Detection of Anti-Toxoplasma gondii Antibodies in Human Sera Using Synthetic Glycosylphosphatidylinositol Glycans on a Bead-Based Multiplex Assay

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General Methods

All chemicals were purchased of reagent grade and all anhydrous solvents were of high-purity grade and used as supplied except where noted otherwise. Reactions were performed in oven-dried glassware under an inert argon atmosphere unless noted otherwise. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates (0.25mm).

1H, and 13C were recorded on a Bruker Ascend 400 (400 MHz) spectrometer in CDCl3 (7.26 ppm 1H, 77.1 ppm 13C), D2O (4.79 ppm 1H), MeOD (4.87 ppm and 3.31 ppm 1H, 49.00 ppm 13C), ESI mass analyses were performed on a Waters Xevo G2-XS Q-TOF with an Acquity H-class UPLC and a Bruker Autoflex-speed MALDI-TOF spectrometer. HPLC supported purifications were conducted using Agilent 1200 systems.

Synthesis of GPI glycans

The GPI1 and mannose 3 were synthesized and characterized according to the previously published protocols.1,2
Synthesis of the Maleimide-linker

\[ \text{3-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-propylammonium trifluoroacetate (5)} ^3 \]

In a 250 mL round bottomed flask, \( N \)-(tert-butyloxycarbonyl)propanolamine (3.17 mL, 18.54 mmol) and diisopropyl azodicarboxylate (4.37 mL, 22.46 mmol) were added to a solution of maleimide 4 (2.0 g, 20.6 mmol) and PPh3 (5.3 g, 20.19 mmol) in THF (100 mL). The solution was stirred at room temperature for 48 h, concentrated and purified by column chromatography using silica gel (4:1 to 2:1 hexanes/EtOAc) to yield a mixture of the \( N \)-Boc protected product and diisopropyl bicarbamate. The reaction mixture was dissolved in a solution of CH\(_2\)Cl\(_2\) (15 mL) and H\(_2\)O (1 mL), and trifluoroacetic acid (TFA, 3.5 mL) was added. The reaction was stirred for 5 h at room temperature and diluted with CH\(_2\)Cl\(_2\) (50 mL) and H\(_2\)O (50 mL). The aqueous layer was extracted with CH\(_2\)Cl\(_2\) (3 x 50 mL) and concentrated to yield the desired product 5 as yellow oil (3 g, 94 %). ATR-IR: 3435, 2959, 2917, 2849, 1707, 1683 cm\(^{-1}\). \(^1\)H NMR (DMSO-d\(_6\), 400 MHz): \( \delta \) 7.81 (br s, 3H), 7.03 (s, 2H), 3.46 (t, 2H, \( J = 6.8 \) Hz), 2.87-2.86 (m, 2H), 1.85-1.65 (app m, 2H), \(^{13}\)C NMR (DMSO-d\(_6\), 100 MHz): \( \delta \) 171.2, 158.5, 134.6, 115.9, 36.7, 34.5, 26.5, HRMS ESI-MS (m/z): [M+H\(^+\)]\(^{\text{cald}}\): 155.0821, obsd: 155.0824.

Beads activity and conjugation assays

Preparation of Mannose 3 conjugate and optimization of glycan loading

The mannose 3 unit was prepared following reported protocol.\(^4\) Man 3 disulfide dimers (4 mg, 0.013 mmol) were dissolved in 2 mL of water and reduced using two equivalents (0.026 mmol) of resin bound Tris-(2-carboxyethyl)-phosphine (TCEP)). After 1 hour, the resin was removed and washed with water and the filtrates were lyophilized.

Scheme S1. Synthesis of the bead-mannose conjugate. a) Resin-bound TCEP, 0.1 M sodium phosphate, 50 mM NaCl, pH 7.0.

The maleimide (5) linker was installed on MagPlex® microspheres using the protocols described in the Luminex cookbook.\(^5\) A permanent magnet was used as a magnetic separator...
during the washing steps. The microspheres were handled in absence of light during all the steps. 200 µL (approx. 2.5 x 10^6 Beads) of the carboxylate microspheres stock suspension were transferred to a microcentrifuge tube, were pelleted using the magnet and the supernatant was removed. The microspheres were resuspended in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH-6.0, vortexed, sonicated for 30 seconds and pelleted. After aspirating the supernatant, the microspheres were resuspended in 200 µL of maleimide (2mg/mL in 0.1 M MES, pH-6.0) followed by the addition of 100 µL of a 10 mg/mL EDC (freshly prepared in 0.1 M MES, pH-6.0) solution and mixed. The microspheres were incubated for 2 h under continuous mixing by rotation at room temperature. The reaction was quenched by adding 500 µL of 0.1M MES, pH-4.5 and mixing. The beads were pelleted and the supernatant was removed. The maleimide coupled microspheres were washed twice with 250 µL of 0.1 M MES, pH-4.5. Finally, the microspheres were resuspended in 250 µL of 0.1 M MES, pH-4.5 and stored in the refrigerator at 4 °C in the dark.

