



Proximity sensitive detection of microRNAs using electrochemical impedance spectroscopy biosensors

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ABSTRACT

This study presents a new strategy and level of mechanistic understanding for ultrasensitive detection of short, non-coding RNAs without target amplification or chemical modification using electrochemical biosensors. Electrochemical impedance spectroscopy (EIS) has been used for probe target interaction detection because of its high utility for sensitive and label-free measurements of the nucleic acid targets as a result of hybridisation. EIS measurements of different probe target combinations in a range of spatial orientations and sequence overlaps showed that bringing the target overhangs closer to the nanometer proximity of the electrode surface improved the EIS signal significantly. Systematic investigations using different lengths of overhangs towards the electrode surface revealed proportionally higher EIS signals with increasing lengths of the overhangs. Our observations could be explained using the Poisson-Boltzmann and Gouy-Chapman model and followed our experimental modelling. In conclusion, the optimized arrangements of our EIS biosensor system enabled us to detect microRNA-122, a known biomarker for liver injury, as well as three common isoforms to a 1 nM (equivalent to 80 fmole) detection limit. This will enable us to develop solutions for the detection of this important blood biomarker at point of care.

1. Introduction

MicroRNAs (miRNAs) are short, 18–25 nucleotides long, single-stranded non-coding RNAs that can regulate gene expression during translational or post-translational modifications (Ambros, 2004). Circulating and exosomal miRNAs are known biomarkers for a wide range of disorders and pathological conditions including sepsis (Wang et al., 2010), drug-induced liver injury (López-Longarela et al., 2020; Starkey Lewis et al., 2011), autoimmune diseases (Nakasa et al., 2008), coronary artery disease (Fichtlscherer et al., 2010), acute myocardial infarction (D'Alessandra et al., 2010), diabetes (Guay and Regazzi, 2013; Joglekar et al., 2016) and cancers (Chen et al., 2008; Heneghan et al., 2010; Mitchell et al., 2008). To use miRNAs as diagnostic biomarkers, it is important to rapidly, sensitively and specifically determine the abundance of these RNA molecules in a clinical context. However, the accurate measurement of circulating miRNAs remains highly challenging due to their short highly homologous sequences (Leshkowitz et al., 2013), and often very low expression levels.

Presently, conventional methods of miRNA detection include

northern blotting (Válczi et al., 2004), quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Chen et al., 2008), microarrays (Li and Ruan, 2009) and sequencing (Hu et al., 2017). Each of these strategies, however, has its own individual limitations. While, qRT-PCR is favourable for high sensitivity and good detection limit and the microarray technique is suitable for highly parallel detection of miRNAs, e.g. genome-wide expression profiles, these methods are complicated by limitations such as complex workflow, high instrumental and energy requirements, cross-hybridisations, error-prone amplification and lack of robust controls (Xi et al., 2016). For northern blotting, the complexity of using radiolabelling makes the detection process expensive and troublesome. Most importantly, these methods are difficult to implement in a point-of-care setting. Alternative methods need to be developed with a special emphasis on low complexity and ease of use while maintaining high sensitivity and specificity. To that end, electrochemical techniques have proven advantageous in terms of rapid detection, simplicity and ease in miniaturization (Roychoudhury et al., 2016). The simple operation based on oxidation and reduction of redox species at electrodes make

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electrochemical techniques convenient for easy integration and real time detection (Roychoudhury et al., 2017). Further combinations of DNA modifying enzymes, such as the recently found clustered regularly interspaced short palindromic repeats (CRISPR)/Cas technology, can improve stability and selectivity during electrochemical assay development (Dronina et al., 2021). Designing target-specific CRISPR RNA improves specificity during miRNA detection, while DNA modifying enzymes can do masking of active sites and protect RNA from RNase activity, temperature differences and other environmental factors (Bruch et al., 2019). Electrochemical impedance spectroscopy (EIS) offers a range of advantages over other electrochemical methods (see Table S11) and allows highly sensitive and label-free measurements of the targets during nucleic acid hybridisation events (Corrigan et al., 2012). In addition to the study of biorecognition events on the electrode surface, EIS can be effectively used to characterize the electrode surface modifications, particularly for functionalisation with molecular probes (Lisdatt and Schäfer, 2008). In general, after immobilizing probe strands on the electrode surface through self-assembly, the sequence-specific detection of miRNA is performed using the EIS. The probes are, however, immobilised in a mixed monolayer consisting of one or more short thiolated molecules, the diluent(s), to enhance the efficiency during the detection of miRNA. The diluents typically help to control the surface density of the probes on the electrode surface, maximizing the number of probes for target binding and prevent non-specific adsorptions and steric hindrances at the electrode surface (Corrigan et al., 2012; Levicky et al., 1998). Following the hybridisation of target miRNA with the probe, there is an increase in negative charge on the electrode surface. This accumulation of negative charge on the surface of the electrode upon target hybridisation causes an increase in charge transfer resistance (R_{ct}) for negatively charged potassium ferri/ferrocyanide redox couple in a faradaic EIS measurement. The increase in R_{ct} is proportional to miRNA target hybridisation. The performance of these EIS-based biosensors for nucleic acid hybridisation is enhanced by using uncharged peptide nucleic acid (PNA) probe molecules (Keighley et al., 2008a). Instead of gradually increasing the charge, hybridisation of PNA probes with the target results in a shift from an uncharged layer to a charged layer and thus a better resolution in the resulting R_{ct} signal upon hybridisation. In addition, because of the absence of electrostatic repulsion between probe molecules, the uncharged PNA probes are more suitable for uniform distribution on the electrode surface as compared to the negatively charged DNA/RNA probes. The optimal probe density on the electrode surface is a crucial factor among various other interfacial parameters such as probe orientation, as the highest sensor signal is typically obtained when the maximum number of probes for target hybridisation are available. In addition, factors such as probe immobilisation chemistry, sensor surface homogeneity, surface passivation and self-assembled monolayer (SAM) organization and defects are likely to be important (Keighley et al. 2008a, 2008b). Altering fundamental parameters in hybridisation events by changing probe length, orientation of probe binding on electrode surface, target length and by creating of overhang sequences at both end of the probe can provide significant changes in the EIS signal and this may lead to the improved detection methodology in miRNA sensing. Most of the previous EIS-based studies mainly focused on SAM layer organisation (Cui et al., 2017; Park et al., 2008; Tamayo et al., 2020), probe immobilisation methods (Li et al., 2021; Peng et al., 2007), probe types (Keighley et al. 2008a, 2008b; Wang et al., 2008), surface probe density (Brothers et al., 2020; Keighley et al. 2008a, 2008b) and surface materials (Chen et al., 2009; Nguy et al., 2017) to improve nucleic acid hybridisation based sensor performance. However, a systematic study is still required on the mechanistic behaviour of the probe-target complex and the effect of its location on the electrode surface in miRNA sensing for elevated impedance responses.

