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Impedimetric Lectin-based Biosensors as Selective Devices for the Detection of Cancer Glycobiomarkers

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Abstract: A common feature seen in different types of cancer, namely in carcinomas, is the alteration in post-translational modifications of proteins, particularly in protein glycosylation. Due to increased or decreased expression of specific enzymes participating in glycosylation of proteins, different glycan structures are formed, which are typical of tumoral cells. When the respective glycoproteins are secreted into the blood stream, these aberrant structures can be used as valuable cancer biomarkers, since they are not synthesized by normal cells.

Glycan structures are efficiently and selectively detected by lectins, which are proteins of natural origin with high affinity for a unique or a very small group of glycan epitopes. Lectins are used for glycan detection as antibodies are used for protein recognition.

Lectin biosensors are attractive devices for the detection of cancer-associated glycobiomarkers in serum since they combine the advantageous aspects of biosensors (portability, easy use in point-of-care analysis, low sample requirement) with the high selectivity of lectin biorecognition. This work presents three lectin-based impedimetric biosensors for the selective detection of specific aberrant cancer-associated *O*-glycans, namely STn, Tn and T antigens, which are well-established pan-carcinoma biomarkers. For these three biosensors, *Sambucus nigra* agglutinin, *Vicia villosa* agglutinin and *Arachis hypogeae* agglutinin were used as biorecognition elements, with specificity for STn, Tn and T antigens, respectively.

Keywords: Cancer, Glycobiomarkers, Truncated *O*-glycans, Biosensor, Lectins, Electrochemical impedance spectroscopy, Screen printed electrodes.

1. Introduction

Cancer is a group of diseases characterized by a wide diversity in location, cellular features and aggressiveness. However, carcinomas (cancers that develop from epithelial cells, in skin or in tissues that line or cover body organs) present some common aspects, one of them being alterations in protein glycosylation [1, 2]. Aberrant glycosylation in cancer cells is due to variations in the expression of genes controlling glycosylation [3] and, as a result, it generates abnormal glycoproteins with glycostructures that are typical of carcinoma cells and not of normal cells, which enables them to be used as carcinoma-associated glycobiomarkers.

Examples of cancer-associated alterations in protein glycosylation are the shortening of *O*-glycan chains, forming the truncated sialyl-Tn (STn), Tn and T antigens, the increased branching and bisecting of *N*-glycans, with the increase in β1,6-branching structures, the increased sialylation and polysialic acid

synthesis, the increased fucosylation, alterations in the synthesis of histo-blood group-related antigens and anomalous appearance of Lewis-type antigens, such as sialyl-Lewis x (SLe^x), in diverse glycoproteins, among others [1].

Many of the aberrant glycoproteins are secreted into the blood stream and, thus, their detection in blood or serum is highly valuable for the diagnosis and monitoring of the disease. Currently, several glycoproteins are approved and used as cancer biomarkers in clinical settings, in the diverse stages of cancer progression, namely for diagnosis, prognosis, staging, therapy monitoring and recurrence detection [4]. Examples include α-fetoprotein (AFP) and AFP-L3 fraction for liver cancer, cancer antigen 125 (CA125) for ovarian cancer, cancer antigen 15-3 (CA15-3) for breast cancer, carcinoembryonic antigen (CEA) for colon, gastric, pancreatic and lung cancers, and prostate-specific antigen (PSA) for prostate cancer. Many other glycoproteins with aberrant glycans have been discovered and are already established as carcinoma biomarkers although their use in the clinic is not yet approved.

Detection of cancer glycobiomarkers in serum presents some analytical difficulties since these aberrant molecules are present in very low amounts, particularly in early stages of the disease, surrounded by a very complex matrix where highly abundant proteins and glycoproteins, such as albumin, transferrin, α-2-macroglobulin, immunoglobulins, among others, may interfere in the determination. The dynamic concentration range of proteins and glycoproteins in serum comprises around ten orders of magnitude, going from mg ml^{-1} to pg ml^{-1} [5]. Therefore, the analytical methods used for cancer glycobiomarker detection must present high sensitivity and selectivity, in order to be useful for clinical applications.

Current tests used for the detection of cancer glycobiomarkers are mainly of the immunoassay type, using the specific interaction between antibody and antigen to detect the glycoproteins by the protein structure. Nonetheless, in this case, only the glycoprotein concentration is determined, and not the aberrant glycosylation, which is a valuable indicator of the presence of tumor cells and may further increase the sensitivity and selectivity of the assays. For instance, the immunodetection of AFP for hepatocellular cancer (HCC) diagnosis presents false positives, as it does not discriminate benign from malign tumors. Hence, it was substituted by a test that quantifies a glycosylated form of AFP – the AFP-L3 fraction. This fraction is fucosylated (presents several fucose units bound to the protein), and fucosylation is significantly increased in the case of HCC, comparing to the benign liver diseases, where the AFP is overexpressed but shows normal glycosylation. This way, the AFP-L3 fraction test allows to distinguish both situations and, thus, reduces the number of false positives [6]. Besides the AFP-L3 fraction, another AFP glycoform named monosialylated AFP (msAFP) enables to differentiate HCC in early stages (when AFP concentrations don't allow the diagnosis) from hepatic cirrhosis (with identical AFP concentration) [7]. In these cases, quantification of total serum AFP does not discriminate HCC from chronic non-malignant liver diseases, but qualitative and quantitative information obtained from the *N*-glycosylation profile of serum AFP significantly improves the performance of AFP as specific HCC biomarker. In addition to AFP glycosylation analysis, the serum levels of fucosylated glycoforms of α-1-antitripsin and transferrin in HCC patients are significantly higher than the levels present in hepatic cirrhosis patients [8].

Lectins are proteins or glycoproteins present in diverse living organisms that selectively recognize and bind to glycan structures, through hydrogen bonds, metal coordination, van der Waals and hydrophobic interactions. They have been employed in different analytical methodologies, such as lectin affinity chromatography [9], lectin arrays [10], enzyme-linked lectin assay (ELLA) [11] and lectin biosensors [12], with the aim of selectively bind to specific glycan structures, enabling to detect them or separate them from complex matrices. Compared to antibodies, lectins are less expensive, easier to be obtained and present longer shelf life [13]. The affinity of lectins towards particular glycan molecules makes them adequate biorecognition agents for the detection of cancer serum glycobiomarkers, as already demonstrated [14].

Among all the possible analytical formats for clinical tests, biosensors present suitable characteristics, as they are portable, of simple-use, require low sample volumes and sample contamination is avoided because each sample is analyzed by an individual biosensor. Therefore, lectin biosensors are attractive devices for the detection of cancer-associated glycobiomarkers in serum since they combine the advantageous aspects of biosensors with the high selectivity of lectin biorecognition [15].

This work presents the construction and validation of three lectin-based impedimetric biosensors for the selective detection of specific aberrant cancer-associated *O*-glycans, namely STn, Tn and T antigens, which are well-established pan-carcinoma glycobiomarkers. For these three biosensors, *Sambucus nigra* type-I agglutinin, *Vicia villosa* agglutinin and *Arachis hypogeae* agglutinin were used as biorecognition elements, with specificity for STn, Tn and T antigens, respectively. A thorough comparison of their characteristics and analytical performance is presented and the advantages and limitations are discussed, considering the intended applications.

