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Electrochemical Biosensing Strategies to Detect Serum Glycobiomarkers

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Authors' contributions

This work was carried out in collaboration between all authors. Author PMSS managed the literature search, wrote the manuscript and developed the figures. Authors LCBBC and MTSC designed, supervised and managed the study performed. All authors read and approved the final manuscript.

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ABSTRACT

Changes in glycosylation profiles have emerged as indicators of diseases. Altered glycans and glycoproteins secreted by pathological tissues are found in human serum and are potential glycobiomarkers for early diagnostic and prognostic of diseases such as inflammation, infection and cancer. To obtain serum samples is a simple procedure and minimally invasive; thus the detection of glycobiomarkers in serum is attractive for clinical applications. Electrochemical biosensors are a friendly strategy for rapid, easy and highly sensitive measuring of glycans and glycoprotein biomarkers. The use of lectins as biorecognition elements in glycobiosensors has provided a specific detection and profiling of glycans linked to glycoproteins. Electrochemical glycobiosensors based on lectin interactions employed to characterize glycan profiles in serum glycoproteins constitute a promising tool in diagnostic and monitoring of diseases.

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1. INTRODUCTION

Glycosylation is a post-translational modification occurring in more than half of human proteome and glycans have important roles in physiological functions and diseases. Changes in glycosylation profiles are frequently observed on cell surfaces and serum glycoproteins in pathological processes, such as immune disorders, infectious diseases and cancer. Evaluations by chromatographic techniques, mass spectrometry, among other approaches in tissues, cells, biological fluids and serum samples reveal quantitative and structural alterations in glycans in diseases when compared with healthy samples [1,2]. Then, the correlation of altered glycans with diseases makes them glycobiomarkers of choice for clinical diagnosis and monitoring of prognosis [3].

The diagnostic of diseases and monitoring during treatment through detection of glycobiomarkers present in bloodstream are attractive in terms of low invasiveness, agility and facility to follow glycan modifications. Analytical techniques based on Enzyme Linked Immunosorbent Assay (ELISA) and other immunoassays are most used to detect and measure serological glycoproteins in clinical laboratories. In addition, the technology of biosensing has been applied successfully to identify and quantify glycobiomarkers with high specificity and selectivity, based on biorecognition events connected to a system of transduction [4]. Biosensors can be constructed using a variety of transductor types, including optical, piezoelectric and electrochemical, to generate the results. Electrochemical biosensors have been detached since they provide rapid diagnostic by a simple manipulation and low cost, overcoming some limitations showed in other technologies.

Lectins, a group of sugar-binding proteins have been largely employed for biorecognition of sugars in binding assays. Lectins recognize carbohydrates with specificity for monosaccharides or oligosaccharides, free or bound to a glycosylation site, and may distinguish the linkage type. The lectin-carbohydrate binding provides quantitative and qualitative information about expression of glycans in biological samples, being valuable tools to characterize and identify changes in secreted glycoproteins [5]. Techniques based on chromatography, electrophoresis, immunoassays and biosensors use lectins as biorecognition agents. Lectin biosensors contain a sensing platform with immobilized lectins to detect glycobiomarkers in serum samples through lectin-glycan interaction with high specificity [6]. Various groups of research have developed lectin biosensors for cancer, infectious diseases and virus or bacterial glycan detections. Lectins of different sources and specificity groups are employed in biosensing due to their versatility and diversity.

Isoforms from a seed lectin of *Cratylia mollis*, denominated Cramoll, belongs to the specificity group of mannose/glucose such as Concanavalin A, Con A [7]. Cramoll biosensors have demonstrated potential to recognize profiles of glycoproteins of different dengue serotypes in human serum [8,9] and glycans of bacterial lipopolysaccharides [10], through electrochemical methods, representing a good alternative for detection of diseases. This review introduces some advances in the lectin biosensor area directed for detection of glycobiomarkers to help in the assays of early diagnostic and monitoring of diseases.

2. GLYCOSYLATION

Glycosylation is the most common post-translational modification of proteins and has fundamental importance in biological processes in eukaryotic organisms. It is predicted that nearly 80% of the human plasma proteins are glycosylated [11], besides of glycans that cover cell membranes. Glycosylation reactions involve the covalent attachment of glycan chains to specific amino acid moieties of proteins during and after translation of polypeptide chains in order to form glycoproteins. The glycosylation profile of a protein is determined by a wide group of enzymes known as glycosyltransferases and glycosidases, residents in the endoplasmic reticulum and Golgi apparatus, which catalyze the extension and transfer of glycan chains to glycosylation site of the protein [12]. They constitute specific groups to a cell type, tissue and organism, resulting in tissue- or cell-specific differences in glycosylation between sites of the same protein [13].

Glycans are linked to human proteins *via* two pathways, *N*- and *O*-glycosylation, with various branching points. *O*-glycosylation is characterized by the attachment of *O*-Linked

glycans through hydroxyl group of serine (Ser) or threonine (Thr) residues, starting with the addition of N-acetyl-galactosamine (GalNAc–O–Ser/Thr) transferred by an N-acetyl-galactosaminyltrasferase in the Golgi apparatus [14]. After, specific transferases elongate different types of core structures, including mucin-type *O*-linked glycans (core 1, 2, 3, 4; as well as T, TF and Tn antigens), *O*-linked GlcNAc and *O*-linked Fuc (Fig. 1). In the *N*-glycosylation, *N*-linked glycans are attached to the amidic nitrogen of asparagine (Asn) residues within the Asn-X-Ser/Thr glycosylation site, being X different of the proline [15]. *N*-glycosylation starts in endoplasmic reticulum, through the synthesis of a *N*-glycan precursor (Glc3Man9GlcNAc2) from a dolichol phosphate to the Asn site of nascent polypeptide chain. This *N*-glycan precursor originates the pentasaccharide tri-mannosyl core (Man3GlcNAc2) that is prolonged to generate three subtypes of mature *N*-glycans: high-mannose, complex and hybrid (Fig. 1). The tri-mannosyl core cleaved by glycosidases is transferred to Golgi apparatus; there other monosaccharides added by specific transferases prolong glycan chains generating a complex branching. The resulting structures originate the subtypes of complex *N*-glycans and hybrid *N*-glycans (Fig. 1) [15,16]. In addition, the subtype high-mannose *N*-glycans contains only mannose residues linked to the tri-mannosyl core [16]. Studies report the complex type *N*-glycans as the most abundant in human serum, while hybrid and high-mannose types are rare. The monosaccharide residues mannose (Man), galactose (Gal), fucose (Fuc), N-acethyl-galactosamine (GalNAc), N-acethyl-glucosamine (GlcNAc) and sialic acid (SA) or neuraminic acid (NeuNAc) compound the *N*- and *O*-glycan chains attached to human proteins [15,16].

