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GLYCOARRAY SECTION

# Cell surface lectin array: parameters affecting cell glycan signature

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**Abstract** Among the "omics", glycomics is one of the most complex fields and needs complementary strategies of analysis to decipher the "glycan dictionary". As an alternative method, which has developed since the beginning of the 21st century, lectin array technology could generate relevant information related to glycan motifs, accessibility and a number of other valuable insights from molecules (purified and non-purified) or cells. Based on a cell line model, this study deals with the key parameters that influence the whole cell surface glycan interaction with lectin arrays and the consequences on the interpretation and reliability of the results. The comparison between the adherent and suspension forms of Chinese Hamster Ovary (CHO) cells, showed respective glycan signatures, which could be inhibited specifically by neoglycoproteins. The modifications of the respective glycan signatures were also revealed according to the detachment modes and cell growth conditions. Finally the power of lectin array technology was highlighted by the possibility of selecting and characterizing a specific clone from the mother cell line, based on the slight difference determination in the respective glycan signatures.

**Keywords** Carbohydrate · Cell lines · Glycomics · Lectin array

## Introduction

A mammalian cell surface is characterized by a complex array of glycans termed glycocalix or cell coat. Glycocalix includes oligosaccharides structures from glycoproteins, glycolipids, proteoglycans and glycosaminoglycans firmly associated or not with the plasma membrane. Cell surface carbohydrates play critical roles in many biological processes. The involvement of these glycans in cell-cell communications, cell-matrix interactions and the modulation of the immune response as well as host-pathogen interactions is now well established [1, 2]. Besides, the nature of cell surface glycans can help to distinguish between cell-types, and for a single cell type, its glycans signature (membrane glycome) can vary during growth, differentiation or pathological transformation [3].

The study of membrane glycome and to a larger extent glycomics appears fundamental to a better knowledge of biological processes. However, an exhaustive analysis of cell glycan signatures is hampered by its complexity and variations during cell life. Analytical techniques such as chromatography, mass spectrometry or NMR are not suitable for a global survey, and flow sorting or histological analysis are limited by the number of samples, which can be analyzed simultaneously. The development of array systems allows now, simultaneous recording of discreet interactions leading to the establishment of characteristic profiles.

Lectins, specific sugar binding proteins, are powerful tools used for glycoconjugate studies and therefore have been largely used for this purpose [4, 5]. Recently, lectin arrays have been introduced in the field of glycomics [6–10]. A lectin array relies on the use of a representative panel of lectins, up to one hundred [11, 12], immobilized on a surface. After incubation with the biological sample, lectin interactions can be measured by different techniques allowing the establishment, at once, of a specific map often termed the glycan signature. Since 2005, the use of lectin arrays has been extended to the study of whole cells. It has been demonstrated that the membrane glycome is specific to a cell type [11, 13–15], varies with differentiation states [14, 16, 17], is linked to the development and differentiation in murine lymphocytes [11], is associated with metastatic

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potential [18] and allows the identification of specific "glyco"markers in mammalian tumors [11]. Specific lectin bindings enabled the isolation of neural progenitor cells [19], endothelial cells [20], and specific tumor cells [11]. Furthermore, lectin arrays can be used to characterize different *E. coli* strains [21] and to predict *E. Coli* tropism [11].

Thus, the membrane glycome can be seen as a characteristic signature (or profile) subject to environmental and/or genetic variations and lectin microarrays should be developed as valuable tools for quality control in biotechnological applications such as cell differentiation monitoring, diagnosis and biomarker discovery. For example, in the biopharmaceutical industry, the glycan signature could be used to validate the cell identity, step by step, through the entire process of recombinant protein production. The use of bacterial or mammalian cells for production of recombinant proteins involves the isolation of a productive clone, *in vitro* propagation, storage in a master bank, *etc.* Specific carbohydrate surface biomarkers could serve to select a specific clone (lectin-aided capture) and to characterize a productive clone during the recombinant protein production process.

However, as pointed out by Arndt *et al.*, [15] and reviewed by Gupta [8], published results clearly show that there are some differences in the glycosylation status of commonly used cells. Many factors could affect membrane glycosylation. The composition of growth media, cell passage number and density, but also cell detachment mode and even the printed spot size (from 500 μm to 120 μm in diameter, allowing binding of 2000 to under 150 cells) should influence obtained results [8]. To our knowledge, the influence of some of these parameters on lectin glycoprofiles obtained with whole cells was never reported. In the present paper, we describe a simple method of a lectin array based on the use of current microtitration plate (96 wells). The influence of some experimental conditions (cell harvesting, cell media growth, cell density) and potential applications to clone selection and/or characterization are presented.