This work was presented in the *6th International Conference on Sensors Engineering and Electronics Instrumentation Advances (SEIA'2020)* that took place in Porto, Portugal, from 23 to 25 September 2020 [16].

2. Materials and Methods

2.1. Chemicals and Materials

The lectin biosensors were constructed on screen printed gold electrodes (Au/SPE 4 mm diameter, 220BT Dropsens). *Sambucus nigra* type-I agglutinin, *Vicia villosa* agglutinin and *Arachis hypogeae* agglutinin (in pure form, all from EY Laboratories) were used as biorecognition elements. Lectin immobilization was performed by covalent binding to self-assembled monolayers (made with 16-mercaptohexadecanoic acid (16-MHDA) and/or 6-mercaptohexanoic acid (6-MHA)) via amine coupling, using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (ECD) and N-hydroxysulfosuccinimide (NHS). Ethanolamine was used to block unoccupied terminal carboxyl groups of alkanethiols (Fig. 1) [17-19]. With the exception of ethanolamine solution that was prepared in water, all solutions were prepared in PBS 1x (buffer solution) with pH = 7. Several glycoproteins were used as glycoprotein models, according to the glycan epitopes carried by them. For the SNA-I biosensor, bovine submaxillary mucin (BSM) and human transferrin (Trf) were the model glycoproteins; for VVA biosensor, asialofetuin (aFet) and asialo-bovine submaxillay mucin (aBSM) were used and for PNA biosensor aFet was the model glycoprotein. Details on the construction procedure and the employed solutions can be found in the respective papers [17-19].

2.2. Impedance Measurement Procedure

The binding event between each lectin and the corresponding aberrant *O*-glycan was monitored by electrochemical impedance spectroscopy (EIS). All impedimetric measurements 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 $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ pair, with a 5 mV sinusoidal excitation amplitude, within a full frequency range from 0.010 Hz to 100 kHz.

Once the biosensors were built, 40 µl of the redox probe (solution of 5.0 mmol l^{-1} potassium hexacyanoferrate(III) and 5.0 mmol l^{-1} potassium hexacyanoferrate(II) trihydrate) was applied on the surface and the impedance was measured. Then, the biosensors were rinsed with PBS and 10-40 µl (depending on the biosensor used) of sample or model glycoprotein solution were incubated for 5-10 min (incubation time), at room temperature. The sensor surface was then washed with 2 % SDS solution and again the impedance was measured in the presence of the redox probe.

The impedance spectra were represented in a Nyquist diagram, from which the formation of the complex and the increase in the resistance to charge transfer (ΔR_{CT}) was 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:

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

The increase in impedance was related to the lectin-glycan complex formation (Fig. 1).

Measurements were performed in duplicates.

2.3. Sample Collection and Processing

Human blood samples were obtained from healthy blood donors and from diagnosed cancer patients, from Centro Hospitalar São João (CHSJ), Medical Faculty of the University of Porto (Porto, Portugal) and Pachuca General Hospital (México). 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 Supporting Information of the respective papers [17-19], as well as the procedure performed for blood sample collection, processing and storage.

3. Results and Discussion

3.1. Biosensor Construction Procedure

Biosensors were built on screen printed gold electrodes. As the lectins were intended to be covalently immobilized on the electrode surface through alkanethiol self-assembled monolayers (SAMs), a gold surface was particularly suited to support the formation of the SAMs, as thiol (-SH) groups from the alkanethiols strongly bind to gold enabling to build robust biosensors. Furthermore, gold is inert and compatible with biological systems [20].

Model glycoproteins were used to optimize the construction and to characterize the analytical performance of all biosensors and, whenever possible, two glycoproteins with very different molecular weight and structure were employed, to simulate the diversity of glycoproteins carrying the glycans of interest present in serum. That was also the reason not to use glycopeptides as these structures don't mimic the real glycoproteins present in the samples.

EIS was the analytical technique chosen to monitor the formation of glycan-lectin complexes since it is well-suited to characterize surfaces, detecting the increase in the resistance of the electrode to charge transfer, caused by the formation of big structures such as the referred complexes. Furthermore, it presents the advantage of label-free detection, which simplifies and reduces the cost of the detection procedure [21].

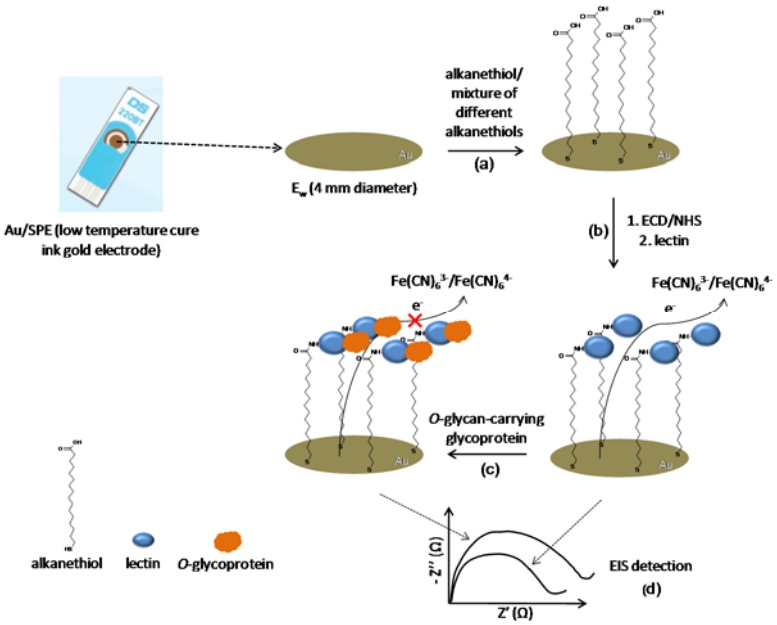


Fig. 1. Schematic diagram describing the construction of each lectin biosensor and detection of aberrant *O*-glycans by EIS: (a) alkanethiol/mixed alkanethiols self-assembled monolayer is formed via incubation of screen-printed electrodes for 24 h; (b) the terminal carboxylic acids of the alkanethiols are activated with ECD and NHS to allow covalent binding with the lectin; (c) the truncated *O*-glycan present in glycoproteins is captured based on the affinity of the lectin to the referred structure; (d) the formation of the complex lectin-truncated *O*-glycan is monitored by the increase in the electrode impedance (by EIS).

Optimization of the biosensor construction process was performed using the univariate method and choosing, for each variable, the conditions that enabled the maximum increase in the impedance after incubating the biosensors with the solutions of the glycoproteins used as models (maximum $\%\Delta R_{CT}$), and with high reproducibility.

The variables optimized were (a) the composition and concentration of the SAM; (b) the concentration of the cross-linker; (c) the amount of lectin immobilized on each biosensor; (d) the blocking procedure.

Starting with the composition and concentration of the SAM, two options were tested, namely one- or two-component SAMs, using a large alkanethiol (16-MHDA) and a short alkanethiol (6-MHA). Different proportions between both alkanethiols were tested, as well as different concentrations, from 0.5 to 40.0 mmol l^{-1}. Table 1 presents the optimum combinations obtained for each biosensor.

Table 1. Optimum composition of the SAM for each lectin biosensor.

