Review paper

Electrochemical biosensing strategies to detect serum glycobiomarkers

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 minimally invasive and a simple procedure; then detection of glycobiomarkers in serum is attractive for clinical applications. Biosensors are a friendly strategy for rapid, easy and highly sensitive measuring of 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 have been employed to characterize glycan profiles in serum glycoproteins and constitute a promising tool in diagnostic and monitoring of diseases.

Keywords: glycosylation; glycans; biomarkers; serum glycoproteins; biosensors; lectin biosensors.

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 the occurrence of 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 easily. 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, biosensing technology has been applied successfully to identify and quantify glycobiomarkers with high specificity and selectivity, based on biorecognition events connected to a transduction system [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, bypassing 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 early diagnostic and monitoring disease assays.

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], in addition to glycan covered 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 a specific group 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 glycoproteins *via* two pathways, *N*- and *O*-glycosylation, with various branching points. *O*-linked glycans are attached through hydroxyl group of serine or threonine residues by 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 (Figure 1). *N*-linked glycans are attached to the amidic nitrogen of asparagine residues within the Asn-X-Ser/Thr glycosylation site, being X different of the proline [15]. *N*-glycosylation is initiated in endoplasmic reticulum, through the synthesis of a membrane lipid-linked *N*-glycan precursor (Glc3Man9GlcNAc2) from a dolichol phosphate to the Asn site of nascent polypeptide chain. *N*-glycans precursor is cleaved up to pentasaccharide tri-mannosyl core (Man3GlcNAc2) that is prolonged to generate three subtypes of mature *N*-glycans: high-mannose, complex and hybrid (Figure 1). The tri-mannosyl core is cleaved by glycosidases and transferred to the Golgi apparatus, where other monosaccharides are added by specific transferases to prolong the glycan chains, generating complex branching. The resulting structures provide the subtypes of complex *N*-glycans, which have complex branching without mannose residues and hybrid *N*-glycans, that contain mannose residues and complex branching [15, 16]. In addition, the subtype high-mannose N-glycans contains only mannose residues linked to the tri-mannosyl core [16]. Studies appointed 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) are the most frequent in the *N*- and *O*-glycan chains attached in human proteins [15, 16].

Inherent heterogeneity and diversity of glycan structures controlled by enzymes contribute to the varied functions that glycosylation carry out in cells and tissues. Glycosylation plays relevant influence in protein synthesis, processing and function, including folding, stability of tertiary structure, protection against action of proteases, increased serum half-life of proteins and reduced nonspecific protein-protein interactions [17, 18, 19]. Moreover, glycosylation mediate a regulation role in many biological processes involving cell-cell and cell-matrix interactions, such as cell proliferation, cell recognition, adhesion, host-pathogen recognition, receptor binding, signaling, fertilization, inflammation and immune responses [19, 20].

