Sensors and Actuators B 252 (2017) 777–784

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

A *Vicia villosa* agglutinin biosensor for cancer-associated Tn antigen

M. Luísa S. Silva[a,b,*], María G.H. Rangel[a]

[a] Centre of Chemical Research, Autonomous University of Hidalgo State, Carr. Pachuca-Tulancingo km 4.5, 42076 Pachuca, Hidalgo, Mexico
[b] LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy of the University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 18 January 2017
Received in revised form 30 May 2017
Accepted 4 June 2017
Available online 10 June 2017

Keywords:
Tn-antigen
Cancer
Vicia villosa agglutinin
Screen-printed-electrodes
Biosensors

ABSTRACT

A label-free biosensor for selective detection and discrimination of the cancer-associated Tn antigen was developed by using *Vicia villosa* agglutinin (VVA) as the recognition element. The VVA biosensor was constructed by immobilizing the lectin on screen-printed gold electrodes. The formation of complexes between VVA and Tn-containing glycoproteins (asialo bovine submaxillary mucin and asialofetuin) were monitored by electrochemical impedance spectroscopy, measuring the impedance increase of the biosensor. The biosensor response was related to the glycoprotein amount applied on the sensor surface and asialofetuin amounts down to 2.5 ng still caused an increase in impedance of 5.9%. Albumin, the most abundant serum protein, did not interfere in the detection of the Tn-glycoproteins up to a concentration of 0.01 mg mL^{-1}. The developed lectin-based biosensor was used to evaluate the Tn-expression in serum samples and allowed to discriminate samples from healthy individuals and patients with different types of carcinomas, where the increased expression of Tn aberrant glycans is well established.

1. Introduction

In the last decades, cancer has progressively become a more frequent disease and is now referred as "cancer epidemic" [1]. The incidence of the disease has grown, with the increase in life expectancy and the increase in exposure to causing factors. This results in the boost of the number of people with cancer, with severe consequences to the patients, their families and the health systems. There is a consensus that prevention is the more logical approach to face this disease and the one that presents more potential to reduce the high incidence rates around the world [2].

Early detection constitutes one type of prevention (secondary prevention) and consists of detecting early phases of the neoplastic development, through population screenings using radiological, histological or biochemical assays. In this context, the discovery and employment of cancer biomarkers for early diagnosis is a major and urgent task, in order to enable detection and treatment of initial cancer lesions, with a higher probability of cure. Although many cancer serum biomarkers have been discovered in the last decades with the help of proteomic techniques, very few are used in the clinic today, due to a low sensitivity or selectivity of the biomarker [3–5].

In cancer cells, protein glycosylation is altered as a consequence of overexpression or subexpression of glycogenes controlling glycosylation, which can result in the expression of antigenically distinct glycoproteins [6]. Truncated *O*-glycans are included in these cancer-specific antigens and the carbohydrate antigen Tn is one of the most immature and truncated *O*-glycans, which are recognized as pan-carcinoma antigens. This structure is widely expressed on glycoproteins produced by cancer cells but not by normal tissues [7–12], which generally produce more complex and branched structures [13]. The discovery of the Tn antigen was a landmark in the history of glycosciences and its abnormal expression was among the first to be correlated with a disease state [10]. The Tn syndrome or Tn polyagglutinability syndrome was firstly described by Moreau et al. [14] and, after its characterization, it was shown that Tn is a truncated form of a major type of glycosylation in animal glycoproteins with the structure GalNAcα1-*O*-Ser/Thr. Besides cancer and Tn syndrome, the Tn antigen is found to be associated with other disorders, such as IgA nephropathy and several parasitosis [10]. In cancer, Tn antigen is present at high levels in breast, colon, lung, bladder, cervix, ovary, stomach and prostate carcinomas and, in many cancers, its expression is associated with metastatic potential and poor prognosis [10].

Diverse biosensors for aberrant *O*-glycans have been proposed and reviewed [15,16], mainly based on the use of lectins as

* Corresponding author at: Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Hidalgo, Carr. Pachuca-Tulancingo km 4.5, 42076 Pachuca, Hidalgo, Mexico.
E-mail address: mluisasilva@portugalmail.pt (M.L. S. Silva).

