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Ultrasensitive and real-time detection of proteins in blood using a potentiometric carbon-nanotube aptasensor

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ABSTRACT

Potentiometric sensing represents the preferred technique in many routine measurements of pH and ions. Unfortunately, the simplicity of the technique has not been exploited so far in high throughput biomolecular sensing. In this work, we demonstrate the capabilities of the hybrid functional material carbon nanotubes/aptamer for the creation of a new generation of nuclease-resistant aptasensors using the potentiometric transduction capabilities of single-walled carbon nanotubes in combination with the recognition capabilities of a protein-specific RNA aptamer. The aptasensor was used to detect and identify disease-related proteins at attomolar concentration values in a rapid and non-expensive way. The variable surface glycoprotein from African Trypanosomes was chosen as an ideal model system for a pathogenic exoantigen protein in a clinical sample. Variations in the electromotive force are achieved in real-time upon the direct addition of diluted real blood samples containing the target protein thus eliminating the need of preliminary matrix removal. This work would open the door to real-time diagnostic assays for a wide range of diseases, but also to the rapid molecular detection of several proteins in truly customizable protein biosensing platforms.

1. Introduction

Detection of proteins is crucial in many areas such as clinical diagnostic, proteomic research and consumer diagnostic products as in home pregnancy tests (Walker, 2002; Zourob et al., 2008). The most commonly used methods rely on highly sensitive and specific label-based immunoassays (Walker, 2002). Among this type of techniques, the methods most widely used in diagnostics rely on westernblot assays, immunoblotting techniques, immunoprecipitation, immunofluorescence and immunosorbent assays (Walker, 2002; Kurien and Scofield, 2009; Scopes, 2010). Although these methods are highly sensitive and specific, they are unsuitable for high-throughput applications because they are labor-intensive, time-consuming and require highly trained staff and expensive equipment. Furthermore, an accurate quantification of the analyte is not possible and false negative results can be obtained if the target protein is not extracted from the matrix effectively.

Nanostructured materials possess outstanding properties, which allow the design of simplified protein detection platforms at lower detection limits within shorter assay times than traditional techniques (Durner, 2010). Interesting approaches on the development of detection platforms based on quantum dots, nanoparticles, nanotubes, nanorods, nanowires and nanosheets that make use of their optical, electrical, magnetic or catalytic properties have recently been explored in protein detection at very low concentrations either for diagnosis of infectious diseases or in proteomic research (Goulart et al., 2010; de la Escosura-Muñiz and Merkoçi, 2010). Biosensors that include nanostructured components as photoluminescent viral nanoparticles (Park et al., 2009), magnetic nanoparticle-based bio-barcodes (Nam et al., 2003) fluorescence-based immunoassays (Konry et al., 2009; Kim et al., 2009), immunobead-based assays for multiplexed protein detection (Rusling et al., 2010), biodetection with localized surface plasmon resonances (Chen et al., 2009), arrays of green fluorescent protein and nanoparticles to differentiate proteins using lineal discriminant analysis (De et al., 2009) or optical resonators (Armani et al., 2007), have demonstrated important advances in ultrasensitive protein detection. However, electrochemical sensing techniques are preferred over others because they are fast, easy to use, cheap, small-sized and easily miniaturized. Recent work has shown that electrochemical techniques in combination with (bio)nanostructured materials as the transducer part achieved detection limits of 0.9 pg mL^{-1} for various proteins in serum using nanowire nanotransistors (Zheng et al., 2005) or detection limits of pg mL^{-1} for several proteins in serum

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using amperometric immunosensors based on single-walled carbon nanotubes (SWCNT) (Yu et al., 2006). Voltammetric sensors have also been used for the detection of pg/mL concentrations of platelet-derived growth factor protein using a surface hybridization assay consisting of a pair of aptamer probes with a short complementary sequence that recognizes different sites of target protein (Zhang et al., 2009), or for the detection of protein-small molecule interactions directly in serum with limits of detection at the nM concentration level (Cash et al., 2009). Other methods allowed biomolecular sensing down to pM concentration (Kara et al., 2010; An et al., 2010), fM concentrations (Moreau et al., 2008; Star et al., 2006; Byon and Choi, 2006; So et al., 2005; Park et al., 2002; Estrela et al., 2010) or even concentrations of 30 aM (Munge et al., 2011) using an amperometric sensor electrode coated with Au nanoparticles with attached antibodies for the detection of cancer biomarker interleukin 8, and in the case of DNA detection also down to the aM concentrations (Kurkina et al., 2011). Potentiometry is one of the most common, cheapest, simplest and portable electrochemical techniques and it is widely used in the detection of ions for many decades. However, its use in label-free protein detection at concentration levels similar to those reached so far in DNA biosensing still remains a challenge.