## Materials and methods

### Materials and reagents

The lectins (see Table 1) were obtained from EY Laboratories (San Mateo, CA) or Vector Laboratories (Burlingame, CA). All other reagents were purchased from Lonza (Basel, Switzerland) and Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Adherent CHO cells (CHO K) were from ATCC (CCL-61). CHO suspension cells (CHO-S) were from Gibco (Ref. 11619012). Clone RO4 is a stable clone, producing IgG1, derived from CHO-S cells. All these cell lines were kindly provided by Promogene (Dijon, France). CHO K-S cells are suspension cells obtained from CHO K under appropriate culture conditions.

### Preparation of the lectin array

The lectins were directly printed in triplicates on Biomat (Rovereto, Italy) fluorescent black 96 wells plates according to GLYcoDIAG technology. Briefly, lectins were allowed to interact with high binding capacity plates. Quantity and contact duration were optimized for each lectin. After washing and saturation with bovine serum albumin (BSA), plates were used immediately or freeze-dried. Each produced batch was controlled by using a set of reference glycoproteins and neoglycoproteins (BSA linked to mono or disaccharides) to ensure the repeatability of the assay itself.

### Cell Culture, harvesting and fluorescent labeling

Cells lines were cultured at 37 °C in a humidified atmosphere of 5 % $CO_2$. Adherent CHO K cells were cultured in RPMI containing 5 % fetal calf serum and 2 mM L-Glutamine and were harvested by treatment for 5 min with PBS containing trypsin (500 μg/ml) and EDTA (200 μg/ml). CHO-S suspension cells and Clone RO4 were cultured in EX-CELL 302 (or ProCHO5) and ProCHO5 medium, respectively, supplemented with 4 mM L-Glutamine. Adherent rendered suspension CHO K-S cells were obtained by culturing CHO K cells in appropriate medium. CHO K-S suspension cells were maintained in ProCHO5 medium. All cell lines were diluted to 2.5 $10^5$/ml 24 h before applying to the lectin array. For CFSE labeling, after harvesting, cells were washed in PBS and collected by centrifugation. The resulting pellet was resuspended at $2\times10^6$ cells/ml and stained with 20 μm CFSE for 15 min at 37 °C. After washing with PBS, cells were resuspended at $2\times10^6$ cells/ml in PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

### Applying to the lectin array and data processing

100 μl (about $2\times10^5$ cells) was incubated for 2 h at 37 °C under gentle agitation (25 rpm). After washing, fluorescence intensity was measured using Alpha Fusion universal microplate analyser (Packard). In parallel, the fluorescence intensity of the serial dilution of each cell line was measured in order to generate a standard curve allowing the normalization of results (expressed as % of bound cells) with respect to the number of cells set down and the level of labeling. Background binding was measured to be less than 0.1 % in each experiment. All values above can be considered as significant binding, ranking from low (<5 %) to high binding (>15 %). For carbohydrate inhibition assays, cells were incubated in the presence of various (see list in Fig. 2) neoglycoproteins (100 μg/ml) synthesized according to Roche *et al.*, [22] and Duverger *et al.* [23].

