MicroRNAs in Ovarian Cancer and Recent Advances in the Development of MicroRNA-Based Biosensors

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Abstract

Ovarian cancer is the most aggressive of all gynaecological malignancies and is the leading cause of cancer-associated mortality worldwide. Over the recent years, there has been a sharp increase in this mortality rate, mostly due to late diagnosis, which can be attributed to the lack of an early and specific biomarker. Under this scenario, recent interest has shifted towards ovarian cancer associated miRNAs which play strong regulatory role in various cellular processes. miRNAs have emerged as promising non/minimally invasive cancer biomarkers for improved diagnostic, prognostic and streamlined therapeutic applications. A large number of miRNA assays have been reported that are based on nucleic acid detection-based techniques such as RT-qPCR, microarrays and RNA sequencing methods. Despite demonstrating commendable analytical performances, these laboratory-based techniques are expensive and hence not ideally suited for routine use in resource-limited settings. In recent years, considerable attention has been dedicated to the development of relatively simple, rapid and inexpensive miRNA biosensor strategies. Among these, electrochemical sensors have shown a great promise towards point-of-care diagnostics, due to their inherent advantages such as simplicity, sensitivity, amenability to high levels of multiplexing as well as low cost. In this paper, we provide an overview of the potential role of miRNAs in ovarian cancer, as well as recent advances in the development of nanotechnology-based, optical, and electrochemical biosensing-strategies for miRNA detection.

1. Introduction

Ovarian carcinomas are the seventh most common cause of cancer-related deaths in females, while they are the commonest cause of gynaecological cancer-associated deaths worldwide. Initially, ovarian carcinomas were thought to be an individual entity, however, it is now known that ovarian cancer refers to several different types of cancer.[1-3] Due to high level of its genetic and molecular heterogeneity, ovarian cancer can be typically sub-classified into at least five different histological subtypes based on the molecular composition, cells of origin, risk factors, clinical features, and treatment procedures.[1, 3] In recent years, it has become evident that each of the major histological subtype is linked with characteristic genetic defects that enable the deregulation of specific signalling pathways in the tumour cells. Only about 20% of ovarian cancers are diagnosed at an early stage (stage-1) because cancers of the ovaries often show no specific symptoms.[4] Since the late diagnosis of ovarian cancer is one of the major reasons for the increased mortality rates, an effective screening strategy that can detect stage-1 ovarian cancer, , could have a significant impact on the improved rates of survival.

Over the years, a number of serological markers have been commonly used to identify subclinical disease and to predict early-stage ovarian tumours which carry a favourable prognosis, however, most of these markers showed low specificity and sensitivity.[2] Tumour markers such as CA-125 (Cancer Antigen-125)[5-8], HE4 (human epididymis protein 4)[5, 6], mesothelin[5, 9], alpha fetoprotein[10, 11], kallikreins[5, 6, 12], osteopontin[5, 6, 9, 13], prostasin[5-7, 9], B7-H4[5, 6], vascular endothelial growth factor (VEGF)[5, 6], lysophosphatidic acid (LPA)[6], transthyretin[5], transferrin[5], interleukins[6, 9, 14], apolipoprotein A1 (ApoA1)[5], inhibin A & B[14-16], beta human chorionic gonadotropin (Beta-hCG)[17], and other protein and autoantibody biomarkers have often been used in combination with radiological scans and biopsies to get a highly precise diagnostic result.[1, 5, 6, 18, 19]. While these biomarkers and their detection approaches are highly reliable and effective; they are time-consuming and laborious for routine

applications. They might also pose a potential cost barrier in low-resource settings due to the need for expensive and sophisticated equipment. Despite the development of these tumour biomarkers and associated conventional detection techniques, an ideal ovarian cancer biomarker with high specificity and absolute sensitivity remains a challenge. In addition, there is a need for distinct and specific biomarkers for each subtype of ovarian cancer to achieve pan-effective diagnostic and treatment strategies.[20, 21] In this regard, small non-coding RNAs, referred to as microRNAs (miRNAs), have shown huge potential to serve as better alternative for sensitive, specific, and non-invasive detection of ovarian cancer.[20, 22, 23]

miRNAs are small, highly conserved noncoding RNAs (~20–30 nucleotide RNA molecules), transcribed by RNA polymerases II and III.[23, 24] They perform significant roles in regulation of post-transcriptional gene expression.[25] They exert their regulatory function *via* translational inhibition and mRNA destabilization, thereby playing a crucial part in a wide range of physiological and developmental processes. Aberrant expression of miRNAs is known to be associated with various diseases including cancer. Over the years, thousands of miRNAs have been found to be associated with different diseases and a number of databases have been developed to to document computer predicted and/or experimentally validated miRNA-disease associations. As per the website Tools4miRs there are at least 34 different miRNA databases available which have collected data based on correlation of miRNAs with various diseases/pathways in humans.[26] Among these, dbDEMC 2.0, OncomiRDB, and miRCancer are the databases with largest collection of miRNAs associated with cancer. miRCancer is based on text mining based identification and collection of experimentally validated miRNAs associated with various cancers. As per the latest update dated 31st October 2019 (based on literature published before 1st January 2019), 8131 miR-cancer relations were identified. So far more than 200 miRNAs are listed on miRCancer website that are associated with ovarian cancer.[27, 28] Several human miRNAs have been found to be involved in cancer initiation and

progression, and may regulate cell adhesion and proliferation, angiogenesis, and apoptosis, dysregulation of which plays a vital role in the pathogenesis and metastasis of ovarian cancer.[25, 29, 30] Besides, miRNAs can act as tumour suppressors or oncogenes, depending on the genes they target and also cellular context. Recent studies have shown that expression of miRNAs is different in diseased tissues and they are differentially enriched in serum, plasma or other types of body fluids, which demonstrates their potential for being an ideal and useful minimally invasive biomarker.[31-33]

Until now, the detection of miRNAs has largely been confined to several classical nucleic acid detection- based methods such as northern blotting, microarray, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), or in recent years next generation sequencing.[34] Despite having good analytical capabilities, the scope of these approaches in the off-laboratory as well as resource-limited settings, where sophisticated and expensive instruments may not be available, is much limited. In addition, there are some other inherent drawbacks of these methods, for example, northern blotting is time-consuming and requires large volume of sample input. Microarray, being a high throughput technique, is usually more suitable for discovery purposes rather than specific diagnostic applications.[35] Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is relatively sensitive, nevertheless, amplification bias and artefacts are among the biggest pitfalls of RT-qPCR based miRNA quantification methods.[34] There is therefore, there is still a scarcity of well-grounded platforms that may be suitable for integration of miRNA screening in routine point-of-care diagnostics.

The trend of detection approaches for cancer biomarkers is rapidly shifting towards sensor-based strategies from the existing conventional techniques.[36] Generally, biosensor based approaches such as strategies based on electrochemical and optical readouts have a high potential to be integrated in to the point-of-care platform.[37] Electrochemical detection

methods, coupled with one or more novel signal enhancement strategies (*e.g.*, nanomaterials-based isolation and signal amplifications), are considered particularly attractive for bioanalysis because of their low cost, high sensitivity, and selectivity, and ease of automatization.[38-41] Metallic nanoparticles (AuNPs, AgNPs), carbon and carbon-based nanomaterials (SWCNT, CNTs, graphene, graphene oxide), quantum dots, magnetic nanoparticles, metal-organic framework (MOFs) are widely exploited in sensing platforms for signal amplification in miRNA detection.[42] Most popular electrochemical techniques used for data acquisition include; voltammetry based such as cyclic voltammetry (CV), square wave voltammetry (SWV), differential pulse voltammetry (DPV), linear sweep voltammetry (LSV), amperiometric like chrono amperometry (CA), and electrochemical impedance spectroscopy (EIS). Among these techniques, EIS analyse the surface of the electrode in a label-free manner.[42,157]

In recent years, microRNA-based detection methodologies have widely been reviewed by many eminent research teams.[42-45] The purpose of the current review is to offer a comprehensive overview of the recent developments in biosensor-based methods (*e.g.*, electrochemical readouts) for miRNA detection, with a special emphasis on their application in ovarian cancer diagnosis. We briefly discuss the subtypes of ovarian cancer and their common biomarkers. We also highlighted miRNA biogenesis, and their diagnostic and prognostic roles in ovarian cancer. A specific discussion on the requirements that are unmet for screening of ovarian cancer related miRNAs, in both research and clinical settings, has also been included. We also reviewed the major technical and biological challenges involved in the existing miRNA detection strategies.