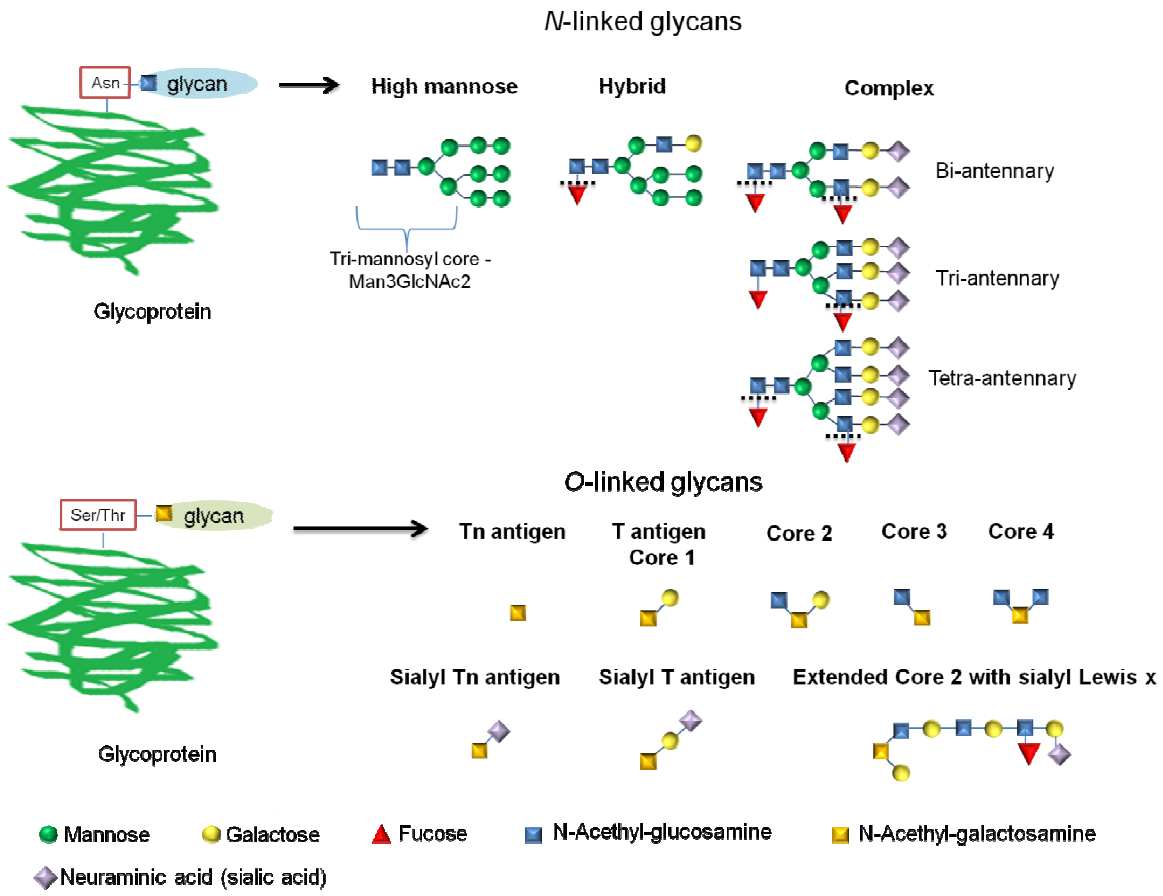

Figure 1. Types of *N*- and *O*- linked glycans found in human serum.

3. GLYCANS ARE POTENTIAL GLYCOBIOMARKERS IN DISEASES

The activities of glycosylation enzymes are factors dependent modulated by cellular dynamics. Changes in cellular environment and physiology often due to diseases, affect enzyme activities, resulting in aberrant glycosylation of proteins [21]. Aberrant 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 the cell surface and secreted in bloodstream [23, 24].

There is strong evidence that these glycosylation changes in the 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 which have a significant decrease in galactosylation for patients with inflammatory arthritis [25, 26]. The serum glycan profiling of fibrosis patients with hepatitis B and C virus infection reveled a significant decrease of bi-antennary and tri-antennary *N*-glycans 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 type 2 diabetes mellitus subjects and provide a serological alternative glycobiomarker [29]. Glycan profiles of whole serum of patients with autoimmune pancreatitis showed elevated levels of agalactosyl 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 and compared with benign disease and healthy individuals to identify cancer glycobiomarkers. Glycosylation aberrant patterns of proteins from cancer cells, tissues and serum commonly involve altered sialylation and fucosylation, change in glycan size and branching, Lewis antigens and truncated *O*-glycans [12]. Lewis antigens (Figure 2) are frequently expressed on membrane glycoproteins of several cancer cells. Cholangiocarcinoma cells expressed highly 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 (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc) antigen and an increased sialylation [33]. Increase of β1-6GlcNAc branches in *N*-glycans added by *N*-acetylglucosaminyltrasferase V (GnT-V) is showed 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 (Figure 1), as observed in breast and colon cancer [23, 36]. T/Tn antigen quantitative assays were developed to detect cancer without previous biopsy using highly purified T antigen for determination of anti-T immunoglobulins in serum samples [37].

