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Authors: Ernesto Rodriguez, Karen Francia, Natalie Brossard, Juan J. García Vallejo, Hakan Kalay, Yvette van Kooyk, Teresa Freire, Cecilia Giacomini

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# Immobilization of β-galactosidase and α-mannosidase onto magnetic nanoparticles: a strategy for increasing the potentiality of valuable glycomic tools for glycosylation analysis and biological role determination of glycoconjugates.

Ernesto Rodriguez [a,b,c], Karen Francia[a], Natalie Brossard[b] Juan J. García Vallejo[c], Hakan Kalay[c], Yvette van Kooyk[c], Teresa Freire[b], Cecilia Giacomini[a*].

[a] Laboratorio de Bioquímica, Departamento de Biociencias, Facultad de Química, UdelaR, Gral. Flores 2124, Montevideo, Uruguay,

[b] Laboratorio de Inmunomodulación y desarrollo de Vacunas, Departamento de Inmunobiología, Facultad de Medicina, UdelaR, Gral Flores 2125 Montevideo, Uruguay,

[c] Department of Molecular Cell Biology and Immunology, VU University Medical Center, De Boelelaan 1108, 1081 HZ, Amsterdm, Netherlands.

***Corresponding author**:

Cecilia Giacomini

Phone: +598 2 9241806

Fax: + 598 2 9241906

Email: cgiacomi@fq.edu.uy

Ernesto Rodriguez: erodriguez@fmed.edu.uy

Karen Francia: karenfranciabogado@gmail.com

Natalie Brossard: nataliebrossard@fmed.edu.uy

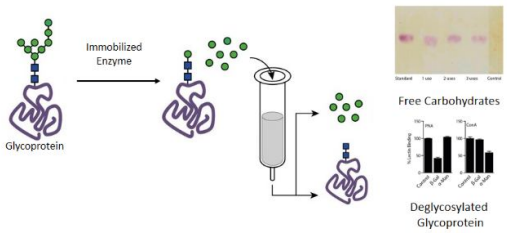
Juan J García Vallejo: jj.garciavallejo@vumc.nl

Hakan Kalay: H.Kalay@vumc.nl

Yvette van Kooyk: y.vankooyk@vumc.nl

Teresa Freire: Tfreire@fmed.edu.uy

Graphical Abstract




**Highlights**

- *A. oryzae* β-galactosidase and *C. ensiformis* α-mannosidase were immobilized.
- Immobilized glycosidases were useful for deglycosylation of model glycoproteins.
- Immobilized glycosidases were re-used without loss of effectiveness.
- Immobilized glycosidases were used for evaluation of biological role of glycans.

## Abstract

Glycans present in biological glycoconjugates have several structural and functional roles. Elucidation of glycan structure and biological function is critical to understand their role in physiological and pathogenic process, enabling the development of diagnostic methods and disease treatment. Immobilized glycosidases are powerful tools for glycan analysis, as they are able to remove specific carbohydrates without altering the protein structure. Here we

describe the individual immobilization of *Aspergillus oryzae* β-galactosidase and *Canavalia ensiformis* α-mannosidase onto agarose and silica magnetic nanoparticles activated with cyanate ester groups. High immobilization yields (70-90 %) were achieved, keeping above 60% of its original activity. Immobilized glycosidases were effective in the selective deglycosylation of model glycoproteins and a *Fasciola hepatica* lysate, evidenced by a decrease in specific lectin recognition of 40-50 % after enzymatic deglycosylation. Immobilized glycosidases were reused for several deglycosylation cycles without loss of effectiveness. Their use was extended to the elucidation of the glycan role of native glycoconjugates. A decrease in the recognition of lactoferrin treated with α-mannosidase by a C-type lectin receptor, DC-SIGN was found. Also the specific deglycosylation of a *F. hepatica* lysate demonstrated the relevance of mannosylated glycans in the induction of Th2/Treg immune responses during the infection. Our results show successful immobilization of specific glycosidases in nano-supports and validate their utility to identify glycans biological functions.

## Abbreviations

AMC: Amino-4-methyl coumarin; BCA: Bicinchoninic acid; BMDc: Bone Marrow dendritic cells; BSA: Bovine Serum albumin; $CD_4^+$ T cells: Lymphocytes T expressing the protein $CD_4^+$; CDAP-$BF_4$: 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate; CLR: Calcium Lectin Receptor; ConA: *Canavalia ensiformis* Concanavalin A lectin; DC-SIGN: Dendritic cell specific ICAM grabbing non integrin; DCs: Dendritic cells; ELISA: Enzyme Linked Immunosorbent Assay; FhTE: *Fasciola hepatic*a total lysate; IL: Interleukin; Gal: Galactose; Man: Mannose; ONPG: ortho-Nitrophenyl-β-D- galactopyranoside; ONP: ortho – Nitrophenol; OPD: ortho-Phenylenediamine; PBS: Phosphate saline buffer; PNA: *Arachis hypogaea* lectin; PNGase: Peptide N-glycosidase; PNPM: para-Nitrophenyl α-D-mannopyranoside; PNP: para-Nitrophenol; TFA: Trifluoro acetic acid; Th: Lymphocytes T helper;

## Key words

β-galactosidase / glycomic analysis / glycosidases /Immobilization /α-mannosidase

## 1. Introduction

Glycans present in biological glycoconjugates such as glycoproteins and glycolipids have several structural and functional roles. They are relevant for maintenance of tissue structure, protection against proteolysis of glycoproteins, protein folding and solubility. Moreover, they play important roles in several biological process such as cell-cell, cell-matrix and cell-pathogen interactions, cell signalling, cell migration, cell adhesion as well as modulation of the immune response and cancer progression [1-3]. Interestingly, changes in glycan profile of cell glycoproteins and glycolipids are associated with the development of cancer, immune deficiencies as well as neurodegenerative and cardiovascular diseases [1,4]. It has also been reported that deficient genes that encode proteins involved in glycosylation pathways lead to serious congenital disorders [1,5]. Furthermore, glycans from different pathogens such as bacteria, virus and parasites are essential for both pathogen infection and immune evasion strategies [6-10]. In fact, helminth parasites modulate the host immune response by inducing a modified Th2-polarized response mediated by carbohydrate moieties [8,9, 11-12]. In addition, our recent results suggest the relevance of terminal mannose (Man) residues of glycoproteins present on the helminth *Fasciola hepatica* in the inhibition of dendritic cell (DCs) maturation and in the induction of a regulatory immune response by this parasite [8,13].

In this context, the elucidation of the structure of glycans as well as their biological function is essential to understand their role in pathogenesis, which in turn will enable the development of new methods for diagnostic and disease treatment. Nevertheless, this constitutes a challenge in the area of glycobiology due to the complexity of glycan structure. Indeed, they are composed of a combination of several monosaccharaides, with different anomeric configuration, linked by diverse glycosidic bonds, which frequently present branches. Thus, a combination of physical and chemical strategies is necessary to achieve a complete structural characterization of glycans [4, 14-16]. The main glycans found in eukaryotic cells are N-glycans attached to an asparagine residue in a consensus sequence Asn-X-Ser/Thr (X can be

any amino acid except proline) and O-glycans attached to Ser/Thr residues [14,17]. Several approaches for glycan analysis include the removal of the glycan portion or the proteolytic digestion of glycoproteins and further analysis by spectrometric methods [4,14,17-18]. Nevertheless, most of these strategies causes the denaturation of the protein backbone of the glycoprotein, preventing its further use for functional analysis. Endo- and exoglycosidases are powerful tools for glycan analysis, as they are able to remove specific carbohydrates without altering the protein structure [19-20]. The evaluation of changes in the biological function of glycoproteins due to specific sugar removal will enable to establish its relevance in the process involved. The immobilization of enzymes can increase their stability, enables their reuse (which compensates the production costs) and allows their easy removal from the reaction mixture [21-23]. The latter avoids the use of drastic conditions to stop the enzymatic reaction, which could affect the glycoprotein structure. Furthermore, it prevents the purification step necessary to separate the glycosidase from the deglycosylated protein so that it not interfere in further assays. All these advantages increase the potentiality of immobilized glycosidases as glycomic tools. Moreover, the use of nano-supports such as magnetic silica nanoparticles could be advantageous as the reduced size of the support suggest a performance of the immobilized enzymes similar to that of the soluble enzyme, minimizing diffusional problems due to immobilization. This is particularly important when working with macromolecular substrates such as glycoproteins [24]. Even though immobilized enzymes have been widely used for several biotechnological processes during the last four decades, there are almost no reports in the literature regarding the use of immobilized glycosydases for glycomic analysis [24-26].

