TITLE:

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.

KEYWORDS:

Aptamers, biosensors, carbon nanotubes, potentiometry, proteins.

MAIN TEXT:

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 western-blot 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 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 platelete-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 $cm^3 \cdot min^{-1}$, 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 Mili-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, $K_3Fe(CN)_6$ 2mM and $K_4Fe(CN)_6$ 2mM, 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 ≥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)-labelled 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) read-out system (Golas et al. 2009). The derived Kd's varied between 278-492 nM, which indicates an approximately 60-fold reduction in the affinity due to the covalent attachment of the 3'-end of the aptamer.

2.3 Electromotive force measurements

Electromotive force (EMF) measurements were automatically taken at periods of 10 s in all cases with a high-input impedance voltmeter model EMF-16 (Lawson Laboratories, Inc., Malvern, PA, USA) using a Ag/AgCl/KCl (3 M) double junction reference electrode containing a 1 M LiOAc electrolyte bridge (type 6.0729.100, Metrohm AG, Herisau, Switzerland) and the SWCNT-based electrode or aptasensor as the working electrode. Constant stirring at 300 rpm was applied during all the potentiometric measurements in an isothermal vessel at 22 ±0.5 °C containing 5 mL of sterile working buffer solution before sample addition. Analyzed protein solutions were prepared from standardized 8.33 µM stock solutions and serially diluted 1:10 in working buffer. Stepwise increasing concentrations of the proteins were introduced into the electrochemical cell by addition of the previously prepared serial dilution and dilution factor correction was applied accordingly. Aptasensors and SWCNT electrodes were always preconditioned in working buffer before every electrochemical measurement. After each round of use with protein-containing aqueous solutions, aptasensors were regenerated in 2 M NaCl for 1 hour, further washed in deionized water for 90 minutes and kept in working buffer until the next use. Aptasensors exposed to blood-containing samples were regenerated in 2 M NaCl overnight under stirring mode, washed generously in deionized water and kept in PBS 1.67 mM pH 7.4 until needed again, and then, aptasensors were again preconditioned in working buffer solution before use.

2.4 Preparation of the aptasensors

The physical support and electric contact for the SWCNT electrodes consisted of a 50 mm long and 3 mm diameter glassy carbon cylindrical rod (HTW Hochtemperatur-Werkstoffe GmbH, Thierhaupten, Germany) covered by a Teflon jacket of 7 mm diameter. One of the exposed ends was successively polished with 25, 10 and 1 µm grain size polishing alumina (Buehler, Lake Bluff, USA) before the SWCNT layer was deposited on the same surface, while the other end served as a connector to the voltmeter (Figure 1a). A 30 µm SWCNT layer (measured with a FEI Quanta 600 ESEM, from FEI Co., Hillsboro, U.S.A., see Figure 1b-c) was deposited by spraying a dispersion of 25 mg of the purified SWCNTs with 25 mg SDS and 100 mL of Milli-Q water homogenized with a tip-sonicator for 30 minutes (amplitude 60%, 0.5 s^{-1}, Ultraschallprocessor UP200S, Dr. Hielscher, Teltow, Germany). Ten milliliters of the SWCNT prepared solution was sprayed onto the glassy carbon surface at high temperature (200 °C) and washed with sterile Milli-Q water intermittently. The residual SDS was removed by heating at 280 °C with air flow rate 100 $cm^3 \cdot min^{-1}$ for 1 h (Teflon jacket was temporarily removed during this step). Finally, the SWCNT electrodes were profusely washed with sterile Milli-Q water, dried overnight at 80 °C, sterilized with UV irradiation and stored at sterile conditions before use. The carboxylic groups on the sidewalls of the deposited SWCNTs were activated with a solution of 100 nmol of EDC and 25 nmol of NHS in 50 mM MES buffer pH 5 for 30 minutes (Jung et al. 2004; Wong et al. 1998). The SWCNT electrodes were then incubated overnight at room temperature in 500 µL of a 1 µM RNA aptamer cl57 solution, which also included PBS 1mM (pH 7.4) and CTAB 0.2 mM. This well-known carbodiimide mediated chemistry was performed in order to form stable amide bonds between the carboxylic groups on the SWCNT sidewalls and the free $-NH_2$ moieties on the 3' end of the aptamers (Figure 1d). Aptasensors where then washed with sterile deionized water and stored at sterile conditions in PBS 1.67 mM pH 7.4 until needed.