2. Histological subtypes of ovarian cancer

Approximately 90 % of primary malignant ovarian tumors can be classified as epithelial ovarian cancer (EOC).[9, 12, 46] EOC ranks fourth as a cause of death in females in the developed world. Histologically, epithelial ovarian cancer can be divided into five major categories- endometrioid, serous, mucinous, clear-cell, and transitional cell (Brenner) carcinomas.[1, 47] There are other rarer subtypes including small-cell carcinoma and non-epithelial ovarian cancers.[1-3] Ovarian endometrioid carcinoma covers 10–20% of all EOC cases.[48] The tumours predominantly occur in women aged 50–59 years, with a median age at diagnosis of 56 years.[2] Among all these subtypes, serous histotype is the most common and can be further classified as either high or low grade, based on the mitotic figures and the extent of nuclear atypia. High-grade serous carcinoma (HGSC) is the most frequently diagnosed.[2, 48] HGSC is similar to high-grade endometrioid carcinoma which is characterised by nearly universal p53 gene abnormalities.[48] Generally, serous tumours are homogeneous in nature depending on their cellular composition and degree of differentiation, whereas mucinous tumours are often heterogeneous, especially the intestinal type. Based on their histopathological features, primary mucinous carcinoma can be further sub-classified as benign, borderline, or malignant.[2, 48] Unlike serous carcinoma, ovarian clear cell carcinomas (OCCA) are reportedly diagnosed as a large pelvic mass at the early stages, which makes it possible for it to be diagnosed early compared to other EOC subtypes. OCCA is currently responsible for less than 5% of all ovarian malignancies and 3.0–12.1% of all ovarian epithelial neoplasms.[48] Sites of origin of some ovarian cancers can also be outside of the ovary; for example, many ovarian HGSCs may have their origin in the fallopian tube whereas other subtypes have been found to emerge from the peritoneum. Additionally, origin of clear-cell and endometrioid carcinomas can be endometrial tissue, which can be situated outside the uterus. Small-cell carcinoma is a very aggressive subtype with undefined tissue origin, and carcinosarcomas are typically diagnosed in younger women, with a median age of 25 years. On the other hand, non-epithelial ovarian cancers

account for ~10% of ovarian cancers, which includes germ-cell tumours and sex cord stromal tumours.[1, 2] **Table 1** provides the major histological subtypes, origin, mutation status, and their diagnostic, prognostic, and treatment options.[2, 9, 12, 13, 38, 49]

Table 1. *Major histological subtypes, origin, mutational status of ovarian cancer and their management strategies.*

Histological subtype [2, 9, 12, 13, 48, 50]	**Possible histogenesis (tissue of origin)**	**Precursor lesion**	**Diagnosis and Prognosis** [22, 23]	**Commonly mutated Genes**[23]	**Familial risk**	**Prevention strategies**	**Treatment options**[2, 9, 12, 13, 48, 50-52]
High-grade serous carcinoma and high-grade endometrioid carcinoma (HGSC)	Fallopian tube fimbria or ovarian cortical inclusion cysts	Serous tubal intraepithelial carcinoma	Usually detected at a later stage After metastatic spread. Poor prognosis if diagnosed in later stages	HR deficiency, p53 mutations, PI3K/RAS, NOTCH signalling	BRCA 1, BRCA 2, BRIP1, PALB2, RAD51C and RAD51D	RRSO, opportunistic salpingectomy, oral contraceptives or tubal ligation	•Platinum-based chemotherapy and polymerase (ADP-ribose) inhibitors •Tumours are initially sensitive to platinum-based chemotherapy, but most patients with advanced-stage cancer will recur
Low-grade serous Carcinoma (LGSC)	Endosalpingiosis or papillary tubal hyperplasia	Serous borderline tumour	Usually detected early. Better prognosis than HGSC	PIK3CA, BRAF, KRAS MAPK	NA	NA	•Chemotherapy in combination with anti-angiogenic agent (for recurrent setting)

							•Hormonal maintenance therapy •MEK inhibitors (currently being tested in clinical trials)
Low-grade endometrioid carcinoma	Endometriosis or endometrioid adenofibroma	Endometrioid borderline tumour	Usually detected early. Better prognosis than HGSC	PIK3CA, BRAF, KRAS	Lynch syndrome (MLH1, PMS2, MSH2 and MSH6)	Tubal ligation, opportunistic salpingectomy or oral contraceptives	•MEK inhibitors (currently being tested in clinical trials) and hormonal therapies
Clear-cell carcinoma (CCC)	Endometriosis or endometrioid adenofibroma	Endometrioid borderline tumour	Usually detected early. Better prognosis than HGSC	ARID1A, PIK3CA	Lynch syndrome (MLH1, PMS2, MSH2 and MSH6)	Tubal ligation, opportunistic salpingectomy or oral contraceptives	•Immunotherapy agents • Can be resistant to platinum-based chemotherapy
Mucinous carcinoma	Unknown	Mucinous borderline tumour, Brenner tumour, teratoma or endometriosis	Usually detected early. Better prognosis than HGSC	ARID1A, PIK3CA, PTEN, MMR deficiency	NA	Tubal ligation or oral contraceptives	•Tends to be insensitive to chemotherapy but is still treated initially with cytotoxic chemotherapy

3. Existing ovarian cancer markers and conventional diagnostic methods

A number of serological tumour markers of ovarian cancer have been identified over the years to diagnose subclinical stages of ovarian cancer. These biomarkers are commonly interrogated

via scans, biopsies or using a combination thereof.[6] **Table 2** depicts a list of ovarian cancer tumour markers and their functions.

Table 2. *Tumour biomarkers in ovarian cancer*

Tumour markers	**Uses**
CA-125[5-8, 19]	• In diagnosis of ovarian carcinomas • In monitoring for recurrence of cancer and to assess the response to treatment • Individual marker is not sufficiently sensitive to detect all cases of early-stage ovarian cancer[5]
HE4 (Human Epididymis Protein 4)[5, 6]	• In diagnosis (more sensitive than CA-125) • In monitoring for recurrence of cancer and to assess the response to treatment • Combination with CA-125 predicts malignancy more accurately than either alone
Mesothelin[5, 9]	• In early diagnosis of early-stage OC • More effective in serous cystadenocarcinoma • Plays a significant role in tumour metastasis, cancer cell survival and proliferation, and drug resistance • Combination with CA-125 found superior in early detection
Alpha-fetoprotein [10, 11]	• In ovarian germ cell tumours • To assess malignancy stage, prognosis, and response to treatment
Kallikreins[5, 6, 12]	• Aberrantly expressed in EOC, specifically, in the more metastatic Type-II tumours • Only have application as biomarkers but also function in disease progression, and therefore as potential therapeutic targets since high levels of Kallikreins are differentially associated with the prognosis of ovarian cancer patients • Not effective in screening the disease at early stage

Osteopontin [9, 13]	• In diagnosis of ovarian neoplasms, mostly in epithelial carcinoma • Can improve the diagnostic accuracy of CA125 (detection of OC is complementary to CA125)
Prostasin[6, 7, 9]	• In screening and detection of early stage OC • Can be used with CA125 and HE4 • Play key role in chemoresistance and can be used as a target for treating/repressing some ovarian tumours in gene therapy
B7-H4[8, 9]	• In screening, early stage diagnosis, and monitoring disease progression • Mostly used in serous, endometrial and clear cell carcinomas • As a potential immunotherapeutic target for patients with EOC • Use either alone, or in combination with CA125
Vascular endothelial growth factor (VEGF)[5, 6]	• Use in tumour progression, peritoneal metastasis, and accumulation of ascites in OC • Use in combination with CA-125 and HE4 for stage 1 OC
Lysophosphatidic acid (LPA)[9, 10]	• In screening, early to late stage diagnosis, and disease progression • A potential target for cancer therapy
Transthyretin[5, 11, 53, 54]	• In detection and monitoring of early-stage ovarian cancer, mostly for EOC (particularly test specificity) • Acts as acute phase reactants • Shows effective sensitivity and specificity in combination with CA-125, ApoA1 and transferrin
Transferrin[5, 11, 53]	• In early stage diagnosis and monitoring. Mostly effective for serous and endometrioid carcinomas • Acts as promoter for tumour development and survival via antiapoptotic effect • Shows effective sensitivity and specificity in combination with CA-125, ApoA1 and transthyretin

Interleukins[9, 14, 55]	• In screening and early stage diagnosis as well as cancer growth, progression, and metastasis • Plays key role in inducing several pathways leading to tumour proliferation, angiogenesis and chemoresistance • Significantly associated with poor prognosis
Apolipoprotein A1 (ApoA1)[5, 15]	• In diagnosis of early stage OC • Shows effective sensitivity and specificity in combination with CA-125 and transthyretin
Inhibin A & B[14-16]	• In mucinous epithelial carcinoma, granulosa cell tumours • In monitoring for recurrence of cancer and to assess the response to treatment
Beta-hCG (Beta Human Chorionic Gonadotropin)[17]	• In ovarian germ cell tumours • To assess malignancy stage, prognosis, and response to treatment

Amongst all these potential biomarkers, sensitivity of HE4 in detecting late-stage ovarian cancer is similar to that of CA-125.[5, 21] Although CA-125 is considered to be the most consistent and clinically applicable serum marker for ovarian carcinoma, it plays a controversial role in distinguishing between a malignant or benign mass, and often fails to show accurate results in the case of postmenopausal women.[5, 6]

Conventional diagnostic approaches for ovarian cancer consist of the history of disease, symptom index diagnosis, physical examination, and imaging diagnosis. Imaging diagnostic procedures mostly include ultrasonic, magnetic resonance imaging (MRI), computed tomography (CT) scan, and positron emission tomography-CT (PET-CT).[6, 33, 56] In addition, several approaches have been appraised to detect ovarian cancer early which include transvaginal sonography (TVS), and a two-stage screening strategy where rising serum markers prompt TVS.[57, 58] Moreover, improvement of previously used transabdominal ultrasonography

(TAU) has given rise to the development of TVS which is more precise and reliable for imaging the ovary[4, 6], as TVS reduces the distance between the probe and the pelvic structures which allows the use of higher frequencies. It also minimises beam deformation by the anterior abdominal wall. Thus, it provides better quality images with a higher resolution. The key advantages of TVS identified over TAU, are that TVS not only provides images with higher resolution but also minimises the discomfort of having a full urinary bladder during scan.[6] The major limitation of TVS is its limited field of view, which is why it is often accompanied by TAU, in order to confirm the possibility of having a mass lying outside the field of view of the TVS transducer.[6, 56] The screening method has certain contraindications for patients who are virgins, have vaginal obstruction or a previous case of premature rupture of membrane. Moreover, the potential cost of such screening exceeds the limits for other screening tests.[6]

