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Using Electropolymerization-based Doping for the Electro-addressable Functionalization of a Multi-electrode Array Probe for Nucleic Acid Detection

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Even though large number of individually addressable electrodes can be effectively assembled in a small area, electrochemical detection methods have a relatively limited ability to detect multiple analytes compared to microdialysis probes and other analytic techniques. Here, we report a facile method for the electro-addressable functionalization of a probe comprising of closely spaced three individually addressable carbon fiber electrodes (CFEs) for the detection of nucleic acids. First, a multi electrode array probe comprising three adjacent CFEs was fabricated through pulling a three-barrel glass capillary with a single carbon fiber in each barrel. Second, electropolymerization based doping was used for the electro-addressable functionalization of the multi-electrode array probe. To demonstrate that the current strategy works, anti-miR-34a was electrografted on only one of three electrodes by the electropolymerization of pyrrole on a specific electrode. A second electrode was coated only with polypyrrole (PPy) and the third was left unmodified. The results demonstrate that the present strategy has great potential for constructing multiplex nucleic acid micro/nano biosensors for local and *in situ* detection of multiple nucleic acid molecules, such as miRNAs at a time.

Keywords Carbon fiber electrode, biosensor, miR34a, miRNA, electrode array, electro-addressable functionalization

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Introduction

The use of microelectrodes in the construction of microbiosensors has many advantages, such as the ability to achieve high temporal and spatial resolution, local detection and detection in small volumes. Increasing the number of electrodes enables the detection of multiple analytes or simultaneous and high-throughput detection of same molecules at multiple points. Even though a large number of individually addressable electrodes can be effectively assembled in a small area,[1–4] electrochemical detection methods have a relatively limited ability to detect multiple analytes, particularly when compared to microdialysis probes and other analytic techniques based on chromatography or electrophoretic separation.[5] There are several reports where multi-electrode arrays were assembled on a substrate to form a biosensor chip to perform multiple analysis. For example, Zhu *et al.* used a multi-point addressable electrochemical device where row and column electrodes were orthogonally arranged to form a 4×4 array for amperometric detection of DNA hybridization.[6] Because of the large distance between electrodes, they were able to modify electrodes with different probes using a pipette. The device had a detection limit of 30 nM. In another study, Erdem *et al.* used a multi-channel screen printed array electrode for the label-free voltammetric detection of microRNAs.[7] Electrode array comprising 16 relatively large carbon electrodes enabled 16 consecutive analyses with a detection limit of 4.3 pmol in 3 μL sample. The treatment of the electrodes with different samples was also done by pipetting due to large distances between the electrodes. A microelectrode array comprising closely spaced electrodes with different labels enables the simultaneous and local detection of different analytes in small volumes, but labeling closely spaced electrodes with different bio-recognition molecules is technically challenging, since pipetting is not an option. In a recent study, Ozsoz *et al.* used polypyrrole (PPy) for the doping of probe miRNA on a large pencil graphite electrode to detect miR-21.[8] PPy is one of the most widely used conducting polymers for biosensor design due to its biocompatibility, high electrical conductivity, wide pH range, fast electrochemical reaction, high surface energy and low cost. Basically, in the study, the miRNA probe was mixed with the pyrrole solution and by electropolymerization of pyrrole, the miRNA probes were electrografted on the electrode surface. The biosensor showed good selectivity and had a detection limit of 0.17 nM. Since pyrrole is electropolymerized only on the electrode through which the electropolymerization potential is applied, we used it for the electro-addressable functionalization of a closely spaced multi carbon fiber electrode (CFE) array micro-probe with a pitch of less than 5 μm for the detection of miR-34a. CFEs are commonly used for local and highly sensitive detection of neurotransmitters such as dopamine or *recording of neuronal* action potentials known as spikes, enabling electrochemical monitoring of the neurochemical activity of the brain.[9–12] In addition to being used in such measurements, CFEs have also been used for the detection of

