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Purification of glycosylphosphatidylinositol-anchored mucins from *Trypanosoma cruzi* trypomastigotes and synthesis of α -Gal-containing neoglycoproteins: application as biomarkers for reliable diagnosis and early assessment of chemotherapeutic outcomes of Chagas disease

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Abstract

Chagas disease (ChD), caused by the protozoan parasite *Trypanosoma cruzi*, affects millions of people worldwide. Chemotherapy is restricted to two drugs, which are partially effective and may cause severe side effects, leading to cessation of treatment in a significant number of patients. Currently, there are no biomarkers to assess therapeutic efficacy of these drugs in the chronic stage. Moreover, no preventive or therapeutic vaccines are available. In this chapter, we describe the purification of *Trypanosoma cruzi* trypomastigote-derived glycosylphosphatidylinositol (GPI)-anchored mucins (tGPI-mucins) for their use as antigens for the reliable primary or confirmatory diagnosis and as prognostic biomarkers for early assessment of cure following ChD chemotherapy. We also describe, as an example, the synthesis of a potential tGPI-mucin-derived α -Gal-terminating glycan and its coupling to a carrier protein for use as diagnostic and prognostic biomarker in ChD.

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Keywords

Anti- α -Gal antibodies; biomarkers; Chagas disease; chemotherapy; diagnosis; chemiluminescent enzyme-linked immunosorbent assay; mucins; carbohydrate synthesis; neoglycoproteins; *Trypanosoma cruzi*

1 Introduction

Chagas disease (ChD), or American trypanosomiasis, is a neglected tropical disease caused by the protozoan parasite *Trypanosoma cruzi*. It affects 6-7 million people in Latin America and kills thousands annually [1,2]. Infected individuals can now be found in other parts of the world, including the U.S. and Europe due to globalized migration [3]. Currently, there are two drugs available to treat ChD, i.e., benznidazole (BZN) and nifurtimox (NFX), which are mainly used in endemic countries in Latin America and, particularly in the case of BZN, in Europe and other nonendemic countries [2,4]. In 2017, the U.S. Food and Drug Administration (FDA) approved BZN for the treatment of ChD in children (ages 2-12) [5]. Even though both drugs are highly effective in the acute stage of the disease, they are less effective for the treatment of chronic ChD [6]. Unfortunately, the chemotherapy with these drugs is also associated with severe side effects, which may result in the discontinuation of the treatment in 10-20% of the patients [6,7]. Moreover, negative seroconversion using conventional serology following chemotherapy takes approximately 10-20 years to occur, which is a very poor prognostic perspective to support the widespread treatment of chronic ChD [8,9]. Due to these reasons, it is estimated that about 1% or less of chronic patients undergo treatment [10,11]. Therefore, lack of reliable biomarkers (BMKs) for assessment of therapeutic efficacy following chemotherapy is a major challenge in ChD. Other major challenges include the lack of effective epidemiological and insect-vector control, accurate diagnosis, and a prophylactic or therapeutic vaccine [12,4,13].

The plasma membrane of infective trypomastigote forms of the protozoan parasite *T. cruzi* is covered by a dense coat of glycosylphosphatidylinositol (GPI)-anchored glycoconjugates, including major glycoprotein families of mucins, mucin-associated-surface proteins (MASP), and *trans*-sialidases (TS), and glycolipids such as glycoinositolphospholipids (GIPLs) [14–18] (Fig. 1, **left panels**). In particular, the highly abundant GPI-anchored mucins of the infective trypomastigote stage (tGPI-mucins) display *O*-glycans containing terminal, nonreducing α -galactosyl (α -Gal) glycotopes, which are absent in human tissues and are therefore highly immunogenic to humans [19–21] (Fig. 1, **middle and right panels**). Approximately 10% of these *O*-glycans are comprised of a single immunodominant linear trisaccharide, Gal α 1,3Gal β 1,4GlcNAc α (Gal α 3LN α), whereas the remaining 90% of the *O*-glycans are branched α -Gal-containing oligosaccharides with as-yet uncharacterized structures [19]. ChD patients produce considerable amounts of lytic, protective anti- α -Gal antibodies (Ch anti- α -Gal Abs) against α -Gal glycotopes on tGPI-mucins, in both the acute and chronic phases of the disease (Fig. 1, **right panel**) [22–24,19,25,26].

In this chapter, we show two approaches toward the development of specific BMKs for the accurate diagnosis of ChD and early assessment of chemotherapeutic outcomes, based on a chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA), using purified α -Gal-

containing tGPI-mucins [25,27–29,24] or synthetic neoglycoproteins (NGPs) [30–32]. The NGPs are based on terminal, nonreducing α -galactosyl (α -Gal) residues known to be expressed in linear and branched *O*-glycans of the tGPI-mucins [19] (Fig. 1, **right panel**). They are promising BMKs not only for diagnosis and early assessment of chemotherapy success (cure), but also as potential vaccine candidates. These α -Gal glycotopes are also highly conserved throughout *T. cruzi* genotypes and field strains, since patients with chronic ChD from very distinct endemic and nonendemic regions universally produce high levels of *T. cruzi*-specific anti- α -Gal antibodies [24,25,29,28,33].

