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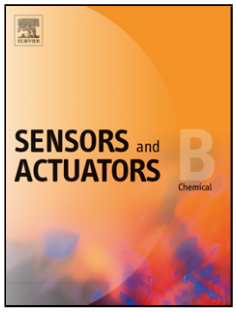

Electrochemistry-assisted Surface Plasmon Resonance Detection of miRNA-145 at Femtomolar Level

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Electrochemistry-assisted Surface Plasmon Resonance Detection of miRNA-145 at Femtomolar Level

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Highlights

- Simple and effective detection of cancer biomarker miRNA-145;
- Label-free detection by combining electrochemical techniques with SPR (eSPR);
- Detection of miRNA-145 at femtomolar level (clinically relevant levels);
- Possibility of application of the methodology in clinical diagnosis context;
- Simple and autonomous performing of the eSPR measurements by the auto-sampler.

Abstract

In this work, we combined electrochemical techniques with SPR (eSPR) for the label-free detection of cancer biomarker miRNA-145. Detection was performed in a simple

two-step assay. In the first step, the gold sensor surface, previously functionalized with a self-assembled monolayer (SAM) of thiolated RNA probes is incubated with the sample containing the target RNA biomarker. In this step, hybridization of RNA fragments with complementary immobilized probes was monitored in real-time by SPR. In the second step, eSPR measurements were performed to improve the sensitivity of the hybridization assay. Potential-induced deposition of a redox probe at the sensor surface resulted in enhanced SPR response promoted by the electrochemical process, thereby allowing the detection of miRNA-145 at femtomolar level (LOD = 0.56 fM), without sample derivatization or post-hybridization treatment for signal amplification. Good linearity was achieved (R^2 = 0.984) over the concentration range from 1.0 fM and 10 nM. Furthermore, the developed eSPR biosensor showed high selectivity towards single-base and two-base mismatch sequences and detection of target miRNA-145 in synthetic human serum was successful achieved.

Keywords: Biomarker; Biosensor; Electrochemistry; miRNA-145; Surface plasmon resonance (SPR)

1. Introduction

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA sequences of ~19-23 nucleotides involved in the regulation of gene expression controlling several crucial cellular and metabolic processes, thus, playing important role in health and disease of all organs and tissues [1, 2]. Several studies have identified dysregulated miRNA signaling pathways in many diseases, particularly in oncological diseases [3]. Therefore, these RNA fragments are currently a rapidly expanding field in clinical diagnostic context as potentially useful biomarkers in cancer diagnosis, prognosis and treatment responses [4-6]. MicroRNA research over the past decade reveals a down-regulation of miRNA-145 expression (<1000 copies per cell) in breast cancer [7-10]. On this basis, it is important to develop new reliable and easy-to-handle methodologies that allow the sensitive detection of circulating miRNA-145 biomarker.

In this work, surface plasmon resonance (SPR) spectroscopy is explored for this purpose, an *in situ* sensitive label-free optical technique that is sensitive to changes in the refractive index very close to certain metal surface (such as gold) [11, 12]. It was used for both, immobilization of thiolated RNA probes on the SPR gold substrates and further monitoring of miRNA-145 hybridization.

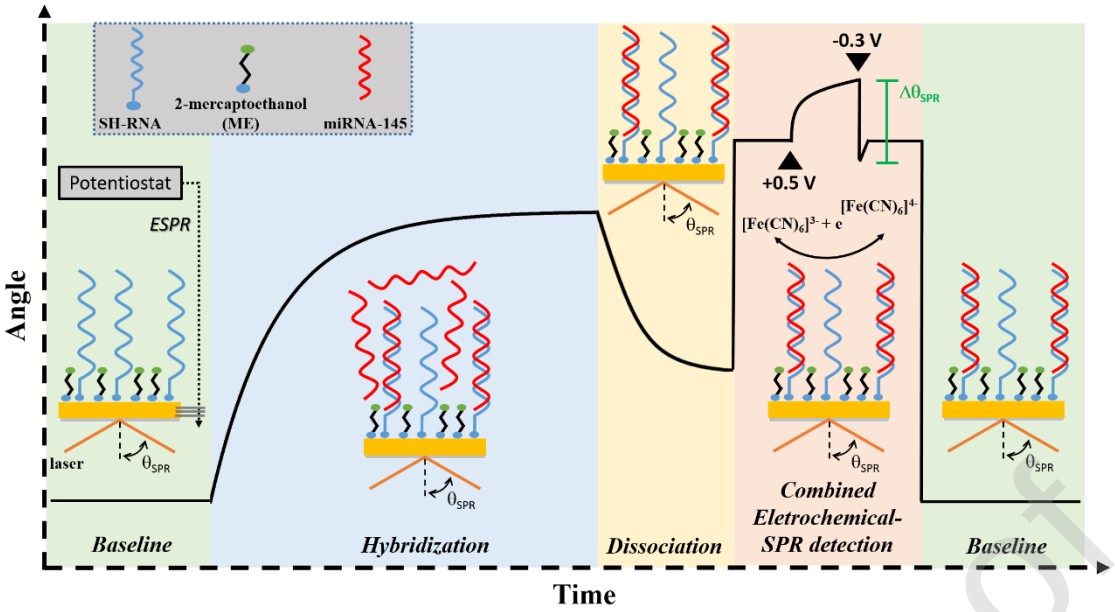
The attachment of thiolated single stranded RNA strands to gold surfaces was achieved by thiol-Au bond formation, a simple and versatile approach commonly used for the preparation of efficient and reusable oligonucleotide probe films [13, 14]. In addition, a secondary thiol was used to prevent non-specific adsorption of RNA fragments to the sensor surface [15-19]. The two-component modification of SPR substrates was performed automatically by the Autolab ESPRIT robotic auto-sampler resulting in rapid, simple and reproducible preparation of sensor surfaces [20]. Subsequently, conditions were established for the specific binding of miRNA-145 to the immobilized complementary strands.

