Microchimica Acta (2022) 189:444
https://doi.org/10.1007/s00604-022-05544-7

ORIGINAL PAPER

Glucometer-based electrochemical biosensor for determination of microRNA (let-7a) using magnetic-assisted extraction and supersandwich signal amplification

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Received: 22 April 2022 / Accepted: 17 October 2022

Abstract

A sensitive and portable biosensor is proposed for simple detection of microRNAs based on a supersandwich hybridization signal amplification strategy and a glucometer transducer. The presence of a target microRNA triggers the cascading hybridization chain reaction to create long supersandwich assemblies containing multiple biotin-labelled DNA probes. Then, large amounts of biotin-modified invertase signal molecules can attach to the supersandwich assemblies to generate an amplified signal for the glucometer readout. With such supersandwich format, a single target microRNA can introduce many biotin-invertase signal molecules, resulting in a one-to-multiple amplification effect. Thus, the accurate quantification of microRNAs can be achieved in a simple detection fashion without the requirement of expensive or precise instrumentation. The linear range of the biosensor for microRNA was from 0.05 to 100 nM with a detection limit of 48 pM. The proposed biosensor can discriminate the target microRNA from its family members with high selectivity and can be successfully applied to the detection of target microRNA spiked in serum samples with a good recovery (96.0–108.0%). Therefore, the proposed biosensor is expected to provide more information for early and accurate cancer diagnosis.

Keywords MicroRNA · Supersandwich hybridization · Portable glucometer · Electrochemical biosensor

Introduction

MicroRNAs are endogenous non-coding single-stranded RNA molecules with lengths of about 18–22 nucleotides, which are involved in the regulation of posttranscriptional gene expression [1]. Thus, microRNAs play crucial roles in several vital biological processes, including cellular proliferation, metabolism, differentiation, and apoptosis [2]. Many studies have demonstrated that the abnormal expression of microRNAs is closely related to various diseases, such as neurodegenerative diseases, autoimmune diseases, cardiovascular diseases, and especially cancers [3, 4]. Let-7 family is one of the most characterized microRNA families containing 12 members (from let-7a to let-7i), which was first discovered in *Caenorhabditis elegans* and acts as a time regulator of cell development [5]. Among them, let-7a is a crucial tumor suppressor, whose expression is found to be down-regulated in many cancers, such as cervical cancer, breast cancer, gastric cancer, and lung cancer [6, 7]. Thus, microRNAs are regarded as important cancer markers. Nevertheless, limited by their low abundance, small size, and high sequence homology between family members, the monitoring of microRNAs is challenging [8–11]. Therefore, it is meaningful to develop quantitative strategies to sensitively and selectively measure microRNAs for the early diagnosis and treatment of cancer [12–14].

A series of successful strategies, including northern blotting, flow cytometry, microarrays, and reverse transcription polymerase chain reaction, have been proposed for the detection of microRNAs [15–18]. However, the requirements of expensive instrumentation and professional operators limit their applications in the field or at home. Therefore, it is desirable to develop convenient and low-cost technology for microRNA monitoring that is directly usable by the public. Glucometers are portable devices for the point-of-care

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Published online: 11 November 2022

testing (POCT) of glucose levels. Motivated by the distinctive merits of glucometers including easy operation, low cost, and reliable quantitative results, Lu's group pioneered the use of glucometers for the detection of non-glucose targets based on a signal switching strategy [19]. Afterwards, glucometers were further extended to detect a series of non-glucose target analytes such as small molecules, metal ions, and proteins [20, 21]. In such attempts, the quantitative determination of non-glucose targets was realized by establishing a functional relationship between target recognition and glucose generation by rational design. The utility of a glucometer as a quantitative device to detect microRNAs greatly simplifies the experimental operation compared to traditional methods. However, the relatively low sensitivity of glucose meters hinders their application in microRNA detection. To overcome this defect, it is necessary to introduce efficient signal amplification strategies.