**Table 1** Specificity of lectins used in this study

| Lectins | Abbreviation | Glycan structure specificity | | |
|---|---|---|---|---|
| | | Very strong binding | Strong binding | Low binding |
| *Canavalia ensiformis* | ConA | High mannose | Hybrid type | Bi-antennary |
| *Pisum sativum* | PSA | Bi-antennary | Triantennary | |
| *Galanthus nivalis* | GNA | High mannose type N-glycans, terminal mannoses | Hybrid type | |
| *Hippeastrum hybrid* | HHA | High mannose type N-glycans, terminal and internal mannoses | Hybrid type | |
| *Amaranthus caudatus* | ACA | Galβ1-3GalNAcα-O-R (T-antigen) | GalNAcα-O-R (Tn-antigen) | Galβ1-4GlcNAc, Galβ1-3GlcNAc |
| *Dolichos biflorus* | DBA | GalNAcα1-3GalNAc (Forssman), GalNAcα1-3(Fucα1-2)Galβ (blood group A) | Galβ1-3GalNAcα-O-R (T-antigen), GalNAcα-O-R (Tn-antigen | |
| *Helix pomatia* | HPA | GalNAcα1-3(Fucα1-2)Galβ (blood group A) | GalNAcα1-3GalNAc (Forssman), GalNAcα-O-R (Tn-antigen) | |
| *Wisteria floribunda* | WFA | GalNAcα1-3GalNAc (Forssman), preference for GalNAcα | GalNAcα1-3(Fucα1-2)Galβ (blood group A), Galβ1-3GalNAcα-O-R (T-antigen) | |
| *Maclura pomifera* | MPA | Galβ1-3GalNAc (T antigen), GalNAcα-O-R (Tn-antigen) | Galα1-6Glc (melibiose), Galβ1-3, 4GlcNAc (N-glycans) | |
| *Artocarpus intergrifolia* | AIA | Galα: Galα1-6 or -3GalNAc | Terminal Galα or β, complex type N-glycans | Lactose |
| *Agaricus bisporus* | ABA | Galβ1-3GalNAc (T antigen), GalNAcα-O-R (Tn-antigen) | | |
| *Arachis hypogaea* | PNA | Galβ1-3GalNAc (T antigen) | Lactose | |
| *Datura stramonium* | DSA | | GlcNAcβ1-4GlcNAc oligomers, Galβ1-4GlcNAc motifs on bi, tri or tetra-antennary complex N-glycans | |
| *Griffonia simplicifolia* | GSLII | Terminal GlcNAc (β1-2, 3 or 4 linkage) in complex type N-glycans | | |
| *Triticum vulgare* | WGA | GlcNAc; GlcNAcβ1-4 oligomers, core of Asn linked oligosaccharides (N-glycans) | Neu5Ac (N or O-glycans) | |
| *Succinyl. triticum vulgare* | SucWGA | | GlcNAc and GlcNAcβ1-4 oligomers but not Neu5Ac | |
| *Lotus tetragonolobus* | LTA | | Lewis X and Fucα1-6GlcNAcβ- motifs of mainly N-glycans and glycolipids | |
| *Aleuria aurantia* | AAL | | Fucα1-6GlcNAc or Fucα1-3Galβ1-3/4GlcNAc | |
| *Maackia amurensis* | MAA | Neu5Ac/Gcα2-3Galβ1-4GlcNAc/Glc- | | |
| *Sambucus nigra* | SNA | Neu5Acα2-6Gal/GalNAc | | |
| *Euonymus europaeus* | EEA | Blood group B and H type 1 and 2 oligosaccharides. | | |
| *Phaseolus vulgaris* | PHA-L | Galβ1-4GlcNAcβ1-6Man of tri- and tetra-antennary | | |
| *Phaseolus vulgaris* | PHA-E | Galβ1-4GlcNAcβ1-2Man of bi-and tri-antennary | | |

## Results and discussion

Direct profiling of whole living mammalian cell surface glycome is relatively poorly documented [11, 13–15, 18, 20, 24]. Many studies using lectin microarrays were conducted on "cell extracts" rather than intact cells [12, 16, 17, 25, 26]. With whole cells, some discrepancies are observed in the results obtained from different laboratories. One major reason could be the size of spotted-lectins. The development of our strategy starts with the same observation as the one of Gupta [8]: a lectin-spot of 120 μm diameter allows the binding of fewer than 150 cells with a "unit-cell" diameter of 10 μm *i.e,*

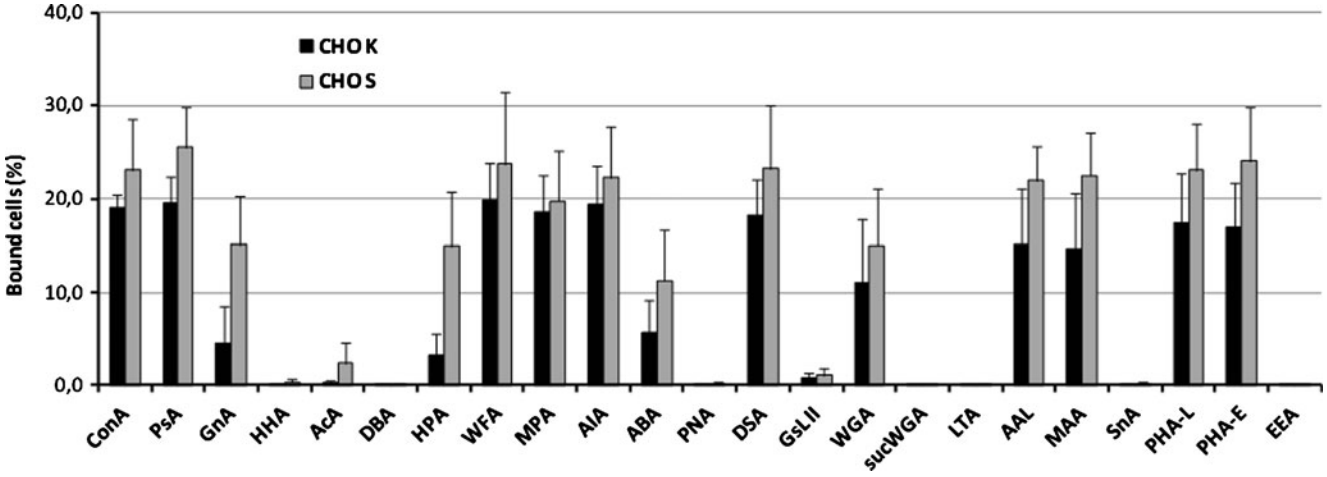


**Fig. 1** Differential profiling of adherent CHO (CHO K) *vs* suspension CHO (CHO S). $2.10^5$ cells were incubated for 2 h at 37 °C; after washing, bound cells were detected by fluorescence. Data are the average±SD of four independent experiments

