Preprint Title	Preparation of glyco-gold nanoparticles carrying synthetic mycobacterial hexaarabinofuranoside and their evaluation *in vivo*
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Publication Date	10 Dez 2019
Article Type	Full Research Paper
Supporting Information File 1	Suppl_Material.pdf; 653.1 KB
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Preparation of glyco-gold nanoparticles carrying synthetic mycobacterial hexaarabinofuranoside and their evaluation *in vivo*

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Abstract

Some bacterial glycans are specific markers for detection and serological identification of microorganisms and are widely used as antigenic components of vaccines. The use of gold nanoparticles as carriers for glyco-epitopes is becoming an important alternative to the traditional conjugation with proteins and synthetic polymers. In this study, we aimed at preparation and evaluation *in vivo* of glyco-gold nanoparticles (glyco-GNPs) bearing the terminal branched hexaarabinofuranoside

fragment (Ara$_6$) of arabinan domains of lipoarabinomannan and arabinogalactan, which are principal polysaccharides of the cell wall of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Specifically, we were interested in checking if the antibodies generated against Ara$_6$-GNPs would recognize the natural saccharides on the cell surface of different mycobacterial strains. Two synthetic Ara$_6$ glycosides with amino-functionalized spacer aglycons differing in length and hydrophilicity were directly conjugated with spherical gold nanoparticles ($d = 15$ nm) to give two sets of glyco-GNPs which were used for immunization of rabbits. Dot assay revealed cross-reactions for the two obtained antisera with the hexaarabinofuranoside with 2-aminoethyl aglycon used for the preparation of glyco-GNPs. Both antisera contained high titers of antibodies specific for *Mycobacteria* as shown by enzyme-linked immunosorbent assay using *M. bovis* and *M. smegmatis* cells as antigens while gave a weak response with *M. phlei* cells and no interaction with *E. coli* cells. The results obtained suggest that glyco-GNPs are promising agents for generation of anti-mycobacterial antibodies.

Keywords

Mycobacterium; lipoarabinomannan; gold nanoparticles; conjugation of glycosides; spacer aglycon

Introduction

Some bacterial glycans [1, 2] are specific markers for detection and serological identification of microorganisms [3-5] and are widely used as antigenic components of vaccines [6-23]. The use of gold nanoparticles (GNPs) [24-26] as carriers for glyco-epitopes is becoming an important alternative [15, 27-48] to the traditional

conjugation with proteins, synthetic polymers and other carriers [15, 17, 38, 49-55].

Immunological properties of GNPs [25, 56, 57] and their use in vaccine development

[58] have recently been reviewed. Glyco-gold nanoparticles (glyco-GNPs) bearing the

residues of tumor associated monosaccharide Tn [37] or disaccharide Thomsen

Friedenreich (TF) [35] antigens, a tetrasaccharide of *Streptococcus pneumonia* type

14 capsular polysaccharide [33, 34, 47] or lipopolysaccharide of *Burkolderia mallei*

[42] have been shown to be promising vaccine candidates.

Tuberculosis (TB), which is caused by the pathogenic bacterial species

Mycobacterium tuberculosis, remains one of the top 10 causes of death worldwide as

nearly two million people die from the disease every year [59-63]. Although

M. tuberculosis has been extensively studied [64], TB presents an ever-growing

challenge, and novel strategies for the prevention and treatment of TB are in urgent

demand [59]. Lipoarabinomannan (LAM) and related arabinogalactan (AG) are two

major structural components of the *M. tuberculosis* cell wall. Previous studies

revealed that LAM and especially its terminal oligosaccharide fragments, conjugated

with proteins [7, 10, 19] or monophosphoryl lipid A [23], are attractive targets for the

development of carbohydrate-based anti-TB vaccines [7, 10, 19, 23, 65, 66].

In this study, we aimed at preparation and evaluation *in vivo* of glyco-GNPs bearing

the terminal branched hexaarabinofuranoside fragment (Ara$_6$, Figure 1), which is

common for arabinan domains of both LAM and AG and has earlier been identified

as one of lead structures [7, 10, 65]. Specifically, we were interested in checking if

the antibodies generated against Ara$_6$-GNPs would recognize the natural

saccharides on the cell surface of different mycobacterial strains.

For the preparation of glyco-GNPs, glycans are usually transformed to glycosides

with *thiol*-functionalized spacer aglycons that are conjugated [43, 44] with pre-formed

citrate-stabilized GNPs [67] or during the *in-situ* synthesis of glyco-GNPs [32, 38, 46].

It is also possible to conjugate suitably derivatized glycans [48] *via* additional functional groups of *thiol*-functionalized ligands attached to the surface of pre-formed GNPs.

We, however, chose an alternative approach that relied on *direct* conjugation of *amino*-functionalized Ara_6 glycosides with the pre-formed citrate-stabilized GNPs that possess no additional ligands. The use of amine-terminating ligands for binding with GNPs is well established [68-71] although not very popular with glycans yet. Indeed, the direct conjugation of amino-functionalized antigenic glycans with pre-formed GNPs has not earlier been reported to the best our knowledge although simple amines, amino acids and peptides [68-78], as well as diethylaminoethyl-dextran [79] and chitosan [80] have been reported to bind to GNPs in pH-dependent manner as the energy of Au–N interaction is intermediate between those of Au–S and Au–O [81] (the affinity of different functional groups to the GNPs's surface decreases in the series Au-S>Au-NH2>Au-COOH [82]) thus making exchange of citrate ligands with amines possible. The amine-capped GNPs are stable enough to be used as targeting agents for drug delivery applications [75], antigens for generation of antibodies [77] or antimicrobial agents [78]. It is shown that such nanoconjugates are non-toxic, effectively penetrate into the cells and can be used as carriers [83]. It has been demonstrated that the amine–gold surface interaction is charge-neutral and stability of amine-capped GNPs is a finite-size effect which is largely kinetic in origin unlike that of thiol-capped GNPs, which are known to possess thermodynamic stability with respect to capping ligand desorption and subsequent particle aggregation [68].

The nature and the length of spacer aglycon are known [84-90] to affect the presentation of carbohydrate ligands that, in turn, determines molecular recognition of glycan moieties including those incorporated in glyco-GNPs [38, 40, 91]. In order to reveal if such an influence would be critical for immunization with Ara_6-GNPs and

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specificities of the elicited antibodies, two synthetic Ara_6 glycosides with amino-functionalized spacer aglycons differing in length and hydrophilicity ($Ara_6C_2NH_2$ (**1**) and $Ara_6C_2EG_7NH_2$ (**2**) [92,93], Figure 1) were conjugated with gold nanoparticles (d = 15 nm) [67,94] to give two sets of Ara_6-GNPs (**3** and **4**, respectively), which were used for generation of antibodies that were then characterized by dot assay and enzyme-linked immunosorbent assay (ELISA), which confirmed their specificity against *Mycobacteria*.

