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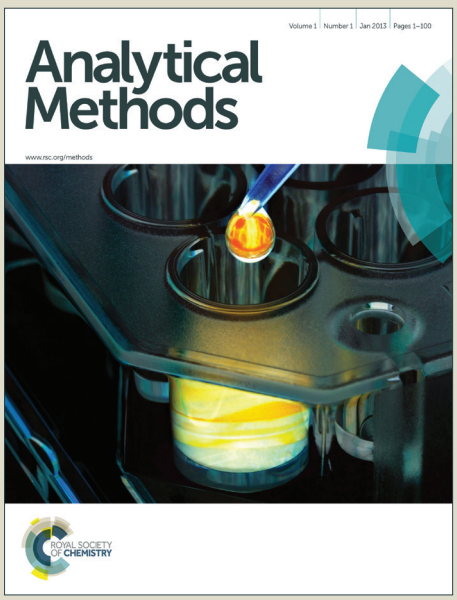

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Cancer Glycan Biomarkers and their Detection - Past, Present and Future

Sergei A. Svarovsky* and Lokesh Joshi‡

Glycoscience Group, National Centre for Biomedical Engineering Science, National University of Ireland Galway, Galway, Ireland

*Email: sergei.svarovsky@gmail.com; Tel: +1 858 750 9788;

‡Email: lokesh.joshi@nuigalway.ie, Tel: +35 391 495 768;

LIST OF ABBREVIATIONS

2D-DIGE – 2-Dimensional difference gel electrophoresis
AALP – Antibody Assisted Lectin Profiling
AFP – Alpha fetoprotein
ALA – Artificial Lectin Array
ASV – Anodic Stripping Voltammetry
ASF – Asialofetuin
CA – Cancer Antigen
CE – Capillary electrophoresis
CEA – Carcinoembryonic Antigen
CFG – Consortium for Functional Glycomics
CLIA – Clinical Laboratory Improvement Amendment
DPV – Differential Pulsed Votammetry
EFF – Evanescent Field Fluorescence
EIS – Electrochemical Impedance Spectroscopy
EIA – Enzyme Immuno Assay
ELISA – Enzyme Linked Immunosorbent Assays
ESI – Electron Spray Ionization
FAC – Frontal Affinity Chromatography
FACE – Fluorophore Assisted Carbohydrate Electrophoresis
FDA – Food and Drug Administration
FET – Field Effect Transistor
FT-ICR -- Fourier Transform Ion Cyclotron Resonance
GAG - Glycosylaminoglycan
GBP – Glycan Binding Protein
HCC – Hepatocellular carcinoma
HILIC – Hydrophilic Interaction Chromatography
HPAEC – High pH Anion Exchange Chromatography
HPLC – High Performance Liquid Chromatography
HTP – High Throughput
IVD – In vitro diagnostics
LFIA – Lateral Flow Immuno Assay
LOC – Lab on a Chip
LIF-CE -- Laser Induced Fluorescence Capillary Electrophoresis
MALDI – Matrix Assisted Laser Desorption Ionization
MS – Mass Spectroscopy
MSn – Tandem mass spectrometry
NHS – N-Hydroxysuccinimide
NIGMS – National Institute of General Medical Sciences
PAD – Pulsed Amperometric Detection
PGA – Printed Glycan Array
PLA – Printed Lectin Array
PSA – Prostate Specific Antigen
PTM – Post-translational modification
RIA – Radio Immuno Assay
QCM – Quartz Crystal Microbalance
SPR – Surface Plasmon Resonance
TF – Thomsen-Friedenreich antigen
Tn – T nouvelle antigen
TOF – Time of Flight

Abstract

Glycans are important in most biological processes, yet the exact insights into their functions remain enigmatic due to the lack of suitable tools for their analysis. Glycoproteins have recently emerged as valuable biomarkers for a wide range of diseases and some of them (e.g. AFP-L3) have already been approved by the US Food and Drug Administration for cancer diagnosis. Increasing attention is now being placed on the examination of glycan modifications because they can increase the predictive values of glycoprotein biomarkers. The main challenge to implementation of glycan biomarkers in clinical settings, however, remains the availability of suitable analytical methods for their identification and detection. The ability to rapidly detect glycan biomarkers that are indicators of pathology such as inflammation, Infection and cancer with high sensitivity and specificity using glycobiosensors will without doubt offer better tools for early-stage disease diagnosis. In this critical review we first provide a brief overview of the existing technologies for glycobiomarker identification and then discuss methods for their detection focusing on the advances in microarray technologies made in the past few years. Current challenges and perspectives on the emerging and future technologies that may help springboard this important field from academic domain to viable diagnostic tools are discussed.

Introduction

The biological significance of protein post-translational modifications (PTM) is well established. Among hundreds of other PTMs, including phosphorylation, sulfation, acetylation, ubiquitination, etc., glycosylation is the most abundant, frequent, and complex. More than half of serum and a vast majority of membrane proteins are believed to be glycosylated.[1] Biological events, such as onset of a disease, are often accompanied by changes not only in the protein expression, but by the rapid and dynamic changes in protein glycosylation patterns, thus making glycans qualitative biomarkers of health and disease. The glycosylation occurs not only on proteins, but is also found on lipids and forms a basis of extracellular matrix. The glycan composition of all glycoconjugates from a single biological source constitutes glycome and the science that studies glycome is termed glycomics. Progress in this area has been slower compared to genomics and proteomics primarily due to the structural complexity of oligosaccharides and the lack of tools to synthesize and analyze them effectively.

For these reasons the area of glycomics has lagged behind for the past few decades. It was not until the cusp of this century when the term glycomics barely grazed the surface of the World's scientific literature. **Figure 1** shows the number of publications since 2000 up to today that contain the words glycomics, glycoproteomics, proteomics, and genomics. The latter continues to boldly dominate the "omics" field with proteomics following in its footsteps. Both areas have matured and have seen modest but steady year-by-year increases. At the same time, the number of publications containing the words glycomics increased nearly 10-fold since the year 2005. Interestingly, the term glycoproteomics appears in the literature for the first time only in 2001, indicating an inception of interest in studying proteins in their entirety. These facts highlight the relative novelty of the field and the rapid increase of research interest in this area. Another interesting observation is the apparent effect of the global economic crisis at the beginning of 2008 particularly on glycomics.

>FIGURE 1<

From this brief analysis, it is evident that compared to genomics and proteomics, glycomics and glycoproteomics are still in their infancy. It is also certain that the undisputed importance of glycans in biological systems will eventually bring these nascent fields to fruition. Unless existing tools from other fields are integrated and unconventional and innovative technologies are developed to speed up the glycan analysis, the process will remain frustratingly slow. This critical review article is intended to provide the reader with a brief retrospective analysis of such approaches, list the currently established analytical methods, with a focus on the past five years, and outline possible future technologies that may help advance this promising field at a swifter rate.

Cancer Glycan Biomarkers – a Historical Perspective

For a long time carbohydrates were considered merely as metabolites or, in the best case, decorations that aid solubility of proteins and provide protection against degradation but confer no functionality. A paradigm shift started to occur only with the discovery of dramatic action of ricin (*Ricinus comunis*, a lectin extracted from castor beans) in agglutinating red blood cells during the first half of 20th century.[2] Now glycans are widely recognized as important antigens in the immune system.[3] The ABO blood group carbohydrates represent well-known earliest examples of such antigens.[2]

In the past four decades, it has become increasingly clear that in addition to immunogenicity, carbohydrates bestow other vital functions onto biomolecules to which they are attached. These functions range from controlling protein folding and clearance rates to mediating events like inflammation, metastasis, and infection to serving as biomarkers of diseases.[4] In fact, an established hallmark of tumorigenesis is the biosynthesis of aberrant glycan chains due to profound changes in metabolism, microenvironment and, as a result, in the expression of glycoprocessing enzymes.[5] These aberrations become more marked as the tumor acquires a more aggressive phenotype. In the two types of carbohydrate linkages to proteins, N- (to asparagine) and O- (to serine or threonine) types, N-linked glycans become larger and more branched while O-linked glycans are truncated and consequently expose underlying peptide epitopes on the covalently attached protein.[6] **Figure 2** shows classic example of the well characterized aberrant glycosylation patterns occurring in MUC1 (CA15-3) breast cancer biomarker during neoplastic transformation. Many cancer associated glycan biomarkers including TF-, Tn-antigens, Lewis antigen family, and their sialylated analogs which are present in vast majority of carcinomas have been described in detail elsewhere.[7] Most of these glycan structures have been identified through long and laborious process of individual observation and validation.

>FIGURE 2<

The field of functional glycomics has recently emerged to address the unmet need in more rapidly identifying and understanding functional relevance of glycans in biological systems.[8] The practical side of this effort is in finding reliable glycan biomarkers and translating them into clinical applications.[9] Despite novelty of glycomics as a field, there has been a long and fruitful

history in applying glycobiomarkers for diagnosis and prognosis. Lectin histochemistry, similar to conventional immunohistochemistry, has been used for decades as an established method to identify glycobiomarkers.[10] Both techniques have been widely used to localize specific glycoconjugates related to tumor progression and metastasis. Although such classical methods have proven their value in studying distribution and functions of glycan biomarkers, nowadays they have limited practical value for the point-of-care use needed for clinical applications let alone large-scale glycomics applications needed to catch up with the developments in genomics and proteomics. This review focuses on general technological aspects and challenges in identifying and using these novel biomarkers more efficiently.