In this work, we immobilized β-galactosidase from *Aspergillus oryzae* and α-mannosidase from *Canavalia ensiformis* onto agarose and magnetic silica nanoparticles. Both of them are relevant tools for glycomic analysis: β-galactosidase for selective removal of terminal β 1-4

and β 1-6 galactose (Gal) [27-28] and α- mannosidase for selective removal of α 1-2, α 1-3 and α 1-6 terminal Mannose (Man) [29-31]. Both glycosidases were applied to the selective deglycosylation of model glycoproteins and a native parasite lysate from *F. hepatica*, highlighting their relevance as glycomic tools. Finally, changes in reactivity towards specific lectins due to deglycosylation process were analysed for deglycosylated samples, as well as their immunomodulatory properties on DCs. The development of immobilized glycosidases described here, contributes to the generation of improved glycomic tool that will enable the elucidation of glycan role in biological processes.

## 2. Materials and Methods

### *2.1 Materials*

*C. ensiformis* (Jack bean) α-mannosidase (α-D-mannoside manohydrolase, EC 3.2.1.24), *A. oryzae* β–galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), fetuin from bovine fetal serum, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP-$BF_4$), o-nitrophenyl-β-D-galactopyranoside (ONPG), p-nitrophenyl-α-D-mannopyranoside (PNPM), o-phenylenediamine (OPD), galactose (Gal), mannose (Man), orcinol, cocktail of protease inhibitors and 7-amino-4-methyl coumarin (AMC) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine lactoferrin was from Murray Golburn Cooperative Co. LTD (Melbourne). Bicinchoninic acid (BCA) and detoxi-gel columns were purchased from Pierce (Rockford, Ilinois, USA). TLC silica plates were from Machery Nagel (Duren Germany). Agarose 4B, PD-10 (Sephadex G25) columns and NAP-5 (Sephadex G25) columns were from GE Health Care (Buckinghamshire, UK). Hydroxylated magnetic nanoparticles (SIMAG-Hydroxyl) were form Chemicell GmbH (Berlin, Germany). Nunc Maxisorp plates were from Roskilde (Denmark). Peptide N- glycosidase (PNGase) was from Promega (Madison, USA), PGS SPE columns were purchased from Supelco (USA), Detergent out beads where from Calbiochem (USA). All the other reagents were from analytical grade.

### *2.2 Enzyme activity assays*

#### *2.2.1 β-galactosidase assay*

Enzymatic activity of β-galactosidase soluble and immobilized onto agarose was assayed as described by Porciúncula Gonzalez et al [32]. Enzyme activity of β-galactosidase immobilized onto magnetic nanoparticles was measured by incubating 50 μL of immobilized enzyme suspension with 1 mL of ONPG 25 mM in 50 mM sodium acetate buffer pH 5.5 (β-gal activity buffer) at room temperature. Every 30 seconds upon 3 minutes, 100 μL of the reaction mixture were withdrawn and immobilized enzyme removed using a magnet. The supernatant was added to 200 μL of 0.2 M sodium borate buffer pH 9.8 and absorbance at 405 nm was measured with an ELISA (Enzyme Linked Immunosorbent Assay) plate reader. ONP concentration was determined using a calibration curve and the rate of ONP formation determined.

One enzyme unit (U) was defined as the amount of enzyme hydrolysing 1 μmol of ONPG per minute in the above defined conditions. Enzymatic assays were performed in triplicate.

#### *2.2.2 α-mannosidase assay*

70 μL of an adequate dilution of α-mannosidase of *C. ensiformis* solution or immobilized enzyme suspension were incubated with 630 μL of 5 mM para-nitrophenyl α-D-mannopyranoside (PNPM) in 50 mM sodium citrate buffer pH 4.5 containing 0.1 mM zinc acetate (α-man activity buffer) at room temperature. Every 30 seconds upon 3 minutes, 100 μL of reaction mixture were withdrawn and added to 200 μL of 0.2 M sodium borate buffer pH 9.8 to stop the enzymatic reaction mixture. Absorbance at 405 nm was measured with an ELISA plate reader. The para-nitrophenol (PNP) concentration was determined using a calibration curve and the rate of PNP formation determined. Immobilized α-mannosidase standard suspension was prepared in 0.1 M phosphate buffer pH 7.5 containing 0.1 mM zinc

acetate (α-man storage buffer). When immobilized onto agarose magnetic stirring was used during the incubation with the substrate. Immobilized enzyme was removed from the reaction mixture before absorbance measure by decantation or by use of a magnet when immobilized onto agarose or magnetic nanoparticles respectively.

One enzyme unit (U) was defined as the amount of enzyme hydrolysing 1 μmol of PNPM per minute in the above defined conditions. All the enzymatic assays were performed in triplicate

### *2.3 Protein quantification*

Protein was determined by the BCA assay as described by Smith et al. [33]. All the protein assays were preformed in triplicate.

### *2.4 Enzyme immobilization*

#### *2.4.1 Support activation*

Agarose activation was carried on as described by Giacomini et al. [34].

Nanoparticles activation was optimized in our laboratory. All the reagents used were pre-cooled to 4°C. Thirty mg of silica magnetic nanoparticles (SIMAG-Hydroxyl) were washed with 5 mL of: phosphate buffer saline (PBS) pH 7.4; acetone : water (3:7v/v) and acetone : water (6:4 v/v). The supernatant was removed by using a magnet. The nanoparticles were suspended in 0.3 mL of acetone : water (6:4v/v), and incubated with 37.5 mg of CDAP dissolved in 1 mL of acetone : water (6:4v/v) under vigorous stirring for 3 min at 4°C. Immediately after, 0.18 mL of 0.2 M triethylamine were added drop wise in a period of 1 to 2 min. Upon 3 min, the supernatant was removed and 1 mL of ice-cold 50 mM HCl was added. After no more than 2 min the supernatant was removed and the nanoparticles washed with 5 mL of ice-cold water.

The activated supports were equilibrated in the corresponding immobilization buffer and immediately used for enzyme immobilization.

#### *2.4.2 β-Galactosidase immobilization*

1.5 g of activated agarose or 10 mg of activated nanoparticles were incubated with 15 mL or 0.4 mL respectively, of varied concentrations of β-galactosidase in 0.1M sodium phosphate buffer pH 8.3 (β-gal immobilization buffer), for 4 hours at room temperature under mild stirring. The supernatant was removed and the immobilized enzyme washed with β-gal activity buffer and stored at 4°C.

*2.4.3 α-mannosidase immobilization*

0.3 g of activated agarose or 10 mg of activated nanoparticles were incubated with 2 mL or 0.4 mL respectively of varied concentrations of α- mannosidase in α-man storage buffer for 4 hours at room temperature under mild stirring. The supernatant was removed and the immobilized enzyme washed with α-man storage buffer and stored at 4°C.

***2.5 Model glycoprotein deglycosylation***

*2.5.1 Asialofetuin degalactosylation with immobilized β-galactosidase*

Asialofetuin was obtained by chemical fetuin desialylation. Thirty mg of fetuin from fetal bovine serum dissolved in 2.55 mL of 0.2 M HCl were incubated in a thermo block at 80 °C for one hour. After being cooled to room temperature it was neutralized with 2.0 M NaOH and dialysed against 50 mM ammonium acetate buffer pH 5.5 for 24 hours. The dialysed asialofetuin was centrifuged at 10.000 rpm for five minutes.

