Comparison of Enzyme-Linked Lectin Sorbent Assay and Flow Cytometry for Profiling Microbial Glycans

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Abstract
The surface of microorganisms is covered with carbohydrates, which makes them unique, self-sustaining glycan probes. Lectins are able to bind to these probes, and this interaction can be exploited for selecting microorganisms or novel lectins. To examine lectin-microorganism interactions, we have previously developed an enzyme-linked lectin sorbent assay (ELLSA) with whole bacterial cells. To further test the validity of this methodology, here we compare it with flow cytometry. For this purpose, we used biotinylated recombinantly produced lectin from Musa acuminata (BanLec), this lectin’s recombinantly produced chimera with green fluorescent protein (BanLec-eGFP) and a lectin from Ricinus communis (RCA120), both biotinylated and FITC labeled. Parallel testing showed equivalent results for the two methods, in terms of the presence or absence of binding, with signal intensity yielding high Pearson correlation coefficient of 0.8 for BanLec and 0.95 for RCA120. The ELLSA method demonstrated multiple advantages, such as reliability and convenience for high-throughput analysis; it also required less lectin and yielded more consistent results. As such, ELLSA proved to be a useful tool for profiling microbial glycan structures or testing novel lectins.

Keywords Glycosylation · Plant lectins · Microorganisms · Yeasts · Salmonella Lactobacillus

Introduction

The cell wall of microorganisms is a key factor for maintaining cell structure, as it gives the necessary rigidity and forms a protective barrier made of various types of carbohydrates, maintaining cell integrity. In microorganisms inhabiting a host, polysaccharide structures have additional roles which are important for the control of microbe-host interactions and immune escape mechanisms. A key event in the colonization and
infection with microorganisms is the interaction between the outer cell surface of the microorganism and the host; and this is where carbohydrates play a prominent role [1].

Traditional analytical tools, such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, high-performance liquid chromatography (HPLC), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and capillary electrophoresis (CE) are commonly applied to solve the precise structure of glycans and to provide information on glycosylation site occupancy and variations of glycan structures [2–7]. However, those methods require the detachment of glycans from the core protein or molecular scaffolds [8] using different enzymatic and chemical fragmentation techniques and a high level of specialization to interpret the data [2]. These are some of the reasons why a relatively small number of surface glycans of microorganisms are fully characterized.

Lectins are carbohydrate-binding proteins involved in many different biological processes including host-pathogen interactions, cell targeting, cell-to-cell communication, induction of apoptosis, cancer metastasis, and differentiation [9, 10]. Many different lectins are known, and due to the relative ease of purification and exceptional stability, plant lectins are well-characterized and readily available analytical tools.

Lectins can bind microorganisms through the interaction of the carbohydrate recognition domain (CRD) with complex carbohydrates on microbial surfaces, such as peptidoglycans, polysaccharides, lipopolysaccharides, and teichoic and teichuronic acids [11, 12]. CRDs consist of at least a single binding site for reversible, specific interaction with free mono and oligosaccharides or glycoconjugates [13, 14].

There is growing interest in developing lectin-based arrays which will selectively recognize glycan epitopes of glycoproteins without the usage of additional purification steps or highly specialized personnel. For the detection of the presence of particular surface carbohydrates on microorganisms and for the examination of lectin specificity, we have previously developed enzyme-linked lectin sorbent assay (ELLSA), with whole microorganisms [15].

The aim of this study was to present validation data of the ELLSA and to compare the obtained results with the results obtained with flow cytometry. Based on differences in cellular composition and morphology, we have selected 33 different microorganisms, among which 27 were bacteria: 7 different Salmonella serovars, 14 bacteria from the phylum Firmicutes and 7 Proteobacteria, and 5 different strains of fungi. In order to test the interaction between carbohydrate structures found on the surface of microorganisms, two plant lectins were selected: the recombinantly produced banana lectin (BanLec, PDB 5EXG) [14] and commercial RCA\textsubscript{120}, purified from seeds of castor plant, Ricinus communis.

