Engineering translational approaches for molecular diagnostics of cancer: Multifunctional nanomaterials and electrochemical sensors for clinically relevant RNA biomarker detection

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Engineering translational approaches for molecular diagnostics of cancer: Multifunctional nanomaterials and electrochemical sensors for clinically relevant RNA biomarker detection

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To my loving parents, wife and son
Abstract

Over the past several years, the human transcriptome has been repeatedly interrogated, and with the recent breakthrough in sequencing technologies, ribonucleic acids (RNAs) comprising different coding and noncoding transcripts, such as messenger RNA (mRNA), microRNA (miRNA), and long-noncoding RNA (lncRNA), are becoming progressively crucial for revolutionising personalised cancer management. Improved diagnostics, prognostics and streamlined therapeutic potentiality makes them an excellent choice as biomarkers. Non-coding RNAs are key regulators of the gene expression network and are involved in the control of a range of important cellular pathways, such as cell cycle, cell proliferation, differentiation, apoptosis, and post-transcriptional regulation. Abnormalities in the expression of these RNAs can affect one or several of these cellular pathways, which might contribute towards initiation and progression of cancer. Despite recent advances in RNA-based fundamental research, their detection approaches are largely confined to laboratory-based molecular biology techniques, such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), microarrays, and RNA sequencing. Although the analytical performance and reliability of these methods are excellent, most of these methods require enzymatic amplification, cumbersome sample pre-treatment, multi-step assay protocol, high maintenance cost, and technical expertise. The development of a simple, sensitive, and low cost method that can be used for rapid detection of RNA biomarkers for a meaningful clinical application at the time and place of patient care (i.e., point-of-care) is of great importance to clinical and translational research.

This PhD project endeavours to engineer such translational approaches to circumvent the aforementioned challenges for developing an inexpensive, sensitive, specific, and portable biosensor platform. This thesis initially studies the biogenesis, diagnostic, and prognostic potential of RNA biomarkers followed by a comprehensive
appraisal of recent progress in the development of RNA biosensors with a special emphasis on electrochemical-detection approaches. We then report on the development of a biosensing platform consisting of four novel readout schemes for the simple, rapid, and inexpensive analysis of various RNA biomarkers (i.e., mRNAs, miRNAs and lncRNAs). First, employing the nucleotides’ affinity towards gold, we developed an amplification-free electrochemical assay for the detection of tumour-specific mRNAs. This straightforward sensor adopted differential pulse voltammetry to enable the readout using simple direct adsorption of magnetically isolated analytes on unmodified disposable electrodes. Subsequent to the development of this proof of concept sensor, we attempted to address the increasing demand for detecting the ultralow levels of RNAs from the complex biological sample via introducing two novel readout strategies for detecting miRNAs. Utilising the coupling of electrocatalytic strength of two in-house synthesised porous graphene oxide-loaded iron oxide (GO/IO hybrid material), gold-loaded nanoporous ferric oxide nanocubes (Au-NPFe2O3NC), and [Ru(NH3)6]3+/[Fe(CN)6]3- electrocatalytic cycle, two ultrasensitive assays were reported, where the detection was achieved by chronocoulometric (CC) charge measurement of surface bound cationic [Ru(NH3)6]3+, which was electrostatically attached to the anionic phosphate backbone of target RNAs. In our final readout strategies, we extended our approach towards a translational- focused assay platform which enabled naked-eye, colorimetric and electrochemical interrogation of lncRNA via 3,3′,5,5′-tetramethylbenzidine (TMB)/Horseradish peroxidase (HRP)-based colorimetric assay.

All of the readout platforms reported herein have shown excellent analytical performance with high sensitivity (LOD for mRNA and miRNA = picomolar to attomolar level, LOD for lncRNA = single cell approaching) and specificity. The applicability of the assays was also demonstrated in complex biological samples (a cohort of cancer cell lines and patient samples) with high reproducibility. The analytical performance of the
assays was also validated with the standard RT-qPCR approach. We believe that our research efforts will lead to the development of a translational-focused point-of-care platform for RNA analysis, which in turn will not only hold the potential to improve patient care and outcomes but might also prove to be a venture of immense commercial significance.
Declaration

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)

Md. Nazmul Islam
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Publications during candidature

Publications arising from this thesis


Conference Proceedings


Publications tangential to this thesis


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Chapter 1
General introduction
1.1 Background and motivation

On a global scale, by 2030 it is estimated that there will be 21.7 million new cases of cancer and 13 million cancer-related deaths. In Australia during 2017, there were approximately 134,174 new cases of cancer diagnosis with 47,753 deaths.\(^1\) Despite significant advancements in cancer diagnostics and treatment, overall mortality of cancer-related disease conditions is still significantly high (approximately 90%). This high cancer burden can be attributed to the lack of efficient and precise diagnostic technologies, as well as stable, specific, and sensitive biomarkers. With the revolutionary advancements in the next generation sequencing technologies, there has been immense interest in RNA-based biomarkers, mainly due to the emerging recognition of their widespread role in various pathophysiological phenomenon and cellular processes, including cell-cycle regulation, apoptosis, and post-transcriptional gene regulation.\(^2\)\(^-\)\(^4\)

There is a growing body of reports that suggest that dysregulated RNA molecules play a central role in the initiation and progression of several diseases including cancers.\(^2\)\(^,\)\(^5\)\(^-\)\(^6\) Furthermore, the release of disease-relevant biomolecules, including RNAs, into various body fluids, such as blood, has also been reported in numerous studies that demonstrate their potentiality in liquid biopsy.\(^7\) Cell transcriptome that is comprised of various coding and noncoding RNA species, such as mRNA, miRNA, and lncRNA, has thus emerged as a prominent non-invasive or minimally invasive biomarker for personalised cancer management.\(^2\) While monitoring mRNA expression in tissues has been an undisputed marker for diagnosis and monitoring of cancer, recent advancements in their efficient detection in body fluids, such as blood and plasma, have opened up new avenues of research. On the other hand, despite being the key molecules responsible for the synthesis of proteins, mRNAs denote only a small portion of the transcriptome. The vast majority of transcripts are in fact non-coding (ncRNAs), for example, miRNA and lncRNA. An increasing number of reports have demonstrated that these RNA species are not only up- or down-regulated in cancers, but that various genomic anomalies such as
copy number variations, single nucleotide polymorphisms, mutations, and alerted epigenetic patterns etc. are also reflected in altered composition of these RNA species. Therefore, monitoring the noncoding RNA (ncRNA) component of transcriptome can prove to be a powerful tool for cancer management.

Despite the huge potential for RNAs in the diagnosis and prognosis of cancer, their detection approaches, to date, are generally confined to several classic nucleic acid detection methods such as Northern blotting, microarray, RT-qPCR, and next generation sequencing. Although there is no concern about the analytical reliability of these approaches, their scope in the off-laboratory and resource-limited settings, where sophisticated and expensive instruments might not be available, is limited. In addition, these methods have other inherent drawbacks. Northern blotting is time-consuming and requires large sample volumes. Microarray, being a high throughput technique, is usually more suitable for discovery purpose rather than specific diagnostic applications. RT-qPCR, in comparison, is relatively sensitive, however, apart from their reliance on extensive laboratory infrastructure and technical support, they rely on lengthy assay steps (time-consuming sample preparation and purification, cDNA conversion, and qPCR).

The development of a well-grounded platform that might be suitable for clinically relevant RNA screening in resource-poor settings is, therefore, a priority. On that note, a great deal of research has been carried out to find relatively robust, sensitive, and specific methods, leading to several biosensor-based RNA sensing approaches coupled with optical and electrochemical readouts. Electrochemical detection methods are considered particularly attractive for bioanalysis, because of their low cost, ease of automatisation, high sensitivity, and selectivity. However, the functionality of electrochemical RNA sensors is still confined to the proof-of-concept studies, and several challenges to transform the technologies into routine clinical applications are yet to be addressed. These challenges include structural instability, non-specific interference from
non-targets, and requirement of ultrasensitivity. In addition, most of the current assays involve complex chemistries, consume large volumes of analytes, and rely on expensive enzymes and labelling agents.

As a matter of fact, most of these electrochemical sensors might not meet the extremely low sensitivity requirement for RNA quantification in clinical samples, in particular due to the lack of adequate signal enhancement abilities in the assay. One way to enhance the readout signal to enable a sensitive RNA detection approach would be the use of nanomaterial-based signal amplification strategies.\textsuperscript{17-18} With the advancement of nanotechnology, in particular, engineered biofunctionalised nanomaterials, nanomaterials have attracted extensive attention in designing of ultrasensitive RNA-sensing platforms. They have been functionalised in biosensors as target immobilisation platforms, signal enhancing probes, electrocatalysts, and tracers, to obtain amplified signals.\textsuperscript{17-19} Moreover, the intrinsic superparamagnetic properties of many of these nanomaterials greatly enhance their utilisation in the magnetic separation and purification of target analytes.\textsuperscript{20}

In the recent years, attention has been shifted towards the fabrication of hybrid composite nanomaterials with superior physicochemical, and electrochemical functionalities, resulting from the synergetic advantages of both the nanoparticles in the composites.\textsuperscript{21-23} As an example, iron oxides (Fe\textsubscript{3}O\textsubscript{4} or Fe\textsubscript{2}O\textsubscript{3}) containing gold nanoparticles (AuNPs) exhibit the combined advantages and serendipitous properties of both iron oxide nanoparticles (high surface area, conductivity, thermal/chemical stability and superparamagnetism) and gold nanoparticles (i.e., affinity interaction of DNA/RNA with gold). In this PhD project, our research endeavour is focused on the development of versatile biosensing platform consisting of four novel readout strategies for RNA biomarker detection. Our aim is to develop a platform that would have the high potentiality to address the criteria set by international experts for a point-of-care diagnostic tool, i.e., a simple, accurate, and portable platform, which enables quick
detection of RNA-based biomarkers using the minimal equipment. Initially, we developed a simple, amplification-free electrochemical approach for mRNA detection, which avoids any sort of electrode modification, and relies on the direct adsorption of magnetically purified RNA samples, on an unmodified disposable gold electrode. After realising this straightforward assay, we further exploited the coupling of intrinsic electrocatalytic activities of two new classes of nanoporous hybrid nanomaterials with multiple electrocatalytic cycles, to obtain a significantly enhanced assay sensitivity while detecting two cancer-associated miRNAs. Our final readout strategy then introduces a translational-focused assay platform, which enables naked-eye, colorimetric and electrochemical estimation of lncRNAs.

1.2 Aims

The overall aim of this PhD study is to design and develop inexpensive, and simple biosensing platforms capable of efficiently detecting a range of important cancer-related RNA targets in clinical samples with high specificity and sensitivity. After a comprehensive literature review on the topic, this research will investigate the development of electrochemical assays for RNA biomarker detection. To address some of the existing limitations of RNA biosensors concerning with poor specificity and sensitivity, two new types of functional nanomaterials with high surface functionality and electrocatalytic activity will be employed in the sensor design. To provide a translational direction, the effort will be extended towards the development of a detection platform for colorimetric (naked-eye) detection of RNA. This will be achieved with the following specific aims.
The specific aims are

- To develop electrochemistry based assays for mRNA, miRNA, and lncRNA detection and quantification;
- To develop alternative naked-eye (colorimetric) assay for lncRNA; and
- To apply the assay platforms for clinical sample analysis.

1.3 Significance of the project

The importance of the research relates to the following scientific advances.

First, one of the major limitations in cancer diagnosis has been the lack of minimally invasive biomarkers, which can be detected and interrogated within complex biological samples with high sensitivity and specificity. In this regard, various species of clinically relevant RNAs that are actively associated with the initiation and progression of cancers might prove to be a promising class of sensitive and specific biomarker. In this thesis, we have selected a set of RNA markers such as mRNA (FAM134B), small ncRNAs (miR-21 and miR-107) and lncRNA (Hox antisense intergenic RNA [HOTAIR]), which have been reported to be highly specific for different stages of various cancers. In addition, these biomarkers have the ability to work as liquid biopsy for non- or minimally invasive interrogation of cancer.

Second, the assays reported in this thesis attempted to meet the increasing demand for detecting the ultralow levels of RNAs from trace amounts of complex biological sample. All the readout platforms have shown excellent sensitivity, ranging from picomolar to attomolar level (1.0 pM, mRNA; 1.0 femtomolar, miR-21 and 100 attomolar, miR-107). Moreover, while analysing assay performance in cancer cell lines, we found an exceptionally low LOD for HOTAIR lncRNA (approaching single cell). Such low LOD is adequate to retrieve disease information from a minute amount of clinical sample. This high sensitivity could be attributed to a number of reasonable assay
components such as a) high catalytic activity and immense sample loading capacity of the newly synthesised nanoporous gold and graphene-based iron oxide materials; b) additional signal enhancement with multiple electrocatalytic cycles, and c) biotin enrichment of the isothermal amplicons.

Third, our assays have addressed the poor specificity issues reported in many RNA analysis platforms. Due to a high level of sequence overlap between various RNA molecules, detection of miRNA often becomes very challenging in the background of abundant non-specific molecules. Repetitive magnetic bead-based washing, isolation, and purification step ensured the high specificity achieved in our assay.

Fourth, our assay platforms have also improved the common concept of RNA biosensing technology, which often relies on sensor fabrications, hybridisation of targets with surface bound capture probes, and use of electroactive ligands. One of the foremost significances here is the use of single-use disposable screen-printed electrodes, which successfully eliminates the utilisation of typical electrochemical cells, as well as counter and reference electrodes, thereby offering a relatively inexpensive (approximately AUD $5) and rapid platform. We have also successfully demonstrated a broad platform technology, which enabled the naked-eye screening of HOTAIR lncRNA. Given the fact that up-regulated HOTAIR is a stable and specific biomarker in most of the cancers, we believe that this assay platform could work as a potential transformative technology for the inexpensive and quick, first-pass screening of a large number of samples specifically in less developed countries, where access to the state-of-art diagnostic facilities remains inadequate. Overall, we believe that our approach can be further extended towards the development of a multiplexed electrochemical device that could have huge potential in miniaturisation and portability of the biosensor with minimal cost, space, and power requirements. The global point of care diagnostics testing market was estimated to be approximately $18.4 billion in 2017, which is expected to rise to around $43.33 billion
during the next five years. It can, therefore, be concluded that if a significant amount of effort is invested in circumventing the remaining challenges in developing fully functional, point-of-care RNA biomarker screening tools, these potential sensors might lead to a multibillion-dollar business. Thus our studies might not only pave the way for improved patient care, specifically in impoverished regions, by developing inexpensive, sensitive, and specific detection platforms for personalised and point-of-care clinical applications but also prove to be a productive commercial venture.

1.4 Structure of the thesis

This thesis includes seven chapters. Chapters 2, 3, 4, 5, 6, and 7 are a collection of journal papers that have been published or are under consideration.

Chapter 1. This chapter introduces the aims, background and significance of this research.

Chapter 2. This chapter presents a comprehensive literature review focusing on biology, clinical significance and detection approaches of RNA biomarkers (e.g., mRNAs, miRNAs, lncRNAs). In addition to the conventional RNA analysis platforms, the review drew a comprehensive appraisal of recent developments of RNA biosensors with a special focus on the electrochemical-detection strategies. The major challenges associated both conventional and current biosensor-based strategies were identified. The current requirements that still need to be met for effective screening of RNA biomarkers in both research and clinical settings have also been discussed. Chapter 2 provides the reasoning for the research described in the subsequent chapters.

Chapter 3. This chapter reports on the development of a new amplification-free electrochemical detection assay for the analysis mRNA. The assay employed the direct adsorption of mRNA onto an unmodified, screen-printed gold electrode, followed by
differential pulse voltammetry (DPV) readout in the presence of a \([\text{Fe(CN)}_6]^{4-/3-}\) redox system. The assay avoids any form of target modification and complex surface fabrication steps, thereby demonstrating an extremely simple platform for mRNA detection. The clinical applicability of the assay was tested via detecting FAM134B mRNA, a potential cancer biomarker, in tissue samples derived from patients with oesophageal carcinomas.

**Chapter 4.** This chapter describes the development of a highly sensitive electrochemical assay for the quantification of miRNA (miR-21), by exploiting the intrinsic catalytic activity of graphene oxide-loaded iron oxide (GO/IO hybrid material) nanoparticles. Upon studying the apparent Michaelis–Menten constant \((K_m^{\text{app}})\) of the nanomaterial towards the reduction of RuHex, chronocoulometric (CC) readout was used to measure charge compensating RuHex molecules, which were bound with a target functionalised GO/IO hybrid-modified, disposable electrode.

**Chapter 5.** Extending on the work from Chapter 4, Chapter 5 discusses the further enhancement in the sensitivity of miRNA detection (LOD = 100 aM) assay. Exploiting the high surface loading and catalytic activity, a newly synthesised, highly porous, gold-loaded superparamagnetic iron oxide nanocubes (Au-NPFe\(_2\)O\(_3\)NC)-modified sensor was designed for CC readout of miRNA (miR-107). To accelerate the detection signal, a coupling of \([\text{Fe(CN)}_6]^{3-/4-}\) and \([\text{Ru(NH}_3)_6]^{3+}\) was used. The applicability of the method was tested and validated in a set of clinical samples.

**Chapter 6.** After successful demonstration of highly sensitive and specific biosensors for mRNA and miRNA in Chapters 3, 4, and 5, the research moved onto a new platform for detecting lncRNA (HOTAIR) via three potentially useful readout approaches (i.e., naked-eye, UV-vis absorbance, and amperometric readouts) under one assay protocol. Instead
of using conventional PCR amplification, a modified recombinase polymerase amplification (RPA) isothermal amplification technique was adopted for the random biotin insertion on HOTAIR amplicons, which were further interrogated in a TMB/HRP-based colorimetric reaction followed by the subsequent naked-eye and other readouts.

Chapter 7, Conclusions are provided in Chapter 7.
1.5 References


This chapter presents an overview of the biogenesis as well as diagnostic and prognostic potentials of clinically relevant RNAs (e.g. mRNAs, miRNAs, lncRNAs). The review further outlined a comprehensive appraisal of recent developments of RNA biosensors with a special emphasis on the electrochemical-detection strategies. The major challenges associated both conventional and current biosensor-based strategies were identified. The current requirements that still need to be met for effective screening of RNA biomarkers in both research and clinical settings have also been discussed.

This extensive literature review provides the basis for our choice of RNA biomarkers, and their detection techniques in the subsequent chapters.

Chapter 2 is included as it appears in Small Method (2017)
Statement of Contribution to Co-authored Published Paper

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**My contribution to the published paper involved:**

- Literature review
- Manuscript preparation.
- Responding reviewers

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RNA Biomarkers: Diagnostic and Prognostic Potentials and Recent Developments of Electrochemical Biosensors

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1. Introduction

Ribonucleic acid (RNA) biomarkers comprising different coding and noncoding transcriptome such as messenger RNA (mRNA), microRNA (miRNA), and long noncoding RNA (lncRNA) are becoming progressively crucial in understanding disease diagnosis and prognosis due to their recognized physiological role in gene expression and regulation. Dysregulation of these RNA biomarkers is involved in the initiation and progression of several diseases including cancers. Among the different types of RNAs, protein-coding RNAs are exclusively represented by mRNAs where aberrant mRNA expression could initiate cancer by altering the DNA methylation pattern and different cellular regulatory pathways such as chromatin modifications, cell adhesion, and cell cycles. A number of recent studies have reported a strong correlation between aberrantly expressed mRNA levels and various cancers including breast cancer, lung cancer, malignant melanoma, and hepatocellular carcinoma. During mRNA biogenesis (i.e., transcription), mature mRNA is produced from pre-mRNA transcripts via a splicing process (i.e., introns removal from nascent RNA followed by the joining of exons). Any alterations in this splicing pattern, referred to as "alternative splicing", may trigger the production of miscellaneous mRNA isomers, which further generates diverse protein variants including oncoproteins. For example, alternatively spliced Fas mRNA isoform (exon 6 is missing) produces a soluble deregulatory protein known as sFas which triggers abnormal cell proliferation in cancer.

These protein-coding mRNA genes represent only a small percentage of the total genome. On the contrary, more than 90% of the genomic DNA generates noncoding RNAs (ncRNAs), consisting of housekeeping (e.g., ribosomal RNA, transfer RNA) and regulatory noncoding RNAs (e.g., miRNA and lncRNA). In recent years, research focus has mostly been shifted toward discovery and translational studies of biomarkers based on miRNAs (19–25 bases) and lncRNAs (200 bases to 100 kilobases), due to their strong ability in regulating the gene expression.
expression. These RNAs do not take part in protein production (i.e., translation) but they actively regulate the post-transcriptional gene expression and remodeling of the epigenome (i.e., DNA methylation and histone modification). They are crucial for a range of controlled biological processes such as cell cycle regulation, pluripotency, retrotransposon silencing, etc. These controlled processes may be affected and eventually could contribute to cancer development when the sequences of noncoding RNAs have anomalies such as copy number variations, single nucleotide polymorphism, and mutations. For instance, it has been reported that dysregulated IncRNA, HOX transcript antisense RNA (HOTAIR), interacted with polycomb repressive complex 2 (PRC2) complex and actively involved in the progression of liver and breast cancer, whereas MALAT1 B7 IncRNA affected the RNA splicing functions and triggered several types of cancers such as breast, lung, uterus, pancreas, colon, prostate, and liver cancer. Until now, nucleic-acid-detection-based techniques such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), microarrays, and RNA sequencing methods have widely been applied for the effective analysis of RNA levels. These methods typically require some forms of amplification steps, cumbersome sample pretreatment procedure, and expensive instrumentation. Additionally, they often cannot amplify RNA sequences without a poly(A) tail due to the use of oligo(dT) primers for the amplification purpose. Additionally, relatively large sample volumes are required for sensing RNA biomarkers in body fluids to avoid sample heterogeneity, resulting from distinct physiological and systemic differences of clinical samples. In this regard, a great deal of research has been carried out to find relatively robust, accurate, and effective methods, leading to several nanotechnology-based RNA sensing approaches coupled with optical and electrochemical readouts. These approaches offer relatively easy sampling procedures, rapid and cost-effective analysis, portability, label-free and amplification-free options. Among these methods, electrochemical methods have evidently achieved the ultrahigh sensitivity and selectivity with the high potential for multiplexed analysis in point-of-care platform. However, the functionality of electrochemical RNA sensors is still confined in the proof-of-concept studies and several challenges are yet to be addressed to transform the technologies into routine clinical applications.

Here, we review the recent developments of RNA bio-sensing approaches, especially electrochemical approaches. The diagnostic and prognostic roles of RNA biomarkers are briefly discussed, followed by a specific discussion on current requirements that still need to be met for screening of RNA biomarkers in both research and clinical settings. We have also highlighted the major technical and biological challenges involved in the existing RNA detection strategies and provided suggestions for the future direction of the field.

2. RNA Biogenesis

The rate and extent of RNA biogenesis in the nucleus and their subsequent role in protein synthesis or gene regulation are subjected to several finely controlled pathways driven by various transcriptional gene expression and remodeling of the epigenome (i.e., DNA methylation and histone modification). They are crucial for a range of controlled biological processes such as cell cycle regulation, pluripotency, retrotransposon silencing, etc. These controlled processes may be affected and eventually could contribute to cancer development when the sequences of noncoding RNAs have anomalies such as copy number variations, single nucleotide polymorphism, and mutations. For instance, it has been reported that dysregulated IncRNA, HOX transcript antisense RNA (HOTAIR), interacted with polycomb repressive complex 2 (PRC2) complex and actively involved in the progression of liver and breast cancer, whereas MALAT1 B7 IncRNA affected the RNA splicing functions and triggered several types of cancers such as breast, lung, uterus, pancreas, colon, prostate, and liver cancer. Until now, nucleic-acid-detection-based techniques such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), microarrays, and RNA sequencing methods have widely been applied for the effective analysis of RNA levels. These methods typically require some forms of amplification steps, cumbersome sample pretreatment procedure, and expensive instrumentation. Additionally, they often cannot amplify RNA sequences without a poly(A) tail due to the use of oligo(dT) primers for the amplification purpose. Additionally, relatively large sample volumes are required for sensing RNA biomarkers in body fluids to avoid sample heterogeneity, resulting from distinct physiological and systemic differences of clinical samples. In this regard, a great deal of research has been carried out to find relatively robust, accurate, and effective methods, leading to several nanotechnology-based RNA sensing approaches coupled with optical and electrochemical readouts. These approaches offer relatively easy sampling procedures, rapid and cost-effective analysis, portability, label-free and amplification-free options. Among these methods, electrochemical methods have evidently achieved the ultrahigh sensitivity and selectivity with the high potential for multiplexed analysis in point-of-care platform. However, the functionality of electrochemical RNA sensors is still confined in the proof-of-concept studies and several challenges are yet to be addressed to transform the technologies into routine clinical applications.

Here, we review the recent developments of RNA bio-sensing approaches, especially electrochemical approaches. The diagnostic and prognostic roles of RNA biomarkers are briefly discussed, followed by a specific discussion on current requirements that still need to be met for screening of RNA biomarkers in both research and clinical settings. We have also highlighted the major technical and biological challenges involved in the existing RNA detection strategies and provided suggestions for the future direction of the field.

