Development of a MEMS-based electrochemical aptasensor for norovirus detection

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Noroviruses (NoVs) are a major cause of acute gastroenteritis worldwide and spread through person-to-person transmission and contaminated food or water. A miniaturised microelectromechanical systems (MEMS)-based electrochemical aptasensor, a biosensor using aptamer as the recognition element for simple, sensitive and rapid detection of NoV has been developed. The novelty of this work is integration of MEMS technology with aptamer to develop a miniaturised and portable electrochemical sensor for environmental pathogen monitoring. An MEMS electrochemical aptasensor device was prepared by immobilising a thiolated DNA aptamer on an on-chip gold (Au) working electrode through the affinity between thiol and Au, and formation of a DNA aptamer monolayer was confirmed by means of cyclic voltammetry as well as visual observation of a fluorescent-labelled aptamer. The MEMS electrochemical aptasensor exhibited rapid and clear square wave voltammetry response against different titres of murine norovirus, an experimental model of human NoV. The possibility of developing a handheld aptasensor platform using a portable electrochemical measurement device is also demonstrated. This research forms initial basis for the development of an electrochemical MEMS-aptasensor platform and its application for virus detection.

1. Introduction: Noroviruses (NoVs), a genetically diverse group of the *Caliciviridae* family, have caused numerous outbreaks of viral gastroenteritis worldwide as the infection can easily spread through person-to-person transmission and contaminated food or water [1]. NoVs are very stable in the environment and highly contagious (i.e. require low infectious dose [2]), warranting development of a real-time, on-site, and rapid detection system. Today, NoVs are generally diagnosed by the reverse transcription-polymerase chain reaction (RT-PCR)-based methods. However, RT-PCR methods are rather complicated, involving complex, and time-consuming operations such as RNA extraction, RT reaction (cDNA synthesis), and PCR amplification conducted by trained personnel, rendering them unsuitable for on-site and rapid detection of NoVs.

Biosensors, analytical devices that rapidly convert biological response into electrical signals, have attracted much attention as a new tool for rapid identification/diagnosis of pathogens in clinical and environmental settings. A biosensor typically comprises two main components: (i) a recognition element that recognises/captures the target analyte and (ii) a transducer that converts the recognition event into a measurable electrical signal. Aptamers, synthetic nucleic acids that fold into unique three-dimensional (3D) conformations capable of binding to a specific target, have shown advantages over other molecules (such as antibodies) as recognition elements for biosensing as they have high affinity, specificity, thermostability, reusability (by reversible denaturation), ease of production and modification with lower cost etc. Microelectromechanical systems (MEMS) technology has been used widely for various applications including sensing devices [3]. In the present Letter, we developed an MEMS-aptasensor i.e., a biosensor using aptamers as recognition molecules, coupled with MEMS technology that enabled fabrication of a minituarised device with high detection sensitivity. Electrochemistry is used as a means of transduction because electrochemical measurement is more sensitive, less expensive, and simpler than other transduction methods such as optical measurement. The unique 3D conformational change of aptamers on target-analyte binding enables the aptasensor to detect NoV particles with high specificity and sensitivity [4]. We fabricated an MEMS electrochemical aptasensor and evaluated its performance with respect to sensor response to murine norovirus (MNV), an experimental model of human NoV.

2. Materials and methods

2.1. Virus and cells: The prototype MNV strain, MNV-1 (clone CW1, ATCC PTA-5935) was propagated on RAW 264.7 (ATCC TIB-71) cell line monolayers with Dulbecco's modified Eagle medium containing 10% foetal bovine serum [5]. The propagated MNV particles were purified with gel chromatography (illustra MicroSpin S-300 HR Columns, GE Healthcare Life Sciences, Chicago, IL, USA), and MNV titre was determined by plaque assay as previously described [6].

