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A PCR-free electrochemical method for messenger RNA detection in cancer tissue samples

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Abstract

Despite having reliable and excellent diagnostic performances, the currently available messenger RNA (mRNA) detection methods mostly use enzymatic amplification steps of the target mRNA which is generally affected by the sample manipulations, amplification bias and longer assay time. This paper reports an amplification-free electrochemical approach for the sensitive and selective detection of mRNA using a screen-printed gold electrode (SPE-Au). The target mRNA is selectively isolated by magnetic separation and adsorbed directly onto an unmodified SPE-Au. The surface-attached mRNA is then measured by differential pulse voltammetry (DPV) in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system. This method circumvents the PCR amplification steps as well as simplifies the assay construction by avoiding multiple steps involved in conventional biosensing approaches of using recognition and transduction layers. Our method has demonstrated good sensitivity (LOD = 1.0 pM) and reproducibility (% RSD = <5%, for n = 3) for detecting *FAM134B* mRNA in two cancer cell lines and a small cohort of clinical samples (number of samples = 26) collected from patients with oesophageal cancer. The analytical performance of our method is validated with a standard qRT-PCR analysis. We believe that our PCR-free approach holds a great promise for the analysis of tumor-specific mRNA in clinical samples.

Keywords:

Messenger RNA detection, Electrochemical detection, Amplification-free method for mRNA detection, Oesophageal cancer biomarker, Tumor-specific mRNA

1. Introduction

Tumor-specific messenger RNAs (mRNAs) are an emerging class of sensitive biomarker for disease diagnosis and prognosis due to their key position at the intersection of genome and proteome where mRNA carries information from DNA to initiate protein synthesis (Ludwig and Weinstein, 2005). mRNA controls different cellular regulatory pathways such as chromatin modifications, cell adhesions and cell cycles (Vogel and Marcotte, 2012). These regulatory pathways could be affected by altered mRNA expression due to the point mutation, alternative splicing, aberrant methylation pattern, inactivation of mRNA open reading frame (ORF) and activation of rapid mRNA degradation, which can cause varieties of diseases including cancer (Cooper et al., 2009; Dixit et al., 2017; Ludwig and Weinstein, 2005). Thus, mRNA expression profiling can be a very powerful way of identifying biomarkers that describe different pathophysiological anomalies including cancer. Over the past several years, a great deal of research reported that the screening and quantification of mRNA levels help in the prognosis and prediction of therapeutic responses of cancer (Mehta et al., 2010; Volinia and Croce, 2013). For example, aberrantly expressed Cyclin D1 and TS mRNA were correlated with poor survival of patients with breast cancer whereas metastases in lung cancer were linked with the altered levels of TERT and EGFR mRNA (Garcia et al., 2008; Miura et al., 2006). Recent studies has established a correlation between altered *FAM134B* mRNA expression and the pathogenesis of gastrointestinal carcinomas especially colon carcinoma and oesophageal squamous cell carcinoma (ESCC) where *FAM134B* acts as an important tissue marker with association between lymph node metastasis and *FAM134B* mutations (Haque et al., 2016; Islam et al., 2017a; Kasem et al., 2014). Although significant progress has recently been made to develop biosensing strategies to detect cancer-related mRNA biomarkers, an effective and simple assay platform that can

sensitively be used to interrogate the level of relatively new *FAM134B* mRNA biomarker remains elusive.

Until recently, the most commonly used method for the detection of mRNA is based on the nucleic-acid-detection techniques such as quantitative reverse transcription PCR (qRT-PCR), microarrays and RNA sequencing methods (Etienne et al., 2004; Wang et al., 2009). While these approaches are highly reliable and analytically sound, they require enzymatic amplification of the target and costly instrumentation. Additionally, to amplify the targets, most of these approaches involve the reverse transcription of mRNA into complementary cDNA which increases the chance of amplification error and artefacts (*i.e.*, due to template switching and partial amplification of target mRNA sequences) (Islam et al., 2017b). Moreover, oligo(dT) primers are commonly being used in reverse transcription which can only work on mRNA with poly(A) tail, therefore these methods are unable to analyze mRNA target sequences having no poly(A) tail. For visualising the results, gel electrophoresis or fluorescence readouts are also required which need expensive instrumental set-up. Together these issues make the nucleic-acid-detection techniques unsuited for their incorporation in miniaturized device for routine clinical application (Haider et al., 2016, Islam et al., 2017b). Previously, a number of electrochemical approaches have been developed for mRNA detection, most of which are based on hybridization of a target sequence with a surface bound receptor probe and the use of enzymes for target amplification and signal enhancement (Aguilar and Fritsch, 2003; Li et al., 2015; Sanchez et al., 2016).

To avoid the challenges of enzymatic amplification of mRNA, several amplification-free assays coupled with optical and electrochemical readouts have been developed (Carrascosa et al., 2016; Pöhlmann and Sprinzl, 2015). Among them, electrochemical methods have shown great promise towards mRNA detection due to their high sensitivity, simplicity, cost-effectiveness and compatibility with miniaturization (Mukumoto et al., 2007;

Mao et al., 2008; Sato et al., 2001; Vasilyeva et al., 2011). Although these methods can accurately detect the levels of mRNA, their diagnostic potential in heterogeneous clinical samples are rather limited due to sample manipulations (*i.e.*, target mRNA is often required to be modified with electroactive ligands) or use of complex sensor fabrication (*e.g.*, nano-structured microelectrode). Therefore, much attention has been focused in recent years on the development of bioassays *via (i)* developing amplification-free approaches for detecting mRNA in heterogeneous population of clinical sample, and *(ii)* designing less complicated electrochemical sensors in a miniaturized format to avoid time consuming and costly sensing steps. Previously, Koo et al. (2016a,b) has developed an amplification-free method that apparently has shown the promise to meet the above criteria while detecting microRNA and prostate cancer specific gene-fusion mRNA. Although this approach uses an unmodified sensor, enzymatic polyadenylation of the target is still required in the assay for sensitivity enhancement. In this method, the use of enzyme for poly(A) tailing of the target mRNA increases the assay cost and analysis time. Moreover, poly(A) enzymes are prone to degradation in room temperature.