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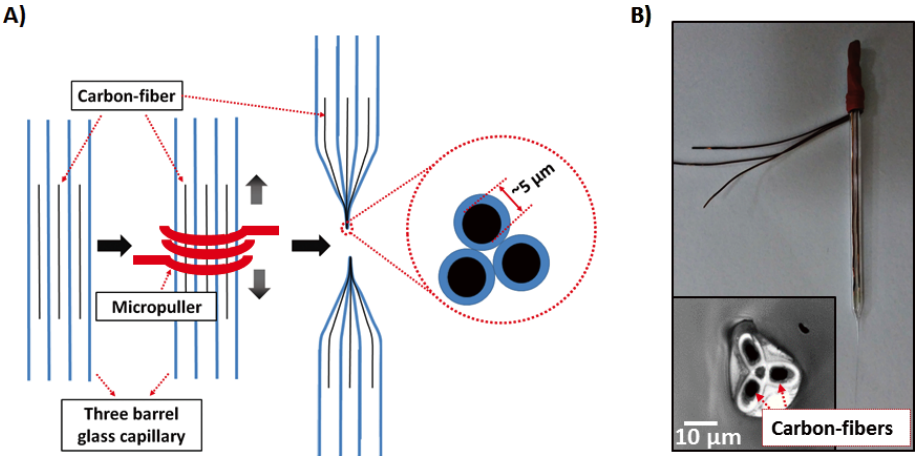

Fig. 1 (A) Schematic illustration showing the fabrication method for a multi electrode array probe. First, three carbon fibers were individually inserted into a three-barrel glass capillary and then the capillary was pulled with a micropuller. (B) Optical image of the probe along with an image of the tip showing the three electrodes.

biologically important molecules with great sensitivity and selectivity by simply modifying the surface of the electrode with a bio-recognition molecule.[13–16] The probe was used in a self-referencing mode[17] for the detection of miR-34a, where one of three electrodes was modified with anti-miRNA34a using PPy. A second electrode was coated with PPy and the third was left unmodified. The results clearly demonstrate that electro-polymerization-based doping works for electro-addressable functionalization of a micro-electrode array probe, and has a great potential for constructing micro-biosensors for the detection of multiple miRNAs at the same time.

Experimental

Probe fabrication

For fabricating a multi-electrode array probe, three copper wires, each connected to a single carbon fiber, were prepared. Each wire was prepared as follows: a single carbon fiber with a diameter of 5 – 6 µm was fixed to an insulated copper wire (Fig. 2).[18] To make the connection, one end of the copper wire was dipped into Ag paste, which was then used for attaching a single carbon fiber. The Ag paste was cured at 180°C for 30 min to make a rigid connection between the carbon fiber and the copper wire. Three copper wires, each fixed with a carbon fiber, were individually inserted into a three-barrel capillary. To fabricate a multi-electrode array probe with a thin and parallel run to the very end of the tip, the three-barrel capillary was pulled (PG10165-4, World Precision Instrument, USA) with a micropuller (PC-10, Narishige, Japan) using the one-stage pull option to seal the carbon fibers in glass (Fig. 1A).[19,20] The heater level that yielded a multi-electrode array probe with the desired characteristics was 68 (heater level displays the current voltage rate calculated from the maximum voltage, 2.5 V, as of 100 percent). In the following step, the excessive unsealed carbon fibers at the tip of the probe were removed with scissors. To fill any possible gap between the carbon-fibers and the sealed glass, the tip of the probe was dipped into resin, which was then cured at 80°C for 40 min. To fabricate micro-disk electrodes, the tip of the probe was ground using a microgrinder (EG-401, Narishige, Japan) (Fig. 1B).

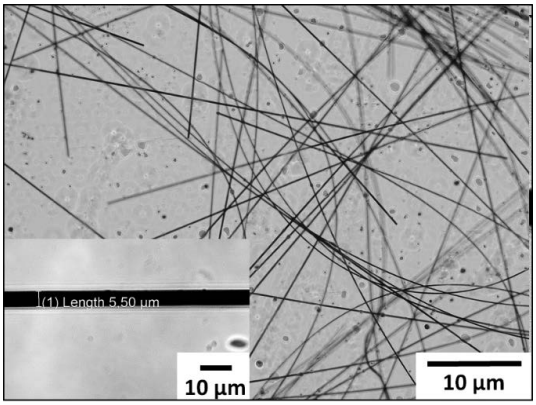

Fig. 2 Image showing both a bundle of carbon-fibers and the diameter of a single carbon fiber.

Electrochemical characterization of probes

The electrochemical behavior of the CFEs was analyzed to assess the usability of the probe. First, cyclic voltammograms (CVs) of each electrode were obtained by sweeping the potential from 0 to +0.6 V *vs.* Ag/AgCl at a scan rate of 50 mV/s in 1 mM ferrocenemethanol ($FcCH_2OH$ + 50 mM PBS). Second, a simultaneous CV of all electrodes was obtained under the same conditions[2,21,22] using COMSOL Multiphysics. Basically, the electrochemical bevavior of the multi-electrode array probe was simulated for validating of the experimental results. First, a model was designed by taking the dimensions of the electrode surface shown in Fig. 1B and the diameter of carbon-fibers in Fig. 2. The final design had a height of 50 µm, a carbon-fiber diameter of 5.5 µm and a glass thickness of 19 µm (Fig. 3A). COMSOL Multiphysics 4.4 was used to obtain CV of multi-electrode array probes for two different cases; in the first case the potential was swept from 0 to +0.6 V through a single electrode, and in the second cases the potential was swept through all electrodes simultaneously. The following parameters were used in the simulation: the number of electrons, $n_{FeCH2OH} = 1$; F: the Faraday constant, $F = 96485.329$ s A/mol; the diffusion coefficient, $D_{FeCH2OH} = 6.7 \times 10^{-6}$ cm^2 s^{-1}; and the analyte concentration, $C_{FeCH2OH} = 1$ mM.[2,24]