1-2 mL of asialofetuin (6 mg/mL) were added to 0.1 g (6 mg/g, 251 U/g) or 10 mg (11 mg/g; 1429 U/g) of β-galactosidase immobilized onto agarose or magnetic nanoparticles respectively and incubated under mild stirring at room temperature for 24 hours. β-galactosidase was removed from the reaction mixture by filtration or by use of a magnet when immobilized onto agarose or nanoparticles respectively, washed with β-gal activity buffer and stored at 4°C for the next use. The supernatant was gel filtrated using Sephadex G25 PD10 column equilibrated with 50 mM ammonium acetate buffer pH 5.5, in order to separate asialofetuin treated with β-galactosidase from the released Gal. Degalactosylated asialofetuin

was subjected to binding lectin assay as described in section 2.7 and the carbohydrate fraction was lyophilized, re-dissolved in minimum water volume and analysed by TLC and HPLC. Control experiments were performed by incubating asialofetuin with non-activated support.

*2.5.2 Lactoferrin demannosylation with immobilized α-mannosidase*

A lactoferrin solution (9.8 mg/mL) was prepared in α-man activity buffer and gel filtrated in Sephadex G25 PD-10 columns. 1-2 mL of lactoferrin (7 mg/mL) in α-man activity buffer were added to 50 mg (2.2 mg/g; 55 U/g) or 10 mg (9 mg/g; 131 U/g) of α-mannosidase immobilized onto agarose or magnetic nanoparticles respectively. The mixture was incubated under mild stirring at room temperature for 24 hours. α- mannosidase was removed from the reaction mixture by filtration or by use of a magnet when immobilized onto agarose or nanoparticles respectively, washed with α-mannosidase storage buffer and stored at 4°C for the next use. The supernatant was gel filtrated using Sephadex G25 PD10 column equilibrated with 50 mM ammonium acetate buffer pH 5.5, in order to separate the lactoferrin treated with α-mannosidase from the released Man. Demannosylated lactoferrin was subjected to binding lectin assay as described in section 2.7 and the carbohydrate fraction was lyophilized, re-dissolved in minimum water volume and analysed by TLC and HPLC. Control experiments were performed by incubating lactoferrin with non-activated support.

***2.6 Deglycosylation of FhTE***

Protein lysate from *Fasciola hepatica* (FhTE) was prepared as described by Rodriguez et al. [8]. To remove endotoxin contamination, the lysates were applied to a column containing endotoxin-removing gel (detoxi-gel, Pierce Biotechnology). The obtained FhTE was gel filtrated in Sephadex G25 NAP columns equilibrated either in 50 mM sodium acetate buffer pH 6.5 or 50 mM sodium phosphate buffer pH 6.5; 0.1 mM zinc acetate containing cocktail of protein inhibitors for degalactosylation or demannosylation process respectively.

0.5 mL (2 mg/mL) of FhTE were incubated with 50 mg of β-galactosidase (251 U/g, 9 mg/g) or α-mannosidase (55 U/g, 2.2 mg/g) immobilized onto agarose, and 0.5 mL (5 mg/mL) of FhTE were incubated with 10 mg of β-galactosidase (1429 U/g, 11 mg/g) or α-mannosidase (131 U/g, 9 mg/g) immobilized onto magnetic nanoparticles. The mixtures were incubated under mild stirring for 24 hours at room temperature. The immobilized glycosidase was removed from the reaction mixture either by filtration or by use of a magnet when the enzyme was immobilized onto agarose or magnetic nanoparticles respectively, washed with β-gal activity buffer or α-man storage buffer for β-galactosidase and α-mannosidase respectively and stored at 4°C for the next use. The supernatant was dialyzed against PBS pH 7.4 using membranes with 3 kDa cut off, and further lectin binding assay were performed as described in section 2.7. The corresponding controls were performed incubating FhTE with non activated supports under the same conditions.

***2.7 Binding lectin assay***

Nunc Maxisorp microtiter plates were coated with model glycoproteins, FhTE or their counterparts treated with glycosidases (1 μg/well) in 0.1 M carbonate buffer pH 9.0 during 18 hours at 4°C. After washing three times with PBS containing 0.1% Tween 20 it was incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. After three washes, plates were incubated with biotin coupled lectins (1-10 μg/mL) for 1 h at 37°C, followed by streptavidin-peroxidase for 45 min at 37°C. Upon three washes, 200 μL of 0.5 mg/mL o-phenylendiamine (OPD) in 0.1 M citrate-phosphate pH 5.0 and $H_2O_2$ (0.003%) were added. Absorbance at 492 nm was determined. The lectins from *Canavalia. ensiformis* (ConA) and *Arachis hypogaea* (PNA), were used in this study.

***2.8 DC-SIGN Binding***

Recognition of lactoferrin and lactoferrin treated with α-mannosidase by DC-SIGN (Dendritic cell specific ICAM grabbing non integrin) was evaluated by ELISA-like assay, using the

recombinant chimeric protein DC-SIGN-hFc (carbohydrate recognition domain of DC-SIGN fused to the constant portion of a human immunoglobulin was prepared as described by Geijtenbeek et al. [35]). ELISA plates were coated with lactoferrin and lactoferrin treated with α-mannosidase (2-5 μg/well) in 50 mM carbonate buffer pH 9.0 during 18 hours at 4°C. After three washes with 20 mM buffer Tris, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4 (TSM) containing 0.1% Tween 20, plates were blocked with 1% BSA in TSM for 1 h at 37°C, followed by incubation with 10 μg/mL of DC-SIGN-hFc for two hours at room temperature. Then, a human immunoglobulin-specific antibody conjugated to peroxidase was incubated for 1 h at room temperature. Finally, it was developed using a solution of the enzyme substrate tetramethylbenzydine (TMB) and 0.003% of $H_2O_2$ in citrate-phosphate buffer 0.1 M pH 5.

***2.9 Sugar analysis***

Released carbohydrates were analysed by TLC and HPLC as described by Porciúncula Gonzalez et al. [36].

***2.10 Lactoferrin N-glycan structure determination.***

Lactoferrin N-glycan analysis was performed as described by Kalay et al. [37] with some modifications. One mg of lactoferrin or lactoferrin treated with α-mannosidase were lyophilized and dissolved in 100 μL of denaturation buffer (0.25 M sodium phosphate buffer pH 8.5, containing 7 M urea, 2 M tiourea, 2% SDS, 1 M β-mercaptoetanol) and sonicated during one hour. Then 100 μL of neutralization buffer (0.25 M sodium phosphate buffer pH 8.5 containing 15% of Igepal CA-630) were added to neutralize the inhibitory effect of SDS on PNGase activity and was sonicated for another 15 minutes. Finally 800 μL of 0.25 M sodium phosphate buffer pH 8.5 and 10 U of PNGase were added and the mixture was incubated for at least 48 h at 37°C. Oligosaccharide purification was performed by using PGC SPE columns of porous activated charcoal and the remaining detergent was removed by incubation with detergent out beads. Purified N-glycans were derivatized with AMC via

reductive amination in the reducing end. Briefly, the oligosaccharides were mixed with 2 mg of AMC and 2 mg of reducing agent picoline borane, after thoroughly vortexing the mixture was incubated at 65°C for 2 hours. Separation, quantification and characterization of glycans was performed using a 2D-LC-MS system with an intercalated nano-Fluorescence detector (excitation/emission wavelength : 350nm/400nm). As first dimension a capillary WAX trap column is used to trap the charged oligosaccharides species. Unbound neutral oligosaccharides were trapped onto a C18 trap column (300 μM x 10 mm, Dionex The Netherlands) and analysed on a long reverse phase nano-LC column (5μ, 75 μM x 3000 mm, prepared in-house). Glycan species were quantified based on fluorescence and structures were identified based on the information obtained from the MS/MS fragmentation pattern.