4. miRNA: the future biomarker for ovarian cancers

Biogenesis of miRNAs

Non-coding RNAs do not participate in protein synthesis but play role in RNA inhibition and other regulatory pathways.[35, 59] Biogenesis of mature miRNA is actually a two-step cleavage process.[34, 35] It initially starts inside nucleus at the inter- or intra-genic locations or from antisense strands of transcribed neighbouring genes. They frequently undergo RNA polymerase II-dependent transcription autonomously from surrounding genes using their own transcriptional initiation sites which later produce a primary-miRNA (pri-miRNA). Pri-miRNAs are further cleaved and processed by a distinct protein complex known as the microprocessor complex which usually consists of a ribonuclease enzyme Drosha and RNA binding protein DGCR8 (also known as pasha). Due to the cleavage of pri-miRNA by microprocessor complex, precursor miRNA (pre-miRNA) is produced. Then the pre-miRNAs

are exported to the cytoplasm by the Ras-related nuclear protein-guanosine-5'-triphosphate (RAN-GTP)-dependent export receptor Exportin-5 (Exp5). Once in cytoplasm, the Pre-miRNA is cleaved at the base of the loop and is further processed to a short dsmiRNA (~22 nt in length) by a second RNase III endonuclease (Dicer) with its dsRNA binding partner, resulting in mature miRNAs, which are incorporated into the miRNA-associated multiprotein RISC (RNA-induced silencing complex). Thus, miRNA usually exerts its inhibitory and regulatory action via RISC which mediates RNA degradation and/or post-translational inhibition.[59-62]

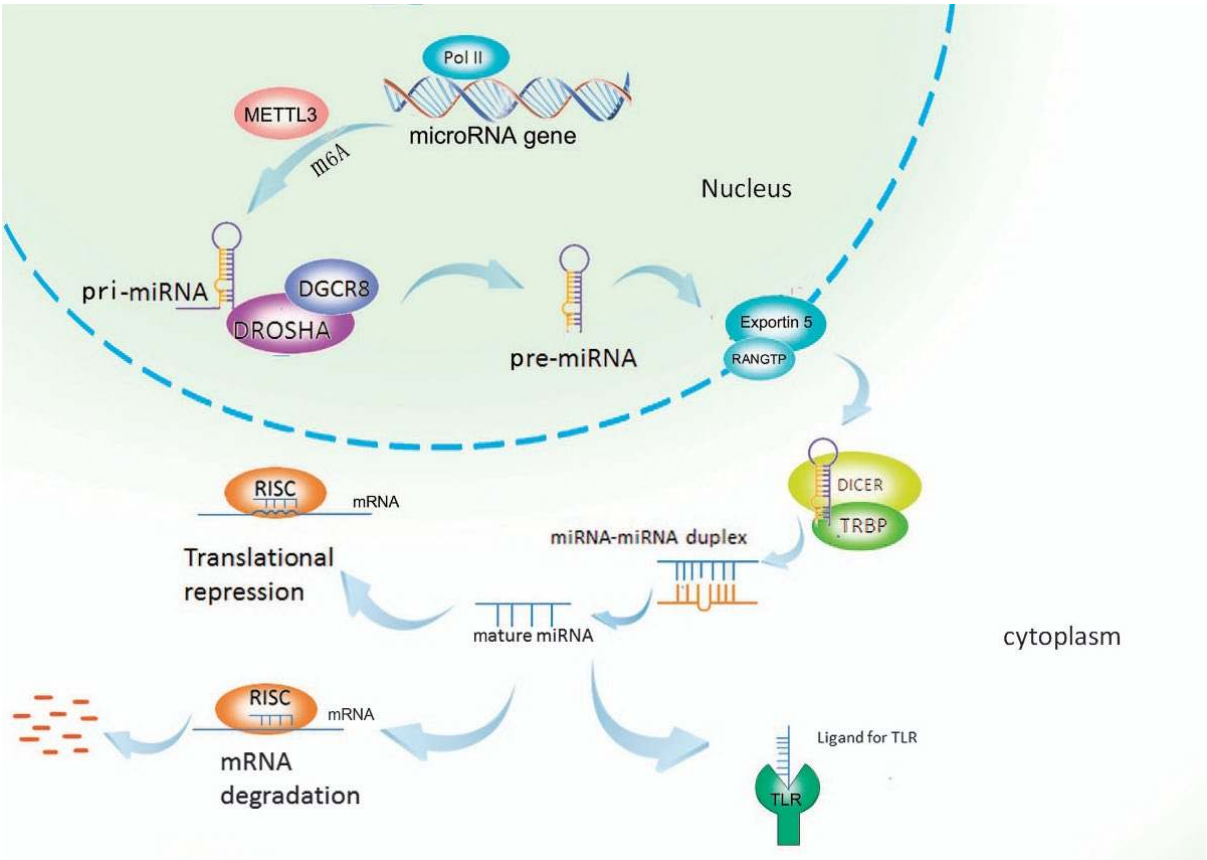


Figure 1: *Biogenesis of microRNA. (a) miRNAs are transcribed by RNA polymerase II (pol II) into pri-miRNA in the nucleus. They are recognized and sliced by Drosha and form a hairpin precursor called pre-miRNA. (b) Pre-miRNA is carried across from the nucleus to the cytoplasm by exportin 5 and is further processed by DICER, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. (c) It produces a transient 19–24-nt duplex. Only one strand*

of mature miRNA duplex (the guided strand) is loaded into a large protein complex called RISC. (d) The mature miRNA leads RISC to cleave the mRNA or induce translational repression, depending on the degree of complementarity between the miRNA and its target. [Reproduced from ref: 25 with permission from Springer Nature Copyright 2016]

miRNAs with known functions in Ovarian Cancer

miRNAs are known to regulate gene expression (*e.g.*, post-transcriptional gene expression, and remodelling of the epigenome, such as methylation and histone modification).[34] Thus, dysregulated miRNAs may affect one or several types of cellular pathways such as cell cycle regulation, pluripotency and retrotransposon silencing, and contribute to various pathological conditions.[63] Over the past years, several studies have linked global and individual miRNA expression patterns with different types of cancers, including ovarian cancers. The first report on the association between miRNA and cancer pathogenesis involved a chronic myelogenous leukemia patient, where miR-15 and miR-16 were found to be under-expressed.[64] Following that, continuous attempts have been made to examine the role of miRNAs in cancers. It has been suggested that miRNAs can be altered during cancer initiation, due to chromosomal rearrangements, alterations in genomic copy numbers, epigenetic modifications, abnormal maturation pathways and their regulation by transcription factors.[29] Studies also demonstrated that miRNA species are differentially expressed among different histotypes of ovarian cancer.[65] As a particular example, let-7 family miRNAs were reported to affect follicular maturation and atresia during ovarian cancer development.[66, 67] **Table 3** lists a group of miRNAs reported to have altered expression in ovarian cancer.[22, 29, 33, 68-71]

Table 3. *miRNA biomarkers in different types of ovarian cancer*

Cancer Type	Upregulated	Downregulated	Regulated

Ovarian Cancer	miR-152, miR-199a-3p, miR-125b-1-3p, miR-140-5p // miR-487b, miR-519e-3p, miR-29c-3p, miR-30a-5p, miR-30e-5p, miR-365a-3p, miR-370, miR-520e, miR-637, miR-99a-5p, miR-214-3p, miR-22-3p, miR-199b-3p, miR-199a-5p, miR-200b-3p, miR-200c-3p, miR-221-3p, miR-223-3p, miR-29a-3p	let-7a-5p, let-7i-5p, let-7b-5p, let-7c, let-7d-5p, miR-155-5p, miR-195-5p, miR-203a, miR-125b-5p, miR-134, miR-154-3p, miR-206, miR-507, miR-514a-3p, miR-21-5p, miR-335-5p, miR-346, miR-493-3p	miR-100-5p, miR-106b-5p, miR-141-3p, miR-15a-5p, miR-200a-3p, miR-222-3p, miR-34b-3p, miR-424-5p
Epithelial Ovarian Cancer	miR-141-3p, miR-182-5p, miR-195-5p, miR-199a-3p // miR-199b-3p, miR-200a-3p, miR-200c-3p, miR-203a, miR-205-5p, miR-21-5p, miR-26a-5p, miR-302b-5p, miR-325, miR-373-3p	let-7c, let-7a-5p, let-7b-5p, let-7d-5p, let-7i-5p, miR-1, miR-100-5p, miR-125b-5p, miR-125b-1-3p, miR-126-3p, miR-133a, miR-140-5p, miR-143-3p, miR-145-5p, miR-152, miR-155-5p, miR-15a-5p, miR-204-5p, miR-22-3p, miR-221-3p, miR-222-3p, miR-224-5p, miR-29a-3p, miR-29c-3p, miR-30a-5p, miR-30a-3p, miR-302b-3p , miR-34b-3p, miR-346, miR-365a-3p, miR-370, miR-	miR-101-3p, miR-103a-3p, miR-105-5p, miR-134, miR-137, miR-147a, miR-154-5p, miR-154-3p, miR-199a-5p, miR-200b-3p, miR-211-5p, miR-214-3p

		375, miR-376a-3p, miR-377-3p, miR-379-5p, miR-410, miR-424-5p, miR-432-5p, miR-492, miR-507, miR-514a-3p, miR-519d, miR-519e-3p, miR-520e , miR-9-5p, miR-99a-5p	
Serous Ovarian Cancer	miR-141-3p, miR-16-5p, miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-21-5p, miR-27a-3p, miR-429, miR-93-5p	let-7b-5p, miR-100-5p, miR-10b-5p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-214-3p, miR-26a-5p, miR-29a-3p, miR-34b-3p, miR-432-5p, miR-514a-3p, miR-99a-5p	miR-199a-3p // miR-199b-3p