2.5 Preparation of Variable Surface Glycoprotein

The bloodstream life cycle stage of *Trypanosoma brucei* (T.*b.*) *brucei* was cultivated at 37 °C in HMI-9 medium (Hirumi and Hirumi 1994) supplemented with 10% (v/v) heat inactivated bovine fetal calf serum. The following trypanosome strains were used: *Trypanosoma brucei* Lister 427 – MiTat serodeme, variant clones 1.4 and 1.2 (Cross 1975). Long slender bloodstream forms of *Trypanosoma brucei* AnTat 1.1 were harvested from infected rats (Michiels et al. 1983). Soluble VSG protein was isolated essentially as described by Cross (Cross 1984). *Trypanosoma brucei gambiense* LiTat 1.1, LiTat 1.3, LiTat 1.5, LiTat 1.6, *Trypanosoma brucei rhodesiense* ETat 1.2 and *Trypanosoma evansi* RoTat 1.2 were kindley provided by Philipp Bücher, Antwerp. All VSG isolates were purified by anion exchange chromatography and analyzed in discontinuous SDS-containing polyacrylamide gels. VSG homodimer formation was verified by size exclusion chromatography and protein folding was analyzed by circular dichroism spectroscopy. VSG stock solutions were stored at -80 °C.

2.6 Clinical samples

Venous blood was drawn from healthy donors in blood collection tubes (Beckton, Dickinson and Company, Sparks, USA) following standard phlebotomy techniques. Blood was used within the following two hours after extraction. Blood samples containing different VSG concentrations were prepared by inoculating the protein accordingly in a sterile manner and used within the same time frame previously mentioned. Ethical review board approval and donor's informed, signed consent was obtained for this study. Blank blood and blood containing VSG were diluted (1:100) in working buffer before inoculating the electrochemical cell during experiments for aptasensor performance measurements.

3. Results and discussion:

To minimize the impact of the blood matrix, the working buffer was tailored by adjusting the osmotic pressure, the ionic strength (I) and pH to near physiological values. During the tailoring process, we performed direct additions of non-diluted blood samples to analyze the solutions' buffering capacities. For this purpose, we recorded EMF response profiles after addition of pure blood to electrochemical cells containing different non-functionalized SWCNTs sensors in buffer with NaCl/phosphates/EDTA/CDTA at the concentrations described in the Materials and methods section. In such a case, the EMF signal stabilized with a slow kinetic mainly driven by the redox reactive species present in blood, stochastic charge transfer between charged red blood cells and the biosensor surface as well as unspecific adsorption phenomena of charged free proteins (Simon et al. 1984; Khubutiya et al. 2010). $Fe(CN)_6^{4-}/^{3-}$ was added at a low concentration of 2 mM (Total I=185 mM) to serve as an electron-transfer agent and as a redox-buffering agent. In this manner, the buffer in the electrochemical cell easily reaches the electrochemical equilibrium with the blood sample, and consequently, signal drift phenomena is substantially minimized (Figure 2). Isotonic buffer conditions ensured that the destruction of red blood cells was minimized. Otherwise, when hypotonic buffers with low ionic strength are used, large quantities of redox-active hemoglobin can be released and undesired collateral redox reactions with $Fe(CN)_6^{4-}/^{3-}$ could take place. Higher ionic strengths (I>185 mM) were also tested, but despite the observed higher buffering capacity, the aptamer-VSG recognition was ionically screened and no response was observed. Finally, unspecific changes in the EMF response were eliminated by diluting blank blood samples (1:100) in working buffer before the addition into the electrochemical cell (Figure 2a). From Figures 2a and 2b, it is evident that a low dilution of blood (1:10 to 1:1000) in buffer without $Fe(CN)_6^{4-}/^{3-}$, prior to the addition to the electrochemical cell did not represent any real solution for the otherwise observed low stabilization time and undesired drift. Consequently, all the potentiometric measurements performed with the aim to assess the aptasensor performance in either real samples or only aqueous buffer solution where therefore carried out with $Fe(CN)_6^{4-}/^{3-}$ at 2 mM.

The aptasensors were exposed to increasing stepwise concentrations of a single antigenic VSG type in sterile buffer (the VSG variant used was VSG117, see supporting information), while the EMF between the aptasensors and a Ag/AgCl double junction reference electrode was recorded in real-time at 22±0.5 °C in stirring mode. This stage was performed to explore the aptasensor response towards the target analyte. After that, the sensor was exposed to blood samples (diluted 1:100 in buffer) containing VSG at different concentrations to analyze the aptasensor response at clinically relevant conditions.

