Lectins as versatile tools to explore cellular glycosylation

Susan Ann Brooks

Department of Biological & Medical Sciences, Faculty of Health & Life Sciences, Oxford Brookes University, Oxford, UK

ABSTRACT

Lectins are naturally occurring carbohydrate-binding proteins that are ubiquitous in nature and highly selective for their, often incompletely characterised, binding partners. From their discovery in the late 1880s to the present day, they have provided a broad palette of versatile tools for exploring the glycosylation of cells and tissues and for uncovering the myriad functions of glycosylation in biological systems. The technique of lectin histochemistry, used to map the glycosylation of tissues, has been instrumental in revealing the changing profile of cellular glycosylation in development, health and disease. It has been especially enlightening in revealing fundamental alterations in cellular glycosylation that accompany cancer development and metastasis, and has facilitated the identification of glycosylated biomarkers that can predict prognosis and may have utility in development of early detection and screening, Moreover, it has led to insights into the functional role of glycosylation in healthy tissues and in the processes underlying disease. Recent advances in biotechnology mean that our understanding of the precise binding partners of lectins is improving and an ever-wider range of lectins are available, including recombinant human lectins and lectins with enhanced, engineered properties. Moreover, use of traditional histochemistry to support a broad range of cutting-edge technologies and the development of high throughput microarray platforms opens the way for ever more sophisticated mapping – and understanding – of the glycome.

Key words: lectin histochemistry; history; carbohydrates; glycosylation; review; methodology; glycomics.

Correspondence: Susan Ann Brooks, Department of Biological & Medical Sciences, Oxford Brookes University, Gipsy Lane, Headington, Oxford, OX3 0BP, UK. E-mail sbrooks@brookes.ac.uk

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Lectins: definition and terminology

The term 'lectin' is derived from the Latin verb *legere*, 'to pick out'. It was first suggested by Boyd and Shapleigh, in 1954,[1] to describe a structurally diverse group of proteins that are able to selectively bind to specific carbohydrate structures on cells, and, through that selective binding, to thereby agglutinate them, as illustrated in Figure 1. A lectin is defined as a 'sugar-binding protein of non-immune origin that agglutinates cells or precipitates glycoconjugates'.[2,3] This implies that lectins are multivalent; two or more sugar-binding sites are required for the cross-linking of cells in agglutination or glycoconjugates in precipitation. It distinguishes lectins from antibodies directed against carbohydrate antigens, which potentially could also act as agglutinins, and from sugar-binding enzymes which generally only contain a single binding site. Their original identification through their ability to agglutinate cells remains in the modern nomenclature of lectins, since many are still referred to as 'agglutinins' or 'hemagglutinins'; for example, the lectin from peanut, *Arachis hypogaea*, is commonly called 'peanut agglutinin' or PNA, and the several lectins from *Phaseolus vulgaris*, the common bean, are referred to as PHA, an abbreviation of phytohemagglutinin – including PHA-L (which agglutinates leukocytes) and PHA-E (which agglutinates erythrocytes). Many of the first lectins to be described were derived from plant sources, notably their seeds, since this material was readily available and seeds are especially rich sources of lectins. However, lectins are ubiquitous in living organisms. Whilst they have been used extensively as tools to explore and characterise cellular glycosylation, especially using the approach of lectin histochemistry, their biological function in the organisms from which they are derived often remains unexamined. However, it is becoming clear that lectin-sugar interactions function in diverse biological processes throughout nature, including cell identity, recognition, signalling and adhesion in health and disease, and are the subject of much research into these phenomena.

Lectins – historical perspectives

The history of lectins is well reviewed by Kocourek[4] and Sharon and Lis.[5] Lectins were first described in the late 19th century during investigations using toxic plant extracts commonly employed in medicine at that time. In 1888-9, Stillmark[6] semi-purified a protein from a toxic extract of castor oil seeds, *Ricinus communis*, and tested its effects on a range of cell types, noting that sometimes he observed a reaction that he described as being like blood clotting, illustrated in Figure 1. He named the protein 'ricin', and also found that the clotting reaction could be inhibited by serum. Other notable early lectin studies include descriptions of a toxic extract from *Abrus precatorius*, the jequirty bean, referred to as 'abrin' agglutinating erythrocytes and precipitating serum, and a toxic extract from the bark of *Robinia pseudoacacia*, the black locust tree, referred to as 'robin', also having agglutinating properties. In these and other early studies, the agglutinating activity of the extracts was wrongly attributed to the toxin, and it was only much later that the non-toxic agglutinating lectins were identified. During the final years of the 19th and early years of the 20th century, a very large number of reports appeared describing agglutinins from diverse sources. Many were from plants, principally from seeds, and especially from species of the *Leguminosae*, *Euphorbiaceae* and *Solanaceae*, but also from biological sources as disparate as invertebrates, snake venoms and fungi.

