A highly sensitive microRNA biosensor based on ruthenium oxide nanoparticle-initiated polymerization of aniline†

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Well-defined voltammetric current peaks of polyaniline were observed at biosensors hybridized with ruthenium oxide nanoparticle-tagged microRNAs and incubated in a mixture of aniline/H_2O_2, which can be used for direct microRNA expression profiling with excellent sensitivity.

MicroRNAs (miRNAs) are ~22 nucleotide (nt) long endogenous noncoding RNAs that regulate fundamental cellular processes through the modulation of gene expression.[1] Despite the existence of quantitative real-time polymerase chain reaction (qRT-PCR) approaches for miRNAs, the extremely small size and high sequence homology of miRNAs means they always require some sort of miRNA extension prior to PCR and so present major challenges to PCR primer design.[2,3] Because of the emerging diagnostic and prognostic values of miRNA, it is critical that miRNA expression profiling methods should be highly sensitive, specific, and only analyze mature miRNAs as they are the active form of miRNAs.

The inherent miniaturization of electrical/electrochemical biosensors and their compatibility with standard microfabrication and semiconductor technologies could in principle provide simple, accurate, and inexpensive platforms for miRNA expression profiling, and thus are exempt from the problems encountered in the optical detection systems.[4] In view of the small size of miRNAs which poses some technical challenges for the adaptation of conventional electrical detection techniques in miRNA study, direct electrical detection of miRNAs themselves may be more advantageous. As demonstrated by Zhang *et al.*[5] silicon nanowire-based field-effect transistor (FET) biosensors are capable of directly and label-freely detecting miRNAs down to femtomolar levels. Unfortunately, as with all other nanowire-based FET sensing devices,[6–8] the analytical signal intensity is very low and even much lower in terms of relative change per unit concentration. For example, a resistance change of only 60% was observed for miRNA concentrations from 1 fM to 1.0 nM, translating into $<1 \times 10^{-4}$ % change per unit concentration, making accurately profiling miRNA expression difficult. Recently, another label-free approach, based on the detection of electroactive guanine was proposed by using inosine-modified capture probes (CPs).[9] After hybridizing with a target miRNA, the oxidation signal of guanine in the hybridized miRNA strands was measured voltammetrically with a detection limit of 5 nM (0.10 pmol). Nonetheless, the label-free methods do not offer the much-needed analytical characteristics for miRNA expression profiling.

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To enhance the sensitivity and lower the detection limit, we believe that a chemical or biological amplification scheme must be employed in the direct detection methods. Indeed, an ultrasensitive nanostructured electrochemical biosensor was developed recently by Kelly's group.[10] By utilizing electrostatic interaction to tag miRNA with $Ru(NH_3)_6^{3+}$ and the electrocatalytic effect between $Ru(NH_3)_6^{2+/3+}$ and $Fe(CN)_6^{4-/3-}$, a detection limit as low as 10 aM was achieved. Although the dynamic range is rather limited, the method may be well suited to detect low copy numbers of miRNAs in a minimal volume. Previously, we showed that detection of miRNAs at sub-picomolar levels could be achieved using an interdigitated electrode[11] and electrocatalysis.[12] However, the sensitivity of those biosensors is not high enough to permit the detection of miRNAs at biological and clinical relevance. For example, to detect circuiting miRNAs in blood without PCR, a femtomolar (~100 copies/μl) sensitivity is needed.[13] In this communication, we present an electrochemical biosensor together with a novel amplification protocol that meets the sensitivity requirements for direct miRNA expression profiling, enabling electrical detections with minimal background and with significantly enhanced specificity and sensitivity. MicroRNA expression profiling is therefore performed in as little as 1.0 ng of total RNA, providing a much-needed platform for miRNA expression analysis.