Recently, our group demonstrated the ultrasensitive detection of bacteria in buffered samples using SWCNT and aptamer-based biosensors (Zelada-Guillén et al., 2009) and even the detection of bacteria in food samples using a filtration system for previous matrix elimination. The excellent potentiometric transduction properties of SWCNTs due to the extremely high surface-to-volume ratio, the material's ability to support charge transfer between heterogeneous phases and its extraordinary double layer capacitance (Yáñez-Sedeño et al., 2010; Düzgün et al., 2011), combined with the quasi-unlimited capability of aptamers (RNA and DNA artificial oligomers) to be tailored in vitro against ions, proteins, viruses and bacteria (Tombelli et al., 2005) convert such a platform into a tool with infinite possibilities in real-time biosensing. However, the use of aptamer-based biosensors (so-called aptasensors) has been mostly limited to non-clinical samples so far, since the degradation of aptamers by nucleases in biofluids such as blood, severely affect their performance. Furthermore, SWCNT are highly sensitive to changes in the ionic environment at their interface as well as to redox conditions in the solution (Zelada-Guillén et al., 2009). As a result, it has remained unclear whether this technique could be directly used to detect traces of small-sized targets such as disease-related proteins, at clinically relevant conditions, without the need of preliminary matrix removal (Zelada-Guillén et al., 2010). We recently selected a family of RNA aptamers that bind to a structurally conserved domain of both, cell-anchored and free (soluble) variable surface glycoproteins (VSG) from African trypanosomes with high affinity and specificity. In addition, the RNAs are highly nuclease-resistant due to the presence of 2′ F-substituted C and U-nucleotides (Lorger et al., 2003). The VSG protein (more information in Supporting information) is an ideal model system for a pathogenic exoantigen protein in clinical samples in view of its high stability in blood serum. Therefore, here we demonstrate the real-time and ultrasensitive identification of medically relevant proteins in a highly complex matrix, such as blood, using a new generation of nuclease-resistant hybrid nanostructured material in potentiometric aptasensors without the need of any sample pretreatment or matrix elimination facilitated by an appropriately tailored buffer.

2. Materials and methods

2.1. Chemicals and solutions

SWCNTs were purchased from HeJi (Zengcheng, China) with $>90\%$ purity, 150 μm average length and 1.4–1.5 nm diameter. The SWCNTs were oxidized in a silica furnace chamber (365 °C, synthetic air flow-rate of 100 $cm^3\ min^{-1}$, 90 min) to remove the amorphous carbon. Afterwards, SWCNTs were refluxed in 2.6 M nitric acid for 4 h to both carboxylate them and oxidize the metallic impurities remaining from the synthesis (Furtado et al., 2004). The SWCNTs were then filtered and thoroughly rinsed with water. SWCNTs were finally were dried overnight at 80 °C.

All the solutions were prepared under sterile conditions using distilled and deionized water previously purified through a Milli-Q system (Millipore, Madrid, Spain) with a resistivity level of 18.2 MΩ cm and pH adjusted accordingly. Molecular biology grade reagents ($>99.5\%$ purity) sodium sodium dodecyl sulfate (SDS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethanesulfonic acid (MES), and cetyltrimethylammonium bromide (CTAB), bovine serum albumin (BSA), human Immunoglobulin G (IgG), KH_2PO_4, Na_2HPO_4, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, NaCl, ethylenediaminetetraacetic acid (EDTA), trans-1,2-cyclohexanediamine-N,N,N′,N′-tetraacetic acid (CDTA) and phosphate buffer solution (PBS) were purchased from Sigma-Aldrich (Tres Cantos, Spain) and used as received. Human α-thrombin was supplied by Haematologic Technologies (Vermont, USA). The working buffer solution used in the potentiometric experiments (NaCl 127 mM, phosphates 1.67 mM, EDTA 2.6 mM, CDTA 2.6 mM, $K_3Fe(CN)_6$ 2 mM and $K_4Fe(CN)_6$ 2 mM, pH 7.4) was prepared starting from a 1:100 dilution of a 0.167 M sterile stock solution of corresponding amounts of KH_2PO_4 and Na_2HPO_4, while the appropriate amounts of NaCl, EDTA, CDTA, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were added under sterile conditions. NaCl was used to adjust both osmotic pressure and ionic strength of the solution. The phosphate buffer was pH adjusted to the normal value of healthy human blood. EDTA was added as an anticoagulant and together with CDTA as efficient chelating agents. In all solutions, both chelators were kept constant in order to chelate normal amounts of free Ca^{2+} and other chelatable cations. Buffer solutions with different concentrations of the components previously mentioned were prepared in the same way.

2.2. RNA aptamer synthesis and binding affinity measurements

The VSG-specific, nuclease-resistant RNA aptamer cl57 (Lorger et al., 2003) with the sequence GGGAGACGAUAUUCGUCCAUCAGCGCGCACCUACUGUGAUGUAGAAGUCACAGCAAGGCCCCGCUGUCCGACUGAAUU was synthesized by run off in vitro transcription in the presence of 2′-F-uridine-5′-triphosphate and 2′-F-cytidine-5′-triphosphate (2 mM each). Full length transcripts were purified in 8 M urea-containing polyacrylamide gels and subsequentially oxidized at the 3′ end with $NaIO_4$ (38 mM) in 50 mM NaOAc pH 4.8, 100 mM NaCl, 10 mM $MgCl_2$. Oxidized RNA was purified by size exclusion chromatography, EtOH precipitated and resuspended in 100 mM $Na_xH_yPO_4$ pH 7.2, 150 mM NaCl. Reaction yields were $\geq 90\%$ as determined by 3′ end labeling using 5′-(^{32}P)-pCp and T4 RNA ligase. Oxidized aptamer preparations (approximately 10 μM) were further converted into a hydrazide derivative by overnight incubation at 4 °C in the dark with 22 mM freshly prepared adipic acid dihydrazide (ADH) in the presence of 110 mM $NaBH_3CN$ in 100 mM $Na_xH_yPO_4$ pH 7.2, 150 mM NaCl (Hermanson, 2008). Hydrazide-derivatized RNA was purified by gel filtration, EtOH precipitated and redissolved in 100 mM $Na_xH_yPO_4$ pH 7.4, 120 mM NaCl, 2.7 mM KCl before storage at −20 °C. Reaction yields were 73%. The aptamer cl57/VSG interaction was routinely tested by nitrocellulose adsorption using 5′-(^{32}P)-labeled cl57 RNA preparations. Depending on the VSG preparation the derived equilibrium dissociation constants (Kd) varied between 0.3 and 14 nM with an average of 7 nM. The affinity of surface-immobilized cl57 preparations to VSG was analyzed in real time using a surface plasmon resonance (SPR)

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