Preparation and immunogenicity of gold glyco-nanoparticles as antipneumococcal vaccine model

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ABSTRACT

Background: Nanotechnology-based fully synthetic carbohydrate vaccines are promising alternatives to classic polysaccharide/protein conjugate vaccines. We have prepared gold glyco-nanoparticles (GNP) bearing two synthetic carbohydrate antigens related to serotypes 19F and 14 of *Streptococcus pneumoniae* and evaluated their immunogenicity *in vivo*.

Results: A tetrasaccharide fragment of serotype 14 (Tetra-14), a trisaccharide fragment of serotype 19F (Tri-19F), a T-helper peptide, and D-glucose were loaded onto GNP in different ratios. Mice immunization showed that the concomitant presence of Tri-19F and Tetra-14 on the same nanoparticle critically enhanced the titers of specific IgG antibodies towards type 14 polysaccharide compared to GNP exclusively displaying Tetra-14, while no IgG antibodies against type 19F polysaccharide were elicited.

Conclusion: This work is a step forward towards synthetic nanosystems combining carbohydrate antigens and immunogenic peptides as potential carbohydrate-based vaccines.

KEYWORDS: gold glyco-nanoparticles; carbohydrate-based vaccines; immunogenicity; *Streptococcus pneumoniae*, capsular polysaccharide fragments.

INTRODUCTION

The possibility to manage microbial infections through nanomedicine is a hot topic in research.[1] There are several examples demonstrating the potential of nanoparticles-based materials for fast, sensitive and specific bacterial detection, as well as of the incorporation of antimicrobial nanomaterials in medical devices to prevent microbial adhesion and infection. Some nanomaterials show strong antibacterial properties and the

development of novel and tailored nanotherapeutics holds great promises to treat infectious diseases.[2] Moreover, nanoparticle engineering is offering significant contributions to immunology, in particular with regards to the understanding of immune mechanisms and in vaccine development.[3] Nanoparticles have been used both as vaccine carriers and adjuvants in formulations against infectious diseases.[4-6] Repetitive antigen display and the ability to potentiate immune responses through enhanced antigen delivery to the immune system are some key points related to nanotechnology-based vaccines.[7]

The growing evidence on the role of carbohydrates both in innate immunity[8] and adaptive immunity[9] has strengthened the interest in these biomolecules. Although carbohydrates are usually T cell independent antigens, and thus unable to induce memory response, they can be converted into potent immunogens by chemical coupling to immunogenic protein carriers (glycoconjugate vaccines).[10] Glycoconjugate vaccines against a number of diseases, mostly bacterial infections, have been already licensed or are in their advanced development.[11] However, their development is based on complex chemical manipulations and time-consuming purification steps, leading to a significant increase in manufacturing costs. Furthermore, there are considerable variations in immunogenicity and safety among various existing carbohydrate-based vaccines against microbes, for example, due to the presence of multifarious glycoforms and unselective methods used for polysaccharide isolation from natural sources. Therefore, identification, characterization, and synthesis of key carbohydrate epitopes capable of inducing a robust antibody response against polysaccharide antigens is a major step in the design of more efficacious glycoconjugate vaccines.[12] The first semi-synthetic human vaccine was developed by Bencomo and collaborators against *Haemophilus influenzae* type b and based on a synthetic oligosaccharide conjugated to tetanus toxoid as a protein carrier.[13]

The risk associated with protein carrier-induced epitopic suppression[14] have pushed researchers to seek alternatives to currently employed glycoconjugate vaccines and to investigate the use of nanotechnology-based approaches to promote the development of new and more efficient vaccine settings.[15], [16]

The importance of multivalent carbohydrate-protein interactions,[17, 18] for example in the early steps of host infection by several bacteria and viruses, makes glycosylated nanomaterials attractive models for presenting glycans in a multivalent fashion, which is abundantly exploited in glycoscience.[19] Nanomaterials loaded with carbohydrate antigens have emerged as synthetic vaccine candidates, as they give the possibility to

tune the loading of well-defined carbohydrates on different scaffolds.[20], [21] Furthermore, besides the intrinsic adjuvant properties of many nanomaterials, other structures can be incorporated onto the nanosystems as active mediators to increase vaccine efficacy, such as cell targeting moieties or Toll-like receptor ligands.[22] Among multivalent scaffolds, gold nanoparticles hold high potential for their relative inertness, low toxicity, and easiness of functionalization especially through thiol-based chemistry.[23] Carbohydrates derivatized with thiol-functionalized linkers can be incorporated as ligands onto gold nanoparticles.[24] The size and the shape of the resulting "gold glyco-nanoparticles" (GNPs) are easily controlled depending on the synthetic methodology, while the carbohydrate density and presentation on the gold surface can be tuned by inserting other thiol-ending ligands.[24], [25] In addition, carbohydrate coating ensures water dispersibility, stability and biocompatibility. Examples related to the use of GNPs as vaccine candidates have been reported, like the GNP constructs containing the tumor associated Tn antigen,[26, 27] a tetrasaccharide of *Streptococcus pneumoniae,*[28] or functionalized with lypopolysaccharide (LPS) to protect against *Burkolderia mallei*.[29]

Capsular polysaccharides (CPS) of encapsulated bacteria are critical determinants of bacterial virulence and have been used in the development of protective vaccines.[30] The gram positive bacterium *Streptococcus pneumoniae* (pneumococcus; Pn) is an important causative agent of severe forms of bacterial infectious diseases. Serotypes 19F (Pn19F) and 14 (Pn14) are among the major groups responsible for pneumococcal infections and included in the current commercial pneumococcal conjugate vaccine.[31] In previous work,[28] some of us prepared GNPs functionalized with the synthetic branched tetrasaccharide repeating unit of the type 14 pneumococcal capsular polysaccharide (Pn14PS), and the peptide fragment (OVA323-339), serving as a T-helper epitope.[32] The immunological evaluation of these GNPs demonstrated their ability to elicit specific and functional IgG antibodies against native Pn14PS, thus promoting uptake and killing of bacteria Pn14.[28]

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Herein, we report on the preparation and immunological evaluation of new types of GNPs containing, together with the OVA323-339 T-helper peptide, i) the trisaccharide repeating unit of serotype 19F pneumococcal polysaccharide (Pn19FPS), and ii) both serotypes 14 and 19F CPS fragments simultaneously displayed on nanoparticle surface. We sought to explore the effect of these GNPs, coated with different antigen patterns, on ~~how~~ the immunological response

and whether this response is affected by the presence of both saccharide antigens from diverse bacterial serotypes loaded onto the same nanoparticle.The main goal of this study was to determine whether these GNPs could induce specific antibodies against both CPSsofpneumococcalserotypes14or19F or to affect the immune activity of one of them. We found that the bi-antigenic GNPs induced anti-Pn14PS IgG antibodies titers of the same order of magnitude of the currently used PCV13 human vaccine.

MATERIALS & METHODS

Synthesis of the ligands (neoglycoconjugates and T-helper peptide).

In order to prepare our new GNPs as a fully synthetic carbohydrate vaccine candidate, the selected components (carbohydrate antigens and T-helper peptide) must be derivatized as thiol-ending ligands in order to be efficiently conjugated to the gold nanocarrier, taking advantage of the sulfur-gold high affinity. The thiol-functionalized 19F trisaccharide **1** (Figure 1) was prepared according to the procedure previously described for the preparation of the thiol-functionalized type 14 tetrasaccharide **2**,[28] through formation of a thiourea bond between the 3-aminopropyl glycoside of Tri-19F, compound **3**, and an amphiphilic bifunctional linker containing an isothiocyanate group at one end and a thioacetate at the other end. Glycoside **3** was in turn obtained as an anomeric mixture (α/β ratio: 2/3, separable by flash chromatography) by glycosylation of *N*-carbobenzyloxy-protected 3-aminopropanol with the corresponding known trisaccharide trichloroacetimidate donor[33, 34] followed by hydrogenolysis (90% yield over 2 steps) (a detailed description of the synthesis is reported in the Supplementary Material).

The D-glucose derivative **4**, glycosylated with a five carbon atoms thiol-ending linker, was prepared as previously described[35] and used as inner component of the GNPs. The inclusion of compound **4** ~~into~~ onto the GNPs improves their water solubility, enables modulating the loading of the oligosaccharide antigens, and favors the correct exposure of the ligands on the organic shell of the GNPs. T-Helper ovalbumin 323-339 peptide (OVAp), derivatized at the *N*-terminus with an additional glycine and a mercapto-propionic acid linker, $HS(CH_2)_2C(O)$GISQAVHAAHAEINEAGR, was obtained from GenScript Corp (Piscataway, NJ, USA).

Preparation of gold glyco-nanoparticles (GNPs).

The GNPs were prepared through a versatile methodology developed by Penadés group[36] and based on a modification of the Brust's procedure.[37] Water-dispersible gold GNPs of 2 nm (average gold diameter) were obtained by adding a 0.025 M aqueous solution of tetrachloroauric acid ($HAuCl_4$, 1 eq.) to a 0.012 M methanolic solution of a mixture of the thiol-derivatized neoglycoconjugates (5 eq. with respect to $HAuCl_4$) in the desired proportion (see Supplementary Material). The resulting mixture was reduced *in situ* with a freshly prepared 1 M aqueous solution of $NaBH_4$ (27 eq.) and the suspension was vigorously shaken for 2 h at 25 °C. The supernatant was removed, the nanoparticles were washed with methanol and then dissolved in milliQ water, purified by dialysis (Slide-A-Lyzer 3.500 MWCO Dialysis Cassette, 9 x 3L water changes) and characterized by ^1H NMR spectroscopy, transmission electron microscopy (TEM) and ultraviolet-visible (UV-Vis) spectroscopy. GNPs produced were well-dispersible and stable in water, could be freeze-dried and easily re-dispersed in water after thawing. Full details of the synthesis can be found in the Supplementary Material.

Mice Immunization

The mouse immunization study was approved by the Animal Care and Use Committee of PT. Bimana Indomedical, Bogor, Indonesia. Inbred 6-week-old female BALB/c mice were maintained at the Animal Laboratory of PT. Bimana Indomedical, Bogor, Indonesia. Five mice per group were immunized intradermal with 6.0 µg of GNPs in mixture with 20 µg of Quil-A® saponin adjuvant (a gift from Dr. Erik B. Lindblad and Brenntag Biosector, Denmark).[28, 32] A booster of 6.0 µg of GNPs antigen was given on day 35 without adjuvant. Blood samples were taken one week after the booster immunization. Commercially available PCV13 vaccine (13-valent pneumococcal conjugate vaccine, cp Pfizer, Inc.) was diluted in saline 1:10 (100 µl per mouse)[38] and used as positive control. PCV13 contains the capsular polysaccharide antigens of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F, individually conjugated to a nontoxic diphtheria CRM_{197}. Saline (0.9% [wt/vol] NaCl in water) was used as negative control.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed to measure the antibody titers to native Pn14PS and to Pn19FPS at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia as described previously.[28]

Briefly, serially diluted sera from immunized animals were incubated for 1 h at 37 °C in flat-bottom plates, coated with 100 µl of purified Pn14PS or Pn19FPS (5 µg/mL). After coating, the plates were blocked with 3% gelatin, then washed and horseradish peroxidase-conjugated goat anti-mouse IgG was added and incubated for 1 h at 37 °C. A ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to visualize the amount of bound peroxidase. The reaction was stopped by the addition of 0.5 M H_2SO_4. Optical density (OD) values were obtained with a micro-titer plate spectrophotometer at 450 nm. Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice.

GNP-2 (Tri-19F:Glc:OVAp=45:50:5) and **GNP-4** (Tetra-14:Glc:OVAp=45:50:5)-coated plates (25 µg/mL) were also used to measure antibody titers towards GNPs components in the sera of immunized mice as described previously.[39]

Dataanalysis

Independentt-testwasusedtodeterminedifferencesinantibodytiterlevelswithap-value≤0.05consideredtobestatisticallysignificant.