Results

Preparation and characterization of conjugates of amino-functionalized glycosides with GNPs

Glycans

The known hexasaccharide fragments of LAM/AG with amino groups at the terminal position of short (C_2) or elongated (C_2EG_7) spacer aglycons (Ara_6 glycosides **1** and **2**, respectively, Figure 1) were prepared as described previously [92, 93] and used without any further modification for direct conjugation with pre-formed GNPs.
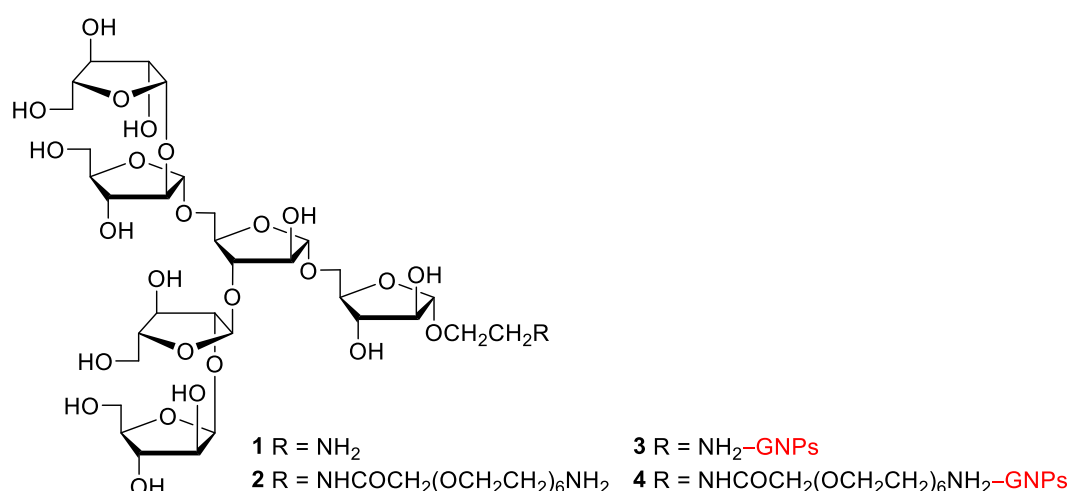

1 R = NH_2 **3** R = NH_2–GNPs
2 R = $NHCOCH_2(OCH_2CH_2)_6NH_2$ **4** R = $NHCOCH_2(OCH_2CH_2)_6NH_2$–GNPs

Figure 1: The structures of hexasaccharide fragments of LAM/AG with amino groups at the terminal position of short (C_2) or elongated (C_2EG_7) spacer aglycons (Ara_6 glycosides **1** and **2**, respectively) and their conjugates with gold nanoparticles (GNPs) (Ara_6-GNPs **3** and **4**, respectively).

Glyco-GNPs

In order to prepare the glyco-GNPs, Ara$_6$ glycosides **1** and **2** (Figure 1), which had amino groups at the terminal position of spacer aglycon, were directly conjugated to pre-formed [67, 94] freshly prepared citrate-stabilized spherical gold nanoparticles (d = 15 nm) (identical to those prepared earlier [77]) to give the corresponding Ara$_6$-GNPs **3** and **4** (Figure 1), which had the same size as the parent GNPs according to the data of transmission electron microscopy (TEM) (Figure 2).

According to estimates of the colorimetric determination of carbohydrates (phenol–sulfuric acid reaction), we can assert that there are no more than 100 glycoside molecules on each nanoparticle on average. And this number of ligands is sufficient for the stability of glyco-GNPs (Figure 3 and 4). The molecular masses of the glycosides **1** and **2** are 853 g mol^{-1} and 1174 g mol^{-1}, respectively. The difference in molecular masses of glycosides is insignificant (1.38 times) for assessing the influence of the size of ligand molecules on the stabilization of GNPs. 1 mL of GNPs contains ~1.6×10^{12} particles. The minimal stabilizing concentrations for both glycosides were found to be 100 µg mL^{-1}. We calculated that glycosides **1** and **2** stabilize the used GNPs at a ratio of ~5×10^3 molecules per GNP and ~3×10^3 molecules per GNP, respectively. Thus, it can be noted that successful conjugation requires more 50-fold excess of glycosides.

Spherical citrate-stabilized GNPs with 15 nm average diameter (which are often considered as standard GNPs for immunochemical studies due to ease of preparation and conjugation with ligands [48, 56, 57] and have successfully been used in our previous studies [56, 57, 77, 78, 95]) were chosen since GNPs of this size, unlike much smaller GNPs (e.g., around 2 nm in diameter), which are often derivatized *in situ* [32, 39, 46, 48], can be easily prepared in advance and kept for further conjugation with a desired ligand. Another advantage of using such GNPs is

their strong light absorption due to localized surface plasmon resonance (LSPR) around 520 nm while small GNP colloids (< 3 nm) do not exhibit an LSPR band and are barely colored. It is known that the color of GNPs solutions dramatically depends on their size and shape as well as on the dielectric properties of the medium surrounding the GNPs, these features being useful in many (bio)analytical applications including monitoring their stability (as described below) [25, 26, 46]. The choice of GNPs size and shape (spheres, nanorods, nanoshells and nanostars) were shown to influence immunogenicity of their conjugates with haptens, the large spherical GNPs (d = 15 and 50 nm) being the optimal antigen carriers and adjuvants for immunization [96].