Here, we show detailed protocols for **(a)** the purification and immunoreactivity analysis of native tGPI-mucins from mammalian tissue-culture derived *T. cruzi* trypomastigotes (TCTs) (Fig. 2a–d); **(b)** the conjugation of 3-thiopropyl α -D-galactosyl-(1 $\rightarrow$ 2)-[α -D-galactosyl-(1 $\rightarrow$ 6)]- β -D-galactoside (Fig. 3a, **structure 4**) to commercially available maleimide-derivatized bovine serum albumin (BSA), which results in a NGP that we named **NGP11b** [31] (Fig. 3a,b); its immunoreactivity to sera and purified anti- α -Gal Abs from chronic ChD patients and healthy individuals (Fig. 3c), and examples of their applications as BMKs for diagnosis and follow-up of chemotherapy (Fig. 3d,e). Taken together, our data support that synthetic α -Gal-containing NGPs could potentially replace native tGPI-mucins in their capability as diagnostic and prognostic BMKs. NGPs could therefore circumvent **(a)** the need of large-scale mammalian cell tissue culture to obtain highly infectious TCT forms; **(b)** the lengthy process of tGPI-mucin purification; and **(c)** the batch-to-batch qualitative and quantitative inconsistency of purified tGPI-mucins. Moreover, since these NGPs are synthetic, rather precise compounds, they have the potential to replace *T. cruzi* lysates as antigens in serological assays, thus eliminating a large degree of non-specificity that crude, non-homogeneous *T. cruzi* lysates suffer from.

2 Materials

2.1 Purification of tGPI-Mucins from mammalian cell-derived *T. cruzi* trypomastigotes (TCTs)

2.1.1 *T. cruzi* trypomastigote cell culture

1. Rhesus monkey kidney cell 2 (LLC-MK2) cells (LLC-MK2 Original, ATCC® CCL-7™, ATTC, Manassas, VA) [34].
2. Ten 175-cm² cell culture flasks (T175).
3. *Trypanosoma cruzi* TCTs, Y strain [35], obtained as described [36].
4. High-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, 0.584 g/L glutamine, 3.7 g/L sodium bicarbonate, and 0.11 g/L sodium pyruvate.
5. Penicillin-streptomycin solution containing 10,000 IU of penicillin, and 10,000 μ g/mL streptomycin.
6. Fetal bovine serum, heat-inactivated for 30 min at 56°C (hi-FBS).

7. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.75 mM KH₂PO₄, pH 7.4.
8. 15- and 50-mL polypropylene conical tubes.
9. Lyophilizer or vacuum centrifuge.

2.1.2 Purification of tGPI-mucins

1. Solvents: chloroform (CHCl₃), methanol (MeOH), *n*- or 1-propanol (1-PrOH), *n*- or 1-butanol (1-BuOH), and H₂O. All these solvents should be liquid chromatography-mass spectrometry (LC-MS)-grade.
2. Ammonium acetate (NH₄Ac), LC-MS grade.
3. 9% 1-BuOH (*see* Note 1).
4. Fraction collector.
5. Masterflex LS low-flow peristaltic pump with 7013-21 drive head and Platinum L/S 13 silicon tubing (item # HV-96404-13, Cole-Parmer, Vernon Hills, IL), or any other peristaltic pump capable of running at a flow rate of 2-15 mL/h.
6. Luer lock, non-jacketed, LC glass column, 8 mL, 10-mm I.D., 100-mm length.
7. Octyl-Sepharose (OS) CL-4B.
8. 0.45-μm low-protein binding PTFE syringe filter.
8. Digital refractometer (AR2000, Reichert) or any other low-volume refractometer.
9. 20-mL borosilicate glass tubes, with polytetrafluoroethylene (PTFE)-lined screwcap, 25-mm O.D., 150-mm length.
10. Gradient maker, 500 mL (model SG500, GE Healthcare).
11. Buffer A: 5% 1-PrOH containing 0.1 M NH₄Ac, pH 7.2.
12. Buffer B: 60% 1-PrOH containing 0.1 M NH₄Ac, pH 7.2.
13. Lyophilizer or vacuum centrifuge.

2.2 Conjugation of the glycan to maleimide-derivatized bovine serum albumin (BSA)

For the conjugation of mercaptopropyl glycosides to maleimide-activated BSA, the conjugation kit “Inject Maleimide Activated Carrier Protein Spin Kit” from Thermo Fisher Scientific was used, and the protocol provided by the manufacturer was followed unless otherwise noted.

1. Inject Maleimide Activated BSA Spin Kit, 5 × 2mg. The conjugation kit is supplied with (a) the carrier protein lyophilized from PBS with EDTA and

¹The 9% 1-BuOH solution is obtained by mixing 1-BuOH and water (1:1, v/v), in a pear-shaped separatory funnel, and vigorously shaking the mixture. Let it rest O/N on the bench, and filter out the lower phase, which contains 9% 1-BuOH, to a fresh amber flask with a PTFE-lined cap. The remaining upper phase (91% 1-BuOH) is transferred to another amber flask. Be careful not to mix the lower and upper phases once partitioned.

stabilizer; pH 7.2; activation: 15-25 moles of maleimide/mole of BSA; **(b)** Inject Maleimide Conjugation Buffer, 0.083M sodium phosphate, 0.1 M EDTA, 0.9 M NaCl, 0.1 M sorbitol and 0.02% sodium azide, pH 7.2; **(c)** Inject purification buffer salts, upon reconstitution this buffer contains 0.083M sodium phosphate, 0.9 M sodium chloride, 0.1 M sorbitol, pH 7.2; and **(d)** Zeba™ spin desalting columns (7K MWCO) containing 0.05% sodium azide.

