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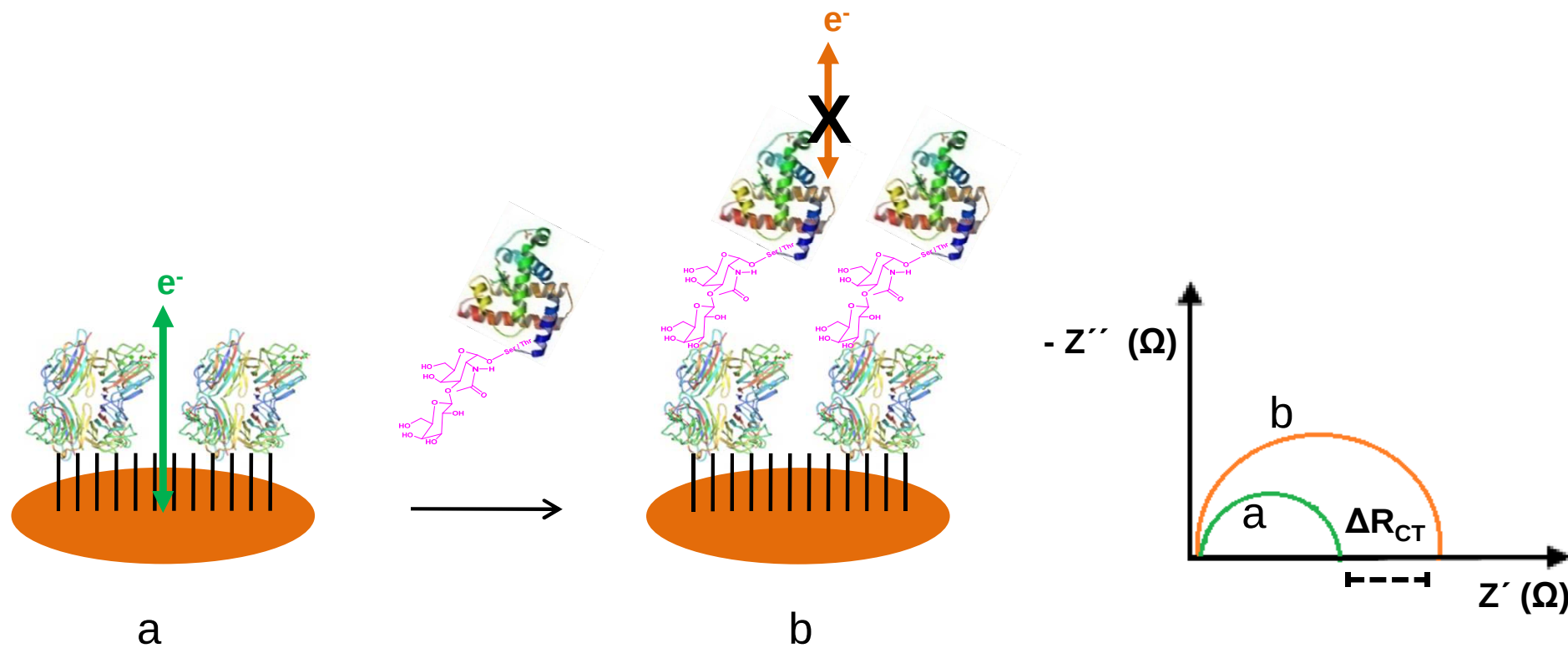
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Graphical abstract



Detection of the cancer-associated T antigen using an *Arachis hypogaea* agglutinin biosensor

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Abstract

An impedimetric biosensor was developed for the selective detection of the cancer-associated T antigen, using the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) as the recognition element. The increase in the biosensor's impedance after sample incubation was indicative of lectin recognition and complex formation between PNA and glycoproteins containing T antigen. When using asialofetuin as model glycoprotein, a minimum amount of 100 ng of glycoprotein could be detected, generating an increase in impedance of 7.2%. Albumin did not cause interference in the detection of T-carrying glycoproteins up to a concentration of 0.01 mg ml⁻¹.

The biosensor was used to evaluate the T-antigen expression in serum samples and was able to discriminate between control samples (of individuals without cancer) and case samples from patients with diverse types of carcinomas (skin, colon, breast, prostate, stomach, kidney, lung, liver and rectum) in which an increase in the expression of T antigen is well-known. The same samples were analyzed with a *Vicia villosa* agglutinin biosensor that has specificity for the cancer-associated Tn antigen, to compare the expression of both antigens in the diverse carcinomas. The results were different for both biosensors, confirming that the use of different lectins allows to monitor different antigen expression. Furthermore, combining different lectins, glycosylation profiles for each carcinoma type can be obtained.

This work demonstrates the feasibility of employing PNA to selectively recognize the T epitope in glycoproteins and the proposed biosensor could be used for high-throughput, label-free profiling of the cancer-associated T antigen in serum samples.