Signal amplification strategies, such as polymerase chain reaction, enzymatic strand displacement amplification, and rolling circle amplification, have been proposed for the amplified determination of microRNAs [22–26]. Although these amplification approaches provide high detection sensitivity, they require the participation of enzymes, which restrains the simplicity of assay protocols. Traditional sandwich hybridization assay is a particularly useful method for bioanalysis, which does not require the participation of enzymes and complex operations. However, in the traditional sandwich hybridization assay, a target DNA molecule hybridizes to a single copy of the signal probe, limiting the total signal gain and thus sensitivity. To overcome this obstacle, an enzyme-free signal amplification strategy, supersandwich assay, was proposed by Plaxco's group [27]. In this assay, a signal probe containing a signal label and a "sticky end" was designed. When a target DNA molecule hybridizes this signal probe, the sticky end remains free to hybridize another target leading to the creation of a supersandwich structure containing multiple signal probes. This assay thus achieves a large signal amplification compared to traditional sandwich assays without additional steps after hybridization. However, signal amplification is achieved by the hybridization between the signal probe and the target molecule with a 1:1 stoichiometric relationship, resulting in a one-to-one amplification effect, which compromises the signal amplification efficiency.

To improve the signal amplification efficiency, Wang's group proposed an ingenious design [28]. In their innovative supersandwich strategy, one target molecule triggered a cascade hybridization chain reaction, resulting in a one-to-multiple amplification effect. Moreover, the supersandwich assay method achieved satisfactory selectivity due to its high selectivity in base matching. Given the unique advantages of sensitivity and selectivity, the supersandwich strategy has been used for the detection of different kinds of targets such as nucleic acid, small molecules, proteins, cell, and microorganism [28–30]. For instance, Zhu group proposed a sensitive cytosensor for detection cancer cells based on the supersandwich signal amplification strategy [29]. Zhang group reported a supersandwich-based approach for sensitive detection of pathogens [30]. Inspired by the successful application of the supersandwich assay for the amplified detection of trace target molecules, the adoption of such a strategy is expected to achieve sensitive and selective detection of low abundance microRNAs.

In this work, a sensitive and portable biosensor for the simple detection of microRNAs was proposed based on the supersandwich hybridization signal amplification strategy coupled with the quantitative ability of a glucometer. The cancer-related microRNA, let-7a, was chosen as the model analyte. In the proposed strategy, a single target microRNA molecule could trigger the cascading hybridization chain reaction between two auxiliary DNA probes to create long supersandwich assemblies containing multiple biotin-labelled DNA probes. Subsequently, abundant biotin-invertase signal molecules were attached to the superhybridization products with the assistance of streptavidin via streptavidin–biotin specific binding. The attached invertase catalyzed the conversion of sucrose into glucose to generate an amplified signal, which was read out by a portable glucometer, leading to a one-to-multiple amplification effect. Thus, a sensitive and portable biosensor for the simple detection of microRNAs was developed and has potential application in practical cancer diagnostics.

Experimental

Materials and reagents

Carboxyl-functionalized magnetic nanoparticles (COOH-MNPs) were supplied by BaseLine ChromTech Research Centre (Tianjin, China). Bovine serum albumin (BSA), 2-(N-morpholino) ethanesulfonic acid (MES), 1-ethyl-3-(3-dimethylamino) propyl carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (sulfo-NHS) were provided by Sigma-Aldrich (St. Louis, USA). Sucrose, streptavidin (STV), and invertase (from baker's yeast) were purchased from J&K Scientific Ltd. (Beijing, China). Test strips and the glucometer (Contour^{TM}TS) were obtained from Bayer Healthcare LLC (Mishawaka, IN). The sulfo–NHS–LC-biotinylation kit was provided by Pierce Biotechnology (Rockford, IL, USA). Serum was provided by Beijing Jiehui Bogao Biotechnology Co., Ltd. (Beijing, China). Ultrapure water with the resistivity of 18.2 MΩ cm was used in the experiment. Oligonucleotides (shown in Table 1) were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Table 1 Oligonucleotides designed in the present study