2. Amicon Ultra 10K centrifugal filters (Millipore).

2.3 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

1. Axima Assurance Matrix-Assisted Laser/Desorption Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF-MS) (Shimadzu).
2. Deionized water.
3. LC/MS grade water.
4. Acetonitrile (ACN), LC-MS grade.
5. Trifluoroacetic acid (TFA)
6. ACN/H₂O (2:1, v/v) with 0.1% TFA.
7. Lyophilized sinapinic acid matrix, reconstituted in 1 mL of ACN/H₂O (2:1, v/v) with 0.1% TFA.
8. Lyophilized underivatized BSA
9. NGP sample.
10. Amber glass vials (4-mL) with PTFE-lined polypropylene caps.
11. Fume hood.
12. Micropipettes and tips.
13. Bicinchoninic acid (BCA) Protein Assay Kit and Bovine Serum Albumin Standard Ampules, 2 mg/mL (Cat# 23209, Pierce, Thermo Fisher Scientific)
14. Microplate spectrophotometer.

2.4. Chemiluminescent Enzyme-Linked Immunosorbent Assay (CL-ELISA)

2.4.1 General CL-ELISA protocol

1. Nunc 96-well, high protein-binding capacity MaxiSorp White Microplate.
2. NGP sample.
3. BSA Fraction V.
4. Biotinylated goat anti-human IgG (H+L) secondary antibody (Thermo Fisher Scientific, catalog # 31770).
5. High-sensitivity NeutrAvidin-horseradish peroxidase (NA-HRP) (Thermo Fisher Scientific, catalog # 31030).

6. SuperSignal™ ELISA Pico Chemiluminescent Substrate (Pierce, Thermo Fisher Scientific, catalog # 37069 or 37070).
7. Carbonate-bicarbonate buffer (CBB), 200 mM, pH 9.6, containing 21.1978 g/L sodium carbonate anhydrous (Na₂CO₃), and 16.8014 g/L sodium bicarbonate (NaHCO₃).
8. PBS.
9. Washing buffer: PBS with 0.05 % Tween 20 (PBS-T) (*see* Note 2).
10. Blocking solution, 1% BSA in PBS, pH 7.4 (PBS-B) (*see* Note 3).
11. 1% BSA in PBS-T (PBS-TB): Add 1g of BSA to 100 mL of PBS-T.
12. Automatic microplate washer.
13. Multichannel pipette and tips (50-200 µL).
14. Reagent reservoirs.
15. 1.5-mL polypropylene microcentrifuge tubes.
16. Aluminum foil.
17. Plastic wrap.
18. Luminometer reader.
19. CO₂ Incubator, at 37 °C.

2.4.2 α-Galactosidase treatment of tGPI-mucins and NGP11b

1. Green coffee bean α-galactosidase (in ammonium sulfate suspension, 9 units/mg protein; G8507, Sigma-Aldrich).
2. 100 mM potassium phosphate buffer (PPB), pH 6.5, ice-cold.
3. 9.9 mM PNP α-D-galactopyranoside (= *p*-nitrophenyl α-D-galactopyranoside) substrate solution (Sigma-Aldrich)

3 Methods

3.1 Purification of tGPI-mucins from *T. cruzi* trypomastigotes

3.1.1 Mammalian tissue-culture-derived *T. cruzi* trypomastigotes (TCTs)

1. Cultivate LLC-MK2 cells to 50%–70% semi-confluence in DMEM containing 10% hi-FBS, and 1% penicillin-200 mM streptomycin solution.
2. Infect subconfluent LLC-MK2 cells on T175 flask with 1×10^8 TCTs (multiplicity of infection (MOI) = 10) and incubate flask at 37°C with 5% CO₂ humid atmosphere for 2-3 days.

²-PBS-T washing buffer is used to eliminate or significantly reduce nonspecific reactivities.

³-Blocking solution (PBS-B) is used to cover free sites on the microplate that the antigen did not bind to in order to eliminate or significantly reduce nonspecific reactivities. Alternatively, 5% skim milk in PBS can be used as blocking solution.

3. 2-3 days post-infection (dpi): remove DMEM from flask, wash cells (three times) with 10 mL PBS, and add 20 mL DMEM containing 10% hi-FBS, 1% penicillin-200 mM streptomycin solution.
4. 6-7 dpi: collect supernatant from infected flask into 50-mL conical polypropylene tubes.
5. Pellet parasites (P1) by centrifuging for 10 min at $1,500 \times g$, 4°C.
6. Incubate parasite pellet at 37°C, for 2 h, to allow TCTs to swim up towards the surface.
7. Carefully collect supernatant and transfer to a fresh 50-mL conical tube (*see* Note 4).
8. Centrifuge supernatant for 10 min, at $3,220 \times g$, 4°C, to obtain a pure TCT pellet (P2). Carefully remove supernatant, avoiding to disturb P2.
9. Transfer P1 and P2 pellets to fresh 15-mL conical polypropylene tubes, wash with 10-15 mL PBS, and centrifuge for 10 min, at $3,220 \times g$, 4°C. Repeat this step until no color (from DMEM) is visible in the pellet.
10. Carefully remove any remaining PBS in the supernatant and freeze pellets at -20 °C (for short-term storage) or -80°C (for long-term storage).
11. Before tGPI-mucin purification, lyophilize P1 and P2.

3.1.2 Purification of tGPI-mucins—The following procedure should be performed with a minimum of 1×10^{10} TCTs, which are obtained from ten T175 flasks of TCT-infected LLC-MK₂ cells at 6-7 dpi.

