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The neoglycolipid (NGL)-based oligosaccharide microarray system poised to decipher the *meta*-glycome

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Abstract

The neoglycolipid (NGL) technology is the basis of a state-of-the-art oligosaccharide microarray system. The NGL-based microarray system in the Glycosciences Laboratory Imperial College London (<http://www3.imperial.ac.uk/glycosciences>), is one of the two leading platforms for glycan microarrays, being offered for screening analyses to the broad biomedical community. Highlighted in this review are the sensitivity of the analysis system and, coupled with mass spectrometry, the provision for generating ‘designer’ microarrays from glycomes to identify novel ligands of biological relevance. Among recent applications are: assignments of ligands for apicomplexan parasites, pandemic 2009 influenza virus, polyoma and reoviruses, an innate immune receptor against fungal pathogens, Dectin-1, and a novel protein of the endoplasmic reticulum, malectin; also the characterization of an elusive cancer-associated antigen. Some other contemporary advances in glycolipid-containing arrays and microarrays are also discussed.

Introduction

Over the last decade oligosaccharide microarrays have become essential tools in glycobiology and are revolutionizing the unravelling of carbohydrate-protein interactions in health and disease processes, infectious and non-infectious. A variety of immobilization strategies have been described for generation of the microarrays [1–9]. This review is focused on the neoglycolipid (NGL)-based microarray system developed in our laboratory, the first microarray system for sequence-defined oligosaccharides.

The NGL technology

The impetus for establishing the NGL technology in 1985 was the need for a sensitive micromethod for direct binding analyses with oligosaccharides released from glycoproteins to study recognition by antibodies and other carbohydrate-recognizing proteins [10]. Inspired by the thin-layer chromatography (TLC)-binding method for glycolipids [11], we designed an approach that involved linking oligosaccharides to an amino-phospholipid tag to make artificial glycolipids, NGLs. Reductive-amination was used to conjugate reducing

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oligosaccharides, and also for reductively released *O*-glycans after mild periodate oxidative cleavage [12]. The resulting NGLs could be immobilized on solid matrices in a highly desirable, clustered state for binding studies; and mass spectrometry could be performed *in situ* in conjunction with the TLC-binding experiments. Fluorescent NGLs were developed for detection of NGLs during chromatography [12] and, oxime-linked NGLs to preserve ring-closed monosaccharide cores. The latter is advantageous for oligosaccharides where core regions are part of the recognition motif [13].

NGL-based microarrays

Following proof-of-concept studies in 2002 [14], the NGL-technology became the basis of a unique state-of-the-art oligosaccharide microarray system [15;16;17] (Figure 1). The technology allows expansion of the library of probes via the ‘designer’ microarray approach, a term used for microarrays of NGLs derived from ligand-bearing glycomes in order to reveal the oligosaccharide ligands they harbor, so that these can be isolated and characterized.

In the NGL-based microarrays, the probes are all lipid-linked and comprise both NGLs (prepared from natural or chemically synthesized oligosaccharides) and glycolipids (natural or synthetic). The library is enriched by its content of chemically synthesized glycolipids from the Akira Hasegawa, Makoto Kiso and Hideharu Ishida collection (<http://www.mizutanifdn.or.jp/20thsympo/pdf/10Kiso.pdf>). The NGL-based microarray system currently contains ~830 sequence-defined probes, with a high content of natural oligosaccharide sequences, some are difficult to synthesize (Figure 2); 492 of these probes are present in the current version of screening microarrays [18]. Other probes are included in various focused arrays for specific projects. A new version of screening microarrays including these will be generated in 2014.

The NGL and glycolipid probes are robotically dispensed onto nitrocellulose-coated glass slides at low fmol levels in a liposome formulation in the presence of carrier lipids [17]. This mode of presentation allows an element of mobility, simulating to some extent the cell surface display of glycans. The applicability of the microarray system to glycolipids and to oligosaccharides derived from them, makes it possible to clinch the role of carbohydrate and ceramide moieties in recognition [19]. Moreover NGLs, like glycolipids, can be incorporated into live cells to evaluate biological significance of microarray binding data [19].