Keywords: T-antigen, Tn-antigen, cancer, *Arachis hypogaea* agglutinin, screen-printed electrodes, biosensors

1. Introduction

Alterations in protein glycosylation are characteristic of tumor cells, and aberrant glycans are found both in *N*- and *O*-glycoproteins (Kudelka et al., 2015; Pinho and Reis, 2015). The first step in *O*-glycosylation is the attachment of an *N*-acetylgalactosamine (GalNAc) residue to a Ser/Thr of the peptide backbone, constituting the Tn antigen (GalNAc α 1-O-Ser/Thr). In normal circumstances, this is followed by attachment of a sialic acid (forming the STn antigen – Neu5Ac- α 2-6GalNAc- α 1-O-Ser/Thr) or, more commonly, further chain extension. In cancer cells, Tn and STn are exposed and chain extension does not occur, due to an increase of expression or activity of glycosyltransferases that attach the first GalNAc or a decrease in the activity of the core β 1,3 galactosyltransferase that catalyses chain extension (Freire et al., 2005). If Tn is extended by addition of galactose (Gal) through β 1,3 linkage, the T (or Thomsen-Friedenreich (TF) – Gal β 1-3GalNAc α 1-O-Ser/Thr) antigen is formed. In normal cells this structure is further extended but it is commonly exposed in cancer cells comprising, together with Tn and STn, the truncated glycans associated with carcinoma cells (Brooks et al., 2008) and recognized as pan-carcinoma antigens because they are detected in practically all epithelial cancer cells (Fu et al., 2016).

The T antigen was discovered in 1920, but it was not until 1975 that it was recognized as a tumor antigen, when the increase in the T-antigen expression was for the first time associated to processes of cell proliferation and metastasis (Barr et al., 1989; Fu et al., 2016; Yu, 2007). T antigen can be found in most human normal tissues, but it is usually masked by covalently bound carbohydrates. In contrast, immunoreactive T antigen is present in the cytoplasm and outer membrane of nearly 90% of the major forms of carcinoma (Springer et al., 1985; Springer, 1997).

Over time, T antigen has been the focus for the development of methodologies that allow detection, diagnosis and even treatment of carcinomas. Immunohistochemical techniques (Baldus et al., 2000; Desai et al., 1995; Dippold et al., 1990) and analytical methods based on liquid chromatography (Campbell et al., 1995) require some type of chemical treatment on the sample before analysis, such as enzymatic digestion and labeling with fluorescent molecules (Svarovsky and Joshi, 2014). A fluorometric bioassay using quantum dots for the detection of T antigen was proposed, showing high sensitivity (10^{-7} M range) and specificity. Nonetheless, the competitive assay requires conjugation of the lectin (PNA) and a somehow laborious sequence of binding steps before fluorescent measurement (Li et al., 2013).

Imaging-based methods for the detection of T antigen have also been developed, namely a nanobeacon for use with fluorescence colonoscopy in the detection of colorectal cancer lesions (Sakuma et al., 2015). Although the methodology could be used for the detection of initial lesions and also for monitoring tumor regression during chemotherapy, it has the drawback of being an invasive procedure that causes discomfort to the patient.

For clinical analysis of biological samples from cancer patients, rapid, simple and non-invasive methods are required. Biosensors possess remarkable characteristics in comparison with traditional methods to be applied in the monitoring of cancer biomarkers in body fluids such as serum. They are portable, use minimum amounts of sample and minimize patient stress (Bohunicky and Mousa, 2011; Soper et al., 2006).

Among the great diversity of biosensors, lectin-based impedimetric devices offer the advantage of not requiring labeling of molecules, which simplifies the construction and measuring procedures (Bertók et al., 2013; Daniels and Pourmand, 2007; Pihílová et al., 2015). In addition, they rely on the selectivity of lectins towards specific glycan structures and can be tailored to different glycan epitopes according to the needs, by employing different lectins as biorecognition agents. Lectins are useful biorecognition elements and allow capturing particular glycan structures from complex samples (Hashim et al., 2017).

Different lectin-based biosensors for truncated O-glycans associated to cancer have been developed (Dai et al., 2006; La Belle et al., 2007; Silva et al., 2014; Silva and Rangel, 2017), but the ones that detected the T antigen were not tested in the analysis of real samples. This research aimed to create a label-free biosensor using PNA for the selective detection of T antigen and to assess its usefulness in the analysis of serum samples from patients with diverse carcinomas.

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 (16-MHDA; Aldrich), 6-mercaptohexanoic acid (6-MHA; Aldrich), ethanol absolute anhydrous (J.T. Baker), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (ECD; Aldrich), N-hydroxysulfosuccinimide (NHS; Aldrich), ethanolamine (ETA) and ethylene glycol (EG) (both from Sigma) and sodium dodecyl sulfate (SDS; J.T. Baker). Pure *Arachis hypogaea* (peanut) agglutinin (PNA; EY Labs) was used as the biosensing agent. PNA is very adequate for the recognition of T antigen, since its high affinity towards the referred glycan has been demonstrated (Wu et al, 2008).

Asialofetuin (aFet) was used as the model glycoprotein in this study, as it carries the T glycan. It was obtained by enzymatic desialylation of fetuin using neuraminidase from *Clostridium perfringens* type VI (Sigma). Briefly, 1.0 mg of fetuin was 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. The product was used without further purification.

Bovine serum albumin (BSA), human transferrin (Trf) and fetuin (Fet) from fetal calf serum (all from Sigma) were used to assess interference of serum proteins and glycoproteins on the impedance measurements.

