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REVIEW

Carbochips: a New Energy for Old Biobuilders

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Microarray technology has come of age for use in high-throughput operations and large-scale studies. It allows rapid and simultaneous detection of thousands of parameters within a single experiment. Recent developments in the field of carbohydrate microarray technology facilitate applications for different types of protein–carbohydrate interactions. These developments included capture molecule immobilization, surface engineering and detection strategies to analyze entire glycomes and glycosylation in vertebrate systems, the most common post-translational modification.

[Key words: carbochip, glycoprotein, surface engineering, glycoproteomics, carbohydrate microarrays]

In the new scientific arena, the unscheduled and uncontrollable behavior of tissue cell societies are paving ways for the development of various research tools. To investigate the intricacies of life, it is important to resolve the mysteries of the ultimate molecular machine: the cell. To fulfill this goal, the “omics” journey, which started with genomics, has come all the way to functional proteomics (Fig. 1).

Glycoproteomics, which was a relatively inactive field in the scientific world a couple of years ago, has now become a major academic and clinical research priority. The microarray, a powerful tool in this omics era providing new opportunities to researchers, has been the major impetus behind this remarkable transformation. There is a brain-teasing structural diversity to glycans and they are bound to proteins in a variety of complex ways (1). The glycans are subject to regulation by more complex structural guidelines (branching events, stereoisomerism and linkage forms) than DNA or proteins. For a given reducing hexasaccharide, the number of possible structural isomers is $1.05 \times 10^{12}$, for a hexapeptide, 4096 and for a hexanucleotide, $6.4 \times 10^7$ (2). In order to drive the molecular engine of various processes (Fig. 2), protein–carbohydrate interactions provide fuel. A recent example of new microarray technology, the carbochip, provided a much better solution for the systematic and high-throughput elucidation of this specific kind of interaction. This new powerful tool has opened the gateway to a knowledge bank which was closed until now. Glycobiology is an umbrella term covering a plethora of areas. According to Dale Cumming of GlycoDesign Inc. (Canada), it is “a bit like the proverbial onion where one goes through one layer and discovers that there are whole new layers of complexity to be addressed”. The integration of various mass spectrometry-founded approaches to carbochip technologies has illuminated the dark corners of glycobiology.

This review aims to summarize recent progress in the area of carbochip technology including capture molecule immobilization, surface engineering, detection strategies, and applications to analyze various protein–carbohydrate interactions.

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Abbreviations: BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; CFG, consortium for functional glycomics; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; FITC, fluorescein isothiocyanate; FucT, fucosyltransferase; HRP, horseradish peroxidase; PHA-I, Phascolus agglutinin I.

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FIG. 1. Ongoing omics journey: various platforms. Representation of the recent ongoing progress of omics technologies. Progressive investigation of new research tools unraveling the dynamic knowledge of life.
I. WHY ANOTHER CHIP?

A microarray can be considered as a collection of miniaturized chemical reaction areas that may also be used to detect proteins or nucleic acid fragments, but carbochips have a more intricate framework than other microarray technologies registered for global glycoproteome cataloging. There is no direct link between gene expression and glycan endproducts. The structure and composition of glycans are guided by sophisticated batteries of biosynthetic enzymes operating principally within the endoplasmic reticulum (ER), the Golgi complex or the plasma membrane and this is the reason why unveiling the enigma of carbohydrate biobuilders is an extremely daunting task. The development of this new search engine has unmasked various pieces of information, which were not previously easily obtainable. Currently, one can investigate various carbohydrate interactions for their use for medical purposes (Table 1).

II. CARBOCHIP ERA TO DATE

Every chip-based experiment involves two components viz., an immobilization surface and the biomolecules to be immobilized. In general for carbochip technology, microspots of saccharide molecules are printed in rows and columns onto a solid support and further displayed to the solutions containing corresponding interacting partners. Interpretation systems are mainly based on fluorescence, radioactivity, chemiluminescence or mass spectrometry. At this juncture, various research groups have followed different technical strategies, and the excitement of innovations and discoveries fuels efforts to develop new and more advanced carbochips. The generally followed characteristics are as mentioned in Table 2.