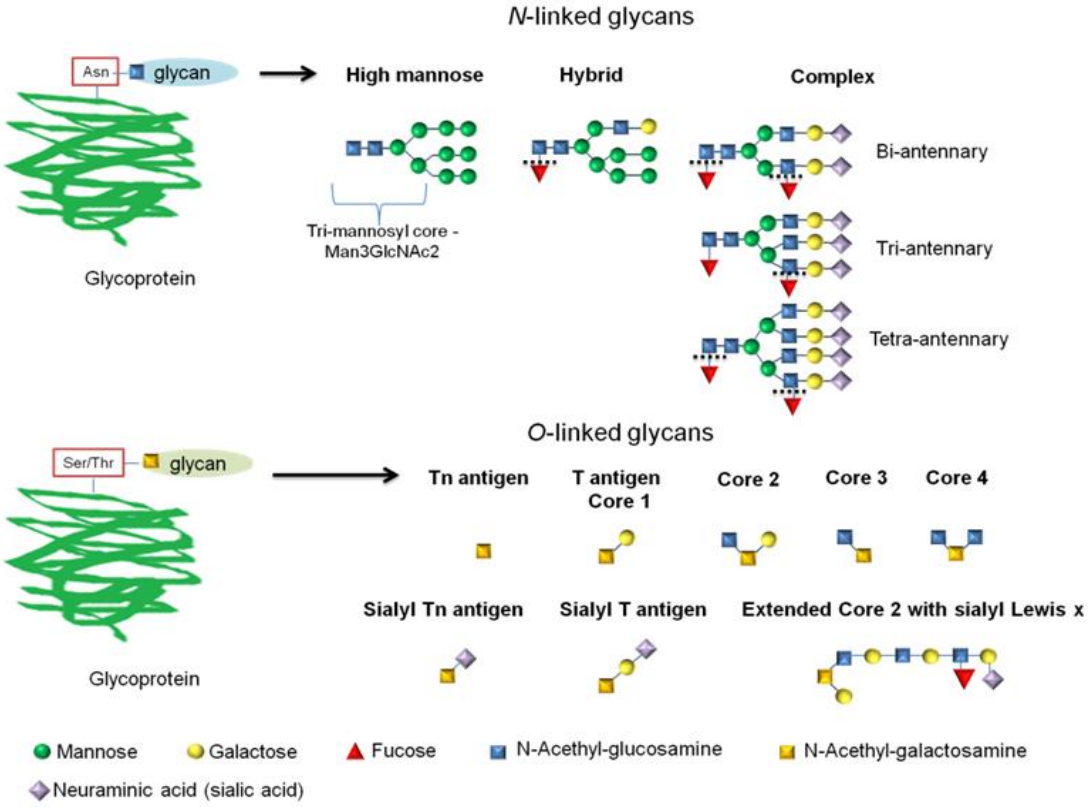

Fig. 1. Types of *N*- and *O*- linked glycans found in human serum

N-linked glycans bind to Asn residue in the nascent polypeptide chain into endoplasmic reticulum. The schematic representation shows the three types of N-linked glycans: high mannose, hybrid and complex type, with the common core structure present in all N-glycans. O-linked glycans bind through Ser or Thr residues present in the protein, starting with the addition of GalNAc. O-glycans can be extended with other monosaccharide residues and generate different core structures, and antigens highly expressed in cancer, such as Lewis antigen, T, Tn and sialyl Tn antigens

The heterogeneity of glycan structures controlled by enzymes contribute to the varied of functions that glycosylation carry out in cells and tissues. Glycosylation plays relevant influence in the synthesis, processing and function of proteins, including folding, stability of tertiary structure, protection against action of proteases, increased serum half-life of proteins and reduction of nonspecific protein-protein interactions [17-19]. Moreover, glycosylation mediates a regulation role in many biological processes involving the interactions cell-cell and cell-matrix, such as cell proliferation, cell recognition, adhesion, host-pathogen recognition, receptor binding, signaling, fertilization, inflammation and immune responses [19,20].

3. GLYCANS ARE POTENTIAL GLYCOBIOMARKERS IN DISEASES

The activities of glycosylation enzymes are dependent and modulated by cellular dynamics. Changes in cellular environment and physiology often due to diseases, affect enzyme activities, resulting in aberrant glycosylation of proteins [21]. Disturbed glycosylation has been linked to various pathologies such as infection, chronic inflammations, immunological and genetic disturbs, cancer and metastasis [12,21,22]. This involves changing of glycosylation sites, increase or decrease of the site numbers, modification in the chemical composition or type of glycan, extra branching of glycans and quantitative alteration of specific glycoprotein on cell surface and secreted in bloodstream [23,24].