An instrumental noise (3 x standard deviation of the average noise (Currie 1995)) equivalent to ±24.6 µV was observed during all experiments. This indicates that EMF changes larger than the previous value could be detected. Potentiometric response in buffer was found to be distinguishable from the instrumental noise at a concentration of 300 aM (Figure 3a). The biosensor response observed at higher concentrations showed two clearly differentiated regions termed region I and II. At VSG concentrations lower than 10 fM steep slope was measured (region I) likely the result of the high affinity interaction between the target protein and the aptamer. Above this value, the response was less pronounced (region II) presumably representing the saturation of available binding sites on the surface of the VSG protein (Figure 3b). For simplicity, both regions can be fitted to a linear model. From the data, it was possible to determine the relative standard deviation (RSD) for the six sensors used as 23%. Importantly, both regions and their related linear approximations can be useful in either quantitative or semi quantitative assays. In any case, immediate changes in EMF were observed at all concentrations. At high VSG concentrations, a stable EMF response was reached after 2-5 minutes. At low VSG concentrations the response took about 10 minutes likely due to the low concentration of target molecules within the solution.

Control assays were performed with non-functionalized SWCNT sensors by measuring EMF responses for increasing VSG concentrations in pure buffer. No response was observed in the concentration range 10^{-16} to 10^{-8} M (100 aM to 10 nM). Selectivity assays were carried out with different serum proteins such as thrombin, immunoglobulin G and BSA (Figure 3c). No significant responses were measured in any case from 10^{-15} M to 10^{-9} M (fM to nM). In addition, we measured the aptasensor potentiometric response and sensitivity for 8 different VSG variants. For simplicity purposes the VSG variants were renamed as VSGa, VSGb … VSGg, VSGh (see supporting information for detailed description). For the first 5 VSG variants

(VSGa-e), the detection results were similar to those observed for the VSG117. The response profiles showed the characteristic separation into regions I and II, with slopes ranging between 150 to 243 µV/Log10[VSG] (region I) and 18 to 54 µV/Log10[VSG] in region II. The last three VSG variant preparations (VSGf-h) did not produce any response. This indicates that the aptasensor is capable of recognizing isotypic VSG types and conserved epitopes between different VSG families, which is important considering the antigenic switching during the course of an infection. Variation in the EMF response slopes might be influenced by differences in the number of negative surface charges between the different VSG antigenic types or by small isomorphic differences between epitopes of different VSG (Rautenberg et al. 1981; Souto-Padrón 2002). The inability of the aptasensor to recognize every VSG variant can be due to a variety of reasons. This includes protein denaturation phenomena during the extraction/purification of the protein as well as glycosylation differences of the different VSG, which might mask the aptamer/protein interaction. Additional experiments are needed to unravel the origin of such a behavior at the molecular level.

Finally, the aptasensors were exposed to stepwise increasing concentrations of VSG in blood at concentrations between 1 pM to 1 nM. This corresponded to a range of VSG concentration between 10^{-16} and 10^{-12} M (aM to pM) in the electrochemical cell after addition of the previously diluted (1:100) blood samples. The sensors responded to the presence of VSG at concentration as low as 10 pM in blood (4 fM in the cell, Figure 4). The total analysis time for blood samples containing the VSG protein ranged between 2 and 10 minutes depending on the time needed for signal stabilization. In order to determine whether the aptasensors are capable of distinguishing between blood samples without target protein and those containing VSG, we performed consecutive additions of blood samples (diluted 1:100) either containing VSG or without it, into an electrochemical cell with the same aptasensor. The change in EMF observed after the addition of VSG-containing blood samples (when a change observed was greater than 3 times the average noise standard deviation) and the absence of response when samples without VSG were added, confirmed that the signal was produced by the recognition of the target protein by the aptasensor. Moreover, during the same experiment, each aptasensor was able to undergo at least 10 consecutive blood sample inoculations into the electrochemical cell without losing its capability to differentiate between those samples containing VSG from samples of blank blood. No difference was observed when different Rh blood groups were analyzed. Parallel control assays were carried out with aptasensors and SWCNT sensors exposed to successive additions of blank blood (diluted 1:100) in buffer, as well as SWCNT sensors exposed to VSG-containing blood 1:100 diluted in buffer at concentrations ranging from 1 pM to 1 nM (which corresponded to a concentration range between the aM and fM order in the electrochemical cell), but no response was recorded. The absence of EMF changes in the previous control experiments confirmed that the observed potentiometric response is driven by the interaction between the aptasensors and the target protein. Recent studies have demonstrated that the aptamers at similar conditions are ionically charged and self-assembled to SWCNT by π-π stacking interactions (Yang et al. 2010; Johnson and Johnson 2008). In the presence of a charged target analyte, aptamers adopt the conformational state needed to recognize the epitopes (Bouvier et al. 2011) and the target is consequently tethered to the nanotubes, thus, switching of the SWCNT surface charge takes place and this process results in a change of the EMF recorded (Zelada-Guillén et al. 2009). In the case of the recognition of the negatively charged VSG protein by the aptasensor, a similar behavior is therefore expected. However, further research is currently being carried out in order to unravel this trend in this type of sensors. The aptasensors were easily regenerated by a 1 h incubation in a 2M NaCl solution, followed by a washing step in deionized water (90 minutes). Preconditioning for the next round of usage was performed overnight in working buffer. No performance loss was observed during