### *2.11 Cell culture*

BMDCs (Bone marrow dendritic cells) were generated as described by Rodriguez et al. [8]. Splenic $CD4^+$ T Cells were purified (Stem Cell Technologies, Canada) from BALB/c mice of 8 weeks old that were previously orally infected with 10 uncapped *F. hepatica* metacercariae (Baldwin Aquatics, USA) for 23 weeks. To analyse the induction of Th2/Treg responses, BMDCs ($2.5\times10^5$/well) were incubated at 37°C and 5% $CO_2$ in 96-well plates with FhTE (75 μg/mL) or medium alone in the presence of LPS (*Escherichia coli* 0111:B4, 0.5-1 μg/mL) overnight at 37°C. Alternative, cells were incubated with FhTE treated with immobilized α-mannosidase or β-galactosidase and then centrifuged at 1,500 rpm for 5 min at 4°C. Stimulated BMDCs were then co-cultured with parasite-specific splenic $CD4^+$ T Cells for 72 h at 37°C. Supernatants were collected and cytokine (IL-4 and IL-10) levels were tested by interleukin specific sandwich ELISA assays (BD Bioscience, NJ, USA).

## 3. Results

### *3.1 Glycosidase immobilization*

*A. oryzae* β-galactosidase and *C. ensiformis* α-mannosidase were immobilized onto agarose and silica magnetic nanoparticles activated with cyanate ester groups. The cyanylating agent used for the support activation was CDAP-$BF_4$ (Figure 1a). This activation method is a less toxic alternative to the traditional activation with cyanogen bromide. Agarose activation with CDAP-$BF_4$ has already been reported [34,38], but it has not been used for the activation of magnetic nanoparticles until now. High Immobilization rates (over 67%) were obtained for both enzymes. β-galactosidase was immobilized fully active as similar enzyme activity yields and immobilization yields were obtained (Table 1). On the other hand, the immobilization of α- mannosidase showed a lower enzyme activity yield than immobilization yield, suggesting either partial inactivation due to the immobilization process or underestimation of immobilized enzyme activity due to diffusional problems. Since silica nanoparticle is a relatively new support for enzyme immobilization, the effect of the enzymatic charge on the immobilization yield was studied. It is well known that extremely high enzymatic loads are responsible for diffusional limitations hindering the access of the substrate to the active site of the enzyme, particularly for macromolecular substrates [34, 39-41]. At low or moderate enzyme load (5-10 mg/g of support), immobilization rates higher than 80% were achieved for both enzymes. Once again, β-galactosidase was immobilized completely active while partial enzyme inactivation was observed for α-mannosidase (Table 1). Nevertheless, as expected, for higher enzymatic loads (around 20 mg/g of support), immobilization rates decreased for both enzymes. Moreover, a decrease in the enzyme activity yield was observed not only for α-mannosidase but also for β-galactosidase (Table 1). One of the advantages of using covalent immobilization strategies is that the enzyme is not released from the support, a phenomenon known as leakage [21]. In order to check the absence of leakage, immobilized β-galactosidase and α-mannosidase were incubated with the corresponding activity buffer

during 24 hours at room temperature under mild stirring. The analysis of supernatants by SDS electrophoresis revealed the absence of enzyme release (data not shown).

In summary, both β-galactosidasase and α-mannosidase were successfully immobilized onto two different supports. Enzymatic derivatives with moderate enzymatic load (average of 2-6 mg/g of agarose or 10 mg/g of nanoparticles) were used for further deglycosylation experiments on different glycoprotein preparations.

### *3.2 Selective deglycosylation of model glycoproteins*

In order to evaluate the functionality of the immobilized glycosidases as effective glycomic tools, the deglycosylation of two model glycoproteins was studied. Asialofetuin, obtained from chemical desialylation of bovine fetal serum fetuin, is a glycoprotein with terminal β-galactose [42, 43]. Therefore, it is useful for degalactosylation studies. On the other hand, bovine lactoferrin was used for the evaluation of demannosylation process as it contains high mannose N-glycans [44]. The deglycosylation process is shown in Figure 1b.

#### *3.2.1 Asialofetuin degalactosylation.*

The degalactosylation process was performed at pH 5.5 and room temperature for 24 hours, followed by the removal of the enzyme either by filtration or by use of a magnet when immobilized onto agarose and magnetic nanoparticles respectively. The immobilized glycosidase was washed and re-used in several degalactosylation cycles. Asialofetuin treated with β-galactosidase was separated from the released monosaccharaides, by size exclusion chromatography. After concentration by lyophilisation, the carbohydrate fraction was analysed by TLC and HPLC. In Figure 2a**,** spots with the same retention factor ($R_f$) than Gal can be observed in the TLC of the carbohydrate fractions corresponding to several degalactosylation cycles performed with the β-galactosidase immobilized onto silica magnetic nanoparticles. Similar results were obtained with the enzyme immobilized onto agarose (data not shown). These results were confirmed by HPLC, where a peak with the same retention

time of Gal appears in all the carbohydrate fractions analysed except for the control (Figure 2b). Therefore, selective release of Gal could be attributed to enzymatic degalactosylation. Moreover, the immobilized enzyme was re-used without loss of effectiveness. Next, the fraction containing the asialofetuin treated with β-galactosidase was analysed using a lectin binding assay with *A. hypogaea* lectin (PNA), which can recognise Gal [45]. A 50% decrease in PNA recognition was observed due to selective enzymatic treatment of asialofetuin with β-galactosidase immobilized either to agarose or magnetic nanoparticles (Figure 2c and 2d).

*3.2.2 Lactoferrin demannosylation.*

Demannosylation process was performed at pH 4.5 at room temperature during 24 hours. Separation of the immobilized enzyme from the reaction mixture and of the lactoferrin treated with α-mannosidase from the released Man was achieved in a similar way as that described for the degalactosylation process in section 3.2.1. The analysis of the carbohydrate fraction by TLC showed spots with the same $R_f$ than the Man standard for all the demannosylation cycles performed with α-mannosidase immobilized onto magnetic nanoparticles, except in the control experiments (Figure 3a). We also confirmed the release of Man by HPLC analysis (Figure 3b), as well as the successful re-use of the immobilized glycosidase (Figure 3a and 3b). Similar results were obtained when the demannosylation process was performed with the enzyme immobilized onto agarose (data not shown). These results confirms the effectiveness of the enzymatic demannosylation process. The fraction containing the lactoferrin treated with α-mannosidase was analysed using lectin binding assay with Concanavalin A from *C. ensiformis* (ConA), which can recognise glucose (Glc) and Man as well as GlcNAc*β*2Man*α*6(GlcNAc*β*2Man*α*3)Man*β*4GlcNAc [45].

A decrease in the recognition of ConA of around 60-80 % and 60 % was observed for α-mannosidase immobilized onto agarose and magnetic nanoparticles respectively (Figure 3c and 3d). When α-mannosidase immobilized onto agarose was used for lactoferrin

demannosylation, loss in ConA recognition was slightly reduced in the second and third use. Nevertheless, α-mannosidase activity of the re-used derivatives was checked, proving that it retained 100% of its initial activity. A complete loss of recognition by ConA was not achieved, probably because not all the Man from the lactoferrin high mannose N-glycans was removed. In order to determine changes in N-glycan structure due to enzymatic demannosylation of lactoferrin, a glycoprofiling analysis was performed. N-Glycans were removed from lactoferrin treated with α-mannosidase by enzymatic hydrolysis with Peptide N-glycosidase (PNGase-F), labelled with a fluorophore and analysed by HPLC-MS. Figure 4a shows the N- glycan HPLC elution profile of bovine lactoferrin where high mannose type structures containing five to nine Man can be observed, as previously described by O'Riordan et al. [46]. After enzymatic demannosylation with α-mannosidase immobilized onto magnetic nanoparticles, a significant decrease in mannose structures containing 8-9 Man residues alongside with an increase in mannose structures containing 1-5 Man residues was evidenced (Figure 4a and 4b). Particularly peaks corresponding to glycans containing one to four Man showed up in the lactoferrin treated with α-mannosidase, while they were negligible in the native glycoprotein. This confirms that immobilized α-mannosidase successfully removed Man residues from lactoferrin although total demannosylation could not be achieved. This is in agreement with the partial loss of recognition for ConA observed for the lactoferrin treated with α-mannosidase. It is well reported in the literature that certain CLR (C-type lectin receptors) such as DC-SIGN can bind mannan as well as glycoproteins with high mannose N-glycans, including bovine lactoferrin. Thus, this interaction can trigger signalling cascades that affect the function of macrophages or DCs [47-50]. In order to evaluate the biological role of Man residues in lactoferrin we analysed the recognition of native lactoferrin and lactoferrin treated with α-mannosidase by DC-SIGN, using the chimeric protein DC-SIGN-hFc. As Shown in Figure 4c a decrease in the recognition by DC-SIGN of the lactoferrin

treated with α-mannosidase compared to the native glycoprotein was observed, demonstrating that the receptor recognition is mediated by oligo-mannose structures. Thus partial demannosylation of lactoferrin decreased recognition by DC-SIGN.

