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Improved coupling of bacterial polysaccharides to macromolecules and solid supports

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(54) Title: IMPROVED COUPLING OF BACTERIAL POLYSACCHARIDES TO MACROMOLECULES AND SOLID SUPPORTS

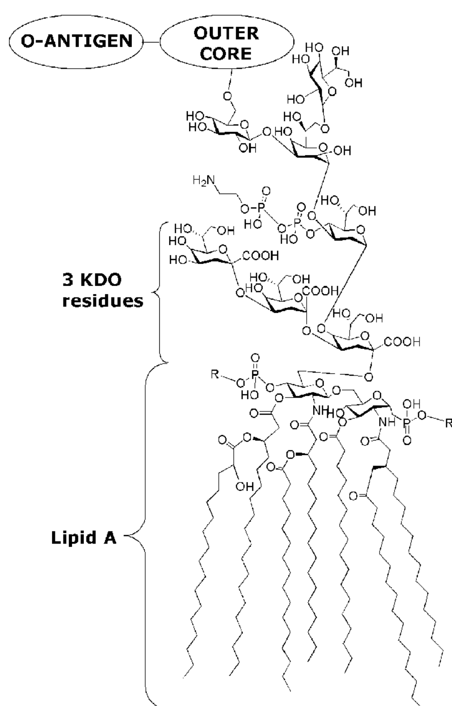

Fig. 1

(57) Abstract: The invention relates to a method of producing a polysaccharide-carrier conjugate comprising coupling a polysaccharide to a carrier, said polysaccharide comprising at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl or a ringclosed ketal or hemi-ketal corresponding thereto, said method comprising the steps of, i) providing a carrier comprising N-hydroxylamine- or N-alkoxyamine groups according to the formula -NHOR, wherein R is H or C_1-C_6 alkyl attached thereto, ii) reacting said N-hydroxylamine or N-alkoxyamine group of the carrier with a keto-carboxy group of said polysaccharide to form a covalent amide bond between the carrier and the polysaccharide. Another aspect of the present invention relates to a compound or solid surface obtained when performing the method of the present invention. A third aspect of the present invention relates to the use of the compound or solid surface in the preparation of an assay device for the detection of antibodies against gram negative bacteria.

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Improved coupling of bacterial polysaccharides to macromolecules and solid supports

Technical field of the invention

5 The present invention relates to bioconjugate chemistry methods for providing molecules or surfaces that are useful within the field of diagnostics, particularly sero-diagnostics. In particular the present invention relates to improved bioconjugation of polysaccharides derived from bacterial antigens containing an alpha-keto acid or ester in their reducing end to solid supports, small molecules 10 and macromolecules comprising an N-hydroxylamine or N-alkoxyamine group and products derived thereof.

Background of the invention

Lipopolysaccharides (LPS) are important antigens, which are prevalent in the 15 outer membrane of bacteria, especially gram negative bacteria. They are amphiphillic molecules comprising a lipid part covalently attached to a polysaccharide part via a single polysaccharide unit which is typically a 2-keto-3-deoxynononic acid (Kdn) residue, or a 2-keto-3-deoxy-D-mannooctonic acid (Kdo) residue. The polysaccharide part comprises an outer and inner region and an O-20 Chain (O-antigen). The O-chain is highly immunogenic and serotype specific, and LPS are therefore good antigens for immunochemical assays for serological detection of antibodies.

The size and amphiphilicity of the native LPS makes it less suitable for chemical 25 ligation onto surfaces, however since the antigenic activity lies in the O-Chain of the polysaccharide part, the lipid part may advantageously be cleaved off the LPS, providing a smaller less amphiphilic polysaccharide (PS) which may more readily be used in immunochemical assay systems.

30 WO 2000/036419 describes a method for immobilising a polysaccharide on a solid surface comprising (a) forming a covalent bond between a PS and a reporter molecule, and (b) immobilising the PS-reporter molecule conjugate via a specific reactivity of the reporter molecule (e.g. biotin or UV sensitive reporter that may

bind to solid surfaces via contact and recognition or UV radiation. The method however relies on traditional carbodiimide mediated amide coupling between the alpha-keto acid of e.g. the Kdo of the PS and an amine moiety on the reporter molecule leading to an alpha-hydroxy or alpha-keto amide bond, which is not particularly stable. Unstable bonds in bioconjugate systems may lead to degeneration or to bleeding of antigen from surfaces or molecules and therefore less reliable analytical results and lower durability of the surfaces or analytical bioconjugates. Furthermore, traditional amide bond formation requires the use of highly reactive amide coupling reagents and bases, which leads to side reactions, and potential antigenic degradation leading to unreliable results. A similar approach to the above is described in Jauho et al., Journal of Immunological methods 242, (2000), 133-143.

It is known that alpha-keto acids will react highly selectively with N-hydroxylamines or N-alkoxyamines to form amide bonds via a decarboxylative mechanism and without the use of reactive activating reagents as described in Bode et al., Angew. Chem. Int. Ed., 45, (2006), 1248-1252, however the reaction is demonstrated for amino acid couplings only to form peptides. The reaction has also been shown to work on the solid phase as described in Fukuzimi et al., J. Am. Chem. Soc., 131, (2009), 3864-3865, but again only for coupling protected amino acids onto peptidyl-resins. Coupling of polysaccharides are not reported in any of the two references.

Hence, an improved method for providing analytical surfaces and compounds in the field of bacterial sero-diagnostics would be advantageous, and in particular a more efficient and/or reliable method of coupling LPS antigens to surfaces and molecules to provide improved analytical sensitivity and reliability would be advantageous.

Summary of the invention

Thus, an object of the present invention relates to conjugation of polysaccharide derived from lipopolysaccharides from Gram negative bacteria, e.g. *salmonella, actinobacillus pneumonia* (Ap) and others to molecules or surfaces such as

microbeads, microtitre plates, organic molecules such as small organic molecules, reporter molecules or nanoparticles.

In particular, it is an object of the present invention to provide an analytic method for the analysis and serotyping of gram negative bacteria in a sample that solves the above mentioned problems of the prior art with respect to regiospecific conjugation to polysaccharides containing an alpha-keto acid or alpha-keto ester moiety under very mild conditions, and provides an improved bonding between the molecule or surface and the polysaccharide antigen and also improved presentation of the antigen.

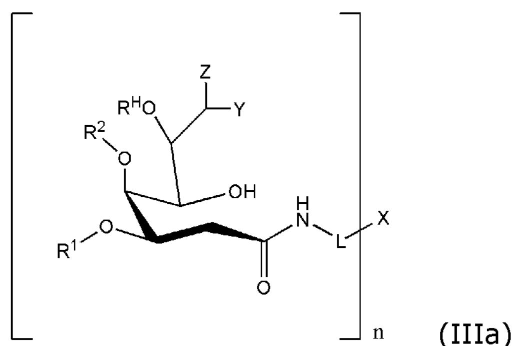
The inventor has surprisingly found that the use of chemoselective amide ligations by decarboxylative condensations of N-hydroxylamine or N-alkoxyamine groups and α-keto acids present in bacterial LPS, provides improved bonding due to the amide bond formed, which does not have a hydroxy or keto group in the α-position.

Thus, one aspect of the invention relates to a method of producing a polysaccharide-carrier conjugate comprising coupling a polysaccharide to a carrier, said polysaccharide comprising at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl or a ring-closed ketal or hemi-ketal corresponding thereto, said method comprising the steps of,

i) providing a carrier comprising N-hydroxylamine- or N-alkoxyamine groups according to the formula -NHOR, wherein R is H or C_1-C_6 alkyl attached thereto,

ii) reacting said N-hydroxylamine or N-alkoxyamine group of the carrier with a keto-carboxy group of said polysaccharide to form a covalent amide bond between the carrier and the polysaccharide.

Another aspect of the present invention relates to a compound or solid surface of the general formula IIIa



(IIIa)

wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group

R^2 is a polysaccharide residue

Y is OR^H or hydrogen

Z is OR^H or CH_2OR^H

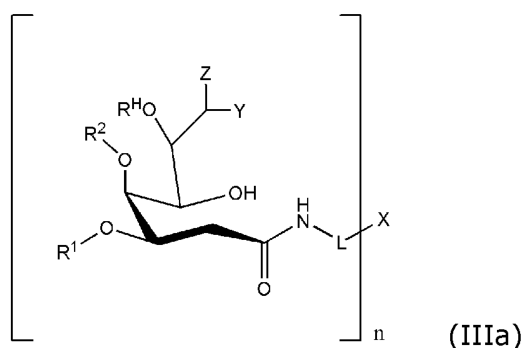
R^H is independently selected from hydrogen and a hydroxy protecting group

L is a covalent bond or a linker molecule

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface,

n is an integer, said integer being 1 or higher.

A third aspect of the invention relates to the use of a compound or solid surface of the general formula IIIa



(IIIa)

wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group

R^2 is a polysaccharide residue

Y is OR^H or hydrogen

Z is OR^H or CH_2OR^H

R^H is independently selected from hydrogen and a hydroxy protecting group

L is a covalent bond or a linker molecule

5 X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface,

n is an integer, said integer being 1 or higher,

for the preparation of an assay device for the detection of antibodies against gram negative bacteria

10

Apart from providing a more stable bond between polysaccharide antigen and the carrier molecule or surface, the ligation method is furthermore reagent free, and no reactive reagents are present when the antigen is ligated to the carrier molecule or surface. This reduces unwanted reactions between other parts of the 15 antigen molecule, which comprises many functional groups that may react with coupling reagents. Also the highly selective Bode ligation methods reduces unwanted side reactions between the carrier and other parts of the polysachharide and any intramolecular reactions in the polysaccharide. This leads to improved presentation of the antigen and thus improved sensitivity.

20

Brief description of the figures

Figure 1 shows an example of a schematic structure of lipopolysaccharide from Salmonella Typhimurium. The Lipopolysaccharide consists of the polysaccharide 25 antigen (inner core + outer core + O-antigen) and the Lipid A residue, which are connected via one of three KDO carbohydrate residues.

Figure 2 shows an example of a delipidated polysaccharide antigen (PS antigen) with retained bioactivity. The Lipid A residue has been cleaved off by mild 30 hydrolysis from the KDO residues of the polysaccharide antigen. The KDO monosaccharide unit in this case represents the monosaccharide unit comprising a keto-carboxy group or a ring-closed ketal or hemi-ketal corresponding thereto of the present invention.

Figure 3 shows an exemplary scheme for the modification of microbeads with N-alkyl alkoxyamine functionality and subsequent reaction with the alpha-keto acid of the antigen KDO residue. This corresponds to the steps of example 1a (A,B) and example 2 (C). The conditions used were A) 1,3- dibromopropane and $NaHCO_3$ were added to amine-functionalized Spherotech microbeads in 50% aqueous ethanol, and stirred at 8 °C for 16 h to obtain an alkylbromide funtionalized microbead. B) R^3ONH_3Cl and $NaHCO_3$ were added to the alkylbromide funtionalized microbead from step A in 50% aqueous ethanol at 8 °C for 16 h to obtain hydroxylamine or alkoxyaminefunctionalized microbeads. C) The hydroxylamine or alkoxyaminefunctionalized microbeads of step B are treated with a delipidated PS antigen in water at 40 °C to obtain PS antigen functionalised microbeads with a highly stable amide connection between the microbead-linker moiety and the PS antigen.

Figure 4 shows a comparison of sensitivity and peak resolution at 300 ng/ml antibody content between 10 month old PS antigen (salmonella Typhimurium) coupled beads via the method of the present invention (TOP GRAPH) and newly prepared antigen beads via carbodiimide coupling (BOTTOM GRAPH). As can be seen from the flow cytometry images the Coefficient Variation or peak broadness (CV) of the carbodiimide beads is much larger compared to antigen beads coupled by the method of the present invention. This indicates that the homogenicity of antigen binding is much lower of the carbodiimide beads compared to the antigen beads coupled by the present method.

Figure 5 shows the same comparison as in figure 4 but at 18.75 ng/ml antibody concentration. The improvements of the beads of the present invention over the carbodiimide beads are also seen here.

Figure 6 shows the same comparison as in figure 4 but at 2.34 ng/ml antibody concentration. The improvements of the beads of the present invention over the carbodiimide beads are also seen here.

Figure 7 shows the same comparison as in figure 4 but at 0.58 ng/ml antibody concentration. The improvements of the beads of the present invention over the carbodiimide beads are also seen here.

Figure 8 shows the same comparison as in figure 4 but at 0 ng/ml antibody concentration. Here the peaks are similar, indicating that the effect does arise from the improved coupling of the antigen.

Figure 9 shows the same comparison as in figure 7 (beads of the present invention in the TOP GRAPH, and carbodiimide coupled beads in the BOTTOM GRAPH), i.e. at 0.58 ng/ml antibody concentration, but the results are shown here as 2D topological graphs. It is seen how the beads of the present invention provide a much more well-defined peak with less noise than for carbodiimide beads.

Figure 10 shows an exemplary scheme for an alternative modification of microbeads with N-alkyl alkoxyamine functionality. This corresponds to the steps of example 1b (A,B). The conditions used were A) Amine modified beads (spherotech, 3.5µm, 0.50mL) was centrifuged (5000rpm, 2min) and the supernatant was removed by decantation. PBS buffer (0.3mL, pH 7.4) was added followed by glutaric aldehyde (50µL, 25% solution in water). The bead mixture was shaken (750rpm, Eppendorf Thermomixer) overnight at room temperature.. B) The mixture was centrifuged and the supernatant removed and fresh PBS buffer (0.3mL) was added followed by sodium cyanaoborohydride (0.16 mmol, 10mg) and O-methoxyamine hydrochloride (0.16 mmol, 14mg). The bead suspension was shaken overnight at room temperature to obtain methoxyamine functionalized microbeads.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

Polysaccharide

In the present context a polysaccharide is a polymeric carbohydrate molecule comprising 2 or more monosaccharide units or derivatives thereof.
Polysaccharides may be linear or branched and the monosaccharide units may be

of natural origin, or they may include synthetic derivatives, such as for example hydroxyprotected monosaccharide units. The monomer units are generally attached to each other via glycosidic bonds, however in the present context other inter-monomer bonds may occur.