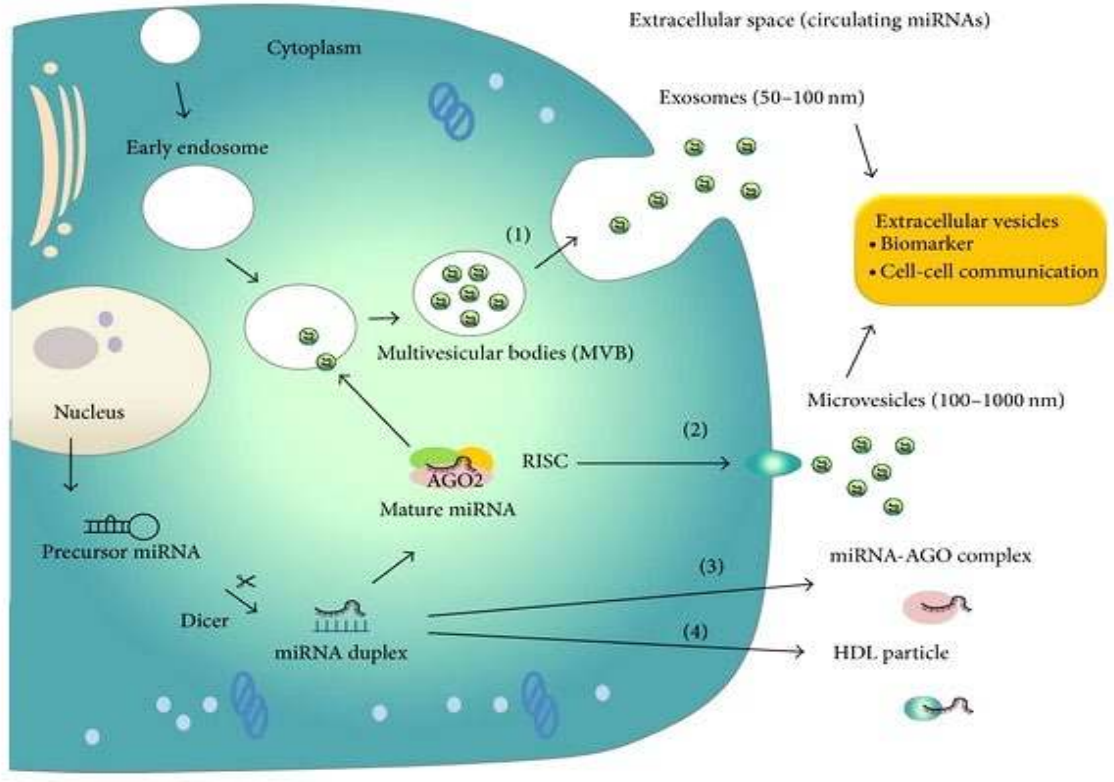
Circulating miRNA: cell-free and extracellular vesicle- derived miRNA

miRNAs circulating in many types of body fluids harbor cancer specific molecular alterations. Analysis of these circulating miRNAs has gained immense clinical significance as a minimally invasive approach for cancer diagnosis, prognosis, and survival prediction. Some of the miRNAs in circulation are packaged in microvesicles such as exosomes, or apoptotic bodies.[72] These vesicle enclosed miRNAs are resistant to ribonuclease mediated degradation and can stably carry the signatures of the originating tumour cells.[20] In this regard, the utility of

exosomes for developing miRNA based liquid biopsy tests for disease detection and monitoring of disease progression, may become a very useful alternative approach.[73-75]

Exosomes carry a unique set of transferable functional biomolecules like proteins, lipids, and nucleic acids, providing a unique way of cell-cell communication, and modulation of recipient cell transcriptome.[76-81] They also play important roles in many biological processes such as: T-cell activation and antigen presentation, coagulation, inflammation, and angiogenesis.[79] Tumour secreted exosomes have been shown to be involved in cancer metastasis, identifying them as potential targets for the development of novel therapies.[79, 82] Moreover, due to their availability in almost all bodily fluids, they can act as non- or minimally invasive disease biomarkers especially for diagnosis, prognosis, and therapy response evaluation in patients with cancer.[79, 83]

As discussed in detail below (section 5), to achieve even a two-fold increase in circulating levels of any particular miRNA, tumour tissues at early stages may need to contribute several



thousand-fold more miRNA to circulation compared to the healthy tissue of the same organ. Therefore, detecting such low levels of cancer specific miRNAs in circulation is extremely difficult. In comparison, it has been reported that cancer cells secrete around 10-fold more exosomes compared to normal cells thus cancer specific exosomes in circulation are often in high abundance. Therefore, targeting exosomes for detection of cancer specific miRNA biomarkers may offer a more reliable approach, provided efficient and highly specific methods for exosome isolation are available. It is worthwhile to mention that exosomal miRNAs have reportedly shown improved specificity and reproducibility in diagnosing various pathological conditions compared to tissue bound miRNAs.[34, 84]

Figure 2: *Release of microRNAs from cells into extracellular space. (1) Fractions of miRNAs sorted into multivesicular bodies (MVBs) that are secreted via exosomes, (2) they are incorporated into microvesicles- produced by the outward shedding of the plasma membrane, (3) associating with RNA-binding proteins, such as AGO2 and release of the miRNA-AGO complexes, and (4) finally, they are carried across and incorporated into high-density lipoprotein (HDL) particles. Extracellular vesicle miRNAs are presumed to be involved in cell-cell communication and thus, can act as an effective biomarker. [Reproduced from Ref: 81 under creative commons license Copyright © 2014 Yu Fujita et al.]*

5. Clinical Translation of miRNA Biomarkers: Challenges and Considerations

Ever since the first report on association of miRNA dysregulation with cancer, there has been a great deal of excitement over their potential use as diagnostic and prognostic biomarkers. Further augmented by later discovery of cancer specific miRNAs in various bodily fluids, several efforts ensued to develop validated and standardised miRNA based liquid biopsy diagnostic approaches. In the past few years, annually more than 3000 studies exploring the diagnostic potential of miRNAs in various diseases have been published. Some miRNA based diagnostic platforms have already been made available to clinicians such as; ThyraMIR by

Interpace Diagnostics, Reveal by Genoptix for thyroid cancer, or miRview™ mets an miRNA panel for "cancers of unknown or uncertain primary origin" (CUP) by (now bankrupt) Rosetta Genomics. Others like miRNA detection panels for various diseases, including cancer, developed by Hummingbird Diagnostics are at the stage of clinical validation. However, comparing the number of commercialised or near to be marketed miRNA diagnostic platforms with the large number of articles published every year, it has now became apparent that translation of miRNA biomarkers from bench to bedside may be constrained by several biological and methodological challenges.[85] To be able to successfully use miRNAs to diagnose and monitor ovarian cancer for both clinical and research purposes, and to achieve desired level of accuracy, sensitivity, cost-effectiveness and portability, several common challenges need to be overcome.[34, 35, 86]

Low Abundance

Low abundance of disease/cancer specific circulating miRNAs is one of the major challenges. It has been estimated that to achieve even a two-fold increase in circulating concentration of any particular miRNA compared to non-diseased individuals, the tumor tissue may need to contribute anywhere between 50-50,000 fold more miRNA to blood as compared to the healthy tissue of the same organ. However, such levels of upregulation are not always possible, especially in tumours of smaller sizes, casting doubts as to the utility of miRNA based liquid biopsy testing for early diagnosis of cancers.[84] Furthermore, for any miRNA biomarker to be specifically associated with a particular cancer it also needs to be ascertained with high level of confidence that the origin of increased expression in circulation is tumour mass itself and not a general non-specific response of neighboring tissue or other body organs to the presence of neoplasm.

Challenges associated with isolaton of exosomes

As exosomes are known to be vehicles for miRNA transport and a valuable source of miRNA biomarkers, much effort has been invested in recent years to develop exosomal miRNA based disease diagnosis and monitoring approaches. However, many of the existing methods cannot effectively separate cancer-specific exosomes from the bulk exosome population thus indirectly hampering the development in exosomal miRNA based platforms. For example, most commonly used exosome isolation methods; ultracentrifugation, size exclusion chromatography, ultrafiltration, or polymer-based precipitation, cannot separate cancer specific exosomes from bulk exosome population. On the other hand, availability of highly specific surface marker and efficient antibodies is a prerequisite for immunoaffinity based separation of specific target exosome population, which in most cases are not well defined (specific marker/s) or readily available (efficient antibodies). Exosome isolation from various bodily fluids is also challenging due to size overlap with lipoprotein, chylomicrons, and microvesicles etc.[87]

Stability of RNAs

RNA is generally unstable at room temperature due to the chance of ribonucleases associated RNA degradation. Both endogenous and exogenous RNases may cause progressive degradation of miRNAs. However, circulating miRNAs are relatively less prone to degradation as they are bound to lipoproteins or RNA binding proteins, thus making them resistant to RNase degradation. miRNAs encapsulated in Extracellular vesicles (EV) are also more stable, as they are protected inside the EV compartment.[86]

Sample preparation and choosing the sample source

Sample source is one of the most important pre-analytical consideration in miRNA analysis. Expression level of miRNAs can vary amongst different sources of samples from the same

individual.[88] It has previously been shown that miRNA concentration in serum is higher compared to plasma samples obtained from the same individual. In vitro haemolysis of blood cells, particularly platelets, could be a reason for this variation.[88]

Low sensitivity

The readily available and clinically relevant miRNA concentration in tissues, serum or other sources are very low. Therefore, highly sensitive methods need to be designed for extracting information from this tiny amount of samples. The efficiency of RNA extraction method could be crucial in this case.[89] It was demonstrated that the majority of variance in RNA detection were derived from the extraction process.[90] Therefore, choosing the right extraction method along with careful optimisation (*i.e*., incubation time, centrifuging speed etc.) of the extraction steps are highly required.

Specificity issues due to the presence of homologous miRNA sequences

Short sequence length of miRNAs increases the probability of finding more than one nucleic acid fragments having similar or partially similar sequence. Especially the miRNAs of the same family often show high levels of sequence similarity.[91] Therefore, accurate and sensitive detection of target miRNAs in the background of various closely related RNA sequences is often compromised and thus jeopardizes the reliability of specific miRNA detection.

Non-specific response from biomolecules

Clinical samples like blood are usually a complex mixture of a large number of biomolecules like lipids, proteins, variety of nucleic acid sequences, extracellular vesicles, whole cells. etc. These biomolecules often interact non-specifically with various components of detection platforms like the sensor surface or the nanoparticles being used in the assay. These non-

specific interactions increase the background signals leading to a high probability of false positive results.