There is strong evidence that these glycosylation changes in serum glycome are a good way to identify potential glycobiomarkers of diagnostic and progression of pathological states. Serum glycoproteins such as immunoglobulins, fetuin, haptoglobin, transferrin, alpha-fetoprotein, and other acute phase proteins showed glycosylation changes in response to inflammatory and immune diseases. Immunoglobulin G contains complex bi-antennary *N*-glycans that have a significant decrease in galactosylation for patients with inflammatory arthritis [25,26]. The glycan profiling in the serum of liver fibrosis patients and infected by hepatitis B virus and hepatitis C virus revealed a significant decrease of N-glycans bi-antennary and tri-antennary as glycobiomarkers to monitor the progress of fibrosis [27]. Multi-branched glycans highly sialylated were detected in elevated levels in serum of ulcerative colitis patients correlated with disease degree [28]. Alpha 1-6-linked arm monogalactosylated and core fucosylated bi-antennary *N*-glycans were reduced in serum glycoproteins from subjects with diabetes mellitus type 2 and provide an alternative of serological glycobiomarker [29]. Glycan profiles of whole serum of patients with autoimmune pancreatitis showed elevated levels of a galactosyl and monogalactosyl bi-antennary glycans, being potential biomarkers of the disease [30].

Studies have evaluated the presence of aberrant glycoforms and quantitative levels of glycosylation in patients with cancer when compared with benign disease and healthy individuals to identify cancer glycobiomarkers. Aberrant glycosylation patterns in proteins from cancer cells, tissues and serum commonly involve altered sialylation and fucosylation, change in glycan size and branching, and the presence of Lewis antigens and truncated *O*-glycans [12]. Lewis antigens (Fig. 2) are frequently expressed on membrane glycoproteins of several cancer cells. Cholangiocarcinoma cells expressed high levels of sialyl Lewis a (Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc) and glycoprotein mucin 5AC was elevated in tissues and serum from cholangiocarcinoma patients [31,32]. Serological *N*-glycome in breast cancer patients showed increased levels of sialyl Lewis x antigen (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc) and an increased sialylation [33]. An increase of β1-6GlcNAc branches in *N*-glycans added by *N*-acetylglucosaminyltrasferase V (GnT-V) is observed in breast and ovarian cancer [23,34]. Glycans of prostate-specific antigen isolated from tumoral cell and sera showed tri- and tetra-antennary structures of *N*-glycans [35]. In cancer cell-surface, *O*-linked glycans of mucin-type glycoproteins are often defective, resulting in the expression of truncated *O*-linked glycans denominated of T (Galβ1-3-GalNAcα-O-Ser/Thr) and Tn (GalNAcα1-O-Ser/Thr) antigens (Fig. 1), as observed in breast and colon cancer [23,36]. T/Tn antigen quantitative assays detected cancer without previous biopsy using highly purified T antigen for determination of anti-T immunoglobulins in serum samples [37].

4. SERUM GLYCOPROTEINS AS BIOMARKERS OF DISEASES

Clinical assays using body fluids such as serum, plasma, urine and saliva are minimally invasive, easily handled, with short time response, being preferred for detection and prognostic evaluation

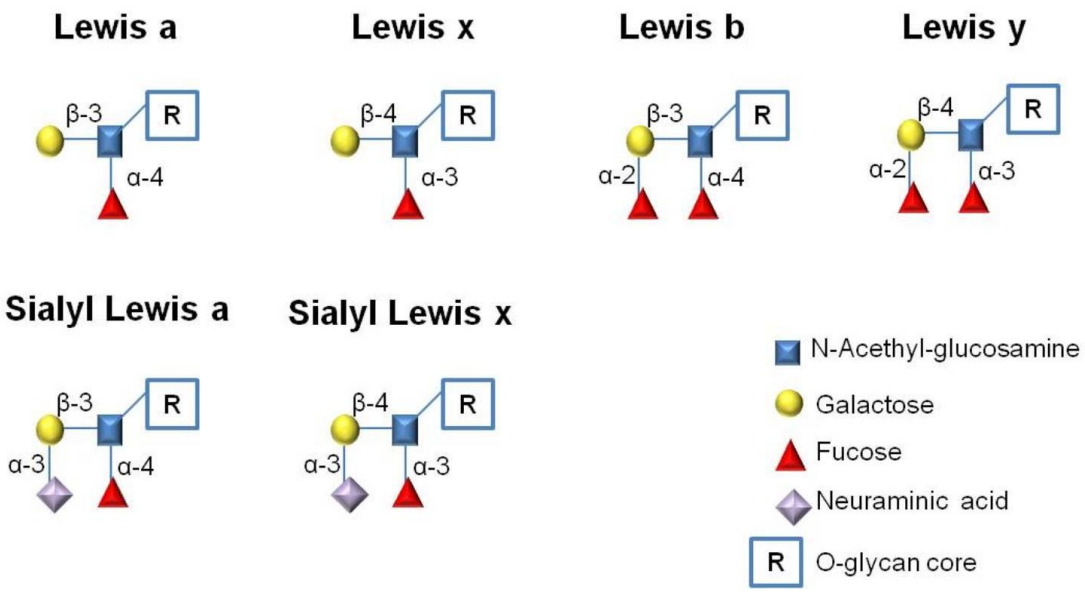

Fig. 2. Terminal Lewis and sialylated Lewis antigens expressed by human tumors

The Lewis antigens can be characterized by the presence of Galβ-3 linkage (Lewis a and Lewis b) or Galβ-4 linkage (Lewis x and Lewis y). The sialylation in the galactose residue can originate sialyl Lewis a and sialyl Lewis x antigens

of various diseases. Glycoproteins secreted in the bloodstream are a considerable part of serum biomarkers and they might show differential quantitative and qualitative level between healthy and pathological samples [3,38]. Some glycoproteins are well known serologic biomarkers for cancer diagnostic that may be detected by laboratory clinical assays, such as the carbohydrate antigens (CA) CA-125 for ovarian cancer, CA15-3 for breast cancer, carcinoembryonic antigen (CEA) for colorectal cancer, alpha-fetoprotein (AFP) for hepatocellular carcinoma (HCC) and prostate-specific antigen (PSA) for prostate cancer (Fig. 3). The early diagnosis of infectious diseases based on serum glycoproteins is also possible. Dengue virus nonstructural 1 (NS1) antigen is a test commercially available to detect dengue infection. NS1 antigen is a glycoprotein present in elevated levels in serum of infected individuals before that specific antibodies emerge [39].