Carrier

In the present context a carrier is a functional entity which may be attached to a means for attaching a polysaccharide using the methods of the present invention. The function of the carrier may be a number of things including immobilisation of the polysaccharide, insolubilizing the polysaccharide-carrier conjugate, providing detectability to the polysaccharide-carrier conjugate, or providing specific binding characteristics to the polysaccharide-carrier conjugate

polysaccharide-carrier conjugate

In the present context a polysaccharide-carrier conjugate is a polysaccharide derivative covalently bound to a carrier. The compounds or solid surfaces of formula (III) and (IIIa) are examples of such polysaccharide-carrier conjugates.

Small organic molecule

In the present context a small organic molecule is a low molecular weight organic compound, with a molecular weight of less than 800 Daltons. The organic compound may include non-organic atoms or ions, i.e. they may include for example an iron (III) ion, or they may be complexed with titanium, palladium or other metal ions. They may also be in a salt form, such as a potassium or sodium salt.

Macromolecule

In the present context a macromolecule is a high molecular weight compound, with a molecular weight of 800 Daltons or more. Macromolecules may include polymers, including micro-beads, quantum dots and nano-particles or biopolymers, such as proteins, antibodies and polysaccharides. It may also be other large synthetic or natural molecules, including for example dendrimers or fullerene derivatives.

Solid surface

In the present context a solid surface is any surface which may bind to a polysaccharide and which belongs in the macroscopic world. By "belonging to the macroscopic world" is meant that it may be physically handled in the laboratory either directly or with tools. It is not microscopic. Examples of solid surfaces include polymeric and glass surfaces. The surface may be integrated in for example well-plates or other laboratory equipment used in for example biological assays.

Linker molecule

In the present context a linker molecule is any molecule that may provide a covalent or non-covalent link between one chemical moiety and another.

Reagent

In the present context a reagent is a chemical entity which actively participates in a chemical reaction, but is not included in the final product of said chemical reaction. A reagent may be a molecular species, an ion or an atom. A reagent may work in a catalytic manor, or it may be consumed in the reaction, and is thus a stoichiometric reagent. Particularly a reagent is not a solvent and it is not any of the starting materials which form the product of the chemical reaction. In a coupling reaction connecting two molecular species, a reagent would thus not be part of these two species (starting materials), but would constitute a third species, which at the most would form intermediate chemical species during the reaction.

C_1-C_6 alkyl

In the present context a C_1-C_6 alkyl is an alkyl group comprising one (-CH_3) to six carbon atoms. The alkyl may be linear or branched. Linear alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl and n-hexyl. Examples of branched alkyls include iso-propyl, 2-methyl-1-butyl, 3-methyl-1-butyl, tert-butyl.

Hydroxy protecting group

In the present context a hydroxy protecting group (PG) is any group that may be attached to a hydroxy group (-OH) to form a protected hydroxy group (-O-PG). Hydroxy protecting groups are generally characterized in rendering hydroxy

groups less susceptible to electrophilic attack, reduction and oxidation, and to be cleavable from said hydroxy group using a well-known reaction.

The Method of providing a polysaccharide-carrier conjugate

5 A first aspect of the present invention is a method of producing a polysaccharide-carrier conjugate comprising coupling a polysaccharide to a carrier, said polysaccharide comprising at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl or a ring-closed ketal or hemi-ketal corresponding thereto, said 10 method comprising the steps of,

i) providing a carrier comprising N-hydroxylamine or N-alkoxyamine groups according to the formula -NHOR, wherein R is H or C_1-C_6 alkyl attached thereto,

15

ii) reacting said N-hydroxylamine or N-alkoxyamine group of the carrier with a keto-carboxy group of said polysaccharide to form a covalent amide bond between the carrier and the polysaccharide.

20 In a preferred method the at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl, or a ring-closed ketal or hemi-ketal corresponding thereto, is selected from the group consisting of a 2-keto-3-deoxynononic acid (Kdn) residue, and a 2-keto-3-deoxy-D-mannooctonic acid (Kdo) residue, and hydroxy-protected 25 derivatives thereof.

The carrier may preferably be selected from the group consisting of a small organic molecule, a macromolecule or a solid surface.

30 The small organic molecule is preferably a molecule detectable via electromagnetic or other forms of radiation or a molecule capable of binding to a such a detectable molecule. The small organic molecule may be selected from the group consisting of a reporter molecule, an antigenic molecule and small immune stimulating peptides. A reporter molecule, may be a fluorophore, an antigenic 35 molecule may be a hapten, and small immune stimulating peptides may be e.g.

muramyl dipeptide or D-amino acid based peptides. Suitable fluorophores include fluoresceine, cyanine dyes, rhodamine dyes, dansyl, dabsyl, EDANS, squarate/squaric acid based dyes, croconic acid based dyes. Suitable D-amino acid based peptides include Ala-D-IsoGln oligomers, poly-D-lysine.

5

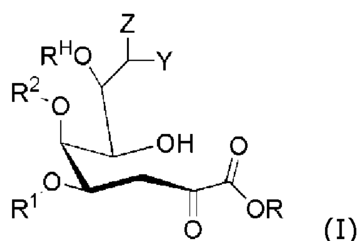
The macromolecule may be selected from the group consisting of a bead, a micro-bead, a nanoparticle, such as gold or silver nanoparticles, a quantum dot, a protein, a dendrimer or dendron, a polynucleotide, an antibody and any combination thereof. Microbeads are particularly preferred. Suitable beads and
10 microbeads include polystyrene microbeads, magnetic beads (Dynabeads), fluoresecent polystyrene beads and combinations hereof. Suitable nanoparticles include quantum dots (inorganic and organic), poly-lactic-glycolic acid (PLGA) particles, dendrimers, dendrons, liposomes, iscoms and combinations hereof.

15 The solid surface may be selected from a polymer surface or a glass surface. The solid surface may for example be the surface of a device used in bioassay applications. Such devices may include surfaces based on polystyrene, polypropylene, polyethylene, PVC or glass. The formate of the surface may include microtitre plates e.g. 96-well-plates, glass-slides, SU8 slides, surfaces for
20 Cantilever or Quartz Crystal Microbalance (QCM) technology.

In a preferred embodiment the N-hydroxylamine or N-alkoxyamine groups are attached to the carrier via a linker molecule. The linker molecule (L) may be branched or linear. Advantages of having a linker molecule between the carrier
25 and the polysaccharide anchor point (the N-hydroxylamine or N-alkoxy amine) includes control of the distance between carrier and polysaccharide via the choice of linker, and also control of “loading”, i.e. number of polysaccharides per carrier, via either control of linker loading, or via choice of branched linkers, which allow for higher loadings and also proximity effects. It has surprisingly been found that
30 the proximity effects are highly present in the bio-assay applications of the present invention, meaning that using branched linkers presents the polysaccharide antigens in close proximity, and this appears to enhance the detection levels of e.g. antibodies binding to these polysaccharide antigens.

In a preferred embodiment the polysaccharide is substantially identical to a lipopolysaccharide (LPS) or the carbohydrate part thereof. Preferably the LPS or carbohydrate part thereof is a bacterial LPS or is derived from a bacterial LPS i.e. it may be a delipidated LPS. In yet another preferred embodiment the
5 polysaccharide is derived from a gram-negative bacterial lipopolysaccharide. The bacteria that express the LPS of the present invention are preferably human or veterinary pathogens. Gram negative bacteria that express the LPS of the present invention include human Gram-negative bacteria selected the group consisting of *Haemophilus sp, Echerichia coli ssp, Salmonella sp, Klebsiella sp, Bordete/la sp,*
10 *Pseudomonas sp, Chlamydia sp, Neisseria sp, Vibrio cholerae, Shigella sp, Proteus sp, Brucella sp, Streptobacillus sp, Yersinia sp, Legionella pneumophila,* and *Serratia marcescens,* especially *Haemophilus influenzae, Salmonella enterica ssp., Klebsiella pneumoniae, Bordetella pertussis, Pseudomonas aeruginosa, Chlamydia psitacci, Neisseria meningitidis, Neisseria gonorrhea, Vibrio cholerae, Shigella*
15 *flexneri,* and *Shigella dysenteriae,* and veterinary or zoonotic bacteria including enterobacteria selected from *Escherichia coli, Salmonella Typhimuriurn, Salmonella Choleraesuis, Salmonella enterica,* and all serotypes hereof, especially *Salmonella Typhimurium, Salmonella Enteritidis, Salmonella Choleraesuis, Salmonella Manhattan, Salmonella Dublin, Salmonella Infantis, Escherichia coli*
20 *spp. including O157,* oedema-disease causing *Escherichia coli, Yersinia enterocolitica,* and *Campylobacter jejuni,* as well as respiratory bacteria selected from the HAP group of bacteria especially *Actinobacillus sp,* in particular the HAP group bacteria *Actinobacillus pleuropneumoniae, Haemophilus somnus, Pasteurella haemolytica, Pasteurella multocida, Haemophilus parasuis* and
25 *Mannheimia sp.*

In a preferred embodiment the polysaccharide is a compound of formula I



(I)

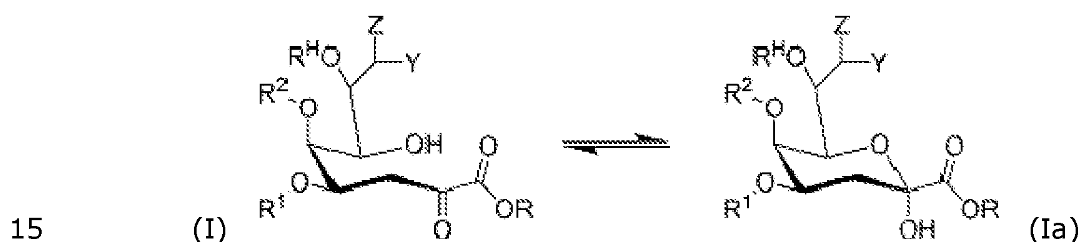
30

wherein

R is hydrogen or C_1-C_6 alkyl
R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group
R^2 is a polysaccharide residue
5 Y is OR^H or hydrogen
Z is OR^H or CH_2OR^H
R^H is independently selected from hydrogen and a hydroxy protecting group.

or a ring-closed ketal or hemi-ketal corresponding thereto. Depending on the
10 chemical and physical conditions the compound of formula (I) may exist in a chemical equilibrium with a corresponding ring-closed form (Ia), as is generally the case for many carbohydrates. The equilibrium is depicted in Scheme 1 below.

Scheme 1

15 

The term "polysaccharide residue" represented by R^2 in formula (I) and (Ia) is intended to mean the part of a polysaccharide (as defined above) which together
20 with the monosaccharide unit comprising a keto-carboxy group and the remaining substituents thereon forms said polysaccharide. Thus, R^2 is preferably a polysaccharide residue derived from a lipopolysaccharide, preferably a polysaccharide derived from a lipopolysaccharide from a gram negative bacterium, preferably of a bacterium as listed above. The monosaccharide unit comprising a
25 keto-carboxy group together with R^1, Y, Z and R^H and part of R^2 form what is referred to as the "inner core" of an LPS. The remaining part of R^2 forms the "outer core" and bound to the outer core is the "O-antigen" (see Figure 1 and 2) also known as the "O-Chain". It is mainly the O-antigen which will bind antibodies to bacteria comprising LPS on their surface. This enables the detection or
30 immobilisation of such antibodies, since the antibody is then effectively attached

to the functional carrier of the present invention, which is a small organic molecule, a macromolecule or a solid surface.

In a preferred embodiment R^2 is a polysaccharide residue comprising 2-1000
5 monosaccharide units, such as 2-500 monosaccharide units, 3-400 monosaccharide units, preferably 4-200 monosaccharide units.

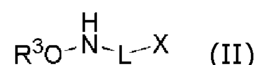
In one preferred embodiment R^H is hydrogen. In another embodiment Y is hydrogen when Z is OR^H, and Y is OR^H when Z is CH_2OR^H. In a particularly
10 preferred embodiment X is a macromolecule or solid surface, Y is hydrogen when Z is OR^H, and Y is OR^H when Z is CH_2OR^H, and L is a linker molecule. R may preferably be hydrogen or C_1-C_3 alkyl.

Suitable hydroxy protecting groups for the present invention are any hydroxy
15 protecting used in carbohydrate chemistry. Such groups may be selected from the group consisting of ethers such as tert-butylethers, benzylethers, dimethoxytritylethers, ,acetals such as e.g. isopropylidene or bezylidene, silylethers such as e.g. TMS, TIPS, TBDMS, TPS, esters such as e.g. acetyl or benzoyl, carbonates such as e.g. t-Butyl carbonate (BocO).
20

The polysaccharide such as for example the compound of formula (I) may generally be provided for example by isolating and hydrolysing a bacterial LPS. Mild acidic hydrolysis will cleave of the lipid part of the LPS, but the antigenic part is retained. This may be referred to as delipidation and it increases the solubility
25 and reduces the toxicity of the resulting polysaccharide. The polysaccharide antigen contains at least one KDO residue (the two conjugated KDO residues may be cleaved off during the acid treatment as well. The single KDO residue will function as a regiospecific handle for the presented conjugation process with N-alkyl alkoxyamines/N-alkyl hydroxylamines. A method of providing such a
30 delipidated polysaccharide is described in WO 00/36419, example 6 and 7 page 34-35.

The carrier comprising N-hydroxylamine or N-alkoxyamine groups attached thereto may preferably be a compound of formula II

35



(II)

wherein

R^3 is hydrogen or C_1-C_6 alkyl.

5 L is a covalent bond or a linker molecule

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface.

R^3 may preferably be hydrogen or C_1-C_3 alkyl. Preferred embodiments of the small

10 organic molecule, the macromolecule and the solid surface are described above.