**Conjugation with Mannose-linker 3**

The 5 x 10^6 microspheres were washed with 0.1 M Sodium phosphate, 50 mM NaCl, pH-7.0 and resuspended in 100 µL of 0.1 M sodium phosphate buffer and 100 µL of freshly reduced mannose 3 (0, 10, 50, 100, 1000 or 2000 µg/mL) in 100 mM Tris, pH-7.4 were added to the beads. The final volume was adjusted to 250 µL with 100 mM Tris buffer pH-7.4. The reaction mixture was incubated at room temperature for 1 h, the microspheres were pelleted and the supernatant was removed. Then, the unreacted maleimide groups on the beads were quenched by adding 100 µL of 50 mM L-cysteine hydrochloride in 100 mM Tris buffer pH-7.4. After 1h, the microspheres were removed from the supernatant using magnetic separator and washed twice with 100 µL of PBS containing 1 % BSA. FITC-Concanavalin A (150 µL) at 2 mg/mL was added to the beads and the mixture was incubated for 2 h. The beads were removed from the solution, washed twice with 100 µL of PBS containing 1 % BSA and resuspended in 250 µL of PBS. The beads were analyzed on a flow cytometer Attune NxT (Invitrogen).

![Graph showing MFI vs. Man 3 Loading](image-url)
Figure S1. Diagram of the loading of Man3 to beads of the region 20

![Diagram showing the loading of Man3 to beads.](image)

Figure S2. FACS analysis showing the increase in fluorescence of the beads using different amounts of Man3. The beads-Man conjugates were incubated with Concanavalin A-FITC conjugate.

**GPI-Loading determination by monosaccharide analysis using HPAE-PAD**

The GPI-Beads conjugates (5x10⁵ beads) were washed with deionized water, incubated with 200 µL of 2 M TFA for 4 h at 100°C and lyophilized. The dry hydrolysis mixture was dissolved in 100 µL of water and diluted 1:10 or 1:20 with water. The monosaccharide content was analyzed using high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac™ PA20 column (3 × 150 mm) (Dionex) and a CarboPac™ PA20 guard column (3 × 30 mm) (Dionex). The separation was performed with isocratic concentration of 10 mM NaOH (J.T.Baker, Devneter, the Netherlands) at 0.5 mL/min flow rate for 15 min at 30°C (figure S1). The quantity of GPI was calculated based on the content of glucose in the injected samples.

![HPAE-PAD chromatographic profile of GPI-bead fraction.](image)

Figure S3. HPAE-PAD chromatographic profile of GPI-bead fraction obtained after incubation with 2 M TFA solution.

The quantification of glucose of the samples was determined from a calibration curve formulated using 2-fold dilutions of a monosaccharides standard mixture having 400 pmol of...
galactosamine, glucosamine and glucose (Figure S2). The monosaccharides used in this study were purchased from Sigma.

Table S1. Determination of Glucose released by acid hydrolysis of GPI-beads conjugates:

| GPI Batch | Dilution | Injection | Glucose in 10 µL (pmol) | Glucose in 10^5 beads (pmol) |
|-----------|----------|-----------|-------------------------|------------------------------|
| Batch 1   | 1:20     | 10        | 13.7244                 | 384.88                       |
| Batch 2   | 1:10     | 10        | 24.8943                 | 348.94                       |

Figure S4. Standard calibration curve of glucose. A second order polynomial curve was used to formulate the standard curve ($y=0.0285x^2+0.0723$, $R^2=0.9962$)