Varying Size of RNAs

miRNA detection becomes challenging in regular RT-qPCR approach due its size match with primers. Additionally, unlike DNA, due to the presence of an extra free oxygen atom in the additional ribose inside the RNA structure, RNAs, are also prone to interactions between nucleotides and often fold into various secondary and tertiary structures[92, 93] on the sensor surface of biosensor-based assays thereby abating the analytical performance of the assay.

Physiological variation in humans

Natural variations in the expression levels of RNA biomarkers, both between and within individuals, are a considerable issue. Factors that can contribute to this variation include gender, race, age and diet of individuals. It has also been revealed that the variation is higher when the sample size is smaller (<100 individuals), and a large cohort of individuals can be recruited to counteract this issue.[35]

6. Conventional detection method of miRNA

Existing and widely acceptable methods for detection and expression analysis of miRNA biomarkers are mostly dependent on nucleic acid detection and amplification-based techniques such as northern blotting, RT-qPCR, microarrays, and RNA sequencing.

Northern blotting

In combination with other RNA markers, northern blotting can perform a quantitative analysis of miRNAs.[33] [33] Usually in a Northern blot, RNAs of different lengths are separated via gel-electrophoresis and later transferred to a membrane, where a chemiluminescence signal is produced when the detection probe hybridizes with the target miRNA.[94] This protocol is capable of quantitating the expression level and size of both the small RNAs and their precursors.[95] However it has certain limitations- it is identified as less sensitive than other analytical methods, as well as the procedure is both time and labour consuming with low-throughput.[33, 42, 96] Besides, this protocol is not suitable for high throughput miRNA analysis, and it requires a very high amount of starting RNA pool (5–25 mg) which is often not practical for routine clinical analysis.[33, 42, 96] Moreover, the method is prone to degradation by RNases and unequal hybridization efficiency of individual probes. Although in recent years the overall protocol of northern blot based assays have been improved, there are still some methods that rely on the use of hazardous chemicals such as formaldehyde, ethidium bromide and radioactive probes etc.[42, 96]

Microarray

Microarray is one of the most preferred methods for analysing the expression of different miRNA species, including their detection and simultaneous high-throughput quantification.[33, 42, 94] It has the ability to distinguish the pattern of expression for all known miRNAs, even those present in poorly differentiated tumours or other cells.[94] In recent days, DNA microarrays have been modified into miRNA microarray technology.[42] The protocol, in general, is based upon the principle of solid phase hybridization of fluorescent labelled miRNAs with oligonucleotide

probes that are immobilized to a platform initially. Later, quantitative information about miRNAs is received *via* the formation of a fluorescent signal upon hybridization in each spot.[42] One example of the high-throughput miRNA analysis is that developed by Chung *et al* which detected a large number of (2222) miRNAs in ovarian cancer samples, where 95 miRNAs were found to be down-regulated and 88 over-expressed.[97] Though this technique has the advantage of high accuracy, it has some significant drawbacks. This method is not cost-effective, especially for small research laboratories and off laboratory settings, making it more suitable for discovery studies rather than clinical applications. Despite having the advantage of flux, it is susceptible to the interference of cross hybridization of homologous miRNA sequences.[42, 98] It has also been observed that the method has a lesser dynamic range and lower specificity than that of miRNA-seq and RT-qPCR.[96] In recent years, a number of commercial microarray platforms with improved performance have been introduced such as miRCURY LNA (Exiqon), GeneChip (Affymetrix), and SurePrint (Agilent).[99, 100] Despite the commercial success, there is still no universally accepted method to analyse, validate and normalize microarray data.[96, 99]

Quantitative real-time PCR technique

Quantitative real-time PCR (qRT-PCR) technique is a well-established and extensively used method for quantifying the expression profiles of miRNAs and their precursors profiling miRNA.[42, 94, 100] and To avoid the issues resulting from the size match of RT-PCR primers and miRNAs, various techniques have been developed which showed improved specificity and sensitivity. For example, stem-loop RT-PCR and poly(A)-tailed RT-PCR.[100] There are other commercially available qRT-PCR kits and primer sets that can also be taken into consideration, such as the TaqMan individual assays (ABI), TaqMan OpenArray (ABI), miRCURY LNA

qPCR (Exiqon), SmartChip human microRNA (Wafergen), miScript miRNA PCR array (SABiosciences/Qiagen), TaqMan TLDA microfluidics card (ABI), Biomark HD system (Fluidigm).[42] One potential weakness of RT-qPCR based approaches is the technique is unsuitable for high-throughput analysis.[42, 96]

RNA sequencing

Next-generation sequencing (NGS) based approaches such as miRNA sequencing (miRNA-seq) enables profiling of all expressed miRNAs without the need for gels.[96, 100] This high-throughput and high-resolution technology may accurately sequence small amounts (e.g., 5 ng) of RNA samples. Many different NGS platforms have been introduced so far and thanks to the technological advances and process streamlining introduced over the years, NGS technology is well on the path towards translation to routine clinical diagnosis. Although each NGS technology is different in terms of platform engineering or the chemistry used to detect successively incorporated nucleotides, in general, NGS is based on the principal that millions of individual DNA strands spatially separated from each other are either clonally amplified beforehand or sequenced as such as single DNA molecules. Sequence of each single DNA molecule, or its clonally amplified pool thereof, is determined by repetitive cycles of polymerase mediated complementary strand extension, or successive oligonucleotide ligation in one platform, where incorporation of each incoming nucleotide generates a specific readable signal.[96, 100, 101] Initially, three platforms were introduced for this purpose: the Illumina (Solexa) Genome Analyzer (GA), the Roche (454) Genome Sequencer (GS), and the Applied Biosystems SOLiD system.[96] The Illumina (Solexa) Genome Analyzer (GA) uses sequencing-by-synthesis method and can sequence up to 75-100 base pairs (bp) with over ~200 million reads. On the other hand, the Roche (454) GS uses pyrosequencing to simultaneous sequence

over 1 million >400 bp reads, whereas sequencing by oligo ligation and detection technique is used by Applied Biosystems SOLiD system to produce 400 million 50 bp reads.[96] The major challenges with miRNA-seq are the resulting large quantities of data (expertise in bioinformatics required) and expensive equipment cost.[96]

Table 4. *Advantages and disadvantages of miRNA detection approaches*

	Methods	Advantages	Limitations
Conventional Techniques	Northern Blotting[42, 96]	• Low-tech • Cost effective • Semi-quantitative	• Low-throughput • Less sensitive • Complex and laborious process • Sample degradation • Carcinogenic risks involved
	miRNA Microarray[42, 96]	• High throughput • Simple process	• Low sensitivity • Lower specificity than qRT-PCR • Expensive • Measure relative abundance only
	Quantitative real-time PCR[42, 96]	• Highly sensitive • Highly specific • Simple process	• Selective performance • Expensive • Cannot identify novel miRNAs • Contamination-prone due to amplification
	Deep Sequencing[42, 96]	• Highly sensitive • High throughput • Highly specific • Rapid process	• Complex steps • Very expensive • Potential underrepresentation of lower copy miRs

			• Requires very high quality and large amount of RNA (at least 10µg)
Electrochemical Techniques[102]	Amperometric and voltammetric	• Highly sensitive • Miniaturization	• Requires labelling • Difficult to detect real sample
Optical Techniques[42]	Surface Plasmon Resonance Impedimetric Colorimetric Fluorescence-based	• Simple process • Free of labelling • Real-time • Direct profiling • Can discriminate single nucleotide difference with a capability of detection inside cell culture extracts	• Difficult to detect real sample • Low sensitivity • Require strategies for amplification • Lacks robustness
Other emerging techniques[42]	Photoelectrochemical	• Cost effective • Highly sensitive • Highly specific	• Difficult to detect real sample • Selective performance

	Paper microfluidics-based LSRR (localised surface plasmon resonance)	• Simple process • Real-time • Direct profiling	• Requires labelling

7. Biosensor- based approaches

Rapid advancements in nanotechnology have resulted in the development of novel nanobiosensors with high potential for point-of-care diagnosis. Generally, a biosensor is composed of a *receptor* (biomolecular recognition species) which recognizes the target analyte with high specificity and the *transducer* (signal-generating and enhancing element) which recognises the biomolecular interaction and converts this interaction into a measurable signal.[35, 103] Over the past several years, a number of novel biosensors comprising of nanopore, optical and electrochemical readout techniques have extensively been developed for the quantification and analysis of miRNAs.[104] However, the main challenges whilst developing an ultrasensitive bioanalytical sensor lie with the sensitivity and selectivity of the platform- including the limit of its background signals as well as its response time.[105] Here, we provide an overview of the prominent recently reported biosensor platforms used for miRNA detection, with an emphasis on the technologies that have particularly targeted ovarian cancer specific miRNA biomarkers. Key advantages and disadvantages of conventional and biosensor-based miRNA analysis approaches are summarised in Table 4.

Optical sensors

Optical biosensing technologies are based on the principle of energy transfer between two light-sensitive molecules. When a donor chromophore reaches its high excitation state it transfers

the energy to the acceptor chromophore through dipole-dipole interactions.[106] However, the energy transfer mechanism depends on the distance between the donor and acceptor chromophore as well as on their orientation.[106-108] Different types of mechanisms are involved in case of transferring energy for the optical sensors which include- Forster Resonance Energy Transfer, Fluorescence Energy Transfer, Resonance Energy Transfer, or Electronic Energy Transfer.[107, 108] Surface plasmon resonance (SPR) and SERS (surface enhanced Raman spectroscopy) based optical readout methods have mostly commonly been employed for ovarian cancer related miRNA analysis.[86]

SPR measures the change in refractive index upon binding of specific biomolecules (e.g., miRNA) to the immobilized probe on the metal surface. This technique is particularly attractive for real-time measurement of binding kinetics, in situ, label and enzyme-free as well.[34, 109] The greatest pitfall of this technique is its low sensitivity. To circumvent the inherent low sensitivity, several signal amplification strategies, for example, use of metallic nano-particles,[110, 111] Graphene oxide-gold nanoparticles,[112] DNA supersandwich,[110, 112] hybridization chain reaction (HCR), etc. have been adopted to make it particularly suitable for nucleic acid detection**.** It has been asserted by *He et.al.* that AuNPs enhance the SPR sensitivity for oligonucleotides more than 1000 times.[113] In 2006, Corn's group described an approach to detect miRNA with a detection limit of 10fM by surface plasmon resonance imaging (SPRI). In this approach, miRNA was hybridized to single-stranded locked nucleic acid (LNA) microarray followed by enzymatic polyadenylation of 3′ end of miRNA. Subsequently, T_{30}-coated Au nanoparticles hybridization to poly(A) tail helped in signal amplification for SPRI. Despite the less 'lower limit of detection', this method suffers from a complex protocol with long hybridization time (~4h) and multiple steps.[114] Recently, Hu et al. reported more sensitive SPRI method with LOD of 0.56 fM of miRNA-15a directly in real human serum samples.

