Advances in nanoarchitectonics enable a wide variety of nanostructured electrodes with tunable shapes and surface for constructing sensitive biosensors. Herein we demonstrate the fabrication of a mesoporous gold (Au) biosensor for the specific and sensitive detection of miRNA in a relatively simple and portable manner. The electrocatalytic activity of the mesoporous Au electrode (MPGE) towards the redox reaction of Fe(CN)$_6^{3-/4-}$ was expansively examined. Leveraging the electrocatalytic activity and signal enhancement capacity of the MPGE, an ultrasensitive and specific electrochemical sensor was developed for the detection of microRNA (miRNA). The target miRNA from spiked samples is selectively isolated and purified using magnetic bead-capture probe followed by the direct adsorption on the MPGE through direct affinity interaction between miRNA and mesoporous Au surface. The MPGE-bound miRNA is then quantified by differential pulse voltammetry (DPV) using [Fe(CN)$_6^{3-/4-}$] redox system (Faradaic current decrease with reference to the bare MPGE). This method evades the cumbersome PCR (polymerase chain reaction) and enzymatic amplification steps. This is a single-step assay building which can detect a wide dynamic linear range (100 aM to 1 nM) of miRNA with an ultra-low limit detection of 100 aM and present high translational potentiality for the development of high-performance detection tools for clinics.
1. Introduction

MicroRNAs (miRNAs) are short (19–25 nucleotides in length), endogeneous, noncoding RNA molecules, presently gaining enormous interest as a cancer biomarker. miRNAs bind to the 3′ untranslated region (3′-UTR) of targeted messenger RNAs (mRNAs) to inhibit translation, functioning as oncogenes and tumour suppressors, thereby regulating post-transcriptional gene expression and remodelling of the epigenome (e.g., DNA methylation and histone modification) (Islam et al., 2017b; Masud et al., 2019; Rosenfeld et al., 2008). They play crucial roles in cancer initiation and progression through their altered expression levels, single-nucleotide polymorphism, copy number variations, and mutations, presenting them as biomarkers for diagnosis and prognosis of various diseases including cancer (Chin and Slack, 2008; Jeffrey, 2008). The prominent functional insights, therefore, triggered the widespread development of advanced strategies for the quantitative detection of miRNAs in clinical practice. RNA sequencing (RNA-seq), microarrays, quantitative real-time PCR (qRT-PCR) have widely been utilized for the analysis of miRNA (Git et al., 2010; Islam et al., 2017b; Pritchard et al., 2012). Despite their reliability, good specificity and sensitivity, they are suffering from PCR amplification bias, fluorescent labelling, and/or complicated protocols (Pritchard et al., 2012). Moreover, these methods are confined to sophisticated laboratories equipped with expensive instrumentation. As an alternative to these techniques, a wide variety of biosensors has been emerged based on the hybridisation of oligonucleotides labelled with different enzymes and redox indicator. Most of these sensors generally involve complicated and monotonous amplification procedures, affluent biomolecules, time-consuming and complicated procedures (Islam et al., 2017b). It is noteworthy to mention that regardless of these advances, the miRNA detection technologies have yet to accomplish their transition into routine clinical practice specifically in resource-limited settings, due to the lack of specific, selective and sensitive detection in a portable, inexpensive point-of-care detection system. Electrochemical strategies have shown to offer rapid, sensitive and accurate miRNA detection exclusive of any preceding amplification process (Hossain et al., 2017; Labib et al., 2016). However, the transduction surface needs to be functionalized with a probe DNA or aptamer, require to employ signalling probe, different redox moiety to get high sensitivity. One of the effective ways to replace that cumbersome functionalization and signal amplification is to integrate engineered nanostructure that could function as both the signal transducer and signal amplifier (Islam et al., 2017b; Masud et al., 2019).