Present

After almost two decades of intensive biomarker research using advanced genomics and proteomics technologies only a handful have been translated into patient care. Of the 1,261 proteins believed to be differentially expressed in human cancers only nine have been approved as tumor-associated antigens by the FDA.[11-13] Importantly, all of these cancer biomarkers are proteins that are glycosylated (some up to 50% by mass) and include mucins CA125 (ovarian cancer), CA27.29 or CA15-3 (breast cancer), and CA19-9 (pancreatic, gastric, colonic, and carcinoma), PSA (prostate specific antigen) and CEA (carcinoembryonic antigen), AFP (α-fetoprotein, implicated in liver cancer) and haptoglobin (multiple cancer types). It is widely agreed that these markers in their current implementation lack the sensitivity (positive prediction) and specificity (negative prediction) required for early detection and therefore are currently not recommended by the American Society of Clinical Oncologists for early detection.[14]

One of the reasons for low performance of these important biomarkers is that the available proteomics technologies have limited power to detect low abundance proteins against the background of high abundance plasma proteins with high accuracy.[12] Development of more sensitive and specific detection of these biomarkers in serum could be one solution for their improved clinical utility. Another solution could be to take advantage of altered glycosylation patterns.[15] While measuring protein levels in biological fluids is not a trivial task, glycan modifications change rapidly, predictably, and dramatically in response to a disease.[16] This makes glycan alterations more reliable qualitative biomarkers in terms of predictive value.[17] Remarkably, these features have not yet been fully explored in the design of commercial *in vitro* diagnostics (IVD) tests.

Table 1 summarizes some of the recent findings related to identification of other glycan biomarkers in various forms of cancer. The main theme appears to be the presence of aberrantly fucosylated and sialylated structures as well as increased branching in cancer associated proteins, an observation known for quite a long time now.[7, 15] The apparent bias of the glycan alterations in disease towards specific set of structures highlights not only an opportunity but also an obvious challenge. In general it is complicated to figure out origin of glycan biomarkers especially when investigating serum proteins. For example glycan biomarkers profiled for breast and ovarian cancers appeared the same for both diseases depicting same trends.[18] Biomarkers developed for commercial use and regulatory approval

must be required to present data supporting validity and clinical utility. In order to present scientifically strong evidence for a particular glycan biomarker, extensive glycobiological studies will be required before passing it into a potential diagnostic pipeline.[19]

In one remarkable if not exceptional example, aberrantly fucosylated liver originated alpha-fetoprotein (AFP-L3) was approved by FDA in 2006 as a biomarker for hepatocellular carcinoma (HCC). This biomarker test is now widely commercially available, and is based on a lectin-antibody sandwich assay using *Lens culinaris* agglutinin to assign the percentage of fucosylated glycoform. It must be noted that the increase in levels of AFP alone was not sufficient for early detection (sensitivity 41-65%).[18] In addition the serum concentration of this biomarker is of little use in the differential diagnosis of HCC versus benign liver disease. This can be considered as a technically modest yet conceptually significant achievement demonstrating definitive clinical utility of cancer glycobiomarkers.

>Table 1<

How Glycan Biomarkers are Discovered

Clinical application of any biomarker traditionally requires significant amount of preclinical validation studies using large sample sets. This requirement was the driving force behind the modern massively parallel genomics and proteomic technologies. It is therefore imperative that similar high-throughput quantification methods are established for glycan biomarkers as well. Unfortunately due to lack of such technologies today many contemporary glycoprofiling efforts are not thorough enough to provide unequivocal statistical evidence if a particular glycomic profile is cancer specific or not.

Today, identification of glycan biomarkers is conventionally achieved by the use of plant lectins in frontal affinity chromatography (FAC),[39] fluorophore-assisted carbohydrate electrophoresis (FACE),[40] laser induced fluorescence capillary electrophoresis (CE-LIF),[41] 2-dimensional difference gel electrophoresis (2D-DIGE),[37] high performance liquid chromatography (HPLC) and its variations such as hydrophilic interaction chromatography (HILIC), RP-HPLC, and UPLC, high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD),[42] all of which are essentially different forms of liquid chromatography (LC), various flavors of mass spectrometry (MS), and their combinations.[41]

Regardless of a combination used, most of these methods require pre-concentration followed by chemical or enzymatic release of glycans from protein core and labeling or other chemical manipulation prior to analysis. Although this process used to represent a serious bottleneck in the past, significant advances made in all aspects of pre-analytical purification and labeling procedures in the past few years make the process less laborious, more robust and even amenable to automation.[43]

Each of the listed methods has its advantages and limitations for glycan biomarker analysis (**Table 2**). Although the choice of an approach depends on the question asked and the depth of understanding required, a complete structural characterization invariably relies on tandem use

of several complementary techniques. A remarkably synergistic approach to discover glycan biomarkers of breast cancer has been recently described by Hancock's group.[44] The analytical throughput of LC methods was relatively low until recent introduction of a high throughput method where up to 96 samples can be analyzed by HPLC in a matter of hours. Owing to high reproducibility of the method, the assignment of glycan biomarkers can be done automatically using a database. For a more detailed account on this and other glycan biomarker discovery technologies we refer the reader to excellent reviews by Marino *et al.*[42] and Vanderschaege *et al.*[41]

Mass spectrometry is currently considered one of the most precise techniques capable of high throughput identification and profiling of glycan biomarkers.[45] Matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF) is often used to identify oligosaccharide structures as a whole. This method affords identification of possible combinations of component monosaccharides and, in itself cannot reveal either the actual isomeric structure or saccharide identities, but still produces useful differential glycomic profiles. Also MALDI is not suitable for simultaneous analysis of sialylated and neutral glycans. The application of MALDI-TOF can be exemplified by glycomic profiling of invasive vs. non-invasive breast cancer cells.[46] Statistically significant differences were found in the overall composition of invasive vs. non-invasive cancer cell lines. In another study comparative glycomic profiles of prostate specific antigen (PSA) in malignant and benign samples have been established.[47] In this case, the initial MALDI-TOF evaluation was complemented with more detailed structural characterization using advanced MS/MS fragmentation techniques that allowed more detailed information about glycan biomarkers to be obtained.

In the Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS instrument, the ions are generated (normally by MALDI) and passed through a series of pumping stages at increasingly high vacuum into an ion trap. Much like in FT-NMR the combination of m/z frequencies is decomposed into a frequency spectrum that in turn is converted into a mass spectrum. To date the FT-ICR MS is the most sensitive method of ion detection which has almost unlimited resolution.[48] This technique was applied for total serum O-glycomic pattern profiling for the discovery of ovarian cancer biomarkers. A unique profile containing 16 cancer-specific glycosignatures was obtained from patients with ovarian cancer.[49] The same approach was applied to discover potential O-glycan breast cancer biomarkers. A principal component analysis had successfully distinguished among breast cancer and normal samples.[14]

Electrospray Ionization (ESI) MS offers a distinctive advantage over MALDI MS in that this type of ionization is mild enough to leave glycan molecules unfragmented, which is favorable for the analysis of unstable glycan biomarkers containing for example ubiquitous sialylation and sulfation modifications. The disadvantage however is that the mass spectrum is more complex to interpret due to possibility of forming multiply charged ions. Also even slight contamination with non-volatile salts is problematic making interfacing with some LC methods difficult. As is the case with MALDI, different analyzers (e.g. TOF or FT-ICR) can be used with ease. Over the past few years ESI-MS was implemented in micro- and nano-chip formats, greatly improving its usefulness for parallel glycan screening.[41]

The most superior advantages of MS over any other method are its relative sensitivity and accuracy (resolution), but it has its own set of serious limitations (**Table 2**). First, pretreatments including liberation of glycans from proteins and lipids and subsequent chemical modification with appropriate reagents are still required, slowing down the identification process. This considerably hinders direct application of MS to clinical samples. Second, the equipment, while significantly more compact and robust than it is was a decade ago, is still prohibitively expensive and requires a high degree of skill in operation and data interpretation. These factors confine the use of MS to specialized centers and other resourceful research environments. Third, beyond the hurdles of sample preparation, the exact structural assignment based on obtained mass spectra constitutes another serious challenge. The process is complex, time-consuming, and, due to high degree of required expertise, also expensive.

Nevertheless, these drawbacks seem to be temporary as the efforts in automation and miniaturization of MS instrumentation, automated sample treatment,[50] and development of methods that do not require release of glycans from protein core are well under way. The past few years also have seen the emergence of many commercial programs and algorithms that assign glycan structures automatically.[51] All these improvements will likely bring MS closer to the clinical use for glycan biomarker identification and detection. It is not outlandish to expect that in a few years fully automated compact machines will appear that will robotically and in high throughput (HTP) fashion purify samples, release and modify glycans, analyze their structure, and map glycan biomarkers to disease conditions.

Future Outlook

In no other field the need for high-throughput methodologies is as apparent as it is in the field of glycomics. In the remaining sections we describe emerging HTP tools for functional glycomics and discuss next generation technologies that may help further advance glycomics.

Currently, an evolving theme for glycan biomarker identification seems to consist of comparative (or differential) analysis of two different samples from the same biological source, e.g. cancer serum sample versus normal serum control. This type of analysis is often referred to as glycoprofiling.[39] As shown in **Figure 3**, this strategy applies equally to LC/MS technologies described above and to any of the microarray technologies below.

>FIGURE 3<

Array-based technologies have rekindled an interest towards less head-on, more “holistic”, if you will, approach to the analysis of biomolecular interactions where instead of one-by-one identification of individual components in a biological system, the latter is analyzed as a whole in a pattern-based recognition process. Such consideration may be particularly rewarding for the analysis of glycan interactions. After all, the amount of information that glycans encode is enormous, their interactions are “fuzzy”, while their structures are difficult to analyze using conventional analytical techniques. Imagine a language that is 20 million times more complex than English (that is by how much theoretically glycan complexity exceeds amino acid complexity[52]). What would communication look like? Would a direct word by word interpretation in this language be practical or would it only be able to exist at subliminal level? Granted, no

holistic method is capable of structural characterization, it is however possible that none is required for glycan biomarker identification as long as the disease biomarker is clearly different from the healthy state and this distinction can be reliably and, most important, reproducibly detected.