Herein, we report an amplification-free detection assay for the analysis of *FAM134B* mRNA in tissues samples from patients with oesophageal carcinomas. Target mRNA sequences were first magnetically isolated and purified from the sample pool, and adsorbed directly onto an unmodified SPE-Au electrode. The level of adsorbed mRNA was then analyzed by differential pulse voltammetry (DPV) in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system. Our method does not require any prior enzymatic amplification of mRNA and it adopts the direct adsorption of mRNA on unmodified electrode surface thus avoids the conventional sensor fabrication steps. This method also uses the relatively inexpensive and commercially available disposable SPE-Au. Moreover, the use of magnetic washing steps in the assay significantly reduces the chance of non-specific target detection.

2. Experimental

2.1 Reagents and Materials

Unless otherwise stated, all the reagents and chemicals used in this study were of analytical grade and purchased from Sigma Aldrich (Sydney, NSW, Australia). UltraPureTM DNase/RNase-free distilled water (Invitrogen, Australia) was used throughout the experiments. Synthetic mRNA, primers and capture probes were purchased from Integrated DNA Technologies (Coralville, IA, USA) and sequences are shown in **Table 1**. SPE-Au (DRP-C250BT) was purchased from Dropsens (Spain). Two oesophageal squamous cell carcinoma (ESCC) cell lines, HKESC-1 and HKESC-4, were kindly provided by our collaborator from the Department of Pathology, University of Hong Kong. These cells were cultured in minimum essential medium alpha (MEMα growth medium, Gibco (Thermo Fisher scientific, Waltham, MA, USA)) medium with non-essential amino acids supplemented with 10% fetal bovine serum (FBS, Gibco), 100 µg/mL penicillin (Gibco) and 100 unit/mL streptomycin (Gibco) in a humidified cell culture incubator containing 5% CO_2 at 37 °C.

2.2. RNA extraction from oesophageal cancer tissue samples

Surgically resected fresh tissue samples (cancer and matched adjacent normal non-neoplastic mucosa near resection margin) from thirteen patients with ESCC were sectioned using a cryostat (Leica CM 1850 UV, Wetzlar, Germany) and stained by haematoxylin and eosin staining. Histopathological confirmation of the tumour and adjacent non-neoplastic mucosae of the oesophagus were confirmed by the author (AKL). Total RNA was then extracted using the standard protocol from all prep DNA/RNA mini kit (Qiagen, Hilden,

NRW, Germany). The concentration and purity of RNA were measured by a spectrophotometer (BioLab, MA, USA) using 260/280 ratio and stored at -80^{o}C.

2.3. Magnetic isolation of mRNA and adsorption on electrode

A volume of 20 µL of streptavidin-labeled Dynabeads MyOne Streptavidin C1 (Invitrogen, Australia) magnetic beads were first washed with 2X binding and wash (B&W) buffer (10 mM Tris-HCl, pH 7.5; 1.0 mM EDTA ; 2.0 M NaCl) and resuspended in 25 µL of 2X B&W buffer. Next, 25 µL of 10 µM biotinylated capture probe was added, followed by the incubation in a thermomixer for 20 min at room temperature to facilitate the functionalisation of capture probes with magnetic beads. Afterwards, the functionalized magnetic beads were washed three times and mixed in 10 µL of 5XSSC buffer (pH 7). For synthetic mRNA capture, 10 µL of previously prepared different known concentration of synthetic mRNA (spanning over 1.0 pM-10 nM) were mixed with 10 µL of capture probes-functionalized magnetic beads. The mixture was incubated on a thermomixer for 20 min at room temperature to allow the hybridization of capture probe and target. After this step, the magnetic beads with attached mRNA targets were separated using a magnet, washed twice and resuspended in 9.0 µL of RNase-free water. This mixture was heated for 2 min at 95°C and the supernatant containing the released mRNA targets were collected immediately using a magnet. The released solution was then diluted to 20 µL with 5XSSC buffer (pH 7.0), and directly put onto the working surface of a SPE-Au and incubated for 20 min. The electrode was then washed with 10 mM phosphate buffer saline (PBS comprised of 137 mM sodium chloride, 2.0 mM potassium chloride, pH 7.4) before electrochemical readout.

2.4. Electrochemical detection of adsorbed target mRNA

All electrochemical measurements were performed on a CH1040C potentiostat (CH Instruments, USA) with the three-electrode system (gold working, platinum counter and silver reference electrode) on each screen-printed gold electrode. Differential pulse voltammetric (DPV) experiments were recorded at -0.3 V to 0.5 V with a pulse amplitude of 50 mV and a pulse width of 50 ms in 10 mM PBS solution containing 2.5 mM [$K_3Fe(CN)_6$] and 2.5mM [$K_4Fe(CN)_6$] electrolyte solution. For synthetic mRNA samples, 5.0 µL (diluted in SSC5X buffer) sample was adsorbed on SPE-Au surface. For clinical samples analysis, 5.0 µL (diluted in SSC5X buffer to get 50 ng of RNA) were used. The electrodes were then washed three times with PBS prior taking DPV measurements. The relative DPV current changes (*i.e.*, $\%I_{\text{Relative}}$, percent difference of the DPV signals generated for captured mRNA (I_{Sample}) with respect to the baseline current (I_{Baseline})) due to the adsorption of mRNA were then measured by using following equation

$$\%I_{Relative} = \frac{\%I_{Baseline} - \%I_{Sample}}{\%I_{Baseline}} \times 100$$

where i_{Baseline} and i_{Sample} are current changes for bare electrode and electrode after sample adsorption respectively.