Advancement of nanoarchitechtonics offers a wide range of size- and shape-controlled mesoporous structure for designing sensitive biosensors (Masud et al., 2019; Soleymani et al., 2009). Having small pore size, high surface-to-volume ratio and intrinsic electrochemical (EC) properties, mesoporous metallic nanostructures can overcome the barriers of structural miniaturization of diagnostics, enabling the development of inexpensive, sensitive portable devices (Ferrari, 2005). Mesoporous metal architecture with electro-conductive framework results in superior signal transduction by providing pore-induced high surface area and hybridisation moicity for the efficient and faster analyte (probe or target) binding, and electrocatalytic signal amplification (Malgras et al. 2016). Mesoporous structure (as electrode material) also offers several advantages for improving the performance of electrodes; i) it has dramatically increased the electrochemically active surfaces, ii) it reduces the amplitude of the bias needed to achieve the high signal-to-noise ratio by lowering the impedance of the electrode, and iii) it can reduce the electrode size, which enables placing of multiple electrodes in a high-density array for advanced spatial resolution (Vafaiee et al., 2019). Au has attained immense attention in biosensing due to its inherent bio-favourability, conductivity, stability and predominantly bio-inspired unique surface chemistry (Koo et al., 2015; Wang et al., 2015). The use of planar Au surfaces or Au nanoparticles (AuNPs) holds great promise for high-throughput electrochemical or optical detection of biomolecules on a single or microfluidic device for multiplexed detection with nominal sample supplies. Additionally, Au enables the direct adsorption of nucleic acids (DNA and RNA) in a sequence-dependent manner through the typical chemisorption and physisorption mechanism (Demers et al., 2002; Li and Rothberg, 2004). This comprises direct interaction of nitrogen atoms of DNA nucleobase rings with Au as well as the partial contribution from the exocyclic amino group and charge transfer between the aromatic ring and Au surface (Masud et al., 2017). Although mesoporous metals have been generally prepared by soft- and hard-templating approaches, mesoporous Au electrode (MPGE) with well-defined pores has not been easily achieved due to the difficulty in controlling the crystal growth of Au. Our group has successfully prepared mesoporous Au films by using stable block copolymer micelles as templates (Li et al., 2015).

Herein, we report on the development of an ultra-sensitive miRNA detection platform using a new class of MPGE (Fig. 1). The MPGE is electrochemically fabricated by using a block polymeric-micelles (polystyrene-block-poly(ethylen oxide); PS-b-PEO). The morphology of MPGE is engineered in such a way that it provides a homogenous porous structure with the enhanced functional surface area for increased electrocatalytic activity and adsorption of a significantly higher amount of miRNA. The electrocatalytic activity of prepared MPGE towards the redox process of \([\text{Fe(CN)}_3]^{3-/4-}\) system have been studied. Target miRNA (mirR-9-2) is initially isolated and purified using a magnetic bead-based isolation strategy followed by the adsorption onto the MPGE surface. The level of adsorbed miRNA is then quantified by differential pulse voltammetry (DPV) in the presence of \([\text{Fe(CN)}_3]^{3-/4-}\) redox system. The MPGE biosensor has attained a wide dynamic linear range (100 aM–1 nM) of miRNA with an ultra-low limit detection of 100 aM without any amplification or surface-modification steps.

2. Experimental Section

2.1. Synthesis and characterization of the mesoporous gold electrode (MPGE)

The MPGEs were prepared by electrodeposition in the presence of the self-assembled polymeric micelles containing gold (III) (Au(III)) ions as electrolyte onto a sputtered gold electrode (SGE) (Au-sputtered on silicon substrate). The PS (18,000)-b-PEO, (7,500) polymeric micelles were acted as pore directing agent and Au(III) as a metal precursor. The electrolyte solutions were prepared by dissolving 10 mg of PS-b-PEO in 3 mL of tetrahydrofuran (THF) at 40 °C. After adding 1 mL of ethanol, aqueous HAuCl₄ (40 mM) solution was slowly incorporated under constant stirring. 2.5 mL of deionized H₂O was then added to form the spherical micelles followed by the gentle stirring for 30 min at room temperature until complete dissolution of the Au precursors. The successive addition of aqueous H₂OAuCl₄ enabled the formation of spherical micelles of PS-b-PEO based on the less solubility of PS core in the water. To understand the formation of the polymeric micelle, Fourier Transform Infrared (FTIR) measurement was carried out during micelle formations and Au ion complexation (Fig. S1a). PS-b-PEO (in THF) exhibits characteristics peaks at 3026 and 2919 cm⁻¹ for the aliphatic chain; at 1604, 1495, 1458, 725, and 693 cm⁻¹ (phenyl) ring for PS and at 1081 cm⁻¹ (C=C-O-C) for PEO moiety. Upon the complexation with Au ions, two characteristics peaks for the aliphatic chain show a negative shift. A new broad peak arises at 3100-3500 cm⁻¹ (hydroxyl group absorption maxima), probably due to the formation of hydrogen bonding between PEO and Au ion.