Fig. 1 shows the working principle of the biosensor. A mixed monolayer of peptide nucleic acid (PNA) CPs and 4-mercaptoaniline (MAn) is self-assembled on a gold electrode, acting as a bioaffinitive interface (a). The interaction of CP with a RuO_2 nanoparticle-tagged target miRNA (b) forms a heteroduplex, bringing the RuO_2 nanoparticle (2–3 nm, Fig. S1 and S2, ESI†) and a high density of negative charges on the biosensor (c), and a mixture of aniline/H_2O_2 in pH 4.0 0.10 M acetate buffer is applied to the biosensor (d). The RuO_2 nanoparticles serve as a catalyst for the polymerization of aniline (Fig. S3, ESI†) and the hybridized miRNA strands act as templates for the deposition of polyaniline (PAn). As a result, deposition of PAn occurs

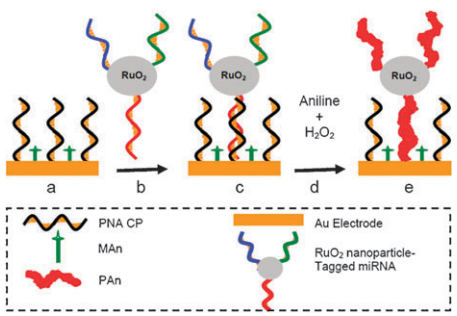

Fig. 1 Illustration of the biosensor based on RuO_2-initiated polymerization of aniline and miRNA-templated deposition of polyaniline.

exclusively at the hybridized miRNA strands (e). If the deposition of PAn is allowed to proceed for a sufficiently long period of time, high sensitivity is expected. The neutral backbone of PNA alleviates the electrostatic interaction between surface immobilized PNA CPs and cationic aniline molecules in the acetate buffer, producing a clean background and a high signal/noise ratio. Additionally, as compared to DNA, PNA CPs often offer a much higher specificity.[14,15]

In first feasibility tests, a mixture of synthetic miRNAs with sequences identical to those of the human let-7 family (Table S1, ESI†) and a biosensor with PNA CPs fully complementary to only one member of the let-7 family, let-7c, were used. Fig. 2a solid line depicts a typical cyclic voltammogram of the hybridized biosensor after a period of 60 min incubation in the aniline/H_2O_2 mixture. Distinct voltammetric activities were observed. Extensive washing and potential cycling thereafter produced no noticeable changes, revealing that the redox-active material PAn[16] is robustly bound to the biosensor. By contrast, in a control biosensor, non-complementary PNA CPs failed to capture any miRNAs from the mixture and thereby no RuO_2 nanoparticles were present on the biosensor surface. Negligible redox activities in the potential window of −0.3 to 0.5 V were observed, which are practically the same as those before the incubation in the aniline/H_2O_2 mixture (Fig. 2a dashed line). In addition, no detectable faradaic currents were obtained when the let-7c biosensor was tested in let-7c pre-miRNA solutions at concentrations as high as nanomolar. As the hybridization process was done at 10 °C below melting without any pre-denature treatment, the miRNA segment remains 'locked-up' in the hairpin of let-7c pre-miRNA due to its high stability. These results clearly demonstrated that the biosensor selectively interacts with its target miRNA and the resulting miRNA–RuO_2 nanoparticle adduct catalyzes the polymerization of aniline and guides the deposition of PAn at the biosensor surface. When the hybridized biosensor is incubated in a mixture of aniline/H_2O_2 at pH 4.0, protonated aniline molecules (pK_a = 4.6)[17] are 'assembled' around the miRNA strands *via* electrostatic interaction between the protonated aniline molecules and phosphates in the miRNA, producing a high aniline concentration around the miRNA strands and providing a favorable environment of high acidity that allows polymerization of aniline in a much less acidic medium than in conventional electrochemical and chemical approaches, and facilitates a predominantly head-to-tail coupling along the miRNA strands so deterring parasitic branching during polymerization.[18] Moreover, no obvious dehybridization was noticed during the incubation in the pH 4.0 acetate buffer. As shown in Fig. 2a, the RuO_2 nanoparticle-initiated PAn displayed only one pair of redox peaks at ~0.1 V. Similar results were obtained for polyelectrolyte-templated PAn films.[18,19] These redox peaks have been assigned as the first redox process of PAn, leucoemeraldine/emeraldine. The absence of the second redox process is believed to be due to the inability of the highly stable PAn/miRNA adduct to oxidize further to pernigraniline. Because of the inferior sensitivity of cyclic voltammetry relative to other voltammetric techniques, such as differential pulse and square wave voltammetry (SWV),[20] the more sensitive SWV was used in quantification experiments to maximize the performance of the biosensor (Fig. 2b).