Other methods

General information about chemicals and techniques, and details of the synthesis of compounds **1** and **3**, the preparation and characterization of hybrid gold nanoparticles **GNP-1–4**, ELISA assays on compound **3**, and determination of IgG subclasses can be found in the Supplementary Material

RESULTS

Preparation and characterization of gold glyco-nanoparticles.

GNP-1, **GNP-2**, **GNP-3** and **GNP-4** (Figure 2) were prepared by *in situ* reduction of an aqueous solution of an Au(III) salt with sodium borohydride in the presence of an excess of the thiol-ending ligands (Figure 1) in order to assure full coverage of the GNP surface.[36] This method allows incorporation of ligands in defined proportions on the same gold nanoparticle, and ensures that their molar ratio in solution is maintained on the nanoparticle surface. Four systems based on gold nanoparticles were prepared and evaluated in this study: the biantigenic hybrid system **GNP-1** coated with the branched tetrasaccharide repeating unit of Pn14 [β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]-β-D-Glc*p*NAc-(1→] (Tetra-14) and the trisaccharide repeating unit of Pn19F [β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→] (Tri-19F) together with D-glucose and

OVAp (40:40:15:5 ratio); **GNP-2** displaying Tri-19F, D-glucose and OVAp (40:50:5 ratio); **GNP-3** carrying Tri-19F and OVAp (95:5 ratio) but lacking D-glucose; and the system used in our previous work, **GNP-4** coated with Tetra-14, D-glucose, and OVAp (45:50:5 ratio).[28]

The new GNPs were water-dispersible and stable for several weeks in aqueous solution. GNPs showed an exceptionally small core with an average diameter (less than 2 nm), as demonstrated by TEM images. In addition, TEM micrographs showed uniform size dispersion of the GNPs and no aggregation. Based on the gold core size and Murray's data,[40] an average molecular formula and the corresponding molecular weight were estimated (Table 1).

UV/Vis spectra gave an indication of the GNPs dimensions:[41] no maximum absorption band at 520 nm was observed, which further confirmed a GNP size less than 2 nm. An example for the GNP characterization is provided in Figure 3 for **GNP-1**. NMR was used to qualitatively assess the presence of organic components at the gold surface. ^{1}H NMR spectra of the initial ligand solution used to prepare the GNPs were recorded and compared with data obtained from recovered supernatants after GNP formation (i.e. analyzing the unreacted ligands). In this way, the theoretical molar ratios of the ligands on the nanoparticles were confirmed experimentally.

Immunological evaluation

Specific antibodies against Pn14PS, Pn19FPS, and Pn23FPS were measured in the sera of immunized mice with a series of GNPs using ELISA. Quil-Awasco-delivered

As adjuvant during primary, but not upon booster immunization.
GNP-1 (Tri-19F/Tetra-14/Glc/OVAp = 40:40:15:5) induced antibodies towards native Pn14PS as coating antigen in higher titers than , **GNP-4** (Tetra-14/Glc/OVAp = 45:50:5; p value=0.004), and PCV13 (vaccine used as a positive control antigen, p value=0.347).

We also observed that immunization with **GNP-2** (Tri-19F/Glc/OVAp = 45:50:5) and **GNP-3** (Tri-19F/OVAp = 95:5) elicited low antibodies level against Pn14PS (Figure 4). In addition, we did not detect any specific IgG antibodies against native Pn19FPS and Pn23FPS (the control polysaccharide coated plate) from the sera of immunized mice with all GNPs except for the sera of mice immunized with PVC13 vaccine (Figure 4).

IgG subclasses against Pn14PS antigen were also detected after the booster immunization had been given at week 5 (Fig. S6, Supplementary Material). **GNP-1**

immunization was found to evoke higher levels of anti-Pn14PS IgG-1, IgG-2a, and IgG2b antibodies subclasses than other GNPs antigens. This data suggests that **GNP-1** ensures a better antigen presentation than other GNPs.

The antibody response to the GNPs was also determined by using GNPs loaded with Tri-19F (**GNP-2**) or Tetra-14 (**GNP-4**) as coating antigens in ELISA assays. We observed that the sera of mice immunized with **GNP-1**, **GNP-2**, and **GNP-3** elicited antibodies against **GNP-2** (loaded with Tri-19F) in higher titers than the sera of mice immunized with PCV13 and **GNP-4** (loaded with Tetra-14, figure 5). **GNP-4**-coated plate bound strongly with the sera of mice immunized with **GNP-1** (loaded with Tetra-14/Tri-19F) and **GNP-4** (loaded with only Tetra-14), and showed low interaction with the mice sera of **GNP-2** and **GNP-3** (both loaded with Tri-19F) as well as the mice sera of PCV13.

DISCUSSION

Based on our previous experience with GNPs carrying the tetrasaccharide (Tetra-14) repeating unit of Pn14,[28] all the new GNPs were functionalized with 5% T-helper OVA323-339 peptide, which resulted essential to boost an efficient and specific antibody response. The GNPs were prepared through a versatile methodology that allows the generation of complex globular shaped gold nanoparticles displaying the carbohydrate ligands at different densities on the gold surface in a controlled fashion.[35, 42] This method requires that all the components to be coupled to the gold surface, i.e. the saccharide antigens, glucose and OVA peptide, are functionalized with a thiol linker (Figure 1) in order to exploit the affinity of sulfur for gold in the *in situ* GNPs formation.[43] The nature and the length of the linker are key factors in controlling the presentation of the ligands and driving the molecular recognition process.[24] A long bifunctional thiol linker, 23-mercapto-3,6,9,12-tetraoxatricosyl isothiocyanate, had been selected to functionalize the saccharide pneumococcal antigens Pn14 and Pn19F for the preparation of **GNP-1** to **GNP-4**. This linker consists of an aliphatic portion of eleven carbon atoms conferring rigidity to the inner organic shell, thus protecting the gold core, and a hydrophilic portion of tetraethylenglycol providing flexibility to the glycans on the GNPs.

The trisaccharide repeating unit (Tri-19F) of Pn19FPS, containing a 3-aminopropyl linker at the downstream residue (compound **3**, Figure 1), was used as the antigenic fragment of Pn19F. Indeed, it was found capable of inhibiting the binding between the 19F polysaccharide and the anti-19F human polyclonal antibody in a classical competitive

ELISA assay. As shown in Fig. S2 (see Supplementary Material), the two anomers of saccharide **3**, compound **3**α and **3**β, were tested separately. They were recognized by the anti-19F antibody, even if with affinity and potency lower than the native polysaccharide. The orientation of the aminopropyl linker did not appear to affect the affinity of the saccharide for antibody binding (IC_{50} 7.44 x 10^{-2} and 2.61 x 10^{-2} mg/mL for **3**α and **3**β, respectively) suggesting the anomeric mixture can be used in the present study without additional purification steps (see Table S2, Supplementary Material).

GNP-1 bears equimolar amounts of Pn14 tetrasaccharide and Pn19F trisaccharide (1:1 ratio), together with OVAp, and D-glucose to improve water solubility and to enable modulating the antigen density. A major goal of the synthesis of **GNP-1** was to reveal whether (and how) the distinct pneumococcal antigens simultaneously displayed on the nanoparticle surface would lead either to the enhancement of their respective biological activity, or to mutual interference reducing desired protective effects. It would, for example, be intriguing to determine whether such multiantigenic nanosystem would be able to evoke an immune response against both serotypes or to enhance the immune activity of one of them.

Both **GNP-2** and **GNP-3** contain only the Tri-19F as the pneumococcal saccharide antigen. While **GNP-2**, displaying Tri-19F, D-glucose and OVAp was prepared analogous to the Pn14 based system (**GNP-4**) that gave the best immunological activation in our previous work,[28] **GNP-3** lacks glucose in order to increase the loading of the trisaccharide antigen.

ELISA assays performed with **GNP-2** (Tri-19F/Glc/OVAp) as antigen-coating (Figure 5A) showed that only sera collected from mice immunized with GNPs loaded with Tri-19F (**GNP-1**, **GNP-2**, and **GNP-3**) recognized the antigen. On the other hand, the same sera were inactive when the native Pn19FPS was coated onto plates. This could be due to inability of the Pn19F trisaccharide repeating unit on the GNPs to function as epitope *in vivo*, although our data demonstrated *in vitro* inhibitory activity of Tri-19F in a classical competitive ELISA assay. This could suggest that either a longer saccharide fragment encompassing more than one repeating unit, which may lead to the formation of a conformationalepitope, [44] is necessary to induce the activation of the immune system. Previously, Safari *et al.* reported that a linear trisaccharide fragment from Pn14PS conjugated to CRM197 protein carrier did not elicit antibodies against native Pn14PS, while the branched tetrasaccharide Tetra-14, corresponding to one structural repeating

unit of Pn14PS, induced a specific antibody response to Pn14PS, demonstrating that a small change in the presentation is of great importance for immunoactivity.[32] We further confirmed these results by showing that sera from mice immunized with **GNP-4** (Tetra-14/Glc/OVAp) were abletorecognize the native Pn14PS in agreement with our previous data,[28] even if the totall gGantibodiestiters were lower than those found with PCV-13 (Figure 4). Strikingly, we found in this study that already the di-valent **GNP-1** exposing two small saccharide fragments (Tri-19F:Tetra-14:Glc:OVAp) was more immunoactive towards native Pn14PS than **GNP-4**, which contains only Tetra-14 saccharide. In addition, the presence of Tri-19F together with Tetra-14 on the same nanoparticle triggered the generation of specific antibodies towards Pn14PS, and the activity was comparable with commercially available PCV13 vaccine. This effect could be ascribed to a better display of the Tetra-14 saccharide antigen on the **GNP-1** surface, whichpromotesenhancedBcellreceptorcross-linking.

Sera from mice immunized with **GNP-2** and **GNP-3**, containing Tri-19F and D-glucose, and solely Tri-19F pneumococcal antigen, respectively, showed similar activities towards **GNP-2** coated plates (Figure 5A). The higher antigen loading of **GNP-3** in comparison to **GNP-2** (95% of Tri-19F in **GNP-3**, 45% in **GNP-2**) did not lead to higher immunoactivity, indicating that the immunogenicity of the GNPs seems not improved by an increased loading of the carbohydrate antigen. These results supplement our previous observations on the importance of a precise saccharide:OVAp ratio on the gold nanoplatform for a robust carbohydrate-directed immune response to occur with GNPs, and suggests that a payload of saccharide antigen higher than 45% does not correlate with higher activities.

Unlike proteins, GNPs as carrier system elicit almost no immune response against themselves. In fact, when **GNP-2** (Tri-19F/Glc/OVAp) is used as antigen to coat the ELISA plate (Figure 5A), mice sera immunized with **GNP-4** are unable to recognize the antigen. This indicates that no significant antibodies against the additional components of the GNPs (OVAp T-helper peptide, Glc and gold) are generated. Further experimental evidence is provided by the results shown in Figure 5B: ELISA plates coated with **GNP-4** (Tetra-14/Glc/OVAp) did not give significant response to sera immunized with **GNP-2** (Tri-19F/Glc/OVAp) and **GNP-3** (Tri-19F/OVAp).

CONCLUSION & FUTURE PROSPECTIVE

In conclusion, we demonstrated that gold glyco-nanoparticles coated with synthetic oligosaccharides corresponding to the repeating units of *Streptococcus pneumonia* CPS type 14 and 19F elicit antibodies against carbohydrate antigens in mice model. An unexpected improvement of immunogenicity against the native polysaccharide type 14 was found after immunization with GNPs bearing both types of oligosaccharide epitopes (Tri-19F and Tetra-14) with respect to the GNPs that contains only Tetra-14. The presence of Tri-19F together with Tetra-14 on the same nanoparticle triggered an immune response comparable with commercially available PCV13 vaccine. Although further tests are needed to elucidate this effect, this work contributes towards the translation of nano-systems based on synthetic oligosaccharides and synthetic peptides into fully synthetic glycovaccines.