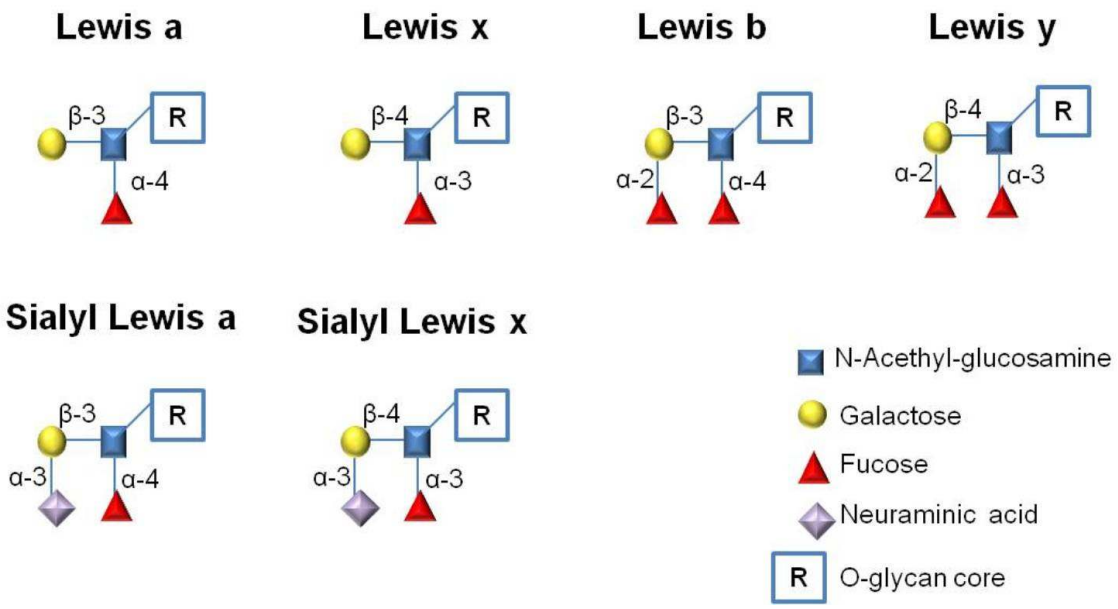

Figure 2. Terminal Lewis and sialylated Lewis antigens expressed by human tumors.

4. SERUM GLYCOPROTEINS AS BIOMARKERS OF DISEASES

Clinical tests using body fluids such as serum, plasma, urine and salive are minimally invasive, easily handled, with short time response, then they are preferred for detection and prognostic evaluation of various diseases. Glycoproteins secreted in the bloodstream are a considerable part of the 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 (Figure 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 for dengue infection. NS1 antigen is a glycoprotein present in elevated levels in the serum of infected individuals before specific antibodies emerge [39].

Current tendencies evaluate changes in the glycosylation patterns of serologic biomarkers appointing them 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 improvement of specificity 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 tumors of prostate [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 the 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 observed high levels of fucosylation in haptoglobin associated with

cancer and 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 glycan fucosylated bi-, tri- and tetra-antennaries 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 involves analyses by robust techniques, such as liquid chromatography and mass spectrometry, which provide detailed information about composition and quantity of residues [47]. However, they require long time of analyses, previous treatment of samples, technical prepare for manipulation, are complex and expensive; then, they become not viable for direct diagnostic of diseases and monitoring of patients in the clinical treatment. Besides, human serum and plasma are considered complex samples, because they contain a mixture of proteins, some very abundant (more than mg/mL) such as albumin, that can mask other protein 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) recognize a glycoprotein biomarker or glycan chains linked to protein, measuring these molecules at very low concentrations in human serum and plasma (Figure 3) with specificity and selectivity [45, 48]. Immunoassays as ELISA commercially available and lectin-based assays have been used for detection and measurement of specific serum glycobiomarkers in research and clinical applications to diagnostic of diseases [48, 49]. Lectins can be immobilized onto the inert support and incubated with serum sample for analysis of a glycobiomarker, as observed in disposable printed lectin arrays, or lectins are employed in enzyme-linked lectin assay for glycosylation profiling of a glycoprotein immobilized onto 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].

