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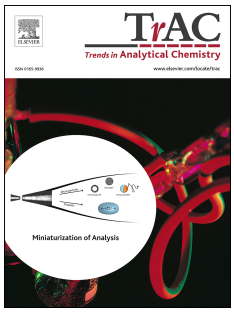

New frontiers in microfluidics devices for miRNA analysis

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New frontiers in microfluidics devices for miRNA analysis

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Abstract

miRNA detection using microfluidics-based devices is one of the most important innovations in biology and modern medicine. miRNAs are small regulatory molecules, whose varied concentrations can indicate diseases or pathologic conditions, boosting their use as reliable modern biomarkers for advanced medical diagnostics. Due to their small size and low concentration (from femtomolar to picomolar), miRNA measurement is quite challenging. Being rapid and affordable analytical devices for high-throughput quantification of miRNAs, microfluidics-based biosensors are promising. Beside the small volumes of required sample, simple handling, and manipulation of the sample, these tools enable amplification and detection of target miRNA/miRNAs in a single device, overcoming common limitations of conventional methodologies. Herein, we review the recent advancements in microfluidic devices applied for miRNA sample handling, manipulation, and measurement. We also discuss their advantages and limitations as well as future trends in this field.

Keywords: miRNA; microfluidic; biosensors; lab-on-a-chip; miRNA sample preparation.

1. Introduction

Micro-RNAs (miRNAs) are small non-coding RNAs with important roles in the post-translational regulation of protein-coding genes. Any dysregulation in their concentrations, either up- or down-regulation, may lead to complications in cell function and consequently leading to diseases, like cancer, infertility, and Alzheimer's [1-3]. They could affect a single or a group of genes, distressing their regulation and tissue-specific expression patterns. Thus, they could be considered as specific biomarkers for a particular or a group of diseases. They are responsible for cell-cell communications. In addition to the intracellular environment, miRNAs also exist in the biological fluids (down to femtomolar concentration), including blood, saliva, tear, urine, milk, and cerebrospinal fluid [4, 5]. Such miRNAs are either free, located inside exosomes and microvesicles, or conjugated to high-density lipoprotein (HDL) cholesterol molecules [4, 6, 7].

Circulating miRNAs have tissue- and/or disease-specific expression levels. They are, therefore, important biomarkers in the diagnosis, prognosis assessment, and monitoring of many diseases [8, 9]. This is mainly because they could be measured in an easy and non/less-invasive manner, through tear, saliva, and even sweat [4, 7, 10]. Due to the abovementioned advantages, many miRNAs are considered for the diagnosis of different types of cancer as well as endocrine, neurological, and cardiovascular diseases [11, 12]. This is while scientists are facing severe challenges for the quantification of miRNAs due to their small size (18-23 nucleotides), high homology, and low quantity (femtomolar to picomolar), as well as high interference with other molecules [13]. Conventional miRNA detection and quantification methods include quantitative real-time PCR (qRT-PCR), Next-Generation Sequencing, and Northern blotting [1, 8]. These methods are expensive, complex, time-consuming, and require expensive instruments and skilled personnel.

This points out the need for alternative simple and cost-effective methods, such as biosensors that have been emerging over the past decade [1, 9, 13, 14]. These alternative tools benefit from different transducers and innovative technologies, including the use of novel nanomaterials and amplification methods, to overcome the limitations of conventional techniques [13, 15, 16]. Moreover, the integration of biosensors into microfluidic devices has led to the fabrication of more sensitive, selective, affordable, portable, and small high throughput instruments, capable

of real-time and rapid detection methods compatible with point of care (POC) applications [13, 15-18].

Microfluidics encompasses miniaturized devices consisting of micro/nano size channels and chambers to handle small volumes of reagents (10^{-9} to 10^{-18} liters) on a chip [19]. The microfluidics' market is constantly growing for applications ranging from cells and stem cells culture monitoring to single-cell analysis, 3D cultures, organ- and lab-on-chips (LOCs) [15, 20]. Microfluidics-based biosensing systems are classified into three classes, namely continuous-flow, droplet-based, and digital microfluidics. The continuous-flow, one of the most common biosensors, is named due to the continuous flow of fluids inside the channels [21]. This is while droplet-based microfluidics, on the other hand, are based on the generation and manipulation of sub-nanoliter-volume droplets within the microchannel environments [22]. Digital microfluidics are based on the manipulation of droplets within a reconfigurable network on the surface [23].

Over the past decade, microfluidics and LOCs have also been applied for miRNA analysis. Many researchers and companies have introduced innovative ideas and designs to improve the analytical performances of these devices. As an example, several materials have been exploited to provide the best support for chip fabrication, including polymers (e.g. PDMS, PMMA), glass, and paper. Sample preparation, pre-treatment, or pre-concentration on *ad hoc* designed microfluidics devices are also crucial to enhance the sensitivity and selectivity of the analytical tools [24, 25]. Microfluidics can also host different methods and strategies to lysis, extract, isolate, and separate the target miRNAs in the samples [14]. This can be achieved using certain structures inside the channels or chambers using innovative fabrication techniques [26, 27]. Moreover, sample manipulation and preparation can also consist of the conjugation of the sample with micro- or nano-particles or incubation, mixing, and sorting with other reagents through, for instance, fluorescence-activated cell sorting and magnetic activated cell sorting [28, 29]. In addition, miRNA-capture biomolecules (aptamers, oligonucleotides, and proteins) can be immobilized upon the detection or capture area of the chip to enhance its separation and detection efficacy [30, 31]. Amplification, enzymatic or non-enzymatic, is another important sample preparation step to enhance sensitivity in miRNA analysis [32, 33].

All those steps can be integrated using different combinations, specific to each chip and depending on the application or particular needs. This is also favorable for each biofluid, which might need a different extraction or isolation technique. Automation and portability are other possible benefits for these devices, especially for portable point of care applications. Finally, multiple miRNA detection is also possible through a high-throughput method for parallel testing, which helps save money and time [34, 35].

Biosensors are mostly categorized based on their transducer types which provide various sensitivity and selectivity potentials [36, 37]. A wide range of transduction systems can be integrated into the microfluidics chips for miRNA detection, such as electrochemistry, fluorescence, Surface-Enhanced Raman Scattering (SERS), Surface Plasmon Resonance (SPR), and colorimetric methods [15, 18]. Some microfluidic devices enable only sample preparation with the detection, using other methods as sequencing and mass spectrometry, performed outside the chip. However, on-chip detection in conjunction with sample preparation is more efficient, time and cost-wise, as well as more convenient for automation [38, 39].

Herein, we aim to provide an overview and discuss the pros and cons of recent advancements in the microfluidics-based miRNA quantification devices and categorize them based on their transducer type into three main groups (i) electrochemical, (ii) optical, and (iii) electrical biosensors. We also discuss existing microfluidics-based sample manipulation and amplification strategies for miRNA analysis, which aim to enhance sensitivity. Later, we discuss the role of microfluidics for (i) miRNA isolation for sequencing & PCR, (ii) miRNA amplification, and (iii) sample preparation before mass spectrometry. To our best knowledge, this review addressed several aspects of miRNA detection using microfluidic devices, going beyond the reviews reported in the literature [40-46].

2. Microfluidic-based miRNA detection

In this section, the microfluidics-based miRNA detection techniques are categorized into three main subsections based on the transducer types namely (i) electrochemical, (ii) optical, and (iii) electrical detection, highlighting the different analytical features including pros and cons, as pointed out in Table 1.

