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Lectins: an effective tool for screening of potential cancer biomarkers

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In recent years, the use of lectins for screening of potential biomarkers has gained increased importance in cancer research, given the development in glycobiology that highlights altered structural changes of glycans in cancer associated processes. Lectins, having the properties of recognizing specific carbohydrate moieties of glycoconjugates, have become an effective tool for detection of new cancer biomarkers in complex bodily fluids and tissues. The specificity of lectins provides an added advantage of selecting peptides that are differently glycosylated and aberrantly expressed in cancer patients, many of which are not possibly detected using conventional methods because of their low abundance in bodily fluids. When coupled with mass spectrometry, research utilizing lectins, which are mainly from plants and fungi, has led to identification of numerous potential cancer biomarkers that may be used in the future. This article reviews lectin-based methods that are commonly adopted in cancer biomarker discovery research.

REVIEW ARTICLE

Lectins: An Effective Tool for Screening of Potential Cancer Biomarkers

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Abstract

In recent years, the use of lectins for screening of potential biomarkers has gained increased importance in cancer research, given the development in glycobiology that highlights altered structural changes of glycans in cancer associated processes. Lectins, having the properties of recognizing specific carbohydrate moieties of glycoconjugates, have become an effective tool for detection of new cancer biomarkers in complex bodily fluids and tissues. The specificity of lectins provides an added advantage of selecting peptides that are differently glycosylated and aberrantly expressed in cancer patients, many of which are not possibly detected using conventional methods because of their low abundance in bodily fluids. When coupled with mass spectrometry, research utilizing lectins, which are mainly from plants and fungi, has led to identification of numerous potential cancer biomarkers that may be used in the future. This article reviews lectin-based methods that are commonly adopted in cancer biomarker discovery research.

Subjects: Biochemistry, Oncology, Proteomics, Medicine

Keywords: Lectin, Cancer, Biomarker, Proteomics, Glycan, Glycosylation

Introduction

Lectins are carbohydrate binding proteins which are found ubiquitously in nature. The term 'lectin' originates from the Latin word *legere,* which means to choose or to select (*Boyd and Shapleigh, 1954*). By binding to carbohydrates, lectins serve diverse biological functions. Plant lectins, which typically cause agglutination of certain animal cells, play important roles in defense against invasion of virus, bacteria or fungi (*Dias et al., 2015*). They are also believed to mediate symbiosis relationship between plants and microorganisms (*De Hoff et al., 2009*), and some may be involved in regulatory and signaling pathways in plant cells (*Chen et al., 2002*).

Lectins have initially been classified based on their binding to different glycan structures. They were categorized either as galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), glucose, L-fucose, mannose, maltose, sialic acid-specific or complex glycan-binding lectins (*Lis and Sharon, 1986*). Later, they are also classified based on the characteristics and numbers of their carbohydrate binding domains, namely merolectins, hololectins, chimerolectins and superlectins (*Peumans et al., 2001*). With the emergence of detail structural properties of lectins being elucidated via the advancement of technology, this classification further evolved into that based on distinct protein folding, domains/structural similarities and evolutionary-relatedness of proteins (*Peumans et al., 2001*). Via this categorization, 12 different lectin families, which include *Agaricus bisporus*

49 agglutinin homologues, amaranthins, class V chitinase homologues with lectin activity,

50 cyanovirin family, *Euonymus europaeus* agglutinin family, *Galanthus nivalis* agglutinin

51 family, jacalins, lysin motif domain, nictaba family, proteins with hevein domains, proteins

52 with legume lectin domains and ricin-B family (*Van Damme et al., 2008*), have been derived.

53 Ricin is believed to be the first lectin discovered in the seeds of the castor bean plant,

54 *Ricinus communis*, in 1888 (*Sharon and Lis, 2004*). Paradoxically, research on lectin only

55 flourished several decades subsequent to ricin's discovery after James Sumner successfully

56 purified a crystalline protein from jack bean (*Canavalia ensiformis*) in 1919. Sumner later

57 showed that the protein caused agglutination of cells such as erythrocytes and yeast. The

58 agglutinin, which is now known as concanavalin A or ConA, was also used for the first time

59 to demonstrate binding of lectins to carbohydrate. To date, there are more than a thousand

60 plant species that have been reported to possess lectins. Most of these lectins are in

61 abundance in seeds (*Lis and Sharon, 1986*; *Benedito et al., 2008*), whilst some are found in

62 leaves, roots, flower, sap, barks, rhizomes, bulbs, tubers and stems (*Dias et al., 2015*).

63 Because of their carbohydrate binding specificities, many lectins have been increasingly

64 applied in different areas of medical research and therapy (*Coelho et al., 2017*). Table 1

65 shows a list of lectins that have been used in cancer biomarker discovery research.

66

67 **Table 1** **List of lectins used in cancer biomarker discovery research.**

Lectin	Abbreviation	Specificity	Glycan Linkage	References
African legume (*Griffonia (Bandeiraea) simplicifolia*) lectin-I	GSLI (BSLI)	α-Gal; α-GalNAc	*O*-linked	*Lescar et al., 2002*
Asparagus pea (*Lotus tetragonolobus*) lectin	LTL	Fucα1-3(Galβ1-4)GlcNAc, Fucα1-2Galβ1-4GlcNAc	*N*-linked	*Pereira and Kabat, 1974*; *Yan et al., 1997*
Koji (*Aspergillus oryzae*) lectin	AOL	α1,6-fucosylated	*N*-linked	*Matsumura et al., 2007*
Castorbean (*Ricinus communis*) agglutinin	RCA	Galβ1-4GlcNAc; terminal β-D-Gal	*N*-linked	*Harley and Beevers 1986*; *Wang et al., 2011*
Champedak (*Artocarpus integer*) galactose binding lectin	CGB	Gal; GalNAc	*O*-linked	*Hashim et al., 1991*; *Gabrielsen et al., 2014*
Champedak *(Artocarpus integer*) mannose binding lectin	CMB	Man	*N*-linked	*Lim et al., 1997*; *Gabrielsen et al., 2014*
Daffodil (*Narcissus pseudonarcissus*) lectin	NPL	α-Man, prefers polymannose structures containing α-1,6 linkages	*N*-linked	*Kaku et al., 1990*; *Lopez et al., 2002*
Elderberry (*Sambucus nigra*) agglutinin	SNA	Neu5Acα2-6Gal(NAc)-R	*N*- and *O*-linked	*Shibuya et al., 1987*; *Silva et al., 2017*
Gorse or furze (*Ulex europaeus*) seed agglutinin-I	UEA-I	Fucα1-2Gal-R	*N*- and *O*-linked	*Holthofer et al., 1982*; *Raj Bharath and Krishnan, 2016*
Jackbean *(Canavalia ensiformis)* lectin	ConA	α-Man; α-Glc	*N*-linked	*Percin, et al., 2012*
Jackfruit (*Artocarpus heterophyllus*) lectin	Jacalin	Gal; GalNAc	*O*-linked	*Kabir, 1995*; *Jagtap and Bapat, 2010*
Lentil (*Lens culinaris*) hemagglutinin	LcH	Man; Glc (Affinity enhanced with α-Fuc attached to *N*-acetylchitobiose)	*N*-linked	*Howard et al., 1971*; *Chan et al., 2015*
Amur maackia (*Maackia amurensis*)	MAL II	Siaα2-3Galβ1-4GlcNAc; Siaα2-	*N*- and *O*-linked	*Konami et al., 1994*;