2.5 Primer design and qRT-PCR

FAM134B mRNA expression in all tested primary ESCC cancers and matched non-neoplastic mucosae were studied via a qRT-PCR. First, RNA was extracted and cDNA conversion was performed as previously reported (Kasem et al., 2014). In brief, reverse transcription reactions were conducted using 1 µg of total RNA in a final reaction volume of 20 µL. cDNA conversion was done using the manufacturer's instructions from miScript reverse transcription kit (Qiagen, Hilden, NRW, Germany). To inactivate the reverse transcriptase mix, 1 µg of total RNA and master mix were incubated for 60 min at 37ºC and then heated up at 95ºC for 5 min. Each cDNA sample was diluted to 30 ng/µL for qRT-PCR.

Samples were then stored at -20ºC. The primer sets for amplification of *FAM134B* (GenBank accession number for variant 1 NM_001034850 and for variant 2 NM_019000), and GAPDH (GenBank accession number NM_002046) genes were designed using Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/) (Primer sequences are provided in Table 1). Then, qRT-PCR was performed in a total volume of 10 µL reaction mixture comprising 5.0 µL of 2XSensiMix SYBR No-ROX master mix (Bioline, London, UK), 1.0 µL of each 10 picomole/ µL primer, 1.0 µL of cDNA at 30 ng/µL genomic DNA at 20-50 ng/µL and 2.0 µL of nuclease free water. Assays were accomplished in duplicate and a no-template (NoT) control was included in all the experiment. The results of the qRT-PCR were analyzed using previously reported methods (Gopalan et al., 2010).

3. Results and discussion

3.1. Detection principle and assay optimization

The principle of amplification-free electrochemical detection assay of *FAM134B* mRNA is schematically presented in Fig. 1. The assay is comprised of three main steps including *(i)* magnetic separation of target *FAM134B* mRNA, *(ii)* heat release of captured target mRNA and *(iii)* adsorption of mRNA on SPE-Au for electrochemical readout. First, streptavidin labeled magnetic beads are functionalized with biotinylated capture probe (which is complementary to the target mRNA) using biotin-streptavidin interaction. These capture probe functionalized magnetic beads are then dispersed into the samples to selectively capture target mRNA by magnetic purification steps. After heat release at 95^{o}C, the isolated target mRNA is directly adsorbed on a SPE-Au. The underlying principle of the direct adsorption of mRNA sequences on unmodified gold surfaces can be explained by the well-explored nucleobases' adsorption affinity towards bare gold surface where mRNA bases are directly adsorbed on bare gold surface in a sequence-dependent manner (Koo et al., 2015).

The amount of the adsorbed mRNA is quantified by DPV in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system.

Previously, it has been shown that the $[Fe(CN)_6]^{4-/3-}$ redox system alone can be used for quantification of surface-bound nucleotide at unmodified gold electrodes (Sina *et al.*, 2014; Zhang *et al.*, 2007). Zhang et al. (2007) proposed an electron transfer kinetic-based mechanism, where density of the nucleotide strands at the electrode surface should be sufficiently low. Under these conditions, the coulombic repulsion between $[Fe(CN)_6]^{3-}$ and negatively charged nucleotide strands (low coverage) at the electrode surface is not effective enough to fully repel $[Fe(CN)_6]^{3-}$ molecules to approach the electrode surface, and therefore generates a significant Faradaic current. Thus, prolonged adsorption of mRNA could result complete saturation of the electrode surface which could lead to the similar current readings for two target samples with significantly different concentrations. To avoid this, adsorption parameters such as adsorption time and pH of the solution should be optimized to get a distinguishable DPV current on the electrode surface. In optimization experiments, we performed our assay to capture *FAM134B* mRNA from 100pM synthetic mRNA sample. We first captured the mRNA on SPE-Au at different adsorption times spanning over 5, 10, 15, 20, 25 and 30 min. We observed that the maximal current response change of approximately 40% was attained at 20 min and no significant changes occurred afterwards (Fig. 2A). Therefore, we selected 20 min as the optimal adsorption time for the rest of the experiments. Next, we tested different pH of the adsorption buffer (5X SSC) to get the maximal response. Using five different buffer pH such as 3.0, 5.0, 7.0, 9.0 and 11, we observed that the optimal relative current response change was spotted at pH 7.0 (neutral) which concludes that mRNA adsorption on gold is hampered in both acidic and basic pH (Fig. 2B). This could be explained by the compromised electrodes properties at acidic pH and probability of mRNA degradation at basic pH of adsorption buffer. This optimized condition facilitates $[Fe(CN)_6]^{3-}$

molecules to diffuse through the electrode surface against the negatively charged adsorbed mRNA molecule resulting a discernible DPV signal. Thus, as indicated in the inset of Fig. 3, the level of adsorbed mRNA on the electrode compared to a bare electrode provides less Faradic current due to the higher columbic repulsion between adsorbed mRNA and the redox system (Koo et al., 2016a,b). This establishes a clear correlation between the resulting Faradic current and the adsorbed target mRNA, where the decrease in Faradaic current with respect to the baseline current (% current response change) is inversely proportional to the level of adsorbed mRNA.

3.2. Assay Specificity

To check the specificity of our assay, we performed our assay using 100 pM miR-107 miRNA as the wrong target, and compared the relative DPV response with that of the 100 pM synthetic target *FAM134B* mRNA. As can be seen in Fig 3 (bar denoted as miR-107), the DPV current changes was almost similar to that of the no-template (NoT) control. This response is also approximately eleven-fold lower than that of the target *FAM134B* mRNA, indicating the high specificity of our assay.