2.1. Electrochemical detection

Electrochemical transduction is one of the most commonly used detection methods in microfluidics-based biosensors, because it is characterized by small size, portability, low cost as well as high sensitivity and selectivity. In addition, thanks to the convergence of cross-cutting technologies, several configurations can be customized considering the wide plethora of supporting materials and electrochemical techniques available [47].

To report an example, Kutluk and colleagues [48] described the realization of a low-cost microfluidic biosensor platform for the monitoring of miRNA-197 (a tumor biomarker candidate) in undiluted human serum samples, based on chips designed through both sandwich and competitive formats (Figure 1A). The obtained results showed that the sandwich assay has superior performance regarding its sensitivity and selectivity, as well as the possibility to operate with very low sample volumes (580 nl) and a sample-to-result time of one hour. This format provided to reach a detection limit of 1.28 nM (0.74 femtomole) in comparison with the competitive format that furnished a detection limit of 4.05 nM (2.35 femtomole). In addition, the sandwich format was capable to provide better discrimination towards single-base mismatch oligonucleotide sequences if compared to the competitive one.

In the last decades, nanotechnology proved its potential to enhance the analytical performances of microfluidic devices when associated with electrochemical transduction systems, as demonstrated by Chand and co-workers [49]. The authors synthetized MoS_2 nanosheets decorated with a copper ferrite ($CuFe_2O_4$) nanoparticle composite to immobilize a molecular probe on such nanomodified support for the electrochemical detection of paratuberculosis-specific miRNAs (Figure 1B). This MoS_2-$CuFe_2O_4$ nanocomposite-modified electrode was demonstrated to generate an amplified signal of the miRNA-specific molecular probe, which was previously biotin-tagged and thiolated with ferrocene thiol. Indeed, upon miRNA interaction with the probe, an increase in the electrochemical signal from ferrocene was registered by means of square wave voltammetric analysis, reaching a limit of detection of 0.48 pM, within a dynamic range from 1 pM to 1.5 nM. The proposed nanomaterial enabled the target miRNAs to be discriminated among different interfering molecules, also in spiked serum and positive clinical

153 samples, highlighting its potential as a portable diagnostic tool, or namely point-of-care, for
154 paratuberculosis assessment in dairy cows.
155 Biotechnology also supported in the last years the design of multiplexing microfluidic devices for
156 miRNAs biosensing in clinical diagnostics. Several microfluidic biosensing configurations have
157 been applied in different areas, devoted not only to the detection of the target but also to its
158 amplification to enhance the sensitivity. More recently, amplification-free formats have been
159 reported in the literature as more effective and sensitive devices powered with CRISPR/Cas
160 technology, which replace amplification steps by a Cas13a-driven signal amplification. Dincer's
161 group [50] realized an integrated electrochemical biosensor for the on-site detection of potential
162 miRNA tumor markers, namely miR-19b and miR-20a, exploiting a CRISPR/Cas13a-powered
163 microfluidics. The specificity was given by a target-specific CRISPR RNA that guided the Cas13a to
164 target RNA (Figure 1C). Upon recognition of the complementary RNA sequence, the reporter RNA
165 was enzymatically cleaved and thus activated target RNA for the quantitative readout. By
166 exploiting this mechanism, a limit of detection of 10 pM was achieved within a readout time of 9
167 min and an overall process time of less than 4 h, using a very small measuring volume equal to
168 0.6 µL. Moreover, this biosensor platform was capable to detect miR-19b in serum samples of
169 children with brain cancer. More recently, the same group [51] implemented the above described
170 device and obtained a multiplexed electrochemical microfluidic biosensor based on four chips
171 designed for the amplification-free and simultaneous quantification of up to eight miRNAs.
172

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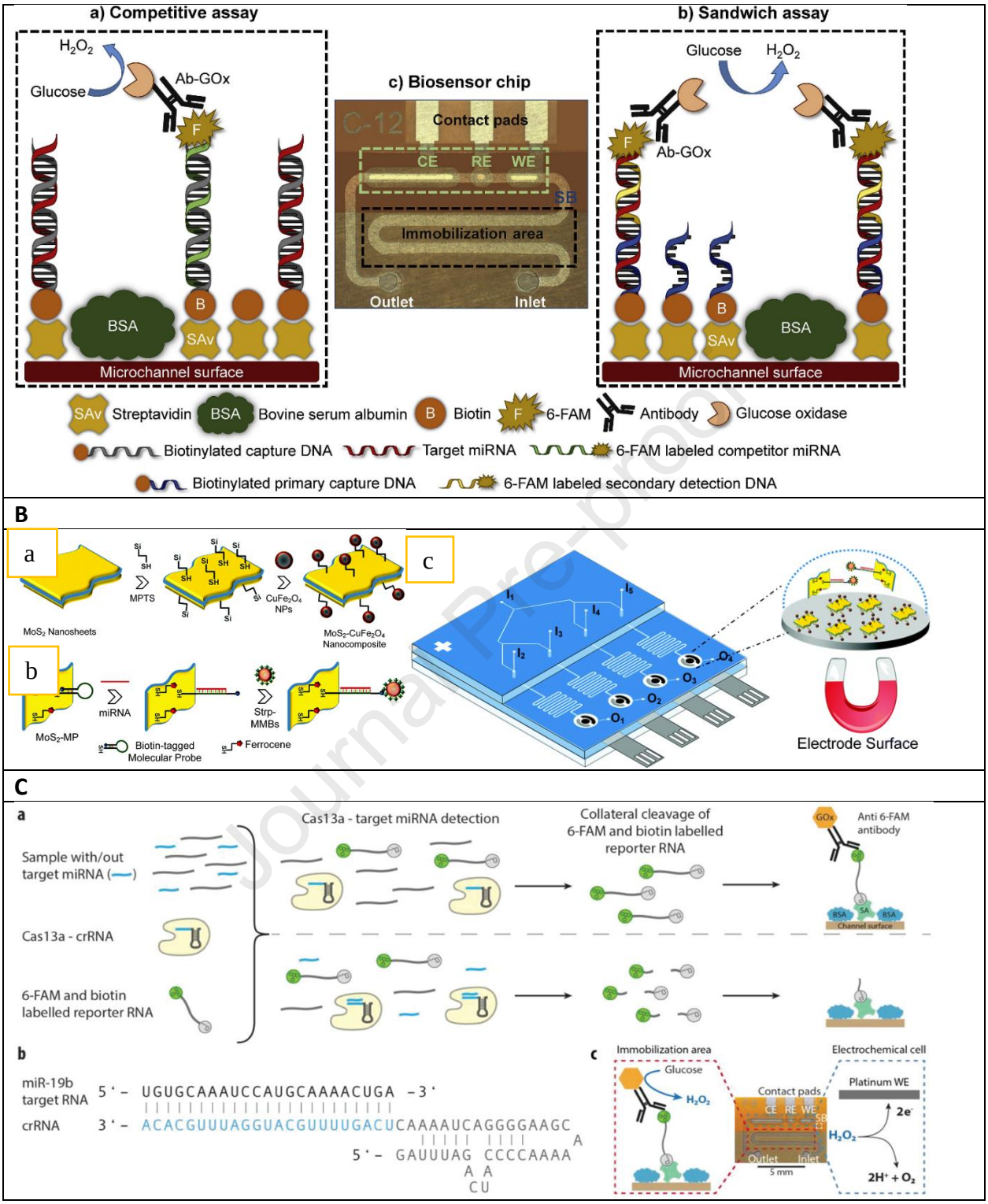

***Figure 1**. Electrochemical methods in miRNA microfluidics biosensors:*
***A**) Illustration of the (a) competitive and (b) sandwich assay formats employed for the detection of target miRNA-197 in the microfluidic biosensor. (c) Image of the microfluidic biosensor, visualizing the immobilization area (black), the electrochemical cell with the counter, reference*

and working electrodes (green), and the stopping barrier (SB), shown in blue, which separates the two chambers [48].

