Analytical Methods

Are glycan biosensors an alternative to glycan microarrays?

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MINI REVIEW

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Complex carbohydrates (glycans) play an important role in nature and study of their interaction with proteins or intact cells can be useful for understanding many physiological and pathological processes. Such interactions have been successfully interrogated in a highly parallel way using glycan microarrays, but this technique has some limitations. Thus, in recent years glycan biosensors in numerous progressive configurations have been developed offering distinct advantages compared to glycan microarrays. Thus, in this review advances achieved in the field of label-free glycan biosensors are discussed.

Introduction

Glycans/carbohydrates can be found on the cell surface or inside cells in the form of glycoconjugates. Carbohydrates play important role in many cellular processes *via* glycan-protein interactions with involvement in immune responses, tumor metastasis, infections by bacteria and viruses, cell signaling, molecular recognition and inflammation.[1-3] Glycans are composed of monosaccharaides, which are connected together *via* glycosidic bonds from building blocks like mannose, glucose, galactose, fucose, sialic acid, N-acetylglucosamine and N-acetylgalactosamine. The glycosidic bond is realized between the anomeric hydroxyl group of one monosaccharide and any of the hydroxyl group of the second monosaccharide. Because of high variability of glycosidic bonds, the number of possible di - and oligosaccharides is phenomenal: more than 10 million tetrasaccharides can be assembled from just nine monosaccharidic building blocks.[4] Different linkage types and branching points make even short oligosaccharides very rich in information coding. The structure of glycans can not be directly read from a template like in case of proteins, which structures are encoded by oligonucleotide sequences. This fact presents substantial barrier for understanding the complex functions of glycans. Not just the primary oligosaccharide sequence, but also the density, distribution and relative orientation of glycans on biological surfaces are really important for the protein recognition.[5]

One of the most important posttranslational modifications of proteins is glycosylation, which is responsible for modulating protein functions both on cellular surfaces and within cells. Changes in glycosylation have been observed in all types of malignant cells and cells affected by other diseases. Alterations in glycosylation are often caused by changed activities of glycosyltransferases and glycosidases and/or by availability of monosaccharide building blocks.[6-8] Glycans often appear conjugated to proteins and lipids in from of glycoproteins, glycolipids and proteoglycans.

The roles of glycans in cellular recognition and function have been recognized in recent years[4, 9] and the detailed study of glycan interactions can be useful for further understanding such functions as well as the development of new therapeutic and diagnostic strategies for many diseases.[1, 10-12] Glycan biochips or microarrays were developed from DNA and protein arrays in 2002[13-17] and soon became a successful tool for highly parallel analysis of interaction of glycans with proteins or cells.[18, 19]

Even though glycan biochips are behind numerous discoveries,[2, 20-26] there are some limitations of this technique such as a need for a labeling step, which can alter selectivity of binding. Moreover, bleaching of a fluorophore can be additional problem of the assay. Label-free detection strategies can overcome such limitations[27] and when a binding event is carried out in a spatial proximity of the transducer, such biosensor devices can offer high selectivity and sensitivity of assays.[28] Thus, in this review assay alternatives to glycan biochips, in a form of label-free glycan biosensors, will be discussed together with key elements of their construction to cover recent advances (2011-April 2014) in this field.

Initial efforts to prepare glycan biosensors were reviewed by Gerlach *et al.* in 2010.[29] In 2012 exciting developments in fabrication of glycan microarrays were summarized, while glycan biosensors were only briefly described.[4] Reuel *et al.* comprehensively reviewed glycoprofiling in a robust way based on various nanoengineered tools with literature cited until the end of year 2011[30] and recently two reviews only marginally mentioning glycan biosensors appeared from this group.[31, 32]

Preparation of glycan biosensors

In order to prepare robust and sensitive glycan biosensors several important issues of their construction have to be addressed including choice of surface, immobilization protocol and a label-free detection platform of analysis.

The surface of the biosensor

There are in general a wide range of different surfaces, which can be applied in glycan immobilization[2, 33, 34], but for construction of glycan biosensors especially gold, various forms of carbon and silicon have been mainly used[9].

