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(54) **Titre : CONJUGUES IMMUNOGENES ET LEUR UTILISATION**
(54) **Title: IMMUNOGENIC CONJUGATES AND USE THEREOF**

(57) **Abrégé/Abstract:**

The provided technology is in the field of conjugating native, non-detergent extracted, outer membrane vesicles (nOMV) to antigens to form nOMV-antigen conjugates, which are particularly useful for immunogenic compositions and immunisation; processes for the preparation and use of such conjugates is also provided.

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(57) Abstract: The provided technology is in the field of conjugating native, non-detergent extracted, outer membrane vesicles (nOMV) to antigens to form nOMV-antigen conjugates, which are particularly useful for immunogenic compositions and immunisation; processes for the preparation and use of such conjugates is also provided.



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IMMUNOGENIC CONJUGATES AND USE THEREOF

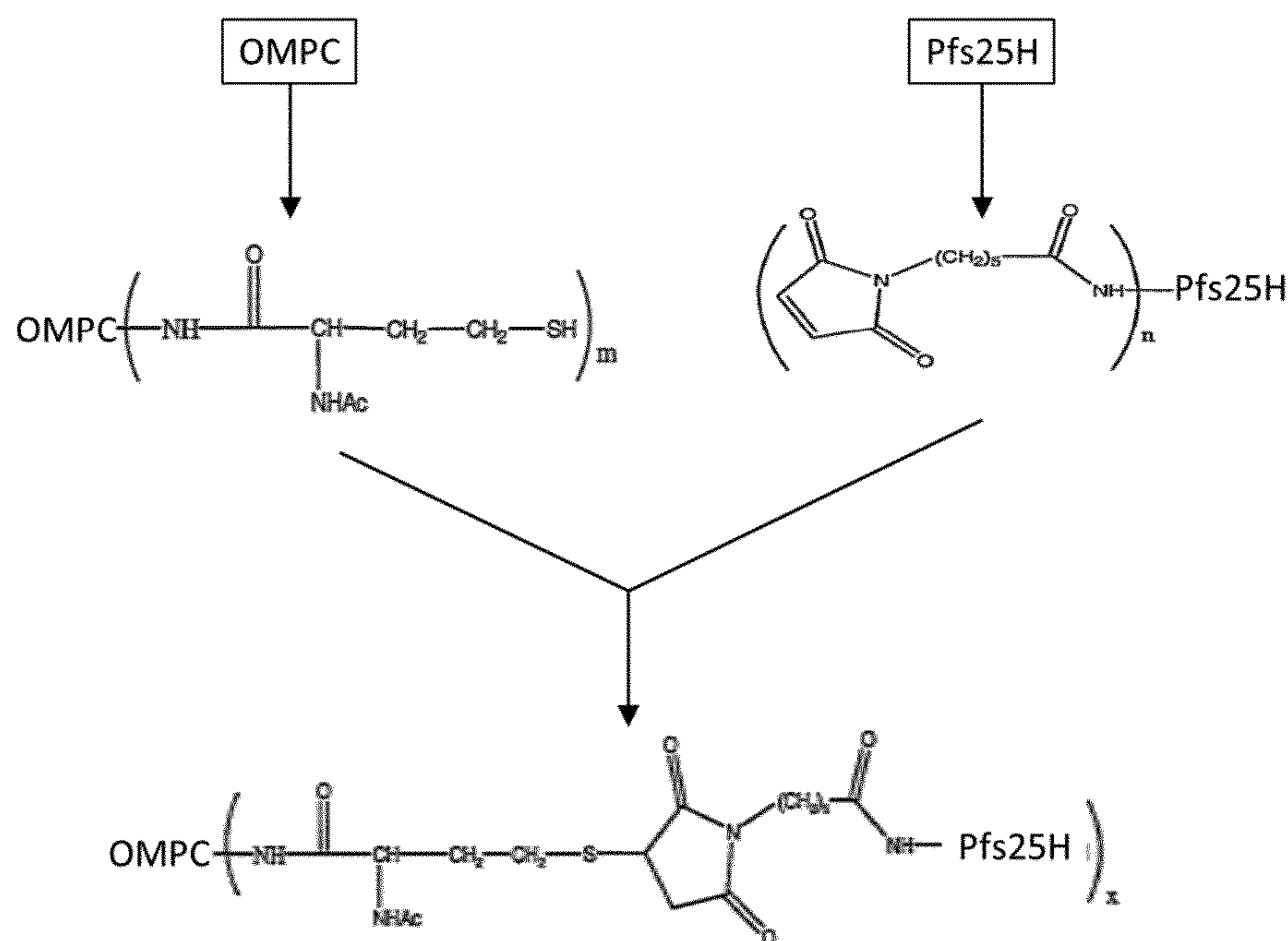
This invention is in the field of conjugating “native”, non-detergent extracted, outer membrane vesicles (nOMV) to antigens, to form nOMV-antigen conjugates, particularly useful for immunisation.

5 BACKGROUND ART

Conjugation of antigens to carriers is an established procedure for improving immunogenicity, especially for saccharides. For instance, bacterial capsular saccharides are naturally T-cell independent antigens which give rise to an immune response that lacks several important properties. Conjugation to a carrier moiety converts these saccharides to T-cell dependent
10 antigens which can then produce an immunological memory effect, and also elicit effective immune responses in young children.

One known source of protein carrier in such conjugates is the Outer Membrane Protein Complex (OMPC) from *N. meningitidis* serogroup B (e.g. see EP-0467714, Merck & co.), which has been included as the carrier in approved *H. influenzae* B conjugate vaccines. OMPC has
15 also been used as the carrier in protein conjugates. According to the prior art, OMPC is conjugated to an antigen via a protein residue, which may be activated or chemically modified in order to better perform the conjugation with the selected antigen.

Wu et al. (PNAS USA 2006; 103(48): 18243–18248) report that conjugation of Pfs25H (a human malarial transmission-blocking protein) to OMPC resulted in a Pfs25H-OMPC conjugate vaccine that was >1,000 times more potent in generating anti-Pfs25H ELISA reactivity in mice
20 than a similar dose of Pfs25H alone. Conjugation of OMPC to Pfs25H protein can be achieved by reacting maleimide-activated Pfs25H with thiolated outer membrane proteins within OMPC (for a general reference see e.g. WO2006/124712), as shown in Scheme 1.



Scheme 1

Even if the process can represent a valid synthetic route, the considered vesicles may be difficult to obtain in a pure form, and they are usually collected via laborious processes. Also, the connection with the selected antigen requires the presence and the activation of a suitable vesicle protein, thus posing an additional challenge in light of the use of detergents or chemicals during the vesicle isolation, which can alter the surface proteins composition. Therefore, there is still the need to provide new conjugates useful as immunogenic compounds which overcome the problems of the prior art, and that are achievable by an easy and convenient procedure.

The Applicant has now found that when nOMVs are connected to selected antigens via saccharide moieties, the thus obtained conjugates are endowed with a remarkable immunogenicity and can be obtained by a reliable and convenient process, as herein below described in more details.

SUMMARY OF THE INVENTION

In a first aspect, the invention refers to an immunogenic nOMV-antigen conjugate, comprising a native, outer membrane vesicle (nOMV) obtained by a detergent free process, having at least a native surface saccharide moiety connected to at least a foreign selected antigen.

In a further aspect, the invention refers to a process for preparing said conjugate, comprising the steps of:

- i) activating at least a nOMV saccharide moiety, generally bond to the nOMV surface, and
- ii) connecting the thus obtained activated saccharide to at least one selected antigen.

According to a preferred embodiment, the nOMV-surface bond saccharides are activated by oxidation, and then connected with the selected antigens, more preferably under reductive amination conditions.

In an additional aspect, the invention also refers to the above conjugate for use as a medicament, particularly as an immunogenic compound, or for the preparation of an immunogenic composition or vaccine.

Still in a further aspect, the invention refers to an immunogenic composition or a vaccine, comprising the above indicated conjugate and at least one pharmaceutically acceptable carrier or adjuvant; and to a method for raising an immune response in a vertebrate, comprising the administration of said composition or vaccine.

In a further aspect, the invention also refers to the use of nOMV for the preparation of immunogenic conjugates.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the -OAg structures for *S. sonnei*.

Figures 2 show anti-Vi (Fig. 2A) and anti-OAg (Fig. 2B) IgG titers after immunisation with fragmented Vi (fVi) saccharide conjugated to *S. Typhimurium* nOMV compared to fVi physically mixed with said nOMV or conjugated to the more traditional carrier CRM197 (formulated with Alhydrogel). CD1 female mice, 5 weeks old (8 per group) were subcutaneously immunised at days 0 and 28 with 1 µg Vi/dose. Titres were measured at days 0, 14, 28 and 42.

Figure 3 shows anti-CTF1232 IgG titers after immunisation with CTF1232 polypeptide conjugated to various carriers (formulated with Alhydrogel). CD1 female mice, 5 weeks old (8 per group) were vaccinated intranasally with 30 µl of vaccine (15 µl per nostril) at study days 0, 21 and 38. Sera were collected at days 0, 14, 35 and 52. Dose was 0.5 µg CTF1232.

Figures 4 show anti-Pfs25 IgG titers after immunisation with Pfs25 polypeptide conjugated to *S. Typhimurium* 1418 ΔtolR nOMV compared to Pfs25 alone or physically mixed with said nOMV. CD1 female mice, 5 weeks old (8 per group) were subcutaneously immunised at days 0 and 28 with 0.1 µg Pfs25/dose (Fig. 4A, with Alhydrogel) or 1 µg Pfs25/dose (Fig. 4B, no Alhydrogel). Titres were measured at days 0, 14, 28 and 42. Fig. 4C shows the anti-OAg IgG titers using 2 and 0.1 µg Pfs25/dose, and corresponding to 10 and 0.5 µg nOMV/dose, respectively (formulated with Alhydrogel). Again, CD1 female mice, 5 weeks old (8 per group) were subcutaneously immunised at days 0 and 28.

Figures 5 show anti-RO6C IgG titers (Fig. 5A) and anti-OAg IgG titers (Fig. 5B) after immunisation with RO6C alone, or conjugated to *S. Typhimurium* 1418 ΔtolR nOMV vesicles (formulated with Alhydrogel). A recycled conjugate was prepared by recycling unconjugated RO6C from the first conjugation batch. CD1 female mice, 5 weeks old (8 per group) were immunised subcutaneously at days 0 and 28, and doses of 1, 4 and 20 µg RO6C were used. For the conjugates, the corresponding doses of nOMV were 13 µg, 52 µg and 258 µg, respectively (4 µg RO6C and 32 µg nOMV for the recycled conjugate). IgG titers were measured at days 0, 14, 28 and 42.

Figure 6a: Anti-Pfs25 IgG response induced in mice (200 µL per dose SC injected at days 0 and 28, bleeds at days 0, 14, 27 and 42) by nOMV conjugates of the invention produced by conjugating *S. Typhimurium* nOMV with Pfs25 antigen, with or without quenching reaction, according to the embodiments of the invention.

Figure 6b: Anti-OAg response induced in mice (200 µL per dose SC injected at days 0 and 28, bleeds at days 0, 14, 27 and 42) by nOMV conjugates of the invention produced by conjugating *S. Typhimurium* nOMV with Pfs25 antigen, with or without quenching reaction, according to the embodiments of the invention.

DETAILED DESCRIPTION OF THE INVENTION

To facilitate an understanding of the present invention, a number of terms and phrases are defined below. Art-recognized synonyms or alternatives of the following terms and phrases (including past, present, etc. tenses), even if not specifically described, are contemplated.

- 5 As used in the present disclosure and claims, the singular forms "a," "an," and "the" include plural forms unless the context clearly dictates otherwise; *i.e.*, "a" means "one or more" unless indicated otherwise.

- The terms "about" or "approximately" mean roughly, around, or in the regions of. The terms "about" or "approximately" further mean within an acceptable contextual error range for the
 10 particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.* the limitations of the measurement system or the degree of precision required for a particular purpose, *e.g.* the amount of a nutrient within a feeding formulation. When the terms "about" or "approximately" are used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the
 15 numerical values set forth. For example "between about 0.2 and 5.0 mg/ml" means the boundaries of the numerical range extend below 0.2 and above 5.0 so that the particular value in question achieves the same functional result as within the range. For example, "about" and "approximately" can mean within 1 or more than 1 standard deviation as per the practice in the art. Alternatively, "about" and "approximately" can mean a range of up to 20%, preferably up to
 20 10%, more preferably up to 5%, and more preferably up to 1% of a given value.

The term "and/or" as used in a phrase such as "A and/or B" is intended to include "A and B," "A or B," "A," and "B." Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

- 25 Unless specified otherwise, all of the designations "A%-B%," "A-B%," "A% to B%," "A to B%," "A%-B," "A% to B" are given their ordinary and customary meaning. In some embodiments, these designations are synonyms.

- The terms "substantially" or "substantial" mean that the condition described or claimed functions in all important aspects as the standard described. Thus, "substantially free" is meant to
 30 encompass conditions that function in all important aspects as free conditions, even if the numerical values indicate the presence of some impurities or substances. "Substantial" generally means a value greater than 90%, preferably greater than 95%, most preferably greater than 99%. Where particular values are used in the specification and in the claims, unless otherwise stated, the term "substantially" means with an acceptable error range for the
 35 particular value.

An "effective amount" means an amount sufficient to cause the referenced effect or outcome. An "effective amount" can be determined empirically and in a routine manner using known techniques in relation to the stated purpose.

As used herein, "heterologous" means the two or more referenced molecules or structures are derived from a different organism. For example, a heterologous antigen is one that is derived from a different organism than the nOMV vesicle to which it is appended. "Homologous" as used herein means the two or more referenced molecules or structures are derived from the same organism.

As used herein, "foreign" means the two or more referenced molecules or structures are not naturally associated with each other. For example, a selected antigen that is herein intended to be "foreign to" a nOMV surface saccharide herein means the antigen is not naturally or innately conjugated to the surface saccharide and is, therefore, not naturally conjugated to the nOMV molecule *even though* the antigen and the saccharide (or nOMV molecule) may originate from the same organism. In this way, a foreign antigen is not necessarily a heterologous antigen but a heterologous antigen is a foreign antigen.

"Sequence identity" can be determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty=12 and gap extension penalty=1, but is preferably determined by the Needleman-Wunsch global alignment algorithm (see e.g. Rubin (2000) *Pediatric. Clin. North Am.* 47:269-285), using default parameters (e.g. with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the needle tool in the EMBOSS package. Where the application refers to sequence identity to a particular SEQ ID, the identity is intended to be calculated over the entire length of that SEQ ID.

The term "w/w%" indicates the weight percentage of a given component, over a different component or over the whole content of a composition, as indicated.

Analogously, the term "% v/v" indicates the volume percentage of a given component, over a different component or over the whole content of a composition, as indicated.

The term "-OAg" (O-antigen) is used within the present invention to indicate an antigen functionality present in the lipopolysaccharides (LPS) or lipooligosaccharides (LOS) on the surface of the considered nOMV, useful for the conjugation with a proper antigen (generally indicated as Ag) according to the invention. In more details, the LPS are generally formed by three different portions, known as: lipidA (responsible for the toxicity of LPS), core oligosaccharide and the -OAg chain, a repetitive glycan polymer and major contributor to the serological specificity of bacteria.

The term “linear or branched C₁-C_x alkyl or alkenyl group” comprises in its meaning a divalent saturated or unsaturated linear or branched alkyl or alkenyl group having 1 to x carbon atoms. For instance, the term divalent C₁-C₁₀ alkyl or alkenyl group comprises in its meaning a divalent saturated or unsaturated alkyl or alkenyl group having 1 to 10 carbon atoms such as methyl, ethyl, vinyl, allyl and the like.

As herein used, the term “saccharide (or sugar) moiety” comprises in its meaning mono saccharides, as well as polysaccharide units. It will be appreciated that saccharide moieties can exist in open and closed (ring) form and that, while closed forms are shown in structural formulae herein, open forms are also encompassed by the invention. Similarly, it will be appreciated that saccharide moieties can exist in pyranose and furanose forms and that, while pyranose forms are shown in structural formulae herein, furanose forms are also encompassed. Different anomeric forms of saccharide moieties are also encompassed.

The term “oligosaccharide” comprises in its meaning polysaccharides having from 3 to 10 monosaccharide units.

Unless otherwise provided, the term “polypeptide” refers to polypeptides of any length capable to act as a selected antigen. The amino acid polymer forming the polypeptide of the invention, may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The term also encompasses an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. Polypeptides can occur as single chains or associated chains.

“Average molecular weight” is intended to indicate the average molecular weight obtained by the ordinary arithmetic mean or average of the molecular masses of the individual component, e.g. aminoacids in case of polypeptide derivatives.

The term “capsular polysaccharides/saccharides” (CPSs) indicates those saccharides which can be found in the layer that lies outside the cell envelope of bacteria, thus being part of the outer envelope of the bacterial cell itself. CPSs are expressed on the outermost surface of a wide range of bacteria, and in some cases even in fungi.

Unless otherwise provided, the term “conjugation” indicates the connection or linkage of the subjected entities, particularly referred to the nOMV and the selected antigen moieties.

By “immunologically effective amount”, it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount can vary depending upon the health and physical condition of the individual to be

treated, age, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively
5 broad range that can be determined through routine trials.

The term "nOMVs" herein indicates vesicle isolated from the medium or sheared from cells, and they are intact membrane vesicles not exposed to detergents or denaturing agents, *i.e.* not detergent extracted. The nOMVs of the invention present the outer membrane proteins (OMP) and lipopolysaccharide (LPS) in their native conformation and correct orientation in the natural
10 membrane environment, and usually lack the cytoplasmatic components.

On the contrary, the term "OMV" or "dOMV" encompasses a variety of proteoliposomic vesicles obtained by disruption of the outer membrane of a Gram-negative bacterium typically by a detergent extraction process to form vesicles therefrom. Outer membrane protein complexes (e.g. OMPC from *Neisseria meningitidis*) may be considered in such definition, since having
15 three dimensional structure and composition similar to dOMV, and being isolated via detergent extraction procedures (see e.g. EP0467714, US4271147, US4459286 and US4830852). The detergent extraction process removes LPS and phospholipids, together with immunoprotective lipoproteins. Such removal changes the native vesicle structure and promotes aggregation. The aggregation may lead to consequent issues in terms of process development (yield, consistency
20 of production and stability). Differently from nOMVs, characterized by defined homogeneous size distribution (typically in the range 20-250 nm, measured by Dynamic Light Scattering DLS technique), the dOMVs have an undefined heterogeneous size distribution (usually in the range 550-5500 nm as measured by Dynamic Light Scattering DLS technique) caused by detergent-induced vesicle aggregation (see for a general reference, Vaccine 28, 2010, 4810). The
25 detergent extraction process also causes contamination of OMV containing composition (e.g. vaccines) with cytoplasmic proteins as a result of bacterial cell lysis.

According to prior art methodologies, dOMVs and nOMVs may be analysed and described in terms of size, shape and overall appearance of impurities or contaminating non-OMV materials (like vesicle aggregates or detergent residues in case of dOMVs) using the Transmission
30 Electron Microscopy (TEM). For detailed references regarding the differences between dOMVs and nOMVs see e.g. van de Waterbeemd (2013) J. Prot. Res. "Quantitative Proteomics Reveals Distinct Differences in the Protein Content of Outer Membrane Vesicle Vaccines"; and J. Klimentova et al. Microbiological Research 170 (2015) 1-9 "Methods of isolation and purification of the outer membrane vesicles from gram-negative bacteria".

As above indicated, in a first aspect, the invention refers to a conjugate comprising a selected antigen connected to a saccharide moiety present on the surface of a non-detergent extractive
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native Outer Membrane Vesicle (nOMV). Of note, the nOMVs in accordance with the present invention are collected and isolated substantially without the use of detergents, differently for instance from dOMVs of the prior art obtained via a deoxycholate extraction or using zwitterionic detergents like Empigen BB (see *e.g.* US4707543) or similar. On the contrary, it has to be highlighted that a detergent extraction step may be undesirable in the present invention, for a series of reasons, among which the fact that a detergent would reduce the amount of lipopolysaccharide (LPS)/lipooligosaccharide (LOS) present on the vesicle, which can be indeed useful for the conjugation with the selected antigen as herein below described.

In further details, the nOMVs are naturally-occurring membrane vesicles that form spontaneously during bacterial growth and are released into culture medium. They can be obtained *e.g.* by culturing bacteria in broth culture medium, separating whole cells from the smaller nOMVs in the broth culture medium (*e.g.* by filtration or by low-speed centrifugation to pellet only the cells and not the smaller vesicles), and then collecting the nOMVs from the cell-depleted medium (*e.g.* by filtration, by differential precipitation or aggregation, by high-speed centrifugation to pellet the vesicles). Strains for use in production of nOMVs can generally be selected on the basis of the amount of nOMVs produced in culture. The present nOMVs are characterised by the fact of being collected and isolated following a detergent-free procedure. Preferably, the present nOMVs are released into the fermentation broth and are purified using a centrifugation and subsequent filtration step (for a general reference see *e.g.* Clin Vaccine Immunol. 2016 Apr; 23(4): 304–314). Still preferably, the present nOMVs are released into the fermentation broth and are purified using the following two consecutive Tangential Flow Filtration (TFF) steps: (i) a microfiltration in which the culture supernatant containing the nOMV is separated from the bacteria, and (ii) an ultrafiltration in which the nOMV are separated from soluble proteins (for a general reference see *e.g.* PLoS One. 2015; 10(8): e0134478). The thus obtained nOMVs can then directly be used within the present invention without additional purification/isolation steps. The presently considered nOMVs have a preferred size distribution comprised from 20 to 250 nm, measured by Dynamic Light Scattering DLS technique.

According to some embodiments, the nOMVs are prepared from wild-type bacteria or from bacteria which have been genetically manipulated generally to increase immunogenicity (*e.g.* to hyper-express immunogens), to reduce toxicity, to inhibit capsular saccharide synthesis, to down-regulate immunodominant antigen expression, and the like. They also may be prepared from hyperblebbing strains. The nOMVs of the invention may also express exogenous proteins on their surface and they may be endotoxin-depleted.

Preferably, the nOMVs to be used in the present invention are produced from genetically-modified bacterial strains that are mutated to enhance vesicle production, and optionally also to remove or modify antigens (*e.g.* lipid A) and/or to over-express homologous antigens or antigens from other organisms. Said preferred nOMVs are also known as Generalized Modules of Membrane Antigens (GMMA) as *e.g.* described in PLoS One. 2015; 10(8): e0134478.