To further confirm the complexation, shape, and structure of the micelles, small-angle neutron scattering (SANS) was employed using the Bilby instrument at ANSTO (Sokolova et al., 2019). The instrument was used in velocity selector mode using neutrons of wavelength 6 Å with detectors positioned at 16 m (rear), 2.5 m (horizontal curtains), and 1.5 m (vertical curtains). The data were reduced and put on an absolute scale relative to the direct beam using Mantid (Arnold et al., 2014), and
then solvent subtracted and modelled using the Igor Pro macros from NIST (Kline, 2006). The micelles containing PS-b-PEO were measured at room temperature under two conditions; (i) the block polymer dissolved in THF and deuterated ethanol, and (ii) aqua-HAuCl₄ was added in the polymeric solution (THF and deuterated ethanol). Small-angle neutron scattering data are presented for both samples in Fig. S2. The scattering data from Fig. S2a were consistent with the sample staying dispersed in the monomeric form, and could be adequately fit with the Debye function representing a linear polymer chain in dilute solution. A radius of gyration of 4 nm was extracted from the fit to the Debye function of the data. Upon the addition of aqua-HAuCl₄ solution, the small-angle scattering data showed the presence of significantly larger particles (spherical micelles) were formed in solution as the polymer self-assembles (Fig. S2b). The data could be adequately fit with a polydisperse sphere with the radius gyration ~13.5 nm.

The as-prepared micelle solution was used as a precursor solution (electrolyte) directly used for electrodeposition. The electrochemical deposition was carried out using a conventional three-electrode system at a constant applied potential without stirring by using an electrochemical workstation. The electrodeposition was performed in a standard three-electrode cell system with a gold-coated silicon wafer substrate (the typical area of 0.09 cm² (0.3 cm × 0.3 cm)) as the working electrode, Ag/AgCl electrode as the reference electrode, and a platinum wire as the counter electrode. The optimal electrodeposition of Au was carried out at room temperature at a constant potential of 0.5 V (vs. Ag/AgCl) for 1000 s without stirring. During the electrodeposition, a

![Fig. 1. Schematic representation of (a) preparation of mesoporous Au electrode (MPGE) via electrodeposition of gold (III)-containing polymeric (block) micelles. The polymeric micelles are formed by dissolving polystyrene-b-poly(ethylene oxide)) (PS-b-PEO) diblock copolymer in THF followed by the addition of ethanol and aqueous gold (III) chloride solution; and (b) Schematic representation of electrochemical detection of miRNA, where target miR-9-2 are magnetically isolated and purified by using a complementary probe-bound dynabead followed by the directl adsorption onto the MPGE via RNA-Au affinity (conventional physisorption and chemisorption mechanism). The DPV interrogation in the presence of [Fe(CN)₆]³⁻/⁴⁻ redox system provide the precise estimation of the concentration of miR-9-2 present in the sample (source). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.).](image-url)
stable current was detected corresponding to the Au reduction (Fig. S3). After the Au deposition, the polymer micelles were removed by immersing the substrates in THF at 40 °C followed by rinsing with deionized water. To check the complete removal of the polymer, the IR-spectra were measured before and after treating the substrate with THF. The corresponding peaks for PS-b-PEO disappeared after the treatment with THF (Fig. S1b).

The complete removal of the micelles gives rise to the MPGE. The uniformly distributed mesopores onto the top surface were seen in the scanning electron microscope (SEM) image (Fig. S4). The SEM image displayed the spherical pores of around 20 ± 5 nm with concave exteriors around the mesopores. X-ray photoelectronic spectroscopy (XPS) was conducted to know the Au oxidation state and surface chemistry (Fig. S5). A doublet at 87.7 and 84.0 eV were obtained from the high-resolution scan of Au 4f, which are separated by 3.7 eV probably because of spin-orbit coupling. This characteristically represented the presence of Au(0) species in MPGE.