Array Based Technologies

No other technology propelled the rapid advances of the "omics" sciences more than microarray technology did. DNA microarray, first introduced in the early 1990s, is the best example of the "omics" revolution and has until now been the platform of choice for massively parallel genomics analyses. The field is now moving towards next generation sequencing. In a similar fashion, polypeptide microarrays are now spearheading the field of proteomics.[53] Glycans are no exception and two array based strategies are now being actively pursued in the glycomics arena as well. In particular, the complementary glycan and lectin microarrays have become exceedingly popular in recent years as rapid glycan profiling tools for the parallel analysis of glycan binding proteins (GBP) and intact glycoconjugates, accordingly.[54]

Printed Glycan Arrays

This already mature technology involves attachment of hundreds of different oligosaccharides of known composition to a surface of a glass slide and is used to identify GBP binding patterns.[55] The chip based format enables screening of multiple binding events on a single slide and requires very small amounts of analyte.

Printed glycan array (PGA) was first conceptualized by Feizi's group in the 80's who had demonstrated that neoglycolipids could be adsorbed onto 96-well plates and used for interrogation of glycan binding proteins (GBP). It was then expanded into PVDF microarray format in 2002.[56] In 2004, Blixt *et al.* introduced a robust technology for covalent attachment of amino-modified glycans onto NHS-activated glass slides.[57] This technology platform has been advanced by the Consortium for Functional Glycomics (CFG) at the Scripps Research Institute in San Diego, a multi-institutional initiative funded by the National Institute of General Medical Sciences (NIGMS) with a purpose to understand role of carbohydrate-protein interactions at the cell surface and cell-cell communications. Multiple other strategies to construct the chip have been suggested since, but principally involve covalent conjugation of modified glycans onto a glass surface via various spacers.[58]

Although it is difficult to predict the actual size of glycome at present, the conservative estimate is that it would be in the hundreds of thousands of distinct structures.[59] Notwithstanding, the PGAs have been quite successful in identifying specific binding partners to various branched glycans, even though the current library consisting of only a few hundred of distinct oligosaccharides is far from comprehensive in covering the whole glycome. It has been argued that the broad sugar binding specificities of GBPs and the fact that only terminal sugar residues are primary determinants of binding, the smaller collections of representative glycans may be sufficient for meaningful evaluation of GBP specificities.[60, 61]

Despite above arguments, PGAs have been highly successful in a variety of practical applications ranging from identification of viral mutations[62] to evaluation of vaccine candidates[63] to the discovery of cancer autoantibody biomarkers.[64-67] The repertoire of methods for their preparation and the new applications of glycoarrays continue to evolve exponentially.[55] Specialized bioinformatics methods for PGA data analysis are being developed in parallel.[68]

Recently the glycoarray concept was taken a step further to include a library of glycopeptides that was used for screening for autoantibodies against breast cancer biomarker CA15-3.[69, 70] Joshi's group developed natural mucin arrays. The glyco-profiles of the whole mucins on the microarray were compared using a panel of lectins and an antibody.[71] This study introduced natural mucin microarrays as an effective tool for profiling mucin glyco-epitopes and highlighted their potential for the discovery of glycobiomarkers.

Printed Lectin Arrays

One of the latest additions in the armamentarium of glycobiologists is printed lectin array (PLA), which technically is a protein microarray composed of carbohydrate binding proteins, such as anti-glycan antibodies and lectins. Lectins are carbohydrate binding proteins of non-immune origin that are involved in diverse biological phenomena ranging from intracellular routing of glycoproteins to cell-cell adhesion and phagocytosis.

In this complementary approach to PGA, lectins or less commonly, anti-glycan antibodies are printed on a solid support in a high spatial density. Interrogation of these arrays with fluorescently-labeled samples creates a pattern of binding that depends on the carbohydrate structures, providing a method for the rapid characterization of glycans on glycoproteins, bacteria, or mammalian cells without a need to deglycosylate, which is unique among other glycoanalysis methods.

Introduced circa 2004, lectin microarray was quickly found to be a highly effective in analyzing complex glycans in both pure and crude forms without the need to release glycans moieties prior to the analysis.[72] The principal advantage of PLA is that multiple glycan-lectin interactions are detected simultaneously, thus opening up unprecedented opportunities for HTP glycoprofiling. Mahal's group first reported a ratiometric lectin microarray approach to accurately analyze differences between glycosylation that occurs in mammalian glycomes during differentiation.[73] In this study glycoconjugates from non-differentiated and differentiated cell types are labeled with different dyes, mixed and applied to a lectin microarray consisting of 58 features. Ratiometric analysis of colors in each spot resulted in distinct, quantifiable, and reproducible binding patterns. Tao *et al* reported a high-throughput PLA for identification of glycosignatures of mammalian cell surface glycans.[74] A comparative analysis of 24 normal mammalian cell lines using an array consisting of 94 plant lectins was performed and compared to glycosignatures of MCF7 breast cancer cells. The cancer cells showed distinctly different binding patterns, ear-tagging potential biomarkers of breast cancer.

In a recent example, diagnostic utility of lectin microarrays was demonstrated for determining glycoprotein profile of cells in pleural effusions of lung cancer patients. Fifty four samples each obtained from lung cancer patients and normal controls were subjected to screening on a 25

lectin array. It was found that SNA lectin binding had the highest sensitivity (92.6%), specificity (100%), and accuracy (96.3%) towards lung cancer samples. It was concluded that SNA may be used as a biomarker to distinguish reactive mesothelial cells from adenocarcinoma cells.[75]

Another study evaluated the glycoproteomic profile of tissues from colon cancer patients using PLA. Level of GlcNAc that *Solanum tuberosum* lectin (STL) bound was found to be elevated in colon cancer, which was verified through lectin histochemistry.[76] Subsequent enrichment of protein fraction with STL lectin and subjecting the sample to MS analysis revealed 72 proteins, of which 17 were exclusively found in cancer tissues.

The persistent problem with lectin microarrays is that lectin-carbohydrate interactions are inherently weak. Therefore the washing steps required for microarray probing must be carefully optimized in order preserve important interactions. For this reason, Hirabayashi's group was first to introduce evanescent field fluorescence (EFF) detection system for the microarray analysis.[77] The EFF detection is extensively used in biosensors for tracking real-time binding events on glass surfaces. Such detection does not require any washing because the evanescent field, which is created by light entering the glass parallel to the surface, extends to only about 200 nanometers from the slide surface and any labeled molecule above that distance is not detected. Unfortunately, currently EFF is not widely available or used because of the cost of instrumentation. The research community is focusing on improving protocols and procedures to make lectin arrays more compatible with conventional nucleic acid microarray scanners that are now available to most molecular biology researchers. All this argues in favor of future developments of cost-efficient wash-free label free detection systems suitable for studying glycan interactions in high throughput. PLAs have been recently reviewed in great depth by Hirabayashi's[78] and Mahal's groups.[79]

Antibody Assisted Lectin Profiling

This technique was pioneered by Haab's group in 2007 and employs spotted antibody arrays to sandwich glycoproteins of interest through their peptide core post-wash followed by profiling of glycan modifications with labeled lectin probes.[33] By profiling both protein and glycan variation in multiple samples using this sandwich assay, cancer-associated glycan alteration on the proteins MUC1 and CEA in the serum of pancreatic cancer patients were reliably determined. Later same group demonstrated, the detection of a glycan variant on MUC5AC from cystic lesions of pancreatic cancer patients using the lectin wheat-germ agglutinin discriminated mucin-producing cystic tumors from benign cystic lesions with a 78% sensitivity at 80% specificity, and when used in combination with cyst fluid CA19-9 gave a sensitivity of 87% at 86% specificity.[80]

In another example, serum samples of cancer, non-cancer and of pancreatitis patients were screened by using lectin/antibody arrays. The method showed excellent reproducibility and allowed discrimination of cancer and non-cancer controls with high specificity and sensitivity. In particular it was found that response of alpha-1-beta glycoprotein to SNA lectin (2-6 linked sialic acid) increased by 69% in cancer patients.[31]

These examples demonstrate the value of glycan variants for biomarker discovery and suggest that these biomarkers could greatly enhance the accuracy of differentiating tumors from normal states.

Hirabayashi's group demonstrated a similar strategy where a target glycoprotein is first enriched from clinical samples by immunoprecipitation with a specific antibody recognizing core polypeptide. The target glycoprotein is then quantified by immunoblotting using same antibody.[81] The glycosylation differences are then determined by antibody-overlay lectin microarray where the glycoprotein is first bound to the lectin array and then detected by sandwiching with fluorescently labeled protein-specific antibody. This strategy mitigates the need for meticulous purification of glycoproteins prior to analysis on lectin microarray.