Unless otherwise stated, solutions were prepared in phosphate buffer saline (PBS 1x) pH 7.4 containing 0.5 mmol l⁻¹ of Ca²⁺, Mg²⁺, Mn²⁺ and Zn²⁺. Divalent metals must be present for carbohydrate binding as they are necessary for PNA to have the active conformation to interact with the T antigen (Moreira et al., 1991; Sun et al., 2011). 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 to build the biosensors.

2.2. Construction of the PNA biosensor

On the gold surface of a SPE, 10 µl of a solution of 16-MHDA and 6-MHA in a ratio of 1:1 (12.5 mmol l⁻¹ each) prepared in ethanol is placed and dried in air for 24 h. The electrode is 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, is dropped onto the electrode and left for 1 h. The electrode is rinsed with PBS and 20 µl of PNA solution (corresponding to 50.0 µg of lectin) is dropped onto the activated surface and left for 1.5 h. Finally, the electrode is immersed in a 20.0 mmol l⁻¹ ETA solution (diluted in deionized water) for 30 min, followed by immersion in a 10% EG solution. The whole process of construction is carried out at room temperature (~ 22°C). Finally, the PNA-Au/SPE biosensor is rinsed with PBS and stored at 4 °C in PBS until use (Figure 1).

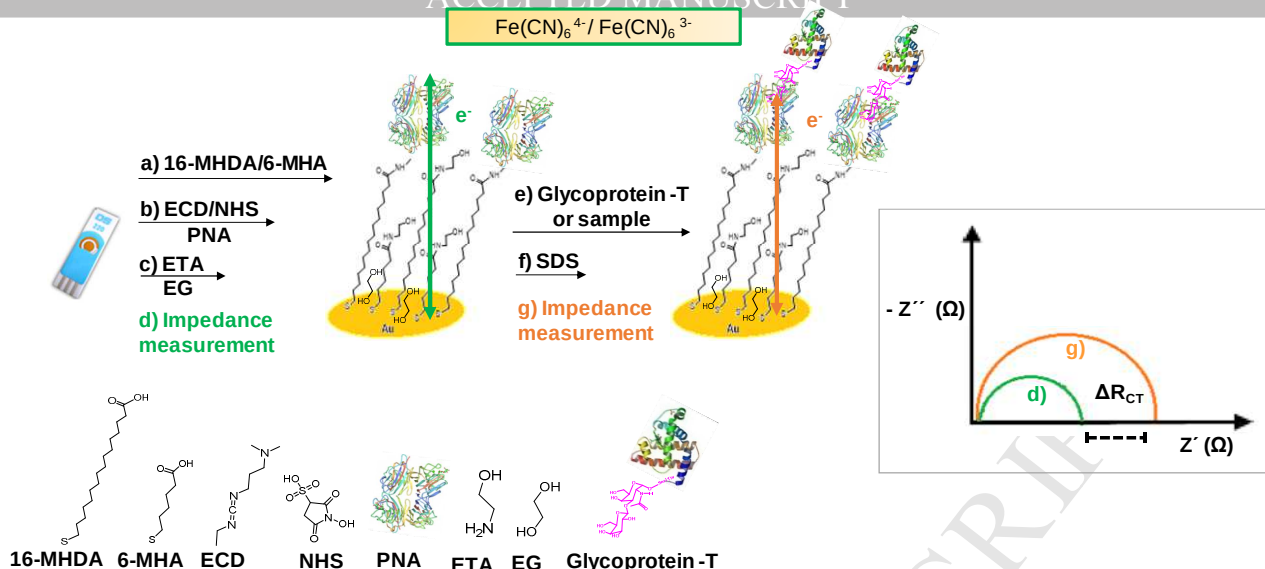


Figure 1 – Schematic diagram of the construction of PNA biosensor and detection of T-carrying glycoproteins by EIS: (a) a mixed self-assembled monolayer is formed via incubation of Au/SPE with 16-MHDA and 6-MHA for 24 h; (b) the carboxylic acid ends of the alkanethiols are activated with ECD and NHS to allow covalent binding with PNA; (c) unoccupied carboxylic acids are blocked with ETA and the electrode surface is blocked with EG; (e) sample is incubated and a complex is formed between PNA and the T antigen present in glycoproteins, based on the affinity of PNA to the referred epitope; (f) the surface is washed with SDS to remove compounds nonspecifically bound. Formation of the PNA-T complex is monitored by the increase in the electrode impedance from the first to the second impedance measurement (ΔR_{CT}).

2.3. Impedimetric and cyclic voltammetry measurements

All impedimetric measurements (performed by electrochemical impedance spectroscopy – EIS) were carried out in an Autolab electrochemical system (Metrohm model PGSTAT 302N) equipped with a FRA32M module and controlled through Nova software version 2.0.

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

Once the biosensor is built, 40 μL of the redox probe is placed and the impedance is measured. Then, the electrode is rinsed with PBS and 10 μL of sample or model glycoprotein solution are incubated for 10 min, at room temperature. The sensor surface is then washed with 2% SDS solution and again the impedance is measured in the presence of the redox probe.