2.2. Fabrication of sensor base: The proposed MEMS electrochemical aptasensor device consists of different layers, in which silicon (Si, 500 μm) substrate serves as the sensor base. A layer of Si dioxide (SiO_2, 1 μm) deposited by plasma-enhanced chemical vapour deposition provided insulation for on-chip sensing electrodes. Before patterning with positive photoresist, the substrate was thoroughly washed by acetone solution and deionised (DI) water to remove the impurities. After that, 5 μm thickness of positive photoresist was spin-coated on the substrate and exposed under ultraviolet light (365 nm i-line). The substrate along with exposed photoresist was then immersed into sufficient amount of AZ400K photoresist developer. The developing was continued for 120 s with agitation. A layer of chromium (Cr, 50 nm) was sputtered on the patterned photoresist by using magnetron sputtering system to promote the adhesion between SiO_2 layer and upper electrode structure. Subsequently, a layer of

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Au (Au, 200 nm) was attached on the Cr layer to function as base electrode layer, serving as the working electrode. The contact pad of the working electrode was wired by using conductive epoxy and the entire sensor base was baked in the oven at 80 °C for 3 h. Finally, non-conductive epoxy was used to cover the sensor base except for the working electrode.

2.3. Immobilisation of DNA aptamer on Au working electrode: Aptamer immobilisation on the Au working electrode through the affinity between Au and thiol was carried out according to a previous study [4], with slight modification. Prior to immobilisation, the fabricated MEMS sensor base was thoroughly washed with DI water and then dried with nitrogen gas (N_2). A DNA aptamer was immobilised on the electrode by drop casting 500 nM high-performance liquid chromatography-purified MNV-specific aptamer (AG3, aptamer nucleotide sequence described in Giamberardino *et al.* [4]) modified at the 5′ position with a 6-hydroxyhexyl disulphide group (5′ Thiol Modifier C6 S-S; Integrated DNA Technologies, Coralville, IA, USA) in 20 mM Tris-ClO_4 buffer (pH 8.6) and incubating for 5 days at 4 °C. Finally, the electrode was incubated with 1 mM β-mercaptoethanol in ethanol for 5 min to back-fill the empty spots of the electrode surface, thus reducing the non-specific adsorption onto the Au working electrode surface.

2.4. Visualisation of aptamer immobilisation: The thiolated AG3 aptamer modified at the 3′ position with 6-carboxyfluorescein (FAM) was immobilised on the electrode following the procedure described above, and the electrode was rinsed with phosphate-buffered saline to remove excess (i.e. non-immobilised) aptamer. The aptamer-derived FAM fluorescence was observed under a laser scanning fluorescent confocal microscope (LSM 700, Carl Zeiss, Oberkochen, Germany).

2.5. Electrochemical measurements: Electrochemical measurements including cyclic voltammetry (CV) and square wave voltammetry (SWV) were performed with a bench-top electrochemical workstation (CH Instruments 760E, TX, USA) connected to a computer or a portable electrochemical system, the WheeStat (Smoky Mountain Scientific, Cullowhee, NC, USA). The working electrode on the MEMS sensor device and commercial counter (Pt) and reference (Ag/AgCl) electrodes were separately connected to the bench-top or portable electrochemical measurement device through crocodile clips. Both CV and SWV experiments were performed in the solution of 25 mM phosphate buffer (pH 7) containing 4 mM $K_3[Fe(CN)_6]$ and 10 µM $[Ru(NH_3)_6]Cl_3$ and all measurements were conducted at room temperature. The open-circuit potential of the system was measured prior to all electrochemical measurements to prevent sudden potential-related changes in the self-assembled monolayer. CV experiments were recorded with initial and final potential of 0.4 V, switching potential of −0.3 V, and scan rate of 60 mV/s. SWV experiments were recorded in the potential range of −0.3 to 0.4 V under the quiescent condition. Parameters of SWV experiment were chosen as step potential of 4 mV, amplitude of 5 mV, and frequency of 10 Hz. The ferricyanide/ruthenium hexamine reporter system is able to enhance the electrochemical signal [7], in which ruthenium hexamine ($[Ru(NH_3)_6]^{3+}$), a cationic electron acceptor, undergoes redox reaction in the vicinity of working electrode. The ferricyanide ($[Fe(CN)_6]^{3-}$), an anionic electron acceptor, is repelled to the solution under the effect of electrostatic force, which aids to regenerate $[Ru(NH_3)_6]^{3+}$ by oxidising the produced Ru^{II} species ($Ru(NH_3)_6)^{2+}$) in the precedent step, thereby amplifying the electrochemical readout [7]. The magnitude of current signal is correlated with the amount of accessible aptamers. All electrochemical measurements were repeated for a minimum of four times for each sensor device or experimental condition.