Current tendencies evaluate changes in the glycosylation patterns of serologic biomarkers as potential candidates for highly specific glycobiomarkers, for early detection and staging of various types of cancers. Researches appoint an increase of core-fucosylation levels of serum AFP of patients with HCC as an indicator more specific for HCC staging [40]. Altered profiles of fucosylation and sialylation in PSA glycans are reported for prostate cancer and fucosylated PSA showed to have potential to substitute the PSA test in the differentiation of aggressive tumors from non-aggressive prostate tumors [41]. Neoglycoforms of CEA in cancer identified as bi-antennary, tri-antennary and tetra-antennary glycans with residues of sialic acid and fucose can improve tumor diagnosis and staging [42]. Haptoglobin is an acute phase glycoprotein produced by the liver and its concentration is elevated significantly in many diseases, including hepatic inflammation, hepatitis and various types of cancer [43,44]. Studies on altered glycosylation of serum haptoglobin show high levels of fucosylation in haptoglobin associated with cancer; fucosylated haptoglobin (Fuc-Hpt) is reported as a biomarker for diagnostic and prognosis of pancreatic cancer and colorectal cancer [45]. Elevated levels of serum Fuc-Hpt also have been associated with prostate cancer. An increase of fucosylated bi-, tri- and tetra-antennaries glycans of serum haptoglobin was observed in prostate cancer patients, correlated with Gleason score [46].

Analytical techniques have allowed the search and characterization of neoglycoforms present in the bloodstream. The structural characterization of glycans by techniques, such as liquid chromatography and mass spectrometry provides detailed information about composition

and quantity of carbohydrate residues [47]. However, they require long time of analysis, previous treatment of samples, and technical preparation for manipulation, complex and expensive; then, they are not viable for direct diagnostic of diseases and monitoring of patients in clinical treatment. Besides, human serum and plasma are considered complex samples containing a mixture of proteins, some very abundant (more than mg/mL) such as albumin, that can mask other proteins expressed in low concentrations (ng/mL and pg/mL range), mainly biomarkers that appear in early diseases.

Techniques based on specific binding assays using biorecognition agents (eg. antibody, lectin) bind a glycoprotein biomarker or glycan chains linked to protein, measuring these molecules at very low concentrations in human serum and plasma (Fig. 3) with specificity and selectivity [45,48]. Immunoassays as ELISA commercially available and lectin-based assays detect and measure specific serum glycobiomarkers in research and clinical applications to diagnostic of diseases [48,49]. Lectins immobilized onto inert supports and incubated with serum sample serve to analyse a glycobiomarker, such as observed in disposable printed lectin arrays; or lectins employed in enzyme-linked lectin assay unravel glycosylation profiling of a glycoprotein immobilized on a surface. However, these techniques frequently require significant sample volumes and several steps for obtaining results. Biosensors based on binding assays have emerged as a rapid, simple and sensitive strategy for serological glycobiomarker measurements [50,51].

5. BIOSENSORS: A STRATEGY FOR DETECTION OF GLYCOBIOMARKERS

Biosensors are analytical devices capable of detecting and measuring a specific analyte using a biological recognition element contacted with a transducer, that converts the recognition event into a signal analytically useful and measurable [52]. A biosensor contains a biorecognition element (bioreceptor) that binds specifically to analyte of interest; a transductor element that detects signals (current, voltage, changes of mass, light or temperature) resultant of the interaction bioreceptor-analyte and converts in an electric signal. Besides, the transductor connected with an appropriate interface process electric signals and display measurable results [53] (Fig. 4).

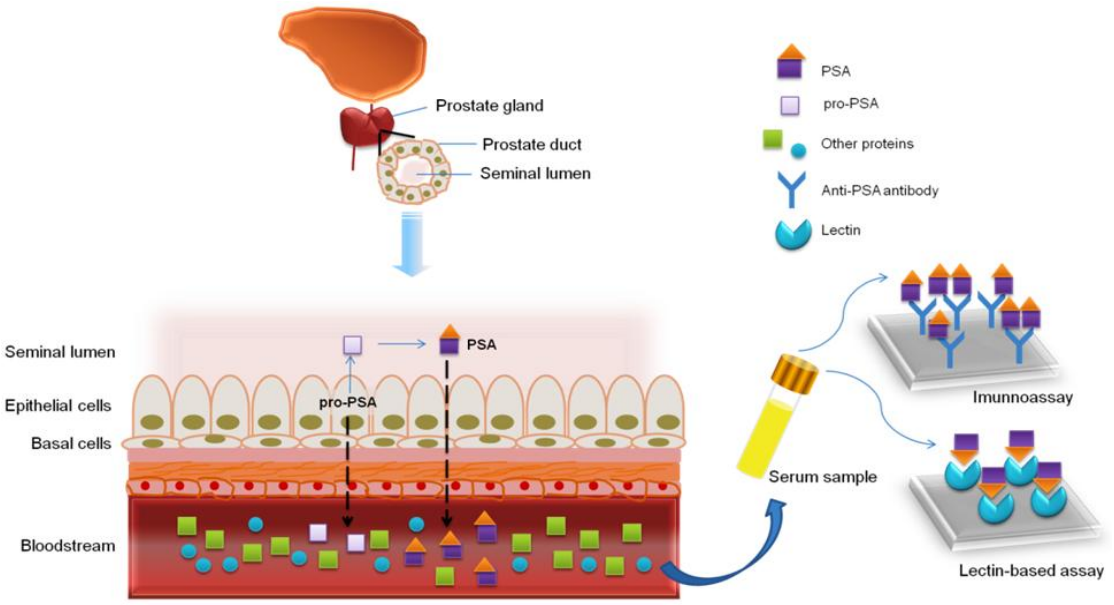

Fig. 3. Schematic representation of secretion from specific prostate biomarker, prostate-specific antigen (PSA), in bloodstream and assays commonly used for specific detection

The prostate gland secrets PSA in the seminal lumen and small concentrations arrive in the bloodstream. In hyperplasic and cancerous conditions, quantitative alterations in the PSA concentration and changes of PSA glycosylation are useful for detection of these diseases. This biomarker analyzed in serum samples through binding assays using antibodies or lectins determine the concentration of PSA and glycan profiles in PSA structure