The application of BanLec was introduced not only due to the specificity for α-glucosyl, α-mannosyl terminal non-reducing units and 3-O-α-D-glucopyranosyl [16], but also because of the binding to ß-1,3-linked glucosyl oligosaccharides and gentiobiosyl groups [15, 17, 18].

The second lectin, RCA\textsubscript{120}, was selected because of the very different affinity in comparison to BanLec. RCA\textsubscript{120} binds ß-galactosyl non-reducing terminal units (Galß), with higher preference to Galß1-4GlcNAc then Galß1-3GlcNAc [19, 20]. Since the main properties of galactans are antiviral, antibacterial, and immunoregulatory activities, they are of considerable biomedical interest [21].

For performing ELLSA, both lectins were biotinylated, and a chimera of the recombinant BanLec with an enhanced green fluorescent protein (eGFP, PDB 4EUL) as well as fluorescein labeled RCA\textsubscript{120} were used for flow cytometric detection.
Materials and Methods

Microbial Strains, Growth Conditions

In this study, the following microbial strains were used: *Salmonella enteritidis* serovar Enteritidis ATCC 13076, *S. enteritidis* serovar Enteritidis CI E, *S. enteritidis* serovar Typhimurium CI B, *S. enteritidis* serovar Typhimurium ATCC 14028, *S. enteritidis* serovar Typhimurium CI 2865, *S. enteritidis* serovar Typhi CI 1243, *S. enteritidis* serovar Typhimurium ATCC 14028, *S. enteritidis* serovar Typhi CI 12, *Candida albicans* ATCC 10259, *C. albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* 79, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231, *Lactobacillus reuteri* DSM 17938, *Lactobacillus species* were propagated in MRS broth (Institute of Virology, Vaccines, and Sera, Belgrade, Serbia) at 37°C, 5% CO₂; *Streptococcus species* were grown in hemo aerobic culturing medium (HEMO-AE, Torlak, Serbia), while other bacterial species were grown in nutritious broth (Torlak) at aerobic conditions at 37°C. Fungal species were propagated in Sabouraud dextrose broth (SDB) (Torlak) in aerobic conditions at 30–35°C. Incubation period for bacterial strains was 16–24 h and for fungal cells 24–48 h. *Escherichia coli* strain DH5α (Invitrogen Thermo Fisher Scientific, Carlsbad, USA) was used as a host to propagate plasmids, and *E. coli* BL21 (DE3)-pLysS (Agilent Technologies Inc., La Jolla, USA) was used for expression of BanLec-eGFP. Bacteria were grown in Luria-Bertani (LB) broth (Torlak) supplemented with antibiotics (ampicillin and chloramphenicol).

Whole Microbial Cells Plate Coating Procedure

The procedure for coating MaxiSorp ELISA plates (Nunc, ThermoFisher Scientific, Denmark) with microorganisms was as previously described [22]. Briefly, overnight cultures were centrifuged (2000×g, 20 min, 4°C for bacterial species and 2000×g, 5 min, 4°C for yeast species), washed with phosphate buffered saline (PBS), centrifuged as described and diluted in PBS to optical density of 0.1 at 620 nm in a final volume of 200 μL in 96 well plate. The suspension of microorganisms was added in a volume of 100 μL per well; the plates were centrifuged (1000×g, 20 min, 24°C), and supernatant was removed. The plates were dried at 50°C for 2 h and stored for no longer than 10 days.