The linker molecule L may preferably be a biradical selected from the group consisting of a C_1-C_{16} alkylene, preferably according to the formula $-(CH_2)_n-$ wherein n is an integer between 1 and 16, oligo-peptides, oligo-amides, or oligo-

15 oxyethylenes such as oligo-oxyethylenes according to the formula $(-CH_2CH_2O-)_n$, wherein n is an integer between 1 and 16. The linker may be attached to the carrier X via a build-in functional group on the carrier such as an amine or alcohol. The three forms of carrier of the present invention (small organic molecules, macromolecules and solid surfaces) are generally commercially available with

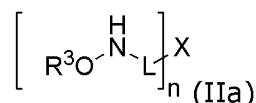
20 various functionalities build in. A functional group on the carrier, such as for example an amine ($-NH_2$) may thus be attached to more than one linker, e.g. X-N-$(L\text{-}NHOR^3)_2$.

Suitable linker molecules include the molecules selected from the list consisting of

25 $-(CH_2)-$ $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$, $(-CH_2CH_2O-)_1$, $(-CH_2CH_2O-)_2$, $(-CH_2CH_2O-)_3$, $(-CH_2CH_2O-)_4$, $(-CH_2CH_2O-)_5$, $(-CH_2CH_2O-)_6$.

Also, in a preferred embodiment, particularly wherein the carrier X is a solid surface or macromolecule, the carrier may comprise a number of N-

30 hydroxylamine or N-alkoxyamine groups attached thereto, i.e. the carrier comprising N-hydroxylamine or N-alkoxyamine groups attached thereto may preferably be a compound of formula IIa



In this embodiment n is an integer, said integer being 1 or higher, preferably an integer between 1 and 10^9, such as between 1 and 10^8, such as between 1 and 10^6. R^3, X and L are as defined for compound II.

Such compounds wherein n is larger than 1 are particularly preferred when the carrier X is a solid surface or macromolecule. Typical loadings for surfaces or macromolecules including resins is 0.1-10 micromolar/gram resin, such as 0.5-2 micromolar/gram resin, e.g. about 1 micromolar/gram resin. When the carrier X is a small organic molecule then n may preferably be 1.

The carrier comprising N-hydroxylamine- or N-alkoxyamine groups attached thereto, such as for example the compound of formula (II) may generally be provided by obtaining a commercially available carrier X, which comprises a number of reactive functional groups, such as for example, amines, alcohols, aldehydes, carboxylic acids, alkyl halogens, and so on. The carrier X is then reacted with an amount of linker molecule, which will eventually define the loading of antigen on the carrier. Any remaining reactive functional groups may be capped or protected. The linker molecule may already comprise the N-hydroxylamine or N-alkoxy-amine, but preferably it comprises a group that may be converted to said N-hydroxylamine or N-alkoxy-amine, such as for example a so-called "leaving group" e.g. a halogen or tosylate. A leaving group may be converted to a N-hydroxylamine or N-alkoxy-amine by reacting it with N-hydroxyl- or N-alkoxylamine hydrochloride ($R^3ONH \cdot HCl$) in the presence of a base. A detailed description of a method of providing such a functionalised carrier is described in W. J. Haap, D. Kaiser, T.B. Walk, G. Jung, Tetrahedron (1998), 54, 3705-3724, in particular page 3722.

In a preferred embodiment the polysaccharide and the carrier comprising N-hydroxylamine or N-alkoxyamine groups are reacted without the use of any reagents. The keto-carboxy group of the polysaccharide and the N-hydroxylamine or N-alkoxyamine groups of the carrier are reacted to form a polysaccharide-carrier conjugate without the use of a reagent. This is a particular advantage of

the present invention. An N-hydroxylamine or N-alkoxyamine will react highly selectively and in high yields with a keto-carboxy group even without the presence of any reagents, thus forming the desired polysaccharide-carrier conjugate of the present invention. Reagents are generally highly reactive and therefore not necessarily very selective, and the addition of a reactive reagent to a polysaccharide antigen comprising a number of sensitive functional groups throughout the carbohydrate structure, may lead to antigen erosion and also coupling of antigenic parts of the polysaccharide directly to the carrier, leading to partial or full inhibition of binding of e.g. antibodies to the antigen. Using the method of the present invention, you obtain a very selective reaction between the keto-carboxy group which is only present in the inner core of the LPS, and the N-hydroxylamine or N-alkoxyamine of the carrier, which is present on the carrier in a controlled number per carrier and in a controlled distance from the carrier via the (optional) linker.

Reagents that are particularly desirable to avoid using the method of the present invention are amide coupling reagents. Thus, the reaction may be performed without the use of coupling reagents known to the skilled person such as DCC, DIPCDI/DIC, TFFH, WSC, BOP, PyBOP, HBTU, TBTU, COMU. Also, oxidative reagents are preferably avoided. Thus the reaction may preferably be carried out without the use of oxidative reagents such iodine, N-iodosuccinimide, N-bromosuccinimide, hydrogen peroxide.

The reagent free coupling of a N-hydroxylamine or N-alkoxyamine with a keto-carboxy group results in an amide bond, and CO_2 and ROH is released (ROH being water in cases where R=H) via decarboxylation and condensation. It is the release of these two highly stable products which is the driving force of the reaction, allowing for reagent free conditions, and which leads to the formation of an amide, rather than a less stable α-keto amide, as provided in the prior art.

The reagent free coupling of the polysaccharide with the carrier comprising N-hydroxylamine or N-alkoxyamine groups may be performed in a number of solvents including water, buffered aqueous solutions, alcohols e.g. ethanol or methanol, DMF, DMSO, and mixtures thereof. The most preferred solvents are

water and buffered aqueous solutions or mixtures of water with the above mentioned organic solvents.

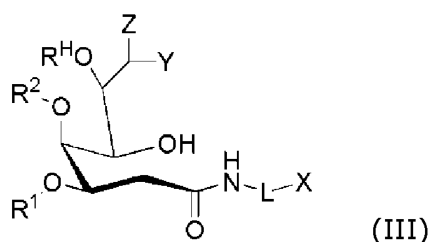
In preferred embodiments the temperature of the reaction mixture is -100 °C to 150 °C, such as -50 °C to 100 °C, -20 °C to 90 °C, -10 °C to 80 °C, 0 °C to 70 °C, 10 °C to 60 °C such as preferably 20 °C to 50 °C.

The herein described process is particularly suitable for the preparation of polysaccharide-carrier conjugates such as compounds of formula (III) or formula (IIIa).

A further embodiment of the present invention is a product obtainable from the process as described above.

The Polysaccharide-carrier conjugate

A second aspect of the present invention is a compound or solid surface of the general formula III



(III)

wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group

R^2 is a polysaccharide residue

Y is OR^H or hydrogen

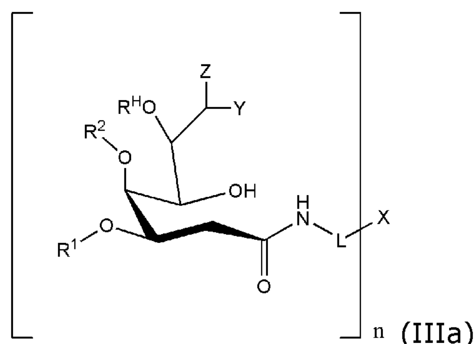
Z is OR^H or CH_2OR^H

R^H is independently selected from hydrogen and a hydroxy protecting group

L is a covalent bond or a linker molecule

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface.

In one embodiment the compound as described above maybe a compound of formula (IIIa)



(IIIa)

5 wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group

R^2 is a polysaccharide residue

Y is OR^H or hydrogen

10 Z is OR^H or CH_2OR^H

R^H is independently selected from hydrogen and a hydroxy protecting group

L is a covalent bond or a linker molecule

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface, and

15 n is an integer, said integer being 1 or higher.

Preferably n is an integer between 1 and 10^9, such as between 1 and 10^8, such as between 1 and 10^6.

20 Such compounds wherein n is larger than 1 are particularly preferred when the carrier X is a solid surface or macromolecule. Typical loadings for surfaces or macromolecules including resins is 0.1-10 micromolar/gram resin, such as 0.5-2 micromolar/gram resin, e.g. about 1 micromolar/gram resin. When the carrier X is a small organic molecule n may preferably be 1.

25

As is evident from the structure of compound (III)/(IIIa), i.e. the polysaccharide carrier conjugate, an amide bond has formed between the N-hydroxylamine or N-alkoxy functionalised carrier and what corresponds to the previous α-keto group of the polysaccharide. This amide bond is much more stable than the α-keto

amide bond described in the prior art. This provides for polysaccharide-carrier conjugates having improved stability, even under e.g. rigorous washing conditions.

5 The preferred embodiments relating to functional groups described in the aspect relating to the method of making the compound of formula (III) or (IIIa), i.e. the polysaccharide-carrier conjugate, from compounds (I)/(Ia) and (II)/(IIa), also applies to the present aspect relating to said polysaccharide-carrier conjugate (III) or (IIIa). Thus, for example, The small organic molecule is preferably a
10 molecule detectable via electromagnetic or other forms of radiation or a molecule capable of binding to a such a detectable molecule. The small organic molecule may be selected from the group consisting of a reporter molecule, an antigenic molecule and small immune stimulating peptides. A reporter molecule, may be a fluorophore, an antigenic molecule may be a hapten, and small immune
15 stimulating peptides may be e.g. muramyl dipeptide or D-amino acid based peptides. Suitable fluorophores include fluoresceine, cyanine dyes, rhodamine dyes, dansyl, dabsyl, EDANS, squarate/squaric acid based dyes, croconic acid based dyes. Suitable D-amino acid based peptides include Ala-D-IsoGln oligomers, poly-D-lysine.
20

The macromolecule may be selected from the group consisting of a bead, a micro-bead, a nanoparticle, such as gold or silver nanoparticles, a quantum dot, a protein, a dendrimer or dendron, a polynucleotide, an antibody and any combination thereof. Microbeads are particularly preferred. Suitable beads and
25 microbeads include polystyrene microbeads, magnetic beads (Dynabeads), fluoresecent polystyrene beads and combinations hereof. Suitable nanoparticles include quantum dots (inorganic and organic), poly-lactic-glycolic acid (PLGA) particles, dendrimers, dendrons, liposomes, iscoms and combinations hereof.

30 The solid surface may be selected from a polymer surface or a glass surface. The solid surface may for example be the surface of a device used in bioassay applications. Such devices may include surfaces based on polystyrene, polypropylene, polyethylene, PVC or glass. The formate of the surface may include microtitre plates e.g. 96-well-plates, glass-slides, SU8 slides, surfaces for
35 Cantilever or Quartz Crystal Microbalance (QCM) technology.

The carrier X may preferably be selected from the group consisting of a macromolecule or solid surface and Y may be hydrogen when Z is OR^H, and Y may be OR^H when Z is CH_2OR^H. In yet another embodiment L is preferably a linker molecule. In other words the definitions and preferred embodiments for R^1, R^2, Y,
5 Z, R^H, L and X mentioned in the first aspect of the invention also applies in the current aspect of the invention.

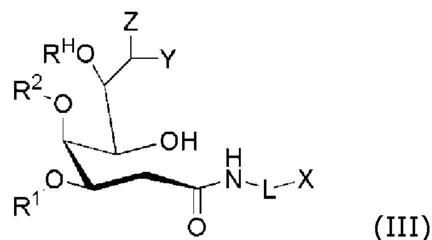
The linker molecule L may preferably be a biradical selected from the group consisting of a C_1-C_{16} alkylene, preferably according to the formula $-(CH_2)_n-$
10 wherein n is an integer between 1 and 16, oligo-peptides, oligo-amides, or oligo-oxyethylenes such as oligo-oxyethylenes according to the formula $(-CH_2CH_2O-)_n$, wherein n is an integer between 1 and 16. The linker may be attached to the carrier X via a build-in functional group on the carrier such as an amine or alcohol. The three forms of carrier of the present invention (small organic molecules,
15 macromolecules and solid surfaces) are generally commercially available with various functionalities build in. A functional group on the carrier, such as for example an amine ($-NH_2$) may thus be attached to more than one linker, e.g. X-N-(L-$NHOR^3$)$_2$.

20 Suitable linker molecules include the molecules selected from the list consisting of $-(CH_2)-$ $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$, $-(CH_2CH_2O)_1-$, $-(CH_2CH_2O)_2-$, $-(CH_2CH_2O)_3-$, $-(CH_2CH_2O)_4-$, $-(CH_2CH_2O)_5-$, $-(CH_2CH_2O)_6-$.

R^2 is preferably a polysaccharide residue derived from a lipopolysaccharide,
25 preferably a polysaccharide derived from a lipopolysaccharide from a gram negative bacterium, preferably of a bacterium as listed above.

Use and Applications

A third aspect of the present invention is the use of a compound or solid surface
30 of the general formula III



(III)

wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting
5 group

R^2 is a polysaccharide residue

Y is OR^H or hydrogen

Z is OR^H or CH_2OR^H

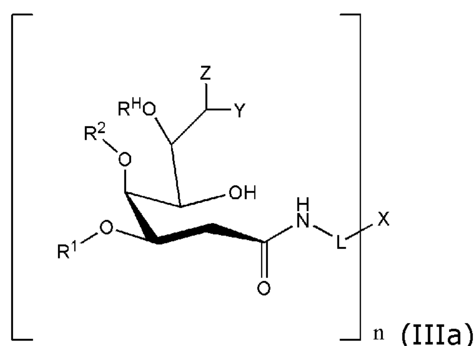
R^H is independently selected from hydrogen and a hydroxy protecting group

10 L is a covalent bond or a linker molecule

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface,

for the preparation of an assay device for the detection of antibodies against gram negative bacteria. Preferably for the detection of antibodies against the gram
15 negative bacteria as listed above.

In one embodiment the compound as described above maybe a compound of formula (IIIa)



(IIIa)

20

wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group

R^2 is a polysaccharide residue

25 Y is OR^H or hydrogen

Z is OR^H or CH_2OR^H
R^H is independently selected from hydrogen and a hydroxy protecting group
L is a covalent bond or a linker molecule
X is a carrier selected from the group comprising of a small organic molecule, a
5 macromolecule and a solid surface, and
n is an integer, said integer being 1 or higher.

