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Neutravidin biosensor for direct capture of dual-functional biotin-molecular beacon-AuNP
probe for sensitive voltammetric detection of microRNA

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Abstract:

We have demonstrated a new approach using a neutravidin-based biosensor combined with a dual-function gold nanoparticle (AuNP) biolabel, for simple and sensitive detection of microRNA-21 (miRNA-21). The selectivity of the biosensor is provided by the intrinsic properties of the dual-functional biotin-MB-AuNP label. The assay procedure is relatively simple, exploiting a one-pot assay concept where the affinity capture of the miRNA-21/dual-functional biotin-MB-AuNP complex, via the strong biotin-neutravidin supramolecular interaction, and simultaneous detection of the captured AuNPs label with stripping voltammetry, is performed in a single step. This electrochemical miRNA biosensor could detect miRNA-21 with limit of detection of 0.1×10^{-12} and a dynamic range from 0.5×10^{-12} to 1.0×10^{-9} M. The performance of the miRNA-21 biosensor was further improved after silver deposition onto the AuNPs, delivering an enhanced detection limit of 4.0×10^{-15} M of miRNA-21, and an extremely wide analytic dynamic range from 10×10^{-15} to 1×10^{-9} M (5 orders of magnitude). This exceptionally broad dynamic range demonstrates the advantage of the one-pot assay approach with direct capture of the dual functional biotin-MB-AuNP via the strong biotin-

neutravidin supramolecular interaction. Furthermore, we demonstrated the detection of miRNA-21 in spiked serum at clinically relevant concentrations. The miRNA biosensor displayed excellent analytical performance for the detection of miRNA and could provide a powerful and convenient tool for biomedical research and applications in cancer diagnostics.

Keywords: neutravidin; biosensor; microRNA; molecular beacon; stripping voltammetry

1. Introduction:

MicroRNAs (miRNAs) are short (18-22 nucleotides) non-coding RNA sequences. They were first identified in nematodes, in 1993, by Lee et al. [1] and to date, over 1000 separate miRNA sequences have been identified in the human [2]. It is becoming clear that miRNAs represent a vast, previously unrecognised level of molecular signaling in eukaryotes, and that miRNAs play an important role in the regulation of protein expression [3] and a significant role in several biological processes including: cell proliferation, developmental regulation, differentiation and epigenetic inheritance [4]. Recent studies have shown that the levels of miRNAs in body fluid can be correlated to the cancer type [5], especially, in prostate cancer (PCa), which is the second most common malignancy and the fifth leading cause of cancer death in men worldwide [6]. In most European countries such as France, The Netherlands, and the Czech Republic, the PCa incidence increased significantly in the early 1990s, and is still increasing [7,8].

Current standard methods for identification and quantification of miRNAs are based on traditional molecular biology techniques (Northern blot, microarray, qRT-PCR). Although these approaches are very sensitive and reliable, they are often expensive, time consuming and need highly trained technicians [9,10]. Hence, there is a real challenge to develop devices able to simultaneously detect and easily quantify different miRNA sequences. Electrochemical biosensors offer the advantage of being amenable to mass fabrication at low cost and hence facilitate decentralised analysis [11].

Various electrochemical methods are available for the determination of miRNA. Gao et. al. reported an amperometric assay for the measurement of miRNAs with a detection limit of 80×10^{-15} M, using an oligonucleotide capture probe immobilised onto an indium tin oxide (ITO) electrode [12]. In the same context, Peng et al. developed an amperometric miRNA biosensor, which delivered a sensitive analysis of miRNA with a detection limit of 2.0×10^{-15} M [13]. Yin et al. described a biosensor which exhibited excellent sensitivity and a low detection limit of 60×10^{-15} M, based on dendritic gold nanostructures and a graphene nanosheet-modified glassy