Expression and Purification of Recombinant BanLec and BanLec-eGFP

The 3D-model of BanLec-eGFP chimeric structure was designed from the crystal structure of a recombinant banana lectin, PDB entry 5EXG [23] (chain A containing 141 amino acids), and the crystal structure of an enhanced green fluorescent protein, PDB entry 4EUL [24] (chain A containing 239 amino acids) as previously described [25]. The expression of BanLec-eGFP was induced in *Escherichia coli* BL21 (DE3)-pLysS with 1 mM Isopropyl-D-thiogalactopyranoside (IPTG) (VWR International, LLC, Radnor, USA) 12 h at 25°C. Purification was done by combining affinity chromatography on branched α-1,6 and α-1,3
glucan polymer (Sephadex G-75 superfine, CV=10 ml) and ion-exchange chromatography (HiTrap ANX column, 1 ml, GE Healthcare, Little Chalfont, UK), as previously described [25]. Recombinant BanLec (GenBank accession number EU0556441) was produced and purified by affinity chromatography according to Gavrovic-Jankulovic [14]. Purity of the proteins was assessed with SDS-PAGE (Supplementary material, S1).

**Lectin Coupling to Biotin and Fluorescein**

Biotinylation and assessing biotinylation efficacy was done as described in our previous research [15]. Biotinylated lectins were analyzed with Tris-Tricine electrophoresis (8% running, 5% stacking gel [26], after which the preparations were stored in 50% glycerol, with 0.1% sodium azide at −20°C. Lectin concentration after biotinylation was assessed with Lowry protein concentration assay [27]. Commercial RCA120 (Sigma Aldrich St. Louis, MO, USA) was dialyzed against 0.1 M sodium carbonate buffer pH 9.0 for 24 h with continuous dialysis apparatus. Fluorescein 5(6)-isothiocyanate (FITC, Sigma Aldrich St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to the concentration 1 mg/ml, and 50 μl was added to the lectin solution. The reaction was conducted at 25°C in the dark, with constant shaking for 2 h; thereafter, NH4Cl was added to a final concentration of 50 mM and incubated for 2 h at 4 °C; subsequently it was dialyzed 24 h against PBS.

**Lectin Binding in ELLSA (Enzyme-Linked Lectin Sorbent Assay)**

The procedure of ELLSA was as described by Dragacevic et al. [15]. Each sample was measured in quadruplicate in order to calculate repeatability. The reproducibility of the ELLSA method was performed in three different laboratories, with preparing fresh plates from newly cultured microorganisms each time.

**Flow Cytometry Analysis of Lectin Binding to Microorganisms**

Overnight culture of yeast cells was centrifuged at 1000×g, 2 min, washed once with PBS, and diluted in PBS. A total of 100 μL (OD620nm= 0.5) was used. Overnight culture of bacterial cells was centrifuged at 2000×g, 20 min, washed once with PBS, and diluted in PBS. A total of 100 μL (OD620nm= 0.5) was used. BanLec-eGFP or RCA120-FITC were added at different amounts, mixed, and incubated for 15 min at 4°C. After washing twice with PBS (3 mL) and centrifugation, the signal was analyzed on FACSVerse (Becton Dickinson, Mountain View, CA, USA). Each sample was measured in quadruplicate in order to calculate repeatability.

**Statistical Analysis**

Graphical presentations were done in GraphPad Prism, Origin Lab, and BD FACSuite software, and Pearson correlation coefficient ($P_{cc}$) was calculated with Origin Lab. Repeatability was calculated according to the formula: $CV_{\text{repeat}} = \frac{\sum (SD(d)) : n : \bar{a}}{100}$, where SD is standard deviation of quadruplicate, $n$ is number of replicates, and $\bar{a}$ is the mean of quadruplicate sample. The coefficient of variation for reproducibility was calculated based on value of quadruplicate of the same sample done in three different laboratories at the institute, according to the formula $CV_{\text{repro}} = \left\{ \frac{\sum (SD(1) + SD(2) + SD(3))}{3} \right\} : 3$.
[(Ā1 + Ā2 + Ā3) : 3]} × 100, where SD(X) is the average SD of the mean values in different laboratories and Ā is the averages of the results from different laboratories.

**Results**

**Validation Experiments**

**Specificity of Lectin Binding to Microorganisms**

Lectin binding specificity was determined by testing interactions between BanLec-eGFP and RCA₁₂₀ to different microorganisms.