3.3. Assay Sensitivity

To test the sensitivity and reproducibility of our assay, a series of magnetically captured *FAM134B* mRNA isolated from different known concentration of synthetic mRNA were adsorbed on the electrode surface (Fig. 3). As indicated in Fig. 3, after adsorbing the mRNA on SPE-Au, a linear increment of relative DPV current response changes was observed with the increase of *FAM134B* mRNA concentration in the dynamic range of 1.0 pM to 10 nM. This might be attributed to the high amount of adsorbed mRNA that creates more coulombic repulsion of $[Fe(CN)6]^{4-/3-}$ system towards the negatively charged mRNA resulting less Faradic current and increased relative current response changes with respect to the NoT control and bare electrode. The linear regression equation was estimated to be *y* (%

current response changes, $\%i_r$) = 10.414 (mRNA concentration) – 7.2867(C) with a correlation coefficient (R^2) of 0.99. The detection limit was found to be 1.0 pM ($\%i_r$ = 12%) with a corresponding signal-to-noise ratio of 3.8 compared to NoT control. Our assay also shows good reproducibility (relative standard deviation, RSD of <5%, for n =3) in synthetic samples. This dynamic range of detection also indicates that our method could potentially be applicable for detecting and analysing *FAM134B* mRNA in complex biological matrixes with varying level of mRNA. We have also checked the stability of the RNA-attached SPE-Au electrodes by using six independent electrodes at one-day interval over 5 days. The electrodes were modified with synthetic mRNA (100 pM) and stored at -4^{o}C. Each electrode was used in each interval (data not shown). The electrode-to-electrode reproducibility (i.e., % RSD) was found to be <6% (for n = 6), indicating the good stability of the sensor with good analytical reproducibility.

The detection limit of the assay is comparable with that of the existing electrochemical methods for mRNA detection. For example, our limit of detection is 25 times better than that of a recent method developed by Sanchez et al. (2016). Moreover, their method incorporates the ligation dependent probe amplification and surface functionalization steps whereas our assay offers a much simpler platform on an unmodified disposable SPE-Au. Our method also shows increased sensitivity compared to one of the earlier electrochemical methods proposed by Aguilar and Fritsch (2003). It is also important to mention that there are few reported electrochemical methods, which offer relatively higher sensitivity such as methods developed by Li et al. (2015) and Koo et al. (2016a). Li et al. (2015) used a special type of enzyme known as duplex specific nuclease and the method developed by Koo et al. (2016a) relied on the presence of poly(A) tails in the target mRNA to achieve the ultrasensitive detection. On the contrary, our assay avoids the use of enzymes for sensitivity enhancement to develop a simple and inexpensive assay. We also believe that

the limit of detection (1.0 pM or 9.0 pg/ml) of our assay is adequate to retract the level of readily available mRNA biomarker from the clinically relevant concentration of target analyte. As indicated in Fig. 5, our assay has shown excellent analytical performance in detecting *FAM 134B* mRNA in a panel of clinical samples derived from oesophageal cancer tissues (discussed in section 3.3 of the manuscript). We believe that several unique features of our assay have attributed to this sensitivity. These are as follows- ***(i)*** complementary capture probe-functionalized dynabeads are dispersed throughout the samples to form an intimate mixture, thus increasing the capture efficiency. The dyanbeads are magnetic, so upon application of a magnetic field, they are washed, purified and collected for detection. This significantly reduces the matrix effect of the complex biological samples and removes the non-specific targets, thus enhancing the assay sensitivity. Additionally, the capture of the target mRNA can be temporally and spatially separated from the electrochemical measurement to alleviate biofouling issues, (ii) while cyclic voltammetric readouts suffer from excessive charging current (*i.e.*, the current needed to apply the potential to the system), DPV is inherently sensitive due to its superior capacitive or background current elimination ability (Ronkainen et al., 2010). Here, the DPV current is measured from the differences of two potential pulses (one just before the application of the pulse and the second at the end of the pulse), which minimizes the effect of charging current (*i.e.*, only faradic current is counted) resulting in more accurate detection of the target mRNA, and ***(iii)*** as our method is free of PCR amplification processes, it avoids PCR amplification bias (*i.e.*, reduced false-positive response).

3.4. Detection of FAM134B mRNA in cancer cell line and patient sample

We further challenged our assay with total RNA extracted from cultured human oesophageal cancer cell lines (HKESC1 and HKESC4), to demonstrate the efficiency of our

assay for detecting *FAM134B* mRNA in cancer cells from different microenvironment. Our assay showed 15 % relative current response changes for HKESC1 RNA and 18 % relative current response changes for HKESC4 RNA which was not significantly higher than that of NoT control (Fig. 4) indicating the down-regulation of of *FAM134B* mRNA in these cell lines. The widely used mRNA expression analysis method, qRT-PCR, was also performed on the extracted RNA derived from these cell lines and the mRNA expression data was found to be in good agreement with our method. These findings raise the possibility of applying our method for analysing *FAM134B* mRNA in heterogeneous clinical samples.