The nature and length of the spacers (linkers) have been selected to modulate the stability of the Ara_6-GNPs prepared as well as to control the presentation of the Ara_6 hexasaccharide epitope on the GNPs surface hence to explore the effect a linker would have on immunogenicity. The very short C_2-linker (only two aliphatic carbon atoms) of glycoside **1** was chosen to allow the Ara_6 hexasaccharide moiety to protrude just a little above the shell of $Ara_6C_2NH_2$-GNPs **3** making recognition of the glycan epitope potentially problematic (for this reason, such short linkers are not popular in neoglycoconjugate chemistry). Thus, glyco-GNPs **3** were initially planned to serve as negative control. The Ara_6 hexasaccharide was also conjugated to a much longer and flexible hydrophilic linker based on heptaethylene glycol (EG_7) that was initially expected to allow better recognition of the glycan epitope on the surface of $Ara_6C_2EG_7NH_2$-GNPs **4** than on the surface of $Ara_6C_2NH_2$-GNPs **3**. The differences in hydrophilicity and rigidity of spacers in glycosides **1** and **2** were also expected to affect the stability of Ara_6-GNPs **3** and **4** formed.
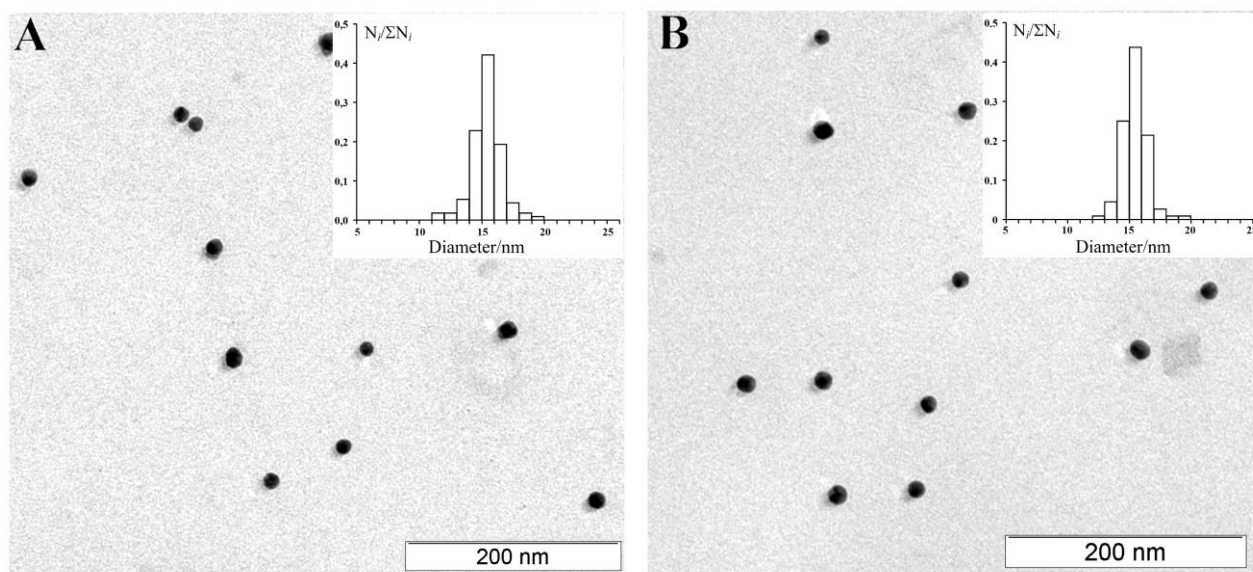
Figure 2: Transmission electron microscopy (TEM) images for prepared Ara₆-GNPs **3** (**A**) and **4** (**B**). The insets show size distribution diagrams with 15 nm average diameter in both cases.

Stability of glyco-GNPs

Gold hydrosols are typical lyophobic colloids that are stable only under low ionic strength conditions. Under physiological conditions, GNPs are thermodynamically unstable and require special stabilization. The sol stability can be increased by coating the GNPs with a ligand layer (by conjugation with ligands). As a result, the particle surface acquires the properties of the stabilizing agent used. Such stabilized GNPs can be lyophilized and become much less sensitive to electrolyte induced coagulation (due to electrostatic, hydrophobic interactions and structural-mechanical stability factor) [57, 97, 98]. The minimal stabilizing concentrations for both glycosides were found to be 100 µg mL⁻¹. This concentration was used to conjugate antigens **1** and **2** with GNPs.

The TEM data of the prepared Ara₆-GNPs **3** and **4** (Figure 2) clearly suggest that coupling of Ara₆ glycosides **1** and **2** with GNPs did not change the size of nanoparticles ($d = 15$ nm) and that aggregation is absent. Addition of NaCl solution to conjugates of GNPs with Ara₆ glycosides **1** and **2** (Ara₆-GNPs **3** and **4**, respectively)

allowed determining the differences in ability of Ara_6 glycosides **1** and **2** to stabilize GNPs nanoparticles at various pH values (Figure 3). The highest stabilizing ability was demonstrated by Ara_6 glycoside **1** with 2-aminoethyl spacer aglycon while Ara_6 glycoside **2** with longer spacer aglycon was slightly inferior and could stabilize GNPs only at a pH of 9.7 and above. For this reason, immunization of rabbits was carried out with solutions of Ara_6-GNPs **3** and **4** at pH ~9.7, at which both Ara_6-GNPs **3** and **4** were stable in saline medium.
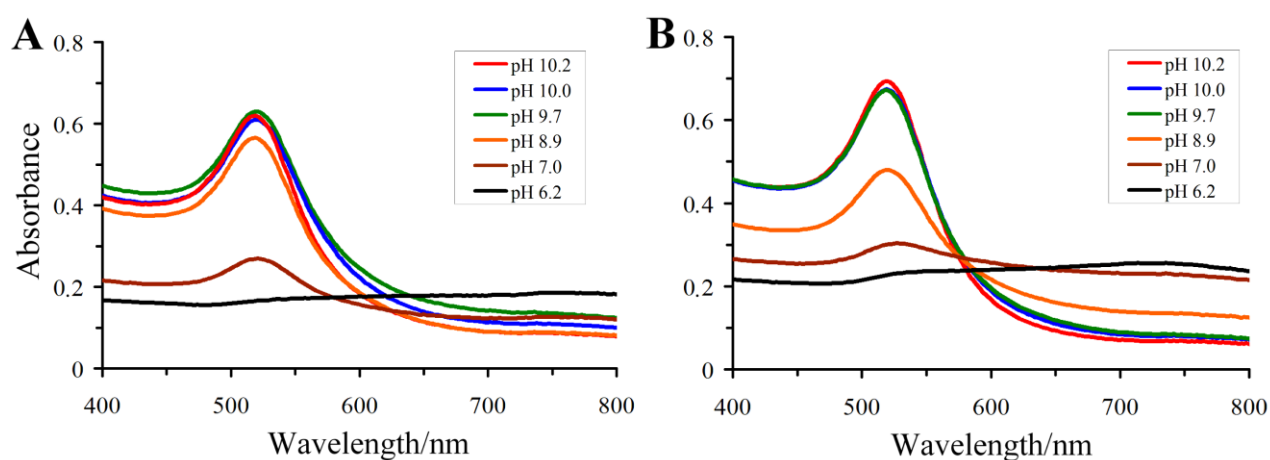

Figure 3: Spectra of solutions of $Ara_6C_2NH_2$-GNPs (**3**) (**A**) and $Ara_6C_2EG_7NH_2$-GNPs (**4**) (**B**), prepared from solutions of containing 100 µg mL^{-1} of glycosides and 0.9% NaCl, at different pH values. Glycan components in glyco-GNPs **3** and **4** are hexasaccharide fragments (Ara_6) of mycobacterial lipoarabinomannan (LAM) with amino groups at the terminal position of short (C_2) or elongated (C_2EG_7) spacer aglycons (Ara_6 glycosides **1** and **2**, respectively; see Figure 1).