Oligonucleotides	Sequence (from 5'to 3')
CP	CTA CTA CCT CAT TT-NH_2
Biotin-S1	TAT GAT GAA ACA ACT ATA CAA CTT T-Biotin
Biotin-S2	GTT TCA TCA TAG TTG TAT AGT TTT T-Biotin
Let-7a	UGA GGU AGU AGG UUG UAU AGU U
Let-7b	UGA GGU AGU AGG UUG UGU GGU U
Let-7c	UGA GGU AGU AGG UUG UAU GGU U
Let-7d	AGA GGU AGU AGG UUG CAU AGU U
Let-7e	UGA GGU AGG AGG UUG UAU AGU U
Let-7f	UGA GGU AGU AGA UUG UAU AGU U

Preparation of the biotinylated invertase (biotin-invertase)

Biotin-modified invertase (biotin-invertase) was prepared according to previous reports [31]. In brief, sulfo-NHS-LC-biotin (67 µL, 10 mM) was added to invertase solution (1 mL, 5 mg/mL) and reacted for 1 h so that the amines of invertase could be covalently cross-linked with NHS esters of sulfo-NHS-LC-biotin. After removing the excess biotin reagents using a desalting column, the biotin-invertase was stored for further use.

Preparation of CP-modified magnetic nanoparticles (MNPs-CP)

The COOH-MNPs were modified with CP using a carbodiimide-coupling strategy, which can cross-link the amines of the CP with the carboxy groups on the COOH-MNPs. Prior to use, the COOH-MNPs were then washed twice with PBS buffer. The COOH-MNPs (100 µL, 5 mg/mL) were incubated with EDC/NHS (400 mM/100 mM) in MES buffer (0.1 M, pH 5.9) for 30 min to activate the carboxyl groups. Following three washes with PBS buffer, CP (100 µL, 1 µM) was added to the activated COOH-MNPs. After 4 h of incubation at room temperature with continuous shaking, the CP-modified magnetic nanoparticles were separated using a magnet and washed thrice to remove un-bound CPs. The resultant MNPs-CP were stored in 1% BSA-PBS at 4 °C until use.

Amplified detection of let-7a using a glucometer

A total of 10 µL let-7a (various concentrations) was incubated with the prepared MNPs-CP (10 µL) for 1 h at room temperature. Then, biotin-S1 (10 µL, 1 µM) and biotin-S2 (10 µL, 1 µM) were added to the solution and reacted for 90 min at room temperature to form supersandwich self-assemblies. Next, the resultant products were sequentially incubated with STV (10 µL, 0.1 mg/mL) and biotin-invertase (10 µL, 0.5 mg/mL) for 30 min. After magnetic separation and washing, the products were incubated with sucrose solution (100 µL, 0.5 M) at 37 °C for 30 min to convert sucrose into glucose [32]. Finally, a commercialized glucometer was used to monitor the level of glucose.

Serum let-7a spike/recovery evaluation

Three different samples containing 0.5, 10, or 100 nM of let-7a spiked in serum were prepared according to previous literatures [33, 34]. The spiked samples were diluted tenfold with PBS buffer and measured by the proposed biosensor described above (amplified detection of let-7a using a glucometer). All experiments were performed in accordance with the relevant guidelines and regulations, and approved by the biomedical research ethics committee at Yunnan Normal University.