Preferably n is an integer between 1 and 10^9, such as between 1 and 10^8, such as between 1 and 10^6.
10

Depending on the nature of X, i.e. whether it is a small organic molecule, a macromolecule or a solid surface, the assay device may be on molecular scale, e.g. polysaccharide antigen coupled to a fluorophore, on a microscopic scale e.g. polysaccharide antigen coupled to a macromolecule such as a polymer bead or on
15 a macroscopic scale, e.g. polysaccharide antigen coupled to a solid surface, e.g. in a well plate.

The method may be applied as valuable tool for mild non-destructive and regiospecific conjugation of polysaccharide antigens in diagnostics, where the
20 antigen may either be conjugated to a solid surface for subsequent bioassay measurement/biosensoring of antibody content in samples by various methods such as flow cytometry or ELISA, or mild and specifically binding to N-alkyl alkoxyamine or hydroxylamine containing macromolecules for the production of specific antibodies against the polysaccharide-macromolecule conjugate.
25

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention. In particular the definitions and functional group limitations provided for the polysaccharide, the carrier, and the individual groups attached to these
30 molecules in the section referring to the method also apply to the aspect describing the polysaccharide-carrier conjugate compound.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.
35

The invention will now be described in further details in the following non-limiting examples.

Examples

Example 1a – Functionalizing microbead carrier with linker and N-hydroxylamine or N-alkoxyamine groups

Commercially available aqueous suspension of Spherotech beads (3.5 µm, 1mL, primary amine surface) was transferred to a 15 mL Sarstedt tube and ethanol (96%, 1mL) was added. The suspension was cooled to 8 °C and sodium hydrogen carbonate (0.5mmol, 42mg) was added followed by 1,3-dibromopropane (0.25mmol, 25µL) and the mixture was shaken overnight at 8 °C. The supernatant was removed and the beads were re-suspended in ethanol (96%, 1mL) and washed 3 times with ethanol and 2 times with MilliQ water. Ninhydrin test of the beads showed a negative result indicating derivatisation of the primary amine groups on the beads. The beads were suspended in milliQ water (1mL) and divided into two 0.5 mL portions. To each bead suspension sodium hydrogen carbonate (0.25mmol, 21mg) was added and to one, a suspension N-hydroxylamine hydrochloride (0.25mmol, 17mg) was added and the mixture shaken overnight at r.t. To the other suspension methoxyamine hydrochloride was added and this suspension was shaken overnight at r.t. The two aliquots of beads were washed with water (3 times) and each portion of beads suspended in 0.5mL MilliQ water. Thus in the current example L = $-(CH_2)_3-$, R^3 = H or CH_3, X is a microbead functionalised with amine groups. See also Figure 3 (steps A and B) for and explanatory reaction scheme.

Example 1b – Functionalizing microbead carrier with linker and N-alkoxyamine groups

Amine modified beads (spherotech, 3.5µm, 0.50mL) was centrifuged (5000rpm, 2min) and the supernatant was removed by decantation. PBS buffer (0.3mL, pH 7.4) was added followed by glutaric aldehyde (50µL, 25% solution in water). The bead mixture was shaken (750rpm, Eppendorf Thermomixer) overnight at room temperature. The mixture was centrifuged and the supernatant removed and fresh PBS buffer (0.3mL) was added followed by sodium cyanoborohydride (0.16 mmol, 10mg) and O-methoxyamine hydrochloride (0.16 mmol, 14mg). The bead

suspension was shaken overnight at room temperature. The beads were washed with MilliQ water (3 times) and resuspended in 0.5mL MilliQ water. The reaction is depicted in Figure 10.

5 Example 2 – conjugation of polysaccharide antigen to microbead carrier comprising N-hydroxylamine and N-alkoxyamine groups

50µL suspension of either N-alkylhydroxylamine microbeads or N-Alkylalkoxyamine microbeads was added to 5 mg polysaccharide antigen (Salmonella Typhimurium containing a KDO residue) derived from delipidated
10 lipopolysaccharide as earlier described (WO 00/36419, example 6 and 7 pp 34-35) weighed off in a 1.5 mL Eppendorf tube and 100 µL MilliQ water was added. The suspension was shaken for 16 h at 45 °C and 750 rpm on an Eppendorf Thermomixer Compact. The beads were transferred to a 96 well filter plate (Millipore, MSBVN1210: 1.2µm Hydrophilic, low protein binding Durapore®
15 membrane) equipped to a Millipore MultiScreen HTS Vacuum Manifold and washed 3 times with MilliQ water , 3 times with 30% aqueous acetic acid (to remove adsorbed non-bound LPS or PS) and 3 times with MilliQ water. The beads were transferred by a spatula to an Eppendorf tube and suspended in 50µL MilliQ water and are ready for use. In the below examples the PS antigen beads functionalized
20 via hydroxylamine functionalized beads are denoted “UB1315B hydroxy b” and the PS antigen beads functionalized via methoxyamine functionalized beads are denoted “UB1315B methoxy b”. See also Figure 3 (step C) for an explanatory reaction scheme (for the KDO residue in this example: R^H = H, R^2 = polysaccharide residue of Salmonella Typhimurium, R^1 = H, Y = H, Z = OR^H).
25

Example 3 – Testing of coupled beads using immune staining and flow cytometric analysis

1. Aliquots of beads: Coupled beads and beads without antigen are stored in Milli Q water at 2-8 °C in aliquots of 107 – 108 beads per ml. Before using the
30 bead preparations, vortex mixing is performed for approx. 1 min. The concentration of “single beads” is estimated by flow cytometry after simultaneous acquisition of “reference counting beads” with known concentration (CountBrightTM Absolute Counting Beads, Molecular ProbesTM). Calculation of bead concentration is based on acquisition of min. 1000 beads. Population of

"Single beads" (i.e. not aggregated beads) is defined from the profile of relative size and granularity in a plot showing forward - and side scatter (FSC-SSC) and for dyed beads also profile of fluorescence intensity. Bead-batches with at least 50% "single beads" of total counts are acceptable.

2. Control beads: For each by flow cytometry analysis, the following controls were used:
-Profiles of Antigen-coupled-beads are compared to control beads having N-alkyl hydroxylamine/N-alkyl methoxyamine "linker", but without antigen.
-Profiles of Antigen-coupled-beads are compared with "positive control beads", coupled with same antigen ("Exiqon-beads" as described in WO 00/36419).
-Beads without "linker", i.e. commercially available microbeads (Spherotech) with primary amine surface groups, are used as 'nude bead' control.

3. Immune staining of beads: To optimize mainly one population of "single beads" the bead preparations are diluted to approx. 106 beads /ml in blocking buffer and receive a 15-min. treatment in an ultrasound bath (DT 52H, Bandelin Sonorex Digital).
Incubations and staining procedures were performed with approx. 5 x 104 "single beads" in each well. Incubations are performed in filter plates (Millipore, MSBVN1210: 1.2µm Hydrophilic, low protein binding Durapore® membrane) and washing procedures performed using a vacuum manifold (Millipore).

Buffers
Blocking buffer: PBS with 0.05% Tween 20 and 1% BSA
Washing buffer: PBS with 0.05% Tween 20

Beads are stained with a panel (different concentrations) of monoclonal antibodies (mAb) recognising Salmonella factor 4 (serogroup B) and 9 (serogroup D), respectively. A reporter signal is obtained after secondary staining of the bound mAb with rabbit-anti-mouse-IgG conjugated with RPE (DAKO R0439). The "reporter antibody" has been titrated and is used in saturating concentration (dilution 1:25).
A graduated level of "reporter signal" is obtained after incubation with the panel of mAbs. A "blank", without mAb, shows "single beads" with "reporter signal" at low

level of fluorescence intensity (auto fluorescence, which has nearly same level of signal as "nude beads" without any staining).

4. Monoclonal antibodies – model system: Peculiar monosaccharides, such as abequose (= factor 4 of Salmonella serogroup B) and tyvelose (= factor 9 of Salmonella serogroup D), constitute strongly immunogenic (haptenic) structures in bacterial lipopolysaccharides.

Monoclonal antibodies recognising factor 4 (serogroup B, S. Typhimurium 4,31A15B6 Jan/Feb 2010) and factor 9 (serogroup D, S. berta 2.36 A14B4C4D2 141210) are produced and validated at DTU-VET. These mAbs are used to test effective coupling of beads and coupling specificity.
-Beads coupled with PS from Salmonella Typhimurium shows positive reaction with mAb against factor 4 (serogroup B), but not with mAb against factor 9 (serogroup D), compared to control beads without PS coupling.
-Beads coupled with PS from Salmonella dublin shows reaction with mAb against factor 9 (serogroup D), but not with mAb against factor 4 (Serogroup B), compared to control beads without PS coupling.

5. Flow cytometry settings and controls: A FACSCanto II flow cytometer (BD Biosciences) equipped with 3 lasers (488 nm, 633 nm, 405 nm) and a HTS plate reader for 96-well plates is used. FACS DIVA 6.02 software is used. For quality control of the flow cytometer, Cytometer Setup & Tracking beads (CST beads) is analysed at daily use, and technical service is performed with regular intervals (maintenance contract). Accept of analysis of CST beads are defined in the DIVA 6.02 software and by the batch identity of the CST beads.

For analysis of stained beads approx. 2000 "single beads" are acquired. These beads are defined from FSC-SSC profiles and bead fluorescence if dyed beads. A threshold level on FSC-SSC is set to exclude noise (events with lower FSC-SSC than the main population of "single beads"). Stained beads are acquired on flow cytometer within 24 hours after immune staining.

Reporter signal (fluorescence intensity of R-Phycoerythrin (RPE)) is measured after excitation with the blue laser (488 nm) and detection of emission using PMT

and 585/42 band pass filter. Instrument settings are adjusted for FSC-SSC to show the population of "single beads" in a pre-defined gate and the reporter signal adjusted for the "blank" (i.e. beads only incubated with reporter antibody) to show a median fluorescence intensity of gated "single beads" at approx. 200 relative units (auto fluorescence).

Using dyed beads for multiplex analysis (e.g. blue particle array kit, Spherotech Inc.), these beads are detected using the red laser (633 nm) and detection of emission using PMT with 780/60 band pass filter. Compensation of spectral overlap between the bead fluorescence and reporter signal is not required in this combination. However using other combinations of dyed beads and reporter fluorochromes may require compensation of spectral overlap.

6. Data and statistic: In a histogram plot showing "single beads" gated in FSC-SSC density plot, median fluorescence intensity (MFI) of reporter signal is calculated. Optimally, the population of "single beads" demonstrate reporter signals mainly in one population (peak), including >90% of the gated beads, and with a %CV < 100 (CV = Coefficient Variation or peak broadness). Bead preparations showing more peaks are not accepted. Bead preparations with more peaks or %CV > 100 for the reporter signal may have an uneven or inadequate coupling of PS.

Table 1: Test of a typical preparation of beads made by conjugation of the KDO carboxylic acid moiety by carbodiimide mediated coupling to a bead with primary amines at its surface (Beads Exiqon). mAb: Monoclonal antibody. MFI: Mean fluorescence intensity. Cells in bold shows the lowest detectable antibody concentration by the bead.

		***Salmonella* Typhimurium PS**	
Mab against *Salmonella* Typhimurium		Beads Exiqon **200810** b	
Concentration mAb ng/ml	Dilution	Reporter signal MFI	Fold increase vs. without mAb
1000,000	1	41101	211
250,000	4	15009	77
62,500	16	4261	22
15,625	64	1231	6
3,906	256	442	2
1,953	**512**	**331**	**2**
0,977	1024	219	1
0,488	2048	207	1
0,244	4096	272	1
0,122	8192	231	1
0,061	16384	202	1
0,015	65536		
0,004	262144		
0,001	1048576		
0	without mAb	195	1

The results in table 1 illustrates that under the given conditions the mAb detection limit is approximately 1.95 ng/ml for the beads of the prior art as described in WO
5 00/36419 (Beads Exiquon).

Table 2: Test of a typical preparation of polysaccharide antigen beads (salmonella Typhimurium) made by the ligation method of the present invention between the KDO α-keto carboxylic acid moiety to beads with N-alkyl methoxylamines
10 (UB1315B methoxy b) or N-alkyl hydroxylamines (UB1315B hydroxy b) at their surfaces (see example 1 and 2) respectively. mAb: Monoclonal antibody. MFI: Mean fluorescence intensity. Cells in bold show the lowest detectable antibody concentration the bead is capable of detecting.

Mab against *Salmonella* Typhimurium		***Salmonella* Typhimurium PS**			
		Beads UB1315B methoxy b		Beads UB1315B hydroxy b	
Conc. mAb ng/ml	Dilution	Reporter signal MFI	Fold increase vs. without mAb	Reporter signal MFI	Fold increase vs. without mAb
1000,000	1	152815	915	101036	532
250,000	4	68015	407	40368	212
62,500	16	21899	131	10104	53
15,625	64	4965	30	2506	13
3,906	256	1038	6	686	4
0,977	1024	616	4	438	2
0,244	**4096**	**340**	**2**	**303**	**2**
0,061	16384	262	2	237	1
0,015	65536	221	1	215	1
0,004	262144	195	1	198	1
0,001	1048576	185	1	191	1
0	No mAb	167	1	190	1

It is seen from the results in table 2 that for the beads of the present invention the detection limit for mAbs of salmonella Typhimurium is as low as 0.24 ng/ml,
5 which is significantly better than the beads of the prior art (see table 1).

Table 3: Test of beads with either N- alkyl methoxylamines (UB1312 methoxy b) or N-alkyl hydroxylamines (UB1312 hydroxy b) at their surfaces without antigen polysaccharide coupled to their surface.

