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Construction and validation of a *Sambucus nigra* biosensor for cancer-associated STn antigen

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ABSTRACT

A label-free electrochemical impedance spectroscopy biosensor for selective detection and discrimination of the cancer-associated sialyl-Tn (STn) antigen was developed by using *Sambucus nigra* agglutinin type I (SNA-I) as the recognition element. The SNA-I biosensor was constructed by immobilizing the lectin on screen-printed gold electrodes. The formation of a complex between SNA-I and STn-containing glycoproteins (transferrin and bovine submaxillary mucin) was monitored by measuring the impedance increase of the biosensor. The increase in electron transfer resistance was linearly proportional to the concentration of the glycoproteins up to 70 ng of transferrin and 40 ng of bovine submaxillary mucin, with a limit of detection of 20 ng for transferrin. Albumin, the most abundant serum protein, did not interfere in the detection of the STn-glycoproteins up to a concentration of 0.2 mg ml^{-1}.

The developed lectin-based biosensor was used to evaluate the STn-expression in serum samples and discriminate samples from healthy individuals and patients with different types of malignant tumors, mostly carcinomas, where the increased expression of STn aberrant glycans is well established.

This work demonstrates the feasibility of employing SNA-I to selectively recognize the STn epitope in glycoproteins and the use of the constructed biosensor was effective in the analysis of serum samples with the ability to discriminate in a fast way between cancer and healthy status. The proposed biosensor could be used for high-throughput, label-free profiling of the cancer-associated STn glycan expression in serum for diagnosis and therapy monitoring.

1. Introduction

Point-of-care detection of disease biomarkers is, nowadays, one of the main challenges in the biosensor research field. Ideally, measurements need to be done at high accuracy and cheaply at point-of-care location to reduce costs, minimize sample degradation, accelerate diagnosis and minimize patient stress (Rusling et al., 2010; Soper et al., 2006). This is particularly important in cancer, since the outcome of the disease is highly affected by the early detection, as well as a personalized, targeted and monitored therapy (Rusling et al., 2010). The use of biomarkers for cancer detection in the clinic could, therefore, decrease the number of cancer related deaths and improve therapeutic efficacy.

It is known that cellular glycosylation profiles change significantly during oncogenesis (Reis et al., 2010). Glycan biosynthesis and diversity are affected by the altered expression of glycogenes controlling glycosylation, which can result in the expression, by the tumor, of antigenically distinct glycoproteins. These aberrant and cancer-specific glycoproteins constitute targets for cancer biomarkers search.

The shortening of *O*-glycan chains is one of the most frequently reported tumor-associated alterations in glycosylation (Kannagi, 2004; Dalziel et al., 2001). These truncated *O*-glycans have been identified as a hallmark of glycosylation in cancer for decades (Hakomori, 2002; Springer, 1984; David et al., 1992). Nevertheless, because of their complex nature, it has been difficult to exploit those changes for clinical use.

The carbohydrate antigens STn, Tn and T are some of the most immature and truncated *O*-glycans, which are recognized as pan-carcinoma antigens. These structures are widely expressed on glycoproteins produced by cancer cells but not by normal tissues (Cao et al., 1996; David et al., 1992), which generally produce more complex and branched structures (Brockhausen et al., 2001).

Glycans are characterized by an enormous complexity and, at the same time, present similar physicochemical properties, which

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is the main limitation for glycomics laging behind advances in genomics and proteomics (Bertozzi and Kiessling, 2001). Identification of glycans has been achieved by the use of lectins or monoclonal antibodies, high performance liquid chromatography, two-dimensional gel and capillary electrophoresis, mass spectrometry and, more recently, optically labeled lectin arrays (Hsu et al., 2006; Rusling et al., 2010; Yoichiro et al., 2006; Zheng et al., 2005). Although these approaches have been partially successful, the field would greatly benefit from additional bioanalytical tools with the capacity to scrutinize glycosignatures rapidly.

Screen-printed electrodes (SPE) have widespread application in environmental, industrial and clinical fields, due to their attractive characteristics such as portability (allowing point-of-care analysis), operation simplicity, reliability and inexpensive manufacture. Furthermore, the use of these small devices requires minute amounts of sample, which is highly desirable in the clinical field (Belluzo et al., 2008; Mascini and Tombelli, 2008; Soper et al., 2006).

Biosensors developed for the detection of cancer-associated glycan epitopes have been based mainly on the use of lectins, taking into account the ability of these natural proteins to recognize and selectively bind to carbohydrate structures (Jelinek and Kolusheva, 2004; Bertók et al., 2013a). Given the selectivity of each lectin for a determined carbohydrate structure, even oligosaccharides with identical composition in simple sugars may be distinguished. Furthermore, the affinity between a lectin and a glycan structure may vary due to small changes in the glycan structure. These properties of lectins allow to isolate glycan structures from complex samples (Jelinek and Kolusheva, 2004; Bertók et al., 2013a).

The detection of carbohydrate-mediated biological interactions may be, then, carried out by different techniques, such as electrochemical impedance spectroscopy (EIS). This technique allows the study of recognition events between molecules by measuring the electrochemical changes induced by binding reactions (Katz and Willner, 2003). EIS presents some attractive features that enable its use in glycan analysis, namely rapidity in label-free assays, simplicity and amenability to miniaturization, which may be translated into low-cost online or on-site detection devices. These advantages make EIS devices especially useful for point-of-care applications (Wang, 2006). In glycomics, label-free modes of operation are desirable since labeling can introduce unwanted variability in the biorecognition event (Gemeiner et al., 2009), besides increasing the assay time and complexity.