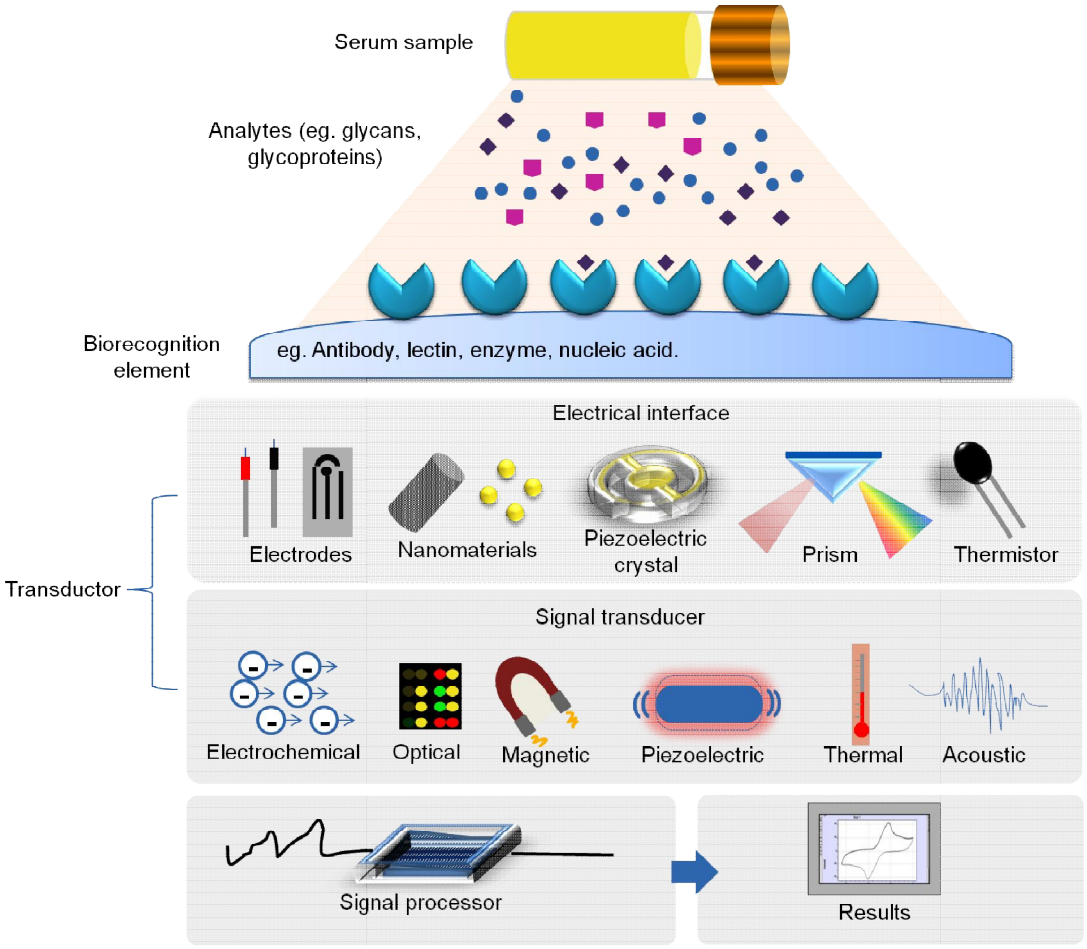

Fig. 4. Schematic representation of a biosensor with a biorecognition element immobilized onto the solid surface in direct contact with an electrical interface of the transductor and a signal processor

The biorecognition element interacts with a specific analyte present in serum sample. The transductor detects the interaction signal converting it to an electrical signal measurable, displaying result

According to type of interaction bioreceptor-analyte, the analytical devices are classified as catalytic or affinity biosensors. Catalytic biosensors based on enzyme activity generate a product [54]. Affinity biosensors involve binding event between the bioreceptor and analyte without chemical transformations, such as antigen-antibody, nucleic acid hybridization and interaction lectin-carbohydrates [53,55]. Biosensors are also classified in relation to the signal detected by transductor (Fig. 4). They can be an electrochemical biosensor, which detect current, voltage, potential or impedance; an optical biosensor, which measure the intensity of the light emitted; a piezoelectric or acoustic biosensor, that detect variation of mass, pressure or elasticity, and a thermal biosensor, which detect changes in temperature [53]. This variety of mechanisms favors a range of designs and applications of biosensors. Electrochemical biosensors are more attractive and predominant in bioanalysis, due to diversity of electrical interfaces and techniques for measurement that collaborate for high selectivity and sensibility; these sensors are of easy construction, portability, possible miniaturization and low cost [56].

Clark and Lyons developed the first biosensor in 1962, for detection of glucose through glucose oxidase enzyme. They elaborated a system known as enzyme electrode, using glucose oxidase immobilized on polyacrylamide film, on a platinum electrode surface; determination of glucose concentration was based on measurement of oxygen consumed [57]. After,

the Yellow Spring Instrument Company developed the Clark and Lyons's technology to introduce in the market the first biosensor of glucose in 1975 [58]. Since then, the biosensor technology has developed different approaches in the detection mode and elaboration of more efficient devices, aiming the various types of analysis [58]. Biosensor technologies focus in the fabrication of self-contained, portable, miniaturized devices and at the same time, in conservation of good analytical properties, such as speed, high sensitivity and selectivity. Biosensors are then of friendly application to diverse areas: environmental monitoring, biosafety and assays for clinical applications [59].

Biosensors or glycobiosensors used to detect glycans and glycoproteins perform analysis of human serum [55]. Different of the complex approaches such as glycoproteomic techniques, which require sophisticated instrumentation and significant volume of sample, biosensor advances result in easy detection and measuring of glycans or glycoproteins in minimal volume of sample, simple manipulation, short-time response, high sensitivity and specificity, and friendly to use outside of a laboratory [59,60]. These possibilities are attractive for development of point-of-care tests useful in early diagnostic, staging and monitoring diseases such as cancer. The detection of biomarkers using glycobiosensors frequently use antibodies and/or lectins. A variety of available glycobiosensors uses antibodies directed to protein as biorecognition element for specific detection and measurement of serum glycoprotein biomarkers [56]. Lectin glycobiosensors developed for characterization of the glycosylation patterns of glycans on serum glycoproteins correlate to diseases, such as degree of sialylation and fucosylation [60,61].