Several early studies, including Stillmark's, reported that cell agglutination could be inhibited or reversed by various substances, including serum. These were the first indications of the sugar-binding properties of lectins, and the presence of sugars on the cell surface, but were not recognised as such at the time. It was not until 1952 that Watkins and Morgan[7] demonstrated that simple sugars, monosaccharides, were able to inhibit lectin binding. Similarly, many early studies, including Stillmark's, noted that only some cell types were subject to agglutination by a particular extract, and some studies reported that erythrocytes from different human subjects reacted differently. However, it was Boyd and Reguera in 1949[8] who were first to specifically recognise a blood group specific lectin – that the lectin from *Phaseolus limensis*, Lima beans, agglutinates human blood group A erythrocytes, but not blood group B or O cells. Intense interest in blood group specific lectins followed, and more than 100 had been described by 1964. Morgan and Watkins[9] took these observations further and provided some of the first clear evidence that there are sugars at the cell surface, and that they are responsible for different ABO blood groups, illustrated in Figure 2. They did so by demonstrating that agglutination of blood group A erythrocytes by the lectin from *Phaseolus limensis* could be inhibited by the N-acetyl-D-galactosamine (GalNAc), while agglutination of blood group O erythrocytes by *Lotus tetragonolobus*, asparagus pea, lectin could be inhibited by alpha-methyl-L-fucose (Fuc).

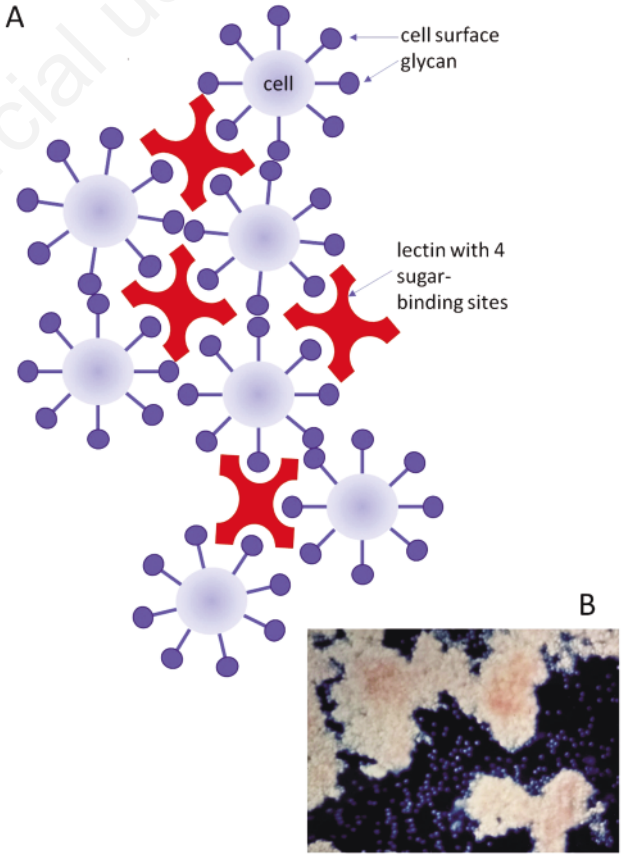

Figure 1. A) Lectins, which have more than one sugar-binding site, recognise cell surface glycans and thereby agglutinate cells in a reaction that resembles clotting. **B**) Blood group A erythrocytes agglutinated by the lectin from *Dolichos biflorus* (horse gram) which recognises terminal GalNAc present on the blood group A determinant.

While some of these early studies reported lectin activity in preparations derived from animal materials, such as invertebrates – for example, the hemolymph of the horseshoe crab (*Limulus polyphemus*), the Californian coastal crab (*Cancer antennarius*), and the American lobster (*Homarus americanus*) by Noguchi[10] and snake venoms by Mitchell and Reichert[11] and Mitchell and Stewart[12] – the first mammalian lectin, a galactose (Gal-)-binding lectin, first identified in rabbit liver, was not described until 1974.[13] It is present in other mammals, including humans, and is responsible for recognising asialylated glycoproteins on aged and damaged erythrocytes, thus removing them from circulation. This discovery was soon followed by the description of electrolectin, a galactoside-binding lectin from the electric organ of the electric eel, by Teichberg *et al.* in 1975,[14] and of galactoside-binding lectins, galectins (formerly known as S-type lectins) from the tissues of birds and animals by Briles *et al.* in 1979.[15] Mammalian lectins are now understood to be key to the mammalian immune response – for example, a mammalian mannose-binding lectin is key to pathogen recognition and initiation of the complement cascade (reviewed by Dommett *et al.*[16]) and selectins function in leukocyte interactions with the lining of blood vessels during the inflammatory response (reviewed by Ley[17]). Our understanding of the function of mammalian, including human, lectins is often far more advanced than our understanding of the biological roles of some of the lectins that were described much earlier – for example, those from plants and invertebrates – which are more commonly employed in lectin histochemistry, and this has been facilitated by advances in molecular biology in the 1990's leading to the development of lectin-deficient animals, for example of mice deficient in members of the selectin family.[18-20]

The development of lectin histochemistry as a means of exploring cellular glycosylation

Prior to the development of the technique of lectin histochemistry, exploration of cellular glycosylation using microscopy was limited to traditional histochemical techniques - such as staining using Alcian blue and periodic acid Schiff - which gave very limited information. During the 1980's the technique of immunocyto- - or immunohistochemistry – an approach which employs a specific antibody to localise a cell or tissue-bound antigen *in situ* and reveals its presence using a visible, often enzyme-based or fluorescent, label became increasingly popular (see Polak and van Noorden[21] for technical details).