2. RNA Biogenesis

The rate and extent of RNA biogenesis in the nucleus and their subsequent role in protein synthesis or gene regulation are subjected to several finely controlled pathways driven by various
cellular factors and enzymes.\textsuperscript{[45–47]} mRNA is synthesized in the nucleus from the DNA template of a target gene via a process known as "transcription." The transcription process is catalyzed by a large molecular enzyme referred to as RNA polymerase II (RNA Pol II). RNA Pol II recognizes and binds to the promoter site of the template DNA with the help of a group of proteins known as general transcription factors (GTFs).\textsuperscript{[48]} First, GTFs (e.g., TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) assemble near the template DNA and assist RNA Pol II to recognize and bind the specific promoter region of the DNA thereby forming the preinitiation complex (PIC). The PIC then recruits DNA helicases, which facilitate DNA to expose their template strand for the initiation of RNA synthesis. After the initiation of transcription, the PIC leaves the template strand and RNA Pol II starts the elongation process and transcribes hundreds of kilobases of pre-mRNA transcripts. After that the RNA Pol II is dissociated from DNA template and started re-initiation of downstream transcription pathways.\textsuperscript{[45,46,49]} The resultant nascent pre-mRNA undertakes three major processing steps to form mature mRNAs, which usually occur co-transcriptionally (i.e., transcription and pre-mRNA processing happens simultaneously). These steps are 5′ capping, splicing and 3′ adenylation.\textsuperscript{[45,50]} Upon completion of the processing steps, mature mRNA is packaged inside a complex consisted of RNA binding proteins and other export factors.\textsuperscript{[45]} Then this complex is transported from the nucleus to cytoplasm through the nuclear pore complex which spans over the nuclear envelope (NPC) for further activities.\textsuperscript{[45]}

Similarly, miRNAs are synthesized in the nucleus by the same transcriptional machinery of mRNA biogenesis.\textsuperscript{[51]} However, after their export into the cytoplasm, they skip the protein synthesis steps and keep a role in gene regulation.\textsuperscript{[37,52]} During miRNA biogenesis (Figure 1), miRNA genes are first converted to hairpin-structured primary miRNA (pri-miRNA) by either RNA pol I or II via transcription process. Pri-miRNAs are then cleaved and processed by a distinct protein complex known as the microprocessor complex, which consists of ribonuclease enzyme Drosha and RNA binding protein DGCGR8 (also known as pasha). In the next step, the miRNAs are then exported to the cytoplasm and further cleaved by Dicer (an enzyme from RNase III family) to form miRNA duplexes. Following strand separation of the duplexes, mature miRNAs are produced, which start to accumulate various proteins and enzymes to form RNA-induced silencing complex (RISC). miRNA exerts its inhibitory and regulatory action via RISC-induced RNA degradation and post-translational inhibition.\textsuperscript{[52]} Although many aspects of the lncRNA biogenesis pathways are relatively less understood, it has been reported that the key processes involved in most of the recently discovered lncRNAs follow the common mRNA biogenesis pathway.\textsuperscript{[51,53]} However, a few other alternative pathways for the biogenesis of lncRNAs have been described, which employ another type of polymerase enzyme known as RNA pol III at the gene promoters and do not undergo a polyadenylation processing step.\textsuperscript{[51,54]}

3. Diagnostic and Prognostic Roles of RNA

RNAs are the key regulators of gene expression network and involved in controlling cell-cycle, cell proliferation, differentiation, apoptosis, metabolism, and post-transcriptional pathways.\textsuperscript{[2–6,8]} Dysregulated RNAs can affect one or several of these cellular pathways, resulting in tumor initiation and progression. Thus, they can be used in diagnosis, prognosis, and
therapy monitoring of cancers. Although the specificity and reproducibility of circulating miRNA biomarkers is a subject to recent contradictory discussion (see Section 3.3), they offer several distinct advantages such as early detection, high stability, and ability to work as liquid biopsy for minimally invasive monitoring of cancer.

**Early Detection:** A number of studies have confirmed that RNA biomarkers have important clinical implications as the early indicator of cancer. This is mostly due to the fact that during the early development of tumor cells, they release significant amounts of RNA into the blood circulation and mostly being upregulated. For example, miR-200 was reported as an effective biomarker for the early detection of ovarian cancer.

**High Stability of Circulating miRNAs:** Compared to mRNAs and lncRNAs, miRNAs have significantly higher stability in a clinical sample and can show robust expression patterns. Several studies have shown that circulating miRNAs are resistant to the degradation by RNases and also unaffected in extreme condition such as freeze–thawing, long-term storage, high pH, and temperature. This high stability can be explained by the fact that they are well protected inside the microvesicles such as exosomes and apoptotic bodies, or attached with RNA-binding proteins.

**Circulating Biomarker for Minimally Invasive Detection of Cancers:** Current techniques for cancer diagnosis commonly require invasive tissue biopsy, which is not always clinically feasible and associated with pain. However, tumor-specific circulating RNAs (miRNAs) available in accessible biological fluids such as serum, plasma, urine, saliva could bypass the need of tissue biopsy. In 2010, Weber et al. assessed 12 different body fluids including urine, saliva, plasma, tears, breast milk, peritoneal fluid, pleural fluid, seminal fluid, amniotic fluid, bronchial lavage, cerebrospinal fluid, and colostrum and showed that miRNAs are present in these body fluids. This wide distribution of miRNA in most biological fluids makes them a promising circulating biomarker for less or noninvasive diagnosis and more personalized monitoring of diseases. The prominent features of using miRNAs as a circulating biomarker are that they are minimally painful, and allow clinicians to quickly assess disease development and response to therapeutics. For example, Debernardi et al. showed a noninvasive profiling of cell-free miRNAs extracted from the urine of patients with pancreatic ductal adenocarcinoma and chronic pancreatitis can efficiently differentiate the early and late stage tumors.

### 3.1. Diagnostic Roles

Different types of cancers have distinctive signature of mRNA expression pattern. For example, Miura et al. demonstrated that the expression of human telomerase reverse transcriptase (hTERT) mRNA and epidermal growth factor receptor (EGFR) mRNA were associated with lung cancer metastasis. In this study, real-time RT-PCR was used to measure the serum hTERT mRNA and EGFR mRNA levels from 112 patients with lung cancer and 80 normal individuals. The levels of the mRNAs were significantly interrelated with tumor size, number, metastasis, and recurrence in lung cancer which elucidated hTERT and EGFR mRNA’s role as diagnostic biomarker for lung cancer. Several other mRNAs were reported as the diagnostic indicators of epithelial ovarian cancer (EOC) due to their specific expression patterns in EOC. For instance, p53, BCL-2, BAX mRNAs are downregulated in EOC while ASAP1 mRNA is overexpressed. A genome wide analyses of mRNA expression in 136 breast cancer patients performed via RNA sequencing (RNA-seq) also showed that a number of 32 mRNAs can sensitively act as the diagnostic biomarkers for breast cancer.

Alternative splicing can also trigger the production of aberrant mRNA isoforms leading to the progression of many diseases including cancer. This could happen due to the mutations in the components of splicing machineries. A number of tumor-specific alternative splicing events have been reportedly considered as the effective diagnostic markers and therapeutic targets in different cancers such as breast and lung cancer. Additionally, mRNA fusion, which is usually generated from gene fusion during aberrant chromosomal rearrangement events, have emerged as the new class of sensitive and specific diagnostic biomarkers for many cancers. For instance, TMPPRSS2-ERG and BCR-ABL mRNA fusions have been used as diagnostic markers for prostate cancer and leukemia, respectively.

In recent years, much has been dedicated to explore the diagnostic significance of miRNAs in cancer. In 2002, Calin et al. drew a relationship between cancer- and tissue-derived miRNA where it was demonstrated that miRNA cluster miR 15/16 was downexpressed in chronic lymphocytic leukemia. In a large cohort study, Volinia et al. screened 540 tumor samples derived from lung, breast, stomach, prostate, colon, and pancreas cancers, and identified 43 dysregulated miRNAs as diagnostic biomarkers. Later, in 2008, the diagnostic role of circulating miRNA in B-cell lymphoma was reported by Lawrie et al. This study reported that the levels of miR-21 and miR-155 were significantly higher in the serum sample derived from cancer patients compared to that of normal sample. Since then, an increasing number of cancer-related circulating miRNA have been identified and studied. For example, Zhou et al. showed the positive diagnostic potential of a group of circulating miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) in a hepatocellular carcinoma (HCC) patient sample. In recent times, there is also an increasing interest on lncRNAs’ research due to their wide biological functions and diagnostic potential. Among different lncRNAs, dysregulated HOTAIR is of great interest due to their robust expression pattern and active involvement in the prognosis, metastasis, and recurrence of a range of cancers. For example, Gupta et al. showed that the rate of HOTAIR transcription in metastatic breast cancer was almost 2000-fold raised compared to that of control. This significantly elevated levels of HOTAIR in cancer cells demonstrates their high diagnostic potential.

### 3.2. Prognostic Roles

Aberrant mRNAs have also been considered as a good indicator to predict and track tumor prognosis. In 2004, Spentzos et al.
showed the positive prognostic behavior of a number of 115 mRNAs present in 68 patients with ovarian cancers where the mRNA profiling data successfully could predict the survival rate of patients. In another genome-wide approach, De Sousa et al. [86] recruited 1100 patients with colorectal cancer where mRNA biomarkers successfully commented on the prognosis of colorectal cancer. In a few other related studies, aberrantly expressed NOTCH1 mRNA was found to be interrelated with poor survival of patients with non-Hodgkin lymphoma [87] while Cyclin D1 and TS mRNA was linked with the poor survival of breast cancer patients. [16]

Compared to mRNA, miRNAs are more stable, [88] which explains their good prognostic behavior in cancer. Over the past several years, a large number of miRNAs have been reported as effective indicators for the cancer prognosis. [89–91] It was demonstrated that overexpressed miR-21 was significantly linked with the poor prognosis and therapeutic outcome of patients with colon cancer, [90] while downregulated let-7 miRNA was correlated with poor survival of patients with lung cancer. [91] In another study, the expression pattern of miR-335 and miR-126 were lost when the condition of patients with breast cancer deteriorated, suggesting their active role in poor metastasis free survival (i.e., survival till the metastasis is detected). [92] Apart from miRNAs, few of the recently discovered lncRNAs also showed fairly good prognostic behavior. [13] For example, Kogo et al. [90] studied the expression pattern of HOTAIR in 100 tissue samples collected from colon cancer and compared them with the matched normal sample where substantial changes in HOTAIR expression was observed. This study was extended to test samples derived from liver cancer where a strong correlation of upregulated HOTAIR with liver metastasis and poor disease prognosis were observed. In a similar approach, Liu et al. [93] detected the overexpression of DANCRC lncRNA in colorectal cancer tissues compared to that of matched normal tissues and their expression was highly correlated with the histologic grade and lymph node metastasis (Table 1 presents an overview of diagnostic and prognostic potentials of RNA biomarkers in various cancers).

3.3. Contradictory Findings on Circulating miRNAs as a Biomarker: Critical Evaluation

Despite recent advances in miRNA biomarker research, circulating miRNAs have not made the translation yet into the clinic, partly due to their contradictory specificity and inconsistent reproducibility issues as cancer biomarkers under different physiological and pathological conditions. [98–104] For example, Haider et al. [98] reviewed a total of 416 circulating miRNA biomarkers in 57 noncancerous diseases and identified that the differential expression of the miR-16, -155, -21, -126, and -223 biomarkers were not specific for any particular diseases, rather associated with at least 10 noncancerous conditions. Witwer et al. [99] also published an extensive review on circulating miRNAs and showed the poor specificity and reproducibility of circulating miRNA as potential cancer biomarkers. In another study, Leidner et al. [100] reviewed the irregular reproducibility of breast cancer related miR-21 and miR-155 biomarkers. Egidi et al. [100] also pointed out the poor specificity issue of circulating miRNA. They studied the expression level of miR-21 and miR-141 in 38 prostate cancer patients after their radical prostatectomy (i.e., surgical removal of prostate gland) and showed that serum samples collected from patients with no prostate glands had significantly elevated level of miRNAs. This finding raised the concern as to whether elevated levels of miR-21 and miR-141 in samples of prostate cancer patients collected after their radical prostatectomy is indicative of prostate cancer or simply related to general disease states such as inflammation.

Although, the miRNAs role as a cancer biomarker is under careful consideration, it is widely accepted that various biological and technical pitfalls associated with miRNA studies, including lack of automation and standard workflows in laboratories, inefficient sampling and extraction methods, and platform dependent variations could result in these inconsistencies. [37,101,102,105] One possible solution for avoiding this variation is to directly screen miRNA that is released from the tumor rather than screening whole plasma or serum. This could be achieved by screening the extracellular vesicles (e.g., exosomes) derived miRNAs which have the capability of representing parental tumor cells. [99]

4. Detection of RNAs: The Challenges

Despite the recent development of RNA detection methodologies, several major challenges still remain. These challenges involve the following considerations.

4.1. Stability of RNAs

RNA is generally unstable at room temperature due to the chance of ribonucleases (RNase) associated degradation. Therefore, both endogenous and exogenous RNases can affect the accuracy of the detection via progressive degradation of the target RNA during incubation steps. One potential solution of this problem is the use of RNase inhibitor in the assay as demonstrated by Frei et al. [106] In their multiplexed quantification assay for RNAs, referred to as PLAYR (proximity ligation assay for RNA), RNase inhibitor was used to avoid RNA degradation. This concern is not factual for RNAs available in various vesicles and biological molecules (e.g., exosomes and apoptotic bodies). The RNAs in these bodies are usually packaged into their structures (i.e., exosomal RNAs are protected by the membrane structure of exosome) and thus become inaccessible to RNases. [63–65]

4.2. Sample Preparation and Choosing the Sample Source

The efficiency of RNA detection is heavily influenced by the type of sample source and preparation method. [102] Wang et al. [107] showed that the expression level miRNA can be different in serum and plasma of the same individual. They found that miRNA concentrations in serum were higher compared to the corresponding plasma samples of the same person. They suggested that the presence of platelet-derived miRNAs originating from the blood coagulation process could be responsible for
this source-dependent variation. In another report, Leidinger et al.\[108\] showed that the influence of ethylenediaminetetraacetic acid (EDTA) on the expression and degradation of miRNA present in blood samples. EDTA has routinely been used as an anticoagulant in blood collection tubes for miRNA analysis in clinical settings. In this method, the sampling tubes were incubated with EDTA at different time intervals (0 min, 10 min, and 2 h) after the blood draw from 6 healthy individuals. It was observed that with increasing incubation time, the levels of miRNAs in the blood sample were significantly altered. The report suggested that transcription and degradation of the miRNAs in the white blood cells and platelets could still be dominating inside the sampling tube, causing a noticeable variation in the miRNA level derived from same individual under two different EDTA-incubation intervals. Therefore, the use of stabilizing reagents in the blood collection tubes is highly recommended to stop unexpected expression and degradation of miRNA (i.e., variations in miRNA expression level).\[102\]

### 4.3. Low Sensitivity due to Ineffective Extraction Methods

The readily available concentration of clinically relevant RNAs in tissues, serum, or other body fluids is very low. Therefore, a highly sensitive and specific method needs to be designed for extracting RNAs from body fluids. The RNA extraction method is also crucial because a little discrepancy in the analysis could result in false-positive responses. This observation was also evident from a study by McDonald et al.\[17\] who demonstrated that the majority of variance in the RNA detection was derived from the extraction process used. Thus choosing the right extraction method along with careful optimization

<table>
<thead>
<tr>
<th>Type of RNA</th>
<th>Target</th>
<th>Sample source and type</th>
<th>RNA expression</th>
<th>Diagnostic and/or prognostic RNA biomarkers</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Messenger RNA (mRNA)</td>
<td>hTERT mRNA, EGFR mRNA</td>
<td>Lung cancer tissue and serum with healthy controls</td>
<td>Downregulated</td>
<td>hTERT mRNA, combined with EGFR mRNA as both diagnostic and prognostic biomarker for pulmonary malignancies</td>
<td>[17][80]</td>
</tr>
<tr>
<td>microRNA</td>
<td>ASAP1 mRNA</td>
<td>10 pairs of epithelial ovarian cancer (EOC) tissue and normal samples</td>
<td>Upregulated</td>
<td>ASAP1 mRNA as prognostic marker of ovarian cancer</td>
<td>[93][80]</td>
</tr>
<tr>
<td>Fusion mRNA</td>
<td>cyclin D1 mRNA</td>
<td>Tissue samples from 151 patients with low-grade B-cell lymphoma</td>
<td>Upregulated in 128 patients</td>
<td>Cyclin D1 mRNA as diagnostic marker of Mantle cell lymphoma (MCL)</td>
<td>[94][80]</td>
</tr>
<tr>
<td>miRNA</td>
<td>Transgenic mouse prostate</td>
<td>Upregulated</td>
<td>Diagnostic marker of prostate cancer</td>
<td>[59][80]</td>
<td></td>
</tr>
<tr>
<td>microRNA</td>
<td>A number of 470 human miRNAs screened</td>
<td>76 tumor and matched adjacent normal tissues derived from radical prostatectomy specimens</td>
<td>10 upregulated and 5 downregulated</td>
<td>miR-96 as prognostic biomarker of prostate cancer</td>
<td>[95][80]</td>
</tr>
<tr>
<td>microRNA</td>
<td>A number of 123 lung cancer and matched normal tissues</td>
<td>35 upregulated and 3 downregulated</td>
<td>Upregulated miR-21, miR-17-5p, miR-191, miR-128b, miR-199a-1 as diagnostic biomarkers for lung cancer</td>
<td>[80][80]</td>
<td></td>
</tr>
<tr>
<td>microRNA</td>
<td>Tissues derived from 46 colon cancer and 8 normal individuals</td>
<td>21 upregulated and 1 downregulated</td>
<td>Upregulated miR-21, miR-191, miR-29b-2, miR-223, miR-128b, miR-24-1, miR-24-2, miR-155, miR-20a, miR-107, miR-32, miR-30c, miR-221, and miR-106a as positive indicators of colon carcinoma</td>
<td>[80][80]</td>
<td></td>
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<tr>
<td>microRNA</td>
<td>A number of 8 miRNAs screened</td>
<td>40 prostate cancer tissue with matched normal tissue samples</td>
<td>2 downregulated</td>
<td>Downregulated miR-205, miR-214 as diagnostic marker for prostate cancer</td>
<td>[96][80]</td>
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<tr>
<td>microRNA</td>
<td>79 breast cancer and 6 normal tissues</td>
<td>15 upregulated and 12 downregulated</td>
<td>Upregulated miR-21, miR-17-5p, miR-29b-2, miR-146, miR-155, miR-181b-1 as diagnostic indicators of breast cancer</td>
<td>[80][80]</td>
<td></td>
</tr>
<tr>
<td>microRNA</td>
<td>197 primary colon tumor and matched normal tissues</td>
<td>37 miRNAs dysregulated</td>
<td>Upregulated miR-21 linked with the poor survival of patients with colon adenocarcinoma</td>
<td>[89][80]</td>
<td></td>
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<tr>
<td>Long noncoding RNA (lncRNA)</td>
<td>DANCR lncRNA</td>
<td>107 colorectal cancer tissues with paired adjacent normal tissues</td>
<td>upregulated</td>
<td>Upregulated DANCR lncRNA as diagnostic marker for colorectal cancer</td>
<td>[93][80]</td>
</tr>
<tr>
<td>Long noncoding RNA (lncRNA)</td>
<td>HOTAIR lncRNA</td>
<td>100 cancerous and matched noncancerous tissues collected from patients with colorectal cancer</td>
<td>upregulated</td>
<td>Upregulated HOTAIR as prognostic marker for colorectal cancer</td>
<td>[90][80]</td>
</tr>
<tr>
<td>Long noncoding RNA (lncRNA)</td>
<td>Serum collected from patients with esophageal cancer (including 42 tumor resection and 8 without surgery) and 20 healthy volunteers</td>
<td>upregulated</td>
<td>Upregulated HOTAIR as potential diagnostic biomarker for esophageal cancer</td>
<td>[97][80]</td>
<td></td>
</tr>
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</table>
(i.e., incubation time, centrifuging speed, etc.) of the extraction steps is necessary for obtaining accurate detection.

4.4. Specificity Issues due to the Cross-Talk between RNAs

RNAs originating from the same family often share similar physicochemical properties and sequences. Consequently, accurate and sensitive detection of RNAs is often compromised by the background response from the closely related sequences of nontarget RNAs. Over the past several years, many approaches have been employed to address this issue. For example, Castoldi et al. used a special type of thermostable probe known as locked nucleic acid in microarray assay to specifically detect single base mismatch and closely related miRNA sequences. In another study, Labib et al. used p19 protein (p19 exclusively binds with double stranded miRNA) in an electrochemical assay to reduce the background responses from closely related nontarget sequences.

4.5. Nonspecific Response from Biomolecules

The clinical sample may have complex biological environments containing various unknown cells and biomolecules such as proteins, which could nonspecifically be attached on the sensor surface resulting in false-positive responses. Therefore, a suitable blocking agent such as mercaptohexanol, mercaptoethanol, poly(ethylene glycol), or bovine serum albumin can be used to prevent nonspecific bindings.

4.6. Varying Size of RNAs

The length of RNA spans over very small (e.g., miRNA, 18–20 nt) to large (e.g., lncRNA > 200 nt). Compared to longer RNAs, detection of short RNAs becomes challenging due to their size match with primers of amplification-based detection techniques. To address this issue, short RNAs can be enzymatically polyadenylated to produce longer sequences before the reverse transcription step in RT-qPCR via using oligo-dT primers. Another possible way to avoid primer match with target RNA is to use a stem–loop primer during reverse transcription, which formed a nicked RNA hybrid via hybridization with the 3’ end of the target RNA sequence. On the other hand, the detection of long RNAs using hybridization-based approaches also a significantly challenging task. This could be explained by the fact that, due to the presence of an extra free oxygen atom in the additional ribose of the RNA structure, long RNAs are prone to interaction between nucleotides and often obtain secondary and tertiary structures on the sensor surface, thereby minimizing the hybridization efficiency.

4.7. Physiological Variation in Humans

Natural variation in the expression levels of RNAs both between and within individuals is a considerable issue in RNA detection in clinical samples, which mostly could be the result of differences in gender, race, age, diet of individuals, etc. It has also been revealed that the variation is more when the sample size is small (<100 individuals), therefore the variation can be lessened by recruiting a large cohort of sample.

5. RNA Biomarker Detection Technologies

5.1. Amplification-Based Molecular-Biology Techniques

Amplification-based nucleic acid detection methods such as RT-qPCR, microarrays, and RNA-seq have been widely used for RNA biomarker detection and expression analysis. In these methods, generally the RNA template is reverse transcribed to complementary DNA (cDNA) using an RT enzyme. RT is a RNA-dependent DNA polymerase enzyme that creates cDNA libraries from mRNA. cDNA is then further amplified by PCR or RNA-seq for detection purpose.

5.1.1. RT-qPCR

RT-qPCR is a sensitive and widely used method for RNA analysis due to the advantages associated with the requirement of relatively less starting RNA, wide dynamic range, and better accuracy. Over the past several decades, a great number of RT-qPCR-based RNA expression analysis methods have been developed and also commonly used in clinical trials. In 2005, Chen et al. developed one of the well-known methods for miRNA analysis, referred to as TaqMan miRNA assay (Figure 21). In this method, a unique stem–loop reverse transcriptase was adopted for cDNA conversion which was followed by TaqMan PCR analysis for the quantification of total 5 miRNAs present in mouse tissues. This method has since been extensively used to quantify miRNAs for different applications. The beautiful feature of this method is that it can differentiate closely related miRNA sequences having as low as one nucleotide difference. Mestdagh et al. described a systematic approach to compare the analytical performance of twelve commercially available miRNA analysis platforms including RT-qPCR, microarray and RNA-seq. Among these methods, the RT-qPCR offers the highest sensitivity for the analysis of miRNA. Although RT-qPCR is sensitive and efficient for measuring relative concentrations of RNA with respect to an internal standard, it has a limitation for the absolute quantification of RNAs. Another potential weakness of this method is that it usually works with a small number of expressed genes and, thus, is not suitable for high-throughput RNA screening. It is also important to note that this method is effective for already established and prevalidated RNA biomarkers while microarray and RNA-seq methods are well-suited for the discovery and validation of novel RNA biomarkers.

5.1.2. Microarray-Based Methods

Being a relatively less expensive method, microarray-based assays can simultaneously profile a large number of RNAs and offer multiplexed analysis. Typically a microarray comprises thousands of spots containing multiple oligonucleotides probes
on a platform, which hybridize with RNAs of interest for large-scale expression analysis (Figure 2III). A significant number of microarray-based methods have been developed for genome-wide analysis of RNAs. In 2004, Rogler et al. developed a reverse format microarray termed an RNA expression microarray (REM). In REM, total RNAs were initially reverse transcribed to produce a cDNA library. These cDNAs were then printed on a solid support of corning gamma amino propyl (GAP) slides. Humidified hybridization chamber containing mixed Cy3- and Cy5-labeled probes were designed to enable simultaneous hybridization of two genes. The applicability of the REM was tested for the specific detection of albumin, Hnf-4 and Igfhp-1 and c-Myc expression. Zhu et al. developed another method which detected aberrantly expressed 303 lncRNAs and 565 mRNAs in Helicobacter pylori infected cells. Very recently, Hu et al. reported another microarray assay to explore the role of miRNAs in gefitinib (a common EGFR inhibitor available as cancer drug) resistance. By using the commercial Agilent miRNA-microarrays, they compared the miRNA expression of a gefitinib-resistant human cell line with its parental cell line (not resistant to gefitinib). The microarray profile revealed that the expression of miR-149-5p was altered in the gefitinib resistant cell line. Further RT-qPCR and biological function tests confirmed the potential involvement of overexpressed miR-149-5p in gefitinib resistance. The microarray method was also used to detect differential expression of ZEB1-AS1 lncRNAs in HCC, where ZEB1-AS1 lncRNA was mostly upregulated in metastatic tumor tissues. Rui et al. performed a microarray profiling of lncRNAs in lymph node metastasis of patients with colorectal cancer. They identified
the dysregulation of a total of 1133 lncRNA transcripts in metastatic lymph node, compared with normal lymph node. In another microarray study, a total number of 3146 differentially expressed lncRNAs and 2208 mRNAs in sinonasal squamous cell carcinoma with respect to noncancerous tissues were identified. These demonstrations suggest that microarrays are more suitable as discovery tools.