Lectin-SPE biosensors for the detection of glycan epitopes using EIS as detection technique have been extensively reviewed (Bertók et al., 2013a; Cunningham et al., 2010; Gerlach et al., 2010; Sánchez-Pomales and Zangmeister, 2011; Zeng et al., 2012). In spite of the well-established desirable properties of lectins to detect specific glycan structures, and the fact that tumor cells secrete altered glycoproteins which can be used as cancer biomarkers, very little has been reported about the development of lectin biosensors for the detection of cancer-associated truncated *O*-glycans in serum. A lectin biosensor for the cancer-related T antigen was developed using the affinity of *Arachis hypogaea* agglutinin (PNA) for this epitope (Dai et al., 2006). From the same group, another PNA biosensor and a *Sambucus nigra* agglutinin (SNA) biosensor were reported, for the detection of glycoconjugates carrying the T and STn epitopes, respectively (La Belle et al., 2007). The proposed biosensors proved to be effective in the detection of glycan-lectin interactions in a label-free fashion, with potential applications in the detection of cancer biomarkers, and the technique was shown to be fast and sensitive. Other SNA-I biosensors employing EIS were also developed and used to detect the STn glycan in the glycoproteins fetuin and asialofetuin (Bertók et al., 2013b, 2013c). The reported biosensors presented high sensitive and wide linear range. In all cases, though, serum samples were not analyzed. Analysis of real serum samples may present additional difficulties in the detection of low-abundance glycoproteins, as cancer biomarkers usually are, due to the very complex serum glycoproteome.

This work describes the construction of a SNA biosensor for the detection of cancer-associated STn antigen and its validation by discrimination between serum samples from patients with different types of carcinomas versus healthy individuals.

2. Materials and methods

2.1. Chemicals and materials

Reagents of p.a. quality were used, without further purification. Deionised water purified by a Millipore Milli Q system (resistivity > 18 MΩ cm) was used throughout.

For biosensor preparation the following reagents were used: 16-mercaptohexadecanoic acid (MHDA; Aldrich), ethanol absolute anhydrous (J.T. Baker), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (ECD; Aldrich), N-hydroxysulfosuccinimide (NHS; Aldrich) and ethanolamine (Sigma). *S. nigra* aglutinin type I (SNA-I; EY Labs), with affinity for NeuAc-α2,6 structures, such as the case of the STn antigen (NeuAc-α2,6-GalNAc-α1-*O*-Ser/Thr) was used as biosensing agent.

Bovine submaxillary mucin (BSM) and transferrin (Trf), both from Sigma, were used as model glycoproteins in this study. Bovine serum albumin (BSA; Sigma) was used to assess interference of high abundant serum proteins on the impedance measurements.

Unless otherwise stated, solutions were prepared in phosphate buffer saline (PBS 1 ×) pH 7.4 containing 0.5 mmol l^{-1} of Ca^{2+}, Mg^{2+} and Zn^{2+}. Divalent metals must be present for carbohydrate binding as they are necessary for SNA-I to have the active conformation to interact with the STn antigen (Zeng et al., 2012). Redox probe solution of 5.0 mmol l^{-1} potassium hexacyanoferrate (III) and 5.0 mmol l^{-1} potassium hexacyanoferrate (II) trihydrate was prepared daily.

Screen-printed gold electrodes (Au/SPE, 220BT, 4 mm diameter, Dropsens) were used as received.

2.2. Biosensor fabrication

A volume of 10 μl of a 25.0 mmol l^{-1} MHDA solution, prepared in ethanol, was drop-coated onto the surface of the Au/SPE and dried in air for 24 h at room temperature (~25 °C). The electrode was then rinsed with ethanol and, after dry, 10 μl of a freshly prepared cross-linker solution, composed of 20.0 mmol l^{-1} ECD and 5.0 mmol l^{-1} NHS, was dropped onto the electrode and left for 1 h. The electrode was rinsed with PBS and 24 μl of SNA-I solution (corresponding to 60.0 μg of lectin) was dropped onto the activated surface and left for 1.5 h at room temperature. Finally, the electrode was immersed in a 20.0 mmol l^{-1} ethanolamine solution (diluted in deionized water) for 30 min at room temperature to block no reacted carboxyl groups of MHDA. After, the SNA-Au/SPE was rinsed with PBS to remove adsorption components and stored at 4 °C in PBS until use (Fig. 1).

2.3. Impedimetric and cyclic voltammetry measurements

All impedimetric measurements were carried out in an Autolab electrochemical system (Eco Chemie model PGSTAT 30) equipped with a FRA module and controlled through FRA software version 2.4.

Impedance measurements were performed at the formal potential of the $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ pair, with a 5 mV sinusoidal excitation amplitude. The EIS was recorded within a full frequency range from 0.010 Hz to 100 kHz.