ETHICAL CONDUCT OF RESEARCH

The mouse immunization study was approved by the Animal Care and Use Committee of PT. Bimana Indomedical, Bogor, Indonesia. Inbred 6-week-old female BALB/c mice were maintained at the Animal Laboratory of PT. Bimana Indomedical, Bogor, Indonesia.

EXECUTIVE SUMMARY

- The preparation of an aminopropyl synthetic trisaccharide related to the capsular polysaccharide of *S. pneumoniae* serotype 19F (Tri-19F, [β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→]) has been reported.
- Suitable chemical derivatization of the Tri-19F amino derivative with a bifunctional linker containing an amino reactive isothiocianate group at one terminus and a thiol functionality at the other terminus was achieved as for an analogous tetrasaccharide related to *S. pneumoniae* serotype 14 (Tetra-14; [β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]-β-D-Glc*p*NAc-(1→]).
- Small gold nanoparticles (~2 nm gold diameter) functionalized with different ratios of neoglycococonjugates Tri-19F and/or Tetra-14 were obtained by modulating the loading and the presentation of these antigenic carbohydrate fragments through the use of 5-(thio)pentyl β-D-glucopyranoside; a thiol-functionalised conjugate of the ovalbumin 323–339 peptide ($OVA_{323–339}$) was also inserted for T-cell activation.

- Immunogenicity studies in mice showed that the induction of specific IgG antibodies against *Streptococcus pneumoniae* type 14 capsular polysaccharide (Pn14PS) can be modulated by the partner ligands of Tetra-14 in the organic shell of the GNPs.
- The co-presence of Tri-19F together with Tetra-14 in one of the GNPs was critical to elicit a high level of specific antibody titers against Pn14PS; on the contrary this effect was not seen towards Pn19FPS.

- No efficient immune response towards type 19F native polysaccharide was elicited with the tested systems.

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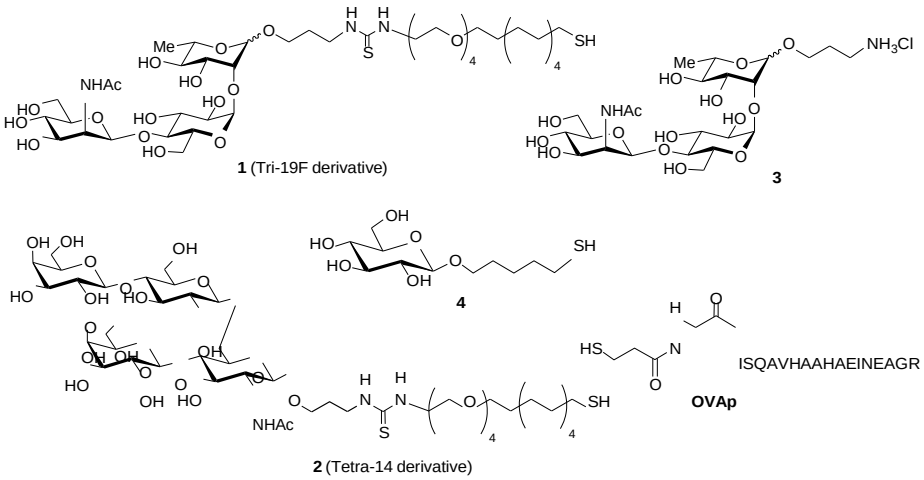


Figure 1. Thiol-ending derivative of the trisaccharide related to serotype Pn19F (compound **1**) and its aminopropyl precursor (compound **3**); thiol-ending tetrasaccharide related to serotype Pn14 (compound **2**); 5-(thio)pentyl β-D-glucopyranoside (compound **4**) used as inner component in the gold nanoparticles; thiol-ending T-helper ovalbumin $OVA_{323-339}$ peptide (OVAp).

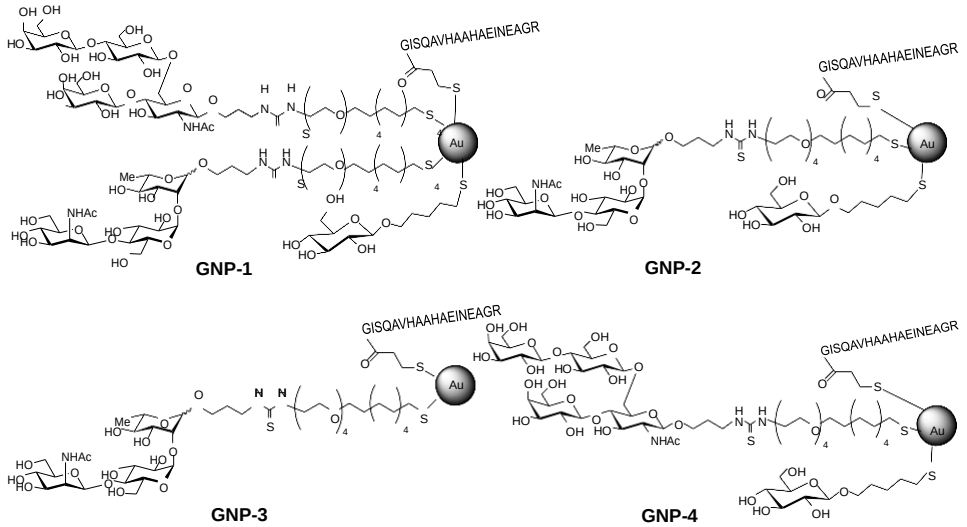
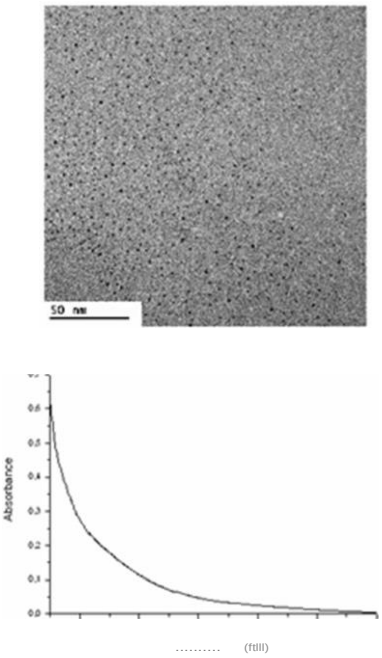


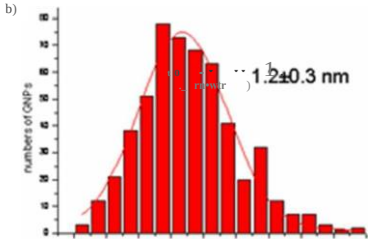
Figure 2 Gold glyco-nanoparticles (GNPs) prepared and used in this work for mice immunization. The GNPs have been functionalized with Pn19F and/or Pn14 saccharide ligands (see Figure 1) in different ratios by using a glucose derivative as inner and modulating component. Approximately 5 % of ovalbumin OVA323-339 peptide is always present.

Table 1: Main properties of the prepared GNPs.

GNPs	Mean gold core (nm)[a]	Average number of gold atoms[b]	Thiol-ending ligands molar ratio[c]	Estimated average molecular formula	Average molecular weight (kDa)
GNP-1	1.2±0.3	79	Tri-19F/Tetra-14/Glc/OVAp 40:40:15:5	Au_{79}(Tri-19F)$_{15}$ (Tetra-14)$_{15}$(Glc)$_6$ (OVAp)$_2$	53.9
GNP-2	1.2±0.3	79	Tri-19F/Glc/OVAp 45:50:5	Au_{79}(Tri-19F)$_{17}$ (Glc)$_{19}$(OVAp)$_2$	41.8
GNP-3	1.2±0.3	79	Tri-19F/OVAp 95:5	Au_{79}(Tri-19F)$_{36}$(OVAp)$_2$	55.6
GNP-4	1.2±0.3	79	Tetra-14/Glc/OVAp 45:50:5	Au_{79}(Tetra-14)$_{17}$ (Glc)$_{19}$(OVAp)$_2$	44.8

[a]Diameter of the gold nanocluster (as measured by transmission electron microscopy).
[b]The average number of gold atoms per nanoparticle was calculated from the size of the gold cluster obtained by transmission electron microscopy.
[c]Molar ratio of conjugates per nanoparticle was determined by analyzing the mixtures using NMR before and after nanoparticle formation (Supplementary Material)


50 nm
Absorbance


b)
1.2±0.3 nm
numbers of QNPs

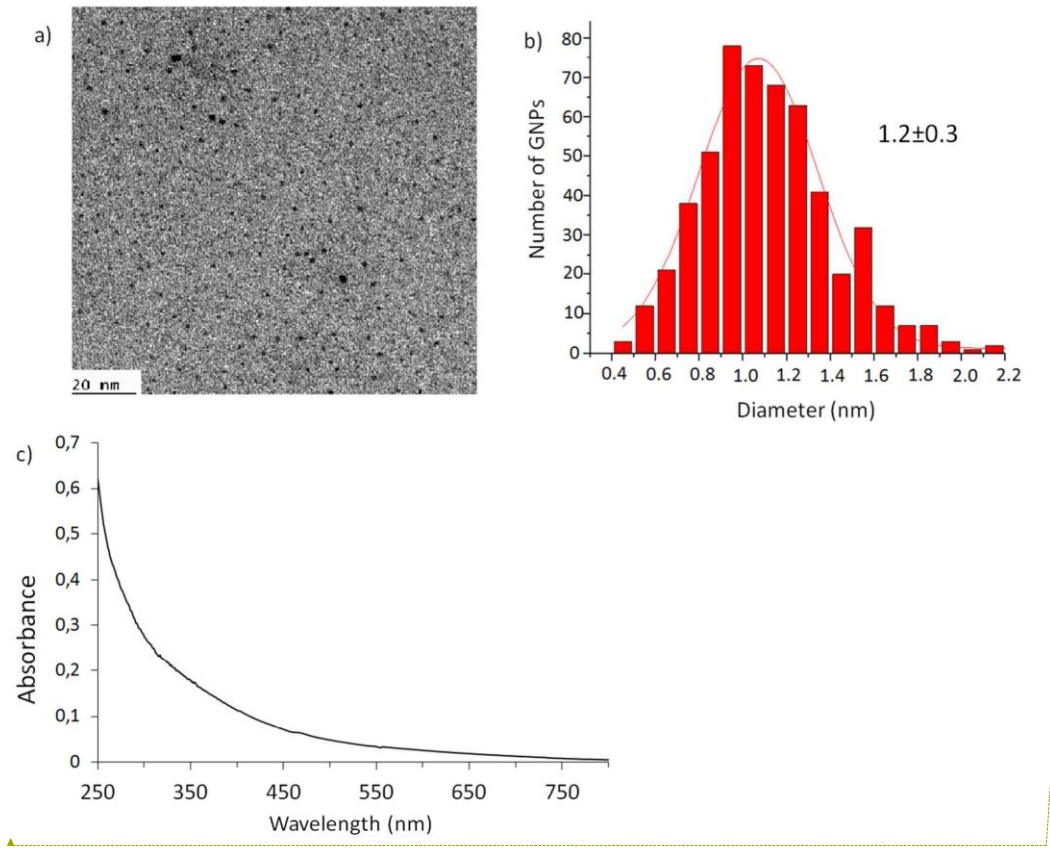


Figure 3: GNP-1 characterization: a) TEM micrograph in H_2O; b) size-distribution histogram obtained by measuring around 400 nanoparticles; c) UV/Vis spectrum (sample concentration 0.10 mg/ml in water); for the characterization of the other GNPs, see the Supporting Information.