### *3.3 Deglycosylation of F. hepatica glycoconjugates*

In order to evaluate the potential of both immobilized glycosidases as glycomic tools to determine the biological function of glycans, we extended their use to native glycoconjugates preparations. We have previously described evidence about the role of *F. hepatica* glycans, a fluke with outstanding importance in human health and farming production, in the immunomodulation of murine DCs. These data suggest that a Man-specific CLR is involved in the recognition of parasite glycans and triggers immunomodulatory signals on DCs [8]. Therefore, the feasibility of using the immobilized α-mannosidase and β-galactosidase immobilized onto magnetic silica nanoparticles as glycomic tool for deglycosylation of a total parasite extract (FhTE) was evaluated. Degalactosylation, demannosylation and control experiments were performed at pH 6.5 as FhTE proteins present in FhTE precipitate at lower pHs. Deglycosylation performance was evaluated by lectin binding assay using PNA and ConA, two lectins that recognize FhTE [8]. Figure 5a shows a decrease of 55% of PNA recognition on the FhTE treated with β-galactosidase with no significant changes on the FhTE treated with manosidase. On the other hand, a loss of 40 % for ConA recognition on FhTE treated with α-mannosidase with no changes on the FhTE treated with β-galactosidase was observed (Figure 5a). These results show the effectiveness of immobilized glycosidases on the removal specific monosacharides from natural glycoconjugates. Next, FhTE treated with β-galactosidase or α-mannosidase were used to evaluate the role of Gal and Man *F. hepatica* glycan moieties in the immunomodulation of DCs. Our group has already reported that *F. hepatica* glycans regulate DCs to induce and expand Th2-like cytokine IL-4 and the regulatory cytokine IL-10 by parasite-specific T cells [8]. Here, the ability of the FhTE treated

with β-galactosidase or α-mannosidase to induce the production of IL-4 and IL-10 by T cells from infected animals was analysed. To this end, BMDCs were pulsed with differential glycosylated FhTE, and incubated with purified splenic $CD4^+$ T cells from infected animals. Interestingly, the incubation with FhTE treated with α-mannosidase, but not FhTE treated with β-galactosidase, reduced the production of both IL-4 and IL-10 (Figure 5b), demonstrating a role of mannosylated glycan structures in the induction of Th2/Treg responses. Altogether, these results show the utility of immobilized glycosidases to determine the biological role of glycans.

**4. Discussion**

In this work, we describe the successful immobilization of *A. oryzae* β-galactosidase and *C. ensiformis* α-mannosidase, two exoglycosidases relevant in glycomic analysis. Both of them were immobilized with good immobilization rates, keeping above 60% of their original activity. As expected, no enzyme leakage was detected and both enzymes could be used in several enzymatic cycles without losing effectiveness. Nevertheless while β-galactosidase was immobilized fully active, α-mannosidase showed a decrease in enzyme activity. The latter suggests either partial inactivation due to immobilization process or diffusional problems. The fact that *C. ensiformis* α-mannosidase is a multimeric enzyme could lead to immobilization through different subunits which could generate distortion in the enzyme structure affecting its catalytic activity [31,51]. On the other hand, partial inactivation could also be caused by immobilization through the ε-amines of lysine moieties near the active site of the enzyme. There are some evidence regarding the presence of lysine moieties of *C. ensiformis* α-mannosidase in or near the active site, while *A. oryzae* exposed lysines are relative far away from the active site [51-52]. Another possibility is the generation of diffusional problems when the immobilization takes place with the active site facing the support. Immobilized glycosidases proved to be effective in the partial deglycosylation of

both, model glycoproteins and native glycoprotein mixture from the trematode *F. hepatica*. This was evidenced by a change in their pattern of lectin recognition after the enzymatic deglycosylation process. No significant differences were observed in the performance of the glycosidases immobilized onto agarose or silica magnetic nanoparticles, suggesting that in spite of its higher particle size, agarose constitutes a suitable support for immobilization of glycosidases for glycomic analysis.

The specific deglycosylation of FhTE allowed us to show the relevance of mannosylated glycans in the induction of Th2 and Treg immune responses during parasite infection. Indeed, *F. hepatica* tegumental coat is highly glycosylated with oligomannose and paucimannose N-glycans [53]. It is worth noting that, even though a complete demannosylation of FhTE could not be achieved, the enzymatic treatment led to a dramatic change in the capacity of FhTE-pulsed BMDCs to induce IL-4 and IL-10 by specific $CD4^+$ T cells from infected animals. This demonstrates the effective use of immobilized enzymes to determine the biological role of glycans. Mannosylated glycans could take part in the development of Th2/Treg responses in different ways. First, mannose-containing glycan structures could be important for the internalization of glycoproteins containing immunodominant peptides, the ones more effective for the development of immune responses. Second, the interaction of glycans with specific receptors could trigger signaling events that lead to a regulatory program in the DCs and a reduced stimulatory capacity. In fact, very recent reports, including our own, have brought insights about the role of *F.hepatica* mannosylated glycans in mediating the regulation of DC-maturation through the mannose-specific receptors [8,54-55].

The results showed in this work are promising considering the implementation of immobilized enzymes as a tool for glycomic analysis, an area where their use is scarce. It should be highlighted that, even when working in the most favourable conditions, complete deglycosylation may not be achieved in a single step. A loss in the recognition of PNA of near

50% was observed after degalactosylation of both asialofetuin and FhTE. PNA can bind to terminal Gal, having a lower affinity for Gal β1-4 GlcNAc ($K_{dis}$ = 4μM) and a higher affinity for Gal β1-3 GalNAc ($K_{dis}$ = 22 nM) [45,56]. On the other hand, *A. oryzae* β-galactosidase hydrolyses preferentially β1-4 galactosides and eventually β1-3 galactosides at a very low rate [27-28]. So remaining Gal β1-3 GalNAc present in asialofetuin could still be recognized by PNA lectin. In the case of α-mannosidase, partial removal of mannose could be attributed to steric hindrance that difficult the access of mannose residues closer to the backbone of the glycoprotein to the active site of the enzyme. This phenomenon is likely to be increased when using immobilized glycosidases. Yet, working with the soluble enzyme will involve an additional step to remove the glycosidase in order to avoid interference in further analysis performed with the deglycosylated sample. Nevertheless, partial demannosylation was enough to lead to a biological effect, as shown by the decrease in lactoferrin recognition by DC-SIGN and the reduction in the expansion of FhTE specific Th2/Treg responses. Our findings highlight that both the presence of Man and its structural conformation, are important for the recognition of specific receptors and the development of biological effects. They also show that partial deglycosylation using immobilized glycosidases, such as the β-galactosidase and α-mannosidase studied here, are potential tools to determine the structure and biological roles of glycan structures.