lectin II		3Galβ1-3GalNAc		*Geisler and Jarvis, 2011*
Orange peel fungus (*Aleuria aurantia*) lectin	AAL	Fucα1-6GlcNAc; Fucα1-3LacNAc	*N*- and *O*-linked	*Hassan et al., 2015*
Peanut (*Arachis hypogaea*) agglutinin	PNA	Galβ1-3GalNAc; Gal	*O*-linked	*Chacko and Appukuttan, 2001*; *Vijayan, 2007*
Chinese green dragon (*Pinellia pedatisecta*) agglutinin	PPA	Man	*N*-linked	*Li et al., 2014*
Poke weed (*Phytolacca americana*) mitogen lectin	PWM	GlcNAc oligomers	*N*-linked	*Kino et al., 1995*; *Ahmad et al., 2009*
Red kidney bean (*Phaseolus vulgaris*) lectin	PHA-L	Bisecting GlcNAc	*N*-linked	*Kaneda et al., 2002*; *Movafagh et al., 2013*
Wheat germ (*Triticum vulgaris*) agglutinin	WGA	GlcNAcβ1-4GlcNAcβ1-4GlcNAc; Neu5Ac	*N*-linked	*Nagata and Burger, 1972*; *Parasuraman et al., 2014*
White button mushroom (*Agaricus bisporus*) lectin	ABL	GalNAc; Galβ1,3GalNAc (T antigen); sialyl-Galβ	*O*-linked	*Nakamura-Tsuruta et al., 2006*; *Hassan et al., 2015*

68

69

70 A biomarker is defined as "a characteristic that is objectively measured and evaluated as
71 an indicator of normal biological processes, pathogenic processes or pharmacologic
72 responses to a therapeutic intervention" (*Biomarkers Definition Working Group, 2001*).
73 Hence, simple parameters from pulse and blood pressure to protein constituents of cells,
74 tissues, blood and other biofluids are classified as biomarkers. Bodily fluids that have been
75 mined for cancer biomarkers thus far include serum/plasma, urine, saliva and other tissue-
76 specific fluids such as seminal fluid, cerebrospinal fluid, bone marrow aspirates, etc. Cancer
77 biomarkers are useful for early detection, diagnosis and prognosis of the disease. They are
78 also heavily relied on in management of patients, and assessment of pharmacodynamics of
79 drugs, risk, as well as recurrence of the disease.

80 Efforts in the search for new cancer biomarkers remain active even in the present day.
81 Currently, there are only a handful of cancer biomarkers that have been officially approved
82 by the US Food and Drug Administration (FDA) for clinical use (*Füzéry et al., 2013*). More
83 are definitely needed for improved detection and diagnosis, particularly when the reliability
84 of many of the FDA approved biomarkers remains a problem due to their limited levels of
85 sensitivity and specificity. For example, CA-125 which is used as a biomarker for ovarian
86 cancer, is also often elevated in other cancers such as those of the breast (*Norum et al.,
87 2001*), lung (*Salgia et al., 2001*) and colon or rectum (*Thomas et al., 2015*). Similarly,
88 prostate specific antigen (PSA), a tissue-specific serum protein that is used in the diagnosis
89 of prostate cancer, is also commonly increased in sera of patients with benign prostatic
90 hyperplasia, thus, posing difficulties in clinically differentiating the two different conditions
91 (*Barry, 2001*; *Thompson et al., 2004*). These limitations, together with the recent
92 development of various state-of-the-art methodologies including genomics, proteomics and

93 bioinformatics, have consequentially propelled research towards identification of new cancer
94 biomarkers that are more sensitive and specific.

95 Amongst bodily fluids that have been mined for cancer biomarkers, serum/plasma is most
96 popular. Serum or plasma has the advantage of being routinely sampled in clinical
97 investigations. However, the extreme complexity and broad dynamic range of protein
98 abundance in serum and plasma pose a formidable challenge in research screening for
99 potential cancer biomarkers, which mostly comprise low abundance glycoproteins. Because
100 of this, many cancer biomarker exploratory studies involving serum or plasma often involved
101 enrichment and/or pre-fractionation of the samples using techniques such as
102 immunodepletion (*Preito et al., 2014*), immunoprecipitation (*Lin et al., 2013*) and size-
103 exclusion chromatography (*Hong et al., 2012*). However, the use of such techniques, despite
104 their wide applications in biomarker discovery investigations, is generally unable to make a
105 significant difference in unmasking proteins of low abundance [*Polaskova et al., 2010*], and
106 may result in concomitant loss of non-targeted proteins (*Bellei et al., 2011*).

107 Interestingly, the majority of cancer biomarkers that are currently being used in the
108 clinical settings are glycoproteins, which are structurally altered in their glycan moieties and
109 aberrantly expressed (*Henry and Hayes, 2012*). However, only alpha-fetoprotein (AFP) and
110 CA15-3 are clinically monitored for their glycan changes in the therapy for hepatocellular
111 carcinoma and breast cancer, respectively. The other cancer biomarkers are being monitored
112 for their total protein levels (*Kuzmanov et al., 2013*). Indeed, changes in glycosylation are
113 believed to be a main feature in oncogenic transformation as glycans are known to be
114 continuously involved in cancer evolving processes, such as cell signaling, angiogenesis,
115 cell-matrix interactions, immune modulation, tumor cell dissociation and metastasis.

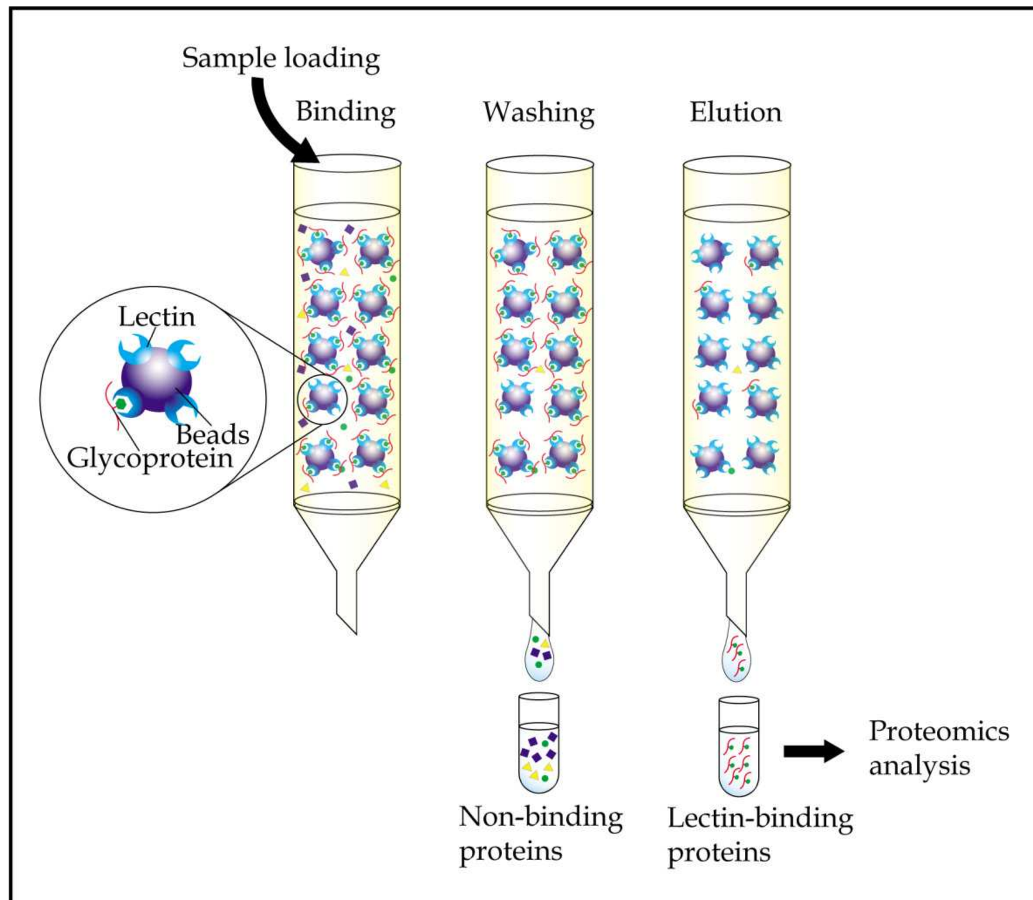
116 Glycosylation changes that are commonly associated with cancer transformation include
117 sialylation, fucosylation, increased GlcNAc-branching of *N*-glycans, and overexpression of
118 truncated mucin-type *O*-glycans (*Pinho and Reis, 2015*). Hence, it is not surprising that
119 lectin-based approaches are becoming more popular in studies screening for novel cancer
120 biomarkers. In this review, the applications of lectins in cancer biomarker discovery,
121 including immobilized lectin affinity chromatography, enzyme-linked lectin assay, lectin
122 histochemistry, lectin blotting and lectin array, are addressed. For lectin-based biosensor
123 analysis, readers are recommended to refer to separate review articles (*Pihíková et al., 2015*;
124 *Coelho et al., 2017*).