In the following study, we aimed to better understand the parameters influencing the performance of sequence specific miRNA electrochemical sensing. The chosen assay works without any target

amplification, post-affinity attachment or complex chemical modification steps. To do this, we explored the mechanistic behaviour of probe-target binding by altering the fundamental parameters of probe length and orientation of probe immobilisation on screen-printed gold electrodes. Specifically, we created probe target combinations with varying positions and degrees of base overhang of the target miRNA towards the electrode interface or at the reverse end.

For our study, we chose microRNA 122 (miR-122, 22 nt length) as the clinically relevant target and designed four different PNA probes and six different probe-immobilisation directions. miR-122 has been identified as a stable and specific biomarker for early diagnosis of drug-induced liver injury (DILI) across species (López-Longarela et al., 2020; Starkey Lewis et al., 2011). The performance of each combination was tested in hybridisation experiments and faradaic EIS readout. In addition, with the obtained EIS signals, we demonstrated the effect of variable overhangs with different lengths on the electrode surface. We further used the optimized probe condition for miR-122 detection at various concentrations in the nanomolar range. Finally, the optimized probe condition was also utilized to determine three common miR-122 isomiR sequences at several nanomolar concentrations.

2. Experimental

2.1. Reagents and chemicals

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl), potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], potassium ferrocyanide [$\text{K}_4\text{Fe}(\text{CN})_6$], sulfuric acid (H_2SO_4), dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Gillingham, UK). 6-mercapto-1-hexanol (MCH) and 1,6-hexanedithiol (HDT) were bought from ProChimia Surfaces (Gdynia, Poland). All of the other chemicals were of analytical grade unless otherwise stated. All of the aqueous solutions were made with deionized water (resistivity $>18 \text{ M}\Omega \text{ cm}$) from a Millipore MilliQ water purification system (Bedford, MA, USA).

2.2. Probes and targets

The peptide nucleic acid (PNA) single-stranded probes were ordered via Cambridge Research Biochemicals (Cleveland, UK) and obtained from Panagene (Daejeon, South Korea). PNA probes ($>95\%$ HPLC purified) were modified with a spacer comprising of three ethylene glycol units (abbreviated as AEEEA) and a terminal thiol group either at N-end (equivalent to 5'-end of DNA) or at C-end (equivalent to 3'-end of DNA) for selective binding on the gold substrates via self-assembled monolayers (SAMs) formation. In the following experiments, four short PNA probes (P-S1₁₀, P-S2₁₀, P-S3₁₀ and P-S4₁₀) of 10 nt length and one full-length probe (P-FL₂₂) of 22 nt length were used for subsequent hybridisation of the RNA targets. P-FL₂₂ probe was made with terminal thiol group at the N-end and the sequence of P-FL₂₂ full-length probe (fully complementary with miR-122 target) was utilized to create four other short probes; P-S1₁₀, P-S2₁₀, P-S3₁₀ and P-S4₁₀ respectively. The P-S1₁₀ probe was made with first 10 bases of P-FL₂₂ probe and N-end terminal thiol modification, while P-S2₁₀ probe was comprised with the last 10 bases of P-FL₂₂ probe and C-end terminal thiol modification for creating 12 nt overhang sequence at the solution end (opposite to the electrode surface) for both the cases during miR-122 (T-FL₂₂) target binding. The P-S3₁₀ probe had the same sequence of P-S2₁₀ (last 10 bases of P-FL₂₂); however the terminal thiol group was placed at the N-end to create the 12 nt overhang target sequence on the electrode interface during target hybridisation. Similarly, the P-S4₁₀ probe was made with the first 10 bases of P-FL₂₂ (same as P-S1₁₀ sequence) and C-end terminal thiol modification for generating 12 nt overhang sequence towards the electrode surface after target hybridisation. All of the probe stock solutions were prepared with 50% (v/v) dimethylformamide

(DMF) aqueous solution and kept at -20°C when not in use. The lyophilized RNA target sequences were purchased from Metabion (Martinsried, Germany). The stock solutions of the RNA targets were made in nuclease-free deionized (DI) water and kept in -80°C during storing. The sequence and structural details of PNA probes and RNA targets are given in Table S1 of the Supporting Information. The concentration of stock solution of the RNA targets were further verified by spectrophotometric analysis (Nanodrop 1000 spectrophotometer; Thermo Scientific) and the measurement details are mentioned in Table S2.

2.3. Electrode preparation

The screen-printed gold electrodes (DRP-C223BT) with gold working track (area 2.01 mm^2 /diameter 1.6 mm), reference silver track and gold counter track, were purchased from DropSens (Oviedo, Spain) and used for electrochemical impedance measurements after probe immobilisation. First, the silver reference track was modified into Ag/AgCl after treating with 50 mM FeCl_3 solution for 30 s followed by rinsing with DI water. Next, the electrodes were cleaned electrochemically by performing cyclic voltammetry in the potential range of 0 – 1.6 V at 100 mV/s scan rate for 10 cycles in presence of 100 mM sulfuric acid solution. The PNA probe solution of particular concentration was prepared with a mixed solution of $100\text{ }\mu\text{M}$ MCH and $200\text{ }\mu\text{M}$ HDT as diluents and 5 mM TCEP as reducing reagent for one-step co-immobilisation and to form ternary MCH/HDT/PNA probe layer. For such purposes, $4.5\text{ }\mu\text{L}$ of mixed probe solution was placed on a cleaned gold working electrode and kept in humid chamber for 16 h for probe immobilisation followed by blocking with 1 mM MCH solution for 2 h to prevent non-specific adsorptions. Later, the electrodes were sequentially rinsed with 50% (v/v) DMSO aqueous solution and DI water and then used for successive electrochemical measurements.

2.4. Electrochemical characterisation and impedance measurements

Functionalised electrodes were characterised using electrochemical impedance spectroscopy (EIS) and the measurements were taken by using an Autolab PGSTAT128N potentiostat/galvanostat electrochemical system (Metrohm Autolab, Utrecht, Netherlands). The EIS measurements were performed using EIS measurement buffer (10 mM sodium phosphate, $\text{pH } 7$, 20 mM NaCl and 0.2 mM potassium ferri/ferrocyanide redox mediator) and each impedance spectrum was recorded in a frequency range of 0.3 Hz – 100 kHz with an AC signal of 10 mV rms amplitude at the measured open circuit potential. All the measurements were conducted after placing the functionalised electrodes inside a measurement cell (MiniFAB, Melbourne, Australia) which can retain a sample volume of $80\text{ }\mu\text{L}$. A multiplexing module was used to connect the measurement cells to the potentiostat, and the multiple measurements were taken in order. The charge transfer resistance (Rct) values were collected from the equivalent Randles' circuit after fitting the data from the respective Nyquist plots in a faradaic EIS measurement. A constant phase element (as non-ideal capacitance) instead of the double layer capacitance (Cdl) in parallel with Rct and Warburg element was used during the design of the Randles' equivalent circuit. The EIS measurements were taken pre and post hybridisation with a 35 or 60 min sample incubation using the probe-functionalised electrodes and the enhancement in Rct value (ΔRct) from pre (baseline measurement) to post (sample measurement) hybridisation was used to plot the impedance data. All the electrochemical impedance studies were performed at least in triplicates ($n \geq 3$) under identical conditions unless otherwise stated.