The impedance spectra are represented in a Nyquist diagram, from which the formation of the complex and the increase in the resistance to charge transfer (ΔR_{CT}) is quantified: $\Delta R_{CT} = R_{CTf} - R_{CTi}$, where R_{CTi} and R_{CTf} are the charge transfer resistance values before and after incubation with samples, respectively. The percentage of increase ($\% \Delta R_{CT}$) was introduced in the formula in order to normalize the experimental results and to enable comparison between different electrodes, and was calculated as follows:

$$\% \Delta R_{CT} = (\Delta R_{CT} / R_{CTi}) \times 100$$

Measurements were performed in duplicates.

Cyclic voltammetry (CV) was used to monitor the fabrication process of the PNA 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 Nova 2.0).

2.4. Sample collection and processing

Human blood samples were obtained from healthy donors (25) and from diagnosed cancer patients (42), from General Hospital of Pachuca (México) from January 2016 to December 2018. The following criteria were considered for sample collection: adults (> 18 years) of both genders, who gave their informed consent to participate in the study. Information about samples is provided in Table 1 – Supporting Information.

The procedure performed for blood sample collection, processing and storage is fully described in Supporting Information.

3. Results and discussion

3.1. Optimization of biosensor construction

Optimization of the biosensor construction was performed using the univariate method and choosing, for each variable, the conditions that enabled the maximum increase in the impedance after incubating the biosensor with a 0.1 mg ml⁻¹ solution of aFet (maximum % ΔR_{CT}), and with high reproducibility.

In the first stage of construction, the type of alkanethiol that would form the self-assembled monolayer (SAM) was studied. Two alkanethiols (16-MHDA and 6-MHA) were used to form the SAM, and different combinations of both alkanethiols were prepared and immobilized at the electrode surface (16-MHDA+6-MHA, in mmol l⁻¹): 25+0; 0+25; 25+12.5; 12.5+12.5; 25+2.5 and 12.5+25. It was observed that the higher % ΔR_{CT} was obtained for the SAM composed exclusively of 16-MHDA (141.3%), followed by the combination 25+2.5 (75.2%) and 12.5+12.5 of 16-MHDA+6-MHA (45.2%). For the other mixed SAMs and for the SAM made of 6-MHA very low increases were obtained. This behavior can be attributed to the length of the alkanethiol chains and the organization of the corresponding SAMs. The short chain alkanethiols form less organized and less dense SAMs. Furthermore, the addition of the lectin to the system causes an increase in the structure disorder, modifies the orientation of the chains due to steric effects and the glycan recognition sites are less accessible (Campuzano et al., 2006; Love et al., 2005). On the other hand, the long chain alkanethiols form a highly ordered and dense SAM which acts as a barrier preventing the free diffusion of the electroactive species towards the electrode surface (Love et al., 2005). This may be the reason why tested SAMs with higher amounts of 6-MHA showed very low increases in % ΔR_{CT} .

To evaluate the behavior of the three best combinations in preventing adsorption of proteins non carrying the T antigen, the same assay was performed substituting aFet by BSA 0.1 mg ml⁻¹ as it mimics human albumin, the most abundant protein in serum samples. The results showed that, for the three tested combinations, lower increases in impedance were obtained for the SAM 16-MHDA+6-MHA 12.5+12.5 mmol l⁻¹ (16.2%). The other two combinations originated increases higher than 40%. For this combination, the ratio of impedance increase between the positive and the negative controls (aFet/BSA) was 2.79, which indicated a good discrimination ability of the biosensor towards glycoproteins carrying the T antigen. Considering reproducibility, best results were obtained for the 12.5+12.5 and 25+2.5 mmol l⁻¹ proportions; therefore the SAM

combination 12.5+12.5 mmol l⁻¹ was selected as optimum.

Concentrations of cross-linker solutions were optimized, using solutions presenting different proportions of both components (ECD+NHS, in mmol l⁻¹), namely 20+5; 20+10; 20+20; 40+5 and 40+10. These proportions were defined according to previous experiments. Higher % ΔR_{CT} and better reproducibility in results was obtained for the combination of 20.0 + 5.0 mmol l⁻¹ (Table 3 – Supporting Information), which was the selected combination. A clear relation between ECD and NHS concentrations and analytical signal could not be established, nor between their proportions and the increase in impedance, analyzed in an independent way. Hence, the effect in impedance must be due to these aspects (concentration of ECD and NHS, their proportion and the electric charges at the biosensor surface) actuating together, which affects the binding of the lectin.

Optimization of PNA amount immobilized on the biosensor was carried out testing 25.0, 50.0, 75.0 and 100.0 μ g of PNA per electrode. The higher increase in impedance was observed for the lower amount tested (102.9%), with a slight decrease for the other amounts, which presented similar % ΔR_{CT} , around 73-79% (Figure 1 – Supporting Information). Nevertheless, for the lower amount, higher standard deviation in results was obtained, which could be justified by the small volume of lectin solution applied (10 μ l), and the consequent associated error. For the other amounts tested, results presented better repeatability. The plateau observed for the amounts of 50.0, 75.0 and 100.0 μ g was attributed to a saturation of the surface coverage of PNA. As the amount of PNA immobilized on the SPE surface increased, the analytical signal slightly decreased, and this could be due to agglutination phenomena at high lectin concentrations, decreasing the number of available sites (Gamella et al., 2009). Thus, for the following experiments, 50.0 μ g of PNA was deposited on the Au/SPE surface.