An integral component of the microarray system is a database that holds all of the microarray data, the experimental conditions and information on saccharide probes and proteins [20] (Figure 3). There is associated interactive software for presentation of microarray data, filtering, sorting and deep mining of every data point. The software is being refactored to accept a wide range of experimental formats, and to be made available to the scientific community.

Since 2003, initially with support from the UK Research Councils Basic Technology Initiative ‘Glycoarrays’, NGL-based microarrays at Imperial College have been available to the scientific community for screening analyses, the remit of the Facility being collaborative

research. Since 2012, the facility has become a Wellcome Trust-funded Biomedical Resource and is entering to a new phase of activity with up-to-date instrumentation and bioinformatics support. The facility will soon launch a new website for on-line requests of microarray analyses and for data sharing.

Some recent applications of NGL-based microarrays

The NGL-based microarray system has been applied in investigation of a considerable number of bio-recognition systems cited in [21]. Below are some highlights.

Apicomplexan parasites

In a program with Steve Matthews's group, NGL microarray analyses have played a central role in studies of adhesive molecules, known as micronemal proteins (MICs), of *Toxoplasma gondii* and other apicomplexan parasites [22–25•]. Information has been gleaned on the way that the apicomplexan parasites use their MICs to decipher and select carbohydrates of the host cells that they invade, with implications for host/tissue tropisms and pathobiology of infections, and designs of therapeutic molecules. Studies began with the structural identification of a novel fold, microneme adhesive repeat (MAR), on the MIC1 of *T. gondii* (TgMIC1). Ligands for TgMIC1 MAR domains remained unknown until microarray analyses revealed binding to a wide range of sialyl oligosaccharide sequences [22], thus providing an explanation for the ability of this parasite to infect almost all warm blooded animals, including humans. A critical threonine residue on TgMIC1 MAR domains was identified as a novel sialic acid-binding motif. Genetical studies have revealed a MAR-containing protein family in *T. gondii* and other apicomplexan parasites, many of these possessing the threonine motif [23]. Among the proteins analyzed, a differing repertoire of sialyl ligands have been identified for MIC13 of *T. gondii* and MIC1 of *Neospora caninum*, also for MIC3 of *Eimeria tenella* (EtMIC3). Among the interesting findings is the lack of binding by EtMIC3 to *N*-glycolyl sialyl (NeuGc) sequences, suggesting an adaptation to the chicken host that lacks NeuGc [24]. A study of the 'apple'-domain-containing protein, TgMIC4, which is closely associated with TgMIC1, has revealed a broad spectrum of galactose-terminating oligosaccharide ligands (many of them asialo-forms of the TgMIC1 ligands) [25•]. The reciprocal binding specificities of the two proteins may result in an amplification of host-cell adhesion.

Viruses

Glycan microarrays have been powerful in determining receptor-binding specificities of known and emerging influenza viruses [26–28]. A special contribution of the NGL-based microarrays was detection of binding by the pandemic influenza A (H1N1) 2009 virus to α 2,3-sialyl in addition to α 2,6-sialyl sequences, a distinctive receptor-binding feature compared to that of seasonal human H1N1 influenza virus (Figure 4) [29]. This difference was exaggerated in hemagglutinin D222G mutants of the 2009 pandemic virus isolated from fatal cases [30], thus providing a link between the ability of the virus to bind to α 2,3-sialyl sequences and severity of disease.

