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Detection of single-nucleotide polymorphisms based on the formation of an electron-transfer impeding layer on an electrode surface

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Zhiqiang Gao*, Wei Shen, Huimin Deng and Yuqian Ren

An electrochemical biosensor utilizing the electron-transfer impeding (insulating) power of a conducting polymer for label-free genotyping single-nucleotide polymorphisms is proposed. Excellent selectivity and sensitivity are accomplished through the engagement of a nuclease clean-up step and a cumulative signal generation/amplification process, respectively.

In the past two decades intensive efforts have been devoted to the study of single-nucleotide polymorphisms (SNPs). Computational analysis has predicted that there are over 15 million SNPs in the human genome occurring on an average of one SNP in every 200 nucleotides.[1] SNPs are believed to be closely associated with various medical conditions and genetic diseases although the majority of them have little impact on human health.[2–4] Information gathered to date suggests that SNPs could be used as a new generation of biomarkers for cancer diagnosis and prognosis,[2–4] presenting clinical scientists with new perspectives in diagnostics and a promising therapeutic frontier.

As the first step toward improving human health, SNPs must be reliably identified and quantified. Currently, two groups of SNP genotyping strategies, namely enzymatic allele discrimination[5,6] and allele-specific hybridizations,[7] have been extensively exploited in the development of the SNP genotyping platforms. Due largely to their excellent selectivity, assays based on enzymatic allele discrimination have attracted considerable attention.[5–11] To increase their sensitivity, amplification strategies, such as the use of DNAzyme,[8] enzyme,[9] ligation chain reaction[10] and rolling circle amplification,[11] have been coupled to the enzymatic allele discrimination-based assays. Satisfactory results were obtained with some of the amplified assays. On the other hand, allele-specific hybridization-based approaches offer high possibilities for constructing simple, robust, and affordable SNP genotyping systems due to their simplicity and straightforwardness. As compared to the enzymatic allele discrimination-based assays, inferior selectivity seriously reduces their potential values in SNP genotyping of real-world samples.

Department of Chemistry, National University of Singapore, 117543, Singapore. E-mail: chmgaoz@nus.edu.sg; Fax: +65 6779-1691; Tel: +65 6516-3887

Because of their prominent relevance to human health, a portable and robust SNP genotyping platform is highly desired for fast and reliable SNP analysis. The development of allele-specific hybridization-based electrochemical biosensors for label-free SNP genotyping is probably one of the promising ways to solve some of the above-mentioned problems encountered in SNP genotyping. Apart from its high sensitivity, electrochemical transduction is advantageous over other transduction technologies due to its inherent high portability, good compatibility with advanced semiconductor technology, independence from solution turbidity, and affordability. In addition, multiplexed detection systems can be conveniently constructed through a simple switching mechanism.

In this communication, an electrochemical biosensor utilizing the electron-transfer impeding (insulating) power of a conducting polymer for label-free genotyping SNPs is proposed for the first time. Excellent selectivity was accomplished through the engagement of a nuclease clean-up step. To significantly enhance the sensitivity of the biosensor, a cumulative signal generation cum amplification process – the horseradish peroxidase (HRP)-catalysed polymerization of aniline and the hybridized SNP target–capture probe (CP) duplex-guided deposition of polyaniline (PAn) – was incorporated in the procedure. The presence of a thin PAn film on the biosensor surface has a significant impact on the electron-transfer process of redox probes in solution. Excellent insulating power of the deposited PAn film was attained when tests were performed in an alkaline medium. Voltammetric detection of SNPs at femtomolar levels was therefore realized through measuring peak-to-peak potential separation (ΔE_p).