6. LECTINS AS BIORECOGNITION ELEMENTS

The term lectin is derived from the Latin word "lectus" (chosen, selected) [62], and was introduced to define a group of proteins that showed selectivity in the interaction with carbohydrates. Lectins are sugar-binding proteins of non-immune origin that exhibit specific recognition and reversible bind to free carbohydrates and link to glycoconjugates (eg. glycoprotein and glycolipid). They are involved in crucial physiological events of protein-carbohydrate interactions, such as adhesion and cell migration [63,64]. Glycobiosensors frequently use lectins as biorecognition elements.

The association constant between lectin and monosaccharides ranges from 10^3 to (5×10^4) M^{-1} and between lectins and oligosaccharides, 10^4 to 10^7 M^{-1}, included values in the same range found for antibody-antigen bindings and enzyme-substrates [65,66]. Lectins bind to a specific monosaccharide or oligosaccharide through their binding sites via hydrogen bonds, Van der Waals interactions, and hydrophobic interactions [66,67]. According to the specificity, lectins are classified in five main groups: mannose and/or glucose, N-acetyl-glucosamine, N-acetyl-galactosamine/galactose, L-fucose and sialic acids [68].

Lectins are distributed among viruses, microorganisms and animals although they were initially found in plants [69-71]. The majority of available lectins, obtained from leguminous plants, comprise the largest number of already characterized proteins, such as seed lectin from *Canavalia ensiformis*, Concanavalin A (Con A), soybean agglutinin (SBA) and *Cratylia mollis* seed lectin (Cramoll). Leguminous lectins, although derived from taxonomically distinct species, have molecular characteristics and physicochemical properties in common, such as Con A and Cramoll, and they exhibit a great variety of specificity for carbohydrates [67,72].

Lectins have been used in glycoanalytical areas, including analysis of glycosylation profiles and detection of glycobiomarkers in serological samples based on lectin-carbohydrate interaction. They are widely used in the characterization of glycans linked to glycoproteins for comparative analysis between healthy and pathological conditions and identification of aberrant glycan profiles as potential glycobiomarkers [24,45]. According to their specificity, lectins can detect alterations in degree of sialylation [73], fucosylation [46], galactosylation [74], mannosylation [75] and the presence of cancer-associated antigens, like sialyl-Tn (STn) antigen, Lewis antigens [4,76], and other changes associated with diseases. However, the main limitation of lectins is their ability of recognizing the same carbohydrate in different substrates, and do not evidence the origin of the alteration. Besides, a lectin can bind to similar carbohydrates belonging to the same group of specificity, reducing the directed recognition in glycoanalysis.

Glycobiosensors have employed various lectin-based models to configure the lectin-glycan interactions, sometimes assisted by antibody and nanomaterials to improve the selectivity and signal amplification. Lectins directly immobilized onto a solid support do recognize glycoproteins by glycan portion, in according to specificity, detecting similar glycans in different glycoproteins. Following this model, a lectin-based glycobiosensor for profiling of the STn antigen in serum samples was developed by immobilizing the lectin *Sambucus nigra* agglutinin type I (SNA-I) on electrode surface which showed potential to discriminate between cancer and healthy conditions [4]. The inverted configuration based on the immobilization of glycan or glycoprotein followed through binding of lectin also is available. A biosensor based on D-glucose was successful used to evaluate Con A with a limit detection of 1.0 pM [77]. This configuration is useful to search lectins in a mixture of proteins.

The two-step sandwich model involves a lectin and an antibody, both to interact with the glycoprotein biomarker. In this case, antibody or lectin immobilized can recognize the glycoprotein, and in the second step, a lectin or secondary antibody added complete the sandwich. A plastic sensor chip based on the technique surface-plasmon field-enhanced fluorescence spectrometry used to detect GalNAcβ1−4GlcNAc-linked prostate-specific antigen applied anti-PSA antibody to capture PSA and the lectin *Wisteria floribunda* agglutinin (WFA) to profile the glycan in serum from patients with benign prostate hyperplasia and prostate cancer patients [74]. The use of the anti-PSA antibody allowed the characterization of glycosylation profile from the specific glycoprotein target present in serum samples. Other sandwich system based on lectin or antibody confined on surface does recognize the glycobiomarker, followed by the addition of lectins coated nanomaterials. The latter compounds are attractive since they increase the amount of secondary biorecognition element and promote the signal amplification. A highly sensitive and selective lectin-based glycol-biosensor developed for comparative analysis of mannose and sialic acid levels on normal and cancer cells used a sandwich system formed by lectins immobilized to detect glycans on cell surface, followed by addition of lectins linked to gold nanoparticles (AuNp) [78]. These strategies provide a characterization of glycosylation profiles on glycoprotein biomarkers with high sensitivity, which is useful in clinical diagnostics, including early detection and staging of cancer and monitoring of patients during treatment.