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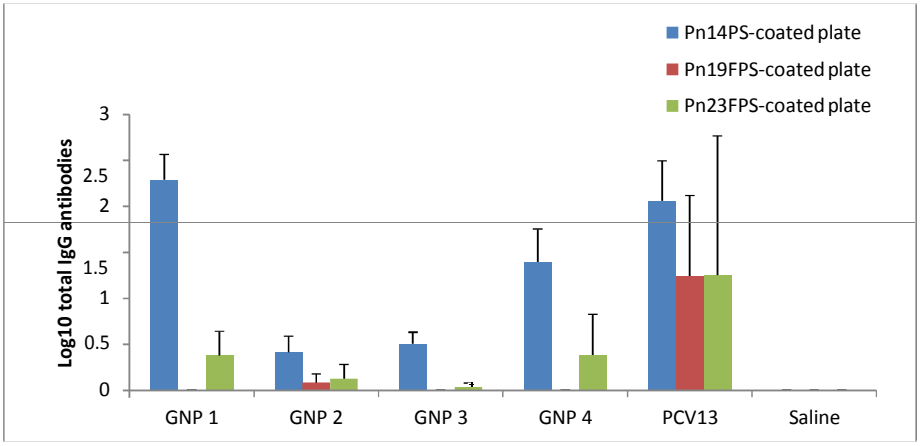
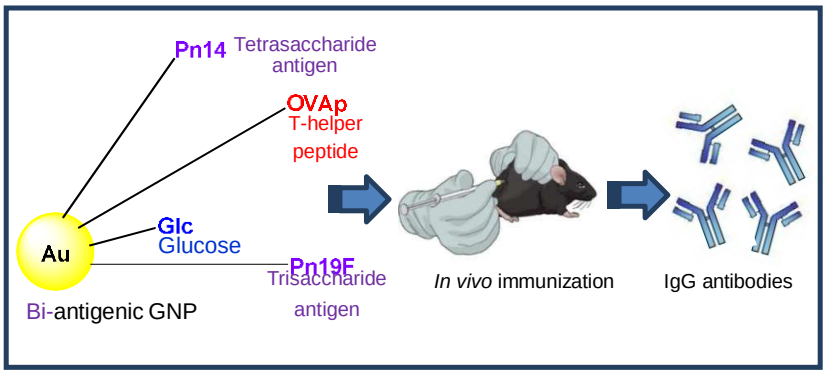


Figure 4. Total IgG antibodies titers recognizing pneumococcal polysaccharide type 14 (Pn14PS) (blue color), Pn19FPS (red color), and Pn23FPS (green color) as coating materials. Group of mice (n=5) were immunized with series of GNPs with adjuvant coadministration at the primary injection. Sera were collected one week after the second booster injection which was given without adjuvant. The GNPs differed in their molar ratio for the saccharide type: glucose: OVA-peptide (Table 1). PCV13 vaccine and saline immunization served as positive and negative control respectively. Antibody titers were expressed as the log10 of the dilution giving twice the absorbance value corrected by buffer

A.

B

Figure 5. Antibodies recognizing **GNP-2** (A) and **GNP-4** (B). Serial dilutions of pooled mice sera (ranging from 1:10 to 1:10000) were incubated on ELISA plates coated with **GNP-2** and **GNP-4**. The sera were obtained from mice previously immunized with series of GNPs (Table 1) and control sera were obtained from mice immunized with PCV13 vaccine (positive control) and saline. Level of antibodies are expressed as optical density (OD) at 450 nm.


Pn14
Tetrasaccharide antigen
OVAp
T-helper peptide
Glc
Glucose
Pn19F
Trisaccharide antigen
Au
Bi-antigenic GNP
In vivo immunization
IgG antibodies

SUPPLEMENTARY MATERIAL

Preparation and immunogenicity of gold glyco-nanoparticles as anti-pneumococcal vaccine model

GENERAL METHODS

All chemicals were purchased as reagent grade from Sigma–Aldrich, except for chloroauric acid (Strem Chemicals), and were used without further purification. Dichloromethane (CH_2Cl_2) and triethyamine (TEA) were distilled from calcium hydride. Methanol (CH_3OH) was degassed with Argon before the use to avoid oxidation of the thiol ending compounds. Air- and moisture sensitive liquids and solutions were transferred *via* oven-dried syringe or stainless steel cannula through septa. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm pre-coated silica gel plate (Merck 60 F_{254}) with visualization under UV-light (254 nm) and by staining with 50 % sulfuric acid (aqueous solution) or *p*-anisaldehyde solution [anisaldehyde (25 mL), H_2SO_4 (25 mL), EtOH (450 mL), and CH_3COOH (1 mL)] followed by heating at 200 °C. Size-exclusion column chromatography was performed on Sephadex LH-20 (GE Healthcare). Flash column chromatography was performed on silica gel high-purity grade, pore size 60 Å, 230-400 mesh particle size. Organic solvents were removed by rotary evaporation under reduced pressure at approximately 40°C (water bath). Purified water was obtained from a Simplicity Ultrapure Water System (Millipore). Nanopure water (18.2 MΩ-cm) was obtained by a Thermo Scientific Barnstead NANOpure DIamond Water System.
All the GNPs were purified by centrifugal filtering with AMICON (10.000 MWCO) and dialyses carried out using Slide–A-Lyzer dialysis cassette (3500 MWCO). UV/Vis spectra were measured with Beckman Coulter DU 800 UV/Vis Spectrophotometer. To perform the measurement a solution of GNPs 0,10 mg/ml in HPLC gradient grade water has been prepared and plastic cuvettes with an internal width of 45mm were used. All UV/Vis spectra were subtracted from blank.

Transmission electron microscopy (TEM) analysis was performed with a Philips JEOL JEM-2100F microscope, working at 200 kV. A single drop (~2 µL) of a GNP aqueous solution (*ca.* 0.05 mg/mL in HPLC gradient grade water) was placed on a copper grid coated with a carbon film (Electron Microscopy Sciences). The grid was left to dry in air for several hours at room temperature before carrying on

the experiment. Statistical determination of gold dimension was performed using Image J program and the average diameter of gold core was correlated to the number of ligands present on GNP.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz (high resolution) spectrometer. Chemical shifts (δ) are given in ppm relative to the residual signal of the solvent used. Specifically 7.26 ppm for $CDCl_3$, 3.31 ppm for CD_3OH and 4.79 ppm for D_2O in ^{1}H NMR spectra and 77.0 ppm (central line) for $CDCl_3$ and 49.0 ppm (central line) for CD_3OD in ^{13}C NMR spectra. Coupling constants (J) are reported in Hz. Splitting patterns are described by using the following abbreviations: *br*, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublet; dt, doublet of triplet. Sugar residues are indicated as g=glucose, m=mannosammine, r=rhamnose.

Mass spectra were recorded with a Thermo Quest Finnigan LCQ™deca ion trap mass spectrometer, or with an Esquire 6000 ESI-Ion Trap spectrometer from Bruker Daltonics. High-resolution mass spectra (HRMS) were obtained using the MALDI technique with a 4700 Proteomics Analyzer (Applied Biosystems) operated in MALDI-TOF-TOF configuration.

Synthesis of thiol-ending ligand.

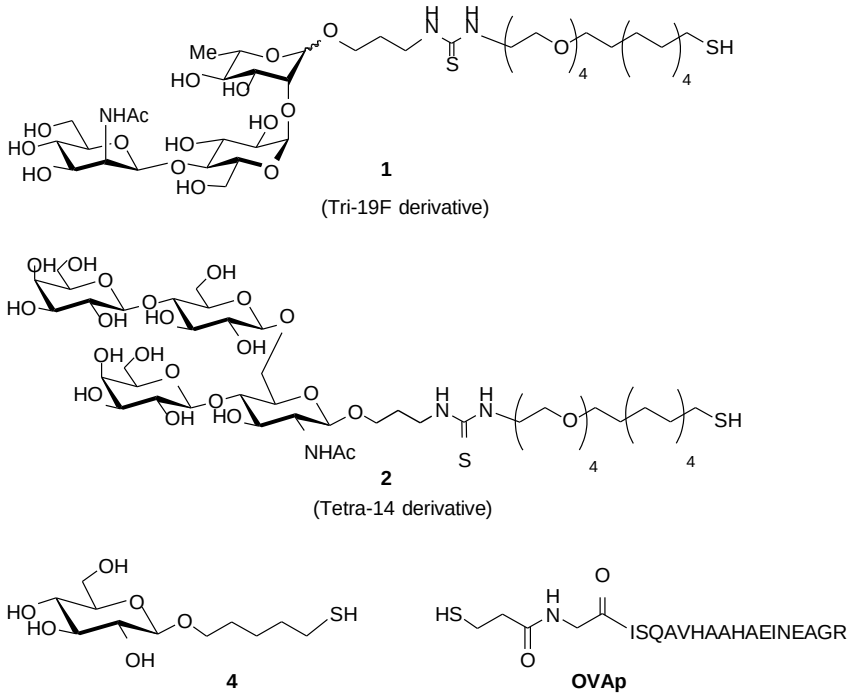


Figure S1. Thiol-ending conjugates used for the preparation of GNPs.

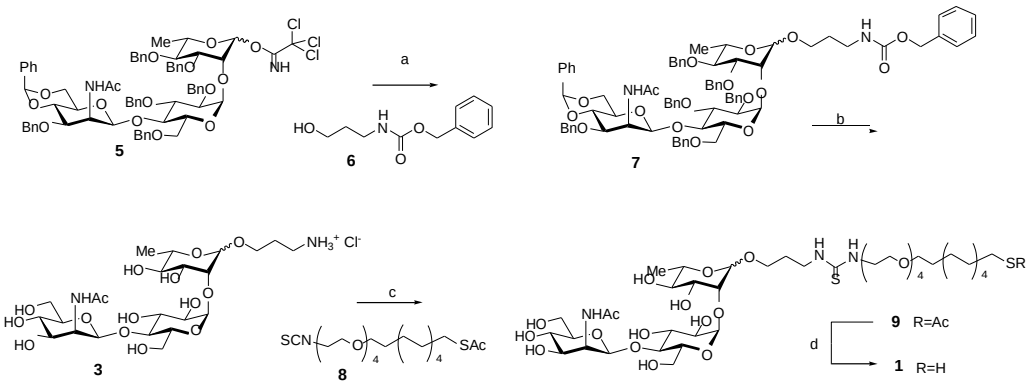
Glucose conjugate **4** was prepared according to the literature.[1] The $OVA_{323-339}$-peptide **OVAp**, consisting of ISQAVHAAHAEINEAGR with an additional glycine and mercaptopropionic acid (MPA) linker at the *N*-terminus, was obtained from GenScript Corp (Piscataway, NJ, USA) and a single batch was used throughout the study.

The thiol-ending conjugate **2** of the tetrasaccharide antigen related to serotype 14, was synthesized as previously reported.[2]

Synthesis of the thiol-ending trisaccharide conjugate related to serotype Pn19F (Scheme S1)

The thiol-ending trisaccharide conjugate **1** was prepared through a glycosylation reaction between known trisaccharide trichloroacetimidate donor **5**[3, 4] and Z-aminopropanol **6**. The reaction, carried out in anhydrous dichloromethane at 0°C,

was promoted with trimethylsilyl triflate (TMSOTf) in the presence of 4Å powder molecular sieves. Product **7** was obtained in high yields (90%) as an alpha/beta mixture (4:6), that can be eventually separated by flash chromatography. Subsequently, compound **7** was submitted to hydrogenolysis with palladium hydroxide on activated charcoal in a mixture of ethyl acetate/methanol/water as the solvent in the presence of an amount (1 eq.) of aq. HCl 0.1 M. The deprotected trisaccharide derivative **3**, isolated as the chloride salt, was coupled with 23-mercapto-3,6,9,12-tetraoxatricosyl isothiocyanate linker **8** in a water/isopropanol/acetonitrile mixture and in the presence of triethylamine, following a previously described procedure.[2] After deprotection of the thioacetyl group, the trisaccharide conjugate **1** was obtained as a mixture of disulfide and thiol, which could be used for the preparation of the GNPs under reductive conditions (Scheme S1).



Scheme S1: a) TMSOTf, m.s. 4Å, dry CH_2Cl_2, 0°C, 90%; b) $Pd(OH)_2$, H_2, $AcOEt/CH_3OH/H_2O$ 1:1:1, HCl 0.1M, rt, quant.; c) TEA 0.05M, $H_2O/iPrOH/CH_3CN$ 1:1:1, rt, 75%; d) CH_3ONa, CH_3OH, rt, 90%.

