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Can glycoprofiling be helpful in detecting prostate cancer?

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Abstract

Glycans are chains of carbohydrates attached to proteins (glycoproteins and proteoglycans) or lipids (glycolipids). Glycosylation is a posttranslational modification and glycans have a wide range of functions in a human body including involvement in oncological diseases. Change in a glycan structure cannot only indicate presence of a pathological process, but more importantly in some cases also its stage. Thus, a glycan analysis has a potential to be an effective and reliable tool in cancer diagnostics. Lectins are proteins responsible for natural biorecognition of glycans, even carbohydrate moieties still attached to proteins or whole cells can be recognized by lectins, what makes them an ideal candidate for designing label-free biosensors for glycan analysis. In this review we would like to summarize evidence that glycoprofiling of biomarkers by lectin-based biosensors can be really helpful in detecting prostate cancer.

Keywords

prostate cancer; electrochemical impedance spectroscopy; lectins; glycans; biosensors; biomarkers

Glycomics

We can define glycomics as a field of science which studies glycome. Analogically to the term “proteome” which is a set of all proteins expressed by a cell, a tissue or an organism, or the term “genome” known as a whole genetic information of the cell, the tissue or the organism, glycome is a set of all glycans, that are present in the cell, the tissue or the organism in particular time (Cummings & Pierce, 2014; Dalziel *et al.*, 2014; Varki & Chrispeels, 1999). Glycans are defined as compounds consisting of a large number of monosaccharides linked via glycosidic bonds (Andre *et al.*, 2014; Solis *et al.*, 2014), however the term “glycan” is often used to describe a saccharide component of glycoproteins, glycolipids or peptidoglycans regardless of their length. Like with nucleic acids or peptides, also glycans are diverse group of molecules consisting of various number and types of monomer building blocks. But unlike nucleic acids or peptides, glycans provide much higher number of combinations and variations as monosaccharide chains can not only be linear but also branched, can be bound either by α or β linkage or can be linked to a polypeptide chain via oxygen (O-linked) or nitrogen atom (N-linked) (Cummings & Pierce,

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2014; Hushegyi & Tkac, 2014; Kluková *et al.*, 2014; Svarovsky & Joshi, 2014; Tkac *et al.*, 2014).

Glycosylation is an *in vivo* process and glycans cannot be cloned and glycosylation reactions are catalyzed by enzymes called glycosyltransferases (GTFs) (Katrlik *et al.*, 2010; Kim *et al.*, 2013; Rillahan *et al.*, 2012; Wang *et al.*, 2013b). GTFs are a wide group of enzymes that catalyze transfer of sugar moieties from a high-energy donor substrate to an acceptor molecules (Gray *et al.*, 2013; Hart & Copeland, 2010; Laurent *et al.*, 2008). To this date, there is approx. 250 GTFs known in human genome and it is believed that genes encoding glycan biosynthesis, degradation or transport proteins form approx. 2% of all genes (Freeze & Ng, 2012; Schachter & Freeze, 2009). According to the atom that links glycan to the protein structure, we distinct two types of glycans according to the linkage between polypeptide and a carbohydrate chain: if the glycan is linked via oxygen, we talk about O-glycans and if it is linked via nitrogen, we talk about N-glycans. There are certain differences between O-glycosylation and N-glycosylation (Marino *et al.*, 2010).

O-glycans can be linked to -OH group of all amino acids, but the most common are Ser and Thr (Fig. 1). Various monosaccharides were reported to be linked to Ser and Thr residues, e.g. N-acetylgalactosamine (Gal-NAc), N-acetylglucosamine (Glc-NAc), xylose (Xyl), mannose (Man) or fucose (Fuc) (Marino *et al.*, 2010). Studies revealed that human O-glycans contribute to many (patho)biological processes such as inflammation, coagulation, virus infections or cancer (Purcell *et al.*, 2008). N-glycosylation is a covalent bond between glycan and amide group of asparagine created during N-glycan synthesis (Fig. 2).

Glycosylation site contains target signal Asn-X-Ser/Thr sequence, where X can be any amino acid except of proline. N-glycosylation in eukaryotic cell is initiated by an addition of a precursor molecule ($Glc_3Man_9GlcNAc_2$) to Asn of target polypeptide chain (so called „core protein“), which is then transferred and modified in endoplasmic reticulum (ER). This precursor takes part in generating the three basic types of N-glycans: high-mannose, hybrid and complex. Enzymes glycosidases, present in ER, cleave three mannoses from glycosylated protein, which is then transferred to Golgi apparatus, where the chains are prolonged by addition of another monosaccharide (sialic acid - SA, GlcNAc, GalNAc and Fuc) by a particular GTF. Golgi apparatus modifications are thus responsible for generating complex and hybrid types of N-glycans. Resulting structure is based on combination of glycosidase (cleavage of terminal monosaccharides) and GTF (addition of terminal monosaccharides) enzymes (Fig. 2) (Marino *et al.*, 2010; Nakayama *et al.*, 2013; Varki & Chrispeels, 1999).

Glycosylation

Glycosylation is the most common post-translation modification of proteins. It is estimated that approximately 50-80 % of all human proteins are glycosylated (Arnaud *et al.*, 2013; Baker *et al.*, 2013; Bertók *et al.*, 2013; Sparbier *et al.*, 2005). Glycans have various functions in the cell signalization, cell growth, differentiation and apoptosis, inflammation processes, immune response etc. (Anthony *et al.*, 2008; Arthur *et al.*, 2014; Burton *et al.*, 2012; Dalziel *et al.*, 2014; Geissner *et al.*, 2014; Haltiwanger & Lowe, 2004; Lepenies & Seeberger, 2014; Song *et al.*, 2013; Svarovsky & Joshi, 2014) and a single glycoprotein can have more glycan

structures (Alley *et al.*, 2013; An *et al.*, 2009). There are also differences in glycan structures of the same glycoprotein expressed in various tissues as glycosylation is often cell type specific and thus detection of aberrant glycan structure in cancer cells compared to non-cancer cells can lead to a discovery of new cancer biomarkers (Hirabayashi *et al.*, 2013; Kim & Misek, 2011; Mechref *et al.*, 2012; Novotny & Alley, 2013; Svarovsky & Joshi, 2014; Tousi *et al.*, 2011). Aberrant glycosylation pattern is a fundamental characteristic of tumorigenesis (Gilgunn *et al.*, 2013; Rudd & Dwek, 1997).

Glycans as cancer biomarkers

As cancer is the leading cause of death worldwide and new ways of treatment are still being developed, there is a growing need for n early and reliable diagnosis of this disease. Among the most widespread causes of cancer deaths worldwide are cancers of breast, prostate, lungs and ovaries (Ferlay & Soerjomataram, 2013). As there are many cancer biomarkers that lack sensitivity or specificity, the research is focused now on the role of glycans and changed glycosylation in tumorigenesis and to study changes in glycan structures in glycoprotein biomarkers. From analysis of serum N-glycome of breast cancer patients, it has been noted that an increased sialylation, an increased levels of sLe^x (sialyl Lewis x) antigen and changes in fucosylation are present. Also differences between breast cancer and benign breast disease were detected as increased levels of agalactosyl biantennary glycans and glycans containing the sLe^x epitope were confirmed in breast cancer samples (Abd Hamid et al., 2008).

Gold standard for prostate cancer detection is PSA (a prostate specific antigen) analysis in serum, but this test is becoming insufficient due to lack of specificity. By analysis of PSA glycans it was revealed that an increase in the amount of core fucosylation and α2-3 linked SA is present in PCa (prostate cancer) samples. Triantennary trigalactosylated glycans and tetraantennary tetrasialylated glycans with outer arm Fuc were less abundant, while tetraantennary tetrasialylated glycans were more abundant in patients with advanced stages of PCa compared to patients with initial stages of PCa (Peracaula *et al.*, 2008; Saldova *et al.*, 2011; Sarrats *et al.*, 2010). Thus, different glycan structure can not only indicate the disease but can also provide information about various disease stages.

By analysis of N-glycome from lung cancer sera, it was detected an increased level of sLe^x, monoantennary glycans and a decreased amount of biantennary core fucosylated glycans. Also changes in sialylation were detected, when an increase level of trisialylated glycans and a decreased amount of disialylated glycans in lung cancer sera was noted (Arnold *et al.*, 2011). Biomarkers in use for diagnostics of ovarian cancer are not reliable when the disease is in its early stages. Glycan analysis of the whole serum revealed an increased sLe^x expression and an increased core-fucosylation of agalactosylated biantennary glycans. IgG (immunoglobulin G) isolated from serum exhibited a decreased galactosylation and sialylation in ovarian cancer samples compared to the healthy control (Saldova *et al.*, 2007).

Prostate and prostate cancer

We know two types of secretory glands in animal bodies, exocrine and endocrine ones. In endocrine glands, the product is secreted directly to the blood stream unlike the exocrine glands where the product is secreted via a duct. In mammalian male reproductory system,

prostate is a walnut-size endocrine gland enclosing urethra and lying between the urinary bladder and the urogenital diaphragm (Faiz & Moffat, 2002). Its weight in human body ranges between 7 to 16 g, having on average approximately 11 g in an adult male (Leissner & Tisell, 1979). Prostate gland has two main functions. First, it surrounds urethra and its muscles can affect the urine flow and second, it has a secretory function where it secretes an acidic fluid containing serine proteases (e.g. PSA) and other semen liquefying factors (Owen & Katz, 2005; Revenig *et al.*, 2014). The volume of ejaculate contains 65-75% of a seminal vesicles fluid, 25-30% of prostate fluid, 5-10% of a vasal fluid and less than 1% of a bulbourethral glands fluid (Revenig *et al.*, 2014).

Benign prostate hyperplasia (BPH)

BPH is non-neoplastic prostate disorder associated with abnormal prostate enlargement which then applies pressure on the urethra. BPH itself does not increase the probability of prostate cancer but these two conditions may occur at the same time. It is also associated with lower urinary tract symptoms (LUTC) such as increased urinary frequency and urgency, nocturia, difficulties with initiating urination, feeling of insufficient bladder emptying, weak and/or interrupted urine stream (Miller & Tarter, 2009). BPH occurs mostly in men after 50 years of age, but it does not occur in men, whose testicles were surgically removed before puberty, indicating that age and hormones are important factors in developing the disorder (Berry *et al.*, 1984; Wang *et al.*, 2014).

Testosterone is a principle androgen in blood, while dihydrotestosterone is the most abundant androgen in a prostate gland (Vis & Schroder, 2009). Development of both, malignant and benign prostate diseases is androgen dependent, however testosterone as the most abundant androgen is not primarily responsible for the development, growth and pathogenesis of prostate (Lepor, 2004). Dihydrotestosterone, a testosterone derivative, is the most potent androgenic hormone in many human organs and tissues and is a decisive androgen in the pathogenesis and progression of various diseases, e.g. BPH, prostate cancer, male pattern baldness and acne (Wang *et al.*, 2014).

Examination for BPH includes prostate palpation *per rectum* – digital rectal examination, blood test (PSA level test), urine test (monitoring of inflammation processes in bladder), trans-rectal ultrasound to evaluate the volume of prostate, non-invasive measurement of urine flow – uroflowmetry and patient´s evaluation of the severity of LUTC. A treatment depends on severity of BPH when early stages are only monitored and treatment is only initiated with later stages of the disease. Surgical removal of prostate is considered as the last option.