Model glycoproteins having either N-linked or O-linked glycans included prostate-specific antigen or podoplanin, were subjected to systematic analysis. Specific signals corresponding to the target glycoprotein glycans were obtained at a sub-picomolar level with the aid of specific antibodies, whereby disease-specific or tissue-specific glycosylation changes could be observed in a rapid, reproducible, and high-throughput manner.[82] Statistical analysis of lectin signals made it possible to select an optimal lectin-antibody pair and facilitate construction of sandwich assay for glycobiomarker detection. This system is close to what is needed for clinical glycobiomarker detection and should provide a powerful pipeline in support of ongoing efforts in glycobiomarker discovery.[83]

Small Molecule Microarrays

In contrast to PGAs, which are limited only by the availability of constantly expanding glycan libraries, PLAs are inherently disadvantaged in that natural lectins are limited in numbers and specificities. Although the analytical range of lectins is diverse, only ca. 80 lectins are available from commercial sources. In addition, most of these lectins are from natural sources (plants and microbes), which introduces variability in their binding affinities dependent on purification, batch and vendor. Many lectins can also be cross-specific, binding multiple glycan structures, which complicates creation of mutually exclusive orthogonal sets of lectins needed for reliable binding pattern analyses.[84]

Arrayed anti-glycan antibodies, which are far more specific towards glycans, have also been used for the detection of PTMs but with limited success. The challenge here is obtaining antibodies against each known glycan, an impossible task considering that production of antibodies is costly, time-consuming, and most important involves animal sacrifice. Also, lectins evolutionarily evolved to transduce glycocode are far more suitable for PLA applications namely *because* they are less specific. A combinatorial use of a few lectins may be far more powerful than a large collection of highly specific antibodies.

Antibodies and lectins are not the only molecules that can bind carbohydrates. Single chain antibody fragments are a distinct alternative to antibodies and lectins for microarray applications.[85] Facile and cost-effective strategy to generate monoclonal lamprey antibodies, called lambodies, that target cancer glycan determinants was recently described.[86] Aptamers, short nucleotides that bind specific proteins and sugars as well as linear and cyclic peptides

have also been shown to bind carbohydrates.[87, 88] Natural antimicrobial peptides, such as defensins, have evolved to bind carbohydrate structures with high affinity and specificity.[89, 90] Fully synthetic lectins have also been described.[91, 92] The field of alternative glycan binders has recently been reviewed by Arnaud *et al.*[93]

One important advantage in using small molecule microarrays (SMM) is that in contrast to proteins, small molecule receptors are cheaper to produce, and offer increased control and stability in the array construction, while affinity issues are resolved by the multiple presentation of the ligands at the surface. Another advantage that becomes relevant especially in the context of glycan screening is that SMMs can be screened in non-aqueous media where the carbohydrate interactions are known to be significantly enhanced.[94] The use of well-defined binding affinity agents would facilitate uniform, robust and reproducible arraying chemistries and allow higher flexibility in screening the arrays. Furthermore, these molecules can be selected, synthesized and arrayed using established protocols, or as shown below synthesized/modified directly on the surface of microarrays.

In our laboratories we have already developed several new tools for large-scale glycomics based on interactions of specially designed luminescent glycoprobes,[95] labeled bacterial envelopes[96] or even whole labeled bacteria [97] with large libraries of random sequence 20-mer peptides arranged in microarray format (**Figure 4**). Our working SMM consists of 10,000 spotted 20 amino acid peptides that are commercially produced on a milligram scale. We chose 20-mers because the length of the peptides is long enough to fold into many patterns of shape and charge, yet short enough to enable cost-efficient chemical synthesis of the peptides. In this format, each peptide on the array serves as a putative ligand. This putative ligand format allows screening of essentially any glycan molecules that have no complementary lectins associated with them. Using this technology we have been able to identify robust bacterial glycosignatures by screening heterogeneous lipopolysaccharides derived from different gram-negative and gram-positive bacteria, which frequently contain extremely rare sugars such as rhamnose, polyfucose and others that have no complementary lectins to detect them. Although in its infancy, this type of technologies is promising to open up new opportunities for the comparative analysis of other complex heterogeneous glycan biomarkers (e.g. mucins and GAGs) that are unyielding to the conventional analytical methods.

>FIGURE 4<

The key to success here depends on the ability to reproducibly synthesize large libraries of putative ligands. Such a possibility was demonstrated in a recent article from Intel group who photolithographically manufactured a silicon microchip containing every possible overlapping peptide within a linear protein sequence covering the N-terminal tail of human histone H2B.[98] The chip was used for high resolution epitope mapping of commercial antibody probes, characterization of specific enzyme activities, and identification of autoantibody reactivity patterns. The advantages of using silicon substrate include (a) possibility of incorporating integrated circuits under each peptide spot for real time detection of binding; (b) absence of intrinsic background fluorescence, and most significant (c) near absence of non-specific binding

to the surface of silicon. As this technology matures, microchips containing millions of putative ligands suitable for carbohydrate analysis may soon become available.

>TABLE 2<

Glycobiosensors

The road from discovery to clinical diagnostics is long and winding.[99, 100] Following the discovery phase, a biomarker enters preclinical validation stage where only highly performant and regulatorily approved high-throughput methods are employed. Radio Immuno Assay (RIA) and Enzyme Immuno Assay (EIA) have been traditionally used for this purpose for decades. Nowadays, biomarker validation is also done from serum using other established multiplexed immunoassay technologies that can be divided into two types: planar and bead-based. The traditional sandwich ELISA, Meso Scale Discovery (MSD), and Quansys Biosciences Q-Plex arrays are some of the representative examples of planar assays that use various plate types, detection modalities, and degrees of multiplexing.

In bead-based assays, immunoreaction occurs not at the surface of a plate, but on micro-sized beads. Each bead contains a unique blend of dyes that acts as a signature of a bead and each bead type is associated with a single analyte. Multiplexing is achieved by combining different bead types into a master mix and incubating it with a sample. When the assay is read, the reader automatically assigns the bead type and the amount of antigen bound. Bead-based technologies include Luminex xMAP technology. Similar offerings are available from BioRad (Bioplex) and BD Biosciences (Cytometric Bead Array, CBA). AlphaScreen by Perkin Elmer is another noteworthy homogeneous bead assay based on proximal transfer of singlet oxygen from a donor bead to a chemiluminescent acceptor bead. The energy transfer happens only when two bead types are bound to each other. The assay essentially replicates a heterogeneous ELISA sandwich assay in liquid phase. The advantages are much bigger dynamic range and elimination of washing steps deleterious for weaker binders. These bead assays have just recently began being used with excellent results in glycobiomarker research.[53, 101-103]

Point of Care Testing

New trends in healthcare have resulted in the need for laboratory testing outside of a main laboratory.[104] While there are many well-established methods available to perform in vitro assays in a centralized laboratory, the challenge is to design of simpler analytical tools that can be used under point-of-care (POC) settings. Other synonyms for POC include bedside, decentralized, near-patient, portable, and peripheral testing. A POC device is a self-contained (not necessarily hand-held) integrated device that can be used for example in a doctor's office or a surgery room by a variety of individuals with minimum or even no training (for CLIA-waived devices). POC testing would be particularly useful in cancer screening where the life-saving potential of early detection at primary points of care is now well established.[105]

POC diagnostics is critically dependent on the combination of the analytical tools and the deep understanding of molecular biomarkers. While the field of portable diagnostics has made

significant strides in the past few years and is well-positioned for the detection of nucleic acid and proteins biomarkers, in glycobiomarker field the development of such biosensors is still in its nascence.

An integrated POC biosensor is composed of three basic elements: (a) a fluidic system for sample manipulation and transport; (b) a biological receptor of appropriate specificity to differentiate analyte from other substances in a sample; and (c) a transduction technology to convert molecular recognition event into a measurable signal (**Figure 5**). The fluidic systems range from simple nitrocellulose membranes used in lateral flow immunoassays (LFIA) to complex and fully integrated lab on a chip (LOC) gizmos consisting of micro-channels, valves, pumps, and mixers.

>FIGURE 5<

Biorecognition

In order to develop a useful biosensor, it is absolutely critical that the nature of the interaction between the biomarker and the biological receptor is well established and characterized on a molecular level. Case in point, the CA15-3 (MUC1) breast cancer biomarker approved by FDA almost 20 years ago is currently measured by immunoassays using antibodies raised against heterogeneous antigen preparations. Since CA15-3 is a heavily glycosylated protein, it would be important to understand which of these antibodies are carbohydrate-dependent, which are protein-dependent, and which are both.[106] There are over 56 different monoclonal antibodies on the market against CA15-3.[107] More than half of these antibodies are directed against protein core. Many of the remaining antibodies appear to be carbohydrate-dependent, but their fine specificities have not yet been firmly established, which could be a reason why this well researched biomarker still lacks specificity and sensitivity to be used in early detection.[14] Furthermore, many other glycoprotein biomarkers including CEA, AFP, PSA, CA125 and others are measured by RIA or EIA immunoassays with monoclonal antibodies that are not carbohydrate-dependent.[107] This fact highlights tremendous opportunities to improve upon existing cancer glycobiomarkers using better recognition molecules.

In the case of glycobiosensors, the biomarker can either be a glycoconjugate or a glycan binding protein. If biomarker is a glycoconjugate, one of the biological receptor necessarily used in today's assays is a lectin acting upon glycan in the glycoconjugate. Notwithstanding the successful use of a lectin in AFP-L3 immunoassay, it must be noted however, that the use of natural lectins in clinical glycodiagnostics on a broader scale may be limited due to low affinities and overlapping specificities. Although a combinatorial use of several lectins may be able to alleviate the problem of non-specificity,[108] use of application tailored recombinant lectins[109] or antibodies or antibody fragments raised against synthetic peptide-glycan epitopes appear to be more promising in the near term.[85,110] In the future it is likely that the ultimate success of glycobiosensors will come from a brand new set of carbohydrate binding reagents that are being

developed. Once the correct set of biorecognition reagents is obtained, the choice of transduction technology and assay development process should be straightforward and no different than that of a traditional immunoassay.