N-(benzyloxycarbonyl)-3-amminopropyl (2-acetamido-3-O-benzyl-4,6-O-benzylidene -2-deoxy-β-D-mannopyranosyl)-(1→4)-(2,3,6-tri-O-benzyl-α-D-glucopyranosyl)-(1→2)-3,4-di-O-benzyl-L-rhamnopyranoside ***7***

Compound **5** (90 mg, 0.07 mmol, 1 eq.) and *N*-(benzyloxycarbonyl)-3-amminopropyl **6** (58 mg, 0.28 mmol, 4 eq.) were dissolved in dry CH_2Cl_2 (1.5 ml, 0.05M) and activated powder molecular sieves 4 Å (50 mg) were added. The suspension was left stirring under Ar atmosphere at room temperature for 15

minutes, then it was cooled to 0 °C and TMSOTf 0.1 M in dry CH_2Cl_2 (140 µl, 0.014 mmol, 0.2 eq.) was added. After 15 minutes, the reaction mixture was neutralized with TEA, filtered over a Celite pad and the solvent evaporated under reduced pressure. Purification of the crude through flash chromatography (Hexane/Ethyl Acetate 6:4) afforded compound **7** (85 mg, 0.063 mmol, 90% yield, white foam) as an anomeric mixture (α/β ratio: 2/3). This elution system allows the separation of the two anomers by flash-chromatography even if a complete separation of the anomeric mixture requires repetitive columns: the α-anomer is less polar and is eluted firstly, while the β-anomer is recovered secondly. At this stage were recovered the necessary amount of pure **7**-α and **7**-β in order to obtain, after hydrogenolysis, **3**-α and **3**-β for Elisa assays (Figure S2).

^{1}H-NMR ($CDCl_3$): δ=1.38 (d, 1.2H, $J_{6r,5r}$=6.2 Hz, CH_3α), 1.42 (d, 1.8H, $J_{6r,5r}$=6.2 Hz, CH_3β), 1.62-1.80 (m, 2H, CH_2), 1.76 (s, 1.2H, CH_3COα), 1.77 (s, 1.8H, CH_3COβ), 3.03-3.10 (m, 1H, H-5m), 3.21-5.15 (m, 35.4H, $OCH_2CH_2CH_2N$, $OCH_2CH_2CH_2N$, 0.4H-1gα, H-2g, H-3g, H-4g, H-5g, 2H-6g, H-1r, H-2r, H-3r, H-4r, H-5r, H-1m, H-2m, H-3m, H-4m, 2H-6m, 7CH_2Ph), 5.48 (s, 1H, CHPh), 5.53 (*br* d, 1H, J=10.9 Hz, NH), 5.63 (d, 0.6H, J=3.6 Hz, H-1gβ), 7.19-7.52 (m, 40H, 8Ph);

^{13}C-NMR (CD_3Cl): δ=17.9 (CH_3β), 18.0 (CH_3α), 23.1 (CH_3COβ), 23.3 (CH_3COα), 29.6 (CH_2α), 29.8 (CH_2β), 38.0 (CH_2Nβ), 38.8 (CH_2Nα), 50.5 (C2m), 65.4 (OCH_2), 66.5 (CH_2Ph-cbzβ), 66.7 (CH_2Ph-cbzα), 67.1 (C5mα), 67.4 (C5mβ), 67.9 (C6gα), 68.2 (C6gβ), 68.4 (C5rα), 68.6 (C5rβ), 69.2 (C5gβ), 69.8 (C5gα), 70.9 (CH_2Ph), 71.2 (CH_2Ph), 71.9 (CH_2Ph), 72.1 (CH_2Ph), 72.7 (CH_2Ph), 73.4 (CH_2Ph), 74.2 (CH_2Ph), 74.7 (CH_2Ph), 75.0 (C2r), 75.3 (CH_2Ph), 75.5 (C4gα), 75.7 (C4gβ), 75.9 (C3m), 78.5 (C4m), 79.0 (C3rα), 79.2 (C3rβ), 79.7 (C2gα), 79.8 (C2gβ), 80.0 (C4rβ), 80.1 (C4rα), 80.3 (C3gβ), 80.4 (C3gα), 96.8 (C1gβ), 96.9 (C1gα), 97.5 (C1r), 99.6 (C1m), 101.6 (CHPh benzilidene), 126.1, 126.3, 126.4, 126.5, 126.6, 127.2-127.9 (16C), 128.0-128.6 (18C), 128.9, 129.0, 129.7, 134.4, 136.6 (2C Cbz α and β), 134.3, 137.4, 137.6 (3C), 137.7 (2C), 137.8, 138.1 (3C), 138.2, 138.3 (2C), 138.4 (3C), 138.5 (3C), 138.7 (2C), 139.5, 139.6 (2C), 139.7, 156.3 (OCONH), 170.4 (CONHAc).

ESI-MS (CH_3OH, positive-ion mode): m/z 1371.5 (100%) $[M+Na]^+$, 1372.5 (90%) $[M+Na+1]^+$, Calcd for $C_{80}H_{88}N_2O_{17}$, m/z 1348.61 [M].

*3-Aminopropyl 2-acetamido-2-deoxy-β -D-mannopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)- L-rhamnopyranoside **3***

The protected trisaccharide **7** (39 mg, 0.029 mmol, 1 eq.) was dissolved in a 1:1:1 mixture of AcOEt/CH_3OH/aq.HCl 0.1M (1 ml, 0.03M) and submitted to hydrogenolysis at atmospheric pressure with palladium hydroxide on activated charcoal (39 mg) as the catalyst. After 48 hours, the mixture was filtrated to remove the catalyst and the filtrate was concentrated under reduced pressure. Product **3** was isolated as the chloride salt (17 mg, quant.) as a white solid after freeze drying.

^{1}H-NMR (D_2O): δ=1.32 (d, 1.2H, $J_{6r,5r}$=6.2 Hz, CH_3α), 1.34 (d, 1.8H, $J_{6r,5r}$=5.8 Hz, CH_3β), 1.97-2.03 (m, 2H, CH_2), 2.09 (s, 3H, CH_3CO), 3.15 (t, 2H, J=7.2 Hz, CH_2N), 3.40-4.05 (m, 15.4H, H-2g, H-3g, H-4g, 2H-6g, 0.4H-2rα, H-3r, H-4r, H-5r, H-3m, H-4m, H-5m, 2H-6m, O*C*H_2), 4.06-4.08 (m, 0.4H, H-5gα), 4.10 (d, 0.6H, $J_{2,3}$=3.1 Hz, H-2rβ), 4.18 (dt, 0.6H, $J_{5,6}$=2.7 Hz, $J_{5,6'}$=10.2 Hz, H-5gβ), 4.57 (*br* d, 1H, $J_{2,3}$=4.4 Hz, H-2m), 4.74 (s, 0.6H, H-1rβ), 4.91 (*br* s, H-1m), 4.93 (d, 0.4H, $J_{1,2}$=1.2 Hz, H-1rα), 5.01 (d, 0.4H, $J_{1,2}$=3.8 Hz, H-1gα); 5.15 (d, 0.6H, J=3.8 Hz, H-1gβ);

^{13}C-NMR (D_2O): δ=16.5 (*C*H_3α), 16.6 (CH_3β), 22.0 (*C*H_3CO), 26.6 (*C*H_2α), 26.8 (*C*H_2β), 37.5 (*C*H_2Nα), 37.6 (*C*H_2Nβ), 53.3 (*C*2m), 59.6 (*C*6gα), 59.8 (*C*6gβ), 60.4 (C3m), 65.1 (O*C*H_2α), 66.6 (*C*4m), 67.3 (O*C*H_2β), 68.9, 69.6, 70.2 (*C*5gβ), 70.3 (*C*5gα), 71.1, 71.2, 71.5 (*C*2gβ), 71.8 (*C*2gα), 71.9 (*C*5rα and *C*5rβ), 72.0 (*C*3rα and *C*3rβ), 72.5 (C4rα), 76.3 (*C*5m), 76.5 (*C*6m), 77.9 (C2rβ), 78.6 (*C*4gα and *C*4gβ), 97.2 (C1rα), 97.5 (C1gα), 99.3 (*C*1mα), 99.4 (*C*1mβ), 99.8 (*C*1gβ), 100.4 (*C*1rβ), 175.4 (*C*ONHAc);

ESI-MS (CH_3OH, positive-ion mode): m/z 587.1 (95%) $[M+1]^+$, 609.5 (50%) $[M+Na]^+$, Calcd for $C_{23}H_{42}N_2O_{15}$, m/z 586.58 [M].

*Synthesis of S-acetylated trisaccharide conjugate **9***

To a solution of 3-aminopropyl trisaccharide **3** (3.91 mg, 6.27 µmol, 1.0 equiv.) in H_2O:iPrOH:CH_3CN (1:1:1, v/v/v, 0.6 mL) a solution of 23-mercapto-3,6,9,12-tetraoxatricosyl isothiocyanate linker **8** (5.82 mg, 12.55 µmol, 2.0 equiv.) in H_2O:iPrOH:CH_3CN (1:1:1, v/v/v, 0.6 mL) was added and the pH was set to basic by addition of triethylamine 0.05 M in H_2O:iPrOH:CH_3CN, 1:1:1 (188 µL, 9.41 µmol, 1.5 equiv.). The mixture was stirred at room temperature for 24 h and then the solvent was evaporated. The crude material was kept in high vacuum to remove the residual triethylamine and then triturated with Et_2O (3 × 2ml) in order to get rid of the excess of the linker. The crude was further purified by Sephadex LH-20 chromatography using as eluent CH_3OH/H_2O 9:1 to afford the trisaccharide conjugate **9** as a white solid (4.95 mg, 4.71 µmol, 75%).

^{1}H-NMR (CD_3OD): δ=1.27-1.38 (m, 17H, CH_3, $7CH_2$), 1.53-1.60 (m, 4H, C*H*$_2$$CH_2SAc, OCH_2C*H*_2$$(CH_2)_9$SAc), 1.81-1.91 (m, 2H, OCH_2C*H*$_2$$CH_2$N), 2.02 (s, 3H, NHAc), 2.30 (s, 3H, SAc), 2.86 (t, 2H, J=7.3 Hz, CH_2SAc), 3.27-3.93 (m, 35.4H, H-2g, H-3g, H-4g, 2H-6g, 0.4H-2rα, H-3r, H-4r, H-5r, H-3m, H-4m, H-5m, 2H-6m, 9C*H*$_2$O, 2C*H*$_2$N), 4.00 (d, 0.6H, $J_{1,2}$=3.8 Hz, H-2rβ), 4.04-4.07 (m, 0.4H, H-5gα), 4.13-4.17 (m, 0.6H, H-5gβ), 4.50 (*br* d, 1H, J=4.1 Hz, H-2m),4.61 (s, 0.6H, H-1rβ), 4.78 (s, 1H, H-1m), 4.83 (d,0.4H, $J_{1,2}$=0.9 Hz, H-1rα), 4.92 (d, 0.4H, $J_{1,2}$=3.8 Hz, H-1gα), 5.11 (d, 0.6H, $J_{1,2}$=3.8 Hz, H-1gβ);

^{13}C-NMR (CD_3OD): δ=18.0 ($2CH_3$) 22.7 (2*C*H_3CO), 27.1 (CH_2), 29.8, 29.9 (CH_3CO), 30.2 (SAc), 30.5 ($2CH_2$), 30.6 ($2CH_2$), 30.7 ($2CH_2$), 37.5 (*C*H_2N), 47.8, 54.9 (C2mα and C2mβ), 61.3, 61.4, 61.9, 66.3, 68.2 (2C), 70.2, 70.7, 70.8, 71.1, 71.3, 71.5, 71.6, 71.7, 71.8, 71.9, 72.4, 73.2, 73.5, 73.7, 73.8, 74.0 (2C), 74.1, 74.2, 74.5 (2C), 78.6, 78.7, 79.9, 80.1, 80.3, 99.2 (C1rα), 99.6 (C1gα), 100.9 (C1m), 101.8 (C1gβ), 102.1(C1rβ), 174.7 (N*C*OCH_3), 190.9 (S=*C*) 197.6 (S*C*OCH_3);

TOF MS ESI (CH_3OH, positive-ion mode): m/z 1072.7 (100%) $[M+Na]^+$, Calcd for $C_{45}H_{83}N_3O_{20}S_2$, m/z 1049.50 [M].