Prostate cancer

Prostate cancer (PCa) is mainly older men’s disease and is the most commonly diagnosed type of cancer and the third leading cause (after lung cancer and large bowel cancer) for all cancer-related deaths amongst males in European Union (EU-27) (Ferlay *et al.*, 2013). PCa is a malignant disease and is frequently asymptomatic or its symptoms are similar to BPH and are therefore underestimated (Miller *et al.*, 2003). Benign tumor cells do not have cancer cell characteristics; do not spread to other parts of the body and cells stay together as a

single mass. Benign tumors are formed by an increased proliferation of normal tissue cells, usually are not life threatening and their surgical removal results in a complete cure.

On the other hand, cancer cells are altered, their proliferation is uncontrolled and their regulatory mechanisms are dysfunctional. Malign tumors can infiltrate and disrupt other tissues and body parts. If cancer cells enter the blood stream or lymphatic system, they can migrate from a primary tumor to other locations and they form secondary tumors. This is called metastasizing and secondary tumors are called metastases (Russell, 2006). When prostate cancer metastasizes, prostate cancer cells can be found first in lymphatic nodes. Later they can spread to other non-adjacent organs such as bones, liver, lungs etc. Secondary tumors have the same cells as a primary tumor, for example, prostate cancer often metastasize to bones – cancer cells in infected bones are prostate cancer cells, not bone cancer cells (Jimenez-Andrade *et al.*, 2010).

As mentioned earlier, male androgenic hormones, play key roles in the origin and progression of PCa (Grossmann *et al.*, 2013). There are also other factors that may influence the development of PCa: age, race, lifestyle, genetic predisposition and obviously sex. PCa affects mostly older men, median age at diagnosis is 67 years (Siegel *et al.*, 2012a). Recent studies also revealed that patients of African ancestry are more susceptible to the disease development than those of European or Asian ancestry. However, patients of Asian ancestry who normally show lower incidence of PCa after relocating to United States had similar incidence of a disease compared to a domestic population (Martin *et al.*, 2013). This is due to environmental factors and lifestyle that are different from those of Asia. Studies also show that previous occurrence of prostate disease among family members, rapidly increases a risk for development of PCa (Kral *et al.*, 2011).

Because PCa in its earlier stages is often asymptomatic, it is very difficult to diagnose it before it reaches more advanced stages. PCa which is enclosed within the prostate gland cannot be detected using digital rectal examination, as the gland is not enlarged. In advanced stages, when cancer tissue presses on urethra and causes symptoms such as back pain and those similar to BPH, digital rectal examination is one of the two main diagnostic procedures. To confirm the PCa diagnosis, trans rectal ultrasound-guided biopsy needs to be carried out. Discovery of PSA (Ablin *et al.*, 1969) was an important milestone for analysis of PCa, however it took more than two decades until PSA serum level test was developed (Catalona et al., 1991) and further few years until it became a gold standard in PCa diagnostics as it was only approved in 1994 (Ueda *et al.*, 2013). However, even though PSA test show abnormal levels, it is not necessarily proof of PCa as elevated levels of PSA can occur not only as a result of PCa, but also as a result of BPH or prostatitis (Végvári *et al.*, 2012). Therefore a biopsy is recommended as the only reliable way to confirm PCa. After the biopsy, the examined prostate tissue is evaluated using the Gleason grading system, which was developed in 1967 by *Gleason et al.* and is based on giving marks from 1 to 5 according to the microscopic appearance and tissue architecture (Gleason *et al.*, 1967). Gleason score (GS) is the most frequently used method to estimate the destructive potential and prognosis of the disease.

GS1 is given to a prostate tissue, formed by single, separate, uniform round glands (Fig. 3A). It resembles a normal prostate tissue as it is very well differentiated. When, the glands are still well-formed, but have more tissue between them (i.e. the amount of stroma has increased) a GS2 is given (Fig. 3B). Most glands are separated by less than one average gland diameter and the shape of the glands is more variable. The glands in Fig. 3C are single, separated, dark and their shape starts to be random. The glands may be closely packed but usually they are irregularly separated, with poorly defined edges. This tissue is formed by relatively uniform glands infiltrating between benign glands. The glands may be separated by > 1 gland diameter and some of the cells begin to invade the surrounding tissue. This corresponds to a moderately differentiated carcinoma and GS3. In Fig. 3D, representing GS4, the majority of glands appear to be dispersed in the surrounding tissue and few recognizable glands are still present. The glands are chopped up and fused, with irregular edges. The glands are without stroma or small fragments of the glands are present. Nuclei are small, hyperchromatic and many cells are invading the surrounding tissue in neoplastic clusters. This corresponds to a poorly differentiated carcinoma. Fig. 3E shows carcinomas with a minimal glandular differentiation, ranging from infiltrating single cells to solid sheets of tumor cells. The tissue does not have any or have only a few recognizable glands. Cells with distinct nuclei appear in sheets within the surrounding tissue. This corresponds to an anaplastic carcinoma. The presence of GS5 and high percent carcinoma at prostatectomy predicts early death (http://www.webpathology.com) (Epstein *et al.*, 2005; Tabesh *et al.*, 2007; Vollmer & Humphrey, 2001).

Since there might be more than only one pattern present in the tissue sample, Gleason grading system has been modified by the International Society of Urological Pathology. With this method, the pattern is given the score from 1 to 5 and then the next most common pattern is graded. Then these two scores are added to get the Gleason score. The lowest score (slowest growing carcinoma) would be 2 and the highest score (fastest growing and dangerous) would be 10 (Epstein *et al.*, 2005). The decision to initiate the treatment is now based on the Gleason score, where score of 2-3 in elderly patient would only require a simple observation, because surgery or radical treatment would not be necessary. As shown in Table 1, new grading method also reflects survivability of patients with PCa (Albertsen *et al.*, 1998).

Treatment options for PCa include watchful waiting (surveillance of a medical condition without any medical intervention), radical prostatectomy, radical external beam radiation, and hormone treatment (Daubenmier *et al.*, 2006).

Prostate cancer biomarkers

A biomarker is a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or a disease. A biomarker may be used to see how well the body responds to treatment for a disease or a condition. It is also called a molecular marker or a signature molecule (http://www.cancer.gov). Biomarkers are very heterogeneous group of molecules as they can be proteins, metabolites, nucleic acids, gene transcripts etc. There are few main outcomes, which biomarkers should provide (Sawyers, 2008): 1) risk assessment – what is the chance of developing the disease; 2)

screening – if early detection affects survivability; 3) prognosis – prediction of outcome in the absence of a therapy; 4) diagnosis – investigation of presence of the disease and its stage; 5) prediction – selecting the most appropriate therapy and 6) monitoring – success rate of the treatment. Moreover, an ideal biomarker should have the following aspects in order to be able to predict or diagnose specific disease or a condition (Pepe *et al.*, 2001): 1) simple and safe assays; 2) provide guidance to ease decision making; 3) establish correlation between an assessment result and a clinical condition (Fletcher *et al.*, 2012) in a sensitive (a positive outcome when the disease is present) and a specific (a negative outcome when the disease is absent) way.

As with every other type of cancer, also in PCa early diagnosis tremendously improves the survivability rate (Albertsen *et al.*, 1998) therefore looking for an ideal PCa biomarker is a way to decrease the mortality and also to decrease the rate of overtreatment and overdiagnosis. There is a wide range of different PCa biomarkers (Fujita *et al.*, 2014; Madu & Lu, 2010; Prensner *et al.*, 2012) including circulating tumor cells, gene transcripts (a prostate cancer antigen 3), gene fusions (TMPRSS2-ERG), enzymes (α-Methylacyl–coenzyme A racemase (AMACR), a prostate specific antigen (PSA), prostatic acid phosphatase (PAP), metabolites (sarcosine), hormones, membrane proteins (caveolin, a prostate specific membrane antigen (PSMA), cell surface proteins (a prostate stem cell antigen (PSCA), exosomes, cytokines, single-nucleotide polymorphisms (SNPs) associated with prostate cancer, an early prostate cancer antigen, haptoglobin (Hp), serum Ca^{2+} etc. However, in the next text only glycoprotein biomarkers are discussed with description of glycan changes as a result of PCa (Table 2).

Prostatic acid phosphatase (PAP)

PAP is a glycoprotein secreted into seminal plasma by a male prostate gland epithelial tissue. It is a non-specific phosphomonoesterase and its level proportionally elevates with PCa progression (Kong & Byun, 2013). The enzyme (EC 3.1.3.2) is a dimer of molecular weight of 100 kDa, consisting of two subunits, 50 kDa each (Ostrowski & Kuciel, 1994). PAP was the first ever biomarker used for PCa diagnostics and had been used for more than 50 years since its discovery in 1938 when it was observed that its level in serum of men with metastatic prostate rises (Gutman & Gutman, 1938). There are two isoforms of PAP, one can be found in a seminal fluid (secretory PAP, sPAP) and the second can be found in an intracellular space (cellular PAP, cPAP) (Muniyan *et al.*, 2013). Physicochemical properties of sPAP and cPAP can vary with differences in an isoelectric point, antigenicity and glycosylation (White *et al.*, 2009). The main function of PAP is to liquefy the seminal fluid (Boron & Boulpaep, 2009), however it has been reported that PAP has also an analgetic effect (Zylka *et al.*, 2008) and PAP gene can act as a PCa tumor suppressor gene (Muniyan *et al.*, 2014). A normal level of cPAP in an adult male is approximately 0.5 mg/g of a prostatic tissue and approx. 1 mg/ml in a seminal fluid. As mentioned before, these values rise in case of PCa presence and a massive expression occurs at late stage of PCa with high GS (Gunia *et al.*, 2009). Even that PAP level test was replaced by PSA level test, nowadays PAP is proposed to be a valuable PCa prediction marker (Taira *et al.*, 2007).

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Prostate specific antigen (PSA)

PSA is a 33 kDa glycoprotein enzyme, produced by epithelial cells of a prostate gland tissue. PSA belongs to a kallikrein-like peptidase family, having chymotrypsine-like protease activity (Wu *et al.*, 2001), able to catalyze digestion of high-molecular weight proteins into bioactive peptides called kinins (Balk *et al.*, 2003). PSA cleaves semenogelin protein in order to liquefy semen coagulum and thus allowing spermatozoa to move freely (Yoshida *et al.*, 2009). PSA is translated as an inactive proenzyme (proPSA) and after cleavage of seven N-terminal amino acids, an active PSA is produced. This active PSA is immediately bound by protease inhibitor α1-antichymotrypsin, creating complexed PSA (cPSA), but a small amount of PSA circulates as free PSA (fPSA) (Zhu *et al.*, 2013). Further, ratio of fPSA level to total PSA level can improve specificity of PCa diagnosis (Mikolajczyk *et al.*, 1997).