125

126 **Immobilized-lectin affinity chromatography**

127 Immobilized-lectin affinity chromatography is a method for separation of glycoproteins
128 based on a highly specific interaction between a lectin, which is immobilized onto a chosen
129 matrix, and its carbohydrate ligands (*Hage et al., 2012*). The technique, when complemented
130 with mass spectrometry analysis, provides a useful tool in research aiming to identify
131 potential cancer biomarkers (Figure 1). By comparing bodily fluid samples of control
132 subjects with those from patients with cancer, glycoproteins that are aberrantly expressed or
133 differently glycosylated from the resulting glycoprotein-enriched eluates can be easily
134 identified. Immobilized-lectin affinity chromatography is currently one of the most widely
135 employed techniques for enrichment of glycoproteins in cancer biomarker research.

136



137

138 **Figure 1 General workflow of immobilized-lectin affinity chromatography.** Bodily fluid
139 of cancer patients can be assayed for potential cancer biomarkers by running it through a
140 chromatography column packed with a gel matrix that is conjugated with a lectin of interest.
141 Non-binding proteins are then washed out, whilst bound glycoproteins are eluted using specific
142 carbohydrate solutions. The lectin bound glycoproteins are finally identified using proteomics
143 analysis.

144

145 By using immobilized-ConA, followed by separation by 2-dimensional gel
146 electrophoresis (2-DE), *Rodriguez-Pineiro et al. (2004)* were able to profile serum samples
147 of patients with colorectal cancer and showed significant altered expression of several *N*-
148 glycosylated proteins that were identified by mass spectrometry. These included up-

149 regulated expression of haptoglobin and lowered expression of antithrombin-III, clusterin,

150 inter-alpha-trypsin inhibitor heavy chain H4, beta-2-glycoprotein I and coagulation factor

151 XIII B chain in the colorectal cancer patients relative to healthy donors. Similarly,

152 *Seriramalu et al. (2010)* reported the lowered expression of complement factor B and alpha-2

153 macroglobulin in patients with nasopharyngeal carcinoma relative to controls using the same

154 technique but a different *N*-glycan binding lectin. In the case of *O*-glycosylated proteins,

155 considerable studies have been reported using champedak galactose binding (CGB) lectin,

156 which has a unique characteristic of binding to the *O*-glycan structures of glycoproteins

157 (*Abdul Rahman et al., 2002*) in serum and urine samples. Cancers that have been

158 investigated using immobilized-CGB lectin include endometrial cancer (*Mohamed et al.,*

159 *2008*) and prostate cancer (*Jayapalan et al., 2012*). However, most of the serum and urine *N*-

160 and *O*-glycosylated proteins that were isolated using the immobilized-lectin affinity

161 chromatography are not directly cancer associated but the body's highly abundant acute-

162 phase reactant proteins (*Pang et al., 2010*).

163 More recently, analyses of enriched glycopeptide eluates of immobilized-lectin affinity

164 chromatography for identification of site-specific glycosylation using mass spectrometry

165 techniques have been reported in studies in search of potential cancer biomarkers.

166 Enrichment of core fucosylated peptides using *Lens culinaris* agglutinin (LCA) after trypsin

167 digestion of glycoproteins, followed by endo F3 partial deglycosylation and nano LC-

168 MS/MS methodologies, has led to identification of glycopeptides that can potentially be used

169 as diagnostic biomarkers for pancreatic cancer (*Tan et al., 2015*). Similarly, enrichment of

170 trypsin-digested glycopeptides using *Aleuria aurantia* lectin (AAL) that was immobilized

171 onto agarose gel, followed by analysis using LC/MS, has resulted in identification of alpha-

172 1-acid glycoprotein with multi-fucosylated tetraantennary glycans as a potential marker for
173 hepatocellular carcinoma (*Tanabe et al., 2016*). In another study, the *Sambucus niagra*
174 agglutinin (SNA) affinity column was used to separate various glycoforms of serum PSA
175 according to the types of sialic acid linkages (*Llop et al., 2016*). This has resulted in
176 identification of α2, 3-sialylated PSA as a marker for discriminating patients with high-risk
177 prostate cancer from those with benign prostatic hyperplasia and low-risk prostate cancer,
178 with higher levels of sensitivity and specificity.

179 Another variant of immobilized-lectin affinity chromatography used in cancer biomarker
180 research is multi-lectin affinity chromatography. Since no single lectin is able to isolate the
181 complete complement of a glycoprotein, a multi-lectin affinity chromatography is gaining
182 popularity because of its greater coverage and depth of analyses. Using a combination of
183 four different types of lectins, including ConA, SNA, *Phaseolus vulgaris* agglutinin (PHA)
184 and *Ulex europaeus* agglutinin (UEA), for sequential multi-lectin affinity chromatography in
185 silica-based microcolumns and nano-LC/MS/MS for identification of proteins, *Madera et al.*
186 *(2007)* successfully profiled glycoproteins from microliter volumes of serum. Along the
187 same line but using ConA, wheat germ agglutinin (WGA) and jacalin that were integrated
188 into an automated HPLC platform and immuno-depleted serum samples, *Zeng et al. (2011)*
189 demonstrated a comprehensive detection and changes in the abundances of post-
190 translationally modified breast cancer-associated glycoproteins. To facilitate a cascading
191 flow of samples from column to column for simultaneous and efficient capturing and
192 enrichment of fucosylated proteins, *Selvaraju and El Rassi (2013)* developed of a platform,
193 which comprised multi-lectin columns driven by HPLC pumps for elucidating differential
194 expression of serum fucome between cancer-free and breast cancer subjects. This method

195 surpasses issues such as loss of samples due to sample preparation and processing (e.g.,
196 dilution) as well as other experimental biases that commonly occur when using other
197 techniques.