III. EFFORTS TO DEVELOP THE MOST EFFECTIVE CARBOCHIP

Although the latest advent in carbochip technology is expected to change the pace and scope of biological research, it is still in its infancy. A schematic overview of carbochip technologies is depicted in Fig. 3. In order to be availed of by the broader research community as a future molecular biology tool kit, it requires further development. The present situation is similar to standing in front of the tree of glycomics and only being able to pluck the fruits from lower branches. To be able to reach out to the top branches, one needs a taller and stronger ladder of carbochips. The ancient biobuilder, glycan, continues to pose a bigger challenge than DNA or proteins. It does not follow the standard molecular protocol of central dogma (DNA → mRNA → protein) and this is the reason why well-documented and user-friendly molecular biology techniques like cloning, sequencing, PCR amplification, and expression strategies cannot be employed with these molecules. The authors are engaged in the study of glycosylation of the enzymes of carbohydrate metabolism (unpublished work).

Carbohydrate microarray technology is plagued by various shortcomings and technical challenges, which are being sequentially discussed in conjunction with various aspects such as sample preparation, surface chemistry, instrumentation, data handling and management.

Large carbohydrate library For broad range characterization and to realize the full potential of this new tool, it is crucial to establish a high-quality storehouse of carbohydrate biobuilders. However, generating a large-scale combinatorial carbohydrate library is a very difficult task, associated with a variety of problematic issues. Using conventional chromatographic techniques such as ion exchange chromatography, the purification of stereoisomers is difficult and blood-derived samples are usually associated with problems such as rapid degradation and erythrocyte agglutination. Since the efficiency of immobilization on a surface is directly proportional to the molecular mass of the biomolecules, only oligosaccharide and polysaccharide preparations can be used. The first carbochip approach by Wang et al. (3) was much more feasible for polysaccharide preparations, which were covered by nitrocellulose-coated glass slides. Using the same approach, Wang et al. (4) raised the
animals such as to synthesize the full complement of known sequences in sugars was attempted. However, it is currently impractical (5), lipid isolation and manipulation is extremely difficult. Glycolipids and glycosaminoglycans (6). Ter-syltransferase (FucT) enzyme, whereas Bryan carbohydrate arrays to study known inhibitors of the fucosylated biological system for the production of carbohydrate (7) postulated that immobilization of the N-glycans of glycoproteins, diverse sequences of glycolipids and glycosaminoglycans (6). Terminal saccharide units are initially recognized by proteins, but it is very difficult to maintain the closed ring structure and the anomericity (e.g., α and β anomeric configurations) of terminal saccharide units during chemical synthesis. Mizuno et al. (7) postulated that immobilization of the glycosyl amino acids allowed the preparation of glycochips that maintained the whole structure of the oligosaccharide. There are branched chain structures in many carbohydrates. The cluster effect (mass affinity due to multivalency) is another significant issue during solid-phase synthesis. Simultaneous multiple selective protection, deprotection and maintaining the blocking efficiency at each step is a prerequisite for the multivalent morphology of sugar structures. This mass affinity is the major obstruction for correct spacing, correct orientation and uniform densities of the fabricated sugar frameworks. It can be considered as a huge barrier to the regular and homogeneous microenvironment that results in lower biological activity and low solvent accessibility of the arrayed samples. High cost and the limited availability of enzymes (e.g., galactosyltransferase) is commonly associated with the enzymatic synthesis of glycan molecules and until now no one has developed a highly efficient genetically modified biological system for the production of carbohydrate molecules. Fazio et al. (8) implemented the cyclo-addition reactions between azides and alkynes. They used carbohydrate arrays to study known inhibitors of the fuco-syltransferase (FucT) enzyme, whereas Bryan et al. (9) used the same array technique for the screening of a library of possible FucT inhibitors.

The carbohydrate synthesis/protein expression core of the consortium for functional glycomics (CFG) recently generated a tool box of over 75 compounds covering a broad range of structures from simple monosaccharides to deca-saccharides. Clumsy nomenclature and difficult structure predictions also impede the characterization of these molecules. For structural analysis, chromatography and mass spectrometry are more often used in combination. The analytical glycomics core of the CFG is focusing on the development and application of ultra-high sensitivity mass spectrometric strategies for the characterization of glycoproteins, which will also allow the identification of isomers that have the same molecular weight but different structures. To avoid the confusion of nomenclature, Glycominds generated a syntax that describes the branched structures of glycans as linear computer-friendly mathematical formulae (10).