Beads without PS-antigen			
Beads UB1312 methoxy b		Beads UB1312 hydroxy b	
Reporter signal MFI	Fold increase vs. without mAb	Reporter signal MFI	Fold increase vs. without mAb
172	1	154	1
167	1	150	1
164	1	147	1
170	1	155	1
166	1	149	1
160	1	141	1

10

The results of table 3 demonstrates that it is the coupling of antigen to the beads of the present invention that facilitates the detection of mAbs, since control beads

(beads witout PS antigen, but including hydroxylamine or alkoxyamine) do not produce any increase in reporter signal MFI.

Table 4: Comparison between 10 month old polysaccharide antigen (salmonella
5 Typhimurium) coupled beads via the method of the present invention and newly prepared antigen beads via carbodiimide coupling (Exiqon method as described in WO 00/036419). See also Figures 4-9.

Mab against *Salmonella* Typhimurium		***Salmonella* Typhimurium PS**			***Salmonella* Typhimurium PS**		
		Beads UB1315B methoxy (Typh PS)			Beads Exiqon S. Typhimurium (150611)		
Conc. mAb ng/ml	Dilution	Reporter signal MFI	**Reporter signal %CV***	Fold increase vs. without mAb	Reporter signal MFI	**Reporter signal %CV***	Fold increase vs. no mAb
1000,000	1,0						
300,000	3,3	83089	**25,0 (32,4)**	704	96817	**46,3 (77,6)**	745
75,000	13,3	28311	**29,4 (35,7)**	240	26216	**64,2 (85,5)**	202
18,750	53,3	7937	**32,5 (39,5)**	67	6048	**63,6 (98,6)**	47
4,690	213,2	1600	**39,8 (43,5)**	14	1431	**69,0 (90,1)**	11
2,340	427,4	708	**40,4 (43,7)**	6	656	**67,6 (>100)**	5
1,170	854,7	330	**38,7 (67,3)**	3	376	**59,9 (79,1)**	3
0,580	1724,1	206	**39,5 (42,4)**	2	219	**60,0 (73,4)**	2
0	no mAb	118	**52,1 (64,6)**	1	130	**51,7 (58,9)**	1

* CV peak (CV single beads)

10 The experiment focuses on coupling homogenicity/effectivity together with durability of the present method. As can be seen from the flow cytometry data of table 1,2 and 4, the sensitivity towards detecting a monoclonal antibody of the antigen beads of the present invention is improved compared to that of Exiqon (carbodiimide) beads, and also the coefficient variation or peak broadness (CV) of
15 the Exiqon beads is consistently higher as compared to antigen beads coupled by method of the present invention, as shown in bold in table 4 and in figures 4-9.

This indicates that the homogenicity of antigen binding is much lower of the Exiqon beads compared to the antigen beads coupled by the present method, in other words there are much fewer beads without antigen coupled to them in a given population of beads when using the method and beads of the present
5 invention.

References

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10 -Bode et al., Angew. Chem. Int. Ed., 45, (2006), 1248-1252
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Claims

1. A method of producing a polysaccharide-carrier conjugate comprising coupling a polysaccharide to a carrier, said polysaccharide comprising at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl or a ring-closed ketal or hemi-ketal corresponding thereto, said method comprising the steps of,

   i) providing a carrier comprising N-hydroxylamine- or N-alkoxyamine groups according to the formula -NHOR, wherein R is H or C_1-C_6 alkyl attached thereto,

   ii) reacting said N-hydroxylamine or N-alkoxyamine group of the carrier with a keto-carboxy group of said polysaccharide to form a covalent amide bond between the carrier and the polysaccharide.

2. A method according to claim 1, wherein the at least one monosaccharide unit comprising a keto-carboxy group according to the formula -C(=O)COOR, where R is either hydrogen or C_1-C_6 alkyl or a ring-closed ketal or hemi-ketal corresponding thereto, is selected from the group consisting of a 2-keto-3-deoxynononic acid (Kdn) residue, and a 2-keto-3-deoxy-D-mannooctonic acid (Kdo) residue, and hydroxy-protected derivatives thereof.

3. A method according to any of claims 1-2, wherein the carrier is selected from the group consisting of a small organic molecule, a macromolecule or a solid surface.

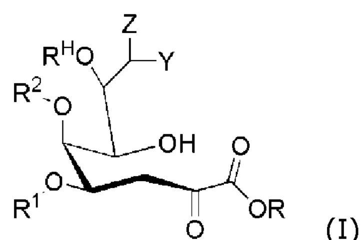
4. A method according to any of claims 1-3, wherein the N-hydroxylamine or N-alkoxyamine groups are attached to the carrier via a linker molecule.

5. A method according to any of claims 1-4, wherein the polysaccharide is substantially identical to a lipopolysaccharide or the carbohydrate part thereof.

6. A method according to any of claims 1-5, wherein the polysaccharide is derived from a gram-negative bacterial lipopolysaccharide.

7. A method according to claim 6, wherein the Gram-negative bacteria is selected from the group consisting of *Haemophilus sp, Echerichia coli ssp, Salmonella sp, Klebsiella sp, Bordete/la sp, Pseudomonas sp, Chlamydia sp, Neisseria sp, Vibrio cholerae, Shigella sp, Proteus sp, Brucella sp, Streptobacillus sp, Yersinia sp, Legionella pneumophila,* and *Serratia marcescens,* especially *Haemophilus influenzae, Salmonella enterica ssp., Klebsiella pneumoniae, Bordetella pertussis, Pseudomonas aeruginosa, Chlamydia psitacci, Neisseria meningitidis, Neisseria gonorrhea, Vibrio cholerae, Shigella flexneri,* and *Shigella dysenteriae,* and veterinary or zoonotic bacteria including enterobacteria selected from *Escherichia coli, Salmonella Typhimuriurn, Salmonella Choleraesuis, Salmonella enterica,* and all serotypes hereof, especially *Salmonella Typhimurium, Salmonella Enteritidis, Salmonella Choleraesuis, Salmonella Manhattan, Salmonella Dublin, Salmonella Infantis, Escherichia coli spp. including O157,* oedema-disease causing *Escherichia coli, Yersinia enterocolitica,* and *Campylobacter jejuni,* as well as respiratory bacteria selected from the HAP group of bacteria especially *Actinobacillus sp,* in particular the HAP group bacteria *Actinobacillus pleuropneumoniae, Haemophilus somnus, Pasteurella haemolytica, Pasteurella multocida, Haemophilus parasuis* and *Mannheimia sp.*

8. A method according to any of claims 1-7, wherein the polysaccharide is a compound of formula I



(I)

5

wherein

R is hydrogen or C_1-C_6 alkyl,

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group,

10 R^2 is a polysaccharide residue,

Y is OR^H or hydrogen,

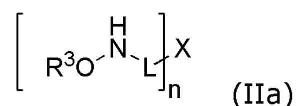
Z is OR^H or CH_2OR^H,

R^H is independently selected from hydrogen and a hydroxy protecting group;

15 or a ring-closed ketal or hemi-ketal corresponding thereto.

9. A method according to any of claims 1-8, wherein the carrier comprising N-hydroxylamine or N-alkoxyamine groups attached thereto is a compound of formula IIa

20



(IIa)

wherein

R^3 is hydrogen or C_1-C_6 alkyl,

25 L is a covalent bond or a linker molecule,

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface,

n is an integer, said integer being 1 or higher.

10. A method according to any of claims 8-9, wherein Y is hydrogen when Z is OR^H, and Y is OR^H when Z is CH_2OR^H.

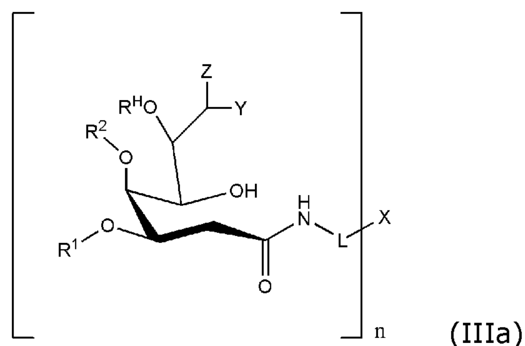
11. A method according to claim 4-10, wherein the linker molecule is a biradical
5 selected from the group consisting of a C_1-C_{16} alkylene according to the formula $-(CH_2)_n-$, where n is an integer between 1 and 16, oligo-peptides, oligo-amides, or oligo-oxyethylenes.

12. A method according to any of claims 8-11, wherein R^2 is a polysaccharide
10 residue comprising 2-1000 monosaccharide units, such as 2-500 monosaccharide units, 3-400 monosaccharide units, preferably 4-200 monosaccharide units.

13. A method according to any of claims 8-12, wherein R^2 is a polysaccharide
15 residue derived from a lipopolysaccharide.

14. A method according to any of claims 8-13, wherein said hydroxy protecting group is selected from the group consisting of ethers such as tert-butylethers, benzylethers, dimethoxytritylethers, acetals such as isopropylidene or
20 bezylidene, silylethers such as trimethylsilyl(TMS), triisopropylsilyl (TIPS), tert-butyldimethylsilyl (TBDMS), tripropylsilyl (TPS), esters such as acetyl or benzoyl, carbonates such as t-Butyl carbonate (BocO).

15. A compound or solid surface of the general formula IIIa



(IIIa)

5 wherein

R^1 is selected from hydrogen, a mono- or disaccharide and a hydroxy protecting group,

R^2 is a polysaccharide residue,

Y is OR^H or hydrogen,

10 Z is OR^H or CH_2OR^H,

R^H is independently selected from hydrogen and a hydroxy protecting group,

L is a covalent bond or a linker molecule,

X is a carrier selected from the group comprising of a small organic molecule, a macromolecule and a solid surface,

15 n is an integer, said integer being 1 or higher.

16. A compound or solid surface according to claim 15, wherein X is a macromolecule or solid surface.

20 17. A compound or solid surface according to any one of claims 15-16, wherein R^2 is a polysaccharide residue derived from a lipopolysaccharide.

18. A compound or solid surface according to any one of claims 15-17, wherein R^2 is a polysaccharide derived from a lipopolysaccharide from a gram negative

25 bacterium.

19. A compound or solid surface according to claim 18, wherein said gram negative bacterium is selected the group consisting of *Haemophilus sp, Echerichia coli ssp, Salmonella sp, Klebsiella sp, Bordete/la sp, Pseudomonas sp, Chlamydia sp, Neisseria sp, Vibrio cholerae, Shigella sp, Proteus sp,*
5 *Brucella sp, Streptobacillus sp, Yersinia sp, Legionella pneumophila,* and *Serratia marcescens,* especially *Haemophilus influenzae, Salmonella enterica ssp., Klebsiella pneumoniae, Bordetella pertussis, Pseudomonas aeruginosa, Chlamydia psitacci, Neisseria meningitidis, Neisseria gonorrhea, Vibrio cholerae, Shigella flexneri,* and *Shigella dysenteriae,* and veterinary or
10 zoonotic bacteria including enterobacteria selected from *Escherichia coli, Salmonella Typhimuriurn, Salmonella Choleraesuis, Salmonella enterica,* and all serotypes hereof, especially *Salmonella Typhimurium, Salmonella Enteritidis, Salmonella Choleraesuis, Salmonella Manhattan, Salmonella Dublin, Salmonella Infantis, Escherichia coli spp. including O157,* oedema-
15 disease causing *Escherichia coli, Yersinia enterocolitica,* and *Campylobacter jejuni,* as well as respiratory bacteria selected from the HAP group of bacteria especially *Actinobacillus sp,* in particular the HAP group bacteria *Actinobacillus pleuropneumoniae, Haemophilus somnus, Pasteurella haemolytica, Pasteurella multocida, Haemophilus parasuis* and *Mannheimia sp.*
20

20. A compound or solid surface according to any of claims 15-19, wherein Y is hydrogen when Z is OR^H, and Y is OR^H when Z is CH_2OR^H.

21. A compound or solid surface according to any of claims 15-20, wherein L is a
25 linker molecule.

22. A compound or solid surface according to any of claims 15-21, wherein the linker molecule is a biradical selected from the group consisting of a C_1-C_{16} alkylene according to the formula $-(CH_2)_n-$, where n is an integer between 1
30 and 16, oligo-peptides, oligo-amides, or oligo-oxyethylenes.

23. A compound or solid surface according to any of claims 15-22, wherein X is a macromolecule or solid surface, Y is hydrogen when Z is OR^H, and Y is OR^H when Z is CH_2OR^H, and L is a linker molecule.

5

24. A compound or solid surface according to any of claims 15-23, wherein said hydroxy protecting group is selected from the group consisting of ethers such as tert-butylethers, benzylethers, dimethoxytritylethers, acetals such as isopropylidene or bezylidene, silylethers such as trimethylsilyl (TMS),
10 triisopropylsilyl (TIPS), tert-butyldimethylsilyl (TBDMS), tripropylsilyl (TPS), esters such as acetyl or benzoyl, carbonates such as t-Butyl carbonate (BocO).

25. The use of a compound according to any of claims 15-24 for the preparation
15 of an assay device for the detection of antibodies against gram negative bacteria.