B) *MoS_2 nanosheet-based electrochemical miRNA sensor: (a) schematic of MoS_2–$CuFe_2O_4$ nanocomposite synthesis, (b) principle of miRNA detection using MoS_2-MP nanocarriers, and (c) structure of the PDMS microfluidic platform showing inlets ($I_{1–5}$), mixing, incubation and sensing zones, outlets ($O_{1–4}$) and integrated electrodes, inset: miRNA detection on the nanocomposite-modified electrode* [49].

C) *Combination of the CRISPR technology along with an electrochemical microfluidic biosensor for miRNA diagnostics. a) Schematic of the off-chip miRNA targeting, including the enzyme Cas13a, the target miRNA (blue), the target-specific crRNA, and the biotin and 6-FAM-labeled reporter RNA, which is immobilized after the cleavage process onto the streptavidin (SA) and BSA blocked channel surface. b) Schematic of the single-stranded target miRNA, miR-19b, and the crRNA, where the complementary sequence is highlighted in blue. c) Working principle and photo of the microfluidic biosensor with its main elements, including the contact pads for the working, reference, and counter electrodes (WE, RE, CE) in the electrochemical cell (marked in blue) and the immobilization area for the assay preparation (highlighted in red), separated by the hydrophobic stopping barrier SB* [50].

Likewise, material sciences have provided smart materials for microfluidics, including paper with leading actor especially in the realization of specific sections of the device devoted to different applications, from target amplification to detection and sample treatment. Yang and colleagues [52] described the fabrication of an integrated ratiometric photoelectrochemical paper analytical device with a hollow double-hydrophilic-walls channel for miRNA-141 quantification (Figure 2A). In particular, a photoanode was integrated onto the paper, together with a photocathode, exploited to improve the selectivity. Dendriform polymeric DNA duplex structures decorated with glucose oxidase (GOx)-mimicking gold nanoparticles were used as a probe for the quantitative detection of miRNA-141 with a linear range between 0.15 fM and 2 nM, and a detection limit of 52 aM (S/N = 3).

With the convergence of nanotechnology and material sciences, Sun et al. [53] realized an electrochemical analytical device for miRNA detection, exploiting paper as the substrate and Au nanorods to modify the chip and cerium dioxide-Au@glucose oxidase (CeO2-Au@GOx) as an electrochemical probe for signal amplification (Figure 2B). The biosensor was able to achieve a linear range from 1.0 fM to 1000 fM and a detection limit of 0.434 fM.

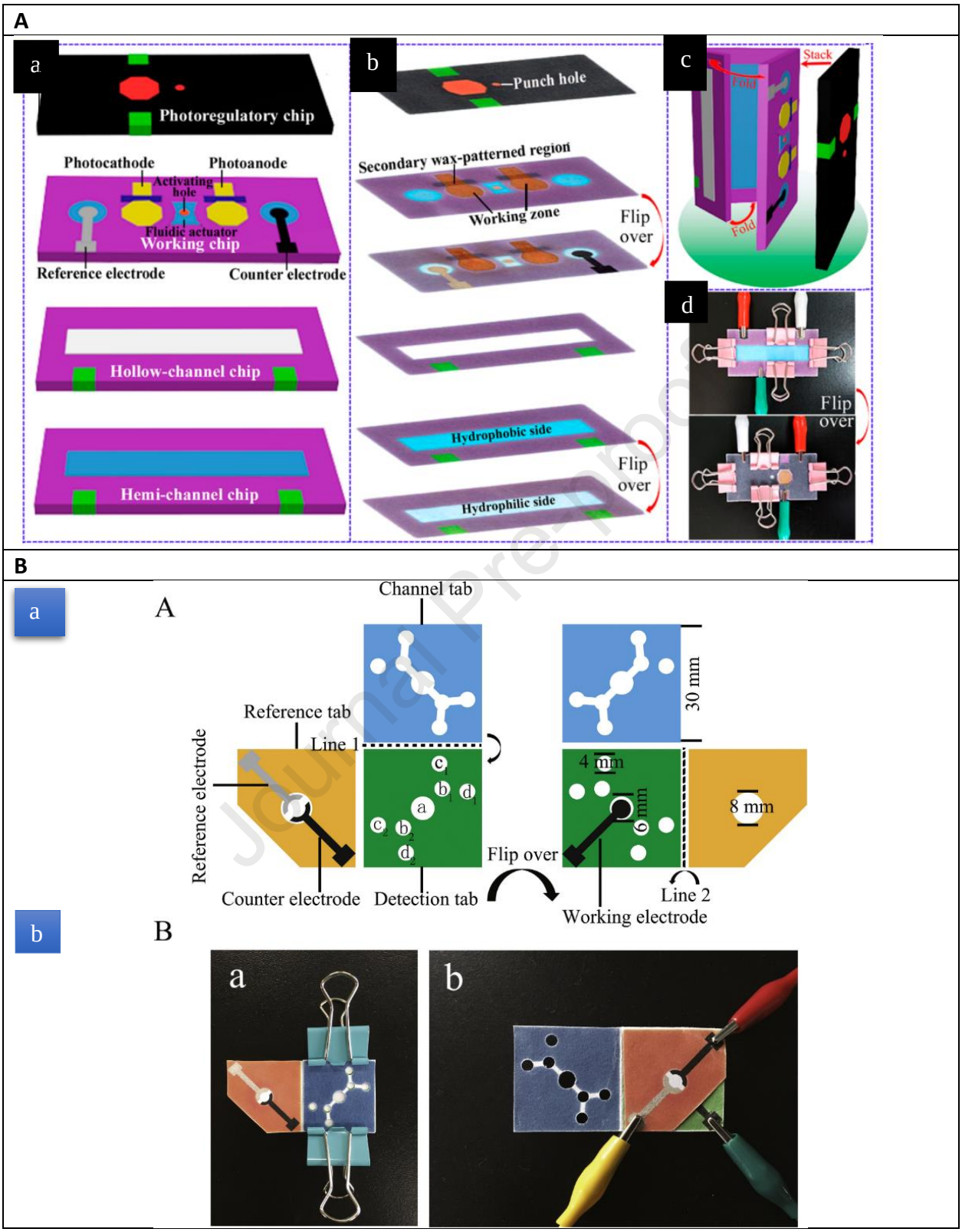

***Figure 2**. Paper-based electrochemical methods of miRNA microfluidics biosensors: **A**) (a) Schematic layout and (b) Photograph of the HDHC paper PEC device; Three-dimensional view of the HDHC paper PEC device (c) before assembly; and (d) after assembly* [52].

B*) (a) The schematic representation, size, and shape of µPADs. (b) The fabrication of microfluidic paper-based analytical devices for miRNA detection, combining chromogenic reaction and electrochemistry* [53].