Biosensor	SAM composition	SAM concentration (mmol l^{-1})
SNA-I	16-MHDA	25.0
VVA	16-MHDA	25.0
PNA	6-MHA + 16-MHDA	12.5 + 12.5

It is known that alkanethiols with longer chains (n > 12 carbons) form SAMs that are denser, more compact and stable, in opposition to the SAMs formed by short chain alkanethiols (n < 12 carbons) [20] because, for longer chains, more Van der Waals interactions between adjacent chains can be established, compared to shorter chains. A mixed SAM, formed by two or more alkanethiols with different chain size, is proposed in several studies with the advantages of allowing to control the thickness of the SAM [22], diminishing its resistivity, and also permitting a reduction of nonspecific adsorption of sample matrix molecules [20, 23].

For all the developed lectin biosensors, it was observed that higher impedance with more reproducible results was obtained when the SAM was prepared from 16-MHDA and that is why this SAM composition was chosen for two of the biosensors. The above mentioned characteristics of short and long chain alkanethiols could explain the better results obtained for a SAM of 16-MHDA and the instability shown for the mixed SAMs and for the SAM exclusively made of 6-MHA.

For the VVA biosensor, a supplementary test was performed to evaluate the biosensor response to albumin, a potential interferent in serum samples, and better results (lower response) was obtained for the mixed SAM, which was chosen as best in this case.

As for SAM concentration, it was found that, for SNA-I and VVA biosensors, the best relation between $\%\Delta R_{CT}$ and reproducibility was obtained for 25 mmol l^{-1} of 16-MHDA. Lower concentrations generated lower $\%\Delta R_{CT}$ and higher irreproducibility in results due to incomplete coverage of the electrode surface and lower amount of lectin immobilized, and higher concentrations originated a decrease in the

$\%\Delta R_{CT}$, which could be attributed to aggregation and auto-oxidation of the alkanethiol molecules that happen at high concentrations, impairing the binding of lectins [24]. Although the same optimization was not performed for the PNA biosensor, as predefined concentrations of the several combinations of alkanethiols were tested, based on the results of the SNA-I and VVA biosensors, it is expected that similar conclusions would be reached for this biosensor. It is worth noting that the total concentration of the mixed SAM for PNA biosensor was the same as the concentration of the 16-MHDA SAMs for the other two biosensors, which denotes high reproducibility on the analytical behavior for the developed biosensors, in spite of the diversity of immobilized lectins.

The covalent coupling of the lectins to the SAMs was performed via standard amine coupling chemistry with the first step being the activation of the terminal carboxylic group of 16-MHDA and/or 6-MHA by a mixture of ECD and NHS (cross-linker solution). Concentrations of cross-linker solution were optimized, using solutions presenting different proportions of both components. Table 2 resumes the optimum concentrations obtained for each biosensor.

Table 2. Optimum composition of the cross-linker solution for each lectin biosensor.

Biosensor	Cross-linker composition	Cross-linker concentration (mmol l^{-1})
SNA-I	ECD + NHS	20.0 + 5.0
VVA	ECD + NHS	20.0 + 5.0
PNA	ECD + NHS	20.0 + 5.0

As can be seen, the concentration of cross-linker solution that originated better reproducibility in results and higher $\%\Delta R_{CT}$ was the same for all biosensors. This result shows high reproducibility, independently of the lectins employed, although the variations in the analytical response for the diverse combinations tested were not the same for all biosensors. Furthermore, it was not possible to establish a clear relation between ECD and NHS concentrations and analytical signal. Thus, the effect of the cross-linker concentration on the response is, probably, the result of the influence of ECD and NHS individual concentrations but also their proportion and their joint actuation, which affects the binding of the lectins.

Optimization of the lectin amount immobilized on the biosensor surface was carried out bearing in mind two aspects, namely (1) the lectin is the biorecognition agent, therefore its amount directly affects the number of glycoproteins captured and, thus, the sensitivity of the devices, and (2) the high cost of the lectins. Hence, the lectin amount immobilized on each biosensor was optimized in order to find the minimum lectin amount that generated the maximum response. Table 3 depicts the optimized lectin amounts obtained for each biosensor.

Although some variation in the optimum lectin amounts is observed for the three biosensors, which could be due to the different structural characteristics of each lectin, the influence of the lectin amount in the analytical signal was identical for all biosensors. As expected, it was observed that for low amounts (25.0 μg), the response was also lower. As this represented very small solution volumes applied on the biosensor surface, more irreproducible results were obtained for lower lectin concentrations. As the lectin amounts increased, the number of sites for complex formation between lectin and glycan also increased and, therefore, the impedance was higher. For the upper amounts evaluated (80.0 – 100.0 μg), the analytical signal showed a plateau or decreased, which could be explained by the saturation of the biosensors surface coverage or by agglutination phenomena at high lectin concentrations, which diminished the number of available lectin molecules for glycan binding [25].

Table 3. Optimum lectin amounts for each lectin biosensor.

Biosensor	Lectin amount (μg) per biosensor
SNA-I	60.0
VVA	75.0
PNA	50.0

To finalize the construction procedure of the lectin biosensors, a blockage step was implemented and optimized, to avoid or minimize the nonspecific interactions between components of the serum sample matrix (namely albumin and other high-abundant proteins) and the biosensor surface, which could increase the analytical signal and generate false positive responses. Besides nonspecific adsorption of high-abundant proteins to the biosensor surface, other interferences could be due to interactions between matrix proteins and non-occupied terminal carboxylic groups from the alkanethiols and interactions between the lectins and glycans other than the glycans of interest. The later type of interaction is inherent to lectins, as they are not totally specific to a single epitope and may bind to a more or less restrict group of glycan structures and, thus, it cannot be avoided when using lectins as biorecognition agents.

To minimize the interaction between non-occupied terminal carboxylic groups from the alkanethiols and proteins and glycoproteins from the sample matrix, ethanolamine was used as blocking agent, and all biosensors were immersed in an ethanolamine solution for 30 minutes at the end of the construction procedure. To minimize nonspecific adsorption of proteins and glycoproteins on the biosensor surface, surfactants such as ethylene glycol and SDS were also used. The VVA and PNA biosensors were immersed in an ethylene glycol solution for 30 minutes, after the ethanolamine blockage step. Furthermore, the PNA biosensor was washed with SDS solution after sample

incubation (before the second impedance measurement), with the purpose of removing the adsorbed proteins and glycoproteins that did not form complexes with the lectin. Table 4 shows the blocking agents used for each biosensor, as well as the blocking conditions.

For all the lectin biosensors, it was shown that incubation with ethanolamine reduced nonspecific interactions. Blank PBS solutions containing no glycoproteins were incubated with the biosensors and the increase in the analytical signal was within the noise level ($\%\Delta R_{CT} \leq 10$ %) for the SNA-I biosensor. As for the VVA and PNA biosensors, in spite of the favorable effect of ethanolamine blockage on the analytical response, it was not enough to reduce the nonspecific interactions, which were finally minimized with the use of the referred surfactants.

Table 4. Blocking procedures employed for each lectin biosensor.

Biosensor	Blocking agent(s)	Blocking procedure
SNA-I	ethanolamine (20 mmol l^{-1})	biosensor immersion for 30 min
VVA	ethanolamine (20 mmol l^{-1}) + ethylene glycol (10 % V/V)	biosensor immersion for 30 min on each blocking solution (sequentially)
PNA	ethanolamine (20 mmol l^{-1}) + ethylene glycol (10 % V/V) + SDS (2 % w/V)	biosensor immersion for 30 min on each blocking solution (sequentially) + washing with SDS solution after sample incubation

The differences in the behavior of the three biosensors is not easily understood, since the construction conditions were similar (though not exactly the same) for all of them. Reasons for this distinct behavior concerning the blocking procedure requirements may rely on the structural characteristics of each lectin, namely their tridimensional configuration, molecular weight and different affinity profile towards the glycan epitopes, which might be present in serum glycoproteins.