Fig. 1 illustrates the working principle of the biosensor: (a) the biosensor is made of a self-assembled monolayer of oligonucleotide CPs on a gold electrode. To improve the stability of the CP monolayer and to prevent non-target related deposition of PAn,

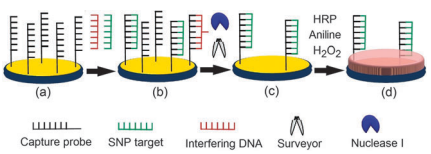

Fig. 1 Schematic illustration of the electrochemical biosensor.

cysteamine is used to fill in the defects of the CP monolayer through thiol–gold interaction. (b) Hybridization with a SNP target under very low stringency conditions brings the SNP target together with some interfering DNA onto the biosensor. (c) Subsequent incubation of the hybridized biosensor in a nuclease cocktail consisting of nuclease I and Surveyor® removes free CPs and all imperfectly hybridized DNA strands. (d) Deposition of PAn is carried out in a mixture of HRP, aniline and H_2O_2. Oxidative polymerization of aniline is initiated by HRP and the deposition of PAn on the biosensor is guided by the hybridized SNP target–CP duplexes. The excellent electron-transfer impeding power of the deposited PAn in the alkaline medium allows SNPs to be conveniently detected by voltammetry. Ultrahigh sensitivity is anticipated when the deposition of PAn is allowed to proceed for a sufficiently long period of time. The reason for engaging a nuclease clean-up step instead of direct voltammetric detection is two-fold: to significantly enhance the selectivity of the biosensor by eliminating all imperfectly hybridized interfering DNA–CP duplexes and to minimize non-hybridization-related deposition of PAn by removing all free CPs from the hybridized biosensor as they can also serve as templates for the deposition of PAn. The presence of free CPs will substantially lift the background signal, and hence the detection limit. In addition, by removing the free CPs from the biosensor surface, a minimal ΔE_p (background) of the redox probes close to that observed at a bare gold electrode was obtained, thus producing a high signal-to-noise (S/N) ratio.

It was reported that HRP-catalysed polymerization of aniline can be used to prepare PAn nanowires on a silicon substrate by using fully stretched DNA strands as templates. It was observed that the DNA strands serve as excellent templates to guide the deposition of PAn around them.[12] We previously utilized the conductivity of PAn after HCl doping as the analytical signal for the detection of microRNAs on a nanogap sensor array.[13] It was found that the stability of the conductivity of the HCl doped PAn deteriorates quickly under ambient conditions. The nanogap sensor array must be examined within 30 min after doping. In addition, strong acids had to be used when working with the conducting properties of PAn.[12] The above-mentioned observations inspired us to rethink the way PAn is employed in the construction of DNA biosensors. In this work, instead of leveraging the electron-conducting properties of PAn, the possibility of utilizing its electron-insulating properties for possible applications in biosensors is exploited.

To test the feasibility of utilizing PAn as a sensitive signal generator for the construction of an ultrasensitive SNP biosensor, a synthetic SNP target at 250 fM was tested at a biosensor coated with fully complementary CPs. Upon hybridization, the SNP target strands were brought to the biosensor by their complementary CPs. Subsequent incubation in the PAn deposition cocktail resulted in the formation of a thin PAn film alongside the hybridized SNP target strands, akin to that of DNA-templated formation of PAn nanowires.[12] Fig. 2 shows typical cyclic voltammograms of the hybridized biosensor under various experimental conditions. Similar to previous observations, distinct redox activity was observed when the biosensor was tested in pH 3.0, 0.10 M phosphate buffer (Fig. 2A). Extensive voltammetric tests revealed that the deposited PAn is robustly bound to the biosensor, owing largely to the surface-bound DNA strands. In addition to firmly holding the CP strands upright,

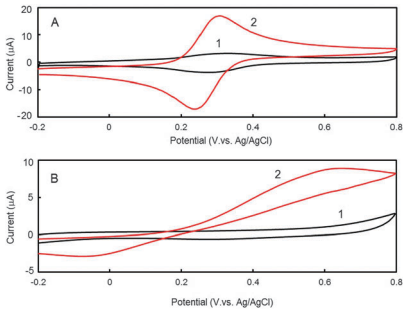

Fig. 2 Voltammograms of the hybridized biosensor in (A) (1) pH 3.0, 0.10 M phosphate buffer and (2) (1) +2.5 mM $Na_3Fe(CN)_6$; and (B) (1) pH 9.0 0.10 M phosphate buffer and (2) (1) + 2.5 mM $Na_3Fe(CN)_6$. 250 fM SNP target, potential scan rate 100 mV s^{-1}.