carbon electrode [14]. Bettazzi et al. developed an amperometric biosensor for miRNA detection based on para-magnetic beads and enzyme amplification [15]. Peng et al. reported an impedimetric miRNA biosensor based on the combination of RuO₂ nanoparticles and the catalytic deposition of poly (3,3'-dimethoxybenzidine) (PDB) [16]. Kilic and his colleagues reported a highly sensitive voltammetric assay for detection of miRNA based on the immobilization of the oligonucleotide capture probes onto a pencil graphite electrode (PGE) [17]. A voltammetric biosensor based on oligonucleotide encapsulated silver nanoclusters (Ag-NCs) that could detect as low as 67×10^{-15} M of miRNA was reported by Dong and coworkers [18]. Bartosik and colleagues developed a miRNA assay by immobilising a biotin-labeled oligonucleotide capture probe onto the surface of streptavidin-coated magnetic beads [19]. Zhou and coworkers developed a miRNA biosensor based on mimicking enzyme catalysis and signal amplification [20]. In this work, gold nanoparticles were electrochemically deposited onto the surface of a glassy carbon electrode. Recently, Liu et al. developed a voltammetric biosensor detecting miRNA levels down to 3.0×10^{-15} M in which the target miRNA was hybridised with a pre-immobilised DNA capture probe onto the surface of a gold electrode [21]. Nevertheless, the above electrochemical methods are based on relatively complex and tedious sensor surface preparation with immobilised oligonucleotide capture probe, and require multiple hybridisation steps.

In the present work, we report the development of a robust neutravidin based biosensor for voltammetric detection of miRNA. The assay selectivity was provided by the smart design of a dual-functional biotin-MB-AuNP probe with a one-pot assay concept, such that the affinity capture of the miRNA-21/dual-functional biotin-MB-AuNP complex occurred via the strong biotin-neutravidin supramolecular interaction, and stimulations detection of the captured AuNPs label was achieved within a single step. The performance of the voltammetric miRNA-21 biosensor was greatly improved after silver deposition onto the AuNPs, which allowed the detection of miRNA-21 with a broad analytic dynamic range from 10×10^{-15} to 1.0×10^{-6} M. The fabrication and binding processes of the miRNA biosensor were characterised with stripping square wave voltammetry.

2. Materials and Methods

2.1 Materials

Sulfuric Acid (H₂SO₄), nitric acid (HNO₃), sodium hydroxide, sodium chloride, sodium citrate, Neutravidin and silver enhancement kit were purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals used in this study were of analytical reagent grade. All solutions

were prepared with ultrapure (18.2 MΩ) water from a Millipore Milli-Q water purification system (Billerica, MA). The sequence of the DNA/LNA MBs was taken from previous reports[22]. The presence of a thiol group at the 3' end of the MB allowed its immobilisation onto the AuNPs, while at the other end the biotin at the 5' was used to facilitate the capture of the biotin-MB-AuNP/miRNA complex onto the transducer surface via interaction with an immobilised Neutravidin layer.

To facilitate the handling of the sample, RNA mimic sequences were taken from miRBase (<http://www.mirbase.org>) and synthesised by biomers.net (Germany). LNA modified Oligonucleotide probes were obtained from Exiqon (Denmark):

MB:

5'-/5BioTEG/GGCCGTCAACATCAGTCTGATAAGCTACGGCCTTTTTTTTTTT/3ThioMC3-D/-3' (in bold and italics are the LNA bases)

miRNA-21:5'-UAGCUUAUCAGACUGAUGUUGA-3'

miRNA-205: 5'-UCCUUCAUUCCACCGGAGUCUGU-3'

miRNA-221: 5'-AGCUACAUUGUCUGCGGGUUC-3'

Oligonucleotide stock solutions (100 μM) were prepared by dissolving the lyophilised synthetic sequences in filtered (filter size: 0.2 μm) MilliQ water. All stock solutions were stored at -20°C. To reduce the risks of deactivation of the thiol group, the stock solution of the MB was divided in aliquots that were stored at -20 °C and defrosted only when needed.

2.2 Instrumentation

Stripping square wave voltammetry (SSWV) was performed using an IviumStat Potentiostat/Galvanostat (Ivium, The Netherlands) with a three-electrode cell. A glassy carbon (GC) electrode (2 mm in diameter, CHI Instruments) was used as the working electrode. An Ag/AgCl KCl 3 M (CHI Instruments) electrode and a platinum wire were used as the reference and counter electrodes, respectively. All the potential values presented are vs. a Ag/AgCl KCl 3 M reference. The voltammetry measurements were performed in 0.1 M sulphuric acid buffer solution before silver enhancement and 10 mM nitric acid as buffer solution after silver enhancement. The amplitude of the applied sine wave potential was 5 mV.

A Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK) using dynamic light scattering was used to measure the size and zeta potentials of the AuNPs and biotin-MB-AuNPs conjugate. The mean size and zeta potential values were calculated by taking an average of 3 repeated measurements and were performed at room temperature (20°C).

2.3 Preparation of gold nanoparticles (AuNPs)

AuNPs were prepared according to the literature [22] by the citrate reduction of HAuCl₄. In brief, 50 mL of 1 mM HAuCl₄ were brought to boil under vigorous stirring. Rapid addition of 5 mL of a 38.8 mM sodium citrate solution to the vortex of the solution resulted in a colour change from pale yellow to burgundy. Boiling was continued for 10 min; the heating mantle was then removed, and stirring was continued for an additional 15 min. After the solution cooled to room temperature it was stored at 4°C.

2.4 Preparation of biotin-MB-AuNP biolabel

The biotin-MB-AuNP conjugate was synthesised in accordance with a previously published protocol [22]. Briefly; 250 µL of the AuNPs (OD 2.3) in 0.1 mM phosphate buffer at pH 7.4 were mixed, in a NaOH treated glass vial, with adequate volume of the MB stock solution to obtain a final DNA-to-AuNPs ratio of 500:1. The solution was then left to react at room temperature, under gentle mixing overnight. The biotin-MB-AuNP mixture was finally subjected to an “aging process” consisting of a stepwise increase of the concentration of NaCl up to 0.3 M; this was followed by an overnight incubation at room temperature under gentle shaking. Finally, the biotin-MB-AuNP conjugates were washed twice by sequential centrifugation (24,000 g, 20 min, 20°C), resuspension in NaCl 0.3 M and 0.1 mM phosphate buffer, pH 7.4 and stored at 4°C until use.

2.5 Fabrication of neutravidin electrode

A glassy carbon electrode (GCE) was first polished with 0.3 and 0.05 µm alumina and then sonicated by ultrasonic cleaning in ethanol and ultrapure water. The clean electrode was dried with high-purity nitrogen. A 5 µL aliquot of 0.25 mg/mL neutravidin solution was drop-cast onto the clean glassy carbon electrode, and the electrode was kept dry at room temperature for 1h. The modified electrode was cross-linked with glutaraldehyde vapour (25% in water on a hot plate at 40 °C) for 45 minutes and then the electrodes were washed with PBS buffer, dried and stored at 4 °C. Prior to use the electrode were re-hydrated for 5 minutes with buffer solution.

2.6 Detection of miRNA-21

Detection of the target miRNA was performed by incubating the neutravidin modified glassy carbon working electrodes in the solution containing miRNA-21 in the presence of the biotin-MB-AuNP label. The solution was prepared by mixing a desired amount of target miRNA with

an optimised concentration of biotin-MB-AuNP in a buffer solution (10 mM phosphate buffer pH; PB containing 500 mM NaCl, pH=7.5) to a final volume of 25 μ L. After incubation, the electrode was rinsed with 25 μ L of 10 mM phosphate buffer solution (pH 7.4), and subsequently detected by SSWV.

2.7 Silver enhancement

After hybridisation, the modified electrode was incubated in the silver enhancer solution (equal volumes (200 μ L) of each solutions A and B from the silver enhancer kit were mixed together) to enhance the electrochemical signal intensity of gold nanoparticles by forming shells of silver around of them. After 10 min, the solution was removed and sodium thiosulfate (0.2 M) was then dropped on the surface for 2 min to stop the silver reaction. Finally, the electrode was thoroughly washed with double-distilled water [23].

3. Results and Discussion

3.1. Design of the voltammetric miRNA biosensor

Scheme 1 illustrates the working principle of the voltammetric miRNA biosensor. The signal generation mechanism is based on a series of simultaneous events within a one-pot reaction by simple addition of miRNA-21/biotin-MB-AuNPs mixture onto the neutravidin modified sensor surface. In the presence of target miRNA-21, hybridisation takes place resulting in the opening of the biotin-MBs. This makes the biotin group that was previously “protected” by the steric hindrance of the stem-loop structure, accessible. The activated biotin-MB-AuNP/miRNA complexes were then available for capture, via supramolecular interaction, onto a neutravidin modified glassy carbon electrode. The binding event resulted in an increase in the peak current at the working electrode/electrolyte interface. The analytical performance of the biosensor was improved by silver deposition onto the captured AuNPs. In the absence of target miRNA-21, the biotin-MB retains its stem-loop structure, thus sterically impeding the interaction between the biotin group and the neutravidin on the electrode surface.