1. Add 2 mL MeOH directly to lyophilized parasite pellet (P1 or P2), vortex briefly and transfer the pellet suspension to a MeOH-rinsed borosilicate glass tube with PTFE-lined cap (*see* Note 5).
2. Add another 2 mL MeOH, followed by 2 mL CHCl₃ and 1.6 mL H₂O (C:M:W, 1:2:0.8, v/v/v); vortex tube vigorously for 2 min.
3. Centrifuge for 10 min at $3,000 \times g$, 4°C.
4. Carefully remove supernatant, which will be enriched with lipids (i.e., neutral lipids and phospholipids) and tGIPLs (~90% of the total), and transfer it to a fresh glass tube with PTFE-lined cap (*see* Note 6).
5. Add 2 mL MeOH and 4 mL CHCl₃ to the partially delipidated pellet, and vortex for 2 min. Centrifuge for 10 min at $3000 \times g$, at 4°C, and transfer supernatant to tubes containing the organic/lipid extracts.

⁴Carefully avoid disturbing the parasite pellet, as P1 will contain substantial LLC-MK₂ cell debris, and large number of TCTs and intra- and extracellular amastigotes (ICA and ECA, respectively). Note that 70-80% of TCTs will remain trapped in P1, which could also be used for tGPI-mucin extraction, provided that high purity is not required. We have systematically observed that P1-derived tGPI-mucins still exhibit high sensitivity and specificity with sera from patients with chronic ChD and Ch anti- α -Gal Abs.

⁵Only use MeOH-rinsed glass Pasteur pipettes and tubes to avoid contamination with lipids and other hydrophobic contaminants (e.g., plasticizers).

⁶Be careful to disturb the parasite debris, which does not pellet very tightly.

6. Repeat step 5 twice.
7. Add 4 mL MeOH, and 2 mL CHCl₃ and 1.6 mL water (C:M:W, 1:2:0.8, v/v/v) to the C:M (2:1, v/v)-delipidated pellet and vortex for 2 min. Centrifuge for 10 min at 3000 × g, at 4°C, and transfer supernatant to the tube containing the organic/lipid extracts obtained from delipidation steps.
8. Repeat step 7 once.
9. Carefully dry the C:M/C:M:W-delipidated pellet and organic/lipid extracts under a low, constant stream of nitrogen gas.
10. Once dried, keep both pellet and organic/lipid extracts at −20°C (short-term) or −80°C (long-term).
11. Add 4 mL 9% 1-BuOH directly to the dry delipidated parasite pellet, and incubate at 4°C, overnight (O/N), while rocking to ensure maximum recovery of GPI-anchored proteins (GPI-APs).
12. Centrifuge for 10 min, at 3,000 × g, 4°C, to pellet the delipidated parasites.
13. Remove and transfer supernatant, enriched in GPI-APs (GPI-AP fraction), to a fresh MeOH-rinsed glass tube with PTFE-lined cap.
14. Repeat step 11-13 twice. However, the second and third 9% 1-BuOH extraction steps should be done for only one-hour each.
15. Centrifuge tube for 10 min, at 3,000 × g, 4°C, to pellet parasites and transfer supernatant to the tube containing the GPI-AP fraction obtained in step 13.
16. Lyophilize the combined GPI-AP-enriched fractions, obtained in steps 13 and 14.
17. To partition GPI-APs from any remaining GIPLs not extracted by C:M:W and C:M, add 1 mL 1-BuOH and 1 mL deionized water to the lyophilized, combined GPI-AP fraction and vortex for 1 min. Centrifuge for 10 min, 3000 × g, 4°C.
18. Remove upper phase (91% 1-BuOH fraction), which is enriched with tGIPLS (~10% of the total) and transfer to a fresh MeOH-rinsed glass tube with PTFE-lined cap. Transfer lower phase (9% 1-BuOH fraction) rich in GPI-APs, to a fresh MeOH-rinsed glass tube with PTFE-lined cap.
19. Repeat steps 17 and 18.
20. Lyophilize the 9% 1-BuOH fraction (enriched with GPI-APs), and store the 91% 1-BuOH fraction (enriched with tGIPLs) at −80°C.
21. Prepare the OS column by packing up to 6-cm resin to the 10-ml glass column (*see* Note 7).

⁷Carefully load OS resin to the glass column. Let resin settle by draining excess solution through the lower column opening. Do not let resin dry, or allow bubbles to form inside the column. Wash the resin with 10-column volumes of Buffer A. Connect the column to the peristaltic pump using a Masterflex Platinum L/S-13 silicone tubing (90 cm) and set flow rate to 12 mL/h. Carefully create a gradient of 30 mL of Buffer A in the left chamber, and 30 mL of Buffer B in the right chamber. Run the gradient to clean the column using a 12-mL/h flow rate. Finally, re-equilibrate the column using 10 column volumes of Buffer A.

