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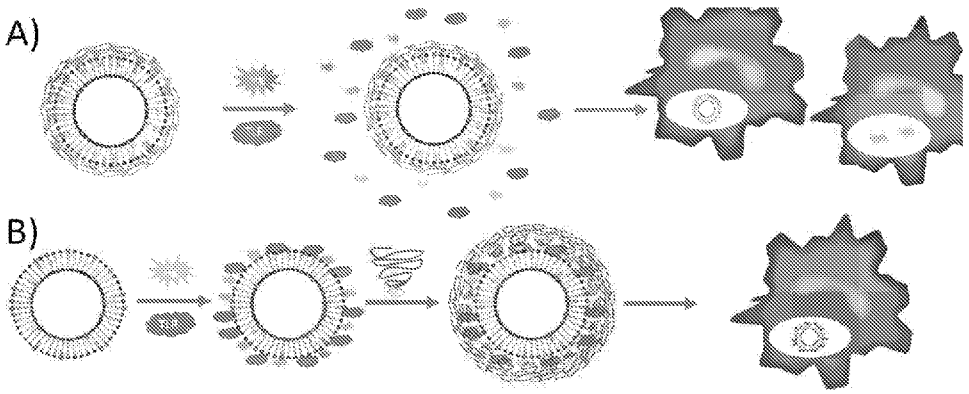


Figure 3

(57) Abstract: A method of controlling the surface charge of liposomes by anchoring hydrophilic coating polymers onto the liposomes by electrostatic interactions using polar tags and adjuvants with changed drainage properties comprising such liposomes.

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1. Adjuvants with modified drainage properties

2. Field of invention

The invention discloses a method of controlling the surface charge of liposomes and thereby the cellular targeting and drainage properties by anchoring hydrophilic coating polymers onto the liposomes by electrostatic interactions using polar tags and a method for coating charged vaccine delivery systems with hydrophilic polymers via attractive electrostatic interactions using oppositely charged residues in the anchoring part of the coating and adjuvants comprising such liposomes to control the net surface charge, drainage properties and cellular targeting.

3 General background

A prerequisite for efficient induction of cellular immunity is the activation of antigen presenting cells (APCs) with ligands for pattern recognition receptors (PRRs). A diverse array of pathogen-associated molecular patterns (PAMPs) binds to the various families of PRRs, e.g. the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs). Different PAMPs induce different types of adaptive immune responses, depending on which signaling pathways are triggered, eventually determiningwhich cytokine milieus and expression of co-stimulatory molecules are induced to direct the T-cell differentiation. Several subtypes of DCs have been identified in mice with different functionalities that can be distinguished via their expression of specific cell surface markers (Figure 2.). In principle, conventional DCs comprise of two subsets, namely 1) lymphoid organ-resident DCs which as the name implies stay in the lymphoid organs and collect foreign matters taken there by the lymph and 2) tissue-migratory DCs which sweep the non-lymphoid organs, sample foreigen matters and transport them to the draining lymph nodes where they can activate naïve antigen-specific T cells. These DC populations have differing preferential activations of the adaptive immune system, which can be exemplified in the superior ability of the resident CD8α+ DCs, which are superior to other DC subtypes to cross-present extracellular antigen on MHC class I, thus initiating CD8 T cell responses (Joffre, Segura et al. 2012). CD8α+ DCs have thus been shown to be the principal APC responsible for the induction of CD8 T cell immunity against infections with both viruses and intracellular bacteria (Belz, Smith et al. 2004, Heath, Belz et al. 2004, Belz, Shortman et al. 2005). It has furthermore been shown that mice deprived of CD8α+ DCs cannot cross-present virus antigens, resulting in a highly abolished virus-specific CD8 T cell immune response (Hildner, Edelson et al. 2008).

It is well-documented that liposomes displaying a positive surface charge (cationic) are more - immunogenic than neutral or negatively charged (anionic) liposomes (Christensen, Korsholm et al. 2007, Korsholm, Agger et al. 2007, Henriksen-Lacey, Bramwell et al. 2010, Christensen, Korsholm et al. 2011). One of their primary merits is that they are taken up more efficiently by APCs because of their ability to adsorb to the anionic heparan sulfate proteoglycan-coated cell surface (Korsholm, Agger et al. 2007). Another quality is the ability to co-localize antigen and adjuvant presented on the liposomal surface, thus ensuring that antigen taken up by APCs will be accompanied with activation of the same cells (Kamath, Rochat et al. 2009, Kamath, Mastelic et al. 2012). A vaccine containing cationic liposomes will typically form a vaccine depot at the injection site (SOI) and be taken up at the SOI) by migratory DCs resulting in a strong induction of systemic cell-mediated immune (CMI) responses (Christensen, Henriksen-Lacey et al. 2012). Cationic liposomes forms depot e.g. they migrate very slowly from the site of injection.

In some cases it is not desirable to have a formation of depots of liposomes at the site of injection (SOI) but it could be advantageous with some drainage to the local lymphnodes. Liposomes are often used as carriers for drugs, e.g. in cancer treatment, carriers of radiographic contrast medium and in such cases it is desirable that the liposomes avoid interaction with the immunesystem. Such use of liposomes are well described in the literature and the most used method of mobilizing liposomes and at the same time giving them a stealth property is to PEGylate the liposomes (Owens and Peppas 2006, Hillaireau and Couvreur 2009). PEGylation is the adsorption or grafting of poly(ethyklene)glycol (PEG) or PEG-containing copolymers to the surface of nanoparticles such as liposomes.

Cationic liposomes e.g. made from amphiphilic quaternary ammonium compounds are wellknown lipids used in adjuvant compositions. Preferred quaternary ammonium compounds are the bromide- , chloride- , sulfate- , phosphate- or acetate- salt of dimethyldioctadecylammonium (DDA) or dimethyldioctadecenylammonium (DODA) compounds. Other preferred quaternary ammonium compounds are 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane and dioleoyl-3-dimethylammonium propane (DODAP) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA).

The cationic liposomes are not colloidally stable in aqueous solution but can be stabilized with a glycolipid in the membrane. A well-known glycolipid for stabilizing the liposomes is

alpha,alpha'-trehalose 6, 6' dibehenate (TDB) or alpha,alpha'-trehalose 6,6'-dimycolate (TDM) which both have immunostimulating properties.

DDA/TDB (CAF01) is a well described and proven vaccine adjuvant and has been in trial with tuberculosis, chlamydia, HIV and malaria antigens. It has also been shown that CAF01 is strongly depot forming.

We have recently identified a particularly powerful CD8 T cell-inducing liposomal adjuvant (CAF09) which consists of the synthetic TLR3 ligand poly(I:C) and the novel immunostimulator monomycolyl glycerol (MMG) (Andersen, Rosenkrands et al. 2009, Andersen, Agger et al. 2009), associated with liposomes based on the cationic surfactant dimethyldioctadecylammonium (DDA) (Korsholm, Hansen et al. 2014). This adjuvant is designed to induce cross presentation of protein antigens via maximum synergy between the PRR activated signaling pathways. Due to its cationic nature this adjuvant however forms a depot at the site of injection and thus primarily targets the migratory DCs which are not able to cross present the antigen on MHC-I.

In some cases depot formation of some but not all antigen and/or adjuvant is desirable. The reason for this can be that the antigen and/or adjuvant should be delivered to APCs not migrating to the site of injection but to lymphnode resident B cells or dendritic cells. In this case increasing the free drainage of the vaccine from the site of injection to the lymphnode would be desireable so the vaccine would be delivered directly to resident DCs able to cross present the antigen and induce a CD8 T cell response. For passive targeting of lymph node-resident CD8+ DCs, a fraction of vaccine has to drain to the local lymph nodes because the vaccine should be delivered to lymph node-resident CD8+ DCs. In this case increasing the self-drainage of the vaccine from the SOI to the lymph node is needed for the vaccine to be delivered directly to lymph node-resident DCs capable of cross-presenting antigen and priming a CD8+ T-cell response.

I other cases it could be desireable to increase the presentation of antigen to B cells in the draining lymphnode. Antigen depot at the site of injection facilitates uptake of the antigen by migratory DCs which will process the antigen before entering the draining lymphnode. B cells will therefore not be exposed to the antigen, resulting in suboptimal antibody responses.Increasing the free drainage of the vaccine antigen to the lymphnode will increase the antigen presentation to B cells, thus activating them to mediate a stronger B cell response. At the same time it is important that a part of the vaccine antigen is presented to migratory DCs to induce the T cell responses. This is illustrated by the fact vaccination of antigen together with

DDA/TDB induces a strong T cell response but relatively low antibody responses, but that parallel injection of free antigen that drains to the same lymphnode significantly increase the antibody responses, and at the same time maintains the T cell responses (Figure 1).

One particular approach for increasing the free drainage into the lymph nodes has been to incorporate PEG-grafted lipids into liposome membranes to form a protective hydrophilic layer on the surface of the liposomes that opposes interaction with host components. We have observed that PEGylation of DDA/MMG/pIC-liposomes with PEG-lipids causes a slightly increased CD8+ T cell response (Figure 2) which may be explained by increased self-drainage of the vaccine to the local lymph nodes, eventually resulting in passive targeting of lymph node-resident, cross-presenting CD8+ DCs.. The kinetics of the free drainage depends on the molecular size and grafting density of PEG, and the half-life can vary from a few minutes up to days.

The use of polyethylene glycol (PEG) derivatized ceramide lipids to form a hydrophilic coating on liposomes is described in *WO96/10391*. Here the hydrophilic coating is thus associated with the liposome membrane via lipid anchoring into the membrane. This anchoring is efficient and the ceramides are, in contrast to the classical PEG lipids based on derivatization of diacylglycerophospholipids, highly stable towards hydrolysis under both acidic and basic conditions.