**Conclusions**

In this work, we describe the immobilization of *A. oryzae* β-galactosidase and *C. ensiformis* α-mannosidase, two exoglycosidases relevant in glycomic analysis. Good immobilization yields (70-90%) were achieved, retaining over 60% of their original activities. No leakage was detected and both enzymes could be used in several enzymatic cycles without losing

effectiveness. They proved to be effective in the partial deglycosylation of both, model glycoproteins and native glycoprotein mixture from the trematode *F. hepatica.*

The specific deglycosylation of FhTE showed the relevance of mannosylated glycans in the induction of Th2 and Treg immune responses during parasite infection. Our results demonstrate the successful immobilization of specific glycosidases in nano-supports and validate their utility to identify glycans biological functions.

**Author Contributions**

ER, TF, CG, conceived and designed the experiments. ER, KF,NB, performed the experiments. ER, KF, HK, TF,CG analysed and interpreted the data. HK, JJGV, YVK, contributed with reagents, materials and analysis tools. ER, TF, CG wrote the paper.

All the authors have approved the final article.

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**Ethics approval**

Mouse experiments were carried out in accordance with strict guidelines from the National Committee on Animal Research (Comisión Nacional de Experimentación Animal, CNEA, National Law 18.611, Uruguay). Adult worms were collected during the routine work of two local abattoir (Frigorífico Carrasco and Frigorífico Sarubbi) in Montevideo (Uruguay). All procedures involving animals were approved by the Universidad de la Republica’s

Committee on Animal Research (Comisión Honoraria de Experimentación Animal, CHEA Protocol Numbers: 071140-001822-11 and 071140-000143-12).

**Declaration of interest:** none

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**Figure Captions**

**Fig 1 Deglycosylation process with immobilized enzymes**

a) Enzyme immobilization mechanism: Agarose and silica nanoparticles were activated with CDAP-$BF_4$ in order to introduce cyanate ester groups capable of reacting with exposed amino groups of the glycosidases. b) Deglycosylation process: Enzymatic degalactosylation of asialofetuin was performed with immobilized β-galactosidase at pH 5.5 at room temperature for 24 h. Enzymatic demannosylation of lactoferrin was performed with α-mannosidase at pH 4.5 at room temperature for 24 h.

**Fig 2 Functional analysis of immobilized β-galactosidase**

a-b) TLC chromatography (a) and HPLC Chromatography (b) of the galactose moiety released from asialofetuin due to degalactosylation with β-galactosidase immobilized onto silica magnetic nanoparticles.

c-d) PNA recognition of asialofetuin treated with β-galactosidase immobilized onto agarose (c) or silica magnetic nanoparticles (d). Elisa plates were coated with asialofetuin (1µg/well) and further incubated with PNA conjugated to biotin and streptavidin-peroxidase.

Standard: 5 mM Gal; Control consisted in the incubation of asialofetuin from bovine serum with non activated silica nanoparticles at room temperature for 24 h; Use 1, Use 2 and Use 3 states for the numbers of cycles the immobilized enzyme was re-used.

Results are expressed as the mean of three independent experiments (± SD, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$).

**Fig 3 Functional analysis of immobilized α-mannosidase**

a-b) TLC chromatography (a) and HPLC Chromatography (b) of the mannose moiety released from lactoferrin due to demannosylation with α-mannosidase immobilized onto magnetic nanoparticles.

c-d) ConA recognition of lactoferrin treated with α-mannosidase immobilized onto agarose (c) or silica magnetic nanoparticles (d). ELISA plates were coated with lactoferrin (1 μg/well) and further incubated with ConA conjugated to biotin and streptavidin-peroxidase.

Standard: 5 mM Mannose; Control consisted in the incubation of bovine lactoferrin with non activated support at room temperature for 24 h. Use 1, Use 2 and Use 3 states for the number of cycles the immobilized enzyme was re-used. Results are expressed as the mean of three independent experiments (± SD, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$).

**Fig 4 Determination of N-glycan structure of differential glycosylated lactoferrin and study of the changes in their recognition by the receptor DC-SIGN**

a) Determination of N-glycan structure of lactoferrin and lactoferrin treated with α-mannosidase immobilized onto silica magnetic nanoparticles: N-glycans where removed from lactoferrin by enzymatic hydrolysis with PNGase F. They were further purified and conjugated to the fluorophore AMC and analysed by HPLC-MS. N-glycan structures were identified using the MS/MS fragmentation pattern of each peak. m/z values corresponding to each glycan moiety are shown in brackets.

b) The graph represent the quantification of identified glycans before and after α-mannosidase treatment.

c) Study of the changes in lactoferrin recognition by the receptor DC-SIGN due to lactoferrin treatment with mannosidase. ELISA Plates were coated with serial dilutions of lactoferrin and lactoferrin treated with α-mannosidase and further incubated with the chimeric protein DC-SIGN-hFc. Binding of chimeric protein was detected with an anti-hIgG antibody conjugated to peroxidase. Asterisks (*) indicate significant stadistic differences (*$p<0.05$).

**Fig 5 Selective treatment of a *Fasciola hepatica* lysate (FhTE) with immobilized α-mannosidase changes its capacity to induce Th2/Treg responses**

a) PNA and ConA reactivity toward FhTE treated with with β-galactosidase and α-mannosidase immobilized onto magnetic nanoparticles was evaluated.

ELISA plates were coated with FhTE (1 μg/well) and further incubated with lectins conjugated to biotin and streptavidin-peroxidase.

b- Production of IL-4 and IL-10 by specific $CD4^+$ T cells from *Fasciola* infected animals, after co-culture with BMDCs pulsed with FhTE/LPS, FhTE + α-Man/LPS or FhTE + β-Gal/LPS. Cytokine levels were analysed by specific ELISA in culture supernatants.

Control consisted in the incubation of FhTE with non activated nanoparticles at room temperature for 24 h. Results are expressed as the mean of three independent experiments (± SD, indicated by error bars). Asterisks indicate statistically significant differences (*$p < 0.05$).