Signal Transduction

The physical signal of the transducer element can be optical, thermal, acoustic, electrochemical or mechanical. The transduction technology should ideally be label free and easy to integrate into a compact self-contained POC system. Optical biosensors are the most widely used in bio-sensing platforms because of their relative ease of use, high sensitivity, and the high information content of the data generated. The vast majority of signal transduction methods described up to this point were optical. Electrochemical detection holds particularly high promise in the POC arena due to high sensitivity, simplicity of instrumentation, and amenability to miniaturization and multiplexing.[111] Electrochemical methods in turn can be subdivided into labeled and label free. The labeled methods generally include Anodic Stripping Voltammetry (ASV), Differential Pulse Voltammetry (DPV), Cyclic Voltammetry (CV), or Square Wave Voltammetry (SWV). Similar to labeled optical methods, all these methods require introduction of an electroactive label (ferrocenyl group, metal nanoparticle, enzyme, etc) to the detector molecule (i.e. lectin) followed by electrical stimulation and detection of response from the label.[112]

Among label-free methods, Electrochemical Impedance Spectroscopy (EIS) in particular is gaining in popularity since its introduction for glycan sensing by our group in 2007.[113] EIS measures the change in the impedance of an electrical circuit due to the binding of analyte to an affinity functionalized electrode. In our labs, EIS has been used for label free detection of lectin-glycan interactions between neo-glycoconjugates and glycoproteins. A chip based biosensor was designed with a three-electrode surface pattern. Lectins deposited onto the gold surface were used to trap carbohydrate ligands covalently attached to gold nanoparticles. An alternating current was passed through a redox probe solution and the difference in impedance between electrodes with and without bound ligands was monitored. Through impedimetric measurement, lectins SNA-I and PNA were demonstrated to selectively bind to TF-antigen coated gold nanoparticles as well as to sialyl and asialo forms of bovine fetuin (**Figure 6**). The specificity of different forms of SNA lectin could also be easily distinguished. The combination of this analysis technique and selectivity of carbohydrate-binding molecules presents one feasible way to miniaturize and modernize rapid identification of glycoconjugates.

>FIGURE 6<

Very recently an ultrasensitive impedimetric glycobiosensor was reported for the glycoprofiling of human serum. The lectin biosensors prepared by immobilization of three different lectins on the gold electrode surface provided high sensitivity of detection of glycoproteins with a detection limit down to the low fM level with a wide linear range. The study suggests lectin biosensors outperform lectin microarrays in terms of sensitivity and utilizable working concentration range with a great potential of the lectin biosensors for searching for new disease biomarkers, which can be present in biological samples at extremely low concentrations.[114] Same group reported sensitivity of glycoprotein detection with immobilized lectins down to attomolar level when the surface of biosensor was further patterned with 20nm gold nanoparticles prior to lectin

immobilization.[115] It is interesting to note that employment of metal nanoparticles as both part of a glycoconjugate to be detected or as a part of biosensor surface led to significant sensitivity enhancements.

Nanoparticles composed of different metals have been used in conjunction with Anodic Stripping Voltammetry (ASV) to produce multiplex sensors for oligosaccharides (**Figure 7**).[116] Lectins were covalently coupled to functionalized gold surfaces. Afterwards, carbohydrate recognition domains of the lectins were occupied by glyconanoparticles during a preparatory incubation step prior to competitive release of the nanoparticle glyconjugates during incubation with a test glycoconjugate sample. Remaining glyconanoparticles on the electrode were quantified by stripping voltammetry in a three-electrode setup. The authors were able to show a discernable current reduction corresponding to the increased displacement of nanoparticle-labeled sugars by the preferential ligand for PNA with a detectable lower limit down to low micromolar range.

>FIGURE 7<

Nagaraj et al have developed a Nanomonitor miniature electronic biosensor for glycan biomarker detection. In this case, glycoprofiling of model protein and of extracts from human pancreatic cancer were analyzed in multiplexed format. The biosensor device consisted of a silicon chip with an array of gold electrodes forming multiple sensor sites working on EIS principle. Lectins were covalently attached to the surface of the electrodes. When specific glycans from a test sample bound to lectins at the base of each nano-well, a perturbation in electrical double-layer resulted in a change in impedance. Based on analytical figures of merit, the Nanomonitor reportedly has excellent potential for development as a point-of-care handheld electronic biosensor.[117]

A combination of electrochemical excitation with optical detection (electroluminescence) also deserves significant attention in biosensor research. In this case, decoupling of excitation and detection methods leads to vastly improved signal to noise ratios. In a study by Han et al, surface of a biosensor was modified with CdS nanoparticles that upon electrical stimulation emit luminescent light. The particles were functionalized with glycans and then probed with corresponding lectin. As the electrical resistance of the interface increases when lectin is bound, the current and amount of emitted light decreases, thus allowing quantitation of the binding event.[118]

Another label-free electrochemical technique involves recent use of glycan functionalized Field Effect Transistor (FET) for the detection of influenza hemagglutinin with impressive 50 attomolar level while the conventional method using antibodies only allowed picomolar level detection.[119] This label free method is based on the perturbation of electric field at the surface of the transistor's gate modified with affinity molecules. The gate acts as a switch for the current flowing from the source to the drain of the transistor. This technology has a potential to be integrated into Intel biochip technology described above to produce massively parallel biosensor arrays that, similar to SPR, can measure binding events in real time.

Mechanical biosensors directly detect the change in mass on the sensor surface due to the binding of biomolecules, viruses, or cells. Quartz Crystal Microbalance (QCM) and microcantilevers are examples of mechanical biosensors. Gruber *et al.* describe a cantilever sensor array with a self-assembled oligomannoside sensing layer to detect interaction with cyanovirin-N, which binds and blocks the HIV virus. This study demonstrated that carbohydrate-based cantilever biosensors are a robust, label-free, and scalable means to analyze carbohydrate-protein interactions and to detect glycan binding proteins at picomolar levels.[120]

Lastly, Pussak *et al.* recently described an exotic example of a force-based detection technique that can be easily adapted to construct carbohydrate biosensors for high-throughput applications. This new technique takes advantage of deformability of soft colloidal particles (SCP) made from polyethylene glycol. When SCPs conjugated with affinity molecules bind to a flat surface functionalized with complementary binding partners, the particles "splatter" forming a distinct contact area with the surface. The binding event is detected by reflection interference contrast microscopy (RICM) to determine the surface energy. In analogy to affinity-based biosensors, the SCP technique allows for direct binding assays as well as inhibition/competition assays.[121]

Conclusions

All cells carry a dense glycocalyx and most secreted proteins are glycosylated. This universal presence underlines the critical roles played by glycans (and their cognate glycan binding proteins, lectins) in biological process including normal physiological and morphological developments as well as disease related pathological processes, including cancer. Thus, detailed knowledge of glycosylation is of growing interest in post-genomic science, clinical research, and biopharmaceutical and diagnostic industries.

Most of today's cancer biomarkers are proteins that are glycosylated. However, it is widely agreed that these markers currently lack the sensitivity and most importantly specificity for early detection and therefore are currently not recommended for early detection. Their use is limited to monitoring response to cancer treatments. Development of sensitive and specific detection of these biomarkers in serum would be a significant step forward to their improved clinical utility.

The biosensor technologies suitable to detect glycan modification in clinical settings are still in their infancy and require further development. From the discussion above, it is clear that by far there is no shortage of technologies for biomarker detection, on the contrary, there are too many. So what are the reasons behind the dismal record of bringing these biomarkers to the market? We believe that at least in the case of glycodiagnostics the first obstacle is the limited access to reliable, specific, and abundant glycan binding receptors (glycoreceptors). Just like monoclonal antibody technology ushered in the era of immunoassays, so the development of new and improved glycoreceptors will be able to revolutionize the glycobiosensor field. Second, to bring together all three components of a biosensor requires an integrated, multidisciplinary team of biologists, chemists, physicists, engineers, and computer experts. This blend of skills is not found in every organization, so a successful biosensor development will result from inter-

institutional collaborations or from industry. Third, the glycobiomarker biology needs more clinical validation before it catches attention of major industry players. The process of validation has been frustratingly slow due to lack of suitable HTP research tools that are still being developed. Finally, in the past few years it has become increasingly clear that no single biomarker can be reliably used for cancer diagnosis. Further improvements using more specific glycan binding receptors and their integration into multiplexed assays with computer-assisted pattern analysis comprise some of the pressing needs in glycobiosensor research.

On a final note, there seems to be a growing consensus that no single biomarker will be sufficient for accurate diagnosis of cancer. For example a panel combining four known biomarkers leptin, prolactin, osteoponin, and insulin-like growth factor II, none of which used separately could distinguish patients from the controls, achieved a sensitivity and specificity of 95% for the diagnosis of ovarian cancer.[12] Therefore it seems inevitable that in the near future coalescence of all three “omics” technologies will lead to integration of protein, gene, and glycan biomarkers into multiplexed platforms. Such integration may someday finally yield a viable cancer diagnostic. Since there are currently no shortage of sensitive transduction and nanotechnology platforms, the success of future glycodiagnostics will ultimately rest upon the availability of new and improved glycan binding probes.

Acknowledgements

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Table 1: Cancer glycan biomarkers published in the period 2008-2013 (second column shows name of a protein or protein ensemble, the third column shows associated glycan alterations).