To further explore the clinical application of our assay, we extended our method to detect different levels of *FAM134B* mRNA from a small cohort of fresh tissue samples derived from oesophageal cancer patients. Total RNA was extracted from twenty-six clinical tissue samples (thirteen oesophageal cancer and thirteen matched non-tumor mucosae). As shown in Fig. 5A, relative current changes for the seven oesophageal cancer tissue samples (1T -7T) are much higher in comparison with their matched non-tumor pairs (1N-7N), indicating the *FAM134B* mRNA over expression. We also observed that the relative current changes of five oesophageal cancer tissue samples (8T-12T) is much lower compared to their non-tumor pairs, indicating the *FAM134B* down-regulation in these five samples. However, the relative current changes of the remaining tumor sample (13T) clearly shows that there are no significant changes in the *FAM134B* mRNA level with respect to its matched normal sample (13N). These data clearly imply that the electrochemical signal generated by our assay was able to distinguish the relative presence *FAM134B* mRNA in RNA samples collected from ESCC patients. It is important to mention that, compared to the smaller RNAs (*e.g.*, microRNAs), specific detection of larger mRNA in a sample containing high level of nonspecific RNAs (with varying size and structures) has generally been considered as difficult to achieve reliably (Islam et al., 2017b). However, the current method has

successfully demonstrated the good analytical efficiency in analysing relatively larger mRNA (i.e., *FAM134B* mRNA) in the clinical tissue samples. To validate our assay findings, we performed a qRT-PCR with the *FAM134B* specific primers to measure mRNA level in these tumor samples where mRNA expression level obtained is calculated as fold change with respect to a housekeeping gene (Fig. 5B). Clearly, the qRT-PCR data are in excellent agreement with our results (Fig. 5A versus 5B). These data validate the analytical accuracy of our method and suggest that our method may help in easy and cost effective screening of *FAM134B* mRNA in cancer patients.

3.5. Overall advantages of the assay

The current method offers several unique advantages over the existing approaches. First, our method does not involve prior enzymatic amplification, polyadenylation, or ligand modification of the target. These features have not only given better accuracy in the analysis by removing the chances of amplification bias but also significantly simplified the assay protocol/time. Second, the adoption of magnetic bead-based intimate mixing and washing steps in the assay enhances the specificity of target mRNA separation (*via* reducing the matrix effect of the complex biological samples and removing the non-specific targets). Third, we used relatively inexpensive disposable screen-printed electrodes (AUD$4 per electrode), which also avoids the tedious cleaning procedures associated with conventional disk electrodes thereby significantly reducing the cost and analysis time of the assay. Fourth, compared to the existing amplification-free electrochemical methods, as our method uses the direct adsorption *of* mRNA on unmodified gold electrode, it simplifies the assay design by avoiding the surface modification steps of the electrode such as probe-based self-assembled monolayer formation and hybridization procedures.

4. Conclusions

We have developed an amplification-free, sensitive and specific electrochemical method for the detection of *FAM134B* mRNA in oesophageal carcinomas. This newly developed electrochemical method avoids the tedious electrode fabrication procedure and was performed by directly adsorbing magnetically separated and purified target mRNA on the screen-printed gold electrode. We successfully challenged our assay performance in a cohort of heterogeneous clinical samples with a head-to-head comparison of mRNA level derived from tumor and non-tumor tissue pairs with good inter-assay reproducibility (% RSD = <5%, for n =3). Though the clinical utility of the assay has successfully been shown only for *FAM134B* mRNA detection in oesophageal cancers, we believe that an optimised format of the current method could also be used for detecting other important RNA biomarkers and thus can potentially be used in routine diagnosis of cancer in the point-of-care platform.

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Table 1. List of the oligonucleotide sequences used in this study.

Target genes and primers	**Oligonucleotide sequences (5'--3′)**
FAM134B synthetic mRNA	AGAGAAAACUUCCUCACCGGGUCGGUC
FAM134B-F	AGAGGTTTTTTAGGAATTTAGAGTTTTT
FAM134B-R	CCATCTTCAACTATACTTCCAAACAAA
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG
FAM134B capture probe (biotinylated)	TGACCGACCCAGTGAGGAAGTTTTCTC- Biotin