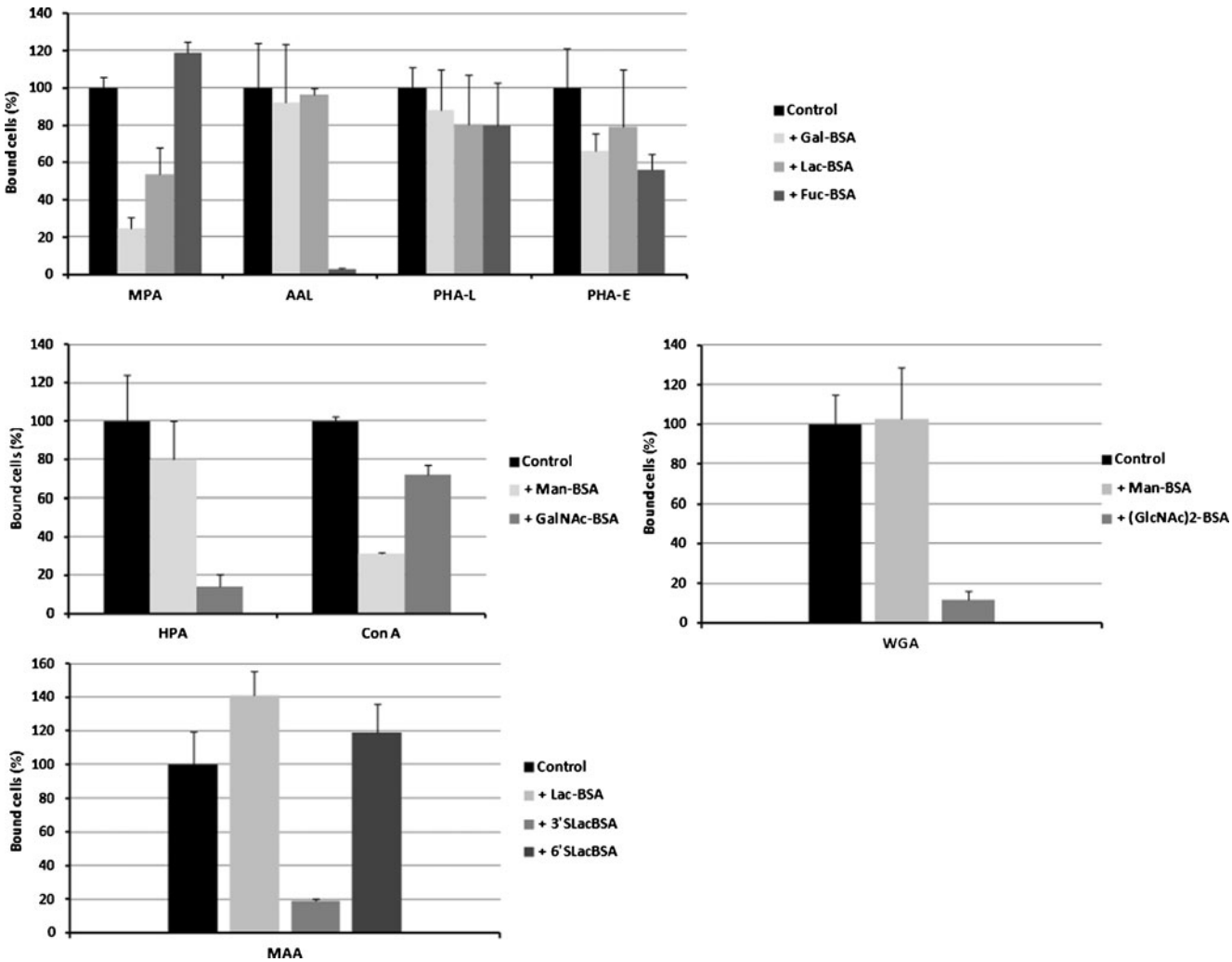


**Fig. 2** Carbohydrate inhibition assays. $2.10^5$ suspension CHO cells were incubated, for 2 h at 37 °C, either in the absence (control) or in the presence of various neoglycoproteins (100 μg/ml); after washing, bound cells were detected by fluorescence. Result of a typical experiment is presented; data are the average±SD of three independent measurements

