminants. The E-DNA sensors reported to date can detect pico moles of ssDNA.[11]

We are now exploring the use of cobalt porphyrin as redox label for genosensor based on gold electrodes (Scheme 1). The porphyrins are attached to the DNA close to the electrode surface thus the distance does not vary greatly upon hybridisation. The analytical signal generation is proposed to proceed *via* a novel mechanism which is not based on a signal-on or –off scheme. The sensitivity of this genosensor towards target DNA is in the femto mole range, offering orders of magnitude lower detection limits.

The synthesis of the porphyrin building block has been reported earlier;[12] the marker was introduced into ssDNA using standard phosphoramidite chemistry and automated SPS (see ESI for synthesis details). The porphyrin-DNA was metallated with cobalt post-synthetically. In order to anchor the DNA stably onto the gold surface, three dithiol and hexaethylene glycol units were added to the 5'-end, which ensures that the DNA is not released from the electrode at high voltages. The general layout and function of the genosensor is shown in Scheme 1 and consists of the porphyrin-DNA being deposited onto the gold surface and embedded in a mercaptohexanol SAM (see ESI for details)†. The presence of the redox active probe (**CoP-ssDNA**) on the electrode surface was confirmed with cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Representative cyclic CVs are shown in Fig. S1A in ESI†. The quasi reversible Co(II)/Co(III) redox processes13 are visible at 0.285±0.022 V and

0.190±0.014 V for electrode modified with **CoP-ssDNA**. DPVs show clearly visible oxidationand reduction peaks (Fig. 2S in ESI) †. The formation of the double helix at the surface of the electrode causes a small (a few mV) shift of oxidation and reduction peaks current towards lower potential (Fig. S1B and Fig. S2B in ESI) †. A similar phenomenon observed for ferrocene in mixed monolayers was reported by *Creger* et al.[14] The main purpose (analytical one) of our work was to find the relationship between concentration of target ssDNA sequence and electrochemical signal. This was achieved by selection of main Co(II)/Co(III) peak observed at ~0.3 V in CV (Fig.1a) as well in DPV (Fig. S2). On the other hand, very small peak observed at ~0.45 V did not fulfil these conditions. Based on literature data [13,15], we can assume that this small peak originated from catalytic activity of CoP leading to water oxidation. The density (Γ) of **CoP-ssDNA** assembled on the Au electrode surface was calculated based on the integration of the voltammetric peaks. The value of Γ obtained is equal to $1.1(\pm 0.3)x10^{-11}$ mol cm-2, which represents $6.6x10^{12}$ molecules cm-2 and is in the same range reported for other redox active DNA probes.7b CVs obtained for **CoP-ssDNA** at different scan rates from 10 to 103 mV s^{-1} (Fig. 1) show that the system becomes more irreversible parallel with increasing scan rate. The linear relationship between the anodic and cathodic peak currents *vs* scan rate indicates that the redox process is not diffusion dependent and confirms the localisation of the **CoP-ssDNA** probe on the surface of the electrode. For finding the relationship between concentration of target ssDNA sequence and electrochemical signal, Osteryoung square – wave voltammetry (OSWV) was applied (Fig. 2), and representative OSWVs after incubation with the 20 mer complementary ssDNA are shown in Fig. 2a. It is worth to underline that with using this technique the small additional peak was almost invisible. Addition of the target ssDNA causes a decrease in the Co(III)/Co(II) Faradic current; the decrease is inversely proportional to the log of concentration of the complementary ssDNA. A non-complementary ssDNAs, on the other hand, induced much smaller changes in the current (Fig. 2b,c). The redox current *vs* ssDNA log of concentration follows a linear trend, from 10 to 80 fM (Fig. 2c). The genosensor displayed good selectivity. The slope of calibration curve for complementary ssDNA was *ca* 2.5 times higher in the comparison to the slope recorded for non-complementary ssDNA (Fig. 2c). The estimated detection limit (DL=2x σ /S, where σ is the standard deviation of the response, S is the slope of the calibration curve)15 is 21 fM. The precision, defined as the closeness of agreement among individual tests,[16] was very good (RSD=3.6%; n=5). Given that for the incubation we used 10 µl of target DNA solution, the effective detection limit of DNA is 10^{-20} moles and

amounts to 1000 DNA molecules; the electrode has a surface area of 2 mm^2 and contains about $1.3x10^{11}$ CoP-DNA molecules.

It should be noted that the sensitivity increased after storing the electrode in buffer solution at 4 °C for three days, which might be the result of restructuring and better ordering of the DNA on the electrode surface (Fig. S3 see ESI)†. The reusability of the genosensor was not tested as our future goal is the development a one-use miniaturized system.