WO99/55306 describes the situation in which lipid anchored PEG stabilizes and increases circulation time of a neutral small uni-lamellar vesicles (SUV) carrying blood coagulations factor FVIII or other proteins/peptides associating to neutral lipids and or PEG, after i.v. injection.

In *WO2009/031911* cationic liposomes PEGylated by lipid anchoring are utilized for gene therapy. Furthermore liposomes serve as delivery system for gene therapy and not for vaccines.

In *WO2009/111088* Neutral liposomes PEGylated by lipid anchoring are utilized for targeting of neutral liposome particles larger than 300 nm to the spleen carrying e.g. cancer therapeutics and/or adjuvants in order to amplify the immune response.

In *US 8,304,565* novel PEG conjugated lipids are described for optimized hydrophilic coating of liposomal formulations of pharmaceuticals and cosmetics. In said application the mechanism of association between liposomes and PEG is based on covalent conjugation of PEG to a lipid anchor

In *WO2012/062867* uncharged liposomes PEGylated by lipid anchoring is utilized as carriers for medicinal products for prophylaxis and therapy.

In *WO2013/033563* liposomes including PEGylated lipids are applied for the delivery of immunogen-encoding RNA. In said application the mechanism of association between liposomes and PEG is based on apolar interactions.

Kaur et al. (Kaur, Bramwell et al. 2012) describes the incorporation of different concentrations of lipid anchored hydrophilic coatings into DDA/TDB liposomes. They report that this leads to an increased drainage of the vaccine to the lymph nodes but also report an altered immune response with decreasing Th1 and increasing Th2 response. This illustrates that PEGylation does increase the potential of PEGylation of Cationic liposomes for targeting cells resident in the lymphoid organs. The reason for the Th2 bias PEGylated adjuvant is suggestively reduced targeting to migratory DCs and thus reduced Th1 induction.

Finsinger et al. (Finsinger, Remy et al. 2000) describes the use of PEG with reactive linkers based on anionic peptides, to coat positively charged nonviral gene vectors by electrostatic interactions. The linkers are designed to be cleaved from the PEG at reduced PH resembling cytosolic environment, thus releasing the DNA when the nanoparticle has entered the cytosol of the cells.

Auguste et al. (Auguste, Armes et al. 2006) describes PEGylation of negatively charged liposomes with PEG-moieties conjugated with cationic block-polymers. The inclusion of dioleoyldimethylammonium-propane (pKa ~ 6.7) enables dissociation of the PEG-polymer upon entrance into the endosomes (pH ~ 5.5).

Schmidt et al. (Tandrup Schmidt, Foged et al. 2016)gives a review of the formulation strategies for liposome-based vaccine adjuvants.

The incorporation of lipid anchored hydrophilic coatings of polymer (as described in *WO96/10391;* Kaur et al. 2012; *WO99/55306; WO2009/111088; US8304565; WO2012/062867; WO2013/033563*) is part of the initial formation of the liposomes, which results in a reduced ability to complex vaccine antigens and immunostimulators to the surfaces of the vaccine delivery system, since the antigens ad-mixed to a PEG coated liposome formulation cannot interact with the liposome surface due to sterically hindrance from the PEG chains . This amount of complexing is an important parameter to obtain an effective vaccine, since co-localization of immunostimulator and antigen is a prerequisite for the induction of

strong T cell induction (Kamath et al./Christensen et al. 2012). –Most vaccine antigens and some immunostimulators are associated to the cationic adjuvant formulation via electrostatic interactions. If the polymer coating is based on hydrophic interactions as is the case with afore mentioned prior art (i.e. *WO96/10391;* Kaur et al. 2012; *WO99/55306; WO2009/111088; US8304565; WO2012/062867; WO2013/033563*), then it is not possible to efficiently associate the antigen and immunostimulators to the adjuvant. This is because the hydrophilic shielding is formed during liposome formation, and thus present when antigen/immunostimulator is add-mixed. The present invention makes it possible to first complex the antigen and immunostimulator to the vaccine delivery system and then coat the charged particle surface hydrophilic coatings constituting an opposite charge or to add-mix the coating together with the antigen to get partial association between antigen and adjuvant.

4. Summary of the invention

This invention covers a method of controlling the drainage of a vaccine from the site of injection and the amount of antigen bound to the liposomes by the use of hydrophilic coatings onto vaccine delivery systems by electrostatic interactions using anionic residues in the anchoring part of the coating. This enables the coating to be added to the vaccine formulation as the very last component, thus ensuring that both antigen and adjuvant components which are complexed to the vaccine delivery system will be shielded by the hydrophilic coating. Polymer shielding using apolar association thus reduces the ability to complex the antigen and immunostimulator to the vaccine delivery system to obtain co-delivery to the desired APCs.

The invention furthermore covers the design of hydrophilic coatings which can be displaced after vaccination. The kinetics of this displacement can be controlled by the strength of the electrostatic interaction, i.e. by adjusting the amount of anionic residues in the anchoring part of the hydrophilic coating intended for a cationic delivery system. This displacement can furthermore be designed to take place only under specific physiologic conditions, such as acidic environment in the lysosomes, by incorporating anchoring parts that will only be electrostatically displaced at low pH.

5. Detailed disclosure of the invention

The present invention discloses a method of controlling the surface charge of liposomes or shielding vaccine delivery systems by anchoring hydrophilic coating polymers onto the liposomes by electrostatic interactions using polar tags. The polar tags are oppositely charged residues attached in the anchoring part of the coat. The hydrophilic coating polymers are chosen from poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol),

poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s.

If the liposomes of the vaccine delivery system are cationic liposomes the polar tags are anionic residues such as 1) polyanionic peptides such as peptides containing two or more aspartic or glutamic acid residues. 2) polyphosphates defined as *n* tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus groups share one oxide and the others phosphorus centres share two oxide centres, 3) polyanionic polysaccharide derivatives such as polyanionic cellulose and 4) synthetic sulphated polymers.

Negatively charged residues in the anchoring part (tags) of the coat are chosen from polyanionic peptides, e.g., peptides containing two or more aspartic or glutamic acid residues, polyphosphates defined as n tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus atoms share one oxide and the others phosphorus centres share two oxide centres, polyanionic polysaccharide derivatives such as polyanionic cellulose and synthetic sulphated polymers.

The invention also discloses adjuvant formulations comprising liposomes coated with hydrophilic polymer with negatively charged residues in the anchoring part of the coat. The liposomes are preferably cationic liposomes such as dimethyldioctadecylammonium (DDA), dimethyldioctadecenylammonium (DODA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane and dioleoyl-3-dimethylammonium propane (DODAP) or N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA). Additionally the liposome can comprise a glycolipid, e.g., TDB or MMG.

The liposomes are coated with the hydrophilic polymer after complexing or associating antigens and immunostimulators to the liposomes.

Preferred cationic lipids used for preparing cationic liposomes are chosen from dimethyldioctadecylammonium (DDA), dimethyldioctadecenylammonium (DODA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane and dioleoyl-3-dimethylammonium propane (DODAP) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA) together with a glycolipid such as TDB or MMG.

The liposomes are coated with a hydrophilic polymer after complexing antigens and immunostimulators to the liposomes.

Another embodiment of the invention is delivery systems or vaccine adjuvants formulations comprising liposomes coated with hydrophilic polymer anchored polar tags according to said method. The hydrophilic coating polymers are chosen from poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s.

Preferred adjuvant formulations comprise cationic liposomes made from dimethyldioctadecylammonium (DDA), dimethyldioctadecenylammonium (DODA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane and dioleoyl-3-dimethylammonium propane (DODAP) or N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA). The cationic liposomes preferably comprises an immunostimulator e.g. a glycolipid such as TDB or MMG. The adjuvant or delivery system comprises liposomes where the surface charge of liposomes has been altered with a coating of hydrophilic polymers chosen from poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s where the hydrophilic polymer has been electrostatically anchored with polar tags such as polyanionic peptides such as peptides containing two or more aspartic or glutamic acid residues, polyphosphates defined as n tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus groups share one oxide and the others phosphorus centres share two oxide centres, polyanionic polysaccharide derivatives such as polyanionic cellulose and synthetic sulphated polymers.

This invention covers a method for coating charged delivery systems with hydrophilic (stealth) polymers via attractive electrostatic interactions using oppositely charged residues in the anchoring part of the coat. This is hypothesised to affect the drainage of the vaccine from the SOI and the degree of adsorption of antigen to the liposomes. This allows for the addition of the shielding after adsorption of antigen/immunostimulator to the liposomes, thus ensuring that both antigen and adjuvant components, which are adsorbed to the vaccine delivery system, may be shielded by the hydrophilic coating. This is in contrast to previously used shielding based on PEG-lipids that may reduce the adsorption of antigen and immunostimulator to the vaccine delivery system, and thus prevent the co-delivery to the desired APCs.

The invention furthermore covers the design of hydrophilic coatings which may be desorbed after vaccination. The kinetics of this desorption process may be controlled by varying the strength of the attractive electrostatic interaction, e.g. by varying the number of charged residues in the anchoring part of the hydrophilic coating intended for an oppositely charged delivery system. This desorption may be designed to take place only under specific physiological conditions.

"Liposomes" are defined as closed vesicles structures made up of one or more lipid bilayers surrounding an aqueous core. Each lipid bilayer is composed of two lipid monolayers, each of which has a hydrophobic "tail" region and a hydrophilic "head" region. In the bilayer, the hydrophobic "tails" of the lipid monolayers orient toward the inside of the bilayer, while the hydrophilic "heads" orient toward the outside of the bilayer. Liposomes can have a variety of physicochemical proper-ties such as size, lipid composition, surface charge, fluidity and number of bilayer membranes. According to the number of lipid bilayers liposomes can be categorized as unilamellar vesicles (UV) comprising a single lipid bilayer or multilamellar vesicles (MLV) comprising two or more concentric bilayers each separated from the next by a layer of water. Water soluble compounds are entrapped within the aqueous phases/core of the liposomes opposed to lipophilic compounds which are trapped in the core of the lipid bilayer membranes.