Orthogonal signal amplification strategy accounted for this sensor to have 10^6 folds sensitivity. Addition of more mass to the target sample spot in surficial direction (in-plane amplification) resulted in 50 percent increased sensitivity and another 50 percent sensitivity was gained from the upward surface (vertical amplification). The method can be extendable to the detection of other miRNAs too.[115] Earlier, Vaisocherova *et. al.* showed simultaneous detection of multiple miRNAs in SPRi biosensor. They detected 0.5 pM miRNA from erythrocyte lysate without an RNA extraction step within an hour.[116] Sipova *et al.* developed a label-free and portable SPR based sensor to detect miR-122[117] with an assay time of 35 minutes which is relatively rapid compared to the existing approaches. In this method, captured miRNA was subsequently recognized by an antibody to realize additional sensitivity which resulted in a LOD of 2 pM. However, one of the major concerns of SPR is the steric hindrance of biomolecule-attached amplification tags used for signal amplification since it may potentially deactivate immobilized molecule in SPR chip. To address this issue, Wang *et.al.* delineated a solution where miRNA target initiated the hybridization chain reaction (HCR) and in situ generated silver nanoparticles (AgNPs) got intercalated into double-stranded probe-miRNA hybrids which favours the increase of SPR angle. As a result, it exhibited much lower detection limit of 0.35 fM.[111]

On the other hand, the generalized working strategy of SERS based biosensor depends on orderly interaction between localized surface plasmon resonances (LSPRs) of nanostructured metallic particles and the electromagnetic fields emitted by the molecules attached closely to the metal surface upon the illumination of light. As a result, enhancement of raman scattering of that molecule takes place. From the classical point of view, SERS can be divided into direct and indirect approaches based on plasmonic nanostructures fabrication. In direct SERS, intrinsic spectrum of target analyte is acquired, whereas signals from raman repoters connected to target is the key to indirect SERS. For nucleic acid biosensors, indirect

approach is more reliable than the direct approach as the later one suffers from reproducibility and sensitivity.[118] However, recent endeavour for direct detection in SERS has been documented with higher sensitivity and reproducibility. For example, Lee *et. al.* electrochemically deposited numerous silver nanocrystals to the inside of uniformly distributed gold nanobowls (Fig. 3) termed as SGBs. The nanogap between Ag nanocrystals and Au nanobowls is responsible for SERS enhancement and signal uniformity respectively. This method offers as low as 1 fM detection of microRNA (miR-34a) in total RNA sample isolated from human gastric cancer cell-line (MKN-45 (miR-34a positive cell lines) without compromising its reproducibility.[119]

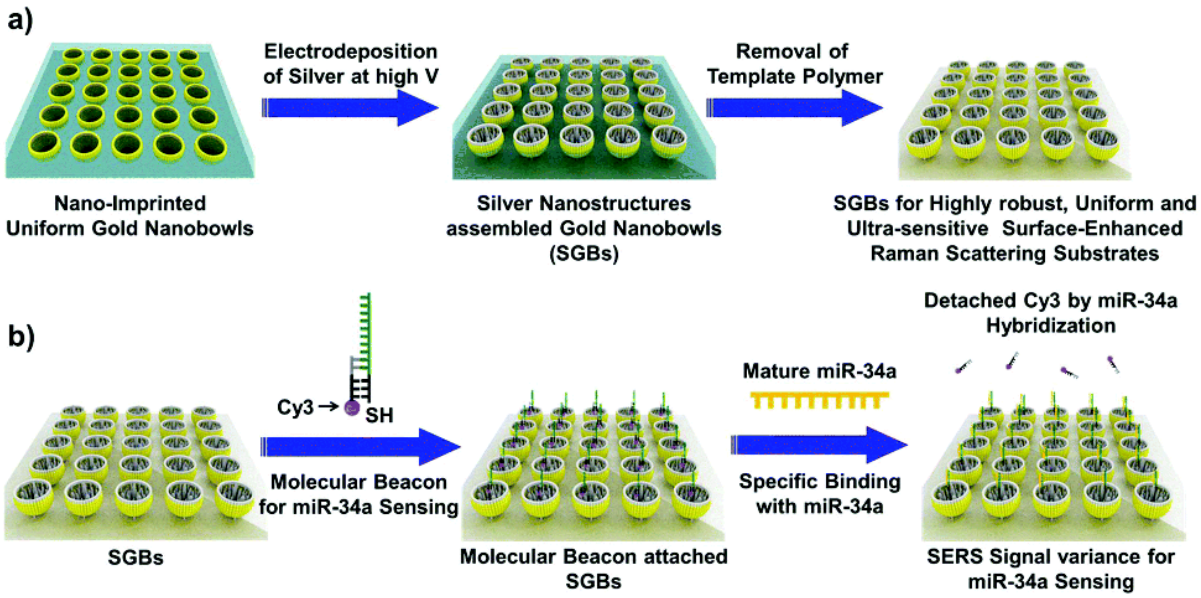


Figure 3. *Schematic illustration of simplified fabrication procedure for* ***a)*** *silver nanostructures grown in gold nanobowls (SGBs) and* ***b)*** *the molecular beacon-based SERS analysis for miR-34a detection. [Reproduced from Ref: 119 with permission from The Royal Society of Chemistry Copyright 2018]*

Wang *et al.* demonstrated the application of a "turn-on" SERS sensing technology, termed as "inverse Molecular Sentinel (iMS)" for multiplexed detection of miRNAs.[120] 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. Highly sensitive (aM) multiplexing in SERS sensor had been achieved by Song *et. al.* using Ag nanorod as SERS substrate and hairpin shaped molecular beacon for miRNA detection.[121] All in all, it can be commented that silver nanostructures were rigorously exploited for SERS signal enhancement.[121-123]

Forster resonance energy transfer (FRET) is another optical method of detetion which is based on radiation-less transfer of electronic excitation from a "donor" molecule to an "acceptor" molecule due to dipole-dipole interaction between the two molecules.[124] One of the latest optical platforms for quantifying absolute miRNAs was demonstrated by Qiu *et al.*[125, 126] They established a ratiometric and single-step detection assay using isothermal amplification of miR-21, miR-132 and miR-146a based on time-gated FRET (TG-FRET) between Tb donors and dye acceptors which resulted in miRNA assays with single-nucleotide variant specificity and detection limits down to 4.2 ± 0.5 attomoles.[125, 126] In actual case, they modified RCA-FRET miRNA assay demonstrated by Wu *et al.* using steady-state detection of two fluorescent dyes as FRET pair.[127] Although their proof of concept study could achieve an LOD as low as 103 aM and steadfast detection above background of 6 fM, the main limitation of this platform is that it was tested on only a small number of samples.[125]

Nanopore-based sensors

Nanopores, a molecular-scale pore structure, are one of the most prominent single molecule sensors that have also been used for miRNA analysis.[128-131] Typically, in the presence of a conducting fluid, when 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. Depending on the presence of target molecules such as miRNAs in the pore, current changes can be measured which facilitate the detection (Fig. 4).[132, 133] Wang *et al.* developed a unique nanopore based approach for detecting miRNAs using hemolysin protein pore.[129] The method relies on the translocation of single-stranded oligonucleotides through the 2-nm sized pore containing a programmable oligonucleotide probe. The method also obtained a highly sensitive detection limit of 100 fM miRNA in blood sample. Additionally, the sensor was successfully tested to differentiate the relative levels of miR-155 in cancer patients.