Although direct label-free SPR measurement of binding events is the common approach for biologically relevant molecules [12], intrinsic characteristic of the SPR technique imposes the lack of sensitivity when analysis involves the binding of small or relatively small molecules (such as microRNAs) to substrates and/or the amount of analyte bound induces insignificant refractive index variation [21, 22]. Thus, signal amplification strategies for microRNA detection are commonly used to overcome these limitations [1, 23]. Most of the amplification procedures require expensive chemical labels and/or

complex amplification schemes sometimes, carrying additional limitations, such as problematic removal of unbound label, high background signal that obscures positive signals (false negative results) and/or non-specific reactions (false positive results) [14, 24].

Although most of the literature reports the applications of SPR method to study biomolecules and their interactions [12], including oligonucleotides hybridization [15, 25, 26], the combination of SPR with electrochemistry (eSPR) for detection of binding events is restricted to few papers. In 2001, Georgiadis and collaborators investigated the effect of applied electrostatic charging on nucleic acid probe immobilization [13] and its interaction with unlabeled DNA target oligonucleotides [14]. More recently, Dallaeire *et al*. [27] reported the detection of oligonucleotides in complex media by the use of immobilized methylene blue (MB)-labeled structure-switching DNA stem-loop. Upon hybridization, the stem-loop structure disrupts and limits the accessibility of the attached redox label to the electrode surface, decreasing its electron transfer efficiency.

In this work, SPR has been integrated with electrochemistry (eSPR) for the simple and sensitive detection of miRNA-145 biomarker in real-time, without the need of labeled-oligonucleotide probes or complex signal-amplification schemes. Briefly, after target RNA hybridization monitored in real-time, the potential-assisted deposition of a redox probe on SPR sensor surface was conducted (see Scheme 1). Unlike the usual binding assay monitored by direct SPR, changes in optical properties resulting from the electrochemical process can afford a larger SPR response, thereby improving the limit of detection. The eSPR measurements were performed in a fully autonomic manner using Autolab ESPRIT facilities. In addition, the equipment provides reproducible hydrodynamic conditions [20] during measurements of binding events which increases mass transport into the diffusion layer of the surface, overcoming typical slow hybridization kinetics that require long incubation times.



Scheme 1. Schematic representation of the combined electrochemical-SPR detection approach used in this work for detection of miRNA-145 biomarker.

2. Material and methods

2.1. miRNAs

The miRNAs (HPLC purified, Eurogentec) used in the experiments were:
- miRNA-145 (23 bases): 5´-GUC CAG UUU UCC CAG GAA UCC CU-3´ and;
- thiol modified RNA sequence complementary to miRNA-145 (SH-RNA, 27 bases): 5´-HS-$(A)_4$-AGGGAUUCCUGGGAAAACUGGAC-3´. Moreover, the following miRNA sequences were used in the selectivity studies:
- one-base mismatch (1-MM) sequence: 5´-GUC CAG UUU UAC CAG GAA UCC CU-3´ and;
- two-base mismatch (2-MM) sequence: 5´-GUC CAG UUU UAC CAG AAA UCC CU-3´.

Lyophilized miRNA sequences were recovered in 10 mM TE (Tris/EDTA) buffer, pH 8.0, 1 mM EDTA. Concentrated solutions of miRNAs (miRNA-145: 5 µM; SH-miRNA: 20 µM) were aliquoted to avoid multiple freeze-thaw cycles and stored in the freezer (-20 °C). Solutions were prepared with sterile/autoclaved materials in a laminar flow cabinet equipped with ultraviolet light. Experimental details related to chemicals, materials and solutions used throughout this work are described in SI.

2.2. Instrumentation

The combined electrochemical-SPR (eSPR) set-up used in this work, operation conditions and experimental details of measurements performed are described in SI.

2.3. Preparation of sensor surfaces

In this work, the modification of the gold substrates (see Scheme S1) was performed by using a full-automated SPR sequence to control the addition of all solutions into the cuvette. First, the baseline was collected with 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, followed by probe immobilization by incubation of the sensor surface for 1 hour with 1 µM thiolated miRNA solution, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, containing 1 M NaCl (immobilization buffer). The gold disk was then washed with buffer flask solution in order to remove unbound material. After

immobilization of SH-RNA, the gold surface was exposed to a 10 mM 2-mercaptoethanol (ME) solution, dissolved in Tris-HCl buffer solution, pH 7.4, 1 mM NaCl, for 1 hour to avoid non-specific binding during the hybridization event.

2.4. MiRNA-145 detection by eSPR

Detection of miRNA-145 was performed by eSPR measurements using an automated SPR injection sequence. After baseline collection in 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, the previously fabricated sensor surface was cumulatively incubated with standard solutions of miRNA-145, with concentration ranging from 1.0 fM to 150 nM, and the hybridization event was monitored in real-time for 15 min by SPR readout. No regeneration of the sensor surface was performed between solutions with different concentration. The hybridization medium chosen was a 10 mM Tris-HCl buffer solution, pH 7.4, and containing 15 mM $MgCl_2$. Then, the sensor surface washed with background solution to remove unbound material.

After that, eSPR measurements were carried out in the following manner: a 5 mM ferro/ferricyanide solution, dissolved in 0.1 M Na_2SO_4 electrolyte solution, was injected into to the measurement channel. After 30 s equilibrium, a potential of +0.5 V (*vs.* Ag/AgCl) was applied to the SPR cell and held for 60s. Then, the potential was swept to -0.3 V, at a rate of 50 mV s^{-1}, and held for 20 s. Finally, buffer flask solution was passed through the gold surface to washout the redox probe and the system was ready to analyze a new sample.

All sensorgramas obtained were normalized to zero and plotted in OriginLab (version 2016). The angle variation obtained in both conditions, under SPR hybridization equilibrium and under electrochemical control (eSPR), for the various miRNA-145 concentrations tested, were used to build the calibration curves.

3. Results and Discussion

3.1. Preparation of sensor surfaces

The attachment of single-stranded RNA probes to the SPR gold detection platforms was achieved via S-Au covalent bond, after functionalization at the 5´end with a thiol group connected by a spacer group composed of four adenine bases (SH-A_4-ssRNA). However, it is necessary to ensure that thiolated oligonucleotide strands are correctly oriented (vertically) on the surface and the concentration of SH-RNA (and/or incubation time) used does not compromise the hybridization of target RNA fragments due to (1) formation of sterically inaccessible excessively dense and compact films or (2) non-specific base adsorption on sensor substrates [15-17]. In addition, it is known that the kinetics of thiolated oligonucleotide immobilization strongly depends on buffer ionic strength [13]. Thus, immobilization conditions of oligonucleotide SAMs, including the ionic strength of the medium, probe concentration, incubation time, etc., should be carefully optimized to control the degree of surface coverage.