Enhanced spontaneous generation of vesicles can be achieved, for example, by targeted deletion of proteins involved in maintenance of membrane integrity. It has been observed that the outer surface of nOMVs substantially corresponds to the outer surface of the bacterium from which they are derived, preserving the membrane antigens (including e.g. lipopolysaccharides, lipooligosaccharides and lipoproteins) in the context of the membrane. Advantageously, the nOMVs used in the invention (unlike detergent-extracted dOMVs) retain these outer membrane components in their native conformation and correct orientation, better preserving immunogenicity against the bacterial strain from which they are derived.

Generally, the nOMVs for use in the present invention may be prepared from any suitable bacterium, where preferred bacteria include, but are not limited to: *Neisseria* (e.g. in particular *N. meningitidis* of any serogroups including A, B, C, X, Y or W135, or from a non-pathogenic *Neisseria*), *Shigella* (such as *S. sonnei*, *S. flexneri*, *dysenteriae* or *boydii*), *Salmonella enterica* serovars (such as Paratyphi A, B or C, Enteritidis, Typhi or Typhimurium), *Haemophilus influenzae* (e.g. non-typable *H. influenzae*), *Vibrio cholerae*, *Bordetella pertussis*, *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, *Escherichia coli*, *Bacteroides* (including *Porphyromonas*), *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Brucella melitensis*, *Campylobacter jejuni*, *Actinobacillus actinomycetemcomitans*, *Xenorhabdus nematophilus*, *Moraxella catarrhalis*, or *Borrelia burgdorferi*.

Particularly preferred bacteria are selected from at least one of: *S. sonnei*, *S. flexneri*, *Salmonella* bacterium, and *meningococcus*, particularly *meningococcus* serogroup B.

Virulent *Shigella* strains possess a 220kb plasmid that mediates virulence properties. This “virulence plasmid” has been shown to encode the genes for several aspects of *Shigella* virulence, including adhesins for target epithelial cells, the invasion plasmid antigens, virF, virG, and the like. A *Shigella* used with the invention may or may not possess a virulence plasmid. Absence of the plasmid can stabilise the strain during industrial culture, attenuate the strain by removing virulence factors (thereby increasing safety of manufacture), avoid the presence of the ShET-2 enterotoxin (encoded by the ospD3 or sen gene on the plasmid), and avoid the presence of msbB2 which is a second copy of the msbB gene responsible for acylation of lipid A. Absence of the virulence plasmid may also disrupt the lipopolysaccharide. However, the biosynthesis genes for the –OAg should preferably be retained, either by maintenance of a mutated virulence plasmid, or by inclusion in a further plasmid or cloning into the bacterial chromosome.

As far as *Salmonella* bacterium is concerned, a particularly preferred strain is selected from: *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Paratyphi A.

Meningococcus bacteria nOMVs are also preferred. Such vesicles can be prepared from any *meningococcal* strain. The vesicles are preferably prepared from a serogroup B strain, but it is

also preferred to prepare them from serogroups other than B, such as one of: A, C, W135 or Y, according to procedures known in the art. The strain may be of any serotype (e.g. 1, 2a, 2b, 4, 14, 15, 16, etc.), any serosubtype (e.g. P1.4), and any immunotype (e.g. L1; L2; L3; L3,7; L3,7,9; L10; etc.). The *meningococci* may be from any suitable lineage, including hyperinvasive and hypervirulent lineages, preferably any of the following seven hypervirulent lineages: subgroup I; subgroup III; subgroup IV-1; ET-5 complex; ET-37 complex; A4 cluster; lineage 3. Most preferably, OMVs are prepared from the strain NZ98/254, or another strain with the P1.4 PorA serosubtype.

In another embodiment, bacteria for preparing nOMVs useful for the invention may be mutant strains which have been manipulated e.g. to enhance vesicle production, to express one or more desired antigen(s), and/or to knockout or modify an undesired gene (e.g. one which encodes a toxin or which encodes an enzyme involved in generating a toxic product, such as endotoxin).

In this direction, other preferred nOMVs for the invention are produced by a *Salmonella* bacterium, particularly a *S. Typhimurium* (also known as *Salmonella enterica* serovar Typhimurium) which does not express a functional TolR protein.

Where the vesicles are prepared from *E.coli*, *Shigella* or *Salmonella* the bacterium may express no more than 4 of TolA, TolB, TolQ, TolR and Pal proteins. Thus at least one protein from the natural five-protein Tol-Pal system may be absent, resulting in a bacterium which, during growth in culture medium, releases greater quantities of outer membrane vesicles into the medium than the same bacterium expressing all 5 Tol-Pal proteins. Preferably TolR is not expressed, but the other four proteins may be expressed (i.e. a Δ TolR strain).

In preferred embodiments, at least one of the five Tol-Pal proteins in *E.coli*, *Shigella* or *Salmonella* is removed e.g. by deletion or inactivation of the gene encoding the protein. Thus the bacterium may express 0, 1, 2, 3 or 4 of TolA, TolB, TolQ, TolR and Pal proteins. Removal of one of the five proteins can suffice, in which case the bacterium expresses only 4 of these proteins. Preferably the TolR protein is removed e.g. by inactivation of a starting strain's tolR gene. Thus a preferred bacterium may be tolA⁺ tolB⁺ tolQ⁺ TolR⁻ Pal⁺.

In some embodiments, the bacterium expresses all five Tol-Pal proteins, but at least one is mutated to cause hyperblebbing. For instance, the TolA, TolQ, TolR and/or Pal protein may be mutated such that the protein retains its membrane localisation but its interactions with other members of the Tol-Pal system are disrupted. The bacterium will thus retain TolA, TolQ and TolR as transmembrane proteins in the inner membrane, and Pal protein as a periplasm-facing lipoprotein in the outer membrane, but at least one of the TolA, TolQ, TolR and/or Pal proteins is mutated and not fully functional.

In addition other mutations may also be present e.g. to give OAg-deficient strains, for instance in those cases where the –OAg functionality is not intended for desired immune response, or in those cases where the –OAg may negatively impact the immunogenicity against the heterologous antigen. In this direction, possible mutations may be $\Delta galU$, $\Delta galE$ or $\Delta wbaP$ in
 5 E.coli, Shigella or Salmonella strains.

In one further preferred embodiment, a *meningococcus* does not express a functional MltA protein. Knockout of MltA (the membrane-bound lytic transglycosylase, also known as GNA33) in *meningococcus* provides bacteria which spontaneously release large amounts of nOMVs into culture medium, from which they can be readily purified. For instance, the vesicles can be
 10 purified using the two stage size filtration process, comprising: (i) a first filtration step in which vesicles are separated from the bacteria based on their different sizes, with the vesicles passing into the filtrate; and (ii) a second filtration step in which the vesicles are retained in the retentate.

In the present invention, it is preferred that -OAg is present on the nOMVs because we observed (e.g. nOMVs from *Salmonella* and *Shigella*) that, the presence of the -OAg on the
 15 surface of said nOMVs is advantageous in providing a bivalent vaccine, as the -OAg can act as a protective antigen. Some preferred strains have penta- or tetra-acylated less toxic LPS, which includes attached –OAg, after the mutation of msbB, htrB, ddg and/or PagP (see e.g. Rossi O et al, Clin Vaccine Immunol. 2016 Apr 4;23(4):304-14 and Rossi O et al, J Biol Chem. 2014 Sep 5;289(36):24922-35).

20 In *Neisseria*, the strain has preferably a modified fur gene. According to this embodiment, mutant *Neisseria* are engineered to reduce or switch off expression of at least one gene involved in rendering toxic the lipid A portion of LPS, in particular of lpxI1 gene. In this way, the resulting nOMVs present a reduced toxicity respect to the wild type strain, since the conversion of acylated lipid A in a less acylated form.

25 Similarly, preferred mutant *Neisseria* for the invention are engineered to reduce or switch off expression of at least one gene involved in the capsular saccharide synthesis or export, in particular of synX and/or ctrA genes. In this way, the resulting nOMVs may present a cross protection versus different serotypes, particularly appreciated by the skilled in the art.

In preferred embodiments a strain may include one or more of the knockout and/or
 30 hyper-expression mutations disclosed e.g. in Fukusawa *et al.* (1999), Vaccine 17:2951-2958. For instance, following the therein guidance and nomenclature, useful genes for down-regulation and/or knockout include: (a) Cps, CtrA, CtrB, CtrC, CtrD, FrpB, GalE, HtrB/MsbB, LbpA, LbpB, LpxK, Opa, Opc, PilC, PorB, SiaA, SiaB, SiaC, SiaD, TbpA, and/or TbpB; (b) CtrA, CtrB, CtrC, CtrD, FrpB, GalE, HtrB/MsbB, LbpA, LbpB, LpxK, Opa, Opc, PhoP, PilC, PmrE, PmrF, SiaA, SiaB, SiaC, SiaD, TbpA, and/or TbpB; (c) ExbB, ExbD, rmpM, CtrA, CtrB, CtrD, GalE, LbpA, LpbB, Opa, Opc, PilC, PorB, SiaA, SiaB, SiaC, SiaD, TbpA, and/or
 35

TbpB; or (d) CtrA, CtrB, CtrD, FrpB, OpA, OpC, PilC, PorB, SiaD, SynA, SynB, SynX and/or SynC.

As above mentioned, the present conjugates are obtained by connecting at least one nOMV surface saccharide moiety (preferably comprised in the related –OAg) to one or more foreign selected antigen, *i.e.* that does not form part of the vesicle.

As far as the nOMV saccharide moiety is concerned, it has to be noted that it can be part of the –OAg functionality naturally present on the surface of the nOMV (*e.g.* in LPS or LOS), or it can be present within a different nOMV surface portion, *e.g.* a CPS, as herein below described in details. Advantageously, any proper antigen may be conjugated to the nOMV to obtain the nOMV-antigen conjugates of the invention, preferably in the form of a (poly)saccharide or polypeptide. In any case, the connection of one or more selected antigen produces an immunogenic conjugate which can raise an immune response which recognises said antigen, and which also recognises one or more components in the nOMV, thereby conveniently useful for the preparation of a multivalent vaccine. Antigens will be included in the present conjugates at a concentration which is high enough to elicit, when administered to a host, an immune response which recognises that antigen. Moreover, the immune response is preferably protective against the pathogen from which the antigen was derived, even more preferably against one of the pathogens listed below.

In one embodiment of the invention, the nOMV is conjugated to at least one homologous antigen, *i.e.* derived from the same organism from which the nOMVs are derived. In a still preferred embodiment, the selected antigen is a heterologous antigen *i.e.* derived from a different organism from the organism from which the nOMVs are derived.

In any case, the antigens are generally selected from any immunogenic polypeptides, *i.e.* polypeptides able to elicit an immune response when administered to a subject. Polypeptides used with the invention will include an amino acid having a residue, or a side chain, with a functional group suitable for conjugation, preferably an amino or a thiol group, even more preferably of general formula: $-NH_2$ or $-SH$. These residues may naturally be present in an antigen, or they may be introduced artificially for the purposes of conjugation. Preferred amino acid residues include, but are not limited to: arginine, lysine, asparagine, glutamine, cysteine and histidine. The most preferred amino acid residue for conjugation is lysine. Its $-NH_2$ side chain in fact can react with an activated oxidised $-OH$ from a nOMV saccharide moiety and react with the thus obtained aldehyde group by reductive amination, according to the process as herein disclosed in details.

Polypeptide antigens are preferably prepared in substantially pure or substantially isolated form (*i.e.* substantially free from other polypeptides). They can be prepared by various means *e.g.* by

chemical synthesis (at least in part), by digesting longer polypeptides using proteases, by translation from RNA, by purification from cell culture (e.g. from recombinant expression or from native culture), and the like. Recombinant expression in an *E.coli* host is a useful expression route. Polypeptide antigens can take various forms (e.g. native, fusions, glycosylated, non-glycosylated, lipidated, disulfide bridges and the like).

Polypeptide antigens used with the invention have a preferred average molecular weight of at least 1 kDa, more preferably of at least 3.5 kDa, even more preferably from 10 to 80 kDa. Still more preferably, the average molecular weight is comprised from 15 to 75 kDa.

Further preferred polypeptide antigens for conjugation to nOMVs according to the present invention comprise an epitope from a fungal, bacterial, protozoan or viral polypeptide. Preferred protozoan polypeptides are from a *Plasmodium* (such as *P.falciparum*, *P.vivax*, *P.ovale*). Particularly preferred bacterial polypeptides are selected from: *E.coli*, *N.meningitidis*, and *Streptococci* (such as *S.agalactiae*, *S.pneumoniae*, *S.pyogenes*).

Preferred *E.coli* polypeptide antigens include CTF1232, 405 and 3526. As a non-limiting preferred example, nOMV from *Shigella* or *Salmonella* can be conjugated to CTF1232, according to the present invention, to generate a bivalent vaccine covering both enterotoxigenic *E. coli* (ETEC) and *Shigella/Salmonella*.

In one embodiment, the considered *N.meningitidis* polypeptides are able, when administered to a mammal, to elicit an antibody response that is bactericidal against *meningococcus*. Preferred *N.meningitidis* polypeptides for use with the invention are selected from at least one of: NHBA, NadA, NsPA, NhhA, App and fHbp, as herein below detailed.

NHBA antigen.

The NHBA antigen was included in the published genome sequence for meningococcal serogroup B strain MC58 as gene NMB2132 (GenBank accession number GI:7227388; SEQ ID NO: 2 herein). The sequences of NHBA antigen from many strains have been published since then. Various immunogenic fragments of the NHBA antigen have also been reported. Preferred NHBA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 2. The most useful NHBA antigens of the invention can elicit antibodies which, after administration to a subject, can bind to a meningococcal polypeptide consisting of amino acid sequence SEQ ID NO: 2. Advantageous NHBA antigens for use with the invention can elicit bactericidal anti-*meningococcal* antibodies after administration to a subject.

NadA antigen.

The NadA antigen was included in the published genome sequence for *meningococcal* serogroup B strain MC58 (see e.g. Tettelin *et al.* (2000) Science 287:1809-1815) as gene NMB1994 (GenBank accession number GI:7227256; SEQ ID NO: 3 herein). The sequences of NadA antigen from many strains have been published since then, and the protein's activity as a *Neisserial* adhesin has been well documented. Various immunogenic fragments of NadA have also been reported. Preferred NadA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 3. The most preferred NadA antigens of the invention can elicit antibodies which, after administration to a subject, can bind to a *meningococcal* polypeptide consisting of amino acid sequence SEQ ID NO: 3. Advantageous NadA antigens for use with the invention can elicit bactericidal anti-*meningococcal* antibodies after administration to a subject. SEQ ID NO: 7 is one such fragment.

NspA antigen.

The NspA antigen was included in the published genome sequence for *meningococcal* serogroup B strain MC58 (see e.g. Tettelin *et al.* (2000) Science 287:1809-1815) as gene NMB0663 (GenBank accession number GI:7225888; SEQ ID NO: 4 herein). The sequences of NspA antigen from many strains have been published since then. Various immunogenic fragments of NspA have also been reported. Preferred NspA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 4; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 4, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 4. The most preferred NspA antigens of the invention can elicit antibodies which, after administration to a subject, can bind to a *meningococcal* polypeptide consisting of amino acid sequence SEQ ID NO: 4. Advantageous NspA antigens for use with the invention can elicit bactericidal anti-*meningococcal* antibodies after administration to a subject.

NhhA antigen.

The NhhA antigen was included in the published genome sequence for *meningococcal* serogroup B strain MC58 (see e.g. Tettelin *et al.* (2000) Science 287:1809-1815) as gene NMB0992 (GenBank accession number GI:7226232; SEQ ID NO: 5 herein). The sequences of NhhA antigen from many strains have been published since e.g. WO00/66741 and

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WO01/55182, and various immunogenic fragments of NhhA have been reported. It is also known as Hsf. Preferred NhhA antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 5; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 5, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 5. The most preferred NhhA antigens of the invention can elicit antibodies which, after administration to a subject, can bind to a *meningococcal* polypeptide consisting of amino acid sequence SEQ ID NO: 5.

Advantageous NhhA antigens for use with the invention can elicit bactericidal anti-*meningococcal* antibodies after administration to a subject.

App antigen.

The App antigen was included in the published genome sequence for *meningococcal* serogroup B strain MC58 (see e.g. Tettelin *et al.* (2000) Science 287:1809-1815) as gene NMB1985 (GenBank accession number GI:7227246; SEQ ID NO: 6 herein). The sequences of App antigen from many strains have been published since then. Various immunogenic fragments of App have also been reported. Preferred App antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 6; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 6, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). Preferred fragments of (b) comprise an epitope from SEQ ID NO: 6. The most preferred App antigens of the invention can elicit antibodies which, after administration to a subject, can bind to a *meningococcal* polypeptide consisting of amino acid sequence SEQ ID NO: 6. Advantageous App antigens for use with the invention can elicit bactericidal anti-*meningococcal* antibodies after administration to a subject.

fHbp antigen.

The factor H binding protein exists as three variants (v1, v2 and v3), and the invention can use any of these as preferred embodiment.

A v1 fHbp preferably comprises (a) an amino acid sequence which has at least k' % identity to SEQ ID NO: 8, and/or (b) a fragment of SEQ ID NO: 8. k' refers to percentage identity and could be defined as any number from 1 to 100. With reference to amino acid or nucleic acid sequences, generally the identity used in the application starts from as low as 40% with specific references to higher percentages, i.e. 70%, 75%, 80%, etc.

The fragment will preferably include at least one epitope from SEQ ID NO: 8. Preferably, the v1 fHbp can elicit antibodies which are bactericidal against v1 strains e.g. against strain MC58 (available from the ATCC as 'BAA-335').

5 A v2 fHbp preferably comprises (a) an amino acid sequence which has at least k'% identity to SEQ ID NO: 1, and/or (b) a fragment of SEQ ID NO: 1. Information about 'k' and fragments are given above. The fragment will preferably include at least one epitope from SEQ ID NO: 1. Preferably, the v2 fHbp can elicit antibodies which are bactericidal against v2 strains e.g. against strain M2091 (ATCC 13091).

10 A v3 fHbp preferably comprise (a) an amino acid sequence which has at least k'% identity to SEQ ID NO: 9, and/or (b) a fragment of SEQ ID NO: 9. Information about 'k' and fragments are given above. The fragment will preferably include at least one epitope from SEQ ID NO: 9. Preferably, the v3 fHbp can elicit antibodies which are bactericidal against v3 strains e.g. against strain M01-240355.

15 Antigens from Group A *Streptococcus* (GAS), Group B *Streptococcus* (GBS) and *Pneumococcus* are also equally preferred. As non-limiting examples, GAS25 (Slo), GAS40 (SpyAD) and GAS57 (SpyCEP) antigens can be incorporated into conjugates in accordance with some embodiments of the invention.

Plasmodium antigens are further preferred. These can be from any suitable species, where preferred species are selected from: *P.falciparum*, *P.vivax* and *P.ovale*.

20 Still another preferred antigen is Pfs25 (SEQ ID NO: 10), which is a sexual stage antigen of *P.falciparum* expressed on the surface of zygote and ookinete forms of the parasite. Another preferred antigen is Pfs48/45, which is a transmission-blocking vaccine candidate. Recently the C-terminal 10 cysteine fragment (10C) of Pfs48/45, containing three known epitopes for transmission blocking antibodies, has been produced as a chimera with the N-terminal portion
25 of GLURP (RO), the asexual blood-stage antigen glutamate-rich protein. The resulting fusion protein (RO10C) elicited high levels of transmission-blocking antibodies in rodents (see Theisen et al. (2014) Vaccine 32:2623-2630). Shing et al. (2015) Vaccine 33:1981-1986 describes a chimera containing truncated 6C-fragments, which increases the yield of correctly-folded conformer. The RO6C construct was able to elicit high titer transmission blocking antibodies in
30 rats. RO6C (SEQ ID NO: 11) is a preferred antigen that can be conjugated according to the present invention.

Another preferred antigen is the *circumsporozoite* protein (CSP; SEQ ID NO: 12).

Shorter peptides from CSP may also be conjugated according to the present invention. For example, the 12 amino acid (NANP)₃ peptide (SEQ ID NO: 13) derived from CSP can be used
35 according to preferred embodiments.

In another still preferred embodiment the antigens are a saccharide species. The invention is in fact also suitable for conjugating one or more selected saccharide antigens to nOMVs, whereby saccharides may be used in their full-length natural form. As an alternative, a particular size fraction can also advantageously be selected. Thus, the saccharides may be fragmented from their natural length, and optionally a size fraction of these fragments can be used. Even further, the saccharides are not limited to saccharides purified from natural sources and synthetic or semi-synthetic saccharides can be used instead.

Preferred saccharide antigens are bacterial capsular saccharides (CPSs). These include, but are not limited to, the capsular saccharides selected from at least one of: *Haemophilus influenzae* type B; *Neisseria meningitidis* serogroups A, C, W135, X and Y; *Streptococcus pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F; *Salmonella* including *Salmonella enterica* serovar Typhi Vi, either full length or fragmented (indicated as fVi); *Streptococcus agalactiae* serotypes Ia, Ib, and III; *Streptococcus pyogenes*, *Shigella* sp, group A and B *Streptococcus* (GAS and GBS respectively). More preferred saccharide antigens are from *Neisseria meningitidis* serogroups A and C. Thus, according to an embodiment of the invention, the nOMV is a GMMA derived from MenB, and the selected antigen is a capsular saccharide from MenA or MenC. In a still additional embodiment, the invention refers to a GMMA from MenB conjugated to a capsular saccharide from MenA antigen via a polysaccharide residue, wherein said GMMA is also conjugated to a MenC antigen via a different polysaccharide residue, thus obtaining a double functionalized GMMA vesicle, as below described in more details.

In any case, and as above mentioned, the selected antigens could be conjugated to nOMVs derived from the same or even from a different bacterial strain, thus providing a multivalent conjugate. In this respect, in a more preferred embodiment of the invention, the nOMV and the saccharide antigen are derived from different bacterial strains.

Other preferred saccharide antigens are β -glucans, particularly useful for protecting against *C.albicans* (for a general reference see Sandlin et al. (1995) Infect. Immun., 63:229-37).

Other preferred saccharide antigens are poly-rhamnose oligosaccharides for protecting against Group A *Streptococcus* (GAS). Native GAS saccharide has a poly-rhamnose backbone substituted with NAcGlcN. Synthetic oligosaccharides of poly-rhamnose, or oligomers with the structure of native GAS saccharide, can be conjugated to nOMVs according to the invention.