*Synthesis of the thiol-ending trisaccharide **1***

To a solution of *S*-protected trisaccharide **9** (4.95 mg, 4.71 µmol, 1.0 equiv.) in CH_3OH (500 µL, 0.01M) solid CH_3ONa (0.500 mg, 9.42 µmol, 2.0 eq.) was added.

The mixture was stirred at room temperature for 4 hours under Ar atmosphere until ^{1}H NMR check analysis attested the complete disappearance of the starting material. The solvent was evaporated and the crude material was purified by Sephadex LH-20 chromatography using as eluent CH_3OH/H_2O 9:1 to afford the trisaccharide conjugate product **1** (7:3 mixture of disulfide and thiol) as a white solid after lyophilisation (4.32 mg, 4.28 µmol, 90%).

^{1}H-NMR (CD_3OD): δ=1.26-1.44 (m, 17H, CH_3, $7CH_2$), 1.53-1.62 (m, 2.6H, 0.6 CH_2CH_2SH, 2 OCH_2CH_2), 1.65-1.71 (m, 1.4H, CH_2CH_2SS), 1.72-1.82 (m, 2H, $OCH_2CH_2CH_2N$), 2.03 (s, 3H, NHAc), 2.49 (t, 0.6H, J=7.3 Hz, CH_2SH) 2.69 (t, 1.4H, J=7.3 Hz, CH_2SS), 3.25-4.17 (m, 37H, H-2g, H-3g, H-4g, H-5g, 2H-6g, H-2r, H-3r, H-4r, H-5r, H-3m, H-4m, H-5m, 2H-6m, $9CH_2O$, $2CH_2N$), 4.50 (d, 1H, J = 3.7 Hz, H-2m), 4.78 (s, 1H, H-1m), 4.81 (s, 1H, H-1r), 4.91 (H-1g), 5.11 (d, 0.6H, J=3.8 Hz, H-1gβ);

^{13}C-NMR (CD_3OD): δ=18.0 (CH_3) 22.7 (CH_3CON), 27.2 (CH_2), 29.4, 30.3, 30.6, 30.7, 31.3, 39.8, 40.9, 54.9 (C2m), 61.4, 61.9, 66.3, 68.2, 71.1, 71.2, 71.6, 71.9, 72.0, 72.4, 74.1, 74.5, 78.6, 79.8, 80.1, 80.2, 99.2 (C1rα), 99.5 (C1gα), 100.6 (C1m), 101.4 (C1gβ), 101.9 (C1rβ), 174.7 ($NCOCH_3$);

TOF MS ESI (CH_3OH, positive-ion mode): m/z=1030.48 (100%) [$M+Na^+$], Calcd for $C_{43}H_{81}N_3O_{19}S_2$, m/z=1007.49 [M].

Competitive ELISA assay

96-Well flat-bottomed plates were incubated overnight at 4–8 °C with a mixture of *S. pneumoniae* 19FPS (Sanofi-Aventis, France) (1 mg/mL) and methylated human serum albumin (1 mg/mL). A solution of foetal calf serum (5%) in phosphate-buffered saline supplemented with Brij-35 (0.1%) and sodium azide (0.05%) was applied to the plates for blocking of nonspecific binding sites. The plates were incubated overnight at 4–8 °C with a solution (1:200) of rabbit anti-19F, used as reference serum (Statens Serum Institut, Artillerivej, Denmark). When alpha- and beta- aminopropyl glycosides, **3**-α and **3**-β, were tested, they were added to each well immediately before the addition of the reference serum. The plates were then incubated with alkaline phosphatase conjugate goat anti-rabbit IgG (Sigma- Aldrich, Milan, Italy), stained with p-nitrophenylphosphate, and the absorbance was measured at 405 nm with an Ultramark microplate reader (Bio-Rad Laboratories S.r.l., Milan, Italy). Control experiments to verify unspecific binding were performed by coating the ELISA plates with colominic acid from Escherichia coli. Results are expressed as means ± SEM of at least three experiments run in triplicate. Data were fitted as sigmoidal concentration-response curves and analyzed with a four-parameter logistic equation by using the software Origin version 6.0 (Microcal Software, Northampton, MA, USA). The IC_{50} value was the concentration (mg/mL) of synthetic compound that inhibits the binding of the native Pn19F CPS to the specific anti-Pn19F antibody by 50% and it was calculated using the same software.

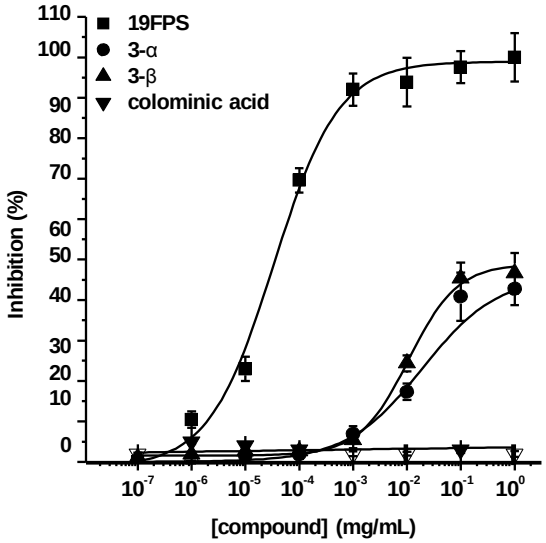


Figure S2 Concentration–response curves of saccharides **3**-α, **3**-β and **19FPS** on the inhibition of the binding between the 19F polysaccharide, coated onto the plates, and the anti-19F human polyclonal antibody were evaluated by a competitive ELISA method. Values are means of at least four experiments run in triplicate.

Table S1. Results of the competitive Elisa assays

Compound	Ic_{50} (mg/mL)	Maximal inhibition[a] (%)
19FPS	8.99×10^{-5}	100
3-α	7.44×10^{-2}	43
3-β	2.61×10^{-2}	47

[a] The maximum inhibition elicited by each compound at 1 mg/ml.

Preparation and characterization of the glyconanoparticles

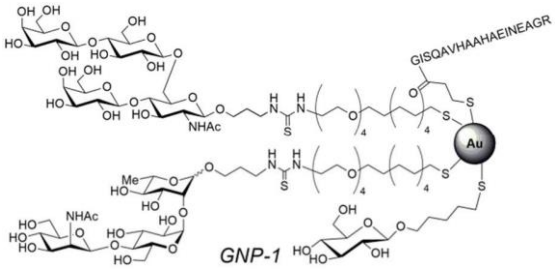
General protocol for the preparation of the GNPs

A methanolic solution 0.012 M of thiol-ending conjugates (5 eq.), *i.e*. the saccharide antigens Pn19F **1** and Pn14 **2**, glucose **4** and **OVAp**, in the desired proportion was prepared. To this mixture, a solution of tetrachloroauric acid $HAuCl_4$ in water (0.025 M, 1 eq.) was added, followed by the addition in four portions under vigorous shaking of an excess of sodium borohydride $NaBH_4$ as reductive agent (1 M in H_2O, 27 eq.). The black suspension formed was shaken for 2 hours at room temperature after which the supernatant was removed by centrifugal filtering with AMICON 10.000 MWCO (5 min, 10.000 rpm, 10 times). The dark concentrated solute was diluted with the minimum volume of water and further purified by dialysis with Slide-A-Lyzer 3.500 MWCO Dialisys Cassette placed in a 2 L beaker full of NANOPURE water under gentle stirring. After changing the water for nine times in three days, the dark solution was freeze-dried to give the GNPs as a dark solid, which can be stored at 4°C for months and redissolved in water prior to use.

The ratio of the ligands on the nanocluster surface was assessed by comparison of the proton nuclear magnetic resonance (^{1}H NMR) spectra of the initial solution used to prepare the GNPs, containing the thiolated ligands in the desired ratios, and of the recovered supernatant solution containing the unreacted ligands after GNPs formation. Spectra were recorded and compared to confirm the expected molar ratio of the components attached on the gold surface of the prepared GNPs. In particular, the ratio of the ligands in the GNPs was evaluated by integrating the signals of the anomeric protons of the glucopyranosyl unit or of the H-2 proton of the mannopyranosyl unit of trisaccharide **1** related to Pn19F antigen, the 4 anomeric protons of tetrasaccharide **2** related to Pn14 which collapse in an unique signal, the anomeric proton of glucose **4** and the methyl groups of isoleucine and valine of $OVA_{323–339}$ peptide conjugate.

The ^{1}H NMR spectra of the GNPs showed the characteristic signals of the organic components confirming their presence on the gold surface, even if the peaks are broader than those of the corresponding free ligands.

GNP-1 (Pn19F/Pn14/Glc/OVA 40:40:15:5)



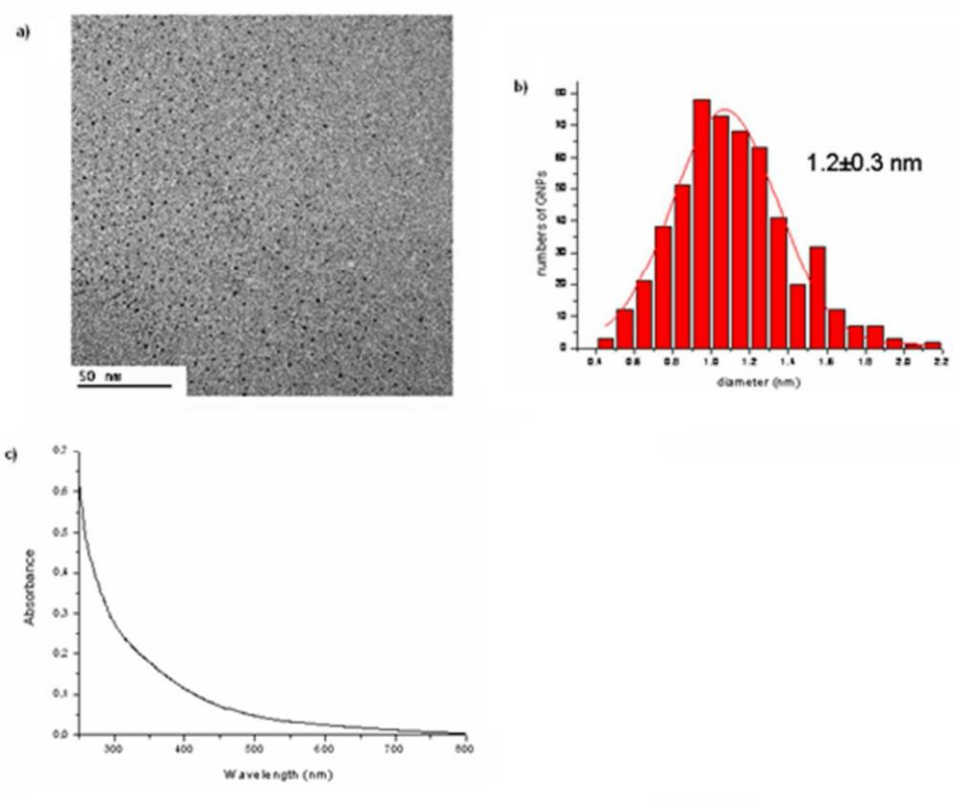
A mixture of compound **1** (1.30 mg, 1.29 µmol, 8 eq.), compound **2** (1.53 mg, 1.29 µmol, 8 eq.), β-D-glucose conjugate **4** (0.15 mg, 0.52 µmol, 3 eq.), and **OVAp** (0.33 mg, 0.17 µmol, 1 eq.) in CH_3OH (273 µl, 0.012M) was prepared.