Optimization of the BBMA with GPI1

To optimize the conditions for the detection of anti-GPI antibodies in sera, we analyzed a series of three positive and five negative sera under different BBMA conditions. Initial experiments were performed to establish the effect of the GPI1 loading on the beads and the type and amount of secondary antibody. The experiments were carried out using modified beads of the region 20 using 25 and 50 µg of GPI1 for loading 2.5 million beads (Figure S5).
Figure S5. Each bar corresponds to the average of the MFI values obtained for the corresponding set of sera samples. The average of the standard deviation is also showed. The GPI-1 was conjugated either using 50 µg or 25 µg for 2.5 million beads. Sera samples were used in 1:100 dilution, a) Detection using a biotinylated secondary antibody and following detection using a 3 µg/mL of antibody (Streptavidin-phycoerythrin). b) Detection using phycoerythrin-coupled secondary antibody; Two concentrations of antibody were used 1.5 µg/mL or 1 µg/mL and two loading of GPI-1, 50 µg and 25 µg. c) Optimization of phycoerythrin-coupled secondary Ab concentration. d) Effect of buffer components EG: Ethyleneglycol, Det: detergent.

From these experiments following parameter were selected:

Amount of GPI1 for loading of 2.5x10⁶ Beads: 50 µg

Detection with phycoerythrin-coupled secondary antibody at 1.5 µg/mL concentration. A standard buffer without ethyleneglycol (PBS pH 7.3, 1% BSA).

Optimization of sera dilution

Eight positive sera were diluted in ratios 1:100, 1:200, 1:500 and 1:1000 and evaluated by BBMA following the procedure in material and methods.
**Evaluation of two GPI-Beads conjugates**

We evaluated the reproducibility of the GPI-bead conjugates for detection of anti-GPI IgM and IgG antibodies in 18 characterized sera. A correlation of the analysis with these two batches showed a great agreement between the conjugates for the detection of antibodies by BBMA.

**Figure S6.** Evaluation of serum sample dilution. The secondary antibody was 1.5 μg/ml.

**Figure S7.** Correlation in the detection of anti-GPI antibodies by BBMA using two batches of the GPI-beads conjugate.
Figure S8. Correlation between IgG and IgM antibodies in a panel of 16 IgM-positive sera. Bio-Plex readout (mfi) vs ELIFA readout (IU or index) are shown for IgG response (a, b) or IgM response (c, d), and GPI1 beads (a, c) or SAG1 beads (b, d), respectively. Results are based on two independent experiments. Shaded areas indicate 95% CI. Linear regression analysis was performed for all sera tested for detection of IgM (c, d) or dependent on the serostatus (acute or non-acute) for detection of IgG antibodies (reverse groupings to figure 4).

Analysis of the sera by Microarrays:

Twenty characterized sera were evaluated using GPI1 printed on microarrays. In agreement with our previous report, these sera contained anti-GPI antibodies binding GPI1.

Printing pattern:

PB: Puffer / Blank

Figure S9. Printing pattern of the glycan array.
Figure S10. Pictures of the glycanarray scans used for determination of IgG and IgM anti-GPI antibodies. Each well correspond to one serum. Some sera also contain antibodies binding an α–1-6-trimannose.
References

1. Götze, S.; Azzouz, N.; Tsai, Y.-H.; Groß, U.; Reinhardt, A.; Anish, C.; Seeberger, P. H.; Varón Silva, D., Diagnosis of Toxoplasmosis Using a Synthetic Glycosylphosphatidylinositol Glycan. *Angew. Chem. Int. Ed.* **2014**, *53* (50), 13701-13705.

2. Tsai, Y. H.; Gotze, S.; Azzouz, N.; Hahm, H. S.; Seeberger, P. H.; Varon Silva, D., A general method for synthesis of GPI anchors illustrated by the total synthesis of the low-molecular-weight antigen from Toxoplasma gondii. *Angew. Chem. Int. Ed.* **2011**, *50* (42), 9961-4.

3. Bertozzi, C. R.; Agard, N. J.; Prescher, J. A.; Baskin, J. M.; Sletten, E. M. Preparation of cyclooctynes and azacyclooctynes for modification of biomolecules in vivo and in vitro by their copper-free strain-promoted [3+2] cycloaddition with azides. US20090068738A1, 2009.

4. Varela-Aramburu, S.; Wirth, R.; Lai, C. H.; Orts-Gil, G.; Seeberger, P. H., Straightforward and robust synthesis of monodisperse surface-functionalized gold nanoclusters. *Beilstein J Nanotech* **2016**, *7*, 1278-1283.

5. Stephen, A.; Sherry, D.; Carlos, G.; Valerie, S., *xMAP® Cookbook*. 3rd ed.; Luminex Corp.: 2016; p 147.