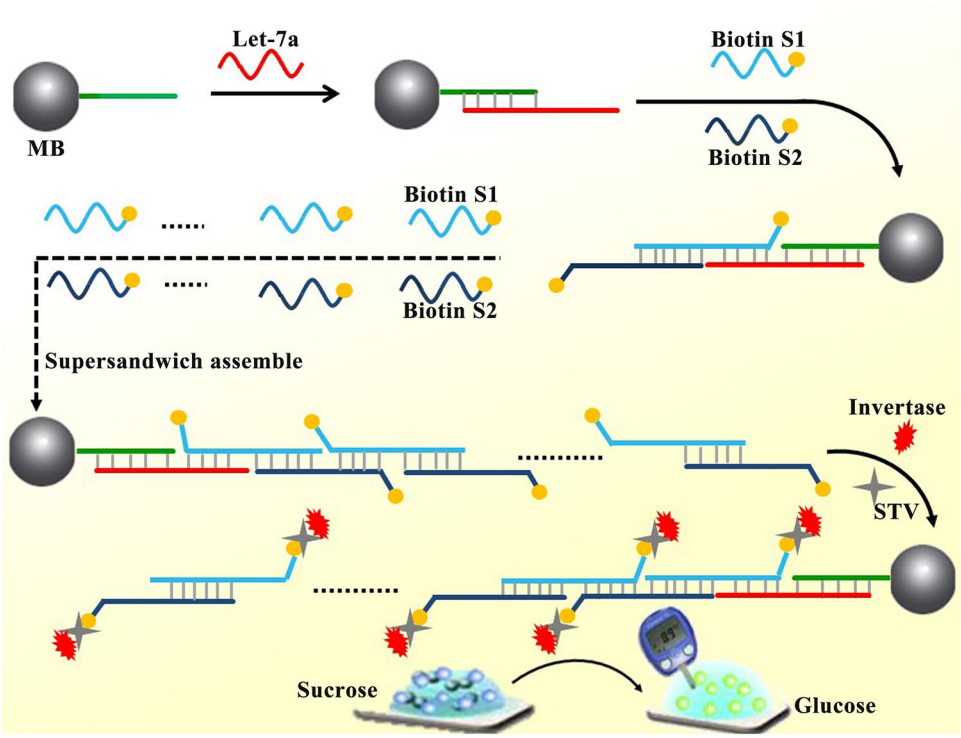
Native polyacrylamide gel electrophoresis (PAGE)

PAGE (16%) was used to assess the formation of the supersandwich structures. The assay was performed in 1 × TBE buffer at 120 V (constant voltage) for 90 min. After staining with Gel-Red for 30 min, the gels were photographed under UV light.

Results and discussion

Design principle of the glucometer-based biosensor for the detection of let-7a

The principle of the glucometer-based sensing strategy for simple detection of let-7a is shown in Scheme 1. Three oligonucleotides were designed for the sensing system, including CP, biotin-S1, and biotin-S2. CP and biotin-S1 could hybridize with partial sequences matching the target let-7a. Initially, amino-modified CPs were attached to the COOH-MNPs by cross-linking the amines of the CP and the carboxylic acid groups of the COOH-MNPs. In the presence of target, one terminus of target probe (let-7a) could hybridize to the CP immobilized on the MNPs, and the other terminus of let-7a could hybridize to biotin-S1. When biotin-S2 was added, the regions at the 3'-terminus of biotin-S2 could hybridize with the 3'-terminus of biotin-S1. The unhybridized 5'-terminus of biotin-S1 then further hybridizes with biotin-S2. The continuous hybridization between alternating biotin-S1 and biotin-S2 triggers cascade hybridization chain reaction, which leads to produce long supersandwich self-assemblies. The hybridization efficiency between S1 and S2 is 99%, which is analyzed by NUPACK. The supersandwich self-assemblies contained



Scheme 1 Schematic illustration of the glucometer-based biosensor for the detection of let-7a

multiple biotin-S1 and biotin-S2, which could introduce a large amount of biotin-invertase via specific interactions between biotin and STV. With the supersandwich format, a single target microRNA could introduce multiple biotin-invertase signalling molecules, resulting in a one-to-multiple amplification effect. The invertase-bound long supersandwiches were isolated and incubated with sucrose solution, which catalyzed the hydrolysis of sucrose to produce a large amount of glucose for quantitative readout by the glucometer. The signal from the glucometer (PGM signal) indirectly indicates the concentration of target let-7a. With increases in let-7a, an increasing amount of supersandwich self-assemblies are produced, so more biotin-invertase is introduced to hydrolyze sucrose into glucose, resulting in a higher reading on the glucometer. Thus, such a strategy allows let-7a to be sensitively detected by commercially available glucometer.