The electrochemical behavior of the electrode surface after each consecutive coating step was monitored by CV as well as by EIS. Figure 2a shows the cyclic voltammograms of the [Fe(CN)₆]^{3-/4-} redox probe for the differently modified surface. The CV for the unmodified electrode showed a quasi-reversible process. As the surface of the SPE was successively modified, the anodic and cathodic current peaks were diminished and the oxidation peak potential moved to more positive values, whereas the reduction peak potential remained almost constant, except for the electrode modified with the SAM and the cross-linker. This was due to the formation of a thick coating on the electrode surface that led to an increase in resistance to electron transfer.

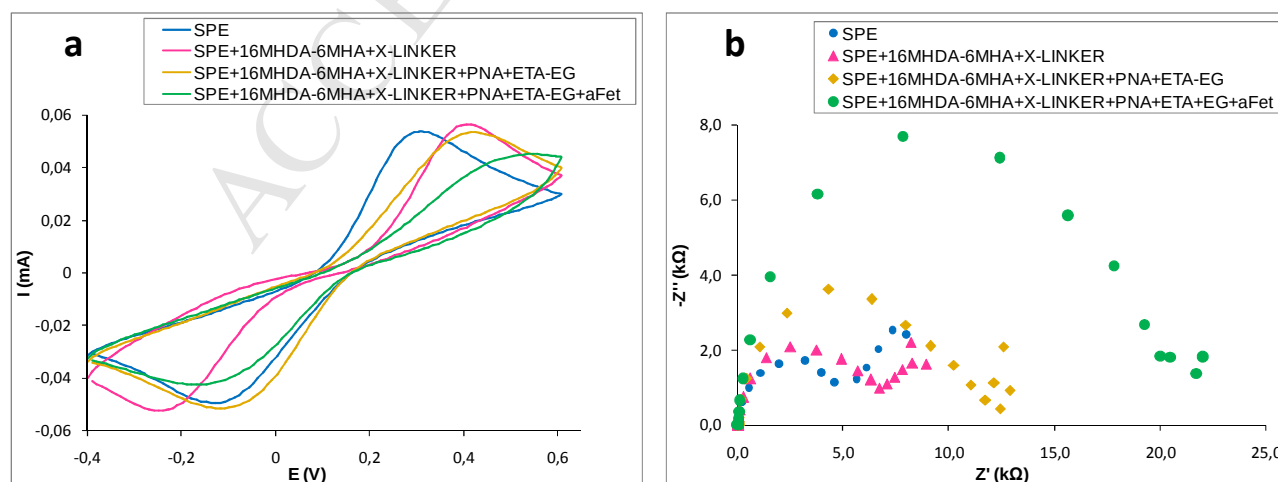


Figure 2 – Electrochemical behavior of the electrode surface: (a) cyclic voltammograms and (b) Nyquist plots for the different stages of biosensor preparation and after incubation

with aFet solution 0.1 mg ml^{-1} , applying $40 \text{ } \mu\text{l}$ of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ 5 mmol l^{-1} on the sensor surface. SAM concentration of 16-MHDA+6-MHA 12.5 mmol l^{-1} each, ECD+NHS concentration of $20.0 + 5.0 \text{ mmol l}^{-1}$, PNA amount $50 \text{ } \mu\text{g}$.

Nyquist plots (Figure 2b) corroborate the CV data. The bare Au/SPE showed the expected fast electron transfer process with a diffusional limiting step. The subsequent layers applied on the electrode caused an increase in the electron transfer resistance, which was further raised after incubation with aFet. The results obtained by both analytical techniques allowed to confirm the formation of PNA-aFet complex. The proposed *Randles* equivalent circuit for the developed biosensor is depicted in Figure 2 – Supporting Information.

Incubation time was optimized by evaluating several incubation times namely 5, 10, 15 and 25 min, using PNA biosensors constructed under the optimized conditions and incubating an aFet solution 0.1 mg ml^{-1} (Figure 3 – Supporting Information). The results showed that, for the tested aFet concentration, the $\% \Delta R_{CT}$ increased with the incubation time up to 10 min and then progressively decreased for the longer incubation times. This suggested that T-antigen binding to PNA proceeded in a relatively fast way. The signal decrease for longer times was due to a partial drying of the biosensor surface exposed to air, which affected the biosensor functioning. To confirm that the increase in impedance was due to the complex formation and not to unspecific interactions, the same assays were performed with PBS instead of aFet and no significant increase in impedance was observed for the incubation times up to 25 min (the maximum signal increase was 4.1% in these assays). Therefore, the incubation time was set at 10 min. Under these conditions, each impedance spectrum was obtained in about 4 min and the total assay time (first measurement, incubation and second measurement) was around 20 min.