3. Results and discussion

3.1. Influence of probe length and probe combinations on EIS signal

To understand the impact of the target orientation and positioning with regards to the electrode surface, we analysed probes of differing lengths hybridising with the miR-122 target (T-FL₂₂) and their influence on the EIS signal (Fig. 1). Specifically, we determined the impact of probe length, probe combinations and probe orientation on the electrode surface on the biosensor signal. A probe-to-probe performance comparison was carried through dual combinations of four different PNA probes (P-FL₂₂, P-S1₁₀, P-S2₁₀ and P-S3₁₀) and two opposite orientation probe-immobilisations. Scheme 1 schematically shows the different probes with their orientation of immobilisation on the electrode substrates and after binding with miR-122 target through hybridisation. Hybridising the P-S1₁₀ and P-S2₁₀ short probes with the miR-122 target created 12 nt overhang stretches of the target RNA at the solution end of the probe, whereas the P-S3₁₀ probe produced 12 nt overhangs at the electrode end of the probe. Along with the analysis of four different probes individually, two other combinations of probes were studied as co-immobilisation of two short probes either in opposite (P-S1₁₀ and P-S2₁₀) or same (P-S1₁₀ and P-S3₁₀) orientation.

In these experiments, we kept the total probe concentration in the immobilisation solution at 1.5 , 6 or $9\text{ }\mu\text{M}$. This was achieved by either using the total concentration for individually immobilised probes or half of the total concentration for each probe in the combined immobilisations (P-S1₁₀+P-S2₁₀, P-S1₁₀+P-S3₁₀). The impedance changes were determined by quantifying the increase in charge transfer resistance (ΔRct) (for overlay of Nyquist plots see Figs. 1A and S3, and Bode plots see Fig. S4). All short probes, individually or in combinations, showed either equivalent or lower responses compared to the full-length probe (P-FL₂₂) except P-S3₁₀ on its own. The results obtained with the P-S3₁₀ probe demonstrated a significant rise in ΔRct signal when compared to P-FL₂₂ probe and all other individual short probes and probe combinations (for p -values see Table S3). These findings suggest that target molecules far away from the electrode, i.e. the overhang stretch on the solution side, made a lesser impact on the EIS signal. Interestingly, when two short PNA probes (P-S1₁₀+P-S2₁₀ and P-S1₁₀+P-S3₁₀) were co-immobilised, they behaved like the full-length probe. This was in contrast to the expectation from the anticipated hybridisation structures (see Scheme 1) with enhanced surface proximity of the RNA target as compared to the individual probes. A reason for this behaviour could lie in a reduced accessibility of the probes for a target leading to less miR-122 bound and thus less ΔRct signal generated. In contrast, the short probe P-S3₁₀ showed almost a double EIS signal compared to the full-length probe (P-FL₂₂). We hypothesize that the 12 nt overhang stretch of the target RNA from the probe onto the electrode surface increased the repulsive electrostatic barrier at the electrode and thus enhanced the ΔRct signal during EIS measurements, acting almost like an electrostatic label in the faradaic EIS measurements with the negatively charged redox couple. Furthermore, we observed a steady EIS signal increase from 1.5 to $9\text{ }\mu\text{M}$ concentration for P-S3₁₀ probe (for p -values see Table S4). This finding implies that the density of overhangs on electrode surface may have an impact on target binding and the resulting EIS signals.

A previous study showed similar for much larger DNA target hybridisation to DNA probes but a theoretical consideration was missing (Riedel et al., 2014). Riedel and colleagues found that higher surface concentrations of probe-target complex mainly contributed to increasing EIS responses rather than creating longer overhangs on the solution side. As a conclusion, our observations suggest that creating overhang and negative charges in a larger distance from the electrode surface have lower influence on EIS signal for negatively charged redox couples. In contrast, overhangs close to the electrode surface are limiting the access for negatively charged redox couple during EIS measurements and thus resulting in higher ΔRct signals.