2.2. Electrochemical detection of miRNA

5.0 μL (diluted in 5X saline-sodium citrate (SSC) buffer) sample was adsorbed on the MPGE surface. After 30 min of incubation, the electrode was washed (gently) with phosphate-buffered saline (PBS) (three times). The differential pulse voltammetric (DPV) experiments were recorded at –0.1 to –0.5 V with a pulse amplitude of 50 mV and a pulse width of 50 ms in 10 mM PBS solution containing 2 mM [K₃Fe(CN)₆] and 2 mM [K₃Fe(CN)₆] electrolyte solution (0.1 M KCl, 10 mM PBS; pH 7.4) before and after adsorbing target miRNA onto MPGE surface. The relative DPV current changes (i.e., %Δrelative per cent difference of the DPV signals generated for captured miRNA (J_mRNA) with respect to the baseline current (J_base)) due to the adsorption of miRNA were then measured by using the following equation;

\[
\%\Delta \text{relative} = \frac{J_{\text{mRNA}} - J_{\text{baseline}}}{J_{\text{baseline}}} \times 100
\]

where J_base and J_mRNA are current density obtained for bare electrode and electrode after miRNA adsorption, respectively.

3. Results and discussion

3.1. Fabrication of mesoporous gold electrode

The MPGE is prepared through electrochemical reduction of Au(III) species present in the micelles containing PS-b-PEO block polymers (Fig. 1a), where the polymeric micelles work as a pore directing agent for forming uniformly sized mesopores (Mai and Eisenberg, 2012). The formation of spherical micelles is characterised by using infra-red spectroscopy (Fig. S1) and SANS (Fig. S2). The SANS data. The SANS data for block polymer (dissolved in THF and deuterated ethanol) are consistent with the sample staying dispersed in the monomeric form, and the Debye function fit representing a linear polymer chain in dilute solution with a radius of gyration of 4 nm. Upon the addition of aqua-HAuCl₄, the SANS data shows the presence of significantly larger particles (spherical micelles) are formed in solution and a fit with a polydisperse sphere indicates the radius to be ~13.5 nm (For details, please see Experimental Section). SGE silicon is used as the substrate (as a working electrode) with a common area of 0.09 cm² (0.3 cm × 0.3 cm).

The optimal electrodeposition of Au is carried out at room temperature at a constant potential of –0.5 V vs. Ag/AgCl for 1000 s without stirring (Fig. S3) (Li et al., 2015). After the Au deposition, it is crucial to remove the micelles from the substrate entirely, which is carried out by dissolving the substrate in THF at 40 °C (Fig. S1). The uniformly distributed mesopores onto the top surface are seen in the scanning electron microscope (SEM) image (Fig. S4). The SEM image displays the spherical pores of around 20 ± 5 nm with the concave framework. X-ray photoelectronic spectroscopy (XPS) characteristically represents the presence of Au(0) in MPGE (Fig. S5). The film thickness can be controlled by varying the electrodeposition time (0 s–2000 s). Both the ECSA and surface roughness is increased with increasing deposition time (Fig. S6). The ECSA of MPGE was calculated using the charge associated with the reduction of gold oxide, determined by integration, which is proportional to the real active surface area of the mesoporous gold surface.