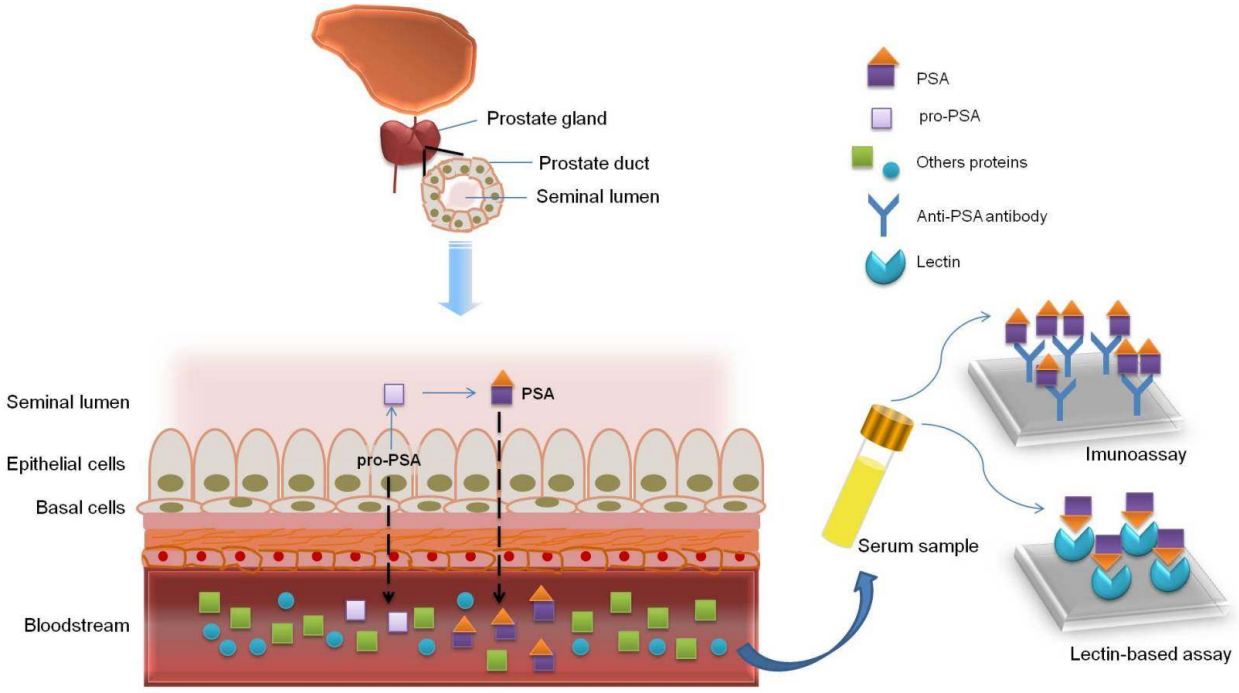

Figure 3. Esquematic representation of secretion from specific biomarker of prostate, the prostate-specific antigen (PSA), in the bloodstream and assays commonly used for specific detection.

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 is connected with an appropriate interface for processing of electric signal and display of measurable results [53] (Figure 4).