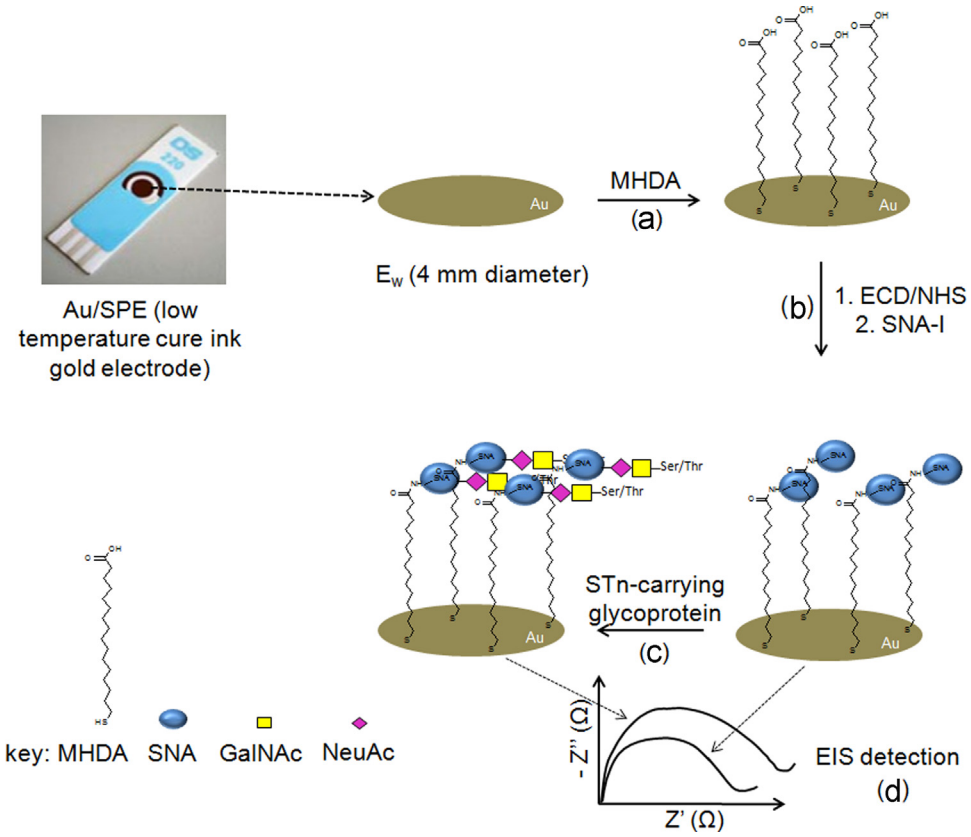

Fig. 1. Schematic diagram of the SNA-I biosensor preparation and detection of STn glycans by EIS: (a) MHDA self-assembled monolayer was formed via incubation of screen-printed electrodes for 24 h; (b) the carboxylic acid end of MHDA was activated with ECD and NHS to allow covalent binding with SNA-I; (c) the STn glycan present in glycoproteins was captured based on the affinity of SNA-I to the referred structure; and (d) the formation of the complex SNA-STn was monitored by the increase in the electrode impedance.

Initially, a blank measurement was performed after dropping 40 μl of the redox probe solution. Then, the electrode was rinsed with PBS solution and the sample was dropped (10 μl) and left for 5 min incubation, at room temperature. Finally, the electrode was rinsed with PBS solution and a second impedance measurement was performed with the redox probe solution. The impedance spectra were plotted in the form of Nyquist plots, and the formation of the SNA-glycoprotein complex was quantified by the increase in the electron transfer resistance $\Delta R_{et}(\Delta R_{et}=R_{et\ f}-R_{et\ i})$, where $R_{et\ i}$ and $R_{et\ f}$ are the electron transfer resistance values before and after incubation with samples, respectively.

Cyclic voltammetry was used to monitor the fabrication process of the SNA-I biosensor. Voltammograms were recorded from −0.4 to 0.6 V at a scan rate of 20 mV s^{-1} and a step potential of 10 mV, at room temperature. All CV measurements were carried out in the Autolab (software GPES version 4.5).

2.4. Sample collection and processing

Human blood samples were obtained from healthy donors and from diagnosed cancer patients, from Centro Hospitalar São João (CHSJ) University of Porto Medical Faculty (Porto, Portugal) and Pachuca General Hospital (México), who gave their informed consent. Information about samples is provided in Table 1, Supporting information.

Immediately after collection, the blood samples were left to clot in a vertical position for 1 h, then centrifuged at 3000 rpm for 10 min, at 4 °C, and serum was transferred to new tubes. To reduce inter-individual variability and enhance common characteristics to all samples (in this case cancer secreted STn antigens), aliquots of serum samples were pooled in two groups: controls and cases, with 25 samples each, and stored at −80 °C until further use.

To reduce the dynamic concentration interval of serum proteins, equalizing the amounts of the more and less abundant proteins, and thus reducing the possible interference of high abundant proteins in the sensor measurements, a combinatorial peptide ligand library (CPLL, Proteominer, BioRad) (Thulasiraman et al., 2005) was used to process samples, according to the manufacturer's recommendations. The processed sera were stored at −20 °C and used in impedimetric analysis. Sera used in this study were thawed two times. Prior to impedance analysis CPLL-processed samples were diluted 1:10 in PBS.

Protein concentration in serum samples was determined after CPLL treatment, by using the Pierce BCA Protein Assay Kit (Thermo), according to the manufacturer's instructions.