Blocking procedures are common in analytical methodologies based on bioaffinity reactions, even when highly-specific biorecognition molecules such as antibodies are used. Additionally, the proteins and glycoproteins present in serum samples tend to adsorb on surfaces, affecting the analysis based on flat supports, such as the case of biosensors. The inclusion of a blockage procedure in the construction inevitably increases the labor and the time required to prepare the biosensors (in these cases, one hour more). Nonetheless, the proposed blockage procedures used accessible reagents, and the blockage procedures did not affect the lectins' ability to recognize and bind to the glycans of interest.

3.2. Optimization of Measurement Procedure

Measurements were performed by EIS in two steps: (1) scanning the first Nyquist plot with a new biosensor, followed by application of the glycoprotein solution or sample for a period of time (incubation time), washing with PBS and then (2) the second impedance measurement (second Nyquist plot).

In this procedure, the sample incubation time was optimized, selecting the time that originated the highest response, which corresponded to the maximum number of complexes formed. Nonetheless, it is desirable that the assay can be performed in the shortest time possible. Therefore, equilibrium between sensitivity and assay time was key to select the best incubation time. Table 5 presents the incubation times elected for each biosensor, optimized with the respective model glycoproteins.

Table 5. Optimum incubation times for each lectin biosensor.

Biosensor	Incubation time (min)
SNA-I	5
VVA	10
PNA	10

Theoretically, a reduced incubation time impairs the formation of a stable complex and a long incubation may degrade the biosensor's surface and cause a loss in its integrity, so it was expected that the analytical signal would increase with the incubation time up to some point and then it would be constant or decrease. For the SNA-I biosensor, an increase in impedance was already observed for 5 min of incubation, which did not increase for longer times. Therefore, it was assumed that the binding between SNA-I and the corresponding model glycoproteins was a fast process. For VVA, the $\%\Delta R_{CT}$ increased with the incubation times with a maximum for 25 min. This suggested that Tn-binding to VVA proceeded in a relatively slow way. The signal seemed to continue increasing, but longer times were not evaluated because, for longer times, the applied solution (40 µl) started to dry, which affected the result, as the biosensor must be always hydrated for lectin activity. For the PNA biosensor, the $\%\Delta R_{CT}$ increased with the incubation time up to 10 min and then progressively decreased for longer incubation times. This suggested that T-antigen binding to PNA proceeded in a relatively fast way. The variation in lectin behavior regarding the incubation time must be related with the intrinsic characteristics of each lectin and the way each one interacts with the respective glycans. Nevertheless, it was possible to select, for all biosensors, a short incubation time without affecting the sensitivity of the measurement.

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 and the best relation between $\%\Delta R_{CT}$ and measurement time was achieved for the interval starting at 10 mHz. Under these conditions, each impedance spectrum was obtained in about 4 min. The same EIS parameters were used for all biosensors.

Together with the time required to scan the Nyquist diagrams, the total assay time (first measurement, incubation and second measurement) was ≤ 20 min.

3.3. Characterization of the Developed Biosensors

According to the experimental data obtained for all biosensors, the Randles equivalent circuit for the developed biosensors was the same and is depicted in Fig. 2. As all biosensors shared the same electrode support, construction procedure and composition, in spite of containing different lectins, it is not surprising that they all had the same impedimetric behavior.

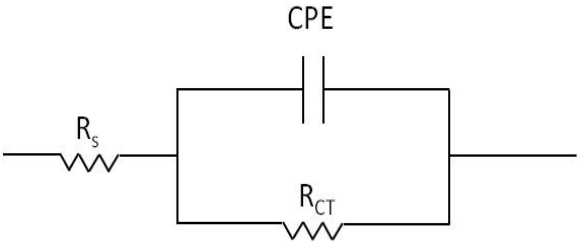


Fig. 2. Randles equivalent circuit for the developed biosensors. R_s – resistance of the electrolyte solution; CPE – constant phase element; R_{CT} – charge transfer resistance.

The surface of VVA and PNA biosensors was observed by scanning electron microscopy (SEM) and the images are shown in Fig. 3. For both biosensors, the surface is characterized by the presence of particles with globular shape, of diverse sizes, that appear conglomerated. The appearance of both biosensors is very similar.

3.4. Analytical Performance of the Developed Biosensors

Once the optimal operating conditions were established, the analytical response ($\%\Delta R_{CT}$) of all biosensors was evaluated at different concentrations of the respective model glycoproteins. For the SNA-I biosensor, the standard additions method was employed, adding successively 10-µl aliquots of standard solutions of BSM or Trf 1.0 mg ml^{-1} on the same biosensor. For the VVA and PNA biosensors, standard solutions of asialofetuin were prepared individually and incubated in individual biosensors, so that each concentration was analyzed by independent biosensors.

For the three biosensors, the impedance increased linearly with the increase in glycoprotein concentration, defining the linear intervals of response. Tables 6-8 show the analytical parameters obtained from the calibration curves for each biosensor, calculated according to IUPAC criteria [26].

Table 6. Analytical parameters obtained from the calibration curve for SNA-I biosensor, using BSM and Trf as model glycoproteins.

Analytical parameter	Value
linear interval	up to 40 ng (BSM) up to 70 ng (Trf)
regression equation	y (Z) = 3010 × (ng Trf) + 288416
correlation coefficient (R^2)	0.9574
limit of detection	20 ng (Trf)

Table 7. Analytical parameters obtained from the calibration curve for VVA biosensor, using aFet as model glycoprotein.

Analytical parameter	Value
linear interval	0.25 to 5.0 µg ml^{-1}
regression equation	y ($\%\Delta R_{CT}$) = 13.416 × (µg ml^{-1}) + 2.831
correlation coefficient (R^2)	0.9964
limit of detection	0.68 µg ml^{-1}
limit of quantification	2.25 µg ml^{-1}

Table 8. Analytical parameters obtained from the calibration curve for PNA biosensor, using aFet as model glycoprotein.

Analytical parameter	Value
linear interval	0 to 150.0 µg ml^{-1}
regression equation	y ($\%\Delta R_{CT}$) = 0.522 × (µg ml^{-1}) + 2.422
correlation coefficient (R^2)	0.9912
limit of detection	11.65 µg ml^{-1}
limit of quantification	61.50 µg ml^{-1}

It should be emphasized that the analytical parameters obtained for each biosensor are only valid for the glycoproteins employed to carry out the measurements and cannot be extrapolated to any other glycoprotein.

Thus, they are merely indicative and will change for each STn-, Tn- or T-carrying glycoprotein assayed because the number of glycan units per molecule may vary. Furthermore, establishing a unique linear range, limit of detection or quantification for each biosensor would require the measurement of each truncated *O*-glycan in free form and not bound to a protein, which is not the physiological form present in serum samples.

Reproducibility of the construction procedure was evaluated by measuring the impedimetric response (R_{CT}) of several bare electrodes (before the fabrication procedure) and after the modification (in the form of biosensors), when measuring the redox probe solution. Table 9 depicts the obtained results.