To achieve a highly sensitive miRNA biosensor, we leveraged on the cumulative nature of the process for sensitivity improvement. A series of SWV tests were conducted after different incubation times and the responses are shown in Fig. 3a. In all cases, an immediate increase in the SWV peak current was observed due to the deposition of PAn. Interestingly, the sensitivity of the biosensor was proportional to the incubation time throughout, indicating that there is little activity loss of the RuO_2 nanoparticles during the incubation. However, there is a balance between high sensitivity and assay time. It was found that 60-min incubation is sufficient to detect miRNA at femtomolar levels. Furthermore, background noises became noticeable after prolonged incubations, probably due to non-specific deposition of PAn. For example, the background current increased significantly from 80 nA after the 60 min incubation to 900 nA after a 120-min incubation. This could cause great difficulties for the detection of miRNA at low concentrations.

The specificity of the biosensor was evaluated by analyzing closely-related members in the let-7 family with the biosensor

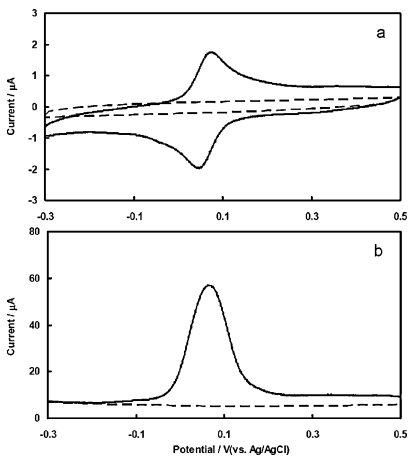

Fig. 2 (a) Cyclic voltammograms of a biosensor (solid line) and a control (dashed line) after hybridization with 5.0 pM RuO_2 nanoparticle-tagged let-7c and 60 min of incubation in 50 mM aniline/100 mM H_2O_2 in pH 4.0, 0.10 M acetate buffer; supporting electrolyte pH 4.0, 0.10 M acetate buffer, potential scan rate 100 mV s^{-1}. (b) Corresponding SWVs of the biosensor (solid line) and the control (dashed line); amplitude 50 mV, step 1.0 mV, frequency 200 Hz.

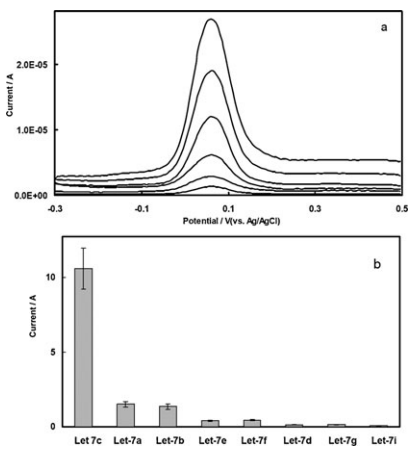

Fig. 3 (a) The dependence of the SWV peak current of 500 fM let-7c on incubation time in 50 mM aniline/100 mM H_2O_2 in pH 4.0, 0.10 M acetate buffer; from bottom to top, 10, 15, 30, 60, 90 and 120 min. (b) Responses of a let-7c biosensor to let-7 family miRNAs at 500 fM; Conditions are as for Fig. 2b.