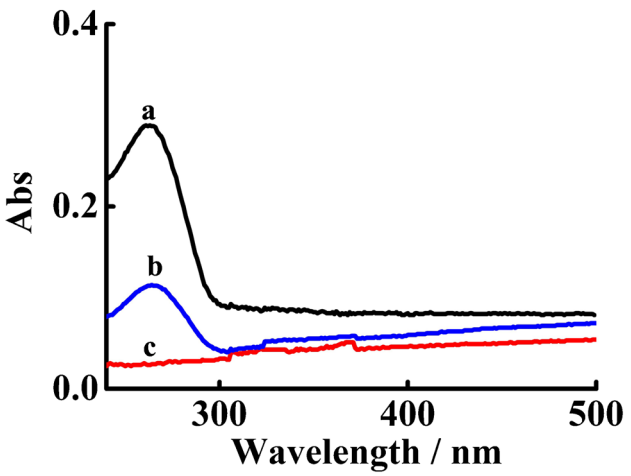


Fig. 1 UV–vis spectra of **a** CP (1 μM), **b** MNPs-CP (0.001 mg mL^{-1} MNPs incubated with 1 μM CP), and **c** MNPs (0.001 mg mL^{-1})

Characterization of MNPs-CP

The conjugation of CP onto MNPs-CP was validated using UV–vis absorption spectrum. As shown in Fig. 1, CP exhibited a characteristic absorption peak at 260 nm (curve a), while MNPs had no characteristic absorption peaks (curve c), which was consistent with the results reported in the literature [35]. When the MNPs were modified with CP, an obvious absorption peak at 260 nm was observed (curve b), which was attributed to the characteristic absorption of the oligonucleotide. Thus, those results demonstrated that CP was successfully conjugated onto the MNPs. The number of CP probes on per MNP is estimated to be 1500 copies, so the binding efficiency of CP probes to MNPs is estimated to be 42.8% (Fig. S1). Moreover, the conjugation of DNA probes onto MNPs and the formation of the supersandwich structures on MNPs were also further verified by dynamic light scattering (DLS) experiments and zeta potential experiments (Fig. S2).

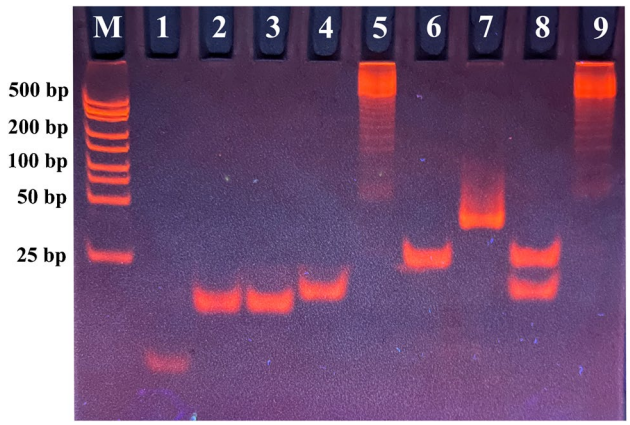


Fig. 2 Native PAGE of the supersandwich assembly. Lane M: DNA marker; lane 1: CP; lane 2: let-7a; lane 3: S1; lane 4: S2; lane 5: S1/S2; lane 6: CP/let-7a; lane 7: CP/let-7a/S1; lane 8: CP/let-7a/S2; lane 9: CP/let-7a/S1/S2. The concentrations of CP, let-7a, S1, and S2 were 2.5 μM, 2.5 μM, 5 μM, and 5 μM, respectively. The time of superhybridization chain reaction was 120 min and this experiment was conducted without MNP participation

Native PAGE analysis of the supersandwich assembly

The formation of the supersandwich structures was verified by native PAGE. As shown in Fig. 2, the distinct bands at different positions in lanes 1, 2, 3, and 4 correspond to CP, let-7a, S1, and S2, respectively. Lane 5 represented supersandwich products between S1 and S2. In contrast to lane 3 and lane 4, it is confirmed that S1 and S2 have been successfully hybridized and DNA supersandwich structures were obtained, which were consistent with reported literatures [27, 28]. Compared to the band for let-7a (lane 2), the incubation of CP with let-7a generated a higher molecular weight product (lane 6), indicating the successful hybridization of CP and let-7a. After adding S1 to the CP and let-7a mixture, another product of higher molecular weight was observed (lane 7), and was attributed to the hybridization between S1 and let-7a to generate a three-stranded DNA complex (CP/let-7a/S1). However, the addition of S2 to the CP and let-7a mixture yielded two distinct products (lane 8), corresponding to the CP/let-7a duplex (high molecular weight band) and S2 (low molecular weight band). The combination of CP, let-7a, S1, and S2 led to the generation of multiple products of successively increasing molecular weights (lane 9). The generation of such bands was due to the cascading effect of the DNA hybridization reaction, indicating the successful generation of DNA supersandwich structures.