Gold surfaces are the most widely utilized for construction of glycan biosensors because gold is a reasonably inert material (in

comparison to silver) and thin gold films are compatible with numerous detection platforms.[35] The most exciting fact about gold is its ability to form self-assembled monolayers (SAMs) *via* spontaneous chemisorption of thiols and disulfides on gold surfaces.[36-38] Formation of SAMs is a very quick process[39] and thiol derivatives having a diverse range of functional groups at its ω-end can be effective applied to tune interfacial properties with ability to control subsequent coupling process at nanoscale. At the same time it is good to take into consideration that SAM with high quality can be prepared only after careful cleaning of gold surface before SAM formation.[40] A two-component SAM formed by incubation of gold surface with a mixture of two thiols in which one thiol bearing a functional reactive group for subsequent immobilization is diluted in the other thiol, is an efficient way to control interfacial density of functional groups.[41, 42] It is beneficial, when a diluting thiol has an additional role besides being a passive diluent i.e. to deliver functional groups resistive to non-specific interaction like oligoethyleneglycol[42] or betaine[41] moieties. The main limitation of SAMs is a narrow electrochemical potential window at which such layers are stable and slow oxidation, when exposed to oxygen and other physical and chemical conditions (i.e. UV exposure).[43]

Various surfaces can be also modified with gold nanoparticles (AuNPs)[44] possessing distinct beneficial properties for a wide range of applications.[45] Glycans can be immobilized on AuNP surface at higher density and with enhanced availability for binding compared to planar gold surfaces.[1] Such features make utilization of AuNPs promising for making glycan biosensors.

Silicon surfaces can be patterned by SAM through silanization[46], what was actually the first case of SAM formation described. Silanization involves hydrolysis of silanes with subsequent condensation reaction with available hydroxyl groups.[47] Since the only prerequisite for silane SAM formation on a surface is presence of available hydroxyl groups, surfaces such as glass, metal oxides, silicon oxide and graphene oxide can be patterned by silane SAM. Like in case of SAM on gold also in this case different functional groups can be delivered to the surface for subsequent immobilization process. Silane SAM patterning is subject to moisture sensitivity resulting in poor SAM coverage,[47] but the process can be optimized taking into account various variables of a coupling procedure.[48, 49]

Silicon structures can be prepared with dimensions at nanoscale and especially silicon nanowires are best suited for construction of various devices, mainly field-effect transistor (FET) based ones, offering a highly sensitive detection with mass production of such nanostructures possible through a well-developed semiconductor fabrication.[50-52]

Carbon surfaces are the first choice conductive surfaces for making biosensors due to good electrical and mechanical properties and their low cost.[53] Carbon surfaces can be quite easily patterned by electrochemical grafting of various chemicals to deposit functional groups of interest for subsequent modification.[53-55] The choice of carbon electrode depends on the application, when roughness, porosity, presence of oxygen functionalities and other aspects have to be carefully considered.[56] Unfortunately, SAMs having comparable parameters as thiolated SAMs on gold or silane SAMs on silane/oxide surfaces cannot be formed on carbon interfaces.

Besides "traditional" carbon forms such as glassy carbon, pyrolytic graphite, graphite, carbon black, etc., other novel forms of carbon are available including carbon nanotubes (CNTs)[57-59], graphene[59-62] or modified diamond[63, 64]. CNTs are rolled-up graphite (single or multi) sheets of carbon, possessing π-conjugative structure with a highly hydrophobic surface. CNTs can be functionalized *via* covalent or non-covalent modifications, but non-covalent one *via* π–π stacking or hydrophobic interactions should be preferred to preserve optical and electronic properties of CNTs.[65] Graphene is a two dimensional material made of single carbon layer with unique electronic, thermal, and physical properties.[60-62] Oxidized form of graphene called graphene oxide can be prepared form graphite in an affordable way with subsequent reduction to graphene using various ways.[66, 67] Oxygen moieties of graphene oxide can be applied for covalent attachment of glycans.[68] Summary of possible conjugation protocol for immobilization of glycans on CNTs and graphene was published by Chen *et al.*.[69]

Glycan immobilization

Even though a non-covalent immobilization of hydrophobized glycans on highly hydrophobic surfaces pioneered by Feizi has been successfully applied in constructing glycan arrays[70], in glycan biosensors covalent and bioaffinity coupling techniques have been applied. There are three main immobilization strategies in constructing a glycan biosensor. The first one involves a synthesis of glycans terminated in an alkanethiol (silane) group for direct formation of glycan SAMs on gold (silicon).[71] The drawback of this strategy is the generation of quite complex glycan structures that may also cause deformed SAM formation. It is very important to take into account that thiolated glycans in mixed SAMs have tendency to make clusters over time frame of few days and that glycan SAM as prepared can be dramatically different from the one after 28 days of storage.[44] The second strategy relies on immobilization of modified glycan on a surface patterned by various functional groups (i.e. NH_2-terminated glycan on COOH-terminated SAM *via* amine coupling chemistry).[71] The third one is based on immobilization of non-derivatized glycans onto surfaces having various reactive groups (i.e. hydrazide or amino-oxy).[72] Bioaffinity-based interaction mostly uses the interaction between surface confined streptavidin and biotinylated glycan.[4] DNA-directed immobilization is another bioaffinity-based coupling method offering quick *in-situ* parallel immobilization of even unstable biomolecules with high surface density and the surface can be regenerated and re-used.[73, 74]

Covalent coupling of glycan derivatives is a popular patterning protocol relying on conjugation chemistries developed for coupling of proteins/DNA. A short description of few coupling protocols applied in covalent immobilization of glycans is provided here.