Stability of the prepared glyco-GNPs **3** and **4** against aggregation followed from the experiments with three different types of solutions of glyco-GNPs **3** and **4**, which (1) contained excess of Ara_6 glycosides **1** and **2** (pH ~9.7; these solutions were used for immunization), (2) prepared in water from glyco-GNPs **3** or **4**, which were purified from the excess of Ara_6 glycosides **1** and **2** by centrifugation, or (3) prepared in 0.9% NaCl from glyco-GNPs **3** or **4**, which were purified by centrifugation, dissolved in 5%

sucrose solution and then lyophilized. Spectra of these solutions were virtually

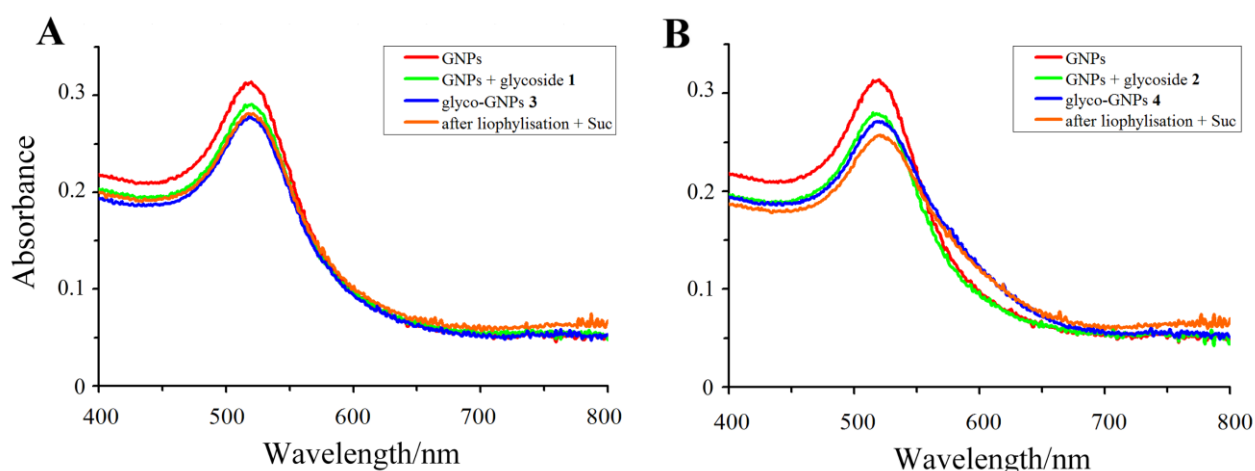identical to each other and very similar to that of the starting GNPs (Figure 4**).**

Figure 4: Spectra of (1) solution of the starting GNPs, (2) solutions of glyco-GNPs **3** (**A**) and

4 (**B**) containing 100 µg mL^{-1} of Ara$_6$ glycosides **1** and **2**, respectively (these solutions were

used for immunization), (3) solutions of glyco-GNPs **3** (**A**) and **4** (**B**) prepared in water from

glyco-GNPs **3** or **4**, which were purified from the excess of Ara$_6$ glycosides **1** and **2** by

centrifugation**,** and (4) solutions of glyco-GNPs **3** (**A**) and **4** (**B**) prepared in 0.9% NaCl from

glyco-GNPs **3** or **4**, which were purified by centrifugation, dissolved in 5% sucrose solution

and then lyophilized.

Detection of glycosides by obtained sera

The glyco-GNPs **3** and **4** obtained were used for hyperimmunization of rabbits. The

interaction of the obtained polyclonal rabbit antisera with the starting glycosides **1**

and **2** was studied by dot-assay on PVDF membrane (Figure 5). Ara$_6$ glycoside **1**

(with a short spacer aglycon) was detected by antisera against both Ara$_6$C$_2$NH$_2$-

GNPs **3** and Ara$_6$C$_2$EG$_7$NH$_2$-GNPs **4**. That is, there was a serological cross-reaction

for the obtained antisera against Ara$_6$-GNPs **3** and **4**. Remarkably, both antisera were

equally effective in detecting Ara$_6$C$_2$NH$_2$ (**1**) in amounts as low as ~60 ng. This

unequivocally proves that both antisera are specific for Ara$_6$ hexasaccharide epitope.

Glycoside **2** (with a long spacer aglycon) could not be detected by the dot-analysis
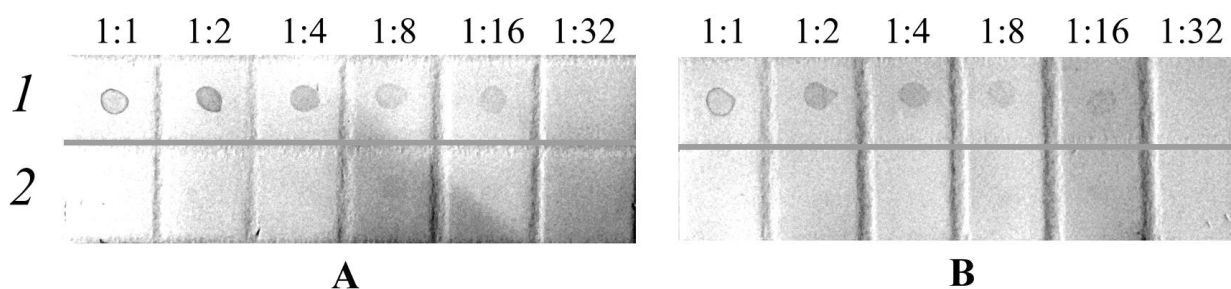
with any of the obtained antisera.

Figure 5: Results of dot assay of Ara_6 glycosides **1** (*1*) and **2** (*2*) with antisera against $Ara_6C_2NH_2$-GNPs (**3**) (**A**) and $Ara_6C_2EG_7NH_2$-GNPs (**4**) (**B**). Solutions of glycosides (1 mg mL^{-1}) in H_2O were titrated 2-fold.