The binding of BanLec-eGFP to microorganisms was detected with ELLSA and flow cytometry to all tested yeast cells and to certain strains of *Salmonella*, as can be seen in Fig. 1a. The binding to yeast cells was not uniform, with *C. albicans* ATCC 10259 showing especially high binding and *S. boulardii* giving a lower signal. The binding was not uniform between the strains of *Salmonella*, with especially pronounced binding to serovar *S. Typhi* 12 and serovar *S. Typhimurium* 2865. No significant binding in both methods was detected for the other microorganisms tested (Fig. 1a).

![Fig. 1 a The binding of BanLec to different microorganisms according to enzyme-linked lectin sorbent assay (ELLSA), gray bars, and flow cytometry, black bars. Microorganisms were stained with 0.05 μg biotinylated BanLec or 1.4 μg of BanLec-eGFP chimera, respectively; b correlation between biotinylated BanLec binding to microorganisms in ELLSA and BanLec-eGFP chimera binding to microorganisms in flow cytometry. Mean values with standard deviations are shown](image)
The signals obtained with ELLSA and flow cytometry were correlated, and in the case of BanLec and BanLec-eGFP chimera, the correlation between the two methods yielded a Pearson correlation coefficient (Pcc) of 0.8 (Fig. 1b). The marked differences between the two methods were in higher FITC-A median values obtained for yeast species and lower values obtained for *Salmonella* with flow cytometry, whereas ELLSA showed less variation in binding, except for *C. albicans* ATCC10259, which gave highest binding with both methods.

In order to examine this phenomenon, we compared the mean values of *Salmonella* and yeast BanLec-eGFP binders in ELLSA and flow cytometry. In ELLSA (Fig. 2a), the differences between *Salmonella* and yeasts were not as pronounced and were not significant. Significant differences were observed with BanLec-eGFP binding to *Salmonella* and to selected yeast cells in flow cytometry (Fig. 2b).

Both ELLSA and flow cytometry detected binding of BanLec to *Salmonella* strains that confirmed the occurrence of mannose derivatives on their surface [28, 29]. The binding of BanLec to yeast cells was also expected because of the occurrence of β-glucan structures in addition to mannose on their surface [30–33].

Thus, we were able to determine surface glycan patterns for different microorganisms that were generally consistent with their known glycosylation.

The presence of galactose containing glycans was detected only on the surface of *L. casei* DG, using both ELLSA and flow cytometry, while the rest of the microorganisms tested showed no binding to RCA120 (Fig. 3a). Therefore, the correlation of signals obtained by RCA120 binding to microorganisms in ELLSA and FITC labeled RCA120 in flow cytometry resulted in high correlation coefficient of Pcc of 0.95 (Fig. 3b).

Both methods managed to detect exceptionally high binding signal between RCA120 and the carbohydrate structures found on the surface of *L. casei* DG.

Fig. 2 BanLec binding to microorganisms in a ELLSA and b flow cytometry. BanLec-eGFP binders were grouped according to microorganism type (*S. Typhimurium* ATCC 14028, *S. Typhimurium* CI 2865, *S. Typhi* CI 1243, *S. Typhi* CI 12) named *Salmonella*; among yeasts (*C. albicans* ATCC 10259, *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763, *C. neoformans* 79) marked—yeasts. Other tested bacteria—other. Mean values with standard deviations are shown. **p = 0.006
Linearity and Precision of ELLSA and Flow Cytometry

In order to make a comparison between the two methods, a single microorganism was selected for each lectin, based on the high binding signal detected. For the binding of Ban-Lec *C. albicans* and for the binding of RCA\textsubscript{120}, *L. casei* DG were selected, and titration experiments were performed with each sample ran in quadruplicate.