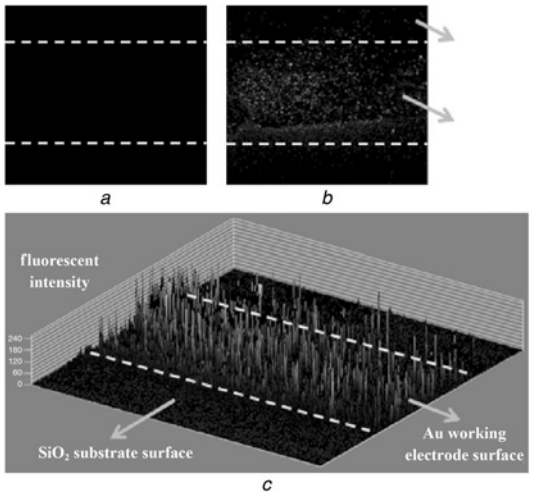

Fig. 1 *LSM images of fluorescent-labelled aptamer immobilised on Au working electrode*
a Top view of bare electrode (control)
b Top view of electrode with fluorescent-labelled aptamer
c '2.5D' view of electrode with fluorescent-labelled aptamer (*z*-axis represents intensity of fluorescence derived from the fluorescent-labelled aptamer immobilised on the surface of Au working electrode)

3. Results and discussion

3.1. Visual confirmation of aptamer immobilisation: An MEMS electrochemical aptasensor was prepared by immobilising the thiolated MNV-specific DNA aptamer on the Au working electrode through the affinity between thiol and Au. To confirm successful aptamer immobilisation, a fluorescent-labelled aptamer was used for immobilisation, which allowed visual confirmation of self-assembled aptamer monolayer formed on the electrode surface. As shown in Fig. 1, fluorescent signal derived from the labelled aptamer was observed only on the Au electrode surface, demonstrating that the immobilisation procedure employed in this Letter produced self-assembled aptamer monolayer as expected.

3.2. Electrochemical evaluation of aptamer immobilisation: The electrochemical characteristics of aptamer immobilisation on the working electrode were investigated by CV with both the bench-top and portable electrochemical measurement devices examining $[Fe(CN)_6]^{3-/2-}$ redox couple in the test solution. As shown in Fig. 2, the bare electrode (without aptamer) presents a quasi-reversible voltammogram with very large redox currents, whereas the redox current for the electrode after immobilising aptamers showed significant decrease of current magnitude. This result, from the electrochemical point of view, confirmed the successful formation of thiolated aptamer monolayer on the electrode surface, as decrease of oxidation and reduction currents was mainly contributed by the blocking of active area of the working electrode. In addition, the bench-top and portable electrochemical measurement devices showed similar CV profile (Figs. 2*a* and *c*, 2*b* and *d*).

Visualisation and electrochemical (i.e. CV) evaluation of aptamer immobilisation confirmed that MEMS electrochemical aptasensor was successfully fabricated via self-assembly of a thiolated aptamer on the Au working electrode surface following a procedure reported previously [4].

3.3. Electrochemical investigation of MNV binding: The response of MEMS electrochemical aptasensor on binding between immobilised aptamers and purified MNV particles was studied with SWV in the test solution. As shown in Fig. 3, a significant decrease in the magnitude of peak current was observed immediately after MNV was added to the test solution to obtain a