Schematic representation of electrochemical detection of miRNA, where target miR-9-2 are isolated and purified by using a complementary probe-bound (biotinylated capture probes and streptavidin-coated magnetic beads) system. The microporous architecture facilitates the increased surface area that accelerates the redox reaction and accessibility of analyte (Saleymani et al., 2009). The redox response of [Fe(CN)₆]³⁻/⁴⁻ system can be utilized for quantification of surface-bound oligonucleotides on Au electrodes. Typically the adsorbed nucleic acids present on the electrode surface causes Coulombic repulsion of [Fe(CN)₆]³⁻/⁴⁻ away from the surface, resulting in the significant lowering of current compared to a bare electrode (Kelley et al., 1999; Koo et al., 2016a). The relative decrease in the Faradaic current (bare vs. Oligonucleotides-adsorbed electrode; % current response change) is inversely linked to the amount of adsorbed species. To understand the responses of prepared MPGE towards the redox process of [Fe(CN)₆]³⁻/⁴⁻ system, herein, we have assessed the electrochemical activity of both MPGE and SGE by measuring cyclic voltammetry (CV). Both electrodes generate a pair of well-defined sharp redox peaks in 2 mM [Fe(CN)₆]³⁻/⁴⁻, presenting a reversible single-electron transfer process (Fig. 2a). Surprisingly, the MPGE generate a pair of well-defined sharp redox peaks in 2 mM [Fe(CN)₆]³⁻/⁴⁻, presenting a reversible single-electron transfer process (Fig. 2a). Surprisingly, the MPGE give rise to significantly enhanced cathodic (jpc) and anodic (jpa) peak currents. Notably, the jpc increases approximately 7.8-times (0.7456 vs. 0.098 mA cm⁻²) with the jpa shifted by 61 mV, whereas the jpa increases approximately 7.3-times (0.9415 vs. 0.13 cm⁻²) with the jpa shifted by 47 mV. It has been demonstrated that the MPGE efficiently boosts both the oxidation and reduction process of [Fe(CN)₆]³⁻/⁴⁻ system, owing to more exposed Au surface through the presence of abundant pores. Moreover, the porous structure shows a negative shifting of both jpc and jpa associated with the redox reaction of [Fe(CN)₆]³⁻/⁴⁻ in comparison with SGE. This suggests a higher affinity as well as the interaction of MPGE towards the redox system (Li et al., 2016). Similar responses are obtained from the DPV measurements (Fig. 2b), where MPGE generated approximately 7-times higher current signal than SGE (0.7682 vs. 0.1124 mA cm⁻²) with a negative shift of potential by 49 mV.

We have performed the CV and DPV measurements of MPGEs with different thicknesses. In both cases, the current reached a plateau after 1000 s of electrodeposition and experienced a polynomial relationship (Figs. 57 and 88). The ECSA follows a linear relationship with the film thickness (i.e., deposition time) (Fig. S6); however, the current density (DPV response in [Fe(CN)₆]³⁻/⁴⁻ redox) shows a polynomial relationship. Based on these results, we select 1000 s as the optimal deposition time for further investigation.