The role of ion effects on the electrochemical properties of monolayers with incorporated redox active sites has been reported mainly for ferrocene.[17] In order to determine the influence of different anions and cations on the value of the generated signal, we evaluated the relationship between the scan rate to anodic and cathodic peak potential measured for **CoP-ssDNA** and **CoP-dsDNA**, in the presence of different supporting electrolytes (CsCl, KCl, NaCl, NaNO3, NaBF4 and NaClO4) at 1.0 M concentrations buffered with 0.01 M sodium citrate (see Fig. S4 ESI)†. For electrolytes containing Cl-, CVs recorded for K+ and Na+ were very similar with a clearly visible reduction process. In the presence of the most lipophilic cation, Cs+, the Co(II)/Co(III) Faradaic current was almost invisible. When different anions (as Na+ salts) were investigated, the reduction processes were better visible in the presence of more lipophilic anions such as NO3- and BF4- (Fig. S4 ESI) †. The shift of oxidation and reduction potential to higher values follows the increase of lipophilicity and size of both cations and anions, and was observed for both ssDNA and dsDNA (Table S1 ESI) †. The values of electron transfer coefficients (α) and electron rate constant (k) measured with different electrolytes are collected in Table S2 (ESI) †. In most cases the α-values are >0.5, meaning that the reduction is favoured,[18] and only the most lipophilic Cs+ has α-values <0.5 and thus favours oxidation. For BF4- the α-values are close to 0.5 indicating energy symmetry for the redox reactions. k values are higher for chlorides, compared to the values measured in the presence of more lipophilic anions. For all electrolytes studied, reduction is superior to oxidation. Based on these observations, NaCl is the most suitable supporting electrolyte as it is the least lipophilic salt for use with **CoP-ssDNA** and **CoP-dsDNA**. Overall, the influence of different anions and cations on the redox properties of cobalt porphyrins is not only governed by their lipophilicity or size, but the affinity towards the cobalt site is likely to be a second parameter in the process. However, the access of the most hydrophilic Cl- and Na+ ions to the Co(II) redox sites seems difficult. Therefore the oxidation and reduction processes are hindered, and because of this, a large decrease of the Co(II)/Co(III) redox current upon hybridisation with complementary DNA is observed.

The majority of the recently developed electrochemical DNA or PNA sensors make use of the hybridisation process, where the induced conformational change from a flexible single strand to a rigid double strand ODN probe alters the distance of the redox label [8,9] (e.g. Fc) to the electrode surface. In our system, the redox centre is located close to the electrode surface, thus the distance change is relatively small. In this respect our sensor does not directly rely on a "signal on/off" mechanism through distance changes but rather through changes in the hydrophobic environment of the attached label. The influence of various parameters such as potential, redox centre to electrode surface distance, solvent, temperature and nature of the interfacial micro environment on redox reactions in electroactive SAMs has been studied extensively.[19] In particular, the solvent effect (stabilisation of a specific state of the redox couple) and double layer effect (prevention of counter-ions from solution entering the SAMs) are described by the *Smith* and *Whit20* model as well as by *Creager*.[14] In consequence of these effects, either the Gibbs free energy ($\Delta G°$) of solvation or the spatial distribution of ions in the interfacial region is changed. For our system the hybridisation process leads to two changes in the electrode surface environment: (i) the **CoP** redox centre is more embedded in the ODN duplex, and (ii) a duplication of the negative charge makes the molecular environment around the porphyrin more polar. Both factors have an influence on the redox reaction by creating a hindrance for the anionic counter ions to be transported to the redox centre, leading to a decrease in Faradic current. This is in-line with reports on using an Fc label to detect biotin-IgG antibody interactions on a glassy carbon electrode, where the association or dissociation of the antibody at the sensing interface caused a modulation of the Fc electrochemistry due to the restriction of counter ions' access to the redox probe.[21]

In summary, we present a new type of electrochemical genosensor based on gold electrodes modified with a cobalt porphyrin DNA probe, where the probe is located very closely to the surface of the electrode. The genosensor displayes very good selectivity andsensitivity at the femtomolar concentration level towards 20-mer ssDNA derived from Avian Influenza Virus type H5N. As a sensing technique SWV was applied, and the changes in Co(II)/Co(III) Faradic current were used as an analytical signal. The signal changes are a consequence of both solvent and double layer effect, and are strongly dependent on the supporting electrolyte. The high sensitivity, low detection limit, selectivity towards complementary DNA and ease of micro-electrode formation shows high potential of the sensor system for applications in medical diagnostics.

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† Electronic supplementary information (ESI) available: Synthesis of the porphyrin-DNA, preparation and characterisation of the Au-electrode. See DOI: 10.1039/c4cc00172a

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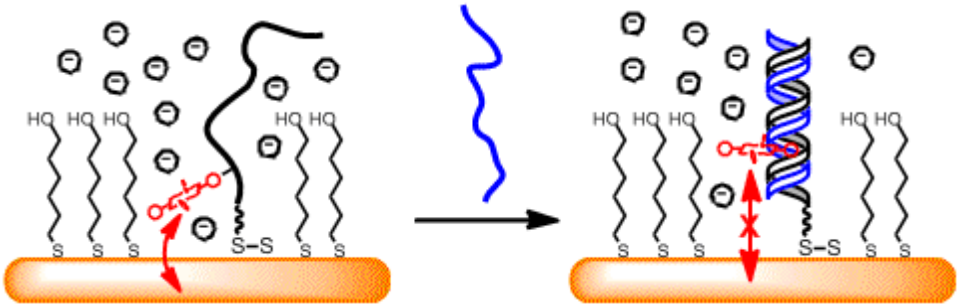
FIGURES