It was soon appreciated that, with minor technical modifications, the same methodology could be used to selectively localise specific carbohydrate structures in cells and tissues using lectins as probes. The technical aspects of lectin histochemistry are reviewed by Roth.[22] As with immunohistochemistry, one major technical advantage of lectin histochemistry is that it can be applied to both freshly harvested cells and tissues and also to fixed and processed paraffin wax embedded tissues, such as routinely processed clinical surgical specimens, thus facilitating retrospective study. This has been especially powerful in clinical studies – described later – where changes in cellular glycosylation can be mapped at a cellular/tissue level and then related to progression of diseases such as cancer over long follow up periods. These types of studies have substantially enhanced our understanding of the function of carbohydrates in health and disease.

Today, more than 100 purified lectins are readily available commercially for use in lectin histochemistry and other applications. The majority are still derived from plant sources, commonly seeds (for example, *Glycine max*, soya bean; *Arachis hypogaea*, peanut) but also bark (for example, *Sambucus nigra*, elderberry), tubers (for example, *Solanum tuberosum*, potato) and bulbs (for example, *Galanthus nivalis*, snowdrop). However, lectins are also commercially available derived from sources as diverse as fungi (for example, *Agaricus bisporus*, mushroom), snails (for example, the lectin derived from the egg laying gland of *Helix pomatia*, the Roman snail), the serum of eels (for example, *Anguilla anguilla*, freshwater eel), and the hemolymph of crustaceans (for example, *Cancer antennarius*, the California coastal crab). Most are purified from their natural sources, but a limited range of recombinantly expressed lectins are also available (for example, from the bacteria *Pseudomonas aeruginosa* and several human lectins, such as members of the galectins). In addition to being available in native, unconjugated form, many lectins, especially those designed for use in histochemistry, are also available biotinylated, conjugated to enzyme (commonly to horseradish peroxidase or alkaline phosphatase) and fluorescent labels, to digoxigenin, and to electron dense labels such as colloidal gold for application in lectin histochemistry at the electron microscope level. With the explosion in the use of confocal microscopy in recent years, facilitating sophisticated live cell imaging and multiple labelling approaches, there has been a concomitant expansion in the range of lectins that are available conjugated to modern fluorescent labels that are compatible with such technologies, such as the 'Alexa Fluor' and 'Dylight' ranges.

Lectins have been extensively employed in histochemistry for many years, and there is a very substantial body of literature describing lectin histochemical studies of animal and human tissues in health and disease. Many of the earlier studies – and the 1990's was a particularly fruitful era for this type of research – were broadly exploratory and descriptive rather than systematic or driven by a defined research question. For example, as a snapshot, during the early 1990's, studies were published exploring lectin histochemistry of normal kidney in human,[23] mouse,[24] rat,[25] hamster,[26] chicken embryo,[27] quail[28] and dogfish.[29] However, such studies remain popular in modern times. For example, Noguchi *et al.*[30] very recently published a descriptive study of lectin histochemistry of the normal cat kidney using a panel of 8 lectins and Jones *et al.*[31] performed a similar lectin histochemical mapping of cat placenta using a panel of 24 lectins. At the same time as the early lectin histochemical studies of normal tissues were becoming popular, the first studies using lectin histochemistry to explore changes in cellular glycosylation in disease such as cancer, and to relate such changes to disease stage, type, or progression began to emerge. Some examples are described in later sections.

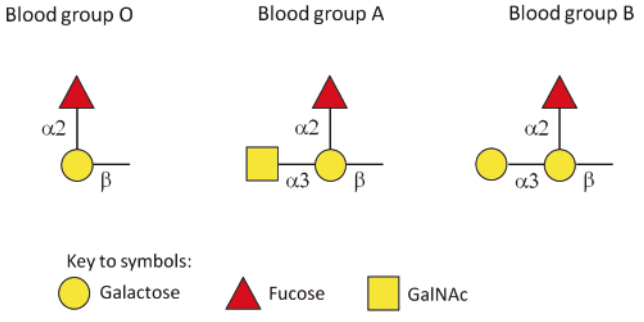

Figure 2. The ABO blood group sugars. All share the common O (or H) determinant Fuc(α1→2)Galβ- which is extended to form blood group A determinant by the addition of (α1→3)GalNAc and blood group B determinate by the addition of (α1→3)Gal.