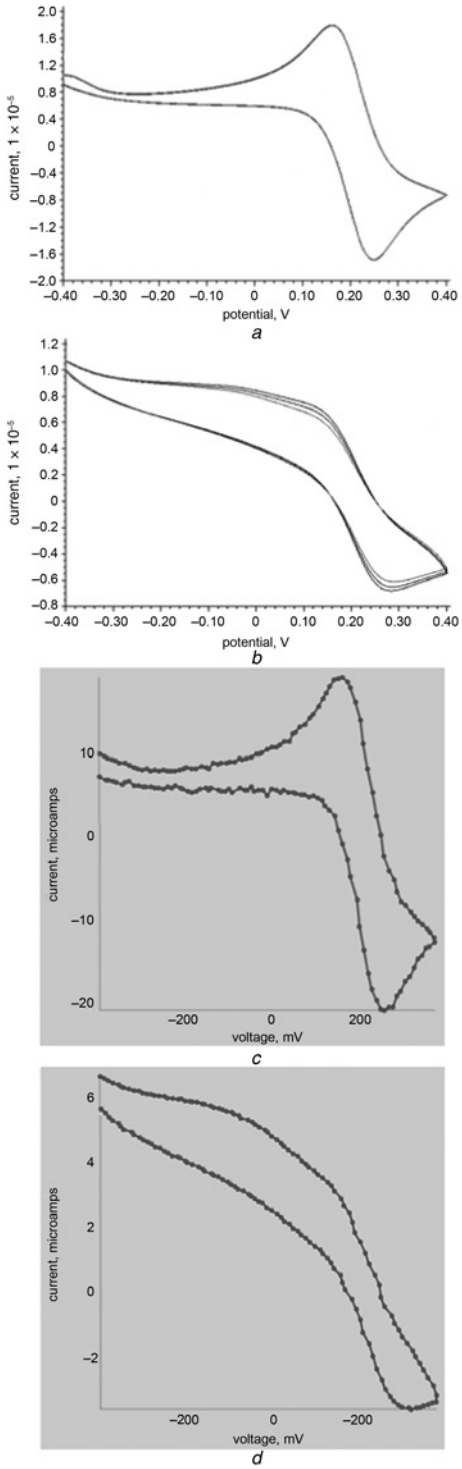

Fig. 2 *Cyclic voltammograms for the MEMS electrochemical aptasensor in the solution of 25 mM phosphate buffer (pH 7) containing 4 mM $K_3[Fe(CN)_6]$ and 10 µM $[Ru(NH_3)_6]Cl_3$*
a Measurement by bench-top electrochemical workstation: bare Au working electrode
b Measurement by bench-top electrochemical workstation: after aptamer immobilisation on the Au electrode
c Measurement by portable electrochemical device: bare Au working electrode
d Measurement by portable electrochemical device: after aptamer immobilisation on the Au electrode

final titre of 50 plaque-forming unit (PFU)/ml. This result demonstrated that MNV particles were captured by the immobilised aptamer, resulting in reduced electron transfer from

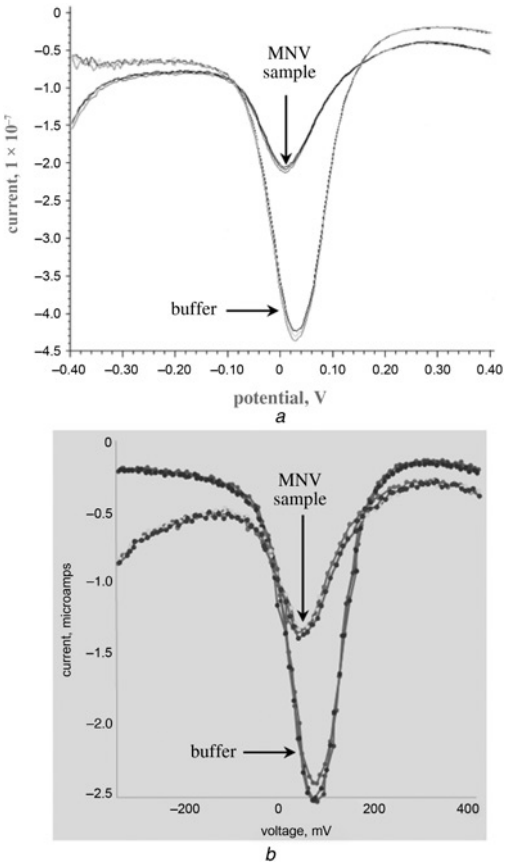

Fig. 3 *Square wave voltammograms for the MEMS electrochemical aptasensor in the solution of 25 mM phosphate buffer (pH 7) containing 4 mM $K_3[Fe(CN)_6]$ and 10 µM $[Ru(NH_3)_6]Cl_3$ with and without inoculation of MNV to obtain a final concentration of 50 PFU/ml*
a Measurement by bench-top electrochemical workstation
b Measurement by portable electrochemical device

the working electrode to cations in the solution. This experiment also manifested strong affinity of the AG3 aptamer toward MNV particles, as reported in a previous study [3]. Again, the bench-top and portable electrochemical measurement devices showed similar SWV profile for the test solution with and without MNV (Figs. 3*a* and *b*), demonstrating the possibility of developing a handheld aptasensor platform using this portable electrochemical measurement device.