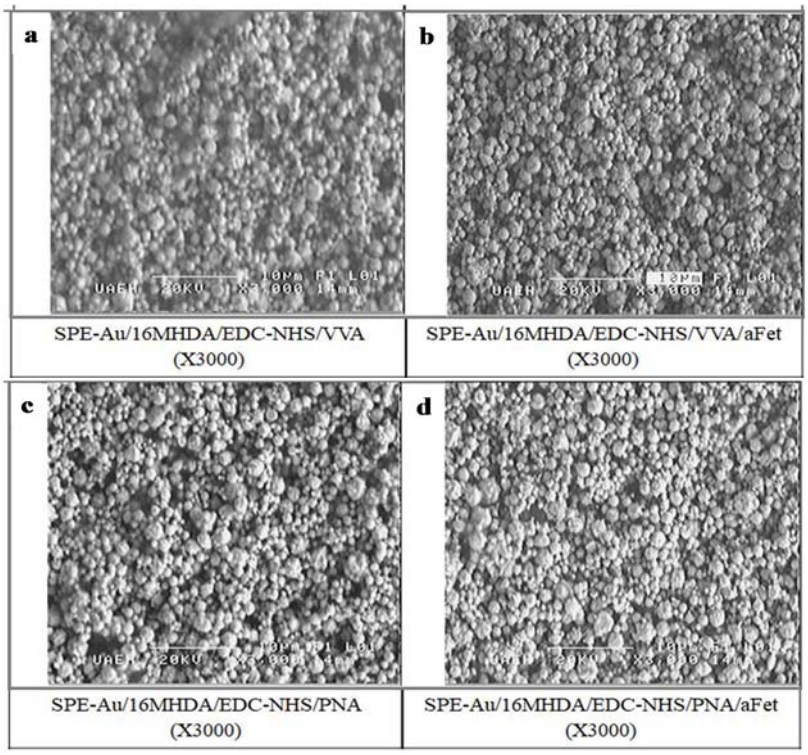


Fig. 3. SEM micrographs of the VVA biosensor (a) before and (b) after incubation with asialofetuin, and of the PNA biosensor (a) before and (b) after incubation with asialofetuin.

Table 9. Results obtained in the evaluation of the reproducibility of the construction procedure.

Biosensor	Number of electrodes tested	%RSD of R_{CT} for bare electrodes (mean)	%RSD of R_{CT} for biosensors (mean)
SNA-I	6	10.6	17.0
VVA	4	16.3	4.2
PNA	7	10.0	5.2

Interestingly, in two of the biosensors, a reduction in %RSD was observed after the fabrication procedure, which means that the modification formed a more homogeneous surface than the original bare surface, which presents an inherent and variable roughness that affects the impedance response. For these cases, the results allowed to conclude that the fabrication methodology was reproducible and the %RSD is acceptable for this kind of disposable biosensors [26]. For the SNA-I biosensor, the results were opposite, which is not easily explainable since the electrodes were all from the same supplier and the fabrication method was the same for all biosensors. Therefore, a higher number of electrodes should be tested for all biosensors, in order to have more consistent results.

The storage stability was also evaluated, since it is a very important factor for the commercialization of analytical devices containing biological molecules that need to maintain their activity, such as biosensors. For the SNA-I biosensor, 6 biosensors were constructed and were used to measure the redox probe solution after each of two consecutive 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. For the VVA biosensor, 8 biosensors were built and used to measure the redox probe after different storage times (on the same day of fabrication and after 3, 6 and 10 days). For each storing time 2 biosensors were analyzed. During storage, the biosensors were kept in PBS at 4 °C. The results showed that the biosensors were stable up to 3 days after construction, considering as reference the analytical response of the biosensor used immediately after preparation. For the biosensors stored during 6 or 10 days, an increase in %RCT values was observed. For the PNA biosensor, 5 biosensors were constructed and one of them was immediately used. The others were stored in PBS at 4 °C for different periods of time (3, 7, 10 and 13 days). The impedance response of the biosensors was measured in the presence of the redox pair and the response of the biosensor used on the construction day was considered as a reference (100 %) for comparison with the stored biosensors. It was shown that the response diminished for the stored biosensors, with higher reductions for longer storage periods (77.5 % of the initial response for 10-day storage).

The increase in the analytical response observed for the SNA-I and VVA biosensors could be explained

by an accumulation of impurities from prolonged contact of the biosensor with the buffer solution [27]. For the PNA biosensor, the decrease in the analytical signal could be justified by a SAM deterioration during long storage times, due to the oxidation of the bound thiolates and their subsequent desorption from the gold surface [20, 28]. Despite the valid arguments that justify the variation in the analytical signal, a dissimilar variation in the impedance was observed for the three biosensors, which is not easily understandable as all of them were stored in PBS solutions prepared from the same reagents and desorption of SAM could happen to all of them. As a general conclusion applicable to all the developed biosensors, they should be preferably used within the 24 h after finishing their preparation (maximum within the first three days) under the used storage conditions. This could be considered a drawback aiming the commercialization of lectin biosensors, and further studies should be performed in order to find better storage conditions.

3.5. Selectivity Studies

A series of cross-reactivity studies was performed to assess the selectivity of the developed biosensors. For selectivity experiments, a response was considered positive (specific interaction) when the $\%\Delta R_{CT}$ was ≥ 10 % (10 % increase in the impedance was set as the background noise limit, considering the response obtained for incubation with PBS solution).

First, the biosensors were constructed without immobilizing the lectins and then they were incubated with the respective model glycoproteins to check the absence of interaction between the glycoproteins and the components of the biosensor other than the lectin. For all biosensors, results showed that there were no significant increases in impedance, demonstrating the absence of significant nonspecific adsorptions even for high glycoprotein concentrations.

In the second row of experiments, the lectin biosensors were incubated with glycoproteins that did not carry the glycan epitopes recognized by each lectin. In this case, no interactions were expected between the lectins and the glycoproteins. Table 10 presents the negative glycoprotein models tested for each biosensor.

For the SNA-I biosensor, the results showed an increase in impedance for fetuin, when 10 ng or higher were incubated. As for the asialo-glycoproteins, an increase in impedance was observed for aBSM and aTrf amounts higher than 15 and 5 μg, respectively. Fetuin is a glycoprotein which is reported to have no STn glycans [29] 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. These results indicate that glycoproteins presenting other glycan structures may interfere in the biosensor response above particular thresholds.

Table 10. Negative model glycoproteins employed in the selectivity studies for each biosensor.

Biosensor	Glycoprotein used as negative model
SNA-I	fetuin asialoTrf asialoBSM BSA
VVA	fetuin Trf asialoTrf BSM BSA
PNA	fetuin Trf BSA

Albumin is the most abundant protein in serum and some adsorption effect may occur at the sensor surface. To assess the interference of high abundant serum proteins on the impedance measurements, bovine serum albumin (BSA) was used to simulate the effect of human serum albumin. It was found that a BSA concentration of 0.2 mg ml^{-1} did not cause a change in the analytical response, but the 2.0 and 20.0 mg ml^{-1} concentrations increased the analytical signal, probably 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.