Fig. 1 shows the sensorgrams obtained for 1 hour exposure of the SPR gold substrates to a solution of 1 µM SH-RNA, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, and containing NaCl at two concentration levels: 1 M (channel 1) and 10 mM (channel 2). Although an increase of the SPR reflected angle was observed in both measuring channels after surface wash with background solution, a lower deposition was observed when thiolated RNA is dissolved in low ionic strength medium (10 mM NaCl) due to the higher electrostatic repulsion between the RNA chains. By opposition, electrostatic repulsions are less intense in the high ionic strength medium (1 M NaCl) and higher degree of surface coverage was reached. The maximum variations of the reflected angle, upon return to baseline, using low and high ionic strength immobilization medium were 95 and 291 m°, respectively.

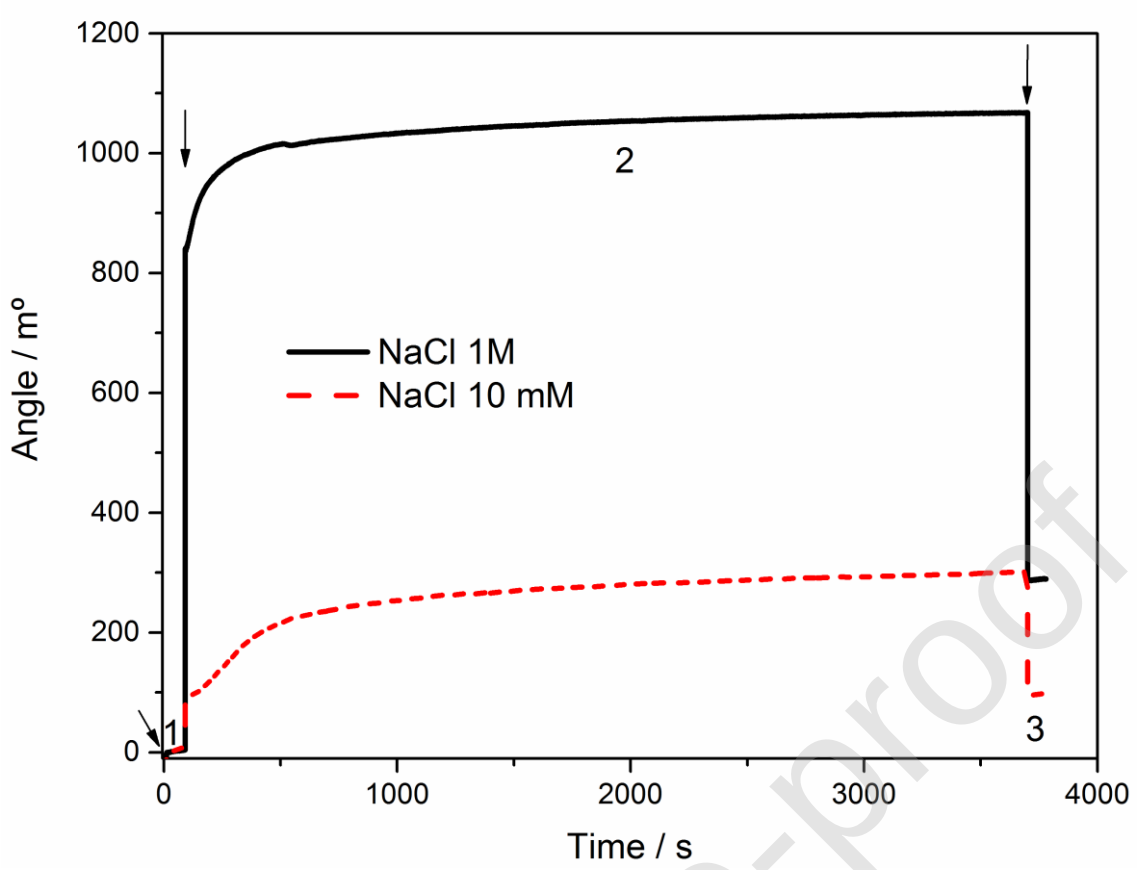


Fig. 1. Sensorgrams obtained for the immobilization on a SPR gold disk of 1 µM SH-RNA, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, containing (——) 1 M or (---) 10 mM NaCl. Line 1: baseline collected in 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, for 90 s; Line 2: immobilization of thiolated RNA for 1 hour; Line 3: wash with Tris-HCl buffer solution for 90 s (return to baseline). The arrows indicate the injection of the different solutions into the measuring system.

The degree of surface coverage can also be controlled by varying the amount of time that SPR gold supports are exposed to the thiol-modified oligonucleotide solution. In this work, the gold substrates were exposed for different time intervals, between 5 min and 6 hours, to a 1 µM SH-RNA solution, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, and containing 1 M NaCl. Fig. 2 shows the variation of the reflected angle obtained, after surface washing with background solution, as a function of the immobilization time. It can be seen that immobilization occurs within 1 hour since angle variation greatly increases with the incubation time due to surface progressive coverage by thiolated RNA strands. For immobilization times greater than 1 hour, no significant increase on surface immobilized probe molecules was observed and saturation tends to occur.