This approach combines the advantages of a dual-functional biolabel and a simple neutravidin electrode for quantitative electrochemical detection of miRNA-21. The concept of using a neutravidin electrode for miRNA detection provides a more stable and robust sensor platform compared with conventional nucleic biosensors based on immobilised single-stranded DNA probes. Moreover, the use of the MB approach is important for detection of the short length miRNA, while the conventional sandwich hybridisation approach is difficult to apply for detection of miRNA, since it is limited by the length of the single-stranded DNA probe.

3.2. Characterisation of the biotin-MB-AuNP label

To follow the steps of biofunctionalisation, dynamic light scattering measurements were used to determine the size of the synthesised citrate-capped AuNPs, biotin-MB-AuNP label and biotin-MB-AuNP/miRNA-21 complexes. The average hydrodynamic diameters obtained were 29 ± 0.6 , 40.3 ± 1.9 and 55.3 ± 2.4 nm, respectively. An increase in hydrodynamic diameter was observed for the biotin-MB-AuNPs compared with the AuNPs; this indicates the successful immobilisation of the MB onto the AuNPs. A further increase in the hydrodynamic diameter was recorded after formation of the biotin-MB-AuNP/miRNA-21 complex, indicating the opening of the MB stem-loop structure.

Zeta potential measurements were performed to study the surface charge densities of the AuNPs, biotin-MB-AuNP label and biotin-MB-AuNP/miRNA-21 complex. Following functionalisation of the AuNPs with MBs, an increase in the zeta potential was recorded from -27.3 ± 2.5 mV (AuNPs) to -35.3 ± 1.4 mV (MB-AuNPs). After hybridisation with target miRNA-21, the zeta potential of the biotin-MB-AuNPs/miRNA-21 complex further increased to -42.3 ± 2.6 mV. These increases in zeta potential values are related to the high negative charges of the MBs and miRNAs.

3.3. Optimisations of the voltammetric miRNA biosensor

In order to improve the performance of the biosensor, optimisation of: (i) the pretreatment time for dissolution of AuNPs into Au ions; (ii) the applied potential during the pretreatment; (iii) the concentration of biotin-MB-AuNPs label; and (iv) the amount of immobilised neutravidin was performed.

SSWV was employed to study the influence of time and potential during the pretreatment on the oxidation peak current of miRNA-21. It is known that an increase in the pretreatment time improves the sensitivity of determination [24]. Hence, the effect of variation of the time was studied over a period of (10, 30, 60, 120, 180, and 240 s), employing a frequency of 20 Hz and a potential of 1 V. Figure 1A shows that the signal response increased with increasing time. Therefore, 180 min was chosen as the preferred time, since it proved a good compromise between the assay time and the signal response.

Keeping the frequency as 20 Hz and the time of pretreatment as 180 s, the optimum potential was determined by scanning a potential window of 0.5 to 1.25 V. As can be seen from Figure 1B, the peak current for detection of 0.5×10^{-9} M miRNA-21 reached its maximum at a potential of 1.25 V, but the transition from 1 to 1.25 V did not significantly influence the sensor

response. Thus, the potential during the pre-treatment was fixed at 1V for further studies, because a higher potential may damage the electrode surface. The final conditions for the SSWV therefore were $E = 1\text{ V}$, $t = 180\text{ s}$ and $f = 20\text{ Hz}$.

The effect of the amount of biotin-MB-AuNP label on the biosensor performance was investigated by detecting $0.5 \times 10^{-9}\text{ M}$ of the target miRNA-21 using different concentration (1.56 to 3.68 mg/mL) of biotin-MB-AuNPs. The peak current increased with increasing amount of biotin-MB-AuNPs from 1.56 to 3.12 mg/mL. When the concentration of the biotin-MB-AuNPs further increased from 3.12 to 3.68 mg/mL, the peak current decreased slightly, probably due to the use of higher concentrations of the biotin-MB-AuNPs, which seems to have a saturation effect on the signal response [22, 25]. Subsequently, 3.12 mg/mL of the biotin-MB-AuNP was used as the optimised concentration. (Figure 1C).