Cancer type	Protein biomarkers	Glycan biomarkers	Ref
Breast	MUC1 (aka CA15-3)	Expression of truncated Tn (O-linked N-acetylgalactosamine (GalNAc) epitope), sialyl-Tn (αNeuAc-2,6-αGalNAc) or TF (βGal-2,3-αGalNAc) antigens	20, 21
	Total serum glycome	Increased sialylation, higher levels of sialyl Lewis^X, significant changes in fucosylation, increase in agalactosylated biantennary glycans	
Colorectal	Complement C3, histidine rich glycoprotein, kinogen-1	Elevated sialylation and fucosylation	22
	Beta-haptoglobin	Increased fucosylation	23
	CEA and CA 19-9	High mannan structures (Hex5–9HexNAc2) and complex type glycans (NeuAc0–4Fuc0–2Hex3–7HexNAc4–7)	24
Liver	Alpha-1-antitrypsin, Alpha-fetoprotein (AFP-L3)	Core (alpha-1,6) fucosylation	25
	C3, CE, HRG, CD14 and HGF	Core fucosylation, elevated fucosylation	26
Lung	Beta-haptoglobin	Expression of sialyl Lewis^X, monoantennary glycans, increased sialylation	27
	Total serum glycome	Increase in sialyl Lewis^X, significant decrease biantennary core-fucosylated glycans	27

Ovarian	CA125	Similar to CA15-3 in breast cancer	[28]
	Acute phase proteins (haptoglobin, alpha1-acid glycoprotein, alpha1-antichymotrypsin)	Elevated sialyl Lewis^X	[28]
	IgGs	Increased expression of sialyl Lewis^X and increased core fucosylation	[29]
	Whole serum glycome	Reduced galactosylation and sialylation	[29]
Pancreatic	MUC1, MUC5AC, MUC16	Increased sialyl Lewis^A	[30]
	Alpha 1-beta glycoprotein	Increased sialylation	[31]
	Amyloid	Increased sialylation	[32]
	Beta-haptoglobin	Increased fucosylation	[33]
Prostate	Beta-haptoglobin	Increased fucosylation and sialylation	[34]
	Beta-haptoglobin	Monosialyl tri-antennary structures	[23]
	PSA	Increase in alpha-2-3 sialic acid and decrease in core fucosylation	[23]
	PSA	Alpha-1,2-linked fucose and beta-N-acetylgalactosaminylation	[35]
	Serum glycoproteins	Changes in high-mannose and fucosylated biantennary complex N-linked glycans	[36]
Stomach	Total serum glycome	Increased sialyl Lewis^X	[37]
	IgGs	Increase in core-fucosylated agalactosyl biantennary glycans	[38]
	Haptoglobin , transferrin, alpha1-acid glycoprotein	Increased sialyaltion	[38]

Table 2: Comparison of different analytical methods used in the discovery of glycan biomarkers.

Method	Basis of Analysis	Benefits	Drawbacks
Liquid Chromatography	Separation by charge and/or by hydrophobic/hydrophilic properties against various stationary phases	Separation of structural isomers allows precise structural characterization Amenable to automation and coupling to MS methods	Low to moderate throughput Complex sample manipulation – pre-concentration, glycan release, labeling, label removal
HPLC **HILIC** **UPLC**	Separation is achieved by differences in hydrophobic/hydrophilic properties	Simple to implement in any laboratory Sensitive and reliable quantitation Neutral and charged glycans analyzed simultaneously Reproducible retention times allow automated structure assignments Broad dynamic range Facile coupling with MS methods	Relatively slow (average run 2hrs) Requires time consuming sample pre-treatment and labeling
HPAEC-PAD	Glycans released from glycoconjugates are ionized in high pH buffer, separated by charge	Label free	High salt content hinders coupling with MS methods Unstable

			baselines Limited sensitivity
CE-LIF	Separation of released glycans by charge in high electrical field gradient	Superior resolution Ultrahigh sensitivity (fM range) Potentially high throughput Very small sample volume	Variable retention times Involves sample labeling Coupling with MS is not straightforward
Mass Spectroscopy	Glycans are released, pre-treated and ionized either by MALDI or ESI, then separated by mass/charge ratio using quadrupoles, TOF, or FT-ICR methods	Fast and efficient Label free High throughput High resolution High sensitivity Small sample volume Provides quick estimate of chemical diversity compositional glycan analysis Can be coupled to LC	Resource intensive Glycans need to be released, and derivatized Differential ionization efficiency makes quantitation difficult Susceptible to salts Identification of isomers not possible Analysis of heterogeneous mixtures is challenging
MALDI	Ions created by laser desorption ionization	Simple to implement on a chip High throughput	Differential ionization makes quantitation difficult task Harsh, glycans

		More tolerant to contamination than ESI	need to be chemically pre-treated Sialylated glycans cannot be directly analyzed
ESI	Ions created in droplets charged by high voltage	Mild ionization leaves labile glycoside linkages intact Easy interface with LC methods High throughput possible	Multiply charged ions complicate analysis Very sensitive to salts, other interferences Lower throughput than MALDI
Microarrays	Two dimensional addressable matrix of distinct binding molecules for multiplex detection of binding partners from a single sample	High throughput Multiplex format Can screen crude samples such as serum Enables parallel screening of complex glycoconjugates including whole cells and tissues	Full structural assignments not possible Need for fluorescent labeling Relatively high sample volumes, with some exceptions (see text)
Glycan Arrays	Glycans or glycoconjugates covalently or non-covalently attached to a surface of a chip. The chip is probed with a solution of labeled glycan binding molecules	Simultaneous high throughput analysis of GBPs' binding patterns in complex biological fluids	Not enough distinct glycans available Weak glycan-GBP interactions may be missed Ambiguities in glycan presentation at the surface

Lectin Arrays	Glycan binding proteins are attached to a surface of a chip which is then probed with labeled glycoconjugates (glycoproteins, glycolipids, bacteria, cells). The binding pattern is analyzed and information about possible structural content is deduced.	Does not require glycan removal from a glycoconjugate prior to analysis. Detects even subtle structural differences Ratiometric methods enable robust differential analysis of complex glycoconjugates. Provides level of information not available by any other methods	Not enough well-characterized lectins available, most of plant origin Relatively low sensitivity Complete structural assignment not possible Reproducibility issues Weak glycan interactants may be lost during washes. Issue addressed by EFF arrays
Small Molecule Arrays	Small molecules (peptides, peptoids, and aptamers) are arraigned either by printing or by direct synthesis at the surface of a microchip. The chip is probed with a sample of labeled glycans, glycoconjugates, glycan binding proteins, antibodies, etc. Binding patterns are analyzed and used as a biomarker. Individual binding moieties can be further processed into artificial binding agents (glycan binders or glycan mimics).	Simple arraying chemistry Dual purpose screening (e.g. glycans and GBPs can be screened on the same array) Unlimited pool of potential binding ligands Differential profiling of unknown samples Provides new binding moieties	Need for purified samples Extra care required for statistical data interpretation Requires larger sample pools for reliable profiling Off-array activity may be difficult to reproduce in solution or on a different surface.

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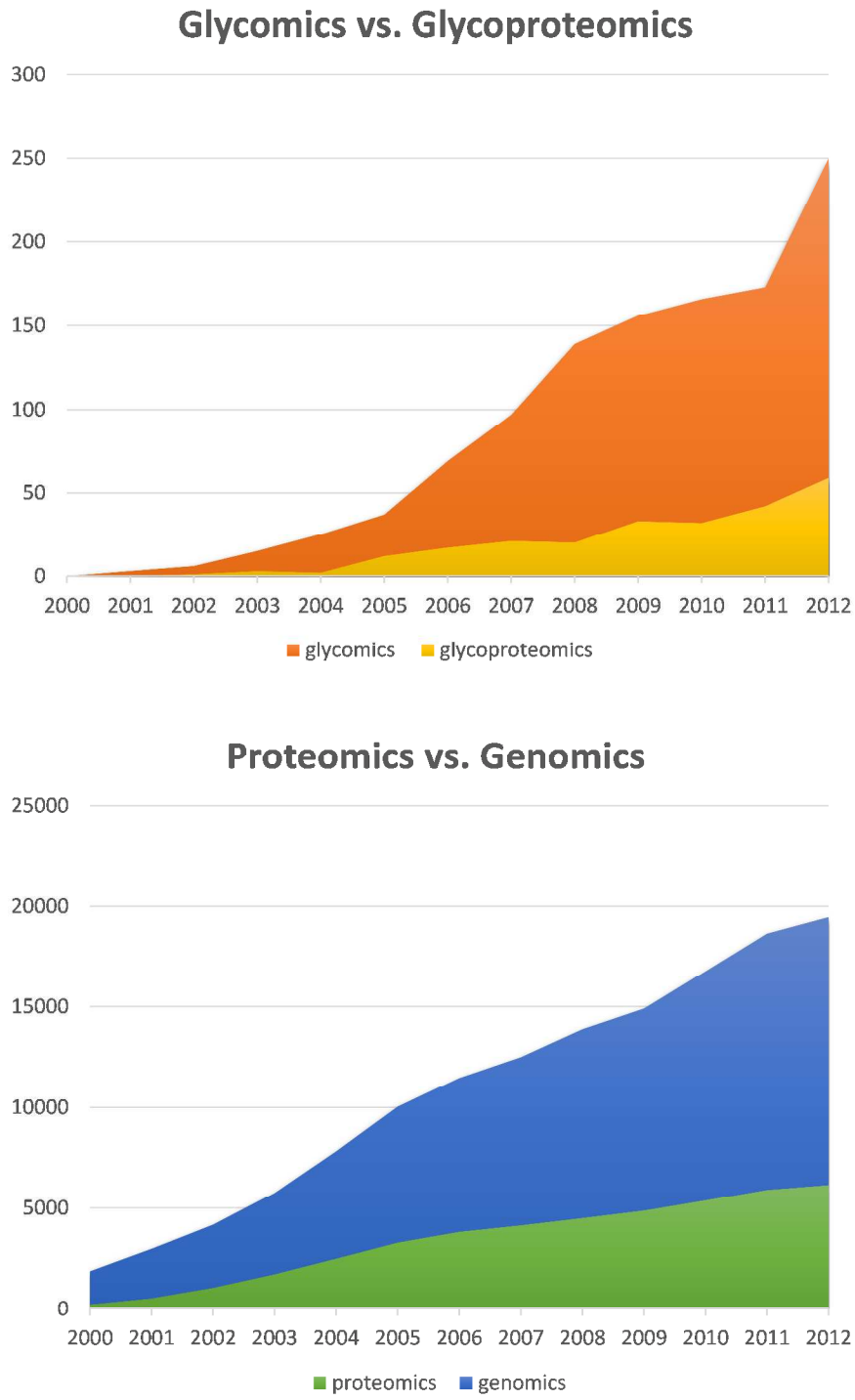

Figure 1. An area graph showing number of publications over 12-year period that contained words genomics, proteomics, glycomics, and glycoproteomics obtained by search in PubMed.