Interaction of the obtained sera with mycobacterial cells

Interaction with the mycobacterial cells of three model cultures (*M. bovis*, *M. phlei* and *M. smegmatis*) has been demonstrated for both obtained antisera against Ara_6-GNPs **3** and **4** by the enzyme-linked immunosorbent assay (ELISA) (Figure 6, Figure S1). Both antisera detected *M. phlei* cells significantly weaker than *M. bovis* and *M. smegmatis* cells. Importantly, neither antiserum gave any interaction with *E. coli* cells. Control experiments showed that the observed specificity of antisera against Ara_6-GNPs **3** and **4** is due to the presence of Ara_6-epitope in Ara_6-GNPs **3** and **4** and is not related to the presence of background anti-mycobacterial antibodies in intact serum of the rabbit used for immunization or antibodies generated against heat killed *M. tuberculosis* cells, which are present in complete Freund's adjuvant (CFA), as no interaction of intact serum of the rabbits or antiserum against LPS of *Azospirillum brasilense* Sp7 generated in the presence of CFA [99] with *M. bovis*, *M. phlei*, *M. smegmatis* and *E. coli* cell suspensions could be detected (Figure 6).
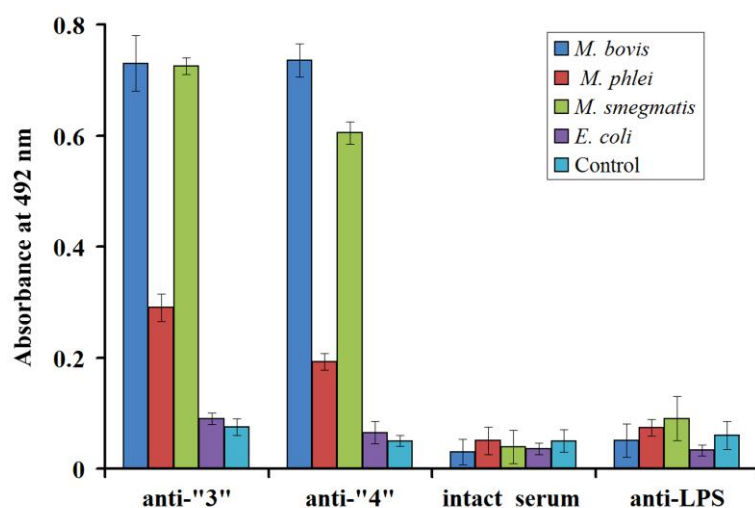
Figure 6: Results of ELISA of *M. bovis*, *M. phlei*, *M. smegmatis* and *E. coli* cell suspensions (10^8 cells mL^{-1}) with antisera against Ara$_6$C$_2$NH$_2$-GNPs **3** (anti-"**3**"), Ara$_6$C$_2$EG$_7$NH$_2$-GNPs **4** (anti-"**4**"), intact serum of the rabbit used for immunization and antibodies against LPS of *Azospirillum brasilense* Sp7 generated in the presence of CFA. Error bars indicate the observed confidence intervals at $p < 0.05$. See also all titration curves in Supporting Information File 1.

Discussion

In this study, hexasaccharide fragments of LAM/AG with amino groups at the terminal position of short (C$_2$) or elongated (C$_2$EG$_7$) spacer aglycons (Ara$_6$ glycosides **1** and **2**, respectively; Figure 1) were conjugated with the pre-formed [67, 94] gold nanoparticles ($d = 15$ nm) to give the corresponding glyco-GNPs with pendant Ara$_6$ moieties (Ara$_6$C$_2$NH$_2$-GNPs (**3**) and Ara$_6$C$_2$EG$_7$NH$_2$-GNPs (**4**), respectively), which were found to be stable in the presence of 0.9% NaCl only in alkaline media (pH 8–10) (Figure 3). Noteworthy is better stabilization of GNPs by Ara$_6$ glycoside **1** with a short 2-aminoethyl spacer aglycon.

It is important to stress that removal of excess of Ara$_6$ glycosides **1** and **2** from solutions of glyco-GNPs (**3** and **4**) or lyophilization of the purified glyco-GNPs **3** and **4**

(provided that they were dissolved in 5% sucrose solution prior to lyophilization) does not impair stability of the prepared glyco-GNPs **3** and **4** against aggregation as follows from similarity of their spectra (Figure 4).

The prepared glyco-GNPs with pendant Ara_6 moieties (Ara_6-GNPs **3** and **4**) were used for immunization of rabbits. Alkalinity of the immunogen (conjugate of an antigen with GNPs) is typical for the immunization of animals, starting with the first published work on the production of antibodies using colloidal gold [100]. The prepared Ara_6-GNPs **3** and **4** were not separated from the excess of ligands **1** and **2** and were used for hyperimmunization of rabbits without additional purification since it was reported that the presence of excess soluble antigen along with the same antigen immobilized on GNPs may be vital for inducing high levels of antibody response in immunization [98]. Although the role of admixture of soluble antigen in inducing protective immunity is unclear yet [98], these results might suggest that leaching of antigen from the prepared glyco-GNPs could be beneficial for the success of immunization. This also suggests that the increased stability of glyco-GNPs does not necessarily mean better immunization. Although one can argue that amine-linked carbohydrate ligands present in Ara_6-GNPs **3** and **4** might be exchanged with various thiols present *in vivo*, immunization with the prepared Ara_6-GNPs **3** and **4** was successful, which indicates that such glyco-GNPs preparations are indeed capable of inducing antibody response. Clarification of these complex issues clearly requires further studies.

The specificity of the obtained antisera against Ara_6-GNPs **3** and **4** was then studied. Dot assay, which is a traditional way for initial rapid assessment of specificity of the antisera obtained, revealed (Figure 5) cross-reactions for the two obtained antisera with the parent hexaarabinofuranoside **1** with 2-aminoethyl aglycon. Therefore, antibodies in high titers of approximately the same specificity against the Ara_6

hexasaccharide epitope are produced by immunization of rabbits with both Ara_6-GNPs **3** and **4**. Of the two Ara_6 glycosides (**1** and **2**), only glycoside **1** with a short spacer aglycon can be used to detect specific antibodies by the dot assay. Lack of interaction between glycoside **2** and the obtained antisera in the dot assay is apparently related to desorption of glycoside **2** with much more hydrophilic spacer aglycon from PVDF membrane during the assay. This explanation is further supported by the fact that it was impossible to use a nitrocellulose membrane for dot assay of the glycosides because of the higher hydrophilicity of this material than that of the PVDF membrane. For both glycosides **1** and **2**, there was no adsorption to the nitrocellulose membrane, and as a consequence, no reaction with specific antisera (data not shown). The combination of these observations makes it possible to conclude that it is Ara_6 hexasaccharide epitope that determines specificities of both antisera.

Both antisera contained high titers of antibodies specific for *Mycobacteria* as shown by ELISA using *M. bovis* and *M. smegmatis* cells as antigens while gave a weak response with *M. phlei* cells and no interaction with *E. coli* cells. Control experiments showed that the observed specificity of antisera against Ara_6-GNPs **3** and **4** is due to the presence of Ara_6-epitope in Ara_6-GNPs **3** and **4** and is not related to the presence of background anti-mycobacterial antibodies, which could be present in intact serum of the rabbit used for immunization, or antibodies generated against heat killed *M. tuberculosis* cells, which are present in complete Freund's adjuvant (CFA) (Figure 6, Figure S1). The importance of using CFA for generation of hapten-specific antibodies has been recently demonstrated [77]. The titers of antibodies against haptens amine-linked to GNPs decreased in the following order: (hapten + GNPs +CFA) > (hapten + GNPs) >> (hapten + CFA). No antibodies against GNPs has been detected in that study [77].