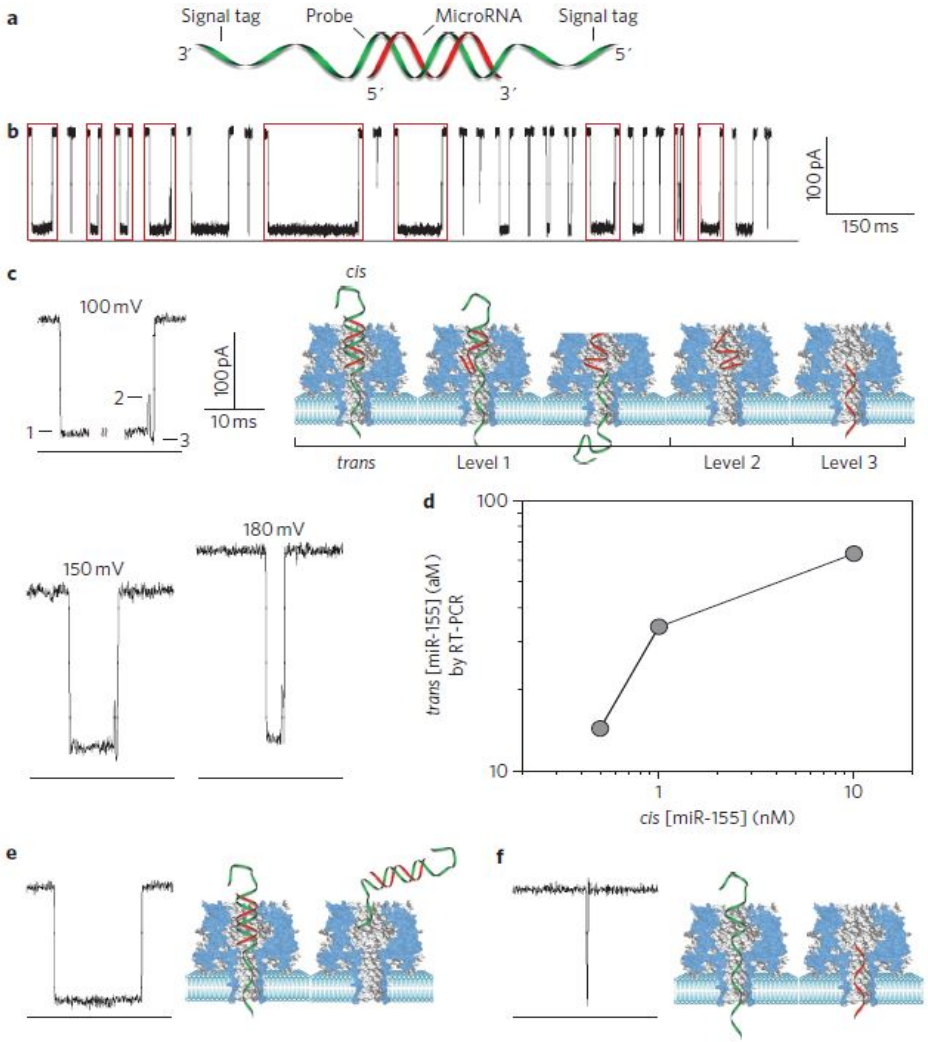


Figure 4. *Nanopore-based detection of cancer-related miRNA. [Reproduced from Ref: 129 with permission from Springer Nature Copyright 2011]*

Electrochemical biosensors

Electrochemical approaches for miRNA detection typically rely on the hybridization of target RNA sequences to complementary surface bound receptor probes (mostly DNA oligonucleotides) on the electrode. The signal transduction step of an electrochemical assay mostly relies on some intrinsic and extrinsic properties such as electroactivity of nucleobases, redox indicators (*e.g.*, methylene blue), covalently bound redox labels (*e.g.*, nanoparticles), reporter enzymes (*e.g.*, phosphatases, peroxidases) etc.[134, 135] Electrochemical sensing platforms are highly sensitive, specific, cost-effective and simple to operate. Morever, because of their portability and amenability to miniaturization they hold a great potential for development of point-of-care testing devices. Being highly sensitive (sub-fM detection limits), these electrochemical platforms may be highly suited for liquid biopsy based analysis of low frequency biomolecules like circulating disease specific miRNAs.[136]

Electrochemical detection of miRNAs is generally read *via* voltametric, amperometric, and impedimetric approaches.[137, 138] One example of such strategies is the direct oxidation based analysis of circulating miRNA bases as demonstrated by Lusi *et al.*[139] In this method, miR-122 was hybridized with its inosine substitute capture probe. Carbon based nanostructured electrode and electroactive polymers were used to increase the electroactive area and reduce the electrical resistance on the electrode. Direct oxidation of guanine during RNA-capture probe hybridization on the electrode surface gave an electrical signal which was read by the differential pulse voltammetry (DPV). This method has a low limit of detection (LOD) of 10 fM. Later, another highly sensitive (LOD= 100 aM) assay was demonstrated where two auxiliary probes were self-assembled to form a one-dimensional DNA concatemers.[140] As

shown in Fig. 5(A), hairpin capture probe was immobilized on the surface of screen-printed gold electrode. They showed that when the target (miR-21) was absent on the sensor surface, the hairpin probe retained its loop structure offering no binding site for the DNA concatemers thereby resulting in ignorable electrochemical signals. However, in the presence of target miRNA, stem-loop of the probe was exposed, allowing hybridization with DNA concatemers. This allowed positively charged RuHex reporter molecule to bind with anionic target-probe-concatemers. This produced a significantly increased electrochemical response.

As discussed earlier, one of the highly concerning issues with the miRNA biosensing is the associated chance of interference from closely related RNA sequences (*e.g.*, different intermediates of RNA biogenesis pathway including pri-miRNA, pre-miRNA and rRNA, dsRNA, miRNA from same family etc.). In 2013, Kilic *et al.* demonstrated a useful method which can overcome this issue.[141] One special type of protein known as p19 was employed, which works as a molecular calliper of small double-stranded RNA (21–23 base pairs) and isolates miRNAs in a size-dependent and sequence-independent manner. Being highly specific for miRNAs, the p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA.[142] Thus, the inclusion of p19 in the reaction mixture may decrease the chance of non-specific detection.

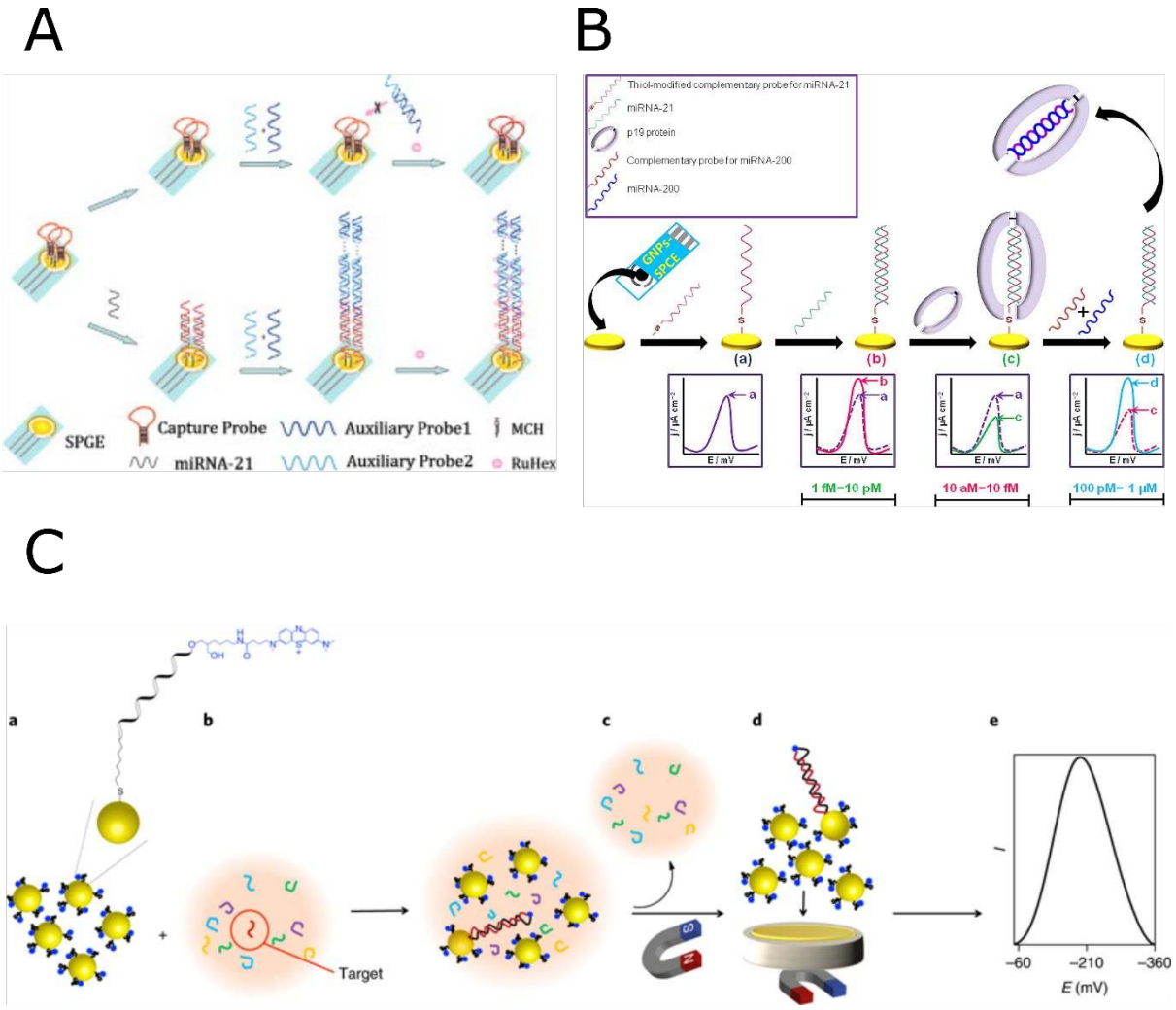


Figure 5. A) *Schematic representation of the concatemer-based electrochemical miRNA biosensor for the detection of target miR-21, [Reproduced from Ref: 140 with permission from Elsevier Copyright 2013]* ***B)*** *Three-mode electrochemical biosensor for multiple miRNA detection on gold nanoparticle modified screen printed carbon electrode. [Reproduced from Ref: 143 with permission from American Chemical Society Copyright 2013].* **C)** *Electrically reconfigurable network of gold-coated magnetic nanoparticles for direct analysis of miRNA in unprocessed whole blood. [Reproduced from Ref: 152 with permission from Springer Nature Copyright 2018]*