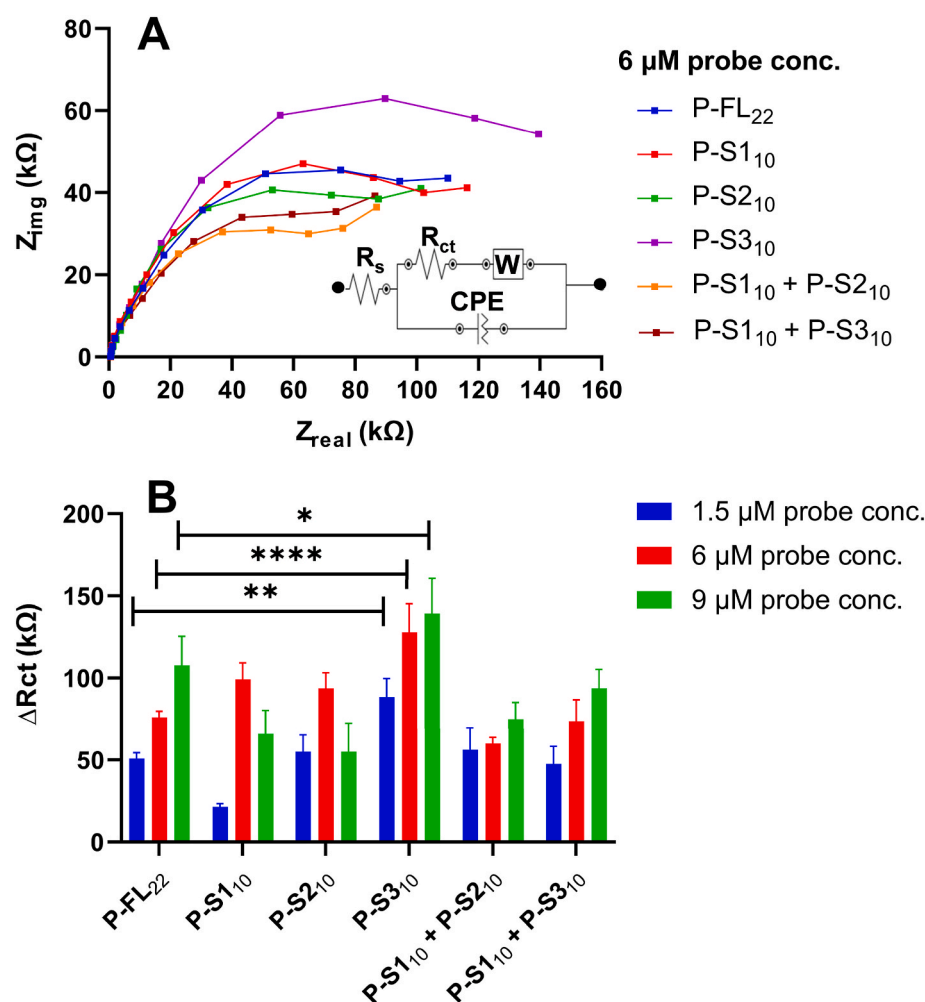
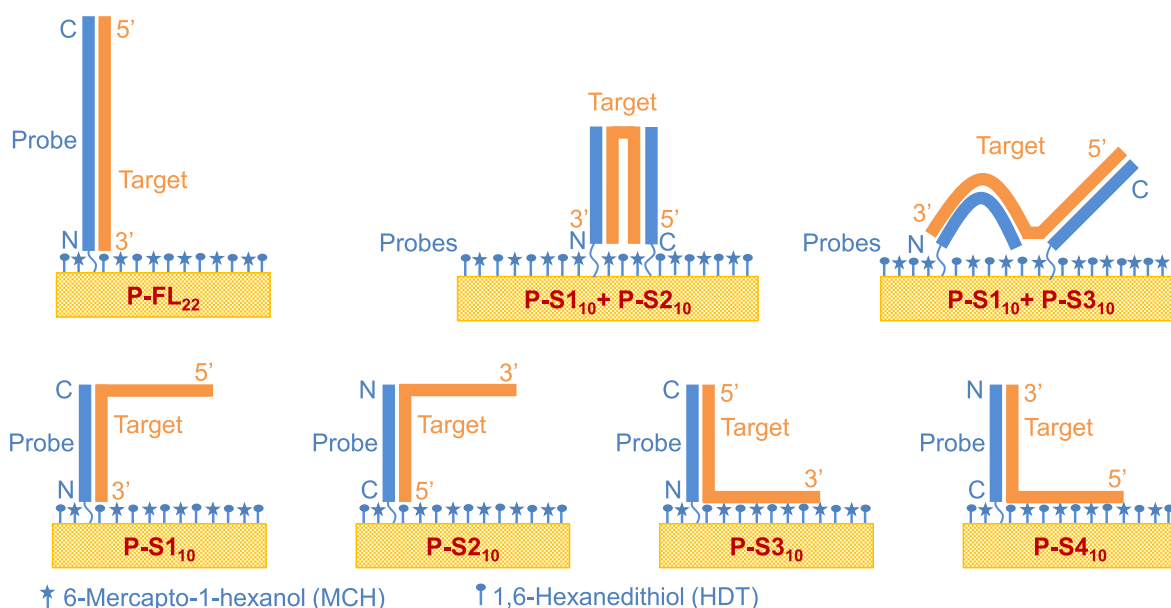


Fig. 1. -Probe to Probe Comparison. (A) EIS spectra (Nyquist plots) of full-length probe (P-FL₂₂), short probes (P-S1₁₀, P-S2₁₀, P-S3₁₀), and short probe combinations (P-S1₁₀+P-S2₁₀, P-S1₁₀+P-S3₁₀) at 6 μM probe concentration after 35 min incubation with 50 nM miR-122 target in EIS buffer, inset shows equivalent Randles' circuit with R_s (solution resistance), R_{ct} (charge transfer resistance), W (Warburg element) and CPE (constant phase element) for fitting Nyquist plots; (B) EIS signals (ΔR_{ct}) of electrodes functionalised with 1.5 or 6 or 9 μM of either full-length probe (P-FL₂₂), short probes (P-S1₁₀, P-S2₁₀ or P-S3₁₀), or short probe combinations (P-S1₁₀+P-S2₁₀, P-S1₁₀+P-S3₁₀) after 35 min incubation with 50 nM miR-122 target. Data represent the mean $\pm$ SD; $n \geq 3$. Statistical significance has been determined by 2way ANOVA test (significance codes: **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.05$).



Scheme 1. Schematic of the proposed structural arrangement of the full-length probe (P-FL₂₂) and four short probes (P-S1₁₀, P-S2₁₀, P-S3₁₀ and P-S4₁₀) and combinations of two short probes (P-S1₁₀+P-S2₁₀ and P-S1₁₀+P-S3₁₀) showing their orientation of immobilisation on electrode surface and positioning after hybridisation to the same target (miR-122) for all. PNA terminal N and C ends are equivalent to DNA/RNA terminal 5' and 3' ends, respectively.

3.2. Influence of probe concentrations on the EIS signal

We further explored the impact of overhang stretch at close proximity to the electrode surface with resulting EIS signals by varying the concentrations of the P-S3₁₀ probe over a greater concentration range. The surface density of probes on gold substrates has a key influence on the sensor performance, i.e. for sensitive detection of the targets (Keighley et al. 2008a, 2008b). Accordingly, we hypothesized that the assay sensitivity would be critically dependent on the probe density and resulting presence of overhanging target RNA. For the protocol of probe immobilisation through self-assembly of thiolated PNA probes onto gold surfaces, we used 6-mercapto-1-hexanol (MCH) and 1,6-hexanedithiol (HDT) as two additional thiolated molecules and diluents to form a ternary MCH/HDT/PNA probe layer. The role of the diluents was to maximize the number of probes accessible for target binding by preventing non-specific interaction and steric hindrance at the electrode surface (Levicky et al., 1998; Movilli et al., 2018). We varied the probe density on electrode surface by changing the probe and diluent concentrations in the immobilisation solution, while keeping the immobilisation time constant. The effect of probe concentration on the EIS signal after target hybridisation on the charge transfer resistance (R_{ct}) is presented in Fig. 2A. Here, a steady increase in ΔR_{ct} up to 9 μM probe concentration in the immobilisation solution was found, confirming the observations shown for this probe in Fig. 1B. A further increase of the probe concentration up to 15 μM caused a decrease in ΔR_{ct} signal again to the 1.5 μM levels. A likely explanation could be that the probes became too closely packed when immobilised and therefore inaccessible for target binding due to steric hindrance (Keighley et al., 2008a; Movilli et al., 2018). Besides this, the steric and repulsive hindrance from the overhang stretches can be another reason of lower EIS signals at high surface probe densities (Riedel et al., 2014). We anticipate that the mole fraction of PNA compared to all thiol compounds (PNA + MCH + HDT) in the immobilisation solution ultimately controls the probe density on the electrode surface after immobilisation. Our data show that a PNA mole fraction of 0.056 corresponding to a 9 μM probe concentration was ideal for immobilisation of P-S3₁₀ probe to achieve high ΔR_{ct} signals.