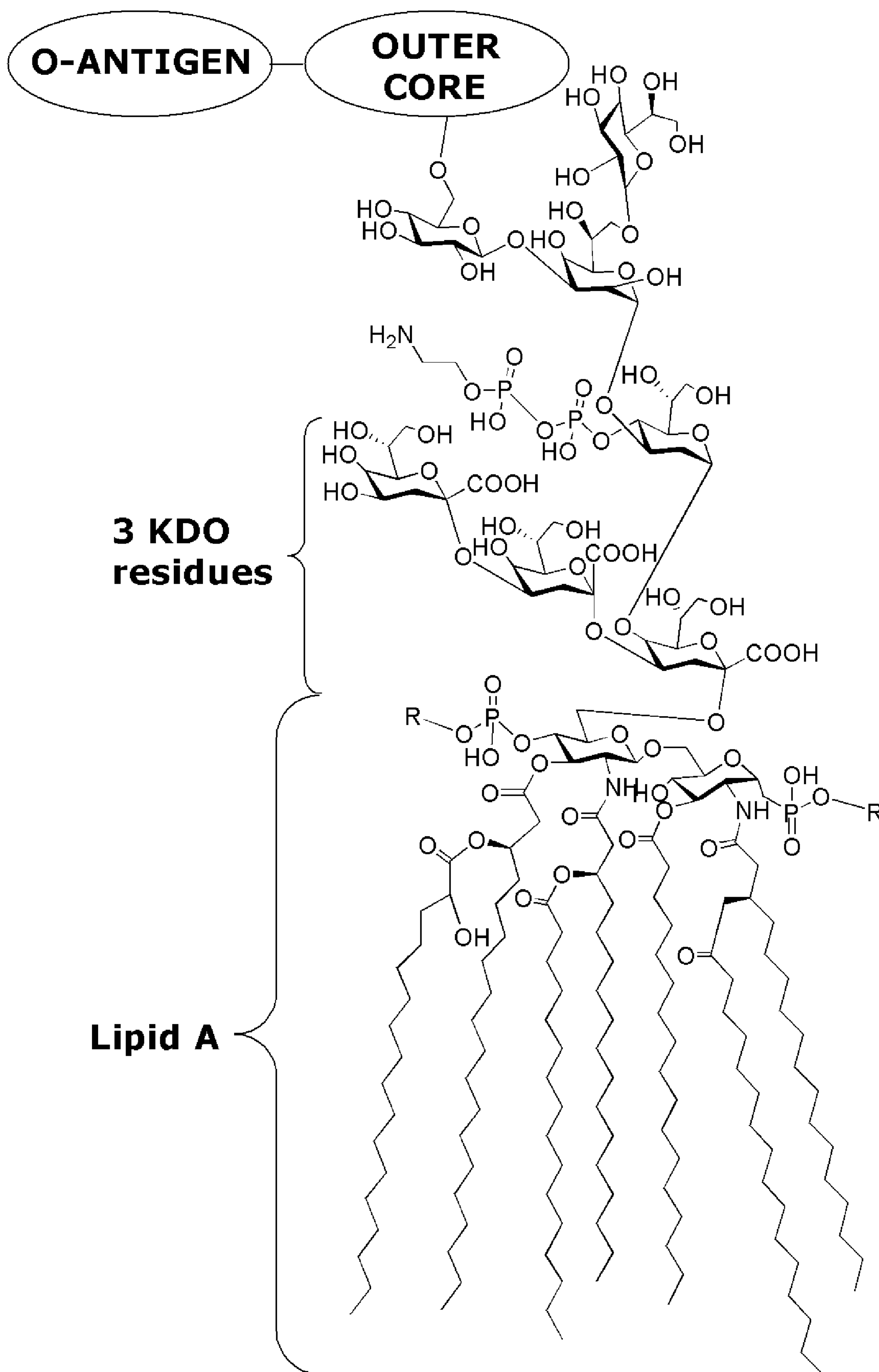


Fig. 1

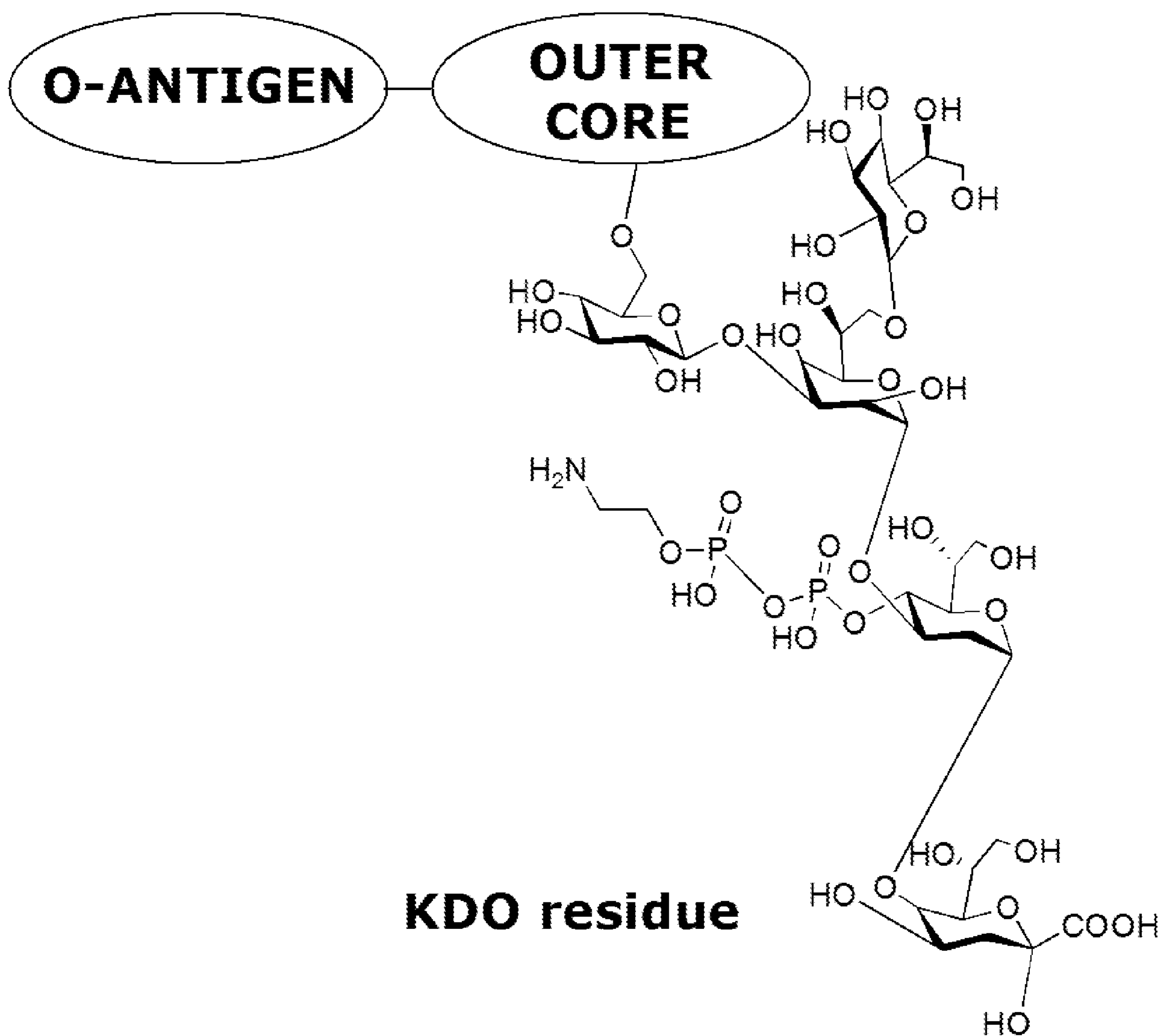


Fig. 2

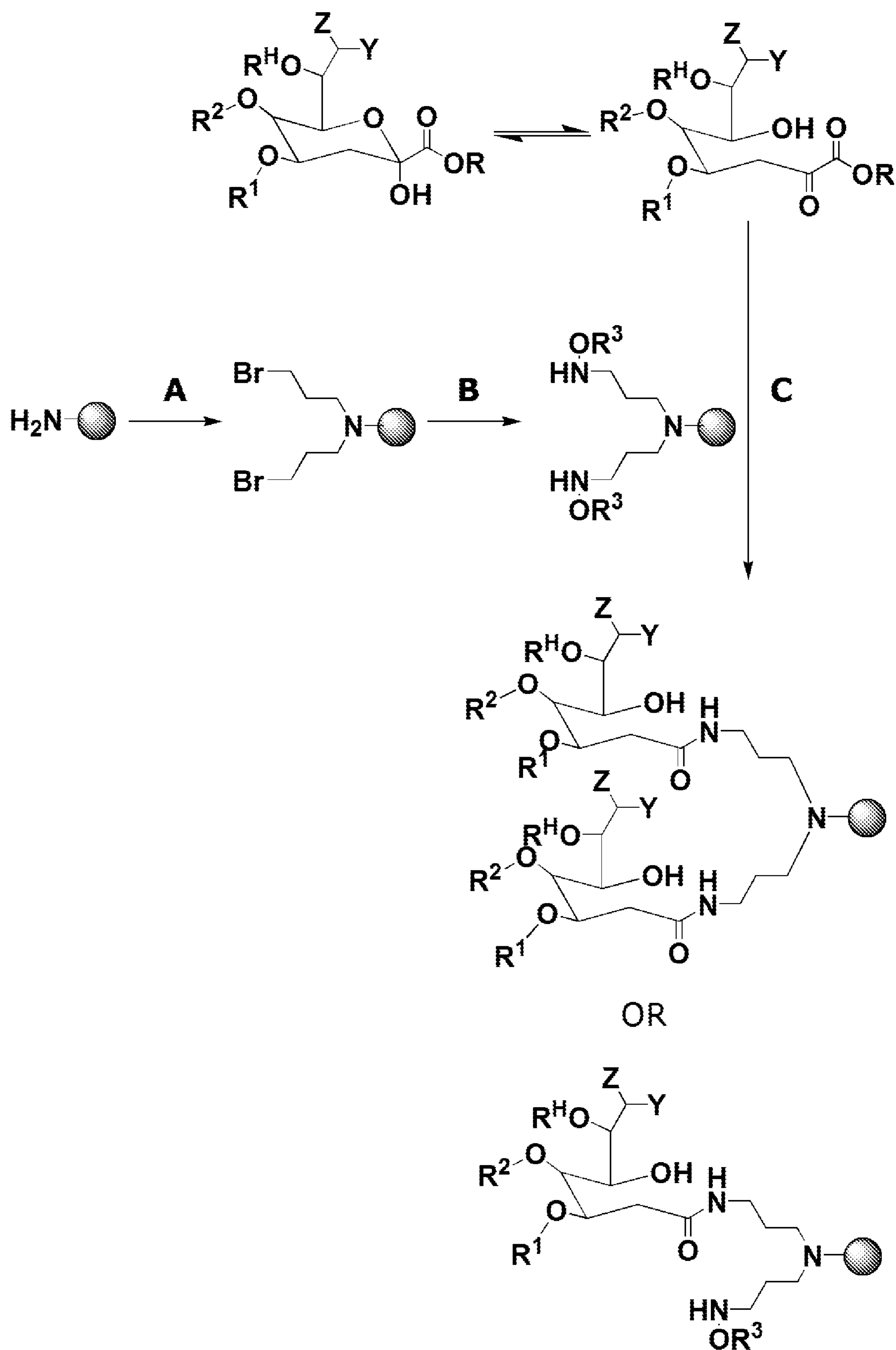
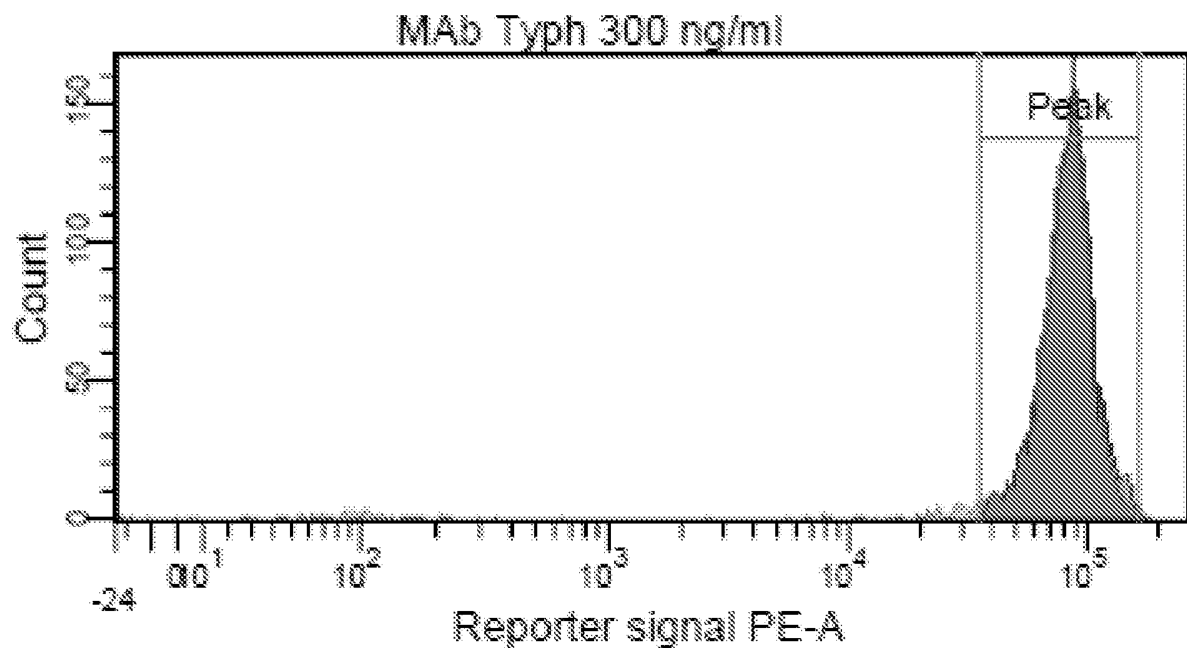