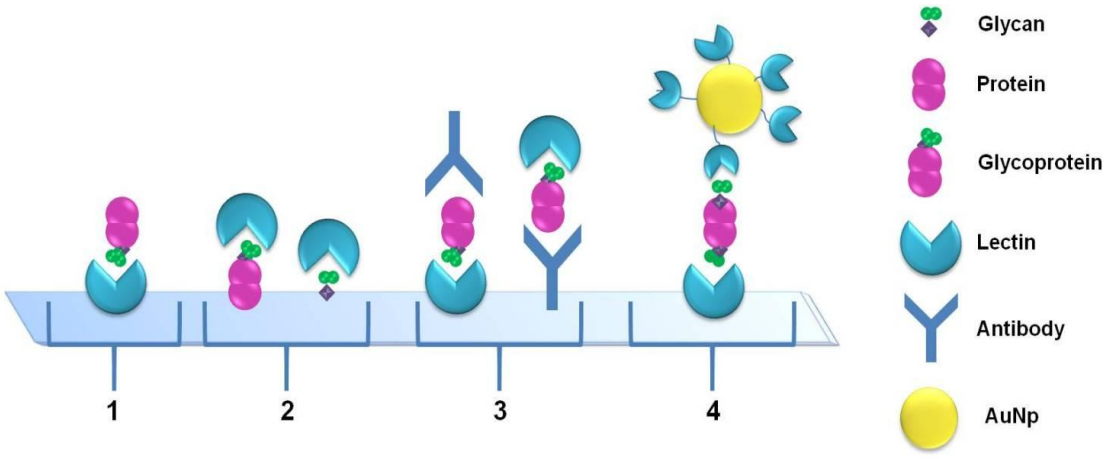

Fig. 5. Configurations of lectin-based assay in glycobiosensing. Direct detection by immobilized lectin (1), reverse detection by incubated lectin (2), lectin-antibody sandwich assay (3) and lectin-conjugate sandwich assay with gold nanoparticles using one lectin type or two different lectins (AuNp) (4)

Lectins are useful for detection of glycans free or linked to proteins. The association with antibody allows the analysis of glycans from a glycoprotein, a more specific assay. Nanomaterials provide a broad field to conjugate lectins, increasing glycan detection

7. ELECTROCHEMICAL GLYCOBIO-SENSORS

Electrochemical methods are the most attractive for sensing biorecognition events due to simple instrumentation, friendly analytical performance and diversity of methods and electrodes available for specific applications [79]. Biosensors based on electrochemical detection are widely applied to glycoanalysis in the field of glycomics to analyze cleaved glycans, together to separation techniques (liquid chromatography, electrophoresis or mass spectrometry) [80]. Currently, developed electrochemical glycobiosensors using a lectin as biorecognition element detect changes in the degree of sialylation, fucosylation, galactosylation and mannosylation in cell surfaces and serological samples [81]. Characterization of glycosylation profile from a sample or a specific glycoprotein by electrochemical methods used glycobiosensors [82,83].

Different electrochemical methods are available for glycobiosensors, including electrochemical impedance spectroscopy (EIS) and voltammetric techniques, such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV) [84-86]. Among these, EIS is widely used in glycobiosensors as an effective method for detection of the biorecognition lectin-glycan on the electrode surface. The measurement applies a small alternating current amplitude on the electrode interface and subsequent detection of change in impedance during binding events. When the binding event happens, a change in impedance is detected in the presence of a redox probe (eg. electrolyte solution of ferricyanide and ferrocyanide), and the change in electron transfer resistance (RCT) is measured. The change in RCT is due to blocking of charge transfer on the electrode surface in the presence of coating, immobilized biomolecules and biorecognition complex [87]. EIS measurements are transformed in an equivalent circuit and a Nyquist plot to obtain information about RCT and lectin-glycan binding. EIS provides a rapid, simple, highly sensitive and label-free detection of glycans in low concentrations, eliminating the use of labels commonly required in non-electrochemical techniques [85]. Voltammetric techniques, also used for label-free detection of lectin-glycan binding, in general, are based on the application of a voltage range in the electrode interface, that in the presence of a redox probe, promote a redox reaction and subsequent generation of a current flow [86]. The electrode surface modification with biomolecules and biorecognition complex changes interfacial electron transfer reaction of the redox probe, resulting in changes of current. The current variation is measured and quantitative information about the target glycans obtained, more rapidly that EIS method. The voltammetric techniques more used for detection of biorecognition events are DPV and SWV due to high sensitivity, quickness (3 to 10 s is necessary for measurement) and minimization of capacitive interference [86].

The first electrochemical biosensor using electrode surface modified with lectins was reported by La Belle and coworkers for detection of cancer-associated T antigen (β-D-Gal-[1→3]-D-GalNAc) [84]. The N-acetyl-galactosamine/galactose specific plant lectin, peanut agglutinin (PNA) was immobilized onto a gold electrode surface previously modified with a mixed self-assembly monolayer, followed by the incubation with nanocrystal CdS-tagged 4-aminophenyl-α-D-galactopyranoside sugar and target sugars (GalNac, Gal and T antigen) to establish a competitive assay. The lectin-sugar recognition monitored by square-wave voltammetric stripping current peaks, decrease with the bind of target glycan. Current peaks decrease for target sugars, in the affinity order T antigen > Gal > GalNAc, achieving a detection of 0.1 μM for T antigen, 1.0 μM for Gal and 2.7 μM for GalNAc. T antigen is a known glycobiomarker for different types of cancer and this electrochemical biosensor based on PNA lectin demonstrated potential to detect and distinguish the T antigen of other similar ones, being a sensitive and simple technique for quantification of T antigen in human serum samples.

EIS analysis requires more steps of processing data; however, this method promoted the detection of very small concentrations of glycobiomarker. An ultrasensitive label-free glycobiosensor reported for measurement of sialylated glycoproteins used the EIS method [83]. A lectin from *Sambucus nigra* (SNA I) specific for sialic acid was covalently attached to a mixed self-assembled monolayer (SAM) consisting of 11-mercaptoundecanoic acid and 6-mercaptohexanol on a gold electrode. The biosensor detected glycoproteins fetuin and asialofetuin through the fraction of sialic acid, with a limit of detection of 0.33 fM for fetuin and 0.54 fM for asialofetuin. Its potential to detect sialylated glycoproteins with very low limit of

detection is attractive for clinical applications in the search of sialylated glycobiomarkers of diseases.