Liposomes have been used as delivery systems in pharmacology and medicine such as immunoadjuvants, treatment of infectious diseases and inflammations, cancer therapy, radiographic contrast medium and gene therapy. In vaccine technology the delivery system is often an adjuvant. An adjuvant is defined as a substance that non-specifically enhances the immune response to an antigen. Depending on the nature of the adjuvant it can promote a cell-mediated immune response, a humoral immune response or a mixture of the two. Since the enhancement of the immune response is non-specific, it is well understood in the field that the same adjuvant can be used with different antigens to promote responses against different targets e.g. with an antigen from *M. tuberculosis* to promote immunity against *M. tuberculosis* or with an antigen derived from a tumor, to promote immunity against tumors of that specific kind. Factors which may have an influence on the adjuvant effect of the liposomes are liposomal size, lipid composition, and surface charge. Furthermore, antigen location (e.g., whether it is adsorbed or covalently coupled to the liposome surface or encapsulated in liposomal aqueous compartments) may also be important. Dendritic cells can be used as antigen delivery vehicles. Loading of antigen to antigen-presenting cells, such as dendritic cells, have shown to be an effective method for generating active T-cells with a role in antitumor immunity.

The term "cationic lipid" includes any amphiphilic lipid, including synthetic lipids and lipid analogs, having hydrophobic backbone and polar head group moieties, a net positive charge at physiological pH, and which by itself can form spontaneously into bilayer vesicles or micelles in water.

Stealth coating is the coating of adjuvant delivery systems with a “hydrophilic coating polymer” shielding the delivery system from the immune system and interstitial proteins.

“Hydrophilic coating polymer” is defined as hydrophilic head group component selected from polymers based on poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein the polymer may be linear or branched, and wherein the polymer may be optionally substituted. The molecular weight for the hydrophilic coating ranges from 350 g/mole to 5000 g/mole.

Anchoring of the polymer is defined as the linking of the hydrophilic coating polymer to the liposomes surface. This classically happens through lipidation of the polymer and insertion of the polymer linked lipids into the liposomal membrane. This anchoring is referred to as apolar association. Anchoring applied in this invention is electrostatic interaction in which the polymer is covalently linked to polar tags of opposite charge than the liposome surface rendering electrostatic association between polymer and liposome membrane possible. The polar tag can be based on peptides, charged polymers, small molecules etc.

The core of the delivery system is comprised of liposomes which are preferably cationic liposomes. which are made from cationic lipids. A preferred cationic lipid comprises a quaternary amine with a halogen counter ion of the formula NR1R2R3R4-hal, wherein R1 and R2 independently each is a short chain alkyl group containing 1 to 3 carbon atoms, preferably methyl groups, R3 and R4 independently each is an alken containing from 12 to 20 carbon atoms, preferable from 14 to 18 carbon atoms, and hal is a halogen atom, not comprising any nucleic acid as adjuvant. In the formula NR1R2R3R4-hal the R1 and R2 groups may e.g. be methyl, ethyl, propyl and isopropyl, whereas R3 and R4 may be dodecenyl, tridecenyl, tetradecenyl, pentadecenyl, hexadecenyl, heptadecenyl, octadecenyl nonadecenyl and eicocenyl groups. However, also other C¬12-C20 alkenes are possible because even though the R3 and R4 groups usually and preferably are straight chain hydrocarbon groups they may in minor degree be branched having e.g. methyl and ethyl side chains. The halogen atom “hal” is preferably bromine or chlorine because the other halogens, fluorine and iodine, may have

undesirable biochemical, physiological and injurious effects, but for some experimental purposes, where such effects can be accepted, they may also be selected.

Other cationic lipid compounds suitable as adjuvant formulations are1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane (DMTAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-distearoyl-3-trimethylammonium-propane (DSTAP) and dioleoyl-3-dimethylammonium propane (DODAP), Cholesteryl 3b-N-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA). The cationic liposomes can be stabilized by incorporating glycolipids such as, but not limited to with trehalose 6'6'-dibehenate (TDB) or (monomycolyl glycerol) MMG.

The adjuvant or delivery system can additionally comprise an immunostimulator. The immunostimulator is preferably selected from the group of so-called pathogen-associated molecular patterns (PAMPs) which comprises e.g. TLR-ligands e.g. MPL (mono-phosphoryl lipid A) or derivatives thereof, polyinosinic polycytidylic acid (poly-IC) or derivatives thereof, flagellin, CpG oligodeoxynucleotides, Resiquimod, Imiquimod, Gardiquimod, nucleotide-binding oligomerization domain NOD-like receptors e.g. muramyldipeptide, C-type lectins e.g. the Dectin-1 ligand Zymosan or the mincle ligand TDM or derivatives thereof (e.g. TDB),and ligands for the RIG-like receptors. The immunomodulator can also be selected from the group of pathogen-associated molecular patterns for which no receptor has been identified yet e.g MMG or derivatives thereof (PCT/DK2008/000239 which is hereby incorporated as reference), zymosan, tamoxifen, or ligands for other pathogen-pattern recognition receptors such as muramyl dipeptide (MDP) or analogs thereof.

The liposomes are made by the thin film method or by high shear mixing as described in WO2013004234.

When used as a vaccine adjuvant an antigenic component is added to the adjuvant dispersion. An antigenic component or substance is a molecule, which reacts with preformed antibody and/or the specific receptors on T and B cells. In the context of vaccination, a molecule that can stimulate the development of specific T or B cells, leading to the formation of a memory population of immune cells that will promote a faster "memory" response if the antigen is encountered a second time by immune cells. Since memory populations are rarely clonal, in practice this means that an antigen is any molecule or collection of molecules, which can stimulate an increase in immune responses when it is re-encountered by immune cells from an individual who has previously been exposed to it.

The antigenic component or substance can be a polypeptide or a part of the polypeptide, which elicits an immune response in an animal or a human being, and/or in a biological sample determined by any of the biological assays de-scribed herein. The immunogenic portion of a polypeptide may be a T-cell epitope or a B-cell epitope.

The hydrophilic coating comprises polymers based on poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s, wherein the polymer may be linear or branched, and wherein the polymer may be optionally substituted. The molecular weight for the hydrophilic coating ranges from 350 g/mole to 5000 g/mole.

Anionic anchor can consist of any anionic chemical/biological compound which will interact with the cationic surface. Examples hereof are: 1) polyanionic peptides such as peptides containing two or more aspartic or glutamic acid residues. 2) polyphosphates defined as *n* tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus groups share one oxide and the others phosphorus centres share two oxide centres, 3) polyanionic polysaccharide derivatives such as polyanionic cellulose and 4) synthetic sulphated polymers.

Furthermore this invention relates to the binding of this coating by the aid of electrostatic interactions, thus permitting the complexing of other vaccine components, such as antigens and immunostimulators, to the cationic liposomes before the formation of a hydrophilic coating.

The *hydrophilic coating* of cationic liposome based vaccine adjuvants *shield* the liposomes from the instant interaction with interstitial proteins after injection. Thus the invention provides enhanced passive diffusion of the vaccine into the lymphatics to reduce/prevent aggregation at the injection site caused by interaction between matrix proteins and the cationic lipid surface by associating hydrophilic polymer coatings by electrostatic interactions obtained by covalently binding an anionic anchor residue to the end of the polymer.

The invention also covers the use of hydrophilic polymer coatings, which upon exposure to extracellular fluid will be shredded from the liposome surface due to i.e. competition with other anionic compounds or pH changes modifying the charge of the anionic anchor and thus the adsorption strength.

Another embodiment of the invention encompass the use of anionic anchors on the hydrophilic coating polymers, which dependent on their size and overall charge are more or less prone to be shredded from the surface of the cationic liposomes upon injection.

Thus in the preferred embodiment the vaccine delivery systems comprises cationic liposomes, such as the DDA based liposomes used in CAF01 and CAF09 which are coated with hydrophilic coatings, such as poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s. The obtained vaccine system, will upon vaccination avoid the immediate formation of a depot at the site of injection, but drain freely to the draining lymph node or blood circulation depending on the size of the particles. The delivery system will carry antigen and immunostimulators such as TDB, MMG, MPL, pIC, CpG, Resiquimod, Imiquimod, MDP etc complexed to the delivery system, which will only be exposed to the in vivo environment when the hydrophilic coating is displaced from the vaccine. This can e.g. happen when the vaccine is exposed to interstitial proteins or when the vaccine ends up in the lysosome of APCs.

Figure legends:

Figure 1: side-by-side immunization creates increased Ab responses

Figure 2: PEGylation of CAF09 increase CD8 T cell responses

Figure 3: Illustration of the invention rationale

Figure 4: Example of anionic tagged PEG: PEG-peptide constructs used for proof-of-concept studies

Figure 5: Physical characterization of Cationic liposomes coated with PEG via electrostatic interactions

Figure 6: Immunological effect of PEGylation of CAF01 via electrostatic interactions

Figure 7: Mice were immunized with the model-antigen OVA, OVA adjuvanted with CAF09 and OVA adjuvanted with CAF09-PEG-peptide (CAF09-PP) Top: Association of OVA with lymphocytes in the draining lymph nodes upon intramuscular immunization. Bottom: Association of vaccine (OVA and/or CAF09) with B cells in the draining lymph nodes.

Figure 8: : Mice were immunized with the model-antigen OVA, OVA adjuvanted with CAF09 and OVA adjuvanted with CAF09-PEG-peptide (CAF09-PP). Inflammatory responses at the site of injection 24 h after immunization.