2.2 Optical detection

The exploitation of optical detection in microfluidics is common, thanks to its easy coupling and rapid response. Optical transduction entails absorption, fluorescence, chemiluminescence, and surface plasmon resonance [54]. Various miniaturized optical components and innovative nanomaterials can be integrated into the microfluidic chips to enhance the sensitivity. As an example, Portela and colleagues [55] exploited nanogap antenna structures to fabricate large-area nanoplasmonic sensor chips by a customized, simple, and low-cost colloidal lithography process. In detail, the authors realized large-area sensor chips of nanogap antennas formed by pairs of gold nanodisks separated by gaps with an average size of 11.6 ± 4.7 nm (Figure 3A). This optical configuration allowed for the detection of miRNA-210, a relevant biomarker for lung cancer diagnosis, through a DNA/miRNA hybridization assay, with a limit of detection of 0.78 nM (5.1 ng mL^{-1}) and without any amplification steps. This underlined the high sensitivity of these plasmonic nanogap antennas for direct and label-free detection of low molecular weight miRNAs. The hydrogel was also exploited for the fabrication of a microfluidic platform for the colorimetric monitoring of miRNAs, without the use of other equipment for fluidics and imaging [56]. To avoid the risk of sequencing bias, a gold deposition-based signal amplification scheme and dark-field imaging were assembled to integrate a previously developed miRNA assay scheme into this platform (Figure 3B). The assay demonstrated a limit of detection of 260 fM, along with multiplexing of small panels of miRNAs in healthy and cancer samples. This versatile platform was able to analyze a wide range of miRNAs in cancer-associated dysregulation with high confidence by exploiting the unique features of hydrogel substrate in an on-chip format and colorimetric analysis.

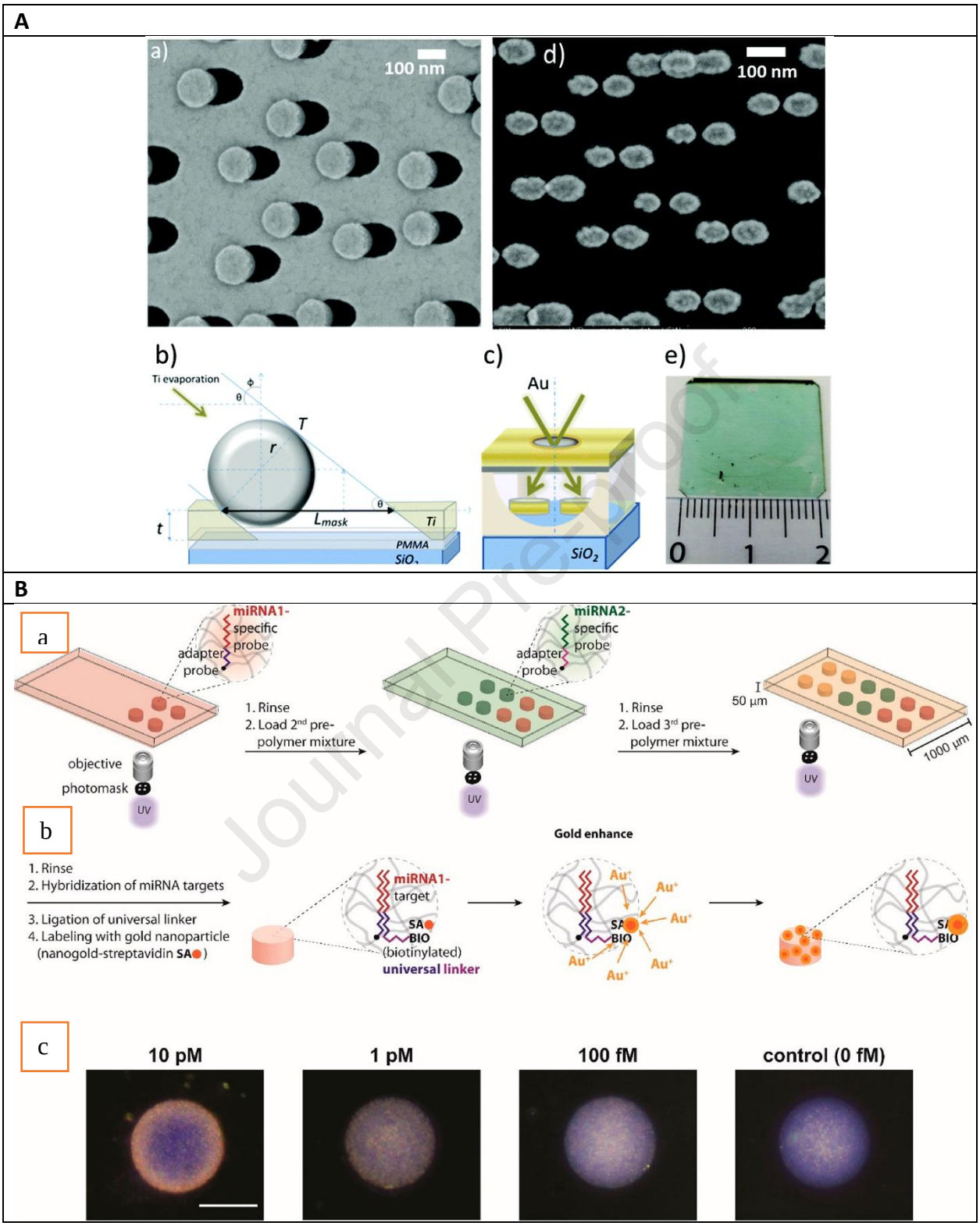

***Figure 3**. Optical methods of miRNA microfluidics biosensors:*
***A**) Fabrication of nanogap antennas. (a) SEM image of the elliptical mask obtained after tilted evaporation of 20 nm Ti; (b) sketch of the flexibility for tuning the length of the long axis of the ellipsoid shape mask; (c) evaporation of gold using two opposite angles with respect to normal*

evaporation; (d) SEM image of Au nanogap antennas over a glass substrate; (e) photograph of a fabricated sensor chip. A ruler (mm) is shown as a reference scale [55].
***B**) (a) Schematics of on-chip hydrogel post synthesis for multiplexing of small miRNA panels using projection lithography with the spatial encoding scheme. (b) miRNA assay scheme: target hybridization, universal linker ligation, gold nanoparticle labeling, and gold ion deposition-based signal amplification. (c) Dark-field images of posts after complete miRNA assay, demonstrating the dose-dependent response of miRNAs. Scale bar represents 100 µm* [56].

2.3 Electrical detection

Electrical detection methods have been exploited in microfluidic-based biosensors for miRNA detection due to their potential for being miniaturized, integrated into a chip, being portable, and having a simple methodology [57]. Field-effect transistor (FET)-based sensors for label-free measurement of specific chemicals and biomolecules with electrical signals have been recently applied for extracellular vesicles (EVs) extraction and thus quantitative miRNA assessment. EVs are carriers of biologically important molecules, including miRNAs. EV extraction, therefore, is one of the main steps in the miRNA measurement. The main benefits of such technology include miniaturization, rapid turnaround time, cost-effectiveness, and mass production as well as durability, stability, high sensitivity and specificity, low signal-to-noise (S/N) ratio, and minimized contamination risk [58, 59].

As an example, an integrated microfluidic system was described in the literature, equipped with highly sensitive FET capable of EV extraction, EV lysis, target miRNA isolation, and miRNA detection within 5 h [60]. The limit of detection was within the physiological fM range for two targeted miRNAs (miR-21 and miR-126) for early diagnosis of cardiovascular diseases. The absence of need for signal amplification in the immunoaffinity-based magnetic beads for high yield EV isolation (54.3 %) is one of the main benefits of this platform over with fluorescent quantification methods using RT-qPCR [61].