Fig. 3



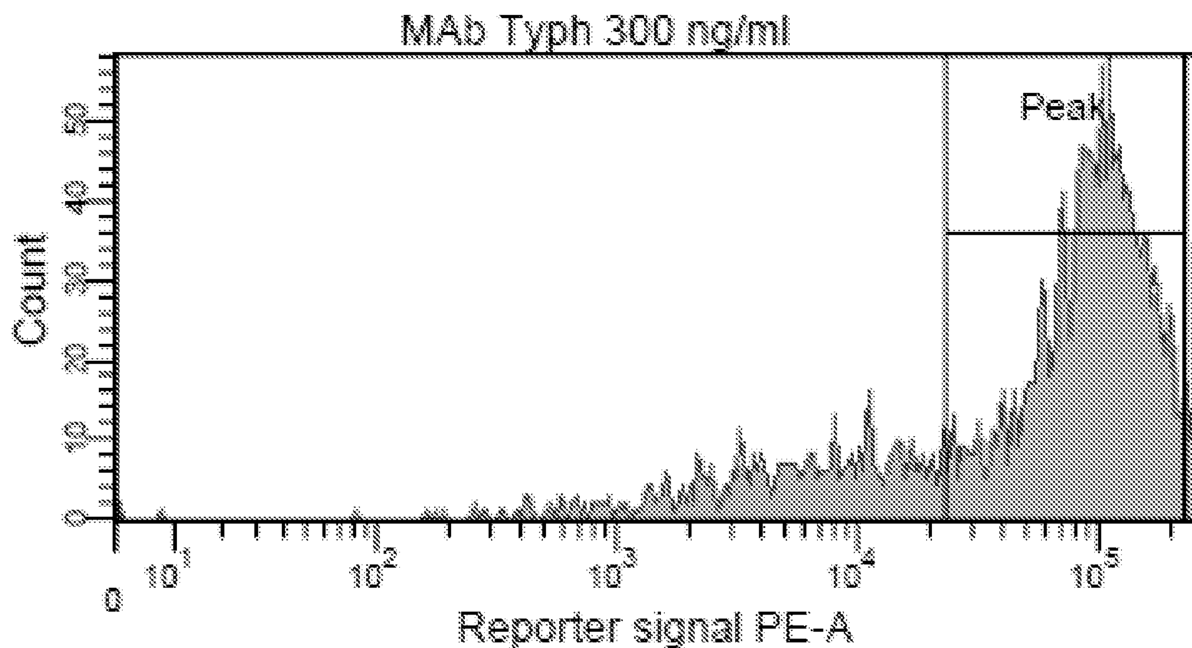
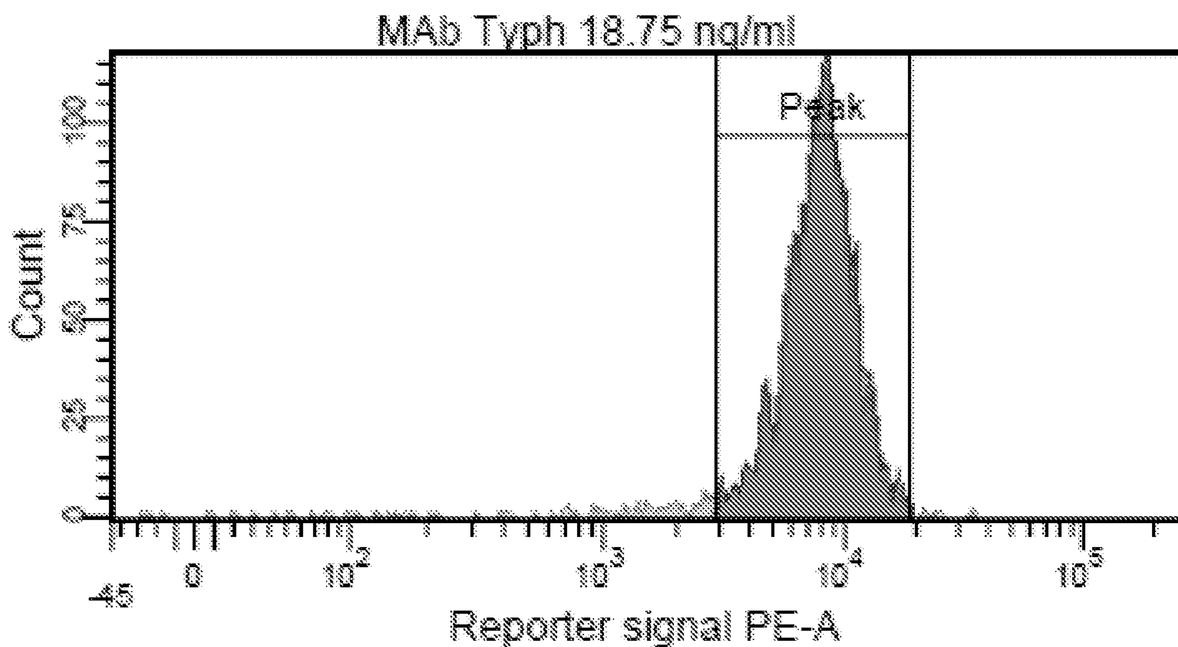


Fig. 4



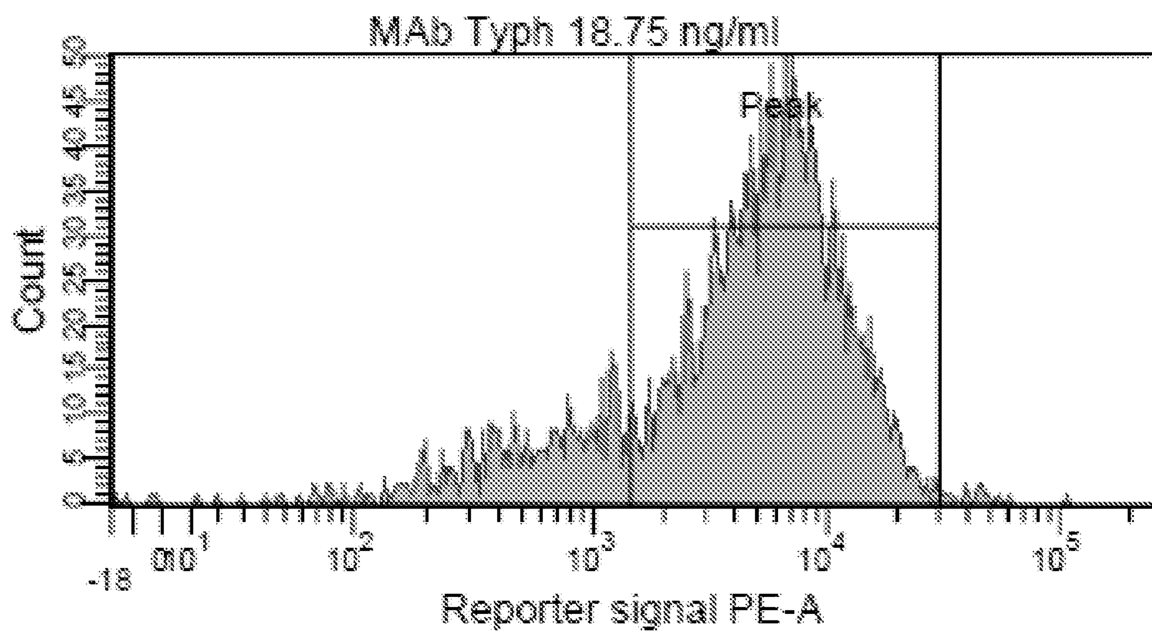
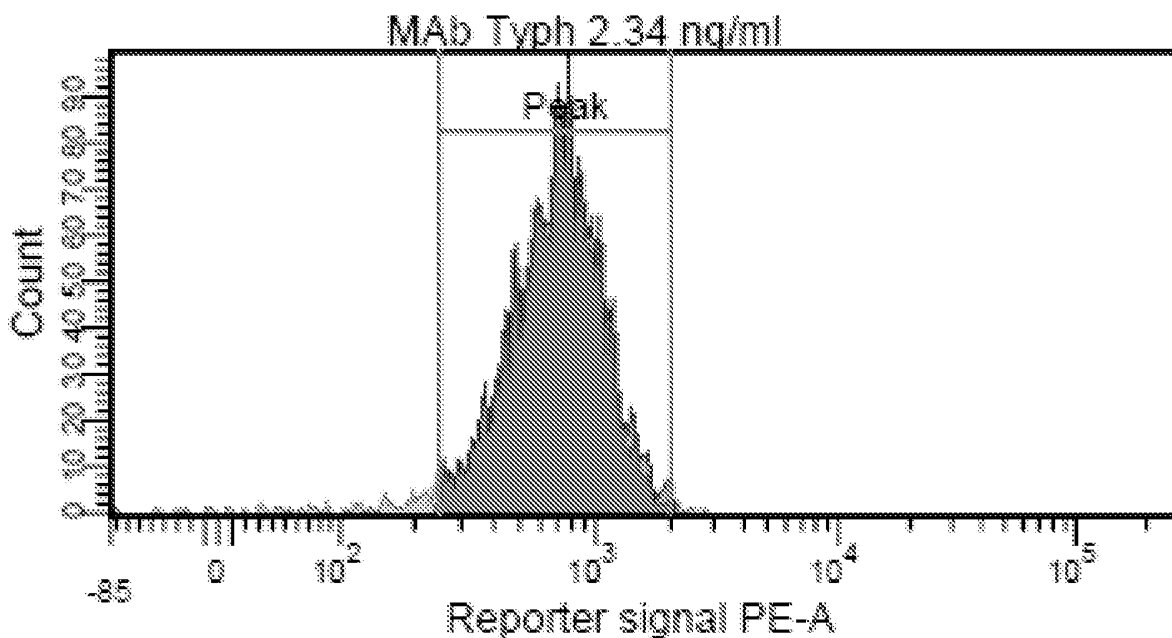


Fig. 5



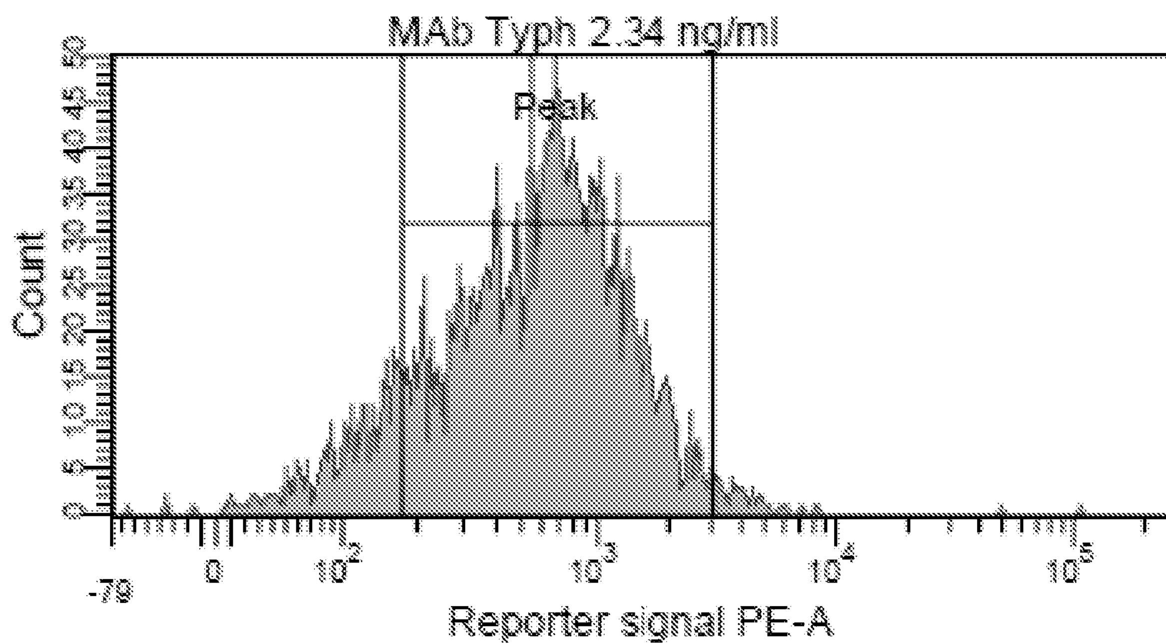
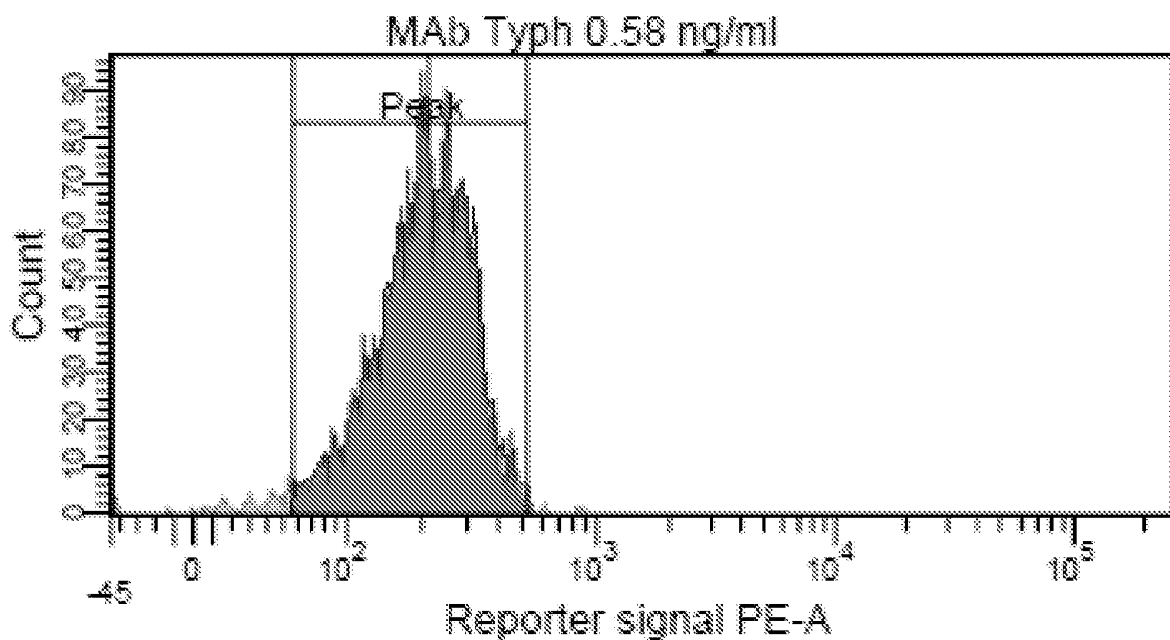