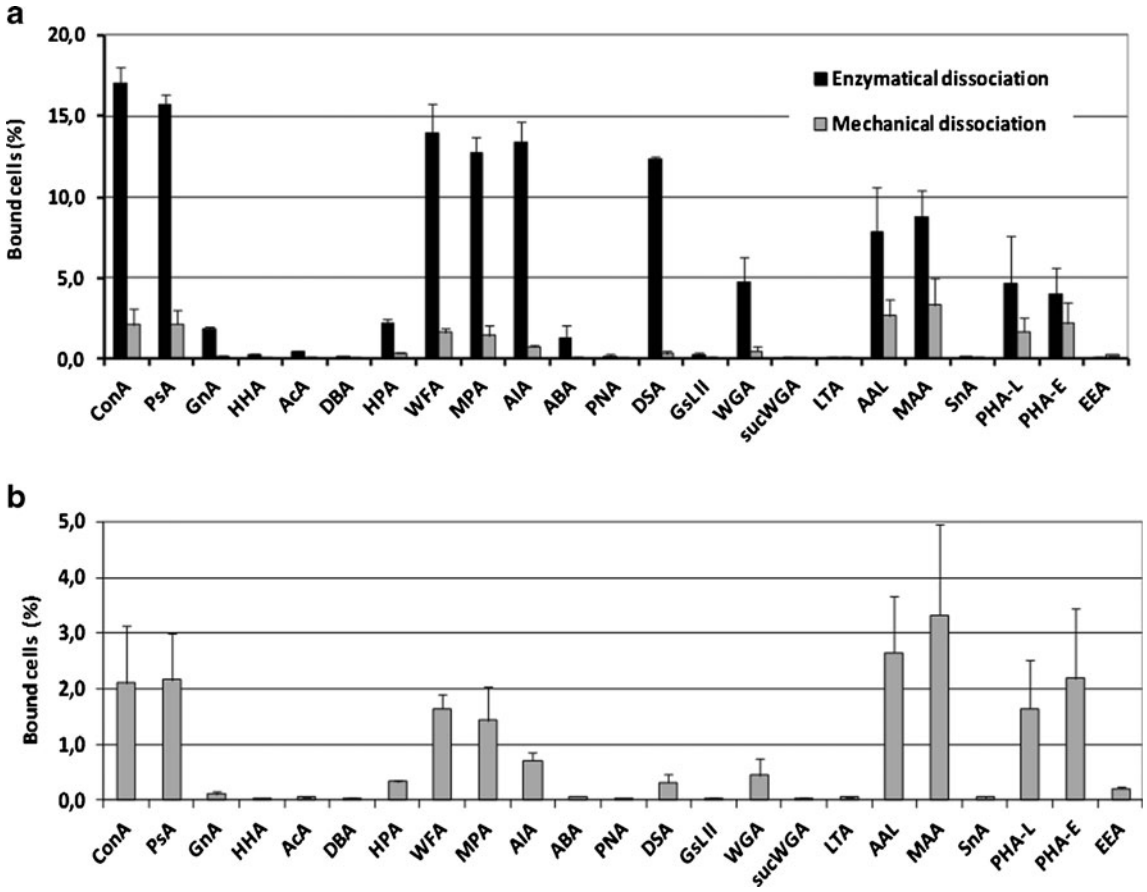


**Fig. 3** Influence of cell dissociation mode. **a** $2.10^5$ adherent CHO cells (CHO K), dissociated enzymatically or mechanically, were incubated for 2 h at 37 °C; after washing, bound cells were detected by fluorescence. Typical experiment is presented; data are the average ±SD of triplicate measurements. **b** Magnified view of results obtained with mechanically harvested cells

starting from $5.10^5$ cells, an individual spot can capture less than 0.03 % of the entire population. So, a minor portion of a heterogeneous population could be selected leading to lectin-spot linked results. To overcome this problem, we adopted the GLYcoDiag lectin array method based on standard 96-wells microplate format.

## A simple method for analyzing the glycan signature of whole cells

As a model, we used CHO cells, which can be cultured both as adherent or suspension population. Harvested living cells were labeled with CFSE and allowed to bind to the lectin