2.5. Desialylation of BSM and Trf

BSM and Trf were enzymatically desialylated using neuraminidase from *Clostridium perfringens* type VI (Sigma). Briefly, 1.0 mg of each glycoprotein were incubated at 37 °C during 24 h with 1.0 ml of neuraminidase solution at a concentration of 3 U ml^{-1}, prepared in acetate buffer pH 5.5.

2.6. 2D gel electrophoresis and western blot analysis

To validate the impedimetric results obtained with the SNA-I biosensor, serum samples were also analyzed by 2D gel electrophoresis. A description of the procedure and reagents used in the 2D electrophoresis and western blot analysis is presented in Supporting information.

2.7. Data chemometric analysis

Impedimetric data obtained with sample analysis were used to generate a Pearson's correlation matrix to identify any correlation between variables. Factor analysis was performed using Minitab (version 13). The scores for the principal components were plotted to determine whether qualitative grouping of serum samples existed in the SNA-I-binding impedimetric responses. More information on *Data Chemometric Analysis* is presented in Supporting information.

3. Results and discussion

3.1. Formation of the lectin-glycoprotein complex at the Au/SPE surface

There are different types of SNAs namely I, II and III, with different specificity for glycan structures, and type I presents the highest selectivity for the STn epitope (Mach et al., 1991). Moreover, SNA from different sources shows different binding behavior as previously reported (La Belle et al., 2007; Pilobello et al., 2005). Due to its affinity for the STn epitope, SNA type I from EY Labs was the lectin chosen to be immobilized at the Au/SPE surface. The immobilization procedure was evaluated by incubating the biosensor with standard solutions of glycoproteins containing the STn glycan, namely BSM and Trf.

Cyclic voltammetry was used to evaluate the electrochemical behavior of the electrode surface after each coating step. Fig. 2A shows the cyclic voltammograms (CV) of the $[Fe(CN)_6]^{3-/4-}$ redox couple for the differently modified surface. As expected, the CV for the bare surface showed a reversible Nernstian charge transfer whereas the absence of current peaks for the subsequent modified surfaces due to the formation of a thick coating indicates the increase in electron transfer resistance.

Impedimetric changes were also monitored after each coating step and the obtained Nyquist plots (Fig. 2B inset) corroborate the cyclic voltammetry data. The bare Au/SPE showed the expected fast electron transfer process with a diffusion limiting step. Formation of the several subsequent layers produced an increase in the electron transfer resistance (Fig. 2B), and the further binding of STn-carrying glycoproteins to SNA-I gave rise to an additional barrier for the access of the redox probe to the electrode surface, increasing even more the electron transfer resistance. The magnitude of this increase in the electron transfer resistance could be related to the concentration of STn-carrying glycoproteins (BSM and Trf) incubated with the biosensor, which was confirmed with the next experiments.

The SNA-Au/SPE was prepared by immobilizing 25 μg of SNA-I on the electrode, and incubated with standard solutions of BSM and Trf to assess the biosensor response. Fig. 1 (Supporting information) compares Nyquist plots obtained before and after incubation with BSM or Trf 0.10 mg ml^{-1}. Complex formation between SNA-I and STn epitope was evidenced by the increase in the electron transfer resistance, and the magnitude of this increase was related to the glycoprotein concentration, for a frequency of 0.075 Hz. The Randles equivalent circuit for the developed biosensor is depicted in Fig. 2 (Supporting information).

3.2. Optimization of biosensor construction and measurement variables

The biosensor construction process was optimized by selecting the value that provided the maximum increase in the impedance after incubation with the glycoproteins BSM or Trf. These two glycoproteins were used in the optimization process because they both carry the STn glycan, but are structurally different, better representing the diversity of glycoproteins found in serum.

The 16-alkanethiol (MHDA) acted as the linker molecule to bridge the Au/SPE to SNA-I and was the first variable to be optimized. In contact with the electrode gold surface, a spontaneous adsorbed monolayer of MHDA was formed, based on the strong interaction between gold and sulfur, and was specially well-suited for controlling and manipulating the reactivity at the interface, since the self-assembled monolayer (SAM) could be further modified into a biologically active surface layer via covalent coupling of SNA-I. The self-assembled process resulted in a well-organized and stable monolayer, with the hydrocarbon tails packed parallel to each other and blocking the transport of species to the underlying gold surface (Wang, 2000). Concentrations from 0.5 to 40.0 mmol l^{-1} were tested on blank electrodes (no lectin immobilized) and ΔR_{et} was determined for each concentration (Fig. 3A). MHDA acted as an insulating layer and, therefore, increasing concentrations resulted in higher R_{et}, suggesting a better coverage of the electrode surface. At the higher concentration, the reduction on ΔR_{et} could be attributed to aggregation and auto-oxidation of the molecule (Li et al., 2012). Hence, the concentration of 25.0 mmol l^{-1} was selected as optimum.