the immobilized cysteamine molecules help to minimize non-specific deposition of PAn on the biosensor. The amino moieties on cysteamine molecules were protonated at the pH for the deposition of PAn. When the hybridized biosensor was incubated in the PAn deposition cocktail, the protonated aniline molecules were not able to indiscriminately access the biosensor due to strong electrostatic repulsion between the protonated cysteamine and aniline molecules. In contrast, the protonated aniline molecules were attracted to the DNA strands *via* electrostatic interaction between the anionic phosphate backbones of the DNA strands and the protonated aniline molecules, producing high concentrations of aniline and protons around the DNA strands. These high concentrations of aniline and protons greatly facilitate the polymerization of aniline in a much less acidic medium than conventional chemical and electrochemical approaches.[12,14] The deposited PAn film displayed only one pair of redox peaks at ~0.30 V (Fig. 2A). The absence of the second redox process is believed to be due to the stabilizing effect brought about by the DNA templates.[14] Additional tests were carried out in the phosphate buffer after spiking 2.5 mM $Na_3Fe(CN)_6$. As seen in trace 2 in Fig. 2A, $Fe(CN)_6^{3-}$ is able to freely exchange electrons with the gold substrate electrode through the PAn due to the excellent electron-conducting properties of the PAn film. As we know, the electrochemical activity and the conductivity of PAn are strongly pH dependent.[15,16] The conductivity of PAn drops precipitously with increasing pH.[16] As shown in Fig. 2B, the redox activity and its electron-transfer ability of the deposited PAn film disappeared completely when tests were conducted at pH ~ 9.0. Instead of being a good electron-conducting layer on the gold electrode, PAn now strongly impeded the electron exchange process between $Fe(CN)_6^{3-}$ and the gold electrode, manifested by the appearance of a totally irreversible voltammogram with a huge ΔE_p of ~600 mV. The widely separated voltammetric peaks clearly indicated that an electron-insulating layer is formed on the biosensor surface at which the electron-transfer is severely impeded. The increased ΔE_p is a direct consequence of the resistance to charge transfer between the redox probes and the substrate gold electrode through the deposited PAn film. In contrast, when a totally non-complementary target (control) was tested at the biosensor after the same treatment, a negligibly small increment of ~20 mV over that obtained with a bare gold electrode was observed, suggesting that there is little resistance to the electron-transfer of the redox probes after a complete removal of the CPs from the biosensor surface by the nucleases. Apparently, the significantly larger ΔE_p observed at

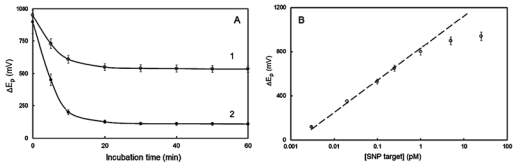

Fig. 3 (A) Effect of nuclease treatment on ΔE_p of (1) the biosensor and (2) control; and (B) calibration curve. Experimental conditions are the same as in Fig. 2.

the hybridized biosensor is a direct consequence of the hybridization and PAn deposition. And the magnitude of ΔE_p directly reflects the electron-transfer impeding power of the deposited PAn film and the key parameter directly associated with the SNP target concentration. Values of ΔE_p can be conveniently extracted from the voltammogram, paving the way for the development of a voltammetric SNP biosensor using ΔE_p as the analytical signal.