Feasibility of the glucometer-based biosensor for the detection of let-7a

To explore the feasibility and supersandwich signal amplification of the glucometer-based strategy for the detection of let-7a, glucometer readings corresponding to different reaction systems were investigated. As shown in Fig. 3, in the absence of target let-7a, the incubation of MNPs-CP with biotin-S1 and biotin-S2 caused an insignificant glucometer reading (column a), which was considered the background signal due to the nonspecific adsorption of invertase. However, the addition of let-7a triggered the cascade hybridization chain reaction to form the concatenated supersandwich structure, which introduced a large amount of invertase that catalyzed sucrose hydrolysis. With the one-to-multiple signal amplification strategy, a considerable increase in the glucometer reading was observed (column b). Conversely, the traditional sandwich strategy (MNPs-CP incubated with let-7a and biotin-S1 without biotin-S2) produced a much lower signal increase due to its 1:1 ratio of CP to biotin-S1 (column c). The signal amplification efficiency of supersandwich self-assembly reaction was improved by about 2.2-times compared with the traditional sandwich strategy (column b vs. c). Furthermore, the incubation of MNPs-CP with let-7a and biotin-S2 but without biotin-S1 resulted in a small glucometer signal, which was similar to the background signal (column d). In that reaction, the long DNA concatamers could not be formed without biotin-S1, and thus, invertase was not introduced to catalyze sucrose hydrolysis. The above results demonstrate the feasibility and supersandwich signal amplification of the proposed strategy.

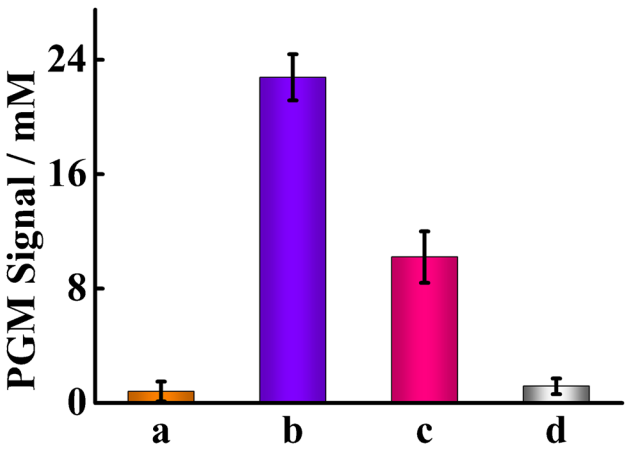


Fig. 3 Glucometer readings corresponding to different reaction systems: MNPs-CP incubated with biotin-S1 and biotin-S2 in **a** the absence of target let-7a and **b** the presence of target let-7a (10 nM), **c** MNPs-CP incubated with let-7a and biotin-S1 (without biotin-S2), and **d** MNPs-CP incubated with let-7a and biotin-S2 (without biotin-S1). Experiments were performed with the concentrations of biotin-S1 and biotin-S2 at 1 μM and the time of the superhybridization chain reaction at 120 min

Analytical performance of the glucometer-based biosensor for the detection of let-7a