5.1.3. RNA-seq

The next-generation sequencing of RNAs (RNA-seq, also known as massively parallel cDNA sequencing) is one of the best alternatives to microarray and RT-qPCR-based methods and has gained a lot of interest in both small- and large-scale analysis of RNAs with greater sensitivity and specificity. Apart from covering a relatively broad range of transcripts, it can also detect mRNA transcript at a single-nucleotide resolution. However, as described by Tavallaei et al. RNA-seq method cannot always reliably quantify circulating miRNAs in a sample with highly varying miRNA distribution pattern.

5.1.4. RNA Detection by Isothermal Amplifications

RT-qPCR exponentially amplifies a small amount of target RNA using multiple heating steps (melting steps) during the thermal cycling which is not appropriate for the interrogation of RNA sequences in live cells. To avoid this issue, isothermal nucleic acid amplification strategies, which work at cellular temperatures could be an alternative choice for RNA detection. In the past decades, various types of isothermal amplification-based RNA detection methods have been introduced including reverse transcription loop-mediated amplification (LAMP),[132–134] RNA primed rolling circle amplification (RPRCA),[135,136] signal mediated amplification of RNA technology,[137] and strand displacement amplification.[138]

RT-LAMP is a one-step isothermal amplification method that generally provides up to 10⁶-fold amplification of single target RNA sequence in 1 h using a single tube. It uses a set of specially designed primers in combination with the mixture of DNA polymerase and RT.[134] The amplified RNA products can then be detected by a suitable readout method (e.g., visualization with SYBR Green I stain). Horibe et al.[132] developed an RT-LAMP-based assay for detecting lymph node metastasis in gastric carcinoma patients. In this method, RNA was extracted from 92 lymph nodes samples of 9 patients with gastric cancer followed by the isothermal amplification of cytokeratin19 (CK19) mRNA. Among 92 lymph nodes samples, 15 were found to be metastasis-positive, which were further validated with a nested RT-PCR assay. Although the sensitivity of RT-PCR and RT-LAMP is similar, the analysis time in RT-LAMP is much faster. On the contrary, the RPRCA method uniquely uses RNA primers instead of DNA primers to avoid the use of reverse transcriptase for detecting small RNAs in a single tube.[135] In RPRCA, usually the target RNA hybridizes to its complementary sequence on a circular DNA template, and is then cleaved by RNase H (30 °C) followed by elongation (via φ29 DNA polymerase at 30 °C), amplification, and detection steps.[135,139] In 2016, Fujita et al.[139] developed another RCA-based method referred to as signal amplification by ternary initiation complexes (SATIC) (Figure 2II). In SATIC, a ternary initiation complex was formed among the RNA, circular DNA template, and a DNA primer, followed by RCA at 37 °C. This caused the amplified products to acquire secondary structures producing multiple copies of G-quadruplex (G4) DNA. The G4s were fluorescence-stained with N3-hydroxethyl thioflavin T (ThT-HE) for the real-time detection of RNA. The method was further improved using two different ternary initiation complexes in the RCA process to enable the visual observation.

5.2. RNA Biosensors

Rapid advancements in nanotechnology have led the development of novel biosensor strategies. Typically an effective biosensor is composed of two major components: i) a receptor (biomolecular recognition species), which specifically recognizes the target analyte, and ii) a transducer (signal-generating and enhancing element) that recognizes the biomolecular interaction and converts this interaction into a measurable signal.[37,140] Over the past several years, several novel biosensors comprising nanopore, optical, and electrochemical readouts have extensively been developed for the quantification and analysis of RNA biomarkers. Starting with a brief outline of optical and nanopore-based biosensors, the remaining part of the review highlights the existing electrochemical sensors.

5.2.1. Optical Sensors

Until now, miRNAs, mRNAs, and rRNAs were reported to be analyzed and quantified by optical biosensing strategies where surface plasmon resonance (SPR) and surface enhanced Raman spectroscopy (SERS) readouts have mostly been employed.[139] In SPR, surface immobilized molecular interaction between target RNAs and specific bioreceptor causes a change in the refractive index, which is measured by the transducer in real-time. It can also explain the molecular interaction of RNA on sensor via analyzing binding kinetics.[139] Sipova et al.[140] developed a label-free and portable SPR sensor to detect miR-122 from mouse liver tissue. In this method, an additional amplification step was used where the captured miRNA was subsequently recognized by an antibody to enhance the sensitivity of the assay (detection limit was 2 × 10⁻¹² mol). The assay (35 min) was relatively rapid. More recently, Huertas et al.[142] designed another SPR sensor for quantifying cancer-specific alternatively spliced variants of Fas mRNA (Figure 3). This approach is comparable with RT-qPCR in terms of sensitivity and reproducibility. The method is also highly specific due to the use of formamide which significantly reduced the chance of cross hybridization between variants of Fas mRNA. It also uniquely incorporated a sample fragmentation step using RNA alkaline hydrolysis prior the readout to avoid accessibility problems of long mRNA isoforms. The viability of the method was further tested in HeLa cancer cell lines.

On the other hand, SERS-based methods depend on the quantification of surface plasmon excitation in metallic
nanostructures of SERS substrates, which has been used for analyzing different RNA biomarkers including fusion mRNA. Very recently, Wang et al. demonstrated the application of a “turn-on” SERS method referred to as “inverse Molecular Sentinel (iMS)” nanoprobes, for multiplexed detection of miRNAs. In this method, the SERS probes used plasmonic-active nanostars as the sensing platform where the “OFF-to-ON” signal switch relied on the conformational change of stem–loop (hairpin) capture probes during target hybridization. The clinical applicability of the assay was tested in breast cancer cell lines using a mixture of the two differently labeled nanoprobes to detect miR-21 and miR-34a biomarkers respectively.

In 2007, Seferos et al. introduced a fluorescence method for mRNA analysis which used a novel intracellular RNA probe termed as “Nanoflares.” Nanoflares has the unique feature of transfecting into the live cells for visualizing and quantifying mRNA. First, 13-nm gold nanoparticles (AuNPs) were functionalized with thiolated oligonucleotides (acting as the recognition element for mRNA transcripts) to develop the nanoflares. Then, a short reporter sequence tagged with cyanine (Cy5) dye was hybridized with functionalized AuNPs. This reporter sequence was considered as “flares” in the nanoflares sensor. In the absence of target, the Cy5 dye is quenched due to proximity to the AuNP surface. However, when target RNA is present, a robust duplex is formed between the RNA and nanoflares which causes the removal of flare from the AuNP, providing measurable fluorescence readout. The method was successfully tested in a synthetic target and a real sample for the real-time quantification of mRNA. One obvious disadvantage of the existing transfecting agents (e.g., lipids and dendrimers) for RNA analysis is that they can produce toxicity inside the cell. Furthermore, oligonucleotides-based transfecting agents are unstable inside the live cells due to their enzymatic degradation, primarily via the action of 3’ exonucleases. This instability of oligonucleotides inside the live cell and other associated issues were critically reviewed by Opalinska et al. Nanofores, on the contrary, are not toxic and are highly stable inside the cells. Nanoflares thus have very high potential in detecting RNAs from live cells and have already been incorporated in several RNA biosensing approaches.

5.2.2. Nanopore Sensors

Nanopore sensing tools are one of the most prominent single-molecule sensors. Typically, in the presence of a conducting fluid, when a potential is held, nanopores produce electric current due to the charge transport in the holes. The produced current is highly sensitive to the size and physical properties of the pore. Thus, detectable current changes can be observed when the target analyte (e.g., RNA) is trapped inside the pore after hybridization with a target specific capture probe. Until now, only miRNAs have been interrogated via nanopores. Wang et al. developed a unique nanopore sensor using α-hemolysin protein pore (Figure 4). The method relied on the translocation of single-stranded oligonucleotides through the 2 nm sized pore functionalized with a programmable oligonucleotide probe. Both ends of this oligonucleotide probe were attached with a poly (dC)30 signal tag, which then became highly specific for target miRNA and thus avoided their cross-hybridization with nonspecific RNAs. Depending on the presence of specific miRNA, a measurable signal was obtained. The method also showed highly sensitive detection limit of $100 \times 10^{-15} \text{m}$. Additionally, the sensor was successfully tested to differentiate the relative levels of miR-155 of healthy and lung cancer patients. A comparative analysis of the advantages, disadvantages, and analytical functionalities of the nanopore sensors along with other major RNA detection platforms is summarized in Table 2.

5.2.3. Electrochemical Biosensors

In recent years, significant progress has been made toward the development of electrochemical methods to interrogate...
Figure 4. A schematic diagram of lung-cancer-related single-miRNA-molecule (miR-155) capture by a nanopore. a) A microRNA (red) hybridized to a probe (green) having signal tags on both ends. b) Diagram of current blocks in the nanopore, in the presence of $100 \times 10^{-9}$ n miR-155 and its capture probe $P_{155}$ on the cis side of the pore. The red boxes denote the multilevel current pattern resulting from the hybridization of target miR-155 with the capture probe. c) A typical multilevel long block of hybridized miR-155 at +100 mV. Right panel: molecular mechanism of hybrid dissociation and translocation. Level 1: Capture of the target-probe hybrid, unzipping of the target microRNA from the probe followed by the translocation of the probe. Level 2: Unzipped microRNA staying inside the pore. Level 3: Translocation of the unzipped microRNA through the pore. Lower panel: multilevel current blocks at +150 and +180 mV where the duration of Levels 1 and 3 is decreased with increasing the potential supporting the functionality of the nanopore model. d) Quantification of miR-155 levels detected by RT-qPCR in trans solutions once the electrical readout is taken. e) A single-level block (from (b)) generated by a trapped miR-155-probe hybrid (without unzipping and translocation). f) A spike-like short block generated by the translocation of unhybridized miR-155 or probe from the cis solution. Reproduced with permission. [155] Copyright 2011, Nature Publishing Group.
Table 2. Major detection platforms for RNA biomarkers.

<table>
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<tr>
<th>Detection platform or assay</th>
<th>Detection principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Amount of RNA required or LOD</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Quantitative reverse transcription PCR (RT-qPCR)</td>
<td>Reverse transcription of RNAs to cDNA followed by quantitative PCR</td>
<td>Established method, sensitive, specific, sensitive, ideal for small RNAs, widely available and compatible with laboratory workflows,</td>
<td>Expensive, not suitable for high throughput RNA screening and discovery studies, effective only against established RNAs</td>
<td>Input RNA, &lt;ng or ng-μg</td>
<td>[102,156,157]</td>
</tr>
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<td>Microarray</td>
<td>Hybridization of RNA with complementary probes prefabricated in the thousands of spots on a microarray platform</td>
<td>Established method, relatively inexpensive, high throughput screening, suitable for discovery studies</td>
<td>Relatively lower specificity and sensitivity, no absolute quantification, cannot efficiently discriminate closely related miRNAs</td>
<td>Input RNA, ng-μg</td>
<td>[102,123,156,157]</td>
</tr>
<tr>
<td>RNA sequencing</td>
<td>Preparation of cDNA library from target RNAs followed by “massively parallel” sequencing of library derived cDNA</td>
<td>Can distinguish miRNAs from closely related RNAs and precursors</td>
<td>Extensive bioinformatics supports required, suitable only for relative quantification</td>
<td>Input RNA, ng-μg &gt; ng</td>
<td>[102,123,156,157]</td>
</tr>
<tr>
<td>Surface Plasmon resonance (SPR)</td>
<td>Measurement of refractive index (RI) changes resulting from the surface immobilized molecular interaction between RNAs and bioreceptor</td>
<td>Real-time and label-free analysis</td>
<td>Low throughput, higher sample input, longer assay time</td>
<td>Input RNA, ng-μg</td>
<td>[39,158]</td>
</tr>
<tr>
<td>Nanopore</td>
<td>The charge transport in the nanopore is halted in the presence RNA target of interest. The resultant blockade current is quantified</td>
<td>Sensitive, single molecule sensor</td>
<td>Complicated sensor fabrication, not suitable for detecting long RNAs</td>
<td>Input RNA, ng-μg</td>
<td>[152,154]</td>
</tr>
<tr>
<td>Electrochemical sensors</td>
<td>a) Cisplatin–biotin labeled mRNA/redox polymer bilayer on a gold electrode. Enzymatic oxidation of glucose oxidase–avidin molecules produce detectable amperometric signal</td>
<td>a) Amplification free, highly sensitive, low sample input (5 ng)</td>
<td>a) LOD, 0.05 × 10⁻¹⁵ μL</td>
<td>Input RNA, ng-μg</td>
<td>[159]</td>
</tr>
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<td></td>
<td>b) Target mRNA induced conformational change of hairpin (HP) probe results in a readable electrochemical signal</td>
<td>b) Amplified signal, efficient capture (HP probe is thousands fold sensitive than linear capture probe)</td>
<td>b) LOD, 0.4 × 10⁻¹⁵ μL</td>
<td></td>
<td>[160]</td>
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<td></td>
<td>c) Target fusion mRNA is captured on amino acid/nucleic acid chimeras (ANAs) capture probe functionalized gold microelectrodes. Voltammetric readings enable the detection in the presence of [Ru(NH₃)₆]³⁺/[Fe(CN)₆]⁴⁻/redox probe</td>
<td>c) Simultaneous screening multiple RNA targets, ANA capture probe facilitates low background response and stable monolayer formation on microelectrodes</td>
<td>c) LOD, 1.0 pg μL⁻¹</td>
<td></td>
<td>[161]</td>
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<td></td>
<td>d) Direct adsorption of magnetically captured target fusion RNAs on the unmodified screen-printed gold electrodes via RNA-gold affinity interaction. Resultant coulombic repulsion between negatively charged RNA and ferricyanide ions produce detectable voltammetric signal.</td>
<td>d) Significantly simplified sensor, avoids conventional surface modification and probe immobilization steps on electrode, relatively inexpensive, highly specific, amplification free, noninvasive screening</td>
<td>d) Not suitable for long RNAs</td>
<td></td>
<td>[41]</td>
</tr>
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Clinically relevant RNAs. Most of these methods are based on the hybridization of target RNA sequences to the surface bound complementary receptor probes (mostly DNA oligonucleotides) on the electrode. The hybridization of RNAs with probes results in a measurable electrochemical signal. Here, signal transduction relies on various factors including intrinsic electroactivity of nucleobases, and the presence of redox indicators (e.g., intercalators such as methylene blue), covalently bound redox labels (e.g., nanoparticles), or reporter enzymes (e.g., phosphatases, peroxidases). Then, the detection of RNAs is mostly read via voltammetric, amperometric, and impedimetric approaches. In parallel to these, few ultrasensitive electrochemical approaches have also been developed using chip-based nanostructured microelectrodes for multiplexed detection of RNA biomarkers. In these chip-based sensors, nanostructured electrodes are usually deposited...
on the surface of the chip using photolithography which work as specific detector for multiplexed analysis.

**Electrochemical Biosensing of Coding RNAs:** Due to the crucial role of coding RNAs (i.e., mRNA) in disease diagnosis and prognosis, several novel electrochemical sensors aiming to detect and analyze mRNA biomarkers have been developed. One of the earliest mRNA-detection methods was demonstrated by Xie et al. (Figure 5I). In this method, first, total extracted RNA was directly labeled with cisplatin–biotin and allowed to hybridize with a thiolated oligonucleotide capture probe, which was functionalized on a gold electrode. Following hybridization of target mRNA on the electrode surface, glucose oxidase–avidin molecules were introduced in the system which conjugated with biotinylated target mRNA via the biotin– avidin interaction. Then, the electrode surface was coated with a cationic redox polymer containing osmium-bipyridine. Since the target mRNA is attached with glucose, the current generated from enzymatic oxidation of glucose-bipyridine. Since the target mRNA is attached with glucose, the current generated from enzymatic oxidation of glucose was directly proportional to the target mRNA concentration in the sample. The viability of this highly sensitive method was tested for the detection of GAPDH and cancer-specific p53 mRNA (LOD, 0.5 × 10^{-15} m). Moreover, a partially complementary capture probe (single base mismatch) was introduced in the sensor, which produced notable drop in the amperometric response, supporting the excellent specificity of the assay.

The hairpin (HP) DNA probe is one of the most versatile oligonucleotide probes for biomarkers screening and is commonly being used in optical and electrochemical assays for RNA detection. The loop area of the hairpin probe is selectively designed to be complementary to the target RNA sequences. When RNA is hybridized with the HP probe, the stem loop structure of the HP opens up allowing the prefunctionalized electroactive reporter molecule to interact, resulting in a detectable signal. For example, Wei et al. incorporated an HP probe on a gold electrode in a "signal on" electrochemical method and successfully detected interleukin (IL)-8 (a potential noninvasive biomarker for oral cancer) mRNA from saliva. In this approach, the 3’ end of the HP probe was tagged with a fluorescein molecule while the 5’biotinylated end was immobilized on the streptavidin modified gold electrode to give the probe a stem loop structure. In the absence of target mRNA, resultant steric hindrance inhibits the binding of the anti-fluorescein-HP conjugate on the electrode surface thereby ceasing further reaction. When target mRNA is present, the hairpin opens up and then, in the presence of the TMB substrate, the bound HRP produces an amplified electrochemical signal. This signal is directly correlated with the amount of target mRNA present in the sample. The method was relatively fast and attained very high sensitivity (≈ 0.4 × 10^{-15} m). The method was later extended for the multiplexed detection of mRNA. In a similar approach, a switchable "on-off-on" technique involving a stem–loop oligonucleotide probe was used for the detection of tumor-specific survivin mRNA.

One of the foremost challenges of RNA biomarker detection in clinical application is to simultaneously screen the very low amount of readily available RNA biomarkers in complex heterogeneous samples, which could contain many nonspecific targets. These challenges could be addressed via the multiplexed and highly sensitive analysis of RNAs by employing novel nanostructured electrochemical sensors. For example, Vasilyeva et al. developed a sensor for the analysis of cancer-specific mRNA fusion using an array of nanostructured gold microelectrodes. In this method, a novel capture probe, termed as amino acid/nucleic acid chimeras (ANAs), was immobilized onto the surface of microelectrodes. ANAs significantly enhance the assay performance compared to the existing neutral capture-probe-based assay. This is due to the fact that ANA has relatively good solubility along with the ability of forming stable monolayer on the sensor. The ANA also specifically can recognize and binds at the junction of gene fusion. In the presence of [Ru(NH3)6]3+/ [Fe(CN)6]3− redox probe, the concentration of hybridized mRNA was measured from the voltammetric readout. To illustrate the applicability of the sensor, chronic myeloid leukemia specific BCR-ABL mRNA fusion was detected in cancer cell lines. When very high concentration of the partially complementary capture probe was used along with the fully complementary BCR-ABL probe, the electrochemical signal for the complementary targets was unaffected, showing the high specificity of the assay.

Apart from BCR-ABL, several other fusion mRNAs (e.g., TMPRSS2-ERG) have attracted significant interest as cancer biomarkers due to their role in cancer development and high specificity. Several other fusion mRNAs (e.g., TMPRSS2-ERG) have attracted significant interest as cancer biomarkers due to their role in cancer development and high specificity. For example, the TMPRSS2-ERG fusion mRNA (originated from mutation between the TMPRSS2 promoter sequence and the ERG coding sequence) is uniquely expressed in ~50% of malignant prostate cancer cases and considered as one of the finest selective biomarkers of prostate cancer for their aberrant role in the inhibition of the apoptosis of prostate gland cells.

More recently, Koo et al. have developed an amplification-free method for detecting prostate cancer specific TMPRSS2: ERG mRNA fusion (Figure 5I). This method avoids the complicated mechanisms underlying the conventional multistep sensor fabrication steps thereby significantly simplifying the sensor design. Moreover, the method recruited patients’ urine samples to extract the TMPRSS2: ERG mRNA for electrochemical interrogation, which offered a significant improvement in the noninvasive screening of prostate cancer patients. In this method, first, RNA was extracted and purified from patient urine samples. TMPRSS2: ERG mRNA transcripts were then selectively captured by the biotinylated capture probe in the solution. Next, streptavidin-coated magnetic nanoparticles were dispersed into the reaction mixture. After magnetic purification, the captured mRNA fusion was directly adsorbed on the commercially available screen-printed gold electrodes via the RNA–gold affinity interaction. The adsorbed mRNA (negatively charged) creates higher Coulombic repulsion toward the negatively charged ferricyanide ions thereby resulting in a decreased electrochemical signal compared to that of control and normal samples. The method showed excellent specificity in
detecting TMPRSS2: ERG mRNA while a negative control cell line (TMPRSS2: ERG fusion gene absent) was used. Until now, the most preferable approach for identifying cancer associated fusion transcripts is the next-generation sequencing (NGS), especially RNA-seq.\textsuperscript{[171]} However, the detection of fusion mRNA by RNA-seq has been limited by the resultant false-positives response.\textsuperscript{[118,175,176]} Moreover, RNA seq for fusion mRNA heavily relies on the complicated data analysis procedure with various type of software packages. This was also supported by a recent study from Kumar et al.\textsuperscript{[176]} who observed substantial variation in the NGS data analyzed by different fusion-mRNA-detection software tools. In this regard, the amplification-free

Figure 5. Electrochemical detection of coding RNAs. I) Schematic illustration of an mRNA detection assay using an mRNA/redox polymer bilayer model: cisplatin–biotin labeled mRNA is hybridized with a thiolated oligonucleotide capture probe functionalized on a gold electrode. Glucose oxidase–avidin molecules are then introduced in the system, which binds the biotinylated target mRNA via a biotin–avidin interaction. Then, the electrode surface is coated with a cationic redox polymer which mediates the enzymatic oxidation of glucose. As the target mRNA is attached with glucose, the amperometric current generated from its oxidation is directly proportional to the mRNA concentration in the sample. II) Steps involved in an amplification-free detection of prostate-cancer-specific TMPRSS2:ERG mRNA using the mRNA–gold affinity interaction: a) TMPRSS2:ERG mRNA is the fusion between the TMPRSS2 promoter sequence and the ERG coding sequence; b) total RNA is extracted from urine samples followed by specific capture of TMPRSS2:ERG mRNA via a biotinylated capture probe. The target is then magnetically isolated from the sample pool using streptavidin-coated magnetic beads; c) the magnetically captured target mRNA is heat-released and adsorbed on an unmodified screen-printed gold electrode. The presence of mRNA is analyzed by differential pulse voltammetry in the presence of the $[\text{Fe(CN)}_6]^{3-}/4-$ redox system. I) Reproduced with permission.\textsuperscript{[159]} Copyright 2004, American Chemical Society. II) Reproduced with permission.\textsuperscript{[41]} Copyright 2016, American Chemical Society.
and nonenzymatic electrochemical method has clear advantages over RNA-seq for acquiring relatively accurate detection of fusion miRNAs.

**Electrochemical Bioensing of Regulatory RNAs (Non-coding RNAs):** The principle of electrochemical bioensing of miRNAs is almost the same as that of DNA or mRNA hybridization biosensors. One example of miRNA detection strategies is the direct oxidation-based analysis of circulating miRNA bases. In this method, miR-122 was hybridized with its inosine substitute capture probe on a carbon-based nanostructured electrode. Electroactive polymers were also used on the electrode to increase the electroactive area and reduce the electrical resistance. Then, direct oxidation of guanine during RNA hybridization resulted in a measurable differential pulse voltammetric (DPV) signal. This method significantly improved the limit of detection of circulating miRNAs in serum (10 × 10^{-15} \text{ m}). The detection limit was further improved (100 × 10^{-18} \text{ m}) in patient serum via the development of a DNA-concatemer-based ultrasensitive electrochemical method. In this approach, two auxiliary probes were self-assembled to form a one-dimensional DNA concatemers. An HP capture probe was immobilized on the surface of screen-printed gold electrodes. In the absence of target miR-21, the HP probe retained its loop structure offering no binding site for the DNA concatemers. This produced little electrochemical signals. However, when miR-21 was present, the stem–loop structure of the HP capture probe opened up, allowing their hybridization with DNA concatemers. Next, the RuHex signal reporter bound to the negatively charged DNA concatemers on the working electrode significantly amplified the electrochemical signal enabling the detection.

Different intermediates of miRNA biogenesis such as pri-miRNA, pre-miRNA, and other small noncoding RNAs such as rRNA share sequence similarities with miRNAs. During miRNA detection, these nonspecific RNA molecules can interfere with the target miRNA via cross-hybridization resulting in false-positive response. This issue was addressed via the incorporation of a special RNA binding protein known as p19 in the sensor. Usually, the p19 protein works as a molecular caliper of small double-stranded RNA (21–23 base pairs) and isolates miRNAs in a size-dependent and sequence-independent manner. Being more specific, the p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA whereas it binds to ssDNA, rRNA, mRNA, ssRNA, or dsDNA due to the presence of an extra oxygen atom in RNA sugar, thereby negatively charged DNA concatemers on the working electrode significantly amplified the electrochemical signal enabling the detection.

Different intermediates of miRNA biogenesis such as pri-miRNA, pre-miRNA, and other small noncoding RNAs such as rRNA share sequence similarities with miRNAs. During miRNA detection, these nonspecific RNA molecules can interfere with the target miRNA via cross-hybridization resulting in false-positive response. This issue was addressed via the incorporation of a special RNA binding protein known as p19 in the sensor. Usually, the p19 protein works as a molecular caliper of small double-stranded RNA (21–23 base pairs) and isolates miRNAs in a size-dependent and sequence-independent manner. Being more specific, the p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA whereas it binds to ssDNA, rRNA, mRNA, ssRNA, or dsDNA due to the presence of an extra oxygen atom in RNA sugar, thereby negatively charged DNA concatemers on the working electrode significantly amplified the electrochemical signal enabling the detection.