Figures

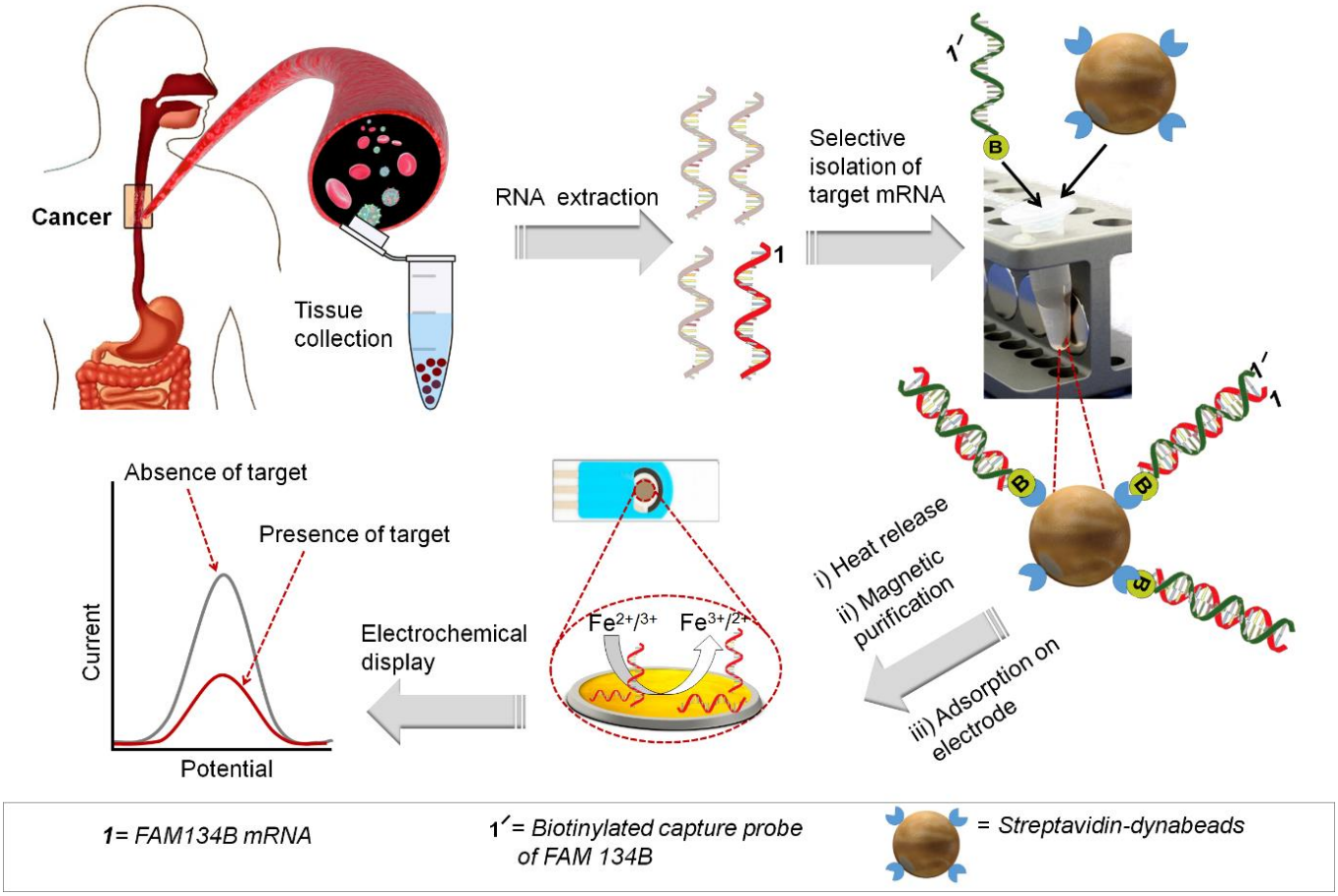

Fig. 1. Schematic of the amplification-free electrochemical detection of mRNA in oesophageal cancer. Magnetically captured target mRNA (top, right) were heat released and adsorbed on an unmodified screen-printed gold electrode (bottom). The relative presence of mRNA was analyzed by differential pulse voltammetry in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system (bottom, left).

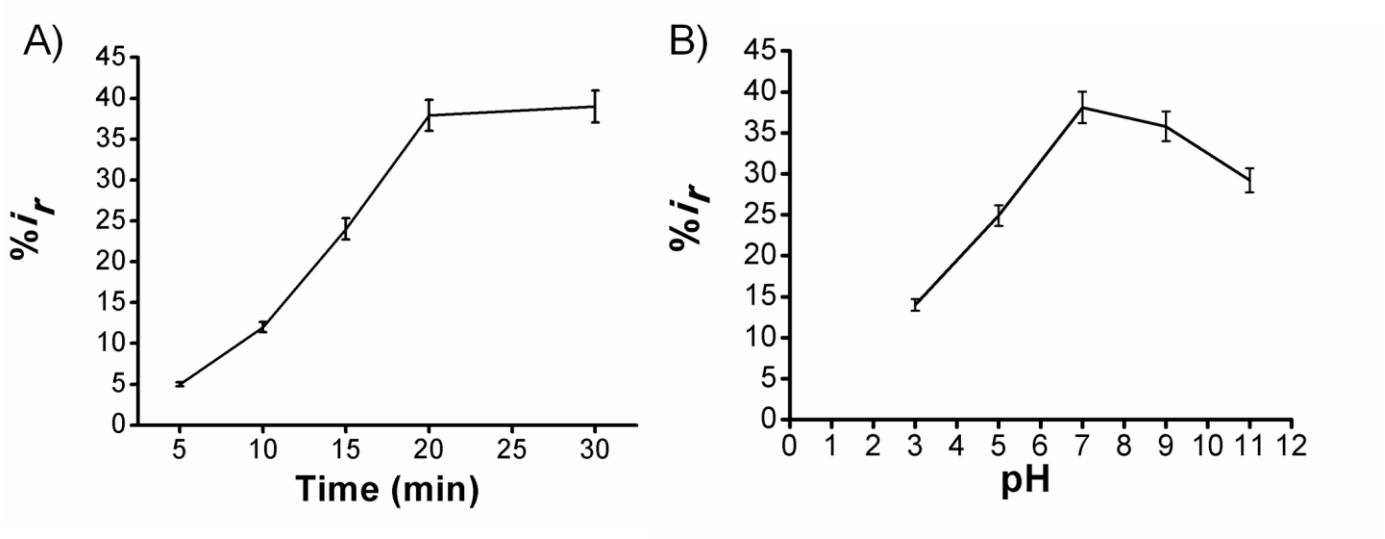

Fig. 2. Optimization of the mRNA detection assay. Relative DPV current changes for 100 pM adsorbed mRNA at designed (A) adsorption time (B) pH of the buffer. Error bars represent the standard deviation of measurements (% RSD = <5%, for n = 3).