To define the nature of the capturing layer, optimisation of the amount of neutravidin on the transducer surface was performed by preparing electrodes with various neutravidin concentrations (0.15, 0.25, 0.5 and 1 mg/mL). The responses of the sensors to $0.5 \times 10^{-9}\text{ M}$ of target miRNA-21 are presented in Figure 1D. A closer analysis showed that the normalised signal for the detection of $0.5 \times 10^{-9}\text{ M}$ miRNA-21 increased with decreasing amount of the neutravidin on the electrode surface from 1.0 to 0.25 mg/mL, while a further decrease in neutravidin concentration to 0.15 mg/mL delivered insignificant improvement to the signal response. Therefore, 0.25 mg/mL of neutravidin was used for preparation of the biosensors.

3.4. Sensitivity and selectivity of the miRNA biosensor

The analytical performance of the electrochemical biosensor was investigated under the optimised experimental conditions. After performing the affinity assay step, the electrode was immersed into a measuring cell containing 0.1 M sulfuric acid. The electrochemical oxidation of AuNPs to Au^{3+} ions was performed at +1 V vs Ag/AgCl for 180 s in a unstirred solution. Immediately after the electrochemical oxidation step, stripping square wave voltammetry (SSWV) was performed. During this step the potential was scanned from +1.0 to 0 V (step potential = 10 mV, frequency = 20 Hz), resulting in an analytical signal due to the reduction of Au ions. The modified GCE displayed a well-defined response at +0.84 V vs Ag/AgCl to Au^{3+} . The detection peak corresponded to the reduction of the Au^{3+} to Au^0 on the surface of GCE.

The SSWV peak currents rose significantly with increasing concentrations of target miRNA-21 (insert Figure 2). A good linear relationship between the peak currents and the miRNA-21 concentration was achieved in range of 0.5×10^{-12} to $1.0 \times 10^{-9}\text{ M}$ with a coefficient of determination (R^2) of 0.985 and the limit of detection (LOD) was calculated to be 0.1×10^{-12}

M (defined as $3\sigma/\text{slope}$; $n=3$) (Figure 2). In addition, the reproducibility of the sensor was further investigated by measuring the target miRNA-21 at 50×10^{-12} and 0.5×10^{-9} M with five replicates. The coefficients of variation for both measured concentrations were 4.2% and 5.5%, respectively (Figure S1). Thus, the biosensor displayed high sensitivity and acceptable reproducibility, and could be applied to quantification of miRNA-21 at low concentrations.

The selectivity of the biosensor was evaluated by analysing closely related miRNAs (miRNA-221 and miRNA-205), which are members of the same miRNA family and have both been associated with cancers: miRNA-205 in breast [26], prostate [27], lung [28] and bladder [29] cancers; and miRNA-221 in bladder [30] and as trocytic tumours [31]. Figure 3 shows that a clear response, was observed in the presence of target miRNA-21 (0.5×10^{-12} M) while no significant signal was observed for either miRNA-205 (with 41% similarity) or formiRNA-221 (with 18% similarity), even at significantly higher concentration (1×10^{-9} M). Interestingly, we did not observe a significant influence of miRNA-21, when this was mixed in large excess (1000 fold) with the other non-targeted miRNAs. It should be noted that miRNAs are specific biomarkers present in blood stream and do not offer the possibility for single-base mismatch detection associated with genetic analysis of nucleic acids. Moreover, the non-target miRNA-205 chosen is one of the miRNAs with the highest similarity to miRNA-21.

3.5. Analytical performance of the silver enhanced miRNA biosensor

Enhancement by precipitation of silver onto AuNP labels have been reported to achieve amplified signals and lower detection limits [32,33]. Silver enhancement technology, using voltammetry techniques, has been previously reported [23]. After the affinity capture of the miRNA-21/biotin-MB-AuNP complex onto the electrode surface, the electrode was incubated in a silver enhancement solution composed of silver ions. The AuNPs act as a catalyst and reduce silver ions into metallic silver in the presence of a reducing agent. The reduced metallic silver deposits on the AuNP surfaces, thus forming an enlarged metallic silver cluster around the AuNPs. Subsequently, silver deposition time followed by voltammetric detection were optimised in order to maximise the sensitivity and to shorten the assay time. Figure 4 shows the effect of the silver deposition time on the SSWV signal response for 0.5×10^{-9} M of miRNA-21. The peak current signal increased against the silver deposition time of 2 to 12 min. Ten minutes was chosen as the preferred time since it proved a good compromise between the silver deposition time and the signal response.