If R_{ct} kinetics upon target hybridisation were considered (Fig. 2B), a signal saturation could be observed starting after 20 min of target hybridisation for 1.5 μM and 30 min for 3 μM . For higher probe concentrations, the signal kept increasing for the whole observation period of 35 min. Interestingly, the steepest R_{ct} increase was found for 6 μM and 9 μM . These data support the concept of optimal probe surface density on the electrode and probe concentration dependency of ΔR_{ct} . Hence, probe concentrations 6 μM and 9 μM were considered as optimum for short probe P-S3₁₀ corresponding to a maximum ΔR_{ct} change upon hybridisation with miR-122 targets.

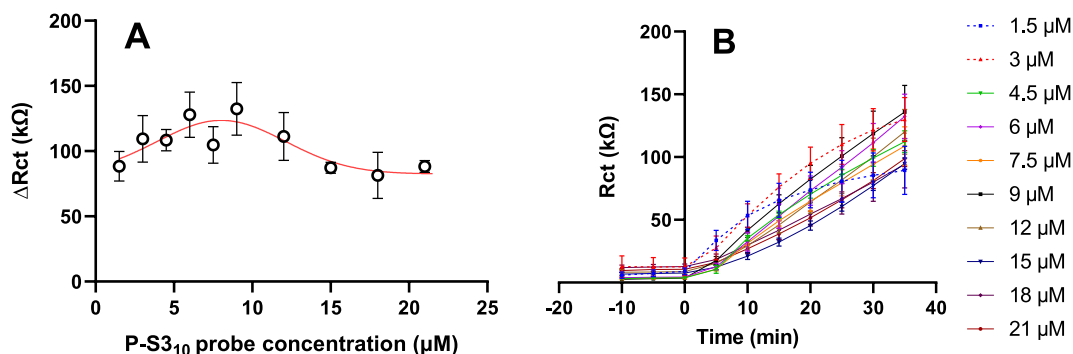


Fig. 2. - Influence of Probe Concentration on Sensor Response. (A) EIS signals (ΔR_{ct}) of electrodes functionalised with different concentrations (1.5–21 μM) of short probe P-S3₁₀ upon miR-122 target hybridisation (50 nM, 35 min). Gaussian function with $R^2 = 0.75$ was applied for nonlinear fitting of EIS signals over probe concentrations; (B) EIS signals (R_{ct}) over time (Baseline: –10 min–0 min; 50 nM miR-122 target addition; Hybridisation: 5–35 min) of electrodes functionalised with different concentrations (1.5–21 μM) of short probe P-S3₁₀. Nonlinear curve fittings have been done using Boltzmann sigmoidal function with $R^2 = 0.99$. All data represent the mean $\pm$ SD; $n \geq 3$.

3.3. Influence of length of target overhang on the EIS signal

To prove the contribution of the RNA target overhang on the electrode side of the probe, we conducted systematic experiments with probe P-S3₁₀ and miR-122 targets of different lengths producing different overhangs (Fig. 3). The length of the target was shortened gradually from the 3' end in 4 nt steps to obtain three shorter targets of 18, 14, and 10 nt (T-S1₁₈, T-S2₁₄ and T-S3₁₀) which we anticipated to form 3' overhangs of 8, 4, and 0 nt on the electrode surface after binding with P-S3₁₀ probe compared to the full length miR-122 target (T-FL₂₂) (Table S1, Scheme S1).

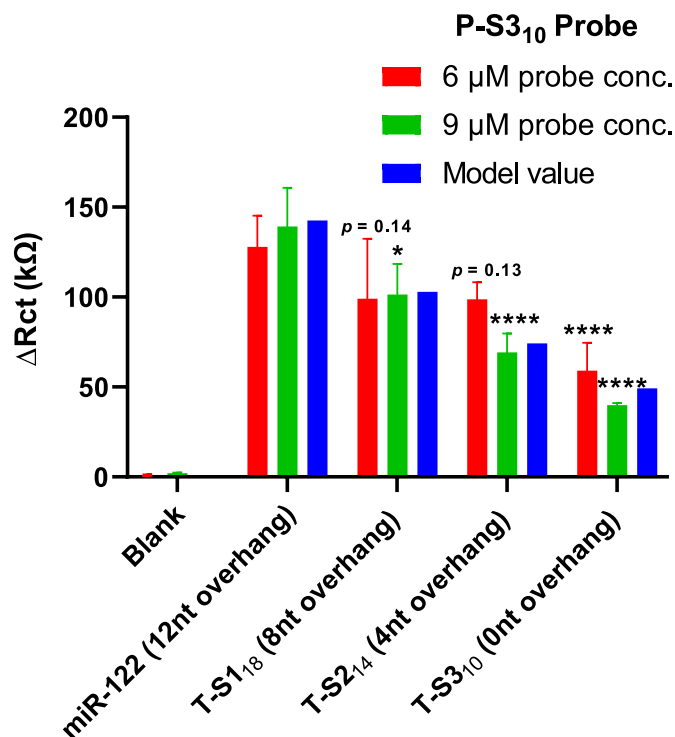


Fig. 3. - Influence of Target Overhang Lengths on Sensor Response. EIS signals (ΔR_{ct}) of electrodes functionalised with 6 or 9 μM short probe P-S3₁₀ after 35 min incubation with 50 nM targets of different lengths creating different length overhangs (miR-122/T-FL₂₂ (22 nt), T-S1₁₈ (18 nt), T-S2₁₄ (14 nt) and T-S3₁₀ (10 nt)). Data represent the mean $\pm$ SD; $n = 4$. Statistical significance has been determined by 2way ANOVA test (significance codes: **** $p \leq 0.0001$, * $p \leq 0.05$, compared to miR-122 signals).

We found that the 12 nt overhang of the full length target caused the highest increase in ΔR_{ct} signal followed by a gradually decreasing signal with decreasing length of the overhang on the electrode surface. These findings support our hypothesis that negative charges close to the electrode surface contribute strongly to ΔR_{ct} . For further confirmation of these observations, we performed a control study where we reversed the orientation of the probe (P-S4₁₀, C-terminally ("3 prime") immobilised instead of N-terminally ("5 prime")) and hybridised again with targets producing overhangs of different length on the electrode side. This time, the target length was reduced in 4 nt steps from the 5' end (Table S1). As shown in Fig. S5, the targets again produced ΔR_{ct} signals which were proportional to the length of the overhangs. (for p -values see Tables S5 and S6). The overlay of Nyquist and Bode plots for two short probes (P-S3₁₀ and P-S4₁₀) at two different probe concentrations (6 and 9 μ M) and with miR-122 and respective short targets also suggest a gradual enhancement in the EIS signals with the increasing overhang lengths (see Figs. S6–S9). These results add further underpinning to our hypothesis of the influence of the target overhangs towards the electrode surface during EIS measurements.