The covalent coupling of SNA-I on the MHDA SAM was performed via standard amine coupling chemistry with the first

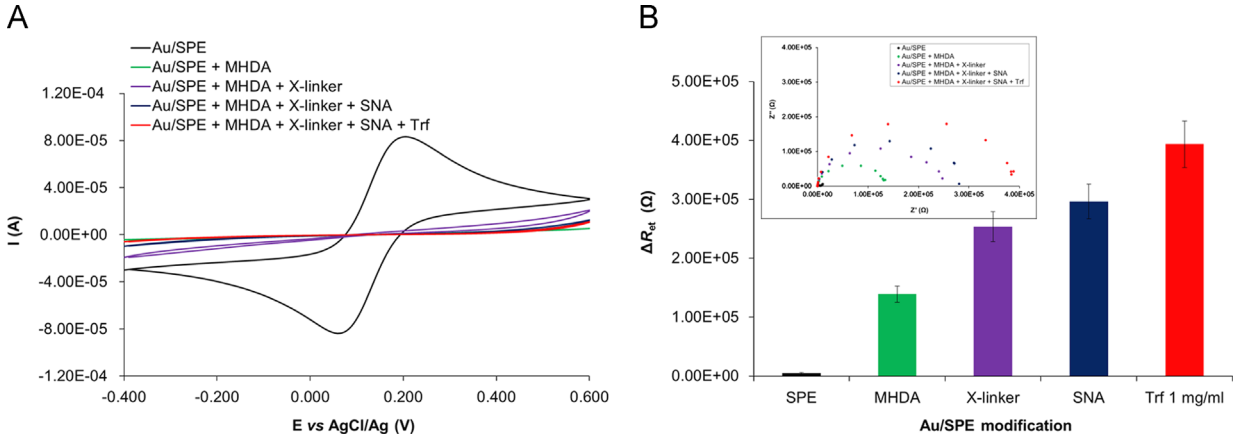

Fig. 2. Electrochemical behavior of the electrode surface: (A) cyclic voltamograms and (B) ΔR_{et} for the different stages of biosensor preparation and after incubation with Trf 1.0 mg ml^{-1}, applying 40 μl of $[Fe(CN)_6]^{3-/4-}$ 5 mmol l^{-1} on the sensor surface. MHDA 25.0 mmol l^{-1}, ECD/NHS 20.0+5.0 mmol l^{-1}, SNA-I 60 μg, scan rate=20 mV s^{-1}, frequency range from 10 mHz to 100 kHz. Error bars indicate the standard deviation of duplicate measurements. Inset: Nyquist plots corresponding to the different stages of biosensor preparation.

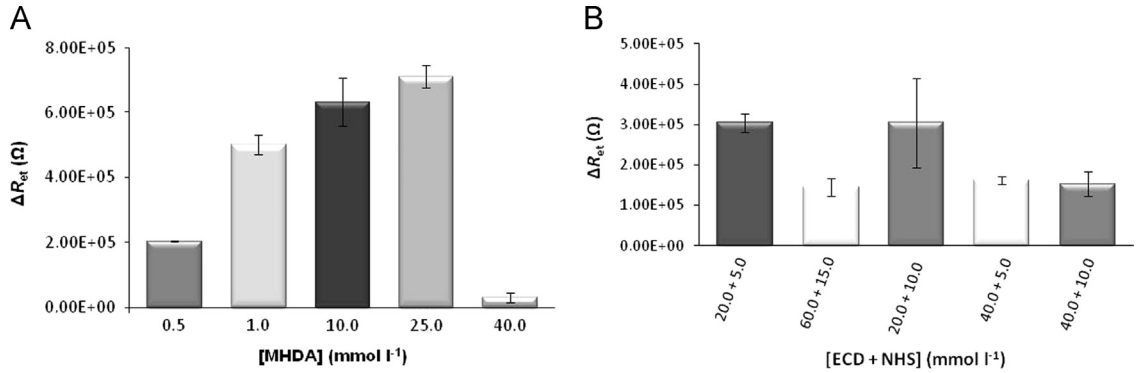

Fig. 3. Dependence of (A) MHDA concentration or (B) cross-linker concentrations on R_{et} at a blank Au/SPE. Error bars indicate the standard deviation of duplicate measurements.

step being activation of the carboxylic group of MHDA by a mixture of ECD and NHS. Concentrations of cross-linker solutions were optimized, using Au/SPE with MHDA (no lectin immobilized) and solutions presenting different proportions of both components. Better reproducibility was obtained for the combination of (ECD+NHS) 20.0 mmol l^{-1}+5.0 mmol l^{-1} and with a higher ΔR_{et}, meaning a better surface coverage. It was observed that the increase in ECD concentration resulted in a reduction in impedance. This can be explained by the fact that after activation of the terminal carboxylic acids of MHDA by co-addition of ECD+NHS, the negatively charged terminal MHDA is replaced by NHS ester. Due to electrostatic attraction, the positively/neutrally charged ester promotes the transfer of the negative redox probe to the electrode surface, so an increased current response (and lower impedance) is observed for higher amounts of ECD immobilized (and consequently higher amounts of NHS esters formed) (Fig. 3B).

The amount of SNA immobilized on the Au/SPE surface is also an important variable since it constitutes the recognition element and, so, it affects the analytical response and sensitivity of the biosensor. On the other hand, since this was the most expensive reagent on the biosensor, it was intended to construct the device with the minimum required amount of SNA that allowed the detection of reduced amounts of STn epitopes in serum samples, as expected for cancer patients, even in advanced disease stage. Different amounts of SNA were tested, specifically 25.0, 60.0, 80.0 and 100.0 μg, and standard solutions of BSM and Trf 0.01 μg ml^{-1} were analyzed. As anticipated, the increase in the amount of SNA up to 60.0–80.0 μg enabled a better detection (higher ΔR_{et}) of complex formation, for BSM and Trf (Fig. 3, Supporting information). The increase in the number of binding sites on the sensor surface due to the increase in SNA concentration reached a plateau for Trf for amounts higher than 60.0 μg and was attributed to a saturation of the surface coverage of SNA. For higher amounts of SNA immobilized on the SPE surface, the analytical signal decreased (for BSM), and this could be due to agglutination phenomena at high lectin concentrations (Gamella et al., 2009). Thus, for the following experiments, 60.0 μg of SNA were deposited on the Au/SPE surface.

