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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-21biosensor 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.

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

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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 paramagnetic 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 (H_2SO_4), nitric acid (HNO_3), 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 use 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/GGCCGTCAA***C***ATCAGTC***T***GATAAGC***T***ACGGCCTT-TTTTTTTT/3ThioMC3-D/-3′ (in bold and italics are the LNA bases)

*miRNA-21:*5′-UAGCUUAUCAGACUGAUGUUGA-3′

miRNA-205: 5′-UCCUUCAUUCCACCGGAGUCUGU-3′

miRNA-221: 5′-AGCUACAUUGUCUGCUGGGUUUC-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 $HAuCl_4$. In brief, 50 mL of 1 mM $HAuCl_4$ 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.