In another work, Kim et al. developed a label-free disposable sensing platform with high sensitivity and specificity for detecting femtomolar levels of miR-21 as an oncogene, let7b as a controller of the androgen-signaling pathway, and miR-1246 as a tumor regulator, without the need for pretreatment or signal amplification in urine samples from patients with prostate cancer **(Figure 4A)** [62]. The FET biosensing module facilitated rapid, direct, and multiplex miRNA detection. In this regard, the surface of the disposable chip was functionalized with reduced

281 graphene oxide nanosheets and then peptide nucleic acid (PNA). The former provided the sp^2
282 carbon-structure domains, which improved the immobilization of PNA probes for capturing
283 miRNA in large surface areas. It also enhanced conductivity and electron mobility, and thus
284 electrical signals and sensitivity.
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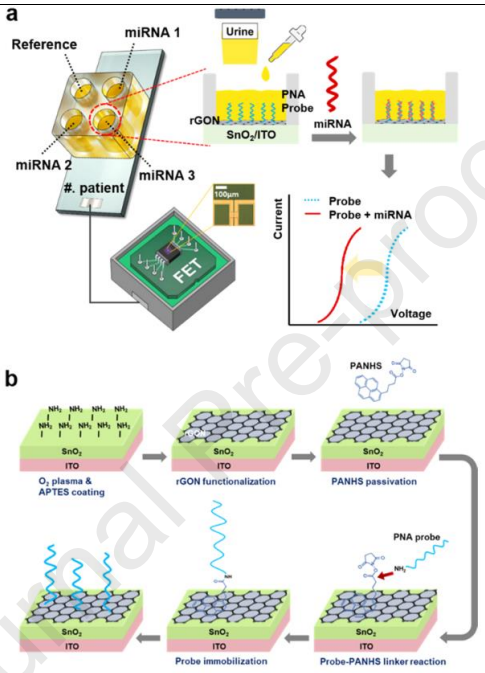

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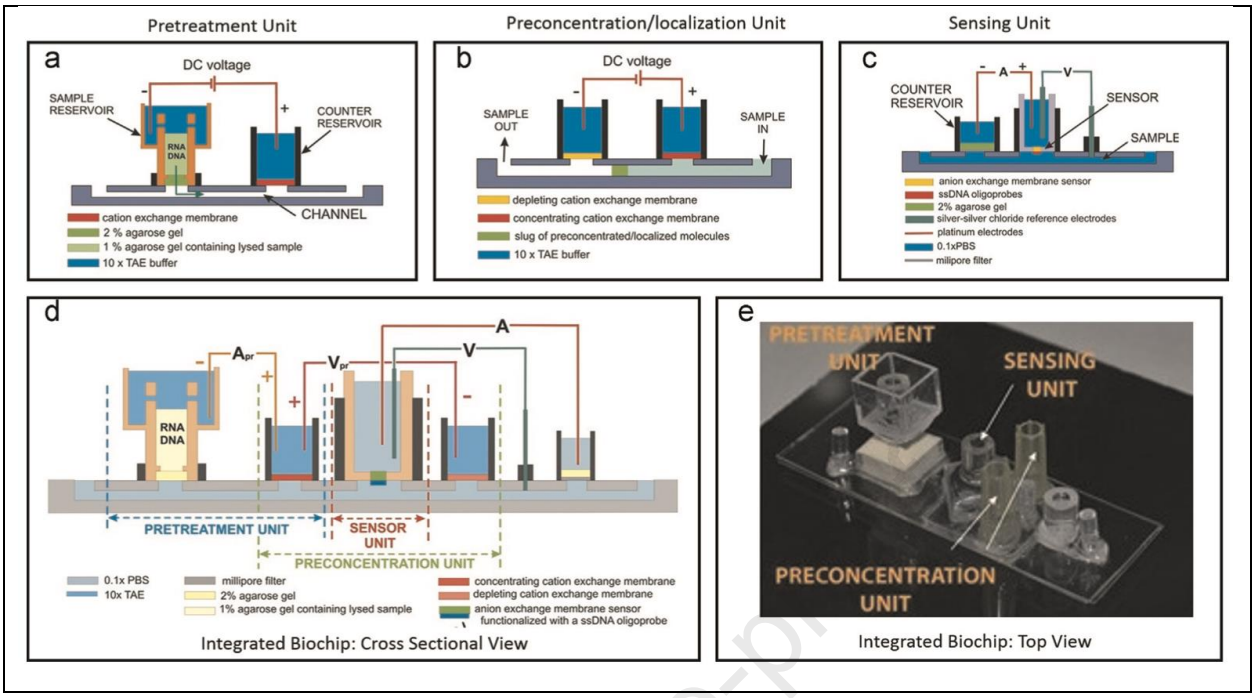

***Figure 4**. Electrical methods of miRNA microfluidics biosensors;*
***A**) (a) Schematic illustration of the urinary miRNA sensing platform based on a disposable and switchable electrical sensor in urine. (b) Process of chemical fabrication and surface modification of the disposable sensor chip* [62].
***B**) schematics of a (a) pretreatment unit ,(b) preconcentration unit, (c) flow-through sensing unit, (d) integrated platform with all electrical connections, (e) Top view of an actual integrated device* [63].

CMOS-compatible silicon nanowire field-effect (SiNW-FET) devices are another high throughput and low-cost tools that can be used for this purpose. The SiNW nanosensors can also be integrated with microfluidics to enable highly selective and sensitive multiplexed and automated detection with rapid analyte delivery, using rather small sample volumes. In a recent study, this system was used for real-time monitoring of miRNA−126 and CEA detection in lung cancer patients [64]. To overcome the complex integration issues accompanied by the use of SiNWs, a simple and low-cost "top-down" method, compatible with commercial semiconductor processes, was applied. In this regard, optical lithography was combined with anisotropic wet etching using tetra-methylammonium hydroxide (TMAH). It was then integrated into a PDMS microfluidic manifold to ensure sensitivity of 0.1 fM for miRNA-126 and 1 fg/ml for CEA detection.

FET sensors, however, suffer from certain shortcomings such as only being capable of detecting the smallest charged molecules and not the nucleic acid molecules, due to the ionic strength

within the electrical Debye layer being 2-3 times higher than the bulk [65]. This requires expensive micro-fabricated electrodes and long assay times due to diffusion-based transport of large nucleic acid molecules to the electrode surface. The use of ion-selective nanoporous membranes is another technology with promising results in miRNA measurement and the possibility to overcome some of these shortcomings. In this regard, Slouka et al. managed to develop a simple, inexpensive, label- and PCR-free microfluidic platform for miRNA measurement in oral cancer using commercially available heterogeneous ion-selective nanoporous membranes **(Figure 4B)** [63]. The platform enabled rapid and selective detection of microRNA 146a biomarkers from oral cancer cell lines in approximately 30 minutes. The three units of the device, pre-treatment, pre-concentration, and sensing, utilized the inherent negative charge of the nucleic acid molecules and the ion-selective properties of cation- and anion-exchange membranes. This is while a DC field was applied to extract the nucleic acid molecules from the sample through an agarose gel, reducing the contamination risk. The cation exchange membrane under DC field was then used to create an electrokinetic filter, needed for concentration and rapid detection of the target. This technique prevented any possible hydrodynamic resistance. The ion depletion feature of the membrane, on the other hand, helped exclude all charged molecules at a precise location in the microfluidic channel, reducing diffusion time to the sensor and resulting in rapid target detection. These steps are much simpler and less expensive than the hydrodynamic, mechanical, acoustic, electric, magnetic bead, and ultrasonic approaches used in other similar LOCs [66, 67]. Moreover, the absence of any electron transfer reaction on the membrane surface has turned such sensors into highly stable measurement tools.

