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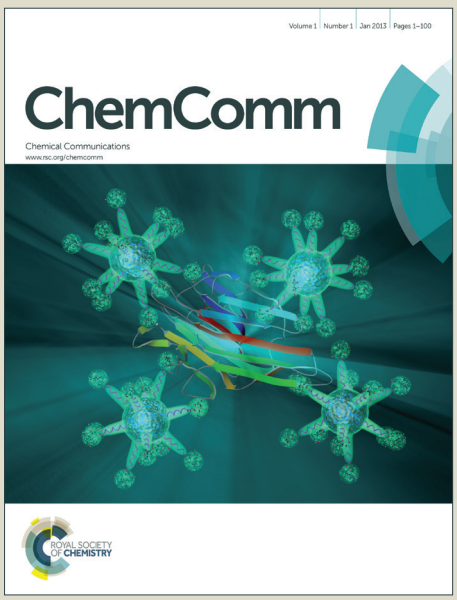

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ARTICLE TYPE

Sensitive Detection of MicroRNA by Chronocoulometry and Rolling Circle Amplification on Gold Electrode

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We developed a quantitative detection scheme for nucleic acids, combining solid-phase rolling circle amplification and chronocoulometry (RCA-CC). Gold electrodes were directly formed on a polystyrene substrate as a cost-effective and flexible biosensor for sensitive detection of microRNA (mir-143) in blood sample.

MicroRNAs, as a group of small non-coding RNA with 18~25 nt in length, widely distribute in diverse organisms and post-transcriptionally regulate protein production through mRNA cleavage or translation inhibition.[1] Over the past decade, much research indicated that microRNAs participate in most of the cellular processes and that changes in their expression are associated with many human diseases.[2] In order to study the regulation and function of this group of small non coding RNAs, many analytical approaches have been reported for sensitive and specific detection of them.[3-6] Although northern blotting[7] and RT-PCR[8] are still regarded as golden standard techniques, many efforts have been made to provide simpler and more cost-effective methods for microRNA quantification. Electrochemical measurement is proven a promising method to serve as devices suitable for point-of-care diagnostics because of its miniaturized instrumentation, simplicity and safety.[9] However, traditional electrochemical detection could not reach the sensitivity requirement for microRNA quantification. Imperative innovations were made to improve the sensitivity of electrochemical methods including introduction of nanoparticle[10], structure[11,12] and signal or template amplification before detection[13-15]. Sensitivity of fM to aM of target microRNA could be achieved by these innovations.

Differential pulse voltammetry (DPV) is found mostly used in electrochemical detection method for microRNA quantification because of its high sensitivity.[10,11,16,17] Moreover square wave voltammetry (SWV)[18], traditional cyclic voltammetry (CV)[12,19] and amperometric[14] detection were also employed as sensitive detection techniques. Chronocoulometry is a convenient electrochemical method for determination of adsorbed reactants on electrode surface.[20] It was successfully employed to detect single stranded or duplex DNA immobilized on gold electrode by taking advantage of the electrostatic attraction of specific redox cations with the nucleotide phosphate backbone.[21] In this report, we are presenting a novel method for microRNA quantification by combing chronocoulometry (CC) with rolling circle amplification (RCA). The principle of this RCA-CC assay was shown in fig.1. An integrated three-electrode biosensor was fabricated on polystyrene substrate by UV directed chemical plating technique.[22] The outline of the fabrication procedure was illustrated in fig.1a and described in more detail in supplementary information. Working electrode (WE) was first modified with thiolated DNA probe 1 and covered with 1mM of 6-Mercapto-1-hexanol (MCH) and 1mg/mL of BSA respectively to seal the excess active groups. Then target microRNA (mir-143) as well as probe 2 and cyclized padlock probe prepared beforehand was added onto electrode surface and hybridized with probe 1. In the presence of phi29 DNA polymerase, RCA amplification occurred and replicons was measured by chronocoulometry in a low ionic strength electrolyte containing Ruhex as a redox marker.

Chronocoulometry is a promising electrochemical method for measurement of nucleic acids (i.e. DNA) molecules adsorbed on the surface of electrode. Unlike other electrochemical methods, an attractive aspect of chronocoulometry is that the double layer charge and charge due to reaction of species adsorbed on the electrode surface can be differentiated from the charge due to reaction of redox molecules that diffuse to the electrode surface. The total current, or charge Q, as a function of time t could be expressed by the integrated Cottrell equation, [20,21]

$$Q = 2FnAC^{b}\left(\frac{Dt}{\pi}\right)^{1/2} + Q_{c} + Q_{ads} \qquad (1)$$

where F is Faraday (96487 coulombs per equivalent), n the number of electrons involved in electrode reaction, A the electrode area (cm^2), C^b bulk concentration (mol/cm^3), D diffusion coefficient (cm^2/s), t time (s), Q_c capacitive charge (coulomb), Q_{ads} charge produced by the adsorbed reactant.