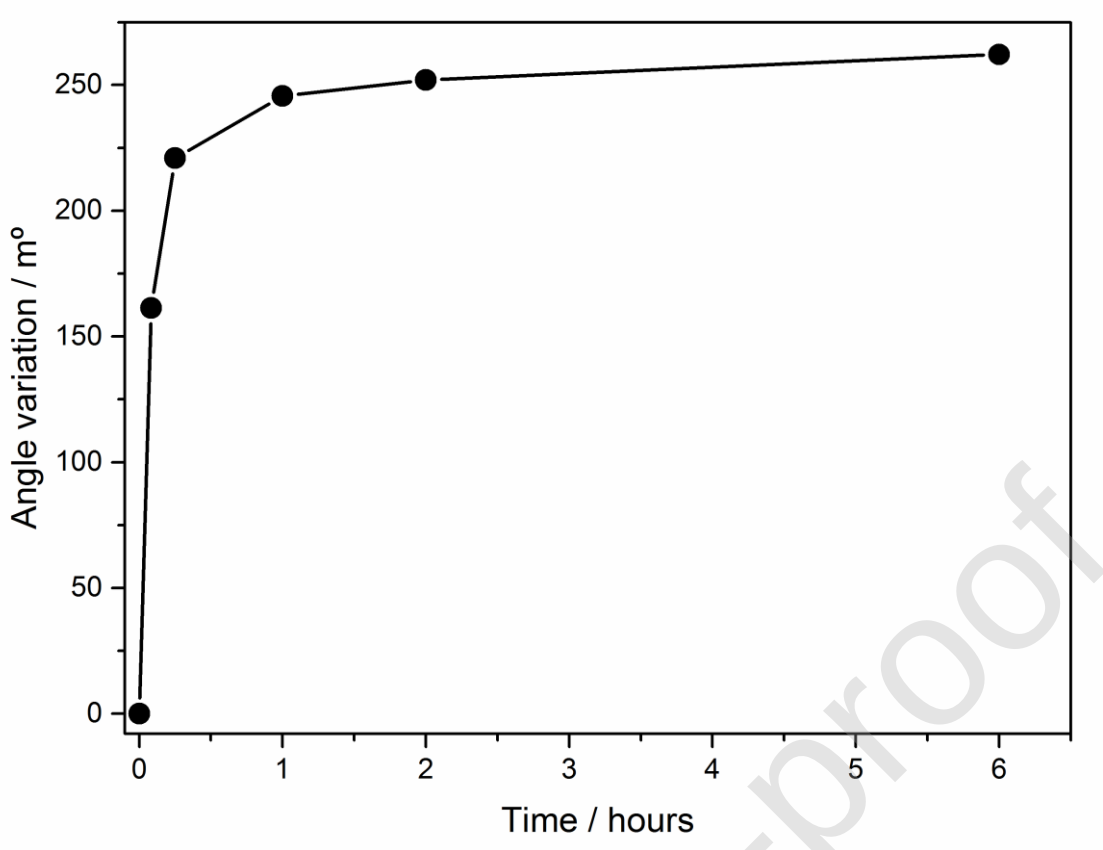


Fig. 2. Graphical representation of the angle variation obtained after immobilization on a SPR gold surface of 1 µM SH-RNA, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, 1 M NaCl, for 5 min, 15 min, 1 hour, 2 hours and 6 hours. The final angle variation values represented were obtained after washing the surface with background solution.

In an effort to quantify the extension of the immobilization process, the maximum degree of surface coverage was estimated by performing three repeated (1-hour) injections of 1 µM SH-RNA solution into the measuring channels (see Fig. S1, SI). The cumulative amount of probe adsorbed resulted in an overall angle variation of 323 ± 14 mº. Typically, a single 1-hour immobilization step resulted in a total SPR angle variation of 239 ± 36 m° (~74% of maximum surface coverage), which was considered a high thiolated RNA density at the sensor surface for effective duplex formation but not excessively dense that impedes capturing miRNA-145 fragments due to steric interactions. Despite the fact that sparser surface coverages (< ~4×10^{12} probes cm^{-2}) promote high hybridization efficiency [13, 28, 29], greater reproducibility of RNA densities and larger SPR signals were achieved for this high probe density regime.

A relevant aspect in the preparation of oligonucleotide detection sensors is the use of a specific molecule, co-immobilized on the sensor surfaces, which has the dual function of contributing to the reorganization of films favoring an upright probe orientation and

minimizing non-specific binding of target oligonucleotides on sensor platforms. 6-mercapto-1-hexanol (MCH) [15-18, 29, 30] and 2-mercaptoethanol (ME) [19] have been extensively used in this context. In this work, both molecules succeed to bind the SPR gold substrates, after previous immobilization of thiolated ssRNA strands. The immobilized thiol-modified RNA probes has a four adenine nucleotide sequence (not participating in the hybridization event) working as a vertical spacer to increase accessibility of target analyte [16]. Thus, the use of shorter length ME as lateral spacer instead of longer MCH should in principle favor hybridization by reducing steric hindrance.

The formation of the two-component monolayer film at the SPR gold surface was performed by means of a pre-programmed SPR injection sequence. A representative sensorgram of the two-step modification procedure is shown in Fig. 3. Initially, the SPR signal recorded after injection of 10 mM Tris-HCl buffer, pH 7.4, 10 mM NaCl, allowed establishing the baseline of the measurement system. Then, 1 µM SH-RNA solution was added to the measuring channel and allowed to interact for 1 hour, followed by surface wash with buffer flask solution to remove non-specifically bound thiolated RNA. For co-immobilization of the ME, the sensor surface was incubated with a 10 mM ME aqueous solution, followed by wash with background solution. The modification process was allowed to occur for 1 hour for effective SAM formation and rearrangement of the film at the surface [15]. Initially, the increase of reflected angle is due to rapid ME adsorption to the surface followed by long and slow angle decrease due to slow desorption of weakly bound thiolated RNA molecules from the surface. After the two-step modification, the sensor surface was stabilized by successive injections of running buffer solution to remove non-specifically bound material at the surface (see Fig. S2, SI). Moreover, cyclic voltammograms collected (in static mode) at the SPR gold surface showed a decrease of $[Fe(CN)_6]^{3-/4-}$ peak current, when compared to the voltammogram obtained at bare gold, due to the blocking effect caused by the two-component film deposited at the electrode surface (see Fig. S3A, SI), confirming the successful assembly of the sensor platforms.