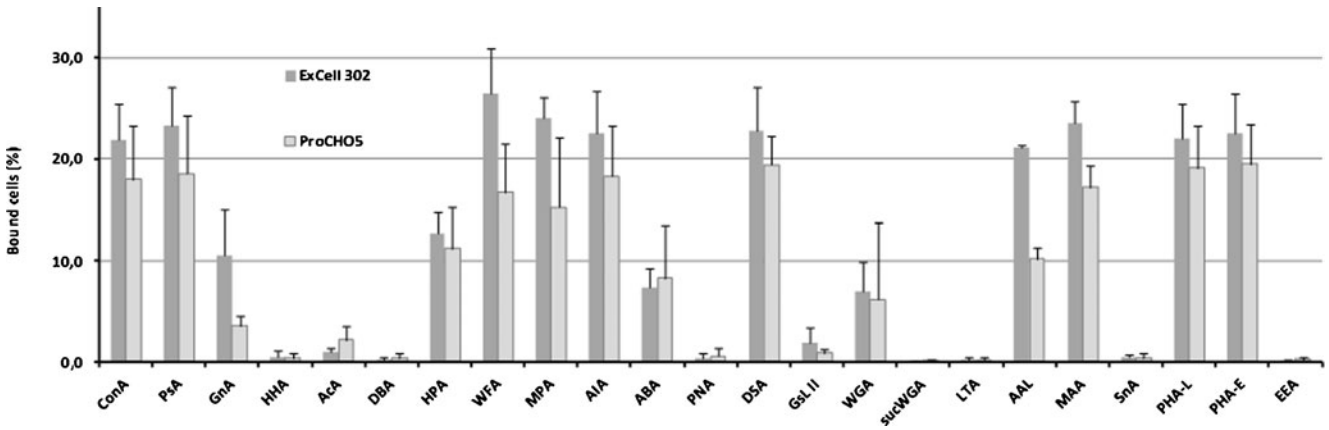


**Fig. 4** Influence of cell culture conditions. $2.10^5$ suspension CHO cells, cultivated either in ExCell 302 or in ProCHO5, were incubated for 2 h at 37 °C; after washing, bound cells were detected by fluorescence. Data are the average±SD of three independent experiments

array (containing a panel of 23 lectins listed in Table 1) for 2 h at 37 °C under gentle agitation. After the washing step, bound cells were quantified using the systematic scaling of cell fluorescence. In this way, results can be normalized from one experiment to another as regards the level of cell line labeling.

Figure 1 shows glycan signature for CHO growth as adherent (CHO K) or suspension cells (CHO S). Interestingly, these two closely related cell lines can reveal distinct binding in our lectin array system. Suspension cells showed more bindings to GNA, HPA and, to a lesser extent, to ACA and ABA lectins. No binding was observed in the absence of adsorbed lectins (data not shown). For each group of lectins, the specificity of the binding was demonstrated by performing experiments in the presence of neoglycoproteins used as inhibitors. As shown in Fig. 2, on one hand, binding to MPA was inhibited by galactose bearing neoglycoprotein but not by the fucose one. Conversely, binding to AAL was only inhibited by fucose-BSA. On the other hand, binding to "more complex lectins" namely PHA-L and PHA-E, was poorly inhibited by the same neoglycoproteins. Binding to MAA and WGA lectins were specifically inhibited by 3'sialyllactose-BSA and $(GlcNAc)_2$-BSA, respectively.

On the structural plan, our observed glycan signature is globally in adequacy with published data obtained for CHO cultivated as monolayer or in suspension [27]. For N-glycans, MALDI-MS analysis showed that CHO (monolayer or suspension) exhibits high mannose structures and complex N-glycans (bi-, tri- and tetra-antennary) with no evidence for hybrid structure. This is confirmed with lectin array profile showing high binding to PHA-E, PHA-L and higher binding to ConA *vs* GNA, this latter mannose specific lectin being restricted to high mannose and hybrid structure (see Table 1). Highest binding to GNA with CHO-S (see Fig. 1) could be related to the presence of more high mannose N-glycans on CHO grown as suspension. Low binding to Gsl-II *vs* DSA (two GlcNAc inhibited lectins) agrees with trace amount of unsubstituted terminal GlcNAc and undectable binding to LTA *vs* AAL (two fucose lectins) strenghtens the absence of N-glycans with more than one fucose residue. O-glycan spectra [27] were quite similar for both CHO cultivated as monolayer or in suspension, with core-1 structure mono-or di-sialylated with Neu5Ac. High binding to MPA *vs* PNA (two T antigen specific lectins) is in agreement with MS results because PNA does not bind to siallylated T antigen (see Table 1).

Compared to the results obtained by Tateno *et al.*, [14] we can observe the same global signature except for some lectins: HHA and ACA for which we have no or poor binding. As previously reported by Arndt *et al.*, [15] the glycosylation signature can vary greatly for commonly used cells from one laboratory to another. Independently of the array-type, which could influence the observed cell-signature, the glycan expressed or "observed" at the cell surface can vary depending on different factors including growth media composition, cell density and even cell detachment method.

## Glycan signature is affected by cell detachment mode

In order to answer the previous question, we compared the glycosylation signature of adherent CHO cells detached by means of enzymatic treatment (trypsinization), chemical (versene solution) or mechanical dissociation (cell scraping). The capacity of mechanically harvested cells to bind to the lectin array was greatly reduced. Figure 3 clearly shows that cell scraping can reduce, up to ten times, the capacity of CHO cells to bind to the lectin array (**a**). Furthermore, as evidenced in the magnified panel (**b**), even if the cell signature is on the whole