198 Recently, *Miyamoto et al. (2016)* reported a comprehensive proteomic profiling of ascites
199 fluid obtained from patients with metastatic ovarian cancer enriched by differential binding
200 to multiple lectins, including ConA, AAL and WGA. Alpha-1-antichymotrypsin, alpha-1-
201 antitrypsin, ceruloplasmin, fibulin, fibronectin, hemopexin, haptoglobin and lumican
202 appeared more abundant in ascites of the patients compared to controls. Further glycopeptide
203 analysis identified unusual *N*- and *O*-glycans in clusterin, fibulin and hemopexin
204 glycopeptides, which may be important in metastasis of ovarian cancer. Similar use of multi-
205 lectin affinity chromatography for enrichment of *N*-linked glycoproteins by *Qi et al. (2014)*
206 has successfully identified human liver haptoglobin, carboxylesterase 1 and procathepsin D
207 as candidate biomarkers associated with development and progression of hepatocellular
208 carcinoma. Whilst the concentrations of human liver haptoglobin and carboxylesterase 1
209 were consistently lower, higher concentration of procathepsin D was detected in the liver
210 cancer tissues. Further in-depth analysis projected the promising use of procathepsin D as
211 serological biomarker for diagnosis of hepatocellular carcinoma.

212

213 Enzyme-linked lectin assay

214 Enzyme-linked lectin assay is a method that adopts the principle of enzyme-linked
215 immunosorbent assay but uses lectin as one of the reagents instead of antibody. This method
216 was introduced by *McCoy Jr. et al. (1983)* in the early eighties. In a direct assay, samples

217 that contain glycoconjugates may be coated directly onto the wells of a microtiter plate,

218 followed by addition of an enzyme-conjugated lectin, which will then bind to their glycan

219 structures (Figure 2, panel A). The enzyme converts a colorless substrate solution to a

220 colored product, that is then measured using a spectrophotometer, and whose intensity is

221 used to estimate the levels of the coated glycoconjugates. Depending on the structures of

222 glycans that need to be detected, specific lectins are carefully selected. Enzyme-linked lectin

223 assay has been used in a plethora of research including those of cancer biomarkers

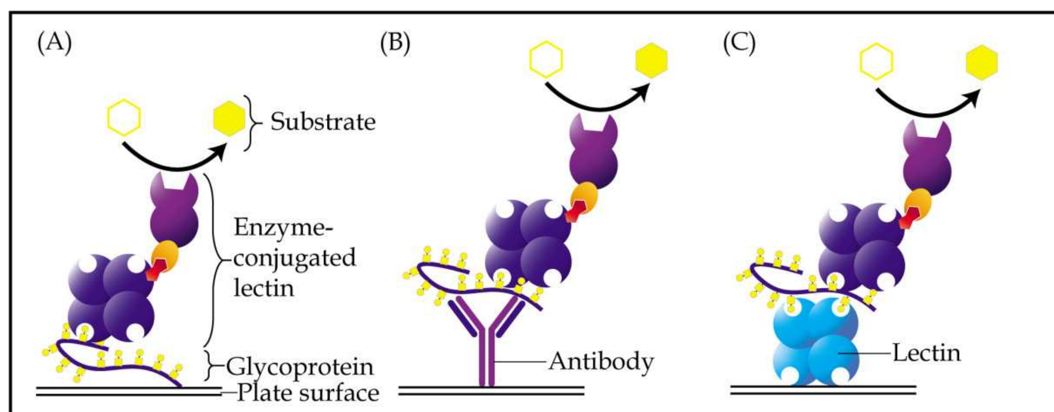
224 (*Kuzmanov et al., 2013*). It is easy to perform, very cost effective and requires minute

225 amounts of samples. One drawback of the direct enzyme-linked lectin assay is that

226 glycoproteins that are detected may not be identifiable unless it is coupled with proteomics

227 analysis or antibody detection.

228



229

230 **Figure 2 Different approaches of enzyme-linked lectin assay.** (A) In the direct assay,
231 coating of samples is performed directly onto the surface of a microtiter plate, followed by
232 addition of enzyme-conjugated lectin. (B) In the hybrid assay, antibody is instead coated onto
233 the plate to capture specific glycoproteins of interest, prior to addition of the enzyme-
234 conjugated lectin. (C) Sandwich enzyme-linked lectin assay is an alternative method involving

235 two different lectins. The first lectin is coated onto plates and used as a capturing reagent,
236 whilst the second lectin is used as detection reagent. For all the aforementioned methods,
237 glycoproteins are usually detected using a lectin that is conjugated to an enzyme, which then
238 converts a specific substrate into a colored product.

239

240 Based on their earlier study that identified a predominantly high molecular weight
241 glycoprotein that binds to peanut lectin (PNA) in the sera of patients with pancreatic cancer,
242 *Ching and Rhodes (1989)* developed a direct enzyme-linked PNA assay for diagnosis of
243 pancreatic cancer. Results obtained from the lectin-based assay were apparently found to be
244 comparable with those derived from using CA19-9 radioimmunoassay, in terms of sensitivity
245 and specificity for pancreatic cancer. In another study, *Reddi et al. (2000)* reported the use of
246 similar enzyme-linked PNA assay to estimate the levels of Thomsen-Friendenreich antigen
247 (T-Ag) in sera of patients with squamous cell carcinoma of the uterine cervix, before and
248 after radiotherapy. The study demonstrated significantly higher levels of T-Ag in the sera of
249 the uterine cervical cancer patients compared to normal individuals, and that the expression
250 of PNA-binding T-Ag were directly proportional to the aggressiveness of the cancer. In a
251 study by *Dwek et al., (2010)*, the specificity of UEA-1 lectin to α1,2-linked fucose sites was
252 capitalized for detection of fucosylated serum free PSA in a direct enzyme-linked lectin
253 assay. Their results demonstrated higher levels of fucosylated serum free PSA in patients
254 with prostate cancer compared to those with benign prostatic hyperplasia.

255 Aside from sera, direct enzyme-linked lectin assay has also been used in the analysis of
256 tissue lysate glycoproteins. In a recent study of breast cancer tissue lysates of different
257 stages, *Wi et al. (2016)* demonstrated increased interaction with ConA, *Ricinus communis*
258 Agglutinin I, AAL and *Maackia amurensis* lectin II (MAL II), relative to normal tissue

259 specimen of the same subjects. This is generally interpreted to show enhanced
260 mannosylation, galactosylation, sialylation and fucosylation of glycoproteins in the breast
261 cancer tissues. In another study, *Kim et al. (2014)* have shown lower levels of fucosylation
262 and sialylation of cytosolic intracellular glycoproteins in cancerous human cervical tissues
263 compared to normal tissue specimens from the same subjects using AAL and SNA lectins,
264 respectively. However, the levels of mannosylation, which was assayed using ConA, were
265 not significantly different between cancer tissues and normal specimens.

266 Subtle changes to the classical enzyme-linked lectin assay protocol have been introduced
267 over the years. An example is the combine use of antibody with lectin to enable detection of
268 glycosylation on a specific protein (*Kim et al., 2008*). In this case, an antibody may be
269 coated directly onto the wells of a microtiter plate, which will allow pre-capturing of a
270 protein of interest from complex samples (Figure 2, panel B). A lectin is then added and let
271 on to bind with the glycan structures of the protein. In this method, prior purification of a
272 glycoprotein is not needed as the antibody utilized specifically isolates the protein of interest
273 from within the samples. This method is also more suitable for glycoprotein antigens, which
274 are generally hydrophilic and cannot be well-coated onto a microtiter plate. The
275 disadvantage of this approach is that a lectin may directly interact with glycan chains of the
276 antibody used, which would then result in high background readings.

