Electrochemical genotyping and detection of single-nucleotide polymorphisms based on junction-probe containing 2′-deoxyinosine†

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A novel electrochemical biosensor for the genotyping/detection of single-nucleotide polymorphisms (SNPs) of trace oral-cancer salivary DNA based on junction probes containing 2′-deoxyinosine (dI) residues is described.

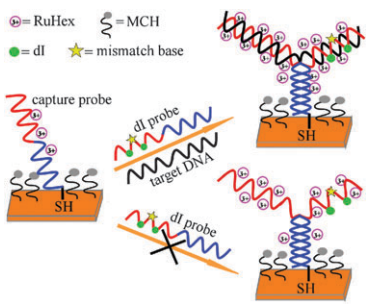
Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variations in individual genomes and are considered a primary influence in genetic diseases.[1] Therefore, considerable attention in genetic studies has been focused on the genotyping and detection of SNPs, which are important biomarkers for genetic diseases, cancer, and infectious diseases such as AIDS and tuberculosis.[2–5] To date, many strategies, including allele-specific hybridization,[6,7] allele-specific cleavage,[8] nucleotide incorporation and oligonucleotide ligation, have been used for the genotyping and detection of SNPs.[9–11] However, these strategies do not prevail owing to relatively high cost, limited specificity, lower resistance to complex matrices, or inadequate sensitivity.

Electrochemical detectors hold potential as a next-generation molecular detection strategy for the genotyping of SNPs due to their high sensitivity, rapid response, low cost and excellent compatibility with miniaturization technologies. In this direction, numerous electrochemical biosensors for SNPs detection have been developed.[12–16] However, previous strategies generally suffer from one or more of following shortcomings: complicated labeling procedures, substantial background current, relatively poor stability of enzyme, and low sensitivity (or poor specificity).

As a "universal pairing base", the deoxyinosine residue (dI, as nucleoside form) has been reported to match with each of the four normal DNA bases, the stability of these matched bases being I:C > I:A > I:T ≈ I:G.[17] The melting temperature of duplexes containing dI varies widely depending on the base to which the dI is paired and the neighboring sequence.[17,18] For the above reasons, it is expected that the SNP typing will significantly increase the allelic differentiation when using an oligonucleotide probe in which two bases adjacent to the SNP site are substituted with dI residues. In addition, it has recently been reported that the "junction probe" strategy, which operates by template-enhanced hybridization processes (TeHPs), has better specificity for the detection of SNPs.[19,20]

Considering all the above merits of the dI residue and junction probes, we herein provide the first report of an electrochemical biosensor for the genotyping/detection of SNPs in trace salivary DNA of oral cancer based on a junction probe containing dI residues. The novel electrochemical biosensor is easy to fabricate and convenient to operate, and affords a robust, specific and sensitive platform for SNP typing. Compared with the existing methods such as polymerase chain reaction (PCR) and micro-arrays,[21,22] the proposed biosensor offers substantial advantages such as good stability, higher sensitivity, better discrimination ability and low cost. The success of this biosensor is therefore a significant step toward the development of salivary diagnosis for clinical detection of oral cancer.

The experimental principle of the biosensor is illustrated in Scheme 1. The capture probe (Cp) contains two regions: the region colored red is complementary to part of the target DNA, while the region colored blue is complementary to the dI probe (Dp). Dp also contains two regions: the region colored blue is complementary to part of Cp, while the region colored red is complementary to target DNA. In Dp, two bases adjacent to the SNP site are substituted by a dI residue. As demonstrated in a previous study,[19] in the presence of target DNA, Cp, Dp and target DNA will hybridize to form a ternary "Y-junction", whereas this structure does not form in the absence of target DNA. The resulting structure that forms after hybridization is then detected by an electrochemical method with $[Ru(NH_3)_6]^{3+}$ (RuHex) as signal molecule. As a result of the formation of the ternary Y-junction, the amount of RuHex electrostatically binding to the surface of gold electrode (GE) increases, and the increase of RuHex leads to a bigger redox signal in the electrochemical measurement. By employing this strategy and the better discrimination ability of the dI residue for different bases, we demonstrate that the



Scheme 1 Experimental principle of the electrochemical biosensor.

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sensor has a higher sensitivity and better specificity for the genotyping and detection of SNPs.