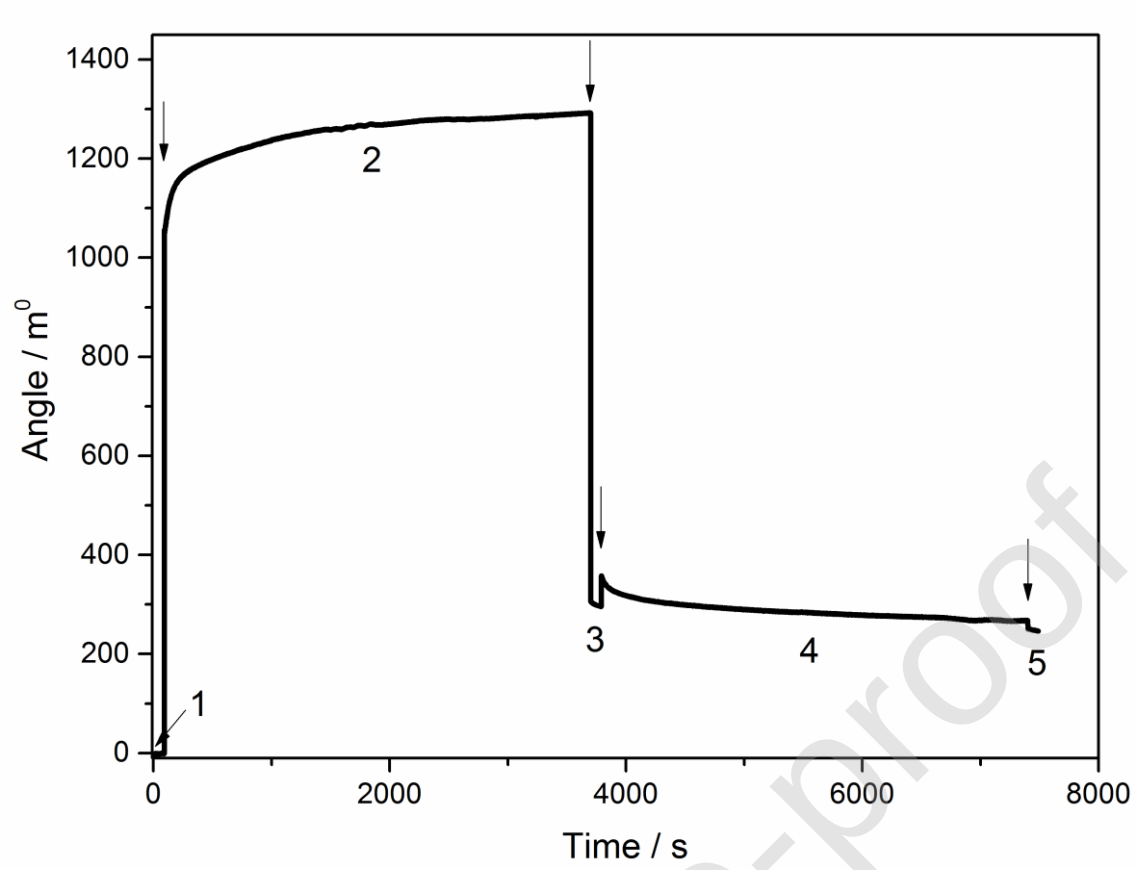


Fig. 3. Representative sensorgram of the SPR gold surface modification with SH-RNA and ME. Line 1: baseline collected in 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, for 90 s; Line 2: immobilization of thiolated RNA (1 μM), dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, 1 M NaCl, for 1 hour; Line 3: wash with Tris-HCl buffer solution for 90 s (return to baseline); Line 4: incubation with 10 mM ME, dissolved in 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, for 1 hour; Line 5: wash with Tris-HCl buffer solution for 90 s (return to baseline). The arrows indicate the injection of the different solutions into the measuring system.

3.2. MiRNA-145 interaction with sensor surface

The effect of medium composition on the extension of the hybridization process was considered in our experiments to enhance detection sensitivity. In this work, miRNA-145 was dissolved in the following buffers for comparison: the sodium chloride buffer (10 mM Tris-HCl buffer solution, pH 7.4, containing 10 mM or 1 M NaCl) and the magnesium buffer (10 mM Tris-HCl buffer solution, pH 7.4, containing 15 mM $MgCl_2$). Experiments performed showed that the sensor response to the miRNA-145 hybridization is much more noticeable for the magnesium buffer when compared to the sodium buffer, even for high salt content (see Fig. S4, SI). This is probably related to the fact that Mg^{2+} ions play an important role in stabilizing oligonucleotide duplexes and increases the

extent of hybridization event [26, 31]. Thus, the magnesium buffer was used as hybridization buffer in our experiments.

The interaction of miRNA-145 with immobilized complementary strands was performed by consecutive injections of solutions with increasing miRNA-145 concentration, from 1.0 fM to 150 nM, into the measuring channel using an automatic SPR measuring sequence.

The sensorgrams obtained for each injected miRNA-145 solution combining both, real-time SPR monitoring of the hybridization event (first step of detection) followed by eSPR measurements (second step of detection), are shown in Fig. 4A. After baseline acquisition in the presence of 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, the hybridization events were monitored in real time for 15 min, following by surface washing of weakly bound miRNA. The hybridization process occurs relatively fast, within few minutes after solution of the complementary target came in contact with the probe-covered sensor surface, and then saturation tends to occur. Thus, a 15 min injection was found to be sufficient for the sensor response to reach plateau equilibrium values.

The graphical representation of the obtained equilibrium angle (maximum angle variation at steady-state conditions) during direct SPR detection as a function of the miRNA-145 logarithmic concentration, shown in Fig. 4B, shows a very narrow response of the sensor for concentration values above 1.0 nM. It seems that the amount bound of relatively low molecular weight miRNA-145 (≈ 7 kDa) is too small to induce significant changes in the SPR resonance angle. This also resulted in high variability of equilibrium angles obtained after triplicate measurements for each concentration tested. For concentrations values higher than 1.0 nM, the cumulative amount of target miRNA specifically adsorbed induces SPR angle shifts to more positive values. A linear relationship between the SPR analytical signal and the miRNA-145 concentration was obtained in the concentration range from 1.0 to 150 nM (see Fig. S5, SI). Moreover, CV data collected under static conditions (see Fig. S3A, SI) showed a decrease of ferro/ferricyanide peak current, while a dip shift of SPR curves, to more positive values (see Fig. S3B, SI), was observed after the injection of 150 nM miRNA-145 into the measuring system, revealing that stable RNA duplexes were formed at the sensor surface.