277 To solve the issue of the non-specific direct interaction of lectin to antibodies in enzyme-
278 linked lectin assays, *Takeda et al. (2012)* have instead used the Fab fragment of anti-human
279 haptoglobin IgG antibody and biotinylated AAL lectin for sandwich detection of fucosylated
280 haptoglobin. Their results showed that the levels fucosylated haptoglobin were significantly
281 associated with overall and relapse-free survival, distant metastasis, clinical stage, and

282 curability of patients with colorectal cancer. When Kaplan-Meier analysis was performed on
283 patients after more than 60 months of surgery, positive cases of fucosylated-haptoglobin
284 showed poor prognosis compared with fucosylated-haptoglobin negative cases. This leads to
285 the suggestion of fucosylated haptoglobin as a prognostic marker in addition to CEA for
286 colorectal cancer. Along the same line, *Jin et al. (2016)* have instead used protein A as the
287 capturing reagent and AAL lectin as detection probe, for assessment of fucosylated
288 circulating antibodies in cervical intraepithelial neoplasia and cervical cancer. Significantly
289 lower levels of fucosylated circulating immunoglobulins were shown in female patients with
290 cervical cancer compared to those with cervical intraepithelial neoplasia or normal subjects.

291 In a reverse contrast strategy, *Wu et al. (2013)* have used SNA lectin to capture sialylated
292 glycoproteins and biotinylated-antibodies to detect clusterin, complement factor H,
293 hemopexin and vitamin D-binding protein to validate the altered levels of the respective
294 glycoproteins in sera of patients with ovarian cancer. The results were consistent with their
295 data that was previously generated using isobaric chemical labeling quantitative strategy. In
296 a similar strategy, *Liang et al. (2015)* have used *Bandeiraea (Griffonia) simplicifolia*-I (BSI),
297 AAL and Poke weed mitogen (PWM) lectins as capturing reagents and biotinylated anti-
298 human α-1-antitrypsin polyclonal antibody in a sandwich enzyme-linked lectin combination
299 assay to validate results of their lectin microarray analysis of serum samples of patients with
300 lung cancer. While galactosylated α-1-antitrypsin was shown to demonstrate remarkable
301 discriminating capabilities to differentiate patients with non-small-cell lung cancer from
302 benign pulmonary diseases, their fucose- and poly-LacNAc-containing counterparts may be
303 used to discriminate lung adenocarcinoma from benign diseases or other lung cancer
304 subtypes, and small-cell lung cancer from benign diseases, respectively.

305 In a slightly different context, *Lee et al. (2013)* have developed a sandwich enzyme-
306 linked assay that uses two different lectins that both bind to *O*-glycan structures of
307 glycoproteins (Figure 2, panel C). The assay, which uses CGB lectin as capturing coated
308 reagent and enzyme-conjugated jacalin as detection probe, was primarily designed to
309 measure the levels of mucin-type *O*-glycosylated proteins in serum samples. When the assay
310 was applied on sera of patients with stage 0 and stage I breast cancer as well as those of
311 normal control women, significantly higher levels of *O*-glycosylated proteins were detected
312 in both groups of breast cancer patients (*Lee et al., 2016*). The specificity and sensitivity of
313 the assay were further improved when the same serum samples were subjected to perchloric
314 acid enrichment prior to the analysis. Further characterization of the perchloric acid isolates
315 by gel-based proteomics detected significant altered levels of plasma protease C1 inhibitor
316 and proteoglycan 4 in both stage 0 and stage I breast cancer patients compared to the
317 controls. Their data suggests that the ratio of the serum glycoproteins may be used for
318 screening of early breast cancer.

319

320 **Lectin histochemistry**

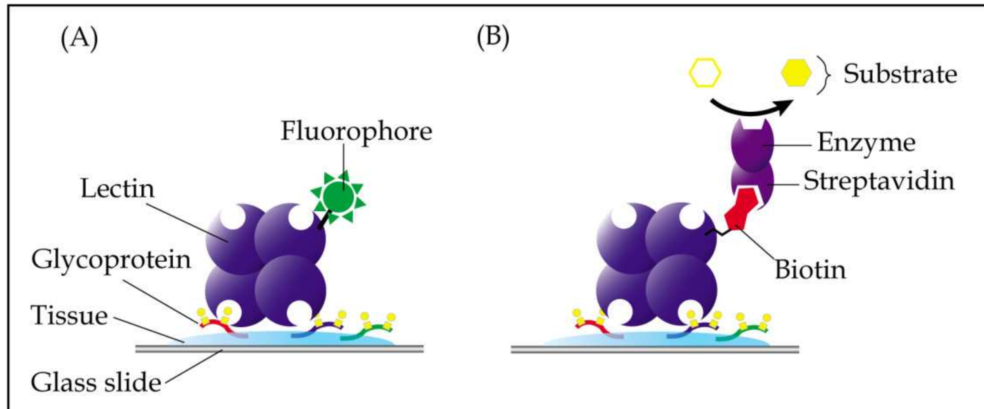
321 Like immunohistochemistry, lectin histochemistry is a microscopy-based technique for
322 visualization of cellular components of tissues except that it uses lectin instead of antibodies.
323 Utilization of labelled lectins in the tissue staining procedure limits the technique to detection
324 of only glycan-conjugated components, as well as those whose glycan moieties are being
325 recognized specifically by the individual lectins. Unlike immunohistochemistry which
326 detects presence of specific antigens based on the specificities of antibodies used, lectin
327 histochemistry provides information concerning glycosylation processes within a tissue

328 sample as well as their intracellular locations. These information can be very useful in the
329 characterization and/or detection of diseases.

330 In lectin histochemistry, labelling can be performed directly or indirectly (*Roth, 2011*).
331 In the direct labelled method, which is generally less sensitive than the direct method, lectins
332 are directly linked to fluorophores, enzymes, colloidal gold or ferritin, depending on the
333 microscopy involved (Figure 3, panel A). On the other hand, the indirect method involves
334 conjugation of lectins with biotin or digoxigenin, which may be detected using enzyme
335 linked-streptavidin or -anti-digoxigenin, respectively (Figure 3, panel B). Apparently, not all
336 chemicals can be used in the fixation and embedding of tissues in lectin histochemistry. For
337 example, the use of formaldehyde in fixation of tissue specimens is known to cause reduced
338 sensitivity of the *Griffonia simplicifolia* agglutinin, whilst ethanol-acetic acid fixation
339 improved its binding (*Kuhlmann and Peschke, 1984*). Paraffin, which causes denaturation of
340 proteins, is also known to result in attenuated binding of lectins due to sequestration of
341 carbohydrates in the glycoproteins that are denatured. However, this can be largely reversed
342 by removal of tissue-embedded paraffin using xylene or by trypsinization, which breaks the
343 protein cross-links and allows the lectins to bind more efficiently (*Brooks and Hall, 2012*).