In other examples, the combination of the electric and acoustic pressures induced by surface acoustic waves (SAWs) are applied in microfluidics [68, 69]. In this regard, two microfluidic platforms for miRNA lysis (~30 min) and detection (~1 h) were developed using sample volumes as low as ~100 µL. SAW-based lysis, which benefits from the combination of dielectrophoretic and acoustic radiation forces, is a promising alternative to traditional chemical or surfactant lysates as it does not interfere with RNA detection downstream, through affecting the buffer pH and ionic strength or disrupting self-assembled layers. It was reported to have a yield of about 38 %. The label-free chip was an ion-exchange nanomembrane sensor sandwiched between two

reservoirs, enabling the measurement of the current–voltage characteristic (CVC) [70]. CVC has shown promising results for miRNA detection as in this technique a dramatic change is observed as large, negatively charged molecules such as RNA are adsorbed to the surface of the positively charged membrane. The nanomembrane sensor was capable of measuring the target microRNA (hsa-miR-550) with a limit of detection of 2 pM for early diagnosis of pancreatic cancer.

Table 1- List of Pros and Cons in function of the different transducers

Transduction	Pros	Cons
Electrochemical	Simple to use Miniaturization Low cost Real-time response Continuous analysis Simultaneous analysis of targets	Need of redox probe in the case of voltametric detection
Optical	Real-time detection Reliability High sensitivity Simultaneous analysis of targets	Sensitive to surrounding environment
Electrical	High sensitivity Label-free detection Real-time response	Detection of smallest charged molecules and not the nucleic acid molecules

3. Microfluidics for miRNA sample preparation, amplification, and other analysis

Microfluidics can be used to manipulate miRNA-containing samples to enhance the sensitivity and selectivity of the quantification process. This can be performed outside the chip, in laboratories using conventional methods such as sequencing and mass spectrometry. Therefore, in this section, we evaluate the literature on microfluidics for miRNA preparation and manipulation, including sequencing, polymerase chain reaction (PCR), other amplifications, and mass spectrometry detection.

3.1. Microfluidic devices to isolate miRNA for Sequencing & PCR

A successful miRNA quantification is highly affected by the isolation methods. Only a few studies have offered novel techniques for extracting miRNAs from biological samples. Most of them have benefited from existing commercial isolation technologies such as ultracentrifugation (UC), UC plus density gradient, qEV size-exclusion chromatography (Izon Science), and the exoEasy Maxi Kit (QIAGEN) [71]. These kits isolate miRNA by combining the sample lysis and silica-based spin-column technologies. These column-based separation methods are mainly suitable for isolating miRNAs in fully equipped labs with skilled personnel and are time-consuming, as they require multiple washing and spinning steps.

To overcome the limitations of such column-based protocols, microfluidic-based devices have become a promising alternative with the capability to improve the yield and purity of the extracted miRNA in a fast, portable, and automated set-up with higher efficiency while requiring less input fluid (Table 2). Generally, microfluidic-based devices can be used to separate EVs and /or exosomes. These cell-cell messengers reflect the state of the parent cell and therefore are commonly used in health assessments. This step could be based on the difference in size, immunoaffinity, and dynamic force separation methodologies. A nanoscale deterministic lateral displacement array (nanoDL), for instance, has demonstrated a superior ~50% yield for both serum and urine samples with the need for smaller sample volumes, up to ~3× higher concentration factor enhancement for both sample types, to ~60× for urine through adjusting the chip design to make the outlet channel width twelve times tighter [71]. Results revealed nanoDLD as a promising alternative for fast, reproducible, and automatable EV-isolation prior to a quantitative PCR. In another attempt, ultrasonic transducer coupled to microfluidic channel was used for EV isolation and processing. This automated acoustic EV trapping technique combined with an optimized RNA sequencing can help detect RNA markers in urine samples in an efficient and robust manner (Figure 5) [72].

The purity and recovery ratios of miRNAs and RNAs isolated from the circulating exosomes could be increased up to 90 % and 84 % respectively, using the charged-based separation microchips [73]. The main concept of such microfluidic devices is to apply positively charged chitosan-

bearing NH_3^+ to separate the negatively charged exosomes. The separated exosomes have to be treated with Trizol buffer before complete extraction of RNAs and miRNAs qPCR quantification. The yield ratio and purity of the isolated EVs could be enhanced by improving the selectivity of the separation system. Immunoaffinity-based microfluidic devices offer better selectivity via the recognition of specific antigens in the EVs. In this regard, conjugated antibodies facilitate the immobilization of the antibodies and release EVs in the microfluidic device. The HBEXO-Chip is an example of a device built based on this principle to isolate EVs in the plasma [74]. This tool was functionalized with anti-Glypican-1 antibodies, which resulted in specific capture of the exosomes through Glypican-1, a tumor-specific surface marker. The captured exosomes were then eluted with glycine-HCl buffer (pH 2.8) and lysed using Trizol as lysis buffer. The organic and the aqueous phases were then separated using chloroform. Trace levels of miRNA in the aqueous phase were finally precipitated using TAKARA precipitating aid. The expression level of the miRNAs was determined using the SYBR® Green method real-time PCR analysis. Compared with the gold standard, the device had the capability to increase the tumor-specific exosomes enrichment ratios by four times. Using this device, hsa-miR-214-3p and hsa-miR-125b-5p were shown to be highly expressed in pancreatic cancer.

Various strategies benefit from conjugated antibodies for the EV separation. Biotinylated antibodies, for instance, recognize common EV surface markers immobilized in a microfluidic system using desthiobiotin [75]. Such device has shown promising capability in capturing EVs from whole blood or cell culture samples. The captured EVs are then released from the microchip using excess amounts of biotin. This competition causes the release of the desthiobiotin-antibody–EV complex from the Neutravidin-coated surface, allowing for EV collection. The processing time was minimized to 1 hr, requiring sample volume as low as 1.2 ml of plasma or 10 ml of cell culture medium. RNAs and miRNAs were, then, extracted using a lysis reagent.

Conjugated antibodies can be also used to recognize EVs in the biological samples without being immobilized on the microfluidic channels [76]. In this regard, antibody-conjugated microbeads for EV recognition are infused in the microfluidic system with negative pressure application after being mixed with the sample. While being pulled through, the magnetically-labeled EVs are captured at the edge of the pores in the chip. EVs could be released for quantification using a

lysis reagent in less than 30 minutes. Although the Immunoaffinity-based microfluidic devices offer high selectivity in separation, their cost is higher compared to other microdevices. On the other hand, the extra step often needed to release the antibodies from the microfluidic chip may increase the separation time.

In all the above-mentioned devices, the process mainly focused on separating the EVs or exosomes from the biological fluids, which were then released from the chip and quantified by qPCR using a lysis buffer. This is while some studies have reported microdevices designed to directly capture miRNAs from biological samples using a simple lysis protocol. This is of great importance as such devices are easier to use because no sample post-separation treatment is required. In this type of separation, the internal surface of the microdevice is modified with positively charged 3-amino-propyl-triethoxysilane (APTES) alone or along with two different neutral poly(ethylene glycol) silanes (PEG-s) [77]. This surface was found to be capable of capturing miRNAs followed by reverse transcription. The resulting cDNA was collected and amplified via real-time PCR. Another study also showed improved performance of the same tool after optimizing the morphological and chemical properties of its surface [78]. The idea behind this modification was to make the surface more positively charged. The optimized device showed high selectivity and a sensitivity of about 0.01 pM in purifying both synthetic and natural circulating miRNAs.