PSA serum level test became a gold standard in PCa diagnosis. Normal concentration of PSA in a blood serum is below 4 ng/ml. Concentration higher than 10 ng/ml is considered to be a high risk of PCa as elevated levels of PSA are not a result of its increased expression but rather of its leakage from a damaged prostate tissue (Gilgunn *et al.*, 2013). However the width of a grey zone 4-10 ng/ml indicates requirement for a biopsy to avoid unnecessary overdiagnoses and overtreatments (Gilgunn *et al.*, 2013). Nevertheless, even that PSA testing dramatically improved PCa detection rate, there are also major drawbacks. The first one is a sensitivity of the method, as the width of a grey zone does not give a certain answer to a question if the prostate cancer is present and what is its stage, and the second one is a specificity, since elevated PSA levels can be also caused by prostatitis or BPH (Bo *et al.*, 2003). However, due to widespread PSA testing, the possibility to discover PCa increased while possibility of dying is slightly decreasing (Fig. 4). Therefore, PCa screening has become controversial and was subsequently ceased, because not all PCa cases may need immediate treatment. For some men, watchful waiting or active surveillance are options. Many men die with PCa instead of it (Edwards *et al.*, 2005).

Study of glycan structures on the surface of PSA glycoprotein could be the solution to the specificity problems. By detection of changes in glycosylation, it would be possible not only to detect PCa but also to evaluate its stage. As aberrant glycosylation is one of the characteristics of tumorigenesis, glycan structures present on the surface of PSA from a healthy donor and from a PCa patient were compared. Different research teams (Peracaula *et al.*, 2003; Sarrats *et al.*, 2010; Tabares *et al.*, 2006; Tajiri *et al.*, 2008) came to a conclusion that there are indeed different glycan structures when compared PSA from a healthy (from seminal fluid) and a PCa sample. Differences are mostly on the outer end of the oligosaccharide chains. Glycans from both sources are mostly disialylated biantennary structures with a core fucose, but their content of GalNAc, SA, and fucose is different. The most significant difference was in SA content, where SA was absent in PSA from cancer cells unlike in PSA from a seminal fluid, where it was present in nearly all glycans. There was also an increase in GalNAc (from 25% to 65%) content in PSA from cancer cells detected. Also to distinguish between PCa and BPH it was noted, that free PSA was decreased in PCa patients compared to BPH patients and that amount of α2,3-linked SA is higher in free PSA from a PCa patient compared to a BPH patient. These findings can be

used in construction of lectin biosensors, since lectins are able to detect sugar moieties on the surface of glycoproteins.

Prostate stem cell antigen (PSCA)

PSCA is a cell surface antigen, anchored *via* glycosylphosphatidylinositol-anchor into the cell membrane. It is a glycoprotein responsible for cell signaling, but its function in cell processes or tumorigenesis has not been fully clarified yet (Raff *et al.*, 2009). Not only is it expressed in the prostate, but also in the stomach, kidney, colon or bladder. A PSCA gene's expression significantly increases in a variety of PCa, bladder or pancreas cancer and decreases in esophageal and gastric cancer. It is also thought to be a tumor suppressor gene in the gastric epithelium (Reiter *et al.*, 1998). Studies reported that more than 94% of prostate tumors were positive for PSCA and also there was a correlation between a PSCA expression and GS or a tumor stage (Raff *et al.*, 2009). Unfortunately, its glycan structure is still not described. Because expression on the surface of PCa cells increases with a tumor progression, PSCA may be a useful molecular target in analysis of advanced PCa (Gu *et al.*, 2000).

Prostate specific membrane antigen (PSMA)

PSMA is an enzyme also known as glutamate carboxypeptidase II, an 84 kDa glycoprotein. PSMA is a transmembrane protein, catalyzing digestion of N-acetyl-L-aspartyl-L-glutamate to glutamate and N-acetylaspartate (Knedlik *et al.*, 2014). PSMA is heavily N- and O-glycosylated (glycans form approx. 25% of the total molecular weight of the protein), there are ten N-glycosylation sites predicted in the primary sequence of human PSMA and the N-glycosylation is crucial for PSMA enzymatic activity and stability (Holmes *et al.*, 1996). PSMA has been reported to be expressed in prostate, nervous system, small intestine and kidney (Knedlik *et al.*, 2014). PSMA is up-regulated in higher grade cancers, metastatic disease, and hormone-refractory prostate carcinomas and therefore can be used as an effective PCa marker. The highest level of PSMA of all cancer tissues was reported in androgen resistant PCa, with expression levels correlating with an increased cancer grade (Bostwick et al., 1998). Glycans of PSMA derived from *in vivo* sources were found to be of a complex type lacking polylactosamine structures. In contrast, cancer cells express only high mannose-type structures (Holmes *et al.*, 1996).

Haptoglobin (Hp)

Haptoglobin is a glycoprotein produced in various tissues such as liver, kidney, lungs etc. It is a well-known acute-phase protein and its expression increases in response to inflammation. Its role is to bind free haemoglobin (Hb) released from erythrocytes when haemolysis and tissue damage occur as free Hb is a source of reactive oxygen species (ROS) (Andersen *et al.*, 2012) and PCa has been linked to an increased oxidative stress (Gupta-Elera *et al.*, 2012). Haptoglobin shows low level of glycan fucosylation in healthy donors and an increased fucosylation and branching in donors diagnosed with high GS PCa. Therefore Hp may be a valuable PCa biomarker to distinguish between low and high-risk disease, and to evaluate the prognosis of a disease progress (Fujita *et al.*, 2014).

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Biosensors

Biosensor is a device integrating immobilized biorecognition elements in a close proximity of an appropriate transducer for bioassays (Fig. 5) (Barfidokht & Gooding, 2014; Matsumoto & Miyahara, 2013; Mu *et al.*, 2014; Nagatsuka *et al.*, 2013; Perumal & Hashim, 2014; Reuel *et al.*, 2013; Sang *et al.*, 2014; Szunerits *et al.*, 2013; Tamayo *et al.*, 2013; Xu et al., 2014; Zeng *et al.*, 2014). Biosensor consists of three main parts: I) a biorecognition element for specific interaction with an analyte), II) a physicochemical transducer to convert a biospecific signal of a biorecognition into a measurable analytical signal and data storage (Fig. 6). All these biosensor components needed for construction of biosensors applicable in glycoprofiling are discussed in the following sections. In the following section only antibodies and lectins applicable in glycoprofiling of PCa biomarkers will be described.

Antibodies

Antibodies (Abs) are proteins (immunoglobulins) produced by the immune system of an organism in response to an exposure to a foreign molecule – antigen (Ag) and characterized by its specific binding to a site of that molecule (an antigenic determinant or an epitope) (http://goldbook.iupac.org). Biosensor using antibody as a biorecognition element is called an immunosensor. In an immunosensor, Ab detects the complementary Ag and formation of the complex Ab-Ag transduces the signal which is measurable in various ways (both labelled and label-free methods) (Gopinath *et al.*, 2014; Kluková *et al.*, 2014). According to their specificity, we recognize monoclonal and polyclonal antibodies. Polyclonal antibodies are molecules that are products of different cell lines and react specifically with the same Ag, but recognizing various epitopes within this Ag. Contrary, monoclonal Abs are produced by a single cell line and have a specific affinity towards the same epitope (Rich *et al.*, 2013). Antibodies can be divided into five classes – A, D, E, G and M. IgG class is widely used for the construction of biosensors as they provide greater affinity and specificity towards Ag than other classes (Zeng *et al.*, 2012b). IgG is Y-shaped molecule, consisting of two large heavy chains (~55 kDa each) and two small light chains (~25 kDa each), where both have constant and variable region and are connected by disulphide bonds (Zeng *et al.*, 2012b). Variable regions are determining the specificity towards the Ag and are localized at both ends of the arms of an antibody (Fab – fragment, antigen binding region). Constant region is present at the stem of an Ab molecule (Fc – fragment, crystallisable region) (Murphy *et al.*, 2008). However, not all proteins bind to Ab in Fab region. For example, bacterial protein A binds to Fc region of IgG and this ability is widely used in a biosensor design, where Ab binds to the immobilized protein A, exposing its Fab regions towards the analyte. This way we can influence orientation of the antibody by avoiding random orientation and thus enhance the biosensor´s sensitivity (Briand *et al.*, 2006). Despite the fact that protein A has five IgG binding sites, it can only bind two molecules of IgG (Yang *et al.*, 2003).

As antibodies are products of an immune system of living biological systems, it takes a long time to produce them in a desirable quantity and also their quality cannot be controlled by human intervention. A way to reduce cost and time of their manufacturing is preparation of recombinant antibodies and their production by recombinant bacteria, yeast or other cell types (Borrebaeck & Wingren, 2011). A genetically engineered Ab is usually a fragment of

the original Ab molecule, but its targeting specificity remains preserved. They consist of variable regions of both the heavy and light chain, connected via an oligopeptide linker. Their great benefit is, that the linker can be modified so it contains for example, metal-binding amino acids (e.g. cysteine), which enables them to self-assemble on the biosensor surface at high density and in a proper orientation (Zeng *et al.*, 2012b). Recombinant bispecific antibodies can also be produced, possessing two binding sites for each antigen, which impart increased avidity (Byrne *et al.*, 2013). Genetically engineered Abs can also be connected with therapeutic molecule such as radionuclides, enzymes, toxins etc. (Holliger & Hudson, 2005).

Lectins

Lectins (from Latin word *legere* = to choose) are non-immunogenic (glyco)proteins that are able to specifically bind carbohydrate structures (http://goldbook.iupac.org). Lectins do not have enzymatic activity and for their ability to agglutinate erythrocytes, they used to be called agglutinins. First lectins were isolated from plants but lectins have also been found in animals and microorganisms (Gabius *et al.*, 2002). Since lectins react specifically with glycosidic residues of other molecules (e.g. cell wall polysaccharides), they found a broad range of applications in characterization of a glycome – glycoprofiling. The bond between the carbohydrate (free or part of a glycoconjugate) and lectin is reversible, based on hydrogen bonds, van der Waals and hydrophobic interactions and does not alter the structure of glycan or a glycoconjugate (Badr *et al.*, 2014). As lectins are very diverse group of proteins, there are more criteria for their classification. Lectins can be classified according to their source (plant, microbial, animal and fungal), their structure (monomeric, dimeric, etc., Fig. 7), presence of carbohydrate moieties in their structure (Table 3), but the most important is their classification according to their specificity for different glycan structures (Table 3).

There are various approaches to analyze glycans, however many of them possess significant drawbacks. Glycans can be characterized by a structural analysis of glycans released from glycoproteins or by an analysis of glycopeptides from proteolysed GPs (Novotny & Alley Jr, 2013). Nevertheless, these methods require chemical or enzymatic alteration of a glycoconjugate molecule and thus can cause loss of some information about the structure or a binding site of glycan. Lectin-based methods enable analysis of intact glycoproteins and even intact, viable cells and thus lectin-based glycoprofiling methods can be an alternative to instrumental analysis.