The emerging functional insights of long noncoding RNAs in gene regulation has triggered extensive studies in the translational clinical research field. Despite the huge clinical significance of lncRNAs, their electrochemical detection is not well explored. Until now, only a few reports on electrochemical bioensing of lncRNAs are available. This could be explained by the fact that lncRNA has a relatively long sequence with a high molecular weight. Moreover, due to the presence of an extra oxygen atom in RNA sugar, lncRNAs tend to interact with the nucleic acid backbone via hydrogen bonding, resulting in secondary or tertiary structures (i.e., hairpin, triplexes, and quadruplexes structures). Taken together these issues, the commonly used RNA-hybridization-based electrochemical sensors cannot recognize and effectively capture the target lncRNAs on the electrode surface. In 2015, Liu et al. used the catalytic amplification ability of single-wall carbon nanotubes coated with Au–Rh hollow nanospheres (Au/Rh@SWCNT) to detect nuclear paraspeckle assembly transcript 1 (NEAT1) lncRNA, which is reportedly overexpressed in patients with HIV. The nanospheres have the combined benefits of RNA binding features of gold, unique electronic properties, and large edge-plane-to-basal-plane ratio of single-wall carbon nanotubes (SWCNTs), and the surpassing catalytic properties of Rh. In this sensor, the gold (Au) electrode was first modified with L-cysteine followed by the formation of a Au monolayer by the electrodeposition of an L-cysteine-containing gold electrode in a colloidal solution of Au nanoparticles. The nanomaterial (Au/Rh-HNP@SWCNT complex) tagged capture probe (RNA fragment containing a (GGGG) quadruplex) was then allowed to bind with each possible site of AuNPs. To avoid nonspecific binding, the sensor was soaked with hexanethiol. Finally the sensor was incubated with hemin solution where quadruplex containing target RNA binds with hemin resulting in a higher (catalytic) redox signal of hydrogen peroxides in the presence of HRP, allowing the quantification of NEAT1.
6. Conclusions

Here, we have provided an overview of the current progress of RNA-detection technologies with special emphasis on the development of electrochemical biosensors. The major challenges involved in these strategies along with the diagnostic and prognostic significance of clinically relevant RNAs have also been discussed. It has been shown that tumor-derived circulating RNAs can work as a liquid biopsy for the minimally invasive diagnosis of cancer, resulting in less patient discomfort. Although, their high stability in clinical samples has triggered huge interest, necessary caution should be taken to ensure the minimal sample preprocessing of these RNA biomarkers to retain their high stability.

From the representative examples of different detection technologies, it is apparent that RNA biosensors usually involve the hybridization of target RNA with a capture probe followed by a suitable readout method such as electrochemical readout. Despite the significant progress made in these electrochemical methods, still major work needs to be performed to achieve a biosensor suitable for a point-of-care platform. This is because most biosensors are merely proof-of-concept demonstrations and highly dependent on complex sensor fabrications and a series of optimization steps in a well-equipped laboratory setup. To translate these laboratory-based proof-of-concept demonstrations to clinical applications in real-world settings, there are many outstanding hurdles. One of the major issues is the false-positive detection of RNAs due to nonspecific bindings. To avoid this, an optimized sample extraction protocol and the development of innovative sensors are urgently required. Nonetheless, we have seen that several unique methods have attempted to address these requirements. For example, novel capture probes such as peptide nucleic acid analogs and microengineered-electrodes-based electrochemical sensors have been designed to avoid cross-hybridization and increase the hybridization efficiency of RNAs where P19 protein was particularly
employed to remove nonspecific targets.[13,161] Moreover, a simplified electrochemical sensor using direct interaction of target RNA and gold electrode has also been developed to circumvent the multisteps sensor fabrication steps.[43] We have also observed the recent trends of using novel nanomaterials to accelerate the signal transduction and obtain amplified and sensitive detection signal.[182] Despite the rapid advances in the biosensing of mRNAs and circulating miRNAs, development of electrochemical sensor for lncRNAs detection is still in its infancy. Considering their huge clinical potentials, more attention needs to be given to develop an effective and reliable biosensor for lncRNAs. We believe that the ultimate requirement for transforming the current approaches to point-of-care platform is the development of fully automated and integrated biosensors capable of high-throughput screening of RNA biomarkers in a heterogeneous clinical sample. As highly efficient RNA biosensors have continued to develop, we foresee that an ideal RNA biosensor with desired clinical applications will be developed in near future.

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Conflict of Interest
The authors declare no conflict of interest.

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In the previous chapter, we have provided a comprehensive literature review on the topic where major challenges associated with current biosensor-based strategies were identified. One of the foremost technical challenges of the existing RNA biosensors are the multi-step sensor fabrication steps, where target RNAs need to be manipulated. This chapter reported a simple assay, which attempted to address aforesaid challenges, thereby developing an amplification-free, and simple electrochemical mRNA detection assay. The assay involves the direct adsorption of mRNA onto an unmodified screen-printed gold electrode followed by differential pulse voltammetry (DPV) readout in the presence of $[\text{Fe(CN)}_6]^{4-/3-}$ redox system. This new method circumvents the PCR amplification steps, simplifies the assay construction and thus paved a simple approach for mRNA sensing. FAM134B mRNA was selected as a model target which was reported to be a potential biomarker for gastrointestinal carcinomas, especially colon adenocarcinoma and oesophageal squamous cell carcinoma (ESCC).
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**My contribution to the published paper involved:**

- Experimental planning and set-up
- Data acquisition and analysis
- Manuscript preparation

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

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

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\section*{A R T I C L E   I N F O}

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\section*{A B S T R A C T}

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 \([\text{Fe(CN)}_6]^{3-/4-}\) 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 \textit{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.

\section*{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 \textit{FAM134B} mRNA expression and the pathogenesis of gastrointestinal carcinomas especially colon adenocarcinoma and oesophageal squamous cell carcinoma (ESCC) where \textit{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 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 (Carrascoa 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, 2016b) 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 tissue 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]³⁻/²⁻ 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 Dropopens (Spain). Two oesophageal squamous cell carcinoma (ESCC) cell lines, HKESC-1 and HKESC-4, were used in this study (Cheung et al., 2007; Hu et al., 2000). 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% CO₂ 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 tumor 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 °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 2× 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 2× 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 5X SSC 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.

<table>
<thead>
<tr>
<th>Target genes and primers</th>
<th>Oligonucleotide sequences (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM134B synthetic mRNA</td>
<td>AGAGAAACCUUUCCUUACCGGCGUCCGUC</td>
</tr>
<tr>
<td>FAM134B-F</td>
<td>AGAGGTTTTTAGAATTAGTGTATT</td>
</tr>
<tr>
<td>FAM134B-R</td>
<td>CCACTTCTCACTATATCTCCAAACAAA</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>TGACACCAACTCTTTAGCTAG</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GCCATGAGTGTGTCATGAG</td>
</tr>
<tr>
<td>FAM134B capture probe</td>
<td>TGAGGACCATGAGCGAATTTCTC-Biotin</td>
</tr>
</tbody>
</table>

Table 1
List of the oligonucleotide sequences used in this study.
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 5X SSC 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 −0.1 to −0.5 V with a pulse amplitude of 50 mV and a pulse width of 50 ms in 10 mM PBS solution containing 2.5 mM [K₃Fe(CN)₆] and 2.5 mM [K₄Fe(CN)₆] electrolyte solution. For synthetic mRNA samples, 5.0 μL (diluted in 5X SSC buffer) sample was adsorbed on SPE-Au surface. For clinical samples analysis, 5.0 μL (diluted in 5X SSC 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., \( \% I_{\text{Relative}} \)) with respect to the baseline current (\( I_{\text{Baseline}} \)) due to the adsorption of mRNA were then measured by using following equation

\[
\% I_{\text{Relative}} = \frac{I_{\text{Sample}} - I_{\text{Baseline}}}{I_{\text{Baseline}}} \times 100
\]

where \( I_{\text{Baseline}} \) and \( I_{\text{Sample}} \) are current obtained for bare electrode and electrode after sample adsorption respectively.

2.5. Primer design and qRT-PCR

FAM134B mRNA expression in all tested primary ESCC 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 37 °C and then heated up at 95 °C for 5 min. Each cDNA sample was diluted to 30 ng/μL for qRT-PCR. Samples were then stored at −20 °C. 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 2X SensiMix 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 biotiny-
labeled 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 °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 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 this condition, 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 100 pM 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 (5× 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 electrode properties at acidic pH and probability of mRNA degradation at basic pH of adsorption buffer. This optimized condition facilitates [Fe(CN)₆]⁴⁻ 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 coulombic repulsion between adsorbed mRNA and the redox system (Koo et al., 2016a, 2016b). This establishes a clear correlation between the resulting Faradic current and the adsorbed target mRNA, where the decrease in

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

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

![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 voltammmograms data corresponding to the 100 pM mRNA, NoT control and bare electrode.](image-url)
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 $y = 10.414 \times \text{mRNA concentration} - 7.2867\, (C)$ with a correlation coefficient ($R^2$) of 0.99. The detection limit was found to be 1.0 PM ($\% RSD = 12\%$) 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 $-4\, ^\circ\mathrm{C}$. 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 retrace 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 FAM134B mRNA in a panel of clinical samples derived from oesophageal cancer tissues (discussed in Section 3.4 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 dynabeads 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, thereby 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).

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.
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 (HKECS1 and HKECS4), to demonstrate the efficiency of our assay in cancer cells from different microenvironment. Our assay showed 15% relative current response changes for HKECS1 RNA and 18% relative current response changes for HKECS4 RNA which was not significantly higher than that of NoT control (Fig. 4) indicating the down-regulation 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 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 non-tumor 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 non-specific 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 carcinoma. 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 optimized 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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References

Subsequent to the development of a proof of concept sensor for an amplification-free mRNA detection in the previous chapter, we extended our approach towards non-coding microRNA (miRNA) detection. One of the major sources of inefficiencies in miRNA biosensing is the requirement of ultrasensitive (due to its low abundance in biological samples) and highly specific (to avoid cross-talk with non-target miRNAs) strategies. To achieve this, we exploited the electrocatalytic activity of a novel superparamagnetic functional GO/IO hybrid nanomaterial. We studied the apparent Michaelis–Menten constant ($K_{m}^{\text{app}}$) of the GO/IO hybrid obtained from the electrochemical version of Lineweaver–Burk model, which was estimated to be sufficiently low, thereby demonstrating the high electrocatalytic activity of GO/IO hybrid materials towards the reduction of RuHex. Utilizing this feature, we designed a highly sensitive (LOD 1.0 fM) miRNA detection platform where the readout was obtained with chronocoulometric (CC) response of charge compensating [Ru(NH$_3$)$_6$]$^{3+}$ molecules bound with magnetically captured target miRNA on the GO/IO hybrid nanomaterials-modified disposable carbon electrode.

Chapter 4 is included as a full article submitted to ChemElectroChem
Statement of Contribution to Co-authored Paper Submitted for Publication

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Novel Graphene Oxide-loaded Iron Oxide Superparamagnetic Nanoparticles for

Ultrasensitive Electrocatalytic Detection of MicroRNA


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We report on the electrocatalytic activity of a new class of superparamagnetic nanoparticle graphene oxide-loaded iron oxide (GO/IO hybrid material) towards the reduction of ruthenium hexaammine(III) chloride (Ru(NH$_3$)$_6$)$^{3+}$, RuHex). Leveraging the electrocatalytic activity of GO/IO hybrid material, and signal enhancement capacity of [Ru(NH$_3$)$_6$]$^{3+}$/[Fe(CN)$_6$]$^{3-}$ electrocatalytic cycle, an ultrasensitive and specific electrochemical sensor was developed for the detection of cancer-related microRNA (miRNA). Using direct affinity interaction between RNA and graphene oxide, magnetically isolated and purified target miRNA were directly adsorbed onto the GO/IO hybrid material-modified screen-printed electrode. The detection was enabled by chronocoulometric (CC) readout of charge compensating [Ru(NH$_3$)$_6$]$^{3+}$ followed by an enhancement in CC charge display via [Ru(NH$_3$)$_6$]$^{3+}$/[Fe(CN)$_6$]$^{3-}$ system. We demonstrated an excellent LOD of 1.0 fM by accurately detecting miR-21 in synthetic samples, and showcased its clinical utility in ovarian cancer cell lines with high sensitivity (10 cells) and good reproducibility (% RSD = < 5%, for $n = 3$).

**Keywords:** Graphene oxide nanoparticles, Magnetic nanoparticles, Iron oxide nanoparticles, Electrocatalysis, Electrochemical detection of MicroRNA
1. Introduction

MicroRNAs (miRNAs) represent a family of short (18–25 nucleotides) and endogenous non-coding RNA species that actively regulate a range of cellular processes.\textsuperscript{[1]} Dysregulated miRNA expression are directly associated with pathogenesis of various diseases including cancer, and thereby emerged as prominent diagnostic and prognostic biomarkers for these pathological conditions.\textsuperscript{[2, 3]} Despite their huge potential in diagnostics and precision medicine, biosensing of miRNAs has proven to be a considerable challenge because of their tiny size, cross interference from non-specific molecules (i.e., lack of specificity against a background of overwhelmingly abundant irrelevant molecules and non-target RNAs with sequence and size similarity), extremely low abundance (0.01% of the bulk RNA pool, or few of molecules per cell).\textsuperscript{[4]} At present, miRNA detection techniques mostly rely on conventional nucleic acid detection assays such as quantitative reverse transcription PCR (RT-qPCR), microrarrays, Northern blot and RNA-sequencing.\textsuperscript{[5]} Despite being reliable in laboratory settings, these conventional techniques are expensive and not suitable in the resource-poor and decentralized settings.\textsuperscript{[4, 6]} Few of the common pitfalls include the need for especially designed primers (e.g., hairpin or oligo-dT in qPCR), high sample volume requirements, platform-dependent variation in the analysis, assay complexity and long analysis time that may range from hours (e.g., PCR) to days (e.g., microarray).\textsuperscript{[4, 6]}

Biosensors-based approaches, such as electrochemical assays, on the contrary, have shown more potential for clinical application due to their inherent advantages of being inexpensive, simple, rapid, and miniaturized.\textsuperscript{[7]} Most of the electrochemical sensors for miRNA however still rely on multiple sensor fabrication steps, some sorts of enzymatic amplification and target RNA modification (e.g., polyadenylation, labelling) which could destabilize RNA and complicate the assay protocol.\textsuperscript{[4, 8]} In addition, even with rigorous target selectivity and
faster analysis time, many of these sensors lack additional signal enhancement steps, thereby failing to achieve the sensitivity levels required for the analysis of miRNA in clinical sample. To enhance the sensitivity of assay, various signal amplification strategies such as rolling circle amplification, hybridization chain reaction amplification, catalyzed hairpin assembly amplification etc. have been incorporated in electrochemical miRNA analysis workflows. Amplification bias and longer analysis time are among the most prominent shortcomings of these signal amplification methods.\[[8]\]

An increasing number of reports however has indicated that the use of functional nanomaterials in electrochemical assays can prove to be an effective alternative strategy for enhancement of assay sensitivity and specificity.\[[9-11]\] Among nanomaterials, there has been a growing interest in the synthesis of magnetic transition metal oxide-based nanoparticles (NPs) due to their unique physicochemical properties such as biofavorable network structures as well as intrinsic enzyme mimetic and electrocatalytic activities.\[[12, 13]\] Such metal oxides have been combined with a second nanomaterial to fabricate hybrid nanocomposites with superior functionalities resulting from the synergetic advantages of both the nanoparticles in the composites.\[[13]\] In addition to that, to meet the specific requirements of the biosensors hybrid nanomaterials have also been engineered with a variety of novel design framework, fabrication and synthesis approaches.\[[14]\] One of such effective approaches is the fabrication of porous structures in the nanocomposite which significantly enhances the functional surface area.\[[15]\] Compared to monometallic and non-porous counterparts of similar mass, these hybrid porous materials exhibit significantly improved surface functionalities (e.g., increased interaction with target analyte) and higher catalytic activities by maximizing the surface dependent mass transport.\[[16]\] Moreover, magnetic properties of these materials allow an intimate magnetic mixing and purification of target analyte which enhances the speed and specificity of the bioassays.\[[17]\] In particular, metallic iron oxide-based hybrid nanocomposites have found a wide
range of potential applications owing to their unique optical, electronic, magnetic, catalytic, and sensing properties.\textsuperscript{[16, 18]} For example, iron oxide nanocomposite loaded with gold nanoparticles have recently been used to develop electrochemical sensors for autoantibody and microRNA.\textsuperscript{[17, 19-21]} Over the past several years, captivating the presence of different functional reactive moieties and exceptional physical properties at biological interfaces, graphene and graphene oxide have also been combined with iron oxide-based hybrid nanomaterials to develop electrochemical bioassays for detecting various analytes that includes NADH, H$_2$O$_2$, nitrite, uric acid, ascorbic acid, dopamine, protein and nucleic acids.\textsuperscript{[22, 23]}

In this paper, we show the electrocatalytic properties of a novel graphene oxide-loaded iron oxide (GO/IO hybrid) nanoparticle towards the reduction of ruthenium hexaammine(III) chloride (Ru(NH$_3$)$_6$Cl$_3$; RuHex) using typical Michaelis– Menten equation for enzyme catalysis. The morphology of GO/IO hybrid materials was engineered in such way that it obtained a highly porous structure with improved functional surface area, which facilitated the adsorption of a significantly higher amount magnetically purified target miRNA on the GO/IO hybrid- modified sensor via RNA-graphene affinity interaction. The level of miRNAs was quantified by chronocoulometric (CC) charge interrogation in the presence of surface bound cationic ruthenium hexaammine(III) chloride ([Ru(NH$_3$)$_6$]$^{3+}$) that was electrostatically attached with the anionic phosphate backbone of the adsorbed target miRNA. The signal was further enhanced with the coupling of ferri/ferrocyanide ([Fe(CN)$_6$]$^{3-/4}$) system (\textit{i.e.,} [Ru(NH$_3$)$_6$]$^{3+}$/[Fe(CN)$_6$]$^{3-}$ electrocatalytic cycle). Electrocatalytic reaction of [Ru(NH$_3$)$_6$]$^{3+}$ or methylene blue with [Fe(CN)$_6$]$^{3-}$ system were previously described.\textsuperscript{[24-27]} We considered miR-21 as a model target to test the applicability of our assay both in synthetic and biological samples, which was reported to have a strong correlation with the progression of ovarian cancer.\textsuperscript{3} Our assay enables a highly sensitive deletion limit of 1.0 fM and 10 cells in the synthetic and ovarian cancer cell line population, respectively.
2. Results and Discussion

The surface morphology of the samples before and after the calcination was observed by SEM (Figure 1A). The detailed synthetic procedures are described in the Supporting Information. The original 2D morphology of the GO sheets is well-preserved even after calcination. In the case of the GO/IO hybrid samples, the surface of GO is homogeneously covered with fine IO nanoparticles. To carefully investigate the crystal structure and the phase purity of the samples before and after calcination, wide-angle XRD measurement was carried out (Figure 1B). In general, GO sheets themselves display two diffraction peaks at 12° and 26° which can be assigned to the interlayer spacing between the GO sheets. After hybridization with the Prussian blue (PB) nanoparticles, however, the diffraction peaks derived from the GO sheets disappear, while several new intense reflections corresponding to PB can be observed (JCPDF no.01-070-0557). This indicates that the PB nanoparticles are located within the stacked GO sheets interlayer spacing which becomes disordered. The optimal calcination results in the formation of an impurity-free γ-Fe₂O₃ phase in the resulting hybrid materials, as identified from the XRD peaks at around 35° and 63°. To evaluate the surface area and porosity the GO/IO hybrids, N2 adsorption-desorption isotherms were carried out (Figure 1C). The surface areas and the pore volumes were calculated to be 120.5 m/g and 0.384 cc/g by the BET and BJH methods, respectively.

To demonstrate the electrocatalytic activity of GO/IO hybrids, the cyclic voltammetric (CV) measurements of GO/IO hybrid-modified glassy carbon electrode (GCE) were carried out in the presence [Ru(NH₃)₆]³⁺/²⁺. As shown in Figure 2A, well-defined cathodic and anodic peaks for the [Ru(NH₃)₆]³⁺/²⁺ system were attained at −250 mV and −180 mV (vs. Ag/AgCl) respectively at the unmodified GCE (GCE/bare). This demonstrates the occurrence of single electron reversible process (ΔE = 70 mV). However, GO/IO hybrid material-modified GCE shows an enhanced cathodic and anodic peak current with higher peak separation compared to
those of bare (Figure 2A). It can be seen that, GCE/GO-IO hybrid material $i_{pc}$ (cathodic current) increased approximately 3.5-times (10.9 vs. 36.62 $\mu$Acm$^{-2}$) with $E_{pc}$ (cathodic potential) shifted by $-77$ mV, whereas $i_{pa}$ (anodic current) increased approximately two-times (6.08 vs. 13.93 $\mu$Acm$^{-2}$) with an $E_{pa}$ (anodic potential) shift of $-24$ mV. These data indicate that GO/IO hybrid samples catalysed both the oxidation and reduction of RuHex where the catalytic reduction was relatively faster. It is believed that the enhanced peak separation at GCE/GO-IO hybrid material is attributed to an enhanced electrocatalytic activity resulting from the combined intrinsic functionalities of Fe$_3$O$_4$ and GO.

To understand the charge transport mechanism, we recorded CVs of both GCE/bare and GCE/GO-IO hybrid as a function of scan rates (10–1500 mVs$^{-1}$). As shown in Figure S1A (Supporting Information), both the $i_{pc}$ and $i_{pa}$ increase with an increasing scan rate spanning from 10 to 1500 mV s$^{-1}$, indicating the stable electrocatalytic activity of GO/IO hybrid materials within the applied range of scan rates. Moreover, with the increasing scan rate, the $i_{pc}$ and $i_{pa}$ peak potentials were shifted towards negative and positive direction respectively, confirming a reversible redox reaction. Figure S1B shows a linear relationship between $i_{pc}$ and $i_{pa}$ with the square root of the scan rate for both the unmodified and modified GCE. This observation suggests that the electrocatalytic redox reactions of RuHex at the GCE/GO-IO hybrid electrode occurred mainly through the diffusion-limited process. Figure S1B also shows that the curve of $i_{pc}$ and $i_{pa}$ versus square root of the scan rate for the GCE/GO/IO hybrid electrode resulted in a steeper slope than that of the unmodified GCE. This further confirms the relatively high catalytic activity of GO/IO hybrid materials towards the redox reaction of RuHex.

To further examine the electrocatalytic activity of GO/IO hybrid materials, chronoamperometric (CA) readout was obtained at the GCE/GO-IO hybrid electrode with the successive addition of RuHex. As can be seen in Figure 2 B and C, after an initial increase in the CA current response, the current reached a plateau. The calibration curve shown here
clearly follows typical Michaelis–Menten equation for enzyme catalysis. The apparent Michaelis–Menten constant (K_m^{app}) obtained from the electrochemical version of Lineweaver–Burk model (Figure 2D) was estimated to be 0.64 mM. It is important to mention that K_m^{app} herein denotes the concentration of RuHex that is required to reach the half of maximum current response (I_{max}) value, thus K_m^{app} can be considered as an indicator of the GO/IO hybrid nanomaterials’ affinity towards RuHex. This significantly low value of K_m^{app} value suggests an increased affinity of GO/IO to RuHex, which also verifies the high electrocatalytic activity of GO/IO hybrid material towards reduction of RuHex.

Figure 3 represents the outline of miRNA detection assay. In the assay, magnetically captured and purified target miR-21 was directly adsorbed on the GO/IO hybrids- modified SPCE surface followed by a CC readout using [Ru(NH_3)_6]^{3+}/[Fe(CN)_6]^{3-} electrocatalytic cycle. Briefly, total RNA was extracted and purified from cell lines. RNA sample was then incubated with miR-21 specific biotinylated capture probe for hybridization. Following target hybridization, streptavidin-labelled dynabeads were dispersed into the sample containing hybridized targets to purify and capture the target analyte via dynabead-based standard separation protocol. The heat-released target miRNA was isolated by another magnetic separation step (See experimental for details) and directly adsorbed onto the GO/IO-modified SPCE using RNA-graphene oxide (GO) affinity interaction. We and others previously demonstrated a number of bioassays which rely on nucleic acid-gold affinity interaction. Similar to the nucleic acid-gold affinity interaction, direct physisorption of nucleotides (DNA/RNA) on graphene surface has been reported to be influenced by the polarizabilities of the individual nucleobases, where van der Wall (vdW) is considered to be the driving force for the adsorption process. A number of studies also showed that the adsorption of nucleic acids on GO surface is influenced by π–π stacking, hydrophobic interaction and hydrogen bonding, and proposed Langmuir–Hinshelwood and Eley–Rideal mechanism could be
The target miR-21 was then quantified by CC charge interrogation via measuring the saturated amount of charge-compensating $[\text{Ru(NH}_3\text{)}_6]^{3+}$ molecules, where positively charged $[\text{Ru(NH}_3\text{)}_6]^{4+/3+}$ stoichiometrically binds to the negatively charged phosphate backbone of miRNA adsorbed on the SPCE/GO-IO surface. To generate a high electrocatalytic signal amplification, $[\text{Ru(NH}_3\text{)}_6]^{3+}$ system was coupled to $[\text{Fe(CN)}_6]^{3-}$ system. $[\text{Fe(CN)}_6]^{3-}$ in the solution-phase further triggers the electrocatalytic reduction of $[\text{Ru(NH}_3\text{)}_6]^{3+}$. Since the $[\text{Fe(CN)}_6]^{3-}$ is a relatively stronger oxidant, it oxidized $[\text{Ru(NH}_3\text{)}_6]^{2+}$ for the regeneration of $[\text{Ru(NH}_3\text{)}_6]^{3+}$ allowing multiple turnovers of $[\text{Ru(NH}_3\text{)}_6]^{3+}$ resulting in a drastic increase in the signal. Thus, the amount of CC charge generated by $[\text{Ru(NH}_3\text{)}_6]^{3+}$ and $[\text{Fe(CN)}_6]^{3-}$ system should have a clear correlation with the concentration of miRNA.