To further examine the performance of MPGE, we conducted the CV measurement at different concentrations (0.5 mM–15 mM) of the [Fe(CN)₆]³⁻/⁴⁻ system (Fig. 2c). With the increasing strength of the redox system, the observed currents increase gradually, revealing that the porous structure provides an enormous surface to accelerate the redox
Fig. 2. Electrocatalytic activity of MPGE in $[\text{Fe(CN)}_6]^{3-/4-}$ redox system. (a) CV curves of SGE and MPGE and (b) DPV responses of SGE and MPGE in 2 mM $[\text{Fe(CN)}_6]^{3-/4-}$ solution (0.1 M KCl, 10 mM PBS; pH 7.4). (c) CV curves at different concentrations of $[\text{Fe(CN)}_6]^{3-/4-}$ on MPGE and (d) bar-diagram of the corresponding currents (cathodic). (e) CV curves at different scan rates from 5 mV s$^{-1}$ to 1000 mV s$^{-1}$ on MPGE in 2 mM $[\text{Fe(CN)}_6]^{3-/4-}$ solution and (f) summary of the corresponding current responses upon the square-root of scan rates. (g) Amperometric responses on MPGE with the successive addition of Fe[$(\text{CN})_6]^{3-/4-}$ solution into the 0.01M PBS (pH-7) and (h) the corresponding calibration plot; inset- Lineweaver-Burk Model. The current densities are normalized by the geometrical area of MPGE.
reactions (Fig. 2d). To elucidate the electrochemical mechanism involving in the MPGE towards \([\text{Fe(CN)}_6^{3-/4-}\) system, we performed CV measurements as a function of different scan rate \((\nu)\) (Fig. 2e). Both the \(j_{pc}\) and \(j_a\) are increasing proportionally with the increased scan rates values (5–1000 m V\(^{-1}\)) and show a linear relationship with \(\nu^{1/2}\) for both \(j_a\) and \(j_{pc}\) which indicates the kinetics towards electrode surface are mainly diffusion-controlled (Fig. 2f) (Bard et al., 1980). Furthermore, chronamperometric (CA) responses of MPGE are recorded upon the successive addition of \([\text{Fe(CN)}_6^{3-/4-}\) solution (Fig. 2g), where the CA response increases steeply. The calibration curve follows the characteristic Michaelis-Menten equation (Fig. 2h). The apparent Michaelis-Menten constant \((K_{M})\) can be obtained from the electrochemical version of Lineweaver-Burk model, and it is estimated to be 1.89 mM. This value is significantly low, suggesting the higher affinity of MPGE to \([\text{Fe(CN)}_6^{3-/4-}\) redox system, further verifying electrochemical activity of MPGE (Dutta et al., 2012). This superior activity is related to its strong electrical field around the centre of the uniform mesopores and on the walls between the pores. In addition to this excellent activity, the MPGE is advantageous with high stability. The MPGE provides stable signal generation over the multiple cycles (30 cycles) of CV measurement using \([\text{Fe(CN)}_6^{3-/4-}\) redox system (Fig. S9). This stability in reaction media (PBS) and strong affinity towards the redox molecule are highly strategic for exploiting MPGE as the impeccable substrate (signal-transduction) for electrochemical biosensing. To further explore the property and validate the electrochemical properties of MPGE, scanning electrochemical microscopy (SECM) with soft-probe scanning in contact mode have been utilized (Fig. S10-S14, for details please see Supplementary data).

### 3.3. MPGE-based detection of miRNA

To explore the functionality of mesoporous Au structure as a proof-of-concept (POC) biosensor, we have utilized as prepared MPGE for miRNA detection. The assay principle for the MPGE integrated amplification-free ultra-sensitive miRNA detection is outlined in Fig. 1b. The assay is mainly comprised of two-steps; (i) magnetic separation and purification of target miRNA sequence using target-complementary capture probe attached-with a dynabead (Islam et al., 2017a) (Fig. 1b), (ii) direct adsorption of purified miRNA on MPGE via RNA-Au affinity interaction through conventional physisorption and chemisorption mechanism followed by DPV interrogation. The magnetic isolation and purification removed all non-specific biomolecules (protein, DNA and RNA) especially non-specific miRNA, facilitates the adsorption of target miRNAs at MPGE only which can provide highly selective electrochemical responses for target miRNA. Herein, miR-9-2 is chosen as a target because of its potentiality as a predictive and diagnostic biomarker for carcinoma (Hildebrandt et al., 2010; Zhou et al., 2012). For instance, a low level of miR-9 is significantly correlated with worse lymphatic invasion and advanced TNM (tumor-node-metastasis) stage in metastatic nasopharyngeal carcinoma (Lu et al., 2014). The magnetic isolation significantly reduces matrix effect from complex bio-system, removes debris and non-specific sequences, thereby provides the pure target miRNA for electrochemical readout. Moreover, bringing target miRNA (only) onto MPGE significantly biofouling issues generally experience in the electrochemical sensor. The purified miRNA (in 5 x SSC buffer) can be directly adsorbed on to the MPGE. The underling principle of the direct adsorption of miRNA sequences on a clean MPGE can be explained by the well-explored nucleobases’ adsorption affinity towards the bare Au surface where miRNA bases are directly adsorbed in a sequence-dependent manner (Koo et al., 2015). The adsorbed miRNAs are then detected by DPV interrogation in the presence of an electroactive \([\text{Fe(CN)}_6^{3-/4-}\) system (0.1 M KCL, 10 mM PBS; pH 7.4). It has been shown earlier that the \([\text{Fe(CN)}_6^{3-/4-}\) redox system alone can be used for quantification of surface-bound nucleic acid attached at Au surface (Sina et al., 2014; Zhang et al., 2007). Under the electron-transfer kinetic-based mechanism the coulombic repulsion between \([\text{Fe(CN)}_6^{3-/4-}\) and negatively charged nucleic acid strands (low coverage) at the electrode surface generate reduced current from \([\text{Fe(CN)}_6^{3-/4-}\) molecules. The amount of current reduction in relatives to bare is directly related to the miRNA present at the electrode surface. To select the optimum electrode thickness, initially, we have challenged the same amount of purified miRNA on to the MPGE with different thicknesses (prepared by different deposition; 0–2000 s). It has been shown that the current responses have been increased with increasing electrode thickness, however, the polynomial fittings express that the current started to became a plateau with the MPGE deposited for 1000 s (Figs. 57 and S8). Therefore we have chosen the MPGE that prepared at 1000 s electrodeposition time for the subsequent experiment for POC assay for miRNA detection.