344

345



346 **Figure 3 Common techniques in lectin histochemistry.** Comparative staining of cancer
347 versus normal tissues may highlight aberrant glycosylation of glycoproteins. (A) In the direct
348 method, glycoproteins are detected in tissue specimens using a lectin that is covalently linked
349 to fluorophores, enzymes, colloidal gold or ferritin. (B) The indirect labelled method, which
350 is generally more sensitive, involves use of a lectin that is conjugated with a hapten, such as
351 biotin or digoxigenin, which are then recognized using enzyme linked-streptavidin or -anti-
352 digoxigenin, respectively.

353

354 Lectin histochemistry has been extensively used in the study of glycosylation changes in
355 cancer tissues. Two lectins have been found useful in distinguishing the different
356 histological grades of mucoepidermoid carcinoma, the most common type of salivary gland
357 cancer (*Sobral et al., 2010*). Whilst ConA was demonstrated to be able to stain all grades of
358 mucoepidermoid carcinoma tissues, staining with UEA-I lectin showed direct correlation of
359 malignancy with the intensity of staining. Another example is cholangiocarcinoma that is
360 attributed to the river fluke infection that commonly occurs in Thailand. In the study of the
361 parasite-induced cancer, *Indramanee et al. (2012)* have used multiple lectins to demonstrate
362 aberrant glycosylation of glycoconjugates in paraffin-embedded liver tissues of patients with
363 primary cholangiocarcinoma. Unique lectin staining patterns derived from the cancer

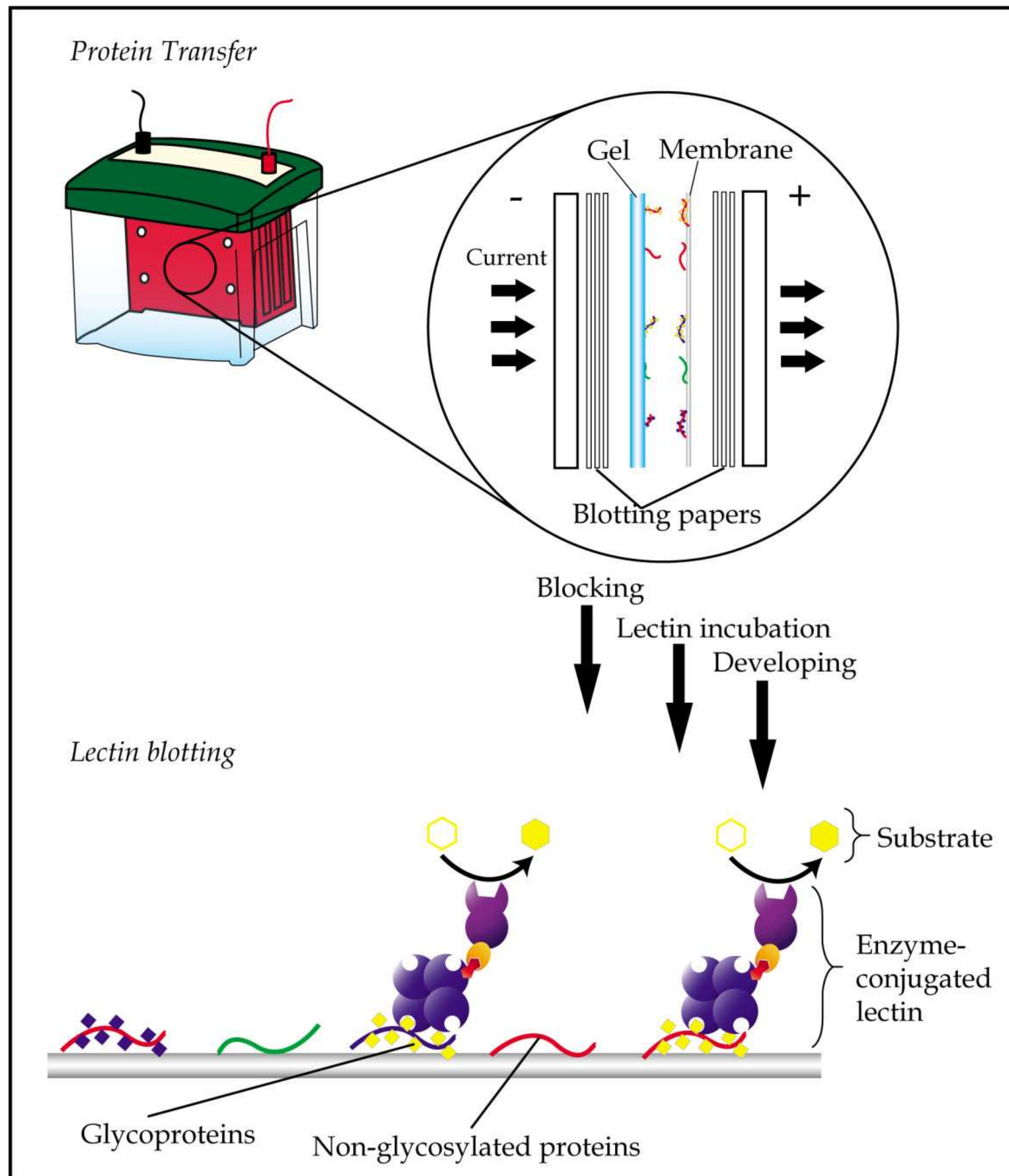
364 patients, relative to non-tumorous tissues, can be utilized as early stage markers for the bile
365 duct cancer. Similarly, SNA has been proposed for use as a prognostic probe for invasive
366 ductal carcinoma based on the different staining patterns that were generated compared to
367 tissue sections of patients with stage 0 breast cancer, ductal carcinoma in situ (*Dos-Santos et*
368 *al., 2014*). In another histochemical study, eight different lectins have been used to identify
369 specific carbohydrates that may contribute to the progression of colorectal cancer
370 (*Hagerbaumer et al., 2015*). The results showed changes in the binding patterns of five of
371 the lectins during advancement of metastasis from adenoma to colorectal carcinoma.

372

373 **Lectin blotting**

374 Lectin blotting is an extension of western blotting that uses lectin instead of antibody to
375 detect glycoconjugates (*Shan et al., 2001*). As in western blotting, samples are similarly
376 resolved using polyacrylamide gel electrophoresis and transferred onto a polyvinylidene
377 fluoride (PVDF) or nitrocellulose membrane but detected using glycan-specific lectin probes
378 (Figure 4). Like histochemistry, visualization of the lectin complex is enabled via the use of
379 conjugates such as enzymes, fluorescent dyes, biotin, digoxigenin, colloidal gold and
380 radioactive isotopes. In lectin blotting, the concentrations of lectins used must be at optimal
381 levels to reduce false-positive binding. Although a powerful tool, this technique is however
382 not quite suitable for routine diagnostics.

383



384

385 **Figure 4 General workflow of lectin blotting.** The method initially involves transferring of
386 proteins that are resolved by gel electrophoresis onto a PVDF or nitrocellulose membrane.
387 This is then followed by subjecting the membrane to washing, blocking and incubation with
388 lectins that are conjugated to an enzyme, a fluorescent dye, biotin, digoxigenin, colloidal gold
389 or radioactive isotopes. Comparative blotting of bodily fluids of cancer patients versus those
390 from cancer negative subjects may highlight presence of aberrantly glycosylated and/or
391 expressed glycoproteins.