Although a clear sensor response was observed for miRNA-145 concentrations higher than 1.0 nM, the overall direct detection methodology lacks the sensitivity necessary for the detection of low levels of miRNA-145 in the context of clinical diagnosis. According

to the literature, detection limits achieved using conventional SPR devices without using amplification strategies are typically above 100 nM [16].

In this work, eSPR measurements were performed after duplex formation with the immobilized probes to enhance detection sensitivity of the hybridization assay. The innovative amplification strategy is very simple and consists in the potential-induced surface deposition of 5 mM ferro/ferricyanide solution resulting in enhanced SPR angle changes. $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox system is commonly used in biosensing applications due to their relative low redox potential (0.15 V *vs.* Ag/AgCl), good biocompatibility and low cost [31]. In the eSPR experiments, the applied potentials are within the limits of the gold electrode double layer region (see Fig. S3A, SI).

The application of the electrode potential of +0.5 V in the eSPR cell (for 60s) forced the flow of negatively charged $[Fe(CN)_6]^{3-/4-}$ molecules into the diffusion layer of the electrode surface where oxidation occurs. In addition, the syringe is continuously aspirating and dispensing the redox probe solution into the cuvette, which increases enormously the mass transport and the accumulation of the species at the surface. Thus, the SPR angle greatly increases in the first seconds after potential application and then tends to reach saturation, since no more redox probe molecules can be accumulated at the sensor surface. Then, the application of a negative electric potential (E = -0.3 V) at the eSPR cell causes the fast desorption of reduced redox probe molecules from the gold substrates due to electrostatic repulsion, causing an abrupt decrease of the SPR angle. The total angle variation resulting from the electrochemical process was used as analytical signal.

The graphical representation of the total angle variation obtained after the application of electrostatic fields (+0.5 V and -0.3 V) against the miRNA-145 concentration logarithm is shown in Fig. 4C. As can be seen, the total angle variation obtained from eSPR measurements decreases with the increasing concentration of miRNA-145 as the hybridization assay proceed, causing a barrier to redox probe migration to the electrode surface. Good linearity was achieved (R^2 = 0.984) over the concentration range from 1.0 fM and 0.10 nM. Beyond this concentration value, an increase in the target miRNA-145 concentration does not decrease the eSPR signal. This is probably due to electrostatic interactions occurring at the electrode/solution interface. Hybridization greatly increases the amount of negative charges in the surface monolayer and further hinders the access of the redox probes to the electrode surface.

The LOD obtained by the eSPR measurements, 0.56 fM, was estimated following the Recommendations of IUPAC for ion-selective electrodes [32] (see Fig. S6, SI). The concentration detection level obtained was of the same order of magnitude as other obtained by commonly used sensitive techniques reported in the literature for miRNAs detection, including fluorescence, colorimetry, electrochemistry and SPR (see Table S1, SI). This work also has a wide concentration range of response comparing with other methods, allowing the detection of circulating miRNA-145 at physiological levels (~fM) for clinical diagnosis and prognosis of breast carcinoma.

To further demonstrate the sensitivity of the developed eSPR biosensor, we compared the LOD of our label-free approach with other SPR methodologies reported in the literature (see Fig. S7, SI). As can be seen, the enhanced SPR response resulting from the electrochemical process (eSPR) provided a suitable amplification strategy to improve detection sensitivity. In fact, from the existing sensing methods, higher sensitivity for miRNA detection was only achieved by SPR sensing based on 2D antimonene nanomaterials [33]. Besides, our detection methodology does not require complex and/or laborious signal-amplification strategies. Most of the reported procedures used to enhance SPR signals rely on secondary antibodies/enzymes (or labeled probes) or metal/magnetic NPs as amplification tags, sometimes, having some limitations associated, namely: need of expensive chemical labels, laborious and time-consuming procedures, problematic removal of unbound label, high background signals (false negative results), non-specific reactions (false positive results), steric hindrance, need of specific reaction conditions, agglomeration of amplification tags, instability, limited mass production of nanomaterials, problematic application in complex biological matrices, among others.