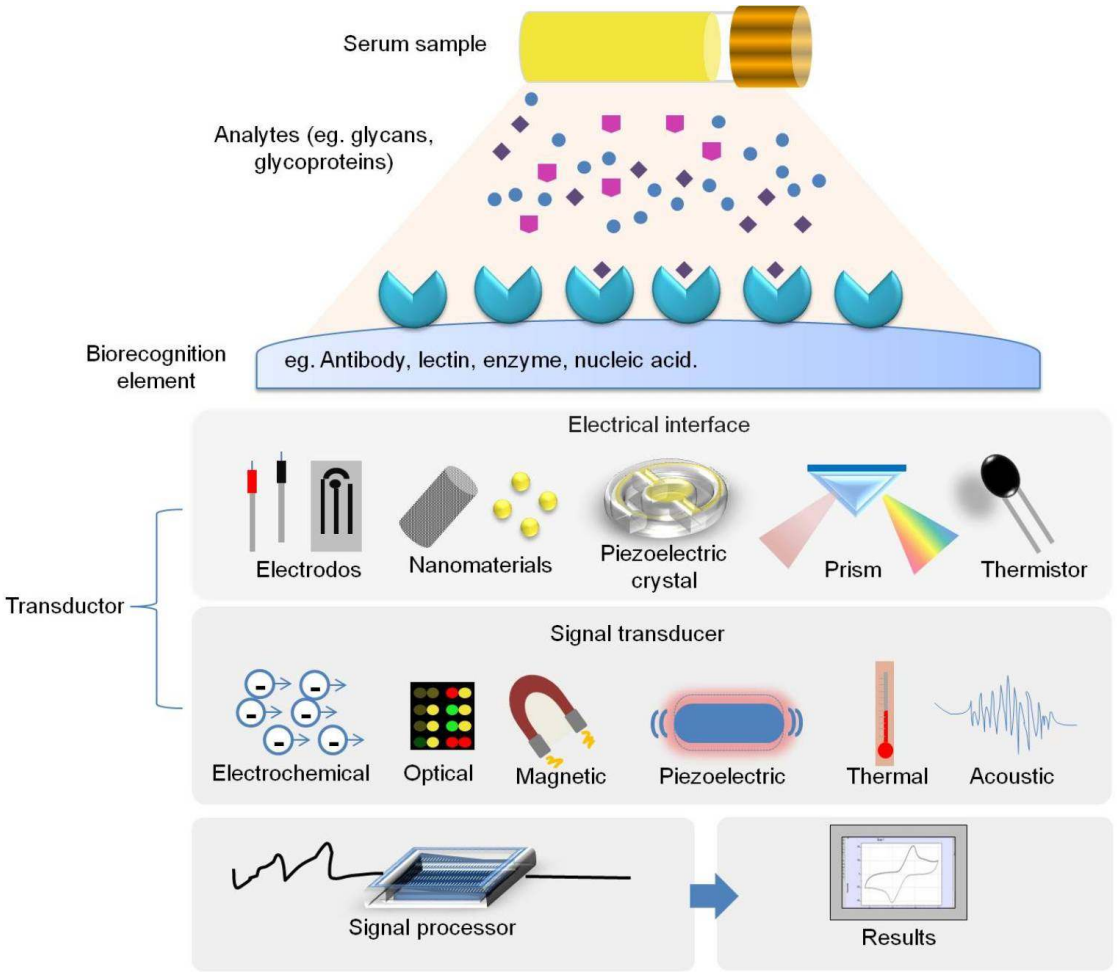

Figure 4. Scheme of a biosensor with a biorecognition element immobilized onto the solid surface in direct contact with an electrical interface of the transductor and a signal processor.

According to type of interaction bioreceptor-analyte, the analytical devices are classified as catalytic or affinity biosensors. Catalytic biosensors are based on enzyme activity with generation of 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 (Figure 4). They can include 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 bioanalyses, due to diversity of electrical interfaces and techniques for measurement that collaborate for high selectivity and sensibility; these sensors are easily of construction, portability, possible miniaturization and low cost [56].

The first biosensor was developed by Clark and Lyons in 1962, for detection of glucose by glucose oxidase. They elaborated a system known as enzyme electrode, using the enzyme glucose oxidase immobilized on the polyacrylamide film, onto a platinum electrode surface, and the determination of the 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 developed of different approaches in the detection mode and elaboration of more efficient devices, aiming the various types of analyses [58]. Biosensor technologies focus in the fabrication of self-contained, portable and miniaturized devices and at the same time, in conservation of the good analytical properties, such as speed, high sensitivity and selectivity. Biosensors are then friendly for applications to diverse areas: environmental monitoring, biosafety and assays for clinical applications [59].