The results obtained suggest that glyco-GNPs bearing oligosaccharide fragments of LAM/AG are promising agents for generation of anti-mycobacterial antibodies. The positive reaction of the obtained antibodies to the cells of all three mycobacterial cultures, in contrast to the cells of *E. coli* culture, confirms the antigenic identity of the Ara_6 epitope and surface cell antigens for all *Mycobacteria* and its specificity for this group of bacteria. Nevertheless, we have identified differences in the interaction of antibodies, obtained with Ara_6-GNPs, with the cells of three mycobacterial cultures by the ELISA assay that may indicate a different presentation of LAM/AG on the surface of *M. bovis* / *M. smegmatis* cells and *M. phlei* cells. This observation suggests the possibility of serological individuality of *M. phlei*, despite *M. smegmatis* and *M. phlei* are phylogenetically close species. Both species belong to the group "rapidly growing *Mycobacteria*", in contrast to *M. bovis* that belongs to "slowly growing *Mycobacteria*" [100]. Serological cross-reaction between the *M. bovis* and *M. smegmatis* cells (in contrast to *M. phlei* cells) was previously noted for antibodies obtained against a tuberculin-GNPs conjugate [101].

Both sets of Ara_6-GNPs (**3** and **4**) that contained Ara_6 glycan epitopes with different spacer aglycons were equally effective in generation of antibodies in rabbits. The fact that even $Ara_6C_2NH_2$-GNPs (**3**) with very short linker (only two carbon atoms) performed well is rather unexpected since it is commonly believed [38, 40, 46, 91] that a rather long spacer aglycon (more than five carbon atoms) is required for correct recognition of glycan moieties and efficacious immunization.

The observed differences in specificity of antibodies generated against the two sets of Ara_6-GNPs (**3** and **4**) suggest substantially different presentation of the same glycan on the synthesized Ara_6-GNPs **3** and **4**, the antibodies against $Ara_6C_2EG_7NH_2$-GNPs **4** with longer C_2EG_7 spacer aglycon being noticeably more selective towards different species of *Mycobacteria*. We hypothesize that this

different presentation of Ara_6 epitope could include different degree of clustering of glycan on the GNPs surface, $Ara_6C_2EG_7NH_2$-GNPs **4** with longer and more hydrophilic C_2EG_7 spacer aglycon being apparently more dispersed over the GNPs surface hence more accessible for interaction. These unusual features of theAra$_6$-GNPs conjugates could be related to differences in structures of solutions [102] of parent amino-functionalized Ara_6 glycosides **1** and **2** used for conjugation with GNPs. We have recently argued [103-105] that the sometimes observed profound influence of the nature of (functionalized) aglycon in a glycosyl acceptor may be related to formation of reaction solutions with modified structure that are featured by the presence of different supramolecular assemblies of reagents (supramers [102]) in solution. In a similar fashion, solutions of $Ara_6C_2NH_2$-GNPs **3** with shorter and more hydrophobic C_2 spacer aglycon might form more dense [106, 107] supramers in aqueous solutions [102, 108-110] which eventually would form more clustered Ara_6-glycan domains on the surface of Ara_6-GNPs.

The results obtained clearly suggest that the choice of spacer aglycon (a linker between glycan and GNPs) may be critical for the specificity of antibodies against the corresponding glyco-GNPs and the studies in this direction seem promising.

Glycoside **2** was previously used for conjugation with mycobacterial proteins for serological detection of antibodies against *M. tuberculosis* [92]. It was shown that conjugation of recombinant proteins MPB-64 and Rv0934 with glycan **2** containing Ara_6 epitope increased the sensitivity of serodiagnosis by 10–15% as compared to the use of unmodified proteins. Here, instead of using protein carriers, conjugation of glycans with GNPs, which is experimentally much easier, was carried out. The obtained Ara_6-GNPs **3** and **4** could potentially also be used for detecting antibodies against *M. tuberculosis*. We demonstrated the immunogenicity of Ara_6-GNPs, which opens the possibility of using similar glyco-GNPs in a new generation of vaccines

aimed at preventing human and animal tuberculosis. Antibodies obtained against Ara$_6$-GNPs could also be used to detect *Mycobacteria* (serodiagnosis) and treat the diseases caused by them (passive immunity).

Generation of antibodies against carbohydrate antigens (epitopes) of *Mycobacteria* linked to GNPs has not been reported to the best of our knowledge. Successful use of glyco-GNPs as vaccines has been described by Parry *et al.* (2013) [37] The authors observed that these nanomaterials generated strong and long-lasting production of antibodies that are selective to the Tn-antigen glycan and cross-reactive toward mucin proteins displaying Tn. Other authors [33, 42, 47] used glyco-GNPs to prepare specific antibodies against carbohydrate antigens or epitopes of bacterial pathogens *S. pneumonia* and *B. mallei* in combination with peptides or proteins that activated the immune response. Safari *et al.* (2012) [33] showed that the mice immunization with glyco-GNPs containing the tetrasaccharide epitope *S. pneumonia* type 14 without T-helper peptide did not result in the activation of the immune response of the animals (mice) and the production of specific antibodies. The situation is rather complex as our analysis of the literature data on the use of thiol- and amino-containing glyco-GNPs suggests. It appears that efficacy of antibodies generation inversely correlates with stability of glycan–GNPs conjugates. The induction of specific immune response (i.e., generation of antibodies) against hapten is T-cell-dependent and requires the uptake (phagocytosis), processing and presentation of epitopes on MHC class II molecules by antigen-presenting cells to the specific T cell. It remains unclear, then, how this process can proceed with a hapten. We cannot now say exactly about the mechanisms of haptens's release in immune cells. We speculate that phagocytosis of antigen and subsequent presentation of the epitopes on the surface of macrophages are important events in this positive regulation. According to the literature data, GNPs contribute to the penetration of

antigens into phagocytic cells [56]. GNPs, in addition to their adjuvant properties, could favor more active uptake of glycans incorporated in glyco-GNPs since free glycans (haptens) cannot be phagocytosed *per se* due to their small size. Probably, substitution of glycans on the surface of glyco-GNPs with endogenous cellular thiols and amines facilitates subsequent translocation of glycans to the surface of macrophage that is required for activation of specific B-cells. Clearly, such substitution is more favored for glyco-GNPs based on amine-terminated glycans as compared to more stable glyco-GNPs based on thiol-terminated glycans (for B-cell activation, the latter require addition of peptide/protein). A low-molecular-mass glycan (hapten) cannot induce cellular immune response alone (without carrier) and a possible specific immune response induced by the unconjugated hapten would be minimal and hardly detected. Fallarini *et al.* (2013) [111] showed that the nanoconjugates are taken up by cells but, afterwards the sugar moieties are detached from the gold surface to be presented on the surface of the cells. We believe that a similar mechanism is implemented in our case. Clearly, this theory requires further studies to support it.