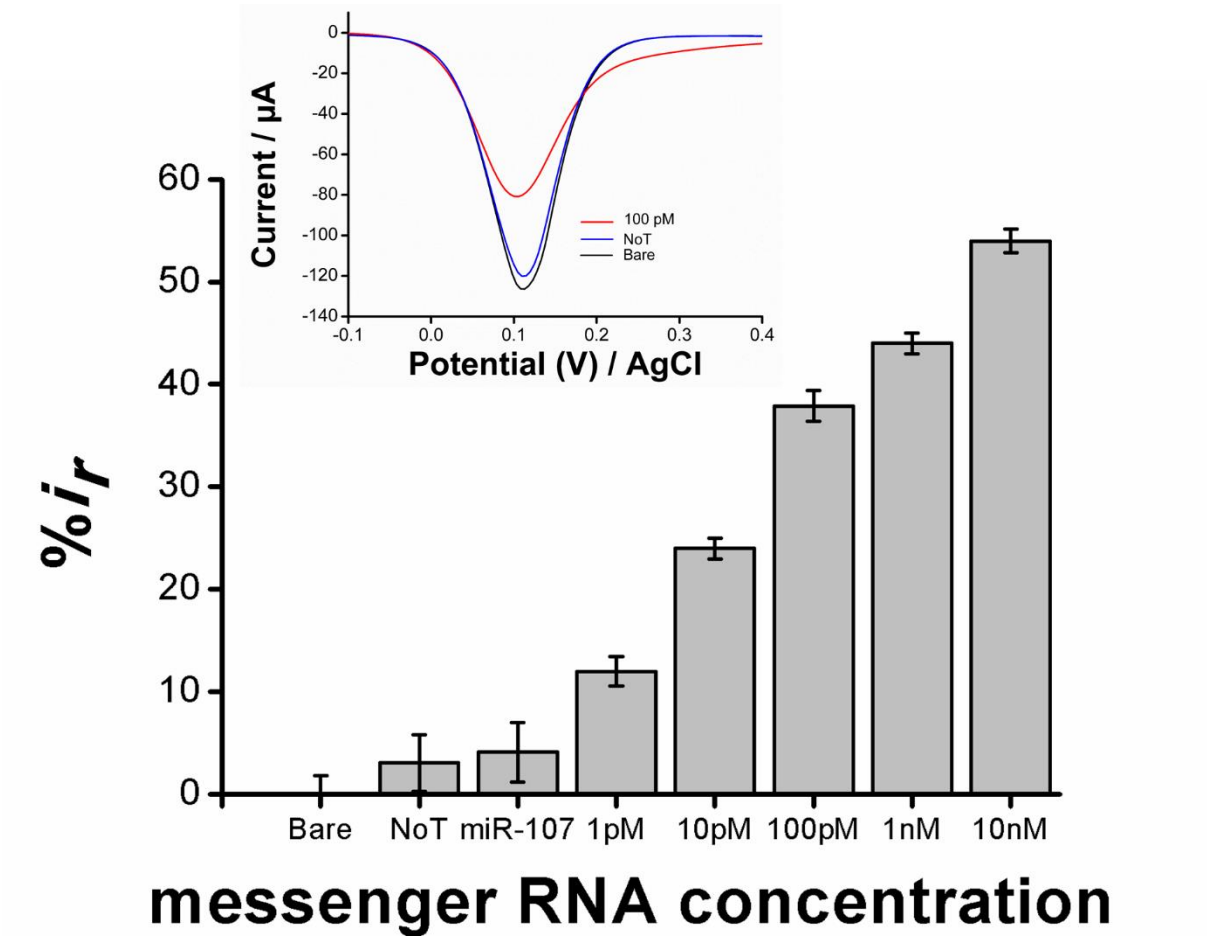

Fig. 3. Sensitivity of the assay. Relative DPV current response changes corresponding to increasing level of synthetic mRNA concentration. The responses for the NoT control and wrong target (miR-107) are also included in the main panel. Error bars represent the standard deviation of the measurements (% RSD = <5%, for *n* = 3). Inset, differential pulse voltammograms data corresponding to the 100 pM mRNA, NoT control and bare electrode.