3.3. Selectivity and interference studies

In order to reduce nonspecific interactions, the following blocking sequence was included in the methodology (Silva and Rangel, 2017): 1) biosensor immersion in ETA 20 mmol l^{-1} solution for 30 min to block carboxyl groups of 16-MHDA and 6-MHA that did not react during the construction of the biosensor (Frederix et al., 2004); 2) biosensor immersion in EG 10% solution for 30 min to cover unoccupied areas of the gold surface that may interact with glycoproteins (Zheng et al., 2003); 3) biosensor washing with SDS 2% solution after sample incubation to remove proteins that were nonspecifically bound to the biosensor surface (Yang et al., 2016). The use of mixed SAMs also enables the reduction of protein adsorption at electrode surfaces (Frederix et al., 2003).

The performance of the developed biosensor in terms of selectivity and the effect of putative interfering proteins was evaluated by cross-reactivity tests, which consisted on the following: a) on the modified electrode (but without lectin) aFet solutions of 1, 10 and $100 \text{ } \mu\text{g ml}^{-1}$ were incubated to examine the nonspecific interaction between the glycoprotein and the sensor components; b) on a complete biosensor, $1 \text{ } \mu\text{g ml}^{-1}$ solutions of glycoproteins that do not contain the T glycan (Trf and Fet) (Iskratsch et al., 2009), were incubated to verify the selective recognition of PNA towards T antigen; c) on a complete biosensor, solutions of BSA 10 and $100 \text{ } \mu\text{g ml}^{-1}$ were incubated, to simulate the effect of human serum albumin, the most abundant protein in the blood serum, and thus assess its interference in the response of the device. Experiments with BSA were also useful to determine the dilution required for serum samples. For selectivity experiments, a negative control glycoprotein was considered to interfere when it caused a change in the response $\geq 10\%$ (the background noise limit established for the biosensor response was 10%, considering the response obtained for incubation with PBS). Figure 3 shows the results.

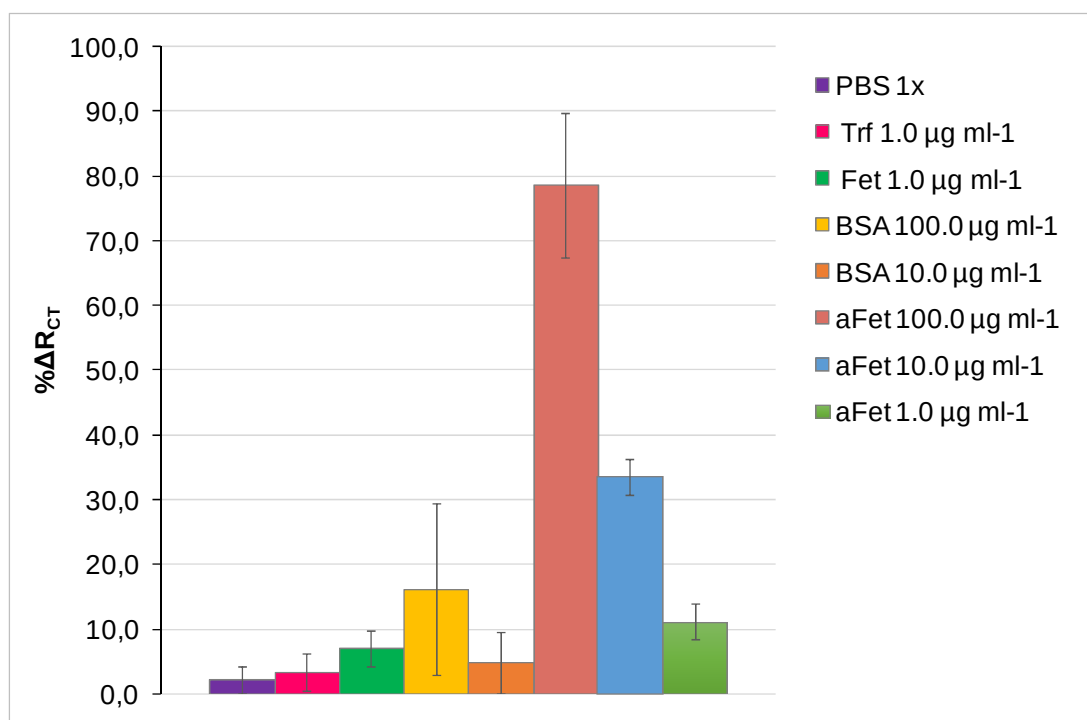


Figure 3 – PNA-biosensor response for several glycoprotein solutions, incubated for 10 min. Error bars indicate the standard deviation of duplicate measurements (two independent biosensors for each solution).

The blocking procedure carried out provided good results, with the higher analytical signal for aFet, as expected, and acceptable signals for negative controls (with the exception of BSA at 0.1 mg ml^{-1} , which was probably adsorbed at the biosensor surface). Considering the sample pretreatments to be performed, the expected amounts of serum glycoproteins and the highly preferential binding of PNA to the T epitope, the interference of glycan structures other than T was estimated to be reduced.