The first one is amine coupling i.e. immobilization of amine modified glycans on surfaces having carboxy groups using carbodiimide (EDC), which activates carboxy group for subsequent reaction with amine (**Fig. 1a**). N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (NHSS) are frequently applied to increase coupling efficiency.[75]

The second coupling protocol is based on Cu^I catalyzed azide-alkyne cycloaddition, or simply 'click chemistry', between azide and alkyne group with formation of a 1,4-disubstituted 1,2,3-triazole product.[76, 77] The reaction can proceed at room temperature, in aqueous solutions in a wide range of pH (4-12) and with a large variety of copper catalysts.[78] All these aspects make this coupling protocol attractive for immobilization of different biomolecules.[79, 80] Salts of Cu^{II} are widely used because they are available with higher purity compared to Cu^I salts and Cu^I salts are generated from Cu^{II} by reduction with ascorbate or electrochemically (**Fig. 1b**).[81] In order to stabilize Cu^I salts during reaction numerous complexes are used to avoid formation of Cu^0.[77, 82] Click chemistry can be effectively applied in an on-demand immobilization process by a redox addressing of electrodes within an array.[83]

Diels–Alder cycloaddition is a chemoselective and a biocompatible glycan immobilization method.[84] The reaction proceeds between diene and double-bonded dienophile (i.e. benzoquinone[17]) in water. Niederwieser *et al.* have attached carbohydrate N-acylmannosamine with alkene terminal group (as dienophile) to 1,2,4,5-tetrazines *via* Diels–Alder reaction without any catalyst.[85] Thiolated glycans can be also coupled to maleimide or alkene terminated SAM surface in a simple and selective way.[86, 87] A novel coupling technique relies on utilization of divinyl sulfone chemistry for conjugation of NH_2- or SH- modified glycans at high pH.[88] Diels-Alder reaction can be used similarly to click chemistry applied in an on-demand immobilization process by a redox addressing of electrodes within an array.[83] Derivatization of glycans for subsequent covalent coupling of glycans to surfaces is not a trivial task since some chemical substitutions can be applied only to a narrow range of glycans and in some cases quite aggressive methods have to be applied affecting glycan structure and subsequent biorecognition process.

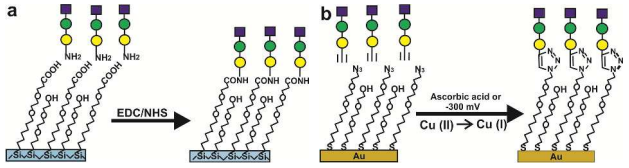

Fig.1: Immobilization of derivatized glycans onto a functionalized biosensor surface. (a) Glycan immobilization onto silanized surface *via* EDC/NHS (amine coupling) and (b) copper catalyzed azid-alkyne cycloaddition (click chemistry) onto thiolated SAM on a gold surface.

Covalent immobilization of natural glycans is usually done *via* hydrazide functionalized surface with a free reducing (aldehyde) end of glycan to form a stable Schiff base[89] and divinyl sulfone and cyanuric chloride coupling chemistry can be applied for coupling of natural, underivatized glycans, as well.[88, 90] The other methods allowing to immobilize natural glycans on surfaces having amino-oxy and other functional groups are possible.[72] Even though immobilization of natural, underivatized glycans can be quite convenient with cheap building blocks involved, such patterning process cannot be controlled on demand as in case of click chemistry or Diels-Alder reaction. The only exception is photo-coupling of natural glycans to perfluorophenyl azide-functionalized surfaces.[91]

Bioaffinity glycan coupling is usually done by immobilization of neutravidin[92] or streptavidin[93] on surfaces either covalently or *via* bioaffinity interaction on biotin modified surface[93]. In next step biotinylated glycans are bound to such patterned surfaces. DNA-modified glycans were also employed in a bioaffinity coupling.[94, 95]

Other glycan immobilization methods include photo-initiated radical reaction between thiols of thiolated glycans and alkene/alkyne modified surfaces[96], immobilization of glycans within DNA duplex scaffold during solid-phase oligonucleotide synthesis[97], click chemistry on an organic monolayer grafted directly to hydrogen-terminated silicon[47], electropolymerization of pyrrole-glycan derivatives[98] and glycan immobilization on conformationally constrained functional peptide monolayers[99].