23. Wash the column with 10-column volumes of buffer A, using a Masterflex Platinum L/S-13 silicone tubing (90 cm) and set flow rate to 12 mL/h in the peristaltic pump.
24. Carefully create a gradient of 30 mL of buffer A in the left chamber, and 30 mL of buffer B in the right chamber (*see* Note 8).
25. Run a 5-60% 1-PrOH gradient to clean the OS column, using a 12-mL/h flow rate. Finally, re-equilibrate the column using 10 column volumes of Buffer A.
26. In parallel, set the fraction collector to collect 60 one-mL fractions.
27. Resuspend dried 9% 1-BuOH fraction (rich in GPI-APs) in 2 mL of buffer A.
28. Gently vortex, centrifuge (10 min, 3,000 × g, 4°C) or filter (0.45-μm low-protein binding PTFE syringe filter) to remove any insoluble debris, and apply the supernatant or filtrate onto the column using a flow rate of 4 mL/h. Be careful not to dry the resin.
29. Run a 5-60% 1-PrOH gradient (prepared as described in step 24) to elute the OS column, using a 12-mL/h flow rate. Collect 60 one-mL fractions. Store them at 4°C.
30. Determine the 1-PrOH concentration in every third fraction by using a refractometer. Generate a standard curve by measuring the refractive index of 5, 10, 20, 30, 40, 50, 60, 70 and 80% 1-PrOH, each containing 0.1 M NH₄Ac, pH 7.2. Use 50-60 μL minimum of each fraction for the measurement. Carefully recover the fraction following measurement, dry the refractometer lens with Kimwipes (or equivalent tissue paper), wash the lens with 100-200 μL 70% ethanol, and wipe-dry.
30. Test reactivity of each fraction (1 μL) to Ch anti-α-Gal Abs (or to ChSP, if purified anti-α-Gal Abs are not available) via CL-ELISA (described in **section 3.4**).
31. Pool fractions showing the strongest reactivities to Ch anti-α-Gal Abs (or ChSP) that elute between 20-30% 1-PrOH. tGPI-Mucins with an alkylacylglycerol (AAG) GPI-anchor typically elute within this range; however, *lyso*-tGPI-mucins that contain a single C16:0-alkyl group elute earlier, between 8-15% (Fig. 2b) [19].
30. Lyophilize tGPI-mucin-enriched fraction, redissolve it in 20% 1-PrOH, and store it at -20°C (short-term) and -80°C (long-term).

3.2 Chemical synthesis of the NGP11b

In this section, we provide an overview of the synthetic strategy to generate a trisaccharide with two nonreducing α-D-galactopyranosyl (α-Gal) moieties as an example of a branched glycan that is likely to exist as a terminal structure on the tGPI-mucins of *T. cruzi* based on

⁸. Use a magnetic stir plate and a small magnetic stir bar in left chamber of gradient maker to slowly mix gradient solutions. It is very important to open the gradient maker divider tab slowly.

partial structural information [19], and the detailed protocol for its conjugation to commercially available maleimide-derivatized BSA (Fig. 3a,b). The key features of the synthetic strategy, which we have applied for the generation of a number of different glycans [30,32], are **(a)** the stereoselective introduction of α -Gal moieties by using Kiso's α -Gal donor equipped with a 4,6-*O*-di-*tert*-butyl silylidene group [37,38]; and **(b)** the installation of a mercaptopropyl group at the reducing end of the glycan, which allows for the convenient conjugation to proteins that have maleimide derivatization. In Fig. 3a, the overall strategy for the synthesis of glycan **4** from monosaccharide building blocks is outlined in abbreviated form. Its detailed synthesis will be published elsewhere. α -Gal acceptor **1** with orthogonal protecting groups PG and PG', and two free hydroxyl groups at positions 2 and 6 is stereoselectively glycosylated with α -Gal donor **2**, which is protected with protecting groups PG'' and PG''', and a suitable leaving group (LG) at the anomeric center. Double glycosylation furnishes the fully protected trisaccharide **3**. Several chemical manipulation steps are necessary to remove protecting groups PG'-PG''', and to convert PG into a mercaptopropyl glycoside (trisaccharide **4**), which easily oxidizes to the sugar-disulfide **5**, unless exposure to oxygen is strictly avoided.

3.2.1. Conjugation of the glycan to maleimide-derivatized bovine serum albumin (BSA)

1. Tris(2-carboxyethyl)phosphine (TCEP, 0.8 mg, 2.79 μ mol) is dissolved in 250 μ L of Imject maleimide conjugation buffer (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M sodium chloride, 0.02% sodium azide, 0.1 M sorbitol, pH 7.2) and added to a 1.5-mL microcentrifuge tube containing the sugar-disulfide **5** (2.7 mg, 2.40 μ mol), and the mixture is agitated on a shaker. After 1 h, the reduction of disulfide **5** to thiol **4** is expected to be complete, and 10 μ L of this solution is removed to determine the thiol concentration (*see* Note 9). The maleimide-activated BSA (2 mg, 15-25 maleimide units/BSA molecule) is reconstituted by adding 200 μ L of ultrapure water. The remaining 240 μ L of trisaccharide **4** solution in conjugation buffer is added to the vial that contains the reconstituted BSA. The vial is flushed with argon, sealed with parafilm, and agitated for 3 h on a shaker at RT. A reaction buffer for the colorimetric determination of the thiol concentration post conjugation is prepared (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA) and a solution of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid) = DTNB] (4 mg DTNB in 1 mL of reaction buffer). After 3 h, 18.3 μ L are removed from the conjugation solution to determine the concentration of unreacted thiol. This sample is diluted to a volume of 250 μ L with reaction buffer, added to a test tube containing 50 μ L of Ellman's reagent solution and 2.5 mL of reaction buffer, and mixed at RT for 15 min. With a spectrophotometer, the absorbance of the sample at 412 nm is measured. Using the molar extinction coefficient of 2-nitro-5- thiobenzoic acid (TNB, $\epsilon = 14,150$

⁹In order to avoid contact of the BSA with TCEP, the reduction can also be accomplished with Immobilized TCEP Disulfide Reducing Gel (Thermo Fisher Scientific) prior to the conjugation. However, using this alternative disulfide reduction procedure does not prevent aggregation of BSA-based NGPs, probably due to the unavoidable exposure to thiols, which are also excellent reducing agents. Regarding removal of aggregated NGP material, *see* Note 10.