coated with PNA CPs only complementary to let-7c. There is only one nucleotide difference (G ↔ A) out of 22 nucleotides between let-7c and let-7a and b, meaning that the CP for let-7c is one base-mismatched for let-7a and b. As shown in Fig. 3b, the peak current dropped by ~89% (selectivity factor of 8) to as low as 1.4 μA when let-7a and b were tested on the biosensor, readily allowing discrimination between the perfectly matched and one-base mismatched miRNAs. Furthermore, much higher selectivity factors of 26, 84 and >200 against let-7e and f (two base-mismatched miRNAs), let-7d and g (three base-mismatched miRNAs), and let-7i (four base-mismatched miRNAs) were observed, respectively (Fig. 3b). The selectivity between perfectly complementary and mismatched miRNA of this biosensor is better than those for PNA-based optical and electrochemical DNA sensors previously reported.[11,14,15] This is probably due to the beneficial effect of nanoparticle tag on the hybridization selectivity.[21,22] In addition, a selectivity factor of >500 was observed when the let-7c biosensor was tested in solutions of let-7c pre-miRNA. The mismatch and pre-miRNA tests agreed well with the voltammetric results obtained earlier and confirmed that the target miRNA was successfully detected with high specificity and sensitivity. Each quantified result represents the specific quantity of a single nature miRNA and not the combined quantity of the entire family or the pre- and mature miRNA. To evaluate the analytical performance of the biosensor, three mature miRNAs, namely miR-720 (17 nt), let-7c (22 nt), miR-1248 (27 nt), covering from the shortest (17 nt) to the longest (27 nt) miRNAs, were used as calibration standards. The 60-min incubation generated a dynamic range of 5.0 fM–2.0 pM with a relative standard derivation of <15% at 0.1 pM (Fig. S4, ESI†). The detection limit, defined as a signal/noise ratio of 3.0, was found to be ~2.0 fM. Compared to previous electrochemical biosensors for miRNAs, the sensitivity was greatly improved by 1–2 orders of magnitude through the combination of direct RuO_2 nanoparticle tagging and amplification-by-polymerization.[11,12] This dual-dependence (template and catalyst) of the signal amplification process substantially lowered non-hybridization-related background noise since neither the non-specifically adsorbed miRNA strands nor RuO_2 nanoparticles are able to initiate the deposition of PAn.

Recent studies have suggested that let-7 miRNAs target oncogenes, such as the RAS family and HMGA2, function as tumor suppressors.[23] Expression levels of three let-7 miRNAs were established by the proposed biosensor and by Northern blot (Table S2, ESI†). With the much improved sensitivity, the biosensor allowed us to directly analyze miRNA expression in real-world samples. It was found that the expressions of let-7b in total RNAs extracted from HeLa cells and lung cancer cells are $2.45 \pm 0.29 \times 10^7$ and $2.73 \pm 0.32 \times 10^7$ copies/μg, respectively, significantly lower as compared to normal cells (Table S1, ESI†). The results were in good agreement with those obtained by Northern blot on the same sample and consistent with recently published data of miRNA expression profiling.[24,25] The lowest amount of total RNA needed for miRNA detection was found to be ~1.0 ng, corresponding to <100 cells.[26] The relative errors associated with miRNA assays on individual miRNAs were generally less than 15% in the concentration range of 10 fM to 2.0 pM, which provides satisfactory accuracy to distinguish slight miRNA expression differences, and cuts down on the required amount of total RNA from micrograms to nanograms.

In summary, we have proposed an electrochemical biosensor for direct detection of miRNAs that utilizes hybridized target miRNA strands as templates to guide the deposition of PAn initiated by RuO_2 nanoparticles brought on the biosensor surface during hybridization. The engagement of amplification-by-polymerization is an integral factor which greatly enhances the sensitivity of the biosensor. While the concept of the dual-dependence amplification strategy has only been illustrated in the electrochemical biosensor, it could be easily extended to a range of other detection techniques such as quartz-crystal microbalance and surface plasmon resonance for sensitivity improvement, enabling the development of a simple, low-cost and portable detection system providing fast, cheap and simple solutions for molecular diagnosis, particularly for point-of-care and field uses.

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