To assess the analytical functionality and specificity of our assay, we investigated a number of control experiments with 100 pM of starting synthetic RNA using (i) sensor modified with GO/IO hybrid materials and IO (i.e., nanoporous iron oxide without GO) (ii) with $([\text{Ru(NH}_3\text{)}_6]^{3+}/[\text{Fe(CN)}_6]^{3-}$ system) and without electrocatalytic cycle (only $[\text{Ru(NH}_3\text{)}_6]^{3+}$ system), and (iii) closely-related non-targets (wrong targets) and no-template (NoT) controls. As shown in Figure 4A (left bar), the total charge density (both the Faradaic and non-Faradaic charges) obtained with Fe$_2$O$_3$ modified (i.e., SPCE/IO) sensor gives a little response in CC data ($4.5 \mu\text{Ccm}^{-2}$). It was expected that in the absence of GO that acted as a platform carrier for RNA molecules in our assay, target miRNA would not be adsorbed on the SPCE/IO surface. As the CC response depends on the amount of target miRNA bound with $[\text{Ru(NH}_3\text{)}_6]^{3+}$ molecules, SPCE/IO gives an expected negligible response. However, this response is slightly higher than that found with bare SPCE (Fig S2, left bar, 4.5 versus 1.7 $\mu\text{Ccm}^{-2}$) and may be comprised of both the Faradaic and non-Faradaic component of the charges, where Faradaic response could related to the possibility of non-specific adsorption of a very tiny amount of redox active $[\text{Ru(NH}_3\text{)}_6]^{3+}$ on the IO.
When we performed the assay with GO/IO- modified SPCE in [Ru(NH₃)₆]³⁺ system (Figure 4A, middle bar), a large increase in the total charge density was observed. This response was ~9 and ~3.6-fold higher, respectively, than those obtained with bare and IO- modified sensors (16.3 vs. 1.8 and 4.5 μCcm⁻²). One of the reasons for this increased response is the large functional surface area of nanoporous GO/IO hybrid materials which can facilitate increased loading of target miRNA molecules. As demonstrated earlier, the intrinsic electrocatalytic activity of GO/IO hybrids towards the reduction of [Ru(NH₃)₆]³⁺ is also a strong contributor to this higher CC charge response. To further enhance the catalytic signal, we coupled [Fe(CN)₆]³⁻/⁴⁻ system with the [Ru(NH₃)₆]³+/⁴⁺, which initiated the electrocatalytic cycle. As can be seen in the Figure 4A (right bar), the coupled system provides a significant enhancement in the charge response. This response is ~4.6-times higher than the response obtained with [Ru(NH₃)₆]³+/⁴⁺ system alone (Figure 4A, 75 vs. 16.3 μCcm⁻²), which demonstrates the superior signal enhancement capacity of the electrocatalytic cycle (i.e., [Ru(NH₃)₆]³⁺/[Fe(CN)₆]³⁻ system) in our assay. This can be explained by the fact that the relatively stronger oxidant [Fe(CN)₆]³⁻ electrocatalytically reduce the surface confined [Ru(NH₃)₆]³⁺ in the solution phase, and initiates a cycle of redox reaction between [Ru(NH₃)₆]³⁺ and [Fe(CN)₆]³⁻, thereby increasing the rate of electron transfer.

To check the assay specificity, we performed our assay using control (SPCE/NP/NoT, no-template control) and non-complementary wrong sequences (synthetic miR-107 and miR-338-3p) with [Ru(NH₃)₆]³⁺/[Fe(CN)₆]³⁻ system. Figure 4B (two bars from left) shows that compared to bare electrode, control gave an enlarged CC response. We predicted that due to the non-specific adsorption of a small number of [Ru(NH₃)₆]³⁺ molecules on the enormous functional area of GO/IO hybrid materials, this charge response (19.5 μCcm⁻²) was observed. However, this has not clearly affected the assay because almost an identical level of CC charge was counted for the two other unrelated miRNAs such as miR-107 (19.3 μCcm⁻²) and miR-
While comparing the charge density responses obtained with control and non-targets with that resulting from target miR-21 (Figure 4B, right bar), a significant increase in the total charge density was observed (19.5/19.3/21.4 vs. 75 μCcm⁻²). This demonstrates that our assay has high specificity and can detect the target RNA sequences in the background of closely related non-complementary non-target RNAs. It is worth noting that, expectedly a higher level of signal enhancement was observed with the electrocatalytic system (NoT vs target, 19.5 vs 75 μCcm⁻², Figure 4B) compared to that of the assay without electrocatalytic cycle (i.e., only [Ru(NH₃)₆]³⁺ was present) (control vs target, 6.9 vs 16.3 μCcm⁻², Figure S2 and S3).

The sensitivity of the assay was evaluated by detecting a designated concentration of synthetic miR-21 spanning from 1.0 fM to 1.0 nM. As presented in Figure 5A and B, the total redox CC charge was increased with an increased concentration of target miR-21 using [Ru(NH₃)₆]³⁺-[Fe(CN)₆]³⁻ electrocatalytic cycle. Because with an increasing concentration of miRNA on the surface of GO/IO hybrid, an increasing number of cationic [Ru(NH₃)₆]³⁺ will bind with the anionic backbone of the miRNAs. This results in an enhanced charge response in the [Ru(NH₃)₆]³⁺-[Fe(CN)₆]³⁻ electrocatalytic cycle. When we plot the concentration of RNA with the charge of RuHex (QRNA) electrostatically bound with target RNA (Figure 5B), the linear regression equation of the assay was estimated to be y (charge density, μCcm⁻²) = 8.7246 (amount of miR-21) + 2.3519, with a correlation coefficient (R²) of 0.9633. This clearly shows the wide dynamic range of our assay, and the limit of detection (LOD) was estimated to be 1.0 fM. This LOD, which is clearly distinguishable from that of control and bare electrodes has a high signal to noise ratio, is comparable or better than most of the existing electrochemical miRNA biosensors.⁷,²⁶ We believe that the enormous functional area, and enhanced electrocatalytic activity of GO/IO hybrid materials, followed by the coupling of [Fe(CN)₆]³⁻ (i.e., electrocatalytic cycle) system that facilitates an increased rate of electron transfer in the
system, have attributed to this low LOD. Our observation was further confirmed when we compared the data with those obtained with the assay performed with [Ru(NH₃)₆]³⁺ system alone (without the catalytic cycle step), where a thousand-fold less LOD was observed (1.0 fM versus 1.0 pM) (Figure 5A, B vs S3). It is noteworthy that despite attaining such high sensitivity and selectivity, we designed an inexpensive and straightforward assay compared to several reported electrochemical methods. Most of these methods rely on some forms of enzymatic amplification processes which complicate the assay. For example, Fang et al. reported an electrochemical assay with a similar detection limit of 2.0 fM. However, this method relies on the use of zinc finger protein and alkaline phosphatase-based enzymatic amplification.[42] Another electrochemical miR-21 sensor reported to have a detection limit of 5.36 fM, also relies on a complex arched probe mediated isothermal exponential amplification reaction.[43] Our method has achieved several hundred folds better sensitivity compared to a recent CC assay reported by Yao et al.[44] which also depend on enzyme based rolling-circle amplification process. Compared to another recent voltammetric approach, we achieved 10-folds better sensitivity.[36] This approach also relies on enzymatic polyadenylation of the target miRNA. In comparison with aforementioned assays, we have not only attained better sensitivity but also avoid the possibility of miRNA degradation (due to target modification) and amplification bias.

To check the applicability of our assay in real samples, we performed our assay on total RNA isolated from a designated numbers of ovarian cancer (SKOV3) and normal non-cancerous (MeT-5A) cell lines. Figure 5C and D show that SKOV3 cells (0, 10, 100, 1000, and 10 000 cells) lead to a gradually elevated charge density profile across the range of 0–10 000 cells. From the electrochemical response, it is evident that our assay can detect miR-21 from only a few number of cells (0-10 cells; 100-300 pg of total RNA). As expected when we analysed our assay in the non-cancerous MeT-5A (1000 cells), we found that the charge density
profile is decreased which is close to that obtained with 10 SKOV3 cells (20.3 vs 16.1 μCcm⁻²) (Figure 4D). Whereas, RNA derived from a similar number of SKOV3 cells (i.e., 1000 cells) gave a ~ 2.5 times higher chronocoulometric response (20.3 vs 48.1 μCcm⁻²). This indicates that miR-21 is overexpressed in the tested SKOV3 cell lines compared to the non-cancerous cells. This preliminary data on cell lines indicate that our method retains its efficiency and sensitivity in analysing miRNA from complex sample, and may become useful in clinical analysis.

The acceptable range of our assay reproducibility (% RSD = <5%, for n = 3) is also comparable or better than most of the existing electrochemical miRNA sensors.⁷ There are several other distinct advantages of our assay. The major development is the utilization of high porosity and catalytic activities of a novel GO/IO hybrid nanomaterials that enables significantly increased loading of RNA samples and subsequent signal enhancement in the readout signal. This alternative signal amplification approach allows us to avoid any form of enzymatic amplification of the target. Another useful aspect of our assay is that we isolate miRNA by magnetic mixing and purification, which may lessen the matrix effects of the biological samples and thus, the assay is less prone to non-specific detection. Moreover, the nanoparticle modified electrodes provide a three dimensional surface for a large amount of target miRNA to diffuse with enhanced kinetics compared to the conventional electrodes. Our assay also uses a single-use (i.e., disposable) and relatively inexpensive SPCE which assists us to avoid the use of conventional disk electrodes, thus the assay is not affected with non-specific response resulting from the multiple surface reactions and excessive capacitive charges of disk electrodes. The elimination of tedious cleaning procedures of disk electrodes also reduces the assay time. Moreover, the direct adsorption of target miRNA on a GO/IO- modified electrode rather than the conventional hybridization-based approach of using recognition and transduction layers allows us to avoid complex conjugation chemistries of sensor fabrication.
The overall features of this electrochemical sensor indicates that the assay can complement with the miniaturized, multiplexed and decentralized analysis of RNA biomarkers with high translational potential.

3. Conclusions

We presented the electrocatalytic activity of a new class of GO/IO hybrid nanomaterials for the development of a highly sensitive (LOD = 1.0 fM) and specific detection platform of miRNA. SPCEs modified with this novel material were used as a platform to adsorb magnetically isolated and purified target miRNA via graphene -RNA affinity interaction. The chronocoulometric signal of surface confined RuHex attached with adsorbed target miRNA was significantly enhanced when the system was coupled with a solution-borne ferricyanide system. The assay also showed excellent reproducibility (% RSD = <5%, for n=3) and specificity (highly selective against closely related non-target) while detecting miR-21 from cancer cells. We envisage that our assay would be potentially useful for ultrasensitive analysis of miRNA in biomedical research, and clinical diagnosis that further can potentially be extended to detect other clinically relevant nucleic acid biomarkers by simply choosing the respective capture probe.

Experimental Section

Isolation of Target miRNA. Total RNA was extracted from SKOV3 ovarian cancer and MeT-5A non-cancerous cell lines using RNeasy Mini Kit (Qiagen, Germany). Target miRNAs were hybridized with a complementary capture probe followed by magnetic bead based isolation and purifications. Target miRNAs were then heat-released from magnetic bead bound hybrid and resuspended in RNAse-free water and stored at - 20 ºC for subsequent experiments (for details, see Supporting Information).
Evaluation of Electrocatalytic Activity of GO/IO Hybrid Materials. To evaluate the
electrocatalytic activity of GO/IO hybrids, GO/IO hybrids were drop-dried onto the surface of
a glassy carbon electrode (GCE). Cyclic voltammetry (CV) was carried out in the presence of
a redox marker, RuHex, over the scan rates of 10–1500 mV s\(^{-1}\). The chronoamperometric (CA)
responses were measured at the GCE/GO/IO hybrid electrode at -0.25V vs Ag/AgCl with the
successive addition of RuHex (ranging from 10 – 1100 µM). The apparent Michaelis-Menten
constant (\(K_{m}^{app}\)) of GCE/GO-IO hybrids was determined using the Michaelis-Menten equation
and the electrochemical version of Lineweaver–Burk equation.\(^{[28, 29]}\) (See Supporting
Information for electrode fabrication protocol and kinetic parameters calculations)

Electrochemical Detection of Target miR-21. For the CC detection of target miRNA, GO/IO
hybrid materials were magnetically bound onto a SPCE using a permanent magnet.
Magnetically purified miR-21 was then directly put onto that GO/IO hybrids followed by an
incubation with RuHex so that positively charged Ru\(^{3+}\) can bind with the negatively charged
phosphate backbone of electrode-bound miRNAs. The charge associated with the electrode-
bound miRNAs was measured by CC in 40 mM Tris buffer (pH 7.4). The number of cationic
redox molecules electrostatically associated with the surface-attached miRNA was calculated
using integrated Cottrell equation (See details in Supporting Information).\(^{[45]}\)

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Conflict of Interest
The authors declare no conflict of interest.

Keywords: MicroRNA detection, Electrochemical detection, grapene oxide/iron oxide
hybrid, electrocatalysis, ovarian cancer, screen-printed electrodes.
References


Figure Captions

**Figure 1.** (A) SEM images of samples prepared with GO:PB = 25:75 (a) before and (b) after calcination. (B) Wide-angle XRD patterns of samples prepared with GO:PB = 25:75 (i) before and (ii) after calcination. (C) N$_2$ adsorption-desorption isotherms of GO/IO sample prepared with GO:PB = 25:75.

**Figure 2.** (A) Comparison of the CVs obtained at an unmodified GCE and GO/IO hybrid-modified GCE in 50 mM RuHex (scan rate, 50 mV s$^{-1}$); (B) amperometric responses of GCE/GO-IO hybrid material with the successive addition of RuHex solution (10 to 1100 μM) into 0.01 M PBS (pH-7); (C) the corresponding calibration plot; and (D) the electrochemical version of Lineweaver-Burk Model.

**Figure 3.** Schematic of the quantification of miRNA assay. Magnetically purified and separated miRNA from the extracted RNA sample pool were adsorbed directly on the magnetically bound GO/IO hybrid- modified SPCE. A significant electrocatalytic signal amplification was achieved via the chronocoulometric (CC) charge interrogation of target miRNA-bound [Ru(NH$_3$)$_6$]$^{3+}$-[Fe(CN)$_6$]$^{3-}$ electrocatalytic assay system.

**Figure 4.** (A) Corresponding charge density data with SPCE/Fe$_2$O$_3$, SPCE/GO-IO hybrid material (without electrocatalytic cycle), SPCE/GO-IO hybrid material (with electrocatalytic cycle) electrodes (Total charge $Q =$ Faradic+ non-Faradic charges of the system); inset, corresponding CC curves ($Q$ vs. $t^{1/2}$). (B) Specificity of the assay. Corresponding charge density data of electrocatalytic cycle for the SPCE/Bare, control, non-complementary miR-107 and miR-338-3p, target miR-21; inset, corresponding CC curves ($Q$ vs. $t^{1/2}$). Each data point
represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5 %, for n = 3).

**Figure 5.** (A) Typical CC curves ($Q$ vs. $t^{1/2}$) for the SPCE/control and a designated concentration of synthetic miR21 (1.0 fM- 1.0 nM) (B) Corresponding calibration plot of $Q_{RNA}$-concentration profile across the range of 1.0 fM to 1.0 nM miR-21; (C) Typical CC curves ($Q$ vs. $t^{1/2}$) for the control and total RNA extracted from a known number of ovarian cancer SKOV3 (10, 100, 1000, 10 000 cells) and non-malignant MeT-5A (1000 cells) cell lines. (B) Corresponding bar diagram of $Q_{RNA}$; $Q_{RNA}$ (corresponding charge of target miRNA bound to surface bound RuHex) = total charge–capacitive charge. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = < 5 %, for n = 3).
Figure 1.
Figure 2
Figure 3
Figure 4
Figure 5
Electrocatalytic activity of superparamagnetic GO/IO hybrid materials, and signal enhancement capacity of \([\text{Ru(NH}_3\text{)}_6]^{3+}/[\text{Fe(CN)}_6]^{3-}\) electrocatalytic cycle have been used to develop an electrochemical platform for the ultrasensitive and specific quantification of miRNA in ovarian cancer.
Supporting Information

Novel graphene oxide-loaded iron oxide superparamagnetic nanoparticles for ultrasensitive electrocatalytic detection of microRNA


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Experimental Section

Reagents and Instrumentations. All the synthetic oligonucleotides were purchased from Integrated DNA Technologies (USA) (Table S1). Reagent grade hexaammineruthenium(III) chloride and phosphate buffered saline (PBS) tablet (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) were purchased from Sigma-Aldrich (USA). Analytical grade hydrochloric acid (HCl) were obtained from Chem-Supply (Australia) and Tris from VWR Life Science (Australia). UltrapureTM DNase/RNase-free distilled water (Invitrogen, Australia) was used. All the electrochemical measurements were performed with a CHI650 electrochemical workstation (CH Instrument, USA). Cyclic voltammetry (CV) and chronoamperometry experiments were done in a 3-mL volume single-compartment cell with a conventional three-electrode system (glassy-carbon working (GCE), platinum auxiliary, and Ag/AgCl reference electrodes, CH Instrument, USA). Chronocoulometry (CC) measurements were carried out on a screen-printed carbon electrode (SPCE) with a potential range between 0.0 and −500 mV with 250 ms pulse width and 2.0 ms sample interval. SPCE was printed on a ceramic substrate (length 33 × width 10 × height 0.5 mm) (DRP-150) and purchased from Dropsens (Spain). In the three-electrode system of SPCE, working (4 mm diameter) and counter electrode are made of carbon and platinum respectively, the reference electrode and electric contacts are made of silver.

Synthesis of Graphene Oxide-loaded Iron Oxide Superparamagnetic Nanoparticles

Synthesis of GO sheets. Graphene oxide was synthesized by the modified Hummer’s method. Sodium nitrate (0.3 g) was firstly dissolved in sulfuric acid solution (10 mL) under constant stirring. Nanographite platelet powder was added to the solution which was further stirred for 30 min. After subsequently adding KMNO₄ (0.30 g), the mixture was aged for 1 h. Finally, H₂O₂ (10 mL) was added to the mixture under constant stirring to obtain GO sheets.
Synthesis of Prussian blue (PB) nanoparticles. A 40 mL aqueous solution containing 3.24 g of FeCl₃·6H₂O and 3.24 g of TSCD was mixed with another 40 mL aqueous solution containing 4.36 g of Na[Fe(CN)₆]·10H₂O, and the mixture was vigorously stirred for 1 h before being statically aged overnight to ensure a complete reaction. Finally, the PB nanoparticles were obtained by centrifugation.

Synthesis of GO/IO hybrid materials. The above-prepared GO and PB suspension was diluted down to 2.0 mg·mL⁻¹ by adding water before being mixed together under sonication with specific weight ratios of 25:75. The mixtures were continuously treated by sonication for 30 min, and then aged overnight. The GO/PB hybrid precipitates settling at the bottom of the vial were washed with water and ethanol several times, before being dried at room temperature. The GO/IO hybrids were obtained by calcining the GO/PB powders at 400 °C at a heating rate of 1.0 °C min⁻¹.

RNA Preparation. SKOV3 ovarian cancer and MeT-5A non-cancerous cell lines were cultured in RPMI-1640 growth medium (Life Technologies, Australia) supplemented with 10% fetal bovine serum (Life Technologies, Australia) and 1% penicillin/streptomycin (Life Technologies, Australia) in a humidified incubator containing 5% CO₂ at 37 °C. The SKOV3 and MeT-5A cells were collected after 4 and 7 days, respectively, for subsequent cell counting and RNA extraction. Total RNA was extracted following the standard protocol from RNeasy Mini Kit (Qiagen, Germany).

Probe Hybridization and Magnetic Purification of RNA. For probe hybridization, 10 μL of RNA sample was mixed with 10 μL of 5× SSC buffer (pH 7.0) and 10 μL of 10 μM biotinylated capture probes. The mixture solution was heated at 65 °C for 2.0 min and placed on a thermomixer for 1.0 h at room temperature (25°C) to allow the capture probe hybridization to target miRNA. Next, 20 μL of streptavidin-labeled (MyOne Streptavidin C1, Invitrogen) magnetic beads were washed with 2× washing and binding (B&W) buffer (10 mM Tris-HCl,
pH 7.5; 1.0 mM EDTA; 2.0 M NaCl) and resuspended in 20 μL of 2× B&W buffer. The preparation was then added to biotinylated capture probes- miRNA complex. The resultant solution was incubated for 30 min at room temperature to allow the formation of dynabead-functionalized target miRNA complex. The magnetic beads bound miRNA complex was separated using a magnet, washed thrice with 2× B&W buffer, and resuspended in 10 μL of RNase-free water. The magnetically captured isolates were heated for 2 min at 95 °C, and the heat-released miRNAs were immediately collected from the supernatant using an external magnet. Then, 5.0 μL of the released miRNA was diluted with 15 μL of 5× SSC buffer (pH 7.0) for electrochemical readout.

Evaluation of Electrocatalytic Activity of GO/IO Hybrid Materials. A GCE was polished using 0.3 and 0.05 mm alumina slurry (CH Instrument, Inc. USA) followed by an adequate wash with water. After sonication with nitric acid and water, the electrode was washed thoroughly, and dried at room temperature. To assess the electrocatalytic activity of GO/IO hybrids, 4.0 μg of a colloidal suspension of GO/IO hybrid materials were drop-dried onto the working surface of the GCE electrode. The electrocatalytic activity GO/IO hybrids towards the reduction of RuHex was studied using cyclic voltammetry with the conventional three-electrode system where the working surface of GCE electrode was modified with GO/IO hybrid materials. The chronoamperometric readout was obtained at -0.25V versus Ag/AgCl in optimum condition. The current response due to the successive addition of different concentrations (10 – 1100 μM) of RuHex was monitored. The apparent Michaelis-Menten constant ($K_{m\text{app}}$) of GCE/GO-IO hybrids was determined from the Michaelis-Menten equation which is as follows.$^1$

$$I = \frac{I_{max} [S]}{K_{m\text{app}} + [S]}$$
where $I$ is the steady-state current, $I_{\text{max}}$ is the maximum current measured under the condition of catalyst saturation, $[S]$ is the substrate concentration, and $K_{m}^{\text{app}}$ (i.e., the substrate concentration needed to reach the half of $I_{\text{max}}$) is the Michaelis-Menten constant used to indicate the affinity of the catalyst towards the substrate. The electrochemical version of Lineweaver–Burk equation was also obtained with the rearrangement of Michaelis-Menten equation which is as follows

$$\frac{1}{I} = \frac{K_{m}^{\text{app}}}{I_{\text{max}}} \frac{1}{[S]} + \frac{1}{I_{\text{max}}}$$

**Electrochemical Detection.** The effective areas of SPCE was determined by the measurement of the peak current obtained as a function of scan rate under cyclic voltammetric conditions for the one-electron reduction of $[\text{Fe(CN)}_{6}]^{3-}$ [2.0 mM $K_{2}\text{Fe(CN)}_{6}$ in 10 mM PBS (0.5 M KCl)] using the Randles- Sevcik equation, as shown before. The chronocoulometry (CC) was performed in 40 mM Tris buffer (pH 7.4) in the absence and presence of 50 μM RuHex with the potential step of 500 mV with the 250 ms pulse width and 2 ms sample interval. For detecting miRNA, SPCE was modified with 4.0 μL of GO/IO hybrid materials (1.0 μg/μL) and magnetically bound on a permanent magnet followed by a PBS wash to remove any unattached or loosely attached nanoparticles on the electrode surface. 4.0 μL of magnetically purified miRNA (diluted in 5× SSC buffer) sample was then directly put onto the GO/IO hybrids modified SPCE surface and incubated for 30 min followed by PBS washing. The electrode was then incubated with 10 μL of 50 μM RuHex so that positively charged Ru$^{3+}$ can bind with the negatively charged phosphate backbone of adsorbed miRNA. After PBS wash, the CC charge derived from the surface-confined RuHex bound with miRNA was measured in 40 mM Tris buffer (pH 7.4). We further measured the CC using 4.0 mM $[\text{Fe(CN)}_{6}]^{3-}$ electrolyte prepared in 40 mM Tris-HCl buffer (pH 7.4). To calculate the number of cationic redox molecules
electrostatically associated with the surface-attached anionic phosphate backbone of miRNA, following integrated Cottrell equation was used:\textsuperscript{4}

\[
Q = \frac{2nFAD_0^{1/2}C_0^*}{\pi^{1/2}} t^{1/2} + Q_{dl} + nF\Gamma_0
\]

Here the total charge is \( Q \) at a time ‘\( t \)’, \( n \) is the number of electrons involved in electrode reaction, \( F \) is Faraday constant (C/equivalent), \( A \) is the electrode area (cm\(^2\)), \( D_0 \) is the diffusion coefficient (cm\(^2\)/s), \( C_0^* \) is the bulk concentration (mol/cm\(^2\)), \( \Gamma_0 \) is represent the amount of RuHex-confined near electrode surface and \( nF\Gamma_0 \) (known as surface excess) is the charge obtained by adsorbed miRNA. CC curves were constructed by plotting the charge versus square-root of time (\( t^{1/2}/s^{1/2} \)) in the presence and absence RuHex. \( Q \) and \( Q_{dl} \) were estimated from the intercept of these two curves at \( t = 0 \) where \( Q \) represents the total charge comprising both Faradic and non-Faradic (capacitive) charges. Hence, the corresponding charge of RuHex deriving from electrostatically bound to surface confined RNA (\( Q_{RNA} \)) can be calculated by subtracting the \( Q_{dl} \) as follows

\[
Q_{RNA} = Q - Q_{dl}
\]
### Table S1. Oligonucleotide Sequences

<table>
<thead>
<tr>
<th>Oligos</th>
<th>5’-Sequences-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21 capture probe</td>
<td>TGACCGACCCAGTGAGGAAGTTTTCTCT/3Bio/</td>
</tr>
<tr>
<td>Synthetic miR-21</td>
<td>ArGrArGrArArArCrUrCrArCrUrGrGrUrCrGrGrUrCrA</td>
</tr>
<tr>
<td>Synthetic miR-107</td>
<td>ArGrCrArGrCrArUrGrUrArCrArGrGrGrCrUrArUrCrA</td>
</tr>
<tr>
<td>Synthetic miRNA-338-3p</td>
<td>UrCrCrArGrCrArUrCrArGrUrGrArUrUrGrUrGrUrG</td>
</tr>
</tbody>
</table>
Figure S1. (A) Typical cyclic voltammograms obtained at unmodified-GCE (top, left) and GO/IO hybrid modified- GCE (top, right) electrodes at different scan rate (50 µM RuHex, 0.01 M PBS, pH 7.0). (B) Corresponding curves for $i_{pc}$ and $i_{pa}$ (current density) as a function of $v^{1/2}$. 
**Figure S2.** Assay specificity without an electrocatalytic cycle. Corresponding charge density data for the SPCE/Bare, control, non-complementary miR-107 and miR-338-3p, target miR-21; inset, corresponding CC curves ($Q$ vs $t^{1/2}$). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for $n = 3$).
Figure S3. Sensitivity without electrocatalytic cycle (A) Typical CC curves ($Q$ vs. $t^{1/2}$) for the SPCE/control and 1.0 pM-1.0 nM of miR-21. (B) Corresponding calibration plot of $Q_{\text{RNA}}$ - concentration profile across the range of 1.0 pM-1.0 nM miR-21. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for $n = 3$).