$HAuCl_4$ (26.2 µl, 0.65 µmol, 0.025M in H_2O, 1 eq.) and sodium borohydride $NaBH_4$ (17.7 µl, 17.7 µmol, 1M in H_2O, 27 eq.) were added to afford 770 µg of **GNP-1** after the work-up and freeze drying.

TEM: average diameter 1.2±0.3 nm (for 534 GNPs);

Average molecular formula estimated based on the size of the cluster obtained from TEM micrographs:
Au_{79} $(C_{43}H_{80}N_3O_{19}S_2)_{15}(C_{49}H_{90}N_3O_{25}S_2)_{15}(C_{11}H_{21}O_6S)_6$ $(C_{79}H_{126}N_{27}O_{27}S)_2$ ~53.9 KDa;

UV/Vis (H_2O, c=0.10 mg/mL): surface plasmon band not observed;



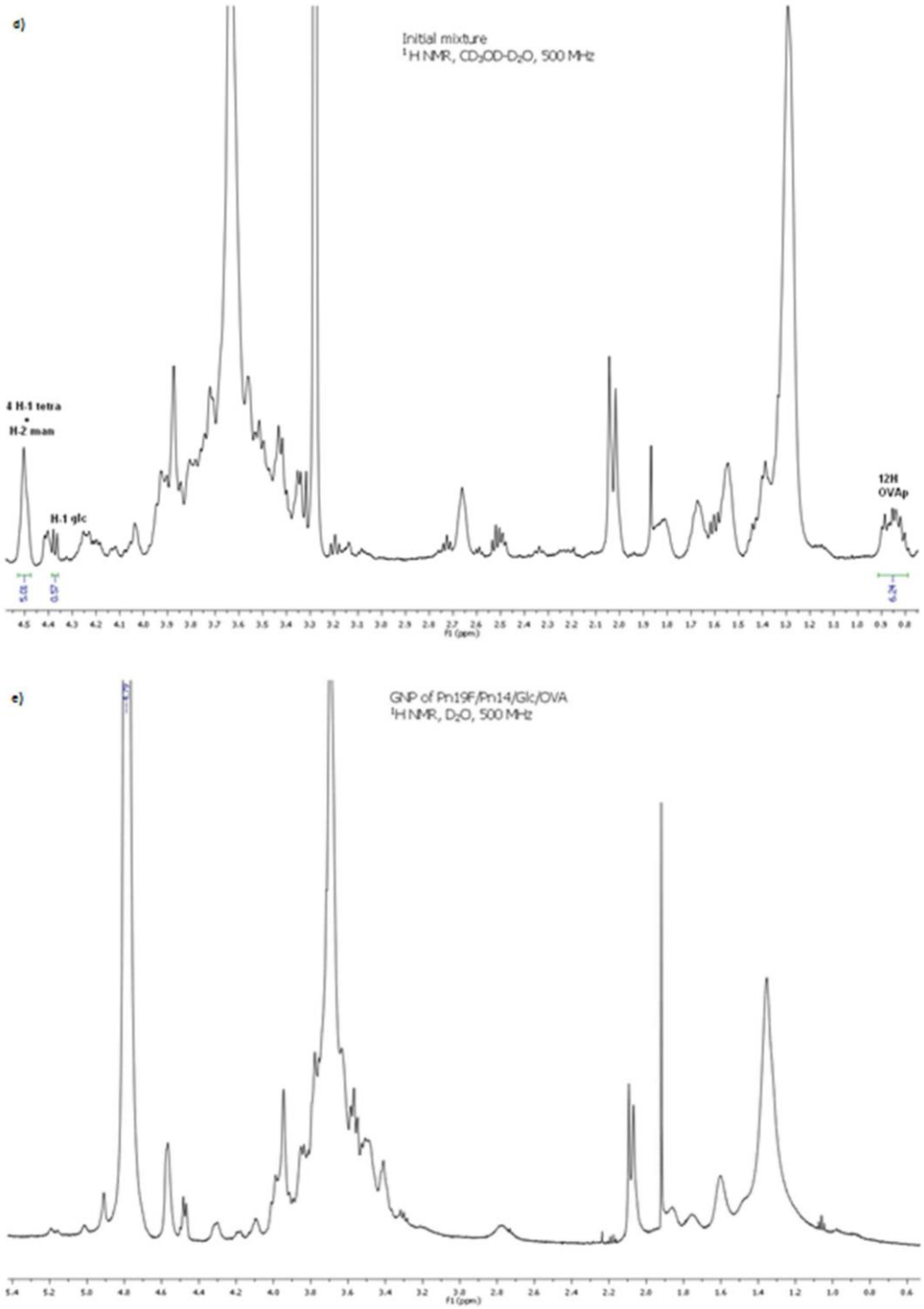
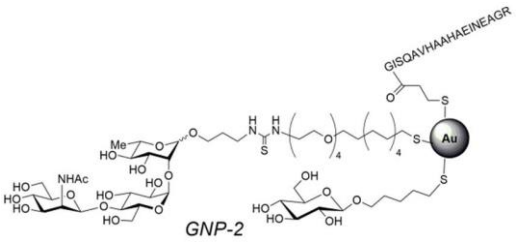


Figure S3: (a) TEM micrographs and (b) histograms of size distribution, (c) UV/Vis adsorption spectrum of gold **GNP-1**. (d) ^{1}H NMR spectrum (500MHz, CD_3OD:D_2O 5:1) of the mixture used to prepare GNP and (e) ^{1}H NMR spectrum (500MHz, D_2O) of **GNP-1** obtained. Integration of selected signals shows that the ratio between, trisaccharide **2**, tetrasaccaride **3**, glucose conjugate **4** and **OVAp** is about 40:40:15:5.

GNP-2 (Pn19F/Glc/OVA 45:50:5)

A mixture of thiol-ending Pn19F trisaccharide **1** (0.96 mg, 0.95 µmol, 9 eq.), β-D-glucose conjugate **4** (0.30 mg, 1.05 µmol, 10 eq.), and **OVAp** (0.20 mg, 0.105 µmol, 1 eq.) in CH_3OH (175 µl, 0.012M) was prepared.

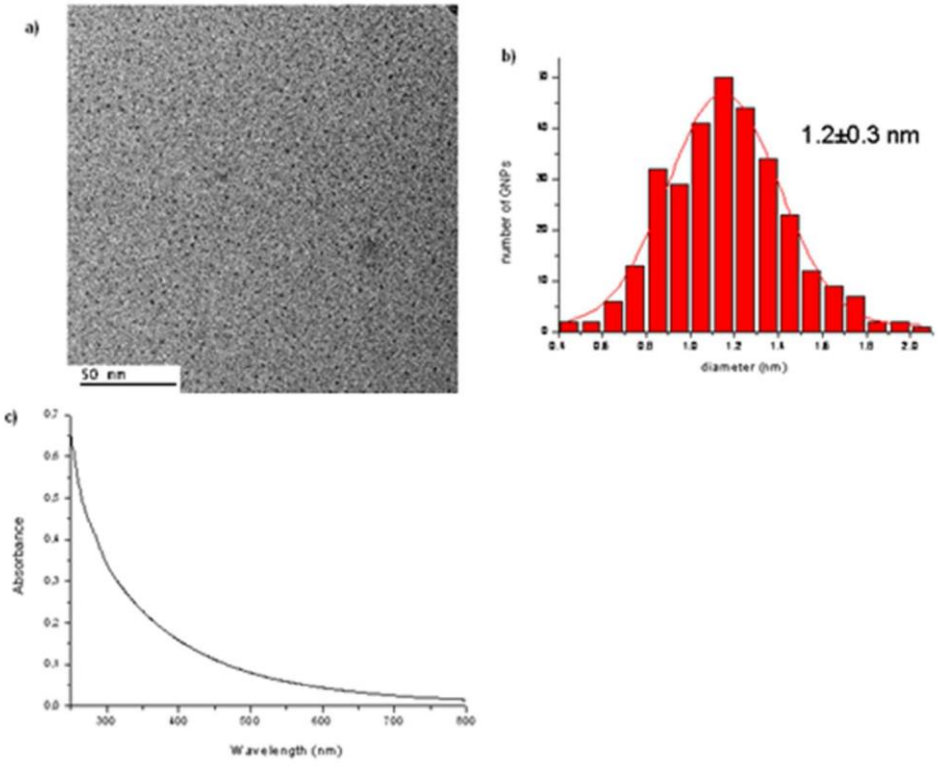


$HAuCl_4$ (16.8 µl, 0.42 µmol, 0.025M in H_2O, 1eq.) and sodium borohydride $NaBH_4$ (11.4 µl, 11.38 µmol, 1M in H_2O, 27 eq.) were added to afford 378 µg of **GNP-2** after the work-up and freeze drying.

TEM: average gold diameter 1.2±0.3 nm (for 309 GNPs);

Average molecular formula estimated based on the size of the cluster obtained from TEM micrographs:

$Au_{79}(C_{43}H_{80}N_3O_{19}S_2)_{17}(C_{11}H_{21}O_6S)_{19}(C_{79}H_{126}N_{27}O_{27}S)_2$ ~41.8 KDa;
UV/Vis (H_2O, c=0.10 mg/mL): surface plasmon band not observed;



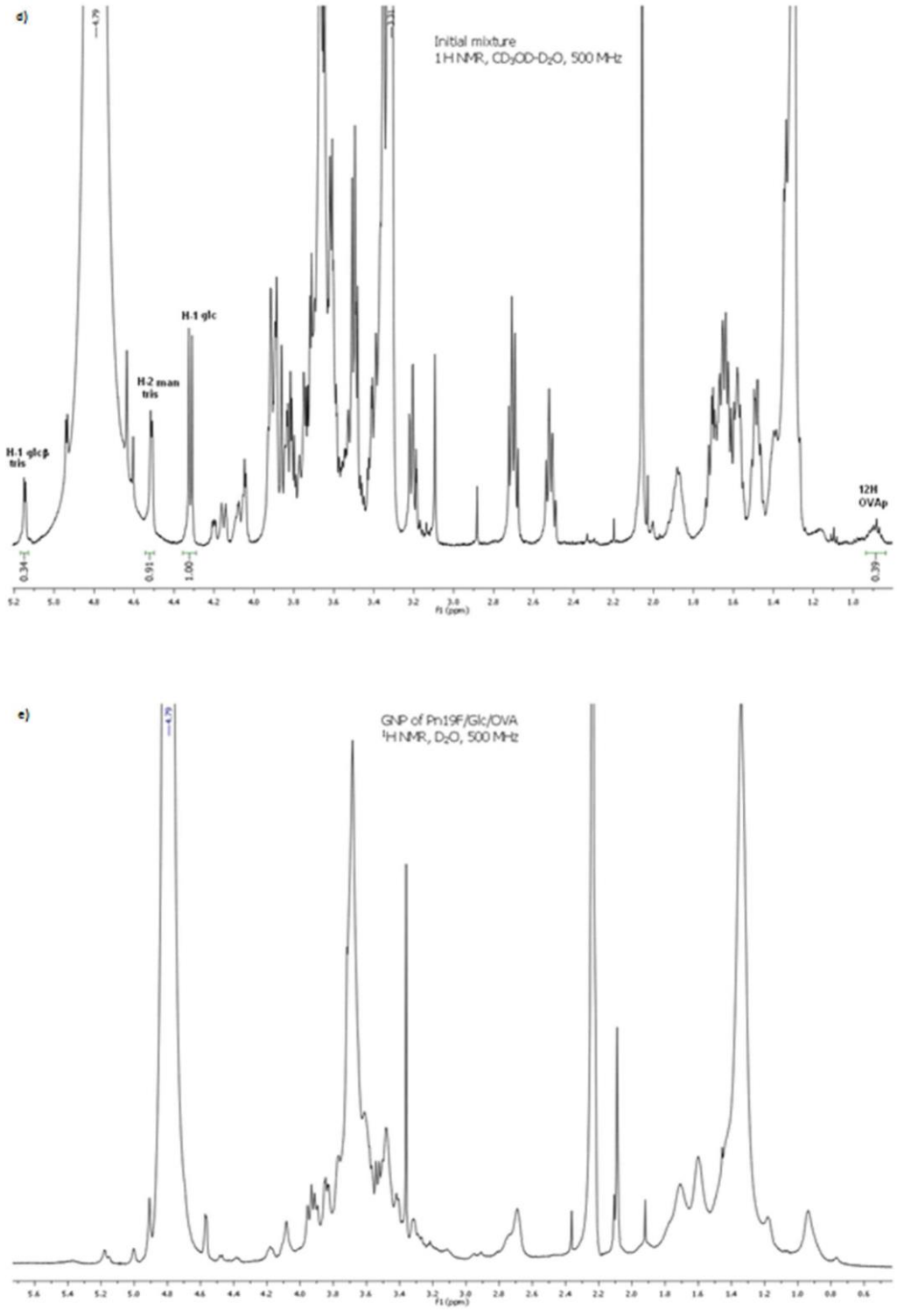
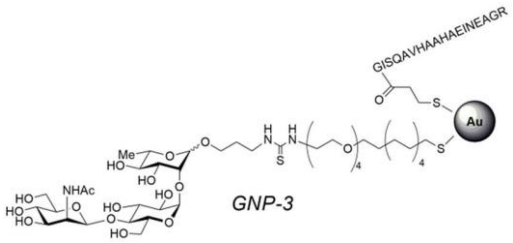


