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Antibody-lectin sandwich arrays for biomarker and glycobiology studies

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Introduction

Protein glycosylation plays an important role in a wide variety of normal and disease-related processes. Because of their frequent association with disease, glycans and glycan-protein interactions are being investigated as targets for intervention and as biomarkers for disease detection [1]. The optimal use of glycan-related information depends upon the ability to characterize the nature and functions of particular glycan modifications and to precisely measure changes in glycan structures on specific proteins. This editorial describes the use of a new approach for measuring glycan variation on specific proteins, the antibody-lectin sandwich array (ALSA). I make the case that ALSA is ideally suited for biomarker studies and particular types of glycobiology studies due to a unique combination of experimental features. The limitations and challenges of the technology are described, as well as a proposed optimal approach to integrating ALSA with other glycoproteomics methods.

Motivation for developing ALSA

The motivation for developing the ALSA approach was to provide capabilities that complement other glycobiology methods. Enzymatic, chromatographic, and mass spectrometry methods have been developing rapidly for the elucidation of glycan sequences on specific proteins. These methods typically involve the isolation of a considerable amount of the protein from a biological sample, followed by the analysis of liberated glycans or glycopeptides. Because of the multiple steps involved in typical glycan analyses and the nature of the experiments and data analysis, both the throughput and the precision of the measurements are low. The inability to obtain precise measurements over multiple samples means that the population variation of a given glycan cannot be accurately determined, so that associations with disease states or conditions are difficult to detect. The high sample consumption limits the use of clinically derived samples, because some clinical samples are available only in small amounts. Therefore, while highly effective for certain aspects of glycobiology, these approaches must be complemented by other technologies in order to pursue a better understanding of how particular glycans are involved in disease.

The antibody-lectin sandwich array

Such complementary technology is the antibody-lectin sandwich array (ALSA) [2, 3]. The method starts with an antibody microarray—essentially identical to those developed for

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multiplexed protein analyses [4]—in which the antibodies on the array target various glycoproteins of interest. A complex biological sample is incubated on the array, resulting in the capture of glycoproteins by the antibodies, after which the array is probed with a labeled lectin. The amount of lectin binding at each capture antibody indicates the amount of a particular glycan on the proteins captured by that antibody. Diverse lectins or glycan-binding antibodies can be used to probe a variety of glycans. The valuable complementary aspects of the ALSA technology arise from two factors: the use of affinity reagents for glycan and protein detection, and the employment of the microarray platform. These aspects of the technology make it ideal for certain types of biomarker and glycobiology studies, as discussed below.

Features of the ALSA platform

Precise and sensitive measurements directly from biological samples

The use of affinity reagents brings well-recognized advantages, such as flexibility in experimental format and the potential for high specificity, sensitivity, and specificity. Lectins and glycan-binding antibodies are widely used affinity reagents for glycan analysis. Plant lectins with high affinities for glycan motifs that occur in animal biology, such as the wheat germ agglutinin with affinity for *N*-acetyl glucosamine and the concanavalin A lectin with affinity for mannose, are particularly valuable. Lectins have been used in a wide variety of experimental formats, including immunohistochemistry, affinity electrophoresis and chromatography, blotting methods, and microarray-based methods [5].

The use of affinity reagents in the ALSA platform means that the measurements can be reproducible and sensitive, even when capturing directly from a complex biological sample such as blood serum [6]. The ability of antibodies to specifically capture low-concentration proteins directly out of complex backgrounds is well appreciated for clinical protein detection, which are based almost exclusively on antibodies. Minimal pre-processing of a sample is critical to achieving high reproducibility, since every processing step introduces variability. The importance of high reproducibility and sensitivity for biomarker studies is that the levels of glycans on a particular protein may be compared between biological samples to determine whether a glycan is altered in a disease condition.

Multiplexing and miniaturization

The usefulness of the microarray platform lies in its multiplexing capability, enabling the acquisition of many data points in parallel, and its miniaturization, which results in very low consumption of reagents and samples. Microarray methods for analyzing RNA and DNA transformed research in gene expression and genetics following their introduction in the early 1990s, but microarrays for studying other molecule types, including proteins, antibodies, lipids, and glycans, developed more slowly due to increased technical difficulty.