References


Chapter 5

Electrocatalytic signal enhancement in microRNA biosensor


In the previous chapter, we have demonstrated an electrochemical method for miRNA detection which achieved the LOD of 1.0 fM. We herein developed another readout platform which enabled a 10-fold better sensitivity, without affecting the specificity and stability of the electrochemical sensor. We have utilized the electrocatalytic activity of gold-loaded nanoporous superparamagnetic iron oxide nanocubes (Au-NPFe$_2$O$_3$NC) for the detection of miR-107. The porous structure of the nanomaterials enhanced the loading capacity (i.e., exposed gold surfaces of the highly porous Au-NPFe$_2$O$_3$NC can adsorb vast amount of RNA sequences), catalytic activity (they maximise surface dependent mass transport and an expedition of cascade reaction by placing catalytic functionality in sequential compartments). In addition to the reduction of target bound charge compensating RuHex molecules, this assay coupled $[\text{Fe(CN)}_6]^{3-/4-}$ system with $[\text{Ru(NH}_3)_6]^{3+}$, thus initiated electrcatalytic cycle thereby attaining a ultrasensitive LOD of 100 aM.

Chapter 5 is included as it appears in *Biosensors and Bioelectronics (2018)*
Statement of Contribution to Co-authored Published Paper

This chapter is in the form of a co-authored published paper. The bibliographic details of the co-authored published paper are:


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**My contribution to the published paper involved:**

- Experimental planning and set-up
- Data acquisition and analysis
- Manuscript preparation

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Title: Gold-loaded nanoporous ferric oxide nanocubes for electrocatalytic detection of microRNA at attomolar level


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A crucial issue in microRNA (miRNA) detection is the lack of sensitive method capable of detecting the low levels of miRNA in RNA samples. Herein, we present a sensitive and specific method for the electrocatalytic detection of miR-107 using gold-loaded nanoporous superparamagnetic iron oxide nanocubes (Au-NPFe2O3NC). The target miRNA was directly adsorbed onto the gold surfaces of Au-NPFe2O3NC via gold-allele affinity interaction. The electrocatalytic activity of Au-NPFe2O3NC was then used for the reduction of ruthenium hexaammine(III) chloride (RuHex, [Ru(NH3)6]3+) bound with target miRNA. The catalytic signal was further amplified by using the ferri/ferrocyanide [Fe(CN)6]3-/4- system. These multiple signal enhancement steps enable our assay to achieve the detection limit of 100 am which is several orders of magnitudes better than most of the conventional miRNA sensors. The method was also successfully applied to detect miR-107 from cancer cell lines and a panel of tissue samples derived from patients with oesophageal squamous cell carcinoma with excellent reproducibility (% RSD = < 5%, for n = 3) and high specificity. The analytical accuracy of the method was validated with a standard RT-qPCR method. We believe that our method has the high translational potential for screening miRNAs in clinical samples.
standard (Islam et al., 2017a). Among other strategies, the high throughput microarray and RNA-seq methods are best suited for discovery and validation of novel miRNA biomarkers rather than diagnostic applications. Moreover, all these methods largely depend on sophisticated and costly laboratory instrument, which is not suitable for the miRNA biomarker screening in resource-limited settings.

In recent years, increasing interest in the simple, miniaturized and cost-effective analysis of biomarkers has led to the development of many electrochemical strategies which generally rely on capture probe-based hybridization or sandwich assay formats (Fang et al., 2017; Labib and Berezovski, 2015). However, these electrochemical assays may not meet the requirement of ultrasensitive miRNA detection in clinical samples, mainly due to the lack of appropriate signal enhancement ability. It has been widely reported that one of the effective ways to increase the sensitivity of electrochemical assays is the use of nano-material-based signal amplification strategies. Nanomaterials have the inherent advantages of high catalytic properties, biomimetic activity, biocompatibility, excellent conductivity and high sample loading capacity (Dong et al., 2013; Xia et al., 2015). Therefore, nanomaterials have widely been used in electrochemical sensors as the tracers, catalysts and electronic conductors to improve the sensitivity of miRNA detection (Dong et al., 2013; Peng et al., 2014; Xia et al., 2013). For example, Gao and Yang (2006) used isoniazid-capped OsO2 nanoparticles for labelling RNA molecules to electrocatalytically amplify the detection signal. Recently, composite nanomaterials, such as bimetallic hybrid nanomaterials (e.g., Fe3O4@Ag), have also been used for amplifying electrocatalytic signal in detecting miRNA (Pang et al., 2016). Very recently, we designed and synthesized a new class of gold-loaded ferric oxide nanocubes (Au-NPFe2O3NC) (Yadav et al., 2017) that exhibit several functionalities. These includes (i) superparamagnetic – the nanocubes are paramagnetic and thus highly suitable for magnetic isolation and separation; (ii) electrocatalyst – the nanocubes possess high electrocatalytic activity (Masud et al., 2017) towards many common electroactive materials such as ruthenium hexaammine(III) chloride (RuHex, [Ru(NH3)6]3+), and (iii) enhanced loading capacity – the exposed gold surfaces of the highly porous Au-NPFe2O3NC can be used to adsorb large amount of target nucleotide sequences via DNA/RNA- gold affinity interaction (Islam et al., 2017b; Koo et al., 2016a, 2016b, 2015; Sina et al., 2014a, 2014b).

The increasing demand for detecting the ultralow levels of miRNAs with electrochemical techniques has also resulted in the integration of more than one signal amplification step in a single assay platform (Fang et al., 2017; Liu et al., 2014). In particular, the nanoparticle-mediated electrocatalysis can be coupled with one or more electrocatalytic cycles to obtain enhanced sensitivity (Zhang et al., 2006). In such electrocatalytic assays, one redox molecule is first electrochemically oxidized or reduced at the electrode surface upon hybridization/intercalation with the target sequence (Zhang et al., 2007). This reaction is then coupled to another redox molecule that regenerates the oxidized or reduced species back to its original state. The resultant sharp increase in the electron flux produces an enhanced electrochemical signal. For example, Barton and Kelley groups have reported a series of studies to detect different DNA-based biomarkers where electroactive methylene blue (MB) or RuHex were coupled to ferri/ferrocyanide ([Fe(CN)6]3-/4-) to obtain the amplified electrocatalytic signal (Boon et al., 2000; Kelley et al., 1999; Lapierre et al., 2003).

In this paper, leveraging the benefits of superparamagnetic, high electrocatalytic activity and surface loading capacity of highly porous framework of Au-NPFe2O3NC and portability of disposable screen-printed carbon electrode (SPCE), we develop an miRNA detection method which pushed the detection limit down to attomolar levels (LOD = 100 aM). It has been reported that, conventional gold disk electrodes often exhibit excess background currents due to their high electrocatalytic properties and capacitive charges (Park et al., 2014). Moreover, due to multiple surface reactions on these conventional electrodes, tedious cleaning procedures are required for their full functionality. On the contrary, our assay uses single-use (i.e., disposable) and relatively inexpensive SPCE as a platform to load the magnetically bound nanocubes, which avoids this time-consuming cleaning procedure thereby reducing the assay time and simplifying the protocol. Moreover, SPCE is portable (three-electrode system is printed on a plastic or ceramic substrate) and has minimal sample requirement which illustrates their potential scope towards the development of miniaturized analytical device suitable for on-site application. In this assay, streptavidin-functionalised dyanabeads were first modified with a biotinylated capture probe. Then, they are dispersed in RNA sample population to isolate and purify the target miRNA. Afterwards, the isolated target miRNA was directly adsorbed on the Au-NPFe2O3NC (via RNA-gold affinity interaction) which was magnetically immobilized on a SPCE. The detection was then achieved by chronocoulometric (CC) charge measurement of surface bound cationic ([Ru(NH3)6]3+/4+) which was electrostatically attached with the anionic phosphate backbone of the adsorbed target miRNA. The signal was further amplified by coupling the reduction of [Ru(NH3)6]3+/4+ with the [Fe(CN)6]3-/4- system (i.e., [Ru(NH3)6]3+/4+/[Fe(CN)6]3-/4- system). We have selected miR-107 as a model target in this assay, which has diagnostic and prognostic roles in many cancers including oesophageal squamous cell carcinoma (ESCC) (Patnaik et al., 2010; Sharma et al., 2013). The clinical applicability of the assay was successfully tested in a panel of eight tissue samples derived from patients with ESCC. The data were validated with a standard RT-qPCR method.

2. Experimental sections

2.1. Isolation of target miRNA

ESCC cell lines (HKESC-1 and HKESC-4) were grown and maintained according to the previously described procedures (Cheung et al., 2007; Hu et al., 2000). The RNA samples from four matched fresh ESCC and non-neoplastic tissues were used for this study (Hu et al., 2017). Total RNA was extracted and purified from the cell lines and tissue samples according to manufacturer’s guidelines (Qiagen, Germany). Target miRNA was captured via dynabead-based miRNA separation procedures with a slight modification of our previous report (Koo et al., 2016b). Briefly, target miRNA were hybridized onto complementary capture probes functionalized magnetic beads (The list oligonucleotide sequences are provided in Table S1). Then, hybridized targets were magnetically purified, heat-released, and resuspended in RNase-free water. (See Supplementary materials for more details)

2.2. Electrochemical detection of miRNA

In this study, we utilized Au-NPFe2O3NC to modify SPCE (SEM images of the bare and modified SPCE are provided in Fig. S1, where the bio-favourable physicochemical properties of AuNPs (i.e. affinity interaction of DNA/RNA with gold) was exploited to capture target miRNAs. Briefly, SPCE was rinsed with an excess amount of Milli-Q water before the adsorption of miRNA. To attach Au-NPFe2O3NC to the SPCE surface, the electrode was placed on a permanent magnet and 4.0 μg of Au-NPFe2O3NC was put and incubated onto the electrode surface for 45 min. The electrode was then washed with 10 mM PBS to remove any unattached or loosely attached particles from the electrode surface. The effective areas of SPCE was determined by using the Randles Sevcik equation (See Eq. (S1) in Supplementary materials) as shown before (Shiddiky et al., 2010, 2009). Fig. S2 shows the typical cyclic voltammogram of bare and modified electrodes. Using CC, the amount of miRNA adsorbed onto the Au-NPFe2O3NC/SPCE surface was calculated from the number of cationic RuHex electrostatically bound with the surface-attached anionic phosphate backbone of miRNA following the integrated Cottrell Eqn (Steel et al., 1998). (See Supplementary materials for detailed experimental)
3. Results and discussion

3.1. Detection principle

The principle of our assay is schematically illustrated in Fig. 1. The assay is comprised of three main steps including (i) magnetic separation and purification of target miR-107, (ii) direct adsorption of target RNA on the Au-NPFe2O3NC, and (iii) CC readout via [Ru(NH3)6]3+/[Fe(CN)6]3− system based electrocatalytic signal amplification. Briefly, total RNA extracted from de-identified ESCC tissue were initially incubated with the miR-107 specific biotinylated capture probe. Upon hybridization of the capture probe with miR-107, streptavidin-labelled dynabeads were dispersed into the reaction mixture which attached with the biotinylated capture probe-target duplex. In this assay, commercially available streptavidin-coated dynabeads is functionalised with biotinylated capture probe and used for the magnetic isolation and purification of target miRNAs from the RNA sample pool. The complex was then magnetically isolated using dynabead-based standard protocol. After heating at 95 °C, the released (target) miRNA was then adsorbed on the Au-NPFe2O3NC, leading to binding onto the surface of Au-NPFe2O3NC which was magnetically held on the SPCE electrode. The [Ru(NH3)6]3+ was electrostatically attached with the surface bound negatively charged miRNAs. The detection was achieved by the chronocoulometric charge interrogation of [Ru(NH3)6]3+/[Fe(CN)6]3− electrocatalytic cycle.

To check the effect of the composition of nanomaterials on the electrocatalytic activity and signal enhancement of our assay, we modified SPCE with NPFe2O3NC (nanoporous iron oxide nanocubes without AuNPs) and Au-NPFe2O3NC and compared the assay performance for detecting miR-107 from 10 pM of synthetic RNA sample. As can be seen in Fig. 2 (left bar) and Fig. S3 (left bar, in Supplementary materials), the total charge density (total charge Q represents the Faradaic and non-Faradaic charges) obtained with the SPCE/NPFe2O3NC and SPCE/bare electrodes were found to be 4.5 and 1.8 μC cm−2 respectively. The low level response for the SPCE/bare electrode could be responsible for the non-Faradaic component of the CC charge at the bare electrode. A slight increase of the CC charge (1.8 versus 4.5 μC cm−2) at the SPCE/NPFe2O3NC-modified electrode could be related to both the Faradaic and non-Faradaic component of the charges. This may be related to the fact that a low amount of redox active [Ru(NH3)6]3+ could be adsorbed on the NPFe2O3NC, leading to the Faradaic component of the charges. When the electrode was modified with Au-NPFe2O3NC (i.e., SPCE/Au-NPFe2O3NC), the total charge binding onto the surface of SPCE/Au-NPFe2O3NC should have a clear correlation with the concentration of target miRNA.

3.2. Electrocatalytic activity of Au-NPFe2O3NC and signal enhancement

The electrocatalytic signal (i.e., CC charge) generated by the [Ru(NH3)6]3+/[Fe(CN)6]3− system after miRNA

\[
[\text{Ru(NH}_3\text{)}_6\text{]}^{3+} + e^- \rightarrow [\text{Ru(NH}_3\text{)}_6\text{]}^{2+} \tag{1}
\]

\[
[\text{Ru(NH}_3\text{)}_6\text{]}^{2+} + [\text{Fe(CN)}_6]^{3-} \rightarrow [\text{Ru(NH}_3\text{)}_6\text{]}^{3+} + [\text{Fe(CN)}_6]^{4-} \tag{2}
\]

In this assay, the enhanced electrocatalytic signal (i.e., CC charge) generated by the [Ru(NH3)6]3+/[Fe(CN)6]3− system after miRNA
density significantly increased for the [Ru(NH₃)₆]³⁺/²⁺ system compared to that obtained with the SPCE/bare (−11-fold higher, 20 versus 1.8 μC cm⁻²) or SPCE/NPFe₂O₃NC-modified electrodes (−4.5 times higher, 20 versus 4.5 μC cm⁻²). The enhancement of the charge response for the SPCE/Au-NPFe₂O₃NC electrode could be explained by considering these two facts (i) large surface loading capacity of highly porous framework of Au-NPFe₂O₃NC and (ii) the intrinsic electrocatalytic activity of Au-NPFe₂O₃NC towards the reduction of [Ru(NH₃)₆]³⁺. The target miR-107 was directly adsorbed onto the exposed gold surfaces of the Au-NPFe₂O₃NC via RNA-gold affinity interaction, which follows conventional pyrophosphorisation and chemisorption mechanism. The surface-bound miR-107 (negatively charged) binds with [Ru(NH₃)₆]³⁺ and thereby generating a higher CC charge (due to the larger contribution from Faradaic component). Recently, we have demonstrated the electrocatalytic activity of Au-NPFe₂O₃NC toward the reduction of [Ru(NH₃)₆]³⁺, where the Au-NPFe₂O₃NC-modified glassy carbon electrode (GCE) offered significantly enhanced cathodic (iœ), and anodic (iα) peak currents for the [Ru(NH₃)₆]³⁺/²⁺ system when compared to that of the unmodified-GCE electrode (Figs. S4 and S5 in the Supplementary materials). We have also shown that the electrocatalytic activity of Au-NPFe₂O₃NC follows typical Michaelis-Menten behaviour as well as Lineweaver-Burk Model for electrocatalysis (Masud et al., 2017). As our assay design requires both gold-RNA affinity interaction for adsorbing target miRNA and electrocatalysis for signal enhancement, we synthesized highly porous Fe₂O₃, which favors the controlled-loading of 2% AuNPs to achieve Au-NPFe₂O₃NC.

To further enhance the catalytic signal, we coupled [Fe(CN)₆]³⁻/⁴⁻ system with the [Ru(NH₃)₆]³⁺/²⁺ system. As can be seen in Fig. 2 (right bar), a 4-fold increase in the CC response was obtained compared to that of the [Ru(NH₃)₆]³⁺ system (20 versus 82 μC cm⁻²) at the SPCE/Au-NPFe₂O₃NC electrode. This response is also 18 times higher compared to that of the SPCE/NPFe₂O₃NC-modified electrode (82 versus 4.5 μC cm⁻²), demonstrating the catalytic signal amplification for detecting miR-107 at the SPCE/Au-NPFe₂O₃NC electrode. We assume that apart from the large surface loading capacity and electrocatalytic activity of Au-NPFe₂O₃NC (towards the [Ru(NH₃)₆]³⁺/²⁺/[Fe(CN)₆]³⁻ system), a few other added features also attributed to this sensitivity, such as (i) SPCE/Au-NPFe₂O₃NC/RNA-bound surface confined [Ru(NH₃)₆]³⁺ could generate more intense CC signal compared to voltammetry where the electrostatic interaction is free of any duplex intercalation and (ii) the double layer charge and charge derived from the surface confined target miRNA can easily be differentiated from the redox charges in diffusion layers allowing more sensitive charge measurements.

3.3. Assay specificity

To check the assay functionality and specificity, we performed our assay in the absence and presence of complementary or non-complementary target miRNAs. In the absence of target miR-107 (i.e., SPCE/Au-NPFe₂O₃NC without miR-107, control), the total charge density for the [Ru(NH₃)₆]³⁺/[Fe(CN)₆]³⁻ system was significantly higher than that of the SPCE/bare electrode (4.5 vs 18.1 μC cm⁻²) (Fig. S3 vs Fig. 3). This charge density for the [Ru(NH₃)₆]³⁺ system was 7.5 μC cm⁻² (Fig. S3, Supplementary materials). This is probably due to the presence of a low amount of [Ru(NH₃)₆]³⁺ on the control electrode via nonspecific adsorption. In the case of the two non-complementary synthetic miRNAs (miR-21 and miR-338-3p), as expected, using the same starting concentration (10 pM), the total charge densities obtained for the miR-21 and miR-338-3p bars represent the charge density at 20 pM of target miR-107. Control and Target-miRNA bars represent the charge density obtained at SPCE/Au-NPFe₂O₃NC electrode before and after adsorbing 10 pM of target miR-107. miR-21 and miR-338-3p bars represent the charge density obtained at SPCE/Au-NPFe₂O₃NC electrode after adsorbing 10 pM of non-complementary miR-21 and miR-338-3p targets respectively. Inset, corresponding CC curves (Q vs. i⁴/²). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = < 5%, for n = 3).

To further enhance the catalytic signal, we coupled [Fe(CN)₆]³⁻/⁴⁻ system with the [Ru(NH₃)₆]³⁺/²⁺ system. As can be seen in Fig. 2 (right bar), a 4-fold increase in the CC response was obtained compared to that of the [Ru(NH₃)₆]³⁺ system (20 versus 82 μC cm⁻²) at the SPCE/Au-NPFe₂O₃NC electrode. This response is also 18 times higher compared to that of the SPCE/NPFe₂O₃NC-modified electrode (82 versus 4.5 μC cm⁻²), demonstrating the catalytic signal amplification for detecting miR-107 at the SPCE/Au-NPFe₂O₃NC electrode. We assume that apart from the large surface loading capacity and electrocatalytic activity of Au-NPFe₂O₃NC (towards the [Ru(NH₃)₆]³⁺/²⁺/[Fe(CN)₆]³⁻ system), a few other added features also attributed to this sensitivity, such as (i) SPCE/Au-NPFe₂O₃NC/RNA-bound surface confined [Ru(NH₃)₆]³⁺ could generate more intense CC signal compared to voltammetry where the electrostatic interaction is free of any duplex intercalation and (ii) the double layer charge and charge derived from the surface confined target miRNA can easily be differentiated from the redox charges in diffusion layers allowing more sensitive charge measurements.

3.4. Assay sensitivity

To evaluate the assay sensitivity, a series of designated concentrations of synthetic miR-107 ranging from 100 aM to 1.0 nM were detected by our assay. As shown in Fig. 4, the CC response generated by [Ru(NH₃)₆]³⁺/[Fe(CN)₆]³⁻ system increased with the increasing concentration of the target miRNA. This can be explained by the fact that, an increasing amount of miR-107 on the SPCE/Au-NPFe₂O₃NC electrode surface will have more anionic phosphate groups which in turn binds with more cationic [Ru(NH₃)₆]³⁺. Thus more [Ru(NH₃)₆]³⁺ will be electrocatalytically reduced to further catalyse the reduction of [Fe(CN)₆]³⁻ in the diffusion layer, thereby resulting enhanced CC response. The linear regression equation of the assay was estimated to be y = 10.85 (amount of miR-107) + 3.1638, with a correlation coefficient (R²) of 0.9984. The limit of detection (LOD) was estimated to be 100 aM and the response is clearly distinguishable from that of control and bare electrodes. It is important to mention that without the use of catalytic cycle step (only with the [Ru(NH₃)₆]³⁺ system), we achieved a detection limit of 100 fM (100 aM versus 100 fM), which is thousand-times less sensitive than that of the [Ru(NH₃)₆]³⁺/[Fe(CN)₆]³⁻ system (see Fig. S6).

The LOD of our method is superior or comparable with those of existing miRNA electrochemical assays (Koo et al., 2016b; Pang et al., 2016; Yao et al., 2014). For example, the LOD of our method is approximately 1000 times better than a recent chronocoulometric detection method reported by Yao et al. (2014). In addition to the superior sensitivity, our assay is relatively inexpensive (enzyme free, low-cost synthesis of Au-NPFe₂O₃NC and simple (via adopting direct RNA adsorption and electrocatalytic signal amplification) whereas the method developed by Yao et al. relies on the enzyme based rolling-circle amplification process. Our approach has also obtained 100-folds better sensitivity than the voltammetric method reported by Koo et al.
Moreover, while the voltammetric method requires the target (RNA) modification (i.e., polyadenylation) using Poly-A enzyme, our approach offers relatively simple (avoids pre-modification of target RNAs) and inexpensive (avoids costly enzymes) assay platform. A comparison of the LOD of our method with that of several other existing electrochemical methods reported in the last five years has been provided in Table S2 of Supplementary materials.

3.5. Analysis of miR-107 in patient derived cell lines and tissues

To determine whether our assay could be applied to biological samples, we tested our method in total RNA sample extracted from human ESCC cell lines (HKESC-1 and HKESC-4) and one non-neoplastic oesophageal mucosa sample (as a control). As shown in Fig. 5A and B, the corresponding bar diagram for Q_{RNA} and typical CC curve demonstrated that the CC response obtained with cancer cells from HKESC-1 and HKESC-4 was significantly higher than that of normal (70 and 54 vs 19 μC cm\(^{-2}\)) suggesting the overexpression of miR-107. We then validated our assay performance in cancer cells with a RT-qPCR. As seen in Fig. 5C, the expression fold change data of a RT-qPCR strongly supports our findings (Fig. 5C vs Fig. 5A).

To further demonstrate the potential clinical applications, we extended our method to analyse miR-107 from the total RNA extracted from cancer tissues with their matched non-cancer tissues from 4 patients. As indicated in Fig. 6A and B, all eight total RNA samples (including non-cancer tissue RNA; N1–N4 and cancer tissue RNA; P1–P4) showed different levels of miR-107 expression. For example, CC charge density obtained for P1 (T) was almost doubled when compared to the matched non-cancer P1(N) (67 vs 24 μC cm\(^{-2}\)). A similar trend was obtained for the other patients samples where CC response derived from cancer samples (P2–P4) had significantly higher charge densities compared to the non-neoplastic counterparts (N2–N4), suggesting an upregulation of miR-107 expression in the ESCC tissue samples. We then validated our assay performance with a standard RT-qPCR method.
which was in excellent agreement with our findings (Fig. 6A vs 6C). The reproducibility of our assay (relative standard deviation, % RSD = < 5%, for n = 3) in analysing clinical sample is also in acceptable range, which shows better (Bettazzi et al., 2013) or comparable (Bartosik et al., 2014; Hong et al., 2013; Koo et al., 2016b; Miao et al., 2016) reproducibility when compared to the recently reported miRNA electrochemical sensors. The level of the reproducibility, sensitivity and selectivity of our assay in analysing patient sample as well as the validation with RT-qPCR analysis indicates the enormous potential of our detection method for screening clinically relevant miRNAs. Also, this approach has future implications in profiling gene expressions and molecular pathways in patients with cancer and other chronic diseases.