Biosensors or glycobiosensors have been used to detect glycans and glycoproteins, in analyses of human serum [55]. Different of the complex approaches such as glycoproteomic techniques, those require sophisticated instrumentation and significant volume of sample, biosensor advances resulting 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 have been developed for characterization of the glycosylation patterns of glycans on serum glycoproteins correlated 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], 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 through 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, 70, 71]. The majority of available lectins are obtained from leguminous plants, comprising the largest number of already characterized proteins, such as Con A, soybean agglutinin (SBA) and *Cratylia mollis* lectin (Cramoll). Leguminous lectins, although derived from taxonomically distinct species, have molecular characteristics and physicochemical properties in common, such as Con A obtained from *Canavalia ensiformis* and Cramoll from seeds of *C. mollis*, and they exhibit a great variety in 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 analyses 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 and 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 it do not clear 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 various lectin-based models to configure the lectin-glycan interactions, sometimes assisted by antibody and nanomaterials to improve the selectivity and signal amplification. The lectin can be directly immobilized onto a solid support to 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 and showed potential to discriminate between cancer and healthy conditions [4]. The inverted configuration based on the immobilization of the glycan or glycoprotein followed by lectin binding 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, the capture antibody or lectin is immobilized to recognize the glycoprotein, and in the second step, a lectin or secondary antibody is additioned to complete the sandwich. A plastic sensor chip based on the technique surface-plasmon field-enhanced fluorescence spectrometry was used to detect GalNAcβ1−4GlcNAc-linked prostate-specific antigen using PSA IgG antibody to capture PSA and the lectin *Wisteria floribunda* agglutinin (WFA) to profile the glycan in serum from patients with benign prostate

hyperplasia and patients of prostate cancer [74]. The use of the PSA IgG antibody allowed the characterization of glycosylation profile from the specific glycoprotein target present in serum samples. Other sandwich system is based on the lectin or antibody confined on surface to 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 glycobiosensor was developed for comparative analysis of mannose and sialic acid levels on normal and cancer cells using 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.

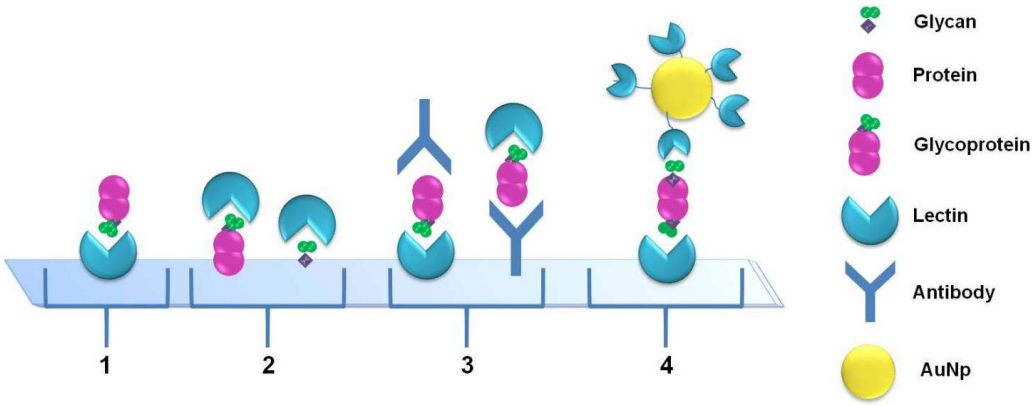

Figure 5. Configurations of lectin-based assay in glycobiosensing. Direct detection by immobilized lectin (1), reverse detection by incubated lectin (2), lectin-antibody sandwich assay (3) and lectin-conjugate sandwich assay with gold nanoparticles using one lectin type or two different lectins (AuNp)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