What do lectins recognise? Defining lectin carbohydrate-binding specificity

As detailed previously, lectins are defined according to their ability to recognise cell surface carbohydrate binding-partners and thereby cross link cells in agglutination. Likewise, their carbohydrate-binding specificity is generally defined as the carbohydrate structure or structures – most commonly the monosaccharide(s), or sometimes di- or trisaccharide – that most effectively inhibits that agglutination. For example, suppliers of commercially available *Phaseolus limensis* (Lima bean) lectin generally define its inhibitory carbohydrate as GalNAc; the inhibitory carbohydrate of *Anguilla anguilla* (freshwater eel) lectin as Fuc; and inhibitory carbohydrates of *Cytisus scoparius* (Scotch broom) lectin as GalNAc >> lactose > melibiose > Gal. For some lectins, for example, the several lectins from *Phaseolus vulgaris* (common bean, phytohemagglutinin, PHA) or *Polygonatum multiflorum* (Solomon's seal) lectin, manufacturers usually simply state that binding is not inhibited by simple sugars, or suggest highly glycosylated proteins as potential inhibitors, such as fetuin and mucin and their asialylated derivatives (inhibitory carbohydrates above cited from the lectin supplier EY laboratories).[32]

It is important when employing lectins as tools in histochemistry, or in other applications, to appreciate that what lectins are actually recognising in cells and tissues is generally far more complex that a simple monosaccharide or disaccharide. A lectin's recognition of its binding partner(s) may not only distinguish between different glycosidic linkages between the monosaccharides in the glycan, but also be influenced by the spatial distribution of the monosaccharides in three dimensions, their linkage and relationship to the polypeptide of the protein, and electrostatic and hydrophobic interactions.[22] Moreover, as described previously, the natural, endogenous binding partners of most lectins in the biological systems in which they have evolved to operate are generally unknown, and are likely to be more complex than their stated 'inhibitory carbohydrate' or 'binding specificity'. Thus, the histochemical labelling distribution of lectins with identical nominal carbohydrate binding specificities may actually be quite distinct. As an example, Roth[22] described how the labelling patterns of the lectins from *Canavalia ensiformis*, (jack bean, known as Concanavalin A or Con A) and *Lens culinaris* (lentil), which are both often defined as being inhibited by mannose and fucose were distinct. Wu and Sugii[33] also exemplified this complexity by classifying 20 different Gal- and GalNAc-binding lectins into six different classes according to the pattern of their inhibition by Tn antigen (α-GalNAc-Ser/Thr) and various Gal- and GalNAc- containing disaccharides.

We have previously reported that the lectin from *Helix pomatia* (the Roman snail, HPA), which has a nominal monosaccharide binding specificity for GalNAc gives quite different histochemical labelling patterns on clinical sections of breast cancers to that obtained with an antibody against the Tn antigen or against GalNAc/the GalNAc-containing blood group A substance,[34] and, moreover, that a panel of 26 different lectins, including HPA, all with reported specificity for GalNAc gave distinct histochemical labelling patterns.[35] The nature of the binding partner(s) of HPA is of interest because, in histochemical studies, it identifies cancers that have metastasised and therefore have poor prognosis, as detailed below. In early studies,[36-38] HPA was reported to selectively agglutinate erythrocytes of blood group A but not blood group O or B. Competitive inhibition studies using simple sugars demonstrated that by far the most effective monosaccharide inhibitor was α-GalNAc and that α-N-acetylglucosamine (α-GlcNAc) had a weaker inhibitory effect.[37] Later[39] the disaccharide GalNAc-α-1,3 GalNAc (the Forssman antigen) was shown to have an even stronger inhibitory effect. In 1991, Wu and Sugii[33] reported the lectin's binding preferences as Forssman antigen (GalNAc-α-1,3 GalNAc) > Blood group A substance (α-GalNAc1-3[α-Fuc1-2]Gal) > Tn antigen (α-GalNAc-Ser/Thr) > α-GalNAc > α-GlcNAc. Both Forssman and Tn antigens are well described in the literature as carbohydrate structures that are frequently exposed on cancer cells, and Tn antigen, in particular is associated with poor prognosis, as described later. Dwek *et al.*[40] explored the binding partner(s) of HPA in breast cell lines and breast cancer samples using complex biochemical analysis of oligosaccharides released from HPA-binding glycoproteins, and identified an incompletely characterised mono-sialylated oligosaccharide, which they referred to as HPAgly-1. The structure of the lectin itself was explored in early studies in the 1960's and 70's by Hammarstrom and colleagues.[37,38,41] It has a molecular weight of 79,000 kDa and consists of six identical polypeptide chains, each with a carbohydrate binding site. More recently, the crystal structure has been elucidated confirming and extending these early studies[42] and detailed analysis of the structural basis for the lectin's recognition of Tn and Forssman antigen has been explored.[43] This detailed analysis, inspired by the ability of the lectin to recognise as yet only partially characterised carbohydrate-binding partners that are linked in clinical studies to the ability of cancers to metastasise, exemplify the limitations of defining a lectin in terms of its simple sugar binding specificity, in this case, 'GalNAc-binding'.