Figure 9: Illustration and examples of how the order of add-mixing of hydrophilic anionic tag and antigen can affect the antigen/adjuvant interaction.

Figure 10: Evaluation of how the order of add-mixing of hydrophilic anionic tag and antigen can affect the immune profile of a vaccine.

Examples

EXAMPLE 1:

To verify that free antigen in addition to adjuvant complexed antigen increases the B cell presentation and activation, while maintaining the strong T cell induction of CAF01, we compared vaccination groups where antigen was administered as 100 percent associated to CAF01 (+CAF01) and 50 percent associated to CAF01 and 50% administered freely as side-by-side immunizations draining to the same lymphnode (Ag/CAF01 s-b-s). As positive control for antibody induction we used Addavax (+sq. emulsion). Bottom figures show that the CAF01 vaccine group also receiving free antigen induces both strong T cell responses and antibody responses, whereas the group with 100% CAF01 association induces one log reduced antibody responses but high T cell responses and the addavax adjuvanted induces strong antibody responses but low T cell responses. These data in figure 1. show that administration of Ag for free drainage to the lymphnodes facilitate increased Antibody responses.

EXAMPLE 2:

In order to verify that PEGylation of the CAF09 adjuvant increase the CD8 responses mice (4-8/group) were immunized s.c. with a dose of 10 µg unadjuvanted OVA (Ag) or OVA adjuvanted with CAF09 (+CAF09) at a dose of 250/50/50 µg DDA/MMG/poly(I:C), or OVA adjuvanted with CAF09 incorporating 10% DSPE-PEG, all in a dose-volume of 200 µl in TRIS-buffer. Mice received three immunizations with two-week intervals, and the immune responses were evaluated eight days after the final immunization. Blood lymphocytes were separated and stained for antigen specific CD8+ T cells using siinfekl tetramer. As illustrated in figure 2, the PEGylation significantly increased the Ag specific CD8 T cell responses.

EXAMPLE 3:

Coating of cationic liposomes with hydrophilic coatings using lipid linkers has do be done during the preparation of the liposomes, which reduces, not only the depot effect after immunization, but also the ability to associate with antigens and immunostimulatory compounds through electrostatic interaction (figure 3 A). the present invention utilizing hydrophilic coating through electrostatic interaction by linking PEG to anionic tags, such as polyanionic peptides, polyphosphates etc. will make it possible to either associate antigens and immunostimulators before coating to enable full association (figure 3B), simultaneously with the hydrophilic coating to obtain partial association of the antigens and immunostimulators in a competitional manner or after the hydrophilic coating to reduce association of the antigens and immunostimulators.

EXAMPLE 4:

Examples of compounds to be used for hydrophilic coatings of cationic surfaces through electrostatic interactions could be one or more polyethylene glycol (PEG) chains linked to peptides of varying charge. Figure 4 illustrates examples hereof where one (monoPEG) or two (bisPEG) chains were associated to peptides with 2-4 repeat sequences of GDGDY thus carrying 4-8 anions.

EXAMPLE 5:

Association of hydrophilic coatings with PEG-peptides illustrates the ability of the anionic tag to bind PEG to the liposomal surface. Increasing peptide size and thus the negative charge reduces the overall surface charge of the formulation in a concentration dependent manner (figure 5 middle top+bottom). At the same time, bisPEGylation (figure 5 middle top) does not reduce the surface charge as much as monoPEGylation (figure 5 middle + bottom) showing that more PEG-peptide can be bound to the liposomes (on molar basis) with reduced PEG-size, suggesting that sterical hindrance is a key factor for amount of PEG-peptide association. Particle size is only slightly increased with PEGylation of the liposomes (Figure 5 left). When added simultaneously with PEG-peptides Protein association to the liposomes can be reduced proportionally with increased negative charge on the PEG-peptides (Figure 5 right).

EXAMPLE 6:

In order to verify that electrostatic association of PEG to adjuvants based on cationic liposomes increase the B cell responses as a consequence of increased free antigen mice (4/group) were immunized s.c. with a dose of 10 µg unadjuvanted CTH522 (Ag) or Ag adjuvanted with Addavax (positive control for Ab responses), CAF01 or CAF01+ different concentrations/charges of PEG-peptide. all in a dose-volume of 200 µl in TRIS-buffer. Mice

received two immunizations with two-week intervals, and the immune responses were evaluated eight days after the final immunization. Ag specific IgG levels were measured in blood and data shows an increase for all PEG-peptide coated CAF01 formulations as compared to uncoated CAF01. Lymphocytes from draining lymphnode were harvested and stained for antigen specific CD19+ B cells as well as Ag specific (Cytokine+) T cells. As illustrated in figure 6, the PEGylation also increass the B cell responses in the draining lymphnode and only slightly reduces the antigen specific T cell responses.

EXAMPLE 7:

The effect of PEGylation of CAF09 on the draining kinetics of the antigen and adjuvant was evaluated by intramuscularly immunizing mice with the model-antigen OVA, OVA adjuvanted with CAF09 and OVA adjuvanted with CAF09-PEG-peptide (CAF09-PP) (Figure 7). The vaccine components were fluorescently labelled and therefore, the association with lymphocytes and B cells in the draining lymph nodes was evaluated by using flow cytometry. At 24 hours after immunization, significantly more antigen was associated with lymphocytes in the mice immunized with OVA/CAF09-PEG-peptide, as compared to mice immunized with OVA/CAF09. This illustrates that the PEG-coating apparently increases the drainage of vaccine from the site of injection to the draining lymph nodes.

EXAMPLE 8:

The effect of PEGylation of CAF09 on the pro-inflammatory response at the site of injection (quadriceps) was evaluated by intramuscularly immunizing mice with the model-antigen OVA, OVA adjuvanted with CAF09 and OVA adjuvanted with CAF09-PEG-peptide (CAF09-PP) (Figure 8). Immunization with OVA/CAF09-PEG-peptide induces significantly stronger pro-inflammatory responses in the quadriceps, as compared to unadjuvanted OVA and OVA/CAF09. The increased pro-inflammatory response may arise from dissociation of poly(I:C), PEG-peptides and/or OVA from the liposomes due to competition with interstitial proteins.

EXAMPLE 9:

Order of add-mixing vaccine Ag and hydrophilic anionic tag can influence the vaccine association to the cationic liposomes: Admixing the Ag before hydrophilic anionic tag will make full association possible. Admixing Ag after, on the other side can block the Ag association whereas admixing Ag and hydrophilic anionic tag simultaneously will facilitate partial Ag association. None of these situations are absolute, and the outcome will be influenced by the nature of the Ag, the pI of the AT, the molecular size of hydrophilic anionic tag and the concentrations of each individual component. One example is shown in the graphs Figure 9,

where 500µg/ml OVA was added to CAF01, either before or after addition of different concentrations of PEG peptide (aa10-bisPEG; PP). In the top-left graph it can be seen that 10 mole% of PP added before OVA enables stabilization of the CAF01 particle size at around 200 nm, whereas no PP leads to significantly increased particle size. The same reduction in particle size is not observed if PP is added after OVA. At the same time, the surface charge is significantly reduced by addition of PP+OVA, and the effect is strongest if PP is added before the antigen (Top right graph). This effect on particle size and surface charge is due to a partial displacement of OVA from the surface of the CAF01 liposomes, increasing with PP concentration (bottom left graph). Addition of OVA before PP does not have a significant role for PP binding to CAF01 (bottom right graph)

EXAMPLE 10:

In order to verify that PEGylation of the CAF01 adjuvant with PEG peptide increase the Ab responses mice (4/group) were immunized s.c. with a dose of 1 µg unadjuvanted CTH522 (Ag) or CTH522 adjuvanted with CAF01 at a dose of 250/50 µg DDA/TDB or CTH522 adjuvanted with CAF01 with 10 mole% aa10-bisPEG (PP) added before or after antigen adsorption to the adjuvant, all in a dose-volume of 200 µl in TRIS-buffer. Mice received two immunizations with three-week intervals, and the immune responses were evaluated 14 days after the final immunization. Spleen lymphocytes were separated and restimulated for 72 hours with CTL522 to quantify T cell responses as measured by IFN-γ secretion (ELISA). Serum was harvested and CTH522 specific Ab titers were determined by Ab ELISA. Addition of the PP after CTH522 resulted in a significant reduction in T cell responses to CTH522 (Figure 10 left graph), whereas the IgG responses increased slightly (Figure 10 right graph). Addition of the PP before CTH522 on the other hand only resulted in a minimal reduction in IFN-γ secretion (Figure 10 left graph), whereas the IgG titers increased with more than a log (Figure 10 right graph).