392

393 In the past, lectin blotting studies have been especially useful in characterization of
394 structures of glycans (*Akama and Fukuda, 2006*), detection and quantification of *N*- and *O*-
395 glycosylated proteins (*Roth et al., 2012*) and detection of altered glycosylation following an
396 abnormality in glycosylation pathways due to disease processes (*Kitamura et al., 2003*). In
397 cancer biomarker studies, lectin blotting is often used for comprehensive profiling of
398 glycosylated proteins in biofluids. For example, the CGB lectin has been extensively used to
399 demonstrate altered abundances of various *O*-glycosylated proteins in serum and/or urine
400 samples of cancer patients that were resolved by 2-DE and transferred onto nitrocellulose
401 membrane. Cancers that have been investigated using the method include endometrial
402 cancer, cervical cancer (*Abdul-Rahman et al., 2007*), breast cancer, nasopharyngeal
403 carcinoma, bone cancer (*Mohamed et al., 2008*), ovarian cancer (*Mu et al., 2012*) and
404 prostate cancer (*Jayapalan et al., 2012*; *Jayapalan et al., 2013*). Similar lectin blotting
405 studies have also been applied on cell lines. Examples are the use of *Pinellia pedatisecta*
406 agglutinin-based lectin blotting analysis to generate unique glycosylation fingerprints for
407 leukemia and solid tumor cell lines (*Li et al., 2014*), and the utilization of ConA and CGB
408 lectin to demonstrate altered released of *N*- and *O*-glycosylated proteins from murine 4T1
409 mammary carcinoma cell line (*Phang et al., 2016*).

410 Another use of lectin blotting is as a means of validation of tumor-specific glycosylation.
411 Based on earlier results that showed elevated levels of mRNA of specific
412 glycosyltransferases in endometroid ovarian cancer tissue relative to normal ovary, *Abbott et*
413 *al. (2010)* have selected three different lectins with distinctive affinities for the respective
414 products of the enzymes to validate glycosylation changes of glycoproteins that are expressed
415 in the ovarian cancer tissues. By extracting intact glycoproteins from the ovarian tissues

416 before isolating the lectin-reactive proteins, the researchers were able to identify a total of 47
417 potential tumor-specific lectin-reactive markers. In another study, *Qiu et al. (2008),* using
418 biotinylated AAL and SNA lectin-blot detection method, were able to validate the
419 differential *N*-linked glycan patterns that are related to the levels of sialylation and
420 fucosylation of complement C3 in colorectal cancer patients, compared to those with
421 adenoma and normal subjects. Similarly, *Park et al., (2012)* have validated earlier findings
422 of aberration of fucose residues in haptoglobin β chain that is associated with progression of
423 colon cancer by generating comparable results using *Lotus tetragonolobus* and *Aspergillus*
424 *oryzae* lectins as detection probes in lectin blotting experiments.

425

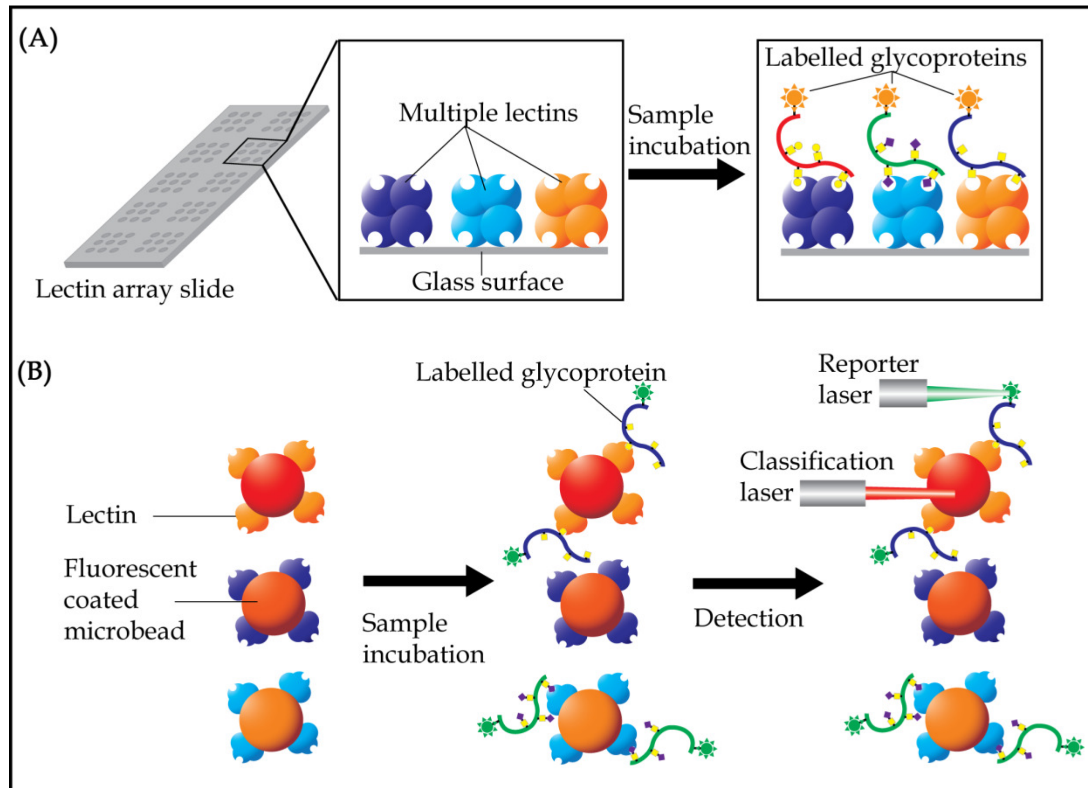
426 **Lectin Array**

427 Lectin array is a technique that was developed for rapid and sensitive analysis of glycans in a
428 high-throughput manner. The technique uses multiple lectins, which are mostly plant-
429 derived, that are immobilized onto a solid support at a high spatial density to detect different
430 carbohydrate content of glycoproteins or glycolipids in a single sample (*Hu and Wong, 2009*;
431 *Hirabayashi et al., 2011*). Display of the lectins in an array format enables observation of
432 the distinct binding interactions simultaneously, which then provides a unique method for
433 rapid characterization of carbohydrates on glycoconjugates (Figure 5, panel A). A glass slide
434 is the most common material used as solid support for the array application. Lectins are
435 coated on the glass surface either by covalent interaction or physical adsorption. Glass slides
436 are usually pre-treated with chemical derivatives such as *N*-hydroxy succinimidyl esters (*Hsu*
437 *and Mahal, 2006*), epoxides (*Kuno et al., 2005*), biotin, streptavidin (*Angeloni et al., 2005*),
438 and 3D hydrogels (*Charles et al., 2004*). Each droplet of lectin is printed onto the glass slide

439 and arranged according to a specific grid map using an array printer. The printed slide is

440 held in place by a multi-well gasket, which allows samples to be loaded into each well.

441



442

443 **Figure 5 Basic concept of lectin array technology.** (A) Multiple lectins are printed onto a
444 slide, which is organized in a grid, single lectin per spot, format. Samples, which are usually
445 pre-labelled with either fluorophore or chromophore, are then allowed to interact with the
446 lectins. Lectin spots, which contain the labelled glycoproteins, will illuminate under an
447 appropriate scanner. (B) In lectin bead array analysis, different fluorescent colored beads,
448 each corresponding to a single lectin, are often used. The conjugated beads are then allowed
449 to interact with samples and the unbound materials being washed out. The beads are then
450 passed through a detector with two laser sources, with the classification laser identifying the
451 specific beads, whilst the reporter laser quantifying presence of the labelled samples.