Table 2- List of microfluidics-based devices utilized for isolating miRNAs from biofluid prior to PCR, their characteristics along with their pros and cons

Device	Isolated target	Sample	Separation technique	Recovery ratio	Post-isolation purification	Advantages	Disadvantages	Ref.
nanoDLD	EVs	Serum, urine	Size-based separation	50%	Required	✓ Low volume needed (µL range)	• miRNA extraction required • No-selectivity • Low recovery	[71]
Microchip	Exosomes	Blood	Chitosan electrostatic-adsorption	84%	Required	✓ Low volume needed (µL range) ✓ Up to 84% recovery ✓ No harsh buffers needed to elute exosomes	• miRNA extraction required • No-selectivity	[73]
HBEXO-Chip	Exosomes	Plasma	Immunoaffinity-based	NA	Required	✓ Highly selective	• Harsh buffers needed to elute exosomes • miRNA extraction required	[74]
Immunoaffinity - based microfluidic	EVs	Whole blood / cell culture	Immunoaffinity-based	NA	Required	✓ Highly selective	• Expensive (need for labelling) • miRNA extraction required	[75]
Microchip for TBI	EVs	Serum / plasma	Immunoaffinity-based	NA	Required	✓ Highly selective	• Expensive (need for labelling) • Complicated miRNA extraction protocol	[76]

Optimized microfluidic	VEs or exosomes	Plasma blood	/	Electrostatic-adsorption	NA	Required	✓ Able to quantify miRNA ✓ High sensitivity	• Pre qPCR reverse transcription required	[77], [78]

Table 3- List of microfluidics-based devices for miRNA amplification prior to PCR, their characteristics along with their pros and cons

Sample	Detection technique	Amplification	Pre-amplification purification	Advantages	Disadvantages	Ref.
Human breast cancer cell line MCF-7	Gel electrophoresis	ddPCR	Required	✓ Low volume needed (µL range)	• Low sensitivity (Gel detection)	[81]
	Fluorescent images	ddPCR	Required	✓ Low volume needed (µL range) ✓ Highly sensitive	• Pre-amplification required	[82]
GAPDH mRNA	Fluorescent images	ddPCR	Not required	✓ Low volume needed (nL range) ✓ Integrated device ✓ Suitable for single cell biomarkers ✓ High-Throughput	• Extended current throughput makes further scaling difficult.	[83]

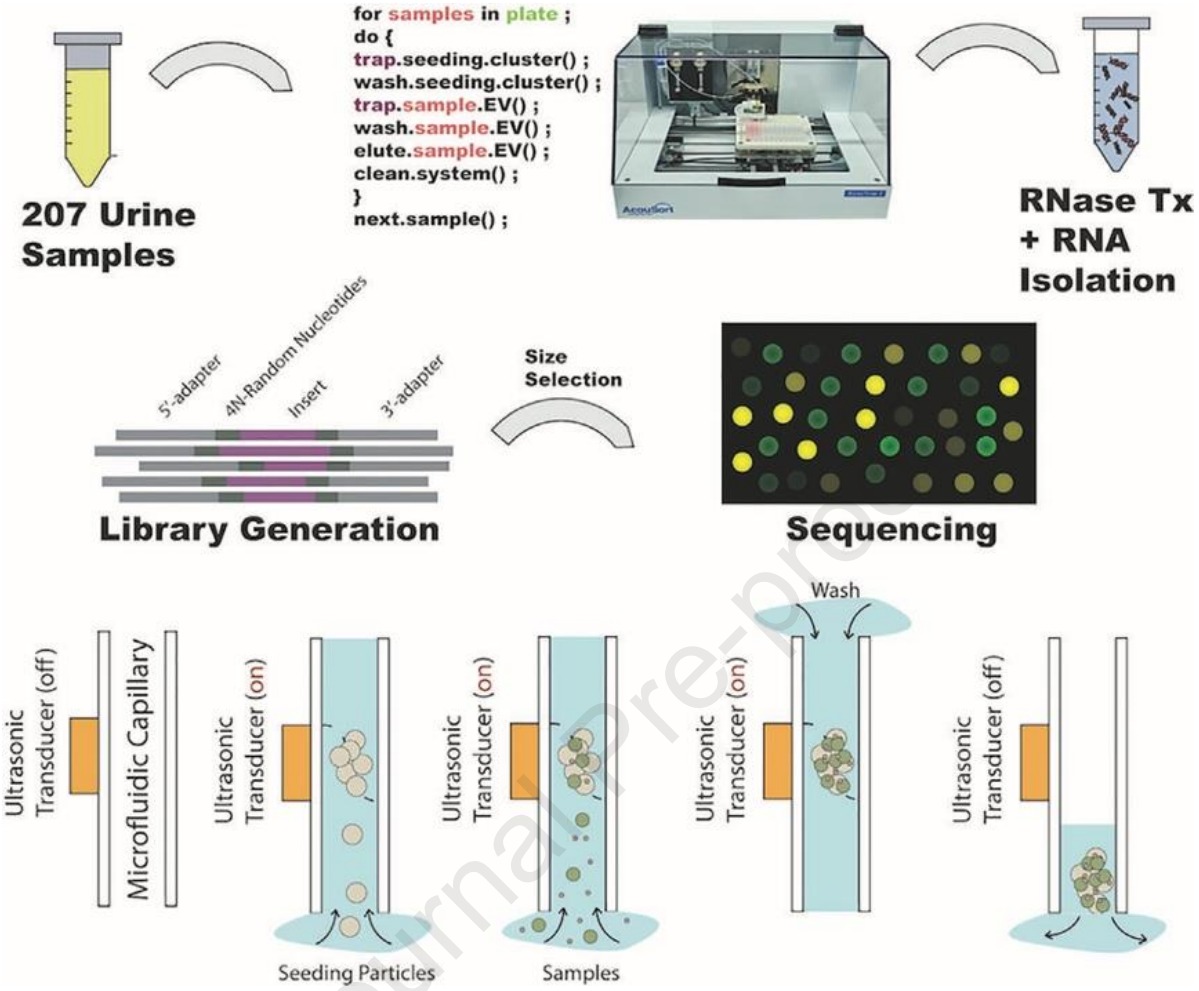

***Figure 5**. Automated microfluidic EV enrichment workflow for small RNA sequencing in urine. (A) Schematic of workflow beginning with urine sample randomization, automated EV isolation by AcouTrap, RNA isolation, small RNA library preparation and finally sequencing. (B) Illustration of acoustic trapping steps [72].*

3.2. Microfluidic devices to amplify miRNA

Droplet Digital PCR (ddPCR) is a recent method used for oligonucleotides amplification based on water-oil emulsion droplet technology. In this technique, a sample is fractionated into thousands of water-oil emulsion droplets, and then PCR amplification of the template oligonucleotides occurs in each individual droplet. Similar to traditional PCR, ddPCR technology utilizes primer or primer/probe assays, DNA template, and thermocycler. However, ddPCR has offered advantages to both traditional and qPCR, including higher sensitivity and absolute quantification without the

need for an external calibration curve [79]. Microfluidic PCR strategy can be miniaturized and portable, automatic, fast and high-throughput which are beneficial to be integrated into lab-on-chip devices for oligonucleotide detection [80]. In contrast, PCR-free strategies, in most cases, might suffer from lower sensitivity; this is while most of these devices are less-complicated and less-expensive, which is compatible with commercialization and easy-handling products [7, 15].