However, for a successful adaptation of ΔE_p as the analytical signal, it must be exclusively associated with the amount of the deposited PAn and the concentration of the SNP target. Unfortunately, the DNA-guided PAn deposition mechanism implies that the presence of a considerable number of free CPs poses an immediate threat as PAn deposition is guided not only by the hybridized target but also by the free CPs, and maybe to a much larger extent by the free CPs due to the presence of plenty of CPs on the biosensor surface. A clean-up step must be performed before the deposition of PAn. As illustrated in Fig. 3A, a substantially higher background ΔE_p and a minute increment in ΔE_p after hybridization were obtained without the nuclease incubation step. In contrast, ΔE_p dropped to a value close to that of $Fe(CN)_6^{4-/3-}$ at a bare gold electrode when the nuclease clean-up step was performed before the deposition of PAn. A significant reduction of ΔE_p was also observed at the hybridized biosensor, but it is largely due to the elimination of the free CPs. The much improved background ΔE_p greatly improved the S/N ratio, permitting the detection of SNP at ultralow concentrations. More notably, the cumulative nature of the signal generation process through the deposition of PAn provided a convenient and highly controllable means to boost the sensitivity of the biosensor. It is expected that the amount of PAn deposited strongly depends on experimental variables, such as aniline and H_2O_2 concentrations, pH and the incubation time in the PAn deposition cocktail. Detailed optimization experiments suggested that the best composition of the PAn deposition cocktail is made of 5.0 μg ml^{-1} HRP, 25 mM aniline, and 5.0 mM H_2O_2 in pH 4.0 0.10 M acetate buffer. Under these conditions, the sensitivity of the biosensor scaled up proportionally with the incubation period in the PAn deposition cocktail, implying that there is little activity loss of HRP during incubation. Taking into consideration both assay time and sensitivity, it was found that a 30 min incubation period is sufficient to push the detection limit down to femtomolar levels. Similar behaviour was also observed when the biosensor was tested in a mixture of SNP target and genomic DNA (salmon sperm DNA), suggesting that there is little uptake of the non-complementary DNA. However, an inevitable increase in ΔE_p was observed after a much prolonged period of incubation due to non-specific deposition of PAn. For example, ΔE_p of the control increased to ~280 mV and lifted the detection limit up to ~20 fM levels after a period of 120 min incubation in the PAn deposition cocktail.

Based on the encouraging results obtained in the feasibility study, attempts were made to utilize the biosensor to genotype SNP using a synthetic target as our calibration standard and a completely non-complementary synthetic DNA as control. A well-studied oncogene, V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS), was chosen as our SNP target since mutations in KRAS are largely SNPs that frequently occur in codons 12 and 13 of exon 2 with SNPs in codon 12 accounting for 80% of the total KRAS mutations. Calibration study showed that there is a linear relationship between the target concentration and ΔE_p from 3.0 fM to 1.0 pM with a relative standard deviation of ~15% at 1.0 pM and a detection limit of 1.0 fM at a S/N ratio of 3.0 (Fig. 3B). The deviation from linearity beyond 1.0 pM is due to the inherent characteristic of the hybridization process on a solid surface. The selectivity of the biosensor was evaluated by analysing the SNPs in codon 12 of exon 2 at a biosensor designed for a particular thymine mutation (MT-T). Mixtures of KRAS MT-T and interfering KRAS genes (KRAS mutation-C, KRAS mutation-A, and KRAS wild-type) were used as our samples. Our experiments showed that practically no interferences (<10%) were observed in the presence of ≤3000-fold interfering KRAS genes. As mentioned earlier, the involvement of the nuclease clean-up step removed all free CPs and mismatched duplexes from the biosensor surface, hence the deposition of PAn and ΔE_p were exclusively associated with the SNP target. Additionally, the engagement of the nucleases greatly simplified the stringency control during hybridization, allowing the target to be captured by the biosensor at its maximal efficiency. Compared to other hybridization-based approaches, the much enhanced selectivity and sensitivity is an attractive feature for further development.

In summary, we have demonstrated that the hybridized target–CP duplex-guided deposition of PAn can be utilized as a signal generation cum amplification strategy to construct a simple and robust biosensor for label-free genotyping of SNPs. Being capable of analysing genomic samples without PCR amplification and/or ligation and with minimal requirements for sample preparation, the proposed biosensor is an attractive candidate for the development of a portable device for routine point-of-care SNP genotyping.

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