The analytical performance of the silver enhanced miRNA biosensors was investigated by varying the target miRNA-21 concentration. After the silver enhancement step, the electrode

was immersed in a measuring cell containing 10 mM nitric acid. Nitric acid is required for the efficient oxidative dissolution of metallic silver. The electrochemical oxidation of metallic silver to Ag^{1+} ions was performed at +1 V vs. Ag/AgCl for 180 s in the non-stirred solution. Immediately after the electrochemical oxidation step, stripping square wave voltammetry (SSWV) was performed. During this step the potential was scanned from 0 to 0.8 V (step potential = 10 mV, frequency = 20 Hz), resulting in an analytical signal due to the oxidation of Ag^{1+} . The modified GCE displayed a well-defined response at +0.27 V vs. Ag/AgCl to Ag^{1+} . The detection peak corresponded to the oxidation of the Ag^{1+} to Ag^{2+} on the surface of GCE.

Experiments showed that the signal obtained from the oxidation peak current of silver could detect miRNA-21 over an extremely wide dynamic range from 10×10^{-15} to 1.0×10^{-9} M covering 5 orders of magnitude of miRNA concentration (Figure 5), where the lowest detectable miRNA-21 concentration was significantly improved by 50 times compared to measurements without silver enhancement. The regression equation of the miRNA biosensor between miRNA concentration of 1.0×10^{-10} to 5.0×10^{-9} M was $Y = 1.62X + 3.47$ (where X is the logarithm of target miRNA-21 concentration (M); and Y is the SSWV peak current (μA)) with a coefficient of determination (R^2) of 0.995, and the limit of detection (LOD) was calculated to be 4.0×10^{-15} M (defined as $3\sigma/\text{slope}$; $n=3$).

A selectivity study of the biosensor was performed after silver enhancement by analysing closely related miRNAs (miRNA-221 and miRNA-205). Figure 6 shows that a clear response, was observed in the presence of target miRNA-21 (10×10^{-15} M) while no significant signal was observed for either miRNA-205 or formiRNA-221, even at significantly higher concentration (1×10^{-9} M). Interestingly, we did not observe a significant influence of miRNA-21, when this was mixed in large excess with the other non-target miRNAs. This result confirms that the addition of silver enhancement did not significantly influence the selectivity of the miRNA-21 biosensors.

3.6. Detection of miRNA-21 in spiked serum sample

Detection of miRNA-21 in spiked human serum samples were investigated using the standard addition method. Serum samples with miRNA-21 concentrations of 50×10^{-15} , 500×10^{-15} , 1.0×10^{-12} and 100×10^{-12} M were prepared and measured. Serum sample with a background level of miRNA-21 equal to 50×10^{-15} M was incubated in the biosensor and the signal fitted to the calibration curve in Figure 5 to calculate an approximate concentration value. After this, solutions containing approximately twice and three times the calculated preliminary concentration were prepared by spiking the sample with adequate volumes of a miRNA-21

stock solution. The responses obtained for the sample and for the spiked solutions were then plotted and miRNA-21 concentration in sample was calculated by extrapolating the linear curve, obtained by plotting the responses vs the nominal concentration of added stock miRNA-21. The measured and the actual concentration of miRNA-21 in the spiked serum samples are compared in Table 1 and the correlation plot (measured concentration using the standard addition method vs the actual concentration) is shown in Figure 7. As it can be seen from Table 1 and Figure 7, there was a good match between the measured experimental values and the nominal concentration of the miRNA-21 in the serum samples (slope of 0.93; $R^2=0.997$). Thus, the developed biosensor allowed accurate detection of miRNA-21 not only in buffer solutions, but also in spiked serum samples.