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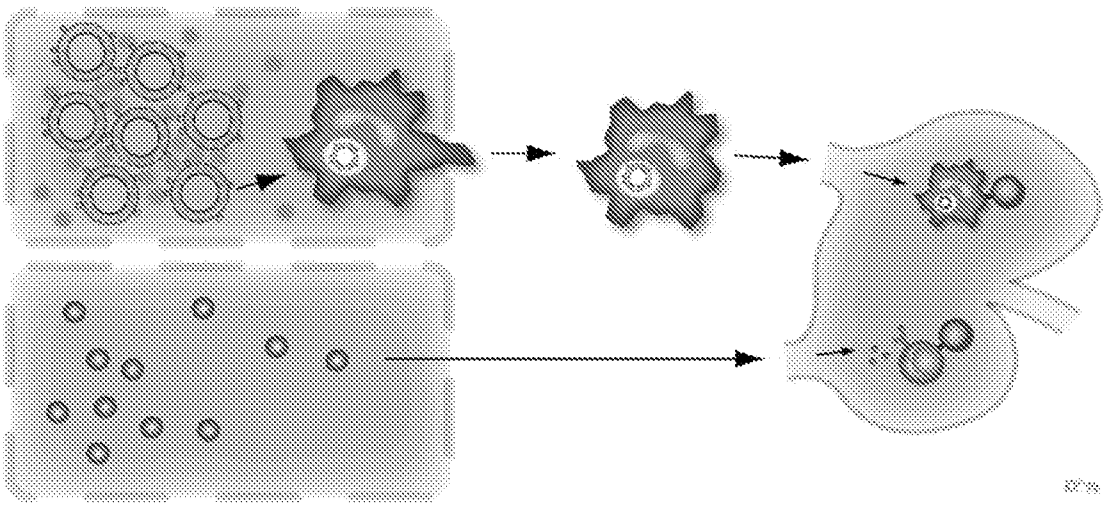
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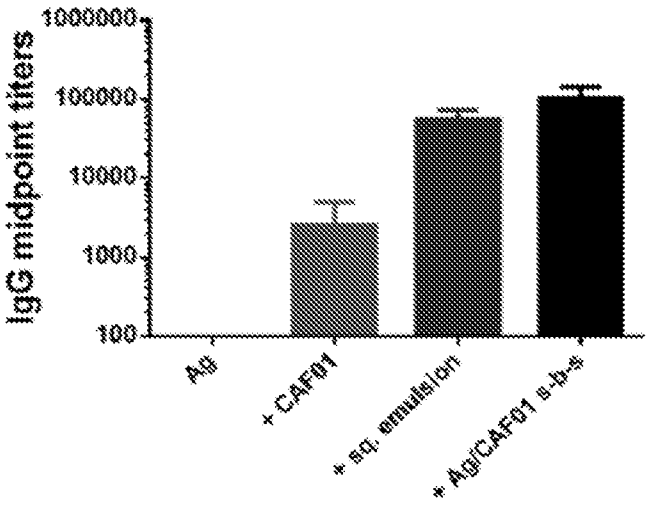
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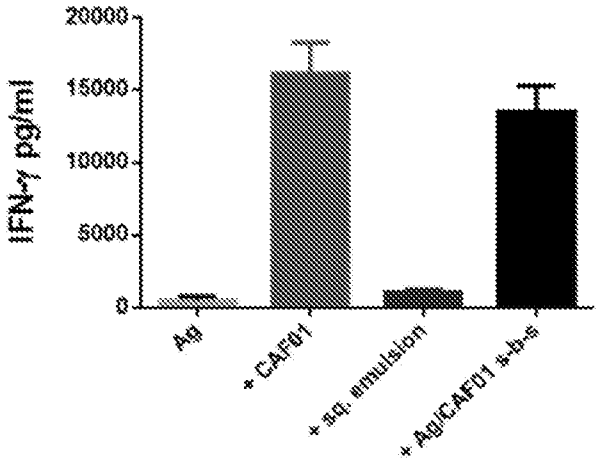
Claims

1) A method of controlling the surface charge of liposomes and shielding vaccine delivery systems by anchoring hydrophilic coating polymers onto the liposomes by electrostatic interactions using polar tags.
2) A method according to claim 1, where the hydrophilic coating polymers are chosen from poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s.
3) A method according to claim 2, where the liposomes are cationic liposomes and the polar tags are anionic residues such as polyanionic peptides such as peptides containing two or more aspartic or glutamic acid residues, polyphosphates defined as *n* tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus groups share one oxide and the others phosphorus centres share two oxide centres, polyanionic polysaccharide derivatives such as polyanionic cellulose and synthetic sulphated polymers.
4) A method according to claim 1-3, where the liposomes are coated with the hydrophilic polymer after complexing antigens and immunostimulators to the liposomes.
5) Adjuvant formulations comprising liposomes coated with hydrophilic polymer anchored with polar tags.
6) Adjuvant formulations according to claim 5, where the liposomes are cationic liposomes.
7) Adjuvant formulations according to claim 6, where the cationic liposomes are made from dimethyldioctadecylammonium (DDA), dimethyldioctadecenylammonium (DODA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1, 2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane and dioleoyl-3-dimethylammonium propane (DODAP) or N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA).
8) Adjuvant formulations according to claim 6-7, additionally comprising a glycolipid such as TDB or MMG.
9) Adjuvant formulations according to claims 6-8, where hydrophilic coating polymers are chosen from poly(ethyleneglycol), poly(oxazoline), poly(ethylene oxide), poly(vinyl alcohol), poly(glycerol), poly(N-vinylpyrrolidone), poly[N-(2-hydroxypropyl)methacrylamide] and poly(amino acid)s.
10) Adjuvant formulations according to claim 6-9, where the polar tags are chosen from polyanionic peptides such as peptides containing two or more aspartic or glutamic acid residues, polyphosphates defined as n tetrahedral PO4 units linked together by sharing oxygen centres, such that end phosphorus groups share one oxide and the others

phosphorus centres share two oxide centres, polyanionic polysaccharide derivatives such as polyanionic cellulose and synthetic sulphated polymers.




IgG midpoint titers
1000000
100000
10000
1000
100
Ag
+ CAF01
+ sq. emulsion
+ Ag/CAF01 s-b-s


IFN-γ pg/ml
20000
15000
10000
5000
0
Ag
+ CAF01
+ sq. emulsion
+ Ag/CAF01 s-b-s

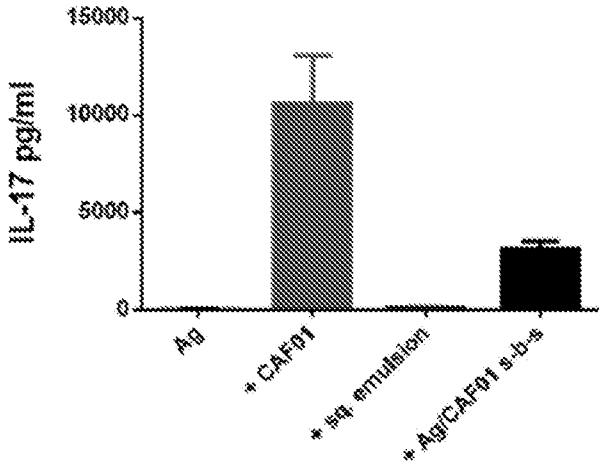

IL-17 pg/ml
15000
10000
5000
0
Ag
+ CAF01
+ sq. emulsion
+ Ag/CAF01 s-b-s

Figure 1

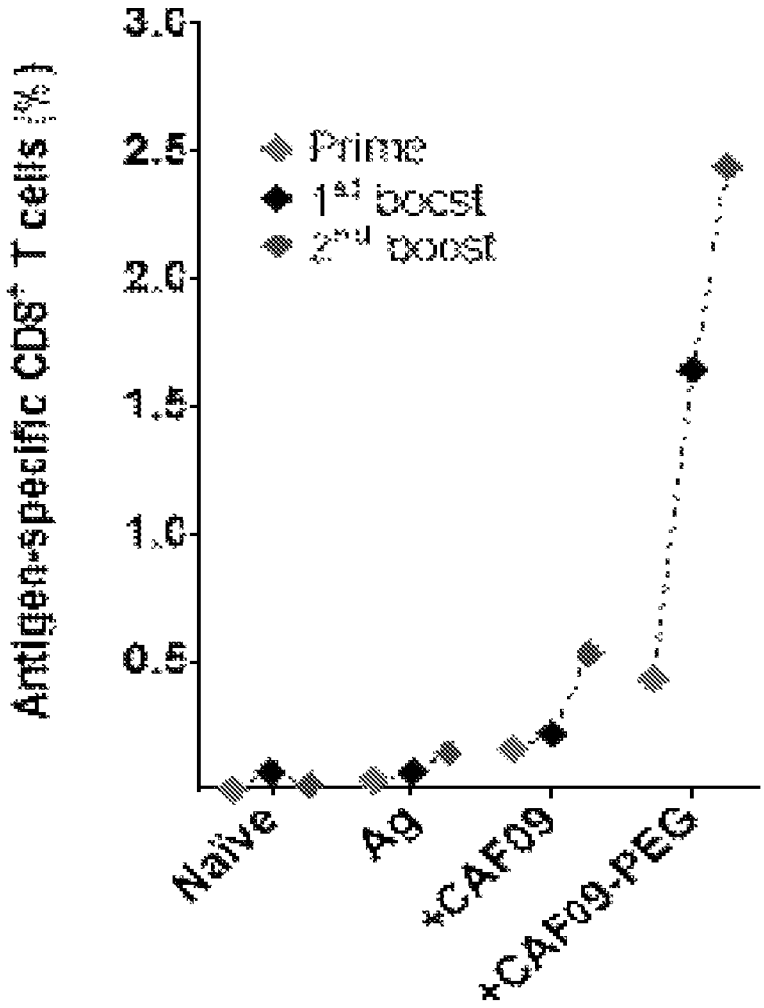

Antigen-specific CD8+ T cells (%)
3.0
2.5
2.0
1.5
1.0
0.5
Prime
1st boost
2nd boost
Naive
Ag
+CAF09
+CAF09-PEG