To examine the superiority of MPGE over SGE for miRNA detection, we have adsorbed the same amount of (10 pM) of magnetically purified miRNA sequence at both surfaces. Fig. 3a and b represent the DPV responses before and after miRNA adsorption at both Au surface. The current response for miR-9-2 at MPGE is significantly higher (0.382 vs. 0.061 mA cm\(^{-2}\)) than SGE (Fig. 3c). More than 6-times higher current generation from MPGE can be related to the large exposed surface area of MPGE obtained from the porous structure. The mesoporous structure provides a higher amount of exposed atomic steps/kinks for adsorbing higher amount of target miRNA and for electrochemical signal enhancement of \([\text{Fe(CN)}_6^{3-/4-}\) redox system. The MPGE adsorbs significantly higher amount of miRNA than SGE through the RNA–Au affinity interaction, resulting in the significantly higher repulsion of \([\text{Fe(CN)}_6^{3-/4-}\) redox molecules from the negatively charged miRNA-confined MPGE surface. To visualize the adsorption of miRNA sequence at MPGE surface, we have recorded the topographic image after miRNA adsorption using SEM and atomic force microscopy (AFM) (Fig. 4a and b). As can be seen in Fig. 4b, the morphology (topography) of the MPGE-miRNA electrode surface is entirely different from the bare MPGE and the presence of miRNA at MPGE detected by AFM (entangled globular shape, elongated shape, etc).

To evaluate the assay specificity, we have challenged the MPGE surface with no-template control (NoT) and wrong-target (non-complementary sequence; miR-338–3p) (Fig. 3d). It has been seen that the NoT and the non-complementary sequence generates negligible current responses (% \(J_r\) = 1.79 and 2.56 respectively), revealing the high specificity of dynabeads based isolation and purification. A very tiny increment of current for miR-338–3p, probably due to the binding of a few amounts of miR-338–3p sequence with the capture probe, however, this value is much lower than the target one (% \(J_r\) = 51.2). The current response for 10 pM of target miRNA is almost 28-times and 20-times higher than both NoT and wrong target, which is due to the sequence-specific capture bring an adequate amount of target miRNA at MPGE surface, in which DPV interrogation in the presence of redox system generates a significant reduction of current in comparison to bare.

Recent studies on disease diagnosis show that the miRNA levels either upregulated or downregulated in disease state (cells, plasma or exosomes) compare to the healthy source. miRNA expression also varies according to the type and stage of the disease, especially cancer. Importantly, the changes in miRNA expression are meagre compared to that of a healthy state, clearly indicating the requirement of a highly sensitive detection system. To determine the detection sensitivity using MPGE, a series of synthetic miR-9-2 with different concentrations ranging from 1 nM to 100 aM are analysed. It has been observed that the charge generated by DPV interrogation at MPGE with increasing concentration of miRNA (Fig. 4c and d). This is attributed to the higher amount of miRNA is isolated and thus adsorbed on to the MPGE surface produces increased coulombic repulsion of \([\text{Fe(CN)}_6^{3-/4-}\) system to the negatively charged miRNA resulting in the reduced Faradaic current and increased relative current response change regarding the control (NoT) and bare MPGE. The linear regression equation is estimated, and the limit of detection is calculated by considering s/n ration equals to three compared to NoT. The MPGE enables a limit of detection of 100 aM with
good reproducibility (relative standard deviation, RSD of < 6%, for n = 3) for detecting magnetically purified miRNA. This dynamic series of detection with attomolar sensitivity indicates the potentiality of MPGE for identifying and analysing miR-9-2 in purified miRNA from complex biological matrices with varying level of miRNA even at an earlier stage.