7 ELECTROCHEMICAL GLYCOBIOSENSORS

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, electrochemical glycobiosensors using a lectin as a biorecognition element have been developed to detect changes in the degree of sialylation, fucosylation, galactosylation and mannosylation in cell surfaces and serological samples [81]. Overall glycosylation profile of the sample or of a specific glycoprotein can be characterized by electrochemical methods used in 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, 85, 86]. Among these, EIS is widely used in glycobiosensors as an effective method for detection of lectin-glycan biorecognition on the electrode surface. The measurement is based on the application of 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 informations about RCT and lectin-glycan binding. By the way, EIS provide 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 have been used for label-free detection of lectin-glycan binding. In general, these techniques 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 modification electrode surface with biomolecules and biorecognition complex changes interfacial electron transfer reaction of the redox probe, resulting in changes of current. The variation of current is measured and quantitative information about the glycan target can be 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, PNA (peanut agglutinin) 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 was monitored by square-wave voltammetric stripping current peaks, which 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.

Although EIS analysis require more steps of processing data, this method promoted the detection of very small concentrations of glycobiomarker. An ultrasensitive label-free glycobiosensor was reported for measurement of sialylated glycoproteins using the EIS method [83]. A sialic acid specific lectin from *Sambucus nigra* (SNA I) was covalently attached to a mixed self-assembled monolayer (SAM) consisting of 11-mercaptoundecanoic acid and 6-mercaptohexanol onto 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 was developed for detection of the cancer glycobiomarker alpha-fetoprotein (AFP) and discrimination of glycosylation profile of AFP in serum samples using lectins as biorecognition elements [81]. First, a lectin biosensor for detection of AFP was mounted through the attachment of wheat-germ agglutinin (WGA) lectin to single-wall carbon nanotubes (SWCN) onto a screen-printed carbon electrode (SPCE). The biosensor was used for binding AFP and achieved a low limit of detection of 0.1 ng/L. Moreover, lectin biosensors were fabricated with different lectins to characterize glycosylation profile of N-glycan linked to AFP and discriminate between healthy and cancer patient's serum samples. 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 changes in charge transfer resistance were monitored. The results suggest an increase of core fucosylation and α2-6 sialylation in AFP from cancer when compared with healthy serum, being a potential tool for label-free profiling of glycan expression in serum samples to provide 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 is based on gold electrode modified with phospholipid membrane and Con A to form a lipid-Con A surface, which was incubated with serum from patients. Electrochemical characterization performed by voltammetry and EIS recorded decreases in the current response 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 by its mannose-binding sites, revealing that the biosensor was able to recognize the dengue serotypes and are useful for detection of dengue infections.

8 ELECTROCHEMICAL GLYCOBIOSENSORS 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 a native forage occurring 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]. The *C. mollis* seeds are sources of four molecular isoforms 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 lectins, and Cramoll 3 is a galactose specific lectin [7, 89]. Cramoll 1 in a characterization study using X-ray crystallography, showed classic tertiary structure of legume lectins. 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 even identical, 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 has showed 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 candidate probe for

histochemical studies. Other study reported an affinity column of Cramoll 1 coupled to Sepharose CL4B as 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 and 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 immobilization process of Cramoll 1,4 unaffected the recognition sites. In this case, the lectin recognized oligomannose glycans chains linked to ovoalbumin. After, Cramoll 1,4 biosensors also were developed to detect dengue serotypes. A gold electrode was modified with Cramoll 1,4, Fe_3O_4 nanoparticles and PVB, and 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 was performed to discriminate serum glycoproteins from DS1, DS2 and DS3; also to detect 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, and 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 the lectin preparation is a potential candidate for recognition of glycobiomarkers.

9. CONCLUSION

Glycosylation is a rich code of informations about physiological and pathological cellular events. Many changes in glycan profiles are significantly correlated with diseases and their detection provide early diagnostic, staging and prognostic with high sensitivity. When these glycobiomarkers are found in serum samples, they can be measured through techniques more friendly that provide rapid results. Lectins are sugar-binding proteins that recognize alterations in glycosylation profiles and therefore are useful in glycoanalytical techniques, among this, the biosensing. Glycobiosensors based on electrochemical detection is a good strategy for development of simple, rapid, portable and sensitive devices that can detect glycans in minimal volume of serum samples to search glycobiomakers in clinical diagnostic.

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