In a program with Thilo Stehle group, NGL-based microarrays are being applied in conjunction with structural studies to assign oligosaccharide ligands for the adhesive proteins of polyoma- and reoviruses [31–33•;34••]. Highly restricted binding of human JC virus VP1 was observed to the α 2,6-sialyl tetrasaccharide sequence, LSTc [31], contrasting with the binding of B-Lymphotropic Polyomavirus (LPyV) to short α 2,3-sialyl *N*-acetyllactosamine or lactose (in sparse display) [34••]. These findings led to X-ray crystallography of the protein-ligand complexes that revealed different modes of sialyl glycan recognition. In the recent study on human BK polyomavirus, a structure-guided mutation of amino acid at position 68 from lysine in BKPyV to serine in SV40 enabled the switch of the specificity of BKPyV from b-series gangliosides (e.g. GD3 and GD1b) to GM1 [33•]. NGL microarray analyses moreover revealed that the mutant is specific for the ‘human’ sialic acid NeuAc, in contrast with SV40 VP1 which has a preference for NeuGc form of GM1 [19], characteristic of simian species and many other nonhuman mammals. The specificity of the mutant thus sheds light on the influence of sialic acid on species tropism. In a joint study with Terrence Dermody on reovirus serotype 1 Lang (T1L) attachment protein σ 1, GM2 was identified as a ligand. Structural studies of the protein-ligand complexes revealed a glycan-binding site distinct from that on the corresponding attachment protein σ 1 of serotype 3 reovirus, pointing to a serotype-specific glycan utilization [32]. These highly selected ligands and modes of binding by the viral proteins will, it is hoped, lead to the development of potential antiviral drugs.

Antibodies

Monoclonal antibody AE3 raised against the major sialomucin glycoprotein of murine mammary adenocarcinoma recognizes an antigen on a variety of human epithelial cancers, and is considered a promising serological cancer biomarker but has eluded characterization. Microarray screening analysis revealed an unpredicted binding to a sulfated glycolipid SM1a that occurs in small amounts in normal kidney [18]. The carbohydrate sequence has not been described hitherto on mucins. The authentic ligand on *O*-glycans is being pursued in our laboratory by the designer microarray approach.

A focused *N*-glycan NGL microarray is among different platforms being used in studies of potent, broadly neutralizing anti-HIV antibodies (bNAbs), cloned from HIV-infected patients that target gp120 *N*-glycans in addition to the peptide backbone [35–37]. These studies are contributing to determining the fine specificities of the bNAbs and informing about vaccine designs. Among striking findings are the bNAbs (PGT121-like) that recognize complex-type *N*-glycans rather than high-mannose type [36•], and the unique glycan specificity of PGT135, namely binding to Man₇(D1) and Man₈ but not Man₉ *N*-glycan sequences [37]. These are in accord with X-Ray crystal structures. Wong and colleagues recently reported the recognition of multi-antennary complex-type *N*-glycans by another bNAb PG16 using a synthetic *N*-glycan array [38•].

Malectin

The NGL microarrays served to elucidate an endogenous ligand for malectin, a novel protein, highly conserved in mammals for which structural studies by Claudia Muhle and colleagues revealed an unpredicted similarity to microbial carbohydrate-binding modules

(CBMs) that bind to glucose oligomers. Location of malectin in the endoplasmic reticulum (ER) led to the design of a NGL-microarray that included gluco-oligosaccharides and also glucosylated high-mannose *N*-glycans of the type that occur in the ER. Microarray analyses not only corroborated the glucan-binding property of malectin, but also revealed a remarkable selectivity for a di-glucosyl high-mannose *N*-glycan, pointing to a function of malectin in the *N*-glycosylation pathway [39].