In order to test if the sensor indeed works as expected, it was characterized by determining the cyclic voltammograms (CVs) of RuHex at different GE surfaces. As shown in Fig. 1, a pair of peaks corresponding to the reduction and oxidation of RuHex was observed in 5 μM of RuHex solution at the Cp-MCH-modified GE (Cp/MCH/GE) (curve b). The peak separation was close to zero and the peak current was linearly proportional to the scan rate, indicating that the electron-transfer reaction of RuHex at the Cp/MCH/GE surface was a surface-confined redox process. This pair of peaks was ascribed to RuHex electrostatically binding to the DNA surface, and reflects the amount of DNA on the GE surface. It worth mentioning that peaks corresponding to the reduction or oxidation of RuHex were not observed on a pure MCH-modified GE (curve c), suggesting that RuHex did not adsorb on MCH-modified GE; in addition, a diffusion current was not detected in dilute RuHex solution (5 μM). This approximately zero background provided a means of obtaining high sensitivity. When Cp/MCH/GE was incubated in the solutions containing target DNA and Dp, a much higher current signal was observed (curve a). This is because that Cp hybridized with target DNA and Dp to form a ternary Y-junction, and as a result of its formation, much more RuHex was electrostatically bound on the GE surface, ultimately resulting in a much higher current signal. The CV results indicated that our biosensor indeed worked as expected. Electrochemical impedance spectroscopy (EIS) was also carried out at different stages of the biosensor preparation (see ESI†), and the results were consistent with those obtained by CV.

In this study, a 24-base complementary target DNA T_1 (salivary DNA of oral cancer) and its one-base mismatch DNA T_2, T_3 and T_4 (T_2 with a T-to-G substitution, T_3 with a T-to-C substitution and T_4 with a T-to-A substitution at position 8 from 5′), and non-complementary DNA T_5 (see ESI†) were used to test the discrimination ability of our biosensor. The LSV (linear sweep voltammetry) results are shown in Fig. 2. The lowest signal was observed for Cp-T_5-Dp/MCH/GE (curve e), implying that Y-junction formation had not occurred in the presence of T_5. In contrast, the highest signal was observed for Cp-T_1-Dp/MCH/GE

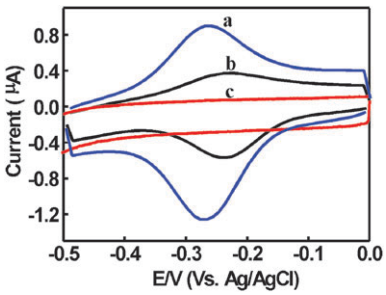

Fig. 1 CVs of RuHex on pure MCH modified GE (c), Cp-MCH modified GE (b) and Cp-target DNA-Dp/MCH modified GE (a). The concentration of target DNA, Cp, Dp and RuHex was 2 pM, 2 μM, 2 μM and 5 μM, respectively. Data was obtained in 10 mM Tris-HCl buffer solution (pH 7.0), with a scan rate of 100 mV/s.

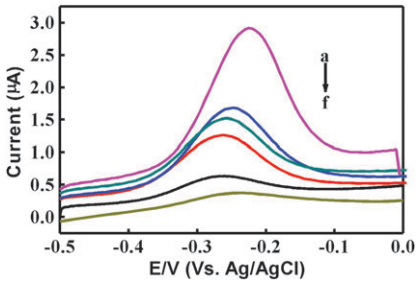

Fig. 2 LSV of 5 μM RuHex at Cp/MCH/GE (f) (background) and after hybridization with Dp and non-complementary sequence T_5 (e), A–A mismatch sequence T_4 (d), A–C mismatch sequence T_3 (c), A–G mismatch sequence T_2 (b) and complementary target sequence T_1 (a). The concentrations of T_1–T_5 were all 10 pM. Other experimental conditions are the same as those in Fig. 1.

(curve a), indicating that T_1 hybridizes with Cp and Dp to form a Y-junction, greatly increasing the density of DNA and negative charges on the electrode surface. As a result, the amount of the RuHex electrostatically binding to the GE surface increased, ultimately resulting in a much bigger current signal. In comparison with T_1, a much lower signal was detected for the one-base mismatch sequence, and the signal decreased in the order $T_2 > T_3 > T_4$, indicating that our biosensor has an excellent discrimination ability for single-base mismatch targets and can be used for the genotyping of SNPs.