The detection limit (LOD) of the assay is highly comparable with the recently reported electrochemical strategies for mRNA detection. For instance, our assay has realized around 9 times lower LOD than recently reported readout by Li et al., though they have utilized base-mismatched catalytic hairpin assembly (CHA) amplification whereas this assay offers much simpler platform without any amplification steps (Li et al., 2019). This assay also shows increased sensitivity compared to recent time reported nanostructure-based strategies such as SPGE (Koo et al., 2016b), silver nanofoam (AgNF) (Kangkamano et al., 2018), amino-graphene-modified glassy carbon electrode (GCE) (Salimi et al., 2019), Au-NPFe$_2$O$_3$/SPCE (Islam et al., 2018b; Masud et al., 2017), DNA-modified Au electrode (Miao et al., 2018b), GO-NPFe$_2$O$_3$/SPCE (Islam et al., 2018a) and Au-μPADs (Sun et al., 2018). It is also imperative to remark that there are some nanotechnology-based methods have also been reported with similar detection sensitivity or even lower range of miRNA (Details comparison are shown in Table S2, see supplementary data) (Guo et al., 2018; Islam et al., 2018b; Miao et al., 2018a, 2018b). However, these methods utilized cumbersome enzymatic amplification, multi-step cascade electrocatalysis, isothermal amplification, enzymatic displacement reaction, and different redox probe. On the contrary, our methods offer straightforward, secure, non-enzymatic, label-free detection and most significantly it requires an inexpensive MPGE. We deem that the LOD of 100 aM of our assay is passable to retract the level of readily available miRNA biomarker from the clinically-pertinent concentration of target samples. We also have confidence in that several distinctive features of our assay have ascribed to this ultra-sensitivity. These are as follows: (i) the tunable mesoporous, concave surface inside the pores, profusion in high-index facets together with assembly of kink/steps sites preferentially facilitates the adsorption of nucleic acid as well significant enhancement of current density towards the redox process, (ii) complementary capture probe and dynabeads-based magnetic collection and purification of target miRNA expressively cuts the matrix effect of the compound biological testers and eradicates the nonspecific targets. Furthermore, the capturing and purification steps of the target miRNA can be spatially separated from the electrode to ease the biofouling issues, (iii) the inherently sensitive DPV readout (its superior capacitive or background current elimination ability curtails the influence of charging current i.e., only Faradaic current is counted) resulting in more accurate detection of the target miRNA, and (iv) PCR or any enzymatic amplification-free processes avoid amplification bias (i.e., less false-positive response). We foretell that the MPGE-based assay could also be radially able to detect any miRNA marker detection and single-step electrochemical readout can easily be translated to automotive device for clinical diagnostics.

4. Conclusion

We have developed a novel biosensor platform, which allows highly sensitive (LOD: 100 aM) detection of miRNA without any amplification or enzymatic process. Compared with the cutting-edge miRNA assays
with different cascade signal amplification, the synthesis of MPGE and design of this work is simple but subtle. The MPGE is highly stable and can be reused for multiple tests. The hollow pore-mediated signal amplification provides the superior adsorption sites for miRNA, catalytic effect and thereby enhances the sensitivity. Besides, the preparation of MPGE is very cost-effective with excellent repeatability and re-usability. This assay offers a single-step detection after isolation of target miRNA from the sources, required less than 1 h, providing the translation capability for designing portable miRNA sensor for clinics. We envisage that the developed method indeed provides a versatile platform for miRNA detection and may find a broad spectrum of applications in biological studies, bioanalysis and clinical diagnosis. Besides, we foretell that the MPGE-based assay could also be radially able to detect any miRNA marker detection and single-step electrochemical readout can easily be translated to an automative device for clinical diagnostics not limited to cancer but also for detecting any infectious disease (pan-demics) based on RNA biomarkers at resource-limited settings.

CRediT authorship contribution statement


Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.
References