To benefit from ddPCR technology in miRNA amplification, several microfluidic devices have been developed (Table 3). It is noteworthy that 3D printing technology has facilitated the fabrication of microfluidic devices for droplet-based miRNA PCR [81]. To simplify the formation of the droplets inside such devices, a T-shaped design provides the needed two inlets and a single outlet. It also helps with the generation of the droplets via fluid shear stress. In this regard, one channel is used to infuse oil as the continuous phase, while the other is for the introduction of the PCR solution as the dispersed phase. The major benefit of this highly controllable and rapid thermal cycling is reduced turnaround time and energy consumption due to the small size of the particles and the fast thermal transfer. Despite the high efficiency of this microchip in miRNA amplification, a pre-amplified miRNA extraction step is still needed. Moreover, the detection step is normally performed outside the chip using gel electrophoresis. The PCR process was also reported to be more effective in commercial thermal cycler.

In another attempt to address these shortcomings, a microfluidic device for both amplification and detection of miRNAs was designed [82]. Soft lithography was used to fabricate the T-shaped microfluidic chip in polydimethylsiloxane (PDMS). Similar to the previous attempts, the chip consisted of two inlets for the PCR solution and oil phase, a reaction chamber, and an outlet for pumping. Droplets were formed through high shear forces, and then generated a monolayer in the reaction chamber for ddPCR reaction. Later on, the hydrolysis of fluorescent probes took place inside the droplets containing the miRNA RT products (cDNA templates). Fluorescent images of the droplets were obtained under the fluorescent microscope. This microfluidic chip platform was shown to be more sensitive and accurate compared with qPCR. Though the number of positive droplets observed in this device (10^5 to 10 copies/µl) was narrower than that of qPCR (10^6 to 10 copies/µl). Its main limitation, however, was the need to extract the miRNAs from the biological samples prior to the amplification.

The lap-on-chip concept has also been applied in the development of an integrated chip for high-throughput digital PCR (dPCR) analysis of single cells [83]. In this tool, the pre-amplification purification step was integrated into the PCR tool. It therefore allowed for parallel processing of single cells and executes, including cell capture, washing, lysis, reverse transcription, and dPCR analysis, in a single chip. After over 1200 single-cell measurements, the device was demonstrated to be highly sensitive and selective; the coefficient of variation (CV) at the single-cell level was as high as 40%.

3.3. Microfluidic devices for sample preparation before Mass Spectrometry analysis

Recently, isothermal signal amplification of miRNA, which benefits from enzyme rather than nucleic acid assisted miRNA target recycling using exonuclease (Exo) and duplex-specific nuclease (DSN), is becoming more and more common [84, 85]. Such step is generally coupled with various detection methods, namely chemiluminescence, fluorescence, surface-enhanced Raman spectroscopy, electrochemical, and magnetic relaxation switch or optomagnetic. The main shortcoming of most of these techniques is the need for labeled DNA probes, which are difficult to prepare, have poor stability, and high cost. Interference from biological sample matrices, producing inaccurate results, is also a common bias in many of these techniques.

Mass spectrometry (MS) is a powerful technique for chemical characterization and measurement of a wide range of molecular classes and sizes with high sensitivity and specificity [86]. This tool has also shown promising results in miRNA analysis [87, 88]. Quantitative assay of miRNAs with MS though suffers from two major disadvantages, as low sensitivity and need for extraction or chromatographic separation prior to MS analysis.

Microfluidic tools have shown high potential to improve nanoparticle and EV enrichment prior to MS, overcoming the need for UC, the current laborious and time-consuming gold standard [89]. These tools apply laminar flow profile and thus need smaller amounts of the sample, and report improved reproducibility of the results through reducing variations in the used rotors and protocols. Various technologies, ranging from nano-DLD [90], immunoaffinity-functionalized microstructures or beads [91, 92], dielectrophoresis (DEP) [93], viscoelastic separation [94], surface acoustic waves (SAW)[95], and acoustic trapping [96] are applied in such microfluidics,

each having their pros and cons. Some of these technologies are already explained in the PCR section above.

Acoustic trapping-based microfluidic tools are among promising alternatives for UC for isolating and enriching EVs. They have shown the significantly higher capacity and throughput, mainly because unlike UC, they are not limited to smaller samples with flow rates < 50 μL/min. The label- and contact-free multinode microfluidic tools benefit from a larger capillary, which has resulted in 40 times higher seed particle capacity by and 25−40 times higher throughput [89]. Using this tool, Broman et al. succeeded to isolate nanogram amounts of miRNA from acoustically trapped urinary EVs for downstream mass spectrometry analysis within 10 min. Vesicle coalescence and aggregation as well as co-precipitation of larger protein complexes, commonly reported with UC, was also not an issue in the novel technology. The main benefit of such tool, apart from the need for low volumes, is the short processing time, which is important for future automated biomarker profiling in clinical samples.

In another study, a simple but sensitive targeted miRNA assay based on the combination of cyclic enzymatic amplification (CEA) and microfluidic voltage-assisted liquid desorption electrospray ionization-tandem mass spectrometry (VAL-DESI-MS/MS) was developed [33]. In this work, the ssDNA probe was modified with a sequence complementary to the miRNA target, aiming to facilitate its preparation as well as improve its stability. The modified ssDNA probe formed a DNA-miRNA hybrid in the sample solution. Duplex-specific nuclease (DSN) was then used to specifically cleave the probe in heteroduplex strands. The repetition of the hybridization-cleavage cycles resulted in the production of a large quantity of CpC molecules that were later quantified by VAL-DESI-MS/MS with accuracy and specificity. miRNA-21, as a model target, was assessed in the 2.5 pM to 1.0 nM range with a limit of detection of 0.25 pM. Therefore, this tool can be used for accurate and cost-effective quantification of targeted miRNAs in biomedical samples.

4. Conclusions and future perspective

In this review, we discussed recent advancements in the microfluidic-based miRNA analytical devices along with their sample preparation and detection strategies. Despite the significant progress in the field, such devices still suffer from several shortcomings including low sensitivity,

high cost, and complexity. Amplification and, in general, microfluidic-compatible sample manipulation methods were described along with the use of nanomaterials in different forms, shapes, and combinations that can help enhance the sensitivity. This latter could be also enhanced through improvements on the device design. With the novel advances in the fabrication techniques such as 3D printing, the cost and time needed to prepare these chips can be reduced, while providing more flexibility in terms of the design of the channels and chambers. Multiplexed arrays are attractive for miRNA analysis, as several miRNAs need to be tested for the detection of the selected disease. Moreover, considering the growing demand for POC and in-home testing, portable and user-friendly miRNA devices are going to be a commercial reality. The advent of washable and reusable chips for miRNA analysis is another way to reduce the cost. Finally, mass production and marketing are still big challenges for microfluidics and LOC devices.

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Highlights

- ✓ Microfluidics devices are used for miRNA sample preparation and detection.
- ✓ Electrochemical, electrical, and optical biosensors combined with microfluidic tools
- ✓ Microfluidics for miRNA sample preparation, amplification, and other analysis

Declaration of interests

☑The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: