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(54) Title: COMPOSITION OF NANOPARTICLES

(57) Abstract: The disclosure relates to a composition of nanoparticles as carrier for pharmaceutically acceptable compounds, a method for the preparation of the composition and the use of the composition for medical purposes, in particular for immunoprophylaxis or immunotherapy. The invention also relates to a vaccine containing the composition.

WO 2021/229015 A1

"Composition of nanoparticles"

FIELD OF THE INVENTION

The present invention relates to a composition of nanoparticles as carrier for pharmaceutically acceptable compounds, the use of the composition for medical purposes, in particular for immunoprophylaxis or immunotherapy as well as to the nanoparticles as such. The invention also relates to a vaccine containing the composition and/or nanoparticles.

BACKGROUND OF THE INVENTION

Nanomedicine and nano-delivery systems are a relatively new but rapidly developing science where materials in the nanoscale range are employed to serve as means of diagnostic or therapeutic tools or to deliver therapeutic agents to specific targeted sites in the body. In particular nanoparticles are known for delivery of chemotherapeutic agents, biological agents, immunotherapeutic agents and as vaccines in the immunotherapy.

Functionalized nanoparticles are well-known as drug-delivery systems, e.g. as carrier for antigens. The literature describes in particular carrier systems in which the antigens are either encapsulated or bound to the surface of the nanoparticles.

WO 2006/037979 A2 describes gold nanoparticles (GNPs) comprising adjuvants and antigens, such as tumor and pathogen antigens, and their use in a range of applications such as for the treatment of cancer and infectious diseases. Also disclosed are immunogenic structures based on nanoparticles or antibodies with carbohydrate

ligands, and their use for therapeutic and prophylactic purposes, and for the isolation and detection of antibodies directed against the carbohydrate structures.

WO 2013/034741 A1 and WO 2013/034726 A1 relate to nanoparticles having an epitopic peptide bound via a linker and which find use as vaccines, e.g. in the prophylactic or therapeutic treatment of a tumor in a mammalian subject.

WO 2011/154711 A1 describes glycated gold nanoparticles that act as carriers for delivery of peptides such as insulin.

WO 2010/006753 A2 discloses monodisperse nanoparticles of silicon dioxide with at least one antigen attached to their surface. The nanoparticles are used for the immunoprophylaxis or immunotherapy of cancer.

Although many nanoparticles have been investigated and developed in particular in the context of treating cancer there is still a high demand on providing substances with improved characteristics, in particular in terms of its adjuvant effects. Furthermore there is a need to provide nanoparticles as a flexible and convenient system to present agents of pharmacological relevance to the immune system of the body.

SUMMARY OF THE INVENTION

It is thus objective of the present invention to provide improved nanoparticles and compositions comprising them in particular for use in the immunoprophylaxis or immunotherapy. It is furthermore an objective of the invention to provide a platform technology, which enables a simple method for the generation of ready-to-use nanoparticles suitable for carrying a broad range of various ligands for the medical treatment. These nanoparticles should have effective immunomodulatory activities.

These objectives are solved by providing a composition comprising nanoparticles with silicon dioxide and functional groups on the surface, which are loaded with pharmaceutically acceptable compounds (preferably any kind of antigen). The compounds represent the payload of the nanoparticles. The nanoparticles have a particle size below 150 nm. The functional groups on the surface of the nanoparticles are suitable for carrying and/or stabilizing negative and positive charges of such compounds. The composition has a zeta potential of at least ± 15 mV.

With a composition according to the invention one fundamental problem of nanoparticles for drug delivery is overcome, which is the lack of stability of the colloidal suspension.

In aqueous systems, surface charges essentially ensure the stability of colloidal systems. These charges can be positive or negative. To substantially reduce or even avoid agglomeration of the particles it is important that there are enough functionalities of the same charge, which leads to electrostatic repulsion. If the charge of the colloidal carrier system is compensated by the adsorption of the oppositely charged molecules, agglomerates will be formed and the colloidal system collapses.

The agglomerates have significantly larger particle diameters, which jeopardizes an effective transport of these large particles within the body and leads to a less effective immunomodulatory efficacy.

The composition according to the invention with nanoparticles having a particle size of below 150 nm and a zeta potential of at least ± 15 mV ensures that the composition is sufficiently stable. In particular under physiological conditions, the nanoparticles dispersed therein can effectively be transported to their site of main activity within the body. The compositions according to the invention thus allow the improved transport of the nanoparticles into the lymphatic system, in particular from the administration site to the lymph nodes in which dendritic cells are located.

When administered by subcutaneous injection the particles according to the invention are too large to enter the blood circulation, hence their fast elimination as well as severe systemic adverse effects are essentially avoided. At the same time they are small enough to penetrate into lymphatic vessels and to enter the dendritic cells by phagocytosis. The same applies when the particles are administered intradermally, intraperitoneally or intramuscularly. It is thus preferred to administer the particles parenterally with the exception of intravenous or intraarterially administration.

The inventors have found that the particles in the composition according to the invention can carry a high number of compounds on the surface. In view of the desired function of the nanoparticles, the compounds are pharmaceutically acceptable. The total amount of the compounds can be up to 5 % by weight with regard to the surface of the nanoparticle in nm^2 (surface loading density). The suitability of the nanoparticles to carry a high amount of compounds is particularly important when high doses of such compounds (e.g. antigens or drug substances) are to be delivered.

The compositions according to the invention comprise nanoparticles with silicon dioxide and functional groups on the surface. The functional groups can be directly or indirectly

connected to the surface of the nanoparticle. In a preferred embodiment of the invention the functional groups are connected to the surface of the nanoparticle via a linker (hereinafter linker compound L). The linker compound L can be connected to the surface of the nanoparticles by any way, in particular by covalent or adsorptive bond, most preferred by covalent bond.

In a particularly preferred embodiment the linker compound L comprises at least one functional group selected from the group consisting of carboxyl (–COOH) or carboxylate ($–COO^-$) group and/or at least one functional group selected from the group consisting of guanidino-group ($–NHC(=NH)NH_2$ or $–NHC(=NH_2^+)NH_2$) or amino-group ($–NH_2$ or $–NH_3^+$). Preferably, it comprises both, a carboxyl (–COOH) or carboxylate ($–COO^-$) group and at least one group selected from the group consisting of guanidino-group ($–NHC(=NH)NH_2$ or $–NHC(=NH_2^+)NH_2$) or amino-group ($–NH_2$ or $–NH_3^+$). Most preferred is that the linker compound L comprises a carboxyl (–COOH) or carboxylate ($–COO^-$) group and at least one guanidino-group ($–NHC(=NH)NH_2$ or $–NHC(=NH_2^+)NH_2$).

Such a linker compound L allows the adsorptive binding of anionic or cationic and also of nonpolar pharmaceutically acceptable compounds (such as e.g. hydrophobic peptides). Therewith the linker provides a platform technology for the simple coupling of a broad range of chemically diverse compounds to the surface of the nanoparticle.

DETAILED DESCRIPTION OF THE INVENTION

The zeta potential of a nanoparticle is commonly used parameter known to the skilled person to characterize the surface charge property of nanoparticles. It reflects the electrical potential of particles. To avoid agglomeration of the particles it is important that there are enough functionalities of the same charge which repel each other. Agglomerates cause the colloidal system to collapse. The resulting agglomerates have significantly larger particle diameters than the individual particles and therefore the desired transport mechanism via the fenestrated endothelium of the lymphatic vessels is no longer guaranteed. It is accepted as a measure for the stability of colloidal systems.

Current characterization methodologies are based on ensemble measurements (e.g. phase analysis light scattering, Doppler velocimetry, streaming potentiometry) that measure the average electrophoretic mobility of particles in suspension (Sci. Rep. 12 Dec. 2017; 7(1): 17479, PMCID: PMC5727177). It is particularly preferred to measure

the zeta potential of the composition according to the invention with Dynamic Light Scattering (DLS) (ZetaSizer Nano ZS, Malvern Instruments, UK).

The zeta potential of the composition according to the present invention has a value of at least ± 15 mV, preferred ± 30 mV, more preferred ± 60 mV and most preferred between ± 25 and ± 40 mV. Therewith the composition, which is desirably a colloidal suspension, is stabilized, which means that the collapse of the system is essentially avoided.

The compositions according to the invention contain nanoparticles with a particle size below 150 nm, preferably 100 nm or less. More preferred are nanoparticles with a particle size of 50 nm or less, most preferred with a particle size between 20 and 30 nm. The particle size defined herein should be interpreted in such a way that a random distribution over the entire range is not present, but instead a defined particle size within the range is selected, of which the standard deviation is a maximum of 15%, preferably a maximum of 10%, wherein the standard deviation always relates to the local maximum in case of a bi- or multimodal distribution.

An indicator for the particle size of nanoparticles is the Z-average diameter. The Z-average diameter measured in dynamic light scattering is a parameter also known as the cumulant mean. It is the primary and most reliable parameter produced by the technique. The Z-average diameter is typically used in a quality control setting according to ISO 22412:2017. Dynamic light scattering techniques will give an intensity weighted distribution, where the contribution of each particle in the distribution relates to the intensity of light scattered by the particle. It is preferred to measure the intensity weighted distributions with a ZetaSizer Nano ZS (Malvern Instruments, UK).

In a preferred embodiment the Z-average diameter of the nanoparticles according to the invention is in the range of ≥5 and <150 nm, preferably ≥15 and ≤60nm, more preferably ≥20 and ≤40nm and still more preferably between 20 and 30nm, measured according to ISO 22412:2017.

The particle size of the nanoparticles and the stability of the composition according to the invention are furthermore confirmed by means of filtration with a sterile filter with maximum 0.2 µm pore size. However, this is practical only for nanoparticles with a diameter less than 50 nm.

The stability of the composition of the invention can be demonstrated by its polydispersity index (PDI). The PDI in general is an indicator for the uniformity of a system; in the context of the invention for the uniformity and stability of the colloidal nanoparticle suspension. The PDI reflects the nanoparticle size distribution. Samples

with a wider range of particle sizes have higher PDI, while samples consisting of evenly sized particles have lower PDI. The skilled person is aware of methods and instruments for the measurement of the PDI, in particular a ZetaSizer Nano ZS (Malvern Instruments, UK).

A PDI greater than 0.7 indicate that the sample has a broad size distribution. A PDI below 0.1 is considered to be monodisperse. The various size distribution algorithms work with data that falls between these two extremes. The calculations for these parameters are defined in the ISO standard document 13321:1996 E and ISO 22412:2008.

The compositions according to the invention show a PDI between 0 and 0.32, preferably between 0.1 and 0.3, more preferably between 0.1 and 0.2, most preferred less than 0.1. The compositions according to the invention having a PDI less than 0.1 and are preferably monodisperse.

In a preferred embodiment the Z-average diameter of the nanoparticles according to the invention is in the range of ≥20 and ≤40nm and a PDI between 0.1 and 0.30 and a zeta potential between ± 20 and ± 40 mV

In a more preferred embodiment the Z-average diameter of the nanoparticles according to the invention is in the range of 20 and 30nm and a PDI between 0.1 and 0.2 and a zeta potential between ± 25 and ± 40 mV.

In a most preferred embodiment the Z-average diameter of the nanoparticles according to the invention is in the range of 20 and 30nm and a PDI less than 0.1 and a zeta potential between ± 25 and ± 40 mV.

It is also possible to measure the particle size of the loaded and unloaded particles by TEM or SEM.

As used herein, the term “transmission electron microscopy (TEM)” refers to a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through it. An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens and appears on an imaging screen, a fluorescent screen in most TEMs, plus a monitor, or on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

As used herein, the term “scanning electron microscope (SEM)” refers to a type of electron microscope that produces images of a sample by scanning the surface with a

focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample. The electron beam is scanned in a raster scan pattern, and the position of the beam is combined with the intensity of the detected signal to produce an image.

In **Fig. 1a** to **1d** show TEM images of the following SiO_2 particles:

Fig. 1a shows a TEM image of SiO_2 nanoparticles with a particle size of 68 nm
Fig. 1b shows a TEM image of SiO_2 nanoparticles with a particle size of 40 nm
Fig. 1c shows a TEM image of SiO_2 nanoparticles with a particle size of 25 nm
Fig. 1d shows a TEM image of SiO_2 nanoparticles with a particle size of 15 nm

Fig. 2 shows a SEM of SiO_2 nanoparticles with a particle size of 150 nm.

In connection with the present invention, a "nanoparticle" is taken to mean a particulate binding matrix which has functionalities on its surface which function as recognition points for antigens ultimately to be bound or adsorbed. The surface here encompasses all areas, i.e. besides the outer surface, also the inner surface of cavities (pores) in the particle. The functionalities may be directly or indirectly bound to the surface.

According to the present invention, the nanoparticle(s) comprise silicon dioxide (SiO_2), optional in mixture with another material. The material thus can also be admixed with further components, where silicon dioxide typically has the highest proportion in a multicomponent system. The nanoparticles of the invention can comprise at least 80% of silicon dioxide, preferably at least 90%.

In a particularly preferred embodiment of the nanoparticles according to the invention, the material comprises silicon dioxide which is essentially pure, i.e. only comprises the impurities to be expected in the course of the preparation process. In a more preferred embodiment of the invention, the nanoparticle material consists of silicon dioxide.

Examples of other materials are metals, a metal chalcogenide, a magnetic material, a magnetic alloy, a semiconductor material, metal oxides, polymers, organosilanes, other ceramics or glass. The metal is selected from the group Au, Ag, Cu, Pt, Pd, Fe, Co, Gd, Ru, Rh and Zn, or any combination thereof.

In a further embodiment of the present invention the nanoparticles have a coating which comprises silicon dioxide. The core may comprise any other material such as metals, polymers or ferromagnetic metals such as Fe_2O_3 or Fe_3O_4. The core can even be devoid of silicon dioxide.

Silicon dioxide nanoparticles according to the invention have been described in, for example, WO 2010/006753 A2, which is expressly incorporated herein by reference.

The silicon dioxide nanoparticles can be prepared using, inter alia, the classical Stöber synthesis, in which monodisperse nanoscale silicon dioxide of defined size can be prepared by hydrolysis of tetraethoxysilane (TEOS) in aqueous-alcoholic-ammonia medium (J. Colloid Interface Sci. 1968, 26, 62).

The process of the preparation of silicon dioxide nanoparticles is described in detail in EP 0216 278 B1 and WO 2005/085135 A1, and consequently these documents are incorporated in their totality into the disclosure content of the present invention by way of reference. At least one amine is preferably used in the medium.

The silicon dioxide matrix of the nanoparticles according to the invention can be either porous or non-porous. The porosity is essentially dependent on the production process. In the synthesis in accordance with EP 0 216 278 B1, non-porous particles, in particular, are obtained.

According to the present invention the nanoparticles contain functional groups on their surface. These functional groups are capable to carry and/or stabilize both negative and positive charges. These charges may belong to pharmaceutically acceptable compounds such as proteins, peptides, polynucleotides, oligonucleotides (RNA, DNA), nucleic acids, small molecules or peptide antigens.

In a preferred embodiment of the invention the pharmaceutically acceptable compounds are selected from the group of Pathogen-Associated Molecular Patterns (PAMPs), Danger-Associated Molecular Patterns (DAMPs), Defective Infectious Particles (DIPs) or epitopes.

Functional groups which are capable to carry and/or stabilize both negative and positive charges are for example –SH, –COOH, $–NH_2$, –guanidino-group (–NHC(=NH))NH_2), $–PO_3H_2$, $–PO_2CH_3H$, $–SO_3H$, –OH, $–NR_3^+X^-$. Preferred functional groups are –COOH, –guanidino-group (–NHC(=NH))NH_2) and $–NH_2$.The functional groups may also be present in their salt form.

Compositions according to the invention comprise nanoparticles which have a surface loading density up to 0.5, preferably between 0.01 and 0.5, more preferred between 0.03 and 0.4, most preferred between 0.05 to 0.3, in relation to the total number of the pharmaceutically acceptable compounds with regard to the surface of the nanoparticle in nm^2 [molecules/nm^2].

This surface loading density is calculated by assuming a perfect sphere and by the molar loading per particle. For example: a spherical particle with a diameter of 25 nm has a surface of 1964 nm² ($A=\pi*d^2$) and a weight of 1.64E-8 ng (with an assumed density of 2000 kg/m³ for amorphous silica). The loading of such a particle with 5 % by weight with the peptide KKKV-Cit-YMLDLQPET (M=1,910.31 g/mol) equals to 4.283E-22 mol. This value multiplied by the Avogadro constant results in 258 single molecules of KKKV-Cit-YMLDLQPET attached to one particle with 1964 nm². This corresponds to a surface loading density of 0.13 molecules/nm².

The compositions according to the invention are formulated to have a pH between 6.0 and 8.0, preferably between 6.5 and 7.8 and more preferably between 6.8 and 7.5, even more preferred between 7.2 and 7.4. The pH of a composition can be maintained by the use of a buffer such as acetate, citrate, phosphate, succinate, TRIS (tris(hydroxymethyl)aminomethane) or histidine, typically employed in the range from about 1 mM to 50 mM. The pH of compositions can otherwise be adjusted by using physiologically acceptable acids or bases.

In one embodiment of the present invention the functional groups are $-PO_3H$ groups. Phosphorylated nanoparticles according to the invention could be for example prepared by reaction of (diethylphosphatoethyl)triethoxysilane with silica nanoparticles under addition of ammonia.

In a preferred embodiment of the present invention the functional groups are connected to the nanoparticle via a linker L. The linker can be connected to the nanoparticles e.g. by way of a covalent or adsorptive bond.

In a preferred embodiment of the present invention the linker compound L comprises at least one carboxyl (–COOH) or carboxylate ($-COO^-$) group as functional group.

In a more preferred embodiment such linker further comprises at least one guanidino-group ($-NHC(=NH)NH_2$ or $-NHC(=NH_2^+)NH_2$) or amino-group ($-NH_2$ or $-NH_3^+$) as functional group.

In a most preferred embodiment of the present invention the linker compound L contains at least a structural unit of the general formula (I)

$$*-(-O)_3Si-(CH_2)_n-CH(COOX)-(CH_2)_p-C(O)-NH-CH(COOX)-(CH_2)_q-Y \quad (I)$$

wherein

X is independently from each other H or a negative charge,

Y	is independently from each other –NHC(=NH)NH_2, –NHC(=NH_2^+)NH_2, –NH_2 or –NH_3^+,
n, p and q	are independently from each other 0 or a number from 1 to 25; and
*–	is the connection point to the nanoparticle.

In a preferred embodiment the linker compounds L contain at least a structural unit of formula (I) wherein n is 3, p is 1 and q is 4 and Y is independently from each other a –NH_2 or –NH_3^+ group.

In a particularly preferred embodiment the linker compounds L contain at least a structural unit of formula (I) wherein n is 3, p is 1 and q is 3 and Y is independently from each other a –NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2 group.

Another aspect of the present invention is to provide a process of preparation of nanoparticles according to the invention wherein

- in a first step (i) hydrolytic polycondensation of tetraalkoxysilanes and/or organotrialkoxysilanes in a medium which comprises water, at least one solubilizer and at least one amine or ammonia take place, where firstly a sol of primary particles is produced, and the resultant nanoparticles are subsequently brought to the desired particle size in a range from 5 to 150 nm in such a way that further nucleation is limited by continuous metering-in of corresponding silane in a controlled manner corresponding to the extent of reaction, and
- in a second step (ii) the nanoparticles from step (i) are reacted with [(3-triethoxysilyl)propyl]succinic anhydride, which in a simultaneous reaction forms an amide with L-arginine or L-lysine.

It is preferred to use in step (ii) of the process L-arginine.

In an alternative embodiment the linker compound L could be also obtained by the reaction of L-arginine with N-(3-triethoxysilylpropyl)maleimide. The reaction is carried out preferably at pH values above 8.

In one embodiment of the present invention it also possible to produce the linker compound L by reaction of [(3-triethoxysilyl)propyl]succinic anhydride with agmatine, histamine, cadaverine or spermidine.

A further embodiment of the present invention are nanoparticles comprising a silicon dioxide based surface with a linker compound L covalently or adsorptive bonded to it, wherein the Linker L contains at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or -NH_3^+) as a functional group. In a preferred

embodiment the Linker L contains at least one guanidino-group (–NHC(=NH)NH_2 or -NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or -NH_3^+) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as a functional group. In a more preferred embodiment the Linker L contains at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (-–COOH) or carboxylate (–COO^-) group as a functional group.

One embodiment of the nanoparticles according to the invention is illustrated in **Fig. 3.**

Fig. 3 shows the schematic drawing of the cross section of a nanoparticle according to the invention. A represents the surface functionalization with the linker compound L, B represents the amorphous SiO_2 shell and C represents the core material, which could be void, water or any other material as well as amorphous SiO_2. The diameter d1 is between 0 and 149 nm and d2 is between 10 and 150 nm.

In one embodiment of the invention the pharmaceutically acceptable compound is conjugated to the nanoparticle by adsorptive or covalent attachment. The adsorptive attachment is preferred.

A further object of the present invention are nanoparticles having silicon dioxide and functional groups on the surface and a particle size below 150 nm, comprising a linker L which is covalently or adsorptive bonded to it, wherein the Linker L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or –NH_3^+) group as a functional group.

A main advantage of the adsorptive binding of the pharmaceutically acceptable compound is that, in contrast to most covalent conjugations, no by-products are formed or remain in the "reaction" mixture. In case of a covalent attachment it could become necessary to chemically modify the molecule which should be attached by adding reactive functional groups to the molecule. This is a fundamental intervention in the structure of the peptide/antigen and constitutes a complex chemical modification in case of RNA- or DNA-based antigens or adjuvants. In addition, a covalent linkage of a known compound that has already been approved by the drug authorities creates a new, independent substance (New Chemical Entity, NCE) which, for regulatory reasons, requires a new approval.

In a preferred embodiment the linker compound L according to formula (I) is able to carry and/or stabilize by way of adsorption pharmaceutically acceptable compounds which have positive or negative charges.

In a particularly preferred embodiment the linker compound L according to formula (I) is able to carry and/or stabilize by way of adsorption pharmaceutically acceptable compounds which have negative charges.

In a more preferred embodiment the linker compound L according to formula (I) is able to carry and/or stabilize by way of adsorption pharmaceutically acceptable compounds which have phosphate or phosphonate groups.

It was surprisingly found that the electrostatic interaction between the arginine group of the linker compound L and the negative charge of the phosphate group of the pharmaceutically acceptable compound possesses a 'covalent-like' stability.

The term "attachment" here relates to any type of interaction between the surface functionality and the antigen, in particular covalent and non-covalent bonds, such as, for example, hydrophobic/hydrophilic interactions, van der Waals forces, ionic bonding, hydrogen bonds, ligand-receptor interactions, base pairing of nucleotides or interactions between epitope and antibody binding site.

In case where the pharmaceutically acceptable compound is hydrophobic, for example a hydrophobic peptide, it is advantageous to increase the hydrophilicity thereof. In case of a protein or peptide this is possible e.g. by an N- or C-terminal extension with polar amino acids. In a preferred embodiment of the invention the protein or peptide is extended with one or more polar amino acids selected from the group comprising aspartic acid, glutamic acid, histidine, lysine, arginine, serine, threonine or tyrosine, preferably lysine, arginine, glutamic acid and aspartic acid more preferred lysine.

The present invention is therefore particularly directed to a tripartite bioconjugate which comprises an N- or C-terminal extension with polar amino acids for enhancing the solubility and which is connected to the linker compound L by adsorptive linkage, a linker unit U and an antigen/epitope which is illustrated in **Fig. 4a**.

A linker unit U according to the invention is for example used in antibody-drug conjugates (ADCs) which contain various types of linkers. There are three main types of chemically cleavable linkers: acid cleavable, reducible disulfides and those cleavable by exogenous stimuli.

Examples of ADCs are Gemtuzumab ozogamicin (Mylotarg® by Pfizer, linker is 4-(4-acetylphenoxy)butanoic acid), Inotuzumab ozogamicin (Besponsa® by Pfizer, linker is condensation product of 4-(4'-acetylphenoxy)-butanoic acid (AcBut) and 3-methyl-3-mercaptobutane hydrazide (known as dimethylhydrazide), Trastuzumab emtansin (Kadcyla® by Roche, linker is 4-[N-Maleimidomethyl]cyclohexan-1-carboxylate) or

Brentuximab vedotin (also known as SGN-035; Adcetris® by Seattle Genetics Inc., linker is the dipeptide valine–citrulline).

In a preferred embodiment of the present invention the peptides comprise as linker unit U comprising an N-terminal extension with an enzymatic cathepsin B-cleavable linker (catB-cleav-linker) to ensure the release of the native unmodified antigen for MHC I or MHC II. The enzymatic catB-cleav-linker comprises one of the cathepsin B sensitive dipeptides Val-Cit, Phe-Cit, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Lys, Ala-Lys or Val-Lys, preferred dipeptides are Val-Cit or Trp-Cit, more preferred is Val-Cit.

In one embodiment the compositions according to the invention comprise phosphorylated nanoparticles and one or more peptides which are conjugated to the nanoparticles by adsorptive attachment, wherein the peptide comprises Val-Cit or Trp-Cit as cathepsin B sensitive dipeptide.

Fig. 4b shows a preferred tripartite bioconjugate according to the invention. The peptide used in **Fig. 4b** is an extreme hydrophobic epitope derived from human NY-ESO-1. For the N-terminal extension the enzymatic (cathepsin B) cleavable peptidic linker Val-Cit was used to ensure the release of the native unmodified antigen for MHC I or MHC II. For enhancing the solubility and the electrostatic attraction to the nanoparticle the extension part “Lys-Lys-Lys” was used. Also possible is the extension for example with Lys-Lys-Lys-Asp or Arg_6.

In a preferred embodiment of the present invention the HPV16 $E7_{82-90}$ epitope LLMGTLGIV is enlarged on the N-terminal site with the enzymatic cleavable linker Val-Cit and the cationic solubilizing sequence Lys-Lys-Lys, leading to KKKV-Cit-LLMGTLGIV.

In another preferred embodiment of the present invention the HPV16 $E7_{11-19}$ epitope YMLDLQPET is enlarged on the N-terminal site with the enzymatic cleavable linker Trp-Cit and the cationic solubilizing sequence Lys-Lys-Lys leading to KKKW-Cit-YMLDLQPET.

A further embodiment is a composition according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as functional groups and one or more peptides or antigens which are conjugated to the linker compound L by adsorptive attachment, wherein the peptide or antigen comprises a linker unit U and a hydrophilic elongation with one or more amino acids.

A preferred embodiment is a composition according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as functional groups and one or more peptides or antigens which are conjugated to the linker compound L by adsorptive attachment, wherein the peptide or antigen comprises a cathepsin B sensitive dipeptide and a hydrophilic elongation with Lys-Lys-Lys.

A more preferred embodiment is a composition according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group (-–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as functional groups and one or more peptides or antigens which are conjugated to the linker compound L by adsorptive attachment, wherein the peptide or antigen comprises Val-Cit or Trp-Cit as cathepsin B sensitive dipeptide and a hydrophilic elongation with Lys-Lys-Lys.

Another embodiment is a composition according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group –HC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as functional groups and the model peptide SIINFEKL which is conjugated to the linker compound L by adsorptive attachment, wherein the peptide comprises Val-Cit or Trp-Cit as cathepsin B sensitive dipeptide and a hydrophilic elongation with Lys-Lys-Lys.

However, this hydrophilic modification of the peptide could lead to a stronger binding to MHC I (i.e. are immunodominant), thereby displacing the target epitope and could provoke an immune response against an epitope not present on the tumor cells. To avoid this effect, there could be the need of a spacer between the hydrophilizing sub-molecule and the epitope.

An optional embodiment of the present invention is the use of so-called self-immolative spacers (PABC, p-aminobenzylcarbamate). Examples of such self-immolative spacers are compounds which comprise the structural unit of 4-aminobenzyl alcohol, 2-aminobenzyl alcohol or 4-hydroxybenzyl alcohol, 2-hydroxybenzyl alcohol (EP-A 0 648 503, WO 2007/031734 A1, WO 2015/162291 A1, US 6,180,095 B1, US 6,214,345 B1).

A self-immolative spacer is defined as a molecular section which is chemically linked to at least two further molecular sections such that when one of the bonds to the molecular sections is released, the remaining bonds are split and the previously attached molecules are released.

If a self-immolative spacer is used, it is preferred that the spacer is placed between enzymatic catB-cleav-linker and the peptide. As an example the following bioconjugate could be produced: (Arg_6)-(Val-Cit)-(PABC)-(SIINFEKL), wherein SIINFEKL is a model antigen.

It was surprisingly found that the enzymatic catB-cleav-linker systems can be used without the need of a self-immolative spacer.

The epitopes used for therapeutic vaccination typically consist of 8 to 11 amino acids (for MHC I) and a maximum of 25 amino acids for MHC II. For those epitopes there is no need for using a self-immolative spacer, because of the small size of these epitopes in comparison to much larger drugs.

The pharmaceutically acceptable compounds preferably are selected from the group consisting of proteins (such as e.g. antibodies or enzymes), peptides, nucleic acids (RNA or DNA), polynucleotides, lipids, polysaccharides or organic compounds ("small molecules", preferably such as e.g. a chemotherapeutic with a molecular weight of 50 to 1000 Da) or mixtures thereof.

Preferably the pharmaceutically acceptable compounds are selected from the group consisting of Pathogen-Associated Molecular Patterns (PAMPs), Danger-Associated Molecular Patterns (DAMPs), Defective Infectious Particles (DIPs) or antigens.

An "antigen" according to the invention is a structure, which is capable of inducing a cellular or humoral immune response. Antigens are preferably proteinogenic, i.e. they are proteins, polypeptides, peptides or fragments thereof. In principle, they can have any size, origin and molecular weight. They contain at least one antigenic determinant or an antigenic epitope.

In an alternative embodiment the antigen is a nucleic acids per se or is encoded by a nucleic acid, which, after transport into the nucleus of antigen-presenting cells, are translated into the proteinogenic antigen which is the presented to MHC molecules.

The nucleic acids can be single- and double-stranded DNA or RNA or oligonucleotides. The nucleic acids may also be a constituent of complexes with lipids, carbohydrates, proteins or peptides.

A "peptide" according to the present invention is composed of any number of amino acids of any type, preferably naturally occurring amino acids, which preferably are linked by peptide bonds. In particular, a peptide comprises at least 3 amino acids,

preferably at least 5, at least 7, at least 9, at least 12 or at least 15 amino acids. In a preferred embodiment the peptide does not exceed a length of 100 amino acids, more preferably, it does not exceed a length of 50 amino acids; even more preferably, it is not longer than 25 amino acids. In a most preferred embodiment the peptide has a length from 8 to 20 amino acids. The peptides can also exhibit posttranslational modifications, as e.g. phosphorylations, glycosylations, lipidations (like myristoylation or Palmitoylation), citrullinations, acetylations (of lysine), hydroxylations (of proline or lysine).

An “epitope” according to the invention, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. T cell epitopes are presented on the surface of an antigen-presenting cell, where they are bound to MHC molecules. In humans, professional antigen-presenting cells are specialized to present MHC class II peptides, whereas most nucleated somatic cells present MHC class I peptides. T cell epitopes presented by MHC class I molecules are typically peptides between 8 and 11 amino acids in length, whereas MHC class II molecules present longer peptides, 13-17 amino acids in length, and non-classical MHC molecules also present non-peptidic epitopes such as glycolipids.

In a more preferred embodiment of the invention the pharmaceutically acceptable compound is a compound comprising at least one Pathogen-Associated Molecular Patterns (PAMPs).

PAMPs are recognized by so-called pattern recognition receptors (PRRs) and have a function as primary sensors of microbial pathogens and initiate immune responses against them. PRRs can be found in different locations, associated to subcellular compartments, such as a membrane-bound context on cellular and endosomal membranes, within the cytosol and extracellularly, in secreted forms present in the bloodstream, lymph fluids and interstitial fluids.

There are four major sub-families of PRR, the Toll-like receptors (TLRs), the Nucleotide-binding Oligomerization Domain (NOD)-Leucine Rich Repeats (LRR)-containing receptors (NRLs), the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) or RIG-I-like helicases (RLHs) and the C-type lectin receptors (CLRs). Further PRRs are within the complement system.

According to the invention PAMPs are preferred which are recognized by TLRs. TLRs can be grouped into membrane-bound TLRs, which are expressed on the plasma membrane, membrane of endocytic vesicles or other intracellular organelles and cytosolic TLRs. TLR 4 has a unique role among the TLRs as it can trigger pathways in

different cellular locations such as the plasma membrane and intracellular compartments. TLRs recognize distinct structures on microbes, often referred to as “PAMPs” (pathogen associated molecular patterns). Ligand binding to TLRs invokes a cascade of intra-cellular signaling pathways that induce the production of factors involved in inflammation and immunity.

Table 1 shows TLR agonists, which are as PAMPs suitable according to the invention.

Table 1: TLR agonists and their PAMPs

Receptor	Pathogen Associated ligands (PAMPs)	Endogenous ligands	PAMPs (synthetic mimetics)
TLR1/2	Triacylated lipopeptides (Bacteria and Mycobacteria)*	Not known	Pam_3CK_4, CAS No.: 112208-00-1
TLR3	ssRNA virus (*WNV*), dsRNA virus (*RSV, MCMV*)	Not known	poly(I:C) CAS No.: 42424-50-0; poly A:U (Polyadenylic–polyuridylic acid (poly(A:U)) is a synthetic double stranded RNA)
TLR4	LPS (Gram-negative bacteria); F-protein (*RSV*); Mannan (*Candida*); Glycoinositolphospholipids (*Trypanosoma*); Envelope proteins (RSV and MMTV)	Hsp60, Hsp70, fibronectin domain A surfactant protein A, hyaluronan; HMGB-1	aminoakyl glucoaminide 4-phosphate (AGP) family, MPL-A (3-O-desacyl-4′-monophosphoryl lipid A); RC-529 (a synthetic lipid A mimetic), MDF2β (murine β-defensin); CFA (complete Freund adjuvant)

TLR7	Viral ssRNA (Influenza, VSV, HIV, HCV), dsRNA	Human RNA	Guanosine analogs; imidazoquinolines, (e.g. Imiquimod (Aldara®); R848 (Resiquimod®); Loxoribine
TLR8	ssRNA from RNA virus	Human RNA	Imidazoquinolines; ssPolyU (synthetic ssRNA CAS No.: 28086-43-3)
TLR9	dsDNA viruses (HSV, MCMV); Hemozoin (Plasmodium); unmethylated CpG DNA (bacteria and viruses)	Human DNA/chromatin, LL37-DNA	CpG Oligodeoxynucleotides (CpG ODN)

More preferred PAMPs according to the invention are selected of the group of TLR3, TLR7, TLR8, TLR9 agonists, most preferred are selected from the group TLR3 and TLR9.

Particularly preferred embodiments are compositions according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) and at least one carboxyl (–COOH) or carboxylate (–COO^-) group as functional groups and one or more PAMPs which are conjugated to the linker compound L by adsorptive attachment, wherein the PAMPs are selected from poly(U) or CpG ODN.

Preferred TLR3 agonists are selected from the group of poly(I:C) (dsRNA, TLR3 agonist, as well as RIG-I agonist), polyI:polyC12U (dsRNA, TLR3 agonist, trade name Ampligen®, INN: Rintatolimod), NAB2 (Nucleic acid band 2, dsRNA isolated from yeast and identified as an agonist of the pattern-recognition receptors TLR3 and MDA-5).

Preferred TLR4 agonists are selected from MPL-A (monophosphoryl lipid A, e.g. from *Salmonella minnesota* R595 or AS04 which consist of MPL-A adsorbed onto a particular form of aluminium salt) or LPS (lipopolysaccharide, e.g. from E. coli 055:B5). Also preferred TLR4 agonists are LPS mimics which are synthetic TLR-4 agonist adjuvants. Examples of peptide sequences that mimic the TLR4 ligand function include

e.g. APPHALS and QEINSSY (A. Shanmugam et al., PLoS ONE, February 2012, Vol. 7, Issue 2; e30839). By introducing of such a peptide sequence into the sequence of the active ingredient polypeptide, the polypeptide may have an adjuvant function.

Another preferred PRR-agonist is poly U (ssRNA, TLR8 agonist (human) and TLR7 agonist in mice.

According to preferred embodiments of the present invention the pharmaceutically acceptable compounds are nucleotide compounds, in particular oligonucleotide agonists including TLR7 agonists including dsRNA structures (WO 2010/105819 A1, WO 2006/063252 A2), short ssRNA TLR7 and TLR8 agonists including such described in WO 2007/031319 A2, TLR 9 agonists such as immunostimulatory CpG dinucleotides as previously described (WO 2001/022990 A2) and in particular the CpG dinucleotide ODN 2006 (Hartmann and Krieg, 2000, The Journal of Immunology, 2000, 164 944 – 952).

Preferred TLR7 agonists are characterized by a G:U base pair in the center of a double-stranded structure which is formed by 4–8 G:C base pairs in addition to the G:U base pair. Examples of suitable TLR 7 agonists are oligonucleotide agents which consist essentially of the nucleotide sequence 5'-GUCCUUCAA-S' (SEQ ID NO:1), preferably taken from the 5' end of this sequence, e.g. 5'-GUCC-3' (SEQ ID NO:2), 5'-GUCCU-3' (SEQ ED NO:3), 5'-GUCCUU-S' (SEQ ID NO:4), 5'-GUCCUUC-3' (SEQ ID NO:5), or 5'-GUCCUUCA-S' (SEQ ID NO:6) (WO 2006/063252 A2).

More preferred TLR7 agonists are described in WO 2010/105819 A1, wherein the RNA poly/oligonucleotide are characterized by a G:U wobble base pair in the center of a double-stranded structure which is formed by 4–8 G:C base pairs in addition to the G:U base pair, preferably G:U and G:C base pairs and A:U base pairs. Preferred poly/oligonucleotides have a structure defined by the following general formula (II) and (III), defining two separate RNA strands containing the cassettes:

$5'X_nG/UV_m\ 3'$ (II), $5'_oW_mU/GY_n\ 3'$ (III),

wherein G/U and U/G are selected that a wobble base pair forms,
with $2 < n \leq 12$, $2 < m \leq 12$ and $2 \leq o < 12$;
X defines any base that forms a Watson-Crick base pair with corresponding bases in Y;
V defines any base that forms a Watson-Crick base pair with corresponding bases in W in an RNA stem structure, and N is any base in a loop.

The total length of these separate RNA strands of Formula (II) and (III) is preferably 5 bases to 45 bases.

Most preferred poly/oligonucleotides having at least one stem-and-loop structure have a structure defined by the following general formula (II) and (III), defining two separate RNA strands containing the cassettes:

$5'X_nG/UV_m\ 3'$ (II), $5'_oW_mU/GY_n\ 3'$ (III),

wherein G/U and U/G are selected that a wobble base pair forms,
with $2 \leq n \leq 12$, $2 \leq m \leq 12$ and $2 \leq o \leq 12$;
with X denoting Gs or Cs that form Watson-Crick bases pairs with corresponding G and C bases in Y, and V represents Gs or Cs that form Watson-Crick base pairs with corresponding bases in W.

Repeats of the base paired cassettes (i.e. multiple G•U base pairs in double stranded structures) will have activity.

A preferred total length of these separate RNA strands is 5 bases to 45 bases.

In a specific embodiment, the single-stranded poly/oligonucleotide contains, preferably consists of, two portions, a 5' portion and a 3 " portion, wherein one of the two portions contains, preferably consists of, n U's, and the other portion contains, preferably consists of, p G's and q A's, wherein n, p and q are integers greater than zero, wherein p+q=m, wherein m is an integer up to 25, preferably equal to or greater than 11 and less than or equal to 21, and wherein n is greater than m. In one embodiment, n is less than or equal to 100. In another embodiment, n is greater than 100, preferably greater than 1000, more preferably greater than 3000, most preferably on the order of 3000-5000. In certain embodiments, n may be different in different molecules in a poly/oligonucleotide preparation of the present invention. In a preferred embodiment, p equals 1 and G is in the center of the portion containing or consisting of G and A's.

Examples of a single-stranded poly/oligonucleotide having at least one stem-and-loop structure include:

C_2UC_2Gn, wherein n is an integer equal to or greater than 4;
preferably greater than 8 to 9.
C_3UC_2Gn, wherein n is an integer greater than 6;
C_2UC_3Gn, wherein n is an integer greater than 6;
C_3UC_3Gn, wherein n is an integer greater than 7;
C_4UC_3Gn, wherein n is an integer greater than 8;
C_3UC_4Gn, wherein n is an integer greater than 8;
C_4UC_4Gn, wherein n is an integer greater than 9;

GnC_2UC_2, wherein n is an integer greater than 5;
GnC_3UC_2, wherein n is an integer greater than 6;
GnC_2UC_3, wherein n is an integer greater than 6;
GnC_3UC_3, wherein n is an integer greater than 7;
GnC_4UC_3, wherein n is an integer greater than 8;
GnC_3UC_4, wherein n is an integer greater than 8;
GnC_4UC_4, wherein n is an integer greater than 9;
A_5GAgUn_7wherein n is an integer greater than 11;
A_6GAUn, wherein n is an integer greater than 12;
A_5GA_6Un, wherein n is an integer greater than 12;
A_6GA_6Un, wherein n is an integer greater than 13;
A_7GA_6Un, wherein n is an integer greater than 14;
A_6GA_7Un, wherein n is an integer greater than 14;
A_7GA_7Un, wherein n is an integer greater than 15;
A_8GA_7Un, wherein n is an integer greater than 16;
A_7GA_8Un, wherein n is an integer greater than 16;
A_8GA_8Un, wherein n is an integer greater than 17;
A_9GA_8Un, wherein n is an integer greater than 18;
A_8GA_9Un, wherein n is an integer greater than 18;
A_9GA_9Un, wherein n is an integer greater than 19;
$A_{10}GA_9Un$, wherein n is an integer greater than 20;
$A_9GA_{10}Un$, wherein n is an integer greater than 20;
$A_{10}GA_{10}Un$, wherein n is an integer greater than 21;
$A_5GA_{10}GA_4Un$, wherein n is an integer greater than 21;
UnA_5GA_5, wherein n is an integer greater than 11;
UnA_6GA_5, wherein n is an integer greater than 12;
UnA_5GA_6, wherein n is an integer greater than 12;
UnA_6GA_6, wherein n is an integer greater than 13;
UnA_7GA_6, wherein n is an integer greater than 14;
UnA_6GA_7, wherein n is an integer greater than 14;
UnA_7GA_7, wherein n is an integer greater than 15;
UnA_8GA_7, wherein n is an integer greater than 16;
UnA_7GA_8, wherein n is an integer greater than 16;
UnA_8GA_8, wherein n is an integer greater than 17;
$UnAgGA_8$, wherein n is an integer greater than 18;
UnA_8GA_9, wherein n is an integer greater than 18;
UnA_9GA_9, wherein n is an integer greater than 19;
$UnA_{10}GA_9$, wherein n is an integer greater than 20;
UnA_9GA_{10}, wherein n is an integer greater than 20;
$UnA_{10}GA_{10}$, wherein n is an integer greater than 21;
$UnA_5GA_{10}GA_4$, wherein n is an integer greater than 21.

Preferably, n is greater than 1000, more preferably 3000, even more preferably on the order of 3000–5000 in the examples above.

TLR7-specific ligands include poly/oligonucleotides in which one strand or a portion of a strand that forms the at least one fully double-stranded section contains, preferably consists of, n G's, and the other strand or a portion of a strand that forms the same at least one fully double-stranded section contains, preferably consists of, p U's and q C's, wherein n is an integer equal to or greater than 5 and less than or equal to 9, wherein p is an integer less than or equal to n, wherein q is an integer, and wherein p+q=n.

In a further embodiment of the invention a RNA poly/oligonucleotide is provided showing selective TLR8 activity, wherein the U nucleoside in the G:U wobble base pair is adjacent on each side to G bases. The RNA poly/oligonucleotide showing selective TLR8 activity may be single stranded or partially double stranded. Preferably the RNA poly/oligonucleotide having G nucleoside in the G-U wobble base pair adjacent to C nucleoside at each side of the G:U wobble base pair, forms a hairpin structure as defined herein, wherein n is between 5 and 100, preferably 20 and 100, and. Said RNA poly/oligonucleotide shows highly selective TLR8 activity. Said RNA poly/oligonucleotide showing selective TLR8 activity, preferably has at least 2 adjacent base pairs on each side of the G:U wobble base pair. The term "selective TLR8 activity" as used herein means the RNA poly/oligonucleotide as defined above shows TLR8 activity but no TLR7 activity.

Preferred TLR9 agonists are selected from CpG ODN (unmethylated DNA with CpG rich motives, TLR9 agonist), as described in US 6,429,199 B, US 2007/0065467 A1, WO 01/22990 A2, US 2003/0100527 A1.

RNA helicases, such as RIG-I, MDA-5 and LGP-2, have been implicated in the detection of viral RNA. In contrast to the TLRs, the RNA helicases are cytosolic and are expressed in a wide spectrum of cell types, including immune cells and non-immune cells, such as fibroblasts and epithelial cells. Therefore, not only immune cells, but also non-immune cells which express one or more of the RNA helicase(s), are capable of detecting and responding to viral RNA. Both the TLR and the RNA helicase systems co-operate to optimally detect viral RNA.

According to the invention pharmaceutically acceptable compounds are RIG-I agonists such as oligonucleotides bearing free, uncapped 5' phosphate described in EP 1 920 755 A1 or EP 3581656 A1. RIG-I agonists according to the invention are selected from the group of 5'ppp-dsRNA, 3p-hpRNA, poly(I:C)/LyoVec complexes that are recognized

by RIG-I and/or MDA-5 depending of the size of poly(I:C); poly(dA:dT)/LyoVec complexes that are indirectly recognized by RIG-I. Other RIG-I agonists are 5′ triphosphate oligonucleotides with blunt end as described in WO 09/141146 A1 or RIG-I agonists based on 5' phosphate oligonucleotides as described in US 2012/0288476 A1.

Another preferred RIG-I agonist is poly(dA:dT) (dsDNA, a synthetic analog of B-DNA). Poly(dA:dT) is recognized by multiple PRRs: Sensing by cytosolic DNA sensors (CDS), including cGAS, AIM2, DAI, DDX41, IFI16, and LRRFIP1, triggers the production of type I interferons. Sensing by the cytosolic DNA sensor AIM2 triggers the formation of an inflammasome and the subsequent secretion of IL-1β and IL-18. Indirect sensing by the cytosolic RNA sensor RIG-I can occur upon the transcription of poly(dA:dT) into dsRNA with a 5'triphosphate moiety (5'ppp-dsRNA) by the RNA polymerase III. This indirect sensing by RIG-I leads to the production of type I interferons.

PRR agonists according to the invention are CDS ligands and STING ligands. Cytosolic DNA sensors (CDSs) detect damaged, mislocalized or pathogenic DNA, typically inducing type I IFN production through the TBK1-IRF3 pathway. Several CDSs have been identified, including cyclic GMP-AMP synthase (cGAS), the IFN-inducible IFI16 protein, and the helicase DDX41. Induction of type I IFNs by cytosolic DNA is mediated by the endoplasmic reticulum membrane protein STING (stimulator of IFN genes).

Suitable CDS ligands according to the invention are dsDNA-EC, G3-YSD, HSV-60 CDS Agonist, ISD, ODN TTAGGG (A151), poly(dA:dT), poly(dG:dC) or VACV-70.

Preferred CDS ligands are dsDNA from pathogens (e.g. *E. coli* K12), dsDNA parts from HIV-1, dsDNA parts from HSV-60 (herpes simplex virus), ISD interferon stimulatory DNA (e.g. from *Listeria monocytogenes*), VACV-70, a 70 bp oligonucleotide containing viral DNA motifs derived from the vaccinia virus DNA.

Another preferred CDS ligand is poly(dG:dC), poly(deoxyguanylic-deoxycytidylic) acid sodium salt. It is a repetitive synthetic double-stranded DNA sequence of poly(dG-dC)•poly(dC-dG). Intracellular poly(dG:dC) is detected by several cytosolic DNA sensors (CDS), such as cGAS, DAI, DDX41, IFI16 and LRRFIP1, triggering the production of type I interferons (IFNs). This induction of IFN appears to be mediated by the endoplasmic reticulum protein STING. Moreover, poly(dG:dC) activates the cytosolic DNA sensor AIM2 (absent in melanoma 2) to trigger inflammasome formation, leading to the secretion of the pro-inflammatory cytokines IL-1β and IL-18.

To achieve stimulation of cytosolic DNA sensors, poly(dG:dC) must be delivered into the cytoplasm, for example by using a transfection agent. Therefore the nanoparticles according to the invention may serve as a transfection agent.

Also suitable are antisense RNA oligonucleotides for immune stimulation as described in US 8,815,503 B or the MDA-5 (melanoma differentiation antigen 5) or NAB2 (Nucleic acid band 2), dsRNA isolated from yeast and identified as an agonist of the pattern-recognition receptors TLR3 and MDA-5.

STING is also a direct sensor of both bacterial and mammalian cyclic dinucleotides (CDNs), activating TBK-1/IRF-3 and NF-κB signaling pathways to induce robust type I IFNs and proinflammatory cytokines.

STING ligands according to the invention are 2'3'-cGAMP, 3'3'-cGAMP, c-di-AMP or c-di-GMP.

Preferred STING agonists are selected from the group of MK-1454 (a synthetic, cyclic dinucleotide (CDN) and agonist of stimulator of interferon genes protein), poly(dG:dC), ADU-S100 (a synthetic, cyclic dinucleotide (CDN) and agonist of stimulator of interferon genes protein), transmembrane protein 173 (TMEM173) (with potential immunomodulating and antineoplastic activities), MIW815 (upon intratumoral administration, the STING agonist MIW815 binds to STING and stimulates STING-mediated pathways).

The composition according to the invention may be used as immunostimulant. The immunostimulant according to the invention preferably contains a TLR3 agonist as pharmaceutically acceptable compound. A further object of the present invention is an immunostimulant comprising the compositions according to the invention. The immunostimulant according to the invention may be administered to humans or animals.

In one embodiment of the present invention the compositions according to the invention is preferably administered before an immunosuppressive event. In case of an administration to animals, preferably cattle, such an immunosuppressive event, for example may be a result of increased stress or fatigue in the animals, which is caused after transport or when the stable is changed. A poor stable climate, a suboptimal supply of feed or water and an insufficient supply of colostrum can also have an immunosuppressive effect. In case of an administration to humans, such an immunosuppressive event is for example an operative surgery.

Therefore, the compositions according to the invention are used for the prevention and/or treatment of Infectious Bovine Rinotracheitis (IBR) or Bovine Respiratory Disease (BRD), preferably BRD, which are often caused by a weakened immune system.

The compositions according to the invention are used for the perioperative immune stimulation in humans.

In one embodiment of the present invention the compositions according to the invention are preferably administered to the patient prior to surgery.

An immunostimulant comprises one or more adjuvants and is used in the adjuvant immunotherapy. As is well known in the art, adjuvants are agents that enhance immune responses. Adjuvants are well known in the art (e.g., see "Vaccine Design: The Subunit and Adjuvant Approach", Pharmaceutical Biotechnology, Volume 6, Eds. Powell and Newman, Plenum Press, New York and London, 1995).

Exemplary adjuvants include complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), squalene, squalane and alum (aluminum hydroxide), which are materials well known in the art, and are available commercially from several sources. In certain embodiments, aluminum or calcium salts (e.g., hydroxide or phosphate salts) may be used as adjuvants. Alum (aluminum hydroxide) has been used in many existing vaccines.

In a specially preferred embodiment of the invention the nanoparticle comprises both, i.e. a compound selected from the group consisting of Pathogen-Associated Molecular Patterns (PAMPs), Danger-Associated Molecular Patterns (DAMPs), Defective Infectious Particles (DIPs) and at least one antigen.

Most preferred is a nanoparticle according to the invention containing a compound comprising a PAMP and an antigen.

A very preferred embodiment is a composition according to the invention comprising SiO_2-nanoparticles having linker compounds L on the surface, wherein the linker compound L comprises at least one guanidino-group ($–NHC(=NH)NH_2$ or $–NHC(=NH_2^+)NH_2$) and at least one carboxyl (–COOH) or carboxylate ($–COO^-$) group as functional groups and one or more PAMPs and one or more antigens or epitopes which are conjugated to the linker compound L by adsorptive attachment, wherein the antigen or epitope comprises a linker unit U and a hydrophilic elongation with one or more amino acids. PAMPs are preferably selected from TLR3 agonists or oligonucleotide agonists including TLR7 agonists including dsRNA structures.

The disclosed compositions and methods are applicable to a wide variety of antigens. In certain embodiments, the antigen is a protein (including recombinant proteins), polypeptide, or peptide (including synthetic peptides). In certain embodiments, the antigen is a lipid or a carbohydrate (polysaccharide). In certain embodiments, the antigen is a protein extract, cell (including tumor cell), or tissue.

In specific embodiments, antigens can be selected from the group consisting of the following:
(a) polypeptides suitable to induce an immune response against cancer cells;
(b) polypeptides suitable to induce an immune response against infectious diseases;
(c) polypeptides suitable to induce an immune response against allergens; and
(d) polypeptides suitable to induce an immune response in farm animals or pets.

In some embodiments, the antigen or antigenic determinant is one that is useful for the prevention of infectious disease. Such treatment will be useful to treat a wide variety of infectious diseases affecting a wide range of hosts, preferably human, but including cow, sheep, pig, dog, cat, and other mammalian species and non-mammalian species. Thus, antigens or antigenic determinants selected for the compositions will be well known to those in the medical art.

Appropriate antigens for use with compositions according to the invention may be derived from, but not limited to, pathogenic bacterial, fungal, or viral organisms, Corona virus (SARS-CoV-2), Bovine Respiratory Syncytial Virus (BRSV), Bovine Respiratory Coronavirus (BRCV), Human Papillomavirus (HPV), Bovine Herpesvirus-1 (BHV-1), Parainfluenza-type3-Virus (PI-3V), *Pasteurella multocida, Mannheimia haemolytica, Histophilus somni, Mycoplasma bovis*.

Examples of infectious disease include, but are not limited to, viral infectious diseases, such as COVID-19, Infectious Bovine Rinotracheitis (IBR), Bovine Respiratory Disease (BRD), SARS and cervical cancer caused by HPV.

Any antigen associated with any of the diseases or conditions provided herein can be used in the compositions and methods described herein. These include antigens associated with cancer, infections or infectious disease or degenerative or non-autoimmune disease. Preferred epitopes are those which are associated with a human papillomavirus infection (such as YMLDLQPET (HPV16 $E7_{11-19}$), FAFRDLCIVY (HPV16 $E6_{52-61}$), TIHDIILECV (HPV16$E6_{29-38}$), RRFHNIAGHYR (HPV18$E6_{125-135}$), KATLQDIVLHLE (HPV18$E7_{5-16}$), HVDIRTLEDLLM (HPV16$E7_{73-84}$), LEDLLMGTL (HPV16 $E7_{79-87}$), LLMGTLGIV (HPV16 $E7_{82-90}$) the cancer antigen *New York*

esophageal squamous cell carcinoma-1 (NY-ESO-1) and neoantigens as a class of tumor-specific antigens.

In another embodiment, antigens associated with infection or infectious diseases are associated with any of the infectious agents provided herein.

In one embodiment, the infectious agent is a virus of Papillomaviridae, virus of the SARS family. In still another embodiment, the infectious agent is SARS-CoV-2 or Human papillomavirus.

In one embodiment the compositions according to the invention are used as vaccines for the personalized cancer treatment.

The compositions according to the invention are used as vaccines for the prevention of HPV or the treatment of HPV positive humans or the treatment of HPV positive tumors. A further object of the present invention is to provide a vaccine comprising compositions according to the invention.

Compositions according to the present invention are preferably stable dispersions.

The nanoparticles can be in dispersed form in any desired solvent, so long as the nanoparticles are neither chemically attacked nor physically modified by the solvent, and vice versa, so that the resultant nano-dispersion is stable, in particular pharmaceutically and physically stable. The dispersion is specifically characterized in that the nanoparticles are in monodisperse and non-aggregated form and have no tendency towards sedimentation, which results in sterile filterability.

Also object of the present invention is a lyophilisate comprising nanoparticles according to the invention. The lyophilisate may comprise additives for example polymers (e.g. polyethylene glycol, polyvinyl pyrrolidone, hydroxyethyl starch, dextran and ficoll) and sugars, (e.g. trehalose, lactose, sucrose, glucose, galactose, maltose, mannose and fructose), polyhydroxy alcohols (e.g. mannitol, sorbitol and inositol), amino acids (e.g. glycine, alanine, proline and lysine) and methylamines (e.g. trimethylamine-N- oxide, betaine and sarcosine). Lyophilisates according to the invention could be resuspended with sterile water before vaccination.

A pharmaceutical composition according to the present invention is any composition which can be employed in the prophylaxis, therapy, control or post-treatment of patients who exhibit, at least temporarily, a pathogenic modification of the overall condition or the condition of individual parts of the patient organism, in particular as a consequence of infectious diseases, septic shock, tumors, cancer, autoimmune

diseases, allergies and chronic or acute inflammation processes. Thus, in particular, it is possible for the pharmaceutical composition in the sense of the invention to be a vaccine and/or an immunotherapeutic agent.

The compositions according to the invention may be formulated as pharmaceutical compositions that may be in the forms of solid or liquid compositions. Such compositions will generally comprise a carrier of some sort, for example a solid carrier such as gelatin or an adjuvant or an inert diluent, or a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution or glycerol or glycols such as propylene glycol or polyethylene glycol may be included.

The compositions according to the present invention optionally may comprise other active ingredients or may comprise one or more of a pharmaceutically acceptable excipient, carrier, buffer, stabilizer, isotonic agent, preservative or anti-oxidant or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the pharmaceutically acceptable compound. The precise nature of the carrier or other material may depend on the route of administration, e.g. orally or parenterally.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the composition according to the present invention will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability.

Preservatives are generally included in compositions according to the invention to retard microbial growth, extending the shelf life of the compositions and allowing multiple use packaging. Examples of preservatives include phenol, meta-cresol, benzyl alcohol, para-hydroxybenzoic acid and its esters, methyl paraben, propyl paraben, benzalkonium chloride, 1-thioglycerol and benzethonium chloride.

The nanoparticle-containing compositions of the invention may be administered to patients by any number of different routes, including enteral or parenteral routes. Parenteral administration of the pharmaceutical composition is preferred. Parenteral administration includes administration by the following routes: intravenous, cutaneous or subcutaneous, nasal, vaginal, rectal, intramuscular, intraocular, transepithelial, intraperitoneal, intracardiac, intraosseous, intradermal, intrathecal, intraperitoneal, transdermal, transmucosal, and inhalational and topical (including dermal, ocular, rectal, nasal, inhalation and aerosol), and rectal systemic routes. In a preferred embodiment, the pharmaceutical composition is for local (e.g., mucosa, skin) applications.

Administration be performed e.g. by injection, or ballistically using a delivery gun to accelerate their transdermal passage through the outer layer of the epidermis. The nanoparticles can then be taken up, e.g. by dendritic cells, which mature as they migrate through the lymphatic system, resulting in modulation of the immune response and vaccination against the epitopic peptide and/or the antigen from which the epitopic peptide was derived or of which it forms a part. The nanoparticles may also be delivered in aerosols. This is made possible by the small size of the nanoparticles.

Particularly preferred is the injection an intradermal, subcutaneous, intramuscular or intravenous injection. The administration can be carried out, for example, with the aid of so-called vaccination guns or by means of syringes. Mucosal administration, in particular mucosal vaccination, can be beneficial for cases were pathogens enter the body via the mucosal route. It is also possible to prepare the substance as an aerosol, which is inhaled by the organism, preferably a human patient and taken up by the nasal and or bronchial mucosa. Other possible forms of mucosal administration are vaginal and rectal suppositories. These forms of administration are cost-effective (no consumables like syringes and needles), safe (very low risk of infection and operating errors), easy to apply and comprised with a high patients compliance (no needlefear). It also addresses the mucosal immune system (MALT) directly.

In one embodiment of the present invention the vaccines comprising the compositions according to the invention are used for a mucosal administration.

The exceptionally small size of the nanoparticles of the present invention is a great advantage for delivery to cells and tissues, as they can be taken up by cells even when linked to targeting or therapeutic molecules. Thus, the nanoparticles may be internalized by APCs, the epitopic peptides processed and presented via class I MHC and class II MHC.

PARTICULARLY PREFERRED EMBODIMENTS OF THE INVENTION

1. Composition comprising nanoparticles which are loaded with pharmaceutically acceptable compounds, having silicon dioxide and functional groups on the surface, wherein
   - the functional groups are capable to carry and/or stabilize both negative and positive charges of the pharmaceutically acceptable compounds,
   - the zeta potential of the composition has a value of at least ± 15 mV,
   - the nanoparticles have a particle size below 150 nm, preferably 100 nm or less.

2. Composition according to embodiment 1, wherein the nanoparticles have a surface loading density up to 0.5, preferably between 0.01 and 0.5, more preferred between 0.03 and 0.4, most preferred between 0.05 to 0.3, in relation to the total number of the pharmaceutically acceptable compounds with regard to the surface of the nanoparticle in nm^2 [molecules/nm^2].

3. Composition according to embodiment 1 or 2, wherein the Polydispersity Index (PDI) of the composition is between 0 and 0.32, preferably between 0.1 and 0.3, more preferably between 0.1 and 0.2, most preferred less than 0.1.

4. Composition according to any of embodiments 1 to 3, wherein the pH value of the composition is between 6.0 and 8.0.

5. Composition according to any of the preceding embodiments, wherein the zeta potential of the composition has a value of at least ± 30 mV.

6. Composition according to any of the preceding embodiments, wherein the Z-average diameter of the nanoparticles is in the range of ≥5 and <150 nm, preferably ≥15 and ≤60nm, more preferably ≥20 and ≤50nm and still more preferably between 20 and 30nm.

7. Composition according to any of the preceding embodiments, wherein the net charge of the loaded nanoparticles is in total different to zero.

8. Composition according to any of the preceding embodiments, wherein the functional groups are connected to a linker L which is linked to the surface of the nanoparticles by way of a covalent or adsorptive bond.

9. Composition according to embodiment 8, wherein the linker compound L comprises at least one carboxyl (-–COOH) or carboxylate (–COO^-) group as functional group.

10. Composition according to embodiment 8 or 9, wherein the linker compound L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or –NH_3^+) group as a functional group.

11. Composition according to any of embodiments 8 to 10, wherein the linker L comprises at least one further functional group L', which is selected from the group –SH, –COOH, –NH_2, –guanidino-group (-NHC(=NH))NH_2), –PO_3H_2, –PO_2CH_3H, –SO_3H, –OH, –$NR_3^+X^-$.

12. Composition according to any of embodiments 8 to 11, wherein the linker compound L contains at least a structural unit of formula (I)

*– (–O)3Si-(CH2)n-CH(COOX)-(CH2)p-C(O)-NH-CH(COOX)-(CH2)q-Y (I)

wherein

X	is independently from each other H or a negative charge,
Y	is independently from each other –NHC(=NH)NH_2, –NHC(=NH_2^+)NH_2, –NH_2 or –NH_3^+,
n, p and q	are independently from each other 0 or a number from 1 to 25; and
*–	is the connection point to the nanoparticle.

13. Composition according to any of the preceding embodiments, wherein the pharmaceutically acceptable compound is conjugated to the nanoparticle by adsorptive attachment.

14. Composition according to any of the preceding embodiments, wherein the pharmaceutically acceptable compound is selected from the group comprising proteins, peptides, polynucleotides, oligonucleotides (RNA, DNA, single stranded or double stranded), nucleic acids or peptide antigens.

15. Composition according to any of the preceding embodiments, wherein the pharmaceutically acceptable compound is selected from the group comprising Pathogen-Associated Molecular Patterns (PAMPs), Danger-Associated Molecular Patterns (DAMPs), Defective Infectious Particles (DIPs) or epitopes.

16. Composition according to embodiment 15, wherein PAMPs contain at least one phosphate group.

17. Composition according to embodiment 15 or 16, wherein PAMPs are Pattern-Recognition-Receptor (PRR)-agonists.

18. Composition according to any of embodiments 15 to 17, wherein PAMPs are selected from the group of TLR agonists, Retinoic Acid Inducible Gene I (RIG-I) Ligands, Melanoma-differentiation-associated gene 5 (MDA-5) Ligands, Cytosolic DNA Sensors (CDS) Ligands, STING Ligands (Cyclic Dinucleotide (CDNs)).

19. Composition according to any of embodiments 15 to 18, wherein PAMPs are selected from the group of 5'-Triphosphate-RNA, Cytosolic DNA Sensors (CDS), 2'3'-cGAMP, 3'3'-cGAMP, c-di-AMP (Bis-(3'-5')-cyclic dimeric adenosine monophosphate), c-di-GMP (Bis-(3'-5')-cyclic dimeric guanosine monophosphate, double-stranded (ds)RNA.

20. Composition according to embodiment 18 wherein the TLR agonists are selected from the group of TLR1/2, TLR3, TLR4, TLR7, TLR8 and TLR9 agonists.

21. Composition according to embodiment 20, wherein the TLR agonists are lipopolysaccharides (LPS), Monophosphoryl Lipid A (MPL-A), Poly(U), CpG Oligodeoxynucleotides (ODN) (unmethylated DNA), Pam3CSK4 (synthetic triacylated lipopeptide).

22. Composition according to embodiment 14, wherein the peptides comprise 8 to 100 amino acids.

23. Composition according to embodiment 22, wherein the peptides are MHC Class I epitopes or MHC Class II epitopes.

24. Composition according to embodiments 22 or 23, wherein the peptide is N- or C-terminally extended with polar amino acids.

25. Composition according to embodiment 24, wherein the polar amino acids are selected from the group aspartic acid, glutamic acid, histidine, lysine, arginine, serine, threonine, tyrosine.

26. Composition according to any of embodiments 22 to 25, wherein the peptides comprise an N-terminal extension with an enzymatic cathepsin B-cleavable linker.

27. Composition according to embodiment 26, wherein the enzymatic cleavable linker comprises one of the cathepsin B sensitive dipeptides Val-Cit, Phe-Cit, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Lys, Ala- Lys or Val-Lys.

28. Composition according to embodiments 26 or 27, wherein a spacer is placed between enzymatic cleavable linker and peptide.

29. Composition according to embodiment 28, wherein the spacer is a self-immolative spacer.

30. Composition according to embodiment 28 or 29, wherein the spacer comprises the structural unit of 4-aminobenzyl alcohol, 2-aminobenzyl alcohol or 4-hydroxybenzyl alcohol, 2-hydroxybenzyl alcohol.

31. Composition according to any of the preceding embodiments, wherein the nanoparticles comprise at least silicon dioxide (SiO_2), optional in mixture with another material.

32. Composition according to any of the preceding embodiments, wherein the nanoparticles consist of SiO_2.

33. Composition according to any of the preceding embodiments, wherein the compositions are stable dispersions.

34. Composition according to any of the preceding embodiments, wherein the pH value of the composition is between 7.2 and 7.4.

35. Composition according to any of the preceding embodiments, wherein the composition is isotonic.

36. Composition according to any of the preceding embodiments, wherein the composition contains further pharmaceutically acceptable additives.

37. Composition according to embodiment 1 for use as vaccines.

38. Composition according to embodiment 1 for use as imunostimulant.

39. Composition according to embodiment 38 for use for the activation of the immune system and as adjuvant.

40. Composition according to embodiment 38 for the use of the prevention and/or treatment of bovine respiratory disease (BRD).

41. Compositions according to embodiment 38 for perioperative immune stimulation in humans.

42. Composition according to embodiment 1 for the personalized cancer treatment.

43. Composition according to embodiment 37 for use as vaccines for the prevention of HPV or the treatment of HPV positive humans or the treatment of HPV positive tumors.

44. Nanoparticles having silicon dioxide and functional groups on the surface and a particle size below 150 nm, comprising a linker L which is covalently or adsorptive bonded to it, wherein the Linker L comprises at least one guanidino-group (-NHC(=NH)NH_2 or -NHC(=NH_2^+)NH_2) or amino-group (-NH_2 or -NH_3^+) group as a functional group.

45. Lyophilisate comprising nanoparticles according to embodiment 44.

46. Vaccine comprising compositions according to embodiment 1.

47. Immunostimulant comprising compositions according to embodiment 1.

EXAMPLES

EXAMPLE 1

Preparation of monodisperse Silicon Dioxide Nanoparticles with 25 nm Diameter

500 mL ethanol absolute were taken from a 500 mL graduated measuring cylinder into a 1000 mL glass bottle with screw cap. 358 mL sterile DI water was added and the mixture was well shaken.
69.6 mL tetraethylorthosilicate (TEOS) were added to the bottle. The bottle was tightly closed and well shaken. After 1 hour waiting time the clear colorless mixture got room temperature (22 °C). Then 9.5 mL ammonia water (25 %) was added quickly.
The bottle was shaken by hand very well for 10 seconds and stored at room temperature.
After 24 hours at room temperature additional 34.8 mL TEOS were added to the mixture (seed-growth process), in order to get a more narrow size distribution. After additional 24 hours at room temperature again 34.8 mL were added to the mixture (seed-growth process continued). After 24 hours at room temperature 50 µL of the reaction mixture were placed in a one-way PMMA cuvette (10 mm width) and diluted with 1.5 mL DI water (0.2 µm filtered). The cuvette was placed in a ZetaSizer Nano for particle size measurement (ZetaSizer Nano ZS, Malvern Instruments, UK).

Applied parameters:

Measurement Angle:	173° Backscatter (NIBS default)
Measurement Cell:	DTS1070 (used for particle size and zeta potential)
Refractive Index SiO2:	1.460
Absorption:	0.010
Dispersant:	water
Refractive Index:	1.330
Viscosity:	0.8872 cP (= sample viscosity)
Temperature:	25 °C

>10 measurement runs for particle size

Results:

Z-average (volume based):	23.86 nm
PDI:	0.108
Intensity Peak:	26.77 nm
pH:	11.0 (freshly calibrated combination electrode)

The nanoparticle suspension was transferred into a 2 liter round bottom flask and the ethanol as well as the ammonia gas was removed by a rotary evaporator with heated water bath. 400 mL sterile DI water was added in portions. The volume was reduced down to 198.15 g.

The solid content (pure nano dispersed silicon dioxide) of the suspension was determined in triplicate via vaporization of 250 µL and weigh out the residue.

Result:

Solid content: 163.3 mg/mL

pH: 8.0 (freshly calibrated combination electrode)

The distillation residue was diluted with 264.5 mL sterile DI water, in order to get a solid content of 70.0 mg/mL.

EXAMPLE 2

Functionalization of the Nanoparticles with L-arginine to get Arginylated Silica Nanoparticles (SiO_2-Arg)

462.65 g of the nanoparticle suspension obtained in Example 1 were placed into a 500 mL glass bottle. A magnetic stir was added and the bottle was placed onto a magnetic stirrer. 17.35 g L(+)-Arginine (CAS No. 74-79-3, PanReac AppliChem, Ph. Eur., USP. product code A1345.0500) were added to the nanoparticle suspension. When the arginine was completely dissolved a pH value of 10.8 was obtained, then 5.34 mL 3-(Triethoxysilyl)propylsuccinic anhydride (CAS No. 93642-68-3, Gelest Corp. product code: SIT8192.6) were slowly added at room temperature. The reaction mixture got very turbid, but cleared up within about 30 minutes. During this time two reactions took place simultaneously:

The ethoxy groups of the silane got hydrolyzed, due to the high pH value. The created silanol groups precipitated onto the silica nanoparticles building a surface coating on top of the nanoparticles. In parallel the amino group of the excess arginine reacts with the succininc anhydride group, forming an amide bond.

The pH value dropped to 9.8, due to the consumption of arginine and mainly due to the formation of a carboxylic group, by opening the cyclic anhydride.

After 24 hours of stirring the suspension was transferred into 20 falcons tubes with a 100 kDa membrane (Pall Corp. Macrosep® Advance, product code MAP100C38). The nanoparticle suspension was centrifuged for 10 minutes at 4,000 rpm (= 2,737 g at the used centrifuge) to a volume at least 5 less than the starting volume. After centrifugation the volume in the falcon tubes was restored with sterile DI water and the centrifugation was repeated 5 times. The centrifugation step is necessary to remove excess arginine and possible unbound reaction products.

After centrifugation the solid content of the collected supernatants was determined in triplicate. The method was the same as in Example 1.

Result:

Yield: 178.3 g
Solid content: 97.16 mg/mL

The nanoparticle suspension was diluted with 168.17 mL sterile DI water to get a final concentration of 50.0 mg/mL.

EXAMPLE 3

Synthesis of Phosphorylated Silica Nanoparticles with 25 nm Diameter

200 mL ethanol absolute were taken from a 250 mL graduated measuring cylinder into a 500 mL pressure-resistant glass bottle with screw cap 143.5 mL sterile DI water were added and the mixture was well shaken. 27.85 mL tetraethylorthosilicate (TEOS) were added to the bottle. The bottle was tightly closed and well shaken.
After 1 hour waiting time the clear colorless mixture got room temperature (22 °C). Then 3.95 mL ammonia water (25 %) was added quickly. The bottle was shaken by hand very well for 10 seconds and stored at room temperature. After 24 hours at room temperature additional 1.5 mL (Diethylphosphatoethyl)triethoxysilane (CAS No. 757-44-8, Gelest Corp. product code SID3412.0) were added to the mixture at room temperature. 10 mL ammonia water (25 %) was added too and the reaction mixture was placed in a water bath at 85 °C for 24 hours.
At this temperature two reactions took place in parallel: The ethoxy groups of the silane got hydrolyzed, due to the high pH value. The silanol groups precipitated onto the silica nanoparticles building a surface coating on top of the nanoparticles. In parallel, but much slower, the phosphoric acid ester got hydrolyzed to yield into the corresponding acid with ammonia as the counter ion. 50 µL of the reaction mixture were placed in a one-way PMMA cuvette (10 mm width) and diluted with 1.5 mL DI water (0.2 µm filtered). The cuvette was placed in a ZetaSizer Nano for particle size measurement.

Results:

Z-average (volume based): 21.3 nm
PDI: 0.144
Intensity Peak: 27.7 nm

The nanoparticle suspension was transferred into a 500 mL round bottom flask and the ethanol as well as the ammonia gas was removed by a rotary evaporator with heated

water bath. 200 mL sterile DI water was added in portions. The volume was reduced down to 120 mL. This volume was placed into 6 falcon tubes with a 100 kDa membrane (Pall Corp. Macrosep® Advance, product code MAP100C38). The nanoparticle suspension was centrifuged for 10 minutes at 4,000 rpm (= 2,737 g at the used centrifuge) to a volume at least 80 less than the starting volume. After centrifugation the volume in the falcon tubes was restored with sterile DI water and the centrifugation was repeated 5 times. The centrifugation step is necessary to remove excess possible unbound reaction products. After centrifugation the solid content of the collected supernatants was determined in triplicate. The method was the same as in Example 1.

Result:

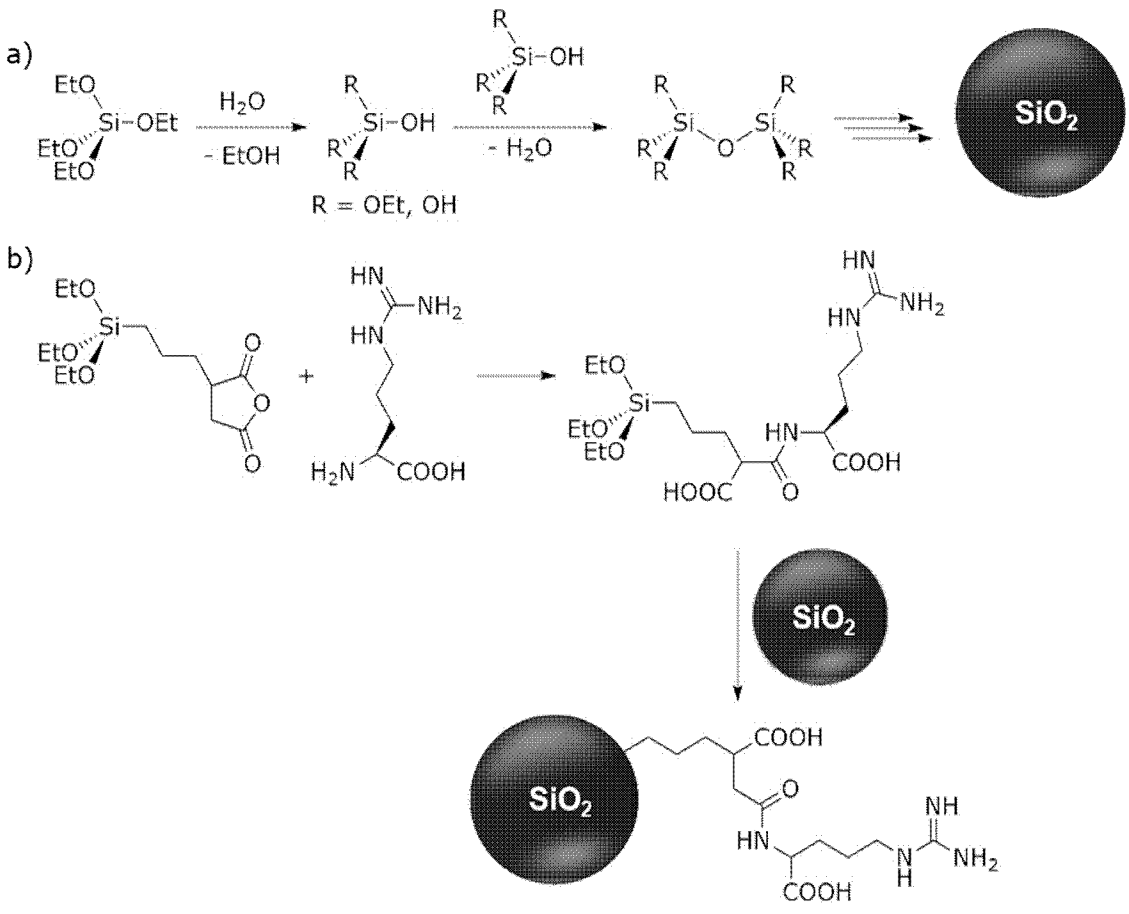
Yield: 83.3 g

Solid content: 104.0 mg/mL

The nanoparticle suspension was diluted with 86.6 mL sterile DI water to get a final concentration of 50.0 mg/mL.

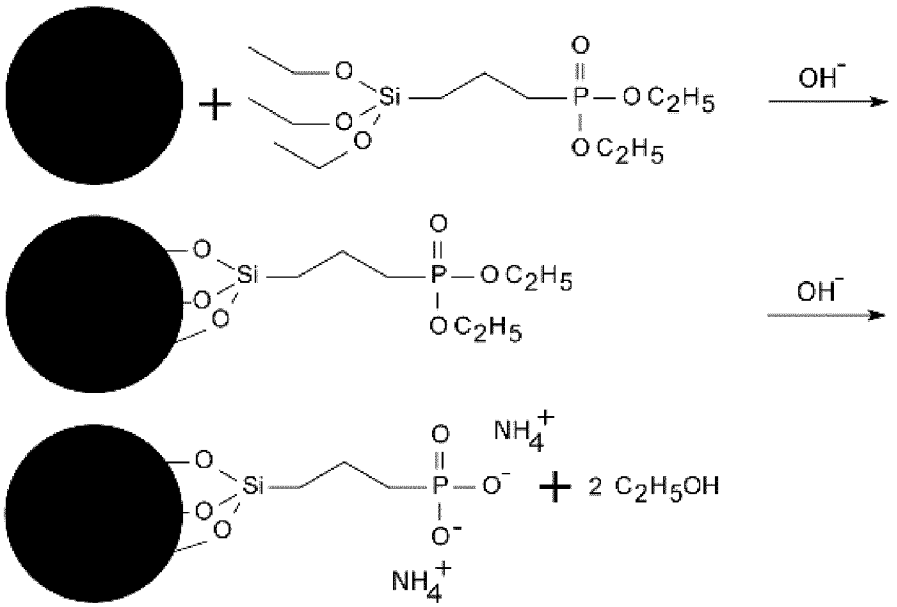
<u>Reaction scheme 1:</u>

Example 1 = a) and Example 2 = b)



Reaction scheme 2:

Example 3



EXAMPLE 4

Preparation of poly(I:C)@ SiO_2-Arg by adsorptive binding of poly(I:C)

A mixture of 200 mL ethanol, 143.5 mL sterile de-ionized water and 27.85 mL tetraethyl orthosilicate (TEOS) was prepared. 3.70 mL ammonia, 25 % (NH_3 in water) were added at room temperature. The reaction mixture was mixed vigorously by shaking for 10 seconds and left at room temperature for 24 hours without stirring. The next day another portion of 27.85 mL tetraethyl orthosilicate (TEOS) was added to the mixture. From the resulting mixture 50 µL were removed and measured by means of dynamic light scattering (DLS) on a ZetaSizer Nano ZS (Malvern). The following results were obtained:

Z-average:	25.53 nm
mean:	20.36 nm
polydispersity index (PDI):	0.136

A portion of 100 ml of the silicon dioxide particles produced before were subsequently concentrated to a volume of about 30 ml on a rotary evaporator and filled up again to 100 ml. This procedure was repeated three times to remove the ethanol and ammonia

from the reaction solution. The resulting suspension was washed five times over a 100 kDa membrane with sterile deionized water. After the last washing step, the solids content of the suspension was determined gravimetrically with 7.9% SiO_2 and the suspension was adjusted to a solids content of 5.0% by adding the calculated amount of water.

500 mg L-arginine (CAS number 74-79-3; company abcr GmbH, Germany) (= 2.87 mmol) was added to 15 ml of the silicon dioxide particles produced in Example 1 (750 mg SiO_2) and stirred while the arginine was completely dissolved. A clear particle suspension was obtained and to this suspension 127 µL (3-triethoxysilylpropyl) succinic anhydride (CAS number: 93642-68-3) (= 0.45 mmol) were slowly added while stirring at room temperature.

2.00 ml of the "argininylated" silica nanoparticles obtained above were mixed with 3.00 ml of a low molecular weight solution of poly(I:C) (poly(I:C)-LMW from InvivoGen, Toulouse, France with a size of 0.2 to 1 kb, CAS number 31852-29-6) with a concentration of 0.833 mg poly(I:C)/mL. After thoroughly mixing with a vortex mixer for 15 seconds and after one hour, the clear suspension was mixed with 250 mg of glucose. The formulation comprises:

100 mg	"argininylated" silica nanoparticles, SiO_2-Arg (c=20 mg/mL)
2.5 mg	poly(I:C)-LMW (c = 0,5 mg/mL)
250 mg	glucose (c = 50 mg/mL) (for isotonization of the formulation)

The mixture was then filled into three sterile 2.0 mL HDPE vials using a 0.2 µm sterile filter. The obtained suspension is clear and completely transparent.
The poly(I:C) loaded particles pass easily a sterile filter. Two types of filters were used: Pall Life Sciences, Acrodisc Supor® Membrane (low protein binding) 0.2 µm, cat. no. PN4602 and VWR 0.2 µm Cellulose Acetate Membrane 0.2 µm, cat. no. 514-0061.

Even after adding larger amounts of poly(I:C), the suspension remains clear and completely transparent.

EXAMPLE 5

Peptide Loading Capacity of SiO_2-Arg Nanoparticles

In a peptide loading experiment 100 µL silica nanoparticles with a diameter of 25 nm (Z-average), a solid content of 20 mg/mL and an arginylated surface were loaded with different amounts of the model peptide KKKW-Cit-SIINFEKL. To simulate physiological

conditions sodium chloride (NaCl) was added to get an isotonic suspension (0.9 % NaCl). Up to 10 % of peptide was added to the nanoparticles. KKKW-Cit-SIINFEKL has an iso-electric point of pH 10.24, indicating cationic properties determined by 4 basic amino acids (Lysine) and 1 acidic amino acid (Glutamic acid).

50 µL of the corresponding particle-peptide-NaCl mixture was diluted with 1.5 mL sterile filtered de-ionized water and measured via dynamic light scattering (DLS) in a ZetaSizer Nano (Malvern Instruments, UK). For every sample # 1 to 10 100 µL SiO_2-Arg stock solution was used. The obtained results are listed in Table 2.

Table 2: Results of the dynamic light scattering

#	[mg/mL] SiO_2-Arg	[mg] peptide	[%] peptide	[µL] Peptide stock[2]	[mg] NaCl	[µL] NaCl stock[3]	z average [nm]	PDI
0	20	0.00	0.00	0.0	0.90	9.00	23.62	0.07
1	20	0.02	1.00	5.1	0.95	9.45	24.00	0.10
2	20	0.04	2.00	10.2	0.99	9.92	24.48	0.10
3	20	0.06	3.00	15.5	1.04	10.39	26.03	0.14
4	20	0.08	4.00	20.8	1.09	10.87	26.91	0.14
5	20	0.11	5.00	26.3	1.14	11.37	28.,70	0.18
6	20	0.13	6.00	31.9	1.19	11.87	59.09	0.27
7	20	0.15	7.00	37.6	1.24	12.39	44.58	0.20
8	20	0.17	8.00	43.5	1.29	12.91	440.5	0.58
9	20	0.20	9.00	49.5	1.35	13.45	1074	0.31
10	20	0.22	10.00	55.6	1.40	14.00	2753	0.21

[1]Stock solution SiO_2-Arg: 20 mg/mL SiO_2-Arg
[2]Stock solution Peptide: 4 mg/mL Peptide (KKKW-Cit-SIINFEKL)
[3]Stock solution NaCl: 100 mg/mL NaCl

Fig. 5a illustrates the Z average versus peptide concentration and **Fig. 5b** the PDI versus peptide concentration.

As shown in **Fig. 5a** and **5b**, up to 5 % by weight of this peptide can be added to the nanoparticles, to keep a stable suspension without agglomeration or precipitation. Compared to bare particles the diameter is increasing slightly (+5 nm) and the PDI indicates a still quite narrow particle size distribution. This optical clear suspension is still passing a sterile filter.
The peptide loaded particles pass easily a sterile filter. Two types of filters were used: Pall Life Sciences, Acrodisc Supor® Membrane (low protein binding) 0.2 µm, cat. no. PN4602 and VWR 0.2 µm Cellulose Acetate Membrane 0.2 µm, cat. no. 514-0061.

With increased peptide loading the particle size increases, indicating the adsorptive peptide binding to the silica nanoparticles surface. At higher peptide concentrations – in the above example 6 % – the nano-suspension is collapsing, indicated by clouding and a measureable increased particle size due to agglomeration. These suspensions do not pass a sterile filter and are very unfavorable for parenteral administration.

EXAMPLE 6

Poly(I:C) Loading Capacity of SiO_2-Arg Nanoparticles

In a loading experiment 50 µL silica nanoparticles with a diameter of 25 nm (Z average), a solid content of 20 mg/mL and an arginylated surface were loaded with different amounts of poly(I:C) solution in RNase/DNase-free water by Gibco™. The poly(I:C) (LMW) was obtained from Invivogen Europe (cat. no. tlrl-picw). To simulate physiological conditions sodium chloride (NaCl) was added to get an isotonic suspension (0.9 % NaCl). Up to 8 % of poly(I:C) were added to the nanoparticles. The colloid remained stable: no precipitation or clouding was observable. The particle sizes and distributions, as well as the zeta potentials confirm the visual observations.

For the Zeta potential was measured according to the Smoluchowski calculation model under the following conditions:
Measurement Temp.: 25 °C
Equilibration Time: 30 s
10 to 100 runs per measurement, 5 measurements per sample (Zeta potential = mean of 5 measurements)

Table 3: Results of the dynamic light scattering

[mg] poly(I:C)	[µL] poly(I:C) stock	Vol [µL]	[mg] NaCl	[µL] NaCl stock	Z ave [nm]	PDI	Peak [nm]	int. Peak [nm]	Zeta potential
-	50	-	0.45	4.50	33.83	0.557	12.69	*)	-
0.00 (0 %)	-	50.0	0.45	4.50	22.79	0.077	20.23	24.47	-27.4
0.01 (1 %)	4.0	54.0	0.49	4.86	22.92	0.067	21.42	24.25	-28.1
0.02 (2 %)	8.2	58.2	0.52	5.23	23.12	0.075	19.96	23.97	-28.7
0.04 (4 %)	16.7	66.7	0.60	6.00	23.35	0.075	20.04	25.23	-34.2
0.06 (6 %)	25.5	75.5	0.68	6.80	23.45	0.08	19.96	34.93	-36.2
0.09 (8 %)	34.8	84.8	0.76	7.63	23.97	0.134	**)	32.15	-37.2

*) two peaks: 27.38 nm (67.8 %) and 362.6 nm (32.2 %)

**) two peaks: 7.691 nm (22.9 %) and 18.4 nm (77.1 %)

Up to a loading of 6 % poly(I:C) LMW the measured Z average values increases due to increase of particle diameter by adsorbed poly(I:C) (**Fig. 6b**). Also the low PDI (<0.1) indicates a smooth particle loading (**Fig. 6a**). At 8 % loading two peaks (at 7.691 and 18.4 nm) appear, also a strong peak broadening of the peak in direction to lower diameter values happens and the PDI value is getting worth (**Fig. 7d**). These data indicate particle "overloading": unbound poly(I:C) generates an additional peak, resp. broadens the measurement peak.

Fig. 7a: Poly(I:C) LMW without nanoparticles

Fig. 7b: SiO_2-Arg nanoparticles without poly(I:C)

Fig. 7c: SiO_2-Arg nanoparticles loaded with 6 % poly(I:C) by weight

Fig. 7d: SiO_2-Arg nanoparticles loaded with 8 % poly(I:C) by weight

EXAMPLE 7

Loading of SiO_2-Arg Nanoparticles with Peptides and Poly(I:C)

In a peptide and poly(I:C) loading experiment 100 µL silica nanoparticles with a diameter of 25 nm (Z average), a solid content of 20 mg/mL and an arginylated surface were loaded with different amounts of the model peptide KKKW-Cit-SIINFEKL and with 50 µg poly IC (LMW) each (20 µL of a poly (I:C) stock solution, having a concentration of 2.5 mg/mL).
To simulate physiological conditions sodium chloride (NaCl) was added to get an isotonic suspension (0.9 % NaCl). Up to 4 % of peptide were added to the nanoparticles. The colloid remained stable: no precipitation or clouding was observable. The particle sizes and distributions, as well as the zeta potentials confirm the visual observations.

Table 4: Results of the dynamic light scattering

[mg] pept.*	[µL] pept.* stock	Vol [µL]	[mg] NaCl	[µL] NaCl stock	[µg] Poly(I:C)	z ave [nm]	PDI	int. Peak [nm]	Peak [nm]	Zeta po-tential
0.04 (2 %)	10.2	110.2	0.99	9.92	50	24.09	0.172	24.57	19.48	-37.1
0.06 (3 %)	15.5	115.5	1.04	10.39	50	27.87	0.279	25.7	21.31	-27.6
0.08 (4 %)	20.8	120.8	1.09	10.87	50	28.47	0.305	25.41	20.76	-25.9

*(pept. = peptide)

EXAMPLE 8

Loading of SiO_2-Arg Nanoparticles with ssRNA (poly(U))

In an ssRNA loading experiment 400 µL silica nanoparticles with a diameter of 25 nm (Z average), a solid content of 50 mg/mL and an arginylated surface (in total 20 mg silicon dioxide) were loaded with 500 µL poly(U) solution with a concentration of 1.0 mg/mL (in total 0.5 mg poly(U) and 100 µL sodium chloride (NaCl) with a concentration of 90 mg/mL was added to get an isotonic suspension (0.9 % NaCl). The loading of poly(U) on silica nanoparticles in this case is 2.44 % by weight. 50 µL of this sample were measure by DLS. The poly(U) was obtained from InvivoGen Europe, Toulouse, France (Cat. Code: tlrl-sspu).

Table 5: Results of the dynamic light scattering

[mg] poly(U)	[mg] SiO2-Arg	[mg] NaCl	[µL] total volume	Z ave [nm]	PDI	Peak [nm]	int. Peak [nm]	Zeta potential
0.5 (2 %)	20.0	90	1000	23.19	0.065	20.21	24.93	-31.6

EXAMPLE 9

Loading of SiO_2-Arg Nanoparticles with unmethylated DNA (CpG ODN)

In a DNA loading experiment 40 µL silica nanoparticles with a diameter of 25 nm (Z average), a solid content of 50 mg/mL and an arginylated surface (in total 2 mg silicon dioxide) were loaded with 50 µL ODN 2395 solution with a concentration of 1.0 mg/mL (in total 0.5 mg ODN 2395 and 10 µL sodium chloride (NaCl) with a concentration of 90 mg/mL was added to get an isotonic suspension (0.9 % NaCl). The loading of on silica nanoparticles in this case is 2.44 % by weight. 50 µL of this sample were measure by DLS.

ODN 2395 was obtained from InvivoGen, France (cat. code: tlrl-2395). It is a 22mer with the structure: 5'-tcgtcgttttcggcgc:gcgccg-3' (bases are phosphorothioate (nuclease resistant), palindrome is underlined)

Table 6: Results of the dynamic light scattering

[mg] CPG ODN	[mg] SiO2-Arg	[mg] NaCl	[µL] total volume	Z ave [nm]	PDI	Peak [nm]	int. Peak [nm]	Zeta potential
0.05 (2.44 %)	2.0	9	100	23.88	0.083	20.76	25.19	-28.5

EXAMPLE 10

Example for Solubility Enhancement of Epitopes

On the N-terminal site of the enzymatically cleaved linker the addition of polar amino acids can be used to enhance the solubility of the whole peptide. The HLA-A:02 immunogenic HPV 16 E782-90 epitope LLMGTLGIV for example is extremely non-polar and has a very bad solubility in water. The use in a human vaccine is difficult. In animal studies researchers dissolve it in DMSO. The solubility after e.g. subcutaneous injection is questionable: dilution might result in precipitation of large peptide particles, which is very unfavorable for transport to lymphnodes.
According to the invention the epitope LLMGTLGIV was enlarged on the N-terminal site with the enzymatic cleavable linker Val-Cit and the cationic solubilizing sequence Lys-Lys-Lys, leading to

KKKV-Cit-LLMGTLGIV.

The complete synthesis was done by automated microwave supported solid phase peptide synthesis (SPPS) in one run.
This peptide has an excellent solubility in water. After endosomal and/or cytosolic cleavage by cathepsin B the native HPV 16 E782-90 is released. Also the remaining KKKV-Cit is not immunogenic, due to the fact it's too short to be presented at any MHC I or MHC II.

EXAMPLE 11

Human TLR-induced Cytokines in the Primary Human Macrophage Model (THP-1)

Production of cytokines after stimulation of differentiated THP-1 cells with poly(I:C), SiO_2-Arg and poly(I:C)@ SiO_2-Arg were performed with Multi-Analyte ELISArray from Qiagen. Nanoparticle diameter is 22.1 nm (Z-average), PDI 0.08.

All experiments were performed with low molecular weight poly(I:C)-LMW from InvivoGen Europe, Toulouse, France.

The screening of cytokines showed significant production of cytokines:

Fig. 8: Cytokine expression (arbitrary units) versus different types of cytokines after 72 h stimulation

TNF-α, IL8 (CXCL8), MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), MIG (CXCL9) After 72 h stimulation with poly(I:C) @SiO_2-Arg. High cytokine release of IL8 (CXCL8), MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), MIG (CXCL9) was also noticed for SiO_2-Arg nanoparticles.

Fig. 9: Cytokine expression (arbitrary units) versus different types of cytokines after 96 h stimulation

After 96 h stimulation, the expression rate of cytokines IL1-β, IL12, IL17A, TARC, IFN-α is increased and high cytokine release of IL8 (CXCL8), MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), MIG (CXCL9) was also noticed for SiO_2-Arg nanoparticles.

To investigate whether the combination of the TLR3 agonist poly(I:C)-LMW and SiO_2-Arg is able to stimulate the cytokine release, the differentiated human macrophage-like THP-1 cells were incubated with poly(I:C)-LMW adsorptively bound to SiO_2-Arg (poly(I:C)@ SiO_2-Arg) or with the individual compounds and then subjected to cytokine-specific enzyme-linked immunosorbent assay (ELISA). The supernatant of the THP-1 cells was analyzed for interleukin 8 (IL-8) and the tumor necrosis factor α (TNF-α) at several time points (**Fig. 9**). IL-8 and TNF-α are important mediators of the innate immune system response, regulating the activity of various immune cells. The release of these two cytokines is proof of a successful stimulation of the immune system.

Fig. 10a and 10b: Cytokine release at different time points after stimulation of differentiated THP-1 cells with poly(I:C) [12.5 µg/ml], SiO_2-Arg [0.5 mg/ml] or the novel adjuvant (poly(I:C) [12.5 µg/ml] bounded on SiO_2-Arg [0.5 mg/ml]).

Fig. 10a: Quantification of IL-8 release was performed using ELISA MAXTMDeluxe Set Human IL-8 from BioLegend, USA.

Fig. 10b: Quantification of TNF-α release was performed using ELISA MAXTMDeluxe Set Human TNF-α from BioLegend, USA.

EXAMPLE 12

Experiments in the influenza A model of the mouse

For the determination of the immunostimulatory potency of TLR3 agonists, a suspension is prepared according to Example 6 was tested in an in vivo study together with other active compounds as immunostimulators with regard to its prophylactic effect against a five-fold LD50 dose of the influenza A virus PR8/34.

The following active compounds were tested:

- Placebo: glucose solution (5 %)
- free poly(I:C) LMW: Low Molecular Weight poly(I:C) comprises short strands of inosinic acid poly(I) homopolymer annealed to strands of cytidinic acid poly(C) homopolymer. The average size of poly(I:C) LMW is from 0.2 kb to 1 kb (InvivoGen, France) in glucose solution (5 %)
- ZelNate®: FDA approved immunostimulant that aids in the prophylaxis of Bovine Respiratory Disease (BRD) due to *Mannheimia haemolytica* (Bayer AG, Germany)
- Poly(I:C)@SiO_2-arginylated prepared according to Example 6
- Silicon nanoparticles unmodified with a diameter of 25 nm in 5 % glucose solution

Table 7: Overview of prophylaxis trials in mice, every group consists of ten mice

Group No.	active compound	composition	H1N1-dose; admin.
A	Placebo	-	50 $TCID_{50}$ (5x LD_{50}); i.n.
B	Zelnate®	100 µL	50 $TCID_{50}$ (5x LD_{50}); i.n.
C	poly(I:C) (LMW)	50 µg in 100 µL	50 $TCID_{50}$ (5x LD_{50}); i.n.
D	SiO_2 (25nm)	2mg SiO_2; (unmodified)	50 $TCID_{50}$ (5x LD_{50}); i.n.
E	poly(I:C)@SiO_2-Arg (25 nm)	2 mg SiO_2-"argynilated" and 50 µg poly(I:C) (LMW) in 100 µL	50 $TCID_{50}$ (5x LD_{50}); i.n.

In all cases the injection volume was 100 µL.

Each animal group (A to E), consisting of ten C57BL/6 mice, was treated 24 hours before administration of the influenza A virus subcutaneously with the respective active compound or placebo. The virus was administered intranasally.

In this study the body weight was used as a reliable and easy-to-measure marker for the animal health. Sick animals eat less and lose weight very quickly. For ethical reasons, the study defined a body weight loss of 25%, based on the body weight on the day of the virus administration, as the termination criterion. In contrast, in many publications from older studies, the "termination criterion" is the death of the animals due to the viral disease.

In this study, the formulation of poly(I:C) prepared according to Example 6, which is adsorptively bound to silica nanoparticles with an “argininylated” surface, showed a surprisingly strong immunostimulatory effect, which resulted in a statistically significantly longer survival rate of the animals of **group E** and to a significantly less weight loss.

The free poly(I:C), which is not bound to nanoparticles (**group C**), with the same concentration of active ingredient, does not show any statistically significant improvement compared to the placebo group (**group A**). The unmodified silica nanoparticles (**group D**) also showed no statistically significant improvement.

From the comparative experiments above, it could be shown that a TLR3 agonist which is adsorptively bound to silica nanoparticles (**group E**), is clearly superior over the free TLR3 agonist **(group C**) which was applied in the same concentration and under identical test conditions. The immune booster ZelNate® (**group B**) also showed no significant improvement in this study compared to the placebo group (**group A**). This also applies to the unmodified silica nanoparticles of **group D**, where no significant effect could be observed (**Fig. 11a and 11b**).

Fig. 12 shows the **clinical scores.** Data are presented as mean clinical score (maximum score = 5) ± SEM (n=10) in relation to days after challenge.

EXAMPLE 13

Generation of HPV16 $E7_{11-19}$ specific CD8 T cell immune response in tumor-free A2.DR1 mice

The immunogenicity of human HPV16 E6/E7-derived, HLA-A2-binding epitopes can only be studied in genetically modified mice. Therefore the in vivo studies were performed using the HLA-humanized A2.DR1 BL6 mice. A2.DR1 mice are a highly sophisticated mouse model since they underwent a multitude of genetic alterations to exhibit the HLA-A2+/HLA-DR1+, H-2-phenotype and shown to assemble functional

$CD4^+$ and $CD8^+$ T cell responses against multiple epitopes restricted by HLA-A2 and HLA-DR1 (*Kruse, S. Therapeutic vaccination against HPV-positive tumors in a MHC-humanized mouse model, Ruperto Carola University Heidelberg, 2019, Dissertation*; *Kruse et al., Therapeutic vaccination using minimal HPV16 epitopes in a novel MHC-humanized, Oncoimmunology, 2019, Vol. 8, 1, p. e1524694*).

The efficacy of various formulations was examined in order to their ability to induce high frequencies of epitope-specific $CD8^+$ T cells after three weekly, *subcutaneous* immunizations (Day 0: Prime, Day 7: Boost, Day 14: Boost, Day 21: Spleen Sampling).

Used substances:
- poly(I:C)-HMW: The average size of poly(I:C)-HMW is 1.5 to 8 kb, InvivoGen, France
- SiO_2-PO_3H_2 according to Example 3

- *Preparation of KKKW-Cit-$E7_{11-19}$ + poly(I:C)-HMW @ SiO_2-Arg:*

2.72 mg (1.48 µmol) KKKW-Cit-$E7_{11-19}$ are dissolved in 592 µL DNase/RNase free distilled water under sterile conditions. The solution is added under vortexing to 2.37 ml of a 50 mg/mL stock solution of SiO_2-Arg. 296 mg solid glucose are added and the solution is mixed until the solid has completely dissolved. Finally, 2.96 mL poly(I:C)-HMW is added as a 1.0 mg/mL stock solution while the sample is vortexed. The vaccine is filtered through a 0.45 µm filter. 1.8 mL each are filled into 3 separate vials (1 vial per immunization) and stored at 4°C.

The influence of the N-terminal peptide elongation of the HPV16 $E7_{11-19}$ (=YMLDLQPET in one letter code for amino acids) as well as the influence of the nanoparticles surface modification were investigated. To evaluate the vaccine-induced HPV16 $E7_{11-19}$-specific $CD8^+$ T cells, the ex vivo restimulated splenocytes were assessed in an IFN-γ intracellular cytokine staining (ICS) followed by flow cytometry (**Fig. 13**).

Fig. 13:
Y-axis:
HPV-001: RW-Cit-$E7_{11-19}$ [50nmol] + poly(I:C)-HMW [50 µg]
HPV-002: RW-Cit-$E7_{11-19}$ [50nmol] + poly(I:C)-HMW [50 µg] @ SiO_2-Arg [2.25 mg]
HPV-003: RW-Cit-$E7_{11-19}$ [50nmol] + poly(I:C)-HMW [50 µg] @ SiO_2-PO_3H_2 [2.25 mg]
HPV-004: KKKW-Cit-$E7_{11-19}$ [50nmol] + poly(I:C)-HMW [50 µg] @ SiO_2-Arg [2.25 mg]

X-axis:
% of Splenic IFN-γ^+/$CD8^+$ T cells

Frequency of IFN-γ positive $E7_{11-19}$ specific $CD8^+$ T cells after ex vivo stimulation of splenocytes with $E7_{11-19}$ in the presence of Golgi apparatus-transport-inhibitors. After subsequent IFN-γ ICS, IFN-γ positive $E7_{11-19}$ specific T cells were determined by flow cytometry. Data are represented as the mean +/- SEM. Each dot represents one mouse.

As shown in **Fig. 13**, three immunizations with nanoparticles conjugated to epitope were able to induce antigen-specific splenic IFN-γ^+ $CD8^+$ T cells at higher frequencies in comparison to epitope injections. Furthermore, the direct comparison of two surface modifications of nanoparticles (HPV-002 vs. HPV-003) has demonstrated, the better performance when the epitope was conjugated to SiO_2-Arg (HPV-002).

Moreover, the influence of the N-terminal peptide elongation (HPV-003 vs. HPV-004) was analyzed, where the "KKKW-Cit-"modification (HPV-004) led to the higher frequencies of epitope-specific $CD8^+$ T cells.

This experiment successfully demonstrated the ability of the HPV16 vaccines according to the invention to induce a high frequency of epitope specific $CD8^+$ T cells. In summary, SiO_2-Arg as well as the "KKKW-Cit-" peptide elongation have shown their beneficial properties over SiO_2-PO_3H_2 nanoparticles and the "RW-Cit-" peptide elongation. Therefore, the combination of both was used for therapeutic efficacy in tumor study.

EXAMPLE 14

Therapeutic efficacy in slow-growing PAP-A2-HPV16 tumor

The final study goal is the development of a therapeutic anti-HPV16 vaccine, which would be given to patients diagnosed with either a precursor lesion or an established cancer. Therefore, the ability of the novel vaccines was tested to induce control of tumor growth in a therapeutic vaccination experiment.

Immunization schedule for early therapeutic treatment study in slow-growing PAP-A2-HPV16 tumor model:

To evaluate the therapeutic efficacy of SiO_2-Arg conjugated with HPV16 E7-derived epitope, the HPV16 $E6^+/E7^+$ PAP-A2 tumor model in HLA-humanized A2.DR1 BL6 mice was used. $1.5 \cdot 10^6$ PAP-A2 cells were injected subcutaneously, which should result in large tumors within 2 to 3 weeks. Starting with day 4 after tumor inoculation, the tumor-bearing mice were treated weekly (3 immunizations total, Prime-Boost-

Boost) with the complete vaccine (HPV-008) or with the individual compounds as controls, until the ethical endpoint (tumor volume 1000 mm^3) was reached.

Fig. 14 shows the survival rate of mice, either receiving the free antigen HPV16 E7 antigen YMLDLQPET + poly(I:C) (HMW, high molecular weight) "free antigen + TLR agonist" or KKKW-Cit-YMLDLQPET + poly(I:C) (HMW) both adsorptively bound to arginylated silica nanoparticles having a diameter of 23 nm.

As shown in **Fig. 14**, the treatment of tumor-bearing mice with KKKW-Cit-YMLDLQPET + poly(I:C) (HMW) both adsorptively bound to arginylated silica nanoparticles resulted in complete tumor regression in 5 out of 9 mice with overall survival rate of 55%. In contrast, 90% of carrier control (free antigen + TLR3 agonist) mice had to be eliminated due to excessive tumor growth. **Fig. 15a** and **Fig. 15b** show the individual tumor growth of both groups.

Taking together the results from therapeutic vaccinations with single compounds and with SiO_2-Arg conjugated with epitope, it can be concluded that the best therapeutic anti-tumor results were achieved with the triple surface-arginylated nanoparticles conjugated with KKKW-Cit-YMLDLQPET vaccination.

EXAMPLE 15

Cross-Presentation Experiments

MHC I cross presentation of $OVA_{257-264}$ after intracellular processing in DC2.4 cells

To demonstrate the functionality of the enzymatic cleavage (W-Cit) site attached to an immunogenic epitope, experiments on cross-presentation of the model antigen $OVA_{257-264}$ (=SIINFEKL in one letter code for amino acids) in murine dendritic cells (DC2.4 cells, immortalized murine dendritic cells) were performed. Therefore, the expression of SIINFEKL on the Major Histocompatibility Complex I (MHC I) after incubation with various OVA-derived constructs, with and without nanoparticles, was determined by antibody (25-D1.16, PE/Cy7 anti-mouse H-2Kb bound to SIINFEKL Antibody, Biolegend, Inc., USA) specific detection of the native epitope presented on MHC I. Free, not MHC I bound epitope or elongated epitopes on MHC I are not recognized by the antibody, which is highly selective to SIINFEKL presented at MHC I.

In the experiment, the $OVA_{257-264}$ presentation efficacy after incubation of $5 \cdot 10^4$ DC2.4 cells with 5 µM solutions of full length protein (OVA = Ovalbumin), N- and C-terminal elongated epitope ($OVA_{247-264}A_5K$, a so called synthetic long peptide (SLP)), N-terminal

elongated epitope with a Cathepsin B cleavable sequence (exemplary shown RW-Cit-$OVA_{257-264}$) or native epitope ($OVA_{257-264}$), each with and without nanoparticles, was compared. Surface phosphorylated silica nanoparticles (SiO_2-PO_3H_2) with an average diameter of 25 nm (comparable size to SiO_2-Arg used in other experiments) were used as carrier. After an incubation time of 6 h, the test substances are removed by washing with phosphate-buffered saline (PBS). In the next step, the cells were incubated with CD16/CD32 antibody in order to block the non-specific binding of the detection antibody to the Fc (Fragment, crystallizable) receptor of the cells. After incubation with 25-D1.16 antibody, the amount of cross-presented $OVA_{257-264}$ was quantified by flow cytometry.

Fig. 16: $OVA_{257-264}$ MHC I expression after 6 h incubation of H2-Kb positive cells with 5 µM solution of native or elongated $OVA_{257-264}$ epitope or full length OVA protein with or without SiO_2-PO_3H_2. Quantification was carried out by flow cytometry after labeling with 25-D1.16 detection antibody.

As shown in **Fig. 16**, only the native epitope and the elongated epitope with an enzymatic cleavage site are presented in significant amounts on the MHC. The uptake and thus the amount of presented epitope can be increased slightly by incubation with peptides ionically bound to nanoparticles. While the native epitope does not necessarily have to be internalized into the cell, since it can also be loaded exogenously onto the MHC molecule, the N-terminal elongated epitope must be internalized to release the native sequence. The detection of the native epitope on MHC I after incubation of the cells with RW-Cit-$OVA_{257-264}$ confirms a proof of the functionality of the enzymatic cleavage site.