3.4. Analytical features

Once the optimal operating conditions were established, the analytical response ($\% \Delta R_{CT}$) of the PNA biosensor was evaluated at different concentrations of aFet in a range from 0 to $150.0 \text{ } \mu\text{g ml}^{-1}$. The analytical signal increased linearly with increasing aFet concentrations, with a regression equation of $y = 0.5224x + 2.4223$ and a correlation coefficient of 0.9912 ($n = 2$ for each concentration, 7 concentration points) (Figure 4 – Supporting information). The limit of detection (LOD) was $11.65 \text{ } \mu\text{g ml}^{-1}$ and the limit of quantification (LOQ) was $61.50 \text{ } \mu\text{g ml}^{-1}$, calculated according to the criteria established by IUPAC (Currie, 1995). Nonetheless, these LOD and LOQ are indicative parameters and will change for each T-carrying glycoprotein assayed because the number of T glycan units per molecule may vary. Furthermore, the levels of total T antigen in serum glycoproteins will depend on the type of carcinoma, tumor size and disease stage (Springer, 1997).

Reproducibility of the biosensor construction was evaluated by comparing the impedance values (R_{CT}) of a $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution between a group of 7 unmodified electrodes and 18 biosensors constructed under the same conditions on different days. The percentage of relative standard deviation (%RSD) was 10.0 for the group of unmodified electrodes and 5.2 for the biosensor group. The results indicate that the biosensor construction process is reproducible and the %RSD is acceptable for this kind of

disposable sensors (Gamella et al., 2009). The decrease in %RSD after the modification could be due to the fact that the several coatings placed on the electrode gold surface reduce the inherent roughness of the bare electrode and homogenize the active surface.

The effect of storage on the biosensor response was examined. A full description of the procedure is presented in Supporting Information. The response diminished for the stored biosensors, with higher reductions for longer storage periods (77.5% of the initial response for 10-day storage) (Figure 5 – Supporting Information). This is possibly due to the fact that the SAM suffers deterioration in its composition during long storage times, due to the oxidation of the bound thiolates and their subsequent desorption from the gold surface, which causes a decrease in resistance (Flynn et al., 2003; Love et al., 2005). Therefore, the biosensors should be preferably used within the first three days after construction (showing 92.8% of the initial response) and should be stored in PBS at 4° C when not in use.

3.5. Sample analysis

The PNA biosensor was validated through analysis of human serum samples from healthy individuals (controls) and from cancer patients (cases). Ten serum samples (1 pool of 25 controls and 9 pools of cases separated by organ) were analyzed using the developed biosensor. Also, the same samples were analyzed with a *Vicia villosa agglutinin* (VVA) biosensor, previously developed by our research group, which detects the cancer-associated Tn antigen (Silva and Rangel, 2017). CPLL-processed samples were diluted in PBS 1:20 for VVA biosensor and 1:40 for PNA biosensor. The obtained results are depicted in Figure 4 and the % ΔR_{CT} obtained for each sample and biosensor is indicated in Tables 4 and 5 – Supporting Information. All results correspond to duplicate independent measurements of each sample.

Controls originated increases in impedance for both biosensors $\leq 13.1\%$. The slight increase in impedance for control samples could be due to some unspecific recognition by the lectins of some non-Tn and non-T glycans present in samples, and also due to some albumin adsorption on the biosensor surface. Nonetheless, the obtained response for controls was very close to the 10% background noise limit established for the biosensor response obtained for incubation with PBS, which reveals high selectivity in the biosensors response.

For cases, differential responses were obtained for different organs and different antigens. According to results, T antigen was highly expressed in skin and breast cancers and showed low expression in kidney, liver and rectum cancers. As for Tn antigen, higher responses were obtained for lung, prostate, breast, skin and rectum cancers, with colon and kidney cancers presenting lower expressions.

Considering collected data from each sample, the pools that comprised cases in advanced stages of disease (with metastasis) were kidney (50% of samples in the pool), lung (33%), breast, prostate, liver and rectum (25% in each case). This could justify the higher increases in impedance for Tn detection (VVA biosensor) for these pools, except for the case of kidney cancer, which revealed low expression of Tn and T even in advanced stages. As for T antigen expression, correlation with advanced stages of disease was not observed, except for breast pool. This raises the hypothesis of Tn antigen being increasingly expressed with the disease progression but that trend is not observed for T antigen.

On the other hand, stomach, colon and skin pools had no metastatic samples included. Nonetheless, high impedance increases were detected for both biosensors in the case of skin cancer, which could mean that T and Tn antigens are highly expressed even in non-advanced stages of the disease. For stomach and colon samples, lower increases in impedance were observed for both antigens which seem to correlate with disease

progression.