Figure S4 (a) TEM micrographs and (b) histograms of size distribution, (c) UV/Vis adsorption spectrum of gold **GNP-2**. (d) ^{1}H NMR spectrum (500MHz, CD_3OD:D_2O 5:1) of the mixture used to prepare GNP and (e) ^{1}H NMR spectrum (500MHz, D_2O) of **GNP-2** obtained. Integration of selected signals shows that the ratio between trisaccharide **2**, glucose conjugate **4** and **OVAp** is about 45:50:5.

GNP-3(Pn19F/OVA 95:5)

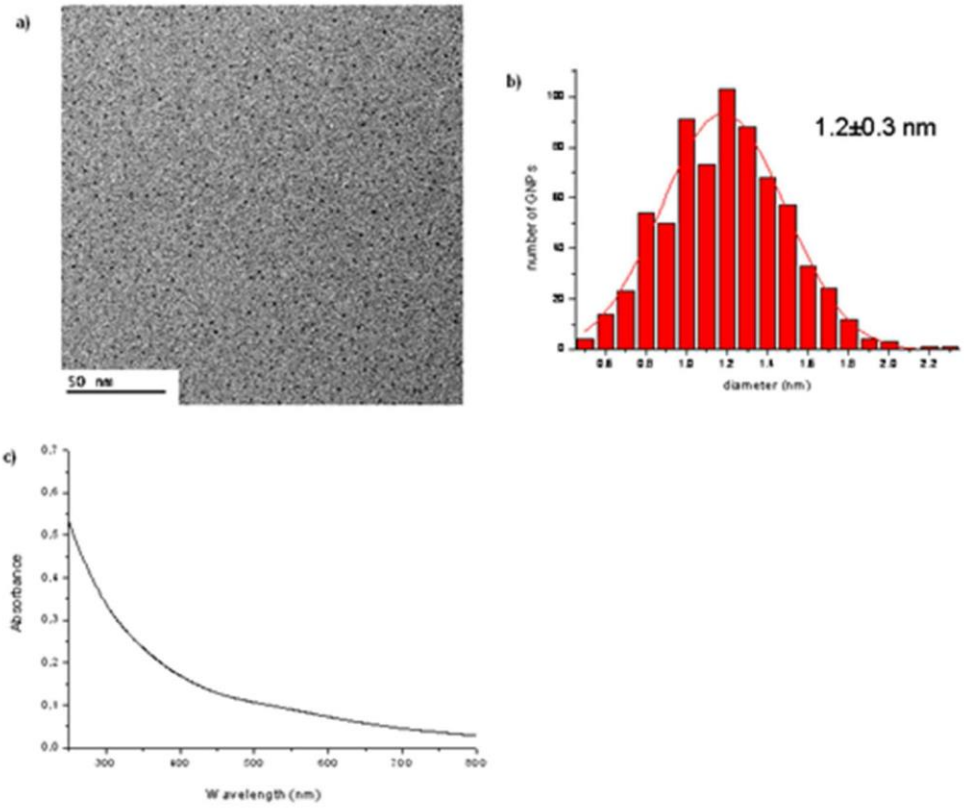
A mixture of thiol-ending Pn19F trisaccharide **1** (1.4 mg, 1.39 µmol, 19 eq.) and **OVAp** (0.14 mg, 0.07 µmol, 1 eq.) in CH_3OH (122 µl, 0.012M) was prepared.



$HAuCl_4$ (11.7 µl, 029 µmol, 0.025M in H_2O, 1eq.) and sodium borohydride $NaBH_4$ (7.9 µl, 7.9 µmol, 1M in H_2O, 27 eq.) were added to afford 336 µg of **GNP-3** after the work-up and freeze drying.

TEM: average diameter 1.2±0.3 nm (for 703 GNPs);

Average molecular formula estimated based on the size of the cluster obtained from TEM micrographs:
$Au_{79}(C_{43}H_{80}N_3O_{19}S_2)_{36}(C_{79}H_{126}N_{27}O_{27}S)_2$ ~55.6 KDa;
UV/Vis (H_2O, c=0.10 mg/mL): surface plasmon band not observed;



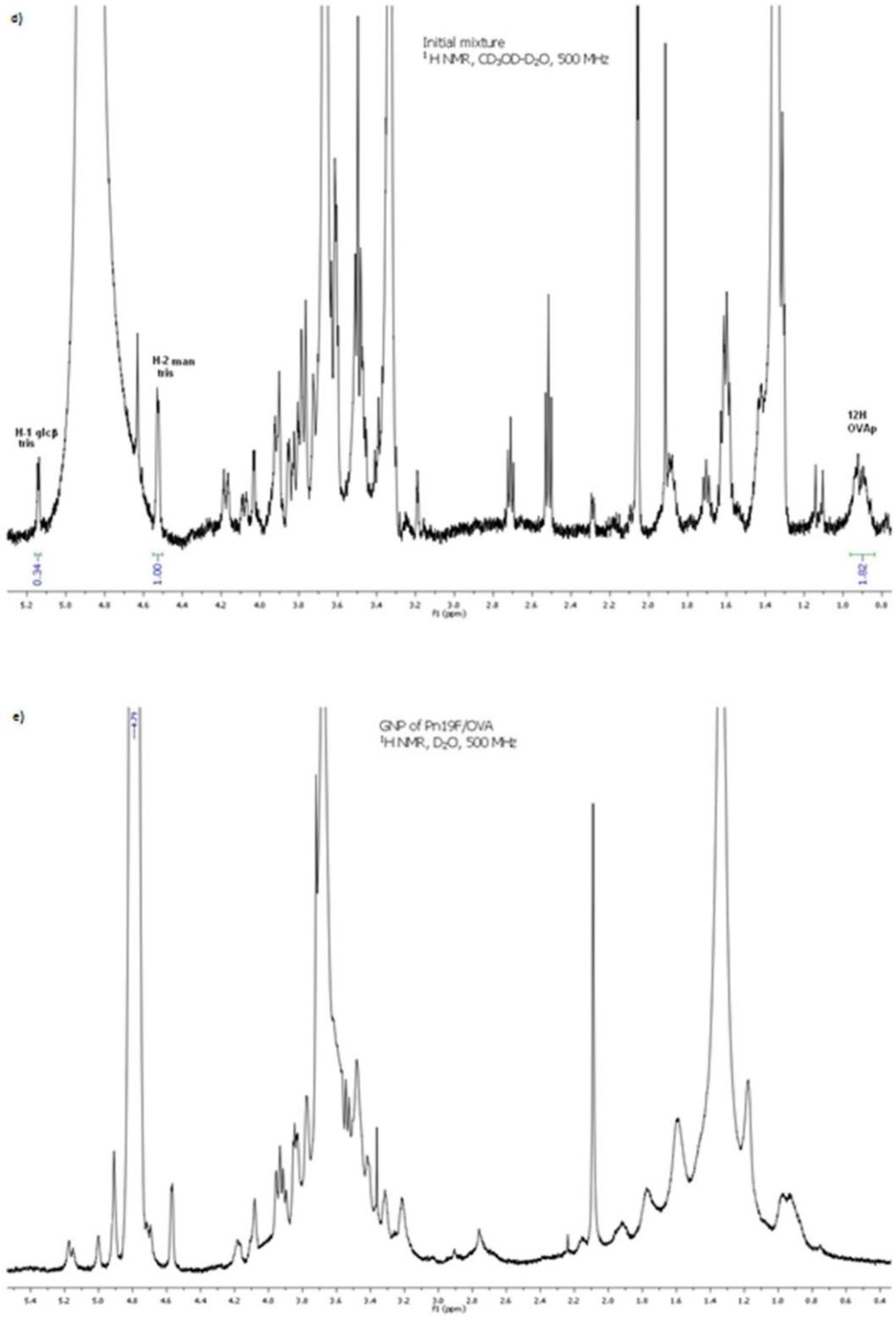


Figure S5: (a) TEM micrographs and (b) histograms of size distribution, (c) UV/Vis adsorption spectrum of gold **GNP-3**. (d) ^{1}H NMR spectrum (500MHz, CD_3OD:D_2O 5:1) of the mixture used to prepare GNP and (e) ^{1}H NMR spectrum (500MHz, D_2O) of **GNP-3** obtained. Integration of selected signals shows that the ratio between trisaccharide **2** and **OVAp** is about 95:5.

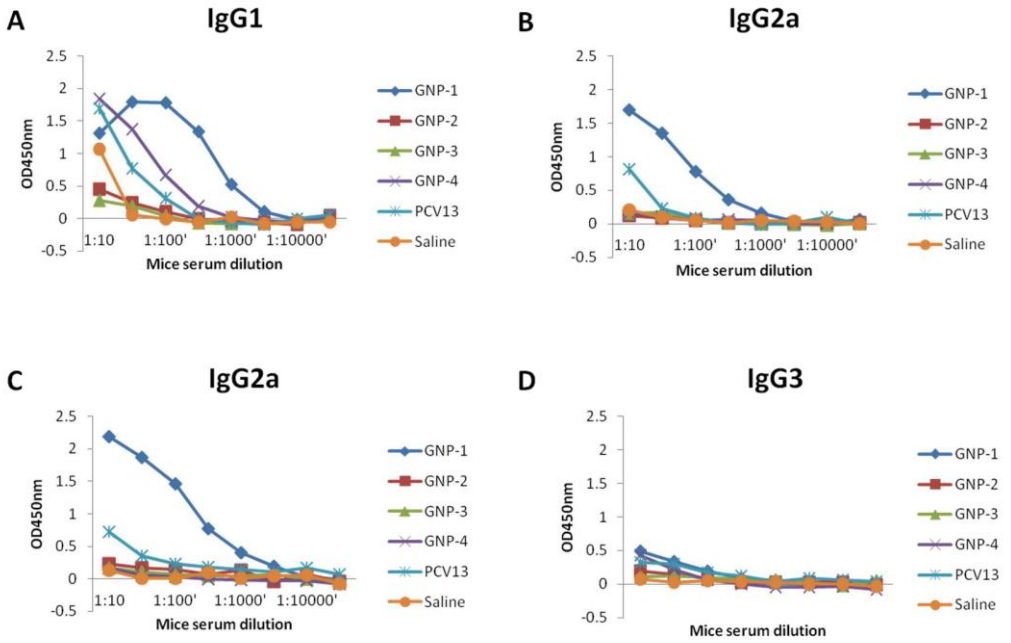


Figure S6. Anti-Pn14PS IgG antibodies subclass distribution. Mice sera were collected after the first booster immunization. ELISA was performed to measure the anti-Pn14PS IgG antibodies subclass distribution: IgG1 (A), IgG2a (B), IgG2b (C), and IgG3 (D).

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