Transducers

Another criterion for a biosensor classification is according to the type of physicochemical transducer chosen to covert a biorecognition event into a measurable signal.From numerous transducing techniques applicable for a biosensor construction, especially electrochemical ones are promising due to high sensitivity of analysis, low cost of analysis, possibility for miniaturization and multiplexing (Bertok *et al.*, 2013a; Kluková *et al.*, 2014; Mislovičová *et al.*, 2009; Paleček *et al.*, 2014). An electrochemical biosensor can detect small changes in the current, voltage or interfacial electrode properties (e.g. impedance, capacitance) caused by a biorecognition event on the electrode surface (Moon *et al.*, 2014). Generally there are two

options how the output signal is generated either in a label mode or without any label. Labeling of an analyte or a biorecognition element is an additional step, which makes the experiment relatively complex and could negatively affect the biorecognition event (Gemeiner *et al.*, 2009; Katrlik *et al.*, 2010; Kluková *et al.*, 2014; Paleček *et al.*, 2014; Zeng *et al.*, 2012a). Therefore novel analytical methods, which can operate in a label-free mode, are needed. Here we will discuss electrochemical label-free methods, which allow sensitive, simple, low cost and fast analysis with promising application in a point-of-care testing. The most sensitive and reproducible methods currently applied for label-free electrochemical assays of glycans are field-effect transistor-based and electrochemical impedance spectroscopy-based sensing (Huang *et al.*, 2013) approaches. Field-effect sensing offers detection limit down to fg/ml (Hideshima *et al.*, 2013; Paleček *et al.*, 2014; Reuel *et al.*, 2012a; Wang & Yau, 2013) and reliable FET biosensors were reported implementing polysilicon thin film transistor (Poly-Si TFT) or complementary metal–oxide–semiconductor (CMOS)-based devices (Estrela *et al.*, 2005; Meyburg *et al.*, 2007). Such devices are not discussed here, but a reader is advised to read recent literature on that topic (Luo & Davis, 2013; Tkac & Davis, 2009).

Electrochemical Impedance Spectroscopy (EIS)

EIS is a popular analytical method having many applications in corrosion monitoring, analysis of electrode kinetics, characterization of membranes, conducting polymers, semiconductors, surface treatment protocols, batteries and fuel cells and is an ideal candidate for biosensor applications (Katz & Willner, 2003; Tkac & Davis, 2009; Xu & Davis, 2014). Therefore it is widely used for characterization of biomaterials, functionalized electrodes and biocatalytic transformations at electrode surfaces and specifically for the transduction of biosensing events at electrodes or within field-effect transistor devices (Ates, 2011; Susloparova *et al.*, 2013). This method is used to determine electrochemical characteristics of the systems, including double-layer capacitance, impedance, determination of the rate of charge transfer and charge transport processes; and a solution resistance (Macdonald & Barsoukov, 2005).

According to the Ohm’s law, resistance is the ratio between voltage, U, and current, I. However, this is only valid for circuits, where direct current (DC) is applied. If this is the case, there is no distinction between impedance and resistance, and resistance can be thought of as impedance with a zero phase angle. Like resistance, impedance is demonstrating the ability of a circuit to resist the flow of electrical current, represented by the “real impedance” term (Z_r), but it also reflects the ability of a circuit to store electrical energy, like a capacitance, reflected in the “imaginary impedance” term (Z_i) (Lvovich, 2012). The total impedance is therefore the sum of an imaginary and a real part (1):

$$Z = Z_r + i\,Z_i \quad (1)$$

Impedance is expressed with symbol Z in ohms (Ω). By modification of the Ohm's law, we can calculate the impedance of the system following equation (2), so the impedance is expressed in terms of a magnitude (Z_0) and a phase shift (Φ).

$$Z = \frac{U_t}{I_t} = \frac{U_0.sin(\omega.t)}{I_0.sin(\omega.t+\varphi)} = Z_0. \frac{sin(\omega.t)}{sin(\omega.t+\varphi)} \quad (2)$$

EIS is based on measuring the current response by applying a small sinusoidal perturbation to an electrochemical cell. Impedance output data at the selected frequencies are consequently transformed into a complex plane Nyquist plot vectors, which by application of an equivalent circuit can provide information about charge transfer resistance (R_{CT}) of a soluble redox probe (usually $[Fe(CN)_6]^{3-/4-}$) (Bertók *et al.*, 2013). Nyquist plot consists of a semicircle and a linear part (Fig. 8). The diameter of a semicircle represents a charge transfer resistance, while a linear part represents a diffusion-limited process at lower frequencies. A biorecognition event (analyte binding) causes an increase of thickness of a molecular layer on the surface, resulting in an increased R_{CT} of the electrode and also in a limited diffusion of the redox probe to the surface, which leads to an increase of the semicircle diameter (Fig. 8) (Chen *et al.*, 2009). This method provides high sensitive and reproducible results, where the detection limit can reach attomolar levels (Bertok *et al.*, 2013b).

Immobilization techniques

One of the key steps in a biosensor preparation is successful immobilization of a biocomponent on the surface of a transducer (Bucko *et al.*, 2012). Appropriate immobilization technique should be selected in order to preserve biocomponent's stability, to retain its binding ability and availability of the biorecognition element for an analyte binding. The most common immobilization techniques are adsorption, microencapsulation, entrapment, cross-linking, affinity and covalent immobilization. Covalent attachment of biorecognition elements on the surface is the most stable method of immobilization but it is necessary to perform it correctly in order to preserve the activity of a biocomponent. One of the best methods for covalent immobilization of biorecognition elements is via formation of a self-assembled monolayer (SAM), which forms spontaneously on some surfaces.

Self-assembled monolayers (SAMs)

SAMs are very often combined with electrochemical detection methods. The formation of a monolayer was first time described by Irving Langmuir in 1917, where he observed that the fatty acid film formed at the interface between air and water was formed by a single layer of molecules (Langmuir, 1917). In biosensor constructions SAM is used as a single layer of molecules attached to the surface of the transducer. SAM-based immobilization provide stable, reproducible and uniform structure while avoiding random orientation of functional groups (Mandler & Kraus-Ophir, 2011). SAMs can be created on different types of surfaces such as glass, silicon oxide or noble metals, e.g. gold, silver or platinum. Molecules that allow formation of SAM include fatty acids, organosilicon or organosulphur compounds, consisting of the head, the chain and the tail with a functional group oriented towards the solution (Love *et al.*, 2005). The head (e.g. thiol, silane, phosphonate) attaches the adsorbate to the substrate, the chain stabilizes the adsorbate when a fully formed film has developed and the tail contains functional groups.

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While a wide range of headgroups have been used in SAM research, the dominant system described in the literature has been alkanethiol-based SAMs formed on gold (Davis et al., 2007; Estrela *et al.*, 2008; Estrela *et al.*, 2010; Guo & Li, 2014). Alkanethiol-gold system has the following features: 1) gold surfaces are relatively inert to oxidation and provide a substrate, when freshly deposited, that is free of oxides and competing adsorbates; 2) strong bond between sulfur and gold (40-45 kcal/mol); 3) sulfur–gold bond is formed preferentially in the presence of other competing chemical functionalities, providing ability to modify the end of the alkanethiol with a variety of functional groups, including –OH, $–NH_2$, –CN, and –COOH; and 4) alkanethiol-based monolayers on gold are easily formed from a dilute solution under ambient conditions.

The ability to use –COOH and $–NH_2$ functional groups allows formation of amide bond between the SAM and the protein. In order to attach the protein to –COOH group, this needs to be activated, which is commonly carried out by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Carboxyl group reacts with NHS in presence of EDC, resulting in formation of a semi-stable ester which subsequently reacts with primary amines of a protein (Bertok *et al.*, 2014; Davis *et al.*, 2009; Hermanson, 2013).

When immobilizing the protein on the SAM, this monolayer acts as a barrier to prevent proteins from a direct contact with the electrode surface, which would cause their denaturation (Cancino & Machado, 2012) and also allow to control density and/or orientation of biorecognition elements on the surface (Gooding *et al.*, 2003). One method to control density of functional groups on the surface is use of mixed SAMs, prepared by co-adsorption of two alkanethiols – one with a long chain (e.g. mercaptoundecanoic acid – MUA) and the other one “diluting” alkanethiol with a shorter chain length and a different terminal group (e.g. 6-mercaptohexanol). Controlled density of biorecognition elements decreases the steric hindrance between bound proteins (Briand *et al.*, 2006). A diluting thiol can also provide functional groups resisting non-specific interactions, what is an important issue when assaying complex samples such as blood, serum and other biological fluids. Latest research is oriented towards use of zwitterion molecules, containing phosphorylbetaine, sulfobetaine or carboxybetaine as a terminal group with anti-fouling properties (Shen & Lin, 2013). It has been reported that construction of SAM by co-assembling MUA and sulfobetaine led to reduction of a non-specific binding to only 6% of a specific binding (Bertok *et al.*, 2013a). Another methods to minimize non-specific interactions is use of oligo- and poly ethylene glycol containing biomolecules, because of their excellent protein-resistant properties and bovine serum albumin as a blocking agent so that the analyte only binds specifically to the immobilized ligand (Davis *et al.*, 2009; Davis *et al.*, 2007; Shen & Lin, 2013).

Nanotechnology in biosensor design

In the last decade, nanotechnology has been extensively introduced into biomedical applications such as biological detection, drug administration, diagnostics or tissue engineering (Mu *et al.*, 2014; Reuel *et al.*, 2013; Reuel *et al.*, 2012b). This allowed a dramatic improvement in the diagnostic/therapeutic values of these methodologies. At

present, the main aim of nanotechnology applications is in cancer research and treatment. Nanotechnology involves study, engineering and applications of nanomaterials, which are materials, consisting of nanoparticles (NPs), with at least one dimension smaller than 100 nm (Daniel & Astruc, 2004). At nanoscale, chemical, physical and biological features of materials are different from those of bulk solid matter and are controlled by quantum effects rather than by a classical physics (Barkalina *et al.*, 2014). Regardless of properties like size, structure, composition and shape, nanomaterials are universally characterized by one key feature – surface area-to-volume ratio. This ratio increases when the size is decreasing and thus the surface phenomena predominate over the chemistry and physics of the bulk solid matter (Jianrong *et al.*, 2004). There are two large groups of nanomaterials - organic and inorganic ones (see Fig. 9).

Most electrochemical studies employ gold NPs thanks to simple synthesis, ability to modify their surface by thiols and biocompatibility. Gold NPs have also ability to conjugate with biomolecules without altering their activity and therefore they are an excellent transducer in biosensing reactions. In general, NPs can play various roles in construction of biosensors but the most notable advantages are (Luo *et al.*, 2006): 1) their immobilization on electrode surfaces generates a conductive high-surface area interface that enables a sensitive electrochemical detection of molecular and biomolecular analytes with a short response time; 2) NPs act as effective labels for the amplified electrochemical analysis of the respective analytes; and 3) conductivity properties of metal NPs enable the design of biomaterial architectures with pre-designed and controlled electrochemical functions.

Applications of electrochemical methods in glycoprofiling of PSA

Methods used nowadays for PSA analysis require complex instrumentation and are therefore carried out within dedicated laboratories which prolongs the time from taking a sample to initiating an appropriate treatment to several weeks. The most commonly used approaches to replace these methods include but are not limited to fluorescent labeling with streptavidin-avidin technology, surface-enhanced Raman spectroscopy, real-time immuno-PCR, surface plasmon resonance or various electrochemical methods based on detecting the changes in potential, current, capacitance, conductance or impedance as a result of a biorecognition event (Healy *et al.*, 2007). Electrochemical biosensors with immobilized lectins proved to be effective in glycoprofiling of quite complex samples such as blood/serum revealing distinct glycoprofile in samples from people with various diseases compared to a glycoprofile from a control group (Bertók *et al.*, 2013; Bertok *et al.*, 2013a; Kluková *et al.*, 2014; Paleček *et al.*, 2014). Such impedimetric lectin-based biosensors after optimization of their preparation at nanoscale or with application of nanomaterials were able to detect glycoproteins down to aM (10^{-18} M) level (Bertok *et al.*, 2013a; Bertok *et al.*, 2013b). Selective glycoprofiling of a biomarker in complex samples has to be performed in a different way. Biomarker has to be “fished out” from a complex sample using immobilized antibodies and only then lectin can be applied to detect composition of glycan attached to the protein biomarker (Fig. 10).