According to a preferred embodiment, the nOMV conjugates of the invention comprise a nOMV surface saccharide moiety directly connected to a selected antigen, where the direct connection may be achieved by activation of the saccharide moiety followed by direct reaction (e.g. via reductive amination) with the selected antigen, as exemplified in the present Example 4. In an

equally preferred embodiment, the nOMV is connected to the selected antigen indirectly, *i.e.* via a linker moiety, as herein described in more details and as exemplified in Example 3.

The conjugates of the invention are immunogenic, as demonstrated by the studies in mice and supported by the herein included experimental part. Advantageously, besides being capable of inducing an immune response against the conjugated antigen, the conjugates of the invention are also capable of inducing an immune response against the nOMV component, thus being good candidates for the preparation of multivalent immunogenic composition thereof. In fact, it has surprisingly been observed that conjugation of the selected antigens through the saccharide moiety present on the surface of the nOMV does not negatively impact the ability of the nOMVs to induce their own immune response, differently from dOMV. Accordingly, the conjugates of the invention may be useful *e.g.* as bi-valent immunogenic agent, suitable for preparing vaccines, with the nOMV and the conjugated heterologous antigen both showing good immunogenicity. Also, it has advantageously found that the conjugates of the invention induce high anti-antigen specific IgG response in mice, with no impact on anti –OAg IgG response, as supported for instance in the present Example 5 and 6. Conjugates of the invention offer further several advantages compared to unconjugated antigens, as for example set forth in Examples 3 to 6.

As formerly set forth, in a further aspect, the invention refers to a process for preparing the above described conjugates, comprising a first step of activating a nOMV-surface saccharide moiety, and a second step of connection of the thus obtained activated vesicle to at least one selected antigen, optionally via a divalent linker.

According to the present process, at least one saccharide moiety on a nOMV is conjugated to one selected antigen (as above described) to form a conjugate of the invention. According to a different embodiment, two or more saccharide moieties are conjugated to two or more different selected antigens, thus providing a nOMV derivative conjugated with two or more different antigens, particularly suitable for the preparation of polyvalent immunogenic compositions. As above indicated, the connection step typically involve activating the nOMV-surface saccharide moiety and/or the selected antigen. Similarly, the connection step may involve introducing a linker between the nOMV-saccharide moiety and the selected antigen, as below detailed. Thus, in one embodiment, the process of the invention comprises the steps of: (i) activating a saccharide moiety on the nOMV surface; and (ii) direct connection of the activated moiety with a selected antigen, to obtain the nOMV conjugates of the invention.

As an alternative embodiment, the process of the invention comprises the steps of: (i) activating a saccharide moiety on the nOMV surface; (ii) connecting the activated moiety to a divalent linker group to form a vesicle-linker conjugate; and (iii) connecting a selected antigen to the vesicle-linker conjugate to form the nOMV conjugates of the invention.

As another alternative embodiment, the process of the invention comprises the steps of: (i) activating a saccharide moiety on the nOMV surface; (ii) connecting a selected antigen to a divalent linker group to form an antigen-linker conjugate; and (iii) connecting the activated moiety of step (i) to the antigen-linker conjugate to form the nOMV conjugates of the invention.

5 As another alternative embodiment, the process of the invention comprises the steps of: (i) activating a saccharide moiety on the nOMV surface; (ii) connecting the activated moiety to a divalent linker group to form a vesicle-linker conjugate; (iii) connecting a selected antigen to a divalent linker group to form an antigen-linker conjugate; and (iv) connecting the linker moiety of step (ii) to the antigen-linker conjugate of step (iv) to form the nOMV conjugates of the
10 invention.

As far as the nOMV saccharide moiety is concerned, it has to be noted that it can be part of the –OAg functionality, or of the core region naturally present on the surface of the nOMV (e.g. in LPS or LOS), or it can be present within a different nOMV surface portions, e.g. a CPS. In all these preferred cases, the process of the invention allows the connection of said saccharide
15 moiety with a selected antigen in a simple and effective way, thus leading to the final nOMV conjugates of the invention, endowed with remarkable immunogenic activity. Advantageously, the presence of the -OAg does not substantially interfere with the response against the selected antigen. Comparative examples 9a-c show that when the present process is applied to dOMV, no conjugation with the antigen occurs, thus preventing the formation of the desired vesicle-
20 antigen conjugate.

Depending on the species from which nOMVs are prepared, various saccharide moieties (including tetraose, pentose and hexose sugars) can be used for activation and subsequent conjugation. Preferably, lipopolysaccharides, via the –OAg portion or core region, or capsular saccharides may be used for activation and subsequent conjugation. Preferred saccharide
25 moieties are selected from at least one of: glucose, galactose, fructose, mannose, ribose, abequose, galactosamine, glucosamine, mannosamine, sialic acid, sulfoquinovose, erythrose, threose, arabinose, rhamnose, sorbose, ribulose, xylose, xylulose, lyxose, tagatose or keto-deoxyoctulosonate. A saccharide moiety on the nOMV is preferably activated by oxidizing a hydroxyl group of the saccharide to form a carbonyl aldehyde functionality, in the presence of a
30 suitable oxidizing agent, such as TEMPO or a periodated salt. This latter is preferably selected from an alkali periodate or a metaperiodate, more preferably NaIO_4 . The oxidizing agent is preferably used as aqueous solution in a concentration ranging from 0.5 mM to 20 mM, preferably from 3 mM to 20 mM, where concentrations from 10 to 20 mM and from 0.5 to 5mM or from 3 to 5mM are still more preferred. Other activation reactions according to some
35 embodiments occurs in the presence of: cyanylating reagents such as CDAP (e.g. 1-cyano-4-dimethylamino pyridinium tetrafluoroborate), carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC and TSTU.

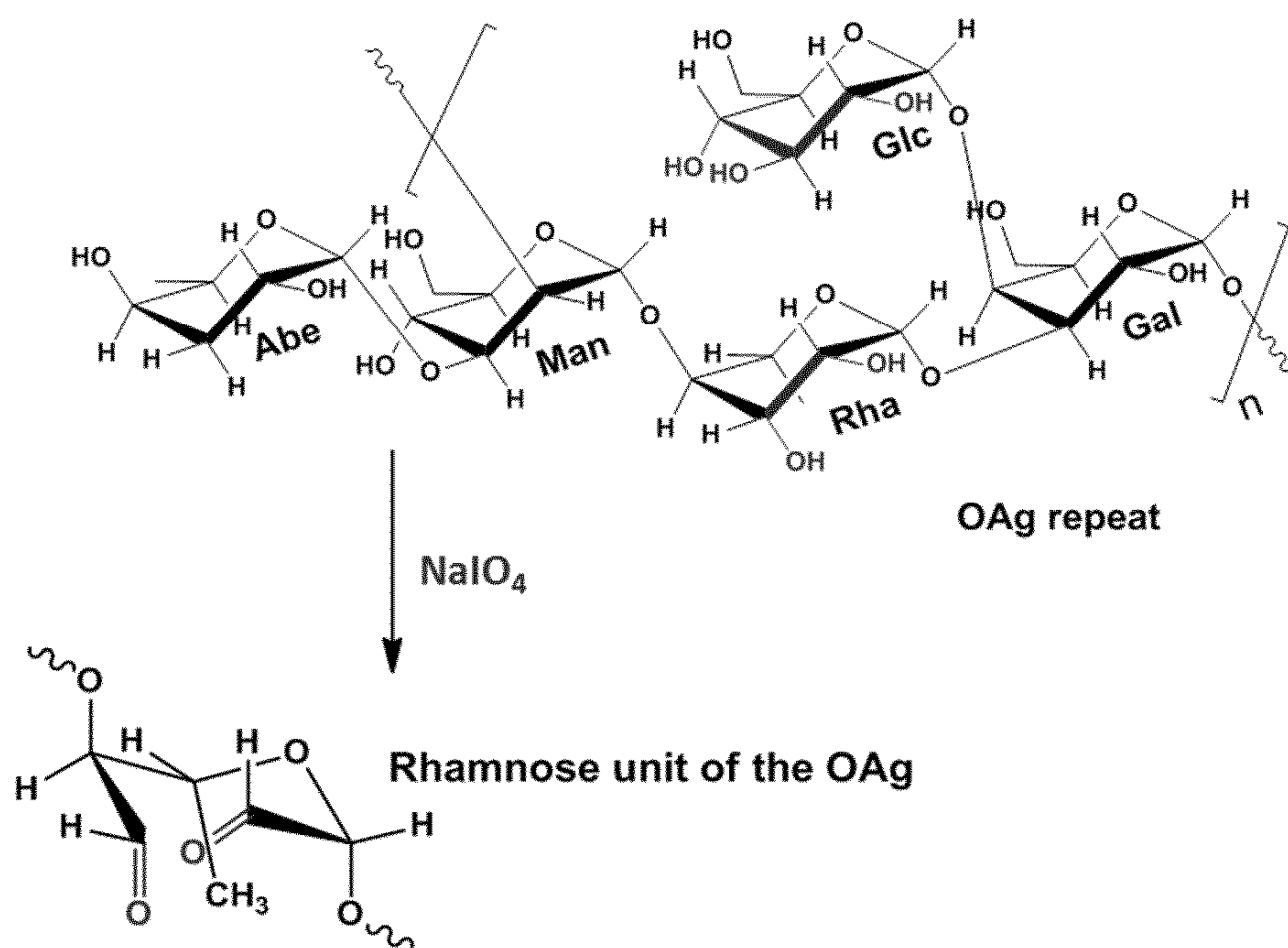
In general, where polysaccharides are oxidised it is not necessary to oxidise all of the available sugars. Indeed, it can be desirable to retain at least part of the natural sugar structures, particularly where these are a useful antigen. Also to be noted is the fact that due to the peculiar nOMVs composition and conformation as above detailed, the polysaccharide moiety can be conveniently activated by the present process leading to the formation of a highly reactive oxidized nOMVs intermediate species. In a preferred embodiment, for a given saccharide moiety of interest, the proportion of oxidised residues can range from 1% to 100%, preferably from 10-50%, or from 20-40%, or from 20-35%, whereas oxidation of 20-35% within an -OAg structure is particularly preferred. In this direction, it has been found that said ranges allow for efficient conjugation with minor or substantially assent impact on the -OAg structural integrity. Also, it has been noticed that higher nOMV oxidation degree corresponds to lower -OAg size, meaning that there is major impact on native -OAg structure and its ability to induce a specific immune response. The proportion of oxidised residues can be determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), by comparing the intact sugar residues pre- and post-oxidation. In this direction, it was found that the concentration of the oxidising agent and of the nOMV, along with the pH may influence the overall conduct of the oxidation step, as for instance indicated in the present Example 2, Table 2c. Thus, in a preferred embodiment, the oxidation agent is used in excess over the starting nOMV, where a molar excess of 3:1 or 2:1 respect to the number of monosaccharides that can be subjected to oxidation is particularly preferred. The oxidizing agent is preferably used as aqueous solution in a concentration ranging from 0.5 mM to 20 mM, preferably from 3 mM to 20 mM, where concentrations from 10 to 20 mM and from 0.5 to 5 or from 3 to 5mM are still more preferred.

The concentration of nOMV is preferably comprised between 0.2 and 5 mg/mL.

Preferably, the pH is comprised between 4 and 8, whereas value from 5 and 7 are particularly preferred. To this extent, the pH may be adjusted using a buffer agent, such as acetate/phospate and the like.

Said parameters can be conveniently set in order to have a preferred degree of oxidation comprised between 20% and 35% over the subjected saccharide moiety. This allows having an efficient further conjugation with the selected antigen, without substantially impacting the saccharide moiety structure.

For instance, Rha residues in an -OAg functionality can be oxidised as e.g. indicated in the below Scheme 2 using NaIO₄.



Scheme 2

The oxidation step is typically performed at room temperature (e.g. from about 15° C to about 40° C), for a suitable time, e.g. comprised from 30 min to 3 h, depending for example on the amount and type of considered nOMV. In any case, it has been found that no substantial nOMV crosslink and/or aggregation occurred. This is of utmost importance also for the effectiveness of the subsequent conjugation step with the selected antigen as herein described in details.

After oxidation, nOMVs can optionally be subjected to a reduction step, for example with NaBH_4 , to stabilise the oxidised nOMV by removing the formed CHO groups. The stabilised oxidised nOMV may then be stored and/or further characterised.

Typically, after the activation step of the present process, the obtained oxidized nOMVs are isolated and purified e.g. by ultracentrifugation at 4° C at 110000 rpm for 30 min, and subsequently reacted with the selected antigen.

Thus in a preferred embodiment, the process comprises the steps of:

- (i) activation of the saccharide moiety on the nOMV surface, preferably by oxidation;
- (i-bis) isolation of the thus obtained oxidised nOMV; and
- (ii) connection of the oxidised nOMV of step (ii) with at least a selected antigen, optionally via a divalent linker.

In a still preferred embodiment, the process is performed in the presence of an alkaline sulphite, preferably Na_2SO_3 . This is particularly advantageous because by quenching the oxidation reaction with Na_2SO_3 , it is possible to perform the process in one step, i.e. avoiding the isolation of the intermediate oxidised nOMV (step (i-bis) above), as exemplified in the Example 5. This

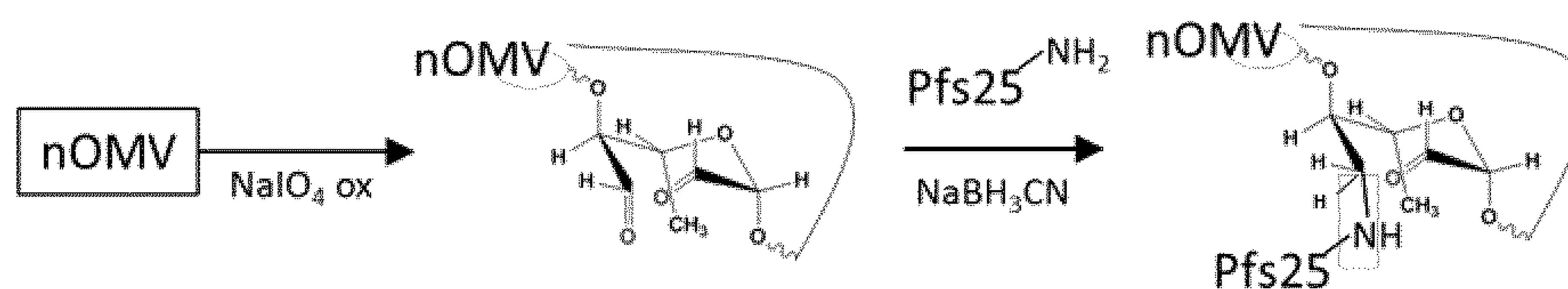
allows saving time, thus obtaining the final conjugate in a simple and effective way. In practice, and according to an exemplified embodiment, after the activation step (i) the reaction is quenched with a proper amount of the alkaline sulphite, and let to react for a proper frame of time (generally comprised from 5 to 20 minutes) in order to neutralize the excess of the oxidizing agent. After that, the selected antigen is directly added to the mixture (*i.e.* without isolation of the oxidised nOMV), according to step (ii), thus obtaining the nOMV conjugates of the invention.

As an alternative, the carbonyl aldehyde group of the saccharide moiety obtained by the oxidation step can be further modified to form a proper functionality which can then be reacted with the selected antigen or with a linker as the case may be (in this case to give a vesicle-linker conjugate which can then be coupled to the selected antigen).

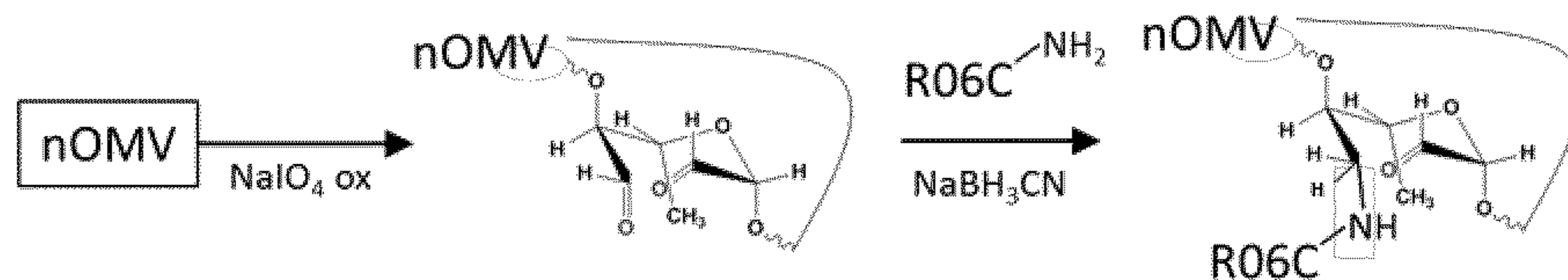
The selected antigen is typically added at 1:1 w/w ratio with respect to the used nOMV, at room temperature, for a proper frame of time, *e.g.* comprised from 2 hours to 24 hours. When the antigen is derivatised with a linker, the reaction is conveniently carried out using an excess of antigen over the nOMV, preferably a 2:1 or more preferably a 3:1 w/w ratio, as indicated for instance in the present Example 3.

In a particularly preferred embodiment, the process of the invention comprises the steps of: (i) oxidation of a saccharide moiety; and (ii) reaction of the oxidized moiety with an amino group on a selected antigen residue. Even more preferably, said selected antigen residue is an amino $-NH_2$ group on a lysine residue within a polypeptide selected antigen.

Preferably, coupling of the oxidized saccharide moiety of the nOMV with the amino group, preferably a free $-NH_2$ group, of the antigen is achieved by reductive amination, more preferably using $NaBH_3CN$, *e.g.* according to procedure known in the art. The $NaBH_3CN$ is used in weight amounts (w/w) comprised from 3 to 1/3, preferably 1 to 1, over the oxidized nOMV. Practically, the $NaBH_3CN$ can be added together with the selected antigen, directly to the oxidised nOMV intermediate product, as generally illustrated in Schemes 3 and 4 below, using, by way of example, a nOMV that is conjugated via an oxidized rhamnose unit to malaria membrane proteins Pfs25 or R06C, respectively.

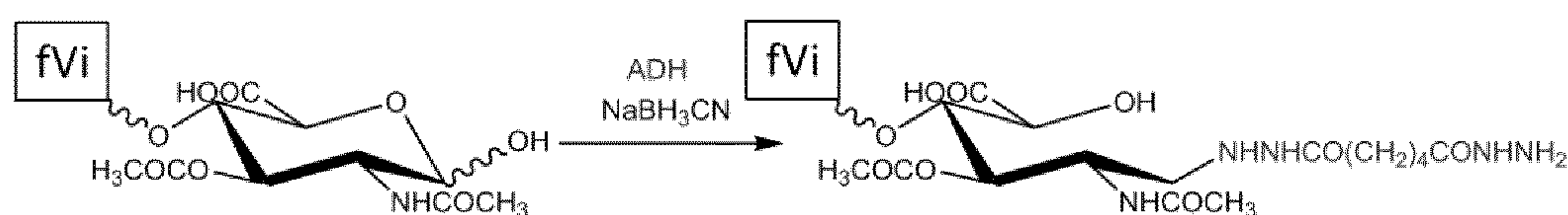
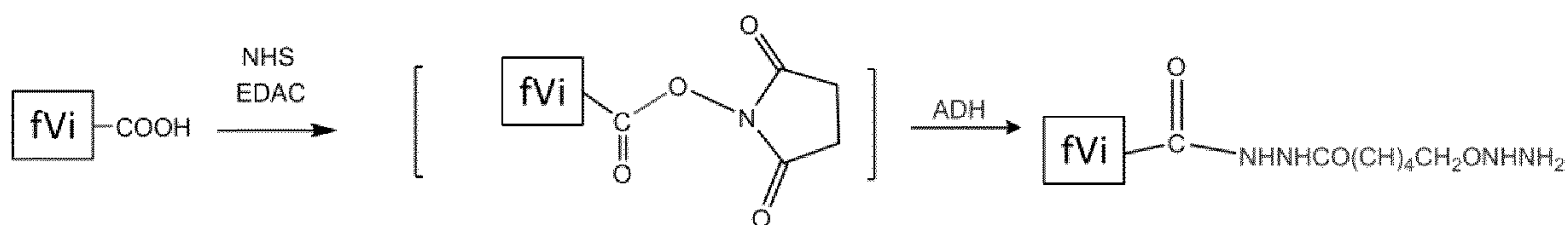


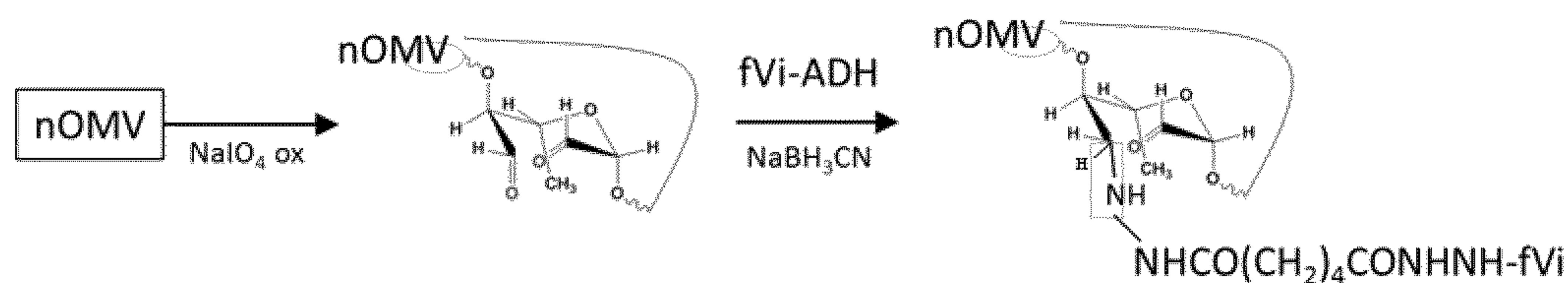
Scheme 3

**Scheme 4**

As an alternative embodiment, the selected antigen may be modified, either by introducing a linker group or by converting a functional group on the antigen into another functional group suitable for the reaction with the activated saccharide moiety on the nOMV, or with a linker of the vesicle-linker conjugate when used. In particular, if the selected antigen is a saccharide, it may be modified by reaction with a linker either randomly (r), meaning that the linker is introduced at multiple points along the sugar chain, or selectively (s), meaning that the linker is introduced at the reducing end of the sugar chain (*i.e.* at only one position). In a preferred embodiment, the linker is selective introduced at the terminal position of the selected antigen, as for instance indicated in the Example 3.