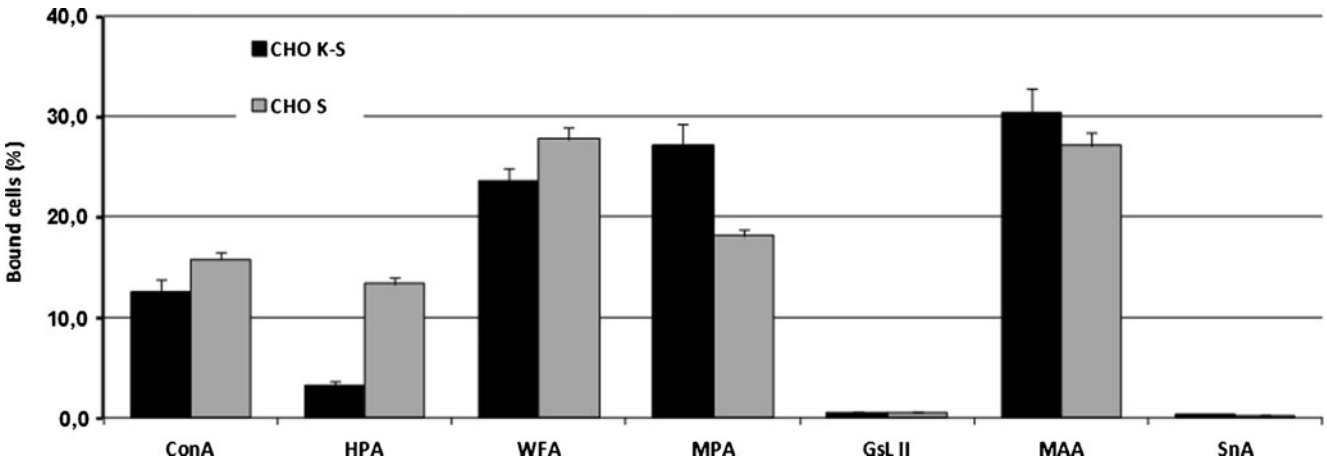


**Fig. 5** Differential binding of adherent CHO cells rendered suspension (CHO K-S) *vs* suspension CHO cells (CHO S). $2.10^5$ cells were incubated for 2 h at 37 °C; after washing, bound cells were detected by fluorescence. Result of a typical experiment is presented; data are the average±SD of three independent measurements

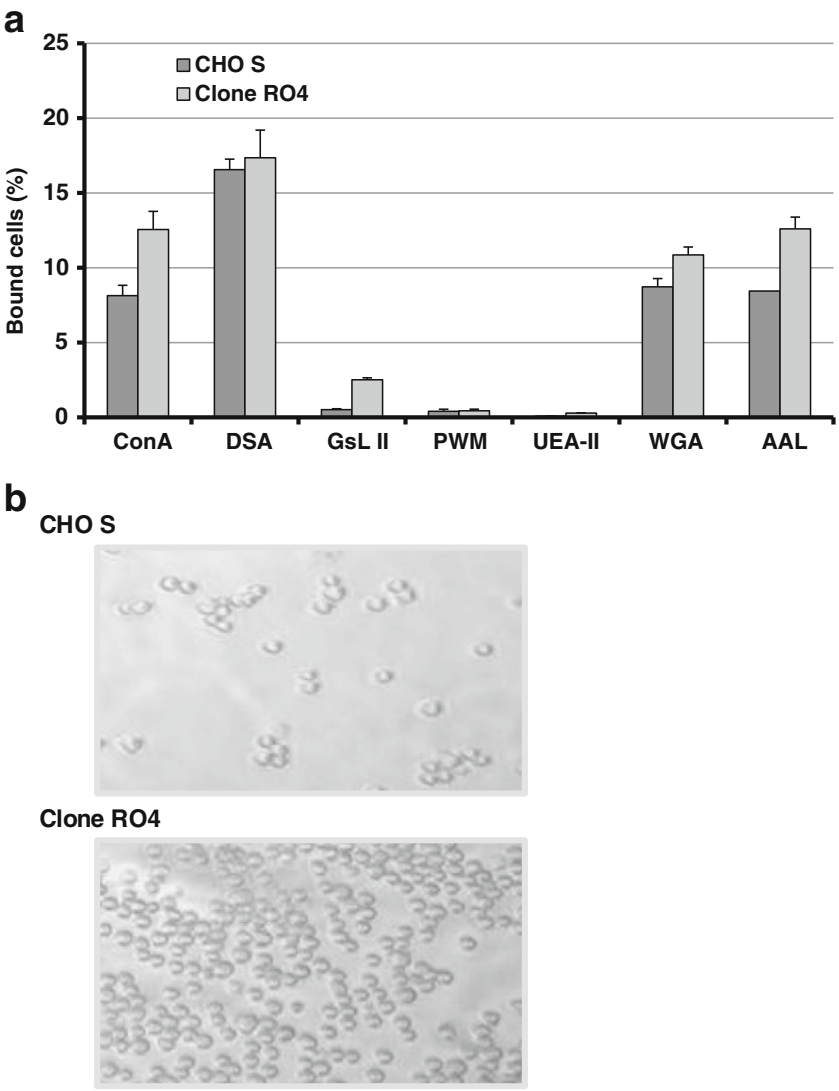


**Fig. 6** Differential binding of CHO cells *vs* engineered CHO cells (clone RO4). $2.10^5$ cells were incubated for 2 h at 37 °C; after washing, **a** bound cells were detected by fluorescence. Result of a typical experiment is presented; data are the average ±SD of three independent measurements or **b** cells were observed by phase contrast microscopy (250 x)