**Enzyme activity profiling**

It is generally used in substrate-affinity mapping of various sugar-modifying enzymes (e.g., glycosyltransferases, kinases and glycosidases) and characterizing the specificities of enzymes.

**Quantitative inhibition assay**

Screening of low molecular weight inhibitors of protein–sugar or sugar–sugar interactions. Main goal in the field of drug discovery is identifying glycomimetic non-natural sugars in order to augment their low bioavailability unlike their natural counterparts. The knowledge obtained can be used for designing novel drugs.

**Post-translational modification**

High-throughput and schematic detection of post-translational modifications viz., glycosylation and glycation.

**Expression pattern analysis**

Determining cell or tissue-specific expression patterns and identification of temporal expression patterns of carbohydrate residues during the process of maturation and development (e.g., cancer cells change their glycosylation pattern to invade other areas).

**Microbial diagnosis**

Simultaneous detection of a wide range of microbial infections using limited quantities of clinical specimens by glycome analysis. This knowledge can be further used in the fight against bioterrorism.

**Biomarker validation**

In pharmacoglycomics, carbochips can be used for the segmentation of patient populations and for searching biomarkers for personalized medicine.

**Discovery of novel carbohydrate-binding proteins**

To identify the novel proteins that contain carbohydrate-binding domains. The sequence data of these proteins can be used in evolutionary studies.

**Target discovery**

Screening of combinatorial peptide libraries (e.g., phage display library). Lectins can be regarded as protein interpreters of the sugar code (25). Carbochips are commonly used for profiling of lectin binding specificities.

**Epitope mapping**

Profiling of broad spectrum antibody specificities.

### Table 1. Applications of carbochips

| Application                           | Description                                                                                       |
|---------------------------------------|--------------------------------------------------------------------------------------------------|
| Enzyme activity profiling             | It is generally used in substrate-affinity mapping of various sugar-modifying enzymes (e.g., glycosyltransferases, kinases and glycosidases) and characterizing the specificities of enzymes. |
| Quantitative inhibition assay         | Screening of low molecular weight inhibitors of protein–sugar or sugar–sugar interactions. Main goal in the field of drug discovery is identifying glycomimetic non-natural sugars in order to augment their low bioavailability unlike their natural counterparts. The knowledge obtained can be used for designing novel drugs. |
| Post-translational modification      | High-throughput and schematic detection of post-translational modifications viz., glycosylation and glycation. |
| Expression pattern analysis           | Determining cell or tissue-specific expression patterns and identification of temporal expression patterns of carbohydrate residues during the process of maturation and development (e.g., cancer cells change their glycosylation pattern to invade other areas). |
| Microbial diagnosis                   | Simultaneous detection of a wide range of microbial infections using limited quantities of clinical specimens by glycome analysis. This knowledge can be further used in the fight against bioterrorism. |
| Biomarker validation                  | In pharmacoglycomics, carbochips can be used for the segmentation of patient populations and for searching biomarkers for personalized medicine. |
| Discovery of novel carbohydrate-binding proteins | To identify the novel proteins that contain carbohydrate-binding domains. The sequence data of these proteins can be used in evolutionary studies. |
| Target discovery                      | Screening of combinatorial peptide libraries (e.g., phage display library). Lectins can be regarded as protein interpreters of the sugar code (25). Carbochips are commonly used for profiling of lectin binding specificities. |
| Epitope mapping                       | Profiling of broad spectrum antibody specificities. |