M- 1 cm-1), the thiol concentration is determined, and the amount of sugar loaded (typically 2.0 μ mol) is calculated.

2. The conjugation mixture is then diluted with ultrapure water to a final volume of 1 mL and filtered using an Amicon Ultra 10K centrifugal filter for desalting. The filter is centrifuged for 20 min at $4,000 \times g$, RT, 1 mL of ultrapure water is added, and the filter is additionally centrifuged for 10 min at $4,000 \times g$, RT. This washing step is repeated twice. The tube with the filtrate is then removed, and 500 μ L of ultrapure water is added to the **NGP11b**. This results in a solution that contains a small amount of aggregated NGP molecules. The solution/suspension is pipetted into a new 15-mL tube, which is then centrifuged at $1,000 \times g$ for 2 min, RT. The supernatant is carefully removed and lyophilized. Then, stock solutions of **NGP11b** are prepared. The protein concentration is determined using a Pierce BCA Protein Assay Reagent kit using a spectrophotometer at a detection wavelength of 562 nm. While this filtration method effectively removes salt and other low molecular weight molecules, we have observed that the resulting NGP sometimes suffers from aggregation. We are currently in the process of changing the protocol to a combination of desalting by filtration and gel permeation chromatography (see Note 10).

3.3 MALDI-TOF-MS

MALDI-TOF-MS has been widely used for determination, with high accuracy and resolution, the molecular mass and composition of proteins, peptides, oligonucleotides, lipids, and numerous other biomolecules, and for analysis of post-translational modifications (PTMs) of proteins [39]. Here, we use MALDI-TOF-MS technique for determination of the shift in molecular mass of a carrier protein (i.e., BSA), following its covalent conjugation to glycan mercaptopropyl derivatives, to give rise to a NGP (described in **section 3.2.1**) [31,32]. This allowed us to estimate the glycan load per protein molecule.

1. Prepare 3 mL of ACN:water (2:1, v/v) with 0.1% TFA solvent; this will be enough to resuspend sinapinic acid matrix, and sample preparation (see Note 11).
2. Using ACN:water (2:1, v/v) with 0.1% TFA, add 1 mL to the lyophilized sinapinic acid matrix stock solution (10 mg/mL) and bring it into solution by either gentle manual inversion or with pipette tip. Do not vortex it (see Notes 12 and 13).

¹⁰Initially, the desalting of the NGP was performed by gel-permeation chromatography using the spin columns supplied by the manufacturer of the conjugation kit, but sometimes the dry NGP sample appeared to be heavier than expected. Then, we replaced the gel-permeation step with a filtration step; however, upon reconstitution of the lyophilized NGP sample, some aggregation was observed. We are currently developing a new NGP purification protocol that entails filtration for the removal of small molecules followed by gel-permeation chromatography for the removal of any microaggregates. Thus, the final, purified NGP sample should be free of any microaggregates that could serve as seeds for further aggregation.

¹¹Prepare ACN:H₂O (2:1, v/v) with 0.1% TFA in small volumes to keep it fresh.

¹²Aliquot in small volumes and store at -20°C for immediate use. We have observed formation of a precipitate in the matrix stock solution within a month. The solution stops functioning with repeated freeze-thaw cycles. If possible, always prepare it fresh.

¹³This product has been recently discontinued, and it is now produced as sinapic acid by Sigma-Aldrich (catalog # 85429).

3. Resuspend lyophilized **NGP11b** in LC-MS or ultrapure water and slowly bring it into solution by either manual inversion or with pipette to a final concentration of 1.0-1.5 mg/mL. Do not vortex it (*see* Note 14).
4. To obtain protein concentration, we use the commercial BCA Protein Assay kit following the manufacturer's instructions and read microplate in a spectrophotometer.
5. Before sample preparation, retrieve metallic plate from the MALDI-TOF-MS instrument with gloves (*see* Notes 15 **and** 16).
6. Use a 5- μ L aliquot of the NGP stock solution for the MALDI-TOF-MS analysis.
7. Prepare pre-loading mix by adding 2.5 μ L of freshly prepared sinapinic (or sinapic) acid and 1.5 μ L of ACN:water (2:1, v/v) with 0.1% TFA to the NGP sample. Mix gently with a pipette tip; do not vortex it.
8. Locate empty sample wells on plate and gently spot 0.5-1 μ L of pre-loading mix in the center of the well.
9. Once completely dried, open instrument door and place loaded plate, and close door once again, and wait for instrument to build up negative pressure.
10. Localize the sample well using the camera window and under acquisition settings, select parameters. Laser power for BSA: 60-80, profiles: 20, shots: 100, with pulsed extraction at known molecular mass (Da), e.g., BSA: 66,430 Da (*see* Notes 17 **and** 18).
11. The glycan load per NGP11b molecule was calculated by subtracting the average molecular mass of BSA from the NGP average molecular mass, then dividing it by the nominal molecular mass of one glycan-linker unit (Figure 3b).

3.4 CL-ELISA

3.4.1 General CL-ELISA protocol

1. Immobilize antigen (Ag) according to the desired concentration. For NGP11b, 125 ng of Ag per well was used (*see* Note 19).