conserved, we can observe a critical loss of some lectin-tagged glycan epitope (DSA, and to a lesser extent AIA and WGA) in case of mechanical detachment. The use of versene solution was hampered by the difficulty to obtain reproducible results; in presence of EDTA alone, binding to the lectin array was reduced by a factor varying from 1.5 to ten (data not shown). Nevertheless some glycoconjugates are lost, we can suppose that enzymatic detachment leads to improved lectin array response by "cleaning" the cell surface allowing better access of glycans to immobilized lectins. Clearly, whatever the cell dissociation mode selected, it must be a highly controlled process to ensure experiment repeatability and comparisons between laboratories.

## Membrane glycome can vary with cell growth conditions

As reported by Senechal *et al.* [28] and Coughlan and Breen [29], cell density can affect expression of membrane glycomarkers. As a consequence, lectin array profiles should be modified during cell culture. We compared CHO glycanic signatures, for both adherent and suspension cell lines, with cultures established 24 h or 72 h previously. Some alterations can be observed in lectin binding with cells maintained in culture for more than 24 h. Glyco-epitope recognized by ACA and WGA, or GnA, HPA, ABA and WGA showed some variations with adherent or suspension CHO cells, respectively (data not shown).

Cell growth media composition, in particular the serum used, can alter protein glycosylation (reviewed in Hossler *et al.*, [30]). The influence of growth media was tested by comparing membrane glycome expressed by suspension CHO cells maintained in two different serum-free media: Excell302 and ProCHO5. In ProCHO5 glycan structures recognized by GnA and AAL are less expressed (Fig. 4).

These observations could, in part, explain discrepancies between the results obtained by Tao [11] and Arndt [15]. Once again, a lectin array established glycan cell signature is dependent on experimental conditions and published results

should clearly include all of these parameters for critical comparisons. Independently of these considerations, lectin arrays, conducted under controlled procedure, remain powerful tools to characterize and/or distinguish closed cell populations.

### Potential of lectin arrays based on standard 96-wells microplate format

In order to prove the potential of our lectin array format, we compared glycan signatures of closed CHO cell lineage cultured under the same strict conditions (growth media, passage number and cell density). Figures 5 and 6 show membrane glycosylation profiles for suspension *vs* adherent rendered suspension CHO cells and a productive clone *vs* non transfected cells. Adherent CHO K cells, grown as suspension ones, retain their specific low binding to HPA (Fig. 5) proving that under the same strict conditions, cells which are genetically identical, have the same glycoprofile. Compared to non-transfected cells, clone RO4 showed a specific higher binding to Gsl-II lectin (Fig. 6a and b). Even though the difference appears small in the histogram representation (Fig. 6a), the larger binding (about 5 times) of RO4 clone to Gsl-II is fully visible by microscopic observation (Fig. 6b). This result opens the fields of the use of such a simple lectin array format to selecting a specific clone (lectin-aided capture), characterizing a productive clone during recombinant protein production process and to a larger extent it offers a simple method to identify specific lectins, useful for purifying cell population by selective capture.

## Conclusion

This study focuses on the conditions that could modify the overall cell surface glycans expression and their interaction with lectins linked on solid surfaces. By using our specific lectin array method, validated by glycans-specific inhibition of a majority of lectins interactions, we show that the glycan signature could vary according to the cell status (in supension or monolayer), the cell dissociation mode and more generally number of cells culture conditions. Moreover, the results obtained from the monolayer rendered supension cells and from the comparison of a clone producing IgG1 from the mother cells, open the way of potentials applications of this simple technology for specific glycans signatures identification related to disease state (biomarker) or expression state (cells or clone selection).

However, it is not easy to deal with the motifs of glycans expressed at the cell surface and compare these interpretations with the glycans motifs identified on the same cells by analytical methods like HPLC or mass spectrometry. Indeed, lectins needs accessibility and recognize clusters of glycans and not the overall glycans expressed at the cell surface. In addition, among the glycoconjugates expressed at the cell surface, lectins could interact mainly with glycoproteins but also with glycolipids. As an example, higher binding to HPA with suspension CHO could be related to differences in glycolipids (lactosides) expression. So, further studies need to be conducted to decipher more precisely these interactions in order to highlight the power of this approach.

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