3.4. Model

To model our system and predict Rct signals depending on the target length (see Fig. 3), we considered a probe surface density of 1×10^{12} probes/cm² based on literature values (Keighley et al., 2008a; Ricci et al., 2007) resulting in calculated 2.01×10^{10} probe molecules on our working electrode (area 2.01×10^{-2} cm²). For the model, we considered that 60% of the surface-attached probes hybridised with miRNA targets leading to 1.21×10^{10} microRNA molecules hybridised on the electrode after adding 80 μ L of 50 nM miR-122 target. For the target molecules with 0, 4, 8 and 12 nt target overhang length we calculated the surface charge density (σ) (C/m²) for each target using 0, 5, 9 and 13 free electrons for 0, 4, 8 and 12 nt overhangs with the elementary charge $e^{-1} = 1.602 \times 10^{19}$ C, as

$$\sigma = \frac{(\text{free electrons per overhang} \times \text{elementary charge} \times \text{no. of miRNA})}{\text{area of working electrode}}$$

We considered the EIS buffer as a symmetrical electrolyte (1:1) and therefore calculated a Debye length (κ^{-1}) of 3.04×10^{-9} m for a 10 mM electrolyte concentration (Masliyah and Bhattacharjee, 2006). By considering an aqueous solution at 298 K (25 °C) and the dielectric permittivity of the solvent (ϵ) as 78.5 and the permittivity of the free space (ϵ_0) as 8.85×10^{-12} C²/Jm, we calculated the change in surface potential (ψ) using the Grahame equation (equation S7) resulting 0, 0.021, 0.038, and 0.055 V for the respective 0, 4, 8 and 12 nt overhangs.

To relate the surface potential (ψ) to the charge transfer resistance (Rct) we used the Butler-Volmer equation (i.e. determining how the electrical current that flows through the electrode is determined by the effective potential on the electrode surface). As we applied bias conditions of 10 mV (E_{app}) that deviate from the equilibrium, the Butler-Volmer equation can be simplified into equation (1) (Tsai et al., 2017).

$$R_{ct} = \frac{E_{app}}{FA k^0 [S]} \exp\left(\frac{\alpha e \psi}{k_B T}\right) \quad (1)$$

where E_{app} is the bias applied to the system (10 mV), ψ is the surface potential, F is the Faraday constant (96485 C/mol), A is working electrode area (2.01×10^{-2} cm²), k^0 is the standard rate constant, $[S]$ is the redox couple concentration at electrode-electrolyte interface, α is the transfer co-efficient and k_B is the Boltzmann constant. We took $\alpha = 0.5$ for a symmetrical energy barrier and $k^0 = 1 \times 10^{-3}$ cm/s, $k_B = 1.381 \times 10^{-23}$ J/K and $T = 298$ K and entered the calculated electrode surface concentration of the redox couple using equation (S1) to calculate the Rct values for 0, 4, 8 and 12 nt overhang length conditions using equation (1). Strikingly, these modelled Rct values were of a similar strength and followed the same trend as the actually measured Rct

values with the different miRNA targets underpinning the validity of our model (Fig. 3). For a detailed explanation of the theoretical models used in this consideration, please see **Supporting Information**.

As a further underpinning of our understanding of the surface processes, we considered the disturbance of the electric double layer (EDL) by the target overhangs. The formation of the EDL and the resulting capacitance changes are common events when the charged electrode and charged ions are separated at the interface. During EIS measurements, capacitive behaviour is more prevalent at lower frequencies. One would ideally expect a -90° phase shift from ideal capacitive behaviour at lower frequencies. Furthermore, a higher shift from -90° can be expected from a non-ideal behaviour of the EDL due to non-uniform charge distribution. In our case, we observed gradually higher shifts from ideal capacitive condition (-90° phase shift) at low frequencies (0.3 Hz) with increasing target overhang lengths for both P-S3₁₀ and P-S4₁₀ probes (Table S7, Figs. S6C–S9C). These results independently of Rct confirm that the charged miRNA molecules are indeed present in the 3.04 nm dimensions of the EDL and contribute according to their length and charge to the disturbance of the EDL.

3.5. Sensitivity and specificity of miR-122 detection

The analytical sensitivity for miR-122 detection of electrodes functionalised with the P-S3₁₀ probe was determined by performing a study with concentrations of 1–100 nM of miR-122. Fig. S10 depicts an overlay of Nyquist and Bode plots at various concentrations, revealing a gradual increase in impedance with increasing target concentration. Using Nyquist plots to determine ΔR_{ct} , the dose response curve (Fig. 4A) shows a steady increase in EIS signal (ΔR_{ct}) with increasing target concentrations and a linear relationship between 5 and 100 nM miR-122. We calculated an assay sensitivity of 4.4 k Ω /nM for miR-122 and a detection limit of 1 nM based on the blank measurements (McNaught and Wilkinson, 1997). Regarding liver injury from drug toxicity, previous studies have shown a mean value of 71.3 million (95% confidence interval 29.3–113.2 million) copies/mL of miR-122 in serum for a group of 18 healthy volunteers (McCrae et al., 2016), and the level of miR-122 has elevated 100–10000 fold in response to hepatocellular injury (McCrae et al., 2016; Starkey Lewis et al., 2011; Vliegthart et al., 2015). 71.3 million copies/mL of miR-122 in healthy individual is equivalent to 118.5 fM and hence it can rise up to 11.85 pM to 1.185 nM in the diseased conditions. Hence, our biosensor could detect miR-122 at clinically relevant concentrations during liver injury.

To investigate the analytical specificity of the assay, we used the P-S3₁₀ short probe functionalised electrodes and exposed them to a complementary (miR-122) and a non-complementary (miR-39) target of same length (22 nt) in high concentrations (50 nM). As shown in Fig. 4B, the P-S3₁₀ probe produced only a marginal increase in ΔR_{ct} signal for miR-39 or the buffer control as compared to a very strong signal for miR-122. An unpaired t -test confirmed the statistical significance between the signals of the two miRNA targets ($n = 4$, $p < 0.0001$). These results confirm that the P-S3₁₀ short probe retained a high level of specificity for the miR-122 target even at half of the full target length. For further confirmation on specificity, we tested miR-122 target with a miR-39 specific probe and compared the results with miR-39 target. The miR-39 probe showed significant enhancement in the ΔR_{ct} signal for the specific miR-39 target as compared to the non-specific miR-122 target ($n = 4$, $p = 0.003$). These results indicate that the probes designed for other miRNA, such as the miR-39 probe, are not specific for our desired miR-122 target, demonstrating the specificity of the sensor.