Label-free platforms of analysis

Fluorescent detection needed for glycan arrays have some limitations discussed above. Moreover, the method does not allow to monitor interaction in real time and offers only a narrow concentration window and low sensitivity of analysis. Thus, alternative methods, working in a label-free mode of operation with high sensitivity and low limit of detection have been continuously integrated into glycan biosensors. The most commonly used label-free detection methods are cyclic voltammetry (CV), differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS), field-effect transistor (FET) sensing, surface plasmon resonance (SPR), quartz crystal microbalance (QCM), cantilever arrays and localized surface plasmon resonance. Only short description of detection methods is provided here and more information can be found in our previous review.[9]

Electrochemical impedance spectroscopy (EIS) can provide interfacial characteristics of a bioreceptive layer using a redox probe.[100, 101] The result of EIS analysis is presented in a Nyquist plot (**Fig. 2**), from which such characteristics can be obtained.[102] The method is sensitive to thickness and density of biomolecules attached to the surface, resulting in change of a resistivity of the biosensor surface.

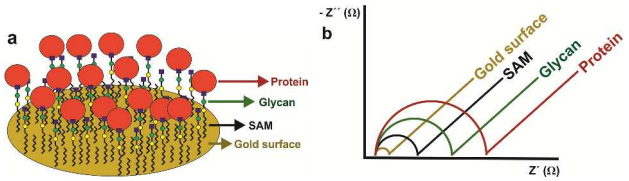

Fig. 2: Scheme of operation of EIS-based glycan biosensor, (a) the structure of an active biosensor surface, (b) a Nyquist plot applied for data analysis.

Cyclic voltammetry (CV) is another available electrochemical technique sensitive to thickness and density of biomolecules attached to the surface resulting in a change of current detected from redox species. CV measures the current by variation of a potential on a working electrode in presence of a redox probe at a defined scan rate[101] and in some cases more sensitive **differential pulse voltammetry** (DPV) technique is applied, as well.

Field-effect transistor (FET) based assay measures changes in a surface-charge density on a surface of a semiconductive channel.[103, 104] When a bio-recognition event takes place, the change of charge state in close proximity to the device surface is directly transduced into an electrical signal.[103] The construction of FET includes three electrodes: source, drain and gate (**Fig. 3**). Source and drain electrodes bridge the semiconductor channel, and gate electrode detects modulation of the channel conductance.[105]

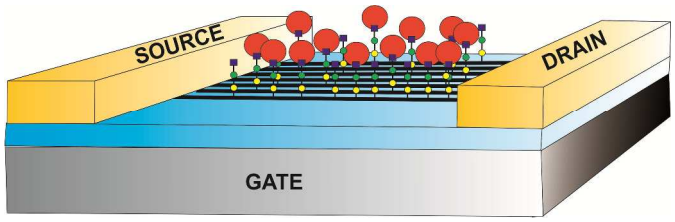

Fig. 3: CNT-based FET device for monitoring of glycan-protein interactions.

Quartz crystal microbalance (QCM) is a mechanical detection method measuring change in a mass on a surface through change in a frequency of an oscillating quartz crystal.[106, 107] There is a proportional dependence between decrease of a frequency of QCM device and increased amount of species attached to the surface with a limit of detection down to ng/cm^2.[108]

Surface plasmon resonance (SPR) is the most widespread optical detection method for following a binding event in real time providing kinetic/affinity constants of an interaction.[109, 110] The SPR detection has some limitations to detect cells/bacteria or very small molecules and the method offers a detection limit down to nM level.[109] **Localized surface plasmon resonance** (LSPR) is a special case of SPR occurring on the surface of metallic nanoparticles providing real-time assays with good reproducibility in a cost-effective way using simple instrumentation.[111] LSPR can be run on a single nanoparticle, what can be used for parallel analysis of up to 500 binding events.[112] **Surface plasmon resonance imaging** is a SPR technique allowing to measure hundreds of biorecognition events simultaneously.[113-115]

Microcantilever arrays detecting minute amount of biomolecules (10^{-18} g)[116-118] attached to a surface is adaptation of atomic force microscopy instrument[117] allowing an array format of analysis in real time (**Fig. 4**). The surface of one side of the cantilever is covered by a biorecognition element, while the other surface is passivated to resist any binding.[119]

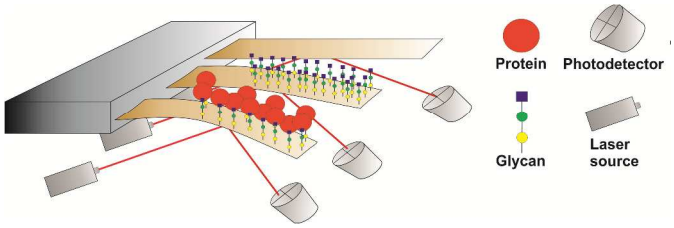

Fig. 4: Detection of glycan-protein interactions with microcantilever arrays.