Figure 2

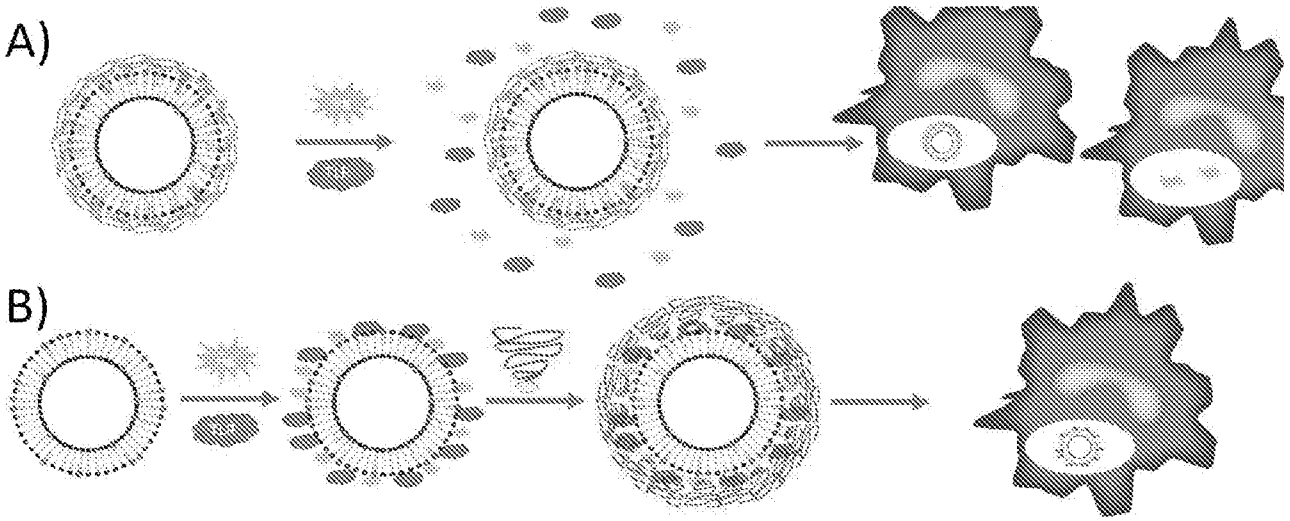
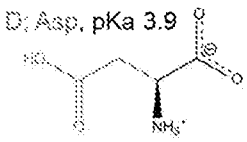


Figure 3

- aa-10-[-4]-monoPEG$_{1000}$
- aa-10-[-4]-bisPEG$_{1000}$
- aa-15-[-6]-monoPEG$_{1000}$
- aa-15-[-6]-bisPEG$_{1000}$
- aa-20-[-8]-monoPEG$_{1000}$
- aa-20-[-8]-bisPEG$_{1000}$

- aa = GDGDY$_n$ (n=2-4)



- PEG =  (n=300-3000)



Figure 4

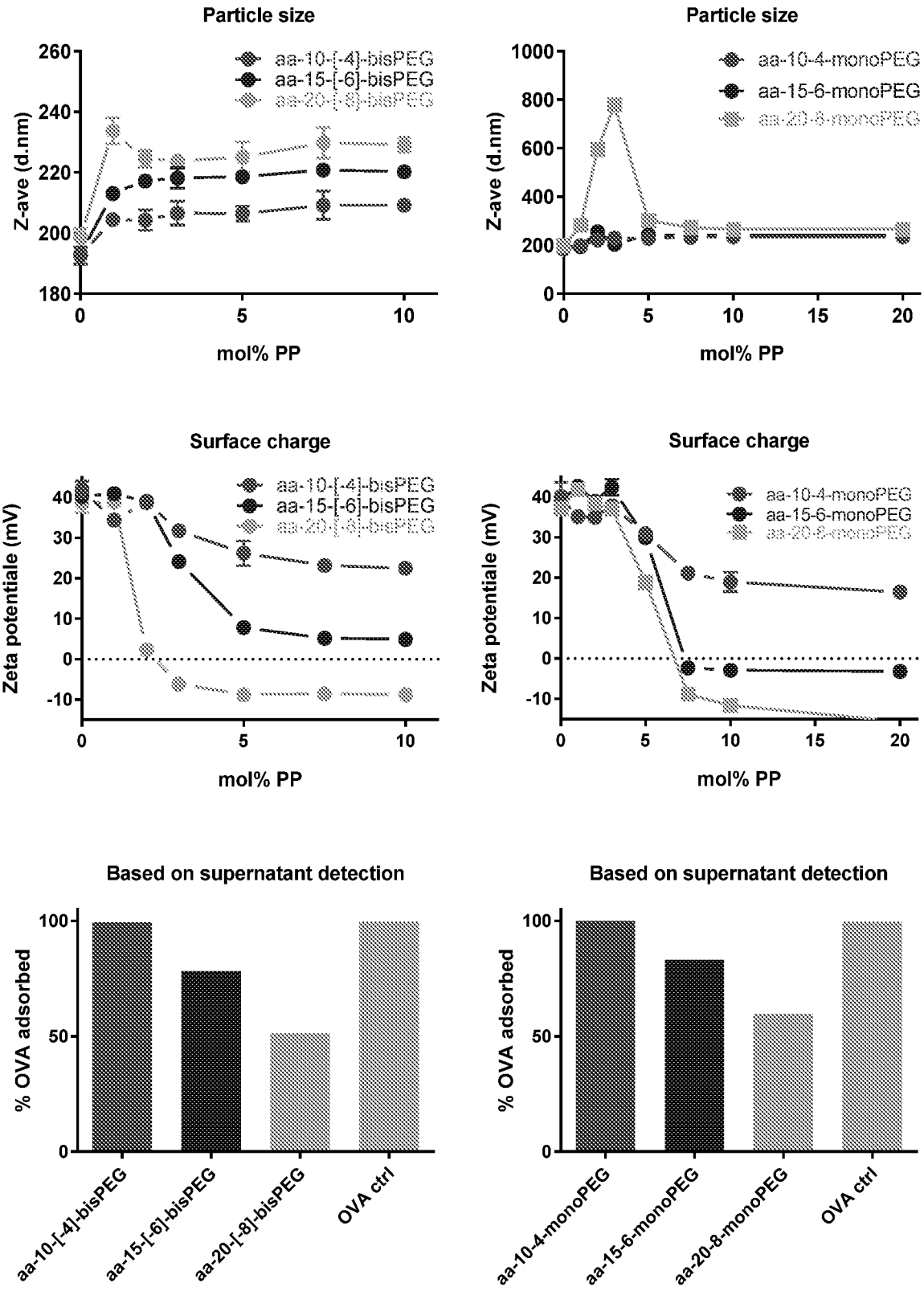

Particle size
Z-ave (d.nm)
mol% PP
aa-10-[-4]-bisPEG
aa-15-[-6]-bisPEG
aa-20-[-8]-bisPEG
Particle size
Z-ave (d.nm)
mol% PP
aa-10-4-monoPEG
aa-15-6-monoPEG
aa-20-8-monoPEG
Surface charge
Zeta potentiale (mV)
mol% PP
aa-10-[-4]-bisPEG
aa-15-[-6]-bisPEG
aa-20-[-8]-bisPEG
Surface charge
Zeta potentiale (mV)
mol% PP
aa-10-4-monoPEG
aa-15-6-monoPEG
aa-20-8-monoPEG
Based on supernatant detection
% OVA adsorbed
aa-10-[-4]-bisPEG
aa-15-[-6]-bisPEG
aa-20-[-8]-bisPEG
OVA ctrl
Based on supernatant detection
% OVA adsorbed
aa-10-4-monoPEG
aa-15-6-monoPEG
aa-20-8-monoPEG
OVA ctrl

Figure 5

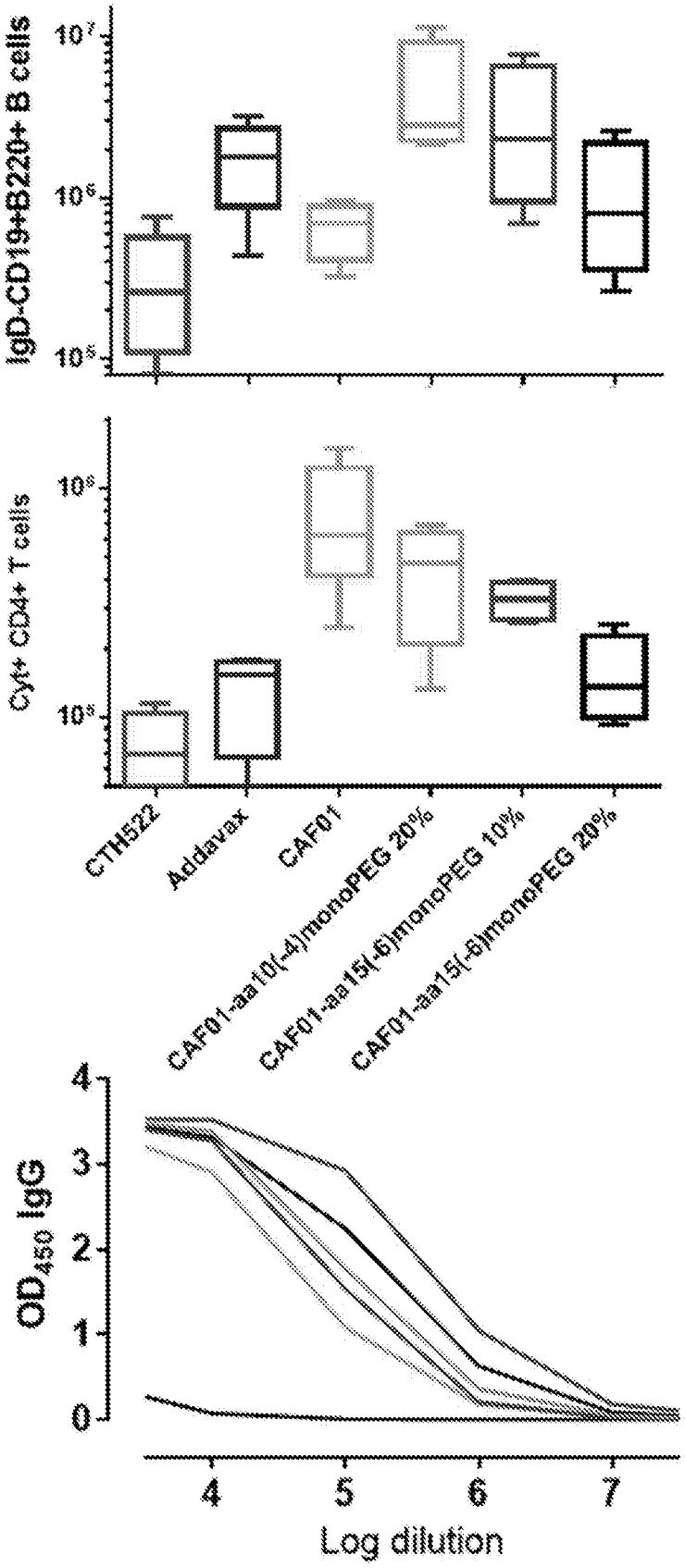

IgD-CD19+B220+ B cells
10^7
10^6
10^5
Cyt+ CD4+ T cells
10^6
10^5
CTH522
Addavax
CAF01
CAF01-aa1(0[-4]monoPEG 20%
CAF01-aa15[-6]monoPEG 10%
CAF01-aa15[-6]monoPEG 20%
OD_{450} IgG
4
3
2
1
0
4
5
6
7
Log dilution