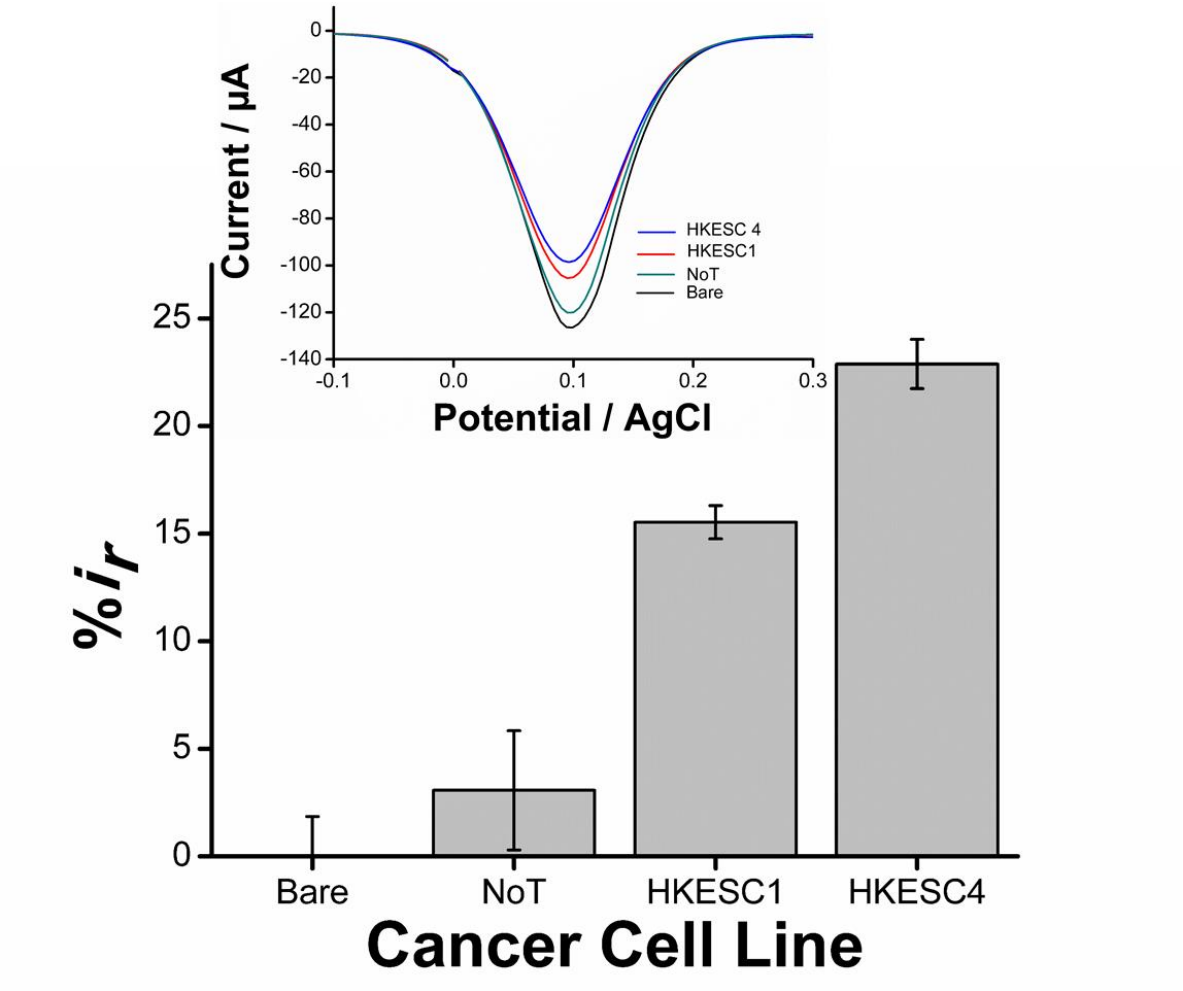

Fig. 4. Relative DPV current response changes corresponding to the differential level of *FAM134B* mRNA present in oesophageal cancer cell lines (HKESC1 and HKESC4). Error bar represents the standard deviation of the experiments (% RSD = <5%, for n = 3). Inset, differential pulse voltammograms data corresponding to the extracted *FAM134B* mRNA in cell lines, NoT control and bare electrode.

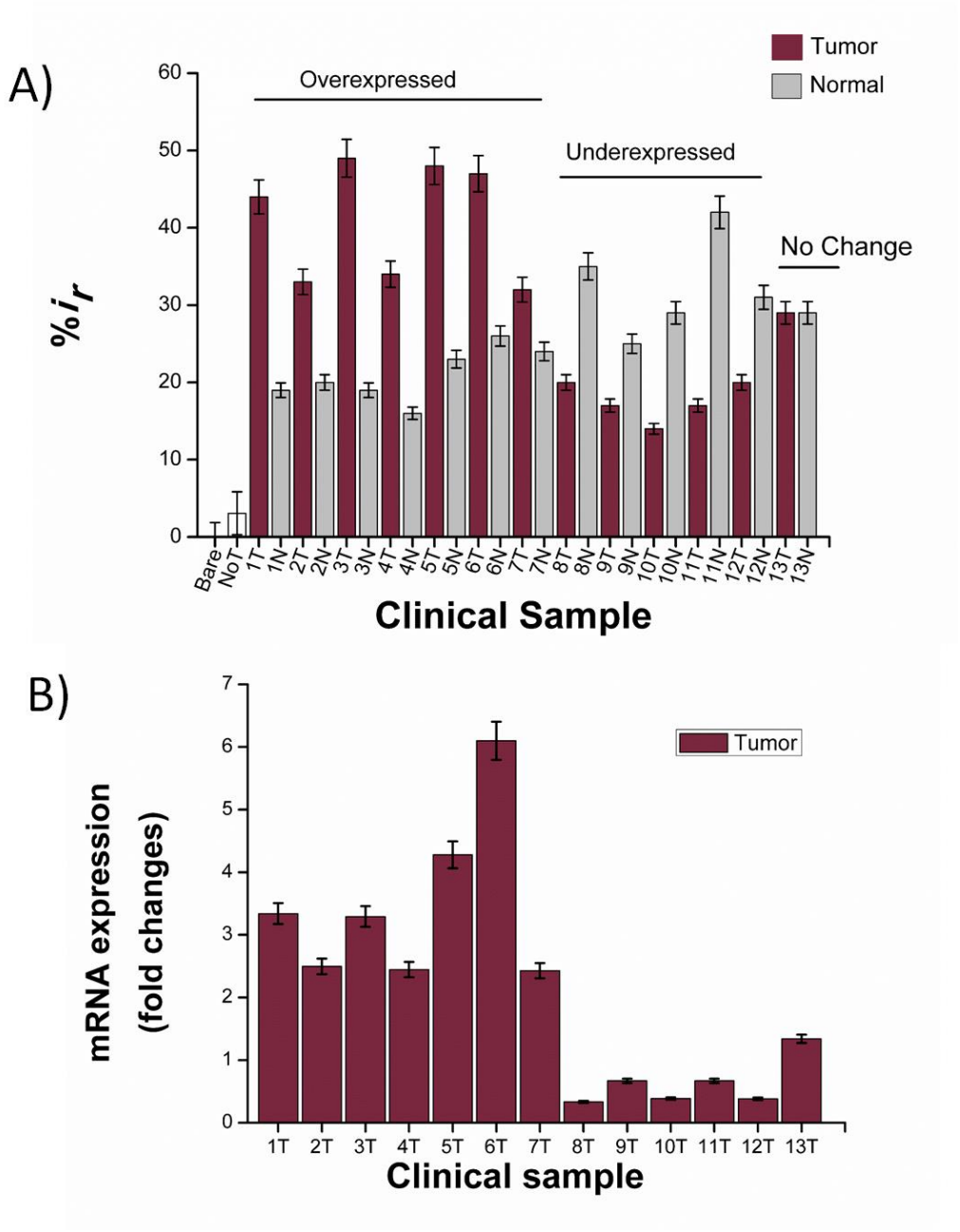

Fig. 5. Clinical application of the assay. (A) Relative DPV current response changes corresponding to the captured *FAM134B* mRNA derived from tumor and matched non-tumor patient tissue samples. (B) Representative mRNA expression analysis data (fold changes) of *FAM134B* mRNA present in tumor tissue samples.