Conductive nanochannels as a novel-label free detection technique was applied in Con A detection down to 10 nM.[120] Onto nanochannels (28 nm in a diameter) made in polymer membranes mannose glycan was covalently linked and upon incubation with Con A, change in nanochannel conductivity was measured.[120]

Application of glycan biosensors

Glycan biosensors have found wide applications in characterization of binding preferences of several lectins, for monitoring of enzyme activities or for detection of bacteria and cancerous cells.

Glycan-protein interactions

Lectins are the most often studies proteins on glycan biosensors. This is quite obvious, especially in the initial development of new strategies for glycan immobilization or novel transducing techniques.

Szunerits *et al.* applied a modified boron-doped diamond for glycan patterning *via* click chemistry of azido-terminated glycans.[63] EIS investigation showed that density of mannose terminated glycans within a mixed SAM was important for binding to Concanavalin A (Con A) lectin, which could be detected down to 5 nM.[63] Loaiza *et al.* applied AuNPs in preparation of glycan biosensors by immobilization of thiolated glycans (terminated in glucose, galactose or mannose) directly on the surface of AuNPs.[121] After glycan immobilization various thiols terminated in –OH, $-SO_3^-$ or $–NH_2$ group were used to block bare spots on the surface of AuNPs. EIS applied for analysis of binding, when optimal immobilization protocol was applied, offered a limit of detection of lectins down to 7 nM.[121] EIS mode of operation was applied for evaluation of Con A binding to a polyaniline modified biosensor surface containing glucose.[122] Such biosensor could detect Con A down to concentration of 0.12 nM.[122]

Pandey *et al.* focused on comparison of binding of lectins to glycans either immobilized on planar or nanoporous gold.[101, 123] Binding of Con A lectin to thiolated mannose attached either to planar gold or nanoporous gold showed differences in its binding investigated by EIS (i.e. Con A was bound with high affinity to a mixed SAM layer at lower glycan density on nanoporous gold compared to planar gold).[123] Moreover K_D for Con A towards mannose immobilized on planar gold surface was in the range 13-15 nM, while on nanoporous gold it was in the range 400-815 nM, depending on mannose density on the surface.[123] Similar results were obtained for binding of soybean agglutinin on globotriose glycan immobilized either on planar or nanoporous gold surface.[101]

CV and DPV techniques were applied to study interaction between two lectins and two glycans (glucose or galactose) immobilized on a gold surface.[124] In this case special hybrids of a glycan with a quinone moiety were prepared terminated in a thiol group for immobilization on gold electrodes with glycan exposed to the solution. Binding of a lectin (i.e. Con A to glucose or a peanut agglutinin - PNA to galactose modified biosensor) on such interfaces resulted in a decrease of current observed either with CV or DPV. Decrease of DPV signal with increased concentration of lectin was proportional with a limit of detection of 75 nM.[124]

Vedala *et al.* and Chen *et al.* introduced FET-based biosensing for analysis of interaction between immobilized glycans and lectins.[125, 126] In the first study glycans conjugated to porphyrin *via* click chemistry were immobilized on the surface of single-walled CNTs (SWCNTs) *via* π- π interactions with four glycan units *per* single porphyrin exposed to the solution.[125] Three different glycans (galactose, mannose and fucose) and three lectins were investigated in the study and lectins could be detected down to 2 nM with K_D values of interaction obtained in low μM range.[125] In the next study from the same group, besides a porphyrin-glycan conjugate also a pyrene-glycan conjugate was applied not only for patterning SWCNT surface, but also for glycan immobilization on graphene.[120] FET device based on SWCNTs offered larger response and better selectivity, when compared to the device based on graphene. Both devices could detect lectins down to low nM level and could provide K_D values of their interaction with immobilized glycans.[120] CNT-modified FET-device with glycan immobilized *via* dendrimer was also applied in detection of Con A down to 10 nM.[127] Silicon nanowire-based FET glycan device was prepared by immobilization of natural glycan on a surface patterned by amino-oxy functional group.[50] When a diluted buffer (0.01xPBS) was used in analysis, two lectins could be detected down to concentration of 1 fM.[50]

A quinone functionalized polythiophene thin film was employed for immobilization of thiolated mannose with subsequent study of interaction between immobilized mannose and various lectins.[128] From 5 lectins studied, only Con A showed a strong QCM and CV response with a limit of detection down to 0.5 nM.[128]