3.4. MNV titre-dependent response of the MEMS aptasensor: To evaluate the potential ability of the MEMS electrochemical aptasensor for virus particle quantification, a series of ten-fold dilutions of MNV (0–1.0×10^4 PFU/ml) were prepared and 10 µl of each dilution was spiked into 10 ml test solution. Separate SWV measurements with the MEMS aptasensor were performed for each virus titre using the bench-top electrochemical workstation. As shown in Fig. 4*a*, well-defined and legible peaks were obtained, and the magnitude of peak current decreased in an MNV titre-dependent manner. More specifically, the magnitude of peak current relative to a baseline (lower peak), decreased linearly with increasing concentrations of MNV over three orders of magnitude (Fig. 4*b*), with the regression equation of $y = -6.03x + 76.99$ ($R^2 = 0.97$), where y is the magnitude of peak current relative to a baseline (nanoamperes) and x is the titre of inoculated MNV (PFU/ml) in $\log_{10}$ scale. This result demonstrated high sensitivity and good linearity of the sensor response against different titres of MNV, indicating promising capability of the proposed MEMS electrochemical aptasensor for MNV quantification.

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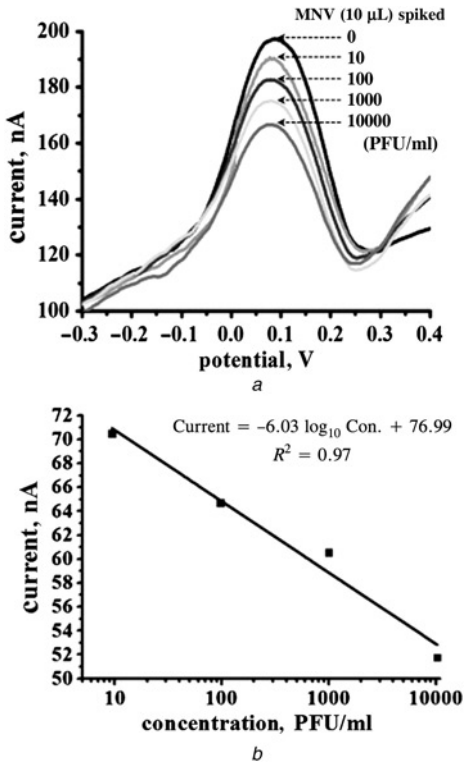

Fig. 4 *MNV titre-dependent SWV response in the 10 ml solution of 25 mM phosphate buffer (pH 7) containing 4 mM $K_3[Fe(CN)_6]$ and 10 µM $[Ru(NH_3)_6]Cl_3$ with inoculation (10 µl of a series of ten-fold dilutions of MNV (0–1.0 × 10^4 PFU/ml))*
a Square wave voltammograms
b Calibration curve representing peak current relative to a baseline (lower peak) against MNV concentration

4. Conclusions: In this Letter, a miniaturised MEMS electrochemical aptasensor was fabricated, characterised, and evaluated for NoV detection and quantification, using MNV as a model virus. The working electrode of the sensor was modified by drop casting an MNV-specific thiolated aptamer, which immobilised the aptamer on the on-chip sensing electrode. Formation of an aptamer monolayer (i.e. successful immobilisation of the aptamer on the Au working electrode surface) was confirmed by means of CV as well as visual observation of a fluorescent-labelled aptamer. The MEMS electrochemical aptasensor exhibited rapid and clear SWV response against different titres of MNV, demonstrating its potential for simple, sensitive, and rapid NoV detection and quantification. In addition, we demonstrated the possibility of developing a handheld aptasensor platform using a portable electrochemical measurement device. This research forms initial basis for the development of an electrochemical MEMS-aptasensor platform and its application for virus detection. It is anticipated that our MEMS aptasensor is simple and portable enough for on-site NoV detection and automation and such electrochemical MEMS-aptasensor platform can be applicable to detection of other microbial pathogens. Our future effort will focus on more detailed evaluations of the proposed sensor, in terms of sensitivity, specificity, applicability for clinical and environmental samples, and further miniaturisation of the electrochemical measurement system toward prototyping a portable aptasensor for on-site virus detection.

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