**Figure 1 (Color only online)**

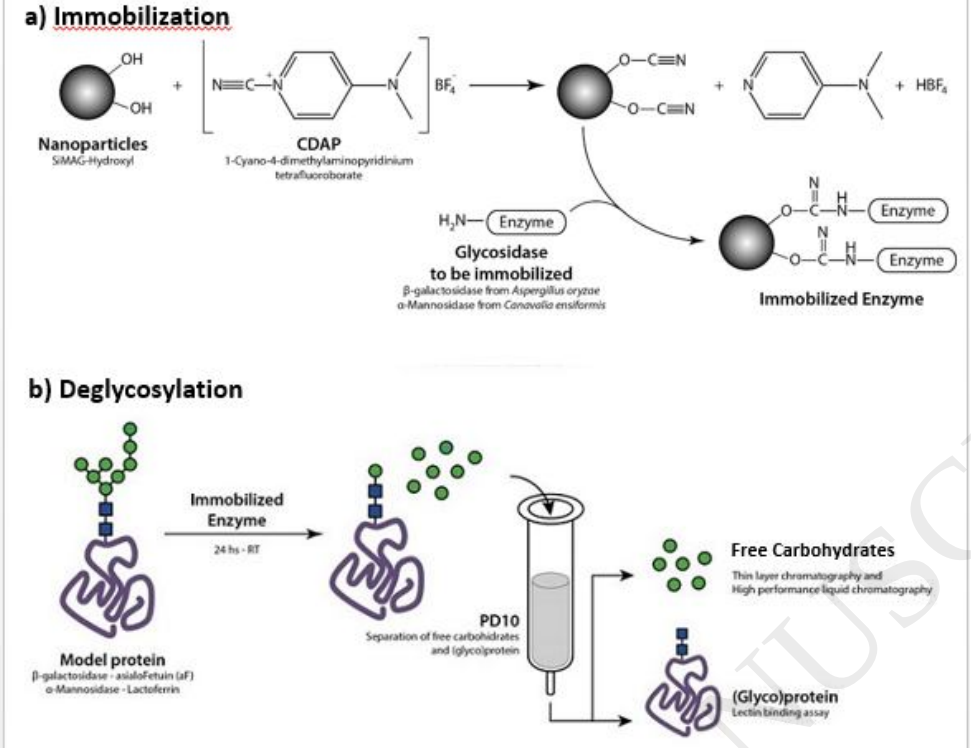
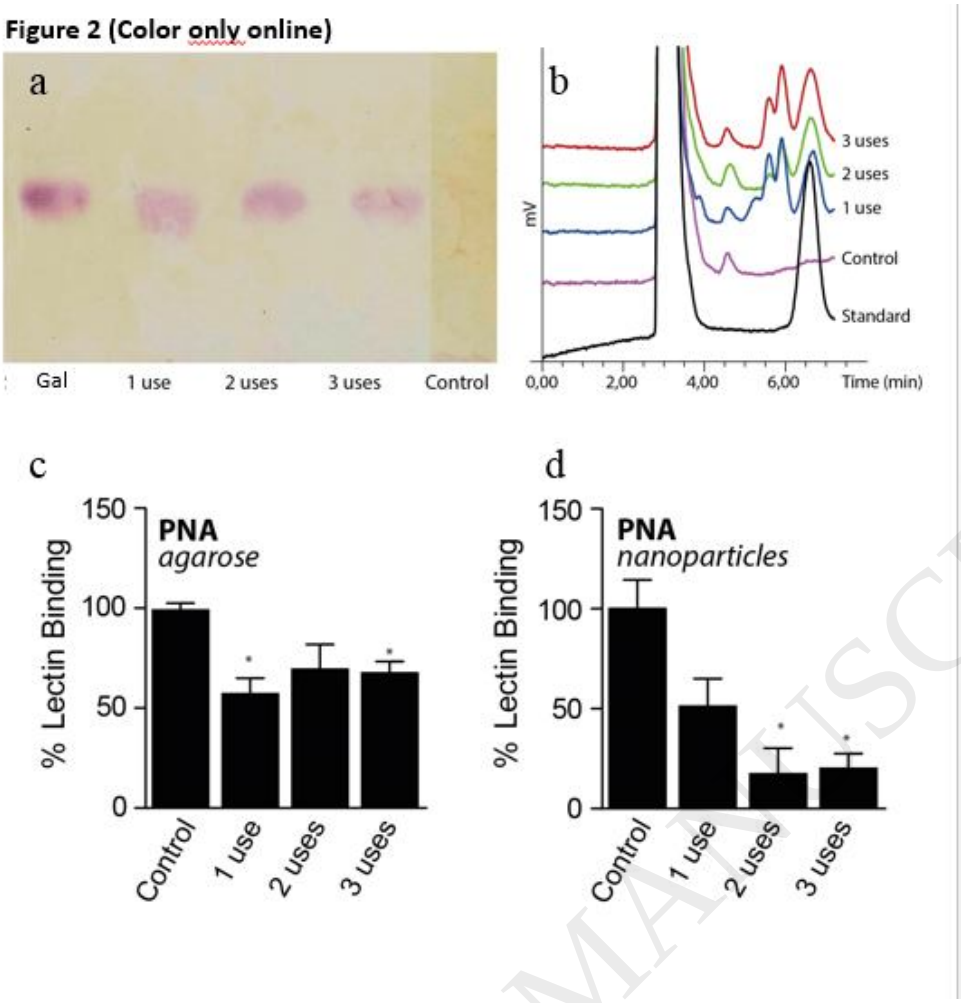
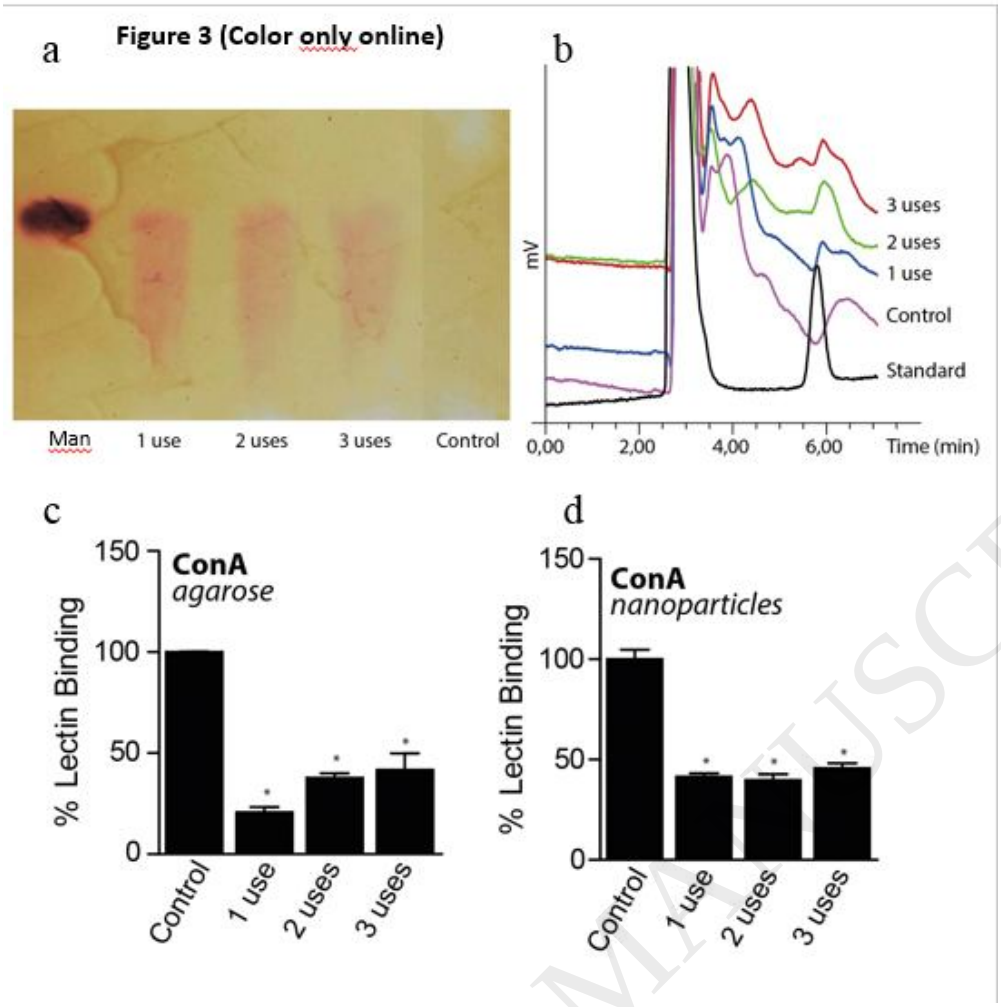