¹⁴ We have noticed synthetic NGPs do not go well into solution and begin aggregation with freeze-thaw cycles, and harsh manipulations. NGP stock solutions containing microaggregates cannot be completely dissolved, and more aggregation seems to occur over time with multiple freeze-thaw cycles and harsh conditions. To reduce aggregation of NGPs, we aliquot them into small volumes (20 μ L), and avoid using the vortex, sonicator, and harsh temperatures when manipulating them. When thawing aliquots, we allow them to thaw on ice completely before use. We believe the best method to preserve NGPs is to lyophilize each time, but this is tedious and material is lost each time.

¹⁵ Do not touch metallic MALDI-TOF-MS plate sample wells without gloves, this can affect readings and introduce contaminants like keratin and lipids from the skin.

¹⁶ Once you retrieve the metallic MALDI-TOF-MS plate, close the instrument door again to avoid losing too much pressure.

¹⁷ These are parameters in which we achieve good spectra for BSA and BSA-conjugated NGPs; however, laser power, profiles and shots should be optimized for different applications and MALDI-TOF-MS instruments.

¹⁸ Make sure to calibrate instrument every new analysis, and to include a protein standard each time an unknown sample is analyzed. Use a protein with a molecular mass in the range of expected mass of your test protein sample.

¹⁹ Titration of the Ag is necessary for optimization of optimal concentration. Excess of Ag may result in the prozone or hook effect, in which considerable reduction in antibody/serum reactivity to the Ag is observed [40].

2. Add Ag on the microplate wells to a final volume of 50 μ L, diluted in 200 mM CBB by using a multichannel pipette (20-200 μ L) and reservoir boat (*see* Note 20).
3. Incubate for 12-24 h at 4°C.
4. Remove the residual Ag by inverting the microplate and tapping it onto absorbent towels. Do not wash.
5. Block free sites with 200 μ L per well of PBS-B (*see* Note 21).
6. Seal the microplate with plastic wrap and incubate for 1 h at 37°C (*see* Note 22).
7. Wash 3 $\times$ with 200 μ L per well of PBS-T using an automatic plate washer (*see* Note 23).
8. Add 50 μ L of human serum at 1:800 dilution, in PBS-TB (*see* Note 24).
9. Repeat steps 6 and 7.
10. Add goat anti-human IgG (H+L) biotinylated antibody at 1:10,000 dilution in PBS-TB.
11. Add 50 μ L of diluted secondary antibody per well.
12. Repeat steps 6 and 7.
13. Dilute High Sensitivity NeutrAvidin-Horseradish Peroxidase (NA-HRP) to a ratio of 1:5,000 in PBS-TB.
14. Add 50 μ L of diluted NA-HRP per well, and cover plate with aluminum foil.
15. Incubate for 1 h at 37 °C.
16. Repeat step 7.
17. Combine SuperSignal ELISA Pico Stable Peroxide Solution, SuperSignal ELISA Pico Luminol Enhancer, and CBB/0.1% BSA in a 1:1:8 ratio (v/v/v).
18. Add 50 μ L per well.
19. Read luminescence immediately, using a microplate luminometer (*see* Note 25).
20. Readings are expressed as relative luminescence units (RLU).

²⁰. Add Ag using a multichannel pipette and reservoir boat to have a good triplicate between experimental samples. Calculate an extra 10% of all solutions, as some will be lost when using a multichannel pipette.

²¹. Prepare it fresh and keep on ice until use.

²². After incubation, if needed, you can store the microplate in PBS-B at 4°C O/N or for no more than 2-3 days, and then continue the experiment.

²³. If no automatic plate washer is available, wash the plate by adding 200 μ L of PBS-T per well with a multichannel pipette, decant and tap microplate onto paper towels to remove excess washing solution. Repeat this step 3 times.

²⁴. We performed a cross-titration of Ch and NHS serum pools against the Ag to determine optimal serum dilution and Ag concentration.

²⁵. When adding the substrate, protect from light and read microplates immediately, as the luminescence signal decreases over time and exposure to light.

3.4.2 α -Galactosidase treatment of tGPI-mucins and NGP11b for Ab specificity evaluation

1. Immobilize Ag and block as described in **section 3.4.1** above.
2. Remove blocking solution and wash wells to be treated with α -galactosidase twice with 100 μ L of PPB to equilibrate pH for the enzyme.
3. Calculate the amount of total enzyme required, considering 0.5 U/well.
4. Centrifuge enzyme at $16,000 \times g$, 4°C for 10 min, and remove ammonium sulfate suspension (*see* Note 26).
5. Resuspend enzyme pellet in freshly made, ice-cold PPB and add 50 μ L to each well.
6. As a control, in an empty well add 50 μ L of the prepared enzyme and 50 μ L of the substrate (PNP α -D-galactopyranoside) to observe an immediate change of color to yellow (*see* Note 27).
7. Incubate at 37°C , O/N, in a humid chamber.
8. Continue CL-ELISA as described in **section 3.4.1**, steps 7-20.

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²⁶. Green coffee bean α -galactosidase is supplied as a suspension in 3.2 M $(\text{NH}_4)_2\text{SO}_4$ solution, pH 6.0, containing BSA. Note that following centrifugation, the enzyme will be in the pellet not in the supernatant.

²⁷. This control test should be done frequently and prior to the Ag treatment to certify the enzyme activity.

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Fig 1.