Other label-free biosensor based on EIS developed for detection of cancer glycobiomarker alpha-fetoprotein (AFP) and discrimination of glycosylation profile of AFP in serum samples used lectins as biorecognition elements [81]. First, a lectin biosensor for detection of AFP was mounted through attachment of the wheat-germ agglutinin (WGA) lectin to single-wall carbon nanotubes (SWCN) onto a screen-printed carbon electrode (SPCE). The biosensor used for AFP binding achieved a low limit of detection of 0.1 ng/L. Moreover, lectin biosensors fabricated with different lectins characterized glycosylation profile of N-glycan linked to AFP and discriminate between serum samples of cancer patients and healthy subjects. The lectins WGA (GlcNAc-specific), LCA (mannose-specific), Con A (mannose-specific), SNA (sialic acid-specific) and DAS (LacNAc-specific) were immobilized onto different SWCN-modified SPCE and the changes in charge transfer resistance were monitored. The results suggest an increase of core fucosylation and α2-6 sialylation in AFP of cancer when compared with healthy serum, being a potential tool for label-free profiling of glycan expression in serum samples and early diagnosis.

A Con A-based biosensor was reported as a strategy for electrochemical detection of abnormal glycoproteins from serum of patients infected with dengue virus serotypes 1, 2 and 3 [82]. This biosensor based on gold electrode modified with phospholipid membrane and Con A to form a lipid-Con A surface, was incubated with serum from patients. Electrochemical characterization performed by voltammetry and EIS recorded decreases in the response of current of the electrodes and notable increases of RCT after incubation with infected sera by dengue virus serotypes 1, 2 and 3. These findings indicate the binding of serum glycoproteins to Con A through its mannose-binding sites, revealing that the biosensor was able to recognize dengue serotypes and are useful for detection of dengue infections.

8. ELECTROCHEMICAL GLYCOBIO-SENSORS BASED ON CRAMOLL 1,4 LECTIN

Cramoll 1,4 is a legume lectin isolated from *Cratylia mollis* seeds that has been employed as biorecognition element in glycobiosensors. *C. mollis* is an occurrent native forage in the semiarid region of Brazil, popularly known as camaratu bean; it belongs to the same Fabaceae family of *Canavalia ensiformis*, the natural source of Con A lectin [7,88]. *C. mollis* seeds are source of four molecular isoforms of lectins with diverse specificity denominated Cramoll -1, -2, -3 and -4, and a preparation containing the 1 and 4 isoforms, known Cramoll 1,4. Cramoll 1, 2 and 4 are glucose/mannose-specific lectins; Cramoll 3 is a galactose specific lectin [7,89]. Cramoll 1 showed classic tertiary structure of legume lectins through X-ray crystallography. Its primary structure consists of 236 amino acid residues with 82% homology with Con A structure, and both have identical carbohydrate-binding sites to the methyl-α-D-mannopyranose, with which they interact by hydrogen bond, and binding sites for Ca^{2+} and Mn^{2+} metals. The secondary structure is composed exclusively of three β sheets; all connected by turns [67].

Cramoll -1 and -1,4 demonstrated its potential for various biotechnological applications such as antitumor action [90]; mitogenic activity [91]; pro-inflammatory and healing of experimental tissue lesions [92] and anthelminthic [93]. The potential of this lectin to recognize glycans and glycoproteins is a valuable tool for detection changes in glycosylation of diseases through tissues and serum samples. Cramoll 1 showed a higher potential to mark neoplastic mammary tissues [94] and Cramoll 1,4 revealed more intense staining in hyperplasia prostate tissues when compared to prostate carcinoma tissues [1], being a potential probe for histochemical studies. An affinity column of Cramoll 1 coupled to Sepharose CL4B was an efficient matrix to isolate serum glycoproteins, such as lecithin-cholesterol acyltransferase [95].

Cramoll 1,4 has been explored in the recognition of serum glycoproteins from patients contaminated with different serotypes of dengue virus using an electrochemical biosensor, a very promising field. Firstly, it was reported the ability of a Cramoll 1,4 biosensor for sensitive detection of glycoproteins in solution [96]. A mix of Cramoll 1,4 with gold nanoparticles (AuNp) and polyvinylbutyral (PVB) was immobilized onto the surface of gold electrodes; the lectin-modified electrode was incubated with solutions containing ovoalbumin. Electrochemical analysis by voltammetric and EIS detected lectin-ovoalbumin interactions in the electrode surface, confirming that the immobilization process of

Cramoll 1,4 did not affect lectin recognition sites. In this case, the lectin recognized oligomannose glycan chains linked to ovoalbumin. Cramoll 1,4 biosensors also were developed to detect dengue serotypes. A gold electrode modified with Cramoll 1,4, Fe_3O_4 nanoparticles and PVB was applied to evaluate the binding with fetuin in solutions and serum glycoproteins from patients infected with dengue serotypes 1, 2 and 3 (DS1, DS2 and DS3) [8]. EIS and voltametric measurements registered the binding of Cramoll 1,4 with fetuin and serum glycoproteins of DS1, DS2 and DS3, showing a differential response for each serotype and higher interaction with glycoproteins from serum contaminated by DS2. Similar approach discriminated serum glycoproteins from DS1, DS2 and DS3; also detected different stages of infection using sera of patients with dengue fever and dengue hemorrhagic fever. An electrochemical biosensor was constructed using a gold electrode, which surface was modified with a nanocomposite of AuNps-polyaniline (PANI) and Cramoll 1,4, subsequently exposed to different sera [9]. Voltammetric and EIS data showed that Cramoll 1,4 biosensor discriminates the different serotypes and stages of dengue infection. Thus, Cramoll 1,4 is a promisor tool in researches for biosensing of serum glycoprotein profiles associated to diseases and this lectin preparation is a potential candidate for recognition of glycobiomarkers.

9. CONCLUSION

Glycosylation is a rich code of information related to physiological and pathological cellular events. Many changes in glycan profiles significantly correlate with diseases and their detection provide early diagnostic, staging and prognostic with high sensitivity. Glycobiomarkers detected in serum samples and measured through techniques more friendly can provide rapid results. Lectins, as sugar-binding proteins recognizing alterations in glycosylation profiles are useful in glycoanalytical techniques, among these, biosensing. Glycobiosensors based on electrochemical detection is a good strategy for development of simple, rapid, portable and sensitive devices detecting glycans in minimal volume of serum samples to search glycobiomarkers in clinical diagnostic.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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