Ethanolamine was used to block the active surface sites, thus reducing the nonspecific interactions of serum components with the biosensor. Two concentrations (10 mmol l^{-1} and 20 mmol l^{-1}) and two incubation times (30 and 60 min) were assayed. For the lower ethanolamine concentration the blocking was better for 1 h incubation, whereas for 20 mmol l^{-1} of ethanolamine there was no difference between the tested time conditions (Table 2, Supporting information). Therefore, the blocking step was carried out by incubating the biosensor with ethanolamine 20 mmol l^{-1} for 30 min.

Since the biosensor was intended to be used in clinical studies, the incubation time was optimized in order to reduce the assay period. Theoretically, a reduced incubation time impairs the formation of a stable complex and a long incubation may degrade the SPE surface and cause a loss in its integrity, so the aim was to find the shorter incubation time that allowed the formation of a stable complex. Au/SPEs were prepared immobilizing 100 μg of SNA and BSM and Trf solutions with concentrations of 0.01, 0.1 and 1.0 μg ml^{-1} were analyzed. Several incubation times were evaluated, namely 5, 15, 30 and 60 min (results for Trf on Fig. 4, Supporting information). The results showed that, for the tested concentrations, there was no significant difference between the different incubation times, and an increase in R_{et} could be obtained for all glycoprotein concentrations with an incubation time of 5 min. This finding suggests that STn-binding to SNA-I proceeded quickly. According to the aim of this study, 5 min incubation time was set as the optimized value.

Finally, the frequency interval defined to run the impedance measurements was also adjusted, in order to have a wide interval but not a very long measurement. Intervals from 5, 10 or 20 mHz to 100 kHz were tested in the analysis of standard solutions of BSM and Trf 0.01 μg ml^{-1} and the best relation between ΔR_{et} and measurement time was achieved for the interval starting at 10 mHz. Under these conditions, each impedance spectrum was obtained in about 4 min and the total assay time (incubation and measurement) was around 10 min.

3.3. *Selectivity and interference studies*

To evaluate the specificity of the biosensor response, a series of cross-reactivity assays was performed. Blank sensors (with no lectin) were incubated with glycoprotein solutions (BSM and Trf) 0.01 and 1.0 μg ml^{-1}. Results showed that, for both concentrations, there were no significant ΔR_{et} changes, demonstrating the absence of significant unspecific adsorptions even for high glycoprotein concentrations (Fig. 5, Supporting information).

Additionally, SNA-I biosensors were incubated with fetuin, asialoTrf and asialoBSM. Fetuin is a glycoprotein which is reported to have no STn glycans (Spiro and Bhoyroo, 1974) and the asialo forms of Trf and BSM were obtained by enzymatic desialylation using neuraminidase, to specifically remove the terminal sialic acids of STn. This way, the STn glycan is no longer complete and the SNA-I is expected to present much lower affinity for the glycan structure. An increase in impedance was evidenced for incubated amounts of fetuin higher than 10 ng. As for the asialoglycoproteins, an increase in impedance was observed for aBSM and aTrf amounts higher than 15 and 5 μg, respectively. These results indicate that glycoproteins presenting other glycan structures may interfere in the biosensor response above particular thresholds. This can be explained by the fact that lectin binding to glycan epitopes is not as specific as, for example, antibody interactions.

Nevertheless, considering the sample pretreatment to be performed, the expected amounts of serum glycoproteins and the highly preferential binding of SNA-I to STn epitopes, the interference of glycan structures other than STn is estimated to be reduced.

Albumin is the most abundant protein in serum and some adsorption effect may occur at the sensor surface. To assess interference of high abundant serum proteins on the impedance measurements, BSA was used to simulate the effect of albumin. Three biosensors were incubated with a Trf solution 1.0 μg ml^{-1} + BSA (0.2, 2.0 and 20.0 mg ml^{-1}). Measurements were made for standard additions of the Trf solution and the slopes were compared for the three BSA concentrations. The results showed that for the lower BSA concentration, there was no interference since the slopes obtained with and without BSA were similar. For the higher concentrations, a decrease in slopes was observed and this could be due to BSA adsorption at the sensor surface, impairing the access of STn to the immobilized lectin. Therefore, it was shown that a sample processing was required in order to reduce the amount of the more abundant proteins and this was performed by using the CPLL treatment.

3.4. Analytical features

Considering the possible interference of serum matrix in the impedance measurements, the analytical parameters were determined using the standard additions method. For that, 10 μl-aliquots of standard solutions of BSM or Trf 1.0 μg ml^{-1} were successively incubated and the increase in the impedance was monitored. It was found that the increase in ΔR_{et} of the SNA-I biosensor was linearly proportional to the incubated amount of BSM and Trf up to 40 and 70 ng, respectively, with sensor saturation for higher glycoprotein amounts (Fig. 4). This is in accordance with the fact that BSM and Trf present different STn contents per molecule. BSM, like other mucins, is highly glycosylated and possess a much more dense *O*-glycosylation profile than Trf, according to Net*O*Glyc 3.1 (Hansen et al., 1995). This way, with a lower amount of BSM, all the SNA-binding sites were occupied and therefore the linear interval was shorter for BSM.