In spite of their often imprecisely defined binding specificity, lectins can nevertheless prove to be useful tools in exploring the glycosylation patterns of cells and tissues. At a fairly superficial level, a small range of lectins with distinct, broad nominal monosaccharide binding preferences can be employed to provide an overview of cellular glycosylation. Several manufacturers supply convenient lectin kits featuring a panel of lectins, often conjugated to enzyme or fluorescent labels, for at least initial identification of glycan motifs. An example would be: *Canavalia ensiformis* lectin (jack bean, Concanavalin A or Con A, nominally mannose-binding), *Dolichos biflorus* lectin (DBA, nominally GalNAc-binding), *Arachis hypogaea* (peanut, PNA, nominally Gal-binding), *Ulex europaeus* lectin (gorse, UEA, nominally Fuc-binding), *Triticum vulgaris* lectin (wheatgerm, WGA, nominally GlcNAc-binding) and *Sambucus nigra* (elderberry, SNA, nominally sialic acid-binding). As an example, Walski *et al.*[44] used a panel of seven fluorescently labelled lectins to map the distribution of glycans in the midgut of the cotton leafworm *Spodoptera littoralis*, a common agricultural pest, using confocal laser scanning microscopy. Following such broad and superficial mapping, lectins with related but distinct binding preferences can then be employed for finer characterisation. As an example, there are several lectins that selectively recognise different linkages of sialic acids. *Limax flavus* (garden slug) lectin will recognise sialic acids on both N- and O-linked glycans, and regardless of linkage, and is therefore helpful in mapping distribution of sialic acids overall.[45] *Maackia amurensis* lectin has a binding preference for α-2,3 linked sialic acid, while *Sambucus nigra* lectin shows a preference for recognise α-2,6 linked sialic acid on O-linked glycan chains (and also recognises terminal GalNAc to some extent) and *Polyporus squamosus* (a type of mushroom) lectin preferentially recognise α-2,6 linked sialic acid on N-linked glycans.[46-48]

Lectin histochemistry reveals changes in glycosylation in cancer, and related to cancer prognosis

Some of the first studies using lectins, including some of the early studies on cell agglutination described previously, were interested in identifying differences between normal cells and cancer cells and first introduced the concept that changes in cellular glycosylation accompany transformation to malignancy, and cancer progression. With the increasing popularity in the use of lectin histochemistry in the 1980's and 1990's, there was a great deal of interest in mapping differences in lectin labelling of cell lines and of clinical samples to identify associations with malignancy and to seek markers of stage or prognosis. That lectin histochemistry could be employed on routinely processed paraffin wax embedded archival tumour samples meant that retrospective clinical studies could be performed seeking associations between cellular glycosylation identified using lectins and long-term patient prognosis.

One of the earliest studies to investigate differences in lectin binding characteristics of normal versus cancer cells was that of Aub *et al.*,[49,50] who showed that a preparation from wheatgerm (*Triticum vulgaris*), was able to agglutinate cancer cells but not normal cells. There followed much interest in the lectin from wheatgerm (wheatgerm agglutinin, WGA), which has a nominal binding specificity for GalNAc and sialic acid, as a histological marker of cancer, and of cancer differentiation and metastasis. Walker[51] reported great consistency in WGA binding to paraffin wax embedded sections of normal and benign hyperplastic breast tissue, but heterogeneity in its binding to breast cancers and a relationship between WGA-positivity and degree of differentiation.[52] Studies have also reported the ability of WGA to distinguish cultured cancer cells of high and low metastatic potential.[53-59]

There has also been much interest in PHA-L, one of the several lectins isolated from *Phaseolus vulgaris*, and which preferentially recognises β-1,6 branched N-linked glycans. PHA-L binding has been repeatedly associated with poor prognosis of cancers, especially colorectal and breast cancer,[60,61] and metastatic potential of cancer cells in animal models.[61-64] This is a rare example where the reason for the association between glycosylation, revealed through lectin labelling, and cancer cell behaviour has been elucidated. Glycosylation of matriptase, an enzyme involved in matrix degradation and therefore invasion of cancer cells, with β-1,6 branched N-linked glycans renders it resistant to degradation and thereby prolongs its activity, thus enhancing the invasive capacity of the cancer.[65]