5 months of use in buffer solutions. However, after the exposure with diluted blood samples, aptasensors needed to be washed thoroughly overnight after the 2M NaCl regeneration step and kept in phosphate buffer before the next round of usage in order to keep the aptasensors functional.

4. Conclusions:

For many decades now, potentiometric ion-selective electrode 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. The hybrid nanostructured nuclease-resistant aptasensor used in this work represents a new step in protein detection due to its specific recognition capacity at attomolar concentrations in real-time (concentration of 4 fM in the cell using blood). The immediate identification of exoantigens in blood would open the door to real-time diagnostic assays for a wide range of diseases related to viruses, bacteria or parasite infections, but also to the rapid molecular detection of several other proteins and protein biomarkers in truly customizable protein biosensing platforms.

ACKNOWLEDGEMENTS:

We thank S. Moreno (Department of Biochemistry, URV), S. Macho and T. Codera (Department of Analytical and Organic Chemistry, URV) and P. Bücher (Antwerp) for support. Philipp Weil is acknowledged for his contribution to the aptamer/VSG affinity measurements and we thank R. Peeling (London School of Hygiene & Tropical Medicine) for suggestions. This study has been supported by the Spanish Ministry of Science and Innovation (MICINN) through project grant CTQ2010-18717 and the Howard Hughes Medical Institute (HHMI) to H.U.G. G.A.Z.-G. thanks the Generalitat de Catalunya (Catalan Government) and the European Social Fund for awarding the FI-DGR fellowship. A.T-K. thanks the Doris Duke Charitable Foundation for awarding the Doris Duke International Clinical Research Fellowship.

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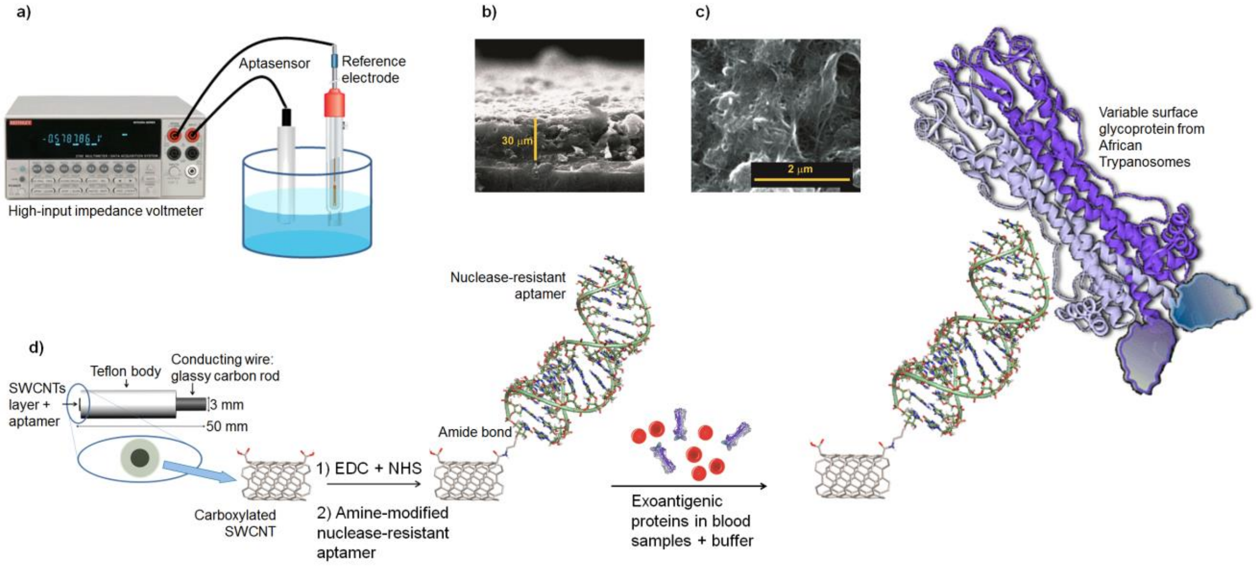
FIGURES:

Figure_1:



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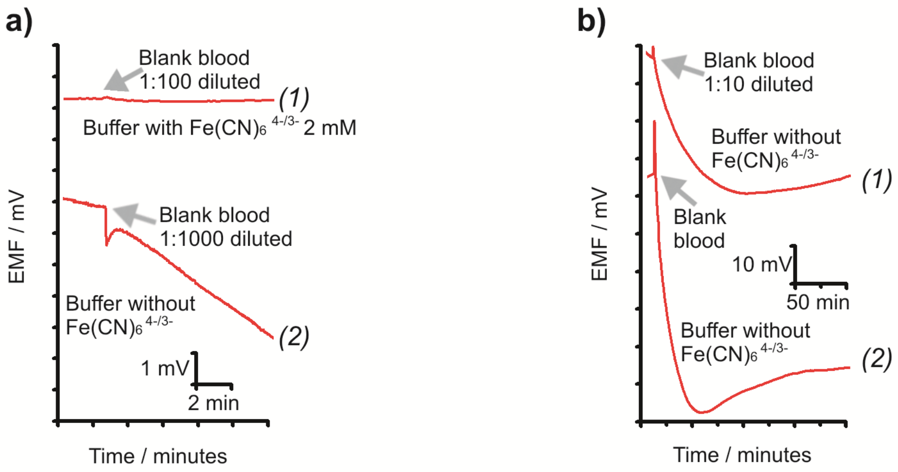

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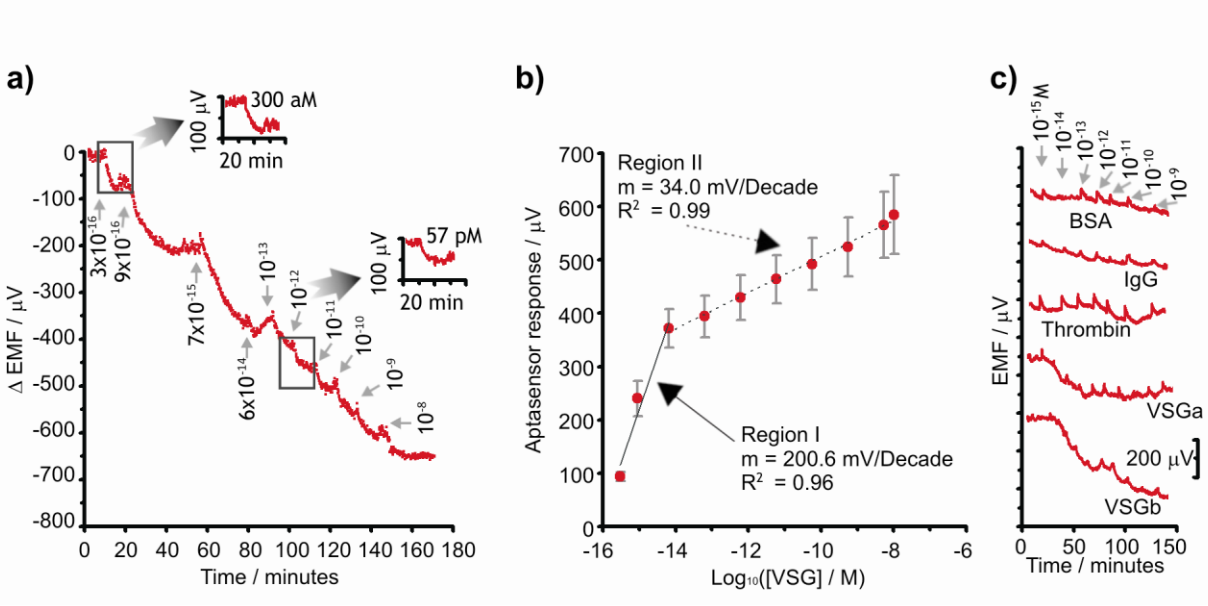