**Figure 2 (Color only online)**



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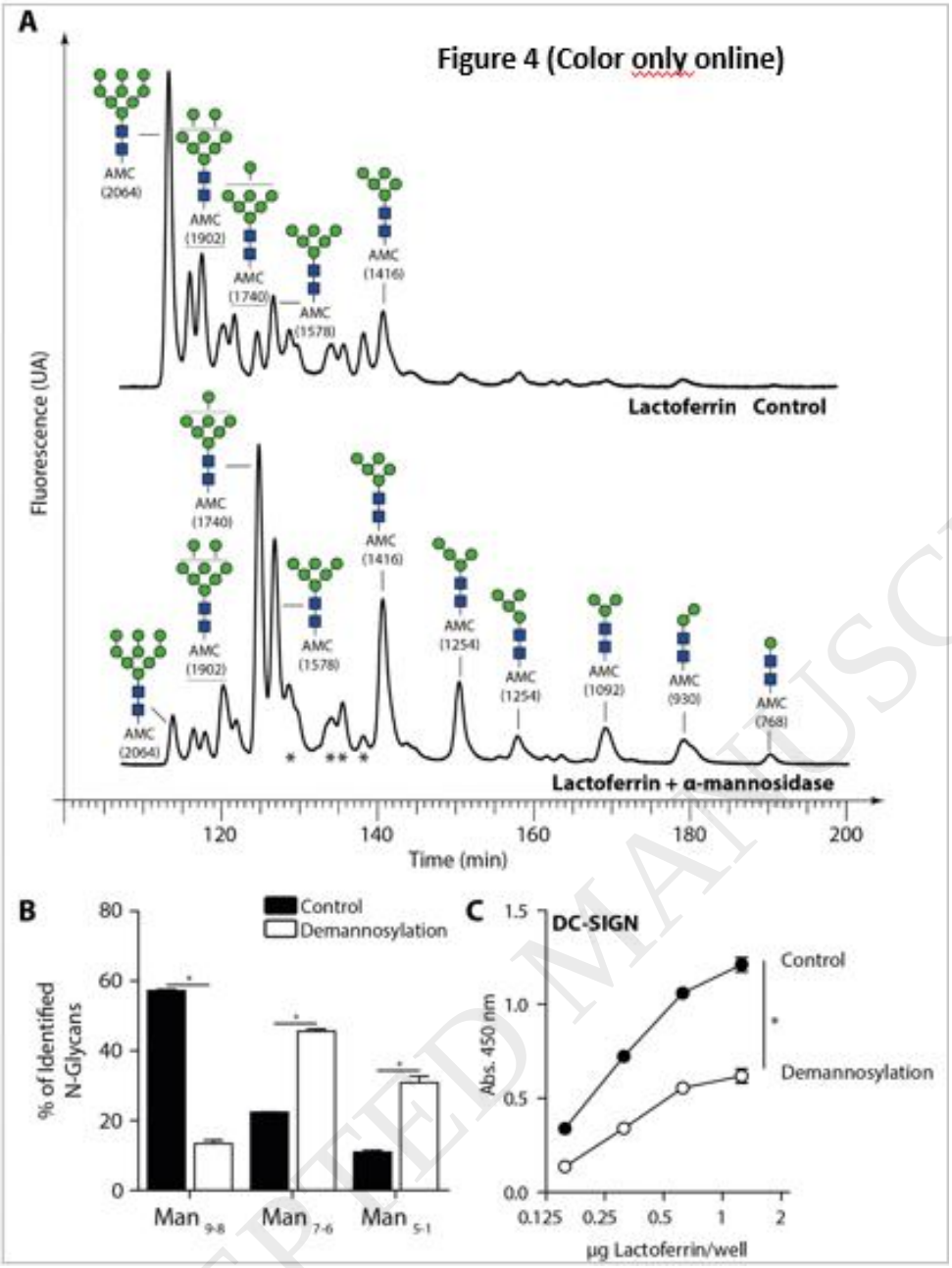

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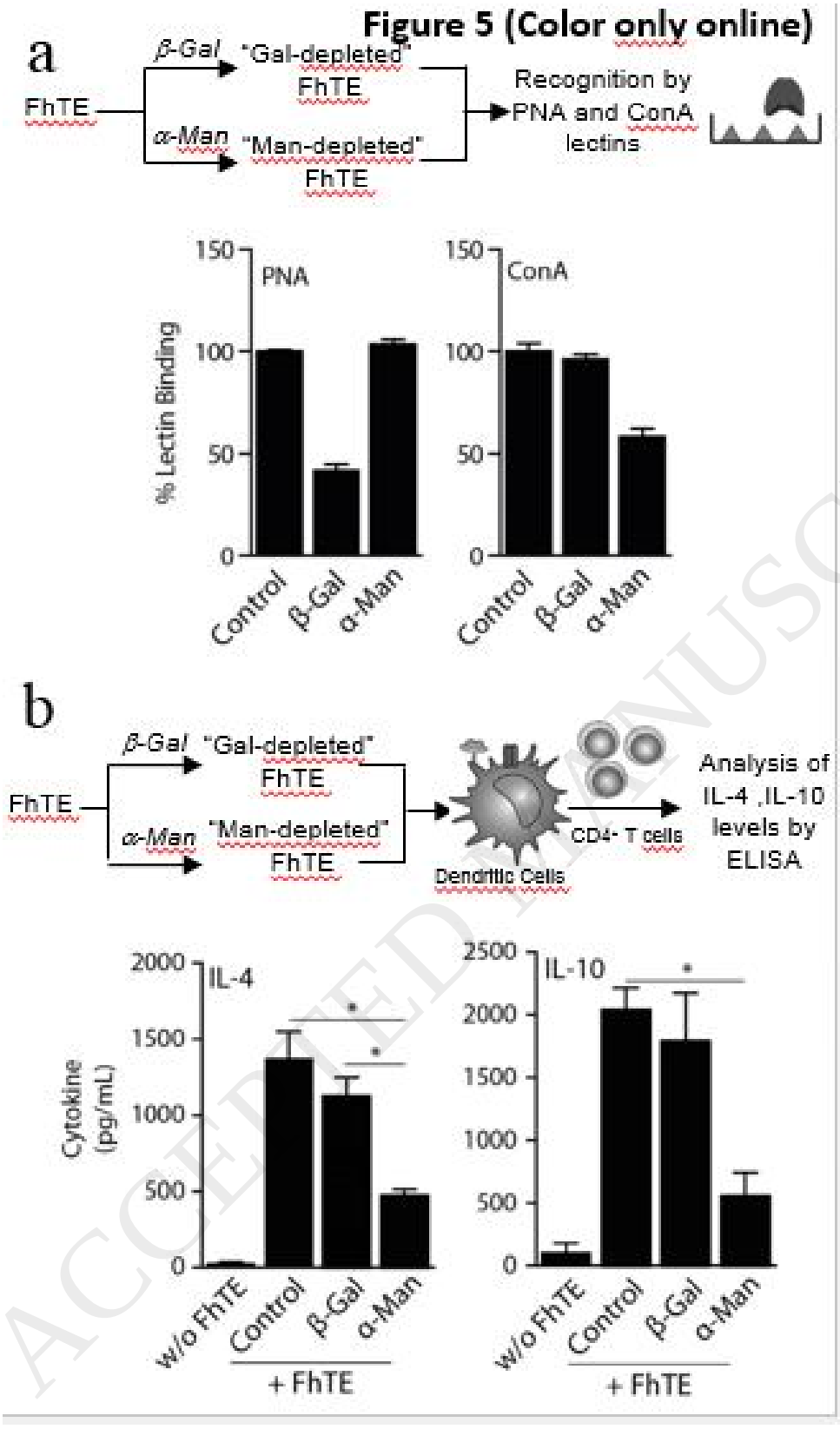

## Tables

### Table 1. Glycosidase Immobilization

| Support | Applied Activity (U/g) | Expressed Activity (U/g) | Expressed activity yield (%) | Applied Protein (mg/g) | Immob. Protein (mg/g) | Immob. yield (%) |
|---|---|---|---|---|---|---|
| **β-galactosidase** | | | | | | |
| agarose | 376 ± 24 | 251 ± 33 | 67 ± 14 | 9 ± 1 | 6 ± 2 | 67 ± 6 |
| nanoparticles | 763 ± 109 | 735 ± 91 | 96 ± 2 | 6 ± 1 | 6 ± 1 | 100 ± 15 |
| | 1520 ± 212 | 1429 ± 98 | 94 ± 7 | 12 ± 2 | 11 ± 2 | 92 ± 15 |
| | 3041 ± 423 | 1716 ± 7 | 56 ± 8 | 23 ± 3 | 19 ± 1 | 83 ± 14 |
| **α-mannosidase** | | | | | | |
| agarose | 84 ± 11 | 55 ± 8 | 65 ± 7 | 2.5 ± 1.0 | 2.2 ± 1.0 | 88 ± 14 |
| nanoparticles | 104 ± 3 | 69 ± 2 | 66 ± 2 | 5 ± 0.7 | 4 ± 0.1 | 80 ± 2 |
| | 201 ± 14 | 131 ± 4 | 65 ± 7 | 10 ± 1 | 9 ± 2 | 90 ± 9 |
| | 438 ± 11 | 132 ± 4 | 30 ± 1 | 20 ± 2 | 13 ± 0.3 | 65 ± 2 |