issue of the involvement of autoimmune responses in the pathogenesis of severe acute respiratory syndrome (SARS). In the neoglycolipid technology applied by Fukui et al. (5), lipid isolation and manipulation is extremely difficult. To overcome these problems, the laboratory synthesis of sugars was attempted. However, it is currently impractical to synthesize the full complement of known sequences in animals such as O- and N-glycans of glycoproteins, diverse sequences of glycolipids and glycosaminoglycans (6). Terminal saccharide units are initially recognized by proteins, but it is very difficult to maintain the closed ring structure and the anomericity (e.g., α and β anomeric configurations) of terminal saccharide units during chemical synthesis. Mizuno et al. (7) postulated that immobilization of the glycosyl amino acids allowed the preparation of glycochips that maintained the whole structure of the oligosaccharide. There are branched chain structures in many carbohydrates. The cluster effect (mass affinity due to multivalency) is another significant issue during solid-phase synthesis. Simultaneous multiple selective protection, deprotection and maintaining the blocking efficiency at each step is a prerequisite for the multivalent morphology of sugar structures. This mass affinity is the major obstruction for correct spacing, correct orientation and uniform densities of the fabricated sugar frameworks. It can be considered as a huge barrier to the regular and homogeneous microenvironment that results in lower biological activity and low solvent accessibility of the arrayed samples. High cost and the limited availability of enzymes (e.g., galactosyltransferase) is commonly associated with the enzymatic synthesis of glycan molecules and until now no one has developed a highly efficient genetically modified biological system for the production of carbohydrate molecules. Fazio et al. (8) implemented the cyclo-addition reactions between azides and alkynes. They used carbohydrate arrays to study known inhibitors of the fuco-syltransferase (FucT) enzyme, whereas Bryan et al. (9) used the same array technique for the screening of a library of possible FucT inhibitors.

The carbohydrate synthesis/protein expression core of the consortium for functional glycomics (CFG) recently generated a tool box of over 75 compounds covering a broad range of structures from simple monosaccharides to deca-saccharides. Clumsy nomenclature and difficult structure predictions also impede the characterization of these molecules. For structural analysis, chromatography and mass spectrometry are more often used in combination. The analytical glycomics core of the CFG is focusing on the development and application of ultra-high sensitivity mass spectrometric strategies for the characterization of glycoproteins, which will also allow the identification of isomers that have the same molecular weight but different structures. To avoid the confusion of nomenclature, Glycominds generated a syntax that describes the branched structures of glycans as linear computer-friendly mathematical formulae (10).