Here, we demonstrated the successful use of glyco-GNPs bearing the hexasaccharide epitope of LAM/AG for activation of specific immune response against carbohydrate antigen in laboratory animals (rabbits) which can be used to develop synthetic protein- and peptide-free glycoconjugate vaccines based on glyco-GNPs.

It should be noted that the interaction of functionalized GNPs with cells of the immune system is still far from being understood in more or less detail and requires further studies [56]. Experiments aimed at elucidating the mechanisms of antibody production in response to the introduction of glyco-GNPs are planned in the near future.

Conclusion

Thus, in our study, the use of GNPs conjugated with glycoside **1** containing a terminal branched Ara_6 hexasaccharide unit of LAM/AG with a short 2-aminoethyl spacer aglycon was most effective in producing specific antibodies by immunizing rabbits. The antiserum obtained by hyperimmunization of rabbits with $Ara_6C_2NH_2$-GNPs conjugate **3** allowed detection of LAM/AG oligosaccharides **1** and **2** as well as cells of *Mycobacteria* with high titers.

Conjugate of GNPs with glycoside **2** containing the Ara_6 hexasaccharide with a longer oligo(ethylene glycol) spacer aglycon showed weak overall efficacy. In addition to the more complex synthesis of the glycoside **2**, this glycoside is poorly absorbed on nitrocellulose and PVDF membranes which significantly complicate the process of detecting specific antibodies by immunodetection. The obtained antibodies against $Ara_6C_2EG_7NH_2$-GNPs **4** did not outperform in immunochemical tests the antibodies against $Ara_6C_2NH_2$-GNPs **3** containing much simpler 2-aminoethyl spacer aglycon.

The results obtained clearly suggest that the choice of a linker between glycan and GNPs may be critical for the specificity of antibodies against the corresponding glyco-GNPs and the studies in this direction seem promising. The conjugates prepared in this study (Ara_6-GNPs) are stable in water (after removal of excess Ara_6 glycoside by centrifugation) or in a saline medium (either at pH ≥8.9 or after lyophilization (in the presence of 5% sucrose) of the conjugate purified from excess Ara_6 glycoside). These features suggest that Ara_6-GNPs (and other conjugates of GNPs with related glycans) might be useful in immunochemical detection of antibodies against surface carbohydrate antigens of *Mycobacteria*.

In conclusion, glyco-GNPs containing fragments of LAM are promising agents for activation of immunological reactions to *Mycobacteria* in humans and animals and generation of anti-mycobacterial antibodies. In the future, similar glyco-GNPs could be used as components of anti-tuberculosis vaccines.

Experimental

Preparation of amino-functionalized Ara6 glycosides 1 and 2

Glycoside **1** [92, 93] with 2-aminoethyl aglycon (Figure 1) was synthesized as described previously [93]. Glycoside **2** [92] with extended amino-functionalized aglycon was synthesized from 2-aminoethyl glycoside **1** by *N*-acylation with *N*-trifluoroacetylated heptaethylene glycol-based amino acid $HO_2CCH_2(OCH_2CH_2)_6NHTFA$ (prepared from 18-crown-6 [112]) in the presence of DMT-MM [113, 114] and *N*-methylmorpholine in MeOH followed by basic deprotection as described previously [92].

Preparation of GNPs and a study of ability of Ara$_6$ glycosides 1 and 2 to stabilize nanoparticles

Gold nanoparticles (GNPs, average diameter $d = 15$ nm) were prepared by the citrate method of Frens [67, 94]. For reduction, 0.01% aqueous tetrachloroauric acid (242.5 mL, $HAuCl_4$; Sigma-Aldrich) in an Erlenmeyer flask was brought to reflux with stirring on a magnetic stirrer. Then 1% aqueous sodium citrate (7.75 mL, Fluka) was added to the flask. Reflux was continued for 30 min to give bright red sol that contained GNPs ($d = 15$ nm) identical to those prepared earlier [77] (hereinafter – GNPs solution).

Determination of ability of Ara$_6$ glycosides **1** and **2** to stabilize GNPs in the presence of NaCl at various pH values was performed as described below. The pH values of

the GNPs solutions were adjusted to 6.2, 7.0, 8.9, 9.7, 10.0, and 10.2 by addition of 0.2 M K_2CO_3 aqueous solution. After that, 100 µL of an aqueous solution of Ara_6 glycoside **1** or **2** (concentration 200 µg mL^{-1}) was added to 100 µL of the obtained GNPs solution. The mixtures were incubated at room temperature for 10 min. Then 10% aqueous solution of NaCl was added to an end concentration of 0.9% (m/v) in each solution. The spectra of the obtained solutions were registered with Tecan Spark 10M microplate reader (Tecan, Austria) at 400–800 nm.

To prepare glyco-GNP, the "gold number" (minimal amount of carbohydrate antigen that protects the sol against aggregation induced by NaCl) for the glycoside solutions was first determined. To this end, 20 µL of aqueous solutions of Ara_6 glycoside **1** or **2** (initial concentration 1 mg mL^{-1}) was titrated twofold on a 96-well microtiter plate. Each well received 200 µL of GNPs solution (pH 9.7) and 20 µL of 1.7 M NaCl aqueous solution. The minimal stabilizing concentration of a glycoside was established visually by change in the color of the GNPs solution from red to blue in the wells of a microtiter plate. The minimal stabilizing concentrations for both glycosides were found to be 100 µg mL^{-1}. This concentration was used to conjugate antigens **1** and **2** with GNPs.

Conjugation was done by simple mixing of components: 4.5 mL of GNPs solution (d = 15 nm) were mixed with 125 µL of 0.2 M K_2CO_3 aqueous solution (pH value of the resulting solution ~9.7) and 0.5 mL of aqueous solution (1 mg mL^{-1}) of Ara_6 glycoside **1** or **2** was added while stirring to give solutions of Ara_6-GNP **3** and **4** (containing 100 µg mL^{-1} of glycosides **1** and **2**, respectively), which were used for immunization.