A sandwich-type SPR sensor based on graphene oxide (GO) covered by dextran was applied for analysis of Con A.[129] The glycan biosensor was able to detect ConA with a detection limit of 86 nM. When after binding of Con A to such layer dextran capped gold nanoparticles (Dex-AuNPs) were added to form a sandwich the glycan biosensor could detect ConA with a detection limit of 3 nM.[129] Maalouli *et al.* prepared SPR glycan biosensor *via* two coupling procedures – standard click chemistry and *via* photo-coupling of natural glycan with perfluorophenyl azide modified surface.[91] Two lectins were detected down to low nM range with

comparable sensitivity of detection on two different glycan surfaces.[91] Fais *et al.* applied imaging SPR with 40 different biotinylated glycans spotted onto neutravidin modified gold surface for interaction with RCA lectin, which could be detected down to nM level.[92] LSPR detection was applied for analysis of two lectins on a surface modified by AuNPs, two different types of dendrimers and two glycans.[130] When optimal conditions were applied, lectins could be detected down to 78 nM.[130] AuNP-modified surface after immobilization of thiolated glycans was applied in LSPR analysis of Con A down to 5 nM.[131]

Antibodies against various glycan antigens can be produced during development of some diseases including various types of cancer. Galban-Horcajo *et al.*, Gildersleeve *et al.* and Campbell *et al.* focused on investigation of a binding preference of various types of antibodies or detection of antibodies against disease antigens present in human samples by glycan arrays.[23, 132, 133] Glycan arrays were applied to understand interaction of antibody raised against glycan antigen present on the surface of a protein of *Bacillus antharicis* causing disease anthrax for future development of a vaccine or for diagnostic purposes[134].

LSPR-based glycan biosensor applicable to the health care sector has been described.[135] In this study, LSPR was applied to the study of antibodies raised against *Salmonella*.

Other proteins such as Alzheimer´s amyloid protein, antiviral protein cyanovirin-N, influenza hemagglutinins and toxins (ricin, Shiga toxin and cholera toxin) have been detected with glycan biosensors.

The first study describing interaction between immobilized glycan (sialic acid) and protein relied on electrochemical detection of such an interaction.[136] More specifically, sialic acid immobilized on the AuNP-modified glassy carbon electrode *via* click chemistry interacted with Alzheimer´s amyloid protein, which was electrochemically detected *via* intrinsic electrochemistry of a tyrosine residue present in the protein. Amyloid protein was detected down to μM level.[136]

A glycan biosensor based on cantilever microarray could detect an antiviral protein cyanovirin-N, which binds and blocks HIV virus.[137] The surface of cantilevers was functionalized *via* thiol-gold chemistry with trimannose, nonamannose and galactose (as an internal control). The binding of CV-N to nonamannose produced a 20% stronger deflection as the binding to trimannose and this protein could be detected down to concentration of 91 pM.[138]

SPR glycan biochips with various sialic acid terminated glycans immobilized on streptavidin modified SPR chip were used to detect influenza hemagglutinins (surface exposed proteins responsible for binding of viruses to host cells).[94] Alternatively biotinylated glycans were immobilized on a SPR chip covered by a single stranded DNA hybridized with a DNA-streptavidin adduct. Such DNA-mediated immobilization allowed effective regeneration of the surface after binding. Hemagglutinins were detected down to nM level, but what is more important affinity constants of interaction were provided, as well.[94]

Hemagglutinins from human influenza virus strains H1N1 and H5N1 could be detected down to unprecedented aM level with glycan FET–based biosensors with a dynamic range covering 10 orders of magnitude.[138] Natural glycans were immobilized on SiO_2 surface modified by amino-oxy functional groups and detection was carried out in a diluted buffer (0.01xPBS). A FET device could detect 60 H5N1 proteins or 6,000 H1N1 proteins corresponding to 1 virus displaying H5N1 proteins or 12 viruses displaying H1N1 proteins.[138]

Lipoic acid derivatized glycans were applied for modification of AuNP-modified surface of LSPR-based glycan biosensor for analysis of three toxins – ricin, Shiga toxin and cholera toxin.[139] Ricin was detected on 20 nm AuNP-modified surface down to 0.7 nM, Shiga toxin down to 0.2 nM on 40 nm AuNP-modified surface and cholera toxin down to 0.4 nM on a surface modified by 40 nm AuNPs.[139] Cholera toxin could be detected down to nM level with SPR-based glycan biosensor with immobilized pentasaccharide (GM1).[140] The surface of gold electrode coated with xyloglucan was incubated with ricin and then with antibodies against ricin conjugated to peroxidase.[141] Such an electrochemical method offered a limit of detection of 35 nM for ricin.[141]

Study of enzymes

Glycan modified surface can be applied for analysis of activities of glycan processing enzymes.[142-144]

Activity of an enzyme glucoamylase (releases of α-D-glucose units from the non-reducing ends) was studied on a surface modified by a glycan acarbose.[145] It was shown that at pH 7.0 the association and dissociation rate constants obtained from SPR for the glucoamylase–acarbose interaction are 10^4 M^{-1} s^{-1} and 10^{-3} s^{-1}.