To obtain the optimal analytical performance, the effect of the biotin-S1/biotin-S2 concentrations and the superhybridization time for the let-7a assay were investigated and the results are shown in Fig. S3. The analytical performance of the glucometer-based biosensor was explored in the optimal analytical performance. As shown in Fig. 4, the glucometer readings corresponding to let-7a had a good linear relationship with the logarithmic increase in its concentration from 0.05 to 100 nM. The regression equation was $y = 13.0 + 8.7 \lg c$ with an R^2 of 0.9972 (y and c represent the readings of the glucometer and concentrations of let-7a, respectively). The limit of let-7a detection calculated by the 3σ rule was 48 pM. The detection performances of the traditional sandwich strategy-based biosensor (without the addition of S2) have been further investigated. The glucometer reading linearly depends on the logarithm of let-7a concentration in the range from 0.5 to 500 nM (Fig. S4). The linear equation is $y = 6.2 + 4.2 \lg c$ ($R^2 = 0.9953$) and the detection limit is calculated to 153 pM. Thus, the supersandwich strategy-based biosensor shows improved sensitivity over the traditional sandwich strategy-based biosensor. Such a detection limit for microRNA is comparable with previous reports with complicated assay procedures (Table S1). However, considering the fact that microRNAs are often at very low concentration in blood, the sensitivity of the biosensor needs to be further improved to match the clinically relevant concentration ranges. In addition, the reproducibility of the assay was evaluated using six independent biosensors (six repetitive measurements) and a relative standard deviation (RSD) of 5.4% at 10 nM was obtained, which indicated satisfactory reproducibility of the biosensor.

The selectivity of the proposed strategy

The assay should be able to distinguish the target let-7a from other let-7 family members, which have high sequence similarity. Thus, the selectivity of the constructed biosensor was investigated. As shown in Fig. 5, compared to the blank, increases in glucometer signals for nonspecific microRNAs (let-7b, let-7c, let-7d, let-7e, and let-7f) were weak, while a strong signal was generated by the target let-7a, which was due to the perfect hybridization between probes. Moreover, the signal for a mixture of let-7 family members (let-7a, let-7b, let-7c, let-7d, let-7e, and let-7f) was similar to that of let-7a. Those results demonstrate the capability of the biosensor to discriminate between perfectly complementary target microRNAs and mismatched microRNAs, suggesting the high selectivity of the biosensor to let-7a.

Serum let-7a spike/recovery evaluation

The potential of the biosensor to monitor let-7a in complex samples was explored by detection let-7a spiked in serums. Three different concentrations of let-7a (0.5, 10, and 100 nM) were separately spiked into serums. The spiked samples were diluted tenfold with PBS buffer and assessed using the proposed strategy. As shown in Table 2,

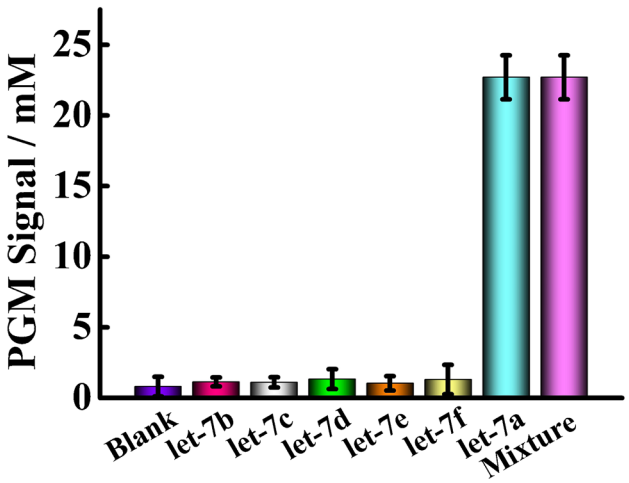


Fig. 5 Glucometer readings for blank, let-7b, let-7c, let-7d, let-7e, let-7f, let-7a, and the mixture of the above microRNAs. The concentrations of all microRNAs were 10 nM