Stability of the glyco-GNPs **3** and **4** (prepared as described above) against aggregation was demonstrated by the following experiments. Solutions of glyco-GNPs **3** and **4** containing 100 µg mL^{-1} Ara_6 glycosides **1** and **2**, respectively, were

centrifuged for 30 min at 12400 g on a Microspin 12 centrifuge (Biosan, Latvia) and the precipitate was resuspended in water to give solutions of glyco-GNPs with spectra virtually identical to the those of starting solutions of glyco-GNPs **3** and **4** and very similar to that of the starting GNPs (Figure 4). Alternatively, the precipitate of glyco-GNPs **3** and **4** purified from the excess of Ara_6 glycosides **1** and **2** by centrifugation (see above) was dissolved in 5% sucrose solution, lyophilized and then the residue was resuspended in 0.9% NaCl to give solutions of glyco-GNPs with spectra virtually identical to the those of starting solutions of glyco-GNPs **3** and **4** and very similar to that of the starting GNPs.

Transmission electron microscopy

Glyco-GNPs **3** and **4** were characterized using a Libra 120 transmission electron microscope (Carl Zeiss, Germany) at 120 kV accelerating voltage at the "Simbioz" Center for the Collective Use of the Research Equipment in the Field of Physical-Chemical Biology and Nanobiotechnology at the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences (IBPPM RAS). Approximately 20 µL of glyco-GNPs suspension was applied to a film (Parafilm, USA) and a formvar coated copper grid (200 mesh) was placed on the drop for 20 min. A thermal attachment was carried out while holding the grid near the incandescent lamp for 2 min. The excess of the liquids was removed by touching the grid to a strip of the filter paper. The grid was washed by a drop of deionized water, dried and then analyzed by TEM. According to TEM data, the prepared Ara_6-GNPs **3** and **4** had the same size ($d = 15$ nm) as the parent GNPs.

Animal immunization and obtaining of antisera

Rabbits were immunized with the Ara_6-GNPs **3** and **4** conjugates (one animal for each type of glyco-GNPs) as follows: 0.5 mL of solutions of Ara_6-GNP **3** and **4** (for the preparation see above) were mixed with 0.5 mL of complete Freund's adjuvant

(CFA, Sigma, USA). Polyclonal antibodies were raised by subcutaneous immunization of chinchilla rabbits with the glycosides–nanoparticle at 10 points along the spinal column, by giving four injections with an interval of 14 days between them. The obtained sera were tested for interaction with the glycosides **1** and **2** by dot assay and with the mycobacterial cells by ELISA.

Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the legislation of the Russian Federation. The use of the animals was also approved by the institution where the experiments were performed.

Dot assay

The specificity of the obtained antisera to both Ara_6 glycosides **1** and **2** was tested by the dot immunoassay (titrated twofold in the microtiter plate) as follows. An aliquot (1 µL) of solution of each antigen (**1** or **2**) in the double dilutions (initial concentration, 1 mg mL^{-1}) was spotted onto a Westran S polyvinylidene fluoride (PVDF) membrane (Whatman), and the membrane was incubated in a dry-air thermostat at 60 °C for 15 min until dry. After spotting and drying, the membrane was then blocked for 12 h with 2% powdered milk diluted in 10 mM PBS. This procedure was performed to prevent the nonspecific antibody adsorption. Then the membrane was incubated in the obtained rabbit antisera**,** diluted 1:50 with the 0.01 M PBS, at room temperature overnight**.** The membrane was washed four times at 15 min intervals with PBS containing 0.02% Tween 20. Then, it was incubated in a solution of peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories; diluted 1:2000) in PBS containing 0.02% Tween 20 and 0.02% powdered milk for 90 min. The membrane was washed four times for 15 min with PBS containing 0.02% Tween 20. After that, the membrane was treated with a

substrate mixture of 0.05% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide in 0.15 M PBS until intense brown dots appeared (Figure 5).

Bacterial cultures and growth conditions

Bacterial cultures of *M. bovis* (vaccine strain BCG), *M. phlei*, and *M. smegmatis* were obtained from the Collection of pathogenic and vaccine microorganisms of animals of Kovalenko Institute of Experimental Veterinary (Moscow, Russia) [115]. Culture of *E. coli* strain K-12 was obtained from the Collection of the Rhizosphere Microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS (Saratov, Russia) [116]. The cells were grown on a solid nutrient medium for isolation of *Mycobacteria* (the Löwenstein–Jensen medium) at 37 °C for 25 days.

Enzyme-linked immunosorbent assay

Detection of serological reactions of antisera with mycobacterial cells was carried out by ELISA in 96-well polystyrene plates using the standard procedure, as previously described [117]. Aliquots (50 µL) of each bacterial suspension in the double dilutions (initial concentration, 10^8 cells mL^{-1}) were immobilized in the wells through simple adsorption, kept for 30 min on a shaker at room temperature. The samples were replaced with 100 µL of 0.05% polyethylene glycol 20000 (PEG), added to each well to block the free binding sites on polystyrene. This solution was replaced with 50 µL of primary antibodies (antisera to glyco-GNPs, or intact serum, or antiserum to LPS of *Azospirillum brasilense* Sp7 [99] as negative control) diluted 1:100 in PBS containing 0.02% Tween 20 and 0.005% PEG (for prevention of nonspecific antibody sorption). After incubation for 40–60 min, the wells were washed three times with 100 µL of PBS containing 0.02% Tween 20, and 50 µL of peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunnoResearch Laboratories, USA; diluted 1:2000) in PBS containing 0.02% Tween 20 and 0.005% PEG was placed in each well. After 30 min incubation, the wells were washed twice with 100

µL of PBS containing 0.02% Tween 20, and peroxidase activity was estimated by adding to each well 50 µL of a substrate mixture of 0.03% *o*-phenylenediamine and 0.02% hydrogen peroxide in 0.1 M sodium citrate buffer (pH 4.5). The enzyme reaction was stopped with 100 µL of 1 N H_2SO_4. The absorbance at 492 nm was read on a Multiskan Ascent analyzer (Thermo). Absorbance values at 492 nm for the wells without bacterial cells were using as the control. Data were processed with Microsoft Excel 2003 software (Microsoft Corp.); 95% confidence intervals are given (Figure 6, Figure S1).

Supporting Information

Supporting Information File 1: Results of ELISA of *M. bovis*, *M. phlei*, *M. smegmatis* and *E. coli* cell suspensions with antisera to $Ara_6C_2NH_2$-GNPs **3** (A), antisera to $Ara_6C_2EG_7NH_2$-GNPs **4** (B), intact serum of the rabbit used for immunization (C) and antiserum to LPS of *Azospirillum brasilense* Sp7 generated in the presence of CFA (D).

Supporting Information File 1: Suppl_Material.pdf

Acknowledgements

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