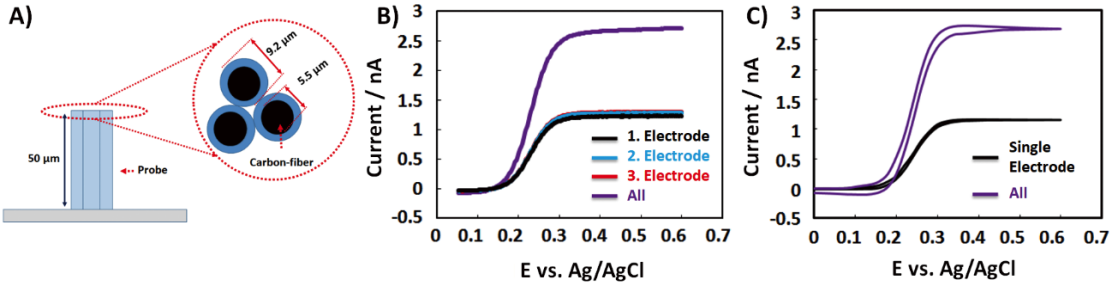

Fig. 3 Model used for simulating the electrochemical behavior of the multi-electrode probe (A). Experimental (B) and simulated (C) CVs of individual carbon-fiber electrodes in the same multi electrode array probe. All electrodes numbered from 1 to 3 demonstrated an identical electrochemical behavior. CV when all connected is also shown as "All" in both graphs.

Electrode surface modification

Anti-miR-34a (5 ppm) was mixed with 0.1 M PPy in 0.7 M KCl at pH 4.80, the probe was immersed into the mixture along with an Ag/AgCl reference electrode. PPy with the anti-miR-34a was electrochemically deposited by sweeping the potential from 0 to +0.8 V *vs.* Ag/AgCl four times at a scan rate of 50 mV/s. Next, a second electrode was modified with PPy in the same way, but without anti-miR-34a. The last electrode was left unmodified. After an electrode surface modification, the probe tip was rinsed in 50 mM PBS at pH 7.4 for 10 s. EIS was then employed for an impedimetric analysis of the electrodes for confirming the modification and comparison after hybridization.

Electrochemical detection via EIS

Basically, EIS was used to monitor changes in the charge transfer resistance (R_{ct}) of a Randles circuit consisting of solution resistance (R_s), double layer capacitance (C_{dl}) and diffusion controlled—Warburg impedance (W).[23] The experiments were performed in a 50 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl solution using an AUTOLAB PGSTAT204 compact and modular potentiostat/galvanostat. The impedance spectra were recorded in a frequency range of 10^1 to 10^5 Hz, which was divided into 98 logarithmically equidistant points. A DC potential of +0.23 V *vs.* Ag/AgCl along with a sinusoidal AC signal of 10 mV was applied throughout all EIS measurements, which were performed in a faraday cage. The data were fitted to an equivalent circuit, and the diameter of first semicircle of the Nyquist plot, corresponding to the R_{ct} value of the electrode surface, was calculated by the fit and simulation option in the AUTOLAB 302 Nova 1.10.3 software.

Results and Discussion

The fabrication of the multi-electrode array probe is illustrated in Figs. 1A and 1B. Basically, three copper wires, each connected to a single carbon fiber (Fig. 2), were prepared, and then individually inserted into a three-barrel capillary. To fabricate a multi-electrode array probe with a thin and parallel run to the very end of the tip, the three-barrel capillary was pulled (PG10165-4, World Precision Instrument, USA) with a micropuller (PC-10, Narishige, Japan) using the one-stage pull option to seal the carbon fibers in glass (Fig. 1A). To fabricate micro-disk electrodes, the tip of the probe was ground using a microgrinder (EG-401, Narishige, Japan) (Fig. 1B). Prior to electrochemical characterization of electrodes, electrochemical behavior of electrodes were simulated using COMSOL Multiphysics for a comparison (Fig. 3A). All three electrodes showed a similar behavior; the maximum current was measured to be ~1.24 nA (Fig. 3B), quite close to the simulated current value of ~1.16 nA (Fig. 3C). We believe that the difference between the experimental and simulated values is normal, considering the likely non-ideal surface conditions of the tips of the probe. When all three electrodes were connected, the maximum current measured was ~2.71 nA (Fig. 3B), approximately 2.2-times the current obtained from a single electrode, and quite close to the simulated value of ~2.74 nA (Fig. 3C). These results clearly indicate that all electrodes were working properly and individually addressable.