A large body of evidence also indicates that synthesis of truncated O-linked glycans by cancer cells is associated with more aggressive biological behaviour, metastasis and poor patient prognosis. The commonest type of O-linked glycosylation (often referred to as 'mucin-type' O-linked glycosylation), begins with the addition of a single α-GalNAc monosaccharide to a serine or threonine residue on the protein. The resulting structure α-GalNAc-Ser/Thr is referred to as the Tn (Thomsen nouvelle) antigen and is always further extended in healthy adult cells, either by the addition of a sialic acid – to form sialyl Tn – which terminates chain extension, or by the sequential addition of further monosaccharides to form a range of core structures that themselves can be further extended, as illustrated in Figure 3. However, Tn antigen is frequently left unelaborated in cancers, where lectin histochemistry studies, most frequently using the lectin from *Helix pomatia* (HPA), indicate that it is associated with metastasis and poor prognosis in many cancer types (for review see[66,67]). Core 1 (β-Gal(1→3)GalNAc, also known as the Thomsen Friedenreich, TF or T antigen) is formed by the attachment of a β-1,3 linked Gal to Tn antigen, and also occurs in a sialylated form, where sialylation prevents further chain extension. The presence of T antigen has also been associated in lectin histochemical studies, using peanut agglutinin, PNA, with aggressive biological behaviour and poor prognosis in cancers, and similar results have also been obtained using immunohistochemistry employing monoclonal antibodies directed against T antigen (for review, see[68]). For example, in an early study, Limas and Lange[69] demonstrated that PNA did not bind to normal urothelium but labelled 10% of non-invasive and 65% of invasive urothelial carcinomas. Rhodes *et al.*[70] reported no PNA binding to normal colonic epithelium, but positivity in almost all hyperplastic polyps and cancers.

Identifying lectin-binding biomarkers in tissues and deciphering their function

As described previously, it is well established that molecules at the cell surface of cancer cells exhibit different glycosylation patterns to those of normal cells, that these differences can be identified and mapped using lectins, and that they are sometimes associated with the biological behaviour and prognosis of cancers. However, there is much value in moving beyond merely describing an association between cellular glycosylation, as revealed by lectin histochemistry, to identification of the actual glycosylated binding partners recognised by the lectin, and the deciphering of their function in biological processes. However, this is by no means a trivial task.

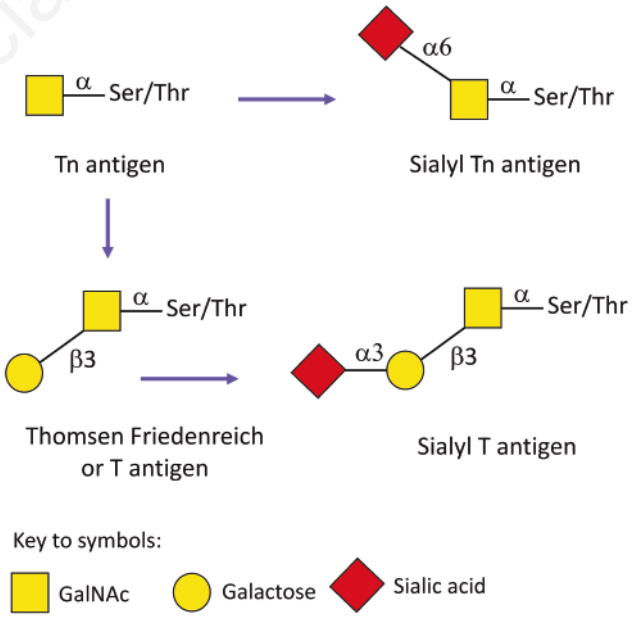

Figure 3. Initiation of 'mucin type' O-linked protein glycosylation begins with synthesis of the Tn (Thomsen nouvelle) antigen, α-GalNAc-Ser/Thr. This may be extended by the addition of an (α1→6) linked sialic acid to form sialyl Tn, and this terminates further chain extension. Alternatively, Tn antigen can be extended by the addition of (β1→3)Gal to form the Thomsen Friedenreich or T antigen, also termed core 1. This can also be sialylated by the addition of a (α1→3) linked sialic acid to form sialyl T antigen, which cannot be further extended. Alternatively, T antigen may be further elaborated to form a range of other core structures (not illustrated) which themselves can be further extended to build more complex O-linked glycans.