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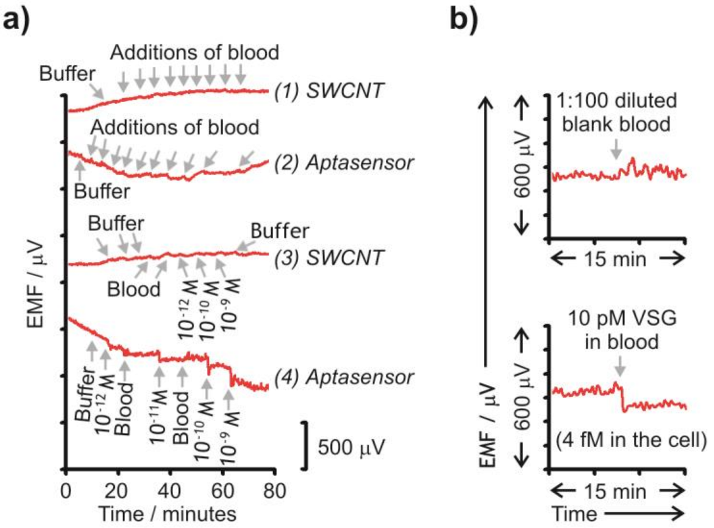

LEGENDS:

Figure 1. a)Experimental set-up for potentiometric measurements; (b and c) ESEM image of the transducing layer formed by the hybrid material SWCNT/aptamer: b) lateral view, the thickness is indicated by the yellow bar; c) surface view, a network of the deposited SWCNTs can be easily distinguished; d) Schematic illustration of the potentiometric aptasensor including the covalent functionalization of carboxylated SWCNTs with the VSG-specific nuclease resistant aptamer following carbodiimide-mediated chemistry and the interaction between aptamer and VSG. For the sake of clarity in the functionalization stage the aptamer is shown away from SWCNT although aptamers are known to strongly adsorb over SWCNTs (Johnson 2008).

Figure 2. In a and b we observed the change in EMF originated in SWCNT-based sensors after the addition of blood (without VSG) at different buffer conditions (I=185 mM): (a 1) blood 1:100 diluted in buffer with $Fe(CN)_6^{4-}/^{3-}$ 2 mM; (a 2) blood diluted 1:1000 in buffer solution without $Fe(CN)_6^{4-}/^{3-}$; (b 1) blood diluted 1:10 in buffer solution without $Fe(CN)_6^{4-}/^{3-}$; (b 2) non-diluted blood, added to buffer solution without $Fe(CN)_6^{4-}/^{3-}$.

Figure 3. a) Performance of the aptasensor when exposed to increasing concentrations of VSG in buffer (ΔEMF indicates the difference between the measured EMF value and the average baseline recorded before VSG addition). b) Potentiometric response obtained for 6 different aptasensors. Solid circles are the average absolute value of ΔEMF; error bars are SD; the slope m and R^2 values for the linear approximation of regions I and II are indicated by arrows. c) Performance of the aptasensors when exposed to stepwise increasing concentrations of different proteins in working buffer solutions: from above to below, EMF recorded when the aptasensors were exposed to increasing concentrations of BSA, IgG, thrombin, and to VSGa and VSGb. Each small arrow in gray indicates the point of protein addition in M units.

Figure 4. a) Change in EMF recorded for the consecutive additions of blank blood 1:100 diluted in buffer to (1) a SWCNT sensor without aptamer and (2) an aptasensor. Change in EMF recorded for the consecutive additions of VSG-containing blood 1:100 diluted in buffer to (3) a SWCNT sensor without aptamer and (4) an aptasensor. b) Change in EMF after addition of either 1:100 diluted blank blood or blood containing VSG (data from curve 4 in a). Gray arrows indicate the point of addition, the concentrations are in M units and represent the concentration of VSG in blood before diluted 1:100, blood additions are addition of blank blood 1:100 diluted. The response observed when VSG-containing blood 1:100 diluted is added to aptasensors (a4 and b) and the lack of signal in the controls (a1 to a3) confirm that the change on EMF is produced by the specific recognition event between the aptasensor and the target protein.