In Fig. 4, the binding of BanLec to *C. albicans* was explored. In ELLSA, Fig. 4a saturation was reached with 0.3 μg of BanLec-B, while with flow cytometry, Fig. 4b saturation was reached with 11.1 μg of the BanLec-eGFP. When converted to moles, effectively a 10 times lower amount was needed to reach a plateau in ELLSA, and again higher standard deviations were obtained with flow cytometry. Linearity range was narrower for ELLSA, than for flow cytometry, but the coefficient of determination $R^2$ was higher (0.98) in comparison to flow cytometry (0.96). Precision was determined by two types of measurements: (1) repeatability with the highest obtained coefficient of variation $CV_{\text{repeat}}$ for ELLSA was 3.9%, while for flow cytometry, it was 8%, (2) reproducibility with the coefficient of variation $CV_{\text{repro}}$ in range between 0.6 and 9.2% for ELLSA depending on microorganisms (Supplementary material, S2).

In Fig. 5, the binding of RCA\textsubscript{120} to *L. casei* DG is shown. In ELLSA (Fig. 5a), the saturation is reached with 2 μg of RCA\textsubscript{120}, while with flow cytometry (Fig. 5b), saturation was reached with 8 μg of the protein. As can be seen in Fig. 5, much higher standard deviations.
were obtained with flow cytometry. Linearity range was narrower for ELLSA, than for flow cytometry, but $R^2$ was higher (0.99) in ELLSA in comparison to flow cytometry (0.95) (Supplementary material, S2).

The highest obtained coefficient of variation CV$_{\text{repeat}}$ for ELLSA was 2.4%, while for flow cytometry, it was 15% (Supplementary material, S2).

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**Fig. 4** a Titration of biotinylated BanLec binding to *C. albicans* with ELLSA; mean values with standard deviations are shown. b Titration of BanLec-eGFP binding to *C. albicans* with flow cytometry; mean values with standard deviations are shown. c Flow cytometric analysis of the interaction of *C. albicans* with different concentrations of fluorescently labeled lectin BanLec-eGFP
Discussion

In this study, we present validation data of the newly developed ELLSA method, compare it with flow cytometry, and test 33 microorganisms with both methods in parallel.

Flow cytometry was chosen in order to compare the results obtained by ELLSA, as it can provide high dimensional quantitative measurements of scattered light and
fluorescence emission from tenths of thousands of individual cells in a single analysis. The measurement of fluorescence is proportional to the amount of fluorochrome-labeled protein bound to cells, which is an appropriate proxy for specific receptor density [34].

Lectins were historically embraced as an omnipotent analytical reagents, but this generally failed to live up to expectations. When it comes to lectin-microorganism interactions, there may be potential, especially if lectin combinations are to be used as microorganism-specific signature. Using lectins as fluorescently labeled probes can be challenging as chemical labeling can cause modification in secondary or tertiary structures and introduce modification to the binding site, changing the binding constant. In case of RCA120, the same chemistry was employed for labeling, so no difference was expected, while in case of BanLec, the addition of eGFP was found to have no effect on the secondary structure of the lectin molecule [25].

Both of the methods tested here are reliant on lectin-microorganism interactions and are therefore also constrained by lectin specificity and affinity and, theoretically, by the structural organization of carbohydrate units. Practically, it means that although a certain lectin has an established specificity towards a certain carbohydrate/s or, in this case, towards an oligosaccharide unit/s, the binding could be dependent on the structural organization of the component on the surface of the microorganism. So, although a positive signal undoubtedly confirms the existence of a particular carbohydrate within the used lectins’ specificity, the negative signal theoretically does not exclude the existence of that carbohydrate unit. The results we have obtained thus far confirm that the absence of a signal implies the absence of a particular structure.

The results obtained by the ELLSA method demonstrated satisfactory results and confirmed the occurrence of galactose on the surface of L. casei DG using RCA120 and mannose structures on the surface of both Salmonella and yeast cells, as well as β-glucan structures on yeast cells using BanLec.