**Surface engineering and instrumentation** Direct and fixed stabilization of diverse glycans onto a slide surface is the essential element of all carbochip-based applications. It is also necessary for the identification of appropriate functional groups, which can promote permanent and quantitative immobilization of target molecules in defined orientations. The linker molecule hypothesis initially proposed by Park and Shin (11) can be readily adapted for diverse categories of substances such as silicon, glass, plastic, metal, fiber optics, filter membranes and so on. Linkers of suitable size make use of whole-cell glycans for correct interaction with the species to be detected and are also helpful in avoiding nonspecific adsorption. The approach proposed by Houseman and Mrksich (12) has significantly reduced the background noise and is principally based on a mixture of two alkanethiols. Ratner et al. (13) also observed very high signal-to-noise ratios in their study. The conjugation strategy
| Spotted molecules | Surface chemistry and engineering of spotted molecules | Molecules/Cells detected | Reference |
|-------------------|------------------------------------------------------|--------------------------|-----------|
| Dextran, inulin, bacterial polysaccharides etc. | Noncovalent spots on the nitrocellulose-coated glass slides | Biotinylated, fluorescein isothiocyanate (FITC)-labeled antibodies, streptavidin-Cy3 and alkaline phosphatase-conjugated secondary antibodies | 3 |
| Cyclopentadiene-conjugated monosaccharides | Gold-coated glass slides converted into self-assembled monolayer surfaces by pretreatment with a mixture of two alkanethiols (inert ethylene glycol and hydroquinone) and sugar conjugates immobilized by the Diels–Alder cycloaddition reaction | Five rhodamine-labeled plant lectins (concanavalin A, *Bendirea simplicifolia* I, *Erythrina cristagalli* lectin, *Ulex europaeus* I and *Galanthus nivalis* lectin) | 12, 26 |
| N-Acetyl glucosamine, lactose, maltose, cellulose etc. converted into glycosylamines and coupled with maleimide linkers | Maleimide-conjugates immobilized by Hetero-michael addition on thiol-containing modified glass slides | Three FITC-labeled plant lectins (concanavalin A, *Erythrina cristagalli* lectin and *Triticum vulgare* lectin) | 11 |
| Glycoprotein, proteoglycan, polysaccharides, glycolipids, and whole organ preparations converted into neoglycolipids by reductive amination with aminolipids | Sugar allyl derivatives noncovalently spotted on microtiter plate | Three phosphatase conjugated anti-lectins, mAb or lectins (concanavalin A, ricin B chain, *Tetragonolobus purpurea* lectin) | 27 |
| Mono-and oligosaccharides conjugated with hydrocarbon chains (under stereochemically controlled conditions) | Long aliphatic hydrocarbon chains (14C) noncovalently immobilized on microtiter plates and azide derivatives added by 1,3 dipolar cycloaddition reactions (*in situ*) | Alkaline phosphatase-conjugated anti- lectin mAb or fluorescence-labeled lectin (ricin B chain, *T. purpureae* I, *Sambucus nigra* lectin) | 8, 28 |
| Mono-and oligosaccharides converted into azide forms | Azide derivatives noncovalently coupled to the microtiter plate | Antiglycan and antilactosaccharide antibodies, various lectins and antibodies (e.g., wheat germ agglutinin, concanavalin A) detected by europium-labeled streptavidin | 17 |
| Bacterial polysaccharides | Linker noncovalently coupled to the microtiter plate | Fluorescence-labeled concanavalin A and cyanovirin N from *Nostoc ellipsosporum* | 14 |
| Biotinylated oligosaccharides | Streptavidin-coated plates (that contained monosaccharides, disaccharides, trisaccharides and larger structures, including both neutral and acidic sugars containing either sialic acids or sulfate) | DC-SIGN and Horseradish peroxidase (HRP)-labeled secondary antibodies | 18 |
| Monosaccharides and oligosaccharides | Carbohydrates were covalently coupled via a flexible linker to the 96-well ELISA plate (microtiter plate) | HRP-labeled lectins concanavalin A, *Ricinus communis* agglutinin 120, *Datura stramonium* agglutinin, wheat germ agglutinin | 7 |
| Fmoc-glycosylasparagines | Fmoc-glycosylasparagines immobilized on 96-well plates (microtiter plate) | Fluorescence-labeled intact cells, chicken hepatocytes, human CD4+ T-cells | 19 |
| Monosaccharides and oligosaccharides | *p*-Aminophenyl glycosides were covalently attached to the glass surfaces in wells via an oligomeric 1,8-diamino-3,6-dioxaoctane linker (17) | Fluorescence-labeled intact cells, chicken hepatocytes, human CD4+ T-cells | 19 |
| Mannose and galactose | *N*-Acetyl lactosamine was noncovalently displayed on the surface of microtiter plates via Cu(I)- catalyzed 1,3-dipolar cycloaddition with lipid alkenyes | Fluorescence-labeled lectins concanavalin A and cyanovirin N from *Nostoc ellipsosporum* | 9 |
followed by Adams et al. (14) achieved adequate spacing between the underlying polymer support and the attached carbohydrate molecules. It also resulted in maintenance of the concentration of immobilized carbohydrate probes. Guo et al. (15) also followed the same strategy and demonstrated that dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) and DC-SIGN-related have distinct ligand-binding properties.

With the evolution of technologies, more sensitive, accurate, cost-effective and rapid automated equipment may be available in future. The array will be highly reproducible (plate to plate, well to well and batch to batch reproducibility), stable and it will be possible to dry-store it for several months. Novel surface slides generated by Willats et al. (16) were stored for three months at room temperature. The array will be highly reproducible (plate to plate, well to well and batch to batch reproducibility). For the detection strategies, the critical point is anticipated as a result of the recent developments. Thus, it is vital to develop a standard protocol for data mining, data handling and management for convenient computational analysis. The Human Protein Reference Database (www.hprd.org) has the potential to fulfill this requirement. This database contains 226 glycosylation sites among the 7168 human proteins annotated as on the date of communication. Moreover, the agreement between glycominds and CFG has been comprehensive and major challenges exist even now for large-scale studies, with the aid of mass spectrometric (MS)-based approaches researchers can now perform high-throughput operations for glycan and protein glycoform cataloging. The identification of glycosylated peptides is generally carried out by matrix-assisted laser desorption/ionization (MALDI)-MS or LC/MS fingerprint analysis, whereas the location of modified amino acids can be obtained from fragmentation spectra generated by nano-electrospray ionization/MS/MS or LC-MS/MS analysis. For more comprehensive characterization of glycoproteins, direct MS-based approaches, such as glyco-catch can be applied (22). In