Considering the VVA biosensor, the indicated glycoproteins were chosen as negative models because fetuin is reported to have no Tn glycans [29], as well as BSM and transferrin. According to the results (Fig. 4), an increase in impedance was evidenced for incubated concentrations of fetuin and BSM of 0.1 mg ml^{-1}. Nonetheless, interference was significantly minimized at 0.01 mg ml^{-1} concentration. These results corroborate the previous conclusion that glycoproteins presenting other glycan structures may interfere in the biosensor's response above particular thresholds. As for BSA, interference was observed for 0.5 and 0.1 mg ml^{-1} concentrations, which could be due to BSA adsorption at the biosensor surface. For 0.01 mg ml^{-1} concentration, BSA did not cause a change in the analytical response (Fig. 4).

Several positive model glycoproteins were also incubated with the VVA biosensor, namely asialoTrf, Trf, asialofetuin and aBSM. All of them generated increases in impedance, as expected (Fig. 4). The different percentages are a result of the variability in molecular structures of the glycoproteins, the number of Tn epitopes that each one carries and the interaction established with the immobilized lectin.

As for the PNA biosensor, on the modified electrode (but without lectin) aFet solutions of 1, 10 and 100 μg ml^{-1} were incubated to examine the

nonspecific interaction between the glycoprotein and the sensor components. As can be seen in Fig. 5, for the lowest concentration tested, there was an interaction between aFet and biosensor components other than the lectin occurs, although in the noise limit. For the higher concentrations the nonspecific interaction increased significantly. On a complete PNA biosensor, 1 μg ml^{-1} solutions of glycoproteins that do not contain the T glycan (Trf and fetuin) [30], were incubated to verify the selective recognition of PNA towards T antigen, and the results showed that, as expected, no interactions occurred (Fig. 5). Finally, albumin interference was evaluated by testing two BSA concentrations, 10 and 100 μg ml^{-1}. The results showed that there was interference only for the higher concentration tested (Fig. 5). The blocking procedure carried out provided good results, with the higher analytical signal corresponding to 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's surface).

Overall, the developed lectin biosensors presented high selectivity towards the glycoproteins carrying the corresponding *O*-glycan epitopes. However, some interaction was also observed for glycoproteins carrying other glycan structures, which can be explained by the fact that lectin binding to glycan epitopes is not as specific as, for example, antibody interactions.

In addition, it should be stressed that EIS is not a selective analytical technique itself, since it detects changes in the electrode resistivity but does not discriminate if the increase in impedance is due to a specific (for example the formation of a complex between the biorecognition agent and the analyte) or nonspecific interaction (for example adsorption of matrix components). Therefore, the selectivity of impedimetric biosensors is exclusively given by the biorecognition molecule present in them.

Nevertheless, considering the sample pretreatments to be performed, the expected amounts of serum glycoproteins and the highly preferential binding of SNA-I, VVA and PNA to STn, Tn and T epitopes, correspondingly, the interference of glycan structures other than the respective ones was estimated to be reduced.

Lastly, the experiments performed with BSA were, furthermore, helpful to define the dilution required, to be performed in sample pretreatment.

3.6. Validation of the Biosensors by Sample Analysis

The lectin biosensors were validated through the analysis of serum samples from healthy blood donors (controls) and from patients with diverse carcinomas (cases).

Serum presents a huge dynamic concentration interval that impairs the detection of low-abundant cancer-secreted glycoproteins in the presence of high abundant proteins. In order to reduce the concentration range of serum proteins and glycoproteins, a combinatorial peptide ligand library (CPLL, Proteominer, Biorad) [31] was used to pretreat all samples, according to the manufacturer's recommendations. The processed sera were stored at -20 °C before being incubated with the biosensors.

For the SNA-I biosensor, prior to impedance analysis, CPLL-processed samples were diluted 1:10 in PBS. Fifteen serum samples (8 controls and 7 cases, one of the cases being a pool of 25 samples from 25 cancer patients) were analyzed (applying 10 μl of diluted sample on the biosensor) and the impedimetric data was processed by Principal Component Analysis (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.

The score values obtained for each sample were plotted and a linear discriminant function was drawn (Fig. 6). As can be observed, a suitable classification of samples in two distinct groups (controls and cases) was achieved with the PCA approach.

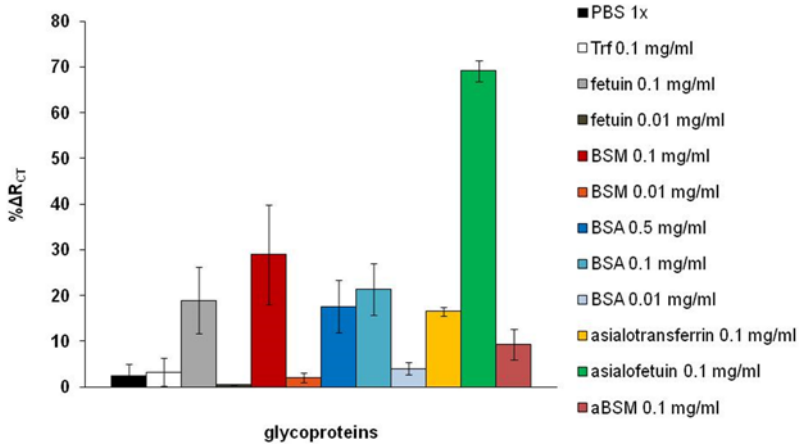


Fig. 4. VVA biosensor responses for several glycoproteins and proteins at different concentrations, incubated for 10 min. AsialoTrf, Trf, asialofetuin and aBSM are the glycoproteins carrying the Tn epitope. Error bars indicate the standard deviation of duplicate measurements (two independent biosensors for each solution).

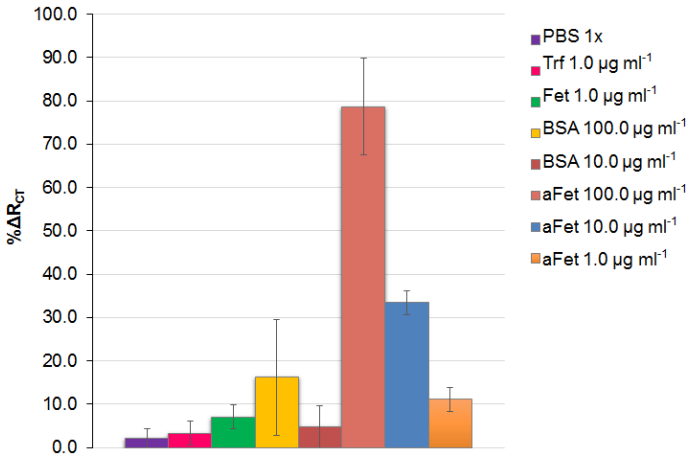


Fig. 5. PNA biosensor responses for several glycoproteins and proteins at different concentrations, incubated for 10 min. Aialofetuin is the glycoprotein carrying the T epitope. Error bars indicate the standard deviation of duplicate measurements (two independent biosensors for each solution).