A good example is that of the association between altered GalNAc-glycosylation, revealed by binding of the lectin from *Helix pomatia*, HPA, and poor cancer prognosis, described previously. This was first revealed using traditional lectin histochemical studies of retrospective clinical cancer samples in the 1980's and 1990's (reviewed in[66,67]). The first papers to illustrate this employed lectin histochemistry for the binding of HPA in large retrospective studies of breast cancers which revealed that not only was HPA positivity of tumours a strong indicator of poor long-term patient survival but, in multivariate analysis including other established prognostic factors, that it was strongly associated with the physical presence of tumour in local lymph nodes, and therefore of metastatic competence.[71-73] Such studies were followed by attempts using affinity chromatography to isolate HPA-binding glycoproteins from cancers, healthy tissues and cell lines and to characterise them using SDS-PAGE and Western lectin blotting. This revealed that HPA recognised a wide and heterogeneous range of GalNAc-bearing glycoproteins in cancer cell lines and clinical cancer tissue samples, indicating that the altered HPA glycosylation profiles seen in cancer represented a broad alteration in O-linked glycosylation mechanisms, rather than altered glycosylation of a single functional biomarker.[74,75] Complex analysis of HPA-binding glycans cleaved from lectin affinity-purified glycoproteins using high performance liquid chromatography has also been reported,[40] again revealing a complex picture. HPA affinity-purified glycoproteins have also been separated by 2-D electrophoresis and identified by matrix assisted laser desorption ionisation mass spectroscopy and confirmed by immunohistochemistry and Western blotting.[76] In this study, a bioinformatics approach was also employed to predict the potential glycosylation sites on target proteins and correlations were made with other established cancer-associated genes, p53 and KRAS mutation, using immunohistochemistry and polymerase chain reaction. In parallel with these studies that were aimed at identifying – in spite of these extensive efforts and sophisticated analysis – the still incompletely characterised glycosylated binding-partners recognised by the lectin HPA, other approaches were focussed on understanding their functional significance in mechanisms of metastasis. Brooks and Hall[77] explored the ability of cancer cells to adhere to, and invade through, basement membrane components using a Matrigel assay system and found no evidence that HPA-binding glycoproteins were functionally involved in these processes. However, later work by Bapu *et al.*[78] demonstrated a functional role of HPA-binding partners on cancer cells in their adhesion to endothelial cell monolayers, indicating a potential function in cancer cell adhesion to the lining of blood vessels and lymphatics during metastasis.

Improving on nature - engineering lectins with enhanced properties

While lectins have long been proven to be useful and versatile probes for mapping glycosylation of cells and tissues, the majority of commercially available lectins are still isolated from natural sources – such as the seeds of plants, or from invertebrates – and, as such, suffer from a degree of batch to batch variability and, occasionally, supply issues due to shortage or seasonality of the natural source material. This limitation is increasingly being overcome by the wider availability of recombinant lectins, often expressed in bacterial or yeast systems (reviewed by Hu *et al.*[79]). Moreover, there is increasing demand for recombinant mammalian and human lectins – such as selectins and galectins – arising from increased interest in their biological function in human health and disease and from the notion that carbohydrate-binding molecules derived from plants and invertebrates may not be the optimal tools for exploring the complexity of the human glycome and its myriad functions.

Advances in molecular biology also mean that there is the potential to improve upon nature and to engineer recombinant lectins with altered or enhanced properties – such as tighter sugar binding or recognition of glycan structures for which no naturally occurring binding partner is known. Markiv *et al.*[80], for example, isolated two naturally occurring isoforms of the lectin from *Helix pomatia*, then expressed recombinant forms that exhibited higher affinity for GalNAc than the naturally occurring product. Since the facility of HPA to recognise altered GalNAc-glycosylation associated with cancer progression is well established, as discussed previously, they suggest that a recombinant, well characterised, homogeneous product with enhanced binding affinity would have greater potential for development of a diagnostic or prognostic test than the native, heterogeneous, naturally occurring form.

There have also been elegant attempts to engineer sialic acid binding lectins with enhanced properties. Maenuma *et al.*[81] introduced mutations into the carbohydrate-binding domain of the sialic acid-binding *Maackia amurensis* lectin, producing thirty-five variants with subtly different carbohydrate recognition properties and which they used to distinguish carcinoma, melanoma, myeloid and fibroblast cells. Yabe *et al.*[82,83] introduced mutations into the carbohydrate-binding domain of the B chain of the Gal-binding lectin ricin such that it instead recognised α-2,6 linked sialic acid. They then further engineered the lectin to have a binding preference for multiple α-2,6 linked sialic acid residues clustered on branched N-glycans, a feature not seen in any known naturally occurring sialic acid binding lectin. These studies illustrate the potential for this type of approach to engineer improved tools for exploring cellular glycosylation, with better defined sugar binding specificities.

Mapping glycan complexity using microarrays

Lectin histochemistry can provide a superficial means of mapping glycosylation of cells and tissues but is limited in the level of detail that can be achieved. Moreover, as described previously, lectins may often recognise more than one carbohydrate binding partner and the structures that they recognise in cells and tissues are often incompletely characterised. A single lectin may recognise different binding partners with different levels of affinity, or have strongest affinity for moieties that are in very low concentrations in a biological sample. Moreover, the cellular glycome is hugely complex and biochemical analysis of carbohydrate structures is technically specialist and extremely challenging, owing to the unique chemistry of carbohydrates (this topic goes beyond the scope of this review, but see Seeberger,[84] Haslam *et al.*,[85] Rudd *et al.*[86] for detailed review). To address some of these challenges, in recent years, there has been an explosion of interest in the development of, and the use of, microarrays as a relatively simple, high throughput approach to mapping the some of the complexity of the glycome without the need for technically intricate glycan analysis. Microarrays allow simultaneous mapping of multiple lectin-glycan interactions from a single sample, or comparison of multiple interactions across several samples. They have the additional advantage that they do not require specialist equipment, highly purified samples, large sample volumes or the cleavage of carbohydrate from protein.