The multiplexing capability allows the testing of multiple candidate biomarkers in single experiments. The antibody microarray typically fills the niche between open-ended discovery research, involving analyses of hundreds or thousands of molecules, and highly focused research on individual molecules. This middle niche has a practical size anywhere from several to dozens, limited usually by the availability of good targets and antibodies. The miniaturization of the microarray format further benefits biomarker research by reducing sample consumption and enabling the efficient or repeated use of precious clinical samples.

Detection of both core protein and glycan levels

In order to properly interpret measurements of a glycan on a protein, one must also know the underlying protein concentration. The observation of a change in glycan level could be due to either a change in the core protein quantity or a change in its amount of glycosylation. Both the protein and glycan levels may be conveniently obtained using antibody microarrays. A sample may be incubated repeatedly on replicate microarrays, each time probed either with a lectin, to characterize glycan levels, or with an antibody, to characterize the core protein levels. The value of using both formats to detect glycosylation differences between samples was shown in previous studies [2, 6], in which it was observed that core protein levels did not change between samples, but the amount of glycosylation did.

High-throughput sample processing

The use of microarrays is further enhanced through the ability to run many microarray assays in a high-throughput mode. Several studies have examined glycosylation changes in biological samples using conventional glycobiology methods, but due to the need for large amounts of material and the time involved to analyze each sample, these studies generally used a small number of patient samples. We have developed a practical way to run low-volume microarrays in high throughput [7]. Multiple, replicate microarrays are printed onto a microscope slide, and the arrays are segregated from one another by hydrophobic, wax borders that are precisely imprinted onto the slide. The borders are imprinted using a stamping device (The Gel Company, San Francisco, CA) and remain on the slide throughout the processing and fluorescence scanning [2, 8]. We typically print arrays at a 4.5 mm spacing (equivalent to that of a 384-well microtiter plate), which allows 48 arrays on a slide in a 4 × 12 grid. Only 6 μl of sample is incubated on each array. The ability to process many samples, with multiple measurements per sample, should be particularly useful in biomarker research.

Limitation and challenges

The ALSA platform has limitations and challenges for future development. One limitation is the lack of information on the exact nature of the glycan structures being measured, including the sequences of the glycans and their attachment sites. A variety of underlying glycan structures may display the target motif of a given lectin, so lectin binding alone does not reveal what overall structures are changing. For that level of detail, MS and chromatography methods are needed. Also, proteins often carry a diversity of glycans at various attachment sites, and lectin binding does not reveal which of those sites bears altered glycans. Furthermore, if proteins in multi-protein complexes were captured on an antibody array, the attachment sites of target glycans would be even more ambiguous. The best method for resolving that ambiguity is MS-based analysis of glycopeptides. A modification to the ALSA technology that would partially address this ambiguity would be to pre-treat samples to break apart protein-protein complexes (or even to cleave proteins into simpler poly-peptide fragments), provided the antibodies could properly capture those cleavage products.

A future challenge for the ALSA approach, and for affinity-based glycoproteomics approaches in general, is to develop a broader array of high-quality, high-affinity reagents for glycan detection. Numerous plant and animal lectins have been isolated, but not all are suitable as analytical reagents due to sub-optimal affinities or specificities. Only a handful of the plant lectins have the affinity required to detect glycans on low-level (1 ng/ml and less) proteins captured from biological samples. Approaches to address this need may be the continued development of glycan-binding antibodies, the continued identification and

development of endogenous lectins as analytical reagents, and the modification and optimization of existing lectins [9].

Future perspective

The features of the ALSA platform can be more effectively used when bolstered with information from MS and chromatography methods. ALSA requires the pre-defining of both the target proteins and glycans, and MS-based proteomics methods can provide information on both. For example, for identifying and validating candidate biomarkers, a glycoproteomics approach could identify and characterize the levels of glycoproteins and their glycans in a small number of samples, using semi-quantitative methods, and assays targeting the best candidates then could be developed on the ALSA platform, which would provide precise measurements of both the protein and glycan levels in larger populations. Such a study would combine both discovery and pre-validation approaches for biomarker discovery, a strategy shown to be effective in a recent report looking at candidate breast cancer biomarkers [10]. MS-based glycan analysis could be further used in combination with ALSA by providing information on the exact sequences of the glycans that are attached to candidate biomarkers, which would then guide the choices of lectins and glycan-binding antibodies to be used as probes in the ALSA platform. Such an iterative process between MS-based discovery/characterization and antibody-based profiling could provide a highly effective, comprehensive biomarker discovery strategy.

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