Selective modification of the antigen is preferably achieved by reaction with adipic acid dihydrazide (ADH) in the presence of NaBH_3CN , as generally shown in Scheme 5 using fVi as the antigen. Random modification of the antigen is preferably achieved by activation of one or more carboxylic acid groups on the antigen, for instance by using NHS/EDAC, and subsequent reaction with ADH, as shown in Scheme 6 using fVi as the antigen. This type of conjugation reaction is illustrated in Scheme 7 below, using, by way of example, a nOMV that is conjugated via an oxidized rhamnose unit to fVi modified to include a $-\text{NH}_2$ by reaction with ADH.

**Scheme 5****Scheme 6**

**Scheme 7**

An alternative embodiment of the invention refers to a process comprising the steps of: a) modifying a selected antigen to include an amino group, preferably -NH₂; b) activating a nOMV by oxidising a hydroxyl group of a saccharide moiety as discussed above, and c) connecting the oxidized saccharide moiety to the modified antigen of step a), preferably, by reductive amination, as discussed above.

An alternative embodiment of the invention refers to a method of making a nOMV-antigen conjugate comprising connecting an oxidized saccharide moiety of an activated nOMV to a modified antigen, thereby making the nOMV conjugates of the invention, wherein the antigen was modified to include an amino group, preferably -NH₂, and wherein the nOMV was activated by oxidising a hydroxyl group of a saccharide moiety. In a further embodiment of the invention, the reacting step is reductive amination.

As above set forth in details, the nOMV conjugates of the invention comprise an activated nOMV surface saccharide moiety directly connected to a selected antigen.

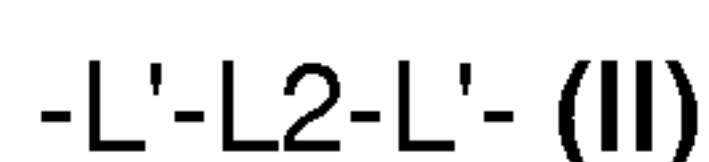
In an equally preferred embodiment, the activated nOMV surface saccharide moiety is connected to the selected antigen indirectly, *e.g.* via a linker unit. This latter will generally be a bifunctional linker, using one functional group to react with the nOMV (via the activated saccharide moiety) and another functional group to react with the selected antigen. The linker can be a heterobifunctional linker or a homobifunctional linker of general formula (I):



wherein:

X and X' groups are independently the same or different as each other, and react with activated nOMV surface saccharide moiety and the selected antigen respectively; and

L is a linking spacer, preferably of general formula (II):



wherein:

the two L' groups are independently the same or different as each other and are selected from: a carbonyl (C=O), ester (-C(O)O-) or amido group (-C(O)NR₁-), wherein R₁ is H or, a straight, or, when comprising at least 3 carbon atoms, a branched cyclic C₁-C₁₀ alkyl group having 1 to 10 carbon atoms (*e.g.* C₁, C₂, C₃, C₄, C₅, C₆, C₇, C₈, C₉, C₁₀); and

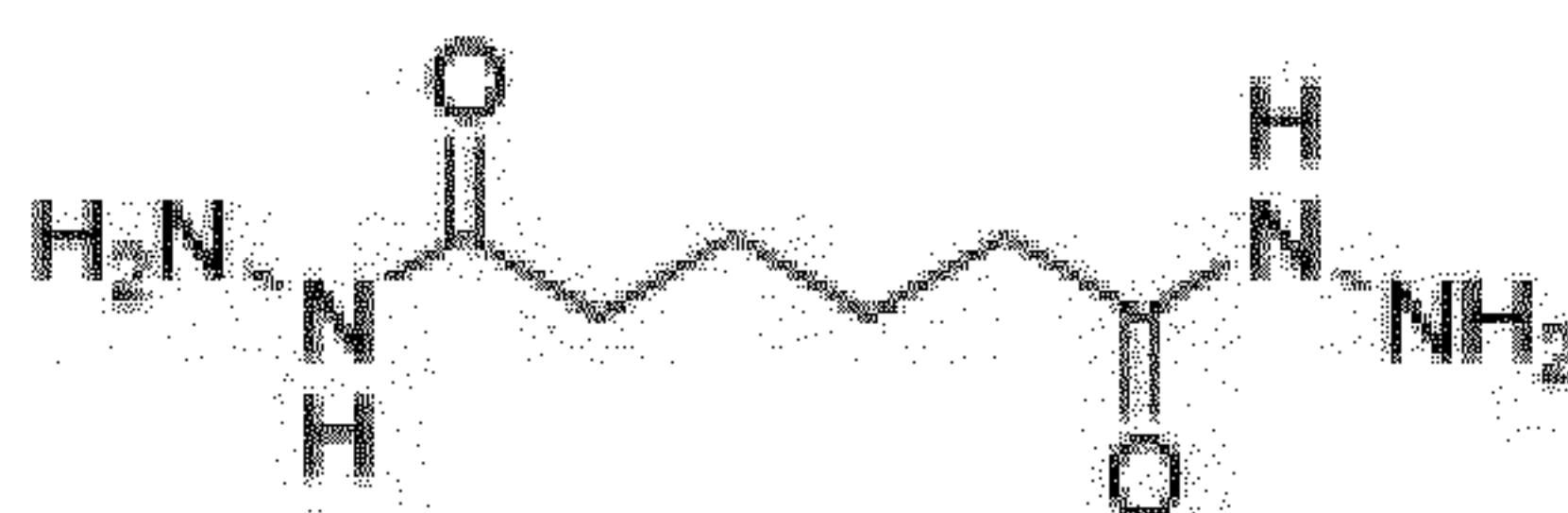
L2 is a straight or branched C1-C10 alkyl group having 1 to 10 carbon atoms, preferably having C4 carbon atom, even more preferably in the form of a straight chain.

X group is preferably selected from: $-\text{NH}_2$, $-\text{NH}-\text{NH}_2$, $-\text{O}-\text{NH}_2$, optionally substituted sulfo-N-hydroxysuccinimide and N oxysuccinimide residue.

- 5 Where the reactions with both the nOMV and the selected antigen involve the same functional groups it is preferred to use a bifunctional linker of general formula (I), wherein both the two X groups are the same.

When the functional groups on the nOMV saccharide moiety and on the selected antigen are both aldehydes it is preferred to use a homofunctional linker having X selected from: $-\text{NH}_2$, $-\text{NH}-$

- 10 NH_2 or $-\text{O}-\text{NH}_2$ reactive group. In a still preferred embodiment, the linker is the adipic acid dihydrazide (ADH) of general formula:



ADH

- 15 The linker may then be reacted with the nOMV and/or antigen by reductive amination as above set forth.

Preferred bifunctional linkers particularly useful for the reaction with amine groups of the selected antigen, are selected from: acryloyl halides, preferably chloride, disuccinimidyl glutarate, disuccinimidyl suberate and ethylene glycol bis[succinimidylsuccinate].

- 20 Other still preferred linkers are selected from: β -propionamido, nitrophenyl-ethylamine, haloacyl halides, glycosidic derivatives linkages, 6-aminocaproic acid.

In a still preferred embodiment, the linker is selected from: N-hydroxysuccinimide, N oxysuccinimide, even more preferably from adipic acid N-hydroxysuccinimide diester (SIDEA).

- 25 When the reaction with the nOMV and the antigen involves different functional groups (such as an amine on the nOMV and a thiol on the antigen,) it will be understood that a heterobifunctional linker will be used capable to selectively react with both the different functional groups. In this case, preferred heterobifunctional linkers are selected from at least one of: succinimidyl 3-(2-pyridyldithio)propionate (SPDP), succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate (LC-SPDP), sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate (sulfo-LC-SPDP), 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene (SMPT), sulfosuccinimidyl-6-[α -methyl- α -(2-pyridyldithio)tolueamideo]hexanoate (sulfo-LC-SMPT), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-
- 30

MBS), N-succinimidyl (4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl (4-iodoacetyl)aminobenzoate (sulfo-SIAB), succinimidyl 4-(N-maleimidophenyl)butyrate (SMPB), sulfosuccinimidyl 4-(N-maleimidophenyl)butyrate(sulfo-SMPB), N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS), N-γ-maleimidobutyryl-oxysulfosuccinimide ester (sulfo-GMBS),
 5 succinimidyl-6-((((4-(iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino)hexanoate (SIACX), succinimidyl 6[6-(((iodoacetyl)amino)hexanoyl)amino]hexanoate (SIAXX), succinimidyl-4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (SIAC), and succinimidyl 6-[(iodoacetyl)amino]hexanoate (SIAX) and p-nitrophenyl iodoacetate (NPIA).

In a further embodiment, the process encompasses the possibility to recycle the unreacted
 10 selected antigen particularly when in form of polypeptide. To this extent, it has been found that the unreacted antigen from the conjugation mixture can be conveniently recycled in the conjugation step, thus improving the overall efficiency of production of the conjugates of the invention, and obtaining final conjugates still endowed with remarkable immunogenicity (see present Example 6).

As above explained in details, the present process allows the preparation of the nOMV
 15 conjugates of the invention in a simple and convenient way, also requiring fewer steps when compared to previous methods for the preparation of similar conjugated derivative s (e.g. starting from dOMV). Thus the invention also refers to a nOMV conjugate obtained (or obtainable) by the process of the invention, according to the above described embodiments.
 20 Particularly, the present process does not necessarily require the expensive step of polypeptide antigen derivatisation, as well as not performing an extraction (e.g. using a detergent) or denaturation of the starting vesicles. Production and purification of nOMVs of the invention in fact is less expensive than for traditional carrier proteins and more robust and consistent than production of dOMV. nOMV used in the invention can be produced at high yields using e.g. two
 25 simple tangential flow filtration steps, and avoiding detergent extraction procedures. In addition, the unreacted selected antigen can be recycled from the conjugation mixture for use in the process, improving the efficiency of preparation of the conjugates, as exemplified in the Example 6. Also, the present invention offers an easy way to prepare a polyvalent vaccine *i.e.* a vaccine which includes multiple immunogens (typically from different pathogens) by properly
 30 choosing the nOMV and the selected antigen as herein described in more details. In fact, due to its versatility, the present process may be conveniently and effectively applied to nOMV from different sources (e.g. *Salmonella*, *Shigella* and meningococcal), being applied with success to both protein and saccharide antigens. Finally, it has to be noted that the present process not only allows for the preparation of highly immunogenic conjugates, but also it does not
 35 substantially change the nOMV integrity and size distribution. This is particularly appreciated by the skilled in the art, because the absence of nOMV aggregates allows for a better yield and overall consistency and robustness of the present process.

The present invention is also useful for the preparation of variously functionalized nOMVs conjugates, allowing multivalent presentation of different antigens on the surface of the selected vesicle.

Thus, according to a preferred embodiment, the invention refers to a conjugate comprising a native outer membrane vesicle as above set forth, having at least a surface saccharide moiety connected to at least a first antigen, wherein said first antigen is connected to a second different antigen according to the general formula (I):



In this direction, the two selected antigens (herein indicated as Ag1 and Ag2) may be coupled together to give an Antigen-Antigen derivative (Ag1-Ag2), which can be subsequently connected to the selected nOMV surface saccharide moiety via a reductive amination procedure as above described.

Alternatively, the nOMV surface saccharide can be first connected to the selected Ag1 via a reductive amination procedure as above described, to give a nOMV-Ag1 conjugate, and subsequently a second Ag2 is connected to said nOMV-Ag1 conjugate, to give the conjugate of the above general formula (I).

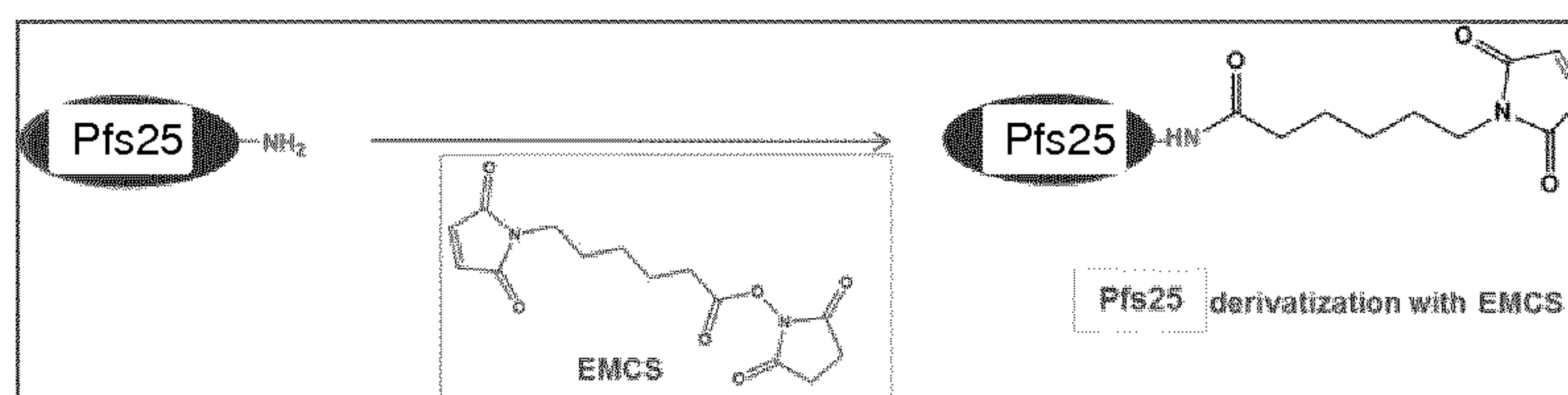
In any case, preferred native outer membrane vesicles are the GMMA vesicles, more preferably from *Neisseria MenB*. The Ag1 and Ag2 can be selected among the preferred antigens as above described, being protein or polysaccharide moieties. Preferably, the antigens used for the multi functionalization as herein contemplated are both proteins or both polysaccharides, or even protein or saccharide.

In any case, the connection between the antigens and the nOMV saccharide moiety can be carried out directly, or by using activating agents, or suitable linkers according to the herein described preferred embodiment.

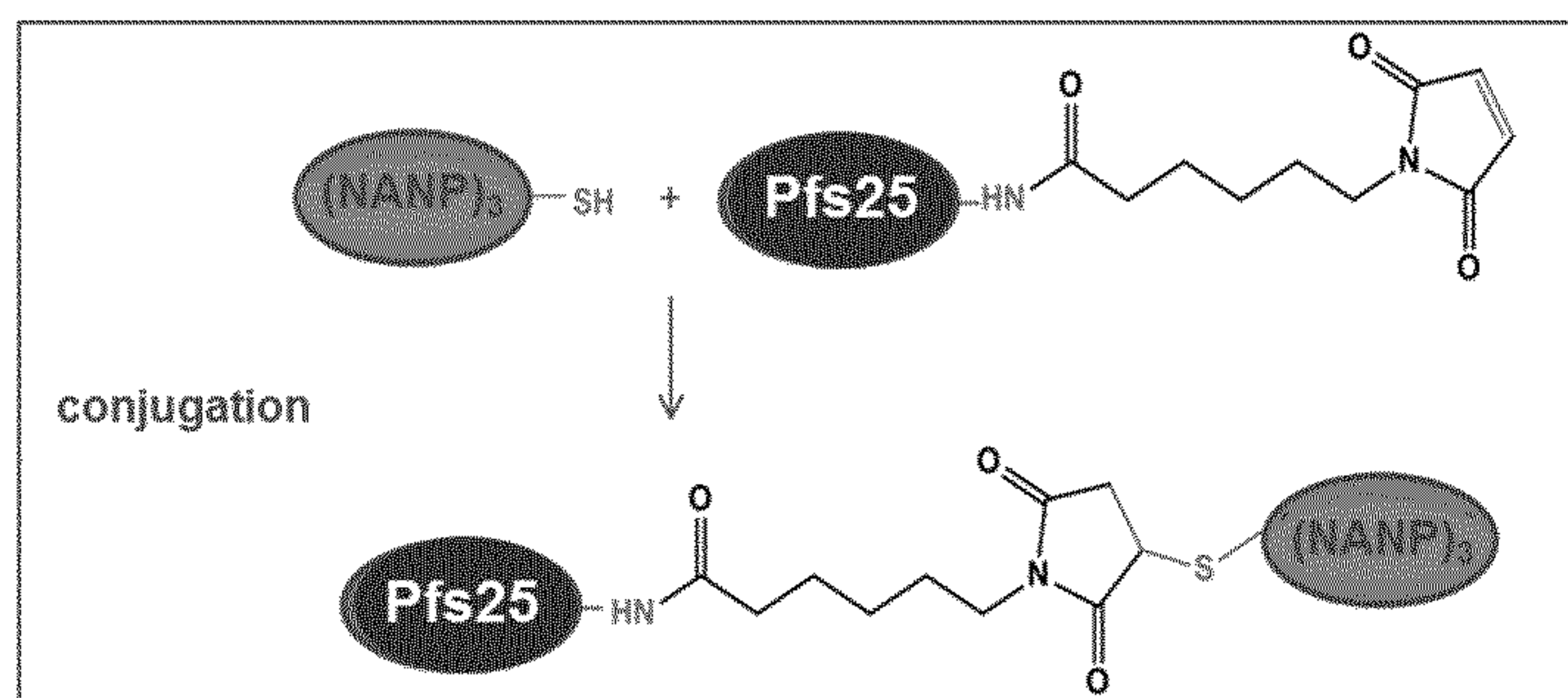
Thus according to a more preferred embodiment, the invention refers to a conjugate of the above general formula (I), wherein Ag1 comprises the (NANP)₃ protein antigen, Ag2 comprises the Pfs25 protein antigen, more preferably having the nOMV particle obtained from *S. Typhimurium*.

In more details, said conjugate is preferably prepared by a process comprising the steps of:

a) activating the Pfs25 antigen using EMCS, to give the below indicated activated Pfs25 intermediate:



b) connecting said activated Pfs25 intermediate with (NANP)₃ to give the Pfs25-EMCS-(NANP)₃ derivative :



c) reacting the Pfs25 portion of such derivative with the native outer membrane vesicle intermediate, via reductive amination reaction according to the above described embodiments to give the GMMA-Pfs25-(NANP)₃ conjugate.

- 5 Western blot analysis confirmed the formation of the conjugate where the Pfs25 is connected to the GMMA surface saccharide moiety, and no aggregation is detected.

According to a further embodiment, the present invention refers to an immunogenic conjugate comprising a native outer membrane vesicle, having at least a surface saccharide moiety connected to a first antigen (Ag1) via a reductive amination procedure as above described, and
 10 at least a surface saccharide moiety connected to a second different antigen (Ag2) via a reductive amination procedure as above described to give a conjugate indicated by the general formula (II):



- According to a preferred embodiment, in the above general formula (II), the nOMV is a GMMA
 15 from MenB, Ag1 is a capsular polysaccharide from meningococcal serogroup C, and Ag2 is a capsular polysaccharide from meningococcal serogroup C.

The conjugates of formula (II) can advantageously provide selective multi functionalization of nOMV, by using a specific functionalization pattern, by using the reductive amination procedure according to the invention. The skilled person will recognize that in light of the versatility of the
 20 proposed technology, the present invention may be suitably used for the multi functionalization of nOMV, preferably GMMA, even with more than 2 different antigens. Beside the possibility of selecting different antigens, the present invention also allows for the introduction of different amount of antigens, thus modulating the antigen/nOMV ratio according e.g. to the selected antigen or nOMV.

- 25 According to a further aspect, the invention refers to the above described nOMV-antigen conjugate for use as a medicament, particularly as immunogenic agent, even more preferably for one or more of the pathogens as herein indicated. In other words, the invention refers to the use of the present nOMV conjugates for the manufacture of an immunogenic composition.

According to a further aspect, the invention thus refers to an immunogenic composition,
 30 preferably a vaccine, comprising a conjugate of the invention and at least one additional pharmaceutically acceptable carrier, excipient or adjuvant. Generally, pharmaceutically acceptable carrier or excipient, can be any substance that does not itself induce the production of antibodies harmful to the patient receiving the composition, and which can be administered

without undue toxicity. Pharmaceutically acceptable carriers and excipient are those used in the art, and can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles, according to the prior art.

- 5 The invention also provides a method for raising an immune response in a vertebrate, preferably a mammal, comprising administering a conjugate of the invention to the mammal or other vertebrate. The invention also provides conjugates of the invention for use in such methods. The immune response is preferably protective and preferably involves antibodies. The method may raise a booster response. The mammal is preferably a human. The subject in
10 which disease is prevented may not be the same as the subject that receives the conjugate of the invention. For instance, a conjugate may be administered to a female (before or during pregnancy) in order to protect offspring (so-called 'maternal immunisation'). Conjugates of the invention may also be used to immunise other mammals e.g. cattle, sheep and pigs (especially against *Salmonella* sp.), and other non-mammal vertebrates including fish and poultry.
- 15 The invention provides conjugates for use in therapy (e.g. as immunogenic compositions or as vaccines). The invention also provides a conjugate for use in a method for raising an immune response in a vertebrate, preferably a mammal. The invention also provides the use of a conjugate in the manufacture of a medicament for raising an immune response in a vertebrate, preferably a mammal. The uses and methods are particularly useful for preventing/treating a
20 variety of diseases, depending on the antigens and nOMVs within the conjugates as above set forth. Preferred conjugates of the invention can confer an antibody titre in a patient that is superior to the criterion for seroprotection for each antigenic component for an acceptable percentage of human subjects. Antigens with an associated antibody titre above which a host is considered to be seroconverted against the antigen are well known, and such titres are
25 published by organisations such as WHO. Preferably more than 80% of a statistically significant sample of subjects is seroconverted, more preferably more than 90%, still more preferably more than 93% and most preferably 96-100%.

Immunogenic compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (e.g. subcutaneously,
30 intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular administration is preferred e.g. to the thigh or the upper arm. Injection may be via a needle (e.g. a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is about 0.5 ml. The invention may also be
35 used to elicit systemic and/or mucosal immunity. Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. A primary dose schedule may be followed

by a booster dose schedule. Suitable timing between priming doses (e.g. between 4-16 weeks), and between priming and boosting, can be routinely determined.