O´Neill *et al.* studied formation of an insoluble glucan (glycan containing only glucose units) from maltotetraose immobilized using *Arabidopsis* phosphorylase AtPHS2 directly on the chip of a SPR biosensor in real time.[146] Interestingly the same enzyme can degrade glucan upon change of reaction conditions and hydrolytic activity of the same enzyme could be monitored, as well. Moreover, the glycan biosensor allowed to follow degradation of glucan by other enzymes such as β-amylase, isoamylase and porcine pancreatic α-amylase. The glycan SPR biosensor could clearly show difference in response to 1 mU/mL or 10 mU/mL of α-amylase.[146]

Bouchet-Spinelli *et al.* have prepared a glycan QCM biosensor for the detection and characterization of glycoside hydrolases.[108] Click chemistry was used for immobilization of maltoheptaose onto thiol modified gold surface. For detection endo enzyme (CGTm625 for oligosaccharide release) isolated from *Bacillus circulans* and an exo enzyme (E.C3.2.1.3 for glucose release) isolated from *Aspergillus niger* were used with a surprisingly low detection limit of 21 pM for the enzymes.[108]

Detection of bacteria, viruses and cancerous cells

Shen *et al.* described two ways for analysis of *E. coli* W1485 strain with glycan biosensors based on QCM detection.[147] The golden QCM chip was modified by thiolated mannose and when such chip was exposed to bacteria, such biosensor could detect *E. coli* cells down to concentration of 3.10^7 cells/mL. In an indirect approach, Con A was firstly deposited on mannose layer and since Con A has 4 binding sites, remaining 3 binding sites were available to bind bacteria. Such an approach allowed to detect bacteria down to concentration of 750 cells/mL.[147] In another study a thiolated glycan was also applied for construction of a glycan QCM-based biosensor.[148] The biosensor was applied in analysis of a lectin, but more importantly in detection of three influenza virus strains H5N1, H5N3 and H1N3 down to concentration of few pM.[148]

A glycan-based label-free biosensor for detection of *E. coli* has been prepared by Guo *et al.*.[149] Thiol terminated glycan α-mannoside was immobilized onto gold surface and binding of a cell line *E. coli*

ORN 178 to this glycan was monitored with EIS. *E. coli* ORN 178 could be detected with a detection limit of 100 cells/mL.[149]

Three *E. coli* strains were detected on golden cantilever array sensors modified by three distinct thiolated glycans.[150] Such detection platform could detect 8 bacterial cells attached to a single cantilever, offering a dynamic working range over 5 orders of magnitude. There were differences in binding of all three bacterial strains to the biosensor observed, as well.[150]

DPV analysis with glycosyl antraquinone derivatives attached to graphene modified surface *via* π-π interaction was applied for analysis of lectins and more importantly for detection of hepatoma cell line Hep-G2.[151] Galactosyl antraquinone modified glycan biosensor could detect cells down to concentration of 5,000 cells/mL.[151]

Challenges ahead

Biorecognition of glycans by its binding partners is to large extent influenced by multivalent binding.[152, 153] Thus, the influence of glycan density and the effect of a neighboring glycan on glycan-protein and glycan-cell interactions have to be taken into account, when designing novel, sensitive and robust glycan biosensors. Moreover, *in-situ* enzymatic synthesis of glycans directly on a biosensor surface has to be implemented to enhance variability of glycans immobilized for construction of biosensors.

Other factors, which have to be considered is a choice of a proper transducing platform for a particular application, controlled immobilization in an array format of analysis and wider utilization of nanomaterials including graphene. Here the most critical aspects, which have to be addressed for future preparation of sensitive, selective and robust glycan biosensors with a label-free mode of operation are provided and discussed.

Immobilization of glycans

Glycan density can affect glycan-protein interactions to high extent. Oyelaran *et al.* and Yu *et al.* focused on evaluation of a glycan density within glycan arrays on strength of binding with proteins. An affinity constant K_D of lectins recognizing glycan antigens can vary by 3 orders of magnitude depending on density of glycans on an array.[154, 155] Interestingly K_D of interaction between monoclonal antibodies and the same tumor antigen was not that dependent on a glycan density.[154] Antibodies present in human serum samples of some individuals bound both high and low density forms of a given glycan, while antibodies from other individuals were bound only to high density glycans.[154] Importantly the length of a diluting thiol for preparing mixed thiolated glycan surfaces have to be controlled for effective interaction with proteins.[156]

The effect of a neighboring glycan on glycan-protein interactions was studied quite intensively. Huang *et al.* developed a flexible protocol, how to study both the effect of neighboring glycans and their separation on binding of lectins.[95] For that purpose a library of glycans attached either to N- or C-end of PNA (peptide nucleic acid) fragments was prepared and by hybridization on a DNA chip they can control both distance and the nature of a neighboring glycan.[95] When lectin Con A was incubated with such array, big differences in the amount of Con A bound were observed.[95] The same group found that a binding preference of two proteins DC-SIGN and gp120 on glycan arrays having 37,485 glycan combinations changed with changed glycan composition.[157]