Designer microarrays – an integral part of the NGL system

The power of the NGL system to pinpoint novel ligands was apparent in early studies of the *O*-glycome of an epithelial mucin within which sulfated ligands were discovered for the selectins (cited in [21]). A more recent test case was Dectin-1, a signaling receptor of the innate immune system against fungal pathogens. Gordon Brown and colleagues showed that Dectin-1 binds to β -glucan polysaccharides. In order to identify the ligands, NGL probes of defined chain lengths and sequences were generated from partially depolymerized ligand-positive glucans (and ligand-negative glucans as controls) [15;16•]. These were arrayed together with mammalian-type sequences. Analyses revealed unique features for Dectin-1: exquisite specificity (calcium-independent) toward fungal-type β 1,3-linked glucooligosaccharides and an unusual chain length requirement of at least a decasaccharide (likely to be a helical epitope). These studies have opened the way to development of novel microarrays for studies of glucan recognition systems such as antibodies being raised for antifungal therapies [40].

Other glycolipid-containing arrays

A number of other microarray systems with glycolipid or lipid-linked glycans have been described, including those with chemically modified and covalently linked glycolipids [1;41•–43•]. Others have used vesicles or liposomes non-covalently [44;45] or covalently [46] immobilized on functionalized surfaces, or by using supported lipid bilayers [47;48]. Another advance is development of micro- and nanoarrays of natural cell membranes and lipid domains enriched in particular gangliosides [49•]. Although relatively low in throughput as described, these platforms would serve well for detailed analyses to validate and follow-up screening microarray results, and in-depth investigations of membrane-associated assemblies and their interactions with binding partners.

Glycan microarrays are usually generated with a single probe at each spot. However analyses of antibody and lectin interactions using combinatorial glycolipid arrays of different pairs of gangliosides have shown that either enhancement or attenuation of the binding signals can occur with different binding partners [50•]. Although the structural basis of this phenomenon requires investigation, these findings are of considerable biological significance as they may occur in natural cell membrane microdomains, and may thus play a role in macromolecular interactions in normal and pathological signalling. Indeed signal amplification was observed using lipid-linked sialyl-lewis^{a/x} and sulfotyrosine in an earlier study with P-selectin, which is known to have structurally distinct binding sites for the two classes of ligand cited in [21]. Similar multicomponent arrays were recently described in studies with covalently immobilized glycans [51].

Conclusions

Oligosaccharide microarrays are gaining momentum and finding applications in analyses of diverse systems of biological and medical importance, and have opened new avenues of research. The Carbohydrate Microarray Facility housed in the Glycosciences Laboratory at Imperial College currently supported by the Wellcome Trust (<http://www3.imperial.ac.uk/glycosciences/>) and that of the Consortium of Functional Glycomics (<http://www.functionalglycomics.org/>) are the two largest (and in some ways complementary) that are offering microarray screening analyses to the broad biomedical community. The current issue of the *Journal* is a signal of the promise of the increasing numbers of other platforms to address specific recognition systems.

In the future, carbohydrate microarray-driven large scale projects can be envisaged where designer microarrays, also the shot-gun microarrays [41• and 52•] will play a major role in delineating recognition systems that decipher the *meta*-glycome [defined as glycomes of diverse organisms (with acknowledgment of Bernard Henrissat for his suggestion of the term)]. From glycomes, many new sequence-defined glycan probes will be generated. These, together with those derived from synthetic glycans of increasing diversity [38•;53••], will result in the much desired expansion of numbers of glycan probes into thousands. These promise to lead to a better understanding of roles of glycans in health and disease processes, and thereby to designs of new therapeutics.

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Figure 1.
Schematic representation of the NGL-based microarray system.

Lipid-linked saccharide probes derived from natural sources or chemically synthesized

Figure 2.
Current composition of NGL-based microarrays.

Figure 4.

Analyses of seasonal and pandemic H1N1 influenza viruses in the NGL-based microarrays showing binding of the seasonal H1N1 virus A/Memphis/14/1996-M only to α 2,6 sialyl sequences (as in upper respiratory tract); pandemic H1N1 viruses A/California/4/2009 and A/Hamburg/5/2009 bound in addition to α 2,3 sialyl sequences (as in lower respiratory tract). Adapted from [29]. This was the first detailed microarray analyses of receptor binding by the 2009 pandemic virus.