For further verification on specificity and to establish a clinical proof-of-concept validation, the developed assay was tested with tissue-extracted samples from a mouse toxicology study. Details on collection and preparation of mouse liver (high miR-122) and kidney (low miR-122) tissue samples and successive extraction of microRNAs are mentioned in **Supporting Information**. To achieve higher detection specificity in clinical samples, we employed P-S3₁₀ functionalised

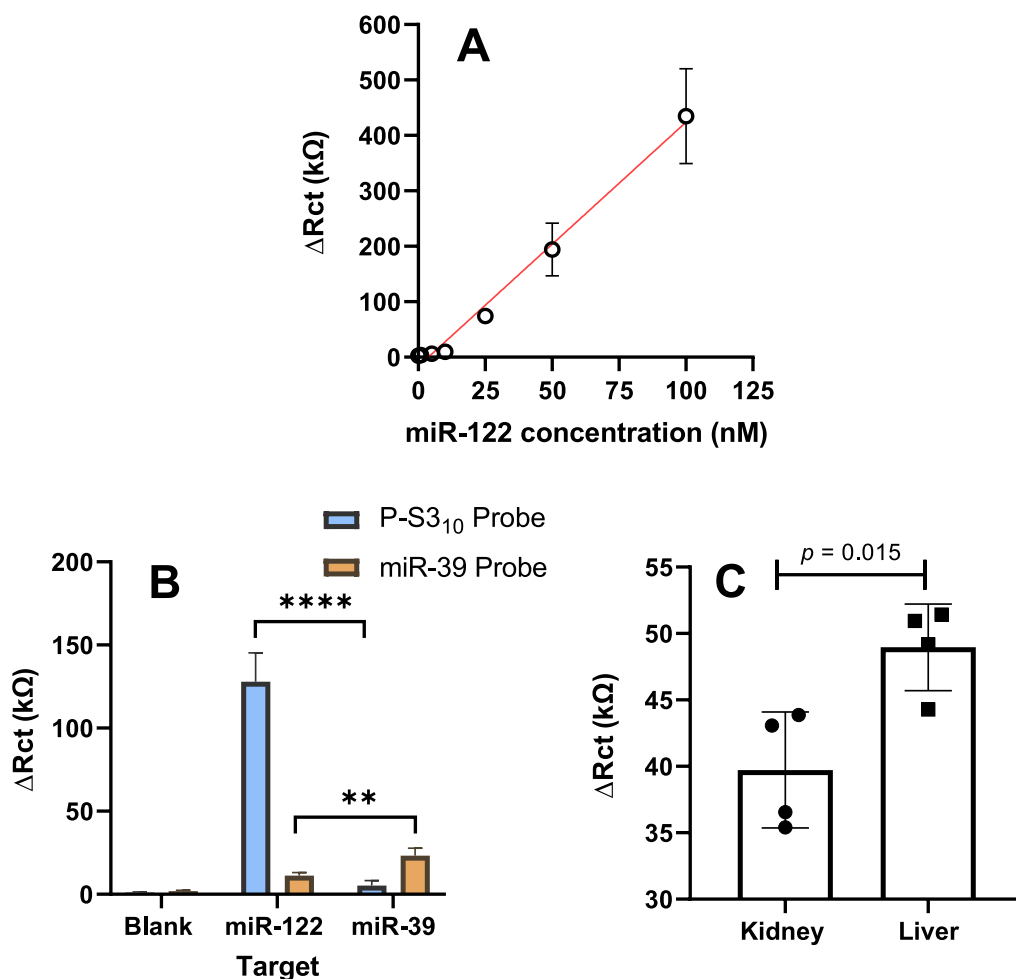


Fig. 4. - miR-122 Sensitivity and Specificity Studies. (A) EIS signals (ΔR_{ct}) of electrodes functionalised with 6 μ M probe P-S3₁₀ after 60 min incubation with 0–100 nM miR-122 target. The following equation has been calculated from the regression line: ΔR_{ct} (k Ω) = 4.401 [(k Ω)(nM)⁻¹] $\times$ [miR122] (nM) – 16.41 (k Ω) with $R^2 = 0.95$; (B) EIS signals (ΔR_{ct}) of electrodes functionalised with 6 μ M of P-S3₁₀ and miR-39 probes after 35 min incubation with miR-122, miR-39 target (50 nM each), or buffer control (blank); (C) EIS signals (ΔR_{ct}) of electrodes functionalised with 6 μ M of P-S3₁₀ after 35 min incubation with mouse kidney or liver tissue-extracted samples with 11.4 times dilution in the background buffer. All data represent the mean $\pm$ SD; $n = 4$. Statistical significance has been determined by unpaired t -test (significance code: **** $p \leq 0.0001$, ** $p \leq 0.01$).

electrodes to detect miR-122 in liver and kidney samples at a temperature (35 °C) close to the melting temperature (T_m) of P-S3₁₀ and miR-122 target binding (see Table S10). Our EIS detection strategy with P-S3₁₀ probe successfully identified miR-122 in the liver samples and produced significantly higher signal ($n = 4$, $p = 0.015$), when comparing with the kidney samples. The utility of our proposed approach for miRNA detection at point-of-care is demonstrated by the selective detection of miR-122 at physiological levels in liver samples.

3.6. Detection of miR-122 isoforms

Conventional miRNA detection is hampered by the physiological occurrence of isoforms of a miRNA species, called isomiRs. IsomiRs are generated from the same precursor during biogenesis or from sample degradation over time (López-Longarela et al., 2020; Telonis et al., 2017). IsomiR sequences are marginally different from their wild type sequence, i.e. by nucleotide trimming from either 5' or 3' end, addition of new nucleotides at 3' end or nucleotide substitutions. The miR-122 isomiRs cannot be measured accurately using conventional PCR assays (López-Longarela et al., 2020). To consider the three common isomiRs associated with liver injury, we designed three synthetic RNA targets (isomiR-1₂₁ (21 nt), isomiR-2₂₀ (20 nt) and isomiR-3₁₉ (19 nt), Table S1) by removing 1, 2 and 3 nt respectively from the 3' end of the miR-122 sequence. These isomiR targets were subsequently incubated in concentrations of 0–100 nM with electrodes functionalised with the P-S3₁₀ probe to produce a dose response curve using Nyquist plots, see Fig. 5A. The overlay of Nyquist and Bode plots at different concentrations for isomiR-1₂₁, isomiR-2₂₀ and isomiR-3₁₉ are shown in Figs. S11–S13. We

found an impedance signal proportional to the isomiR concentration and a linear relationship between 5 and 100 nM with a regression co-efficient (R^2) above 0.97 for all three isomiRs. The analytical sensitivity for the respective isomiRs of electrodes functionalised with the P-S3₁₀ probe was calculated as 2.89, 2.73 and 3.18 k Ω /nM for isomiR-1₂₁, isomiR-2₂₀ and isomiR-3₁₉, respectively. The limit of detection (LoD) was 1 nM for all three isomiRs.