This p19 protein was used in another versatile electrochemical miRNA sensor reported by Labib *et al.* which was also known as three mode electrochemical sensor (Fig. 5B).[143] The sensor was designed based on three different modalities; *(i)* hybridization (*ii)* p19 protein

binding, *(iii)* and protein displacement modes. Thiolated probes immobilized on gold nanoparticle functionalised screen-printed carbon electrode (GNPs-SPCE), were use to capture target miR-21. First mode depended on increase in square wave voltammetry (SWV) response after binding of target miRNA with the capture probes (linear range of detection 1 fM to 10 pM) In the second mode, a p19 protein binding-based reduction in the current improved the detection range from 10 aM to 10 fM while in the third mode hybridization of the second miRNA with its complementary probe dissociated the p19 protein from the previous resulting a shift-back in the signal.The linear detection range of this part is from 100 pM to 1 μM. The sensor can distinguish miRNAs with different A/U and G/C content and differentiate between a fully matched miRNA and a miRNA with single base mutation. Over the past years, many other sensitive approaches have been reported. For example, Peng *et al*. developed an amperometric miRNA biosensor based on a self-assembled monolayer (SAM) of peptide nucleic acid (PNA) capture probes immobilized onto a gold electrode.[144, 145] The main advantage of using PNA is that it makes the electrostatic interaction with the cationic aniline molecules which reduces the non-specific background current successively. This approach allows for a sensitive analysis of miRNA with an LOD of 2 fM.[145] In 2012, another sensitive (LOD 0.06 pM) approach was developed which was based on dendritic gold nanostructures and graphene nanosheets modified glassy carbon electrode, and thiol-modified locked nucleic acid (LNA) hairpin molecular beacon (MB) probe.[144, 146] Cai *et al.* used functional allosteric molecular beacons as sensing platform (LOD 13.6 aM), while Wu *et al.* employed conductive self-assembled multilayer of nafion, thionine and Pd nanoparticles as enhancer and linker (with a detection limit of 1.87 pM).[147, 148] Electrochemical impedance spectroscopy (EIS) has also been used for miRNA analysis based on the principle of measuring the effective resistance of an electric circuit or component to alternating current signal of different frequencies arising from the combined effects of ohmic resistance and reactance.[144, 149-151]

A very simple yet highly sensitive platform for electrochemical detection of miRNA has recently been reported.[152] The method employs target miRNA specific probe modified gold-coated magnetic nanoparticles as dispersible capturing vehicles to isolate target miRNA from unprocessed blood. Subsequent collection of nanoparticles on the surface of gold electrode and electric field induced reconfiguration. Hybridization of miRNA to the target probe suppresses the current due to increased distance between the DNA and Au@MNPs and also possibly by redirecting the hybridized nanosensors close to the electrode surface where they act as barriers for the tunnelling of current (Figure 5C). The method achieved highly sensitive miR-21 detection across a broad range (10 aM to 1 nM). Although this method was only tested in a clinically relevant xenograft mouse model of human lung cancer, the versatility of the system as well as the predominant role of miR-21 in ovarian cancer suggests that the platform may be useful in ovarian cancer detection and monitoring.

More recently, an amplification-free electrochemical assay for detecting exosome derived miR-21 in complex biological samples has been developed at our group.[153] (**Fig. 6**)

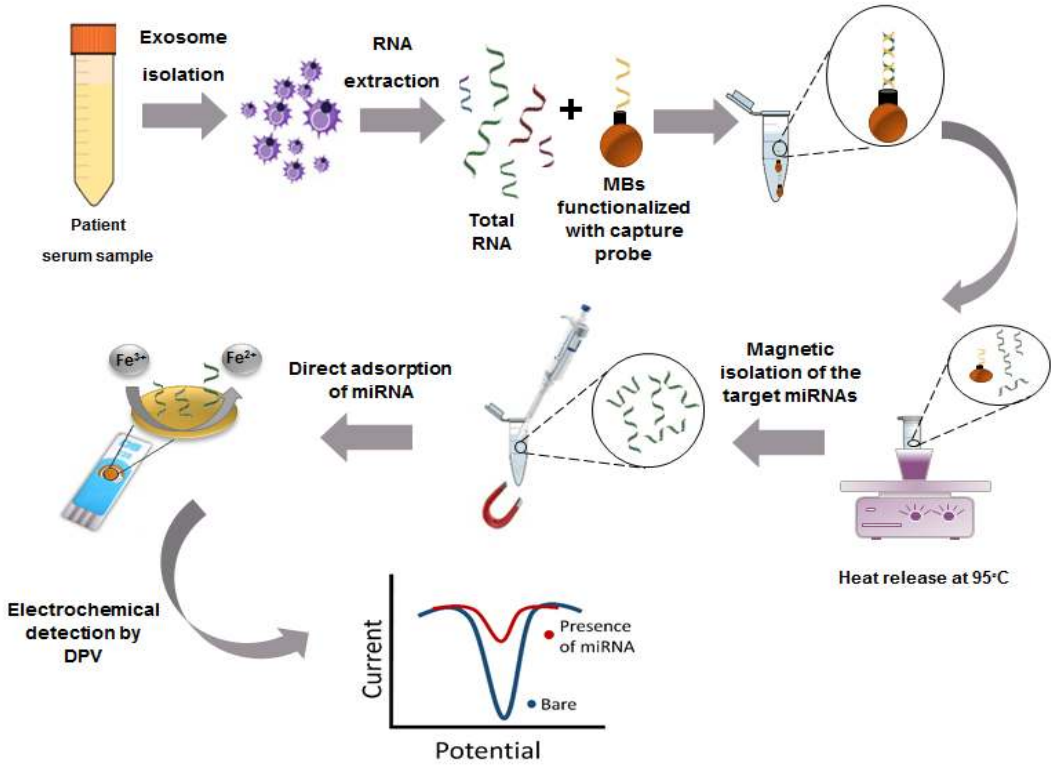


Figure 6: *Schematic representation of the assay for the detection of exosomal miRNA-21 in cancer serum samples developed by Boriachek et al.*[153] *[Reproduced from Ref: 153 with permission from The Royal Society of Chemistry Copyright 2018]*

The method relies on the capture of target miRNA by hybridization with biotinylated complimentary probes attached to streptavidin-coated magnetic beads. Post-hybridization captured miRNA species were heat released from the hybrid and adsorbed directly onto the gold surface of the disposable screen-printed gold electrode *via* RNA-gold affinity interaction. The level of the target miR-21 was subsequently measured *via* DPV response in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system. The functionality of the assay was tested in exosomes samples derived from a cohort of cancer patient samples. Since, miR-21 is one of the potential biomarkers for ovarian cancer, we assume that the method has high potential for ovarian cancer related exosomal miRNA analysis. There have also been continuous efforts to develop a more sensitive and specific assay for detecting microRNAs using electrocatalytic detection technique. In their recent study, Shiddiky *et al.* used gold-

loaded nanoporous superparamagnetic iron oxide nanocubes (Au-NPFe_2O_3NC). The target miRNA (miR-107) was directly adsorbed onto the gold surfaces of Au-NPFe_2O_3NC *via* gold-RNA affinity interaction.[49] The assay used the electrocatalytic property of Au-NPFe_2O_3NC to reduce ruthenium hexaammine (III) chloride (RuHex, $[Ru(NH_3)_6]^{3+}$) bound with miR-107. The amplification of the catalytic responses was detected using the ferri/ferrocyanide $[Fe(CN)_6]^{3-/4-}$ system. The assay involved multiple signal enhancement steps and the LOD was reported as low as 100 aM, which till now could be considered as one of the more precise platforms, with better or comparable reproducibility for miRNA detection compared to most of the conventional miRNA sensors.[49]

As discussed earlier, electrochemical biosensors are highly suitable for liquid biopsy and point-of-care applications therefore extensive research is underway for development of novel electrochemical miRNA biosensing platforms and several methods have been reported recently. For example, Zeng et al. developed an electrochemical miRNA biosensor whereby DNA tetrahedral nanostructures were used as miRNA capture probes. Hybridization of target miRNA was detected by using biotin labelled signal probes (complementary to other part of miRNA) which leads to the capture of poly-HRP40 on the gold electrode and subsequently the classic TMB/H_2O_2 catalytic reaction, which in turn can be amperometrically detected and reflects the target miRNA concentration. The authors further expanded the platform for multiplex detection of four prostate cancer related miRNAs; miR21, miR155, miR196a, and miR210 using a 16-channel disposable SPGE. The authors reported a detection sensitivity as low as 10 fM and successfully profiled serum levels of the four miRNAs in prostate cancer patients as well as healthy individuals.[154] Yang et al. used methylene blue (MB) labelled DNA probes whereby target induced strand displacement amplification and DNAzyme cleavage releases the bound MB molecules. The resulting decrease in MB oxidation peak is thus directly proportional to miRNA concentration.[155] Liang et al. on the other hand developed an

electrochemical miRNA biosensor based on target miRNA initiated cascacde hybridization chain reaction which leads to the assembly of DNA nanostructures on the electrode surface which can be subsequently detected by using various DNA interacting electroactive compounds like $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$, $Ru(NH_3)_6^{3-}$, or MB. The method could detect miR21 up to lower detection limit of 11 pM.[156] More recently, Han et al. utilized the cross-shaped DNA origami, that comprised of target specific probes, immobilized on chitosan coated gold electrodes to capture the target miRNA which was subsequently detected by increased MB binding and proportional increase in oxidation peak current. However, the method only exhibited a linear range between 1.0 pM and and 10 nM concentrations with an LOD of 79.8 fM.[157]

Conclusions

We have reviewed the potential role of miRNAs as an accurate and effective biomarker for ovarian cancer. The biogenesis, diagnostic and prognostic significance of clinically relevant miRNAs has also been discussed. We briefly outlined the conventional approaches for ovarian cancer detection as well as miRNA analysis. We have identified major challenges faced by conventional miRNA analysis techniques and have also discussed probable solutions. Subsequent to this brief introductory discussion, current biosensor-based miRNA detection strategies are comprehensively reviewed with a special focus on electrochemical approaches and the platforms that have targeted ovarian cancer specific miRNAs. Moreover, we discussed that compared to cell-bound miRNAs, circulating miRNAs packaged into exosomes have more potentiality towards the specific and sensitive detection of ovarian cancer. With the advent of new technologies over the past few years miRNA detection approaches have seen a considerable progress. Despite this progress, an integrated, automated and relatively fast biosensing platform is still required for transforming these proof-of-concept methods to routine

analysis of miRNA biomarkers. With the regulatory importance and clinical potential of miRNAs being increasingly recognised, the appetite for technologies enabling sensitive and low-cost miRNA analysis will likely continue to grow. Several recent innovations such novel nanomaterials, advancements in micro- and nano-scale fluid handling platforms, as well as electrochemistry could herald revolutionary changes in ovarian cancer management strategies, by enabling low cost near-patient miRNA analysis.

Conflict of interest

There are no conflicts to declare.

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