The detection limit for Trf was 20 ng, calculated from the regression equation (Miller and Miller, 2000). This is an indicative parameter and will change for each STn-glycoprotein assayed because the number of STn glycan units per molecule vary, depending on the glycoprotein. Cut-off limits for CA72-4 are reported to be 6 U ml^{-1} in clinical radioimmunoassays (Guadagni et al., 1992) and ELISA assays (Immuno-Biological Laboratories). Anyway, the levels of total STn in serum glycoproteins will depend on the type of carcinoma, tumor size and disease stage (Guadagni et al., 1995).

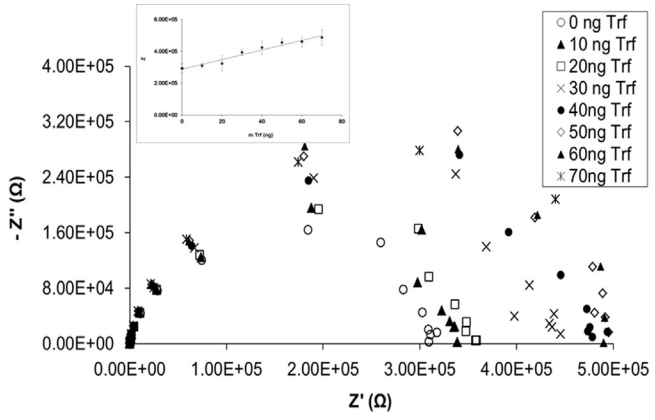

Fig. 4. Nyquist plots for standard additions of Trf 1.0 μg ml^{-1}. Each successive addition corresponded to 10 ng of Trf. Inset: calibration plot corresponding to the absolute impedance (Z) at the SNA-I biosensor upon incubation with increasing amounts of Trf. Amount of SNA immobilized 60 μg, frequency 0.562 Hz. Error bars indicate the standard deviation of duplicate measurements. Calibration equation: $y = 3010x + 288416$, $R^2 = 0.9574$.

Repeatability of the responses obtained with six different SNA-Au/SPE sensors prepared in the same manner was tested by measuring the impedance for the redox solution. A RSD value of 17.0% was obtained. The same experiment was performed using six bare electrodes, with a RSD value of 10.6% being obtained. The RSD value for the SNA biosensors was high, but this was not entirely related with the sensor preparation process, since some irreproducibility was already observed for the bare electrodes.

The storage stability of the SNA-Au/SPE biosensor was also examined. Six individual biosensors were prepared and used to measure impedance of the redox probe after each of 2 successive weeks. Between measurements, the biosensors were kept in PBS at 4 °C. It was observed that the biosensors were not stable since an increase in the impedance was observed after one week, which increased even further after the second week. Therefore, it was concluded that the biosensors should be preferably used within the 24 h after finishing their preparation. In that period of time, they should be kept in PBS at 4 °C.

3.5. Recovery assays

Recovery assays were performed to evaluate the accuracy of the developed biosensor. A 1.0 μg ml^{-1} Trf solution doped with BSA 2.0 mg ml^{-1}, and the CPLL-processed sera samples, undiluted or diluted 1:10 with PBS, in both cases doped with BSA 2.0 mg ml^{-1} were analyzed. Double amount of Trf was added (20.0 and 30.0 ng) and the recoveries were calculated from the obtained results (Table 3, Supporting information). Recoveries were 117.5 ± 2.1% for the Trf solution, 89.4 ± 3.3% for the diluted serum sample and 94.9 ± 29.9% for the undiluted serum sample. Worst recovery values were obtained for the undiluted sample, which seems to be due to the natural presence of albumin in addition to the BSA amount added in the tested concentrations. In the undiluted serum sample, the amount of albumin was expected to be 5.0 mg ml^{-1} at most (considering that the CPLL treatment reduced the total protein content in a factor of around 10). These results are in accordance with the interference studies described above. For the diluted sample, more acceptable recovery results were obtained (for this kind of disposable sensors) and treated sera diluted 1:10 were used throughout the following sample analysis.

3.6. Sample analysis and classification by PCA

The SNA-I biosensor was validated through analysis of serum samples from healthy individuals (controls) and from cancer patients (cases). Fifteen serum samples (eight controls and seven cases) were analyzed using the developed biosensor and the impedimetric data was processed by PCA. The obtained Pearson's correlation matrix and the Bartlett's sphericity test indicated that there was a correlation between variables and that the new variables could be obtained by combining some of the original variables.

Table 4 (Supporting information) shows the eigenvalues for the correlation matrix along with the percentages of variance explained by each factor. More details about *Sample Analysis and Classification by PCA* are presented in Supporting information.