http://dx.doi.org/10.1016/j.snb.2017.06.021
0925-4005/

biorecognition elements towards specific carbohydrate structures. A lectin biosensor for the cancer-related T antigen was developed using the affinity of *Arachis hypogaea* agglutinin (PNA) for this epitope [17]. From the same group, another PNA biosensor and a *Sambucus nigra* agglutinin (SNA) biosensor were reported, for the detection of glycoconjugates carrying the T and STn epitopes, respectively [18]. The proposed biosensors proved to be effective in the detection of glycan-lectin interactions in a label-free fashion, with potential applications in the detection of cancer biomarkers, and the technique was shown to be fast and sensitive. Other SNA-I biosensors employing EIS were also developed and used to detect the STn glycan in the glycoproteins fetuin and asialofetuin [19,20]. The reported biosensors presented high sensitive and wide linear range. In all cases, though, serum samples weren't analyzed. Analysis of real serum samples presents additional difficulties in the detection of low-abundance glycoproteins, as cancer biomarkers usually are, due to the very complex serum glycoproteome. Our group has previously developed and validated a STn biosensor, which has demonstrated to be useful for point-of-care detection and monitoring of carcinomas, as it was sensitive to differences on STn expression of the serum glycoproteome and allowed a discrimination between samples (controls and cases) [21]. The fact that lectins are not completely specific is more an advantage than a limitation because the lectin biosensor can target a wide group of glycoproteins carrying the cancer-specific glycan, increasing the sensitivity of the biosensor, since it is not confined to the targeting of one single protein, as occurs in immunosensors. Also, lectins are more stable than antibodies, which are prone to denaturation. Finally, since sugars are poorly immunogenic, antibodies for glycan structures exhibit lower affinity compared to antibodies for protein epitopes. Label-free detection is an advantage of EIS measurements because the labeling process might affect the bioaffinity between probes and their target. Also, EIS provides very low detection limits.

Ideal biosensors produce measurements in a cheap way and with high accuracy, at point-of-care location, to reduce costs, minimize sample degradation, accelerate diagnosis and minimize patient stress [22,23]. Screen-printed electrodes (SPE) have widespread application in the biosensor development field, due to their attractive characteristics such as portability (allowing point-of-care analysis), operation simplicity, reliability and inexpensive manufacture. Furthermore, the use of these small devices requires minute amounts of sample, which is highly desirable in the clinical field [23–25].

This work describes the construction and optimization of a *Vicia villosa* (VVA) biosensor for the detection of cancer-associated Tn antigen. The analytical behavior of the device was evaluated through a full selectivity study and its analytical features were determined. The biosensor validation was performed by sample analysis and discrimination of serum samples from patients with different types of carcinomas *versus* healthy individuals. *Vicia villosa* was the lectin chosen as recognition element since the affinity of its subunit B towards the Tn epitope is well-established [26,27].

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), ethanol absolute anhydrous (J.T. Baker), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (ECD; Aldrich), *N*-hydroxysulfosuccinimide (NHS; Aldrich), ethanolamine and ethylene glycol (EG) (Sigma). *Vicia villosa* aglutinin (VVA; EY Labs), with affinity for α-D-GalNAc structures, such as the case of the Tn antigen (GalNAc-α1-*O*-Ser/Thr) was used as biosensing agent.

Asialo-bovine submaxillary mucin (aBSM) and asialofetuin were used as model glycoproteins in this study. Asialotransferrin was also used as positive control in selectivity studies. Bovine serum albumin (BSA), fetuin from fetal calf serum, human transferrin, mucin from bovine submaxillary glands (BSM) (all from Sigma) were used to assess the 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 mM of Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}. Divalent metals must be present for carbohydrate binding as they are necessary for VVA to have the active conformation to interact with the Tn antigen [28]. Redox probe solution of 5.0 mM potassium hexacyanoferrate(III) and 5.0 mM potassium hexacyanoferrate(II) trihydrate was prepared daily.

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

2.2. Biosensor fabrication

Ten microliters of 25.0 mM 16-MHDA solution, prepared in ethanol, was drop-coated onto the surface of the Au/SPE and dried in air for 24 h at room temperature (~22 °C). The electrode was then rinsed with ethanol and, after dry, 10 μL of freshly prepared cross-linker solution, composed of 20.0 mM ECD and 5.0 mM NHS, was dropped onto the electrode and left for 1 h at room temperature. The electrode was rinsed with PBS and 30 μL of VVA solution (corresponding to 75.0 μg of lectin) was dropped onto the activated surface and left for 1.5 h at room temperature. Finally, the electrode was immersed in 20.0 mM ethanolamine solution (diluted in deionized water) for 30 min at room temperature to block unoccupied carboxyl groups of 16-MHDA, followed by immersion in EG solution 10% in PBS, to block the electrode gold surface. The VVA-Au/SPE was rinsed with PBS and stored at 4 °C in PBS until use (Fig. 1).

2.3. Impedimetric and cyclic voltammetry measurements

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

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

Initially, a blank measurement was performed after dropping 40 μL of the redox probe solution. Then, the electrode was rinsed with PBS solution and the sample was dropped (40 μL) and left for 10 min incubation, at room temperature. Finally, the electrode was rinsed with PBS solution containing sodium dodecyl sulfate (SDS) 2% and a second impedance measurement was performed with the redox probe solution.

The impedance spectra were plotted in the form of Nyquist plots, and the formation of the VVA-glycoprotein complex was quantified by the increase in the charge transfer resistance ΔR_{CT} ($\Delta R_{CT} = R_{CTf} - R_{CTi}$), 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. The $\%\Delta R_{CT}$ was calculated as follows:

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