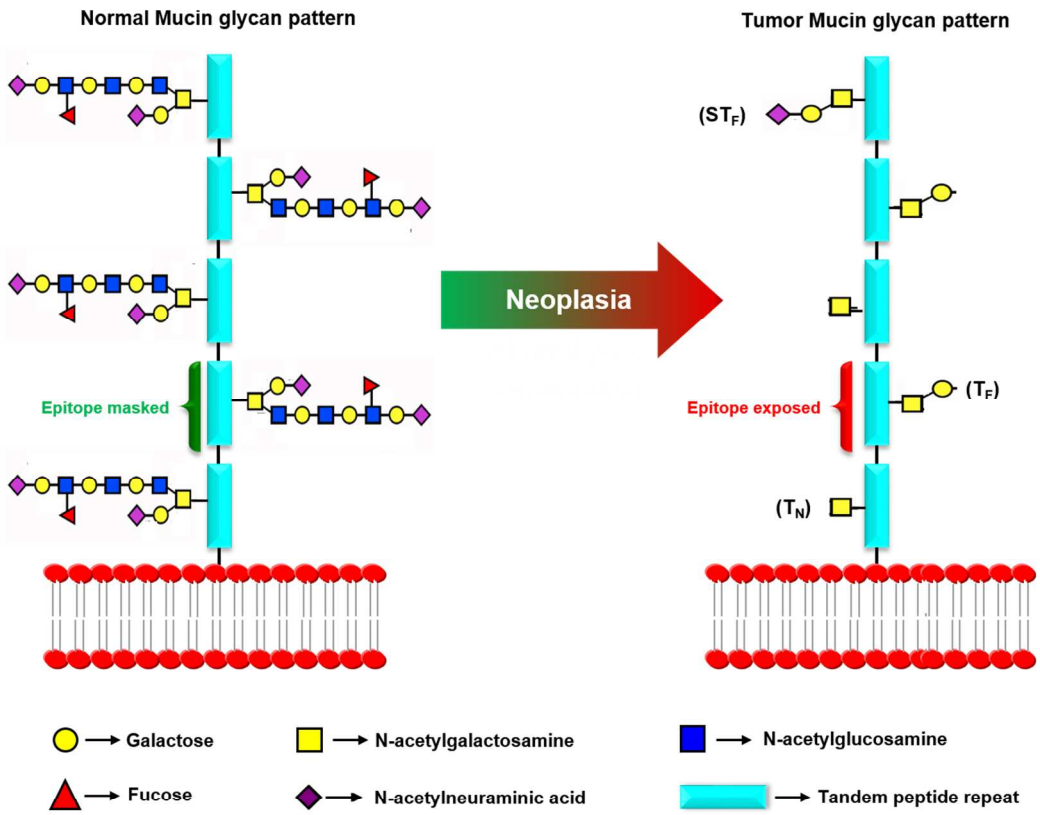

Figure 2. Mucins are comprised of several 16-20 amino acid peptide repeats (blue rectangles) each with clusters of O-glycosylated Ser and Thr residues. During neoplastic transformation O-linked glycans are under-processed due to changes in activity of glycoprocessing enzymes into tumor associated glycan biomarkers such as Tn and TF. (Courtesy of Dr. Joseph J. Barchi, Laboratory of Chemical Biology, National Cancer Institute).

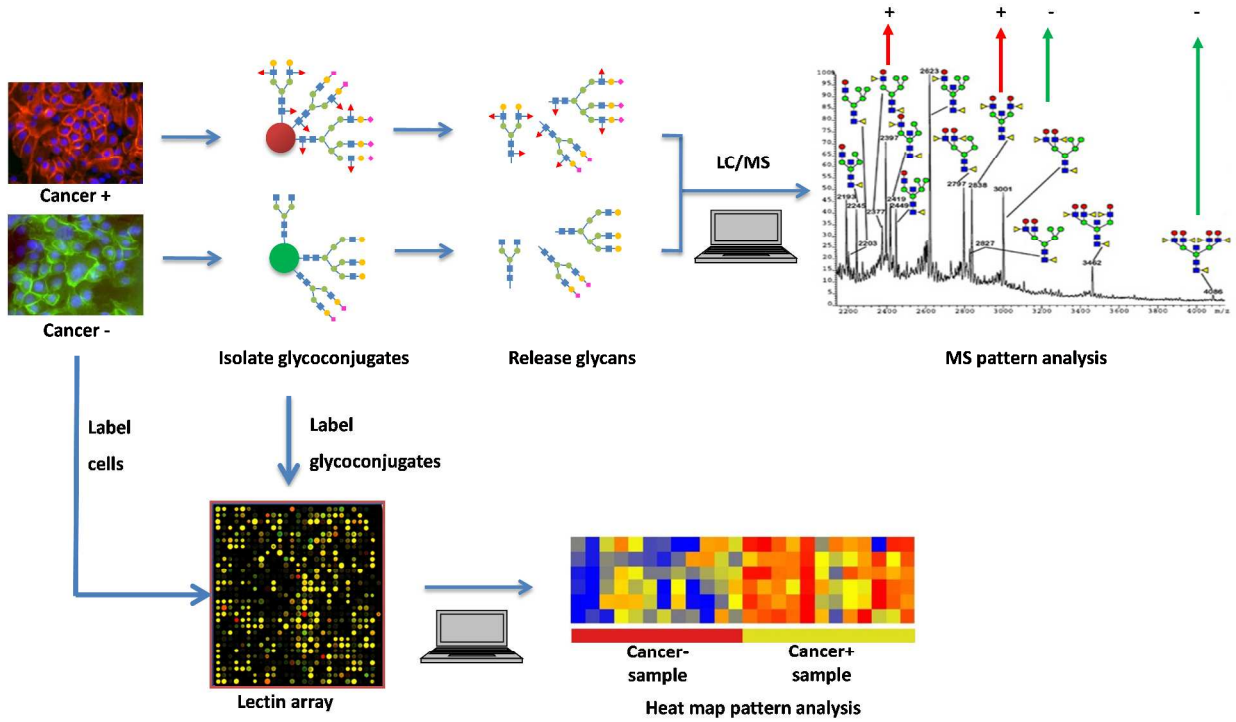

Figure 3. Current strategies for glycan biomarker identification include multiple time-consuming steps of glycoconjugate isolation from cells or tissues, the release, pre-concentration, and mass spectrometric analysis. Each of these steps requires multiple procedures and method of analysis. On the other hand, lectin microarrays do not require glycan release and mixtures of labeled glycoconjugates including whole cells can be analyzed without laborious purification steps. Both techniques yield differential "glyco-fingerprints" that are used to identify cancer specific glycan biomarkers.

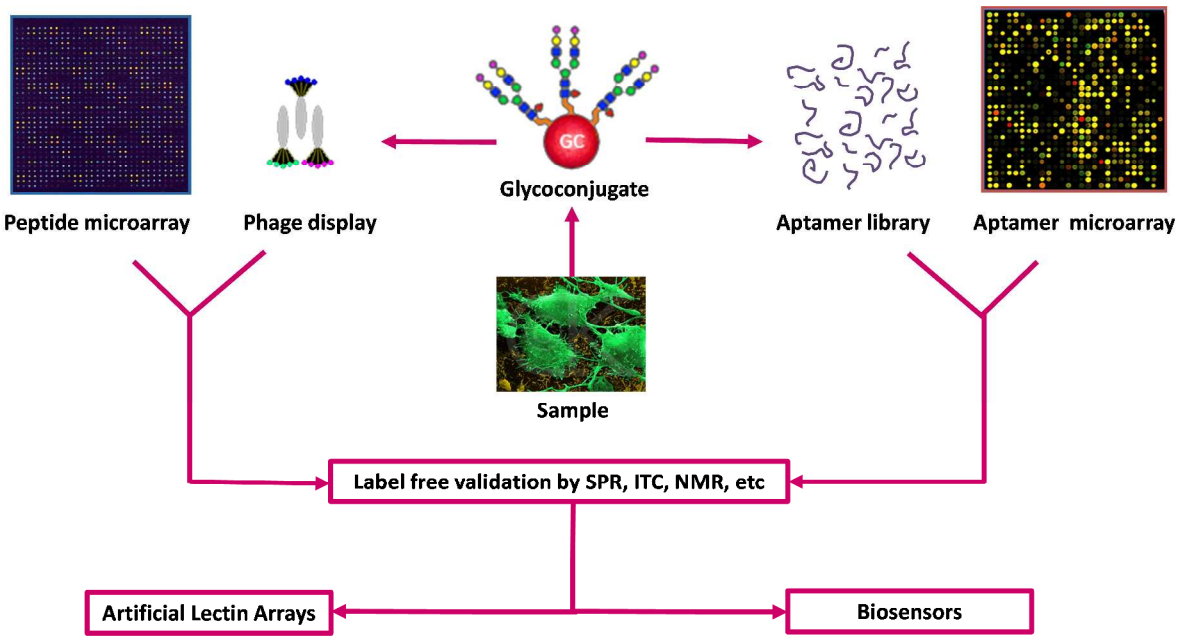

Figure 4. A schematic representation of selection and use of artificial glycan recognition elements. Glycans of interest are extracted and blotted onto luminescent or magnetic nanoparticle scaffolds. The resulting neoglycoconjugates are screened against peptide or aptamers microarrays or phage display or SELEX-based aptamers libraries. Selected hits are validated by available biophysical methods for affinity and specificity and used in the construction of artificial lectin microarrays or incorporated directly into glycobiosensors.