Among the seven different Salmonella serovars tested, there was considerable difference in signal intensity; however, there is also significant difference in structure between the serovars, such as in the structure of flagellum, carbohydrates, and lipopolysaccharides. For example, Salmonella Typhi contains mannose-rich LPS and binds mannose binding lectin (MBL), while other Salmonella serovars can lack mannose rich O-polysaccharide in LPS and as such do not bind MBL [28]. Similarly, fungal cell wall varies from strain to strain and consists not only of β-1,3 glucans (laminaribiose oligomer) and β-1,6 glucans (gentiobiosyl groups), but also of mannosylated structures, while BanLec has affinity to all. The differences obtained in BanLec binding to bacterial (Salmonella) and yeast cells between the methods can be a result of the different cell sizes of these microorganisms and, therefore, the higher number of fluorochromes bound per fungal cell, in comparison to Salmonella, which gives a higher signal in flow cytometry. This is not so extreme in ELLSA, since the surface area for binding is roughly the same and of even more importance; the signal is amplified with aid of an enzyme. Another possible explanation would be that in flow cytometry, live cells are used, while in case of ELLSA, the cells are heated and dried at 50 °C.

The results obtained by detecting selective binding of RCA120 to L. casei DG out of 32 other microorganism is in line with the results published by Freitas et al. in 2003 [35] which showed that L. casei DG has galactose on its surface, while the rest of lactobacilli tested had completely different glycan patterns, and which concluded that different bacterial strains develop unique surface glycan structures. This may be true for some bacterial species, such as S. pneumoniae which has over 95 serotypes with different
polysaccharide capsules, with different characteristics [36], but it may not be applicable for other bacterial species, and surface glycan structures may also be species specific.

Data presented here shows that a much lower amount of lectin is needed to reach a plateau in ELLSA, which results in smaller quantity of lectin needed for detecting surface glycans of microorganisms. Throughout the experiments presented, both standard deviations and CV\textsuperscript{repeat} were higher in data obtained from flow cytometric analysis, while coefficients of determination were lower, proving ELLSA to be a more reliable method. The wider linearity range observed in flow cytometry does not represent an obvious advantage. Both comparison studies resulted in high correlation coefficients between the two methods tested, which confirms the results obtained with the ELLSA method.

Although both methodologies gave equivalent results in terms of binding versus non-binding, certain differences in signal intensity were noted. This can be related to difference in glycosides structure, but also due to technical differences between two methods. The main difference between the two methods is the preparation of microtiter plates and staining dead cells in ELLSA, versus staining of live cells, in flow cytometry. Since plate preparation includes procedures such as heating and drying of microbial cells, those procedures could slightly alter polysaccharides found on microbial surface and be responsible for slight difference in the obtained results. However, our results show that the difference between the methods is not drastic, implying that exposed saccharide structures in live versus those in dead microbial cells do not differ significantly and that there are generally no hidden glycan epitopes, at least in case of the two lectin specificities tested here.

Conclusions

The results obtained show that ELLSA was more sensitive than flow cytometry in determining the presence of glycan structures with use of lectins, required lower amount of lectin necessary for the analysis, and had higher repeatability and reproducibility of results, evidenced by lower standard deviations. ELLSA was also more convenient for high-throughput screening as it required less manual labor per analysis.

As mentioned previously, plate preparation necessary in ELLSA could be the potential drawback of the ELLSA method as it includes heated and dried microbial cells and takes approximately an additional day to complete. However, the potential benefits of ELLSA method outweigh the potential drawbacks.

The results presented here open up possibilities of using ELLSA, not only for glycosylation profiling of microorganisms, but also in testing novel lectin specificities, for potential therapeutic usage, such as for testing potential novel antiviral lectins [37], and for comparing lectin molecules as well. Therefore, the ELLSA method could be adopted as a complementary tool for lectin typing in the development of protein therapeutics.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12010-021-03772-w.

Availability of Data and Material Data and material are available upon reasonable request to the corresponding author.

Code Availability Not applicable.

Author Contribution Rajna Minić and Natalija Polović made substantial contributions to the conception and design of the study. Material preparation and data collection were done by Luka Dragačević, Zorana Lopandić, and Veljko Blagojević. Data analysis and interpretation were done by all authors. The first draft
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**Declarations**

**Ethical Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

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