Infections affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectable, either
 5 as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition may be prepared for topical administration e.g. as an ointment, cream or powder. The composition be prepared for oral administration e.g. as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration e.g. as an inhaler, using a fine powder or a
 10 spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration e.g. as drops. Compositions suitable for parenteral injection are most preferred. The composition is preferably sterile. It is preferably pyrogen-free. It is preferably buffered e.g. at between pH 6 and pH 8, generally around pH 7. Compositions of the invention may be isotonic with respect to humans. Immunogenic
 15 compositions comprise an immunologically effective amount of a conjugate of the invention, as well as any other of other specified components, as needed. Dosage treatment may be a single dose schedule or a multiple dose schedule (e.g. including booster doses). The composition may be administered in conjunction with other immunoregulatory agents.

Adjuvants which may be optionally used in compositions of the invention include, but are not
 20 limited to insoluble metal salts, oil-in-water emulsions (e.g. MF59 or AS03, both containing squalene), saponins, non-toxic derivatives of LPS (such as monophosphoryl lipid A or 3-O-deacylated MPL), immunostimulatory oligonucleotides, detoxified bacterial ADP-ribosylating toxins, microparticles, liposomes, imidazoquinolones, or mixtures thereof. Other substances that act as immunostimulating agents are disclosed for instance in Watson, *Pediatr. Infect. Dis. J.*
 25 (2000) 19:331-332. The use of an aluminium hydroxide and/or aluminium phosphate adjuvant is particularly preferred. These salts include oxyhydroxides and hydroxyphosphates. The salts can take any suitable form (e.g. gel, crystalline, amorphous, etc.).

Conjugates of the invention which include nOMVs from one pathogen and a selected antigen from a second pathogen can be useful as multivalent vaccines. Pairs of pathogens which may
 30 be combined (one as antigen, and the other as a nOMV vesicle) include, but are not limited to: *N. meningitidis* and non-typhoidal *Salmonella* (e.g. *Salmonella Typhimurium* or *Salmonella Enteritidis*); *P. falciparum* and non-typhoidal *Salmonella*; *Salmonella Typhi* and non-typhoidal *Salmonella*; ETEC and *Shigella* sp.; *Group A Streptococcus* (GAS) and *N. meningitidis*; and GAS and non-typhoidal *Salmonella*.

35 Preferred pairings of the invention are indicated in the following Table A.

Table A: preferred nOMV-Ag combinations

nOMV	Antigen
<i>Salmonella</i> Typhimurium	<i>Neisseria meningitidis</i> fHbp
<i>Salmonella</i> Typhimurium	Plasmodium falciparum CSP
<i>Salmonella</i> Typhimurium	Plasmodium falciparum Pfs25
<i>Salmonella</i> Typhimurium	Plasmodium falciparum RO6C
<i>Salmonella</i> Typhimurium	Plasmodium falciparum RO10C
<i>Salmonella</i> Typhimurium	<i>Escherichia coli</i> CTF1232
<i>Salmonella</i> Typhimurium	S. Typhi Vi saccharide
<i>Neisseria meningitidis</i>	<i>Neisseria meningitidis</i> fHbp
<i>Neisseria meningitidis</i>	Poly-rhamnose oligosaccharide
<i>Shigella</i> , preferably <i>sonnei</i>	<i>Escherichia coli</i> CTF1232
<i>Neisseria meningitidis</i> B	Capsular saccharide from MenA
<i>Neisseria meningitidis</i> B	Capsular saccharide from MenC

Thus, the present nOMV-antigen conjugates are particularly useful as immunogenic agents against the pathogens listed in Table A.

5 Conjugates of the invention which include nOMVs from one pathogen and a selected antigen from a second pathogen can be useful as immunogenic compounds for the preparation of multivalent vaccines. Thus the invention provides a composition comprising a conjugate of the invention and one or more of the following further antigens:

- a saccharide antigen from *N. meningitidis* serogroup A, C, W135 and/or Y,
- 10 - a saccharide antigen from *Streptococcus pneumoniae*,
- an antigen from *hepatitis A* virus, such as inactivated virus,
- an antigen from *hepatitis B* virus, such as the surface and/or core antigens,
- a diphtheria antigen, such as a *diphtheria* toxoid e.g. the CRM197 mutant,
- a tetanus antigen, such as a *tetanus* toxoid,
- 15 - an antigen from *Bordetella pertussis*, such as *pertussis* holotoxin (PT) and *filamentous haemagglutinin* (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3,
- a saccharide antigen from *Haemophilus influenzae* A or B,
- polio antigen(s) such as IPV,
- 20 - measles, mumps and/or *rubella* antigens,
- *influenza* antigen(s), such as the haemagglutinin and/or neuraminidase surface proteins,
- an antigen from *Moraxella catarrhalis*,
- an protein antigen from *Streptococcus agalactiae* (group B *streptococcus*),
- a saccharide antigen from *Streptococcus agalactiae* (group B *streptococcus*),
- 25 - an antigen from *Streptococcus pyogenes* (group A *streptococcus*),
- an antigen from *Staphylococcus aureus*.

The invention will be now described by the following experiment part, without posing any limitation to its scope.

EXPERIMENTAL PART

5 EXAMPLE 1: nOMV production

Preferred nOMVs used in the present experimental part are GMMA prepared from Δ tolR strains of *S. Typhimurium* or *S. sonnei*, as e.g. disclosed in Clin Vaccine Immunol. 2016 Apr; 23(4): 304–314 and PLoS One. 2015; 10(8): e0134478 respectively.

Characteristics of said nOMVs were as indicated in the following Table 2.

10 Table 2: characteristics of purified nOMVs prepared from Δ tolR strains of *S. Typhimurium* or *S. sonnei*.

	<i>S. Typhimurium</i> 1418 (Δ tolR)	<i>S. sonnei</i> 1790 (Δ tolR Δ htrB)
Diameter (nm)	131.5	140
Surface charge (mV)	-14.1	-9.87
Lipid A / mg vesicles	172.8	155.4
OAg / total protein weight ratio	0.84	0.039

EXAMPLE 2: nOMV oxidation

15 A variety of oxidation conditions were tested. For example, for oxidation of nOMV Δ tolR 1418, NaIO₄ concentrations in the range 5-20 mM were tested. The effect of oxidation on the -OAg chain length was assessed. Chain length was reduced as oxidation progressed. Higher NaIO₄ concentrations tended to reduce the OAg size. It was verified that increasing NaIO₄ molarity not only led to higher oxidation levels, from 5 to 45% (with rhamnose the main sugar involved in the process), but also reduced -OAg chain length. At the same time there was no change in vesicle size distribution, and integrity of the vesicles was maintained.

20 This was verified by dynamic light scattering (dls), nanoparticle tracking analysis (NTA) and high performance liquid size exclusion chromatography/multi-angle light scattering (HPLC-SEC/MALS; see Table 2a). It was also verified that NH₂ groups on nOMV did not react with produced CHO groups in conditions of reductive amination resulting in bigger cross-linked particles (Table 2b).

Table 2a – analysis of oxidised nOMV (obtained by reaction with 10 and 20 mM NaIO₄) by dls and HPLC-SECMALS.

Sample	Z-Average (Pdl) (nm by dls)	Rw (nm by MALS)
1418 ΔtolR nOMV	131.5 (0.219)	30.5
1418 ΔtolR nOMVox 10mM	129.5 (0.273)	27.4
1418 ΔtolR nOMVox 20mM	135.8 (0.224)	30.5

Table 2b - no reaction of nOMV under conditions of reductive amination verified by dls and HPLC-SEC/MALS.

Sample	Z-Average (Pdl) nm by dls	Rw nm by MALS
1418 ΔtolR nOMVox 20mM NaIO ₄	135.8 (0.224)	30.5
1418 ΔtolR nOMVox 20mM NaIO ₄ after reaction with NaBH ₃ CN	131.7 (0.291)	31.2

In conditions of reductive amination, without nOMV, the foreign antigen to conjugate does not give any aggregation, as verified for several antigens (e.g. fHbp, Pfs25 and CTF1232).

In addition, it was verified that NaBH₃CN does not reduce S-S bonds, which could otherwise affect conformation of proteins such as Pfs25. Pfs25 was treated with NaBH₃CN by mimic conjugation conditions, and the same reaction was performed with DTT as a reducing agent for comparison. After mixing overnight at room temperature, analysis by SDS PAGE and HPLC-SEC of Pfs25 treated with NaBH₃CN, in contrast to Pfs25 treated with DTT, showed no changes of the protein compared to fresh Pfs25. The same results were confirmed by MALDI-MS analysis, when the protein was treated with iodoacetamide (IAA) in the presence of NABH₃CN or DTT.

Further experiments on *S. Typhimurium* triple mutant nOMV (*S. Typhimurium* 2192 nOMV ΔtolR ΔPagP ΔmsbB) showed that low NaIO₄ concentrations (3-5 mM) were also sufficient to obtain good oxidation levels. Exemplary results are shown in Table 2c, using nOMV concentration in

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the range 0.2-4 mg/mL, pH in the range 5-8, and NaIO₄ concentration in the range 0.5-5 mM. The resultant vesicles were assessed for % nOMV recovery, % rhamnose oxidation, nOMV size, and OAg size.

Table 2c *S. Typhimurium triple mutant nOMV oxidation.*

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
	A:[nOMV]	B:[NaIO ₄]	C:pH	% nOMV recovery (micro BCA)	% Rha oxidation (HPAEC-PAD)	nOMV size (Z average r dls)	OAg size (MP dRI)
	μg/mL	mM		%	%	nm	kDa
1	2100	2.75	6.5	72	10	50.28	20907
2	2100	2.75	8	75	6	50.77	24215
3	4000	0.5	5	79	0	51.52	31148
4	2100	2.75	5	80	13	48.09	17935
5	200	5	5	87	58	42.39	6826
6	2100	2.75	6.5	77	7	50.4	20276
8	4000	2.75	6.5	81	3	50.89	25221
9	2100	5	6.5	82	23	47.56	10170
10	2100	2.75	6.5	80	10	49.8	18493
11	2100	0.5	6.5	85	1	50.39	30592
12	200	0.5	5	83	7	50.19	25131
13	2100	2.75	6.5	82	9	50.85	18493
14	2100	2.75	6.5	78	10	49.07	20589
15	200	0.5	8	77	2	51.42	30347
16	200	5	8	72	20	47.69	10980
17	2100	2.75	6.5	83	8	49.09	20536
18	4000	5	8	80	11	48.44	18212
19	4000	0.5	8	78	1	50.86	30208

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20	4000	5	5	86	15	48.6	16611
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Similar rates of nOMV recovery were seen for all reaction conditions, as tested by micro bicinchoninic acid protein assay (micro BCA). It was further verified that none of the reaction conditions tested gave rise to nOMV crosslinking or aggregation.

The percentage rhamnose oxidation was affected by both nOMV concentration and NaIO₄ concentration, with lower nOMV concentration and higher NaIO₄ concentration tending to give higher rates of rhamnose oxidation. Accordingly, in general, the nOMV concentration and/or the NaIO₄ concentration can be manipulated to achieve the desired rate of rhamnose (or other sugar) oxidation.

To compare vesicles with different -OAg size and levels of rhamnose oxidation, the vesicles from runs 5, 14 and 16 of Table 5 were treated with NaBH₄ to remove aldehyde (CHO) groups and stabilise the nOMV vesicles, and the rhamnose oxidation level and -OAg size were assessed again after treatment. The results are shown below in Table 2d.

Table 2d: runs 5, 14 and 16 treatment with NaBH₄.

Conditions for oxidation	% Rha ox expected	OAg size expected	Pre reduction		Post reduction	
			% Rha ox obtained	OAg size obtained	% Rha ox obtained	OAg size obtained
Run 5	58	6826 Da	58	7481 Da	53	6039 Da
Run 16	20	10980 Da	14.4	18162 Da	16	16528 Da
Run 14	10	20589 Da	13	24224 Da	12	21163 Da

The results show that the reduction step did not affect -OAg length or the degree of nOMV oxidation. Similar results were obtained in a separate experiment.

EXAMPLE 3: nOMV-Ag conjugation step (fVi- *S. Typhimurium* nOMV; indirect conjugation via linker)

fVi was modified by reaction with an ADH linker, either randomly (r) or selectively (s). The modified fVi was then conjugated to oxidised *S. Typhimurium* nOMV using reductive amination. When fVi was randomly activated with ADH, a fVi to nOMV 1:1 weight ratio was used in conjugation, while a 3:1 weight ratio was used when fVi was terminally derivatised with ADH. The conjugates were characterized using micro BCA/Lowry assay to determine the total protein content (nOMV recovery); high-performance anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD) was used to determine total Vi content (no nOMV interference); high performance liquid chromatography size-exclusion chromatography (HPLC-SEC using a TSK gel 3000 PWxl column) was used to estimate the free Vi % using differential Refractive Index (dRI) and dynamic light scattering high performance liquid chromatography

size-exclusion chromatography multi-angle static light scattering (DLS/HPLC-SEC MALS) was used to determine size. The effect of conjugation conditions of this conjugation method on the final number of fVi chains per nOMV was assessed considering the HPAEC-PAD collected data along with the number of nOMV by Nanoparticle Tracking Analysis (NTA).

5 The results are given in Table 3.

Table 3: influence of the NaIO₄ concentration and pH on fVi-nOMV conjugates.

run	fVi avMW / kDa	r/s	% fVi RU activated (r) /% fVi chains activated (s)	[NaIO ₄] for nOMV oxidation	pH of conjugation	nOMV recovery %	w/w ratio fVi/nOMV	chains fVi /particle nOMV
1.	48.5	r	24.4	20	4.5	30.7	0.45	97
2.	48.5	r	24.4	20	6	43.8	0.35	76
3.	48.5	r	30.4	20	7.2	58.8	0.2	43
4.	48.5	s	>95	10	7.2	69	0.08	17
5.	48.5	s	>95	20	4.5	48.9	0.44	95
6.	48.5	s	>95	10	4.5	80	0.43	93
7.	23	r	11	20	4.5	80	0.08	37
8.	23	r	11	10	4.5	89.4	0.07	32
9.	23	r	11	10	6	77	0.04	18
10.	8	r	23.8	10	4.5	100	0.12	158
11.	3.8	r	15.5	20	4.5	100	0.05	138
12.	3.8	r	15.5	10	4.5	67.6	0.07	194
13.	3.8	r	15.5	10	6	82	0.02	55
14.	3.8	r	16.2	10	7.2	74	0.01	30

r: random linker (ADH) introduction along the fVi antigen.

s: selective linker (ADH) introduction at fVi antigen terminal end.

RU: repeating units.

avMW misured by HPLC-SEC by using dextran standards.

10

Performing reductive amination at pH 4.5 with 48.5 kDa fVi, resulted in higher fVi/nOMV ratios and more fVi chains per nOMV at both 10 mM and 20 mM of NaIO₄, than at higher pH. With

randomly modified fVi, precipitation occurred at pH 4.5; precipitation was avoided by working with selectively modified fVi. In general, selective chemistry was identified as a way to improve nOMV recovery and avoid precipitation. Precipitation was not observed for fVi with an avMW $\leq$ 23 kDa at low pH with randomly modified fVi chains.

- 5 Oxidation of nOMV with 10 mM of NaIO₄ instead of 20 mM resulted in improved conjugate recovery. Increasing the concentration of NaIO₄ did not impact the characteristics of the final conjugates.

When using 23 kDa fVi, lower fVi to nOMV ratios were obtained, which could be related to the lower derivatization percentage of fVi-ADH used for conjugation (11% of fVi repeating units).

- 10 pH and activation degree of fVi-ADH were identified as variables for modulation of the number of fVi chains linked per nOMV.

EXAMPLE 3a: Vi-S. Typhimurium nOMV conjugates (indirect conjugation via linker)

- 15 The conjugates 1 and 5 according to Table 3 above, having Vi:nOMV w/w ratios 0.45 and 0.44 respectively,) were tested in mice. For comparison, CRM197 was also used as a carrier, and simple mix of fVi + nOMV was also tested.

Mice were immunised subcutaneously at days 0 & 28 with the conjugates (1 μ g Vi dose) and an Alhydrogel adjuvant. Anti-Vi IgG titers were measured at days 0, 14, 28 and 42, and results are shown in Figure 2A. nOMV was non-inferior to CRM197, but was significantly better than the unconjugated mixture.

- 20 IgG titers against the -OAg were also assessed. Figure 2B shows anti-OAg titers for the vesicle alone or conjugated to Vi. Conjugation reduced the -OAg response by a small amount, but responses remained significant.

EXAMPLE 4: nOMV-Ag conjugation step (ETEC CTF1232- S. Typhimurium Δ tolR 1418 or S. *sonnei* 1790 nOMV; direct conjugation without linker)

- 25 CTF1232 is an *E.coli* antigen (SEQ ID NO: 14 with a C-terminus hexa-histidine tag that was conjugated to the two types of nOMV vesicles. The polypeptide includes five lysine residues which can be used for linking to oxidised saccharides in the vesicles.

- 30 For conjugation to CTF1232, the vesicles were oxidised in 100 mM sodium acetate (pH 5) with sodium periodate (20 mM for *S. Typhimurium*, 40 mM for *S. sonnei*) for 2 hours in the dark at room temperature. Oxidation in *S. Typhimurium* was preferential in rhamnose (Rha) residues, with about 30% of Rha units oxidized (calculated relative to mannose). Oxidation in *S. sonnei* impacted the core region of the LPS molecules.

500 μ g oxidised vesicles (total measured protein) from either *S. Typhimurium* Δ tolR 1418 or *S. sonnei* 1790 nOMV were reacted with 500 μ g CTF1232 with 1-2 mg NaBH₃CN at room

temperature over a weekend. Based on unreacted antigen quantification after conjugation, the antigen presence on the nOMV surface was calculated as <36% in *S. Typhimurium* and <31% in *S. sonnei*.

The results showed that reductive amination chemistry is suitable for conjugation of polypeptide antigens to vesicles. CTF1232 antigen was conjugated to the oxidised LPS of the two vesicles.

Mice were immunised with the protein alone, a mixture of the protein and *Shigella* nOMV, or the conjugates. Alhydrogel adjuvant was used in all groups. Immunisations were administered intranasally on days 0, 21 & 38 and immune responses were assessed on days 0, 14, 35 and 52. Anti-CTF1232 IgG titers are in Figure 3.

At day 14, 1418nOMV-CTF1232 conjugate was able to induce a response significantly higher than the protein alone ($p = 0.0005$) or physically mixed with nOMV ($p = 0.042$) (Kruskal-Wallis test with Dunn's post hoc analysis). In addition to the improvement in anti-CTF1232 titers, the conjugate has the further advantage of being a bivalent vaccine. No major differences were observed between 1790nOMV-CTF1232 and 1418nOMV-CTF1232 conjugates, meaning that both *Salmonella* and *Shigella* nOMV can work as good carriers for ETEC antigen.

EXAMPLE 5: nOMV-Ag conjugation step (Pfs25-*S. Typhimurium* nOMV conjugation in the presence of Na_2SO_3)

Malarial antigen Pfs25 was conjugated to the *S. Typhimurium* nOMV vesicles by two different chemistries: to proteins via SH-maleimido or click chemistry; or to NaIO_4 oxidised OAg. For the linkage to oxidised -OAg, a 1:1 ratio of nOMV:Pfs25 was used, at a Pfs25 concentration of 2.6 mg/ml in PBS with overnight incubation at room temperature. Conjugate formation was also obtained when excess NaIO_4 was quenched with Na_2SO_3 (a concentration of 10 mM was used in this experiment, for 10 minutes), followed by direct addition of Pfs25 into the same pot (0.2 mg/ml final concentration). Figure 4A shows anti-Pfs25 IgG titers in response to: the three conjugates; Pfs25 alone; or Pfs25 physically mixed with the vesicles. All the constructs were formulated with Alhydrogel. Mice were immunised subcutaneously at days 0 & 28 at 0.1 μg Pfs25/dose. Anti-Pfs25 IgG titers were measured at days 0, 14, 28 and 42.

Pfs25 alone induced an anti-Pfs25 IgG antibody response significantly lower than nOMV-SH-Pfs25 ($p=0.001$) and nOMV-ox-Pfs25 conjugate ($p=0.0095$). Pfs25 physically mixed with nOMV similarly induced a lower response than nOMV-SH-Pfs25 and nOMV-ox-Pfs25 ($p = 0.0038$ and $p = 0.0282$ respectively) (Kruskal Wallis test with Dunn's post hoc analysis). Interestingly Pfs25 linked through the sugar component on nOMV (nOMV-ox-Pfs25 conjugate) induced a similar antibody response to Pfs25 linked to proteins on nOMV (nOMV-SH-Pfs25 and nOMV-click-Pfs25) and higher than Pfs25 alone or physically mixed to nOMV.

Sera from Pfs25-NOMV conjugates showed transmission blocking activity when tested by standard membrane-feeding assay (SMFA; transmission reducing activity > 90% at 1:8 dilution and maintained at 1:16 dilution for nOMV-SH-Pfs25 and nOMV-ox-Pfs25).

The linkage of Pfs25 on nOMV did not impact on the anti-OAg IgG response. Also the conjugate obtained by reductive amination, where the chemistry used has an impact on -OAg structure and length, maintained high anti-OAg IgG titers. Accordingly, the presence of a foreign antigen on *S. Typhimurium* nOMV does not impact on anti-OAg IgG responses (see Figure 4C).

In a second study, the immunogenicity of Pfs25-nOMV conjugate (produced by reductive amination) was compared to Pfs25 physically mixed to nOMV at a dose of 1 µg Pfs25 without Alhydrogel.

Figure 4B shows the anti-Pfs25 IgG response induced in mice by Pfs25-nOMV conjugate compared to Pfs25 physically mixed to nOMV with no Alhydrogel, using the same immunisation schedule as for Figure 4A.

The conjugate was able to induce a significantly higher anti-Pfs25 IgG response than the protein mixed with nOMV ($p = 0.0002$; Mann-Whitney two-tailed analysis).

EXAMPLE 6: RO6C- *S. Typhimurium* nOMV conjugates (recycling step)

Plasmodium RO6C antigen was conjugated to oxidised *S. Typhimurium* nOMV vesicles using reductive amination. A further conjugate was produced by recycling unreacted RO6C from the first conjugation batch and re-using it for conjugation. The ratio of RO6C to total protein was measured by competitive ELISA, and was 7.2% for the non-recycled conjugate and 11.1% for the recycled conjugate. For comparison, RO6C alone was used. All the constructs were formulated with Alhydrogel.