| Spotted molecules | Surface chemistry and engineering of spotted molecules | Molecules/Cells detected | Reference |
|-------------------|--------------------------------------------------------|-------------------------|-----------|
| Mannose, galactose, high mannose oligosaccharides like linear trimannoside, hexamannoside and branched trimannoside | Synthetic oligosaccharide structures immobilized covalently on maleimide-activated BSA-coated glass slides by way of a hydrophilic thiol linker, whereas the remaining maleimide groups on the surface were subsequently blocked with 3-mercaptopropionionic acid | FITC-labeled concavalin A BODIPY-labeled cyanovirin N | 13 |
| Sulphydryl-containing ethylene glycol-derivatized natural and modified glycoproteins as well as neoglycoproteins | Prepared samples fabricated by reacting amine-modified glass slides with ethylene-glycol disuccinimide to form a hydrophilic, amine-reactive surface. Slides were subsequently quenched in a solution of BSA to inactivate remaining succinimidy1 groups | Fluorophore-labeled proteins DC-SIGN, antibody 2G12, cyanovirin N, scytovirin, soluble CD4 | 21 |
| Collection of 51 carbohydrate antigens including both microbial polysaccharides and cellular glycan complex carbohydrates | Nonevalent spots on the nitrocellulose-coated glass slides | Horse antisera containing anti SARS coronavirus antibodies and anti Streptococcus pneumoniae type 18 polysaccharide antibodies, anti asialoorosomucoid (ASOR) IgG antibodies, lectin PHA-L/avidin FITC, lectin Griffonia simplicifolia 1-B4/avidin FITC | 4 |

* Neoglycolipids (NGL) technology was employed for sample preparation.
* RANTES, Regulated on activation normal T-expressed and secreted.
* Ligand-positive components were further determined by a deconvolution strategy (which includes TLC and mass spectrometry) (see Fig. 3).

In the foreseeable future, a huge data explosion can be anticipated as a result of the recent developments. Thus, it is vital to develop a standard protocol for data mining, data handling and management for convenient computational analysis. The Human Protein Reference Database (www.hprd.org) has the potential to fulfill this requirement. This database contains 226 glycosylation sites among the 7168 human proteins annotated as on the date of communication. Moreover, the agreement between glycominds and CFG has been comprehensive and major challenges exist even now for large-scale studies, with the aid of mass spectrometric (MS)-based approaches researchers can now perform high-throughput operations for glycan and protein glycoform cataloging. The identification of glycosylated peptides is generally carried out by matrix-assisted laser desorption/ionization (MALDI)-MS or LC/MS fingerprint analysis, whereas the location of modified amino acids can be obtained from fragmentation spectra generated by nano-electrospray ionization/MS/MS or LC-MS/MS analysis. For more comprehensive characterization of glycoproteins, direct MS-based approaches, such as glyco-catch can be applied (22). In
this methodology, sequential lectin affinity columns are used for glycopeptide enrichment, which are further resolved by MALDI-TOF or Edman degradation. This method may further be coupled with database searching. Alternatively, an another MS based technique termed β elimination followed by Michael addition with dithiothreitol (BEMAD) can also be used for the identification of O-GlcNAc-modified sites (23).

This in vitro methodology relies on mild β elimination followed by Michael addition with DTT. This method is suitable for the quantification of glycosylation by mass spectrometry. Methods to quantify post-translational modifications have been recently updated (24).

To conclude, the integration of various MS-based approaches into carbohydrate microarray technologies will generate more ideas for tackling the enormous task of unraveling the huge amount of biological information that is currently being obtained, which will increase the understanding of the molecular machinery of life.

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