Schematic representation of *T. cruzi* trypomastigote surface coat. (**Left panels**) *T. cruzi* contains a complex cell surface consisting of several glycoconjugates inserted into the plasma membrane via a GPI anchor. These glycoconjugates include GIPL and GPI-anchored proteins (GPI-APs) (tGPI-mucin, TS, and MASP). (**Middle panel**) tGPI-mucin contains a protein core heavily glycosylated with *O*-glycans linked to threonine (Thr) (rarely to serine) residues via an α -N-acetylglucosamine (α -GlcNAc). (**Right panel**) tGPI-mucin specifically contains terminal, nonreducing α -galactopyranose (α -Gal_p) residues, which serve as the major targets for the highly abundant, lytic anti- α -Gal Abs, produced during both the acute and chronic phases of ChD. A small portion of these *O*-glycans (10%) exist as linear trisaccharides (Gal α 1,3Gal β 1,4GlcNAc α), while the remaining 90% of the structures are branched and are as-yet to be structurally characterized. The hypothetical linkages (*) of the branched *O*-glycans are based on partial liquid chromatography-tandem mass spectrometry (LC-MS/MS) data analysis, following beta-elimination and permethylation of tGPI-mucin-derived *O*-glycans (Almeida et al., unpublished data).

Fig. 2.

Purification and immunoreactivity analysis of tGPI-mucins. **(a)** General scheme for purification and functional analysis of tGPI-mucins. Parasites are first delipidated, followed by extraction of GPI-APs with 9% 1-BuOH. GPI-APs are enriched by 1-BuOH/ H_2O partition, and tGPI-mucins are purified by hydrophobic-interaction chromatography (HIC) with Octyl-Sepharose. The tGPI-mucin-containing fractions are detected by CL-ELISA using Ch anti- α -Gal Abs. Positive fractions are combined, lyophilized, resuspended in 1 mL 20% 1-PrOH and quantified by *myo*-inositol analysis, as described [19]. The purified tGPI-mucins are titrated with a pool of sera from patients with chronic ChD (ChSP) and a pool of normal human sera (NHSP), before further assays with individual sera from ChD patients. **(b)** Chromatography elution profile of tGPI-mucins on OS-HIC. α -Gal-Enriched fractions (*lyso*-tGPI-mucins, tGPI-mucins, and tGIPLs) are located by CL-ELISA with Ch anti- α -Gal Abs. RLU, relative luminescence units. **(c)** Titration of the tGPI-mucins was performed with a pool of sera from patients with chronic ChD (ChSP) (n=10) and a pool of normal human sera (NHSP) (n=10), at different dilutions. Ch and NHS anti- α -Gal antibodies were purified from ChSP and NHSP, as described [19]. **(d)** Reactivity of purified tGPI-mucins with individual sera from patients with chronic ChD (ChS), ChSP, and NHSP. The tGPI-mucin

CL-ELISA titer was calculated by dividing the test sample average RLU value by the cutoff, calculated as the mean value of 10 NHSP + 10 SD. Horizontal black line: titer equal to 1.0. Green dashed line: titer <1.0 and equal or greater than 0.9 [24]. Data interpretation: positive result: titer ≥ 1.0; inconclusive result: titer <1.0, >0.9; negative result: titer <0.9. ChS and ChSP samples (**c**, **d**) were from IMT/FM/UCV, and NHSP sample (**d**) was from ISGlobal-Barcelona. Sera were collected from patients strictly following the International Ethical Guidelines for Biomedical Research Involving Human Subjects and protocols approved by the Institutional Review Boards of IMT/FM/UCV and ISGlobal-Barcelona.

Fig. 3. Synthesis of the α -Gal-containing NGP11b, and comparative immunoreactivity analysis between NGP11b and tGPI-mucins. **(a)** Synthesis of the trisaccharide 3-thiopropyl α -D-galactosyl-(1 $\rightarrow$ 2)-[α -D-galactosyl-(1 $\rightarrow$ 6)]- β -D-galactoside and its conjugation to maleimide-derivatized BSA to afford the NGP11b. **(b)** MALDI-TOF-MS spectra of BSA and NGP11b. Singly ($[BSA]^1+$ and $[NGP11b]^1+$) and doubly ($[BSA]^2+$ and $[NGP11b]^2+$) charged molecular ions of BSA and NGP11b are indicated. m/z , mass to charge ratio. **(c)** CL-ELISA immunoreactivity of ChSP, NHSP, Ch anti- α -Gal Abs, and NHS anti- α -Gal Abs

to immobilized NGP11b. A dose-dependent reactivity was observed with ChSP, NHSP, and Ch anti- α -Gal Abs. A clear differential immunoreactivity was observed between these sera and Abs. **(d)** Reactivity of sera from three individual chronic ChD patients to tGPI-mucins and NGP11b, before and after α -galactosidase (α -Galase) treatment. The considerable reduction in reactivity following enzymatic treatment indicates that specificity of individual Ch sera is directed mainly to the terminal, nonreducing α -Gal residues or α -Gal-containing glycotopes present in both tGPI-mucins and NGP11b. **(e)** Follow-up of patients before (–) and 24 months after (+) benznidazole (BZN) treatment, using tGPI-mucins or NGP11b as Ags in CL-ELISA. ChSP **(c)** and individual ChS samples (Patients # 45, 60, and 221) **(d)** were from IMT/FM/UCV. NHSP **(c)** and individual ChS samples (Patients # 4, 59, and 60) **(e)** were from ISGlobal-Barcelona. Serum samples were collected strictly following the International Ethical Guidelines for Biomedical Research Involving Human Subjects and protocols approved by the Institutional Review Boards of IMT/FM/UCV and ISGlobal-Barcelona.