Our proposed design employs lectin instead of a secondary antibody and thus enables to detect different PSA glycoforms in their native form without any labeling. As these glycoforms vary depending on the stage of PCa, lectin-based PSA immunoassay can be a

valuable detection technique in PCa diagnostics. A proposed concept allows cost-effective, rapid and sensitive evaluation of patient's condition without large laboratory instrumentation and thus is a promising tool for a point-of-care cancer diagnostics. Extensive review of the literature showed that such a concept has not been described yet for glycoprofiling of PCa biomarkers (Paleček *et al.*, 2014). Recent results from our group, however, suggest that this assay protocol is viable with ability to glycoprofile PSA biomarker down to 10 ng/mL concentration (a threshold in PCa diagnostics) (unpublished results). A work to glycoprofile much lower concentration of PSA is under way in our laboratory. The main reasons for choosing electrochemical detection platform was already mentioned but summary of basic biosensor characteristics for analysis of PSA as shown in Table 4 convincingly demonstrate benefits of using electrochemistry in PSA analysis with perspective in a sensitive glycoprofiling of PSA. Moreover, application of various nanomaterials or sandwich configurations may enhance desirable properties of electrochemical immunoassays even further.

Summary of electrochemical immunosensor characteristics in Table 5 show that detection limit of various electrochemical detection platforms is well below 10 ng/mL, what is a threshold concentration in PCa diagnostics and thus PSA can be reliably detected in the clinically relevant concentration range. Moreover, from different electrochemical methods applied so far in PSA detection, EIS seems to be sensitive as other electrochemical methods but additional advantage of using EIS is a label-free mode of operation.

Conclusions

We can conclude that changes in the glycan moiety on the protein backbone of PCa biomarkers can really indicate PCa or indicate the stage of the disease. Even though sophisticated instrumental techniques can detect glycan changes in a reliable way, such approach can hardly be multiplexed and thus more user-friendly approaches are currently sought. Application of lectin-based biosensors is a viable alternative since such devices can detect changes in the glycan composition on the surface of intact PCa biomarkers. From numerous detection schemes available for construction of biosensors, especially electrochemical ones provide features for highly sensitive and robust biomarker detection. Moreover, EIS provides a label-free mode of detection with sensitivity still comparable to other electrochemical techniques requiring use of redox/enzyme labels. Finally, a sandwich configuration with antibody immobilized on the electrode surface in a controlled way applied for selective "fishing" of an analyte from complex samples followed by incubation of the biosensor with lectins is a feasible way for glycoprofiling of PCa biomarkers. Our unpublished results suggest that this sandwich concept can work with PSA at clinically relevant concentration and the method has a potential to quantify concentration of PSA in the sample and at the same time allows detecting glycan composition of this biomarker.

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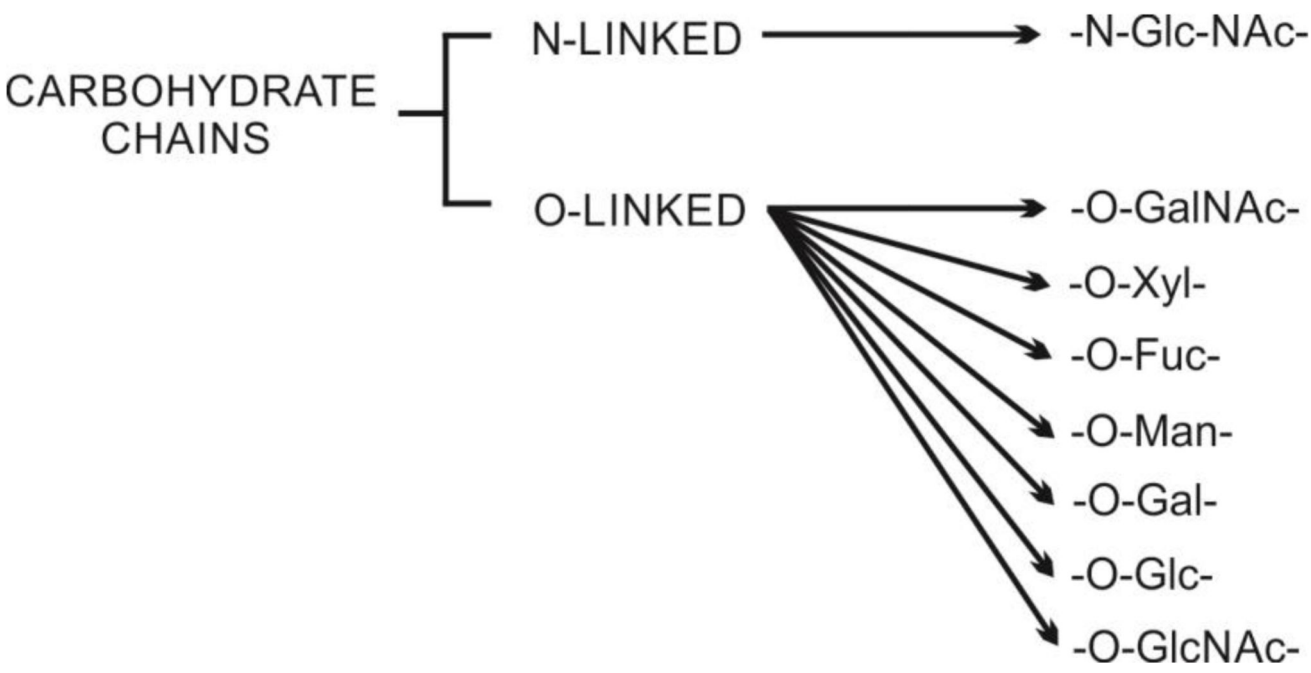

Figure 1.

Classification of glycoprotein carbohydrate chains linked to proteins through $–NH_2$ group with N-acetylglucosamine attached (Glc-NAc, N-linked glycans) or to –OH group on proteins with N-acetylgalactosamine (GalNAc), xylose (Xyl), fucose (Fuc), mannose (Man), galactose (Gal), glucose or GlcNAc attached. Adapted from (Nakayama *et al.*, 2013).

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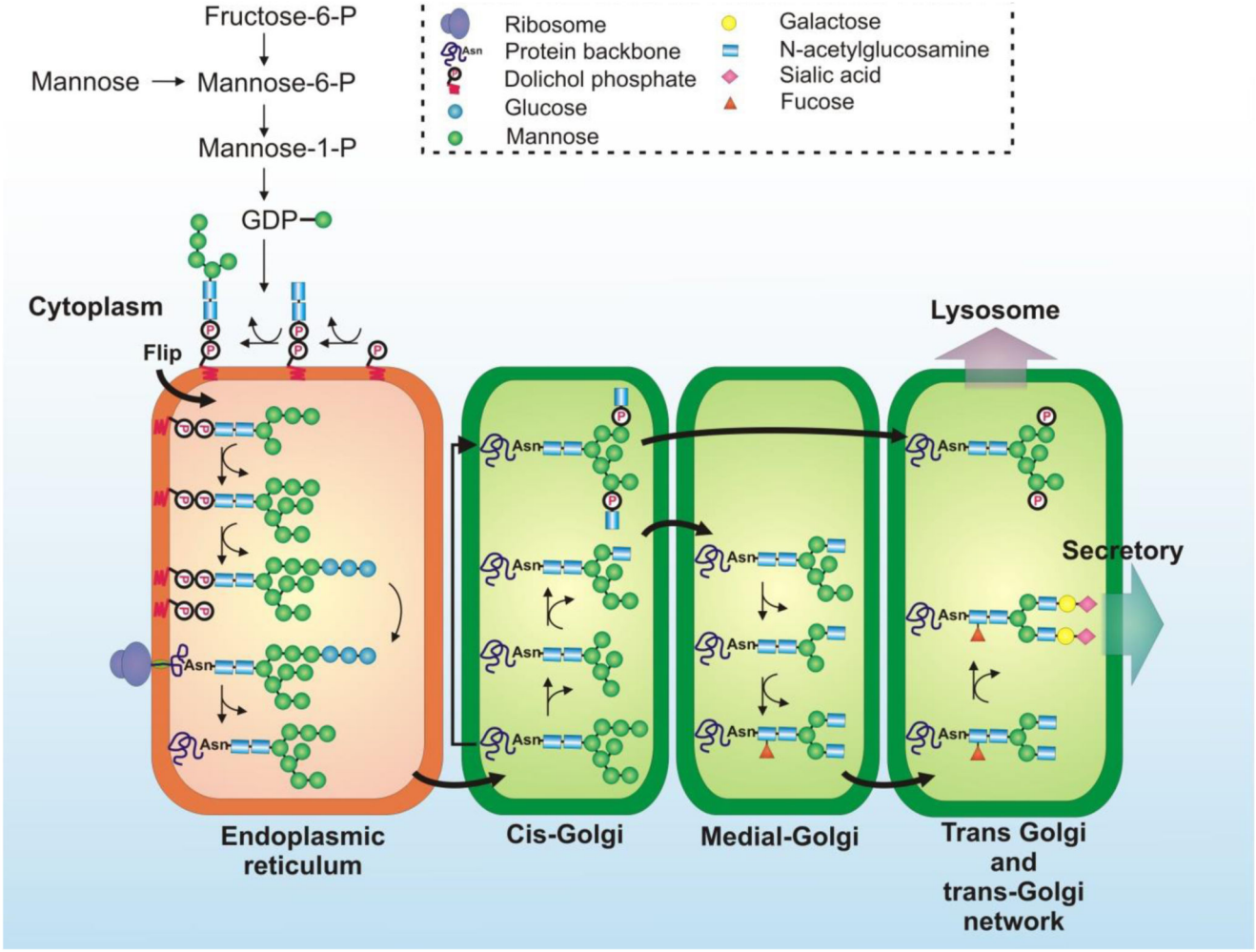

Figure 2.

Synthesis of N-glycans. In the initial stage a glycan precursor $Glc_3Man_9ClcNAc_2$ is formed attached to the membrane and then transferred to the protein backbone. In the next step two Glc and one Man units are trimmed and the glycan attached to the protein backbone is then further processed in the Golgi apparatus. If the glycan has a correct composition it is secreted away from the Golgi apparatus for proper functioning inside the cell. When glycan has an incorrect composition it is transferred to lysosome to be recycled. Adapted from (Nakayama *et al.*, 2013).