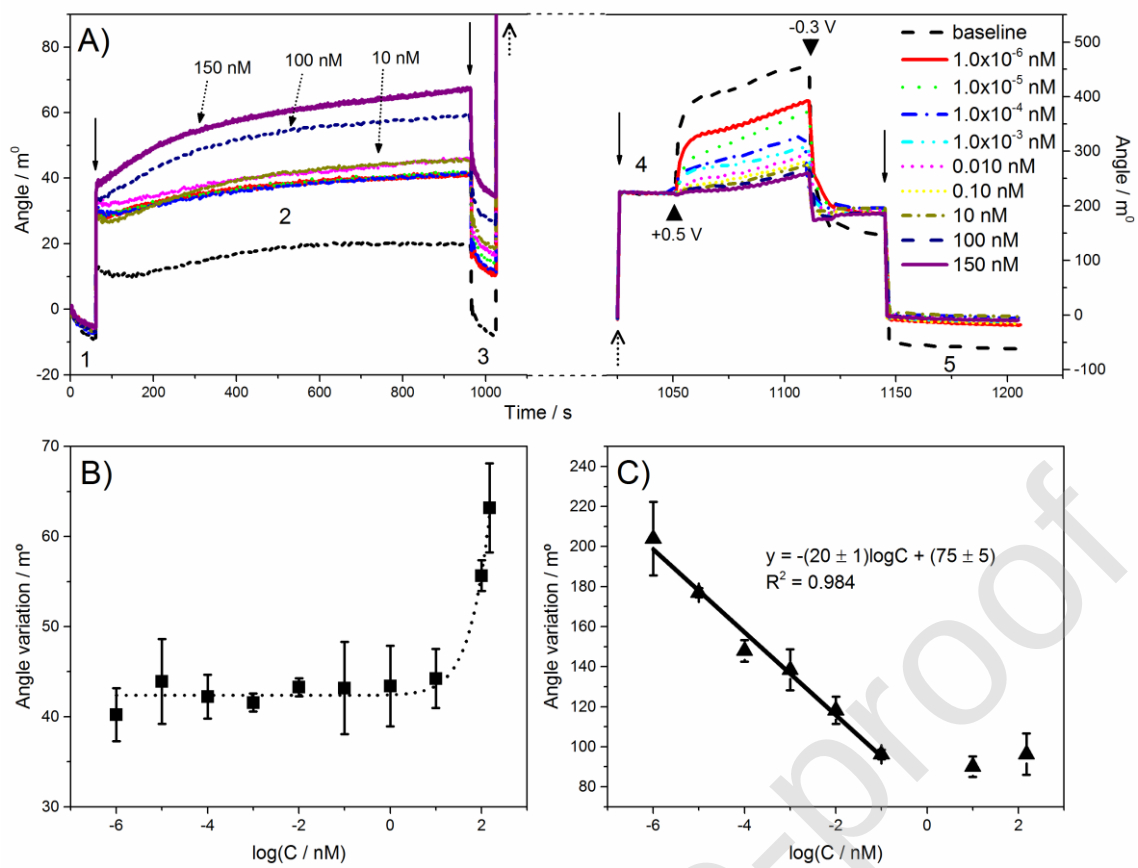


Fig. 4. (A) Real-time monitoring of the interaction between immobilized RNA strands on SPR gold substrates and miRNA-145 by SPR (left) followed by eSPR measurement in the presence of a redox probe (right). The concentrations of miRNA-145 tested ranged from 1.0 fM to 150 nM. Line 1: baseline collected in 10 mM Tris-HCl buffer solution, pH 7.4, 10 mM NaCl, for 60 s; Line 2: real-time monitoring of the interaction between surface immobilized RNA with miRNA-145 for 15 min; Line 3: wash with Tris-HCl buffer solution for 60 s (return to baseline). For comparison, the response obtained after injection of hybridization buffer (without miRNA-145) into the measuring channel (dashed line) is also shown in the figure. Line 4: combined electrochemical control and SPR detection after injection of 5 mM ferro/ferricyanide redox probe into the measuring channel. After 30 s equilibration at open circuit, the potential of +0.5 V (*vs.* Ag/AgCl) was applied and held for 60 s. Then, the potential swept to -0.3 V, at a rate of 50 mV s^{-1}, and held for 20 s. Line 5: wash with Tris-HCl buffer solution for 60 s (return to baseline). The arrows indicate the injection of the different solutions into the measuring system. (B, C) Graphical representations of the total angle variation as a function of the logarithm of the miRNA-145 concentration obtained under conditions of (B) SPR hybridization equilibrium and (C) combined electrochemical control-SPR monitoring, in the presence of a redox probe.

Control experiments were carried out to evaluate the possibility of non-specific attachment to the sensor surface. In these experiments, hybridization buffer (without miRNA-145) was successively injected into the measuring channel and the SPR (first step of detection) and eSPR (second step of detection) responses recorded (see Fig. S8,

SI). The resulting SPR and eSPR angle variations remained constant after incubation of the sensor surface with hybridization buffer, revealing poor contribution of non-specific binding of buffer and redox probe solution to the detected signal. Furthermore, experiments with similar concentrations of non-complementary (NC) target miRNA were also performed to ensure that non-specific binding is negligible (see Fig. S9, SI).

Reusability of the SPR Au substrates was also investigated in this work by regeneration attempts of the sensor surface after incubation with HCl (2 – 10 mM) and urea (1 M) aqueous solutions to denaturate RNA duplexes. Unfortunately, signals arising after surface treatment indicated that initials conditions were not fully restored and RNA fragments probably still hybridized at the surface. Thus, the use of (low cost) disposable SPR gold substrates was found to be the most effective route for miRNA detection using the proposed methodology.

In this work, real-time hybridization performed under electrochemical control was also tested to improve detection sensitivity. Application of an electrostatic field (E = + 0.3 V) and CV assistance to hybridization experiments were performed to easily induce electrostatic forces at the surface-immobilized RNA and increase the speed and extension of the interaction. However, these eSPR measurements fail in restored initial conditions after changes induced at the sensor surface by the electrochemical process (see Figs. S10 and S11, SI).

In this work, the selectivity of the eSPR biosensor was investigated by measuring the responses to three types of miRNA sequences, one-base mismatch (1-MM), two-base mismatch (2-MM) and complementary sequences (miRNA-145), under the same experimental conditions and at the same concentration, of 150 nM. This high concentration was chosen to provide both responses, SPR and eSPR, to the hybridization event. The results obtained, based on duplicate measurements, are depicted in Fig. 5.

After incubation of the sensor surface with the 1-MM miRNA sequence, a small decrease (of 20%) of the SPR equilibrium angle was registered, when compared to the perfectly complementary sequence, while a more pronounced decreased, of 33%, was observed for the 2-MM sequence (see Fig. 5A). By opposition, the overall eSPR angle variation increases with the increasing amount of duplexes with mismatched base pairs, causing a decrease in electrode surface blocking to the redox probe upon hybridization (see Fig. 5B). The results obtained clearly indicate that the thiolated RNA probes immobilized on the sensor surface have a very good sequence specificity toward the target miRNA-145.