Mice were immunised subcutaneously at days 0 and 28, and doses of 1, 4 and 20 µg RO6C were used. The recycled conjugate was tested at 4 µg RO6C dose. Anti-RO6C IgG titers were measured at days 0, 14, 28 and 42, and results are shown in Figure 5A. At day 42, a higher anti-RO6C IgG response was induced by the nOMV-RO6C conjugate compared to RO6C alone (Mann Whitney test, $p = 0.05$ at 1 µg dose, $p = 0.03$ at 4 µg dose and $p = 0.04$ at 20 µg dose). In addition, the nOMV-RO6C conjugate elicited an anti-RO6C IgG response in a dose dependent manner (Spearman rank, $p = 0.001$, day 42). All the constructs (at all doses) showed the ability to boost the response (day 14-day 42). Non-recycled and recycled conjugates at 4 µg dose were compared by Mann Whitney two-tailed test, showing the ability of the recycled conjugate to induce a response not inferior to the non-recycled one.

IgG titers against the -OAg were also assessed. Figure 5B shows anti-OAg IgG titers for the non-recycled and recycled vesicles. The nOMV doses corresponding to the RO6C doses of 1, 4

and 20 µg were 13 µg, 52 µg and 258 µg, respectively. For the recycled conjugate, the nOMV dose was 32 µg (corresponding to an RO6C dose of 4 µg).

EXAMPLE 7a-c: comparative examples

Example 7a: reaction of dOMV (from *Neisseria meningitidis* B) with fHbp v3 (no reaction)

5 The dOMVs of the present example have been prepared by a detergent extraction process, where the deoxycholate is used as selected detergent. The thus obtained detergent extracted vesicles have been reacted with the selected antigen (fHbp) according to process of the present invention. In particular, dOMV, at the concentration of 0.96 mg/mL, were incubated with NaIO₄ 10 mM for 30 minutes at room temperature, in the dark. Excess of NaIO₄ was quenched with
10 Na₂SO₃ at a final concentration of 20 mM, for 15 minutes at room temperature. fHbp (w/w ratio of dOMV to fHbp 1:1 and with dOMV concentration of 0.335 mg/mL) and NaBH₃CN (3 mg) were directly added to the reaction mixture. After overnight gently mixing at room temperature, the conjugate was purified by ultracentrifuge (110000 rpm 4°C 1h), re-suspended in PBS and analysed by SDS PAGE/western blot.

Example 7b: reaction of nOMV (from *Neisseria meningitidis* B) with fHbp v3 (formation of the nOMV-fHbp conjugate of the invention)

The nOMVs of the present example have been prepared without using any detergent, as described in Koeberling et al. Vaccine (2014) 32:2688. The thus obtained extracted vesicles have been reacted with the selected antigen (fHbp) according to the process of the present
20 invention. In particular, nOMV at the concentration of 0.96 mg/mL were incubated with NaIO₄ 5mM for 30 minutes at room temperature, in the dark. Excess of NaIO₄ was quenched with Na₂SO₃ at a final concentration of 20 mM, for 15 minutes at room temperature. fHbp (w/w ratio of dOMV to fHbp 1:1 and with dOMV concentration of 0.335 mg/mL) and NaBH₃CN (3 mg) were directly added to the reaction mixture. After overnight gently mixing at room temperature, the
25 conjugate was purified by ultracentrifuge (110000 rpm 4°C 1h), re-suspended in PBS and analysed by SDS PAGE/western blot. SDS PAGE/anti-fHbp western blot analysis confirmed conjugate formation by reductive amination chemistry only with nOMV, but not with dOMV. 10% SDS page gel.

Example 7c: reaction of nOMV (from *Salmonella*) with fHbp v1, following the procedure of example 7b.

The same experiment as Examples 7b has been performed using nOMV from *Salmonella* Typhimurium, and similar results have been collected, obtaining the nOMV-fHbp conjugate of the invention.

EXAMPLE 8: preparation of multi functionalized nOMV using (NANP)₃-SH-Pfs25, according to the invention.

Pfs25 protein was derivatised with EMCS linker according to the following procedure. Pfs25, in PBS buffer at the concentration of 2.6 mg/mL, was added of EMCS linker (molar ratio EMCS linker to Pfs25 Lys residues of 0.3). The reaction was mixed at room temperature for 4h. Resulting derivatised protein (Pfs25-EMCS) was purified by PD10 column against NaH₂PO₄ 10 mM pH 6. Analysis by MALDI-TOF MS revealed an average of 4 EMCS linkers introduced per Pfs25 molecule. (NANP)₃ was added to the solution of Pfs25-EMCS to have (NANP)₃ to EMCS linkers molar ratio of 3:1 and Pfs25 concentration of 0.7 mg/mL. The reaction was mixed at room temperature overnight. After this time, the Pfs25-(NANP)₃ derivative was purified by Vivaspin 10K against PBS buffer. Analysis by SDS PAGE/western blot and MALDI-TOF MS confirmed product formation. *S. Typhimurium* nOMV at the concentration of 2.1 mg/mL in NaH₂PO₄ 100 mM pH 6.5 were incubated with NaIO₄ 5mM for 30 minutes at 25°C, in the dark. Excess of NaIO₄ was quenched with Na₂SO₃ at a final concentration of 10 mM, for 10 minutes at room temperature. Pfs25-(NANP)₃ (w/w ratio of nOMV to Pfs25-(NANP)₃ 1:1 and with nOMV concentration of 0.45 mg/mL) and NaBH₃CN were directly added to the reaction mixture. After overnight gently mixing at room temperature, the conjugate was purified by ultracentrifuge (110000 rpm 4°C 30 min), re-suspended in PBS and analysed by SDS PAGE/western blot, that confirmed conjugate formation.

EXAMPLE 9: *in-vivo* data of the conjugates of the invention obtained by conjugating a *S. Typhimurium* nOMV particle to Pfs25 antigen with or without quenching, according to the embodiments of the invention.

CD1 female mice were immunised subcutaneously at days 0 and 28 with 2.5 µg total protein *S. Typhimurium* nOMV particles conjugated to Pfs25 antigen with or without the quenching step (see Example 5). Both conjugates showed a Pfs25 to total protein w/w ratio close to 20% by competitive ELISA and were adsorbed on Alhydrogel (0.7 mg/mL Al³⁺). Anti-Pfs5 and anti-OAg IgG titers were measured at days 0, 14, 27 and 42. At all the time points the two conjugates induced similar anti-Pfs25 IgG response (Mann Whitney test), as shown in Figure 6a. Also, the conjugates were able to induce similar anti-OAg IgG response, as indicated in Fig.10b. The step of quenching in the conjugation by reductive amination can be introduced with no impact on the immune response induced in mice, thus avoiding GMMA oxidation intermediate purification.

EXAMPLE 10: reaction of nOMV (from *Neisseria meningitidis* B) with MenC (formation of the nOMV-MenC conjugate of the invention)

MenC polysaccharide was solubilised in AcONa 100 mM pH 4.5 at the concentration of 40 mg/mL. ADH linker and NaBH₃CN were added with a w/w ratio 1:1.2:1.2 MenC/ADH/NaBH₃CN respectively. The mixture was heated at 30°C overnight, and then desalted by G10 column. Characterization by TNBS colorimetric method and HPAEC-PAD showed 100% derivatization.

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MenB GMMA overexpressing fHbp, at the concentration of 8.5 mg/mL in NaH₂PO₄ 100 mM pH 6, were incubated with NaIO₄ 5 mM for 30 minutes at room temperature, in the dark. Excess of NaIO₄ was quenched with Na₂SO₃ at a final concentration of 10 mM, for 15 minutes at room temperature. MenC oligosaccharide, previously terminally derivatised with ADH linker (w/w ratio of GMMA to MenC 1:10 and with GMMA concentration of 7.7 mg/mL) and NaBH₃CN were directly added to the reaction mixture. After overnight gently mixing at room temperature, the conjugate was purified by ultracentrifuge (110000 rpm 4°C 1h) and re-suspended in PBS. Analysis by SDS PAGE/western blot confirmed conjugate formation and analysis by micro BCA and HPAEC-PAD revealed a weight ratio of MenC polysaccharide to protein equal to 0.11.

10 **EXAMPLE 11: reaction of nOMV (from *Neisseria meningitidis* B) with MenA (formation of the nOMV-MenA conjugate of the invention)**

MenA OS was solubilised in AcONa 100 mM pH 6.5 at the concentration of 40 mg/mL. ADH linker and NaBH₃CN were added with a w/w ratio 1:1.2:1.2 MenA/ADH/NaBH₃CN respectively. The mixture was heated at 30°C for 5 days, then desalted by G10 column. Characterization by TNBS colorimetric method and HPAEC-PAD showed 90% derivatization. Conjugation to MenB GMMA overexpressing fHbp was performed as described for MenC polysaccharide.

EXAMPLE 11: preparation of conjugates of nOMV (from *Neisseria meningitidis* B) with MenA and MenC

Same conjugation conditions described for the synthesis of MenA- and MenC-MenB GMMA conjugates were used for conjugation of both polysaccharides on same GMMA particle. GMMA were oxidised as previously described and, after quenching with Na₂SO₃, MenA-ADH and MenC-ADH were added simultaneously with a w/w ratio 8:8:1 MenA/MenC/GMMA respectively.

SEQUENCE LISTING**>SEQ ID NO: 1 [fHbp v2]**

VAADIGAGLADALTAPLDHKDKSLQSLTLDQSVRKNEKLKLAQAQGAEKTYGNGDSLNTGKLKN
DKVSRFDFIRQIEVDGQLITLESQGEFQIYKQDHSVVALQIEKINNPDKIDSLINQRSFRVSGLG
EHTAFNQLPDGKAHEYHGKAFSSDDAGGKLTYTIDFAAKQGHGKIEHLKTPEQNVFLAAELKA
DEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKEVHEIGIAGKQ

>SEQ ID NO: 2 [NHBA]

MFKRSVIAMACIFALSACGGGGGGGSPDVKSADTLSPAPVSEKETEAKEDAPQAGSQGGG
APSAQGSQDMAAVSEENTGNGGAVTADNPKNEDVAQNDMPQNAAGTDSSTPNHTPDPM
LAGNMENQATDAGESSQPANQPDMANAADGMQGGDDPSAGGQNAGNTAAQGANQAGNNQA
AGSSDPIPASNPAPANGGSNFRVLDLANGVLIDGPSQNTLTTHCKGDSCSGNNFLDEEVQLKS
EFEKLSADADKISNYKKDGKNDKVFGLVADSVQMKGINQYIIFYKPKPTSFARFRRSARSRRSLP
AEMPLIPVNQADTLVDGEAVSLTGHSNIFAPEGNRYLTGAEKLPGGSYALRVQGEPAGE
MLAGAAVYNGEVLHFHTENGRPYPTRGRFAAKVDFGSKSVSDGIIDSGDDLHMGTTQKFKAAIDG
NGFKGTWTENGSGDVSGKFYGPAGEEVAGKYSYRPTDAEKGFGVFGAGKKEQD

>SEQ ID NO: 3 [NadA]

MKHFPKSVLTTAILATFCSGALAATSDDDVKKAATVAIVAAYNNGQEINGFKAGETIYDIGEDGTI
TQKDATAADVEADDFKGLGLKKVVTNLTKTVNENKQNVDAKVKAASEIEKLTTKLADTDAALA
DTDAALDETTNALNKLGENITTFEETKTNIVKIDEKLEAVADTVDKHAEAFNDIADSLDETNTKA
DEAVKTANEAKQTAEETKQNVDAKVKAETAAGKAEAAAGTANTAADKAEAAVAKVTDIKADIA
TNKADIAKNSARIDSLDKNVANLRKETRQGLAEQAALSGLFQPYNVGRFNVTAAVGGYKSESA
VAIGTGFRFTENFAAKAGVAVGTSSGSSAAYHVGVNVEW

>SEQ ID NO: 4 [NspA]

MKKALATLIALALPAAALAEAGSGFYVQADAAHAKASSSLGSAKGFSPRISAGYRINDLRFAVDY
TRYKNYKAPSTDFKLYSIGASAIYDFDTQSPVKPYLGARLSLNRASVDLGGSDSFSQTSIGLGVL
TGVSYAVTPNVLDAGYRYNYIGKVNTVKNVRSGELSAGVRVKF

>SEQ ID NO: 5 [NhhA]

MNKIYRIIWNALNAWVVVSELTRNHTKRASATVKTAVLATLLFATVQASANNEEQEEDLYLDP
VQRTVAVLIVNSDKEGTGEKEKVEENSDWAVYFNEKGVLTAREITLKAGDNLKIKQNGTNFTYS
LKKDLTDLTSVGTEKLSFSANGNKVNITSSTKGLNFAKETAGTNGDTTVHLNGIGSTLTDLLNT
GATTNVTNDNVTDDEKKRAASVKDVLNAGWNIKGVKPGTTASDNVDFVRTYDTVEFLSADTKT
TTVNVESKDNGKKTEVKIGAKTSVIKEKDGLVTGKDKGENGSSTDEGEGLVTAKEVIDAVNKA
GWRMKTITANGQTGQADKFETVTSNTVTFASGKGTTATVSKDDQGNITVMYDVNVGDALNV
NQLQNSGWNLDKAVAGSSGKVISGNVSPSKGKMDETVNINAGNNIEITRNGKNIDIATSMTPQ
FSSVSLGAGADAPTLSDGDALNVGSKKDNKPVRITNVAPGVKEGDVTNVAQLKGVAQNLNN
RIDNVDGNARAGIAQAIATAGLVQAYLPKSMMAIGGGTYRGEAGYAIGYSSISDGGNWIIGT
ASGNSRGHFGASASVGYQW

>SEQ ID NO: 6 [App]

MKTTDKRTTETHRKAPKTGRIRFSPAYLAICLSFGILPQAWAGHTYFGINYQYYRDFAEKNGKF
AVGAKDIEVYNKKGELVGKSMKAPMIDFSVSRNGVAALVGDQYIVSVAHNGGYNNVDFGAE
GRNPDQHRFTYKIVKRNNYKAGTKGHPYGGDYHMPRLHKFVTDAEPVEMTSYMDGRKYIDQN
NYPDRVRIGAGRQYWRSEDEPNNRESSYHIASAYSWLVGGNTFAQNGSGGGTVNLGSEKIK
HSPYGLPTGGSGFDGSGSPMFIYDAQKQKWLINGVLQTGNPYIGKSNGFQLVRKDWFYDEIFA
GDTHSVFYEPQNGKYSFNDDNNGTGKINAKHEHNSLPNRLKTRTVQLFNVSLSETAREPVYH
AAGGVNSYRPRLNNGENISFIDEGKGELITSNINQGAGGLYFQGDFTVSPENNETWQGAGVHI
SEDSTVTWKVNGVANDRLSKIGKGTLHVQAKGENQGSISVGDGTVIDDQQADDKGKKQAFSEI
GLVSGRGTVQLNADNQFNPDKLYFGFRGGRLDLNGHSLSFHRIQNTDEGAMIVNHNQDKEST
VTITGNKDIATTGNNNSLDSKKEIAYNGWFGKEDTTKTNGRLNLVYQPAEDRTLTLSSGGTNLN
GNITQTNGKLFFSGRPTPHAYNHLNDHWSQKEGIPRGEIVWDNDWINRTFKAENFQIKGGQAV
VSRNVAKVKGDWHLSNHAQAVFGVAPHQSHTICTRSDWTGLTNCVEKTITDDKVIASLTKTDIS
GNVDLADHAHLNLTGLATLNGNLSANGDTRYTVSHNATQNGNLSLVGNAQATFNQATLNGNT

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SASGNASFNLSDHAVQNGSLTSLSGNAKANVSHSALNGNVSLADKAVFHFESSRFTGQISGGKD
 TALHLKDSEWTLPSGTELGNLNDNATITLNSAYRHDAAGAQTGSATDAPRRRSRRSRRSLLS
 VTPPTSVESRFNTLTVNGKLNGQGTFRFMSELFgyrSDKLKLAESSEGTYTLAVNNTGNEPAS
 LEQLTVVEGKDNKPLSENLFNLQNEHVDAGAWRYQLIRKDGEFRLHNPVKEQELSDKLGKAE
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 KQREAETRPATTAFFPRARRARRDLPLQLPQPQPQPQRDLISRYANSGLSEFSATLNSVFAVQD
 ELDRVFAEDRRNAVWTSGIRDTKHYRSQDFRAYRQQTDLRQIGMQKNLGSGRVGILFSHNRT
 ENTFDDGIGNSARLAHGAVFGQYGIDRFYIGISAGAGFSSGSLSDGIGGKIRRRVLHYGIQARYR
 AGFGGFGIEPHIGATRYFVQKADYRYENVNIATPGLAFNRYRAGIKADYSFKPAQHISITPYLSLS
 YTDASGKVRTRVNTAVLAQDFGKTRSAEWGVNAEIKGFTLSLHAAAAGKGPQLEAQHSAGIKL
 GYRW

>SEQ ID NO: 7 [NadA fragment]

ATNDDDVKKAATVAIAAAYNNGQEINGFKAGETIYDIDEDGTITKKDATAADVEADDFKGLGLKK
 VVTNLTKTVNENKQNVDAKVKAASEIEKLTTKLADTDAALADTDAALDATTNALNKLGENITTF
 AEETKTNIVKIDEKLEAVADTVDKHAEAFNDIADSLDETNTKADEAVKTANEAKQTAEETKQNV
 AKVKAETAAGKAEAAAGTANTAADKAEVAAKVTDIKADIATNKDNIKKANSADVYTREESD
 SKFVRIDGLNATTEKLDTRLASAEKSIADHDTRLNGLDKTVSDLRKETRQGLAEQAALSGLFQP
 YNVG

>SEQ ID NO: 8 [fHbp v1]

VAADIGAGL ADALTAPLDH KDKGLQSLTL DQSVRKNEKL KLAAGAEKT YGNGDSLNTG
 KLKNDKVSFR DFIRQIEVDG QLITLESCEF QVYKQSHSAL TAFQTEQIQD SEHSGKMKVAK
 RQFRIGDIAG EHTSFDKLPE GGRATYRGTA FGSDDAGGKL TYTIDFAAKQ GNGKIEHLKS
 PELNVDLAAA DIKPDGKRHA VISGSVLYNQ AEKGSYSLGI FGGKAQEVAG SAEVKTVNGI
 RHIGLAAKQ

>SEQ ID NO: 9 [fHbp v3]

VAADIGTGLADALTAPLDHKDKGLKSLTLED SIPQNGTLTSLAQGAEKTFKAGDKDNSLNTGKL
 KNDKISRFDVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSLINQRSFLVSG
 GGEHTAFNQLPGGKAEYHGKAFSSDDPNGLHYSIDFTKKQGYGRIEHLKTLEQNVELAAEL
 KADEKSHAVILGDTRYGSEEKGTYHLALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ

>SEQ ID NO: 10 [Pfs25]

KVTVDTVCKR GFLIQMSGHL ECKCENDLVL VNEETCEEKV LKCDEKTVNK PCGDFSKCIK
 IDGNPVSYAC KCNLGYDMVN NVCIPNECKQ VTCGNGKCIL DTSNPVKTGV CSCNIGKVPN
 VQDQNKCSKD GETKCSLKCL KEQETCKAVD GIYKCDCKDG FIIDQESSIC T

>SEQ ID NO: 11 [RO6C]

AERSTSENARNKRIGGPKLRGNVTSNIKFPSDNKGKIIRGSNDKLNKNSEDEVLEQSEKSLVSENV
 PSGLDIDDIPKESIFIQEDQEGQTHSELNPETSEHSKDLNNNGSKNESSDIISENNKSNKVQNH
 ESLSDLELLENSSQDNLDKDTISTEPFPNQKHKDLQQDLNDEPLEPFPTQIHKDYKEKNLINEED
 SEPFPRQKHKKVDNHNEEKNVFHENGSAANGNQGSLKLKSFDEHLKDEKIENEPLVHENLSIPN
 DPIEQILNQPEQETNIQEQLYNEKQNVEEKQNSQIPSLDLKEPTNEDILPNHNPLENIKQSESEIN
 HVQDHALPKENIIDKLDNQKEHIDQSQHNINVLQENNINNHQLEPQEKPNIESFEPKNIDSEIILPE
 NVETEEIIDDVPSPKHSNHETFEETSESEHEEAVSEKNAHETVEHEETVSQESNPEKADNDG
 NVSQNSNNELNENEFVESEKSEHEARSKPKYEKKVIHGCNFFSSNVSSKHTFTDSLDISLVDDSA
 HISCNVHLSEPKYNHLVGLNCPGDIIPDCFFQVYQPESEELEPSNIVYLD SQINIGDIEYYEDAEG
 DDKIKLFGIVGSIPKTTSFTCICKKDKKSAYMTVTIDSARSHHHHHH

>SEQ ID NO: 12 [CSP]

MLFQEYQCYGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWYSLKKNSRSLGENDDG
 NNNNGDNGREGKDEKRDGNNEDEKLRKPKHKKLKQPGDGNPDPNANPNVDPNANPNVD
 PNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPN
 NANPNANPNVDPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPN

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ANPNANPNANPNANPNANPNANPNANPNANPNKNNQGNGQGHNMPNDPNRNVDENANANNAVKN
NNNEEPSDKHIEKYLKKIQNSLSTEWSPCSVTCGNGIQVRIKPGSANKPKDEL DYENDIEKKICK
MEKCSSVFNVVNSSIGLILEHHHHHH

>SEQ ID NO: 13 [(NANP)3]

NANPNANPNANP

>SEQ ID NO: 14 [CTF1232]

QDQRYISIRNTDTIWLPGNICAYQFRLDNGGNDGFGPLTITLQLKDKYGQTLVTRKMETEAFG
DSNATRTTDAFLETECVENVATTEIIKATEESNGHRVSLPLSVFDPQDYHPLLITVSGKNVNLEH
HHHHH

CLAIMS

1. An immunogenic conjugate comprising a native outer membrane vesicle (nOMV), having at least a surface saccharide moiety connected to at least an antigen.
- 5 2. The immunogenic conjugate according to claim 1, comprising a nOMV having at least a surface saccharide moiety connected to a first antigen, wherein said first antigen is connected to a second different antigen.
3. The immunogenic conjugate according to claim 1, comprising a nOMV having at least a surface saccharide moiety connected to a first antigen, and at least another surface
- 10 saccharide moiety connected to a second different antigen.
4. The immunogenic conjugate according to claims 1-3, wherein said nOMV is obtained by a detergent free process, being released into the fermentation broth and purified using a centrifugation and subsequent filtration; or being released into the fermentation broth and purified using two consecutive Tangential Flow Filtration (TFF) steps.
- 15 5. The immunogenic conjugate according to the preceding claims, wherein said nOMV is produced from wild type bacteria or from genetically-modified bacterial strains that are mutated to enhance vesicle production, and optionally also to remove or modify antigens and/or to over-express homologous antigens or antigens from other organisms.
6. The immunogenic conjugate according to the preceding claims, wherein said nOMV is
- 20 obtained from a bacteria selected from: *Neisseria*, *Shigella*, *Salmonella enterica* serovars, *Haemophilus influenzae*, *Vibrio cholerae*, *Bordetella pertussis*, *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, *Escherichia coli*, *Bacteroides*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Brucella melitensis*, *Campylobacter jejuni*, *Actinobacillus actinomycetemcomitans*, *Xenorhabdus nematophilus*, *Moraxella catarrhalis*, or *Borrelia burgdorferi*.
- 25 7. The immunogenic conjugate according to the preceding claims, wherein the antigen is an immunogenic polypeptide or a capsular polysaccharide.
8. The immunogenic conjugate according to the preceding claims, wherein said nOMV and antigen are derived from the same or different bacterial strain.
- 30 9. The immunogenic conjugate according to the preceding claims, wherein said nOMV and at least one antigen are selected as follows:

nOMV

Salmonella Typhimurium
Salmonella Typhimurium
Salmonella Typhimurium
Salmonella Typhimurium
Salmonella Typhimurium
Salmonella Typhimurium
Salmonella Typhimurium
Neisseria meningitidis
Neisseria meningitidis
Shigella

Selected antigen

Neisseria meningitidis fHbp
Plasmodium falciparum CSP
Plasmodium falciparum Pfs25
Plasmodium falciparum RO6C
Plasmodium falciparum RO10C
Escherichia coli CTF1232
S. Typhi Vi saccharide
Neisseria meningitidis fHbp
Poly-rhamnose oligosaccharide
Escherichia coli CTF1232.