Figure 6

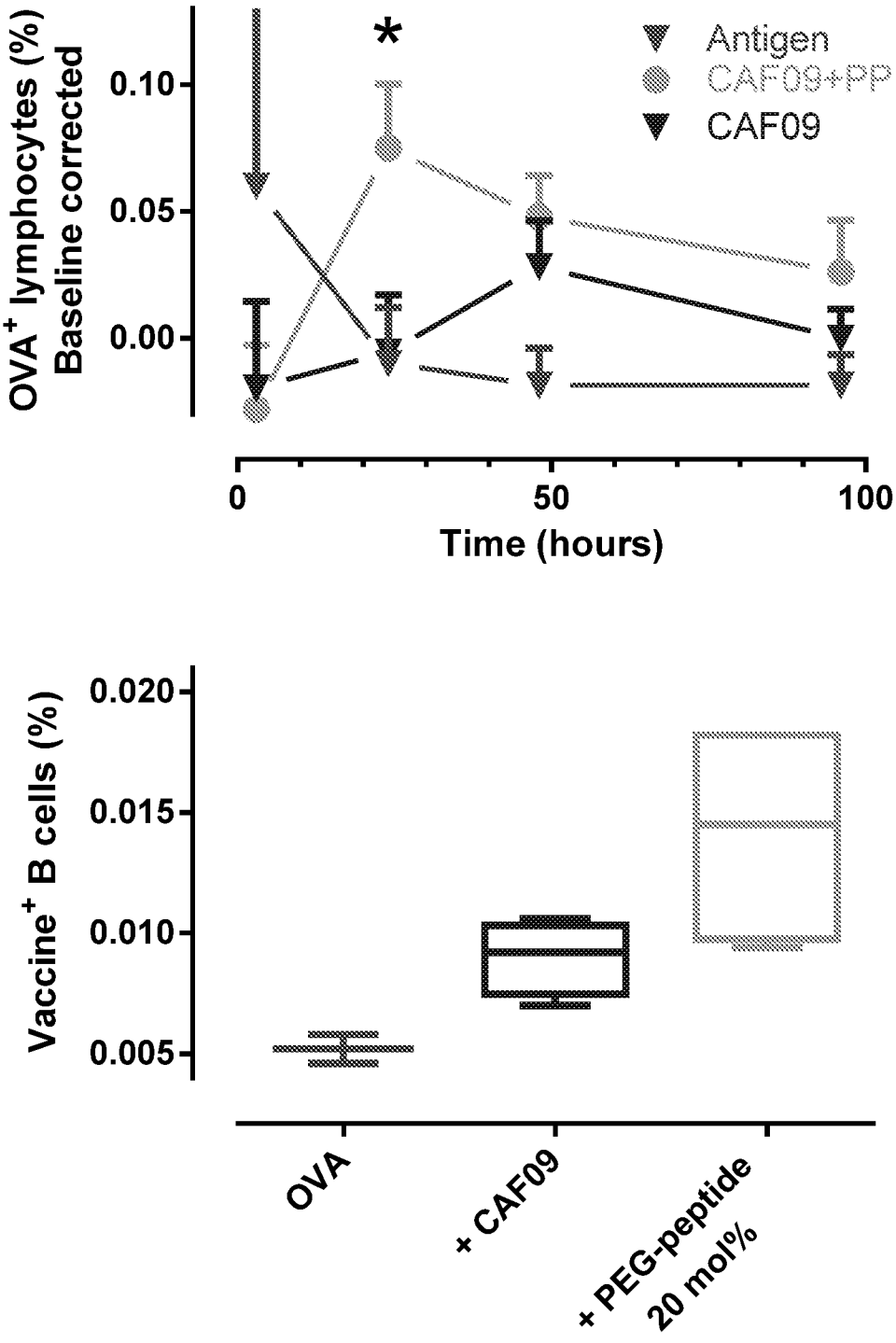

OVA+ lymphocytes (%)
Baseline corrected
0.10
0.05
0.00
*
Antigen
CAF09+PP
CAF09
0
50
100
Time (hours)
Vaccine+ B cells (%)
0.020
0.015
0.010
0.005
OVA
+ CAF09
+ PEG-peptide
20 mol%

Figure 7

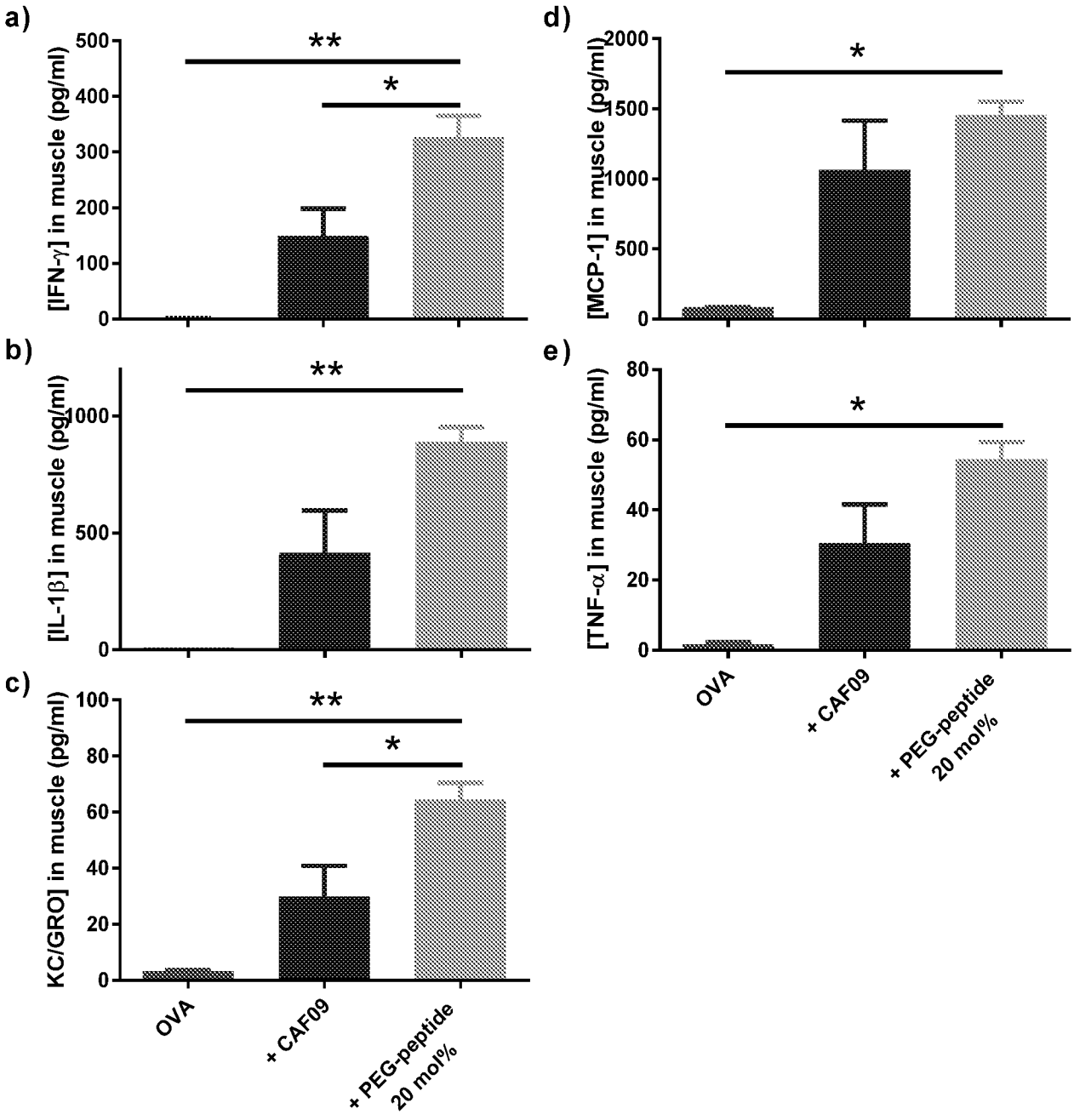

a)
[IFN-γ] in muscle (pg/ml)
500
400
300
200
100
0
**
*
b)
[IL-1β] in muscle (pg/ml)
1000
500
0
**
c)
KC/GRO] in muscle (pg/ml)
100
80
60
40
20
0
**
*
OVA
+ CAF09
+ PEG-peptide 20 mol%
d)
[MCP-1] in muscle (pg/ml)
2000
1500
1000
500
0
*
e)
[TNF-α] in muscle (pg/ml)
80
60
40
20
0
*
OVA
+ CAF09
+ PEG-peptide 20 mol%