452

453 By using an array of 45 different lectins to determine predictive biomarkers of colorectal
454 cancer, *Nakajima et al. (2015)* were able to identify 12 lectins that showed increase binding,
455 whilst 11 more lectins demonstrated low binding of glycoproteins in the colorectal cancer
456 tissues compared to normal epithelia. Amongst the lectins, *Agaricus bisporus* lectin which
457 was selected for further validation by the researchers, showed strong potential to be used as a
458 new predictive biomarker for distant recurrence of curatively resected colorectal cancer. A
459 similar approach performed on tissue extracts of gastric cancer demonstrated high
460 interactions of 13 lectins with tissue glycoproteins, whilst 11 others showed low interaction
461 (*Futsukaichi et al., 2015*). In both these studies, the altered interaction of lectins only
462 reflected the general presence of glycoproteins that were differently glycosylated without
463 providing any information on the precise glycoproteins that are affected.

464 In an earlier study, *Wu et al. (2012)* have used lectin array to screen for altered
465 fucosylated proteins in serum samples of patients with ovarian cancer. Based on the results,
466 the researchers then immobilized the lectins that showed differential interactions and used it
467 as affinity chromatography to isolate serum glycoproteins with aberrant glycan structures and
468 determine their protein identities. This strategy has led to the identification of four serum
469 glycoproteins with altered fucose residues. Recently, a different lectin array strategy was
470 also developed to serve as an analytical technique for determination of differences in
471 glycosylation of proteins that are isolated from serum samples (*Sunderic et al., 2016*). In this
472 study, the glycan content of serum alpha-2-macrogobulin, which was isolated from serum
473 samples of patients with colorectal cancer, was studied using the lectin array. From a set of
474 14 fluorescent labelled lectins that were used in the analysis, statistically significant
475 differences between two groups of patients with colorectal cancer and cancer negative

476 individuals were found for five of the lectins. When taken together, the results generally
477 showed that the alpha-2-macrogobulin of patients with colorectal cancer have higher content
478 of α2,6 sialic acid, GlcNAc and mannose residues, and tri-/tetraantennary complex type high-
479 mannose *N*-glycans.

480 Since its inception, the technology of lectin array has been through several modifications
481 to improve detectability of glycoproteins in biological samples. The array may involve prior
482 pre-capturing of a glycoprotein of interest using antibody, and the subsequent detection of
483 glycans using pre-labelled lectins (*Kuno et al., 2011*; *Li et al., 2011*). This approach allows
484 detection of the total glycan content of a specific glycoprotein and also reduces the need for
485 prior glycoprotein purification. Lectin array is not limited to glass slide as its solid support.
486 *Wang et al. (2014)* have used fluorescent dyes coated microbeads, which allows multiplex
487 detection in a single reaction vessel that greatly improves detection sensitivity compared to
488 the standard lectin arrays. More recently, an alternative approach which involves printing of
489 purified samples onto a chip surface has also been reported (*Sunderic et al., 2016*).

490 Lectin array analysis can also be performed on magnetic beads (Figure 5, panel B).
491 Known as lectin magnetic bead array, the technique was first introduced as a robust and high-
492 throughput pipeline for glycoproteomics-biomarker discovery in 2010 (*Loo et al., 2010*).
493 The method is based on use of multiple lectins that are conjugated to magnetic beads to
494 isolate glycan specific proteins. These lectin-conjugated beads are incubated with protein
495 samples, washed and the bound glycoproteins are then eluted in appropriate buffers for
496 subsequent proteomics analysis. By coupling a mass spectrometer to the one-step
497 glycoprotein separation and isolation procedure, profiling of glycan-specific proteins may be
498 achieved without much loss of proteins. This increases the probability of identification of

499 proteins of lower abundances that have biomarker potentials. Nevertheless, a few
500 methodological concerns need to be carefully considered when using the lectin bead array.
501 These include surface functionality and diameter of the beads, conditions of buffers and
502 duration of trypsin digestion protocols for optimal isolation of lectin-binding proteins. In this
503 technique, understanding of the specificities of lectins is also imperative as most glycosylated
504 proteins are expected to have multiple glycosylation sites for interaction with the lectins.

505 Using a panel of 20 lectins in a magnetic bead array that was coupled to a tandem mass
506 spectrometer, *Shah et al. (2015)* have demonstrated unique lectin-glycoprotein interactions in
507 serum samples that may be used to distinguish three groups of subjects comprising healthy
508 volunteers, patients with Barrett's esophagus and patients with esophageal adenocarcinoma.
509 Their results demonstrated the possibility of using apolipoprotein B-100 to distinguish
510 healthy volunteers from patients with Barrett's esophagus. The use of *Narcissus*
511 *pseudonarcissus* lectin in the assay was able to differentiate differently glycosylated
512 apolipoprotein B-100 in the two groups of subjects. On the other hand, patients with
513 Barrett's esophagus were markedly distinguishable from those with esophageal
514 adenocarcinoma via differences in the glycosylation of AAL-reactive complement
515 component C9, whilst PHA-reactive gelsolin was shown to have potential in differentiating
516 healthy subjects from patients with esophageal adenocarcinoma.

517

518 Challenges in Lectin-based Biomarker Research

519 Development and progression of cancer are associated with altered glycosylation and
520 aberrantly expressed glycoproteins. Hence, the use of lectin-based assays and strategies that

521 are discussed in this review article, together with the emergence of proteomics technology,
522 has led to identification of hundreds of putative glycopeptide biomarkers that can be utilized
523 in clinical practice. However, the translation of biomarkers from discovery to clinically
524 approved tests is still much to be desired. This is mainly attributed to the lack of follow-up
525 characterization and validation investigations of the potential biomarkers, which is an
526 absolute requirement to ensure that the discovery phase experiments are not flawed and that
527 detection of the biomarkers is reproducible, specific and sensitive (*Diamandis, 2012*;
528 *Drucker and Krapfenbauer, 2013*). A potential glycopeptide biomarker has to be validated
529 using hundreds of specimens to become clinically approved tests. Hence, this is certainly not
530 possible in cases of rare cancers.

531 In some cases, validation may not be successful with the use of a single cancer biomarker
532 in a single assay. One solution is to explore the simultaneously use of several different
533 biomarkers for development of a highly specific and sensitive assay (*Pang et al., 2010*).
534 Hence, there is an urgent need to consolidate data on availability of all putative glycopeptide
535 biomarkers that have been unmasked from the discovery phase studies for every different
536 application in every cancer. In addition, new high throughput assays for simultaneous
537 detection of multiple biomarkers are also required. The recent technological advances in
538 chip-based protein microarray technology (*Sauer, 2017*) may provide with the solution, and
539 therefore ought to be explored for simultaneous validation analysis of the different
540 biomarkers in a single experiment.

541 In many other cases, identification of the potential glycopeptide biomarkers using lectin-
542 based strategies may involve complex separation techniques such as 2-DE, which is
543 laborious and expensive for large scale validation studies. 2-DE comes with the advantage of

544 knowing the actual experimental molecular weight of a glycopeptide biomarker, which is not
545 possibly attained from liquid-based separation methods. This is important as many tumor
546 associated glycopeptides are known to be truncated products of native glycoproteins (*Pinho*
547 *and Reis, 2015*). For these potential biomarkers, validation experiments would need to
548 involve a different indirect high-throughput technique using both lectin as well as an
549 antibody that is capable of differentiating truncated glycopeptides from their native
550 glycoprotein structures. However, such antibodies are usually not available commercially,
551 and generating them is time consuming, costly and involves substantial laboratory work.

552

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