Neisseria meningitidis B
Neisseria meningitidis B

Capsular saccharide from MenA
Capsular saccharide from MenC

10. The immunogenic conjugate of claim 9, wherein said nOMV and at least one antigen are selected as follows:

nOMV

Neisseria meningitidis B
Neisseria meningitidis B

Selected antigen

Capsular saccharide from MenA
Capsular saccharide from MenC

- 5 11. The immunogenic conjugate of claim 2, wherein said first antigen is Pfs25, and said second antigen is (NANP)₃.
12. The immunogenic conjugate of claim 11, wherein the nOMV is obtained from *Salmonella Typhimurium*.
- 10 13. The immunogenic conjugate of claim 2, wherein said first antigen is a capsular saccharide from MenA, and said second antigen is a capsular saccharide from MenC.
14. The immunogenic conjugate of claim 13, wherein the nOMV is obtained from *Meningococcus* serogroup B strain, preferably expressing fHbp v1 and 2.
- 15 15. The immunogenic conjugate according to claims 1-14, wherein the nOMV saccharide moiety and the at least one antigen are connected together via a bivalent linker.
16. The immunogenic conjugate according to the preceding claims, wherein said nOMV is a GMMA vesicle.
17. A process for preparing the immunogenic conjugate according to claims 1-16, comprising the steps of:
- 20 i) activating at least a nOMV surface saccharide moiety, and
- ii) connecting the thus obtained activated saccharide to at least one antigen, optionally via a divalent linker.
18. The process according to claim 17 wherein the activation step i) comprises the oxidation of one or more hydroxyl group of said nOMV said saccharide moiety to aldehyde functionality.
19. The process according to any one of claims 17-18 in the presence of an alkaline disulfite.
- 25 20. An immunogenic composition comprising an immunogenic conjugate according to claims 1-16 and at least one pharmaceutically acceptable carrier or excipient.
21. The immunogenic conjugate or composition according to any one of claims 1-16 or 20, for use as a medicament.
22. The immunogenic conjugate or composition for use according to claim 21 to induce an
- 30 immune response in a vertebrate.
23. A vaccine comprising the immunogenic conjugate or composition according to any one of claims 1-16 or 20.
24. Use of nOMV for the preparation of immunogenic conjugates.
25. Use of nOMV according to claim 24, wherein said immunogenic conjugates are as defined
- 35 in any one of claims 1-16.