4. Conclusion

A simple and highly sensitive neutravidin-based biosensor combined with a dual-functional gold nanoparticle (AuNP) biolabel have been developed for microRNA-21 detection. The dual functional probe was comprised of AuNPs coupled with a biotinylated molecular beacon (biotin-MB) for both biorecognition and signal generation. Although the concept of the MB-AuNP label system has been demonstrated with great success for solid-phase affinity bioassays for rapid and simple visual detection of various analytes, it only provided semi-quantitative results and suffered from relatively low sensitivity. We report for the first time the combination of such a dual-functional biolabel for quantitative electrochemical detection of miRNA. In the presence of target miRNA-21, hybridisation takes place resulting in the “activation” of the biotin-MB; this event makes the biotin group, which was previously “protected” by the steric hindrance of the MB stem-loop structure, accessible. Simultaneously, the activated biotin-MB-AuNPs/miRNA complexes become available for capture, via supramolecular interaction onto a neutravidin-modified electrode, for electrochemical transduction within a single step. The captured AuNPs were detected by stripping square wave voltammetry. The performance of the miRNA biosensor was further improved by silver enhancement delivering a detection limit of 4.0×10^{-15} M miRNA-21, and an extremely wide analytic dynamic range from 10×10^{-15} to 1.0×10^{-9} M (5 orders of magnitude). The biosensor offers a combination of simple operation, high sensitivity and wide analytical range for the detection of miRNA and could provide a powerful and convenient tool for biomedical research and applications in cancer diagnostics.

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Figure Captions

Table 1. Actual and measured concentration of target miRNA-21 in spiked serum samples.

Scheme 1. A) Illustration of the design of the miRNA biosensor and B) the working principle of the neutravidin-based biosensor combined with a dual-functional gold nanoparticle (AuNP) probe for miRNA detection.

Figure 1. Optimisation of the miRNA biosensor: (A) the pretreatment time for stripping voltammetry ($E = 1 \text{ V}$ and $f = 20 \text{ Hz}$); (B) the pretreatment potential ($t = 180 \text{ s}$ and $f = 20 \text{ Hz}$); (C) the amount of the biotin-MB-AuNPs biolabel; and (D) the amount of immobilised neutravidin onto GCE ($E = 1 \text{ V}$, $t = 180 \text{ s}$ and $f = 20 \text{ Hz}$).

Figure 2. Calibration curve shows the SSWV reduction peak current as a function of miRNA-21 concentration ($n=3$); (Insert) signal response curves of the biosensor obtained with various miRNA-21 concentrations ($E = 1 \text{ V}$, $t = 180 \text{ s}$, $f = 20 \text{ Hz}$ and 0.1 M sulphuric acid).

Figure 3. Selectivity studies of the biosensor for detection of target miRNA-21; non-specific miRNA-221 and miRNA-205; and miRNA-21 mixed with miRNA-221 and miRNA-205.

Figure 4. The blank and SSWV response of $1 \times 10^{-9} \text{ M}$ miRNA-21 in 10 mM nitric acid with different silver deposition times.

Figure 5. Calibration curve shows the SSWV oxidation peak current after silver enhancement as a function of miRNA-21 concentration ($n=3$); (Insert) signal response curves of the biosensor obtained with various miRNA-21 concentrations concentrations ($E = 1 \text{ V}$, $t = 180 \text{ s}$, $f = 20 \text{ Hz}$ and 10 mM nitric acid).

500 **Figure 6.** Selectivity studies of the biosensor after silver enhancement for detection of target
501 miRNA-21; non-specific miRNA-221 and miRNA-205; and miRNA-21 mixed with miRNA-
502 221 and miRNA-205.

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504 **Figure 7.** A correlation plot between the measured and the actual concentration of miRNA-21
505 in spiked serum samples ($E = 1\text{ V}$, $t = 180\text{ s}$, $f = 20\text{ Hz}$ and 10 mM nitric acid).

506 **Table**

507

508 **Table 1**

Actual concentration	Measured concentration (n=3)
$50 \times 10^{-15} \text{ M}$	$(56 \pm 6.35) \times 10^{-15} \text{ M}$
$500 \times 10^{-15} \text{ M}$	$(577 \pm 34.2) \times 10^{-15} \text{ M}$
$1.0 \times 10^{-12} \text{ M}$	$(1.4 \pm 0.37) \times 10^{-12} \text{ M}$
$100 \times 10^{-12} \text{ M}$	$(108.5 \pm 5.7) \times 10^{-12} \text{ M}$

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Scheme

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Scheme 1

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Figures

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Figure 1

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