From Faraday's Law, Q_{ads} in equation (1) could be expressed as,

$$Q_{ads} = nFA\Gamma \qquad (2)$$

where Γ is quantity of adsorbed reactant (mol/cm^2). The saturated suface excess of redox marker (Ruhex) is converted to DNA probe surface density with the relationship,

$$\Gamma_{DNA} = \Gamma(z/m)(N_A) \qquad (3)$$

where DNA is the probe surface density in molecules/cm^2, m is the number of bases in the probe DNA, z is the charge of the redox molecule (for Ruhex, z is 3), and N_A is Avogadro's number. By using ruthenium(III) hexaammine (RuHex), a cationic redox marker which could be electrostatically trapped onto the phosphate groups of DNA probe, chronocoulometry is able to

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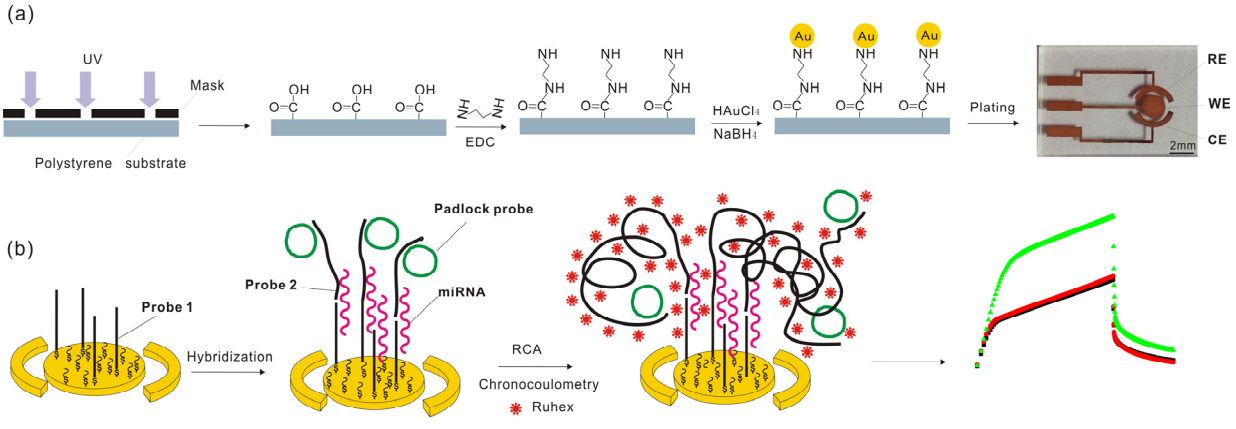

Fig.1 Schematic illustration of the method. Integrated gold electrodes were fabricated on polystyrene substrate by chemical plating (a); Principle based on rolling circle amplification and Chronocoulometry (RCA-CC) for microRNA detection.

give direct signal proportional to number of phosphate groups of nucleic acids at the electrode surface. Based on equation (3), similar strategy could be used for quantification of single stranded and duplex DNA as well as RCA products (Fig.S1, ESI†). We investigated the behaviour of DNA as well as solid-phase RCA products on the gold electrode by chronocoulometry and cyclic voltammetric (Fig.S2, ESI†). Moreover this strategy provides a potential approach for absolute determination of probe density on electrode surface under a reasonable assumption that when Ruhex is the only cation present in appreciable concentration (such as 50 μM), complete saturation of electrostatic adsorption between phosphate groups and Ruhex is achieved. Based on this strategy, we quantitatively investigated the immobilization, hybridization and RCA amplification on gold electrode surface (figure2). When DNA probe 1 modified working electrode hybridized with 10 nM of target microRNA and DNA probe 2, we could get a little increase of chronocoulometry signal on hybridization. While after four-hour RCA amplification, an obvious increase was observed due to adsorption of Ruhex to RCA products on electrode surface.

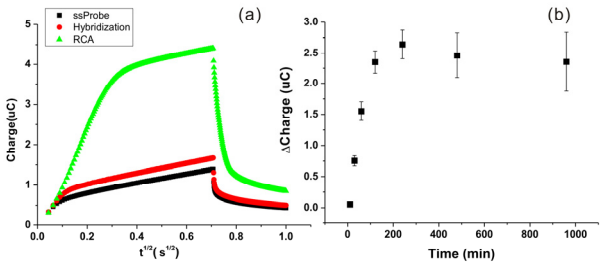

Fig.2 Chronocoulometry plot of immobilization of single stranded DNA probe on gold electrodes, hybridization with 10 nM of target molecules and four-hour RCA amplification (a); Investigation of reaction time of RCA amplification including 10, 30, 60, 120, 240, 480 and 960 minutes respectively (b). ΔCharge is the difference of charge between RCA amplification and hybridization.