Fig. 6



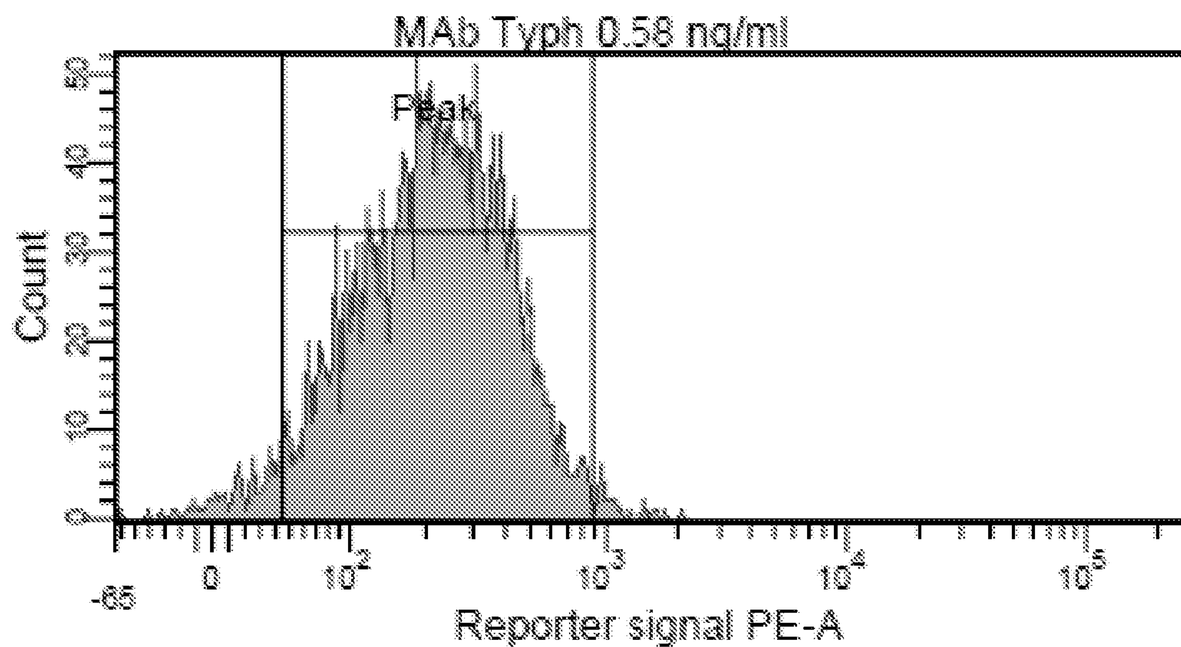
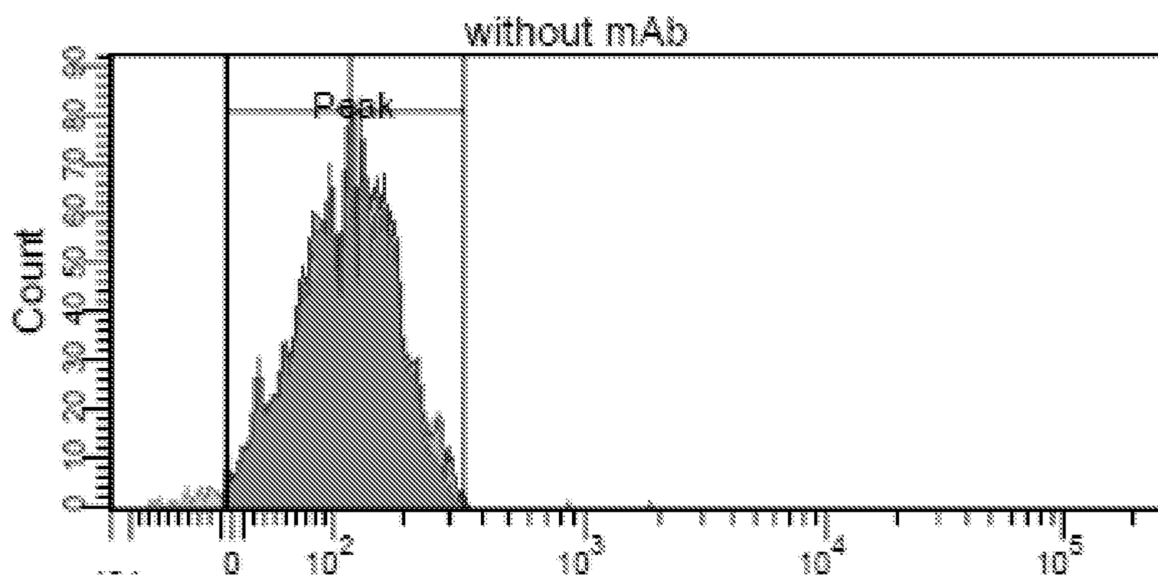


Fig. 7



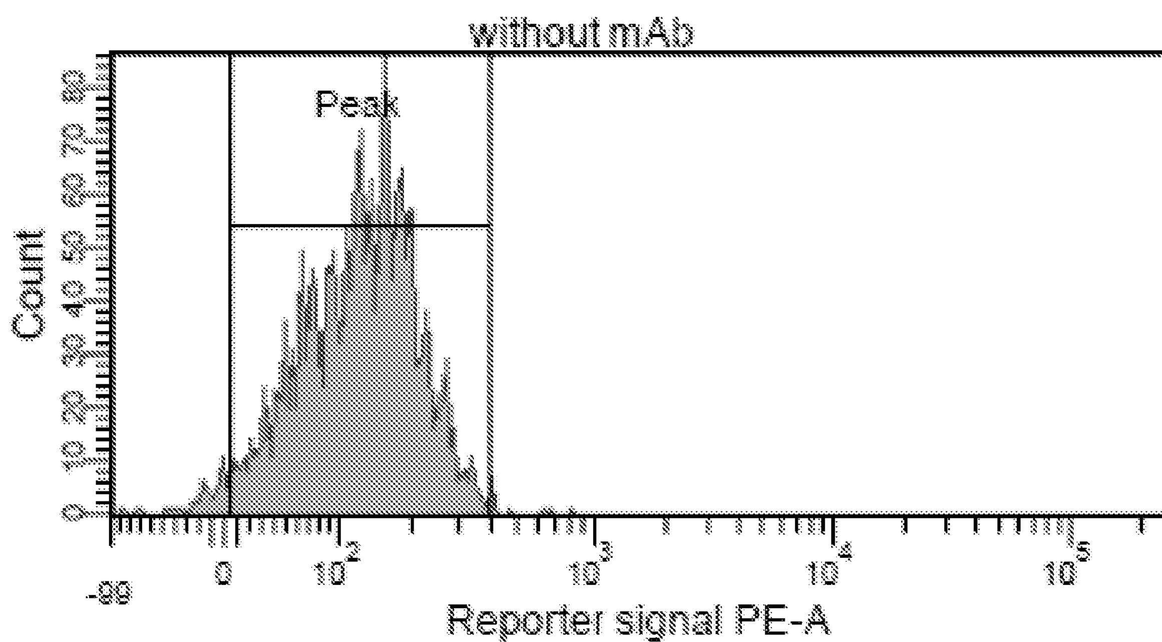
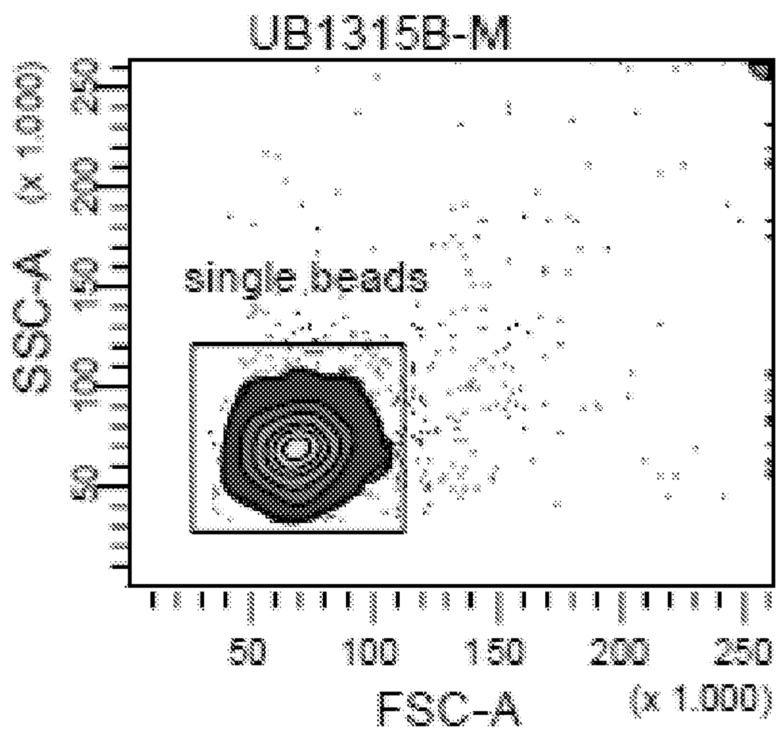


Fig. 8



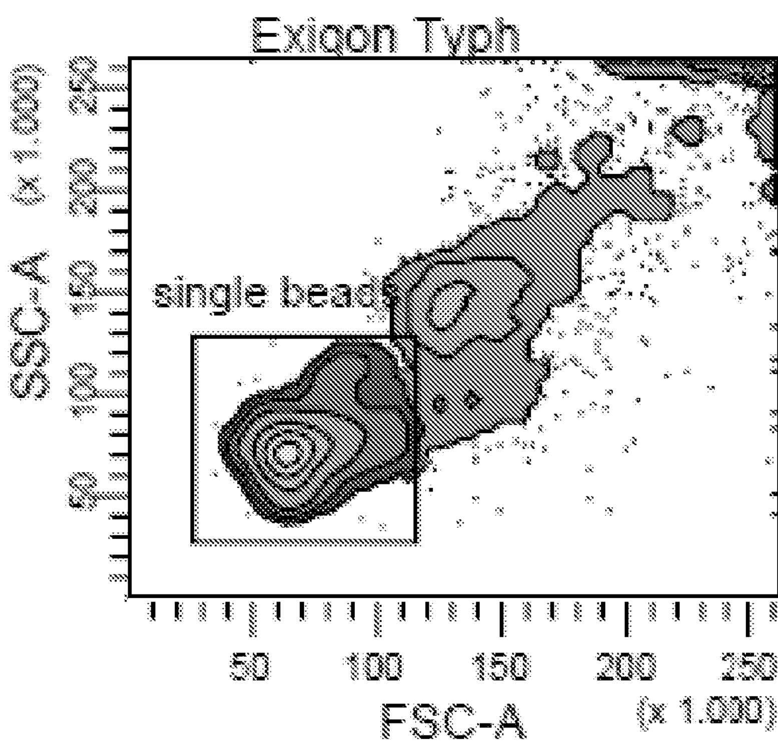


Fig. 9

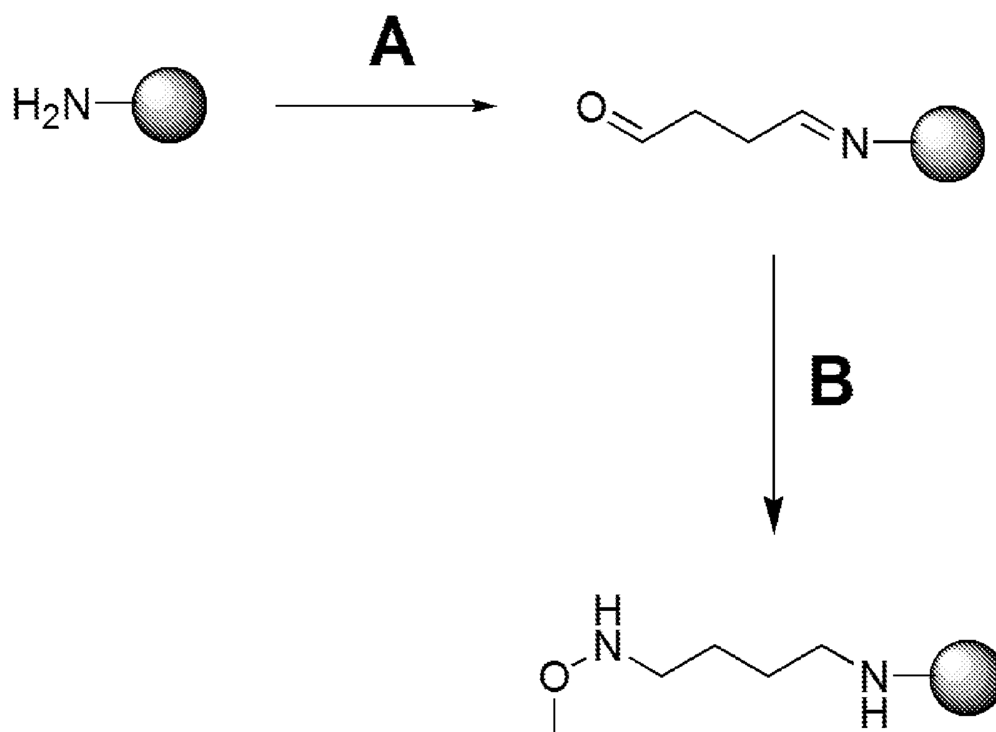


Fig. 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2012/050394

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 23 21 752 A1 (ALBERT AG CHEM WERKE) 14 November 1974 (1974-11-14) Abstract;Formula I; Compounds 1-23	15-24
A	JEFFREY W BODE ET AL: "Chemoselective Amide Ligation by Decarboxylative Condensations of N-Alkylhydroxylamines and alpha-Ketoacids", ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, WILEY VCH VERLAG, WEINHEIM, vol. 45, no. 8, 13 February 2006 (2006-02-13), pages 1248-1252, XP002590575, ISSN: 1433-7851, DOI: 10.1002/ANIE.200503991 [retrieved on 2006-01-17] cited in the application reaction 1	1-25

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 2013	13/02/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hohwy, Morten

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2012/050394

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/013735 A2 (GORVERNMENT OF THE UNITED STAT [US]; VINOGRADOV EVGUENII [CA]; KUBLER-) 31 January 2008 (2008-01-31) abstract; Fig. 1	1-25
A	WO 00/36419 A1 (EXIQON A S [DK]; JAKOBSEN MOGENS HAVSTEEN [DK]; BOAS ULRIK [DK]; JAUHO) 22 June 2000 (2000-06-22) cited in the application p. 17-19	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2012/050394

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