3.6. Advantages of the assay

The assay offers an important progress towards the ultrasensitive detection of miRNA. First, utilization of highly porous structure of Au-NPFe₂O₃NC significantly enhances the capture efficiency via loading vast amounts of RNA on the gold surfaces. Additionally, it offers the direct and fast electron transfer between the target and electrode surface attributing the high sensitivity of the assay. Second, unlike the traditional heterogeneous hybridization-based detection, our assay exploits magnetic nanoparticle-based intimate mixing, separation and purification of miRNA which reduce the matrix effects of the biological samples. Thus, the method is less susceptible to non-specific detection. Third, the direct adsorption of target miRNA on an Au-NPFe₂O₃NC-modified electrode rather than the conventional RNA biosensing approach of using recognition and transduction layers, substantially simplifies the detection method by avoiding the complex chemistries underlying each step of the sensor fabrication. Fourth, RuHex bound electrostatically with the target, thereby avoiding the need of expensive electrochemical tags or labelling. Finally, electrochemical detection can complement with the miniaturized and multiplexed analysis in non-laboratory settings with high translational potential.

4. Conclusions

In conclusion, we have developed a sensitive electrochemical method for detecting small non-coding RNA (miRNAs) using multiple electrocatalytic signal amplification steps. Magnetically separated and purified target miRNA was directly adsorbed on novel Au-NPFe₂O₃NC nanocomposites with high electrocatalytic activity. Further chronocoulometric charge interrogation of surface-bound RuHex (via interaction between [Ru(NH₃)₆]³⁺ and miR-107 adsorbed onto the SPCE/Au-NPFe₂O₃NC) coupled with the [Fe(CN)₆]³⁻/⁴⁻ system result in a significant signal enhancement via electrocatalytic cycle. The assay enabled the ultra-low level of limit of detection of 100 aM and showed high reproducibility (% RSD = < 5%, for n = 3) and specificity towards detecting a known cancer-related miR-107, in cell lines and tissue samples collected from patients with ESCC. The application of our assay on both the cell lines and clinical samples suggests the future use of screening other cancer biomarkers including miRNAs (depending on the selection of specific capture probe). Considering the versatility of our assay, we believe that with further optimisation, this new strategy based on nanocomposite and electrocatalytic cycle can potentially be applicable for the determination of a wide variety of clinically relevant DNA or RNA biomarkers for other human cancers and chronic diseases.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.09.027.
References

Supplementary materials

Gold-loaded nanoporous ferric oxide nanocubes for electrocatalytic detection of microRNA at attomolar level

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1. Detailed experimental

Materials and Reagents

Reagent grade hexaammineruthenium(III) chloride and phosphate buffered saline (PBS) tablet (0.01M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25°C) were purchased from Sigma-Aldrich (USA). Analytical grade hydrochloric acid (HCl) and Tris were obtained from Chem-Supply (Australia) and VWR Life Science (Australia) respectively. Screen-printed carbon electrode (SPCE) with a three-electrode system printed on a ceramic substrate (length 33 × width 10 × height 0.5 mm) (DRP-150) from DropSens (Spain). In the three-electrode system, working (4 mm diameter) and counter electrode are made of carbon and platinum respectively while the reference electrode are made of silver. UltrapureTM DNase/RNase-free distilled water (Invitrogen, Australia) was used in all aqueous solution. Oligonucleotides were purchased from Integrated DNA Technologies (USA) (oligonucleotides sequences are shown in Table S1).

NPFe$_2$O$_3$NC and Au@NPFe$_2$O$_3$NC were prepared according to our recently reported protocols (Yadav et al., 2017; Masud et al, 2017). Briefly, NPFe$_2$O$_3$NC were obtained by calcination of prussian blue (PB) nanocubes. To load the Au nanoparticles for preparing Au-NPFe$_2$O$_3$NC, NPFe$_2$O$_3$NC (250 mg) were dispersed in water containing sodium citrate under stirring, followed by addition of 3 mL of 10 mM HAuCl$_4$ aqueous solution, incubation under ice-water bath till its temperature was stable. Then, sodium borohydride solution was added
under vigorous stirring for 10 min followed by and the product was washed and collected by successive centrifugation.

**Magnetic Separation and Purification of miRNA**

10 μL of RNA sample was mixed with 10 μL of 5X SSC buffer (pH 7) and 15 μL of 10 μM biotinylated capture probes. The mixture solution was heated at 55°C for 2 min followed by a 1 hour incubation on thermomixer at room temperature (25 °C) to allow the hybridization of the capture probe to target miRNA. Next, 20 μL of streptavidin-labeled Dynabeads (MyOne Streptavidin C1, Invitrogen) magnetic beads were washed with 2X binding and washing (B&W) buffer (10 mM Tris-HCl, pH 7.5; 1.0 mM ethylenediaminetetraacetic acid (EDTA); 2.0 M sodium chloride) and resuspended in 20 μL of 2X B&W buffer. Then, the resuspended magnetic beads were added to the prepared biotinylated capture probes-miRNA complex. After incubation of the resultant mixer for 30 min at room temperature to allow the formation of streptavidin dynabeads-biotinylated capture probe-target miRNA complex. After that, the magnetic beads bound miRNA complex was isolated with a magnet, washed thrice with 2X B&W buffer, and resuspended in 9.0 μL of RNase-free water. The magnetically captured isolates were then heated for 2 min at 95 °C, and immediately placed on a magnet to collect the supernatant containing the captured target miRNA. After that, 5.0 μL of the released miRNA was diluted with 15 μL of 5X SSC buffer (pH 7.0) for further electrochemical measurements.

**Electrochemical Detection of Adsorbed miRNA**

All electrochemical measurements were done with a CHI650 electrochemical workstation (CH instrument, USA). Cyclic voltammetry (CV) was performed in 10 mM PBS solution containing 2.0 mM [K₃Fe(CN)₆] electrolyte solution. The effective areas of SPCE was determined by the measurement of the peak current obtained as a function of scan rate under
cyclic voltammetric conditions for the one-electron reduction of \([\text{Fe(CN)}_6]^{3-}\) [2.0 mM in PBS (0.5 M KCl)] and by using the Randles -Sevcik equation (Eq S1)

\[ i_p = (2.69 \times 10^5) n^{3/2} AD^{1/2} C \nu^{1/2} \]  

(Eq S1)

where, \(i_p\) is the peak current (A), \(n\) is the number of electrons transferred \((\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}, n = 1)\), \(A\) is the effective area of the electrode (cm\(^2\)), \(D\) is the diffusion coefficient of \([\text{Fe(CN)}_6]^{3-}\) (taken to be \(7.60 \times 10^{-5}\) cm\(^2\)s\(^{-1}\)), \(C\) is the concentration (mol/cm\(^3\)), \(\nu\) is the scan rate (Vs\(^{-1}\)).

For detecting miRNA, 4.0 \(\mu\)L of Au-NPFe\(_2\)O\(_3\)NC (1 mg/mL) was added on the SPCE surface. The electrode was then placed on a permanent magnet (see Fig. 1). 5.0 \(\mu\)L of magnetically separated target miRNA sample were then directly put onto the magnetically bound Au-NPFe\(_2\)O\(_3\)NC surface and incubated for 30 min followed by PBS washing. The electrode was then incubated with 15 \(\mu\)L of 50 \(\mu\)M RuHex. After washing with PBS, chronocoulometry (CC) measurement was carried out with the potential step of 5.0 mV with the 250 ms pulse width and 2.0 ms sample interval, in 4.0 mM \([\text{Fe(CN)}_6]^{3-}\) prepared in 40 mM Tris buffer (pH 7.4). Using CC, the amount of miRNA adsorbed onto the Au-NPFe\(_2\)O\(_3\)NC/SPCE surface was calculated from the number of cationic RuHex electrostatically bound with the surface-attached anionic phosphate backbone of miRNA. The total charge \(Q\) at a time ‘\(t\)’ can be expressed by the integrated Cottrell Equation (Eq S2)

\[ Q = \frac{2nFA_0^{1/2} C_0^{*} t^{1/2}}{\pi^{1/2}} + Q_{dl} + nFA \Gamma_0 \]  

(Eq S2)

where, \(n\) is number of electrons involved in electrode reaction, \(F\) is Faraday constant (C/equivalent), \(A\) is the electrode area (cm\(^2\)), \(D_0\) is the diffusion coefficient (cm\(^2\)/s), \(C_0^{*}\) is the bulk concentration (mol/cm\(^3\)), \(\Gamma_0\) is represent the amount of RuHex-confined near electrode surface and \(nFA \Gamma_0\) (known as surface excess) is the charge obtained by adsorbed target
miRNA. CC curves were constructed by plotting the total charge versus square-root of time ($t^{1/2}/s^{1/2}$) in the presence and absence RuHex. $Q$ and $Q_{dl}$ were estimated from the intercept of these two curves at $t = 0$. Here, $Q$ represents the total charge derived from both Faradaic and non-Faradaic (capacitive) charges. Hence, the corresponding charge of RuHex (electrostatically bound to surface confined RNA) $Q_{RNA}$ can be calculated from Eqn. S3,

$$Q_{RNA} = Q - Q_{dl} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \text{(Eq S3)}$$

**RNA Extraction from Cell Lines and Tissue Samples**

Total RNA was extracted and purified from all ESCC cell lines and tissue samples according to manufacturer’s guidelines (Qiagen, Germany). Agarose gel (1.5%) electrophoresis was performed to evaluate the quality and quantity of RNA. Purity was verified via Nanodrop spectrophotometric analysis (BioLab, USA). The concentration of RNA was noted in ng/µl and stored at -80°C until assayed.

**Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR)**

The cDNA conversion was performed using miScript Reverse Transcription kit (Qiagen, Germany). For RT-qPCR analysis, each cDNA sample was 30 ng/µL and stored at -20°C until further use. To quantify the expression level of miR-107, RT-qPCR was performed in a total volume of 10 µL reaction mixture containing 5.0 µL of 2XSensiMix SYBR No-ROX master mix (Bioline, UK), 1.0 µL of each 1.0 µmole/µL primer, 1.0 µL of cDNA at 30 ng/µL and 2.0 µL of nuclease-free water. Thermal cycling was initiated with a first denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation), 55°C for 30 s (annealing), and 72 °C for 30 s (extension). The expression levels of miR-107 were normalized
using the endogenous U6 control gene, which was amplified in the same run following the same procedure described above. Assays were done in triplicate in the presence of a non-template control in each PCR assay. The analysis of miR-107 expression was achieved following previous method (Gopalan et al., 2016).

2. Supplementary tables

**Table S1. Oligonucleotide sequences**

<table>
<thead>
<tr>
<th>Oligos</th>
<th>5’-Sequences-3’</th>
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<tr>
<td>Biotinylated miR-107 capture probe</td>
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<td>Synthetic miR-107 sequence</td>
<td>ArGrCrArGrArUrUrGrGrArGrGrArUrUrGrUrUrGrCrA</td>
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<tr>
<td>Synthetic miR-21 sequence</td>
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<tr>
<td>Synthetic miRNA-338-3p sequence</td>
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<td>miR-107 F</td>
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</tr>
<tr>
<td>U6R</td>
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<tr>
<td>Assay</td>
<td>Target</td>
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<td>-------</td>
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<tr>
<td>SWV using an interpenetrated network of CNTs and CP</td>
<td>miR-141</td>
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<tr>
<td>Amperometry using Pd NPs as enhancer and linker</td>
<td>miR-155</td>
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<td>Chronoamperometry based on magnetic bead based capture</td>
<td>miR-222</td>
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<td>DPV using Poly(A) extensions and gold-RNA affinity</td>
<td>miR-107</td>
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<tr>
<td>DPV using a redox complex of Os(VI)bipy</td>
<td>miR-261 and miR-522</td>
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<td>DNA concatamers based DPV detection</td>
<td>miR-21</td>
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<td>Potentiometry using microelectrode array</td>
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<td>Detection of miRNA based on an arched probe mediated IEA</td>
<td>miR-21</td>
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<td>Detection of MicroRNAs with MFNB</td>
<td>Let-7b</td>
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<td>Method based on the use of Using a ZFP specific to DNA–RNA hybrids</td>
<td>miR 21</td>
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<tr>
<td>Based on Ir (III) complex as catalyst</td>
<td>miR-21</td>
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</table>

**Abbreviations:** SWV: Square wave voltammetry, CNTs: carbon nanotubes; CP: conductive polymer; NPs: nanoparticles; DPV: differential pulse voltammetry; Os(VI)bipy: osmium (VI) and 2,2′-bipyridine; IEA: isothermal exponential amplification; MFNB: morpholino-functionalized nanochannel biosensor; ZFP: zinc finger protein; Ir: Iridium.
3. Supplementary figures

**Fig. S1.** SEM image of bare screen-printed carbon electrode (a, b) and Au-NPFe$_2$O$_3$NC-modified screen-printed carbon electrode (c, d).

**Cyclic voltammetry at bare and modified SPCE**

The modification of the SPCE with Au-NPFe$_2$O$_3$NC and target RNA was followed by the electron transfer reaction of the [Fe(CN)$_6$]$^{3-/4-}$ process by cyclic voltammetry. As can be seen in the CV data below, a pair of well-defined cathodic and anodic peaks for the [Fe(CN)$_6$]$^{3-}$ system were obtained at -60 mV and +220 mV (vs. Ag/AgCl) at bare SPCE, where Au-NPFe$_2$O$_3$NC-modified SPCE (SPCE/Au-NPFe$_2$O$_3$NC) shows relatively less cathodic and anodic peak current with enhanced peak separation. When 10 pM of synthetic RNA was adsorbed on the SPCE/Au-NPFe$_2$O$_3$NC, the current is further reduced. The peak separation between cathodic and anodic peaks has also enhanced. These CV responses could be explained by the fact that due to the coulombic repulsion among the negatively charged [Fe(CN)$_6$]$^{3-}$ with negatively charged RNA strands and iron oxide nanocubes, [Fe(CN)$_6$]$^{3-}$ molecules tend to repel out from the electrodes which hinders the electron transfer process thereby reducing the
current response and increasing the peak separation. These results indicate successful stepwise modification of Au-NPFe$_2$O$_3$NC and RNA on the SPCE surface.

![Cyclic voltammograms for bare, Au-NPFe$_2$O$_3$NC, and Au-NPFe$_2$O$_3$NC/RNA-modified SPCE in 10 mM phosphate buffer saline containing 2 mM K$_3$[Fe(CN)$_6$]. Scan rate 100 mV/s.](image)

**Fig. S2.** Cyclic voltammograms for bare, Au-NPFe$_2$O$_3$NC, and Au-NPFe$_2$O$_3$NC/RNA-modified SPCE in 10 mM phosphate buffer saline containing 2 mM K$_3$[Fe(CN)$_6$]. Scan rate 100 mV/s.
Fig. S3. **Assay specificity.** Charge density obtained for the synthetic miR-107 at designated electrodes without electrocatalytic cycle (in the presence of only [Ru(NH$_3$)$_6$]$_3^{3+}$). Left bar represents the charge density obtained at the bare SPCE after adsorbing 10 pM of target miR-107. Control and Target-miRNA bars represent the charge density obtained at SPCE/Au-NPFe$_2$O$_3$NC electrode before and after adsorbing 10 pM of target miR-107. miR-21 and miR-338-3p bars represent the charge density obtained at SPCE/Au-NPFe$_2$O$_3$NC electrode after adsorbing 10 pM of non-complimentary miR-21 and miR-338-3p targets respectively. Inset, corresponding CC curves ($Q$ vs. $t^{1/2}$). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for $n = 3$).
Fig. S4. Comparison of the two cyclic voltammograms obtained at an unmodified GCE and Au-NPFe$_2$O$_3$NC-modified GCE in 50µM RuHex (scan rate, 50 mVs$^{-1}$).
Fig. S5. Corresponding curves for $i_{pc}$ and $i_{pa}$ (current density) as a function of $ν^{1/2}$ at unmodified-GCE and Au-NPFe$_2$O$_3$NC modified-GCE electrodes at different scan rate (50 μM RuHex, 0.01 M PBS, pH 7.0).
**Fig. S6. Sensitivity without electrocatalytic cycle.** (A) Typical CC curves ($Q$ vs. $t^{1/2}$) for the (b-g); 10 fM-1.0 nM of miR-107. Curves a is for the charge density obtained at SPCE/Au-NPFe$_2$O$_3$NC electrode before adsorbing target miR-107. (B) $Q_{RNA}$-concentration profile across the range of 10 fM to 1.0 nM miR-107. Inset, corresponding calibration plot. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for $n$ = 3).
References


Chapter 6
Towards point-of-care detection of long non-coding RNA

The purpose of our final assay platform was to reflect on the extent to which the RNA biosensor might move towards more personalized diagnostics to enable a simple translational-focused platform. In this regard, after successful demonstrations of three sensitive electrochemical assays for mRNA and miRNAs, we developed a detection platform for HOTAIR lncRNA, which uniquely integrated three useful readout approaches (i.e., naked-eye, UV-vis absorbance and amperometric readouts) under one assay protocol. Given the fact that up-regulated HOTAIR is a promising class of biomarker in most of the cancers, this assay can be a potential transformative technology for the inexpensive and quick first-pass screening (via naked eye) of a large number of samples specifically in resource-limited settings. The assay relies on biotin enrichment of isothermally amplified HOTAIR amplicons, followed by their magnetic separation and subsequent TMB/HRP-based colorimetric reaction. In addition to the visual detection, UV-vis and amperometric measurements were performed for more quantitative estimation of HOTAIR.

Chapter 6 is included as it appears in Analyst (2018)
Statement of Contribution to Co-authored Published Paper

This chapter is in the form of a co-authored published paper. The bibliographic details of the co-authored published paper are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the paper.

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My contribution to the published paper involved:

- Experimental planning and set-up
- Data acquisition and analysis
- Manuscript preparation.

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**Naked-eye and electrochemical detection of isothermally amplified HOTAIR long non-coding RNA**


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Naked-eye and electrochemical detection of isothermally amplified HOTAIR long non-coding RNA †

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(†Electronic supplementary information (ESI) available. See DOI: … … … )
Abstract

An inexpensive, simple and rapid sensor platform capable of detecting cancer-related long non-coding RNA (lncRNA) with high accuracy is of great interest in the field of molecular diagnostics. Herein, we report on the development of a new colorimetric and electrochemical assay platform for long non-coding HOX transcript antisense intergenic RNA (HOTAIR) detection. Isothermal reverse transcription-recombinase polymerase amplification (RT-RPA) was performed to amplify HOTAIR sequences from a RNA pool extracted from a designated number of ovarian cancer cells and a small cohort of plasma samples derived from patients with ovarian cancer. During RT-RPA, biotinylated dUTPs were randomly incorporated in the amplified product. Subsequently, HOTAIR amplicons were magnetically purified and isolated followed by a horseradish peroxidase (HRP)-catalyzed colorimetric reaction in the presence of 3,3′,5,5′-tetramethylbenzidine (TMB)/H₂O₂ system. We finally introduced three potential readout methods for HOTAIR detection – (i) naked-eye visualisation of the color change for a quick screening of the target, (ii) quantitative absorbance measurement by UV-vis, and (iii) amperometric quantification using the electrochemical properties of TMB. The assay has shown excellent reproducibility (% RSD = < 5%, for n = 3) and sensitivity (10 cells/mL) while detecting HOTAIR in cancer cell lines and patient samples. Expression of HOTAIR in clinical samples was also verified with a standard RT-qPCR method. We believe that our proof of concept assay may find potential relevance for routine clinical screening of cancer-associated lncRNAs.
Introduction

Advances in genomic sequencing technologies have revealed fascinating complexity of transcriptome. A vast majority of eukaryotic transcriptome was found to be comprised of non-coding RNAs (ncRNAs) which play crucial role in regulation of gene expression.\textsuperscript{1} Taxonomically, ncRNAs may be categorised as housekeeping (\textit{e.g.}, ribosomal RNA) and regulatory RNAs (\textit{e.g.}, microRNA and long ncRNA).\textsuperscript{1, 2} Long ncRNAs (lncRNAs), arbitrarily defined as \textasciitilde200 nt or longer transcripts, represent a subgroup of regulatory ncRNAs involved in chromatin remodelling, epigenomic modulation, as well as regulation of post-transcriptional gene expression and are increasingly being recognised as pivotal regulators of physiology and pathology.\textsuperscript{3, 4} An increasing number of studies have highlighted the significance of lncRNA as diagnostic and prognostic biomarkers for different types of cancers.\textsuperscript{5-7} HOX transcript antisense intergenic RNA (HOTAIR), a 2158 nucleotide long transcript, is among the very few well-characterised lncRNAs which has been reported to be aberrantly expressed in multiple cancers.\textsuperscript{8} HOTAIR influences the chromatin dynamics by interacting with histone modifiers thereby influencing gene expression. Specifically, it binds with the Polycomb Repressive Complex 2 (PRC2) and histone demethylase complex [LSD1 (lysine-specific demethylase 1), and directs their epigenetic modification and gene silencing. In addition to this scaffold function, HOTAIR exerts a regulatory role on the maintenance of protein levels \textit{via} the ubiquitin-proteasome pathway.\textsuperscript{9-11} Through these activities, aberrantly expressed HOTAIR may dysregulate multiple genes involved in the pathogenesis of cancer, and thereby promoting initiation, growth and invasiveness of tumor.\textsuperscript{10} A number of recent evidence suggests that up-regulated HOTAIR is associated with proliferation and invasion of tumor cells in breast, liver, ovarian, colorectal, and pancreatic cancers. Moreover, high expression of HOTAIR has been found to be correlated with survival and prognosis of cancer.
patients. Consequently, HOTAIR has become an emerging class of diagnostic or prognostic biomarker for several types of cancers.

A functional, specific and sensitive detection platform that can readily detect HOTAIR in clinical samples may thus open a principal avenue in the diagnostics, prognostics and overall cancer care. Over the past few years, several molecular biology-based approaches such as Northern blotting, microarray, quantitative reverse transcription PCR (RT-qPCR), and next generation RNA-seq have been used for analysis of HOTAIR and other lncRNAs. Despite being a specific and commonly used method, Northern blotting is susceptible to RNA degradation and requires relatively large amount of sample input. Moreover, the use of radioactive probes and excess formaldehyde in some of the conventional Northern blotting approaches limit their applicability. Although RT-qPCR is considered as more sensitive and reliable, it relies on extensive and expensive instrumentation, and is often affected by the amplification-bias and longer analysis time.

In this regard, a relatively rapid, sensitive, and inexpensive biosensor-based HOTAIR detection could alleviate some of the above-mentioned issues and may represent an appealing alternative for routine analysis of lncRNA. Electrochemical biosensors have shown great promise in diagnostic applications due to their relatively high sensitivity and specificity, cost-effectiveness and compatibility with the miniaturization. Optical assays comprise another group of highly potential biosensor platform that is suitable for rapid, direct and label-free analysis of RNA. Among optical strategies, colorimetric method is highly amenable for patient-centric diagnostics in resource-limited settings, where naked-eye evaluation could be useful for the first-pass screening of the analyte. One of the widely used colorimetric systems is the horseradish peroxidase (HRP)/H$_2$O$_2$ coupled with 3,3',5,5'-tetramethylbenzidine (TMB) substrate. This assay generates a coloured byproduct to signal the presence of target, given that the target biomolecules are attached with HRP. Over the
past several years, it was shown that HRP and other enzyme-immobilized nanoparticles (e.g., catalase coated metal-organic framework) have the ability to enhance the performance of bioassays.\textsuperscript{22-25} Another important element of these colorimetric assays is their chromogenic substrates (e.g., TMB) which often possess the electrochemical properties, hence it can be further interrogated \textit{via} an electrochemical quantitirication approach. Recently few combined colorimetric and electrochemical assay platforms have been demonstrated for RNA detection.\textsuperscript{26-28}

Despite these developments, not many examples of lncRNA biosensing have been reported to date.\textsuperscript{1, 29-31} In addition, to the best of our knowledge, there has been no report on the development of biosensor for the analysis of HOTAIR. One of the major reasons that complicates the biosensing of lncRNA is their structural instability on the sensor interface (\textit{i.e.}, strong folding tendency of lncRNAs into various secondary or tertiary structure).\textsuperscript{1} Therefore, biosensing of lncRNA with a conventional RNA-hybridization-based system remains a critical challenge. The present study avoids conventional RNA sensor designs, and reports on the development of a simple colorimetric and electrochemical assay platform for HOTAIR detection. We employed a simple and rapid recombinase polymerase amplification (RPA) isothermal amplification technique to produce biotinylated HOTAIR products. The target amplicons were further magnetically purified using streptavidin coated HRP and dynabeads, and their level was evaluated with naked-eye and UV-vis \textit{via} horseradish peroxidase (HRP) -catalyzed colorimetric readout in the presence of TMB/ H\textsubscript{2}O\textsubscript{2} system. We further used the electroactive properties of TMB to develop an alternative electrochemical assay for more rigorous quantification of HOTAIR. The applicability of our assay has been successfully tested in ovarian cancer cell lines and a small cohort of plasma samples derived from patients with ovarian cancer. The analytical performance of our assay was also found to be in good agreement with the RT-qPCR assay.
Experimental

Reagents and instruments

All the synthetic oligonucleotides were purchased from Integrated DNA Technologies (Singapore) (Table 1). 1-Step TMB substrate solution, HRP-conjugated streptavidin, biotin-11-dUTP solution and Dynabeads MyOne Streptavidin C1 were purchased from Thermo Fisher Scientific (Australia). Reagent grade Tween20, triton-X, and phosphate buffered saline (PBS) tablet (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) were purchased from Sigma-Aldrich (USA). Ultrapure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used for preparing aqueous solutions. The TwistAmp basic RT-RPA kit (Twist-DX, UK) with pre-included reverse transcriptase was used for isothermal amplification.