After confirming the integrity of the electrodes, the multi-electrode array probe was modified for miR-34a detection using electropolymerization based doping (Fig. 4A). It has been already demonstrated that by entrapping DNA, the co-deposition of the pyrrole monomer is more effective than physical adsorption.[8] The positively charged polymer is thought to contribute greatly to the entrapment of negatively charged DNA probes. miR-34a, a non-coding miRNA, is a direct transcriptional target of the onco-suppressor p53, which plays a critical role in genomic stability, apoptosis, and the inhibition of angiogenesis.[24,25] To show that each electrode could be used for a different purpose, only one of three electrodes was modified with anti-miR-34a using PPy. According to the results, the impedance response of the unmodified electrode exhibited a nearly straight line, reflecting a mass diffusion-limiting step of the electron-transfer process. The semicircle shape of the PPy modified electrode (R_{ct}: 7.68 MΩ or 7.29 Ω·cm^2) plot was lower than that of an anti-miR-34a + PPy modified electrode (R_{ct}: 22.5 MΩ or 21.37 Ω·cm^2) because anti-miR-34a increases the resistance. In other words, these results clearly indicate that anti-miR-34a was successfully deposited electrochemically to one of the electrodes.[8]

The modified probe was immersed into a 50 mM PBS (pH 7.4) solution containing 10 ppm of miR-34a at room temperature for 1 h to allow hybridization of miR-34a with anti-miR-34a on the electrode surface. The volume of the analyte solution was 70 µL. Once this step was complete, the probe was washed in 50 mM PBS at pH 7.4 for 10 s. Impedimetric analysis of the electrodes was performed in a 5 mM $K_3Fe[(CN)_6]$ + 0.1 M KCl solution to verify miR-34a detection. Figure 4B shows that the data of the unmodified (almost no semicircular shape) and PPy (R_{ct}: 6.63 MΩ or 6.29 Ω·cm^2) modified electrodes remained the same, whereas the semicircular shape of the anti-miR34a modified electrode increased substantially after hybridization (the R_{ct} value increased from 22.5 (21.37 Ω·cm^2) to 34.5 MΩ (32.76 Ω·cm^2)). In other words,