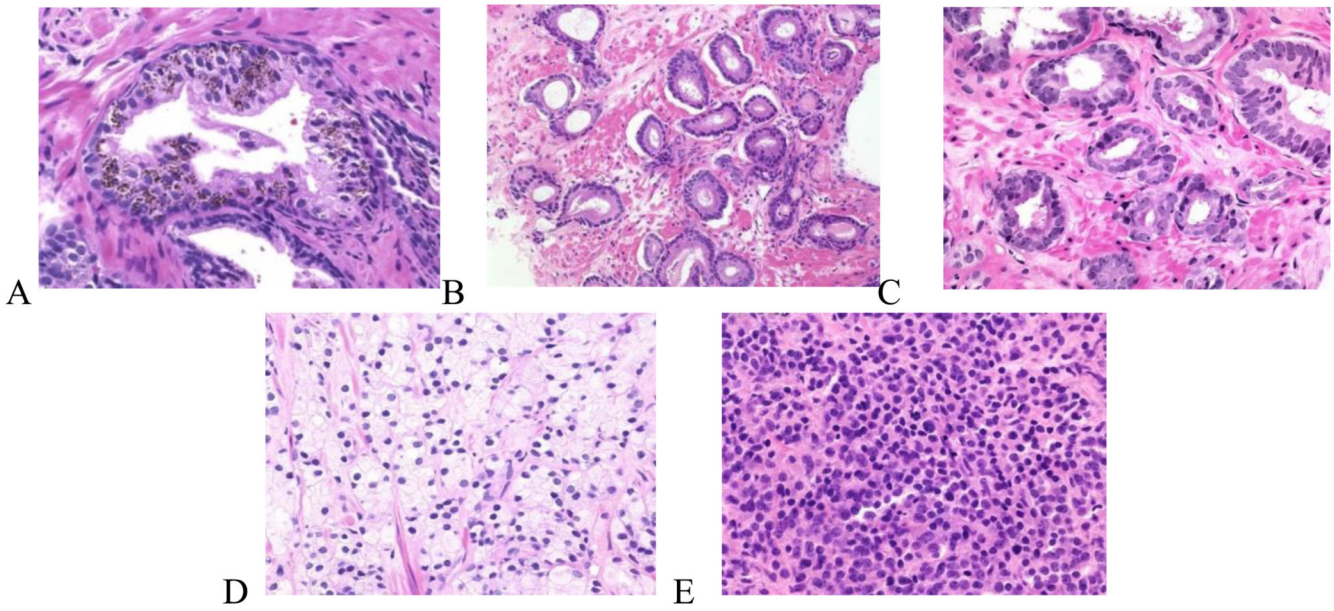

Figure 3.

Stained prostate tissue samples representing various stages of prostate cancer. Further details and description of changes in the tissue samples with progression of the disease is provided in the text. Reproduced with permission from http://www.webpathology.com.

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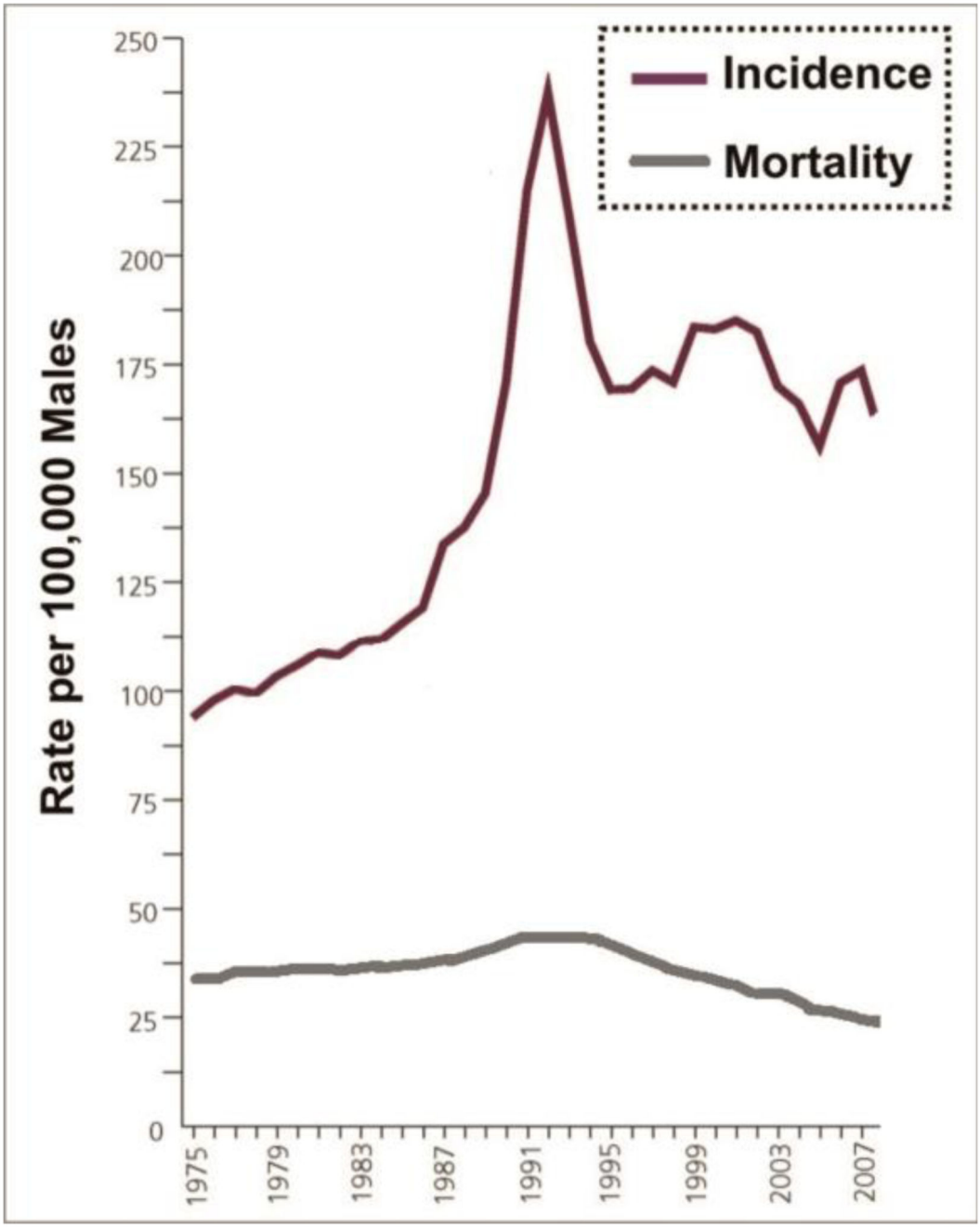

Figure 4.
Incidence *versus* mortality before and after PSA screening in years 1975-2007. Adapted with permission from (Siegel *et al.*, 2012b).

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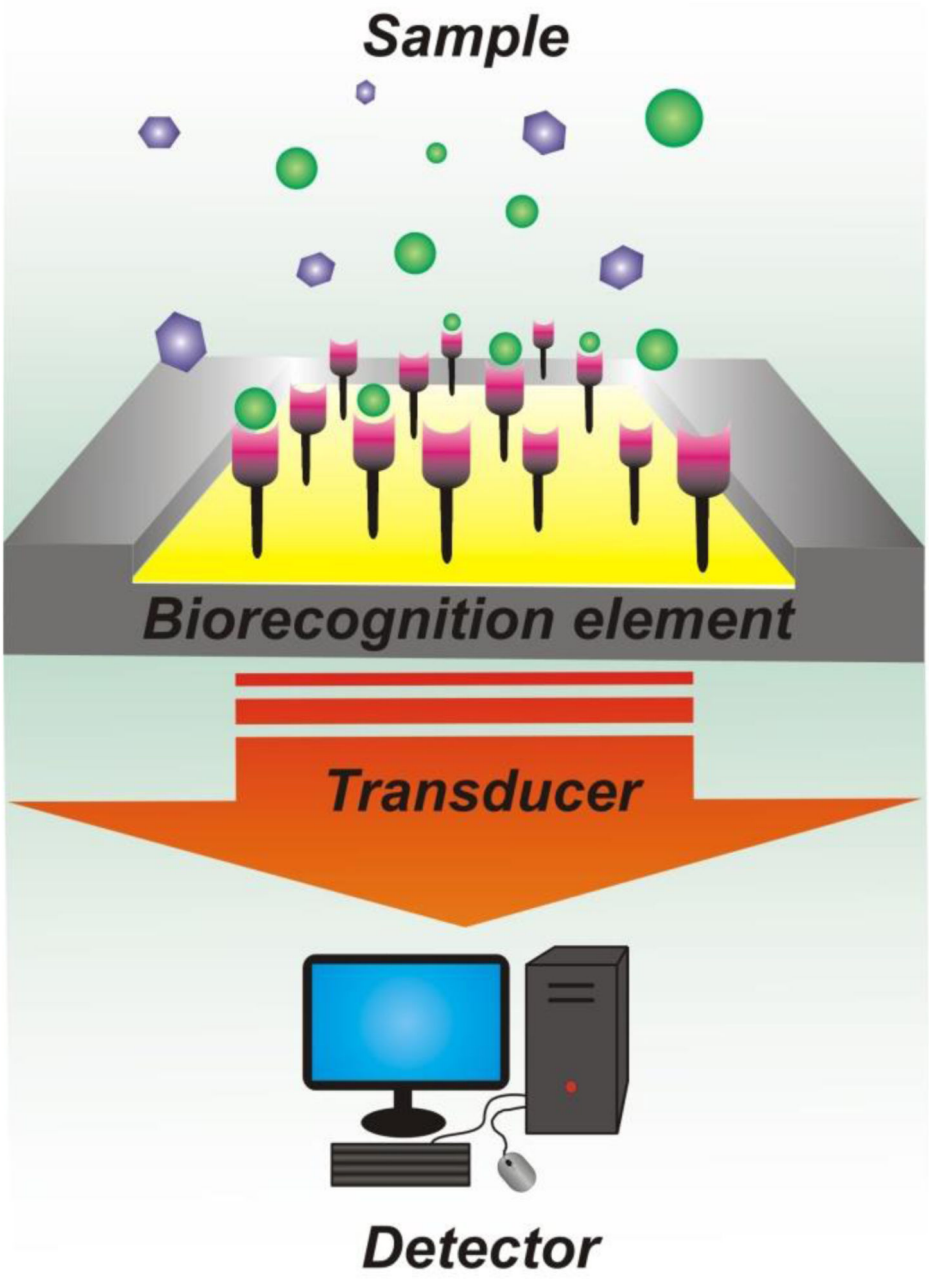

Figure 5.
A simplified scheme of a biosensor consisting of a biorecognition element immobilized in a close proximity on the transducer surface.