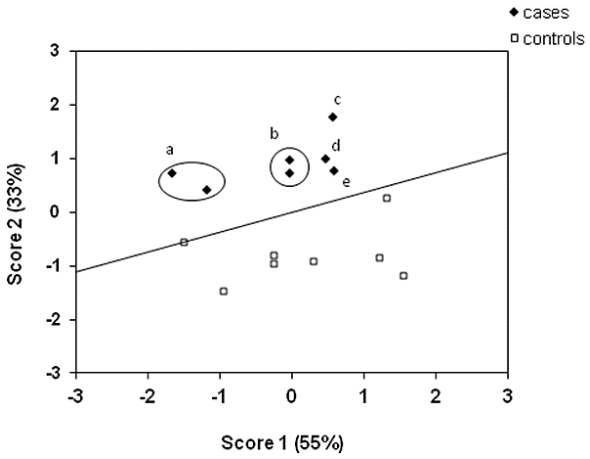


Fig. 6. Graphical representation of the first two scores of a PCA performed on the impedimetric data from sample analysis. Each point represents an individual analysis of a sample; (a) breast carcinoma, (b) retroperitoneal located malignant tumor, (c, e) pools with 25 different cancer samples, and (d) cervical-uterine carcinoma.

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 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.

Although the analysis of a higher number of samples would be required to allow more consistent conclusions, the obtained results clearly showed an increase of STn expression in serum glycoproteins of cancer patients, with different types of carcinomas, and this altered level of expression could 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.

The same samples were further analyzed by Western blot, following bidimensional electrophoresis, using TKH2 antibody, with specificity towards the STn epitope. The results showed that immunoreactivity for STn antigen was higher for the cancer samples [17], compared to the control group, corroborating the results obtained with PCA analysis.

To validate the VVA biosensor, 10 serum samples (4 controls, 4 cases and a pooled sample of each type, containing 25 samples each) were analyzed, incubating 10 µl of diluted sample (1:20 in PBS) on the biosensor. By pooling samples, the individual characteristics and variability are minimized and the common aspects, in this case the amount of Tn antigen, is enhanced [32, 33]. By doing so, the detection of the truncated *O*-glycan was facilitated and a more reliable detection was assured.

For each CPLL-treated sample, protein concentration was determined in order to apply the same amount of total proteins on each biosensor and, this way, the differences in the analytical response were due to distinct glycan amounts and not to different protein amounts. Samples were analyzed in a blind way, to avoid bias. For this, all samples were identified with a random numeric code and the person that carried out the analysis had no knowledge of which were controls and which were cases.

The results obtained in sample analysis are indicated in Table 11 [18].

Control samples generated lower increases in the analytical response, compared to case samples, although higher than the noise limit level. This could indicate some nonspecific recognition of non-Tn glycans by the lectin. Adsorption of high-abundant proteins could also contribute to the increase in the analytical signal, but it would equally affect control and case samples, as the same protein amount was applied on the biosensors.

Table 11. Results obtained in sample analysis, for the VVA biosensor.

Sample	Sample type	$\%\Delta R_{CT}$
1	control	9.54
2	control	19.81
3	control	13.85
4	control	19.73
5	pool 25 controls	-9.96
6	case	48.19
7	case	57.45
8	case	36.44
9	case	56.03
10	pool 25 cases	27.36

A *t* Student test was performed on the sample results to compare both experimental means. The calculated |t| value (4.32) was higher than the critical t value (2.31, two tail), for a confidence level of 95 % (n = 5). Thus, the null hypothesis was rejected and the test concluded that both sample sets presented different means.

Also, a Mann-Whitney *U*-test was carried out to compare both groups of samples. According to the table, for a one-side test at P = 0.05, the test statistic (0) was lower than the critical value (4), meaning that the null hypothesis was rejected and that the case samples presented higher Tn amounts. The obtained impedance data allowed to correctly classify the samples in two distinct groups (Fig. 7) [18].

In spite of the limited number of samples analyzed, which does not allow a more consistent conclusion, the results clearly showed an increase of Tn expression in serum glycoproteins of cancer patients, with different types of carcinomas, and this increased level of expression could be detected by the developed biosensor.

For the PNA biosensor, 10 serum samples (1 pool of 25 controls and 9 pools of cases grouped by organ) were analyzed using the developed biosensor. Also, the same samples were analyzed with the VVA biosensor. CPLL-processed samples were diluted in PBS 1:20 for VVA biosensor and 1:40 for PNA biosensor. The obtained results are represented in Fig. 8 [19].

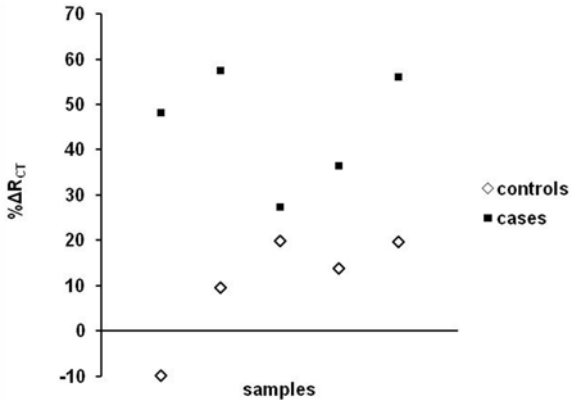


Fig. 7. Results obtained in the Mann-Whitney *U*-test for the VVA-analyzed samples.

For all controls, the $\%\Delta R_{CT}$ was ≤ 13.1, which is slightly higher than the noise level. This could be due to some nonspecific recognition of some non-Tn and non-T glycans present in the samples by the lectins, and also some adsorption by high-abundant proteins. Nonetheless, it was an acceptable increase for control samples.

As for the cases, diverse responses were obtained for the distinct pools, for VVA and PNA biosensors. As can be seen in Fig. 8, the Tn antigen was highly expressed in lung, prostate, rectum, breast and skin carcinomas and showed low expression in colon, kidney, stomach and liver carcinomas. For the T antigen, higher responses were obtained for skin, breast and lung carcinomas, while kidney, liver, rectum, colon and prostate cancers generated lower responses.

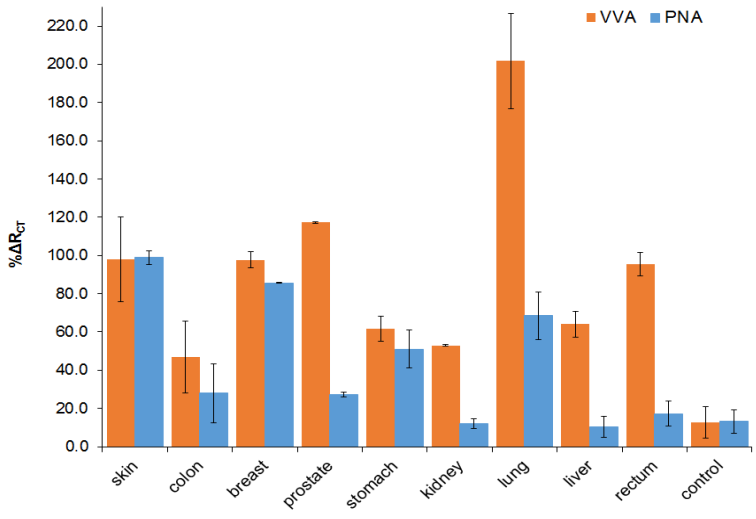


Fig. 8. Results obtained in the analysis of serum samples, with the VVA and the PNA biosensors. Error bars indicate the standard deviation of duplicate measurements (two independent biosensors for each solution).

Considering the clinical data compiled for each blood sample, the pools that included 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). The presence of samples in advanced progression of cancer could justify the higher increases in impedance for Tn detection for these pools, except for the case of kidney cancer, which revealed low expression of Tn. As for T antigen expression, correlation with advanced stages of disease was not observed, except for breast pool. These findings raise 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, higher responses were detected for both biosensors in the case of skin cancer, which could mean that T and Tn antigens are highly expressed in this type of carcinoma, 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.