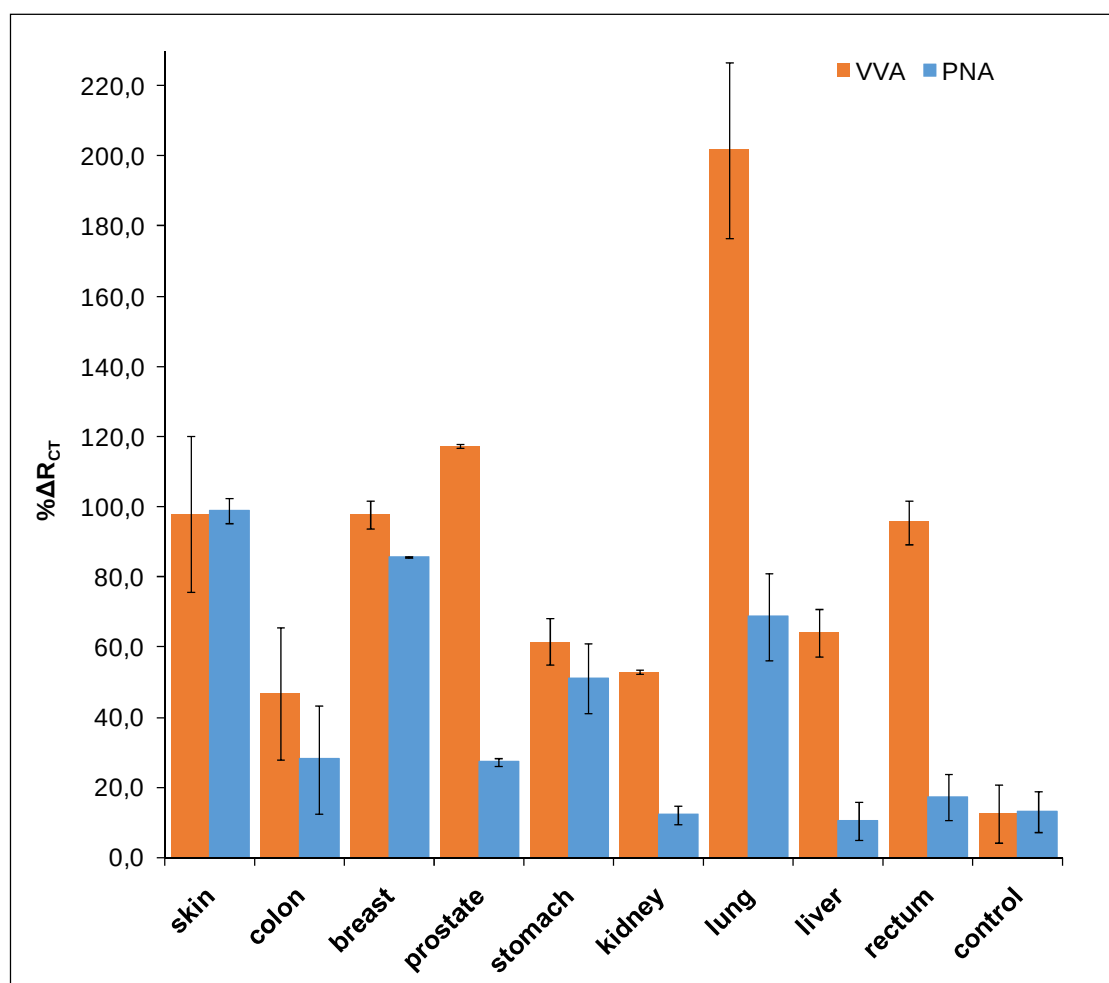


Figure 4 – Results obtained in sample analysis for PNA and VVA biosensors. Error bars indicate the standard deviation of duplicate measurements (two independent biosensors for each sample).

A thorough and detailed comparison between obtained results and published data is presented in Supporting Information.

4. Conclusions

Herein a novel label-free PNA biosensor is proposed, which presents high selectivity for the cancer-associated T antigen. The total assay time was 20 minutes, including the sample incubation time. The biosensor construction is simple and reproducible, and minimum amounts of sample are required for the analysis. The construction procedure used for lectin immobilization did not impair its recognition and binding to the target glycan. An important limitation of the proposed biosensor is the storage stability. Further studies must be carried out to improve long-term stability which would enable mass production and storage.

Results obtained for serum samples from controls and from patients with diverse types of carcinomas allowed to discriminate samples in a correct and clear way. Controls generated very low increases in impedance, which were close to the noise limit established for this study. Agreement with reported data was achieved for the majority of

carcinoma samples, which validates the performance of the developed biosensors. Nonetheless, 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 T antigen in serum glycoproteins for different disease stages. Samples could not be analyzed without a pretreatment, which impairs a complete point-of-care analysis, but the performed pretreatment showed to be effective to avoid interferences of sample matrix.

The proposed PNA biosensor showed to be sensitive to differences on T-antigen expression on serum glycoproteome for carcinomas of different location and constitutes a potentially useful tool for the clinical detection and monitoring of cancer glycobiomarkers, as an alternative or complement to other routinely-used analytical assays.

Association of two lectin biosensors towards different cancer-associated antigens enables to obtain a glycosylation profile for each type of carcinoma, and may allow to increase sensitivity and specificity in the detection of cancer glycobiomarkers. Differential expression of T and Tn expression for the same organ location was observed in this study, by employing two different biosensors.

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6. References

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Highlights

1. A novel PNA biosensor for the cancer-associated T antigen is proposed.
2. The complete assay is fast, simple, label-free, reproducible and selective.
3. The PNA biosensor accurately discriminates controls and carcinoma samples of different organ location.
4. The proposed PNA biosensor allows to get T-antigen expression profiles for different types of carcinomas.

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Credit Author Statement

Maria Luísa S. Silva performed the following roles: conceptualization, project design, administration and financing, project supervision, experimental design, data analysis and interpretation, article writing, review and editing.

María G. H. Rangel performed the following roles: experimental execution, data analysis and interpretation, article writing.