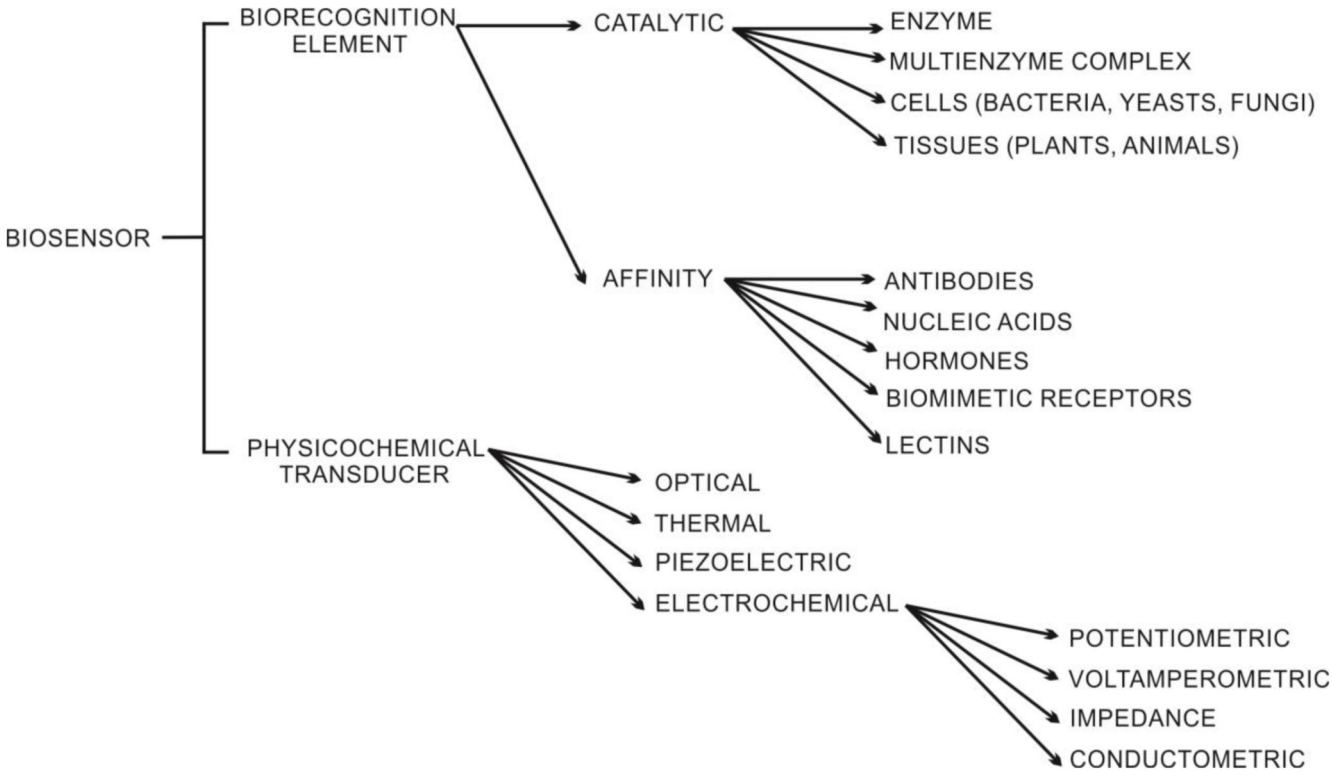

Figure 6.

An overview of a biosensor classification describing the most often applied biorecognition elements and physicochemical transducers.

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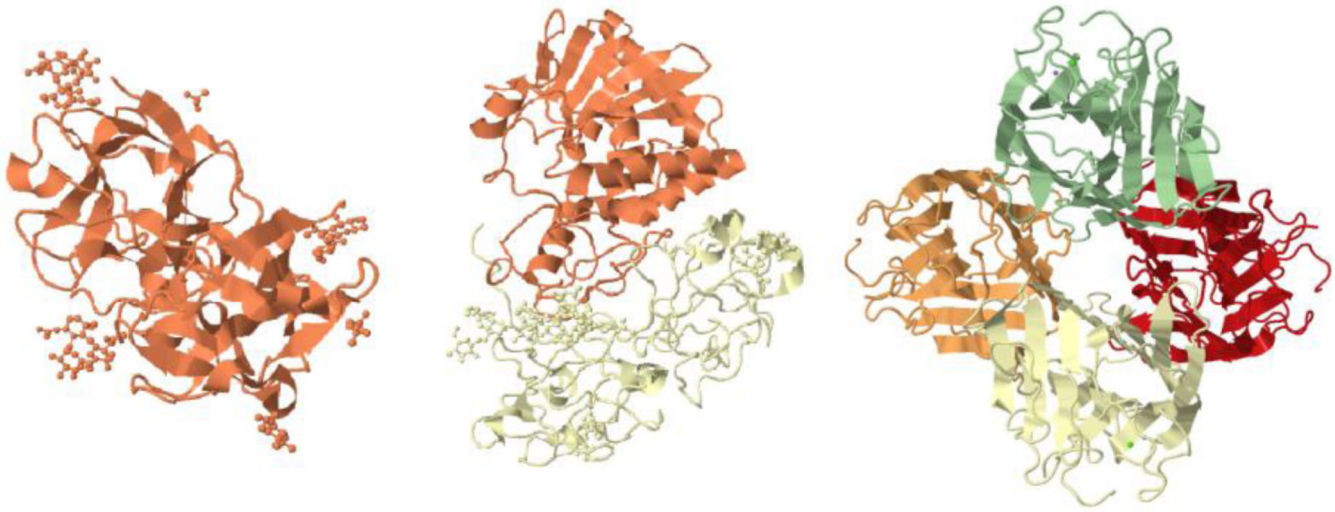

Figure 7.
Various lectins with monomeric (*Sambuccus nigra* agglutinin), dimeric (*Ricinus communis* agglutinin) and tetrameric (Concanavalin A) binding sites (from left to right).

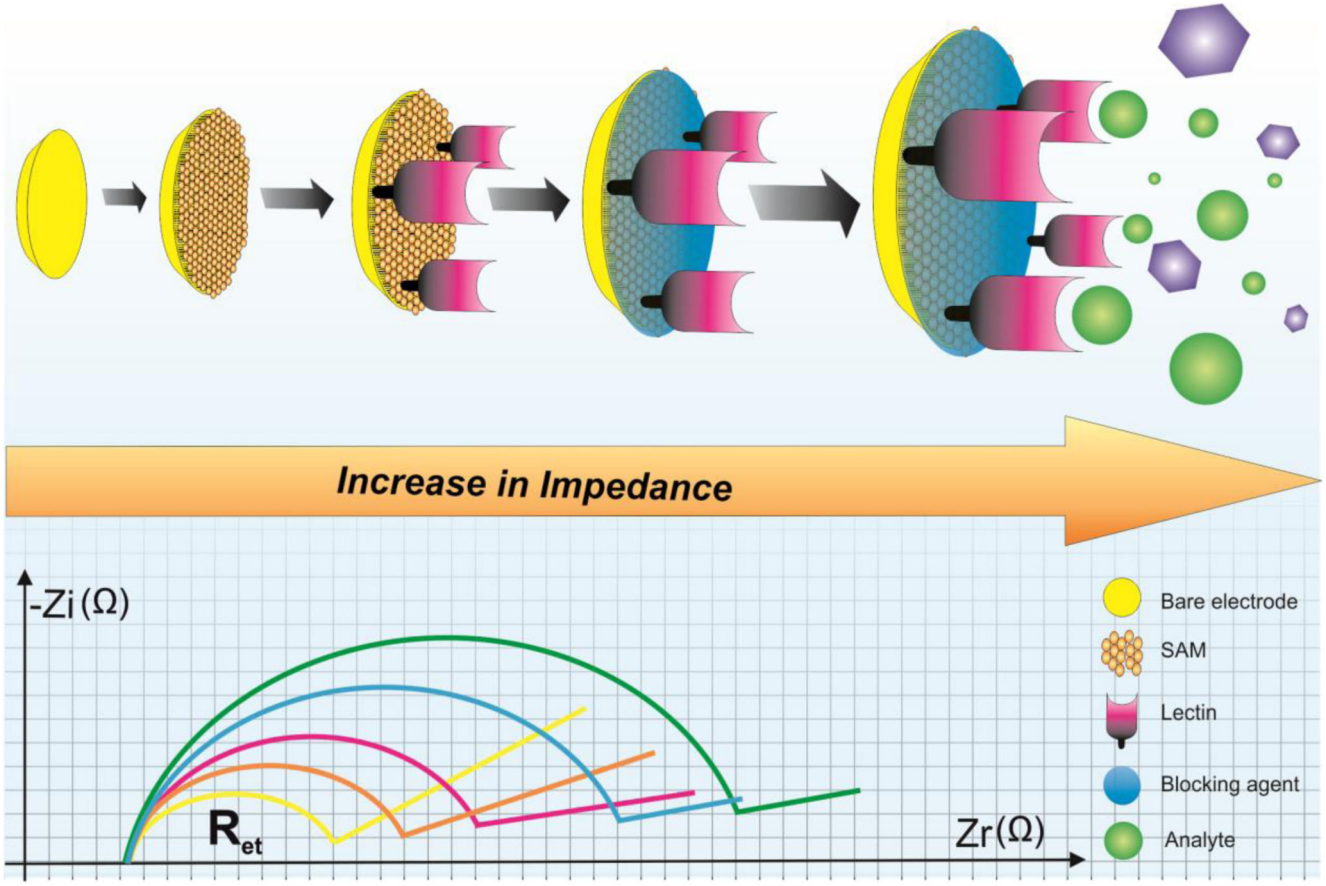

Figure 8.

A scheme of a build-up of the biosensor by layer-by-layer approach, by forming SAM layer on gold, by immobilization of lectin, by blocking of the surface to resist non-specific interactions and finally application of the biosensor to detect its analyte (up). A Nyquist plot used for providing interfacial characteristics (i.e. R_{CT} in a form of a semicircle) of the interface after every building step and finally after interaction with its analyte (down). Further details are provided in the text.

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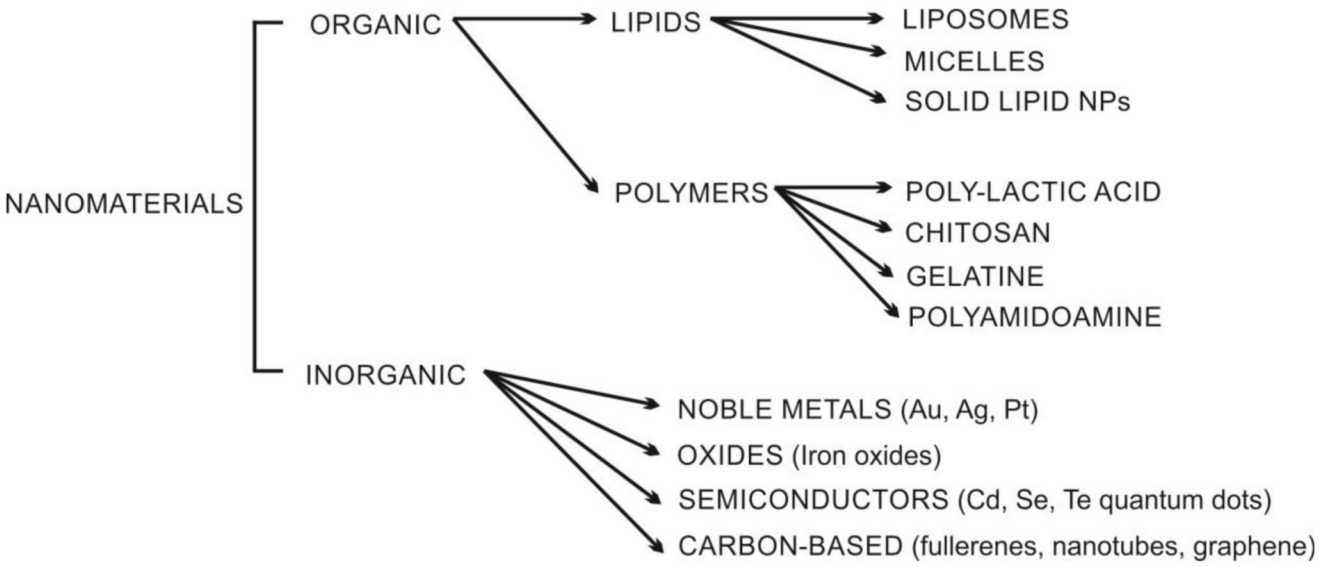

Figure 9.
Classification of the most commonly applied nanomaterials dividing into two main categories – organic and inorganic ones.