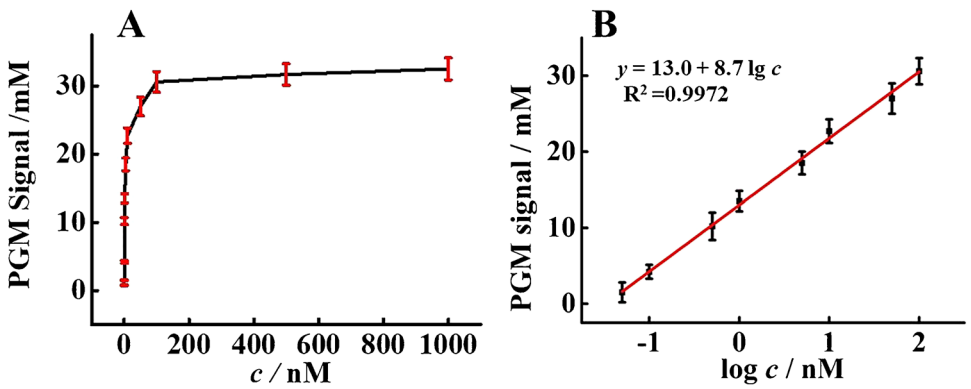


Fig. 4 **A** The plot of glucometer reading vs. concentration of let-7a, the concentrations of let-7a were 0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 nM. **B** The calibration plot of glucometer reading vs. logarithmic concentration of let-7a in the range of 0.05 to 100 nM. Error bars, SD, $n = 3$. The PGM signal for let-7a at 0 nM was 0.8 mM, which was considered the background signal

Table 2 Recoveries of the proposed biosensor for let-7a spiked in serum ($n = 3$)

Samples	Added (nM)	Found[a] (nM)	Recovery (%)	RSD (%)	Found by reference method (nM)	Ref
1	0.5	0.48	96.0	4.2	0.48	[33]
2	10	9.7	97.0	2.7	9.8	[34]
3	100	108.00	108.0	4.8	110	[34]

[a]The average value of the three measurements

the recoveries of let-7a were in the range of 96.0–108.0% with the RSD of 2.7–4.8%, which were well consistent with the results reported in the literature [33, 34]. The recovery results were quite satisfactory considering the complexity of the spiked serum samples. All of these results fully demonstrated the capability of the proposed biosensor for accurate quantification of let-7a in complex biological samples.

Conclusion

In this work, a portable and sensitive biosensor has been proposed for the simple detection of trace microRNAs based on the supersandwich signal amplification approach and a glucometer transducer. The presence of a single target microRNA molecule can trigger cascading hybridization chain reaction to produce a supersandwich structure containing multiple signal probes, leading to a one-to-multiple signal amplification effect. The amplified signal can be detected by a portable glucometer compared with other microRNA detection methods that require expensive instrumentation and professional operators. Using glucometer as quantitative device to detect microRNAs greatly simplifies the experimental operation. And the estimated cost of the proposed glucometer-based biosensor for microRNA testing was approximately 0.5325 dollars (Table S2), thus providing a cost-effective platform to conduct high-performance POCT. Given the distinctive merits of glucometer endows the glucometer-based biosensor with attractive advantages in terms of low cost, easy operation, rapid response, and reliable quantitative results, which will significantly promote the development of the facile detection platform for microRNA. However, the intrinsic detection range of glucometer and the steric hindrance effect of MNPs limit the total signal gain and thus sensitivity. Future work focused on the development of more efficient signal amplification strategies and signal readout devices may potentially make this biosensor a more sensitive and versatile platform for detecting various microRNA biomarkers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00604-022-05544-7.

Author contribution LW: conceptualization, investigation, formal analysis, writing—original draft preparation.
TS: investigation, formal analysis.
LP: investigation, formal analysis.
JZ: software, validation.
RH: investigation, formal analysis, visualization.
YY: conceptualization, supervision.
JY: writing—reviewing and editing, supervision, funding acquisition.
YZ: writing—reviewing and editing, supervision, funding acquisition.

Funding This work was supported by the National Natural Science Foundation of China (No. 21961046, No. 21362046, and No. 21062030), Yunnan Fundamental Research Projects (grant NO. 202201AU070056), Scientific Research Foundation Project of Yunnan Provincial Department of Education (No. 2021J0436), and PhD Scientific Research Foundation of Yunnan Normal University (No. 2020ZB009), College students' Innovative Entrepreneurial Training plan program of Yunnan Province (No. S202110681054), Graduate Students' Scientific Research Innovation Foundation of Yunnan Normal University (No. YJSJJ22-B64).

Declarations

Conflict of interest The authors declare no competing interests.

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