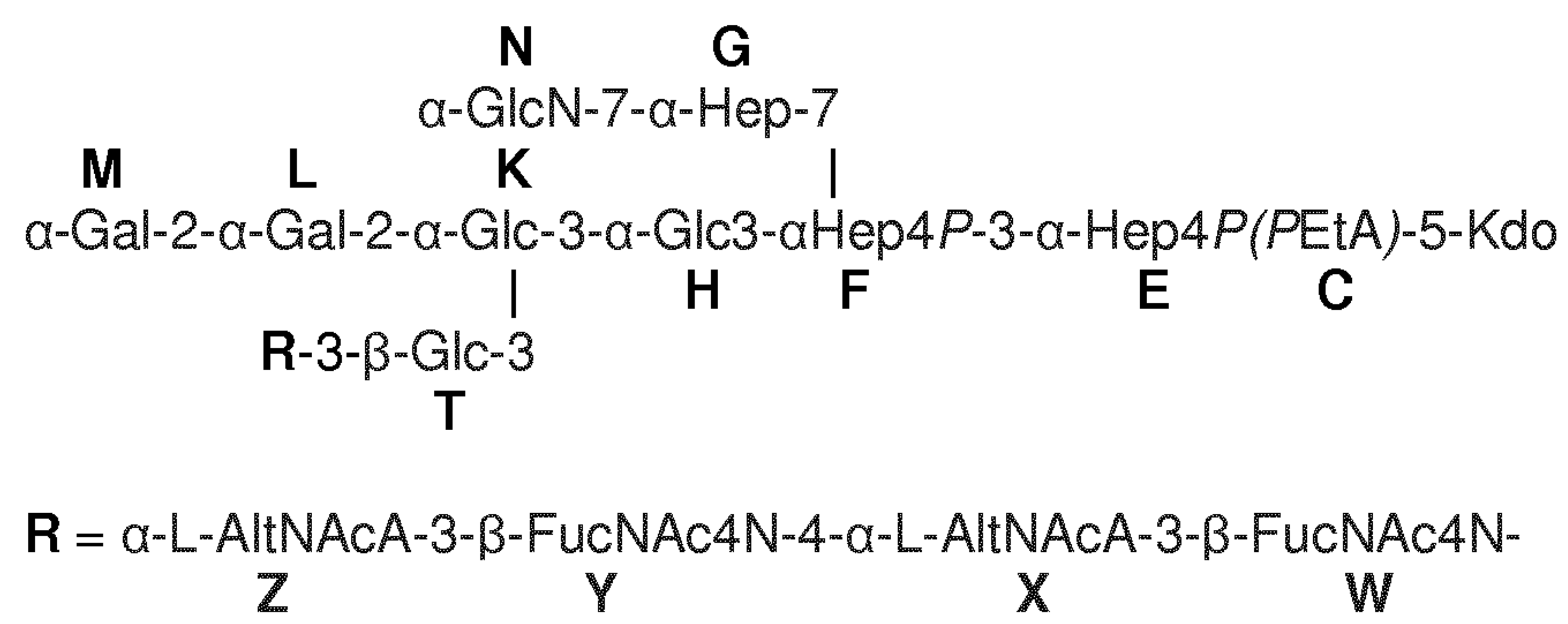


FIG. 1

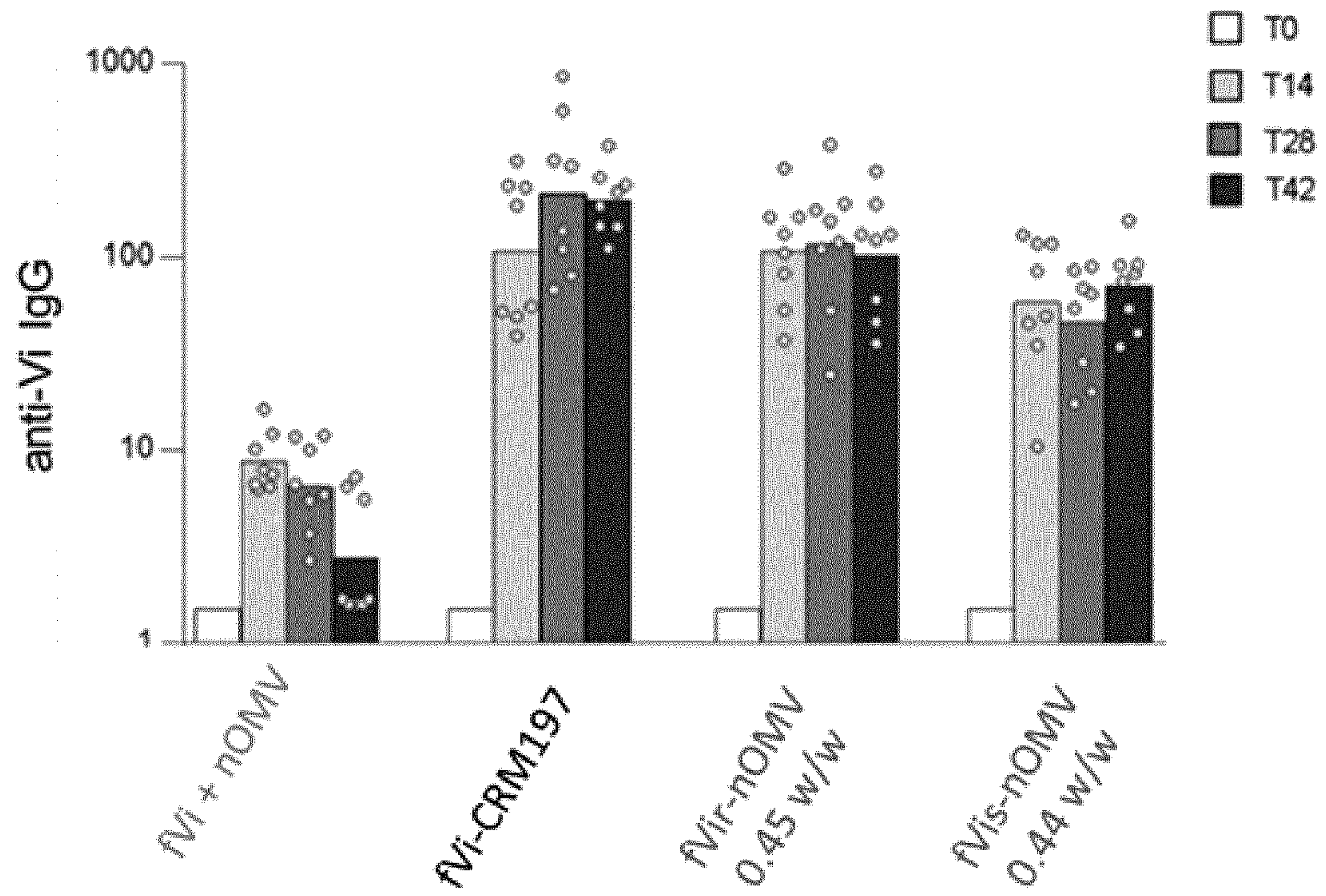


FIG. 2A

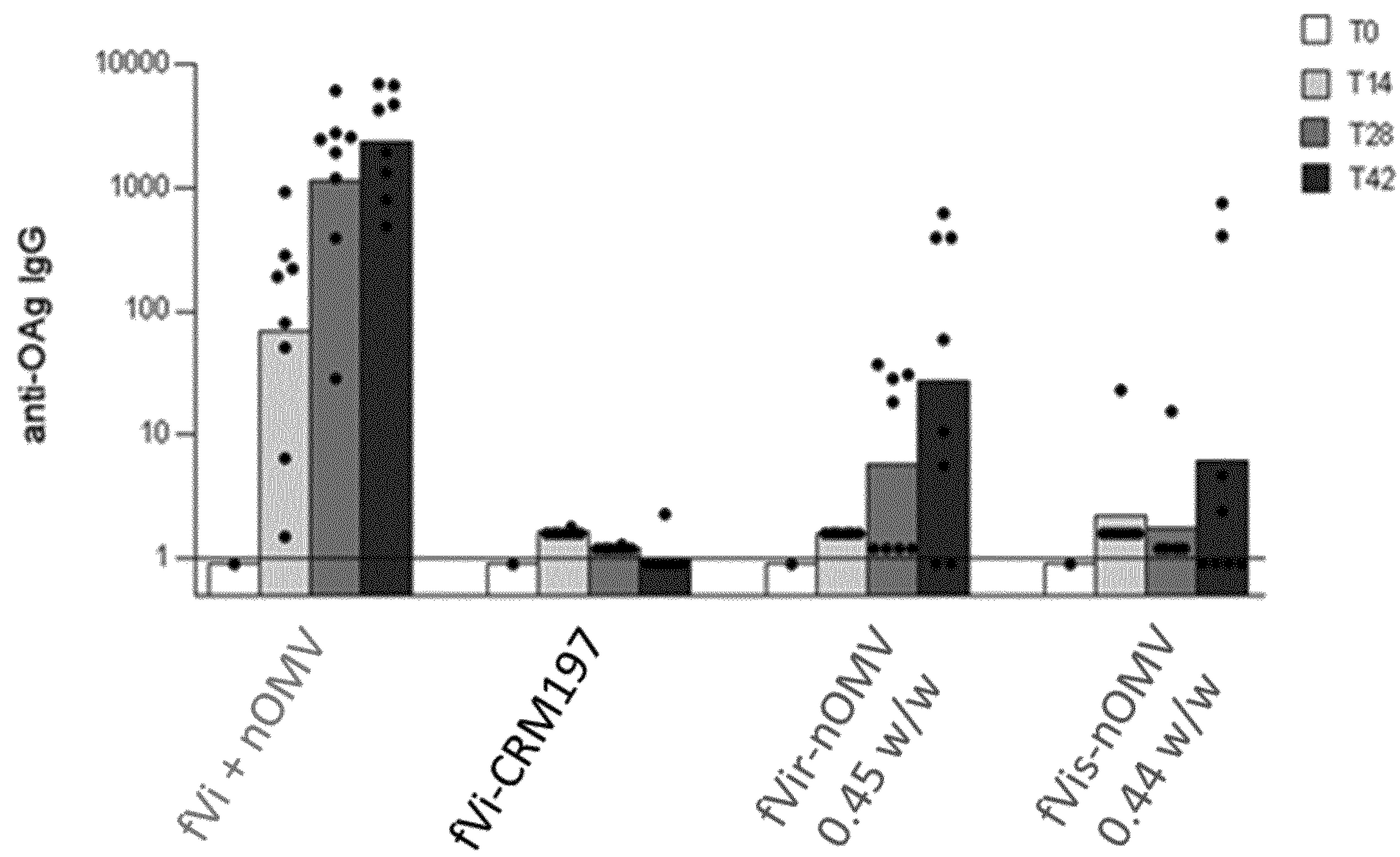


FIG. 2B

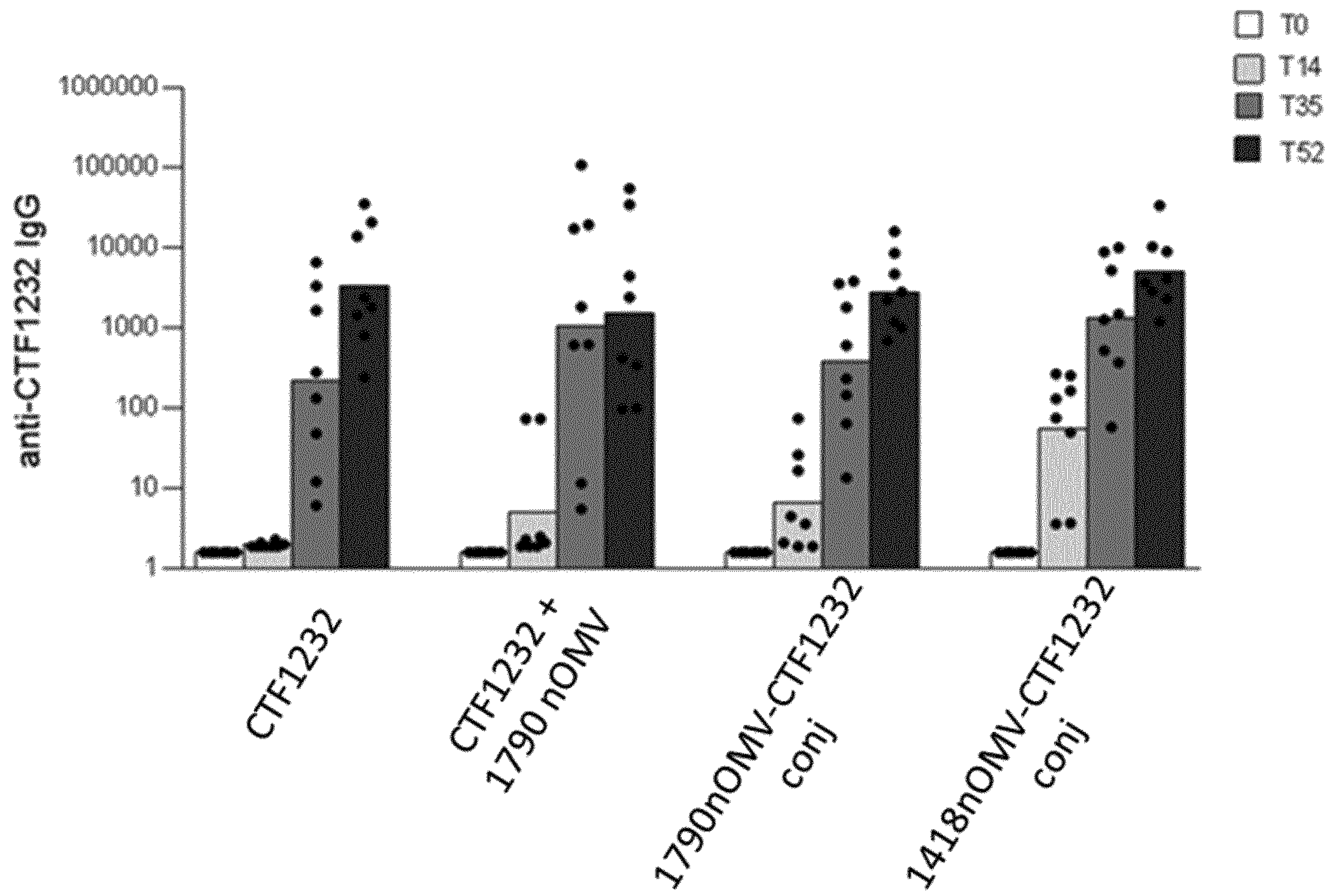


FIG. 3

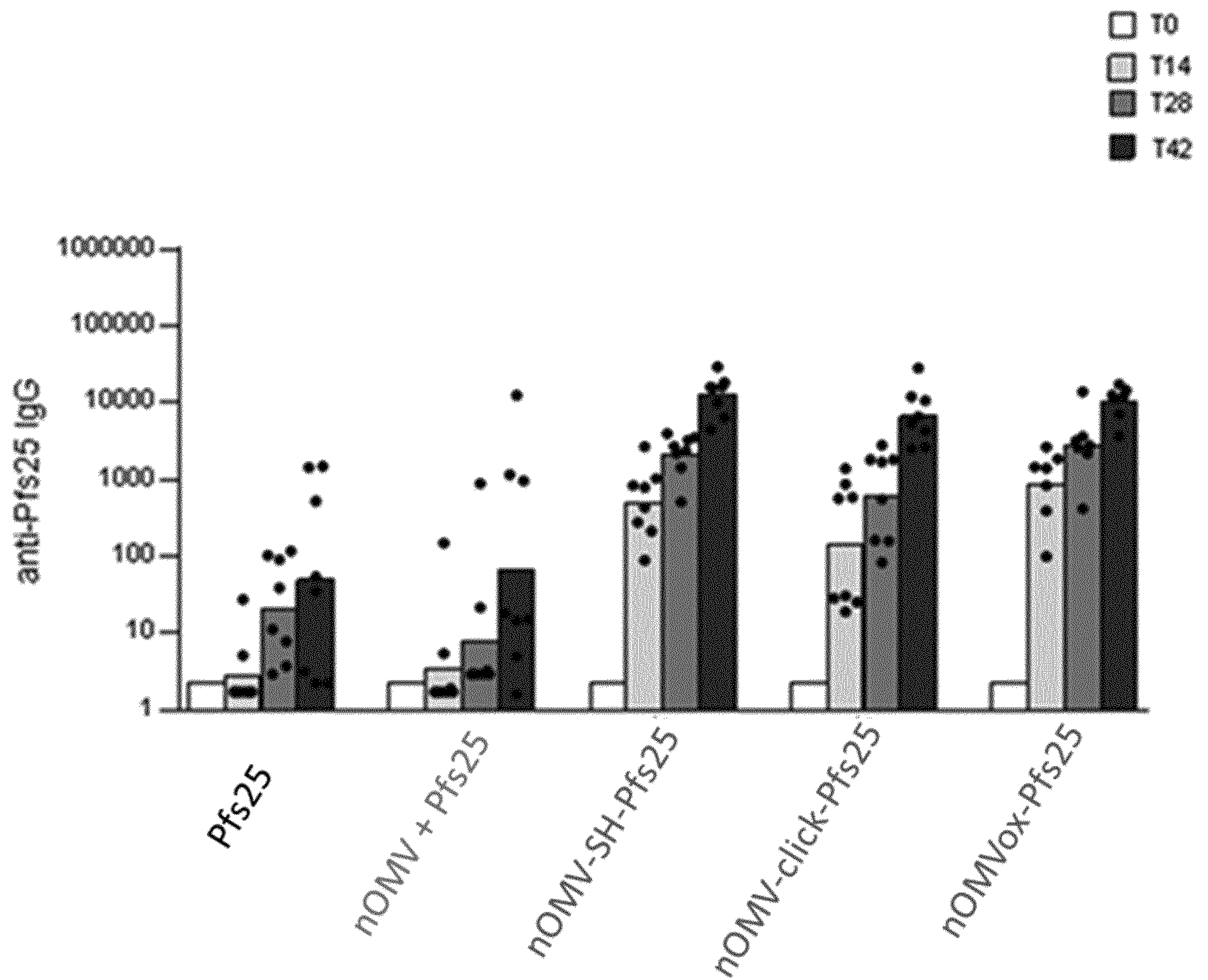


FIG. 4A

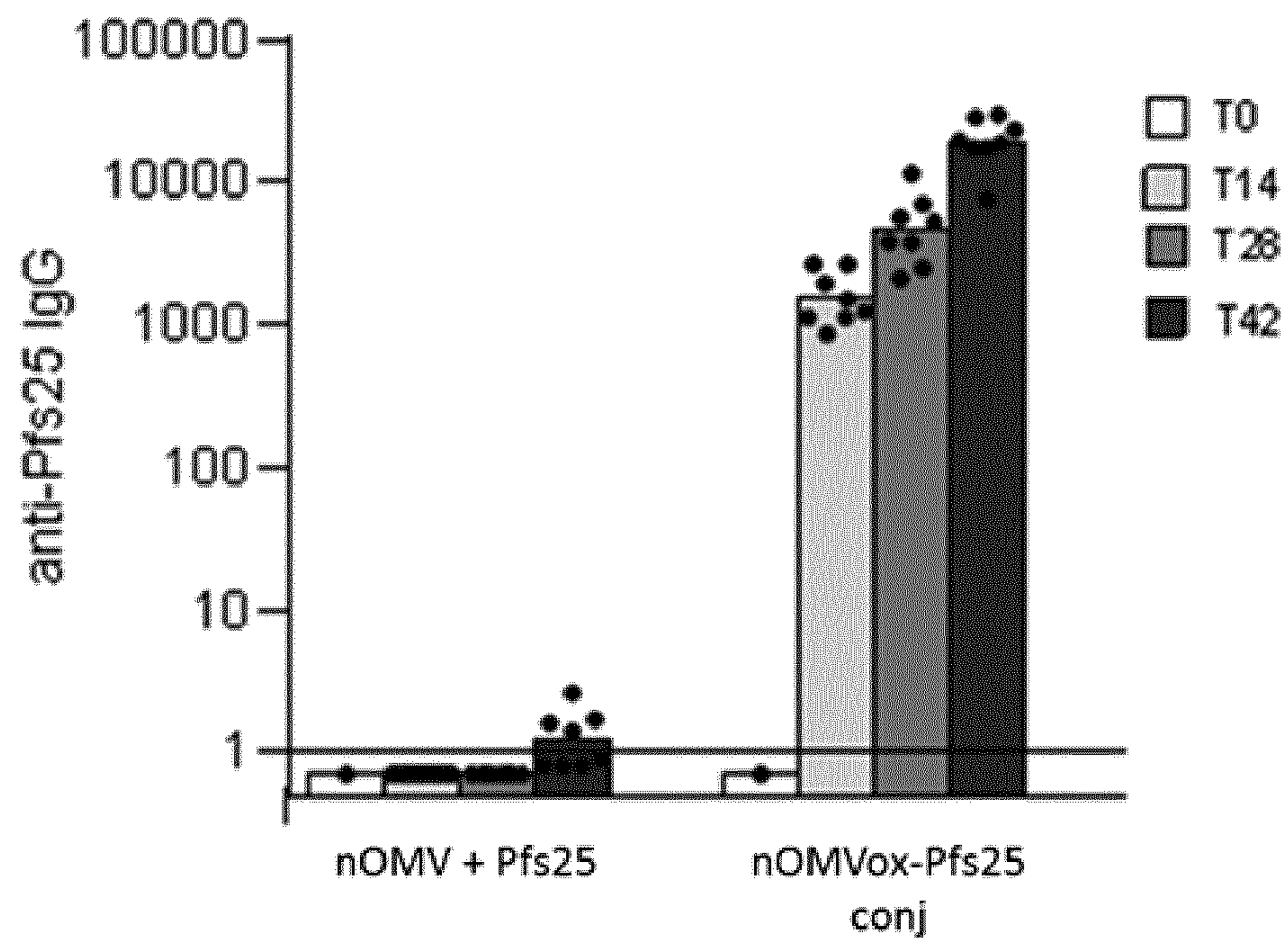


FIG. 4B

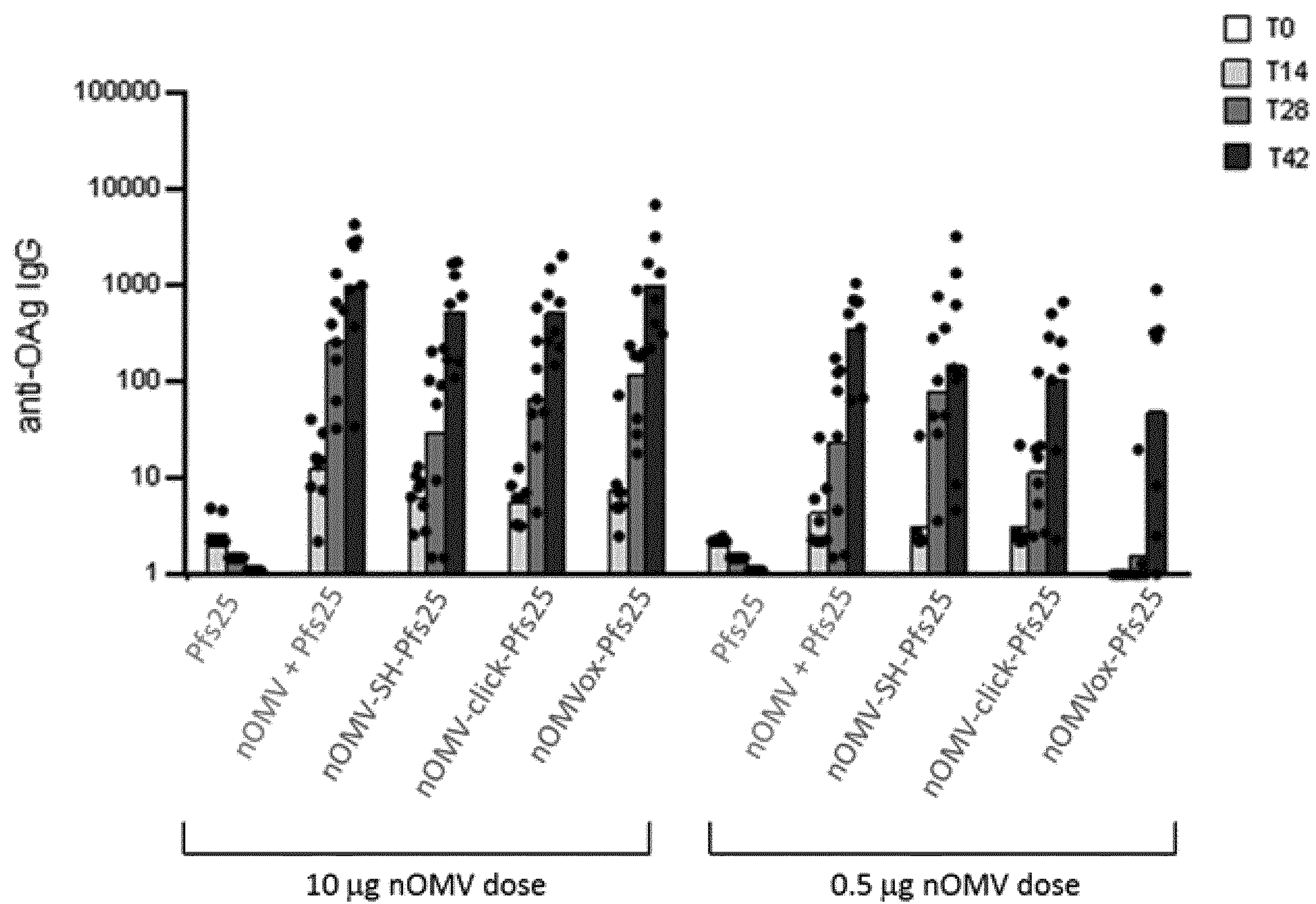


FIG. 4C

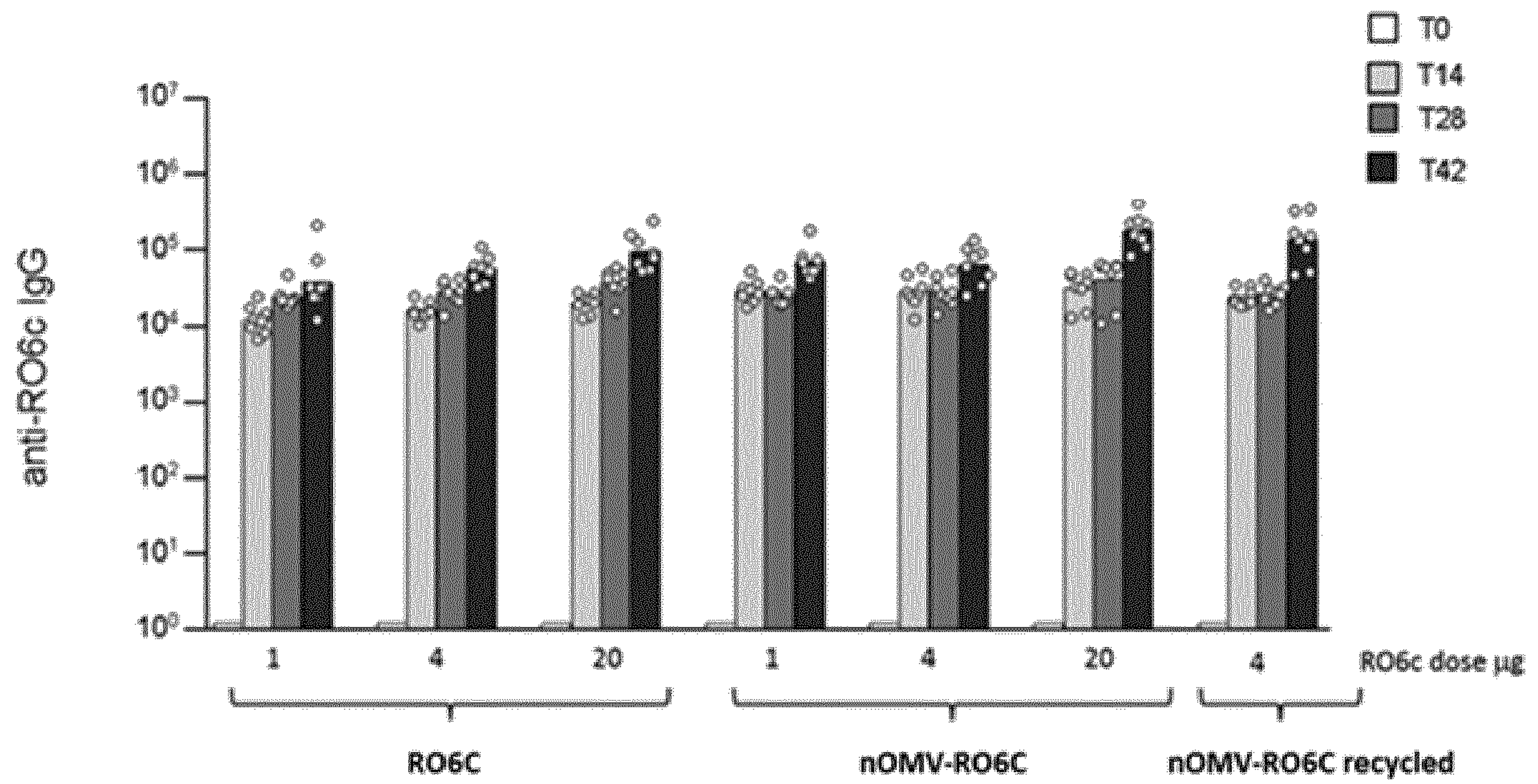


FIG. 5A

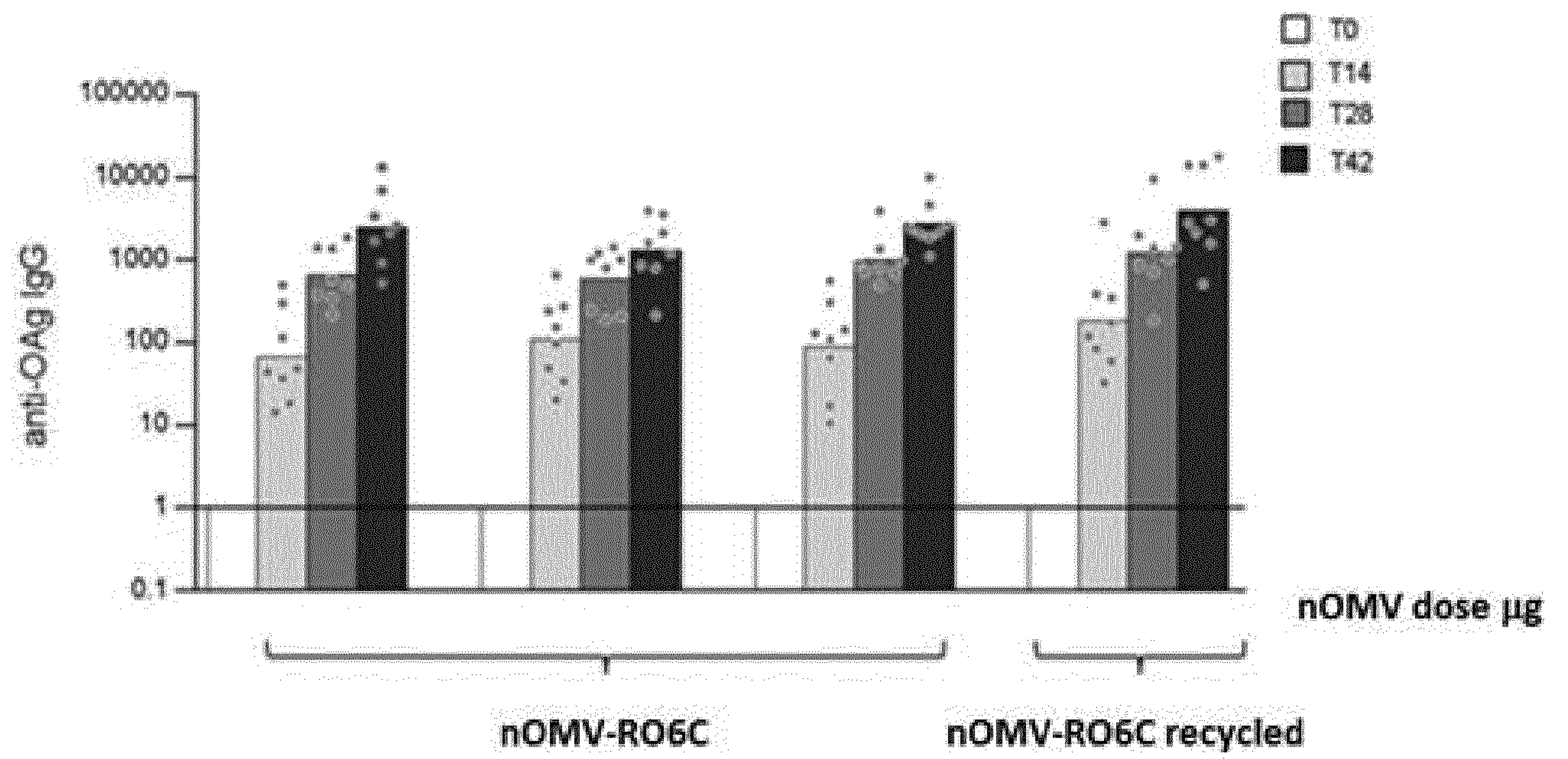


FIG. 5B

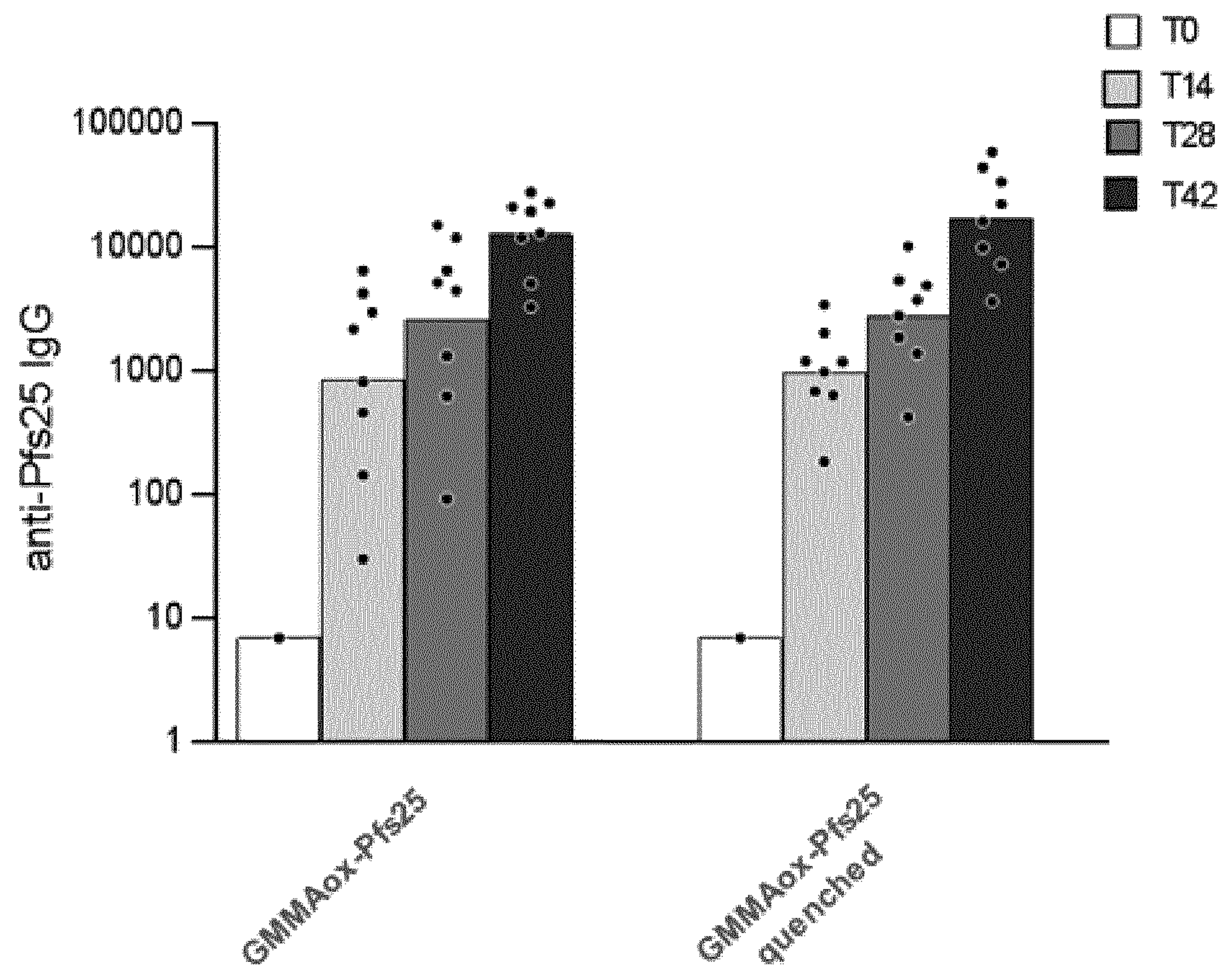


FIG. 6A

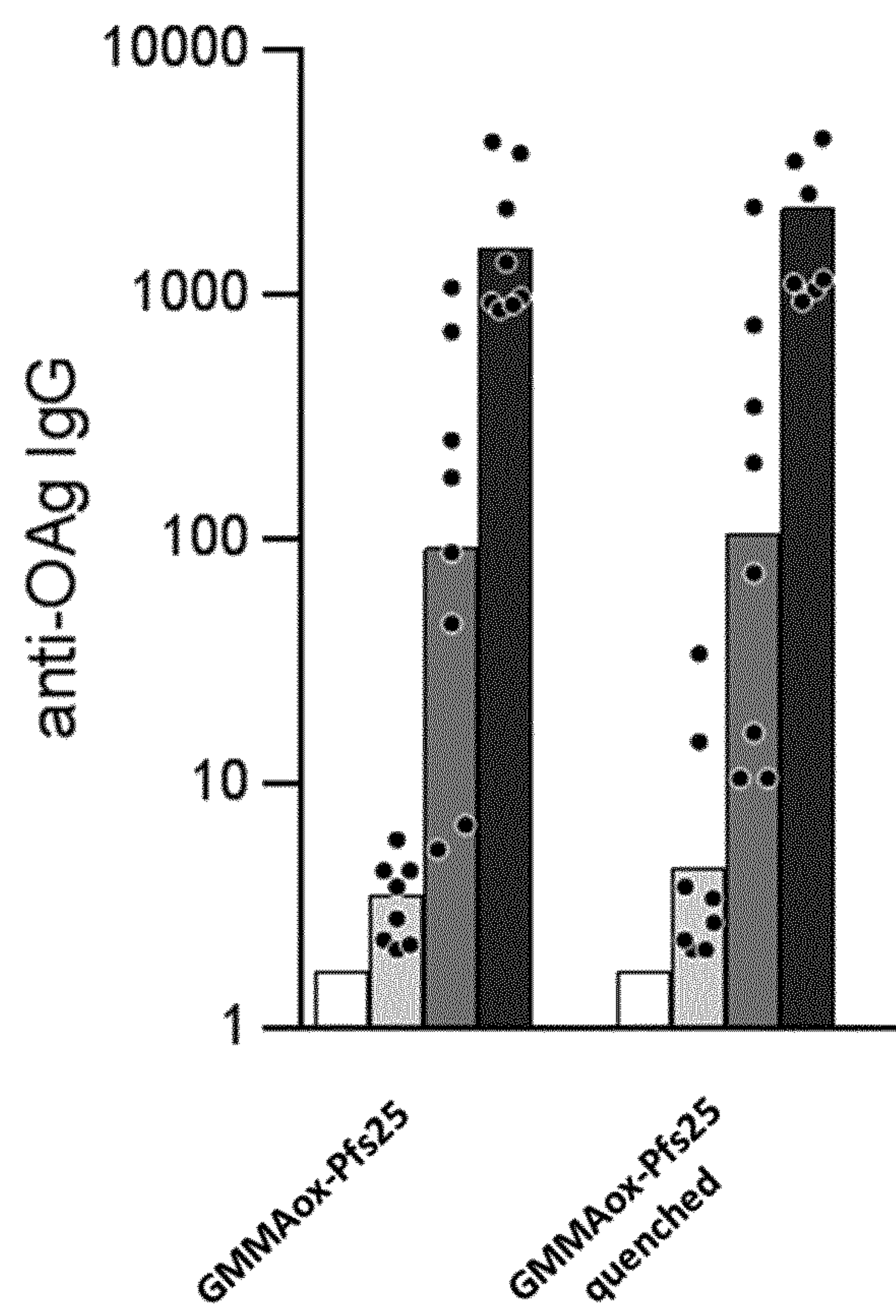


FIG. 6B