Several different microarray formats exist, designed to explore different types of research questions. Lectin microarrays are increasingly popular and, typically, feature between about 25 and 100 different lectin spots. Such arrays may feature both lectins derived from natural sources, such as those typically used in lectin histochemical studies, but also recombinant lectins (see Pilobello

et al.[87]). Some of the larger arrays contain some unusual lectins that have been little reported in traditional lectin histochemistry approaches. Lectin microarrays will capture glycosylated molecules from complex samples and provide a snapshot comparison of their glycomic profile. For example, glycomic profiling of mammalian[87,88] and bacterial[89] cells have been reported using this type of approach. In some more sophisticated studies, glycoconjugates identified in lectin microarrays as being of interest have then been further explored using techniques such as surface plasmon resonance (for example,[90]) or mass spectrometry (for example,[91]). Kuno and colleagues[92,93] developed a lectin microarray that enables direct observation of lectin-carbohydrate interactions in real time and under equilibrium conditions, on the basis of an evanescent-field fluorescence-assisted detection principle. The approach has the advantage that it does not require the repeated washing steps of a conventional array and is therefore better suited to observing weak lectin-carbohydrate interactions. They have reported glycomic analysis of Chinese hamster ovary cells and their glycosylation defective mutants, thus demonstrating the platform's utility for analysis of complex biological samples.[94]

Alternatively, microarrays featuring immobilised carbohydrate structures are available that can be used to detect the presence of carbohydrate-binding molecules, including endogenous lectins, in a sample (for example, see[95,96]) and microarrays of immobilised antibodies will capture specific target molecules in a complex sample that can then be interrogated for their glycosylation by overlaying with labelled lectins (for example,[97]). Some applications of these approaches are reviewed by Kim *et al.*[98]

Since changes in glycosylation are well documented in disease, including cancer, described previously, there has been much interest in the use of microarrays for high-throughput screening of biofluids, such as serum or plasma, for potential disease biomarker discovery and development of screening assays (reviewed in Yu *et al.*,[99] Dang *et al.*[100]). Tikhonov *et al.*[101] review how glycan microarrays can be employed for screening of patient serum for the presence of antibodies against tumour-related glycans as a potential early diagnostic or disease staging assay. One technical limitation in this type of approach is that such samples are likely to contain a hugely heterogeneous range of glycosylated biomolecules, only a very small proportion of which are derived from the diseased tissue itself, or are of relevance to the disease under study. Therefore, samples may need to be processed in some way prior to the microarray analysis to enrich them in relation to the biomarker(s) of interest, or the microarray analysis may need to be performed in tandem with other approaches, including traditional lectin histochemistry. As an example of this, Huang *et al.*[102] mapped the glycosylation profiles of fresh, frozen gastric ulcer samples in comparison to gastric cancers using a 37-lectin microarray. They then went on to explore potential lectins of interest using lectin histochemistry and identified two of them, from *Maclura pomifera* and *Vicia villosa,* as being potential biomarkers for gastric cancer. Nakajima *et al.*[103] took a similar approach to charting the glycosylation profiles of glycoproteins extracted from formalin fixed, paraffin wax embedded colorectal cancers using a 45-lectin microarray. Here, they followed up promising results from the microarray using lectin histochemistry and identified *Agaricus bisporus* (mushroom) lectin binding as being associated with risk of disease recurrence. This study, in particular, since it employed routinely fixed and processed clinical specimens, exemplifies the potential of this approach for biomarker identification in retrospective studies with long follow up. Jiang *et al.*[104] started from a point of first identifying a candidate serum biomarker, GP73, which had been reported to be over-expressed and differently fucosylated in patients with liver disease. They concentrated GP73 from patient serum using immunoprecipitation before applying samples to a 50-lectin antibody overlay microarray and following this up with Western blotting and ELISA. Using this approach, they were able to identify a profile of five lectins that could distinguish between samples taken from patients with cirrhosis of the liver and liver cancer.

Conclusions

We are still appreciating and exploring the complexity of the 'sugar code' that underlies much of the biology of living organisms, including ourselves. The unique chemistry of carbohydrates means that it is many orders of magnitude more diverse than that achievable by nucleic acids or proteins, and technically far more challenging to analyse. Lectin histochemistry has long provided a straightforward and easily accessible technique to map glycosylation of cells and tissues during development, in health and disease, and has both identified glycosylated biomarkers associated with progression of diseases such as cancer and also paved the way for greater understanding of the processes underlying its biology. Whilst lectins are long standing stalwarts of glycobiology research, their function in the organisms that produce them are still often unexplored and their fine binding specificity not fully characterised, which has meant that in some respects they are yet to reach their full potential as versatile tools to explore the glycome. Recent advances in technology and the use of traditional lectin histochemistry to complement a range of cutting-edge approaches heralds a bright future for lectin-based glycobiology research.

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