Countess II Automated Cell Counter (Thermo Fisher Scientific, Australia) was used for counting the cells. A DynaMag 2 magnetic separation rack was acquired from Thermo Fisher Scientific (Australia), and microtube mixer from Eppendorf (Germany) was also employed. The absorbance data at 652 nm was taken using a spectrophotometer (Fluostar Omega microplate reader, BMG Labtech, Germany). All electrochemical measurements were performed with a CHI650 electrochemical workstation (CH Instrument, USA). Cyclic voltammetry (CV) and chronoamperometry experiments were carried out on the screen-printed gold electrode (SPGE). SPGE (220BT) were purchased from Dropsens (Spain) which is comprised of gold working and auxiliary electrodes with Ag-modified reference electrode printed on a ceramic substrate.

Preparation of RNA from cell line and ovarian cancer samples
SKOV3 ovarian cancer and MeT-5A non-cancerous cell lines were cultured in RPMI-1640 growth medium (Life Technologies, Australia) supplemented with 10% foetal bovine serum (Life Technologies, Australia) and 1% penicillin/streptomycin (Life Technologies, Australia) in a humidified incubator containing 5% CO₂ supply at 37 °C. The SKOV3 and MeT-5A cells were collected after 4 and 7 days, respectively, for subsequent cell counting and RNA extraction.

Staged samples (cross-sectional) were collected at the Ochsner Baptist Medical Center in the clinical trials and obtained via The UQ Centre for Clinical research (UQ IRB 2016000300). Plasma samples were obtained in accordance with the declaration of Helsinki and approved by the Ethics Committee of The University of Queensland and the Ochsner Medical Center (New Orleans, USA). Plasma was separated from whole blood by centrifugation (2000g x 10 min at Room temperature) and stored at -80°C until analyses. Ovarian cancer samples were collected prospectively, assigned according to the histotype classification (e.g. stage I, and stage III), and stored at -80°C in the Biobank units. Only patients with epithelial ovarian cancer high-grade serous subtype (n=3) and benign samples (n=2) were included in this study (Table S1). RNA was extracted using the miRNeasy Mini Kit (Qiagen, Australia) and TRIzol LS Reagent (Life Technologies, Australia) as the lysis solution. A spectrophotometer (SPECTROstar Nano Microplate Reader, BMG Labtech) was used to quantify RNA concentration. Following a cleanliness check and blank measurement using RNase-free water, 2.0 uL of sample was pipetted onto each microdrop well on an LVVis plate. RNA concentration was measured using MARS data analysis microplate reader software.

RT-RPA and colorimetric assay
Synthetic primer sequences for amplifying HOTAIR (for RT-RPA), and GAPDH (housekeeping gene) were designed (Table 1). RT-RPA was performed using TwistAmp Basic RPA kit (Twist-DX, UK) according to the manufacturer’s instructions with slight modifications. In brief, unless otherwise stated, the RT-RPA master mix contained 29.5 µL of rehydration buffer in one sachet of the kit, 4.8 µL of each primer (500 nM), 4.0 µL of 40 nM biotinylated dUTPs and 11.7 µL of RNase free water to make a 50 µL reaction volume. The volume was aliquoted in 4 tubes and 1.0 µL of template RNA (15 ng) and 0.625 µL of 280 mM MgAc were added before incubating at 43°C for 20 min. The amplified products were immediately stored at -20°C for further replicate analysis and gel electrophoresis.

2.0 µL of RT-RPA products was taken and mixed with 1.0 µL of 1: 2000 diluted streptavidin (SA)-HRP, 1.0 µL of SA-magnetic beads and 10 µL of wash buffer-1 (0.5% triton-X in 10 mM PBS) for 10 min. A magnetic rack was subsequently used to separate the beads. The beads were washed twice with wash buffer-2 (0.1% Tween20 in 10 mM PBS). After magnetic purification of the beads, 35 µL of 1-Step TMB substrate solution was added to the beads and incubated for 10 min to observe the color change. Next, using an external magnet, the clear blue solution was separated, and absorbance (at 652 nm) data was taken with a spectrophotometer. Please note that, we have demonstrated current proof of concept electrochemical and colorimetric assays using RT-RPA HOTAIR amplicons only. The methods however can be extended towards the biosensing of housekeeping gene (e.g., GAPDH) for a relative quantification HOTAIR.

**Electrochemical readout**
CV was performed in 10 mM PBS solution containing 2.0 mM [K$_3$Fe(CN)$_6$] electrolyte solution for determining the effective surface area of the electrode as shown before $^{32,33}$ (see ESI). For electrochemical detection of RT-RPA products, 1.0 µL of stop solution was added to the blue colored solution described above which turned yellow upon the cessation of the reaction. Then, 35 µL of the resulting mixture was pipetted onto the SPGE surface for amperometry measurements at 150 mV for 80 s.

**Results and discussion**

The assay protocol for the isolation and detection of HOTAIR is schematically presented in Fig. 1. In our assay, isothermal RT-RPA amplified HOTAIR was detected using TMB-based colorimetric and electrochemical readouts. Briefly, total RNA was extracted from cell lines and plasma samples derived from patients with ovarian cancer. During RT-RPA, biotinylated dUTP bases were randomly inserted into the amplified strands. Following RT-RPA, SA-coated Dynabeads and SA-HRP were dispersed into the amplified product, which selectively bind to the biotinylated strands of HOTAIR due to the well-established high-affinity interaction between biotin and SA.$^{25,34,35}$ The target RNA was then isolated and purified with multiple magnetic washing steps via Dynabead-based magnetic separation protocol. Upon addition of the 1-step TMB solution, the HRP present in the isolated HOTAIR-Dynabeads-HRP conjugates triggers oxidation of TMB that generates a blue-colored charge transfer complex. This facilitated the naked-eye observation of the presence of HOTAIR. The intensity of the colored complex is likely to be proportional to the amount of captured HRP present in the conjugates, which is in turn proportional to the amount of HOTAIR in the amplified RNA sample. The color intensity was also quantified by UV–vis at 652 nm. With further addition of a stop solution (acid), the blue colored product converted to
a stable electroactive yellow (diimine) complex which enabled an alternative amperometric quantification of HOTAIR.

The attachment of biomaterials with biotinylated HOTAIR amplicons was investigated by an AFM (Fig S1). The image shows distinct differences between the control (no biotinylated amplicon, Fig S1A) and sample (biotinylated HOTAIR amplicons/SA-Dynabeads/SA-HRP). To demonstrate the assay specificity, we performed our assay with RNA extracted from the same number (10000 cell) of two different cell lines. SKOV3 is an ovarian cancer cell line where HOTAIR has been reported to be overexpressed,\textsuperscript{36} while MeT-5A is a non-cancerous cell line. Apart from the no-template (NoT) control, we performed an additional control experiment where we did not add biotinylated d-UTP during the RT-RPA (No b-dUTP). As can be seen in Fig. 2A, the control experiment without target (NoT) produces no color and a very low response in the UV-vis measurement (absorbance at 652 = 0.034). The second control experiment, where we did not include b-dUTP, also did not generate any noticeable color change, and the UV-vis measurement showed a similar level of low response (absorbance at 652 = 0.075). These control studies clearly demonstrate that our method completely relies on the presence of biotinylated HOTAIR in the amplified products, followed by their magnetic separation via SA-biotin interaction. The data further confirms that our magnetic separation protocol and the overall assay was not susceptible to non-specific interaction from other types of non-specific RNAs present in the bulk RNA samples, thereby attributing high specificity to the assay.

When the assay was run with SKOV3 cancer cell lines, it consistently produced an absorbance which was ~ 50 times higher than that of the NoT (Absorbance at 652 = 1.809 vs. 0.035). However, in the assay with non-cancerous Met-5A cell line, a 5-times lower response in the UV-vis data was recorded (Absorbance at 652= 1.809 vs. 0.352). Also as shown in the picture (Fig. 2A inset), it is evident that all the control and cell line samples can be visually
discerned with naked-eye. When we performed the electrochemical experiment, we found a similar trend of the amperometric response (Fig. 2B), where the current response obtained with SKOV3 is several folds higher than that of No-b-dUTP and NoT controls (2.2 vs 0.22 and 0.1 µAcm⁻²). Together these control experiments clearly demonstrate the excellent level of specificity of our assay towards the detection of HOTAIR. Furthermore, we performed gel electrophoresis of the RT-RPA products to verify the HOTAIR primer specificity. As can be seen in Fig. S2, from the same starting amount of RT-RPA product, clear band for a specific 170 bp products were observed for both SKOV3 and Met-5A samples, while the control experiments without the template (NoT) did not generate any RPA products.

To evaluate the sensitivity of our assay, total RNA isolated from a known number of SKOV3 cell line samples obtained via serial dilution (10:1, 100:1, 1000:1 and 10000:1 cells/mL) were tested. From the picture of Fig. 3A (left panel), a gradual increment of the color intensity with increasing cell numbers could be visually observed. Moreover, by naked-eye, the color corresponding to the RNA sample of 10 cells (light blue) can easily be visually discriminated from that of the NoT (no color change). Afterwards, when the subtle color changes of the assay were quantified via UV-vis readout, a similar increasing trend of the absorbance value was observed in RNA sample collected across the range of 0–10 000 SKOV3 cells (Fig. 3A, right panel). This linear increase of the colorimetric response is due to the presence of higher amount of RT-RPA biotinylated HOTAIR products with the increasing number of cancer cells. These amplicons in turn can attract a large number of streptavidin-HRP, which subsequently accelerate the rate of TMB oxidation, thus can increase the intensity of blue colored complex. As can be seen in Fig. 3A, the linear regression equation for the colorimetric assay was estimated to be \( y = 0.0468x - 0.1006 \), with the correlation coefficient \( (r^2) \) of 0.9602. The data also shows that our assay can successfully detect
HOTAIR from RNA samples extracted from a very low number of cells (10 cells / mL), with a very high signal to noise ratio of ~ 4.5 (Absorbance at 652 nm = 0.034 vs 0.156).

In the case of electrochemical readout, similar to the UV-vis data, a gradual increase in the current density profile with increasing amount of cells was recorded (Fig. 3B). The linear regression equation for amperometric readout was estimated as \( y = 0.49 \times + 0.176 \), with the \( r^2 \) of 0.9942. We also found that this readout could detect HOTAIR from RNA samples extracted from as low as 10 cells/mL, however with a relatively better signal to noise ratio of 6.8. It is noteworthy that this low level of LOD is adequate for clinical screening of HOTAIR.

To the best of our knowledge, until now, there are no previous reports on the colorimetric and electrochemical detection of HOTAIR. However, there are only a few reports on the electrochemical detection of other lncRNAs. Among these, assays developed by Pu and colleagues can be considered as some of the most prominent and have been reported to detect ‘highly up-regulated in liver cancer’ (HULC) and ‘nuclear paraspeckle assembly transcript 1’ (NEAT1) long non coding RNA.\(^{29,30}\) In these reports, to enhance the assay performance, electrochemical sensors were functionalised with especially designed nanostructured materials and components (e.g., green L- cysteine electrodeposition, tagging with Au–Rh hollow nanospheres, designing PtPd nanodendrite/nano-flowerlike graphene oxide, etc.). Our assay avoids such time consuming and multi-step sensor fabrication procedures and rather adopted a much simpler naked-eye colorimetric assessment followed by an electrochemical readout. By employing unmodified and easy-to-use disposable electrodes our report offers a sensitive and selective platform for lncRNA detection.

To demonstrate the applicability of our assay in complex biological samples, we further performed our assay on five plasma samples derived from patients with ovarian
cancer. Only patients with epithelial ovarian cancer high-grade serous subtype (n = 3) and benign samples (n = 2) were tested (Table S1). As shown in Fig. 4A, compared to the NoT control, absorbance data corresponding to the high grade epithelial ovarian cancer samples (P1, P2, P3) produced at least 75-times higher response (absorbance at 652 nm = 0.034 vs. 2.882/2.702/2.519). It is also noticeable that the absorbance obtained from P1, P2, P3 was at least two times higher than that of the benign samples (P4 and P5). The current density profile obtained for the electrochemical readout (Fig. 4B) also followed the similar trend of colorimetric data. The data derived from these clinical samples also showed an excellent inter-assay reproducibility (RSD < 5%, for n = 3) for the detection of HOTAIR which is better or comparable with most of the existing RNA sensors.1,25,34,35,37,38 We also verified HOTAIR expression in clinical samples via RT-qPCR (see ESI).

Conclusions

We have developed a simple naked-eye colorimetric and electrochemical approach based on an innovative merger of isothermal RT-RPA and HRP-catalyzed colorimetric and electrochemical readouts. Isothermally amplified and magnetically purified and isolated HOTAIR sequences were detected by HRP-catalyzed colorimetric reaction in the presence of TMB/H2O2 system. The initial readout was obtained with a visual observation of the color change of the assay which allows a quick first pass screening (yes/no answer) of HOTAIR. This followed by a quantitative absorbance measurement by UV-vis (at 652 nm). The versatility of the developed assay platform was further demonstrated with an amperometric readout which quantified HOTAIR with more precision. The data obtained with our proof of concept assay which integrates these three useful readout platforms were found to be in good accordance with a standard RT-qPCR while the assay also showed excellent reproducibility.
and sensitivity (10 cells/mL) in cancer cell lines and patient samples.

Overall, there are some distinct advantages in our assays. First, the assay was able to detect HOTAIR in the human plasma samples, which demonstrates its high potentiality towards liquid biopsy of cancer. Second, RT-RPA isothermal assay allows us to avoid expensive and time-consuming PCR amplification setup, and enable rapid amplification of HOTAIR within 20 minutes at a manageable temperature in the presence of minimal resources. Third, multiple magnetic mixing and purification steps to isolate the HOTAIR amplicons may reduce the matrix effects of the biological samples attributing to the diminution of non-specific detection. Fourth, the assay eliminates the need for tedious cleaning procedures of traditional disk electrodes as it used inexpensive disposable SPGE. Finally, considering the minimal resource requirement of the colorimetric sensor, along with the versatility and sensitivity of the electrochemical method, we envisage that our assay would find potential clinical application for sensitive and specific analysis of lncRNA for screening human diseases.

Acknowledgement

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Notes and References


**Figure Captions**

**Fig. 1.** Schematic of the colorimetric and electrochemical detection of HOTAIR assay. Isothermal RT-RPA was performed on extracted total RNA. During amplification, biotinylated dUTP were inserted into the amplicons. Using SA-Dynabeads, SA-HRP, the biotinylated HOTAIR was magnetically purified separated. Subsequently, employment of HRP/TMB based colorimetric reaction facilitated naked-eye and UV-vis readout while the electrochemical properties of TMB enabled a more precise electrochemical quantification of HOTAIR.

**Fig. 2.** Specificity of the colorimetric and electrochemical assay. (A) Absorbance (UV-vis) obtained for SKOV3 (ovarian cancer), MeT-5A (non-cancerous) cell lines, no biotinylated d-UTP bases (No b-dUTP) and no-template (NoT) controls (inset: pictures of the naked-eye detection); (B) representative i–t curves of amperometric current density for *HOTAIR* detection. Error bars represent the standard deviation of three independent experiments.
Fig. 3. Sensitivity of the assay. (A) Picture for the naked-eye detection of HOTAIR derived from total RNA of a known number of (0 - 10000) SKOV3 cancer cells (left panel); corresponding bar diagram for absorbance at 652 nm(right panel). The inset shows the analogous linear calibration plot. (B) Corresponding i-t curve (left panel) and bar diagram (right panel) for the amperometric current density obtained with RNA extracted from a designated number of SKOV3 cancer cells. The inset provides the analogous linear calibration plot. Error bars represent standard deviation of three independent experiments.

Fig. 4. Clinical sample analysis. (A) Corresponding bar diagram for absorbance at 652 nm (inset: picture of the naked-eye screening); and (B) corresponding i-t curve for the amperometric current density obtained with RNA extracted from patient samples (P1, P2, P3 = high-grade serous subtype and P4, P5 = benign samples) and NoT control. Error bars represent standard deviation of three independent experiments.
**Table 1.** Oligonucleotide sequences used in experiments

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<td>HOTAIR Rev Primer Sequence</td>
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Fig. 1

Fig. 2
Fig. 3
Fig. 4

A naked-eye, colorimetric and electrochemical detection of HOTAIR long non-coding RNA has been demonstrated.
Electronic supplementary information (ESI)

for

Naked-eye and electrochemical detection of isothermally amplified HOTAIR long non-coding RNA †

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Experimental

Determination of surface area of electrodes

The effective areas of SPGE was determined by the measurement of the peak current obtained as a function of scan rate under cyclic voltammetric conditions for the one-electron reduction of \([\text{Fe(CN)}_6]^{3-}\) [2.0 mM \(\text{K}_3\text{Fe(CN)}_6\) in 10 mM PBS (0.5 M KCl)] using the Randles- Sevcik equation (Eqn. S1), as shown before.¹

\[
i_p = (2.69 \times 10^5) n^{3/2} AD^{1/2} C_v^{1/2} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \text{(Eqn. S1)}
\]

Atomic Force Microscopy (AFM) Measurements

A Cypher scanning atomic force microscope (Asylum Research, Santa Barbara, CA) was used to image the samples (Fig S1). The cantilevers used were from Etalon series (TipsNano, Tallinn, Estonia) with a nominal Resonant frequency of 140 kHz. All the measurements were performed in tapping mode method at room temperature.

RT-qPCR

The cDNA conversion was performed in a 20 µL reaction using miScript Reverse Transcription kit (Qiagen, Germany) according to the manufacturer’s instructions and the converted product was stored at -20°C until further use. To verify the expression of HOTAIR, RT-qPCR was performed in a total reaction volume of 50 µL containing 25 µL of 2XSensiMix SYBR No-ROX master mix (Bioline, UK), 1.0 µL each of 10 µM primer, 3.0 µL of cDNA at 5.0 ng/µL and 19 µL of nuclease-free water. Thermal cycling was initiated with a first denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation), 55°C for 15 s (annealing), and 72 °C for 15 s (extension). All samples were run in triplicate and no template control was also included in the PCR assays.
Table S1. Patient sample information

<table>
<thead>
<tr>
<th>Sample id</th>
<th>Sample category</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Cancer</td>
<td>Mucinous borderline tumour, endocervical type, left ovary only</td>
</tr>
<tr>
<td>P2</td>
<td>Cancer</td>
<td>High-grade papillary serous carcinoma</td>
</tr>
<tr>
<td>P3</td>
<td>Cancer</td>
<td>Papillary Serous Carcinoma</td>
</tr>
<tr>
<td>P4</td>
<td>Benign</td>
<td>Benign Mucinous Cystadenoma</td>
</tr>
<tr>
<td>P5</td>
<td>Benign</td>
<td>Benign Haemorrhagic Cyst Left Ovary-Normal Right Ovary</td>
</tr>
</tbody>
</table>
Fig. S1. Corresponding AFM 3D images of A) conjugates without RT-RPA amplified biotinylated target (dynabads only), and B) biotinylated target amplicons/SA-HRP/SA-dynabeads complex.
**Fig. S2.** Corresponding gel electrophoresis images after RT-RPA for NoT, and RNA amplicons derived from SKOV3 and Met-5A cell lines.

**Notes and References**

Chapter 7
Conclusions

The overall focus of this PhD study was to design and develop inexpensive, simple biosensing platforms for the analysis of various clinically relevant RNA species. This thesis presents the efforts undertaken to develop practical biosensor platforms capable of efficiently detecting a range of important RNA targets in clinical samples, with high specificity and sensitivity. Chapter 1 provides background and summarises the specific motivation for the research work. The extensive literature review presented in Chapter 2 provides an overview of the up-to-date developments in RNA biosensing technologies, with a special emphasis on electrochemical-detection approaches. The chapter further highlights the major technical and biological challenges of existing RNA detection techniques, as well as the future perspective of the field. Chapter 3 represents an amplification-free electrochemical approach for the detection of mRNA. Chapters 4 and 5 report on the intrinsic electrocatalytic activity of two new classes of graphene oxide and gold nanoparticles coated hybrid nanocomposites, for the development of highly specific and ultrasensitive miRNA biosensors. Chapter 6 introduces a translational-focused versatile assay platform that enables naked-eye, colorimetric, and electrochemical interrogation of lncRNA.

In brief, the thesis describes the design and development of a biosensing platform consisting of four novel readout schemes for the simple, rapid, and inexpensive analysis of various components of transcriptome, i.e., mRNA, miRNA, and lncRNA. Considering the well-established significance of mRNAs as disease biomarkers, initially we developed a simple electrochemical biosensing strategy for clinically relevant mRNA detection in cancer. Magnetically purified and directly adsorbed mRNA was quantified by differential pulse voltammetry (DPV) in the
presence of an $[\text{Fe(CN)}_6]^{4/-3}$ redox system. One of the significant developments to emerge from this assay was that it eliminates the need for multi-step electrode modification and sensor fabrication procedures, and exerts the action in an amplification-free manner using easy-to-operate unmodified disposable gold electrode. As a model target we assayed FAM134B mRNA, which is a potential biomarker for gastrointestinal carcinomas.

These mRNAs represent only a small portion of the human transcriptome, while the vast majority of transcripts known as non-coding RNAs, such as miRNA, lncRNA, also have enormous potential as candidate biomarkers. After establishing the proof of concept strategy for mRNA detection, we extended our effort towards biosensing of small regulatory miRNAs. Major sources of inefficiencies in miRNA biosensing are some of its inherent features, such as the requirement of highly sensitive (due to its low abundance in biological samples) and specific (to avoid interference from other non-target miRNAs with sequence similarity and size homology) strategies. In this regard, this thesis presents the efforts that have been undertaken for the development of highly specific and sensitive platform for miRNA analysis. Two novel nanoporous GO/IO hybrid and Au-NPFe$_2$O$_3$NC nanomaterials were synthesised to achieve this goal. Exploiting the benefits of superparamagnetism, high electrocatalytic activity, and enormous surface loading capacity of highly porous framework of these nanomaterials, two portable alternative electrochemical (chronocoulometric) readout strategies were developed for the detection of miR-21 and miR-107, with an ultrasensitive detection limit of 1.0 fM and 100 aM. The developed assays thus provide a framework to meet the high sensitivity and specificity requirement of future bioassays. Overall these two assays strengthen the idea that adoption of $[\text{Ru(NH}_3)_6]^{3+/6-}/[\text{Fe(CN)}_6]^{3-}$ electrocatalytic cycle in chronocoulometric readouts might prove to be lucrative in related bioassays for high signal enhancement.
The purpose of our final assay platform was to reflect on the extent to which the RNA biosensor can move towards more personalised diagnostics of cancer and enable a user-friendly and inexpensive approach that is suitable for point-of-care settings. In this regard, after successfully challenging the sensitivity and specificity requirements of miRNA in previous two assays, we move on to report on a new platform for the detection of lncRNA where three potentially useful readout approaches (i.e., naked-eye, UV-vis absorbance and amperometric readouts) were integrated under one assay protocol. In the assay, HOTAIR lncRNA was selected as a model target amplified with an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) step, where biotinylated nucleobases were arbitrarily inserted into the amplified HOTAIR products. With a subsequent TMB/HRP-based colorimetric reaction, the presence of HOTAIR were visualised with the naked eye, followed by more quantitative absorbance and amperometric measurements. This particular assay, which is capable of giving a yes/no answer for the presence of target RNAs, appears to be the first study for colorimetric detection of lncRNA, where naked-eye screening can be exploited as a first-pass test for a large samples screening, followed by more precise quantification using other readouts. All of the platforms developed in this PhD were demonstrated for their applicability in complex biological samples (a cohort of cancer cell lines and patient samples), to ensure their translational potential. Importantly, the analytical performance of the assays was validated with the standard RT-qPCR approach.

Despite numerous investigations and progress in this field, there are still many unmet biological and technical challenges that need to be considered, to fully integrate developments into a point-of-care platform. Compared to DNA, RNAs are highly unstable at room temperature and more prone to RNase-induced degradation. RNA samples, thus, require extensive care during extraction, storage, and handling. Another key consideration should be the associated false-positive response of relatively smaller
RNAs (miRNAs), which could result from the cross talk amongst targets and closely related, non-target RNA sequences. In our assays potential non-specific interferences were minimised using innovative transduction schemes. However, the scope for improving the specificity considering several other innovative strategies is still “wide open”. In regards to the longer RNAs (mRNA, lncRNAs), their strong folding tendency, which might substantially affect their in situ hybridisation with capture probe, seems to be a great obstacle for the development of a straightforward, hybridisation-based sensor. Further research is needed to introduce reasonable ways of tackling the instability of longer RNAs on sensors. Moreover, to be able to translate the proof-of-concept bioassays to clinics, each of the assays should be applied in a large cohort of patient samples within controlled trials. Because cancer is a multifaceted complex disease, and there is no single biomarker that can provide detailed information about a particular cancer, a combined analysis of a group of sensitive biomarkers is recommended. Therefore, there is a definite need for the development of a multiplexed device platform that could simultaneously analyse a group of RNA biomarkers, with high sensitivity, specificity, and speed. In addition, to avoid the aforementioned stability issues of RNA molecules, full automation of the device is required; a system that can function with minimal human intervention. With the recent tremendous advances in the fabrication technologies for microelectromechanical and microfluidic systems, we hope that this will become a reality in the future.