IsomiR sequences are usually formed by degradation of the wild-type micro-RNA over time. Hence, under physiological conditions the miR-122 and isomiR sequences are generally present with different proportions in a sample. The impact of the presence of three isomiRs with miR-122 target was determined by measuring EIS responses (ΔR_{ct}) for four targets (miR-122 and three isomiRs) individually with 50 nM target concentrations and as a mixed sample of four targets with equimolar proportion of each target (Fig. 5B). The observed signals were lower for the individual isomiRs as compared to the full-length miR-122 target, likely owing to their shorter overhangs on the electrode surface. As expected, a solution of same total concentration (50 nM) formed by equal proportions (12.5 nM) of each of the four targets led to a signal increase between the individual isomiRs and the full length target.

In addition, we observed the responses of miR-122, isomiRs and miR-122 + isomiRs mixture using P-S2₁₀. All three isomiRs produced ΔR_{ct} values in similar range during measurements with both types of probe (P-S3₁₀ and P-S2₁₀) (Fig. 5B and Fig. S14A). The miR-122 responses were higher for P-S3₁₀, as compared to P-S2₁₀ due to the formation of target overhangs on the electrode surface. Signal intensity of miR-122 wild type, as well as the signal differences in between wild type and isomiRs, were higher in case of P-S3₁₀, when comparing with P-S2₁₀. To

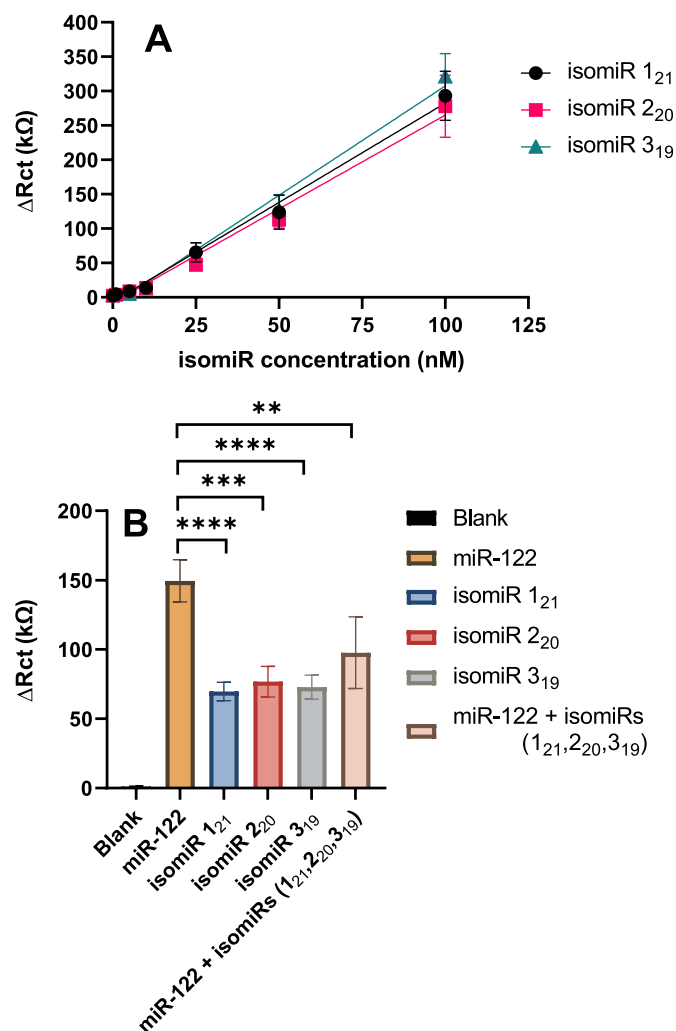


Fig. 5. (A) - Dose Response Curves isomiRs. EIS signals (ΔR_{ct}) of electrodes functionalised with 6 μM probe P-S₃₁₀ after 60 min incubation with 0–100 nM isomiR-1₂₁, isomiR-2₂₀ or isomiR-3₁₉ target. The following calibration equations have been calculated for respective isomiRs: **isomiR-1₂₁**, $\Delta R_{ct} \text{ (k}\Omega\text{)} = 2.89 \text{ [(k}\Omega\text{)(nM)}^{-1}] \times \text{concentration (nM)} - 6.35 \text{ (k}\Omega\text{)}$ with $R^2 = 0.97$; **isomiR-2₂₀**, $\Delta R_{ct} \text{ (k}\Omega\text{)} = 2.73 \text{ [(k}\Omega\text{)(nM)}^{-1}] \times \text{concentration (nM)} - 7.49 \text{ (k}\Omega\text{)}$ with $R^2 = 0.97$; **isomiR-3₁₉**, $\Delta R_{ct} \text{ (k}\Omega\text{)} = 3.18 \text{ [(k}\Omega\text{)(nM)}^{-1}] \times \text{concentration (nM)} - 10.61 \text{ (k}\Omega\text{)}$ with $R^2 = 0.97$; **(B) - Individual miRNA Targets and Target Mixtures.** EIS signals (ΔR_{ct}) of electrodes functionalised with 6 μM short probe P-S₃₁₀ after 35 min incubation with buffer control (blank), 50 nM of individual targets (miR-122, isomiR-1₂₁, isomiR-2₂₀, isomiR-3₁₉) or 50 nM equimolar mixture of all 4 targets (miR-122, isomiR-1₂₁, isomiR-2₂₀, isomiR-3₁₉). All data represent the mean $\pm$ SD; $n \geq 3$. Statistical significance has been determined by one-way ANOVA test (significance codes: **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$).

develop a strategy for identifying the presence of isomiRs along with wild type miR-122, we used the signal ratios of P-S₃₁₀ and P-S₂₁₀ for respective targets. As shown in Fig. S14B, the signal ratio of P-S₃₁₀ and P-S₂₁₀ is highest for the wild type miR-122 (mean value 1.63, SD 0.27, $n = 3$) among all the measurements. The isomiR sequences, as well as the equimolar mixture of miR-122 and isomiRs, produced mean signals ($n = 3$) below 1.45. Therefore, we set a threshold of 1.6, and if the signal falls below that, we suspect the presence of isomiRs in an unknown sample alongside the wild type sequence.

4. Conclusion

We have demonstrated the sensitive detection of miRNA 122 and

three of its important isomiRs with a limit of detection of 1 nM using electrochemical impedance spectroscopy based biosensors. The sensor performance critically depended on the probe immobilisation density and orientation. Interestingly, we found that some shorter probes produced stronger signals. This was attributed to miRNA target overhangs towards the electrode surface and experimentally evaluated. We hypothesized that these overhangs produce an additional electrostatic barrier which reduced the concentration of negatively charged redox couples contributing to the EIS signal. Theoretical considerations suggest this effect as well as the concentration of negatively charged redox species decays exponentially with the distance away from the electrode during the measurements.

CRedit authorship contribution statement

Apan Roychoudhury: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **James W. Dear:** Resources, Writing – review & editing, Funding acquisition. **Till T. Bachmann:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Funding acquisition.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2022.114404>.

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