Based on equation (2) and (3), we could calculate surface density of DNA probe 1 immobilized on working electrode ranging from 1×10^{12} to 6×10^{12} molecules per square centimetre (Fig.S3, ESI†). About 10% of probe1 (~5.17×10^{10} molecules) was observed to be hybridized with target microRNAs in the presence of 10 nM of mir-143 and probe 2. We investigated amplification time from 10 minutes to 16 hours (figure2b). RCA reaction was observed in a rapid rate before 2 hours, while after four-hour amplification the products detected by chronocoulometry did not increase any more. On the contrary, the signal was getting lower slightly because of non-specific amplification of the control. Moreover, we further calculated how many nucleotides have been produced after RCA amplification and estimated the reaction rate approximately. In our experiment, we found that electrochemical signal was proportional to amplification time within one hour and the average reaction rate obtained was about nine nucleotides per minute which is much lower than 53 nucleotides per second reported by Lizardi et al[23], indicating that reaction efficiency of RCA amplification on solid surface is much lower than that in solution.

Mir-143 is proven a typical microRNA which targets KRAS oncogene and acts as a tumour suppressor for variety of cancers.[24] We chose mir-143 as target microRNA to demonstrate the principle of our method. Sensitivity of this RCA-CC assay was examined by putting in stepwise diluted target mir-143 ranging from 10 fM to 10nM on the surface of gold electrodes. Although microRNAs could be detected with only hybridization at high concentration (100 nM), the sensitivity of hybridization detection could be improved to ~1 nM by adding DNA probe2 (43 nt) in addition to microRNA. After RCA amplification, directly measured detection limit was 100 fM, and the signal gain is linear over the range from 100 fM to 1 nM (fig.3a and fig.S4, ESI†). Sensitivity of RCA-CC assay was consisted with another chronocoulometry assay which employed gold nanoparticle for signal amplification.[25] However, it seems much lower than fluorescent detection method.[5] In the future study, we could further improve the sensitivity by employing multiple amplication[26] or branched RCA[6]. Then, we investigated the specificity of this assay by exposing the immobilized electrode to three microRNAs (mir-1, mir-122 and mir-145) as well as mir-143 at same concerntration. The method exhibited good performance on selectivity (Fig.S6, ESI†).

Subsequently, we investigated the practicality of this method to determine mir-143 in real world sample. It was reported that mir-143 is a biomarker of human prostate cancer and could be detected in blood as other circulating microRNAs.[27] Certain

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amount of synthesized mir-143 was spiked into healthy human blood sample, then total RNAs including spiked mir-143 were isolated and detected with RCA-CC assay. Without consideration of purification efficiency, as low as 1 pM of mir-143 in blood sample was detectable (fig.3b) and a dynamic ranged from 1 pM to 1 nM was obtained.

In conclusion, we developed a novel microchip containing integrated gold electrodes fabricated on polystyrene substrate which could be a promising biosensor. Unlike traditional gold electrode fabricated on glass substrate which requires ultraclean room and expensive instruments, this polymer electrodes are low-cost and could be easily fabricated in a ordinary chemistry laboratory. Moreover, we developed a method for sensitive nucleic acids detection by combining solid-phase rolling circle amplification with chronocoulometry. Compared with other electrochemical methods, an attractive aspect of chronocoulometry is that it could perform absolute quantification of bio molecules on the surface of electrodes, therefore having great potential for molecular diagnostics and other application, for example study on enzymatic activity at molecular level.

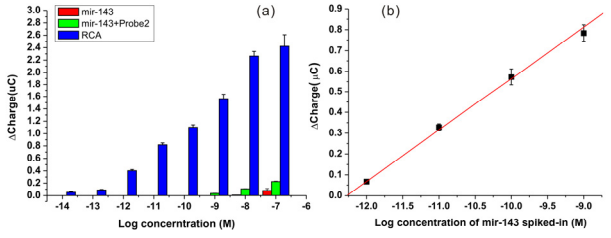

Fig.3 Sensitivity of RCA-CC assay for detection of mir-143. Stepwise diluted target microRNAs were put onto the DNA modified gold electrodes. The concentration of mir-143 ranged from 10 fM to 100 nM. The concentration of DNA probe2 added for initiation of RCA amplification was 10 nM and RCA reaction was performed at 30 °C for two hours(a); (b) Detection of mir-143 spiked in human blood sample. The concentration of total RNA put onto the gold electrodes was ~500 pg/μl. ΔCharge is the difference of charge before and after RCA amplification.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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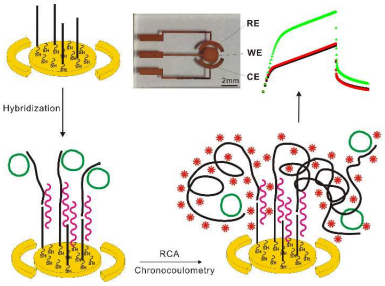

Integrated gold-electrode fabricated on polystyrene substrate was developed for quantitative detection of nucleic acids by Rolling circle amplification and chronocoulometry

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