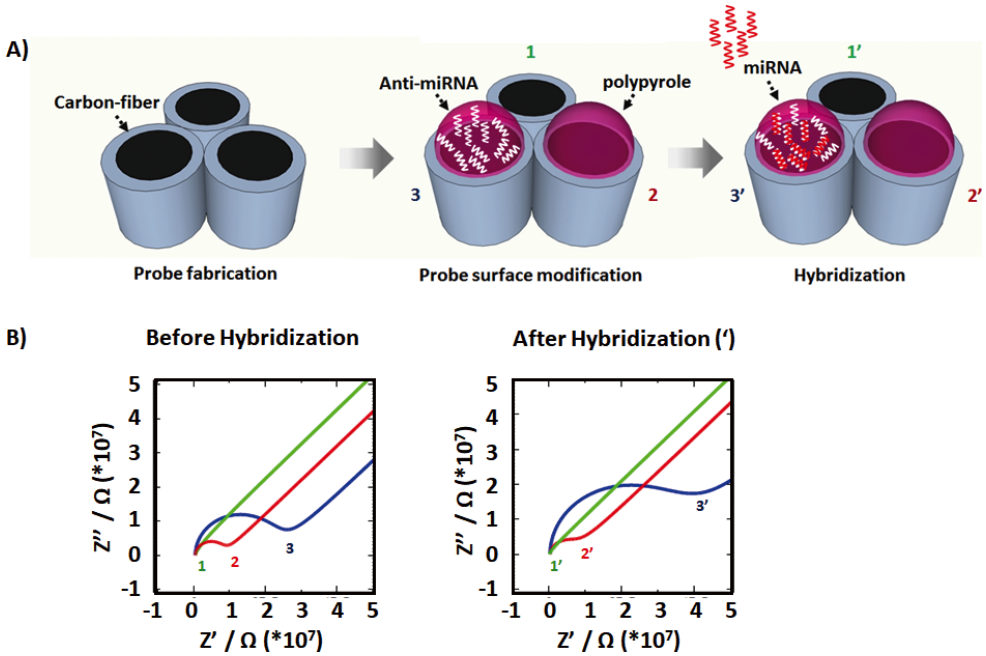

Fig. 4 (A) Modification of the multi electrode array probe for miR-34a detection. The probe was used in "self-referencing mode" for detection of miR-34a, in which case the first electrode (1) was left unmodified while second (2) and third (3) carbon-fiber electrodes were modified with PPy and anti-miRNA34a + PPy, respectively. (B) Electrochemical impedance spectroscopy (EIS) results before (1, 2 and 3) and after (1′, 2′ and 3′) hybridization. Numbers in the graphs represent the numbers assigned for the electrodes in the illustration.

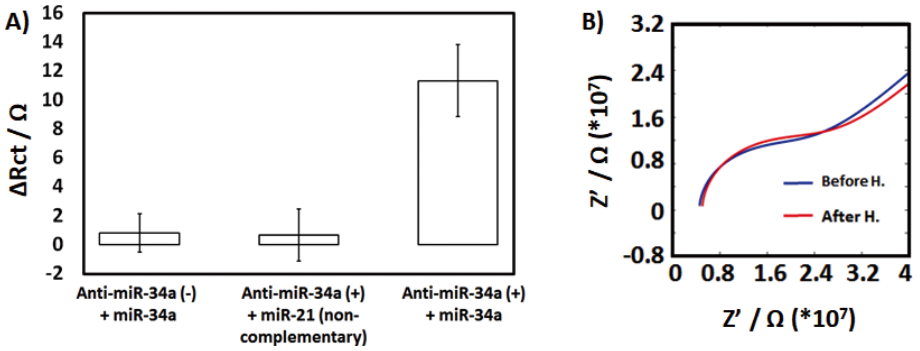

Fig. 5 (A) Average ΔR_{ct} values obtained for both anti-miR-34a (–) and (+) modified electrodes after hybridization with miR-34a and non-complementary miR-21. (B) Nyquist plot of a probe whose surface is modified with PPy + anti-miR-34a before and after hybridization with miR-21.

miR-34a was successfully detected using a modified electrode in a multi-electrode array probe. The main goal of the study was electro-addressable functionalization of the closely-packed electrodes using pyrrole rather than quantitation. The obtained results prove the applicability of the strategy. In order to show the bio-selectivity of the biosensor, a probe whose surface was modified with PPy + anti-miR-34a was dipped in a solution containing 10 ppm non-complementary miR-21 for 1 h at room temperature, no significant difference was found in the charge transfer resistance (ΔR_{ct}) (Figs. 5A and 5B), even though there were 7 matching bases. To confirm the repeatability, the experiment was repeated three times, and average ΔR_{ct} values were obtained for PPy and PPy + anti-miR-34a modified electrodes after hybridization (Fig. 5A). In addition, the probe surface was modified only with PPy to show that the signal is coming from the hybridization, and no significant difference was observed (Fig. 5A). Using the probe in this manner enables self-referencing, since all three electrodes share the same conditions. Self-referencing helps to remove interference of the analytical signal, and to make detection more accurate.

Conclusion

In conclusion, we used electropolymerization-based doping for the electro-addressable functionalization of a multi-electrode array probe with three electrodes for the detection of miR-34a, a non-coding miRNA that has a significant impact on apoptosis.

The fabrication method used in this study yielded a multi-electrode array probe comprising three electrodes, all electrodes demonstrated an identical electrochemical behavior. The probe was used in a self-referencing mode for the detection of miR-34a with one of the electrodes modified with PPy + anti-miR-34a. Instead of using separate electrodes for both the test and control groups, as used in a conventional analysis, a single probe with three electrodes, each assigned for a different measurement, was used in this mode. Because all three electrodes shared the same microenvironment, all measurements were completed with a single probe, which made the analysis comparatively easy and accurate. Due its size, the probe can be easily used in very small volumes, such as single-cell analysis as well as *in situ* detection. This proof-of-concept study clearly shows that electropolymerization based doping can be easily employed for the electro-addressable functionalization of electrodes to detect a large number of different miRNAs at the same time, since it is possible to fabricate micro or even nano multi-electrode array probes with more electrodes using capillaries with more barrels.[26,27] In brief, the miniaturization of a miRNA sensor is meaningful for constructing a multiplex miRNA sensor, *in vivo* monitoring of local miRNA and reducing the sample volume.

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