Scheme 1. Schematic illustration of signal generation mechanism of metallo-porphyrin-DNA genosenor

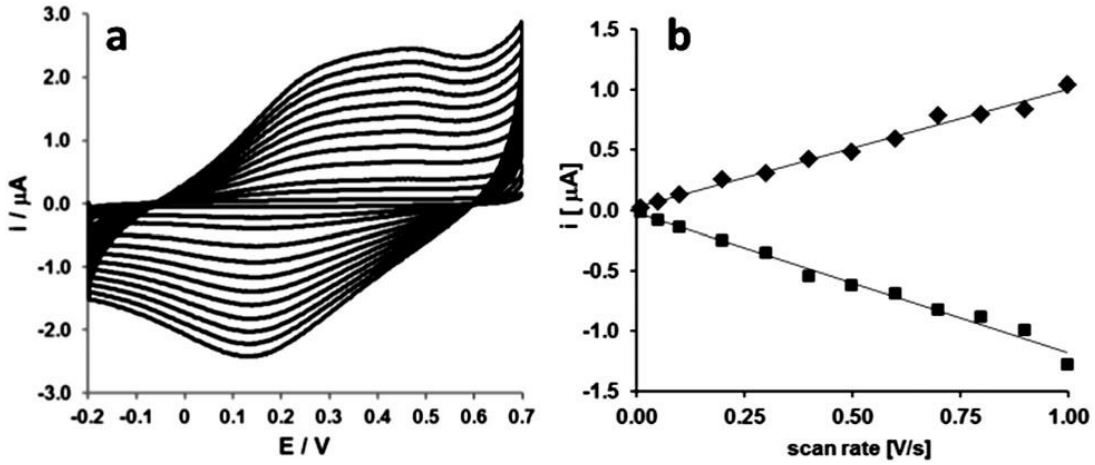


Fig. 1. (**a**) Cyclic voltammograms of **CoP-ssDNA** on Au-electrode at scan rates from 10 to 1000 mV s-1; buffer conditions: 1.0 M NaCl, 0.01 M sodium citrate, pH 7.0; (**b**) plots of anodic (♦) and cathodic (■) current *vs* scan rate.

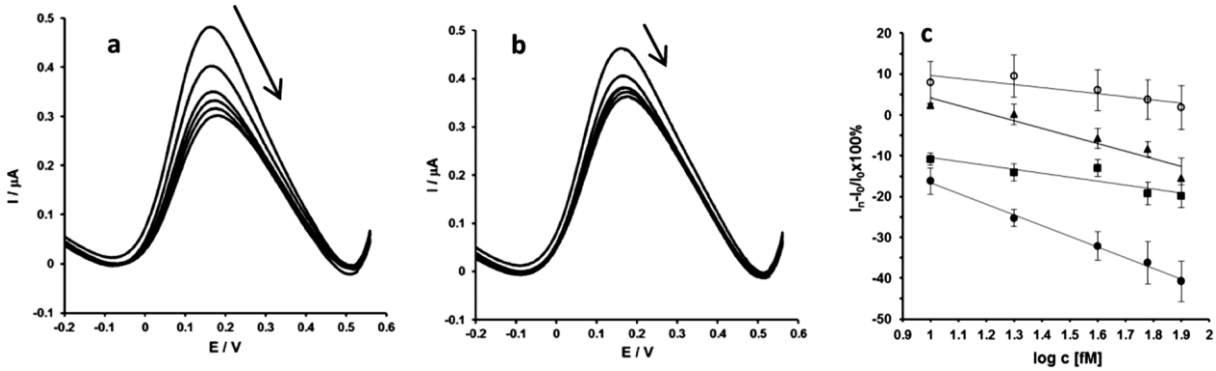


Fig. 2. Representative Osteryoung square-wave voltammograms (OSWVs; 1 mV, square-wave frequency: 50 Hz and amplitude: 25 mV) obtained for electrode modified with Co-porphyrin-ssDNA probe: 5`-APT TGG AGC TAT AGC AGG TT-3` (**a**) after hybridisation with 20-mer complementary ssDNA and (**b**) in the presence of sequence with three complementary bases at concentrations of 0, 10, 20, 40, 60 and 80 fM (buffer conditions: 0.9 M NaCl, 0.09 M sodium citrate, pH 7.0). (**c**) Relative intensity of redox Co(II)/Co(III) current *vs* log of concentration of 20-mer complementary ssDNA 5`- **AAC CTG CTA TAG CTC CAA AT**-3` (●); sequence with three complementary bases: 5`- GGA G**T**T **C**CT CTC TCA TC**A** TC-3` (■); sequence with one complementary base.: 5`-G**A**A GAA GAG AGA GGA ACT CC-3` (▲); fully non-complementary sequence.: 5`-TTG GAC GAT ATC GAG GTT TA-3.