The score values obtained for each sample were plotted and a linear discriminant function was drawn (Fig. 5). As can be observed, a suitable classification of samples in two distinct groups (controls and cases) was achieved with the PCA approach. For the control group, a disperse distribution was observed, and the samples could not be clustered according to age or gender. As for the case samples, some clustering was observed for breast

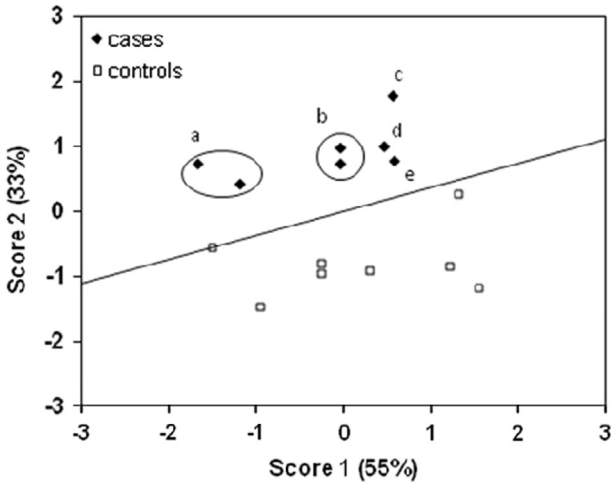

Fig. 5. Graphical representation of the first two scores of a PCA performed on the impedimetric data from sample analysis. Each point represents an individual analysis of a sample; (a) breast carcinoma, (b) retroperitoneal located malignant tumor, (c, e) pools with 25 different cancer samples, and (d) cervical-uterine carcinoma.

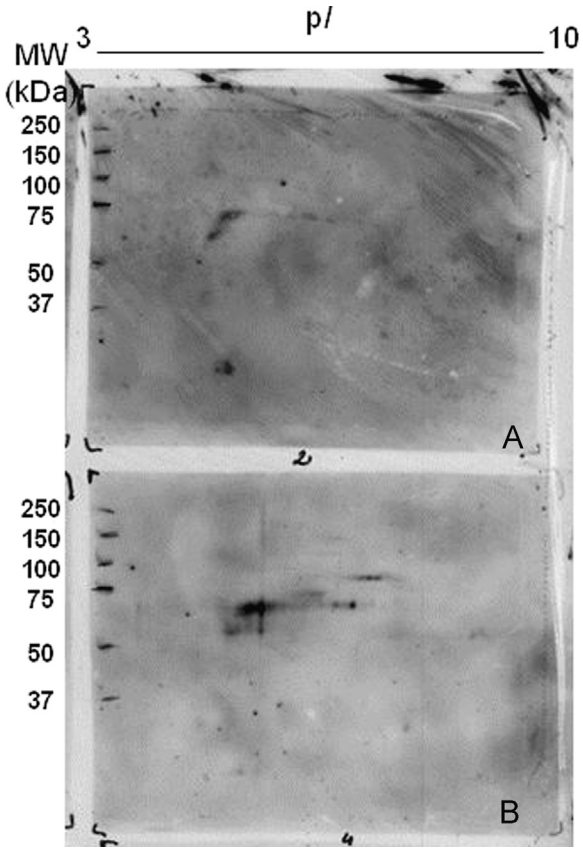

Fig. 6. 2D gel electrophoresis and western blot analysis for STn antigens of pooled sera from (A) healthy individuals and (B) cancer patients. One of the axes corresponds to isoelectric points (p*I*) of the glycoproteins and the other one corresponds to the molecular weight (MW). Western blot was performed with TKH2 antibody, 1 min exposure.

and retroperitoneal carcinomas, but this could not be related to the stage of the cancer, since samples were obtained from patients with different disease progression. The analysis of a higher number of samples is required to allow more consistent conclusions. However, the results clearly show an increase of STn expression in serum glycoproteins of cancer patients, with different types of carcinomas, and this altered level of expression can be detected by the developed biosensor.

One of the control samples was right on the discrimination line, which would require a confirmative analysis by a different technique. Actually, in many cases chemical pattern recognition can be performed as a form of screening, with doubtful samples being subjected to more sophisticated confirmative tests.

3.7. Sample analysis by 2D gel electrophoresis

To validate the results obtained by EIS, the CPLL-processed pooled samples (controls and cases) were also analyzed by two-dimensional (2D) gel electrophoresis. The equalized serum protein samples from the different clinical groups were subjected to 2D electrophoresis with a first dimension separation according to the protein isoelectric points and the second dimension separation was according to protein molecular weight. Fig. 6 shows the 2D maps obtained. The 2D immunoblotting revealed that the immunoreactivity for the STn antigen was higher for the cases group (b) when compared with normal control group (a). The differential expression of STn in the distinct pooled samples corroborated the results obtained with the SNA-I biosensor and the PCA analysis.

4. Conclusions

Herein a novel SNA-I biosensor is proposed, based on screen-printed gold electrodes, modified with a lectin with high selectivity for the cancer-associated STn antigen. The detection of the glycan present in serum glycoproteins was performed by EIS, and the complete assay could be carried out in around 10 min, including the incubation time.

PCA performed on the data obtained with serum samples from controls and from patients with diverse types of carcinomas allowed to classify the samples in a correct way. Analysis through western blot with TKH2 allowed the validation of the biosensor results. The analysis of a higher number of samples is required to allow more consistent conclusions and to permit the establishment of correlations between the biosensor response and the level of expression of the STn antigen in serum glycoproteins for different disease stages, including early diagnosis, and therapy monitoring. Nevertheless, the proposed SNA-I biosensor showed to be sensitive to differences on STn expression of the serum glycoproteome and constitutes a potentially useful tool for the clinical detection and monitoring of cancer.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.02.006.

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