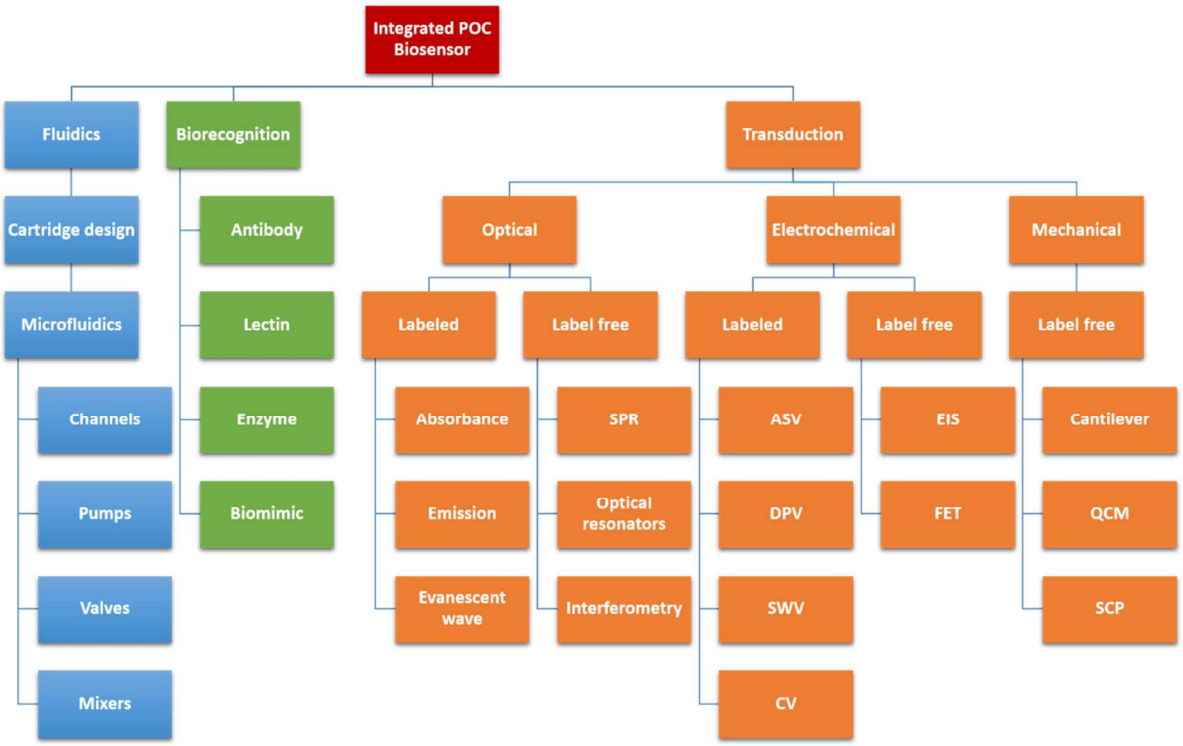

Figure 5. Main components of an integrated point-of-care biosensor.

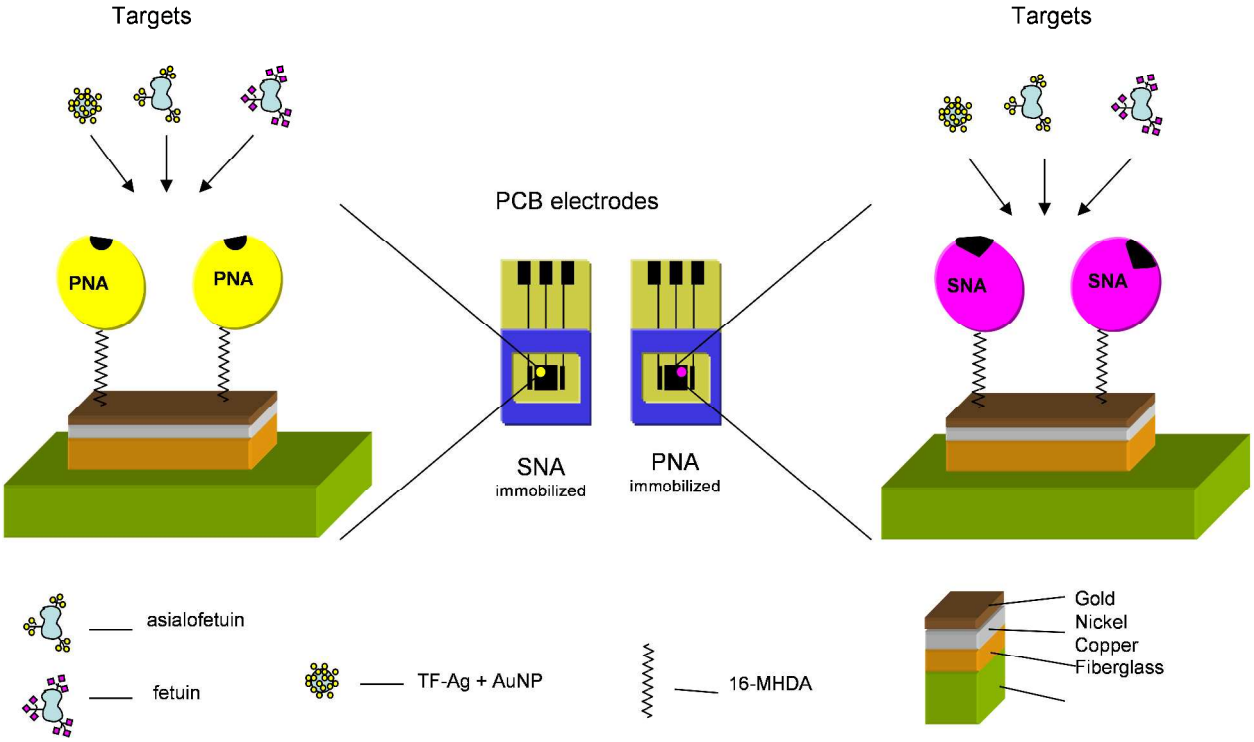

Figure 6. Label free EIS glycobiosensor. Printed circuit board (PCB) electrodes consisting of layered copper/nickel/gold films are covalently functionalized with either PNA or SNA lectins and probed with serial dilutions of TF-antigen encapsulated gold nanoparticles, asialofetuin (ASF) and fetuin (FET) resulting in 13-150fM limits of detection of TF-antigen.[113]

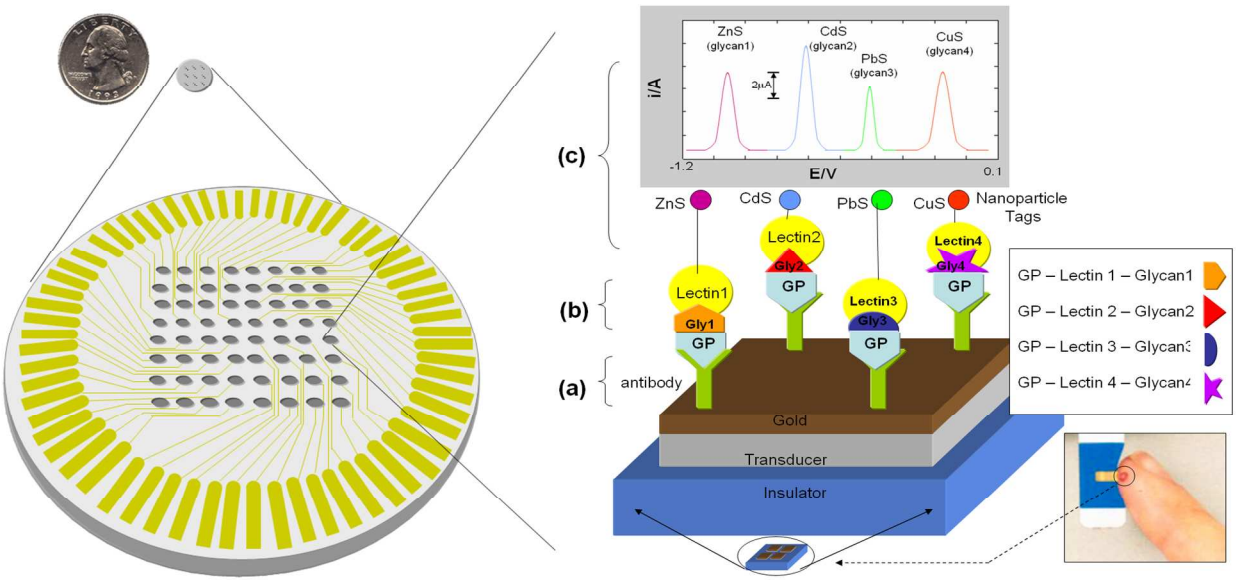

Figure 7. A prototype of multiplexed amperometric glycobiosensor where cancer-specific glycan biomarkers are detected using nanoparticle-based sandwich assay – these are highly enhanced electrochemical ELISA-like reactions for rapid and sensitive detection of glycan biomarkers. Different lectins or other GBPs are encoded by nanoparticles of varying composition (ZnS, CdS, PbS, CuS). Biosensor surface is functionalized by analyte glycoprotein either directly or via antibody capture. The sandwich assay is then performed. Particles that are not bound are washed away while bound particles are dissolved and the current at the potential corresponding to each ion (Zn^{2+}, Cd^{2+}, Pb^{2+}, or Cu^{2+}) is measured. An area under each peak is correlated to number of particles bound [Reproduced with permission from [116]].

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