Scheibe *et al.* showed a large change of a K_D value for *Ricinus communis* agglutinin (RCA) with changed distance between glycans or composition of glycans (2.7–256 μM).[158] Sato *et al*. studied the influence of glycan density and the height difference between thiolated glycan and diluting thiol on the kinetics of adsorption/ desorption of Con A by SPR.[159] The study showed that the optimal height difference is 6 carbons of aliphatic chain (i.e. ≈0.7 nm^{42}) and 10% of thiolated glycan within mixed SAM allowed multivalent Con A binding.[159] In another study besides glycan density, also thickness and epitope glycan density within a glycopolymer brush showed significant influence on RCA binding investigated by SPR.[160] Mori *et al.* also showed a significant influence of glycan cluster density on kinetics of adsorption/desorption and on the amount of Shiga toxin bound to the glycan surface investigated by QCM.[161]

Liang *et al.* prepared glycan surfaces with heterogeneous glycans by spotting a mixture of two glycans (Gb5 glycan and 6 other glycans) at different ratio onto slides.[162] Such array was tested in binding of anti-Gb5 antibody revealing dependence of binding of the antibody on the density of a neighboring glycan. A more precise control of a ratio of mixed glycan was realized by attachment of glycans to dendrimers. When such arrays were incubated with a HIV neutralizing antibody (2G12), a small difference in K_D was observed (13.5-47.4 nM).[162]

In-situ glycan synthesis

Glycan patterning can also be done *via* enzymes directly *in situ* on the array surface when traditional ways of preparing glycans cannot be applied or proceed with low efficiency.[18, 144, 163] This is for example the case of sialic acid terminated glycans, which can be prepared by the action of sialyltransferases.[163, 164] Without any doubts engineered glycan processing enzymes with novel properties can help to prepare a wider range of glycans to be integrated into biosensor devices.[165] So far glycan biosensors have not been prepared by *in-situ* enzymatic synthesis.

Transducing platforms

Ultrasensitive analysis by glycan biosensors is possible mainly using EIS, FET and cantilever arrays, which can detect proteins down to fM-aM level or down to a single virus/cell particle. QCM and SPR are techniques, which can be successfully utilized for characterization/optimization studies. Moreover, microcantilever arrays can offer parallel format of analysis.

Highly parallel analysis similar to glycan arrays is possible only using SPR imaging method and maybe using LSPR with detection monitored on a single nanoparticle. Microcantilever arrays can provide some level of multiplexed analysis, but most likely analysis of hundreds of interactions simultaneously would not be feasible. EIS and FET could be run in a multiplexed format, but analysis of more than few tens of interactions at the same time would be problematic.

Addressed glycan immobilization within EIS/FET arrays as the most sensitive platforms of detection is possible by redox triggered immobilization on demand. In such case inactive functional groups are activated by a potential to make such groups highly reactive and ready for bioconjugation.[54, 83, 166-168]

Enhanced selectivity of detection by glycan biosensors in presence of other components in real samples has to be seriously considered by designing highly resistive surfaces.[169] We tried to solve this problem by application of betaine-[41] or oligoethyleneglycol-[42, 170] containing thiols resistive to non-specific binding.

Wider application of graphene and other nanomaterials is what is missed for preparation of more sensitive, selective and robust glycan biosensors. Graphene will be without any doubt more and more often applied for preparation of diverse range of biodevices since graphene can be prepared in many different ways with ability to tune its interfacial properties by a preparation protocol. Moreover, some studies already indicate that even sensitivity of SPR can be enhanced by application of graphene[171] and other nanomaterials[172].

Conclusions

From literature survey done in this review paper we can really confirm that glycan biosensors can be an alternative to glycan arrays especially, when sensitivity of analysis is an important issue. When highly parallel analysis of glycan-protein and glycan-cell interactions even at low sensitivity is important then glycan arrays can not be outperformed by glycan biosensors. Only SPR imaging and LSPR working in a special mode can detect simultaneously hundreds of interactions in a label-free mode in real time. Even though glycan biosensors provided very useful information about glycan-protein and glycan-cell interactions there is still a plenty of room for their improvement, especially for the most sensitive ones, which have been introduced only in recent years. It has to be emphasized that electrochemical techniques are very promising to achieve low limits of detection in label-free mode of operation, but a lot of work is needed to achieve at least some degree of multiplexed electrochemical analysis. Furthermore, electrochemical glycan biosensors should be available in a form of a compact device to laboratories worldwide to become alternative to widely available glycan array devices for special applications, when highly parallel analysis of glycan-proteins interactions is not crucial. It can be anticipated that application of both glycan arrays together with glycan biosensors will provide a synergetic effect for better understanding of glycan-protein and glycan-cell interactions in the future.

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