To quantitatively evaluate the discrimination ability of our sensor, we defined the single-base mismatch discrimination factor as the ratio of net LSV signal (the peak current of target minus that of the background) obtained with complementary target DNA T_1 to that obtained with one-base mismatch DNA. A larger discrimination factor is thus indicative of improved specificity for SNPs. From the data shown in Fig. 2, we calculated that the discrimination factor was in the range of 2.0–3.0 for a one-base mismatch (in a 24-base DNA sequence). The best discrimination factor (3.0) was observed for the A–A mismatch, an intermediate value (2.4) for the A–C mismatch and the lowest (2.0) for the A–G mismatch (see Fig. 3). Recently, some sensing strategies such as "triple stem" and "gap ligation" have been developed to improve the discrimination ability of electrochemical DNA biosensors.[15,23] However, these strategies need complicated labeling and fabrication procedures, enzyme-aided reactions, and have relatively low sensitivity.

The electrochemical DNA sensor based on the electrostatic interaction of DNA and RuHex is easy to fabricate,

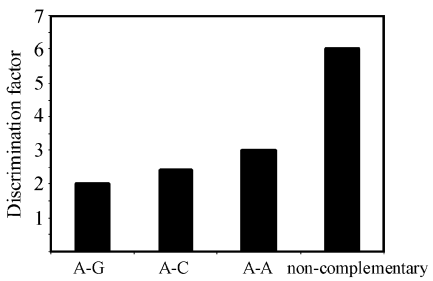

Fig. 3 The discrimination factors of the electrochemical biosensor for a single-base mismatch in 24-base salivary DNA of oral cancer. Data shown in Fig. 2 were used for the calculation.

Table 1 Determination of target DNA in artificial saliva sample ($n = 5$)

DNA T_1 added (pM)	Detection results (pM)	Recovery (%)	RSD (%)
2.00	2.10	105	6.2
5.00	4.95	99.0	5.1
10.0	9.82	98.2	4.8

convenient to operate and has good stability. However, this type of sensor has been reported to have a relatively low sensitivity and bad discrimination even if coupled with complex equipment such as nanoelectrodes.[24] In this study, by employing the strategy of junction-probe and dI residue, the sensitivity and the discrimination ability of the sensor have been greatly improved. The discrimination factor of 3.0 for the detection of a one-base mismatch is much better than that based on a single-stem molecular beacon probe or double-stem pseudoknot.[14] The detection limit of 1.3×10^{-13} M is much lower than that of the "triple stem" sensor previously reported.[23] Of particular note, our biosensor can be used for the genotyping of SNPs in real samples.

The sensitivity of the electrochemical biosensor was investigated by varying the concentration of target DNA T_1 in the range 2.0×10^{-12} to 1.0×10^{-11} M (the concentration of dI probe and capture probe was kept at 2 μM, respectively). The currents responsing to 5 μM RuHex after the sensor was hybridized with different concentrations of target DNA T_1 were determined with LSV. The average current showed a good linear correlation, with the concentrations of T_1 in the range 2.0×10^{-12} to 1.0×10^{-11} M. The regression equation was:

$$I\,(10^{-7}\ \mathrm{A}) = 0.2712\,C\,(\mathrm{pM}) + 8.0821 \quad r = 0.9954$$

The detection limit ($3\sigma/S$, where σ is the standard deviation of the blank solution, $n = 8$) was calculated to be 1.3×10^{-13} M for complementary DNA T_1. The relative standard deviation (RSD) of 8 different sensors, which were fabricated on the same electrode for different lengths of time, for the detection of 5.0×10^{-12} M target DNA T_1 was 6.0%, and that of 3 different sensors fabricated on three different electrodes was 6.4%.

In order to evaluate the applicability of our biosensor, an artificial saliva sample was composed by adding an amount of DNA T_1 in unstimulated saliva collected from a normal person, and the concentration of T_1 in it was determined with our biosensor. Unstimulated saliva samples were collected with previously established protocols.[25] From the results shown in Table 1, it was clear that the electrochemical biosensor has a strong resistance to the complex matrix of saliva, and can be used to detect target DNA in real saliva sample.

In summary, a highly specific and sensitive electrochemical system for the genotyping/detection of SPNs based on junction probes containing dI residues was developed for the first time. The substitution of bases with dI residues significantly increased the discrimination for single-base mismatch. The high sensitivity and discrimination ability for the detection of single-base mismatches, as well as ease of fabrication, operational convenience, stability and reusability, make this a promising alternative for the SNP genotyping/detection of salivary DNA of oral cancer in clinical diagnosis.

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