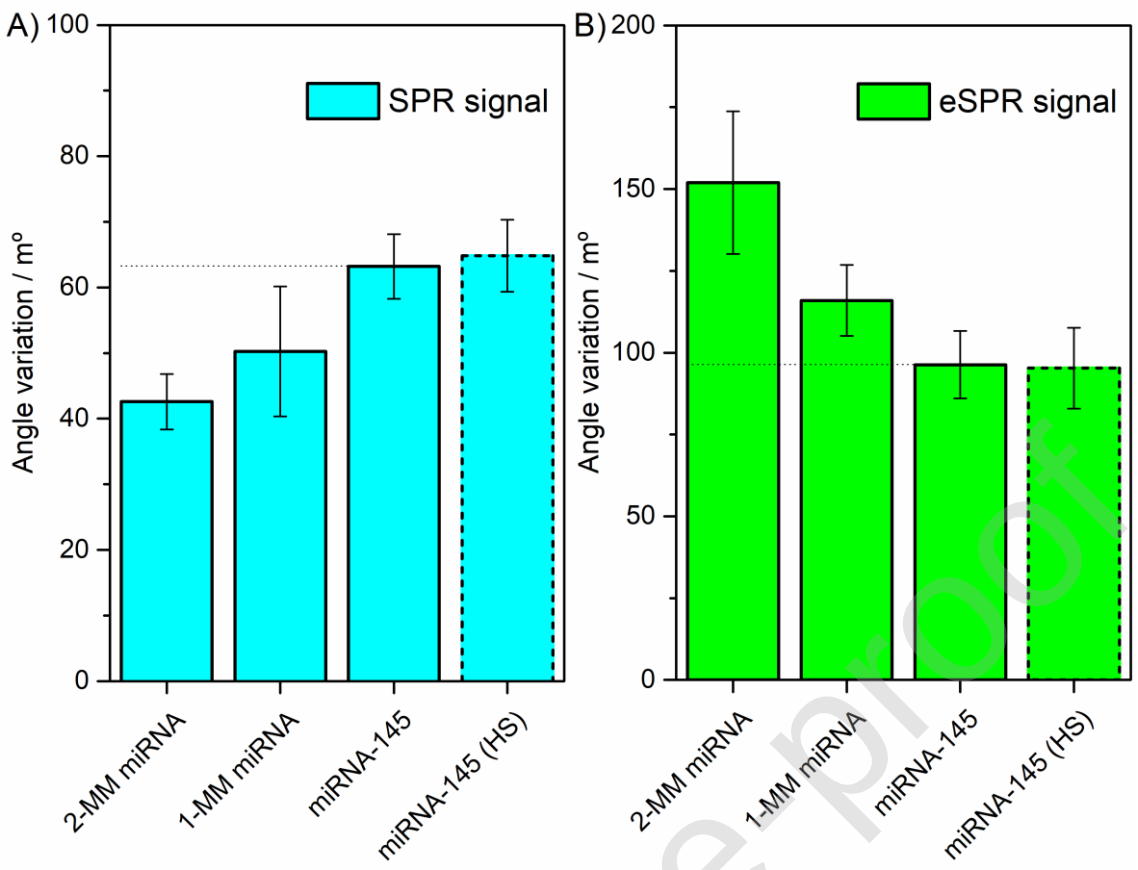


Fig. 5. Selectivity study (solid lines) performed by collecting the A) SPR and B) eSPR responses to detection of 150 nM of one-base mismatch (1-MM), two-base mismatch (2-MM) and complementary (miRNA-145) sequences, in buffer solution. In addition, the analysis of 150 nM miRNA-145 dissolved in diluted (1:15, V:V) synthetic human serum (HS) matrix (dashed lines) was also included in the figure.

In this work, the eSPR detection of target miRNA-145 in synthetic human serum (HS, Cormay serum HN, ref. 5-172, PZ CORMAY S.A., Poland) was also investigated. Prior to the detection, the most abundant high molecular weight serum proteins (HSA and globulins), that strongly dominates the SPR response and makes the detection of low size miRNAs challenging, were removed by centrifugal filtration [34, 35] for 10 min at 7500g using 10K filters (Amicon® Ultra-2 mL. Millipore, Merck). After that, the serum fraction passed by the membrane was collected and diluted 15-fold in hybridization buffer. Noteworthy, for less diluted serum samples, nonspecific binding of interfering proteins with size <10 kDa produced a false positive SPR response.

The SPR and eSPR angle variations obtained after incubation of the sensor surface with diluted HS spiked with miRNA-145 standard, to obtain a final concentration of 150 nM, are also shown in Fig. 5. As can be seen, the experimental responses to the miRNA-145

in diluted HS are very similar to that obtained in buffer solution, indicating that nonspecific binding from complex serum matrix can be neglected under the specified experimental conditions. Thus, the features of high selectivity against similar sequences to that of miRNA-145 and the possibility of its detection in complex biological sample matrices, without losing detection ability, can be very attractive for the successful application of the developed eSPR biosensor in cancer diagnosis context.

4. Conclusions

This work is distinguished by the use of label-free electrochemistry combined-SPR (eSPR) measurements for the sensitive detection of cancer biomarker miRNA-145, achieved by simple deposition of a redox probe at the SPR gold surface after application of an externally controlled electrochemical field to the eSPR cell. Signal amplification arises from enhanced SPR response induced by electrochemical phenomena, without the necessity of expensive markers or complex strategies for signal amplification.

In the first step of the detection, direct SPR detection was used to monitor miRNA hybridization with surface immobilized probes. Although intrinsic size limitation/amount of miRNA bound makes accurate direct measurement rather challenging, the real-time monitoring of the hybridization event allows establishing equilibrium conditions and further optimize incubation times for quicker analysis. In the second step, eSPR measurements were performed to produce detectable response upon hybridization, providing a detection level of miRNA-145 of 0.56 fM, which is 2-3 orders of magnitude lower than the expected by direct SPR measurements (of few nM). The high sensitivity achieved has great diagnostic significance as downregulated miRNAs usually exists in serum at ~fM levels. Moreover, the selectively of the eSPR biosensor was very good, since it was able to discriminate the duplexes formed with single and two mismatching base pairs, and the possibility of extending the application of the biosensor for complex biological sample analysis was demonstrated by the effective detection of target miRNA-145 in synthetic human serum samples.

In this work, the combination of SPR and electrochemical detection in the same spot brought in several advantages to the miRNA detection methodology, namely: (1) technically simple set-up to perform eSPR measurements; (2) rapid, easy, automated, reproducible *in situ* preparation of sensor platforms and detection procedures, due to reproducible hydrodynamic conditions (continuous aspirating/dispensing of solutions by

the syringes) provided by the SPR robotic auto-sampler and using pre-programmed injection sequences; (3) safe and improved handling of biological solutions by the auto-sampler needles, minimizing the risk of sample contamination and errors associated with the manual volumetric transfers;

More work is currently undergoing to extend the use of eSPR technique to the detection of other biomolecules, but with the dual-purpose of enhancing detection sensitivity and collect the response from both detection systems, SPR and electrochemical techniques, in the same assay.

CRediT author statement

José A. Ribeiro performed writing – original draft and conceptualization. Carlos M. Pereira and M. Goreti F. Sales performed writing – review and editing, conceptualization and supervision.

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.

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