To assess the quality of the obtained results, a thorough review of published data on the expression of Tn and T antigens in different carcinomas was carried out and the findings were presented in the original paper [19], as well as herein. According to published data, primary breast carcinomas show high expression of Tn and T antigens [34, 35], with Tn expression presenting a highly significant correlation with clinicopathological tumor stage, while T antigen expression has no association with prognostic factors (Desai, 2000; Springer, 1997). The results obtained with the lectin biosensors are in accordance with the referred data.

T antigen is also highly expressed in lung, upper gastrointestinal and genitourinary carcinomas and in melanoma [36]. Although high responses were obtained with the PNA biosensor for lung, stomach and skin cancers, corroborating the published data, expression in kidney samples was low, in opposition to what was expected. In the results obtained with the lectin biosensors, both T and Tn showed high expression in skin cancer, in accordance with the literature [37, 38].

T and Tn antigens are expressed in colon cancer, and Tn expression is associated with poorly differentiated adenocarcinomas and mucinous carcinomas [35, 39]. Unlike Tn, T antigen was preferentially expressed in well-differentiated adenocarcinomas and, compared to Tn, T antigen was found in fewer mucinous carcinomas. In another study, T antigen was found to be associated to colorectal carcinoma, exhibiting qualities of a prognostic marker [40]. In another study, detection of high expression of T and Tn antigens in colorectal cancer cells was effectively accomplished by using lectin staining with PNA and VVA, among other lectins [41]. In our results, for colon and rectum carcinoma samples, Tn was more expressed than T antigen, particularly in rectum cancer sample.

In other papers, it was reported that the Tn antigen was highly expressed in prostate cancer and the T antigen was expressed in breast, colon and prostate cancers [42, 43]. Similar result was obtained in the present study for Tn expression in prostate samples and for T expression in breast samples, but data for T expression in colon and prostate was not elevated, when compared to other case samples.

The T antigen was found to have relatively high expression in pulmonary adenocarcinoma, with immunoreactivity for 48 % of the patients analyzed. This T antigen immunoreactivity was not correlated with gender, tumor size, stage of the disease and metastasis, but it was shown to be a marker of poor prognosis [44]. As for Tn antigen expression, it is expressed in around 70 % - 100 % of the lung cancer cases [35, 38]. In our results, lung cancer was among the samples for intermediate response regarding T antigen, in spite of the fact that lung pool had 33 % of metastized samples. On the contrary, the lung cancer sample showed the highest result in Tn detection, corresponding to a high expression of this antigen.

In other studies, T antigen immunoreactivity was evaluated and correlated with gastric cancer in about 60 to 100 % of the analyzed carcinomas [35, 45]. The authors found that T and MUC1-T immunoreactivity defined a high-risk subgroup of stage I patients in gastric cancer. Tn antigen was also reported to be highly expressed in stomach cancer [35, 38]. Our results for T expression in stomach carcinomas revealed an intermediate response, which was slightly higher for Tn detection in the same sample.

Kidney and liver carcinomas showed higher expression of Tn antigen, with lower expression of T antigen. These results are in agreement with reported data [46, 47].

To resume the comparison between the obtained results with VVA and PNA biosensors and the evidence already published in other studies, the data generated by the developed biosensors is, generally, in agreement with the current knowledge on the expression of Tn and T antigens in diverse types of carcinomas. A clear distinction is observable between the responses for every case sample and the control sample, which enables to conclude that the developed biosensors were able to correctly distinguish controls from cases. The expression of Tn and T antigens varies, depending on the organ of the primary tumor.

The results could have been affected by some bias, namely: (1) the age of healthy donors versus cancer patients is not equivalent; (2) besides the presence of cancer, healthy status of controls and cases was not equivalent, since some cancer patients presented other chronic diseases such as diabetes or hypertension; (3) analysis of sample pools containing samples in different disease stages impairs correlation of results with the disease stage. Geographical and technical bias were not plausible since all samples were collected on the same geographical location, on the same

population, and the process of sample collection, processing and storage was carried out exactly under the same procedure, by the same person. All samples were submitted to the same cycles of freezing/thawing.

Also, some limitations on this study, concerning sample analysis, must be pointed out. First, a higher number of samples should be analyzed, in order to have more consistent and statistically robust results. The justification for the reduced number of samples used to validate the biosensors relies on the difficulty to have access to cancer patients that gave their consent to donate the blood sample, as well as other constraints related to burochratic and ethical permissions from the participating hospitals.

Second, every analytical methodology newly developed should be validated by performing sample analysis with a different and already well-established method, based in a different type of detection, so that results obtained by both methods can be compared. In this study, only the samples analyzed by the SNA-I biosensor were further analyzed by 2D-electrophoresis and Western blotting, and the same should have been performed for the samples analyzed by the VVA and PNA biosensors. Although that was the desire of the authors, logistical difficulties in the access to the equipment and reagents required to perform Western blot assays have impaired to carry out the analysis.

Third, it would be very interesting to perform sample analysis using the three lectin biosensors simultaneously on the same samples (as was carried out for the VVA and PNA biosensors). The development of this work was extended through several years and the first idea was to develop and validate each biosensor individually. Nonetheless, the idea of testing the same samples with the three biosensors came during experimentation, as a way to characterize the profile of truncated *O*-glycan expression in diverse carcinomas, using lectin biosensors, which is not yet done, up to our knowledge.

4. Conclusions

Considering the work carried out to develop the three lectin-based impedimetric biosensors, and the results obtained throughout their characterization and validation, a number of general conclusions can be enunciated:

- All biosensors were constructed following the same general procedure, demonstrating its high versatility. It could be adapted to any lectin impedimetric biosensor, does not require very expensive reagents, except the lectin, nor extreme fabrication conditions. Furthermore, the construction procedure was show to be suitable to immobilize the lectins, as they kept their activity and affinity towards their specific glycan structures.

- The use of gold screen printed electrodes as support to the lectin biosensors was adequate, allowing for an easy construction of the biosensors, and giving them the characteristics of portability and simple use.

- The employed lectins showed high selectivity towards the STn, Tn and T antigens, and were able to discriminate different glycan structures present in glycoproteins. Moreover, the three biosensors correctly discriminated samples from healthy donors and from cancer patients with different carcinomas, showing high selectivity towards the antigens STn, Tn and T in real sample analysis. The obtained results are in agreement with the data already published concerning the expression of truncated *O*-glycans in diverse types of carcinomas. Ultimately, the use of thee three lectin biosensors in the analysis of the same sample could allow to characterize the glycosylation profiles of glycoproteins in the different types of carcinomas, according to the organ of primary tumor location.

- The developed lectin biosensors constitute potentially useful tools for the clinical detection and monitoring of cancer glycobiomarkers, as alternatives or complement to other well-established and routinely-used analytical assays.

- The developed lectin-based impedimetric biosensors were label-free, which enabled to carry out a simple, fast and economical measurement procedure.

- These works open the possibility to develop a multi-lectin biosensor, capable of performing a multiplexed analysis, where several aberrant glycans could be detected simultaneously on the same sample and on the same biosensor, allowing to reduce analysis time, sample volume requirement and costs.

- The storage stability of the lectin biosensors was limited to three days after their construction, which is a major drawback for their commercialization and implementation in clinical context. Further studies must be carried out to improve this stability parameter.

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