Figure 8

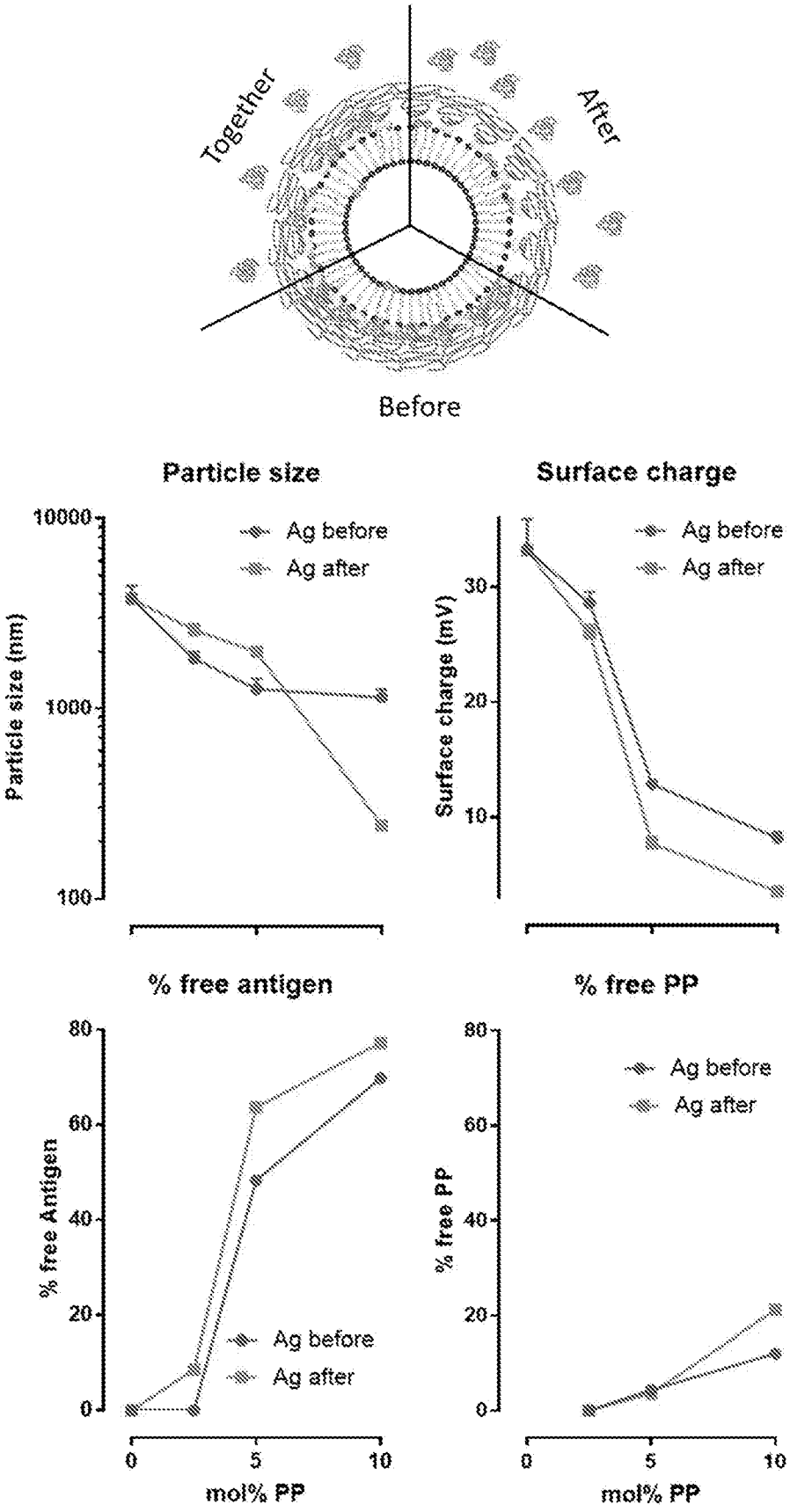

Together
After
Before
Particle size
Particle size (nm)
10000
1000
100
Ag before
Ag after
Surface charge
Surface charge (mV)
30
20
10
% free antigen
% free Antigen
80
60
40
20
0
0
5
10
mol% PP
% free PP
% free PP

Figure 9

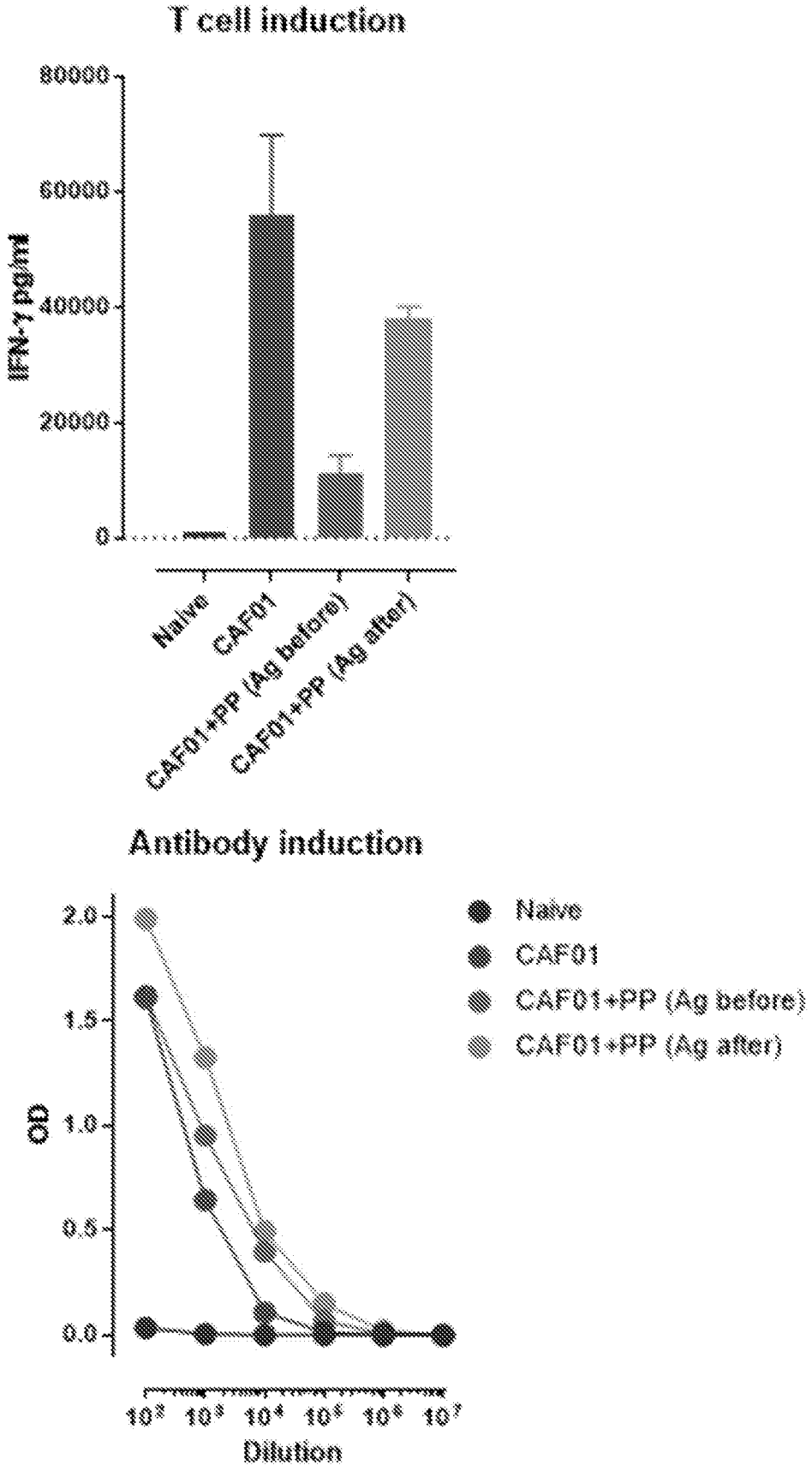

T cell induction
IFN-γ pg/ml
80000
60000
40000
20000
0
Naive
CAF01
CAF01+PP (Ag before)
CAF01+PP (Ag after)
Antibody induction
OD
2.0
1.5
1.0
0.5
0.0
Naive
CAF01
CAF01+PP (Ag before)
CAF01+PP (Ag after)
10² 10³ 10⁴ 10⁵ 10⁶ 10⁷
Dilution

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2017/050208

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/127 A61K39/00 A61K47/50
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)
A61K

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, 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	FAN YUCHEN ET AL: "Cationic liposome-hyaluronic acid hybrid nanoparticles for intranasal vaccination with subunit anti", JOURNAL OF CONTROLLED RELEASE, vol. 208, 11 April 2015 (2015-04-11), pages 121-129, XP029158899, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2015.04.010	1-10
Y	the whole document	1-10

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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
2 October 2017	18/10/2017

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	Raposo, Antonio

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2017/050208

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 00/43043 A1 (UNIV GEORGETOWN [US]; XU LIANG [US]; CHANG ESTHER H [US]) 27 July 2000 (2000-07-27) page 5, lines 11-14 page 6, lines 30-31 page 7, lines 1-10, 13 page 8, lines 4-8, 13-28 page 9, lines 3-7, 9-20 -----	1-10
Y	SIGNE TANDRUP SCHMIDT ET AL: "Liposome-Based Adjuvants for Subunit Vaccines: Formulation Strategies for Subunit Antigens and Immunostimulators", PHARMACEUTICS, vol. 8, no. 1, 10 March 2016 (2016-03-10), page 7, XP055404844, DOI: 10.3390/pharmaceutics8010007 the whole document -----	2,6-9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2017/050208

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0043043 A1	27-07-2000	AU 2620000 A	07-08-2000
		CA 2358497 A1	27-07-2000
		EP 1144012 A1	17-10-2001
		JP 2002535290 A	22-10-2002
		WO 0043043 A1	27-07-2000