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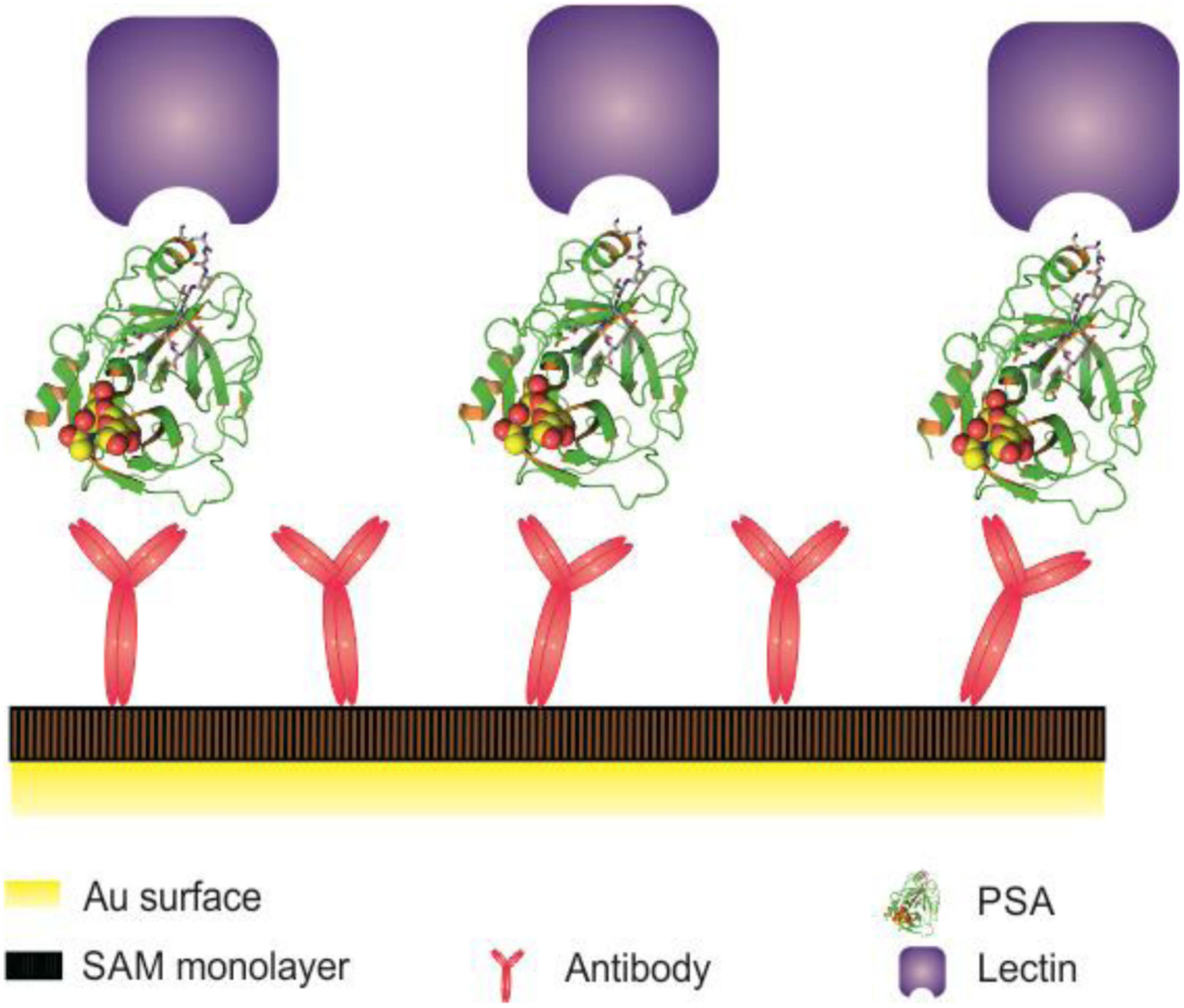

Figure 10.
Sandwich configuration of the proposed lectin-based PSA immunosensor. Antibody is covalently immobilized on a SAM surface, followed by incubation with a sample containing PSA and finally a glycoprofiling of PSA is completed by injection of lectin.

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Table 1

Death rates of untreated PCa related to reached Gleason score (Albertsen *et al.*, 1998)

Gleason score	Death by 15 years
2-4	4 - 7%
5	6 - 11%
6	18 - 30%
7	42 - 70%
8-10	60 - 87%

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Table 2

PCa biomarkers with changes in the glycosylation of biomarkers as a result of a disease.

Biomarker	Glycosylation change	Ref.
PAP	↑Gal, ↓high Man glycan	(White *et al.*, 2009)
PSA	↑ α2,3-linked SA, ↑ GalNAc	(Peracaula *et al.*, 2003; Sarrats *et al.*, 2010; Tabares *et al.*, 2006; Tajiri *et al.*, 2008)
PSCA	N/A	(Gu *et al.*, 2000)
PSMA	↑ Man	(Holmes *et al.*, 1996)
Hp	↑Fuc, ↑ branching	(Fujita *et al.*, 2014)

Table 3

List of the most common lectins, their abbreviations, specificity and selected characteristics, taken from https://www.vectorlabs.com/data/brochures/K4-K7.pdf

Lectin	Abbreviation	M_w	SU	GP	Specificity
Aleuria aurantia	AAL	72	2	☒	Fucα6GlcNAc
Amaranthus caudatus	ACL, ACA	66	2	☒	Galβ3GalNAc
Bauhinia purpurea	BPL, BPA	195	4	☑	Galβ3GalNAc
Concanavalin A	Con A	104	4	☒	αMan, αGlc
Datura stramonium	DSL	86	1	☑	$(GlcNAc)_{2-4}$
Dolichos biflorus	DBA	111	4	☑	αGalNAc
Erythrina cristagalli	ECL, ECA	54	2	☑	Galβ4GlcNAc
Euonymus europaeus	EEL	140	4	☑	Galα3Gal
Galanthus nivalis	GNL	50	4	☒	αMan
Hippeastrum hybrid	HHL, AL	50	4	☒	αMan
Jacalin	Jacalin	66	4	☑	Galβ3GalNAc
Lens culinaris	LCA, LcH	50	4	☒	αMan, αGlc
Lotus tetragonolobus	LTL	107	4	☑	αFuc
Lycopersicon esculentum	LEL, TL	71	1	☑	$(GlcNAc)_{2-4}$
Maackia amurensis I	MAL I, MAL	130	2	☑	Galβ4GlcNAc
Maackia amurensis II	MAL II, MAH	130	2	☑	Neu5Acα3Galβ4GalNAc
Maclura pomifera	MPL	44	4	☒	Galβ3GalNAc
Narcissus pseudonarcissus	NPL, NPA, DL	59	4	☒	αMan
peanut	PNA	110	4	☒	Galβ3GalNAc
Pisum sativum	PSA	53	4	T	αMan, αGlc
Ricinus communis I	RCA I, RCA_{120}	120	2	☑	Gal
Ricinus communis II, ricin	RCA II, RCA_{60}	60	1	☑	Gal, GalNAc
Sambucus nigra	SNA, EBL	140	4	☑	Neu5Acα6Gal/GalNAc
Solanum tuberosum	STL, PL	100	2	☑	$(GlcNAc)_{2-4}$
Sophora japonica	SJA	133	2	☑	βGalNAc
soybean	SBA	120	4	☑	α>βGalNAc
Ulex europaeus I	UEA I	63	2	☑	αFuc
Vicia villosa	VVL, VVA	144	4	☑	GalNAc
wheat germ	WGA	36	2	☒	GlcNAc
succin. wheat germ	Succin. WGA	36	2	☒	GlcNAc
Wistera floribunda	WFA, WFL	116	4	☑	GalNAc

Abbreviations: M_W: molecular weight in kDa; SU: number of subunits; GP: glycoprotein; T: trace; Fuc: fucose; Gal: galactose; GalNAc: N-acetylgalactosamine; Glc: glucose; GlcNAc: N-acetylglucosamine; Man: mannose; MeαGlc: α-methylglucoside; MeαMan: α-methylmannoside; Neu5Ac: N-acetylneuraminic acid (sialic acid), Succin.: succinylated.

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Table 4

Comparison of different methods in PSA detection, adapted from (Poon et al., 2014)

	ELISA	F. microscopy	SPR	Electrochemistry
Sample consumption	100 µl	20 µl	100 µl	10 µl
Detection Limit	3 pM	6 fM	549 pM	15 fM

ELISA-Enzyme linked immunoassays, F. microscopy-fluorescent microscopy, SPR-surface plasmon resonance

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Table 5

Overview of selected PSA immunosensors, updated from (Kavosi *et al.*, 2014)

Electrode	Detection	DL [ng/ml]	LR [ng/ml]	Ref.
GCE/GS/Th	Amper.	0.001	0.002-10	(Yang *et al.*, 2010)
GCE/MWCNTs/polytyrosine	CV	0.002	-	(Yuan *et al.*, 2010)
GCE/AuNPs/MWCNT–CAS	CV	0.007	0.01-3	(Tian *et al.*, 2012)
GCE/AgNPs@MSNs	CV	0.015	0.05-50	(Wang *et al.*, 2013a)
OECT/AuNPs	CV	0.001	0.1-100	(Kavosi *et al.*, 2014)
SPE/Polymer GE/Fc-peptide	DPV	0.2	0.5-40	(Zhao *et al.*, 2010)
GCE/ILs/CNTs	DPV	0.020	0.2-40	(Salimi *et al.*, 2013)
GCE/ILs/CNTs/Dendrimer	DPV	0.001	0.05-80	(Kavosi *et al.*, 2014)
GE/AuNW/PPy	DPV	$0.3.10^{-6}$	10.10^{-6}-10	(Moon *et al.*, 2014)
Graphene-modified GCE	ECL	0.008	0.010-8	(Xu *et al.*, 2011)
Au-IDμE	EIS	0.001	0.001-0.1	(Chornokur *et al.*, 2011)
GE/PANI/AuNPs	EIS	$0.6.10^{-3}$	0.001-100	(Dey *et al.*, 2012)
AuE	EIS	0.001	10^{-3}-10^{3}	(Suaifan *et al.*, 2012)
GCE/ILs/CNTs/Dendrimer	EIS	0.5	Up to 25	(Kavosi *et al.*, 2014)
GDE/cysteamine	LSV	0.007	0.01-100	(Qu *et al.*, 2010)
AuE/AuNP-hydroxyapatite-Chit	Pot.	2.6	3.5-30	(Shen *et al.*, 2011)
GS/Quantum dot	SWV	0.003	0.005-10	(Yang *et al.*, 2011)

DL-detection limit, LR-linear range, Amper.-amperometric detection; CV-cyclic voltammetry; DPV-differential pulse voltammetry; ECL-electrochemical luminescence; EIS-electrochemical impedance spectroscopy; LSV-linear sweep voltammetry; Pot.-potentiometric detection; SWV-square wave voltammetry; GCE-glassy carbon electrode; GS-graphene sheets; Th-thionine; MWCNTs-multiwalled carbon nanotubes; CAS-cross-linked starch; AgNPs@MSNs -silver nanoparticles on mesoporous silica nanoparticles; OECT-organic electrochemical transistor; AuNPs-gold nanoparticles; SPE-screen printed electrode; Fc-ferrocene; ILs-ionic liquids; CNTs-carbon nanotubes; AuNW-gold nanowires; PPy-polypyrrole; Au-IDμE-gold interdigited μ-electrode; PANI-polyaniline nanowires; AuE-gold electrode; GDE-gold disc electrode.