EXAMPLE 16

Stability Measurement in Human Serum

HEK-Blue™ hTLR3 Cells are designed to measure the stimulation of human TLR3 by human TLR3 by monitoring the activation of NF-kB. HEK-Blue™ hTLR3Cells were obtained by co-transfection of the hTLR3 gene and an optimized secreted embryonic alkaline phosphatase (SEAP) reporter gene placed under the control of an NF-kB and AP-1-inducible promoter intoHEK293 cells. Stimulation with a TLR3 ligand activates NF-kB and AP-1 which induce the production of SEAP. Levels of SEAP can be easily determined with HEK-Blue™ Detection, a cell culture medium that allows for real-time detection of SEAP. The hydrolysis of the substrate by SEAP produces a purple/blue color that can be easily detected with the naked eye or measured with a 96 well plate reader. (Invivogen, see https://www.invivogen.com/hek-blue-htlr3).

Poly(I:C) and poly(I:C)@SiO_2-Arg were exposed for 60 minutes at 37 °C to human serum (HS). The serum was used in concentrations of 5, 10 and 20 %. A serum concentration of 20 % corresponds quite well to the composition of peripheral lymph.

Poly(I:C) and poly(I:C)@SiO_2-Arg were added separately to human serum to get a concentration of 1 µg poly(I:C)/mL. In case of poly(I:C)@SiO_2-Arg the SiO_2 concentration was 40 µg/mL. The poly(I:C) payload at SiO_2-Arg in this case was about 2.5 %. After exposure to HS 20 µL of the medium were taken and added to 180 µL HEK-Blue™hTLR3 cells in HEK-Blue™ Detection medium. This mixture was incubated for 13 hours at 37 °C and the plate was analyzed in a 96 well plate reader (Tecan Reader, Type: Infinite M200 Pro).

The results are shown in **Fig. 17**.

The calculated half-life of free poly(I:C) in 20 % human serum is under the chosen conditions 24.2 minutes (18 % of initial concentration after 60 minutes). Half-life calculation:

$t_{½}$ = ln 2/ (ln 100/ln 18) * 60 [min]

The half-life for poly(I:C)@SiO_2-Arg under the same conditions is calculated to 395 minutes (90 % of initial concentration after 60 minutes)

$t_{½}$ = ln 2/ (ln 100/ln 90) * 60 [min]

So the attachment of poly(I:C) to arginylated silica nanoparticles increases the half-life by a factor of 16 (=395 / 24.2).

EXAMPLE 17

Stability Measurement in Bovine Serum

HEK-Blue™ hTLR3 Cells are designed to measure the stimulation of human TLR3 by human TLR3 by monitoring the activation of NF-kB. HEK-Blue™hTLR3Cells were obtained by co-transfection of the hTLR3 gene and an optimized secreted embryonic alkaline phosphatase (SEAP) reporter gene placed under the control of an NF-kB and AP-1-inducible promoter intoHEK293 cells. Stimulation with a TLR3 ligand activates NF-kB and AP-1 which induce the production of SEAP. Levels of SEAP can be easily determined with HEK-Blue™ Detection, a cell culture medium that allows for real-time detection of SEAP. The hydrolysis of the substrate by SEAP produces a purple/blue color that can be easily detected with the naked eye or measured with a 96 well plate reader (Invivogen).

Poly(I:C) and poly(I:C)@SiO_2-Arg were exposed for 60 minutes at 37 °C to fetal bovine serum (FBS). The serum was used in concentrations of 5, 10 and 20 %. A serum concentration of 20 % corresponds quite well to the composition of peripheral lymph.

Poly(I:C) and poly(I:C)@SiO_2-Arg were added separately to bovine serum to get a concentration of 1 µg poly(I:C)/mL. In case of poly(I:C)@SiO_2-Arg the SiO_2 concentration was 40 µg/mL. The poly(I:C) payload at SiO_2-Arg in this case was about 2.5 %. After exposure to FBS 20 µL of the medium were taken and added to 180 µL HEK-Blue™ hTLR3 cells in HEK-Blue™ Detection medium. This mixture was incubated for 13 hours at 37 °C and the plate was analyzed in a 96 well plate reader (Tecan Reader, Type: Infinite M200 Pro).

The results could be seen from **Fig. 18**:

While the free poly(I:C) shows a clear signs of degradation at higher serum concentrations, poly(I:C)@SiO_2-Arg shows a significantly high stability. Half-life calculation for poly(I:C)@SiO_2-Arg is useless, due to a very low decay rate.

CLAIMS

1. Composition comprising nanoparticles which are loaded with pharmaceutically acceptable compounds, having silicon dioxide and functional groups on the surface, wherein
   - the functional groups are capable to carry and/or stabilize both negative and positive charges of the pharmaceutically acceptable compounds,
   - the zeta potential of the composition has a value of at least ± 15 mV,
   - the nanoparticles have a particle size below 150 nm.

2. Composition according to claim 1, wherein the nanoparticles have a surface loading density up to 0.5, in relation to the total number of the pharmaceutically acceptable compounds with regard to the surface of the nanoparticle in nm^2 [molecules/nm^2].

3. Composition according to claim 1 or 2, wherein the Polydispersity Index (PDI) of the composition is between 0 and 0.32.

4. Composition according to any of the preceding claims, wherein the zeta potential of the composition has a value of at least ± 30 mV.

5. Composition according to any of the preceding claims, wherein the net charge of the loaded nanoparticles in total is different to zero.

6. Composition according to any of the preceding claims, wherein the functional groups are connected to a linker L which is linked to the surface of the nanoparticles by way of a covalent or adsorptive bond.

7. Composition according to claim 6, wherein the linker compound L comprises at least one carboxyl (–COOH) or carboxylate ($–COO^-$) group as functional group.

8. Composition according to claim 6 or 7, wherein the linker compound L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or –NH_3^+) group as a functional group.

9. Composition according to any of claims 6 to 8, wherein the linker L comprises at least one further functional group L', which is selected from the group –SH, –COOH, –NH_2, –guanidino-group (–NHC(=NH))NH_2), –PO_3H_2, –PO_2CH_3H, –SO_3H, –OH, –$NR_3^+X^-$.

10. Composition according to any of claims 6 to 9, wherein the linker compound L contains at least a structural unit of formula (I)

*– (–O)3Si-(CH2)n-CH(COOX)-(CH2)p-C(O)-NH-CH(COOX)-(CH2)q-Y (I)

wherein

X	is independently from each other H or a negative charge,
Y	is independently from each other –NHC(=NH)NH_2, -–NHC(=NH_2^+)NH_2, –NH_2 or –NH_3^+,
n, p and q	are independently from each other 0 or a number from 1 to 25; and
*-	is the connection point to the nanoparticle.

11. Composition according to any of the preceding claims, wherein the pharmaceutically acceptable compound is conjugated to the nanoparticle by adsorptive attachment.

12. Composition according to any of the preceding claims, wherein the pharmaceutically acceptable compound is selected from the group comprising proteins, peptides, polynucleotides, oligonucleotides (RNA, DNA, single stranded or double stranded), nucleic acids or peptide antigens.

13. Composition according to any of the preceding claims, wherein the pharmaceutically acceptable compound is selected from the group comprising Pathogen-Associated Molecular Patterns (PAMPs), Danger-Associated Molecular Patterns (DAMPs), Defective Infectious Particles (DIPs) or epitopes.

14. Composition according to claim 13, wherein PAMPs are selected from the group of TLR agonists, Retinoic Acid Inducible Gene I (RIG-I) Ligands, Melanoma-differentiation-associated gene 5 (MDA-5) Ligands, Cytosolic DNA Sensors (CDS) Ligands, STING Ligands (Cyclic Dinucleotide (CDNs)).

15. Composition according to any of claims 12, wherein the peptides comprise a C-terminal extension with an enzymatic cathepsin B-cleavable linker.

16. Composition according to claim 1 for use as vaccines or as imunostimulant.

17. Nanoparticles having silicon dioxide and functional groups on the surface and a particle size below 150 nm, comprising a linker L which is covalently or adsorptive bonded to it, wherein the Linker L comprises at least one guanidino-group (–NHC(=NH)NH_2 or –NHC(=NH_2^+)NH_2) or amino-group (–NH_2 or –NH_3^+) group as a functional group.

Figure 1a

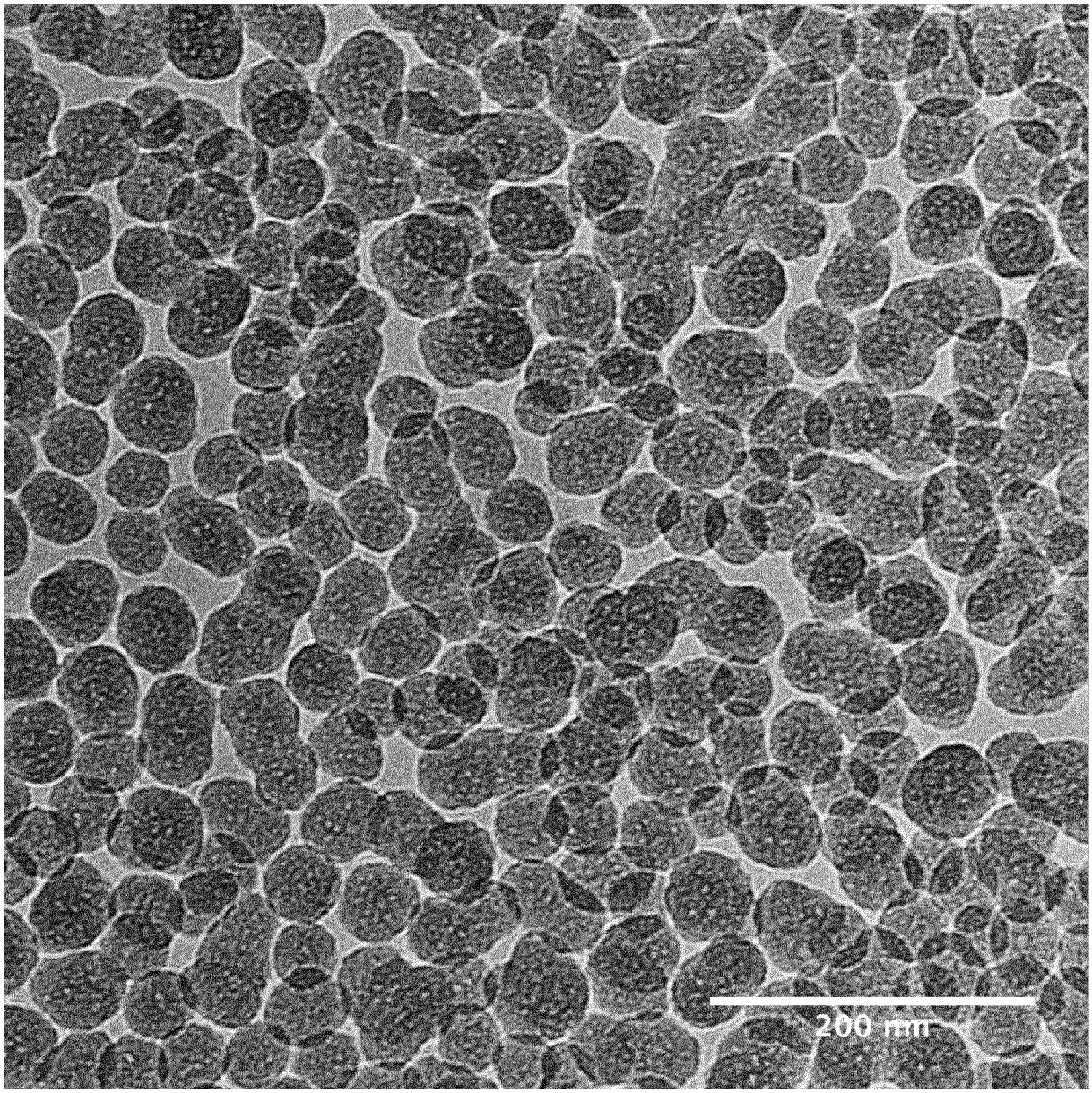


Figure 1b

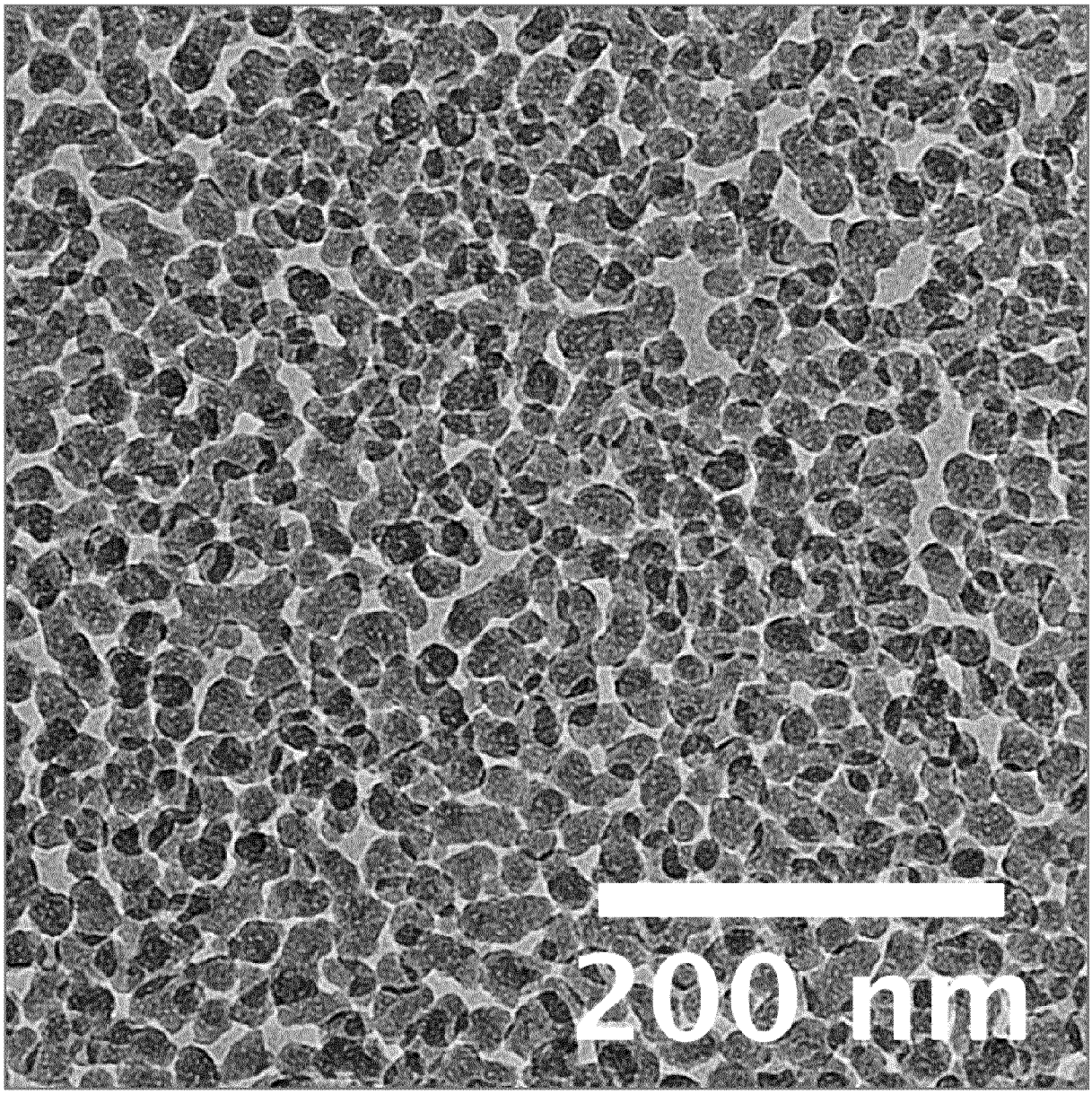


Figure 1c

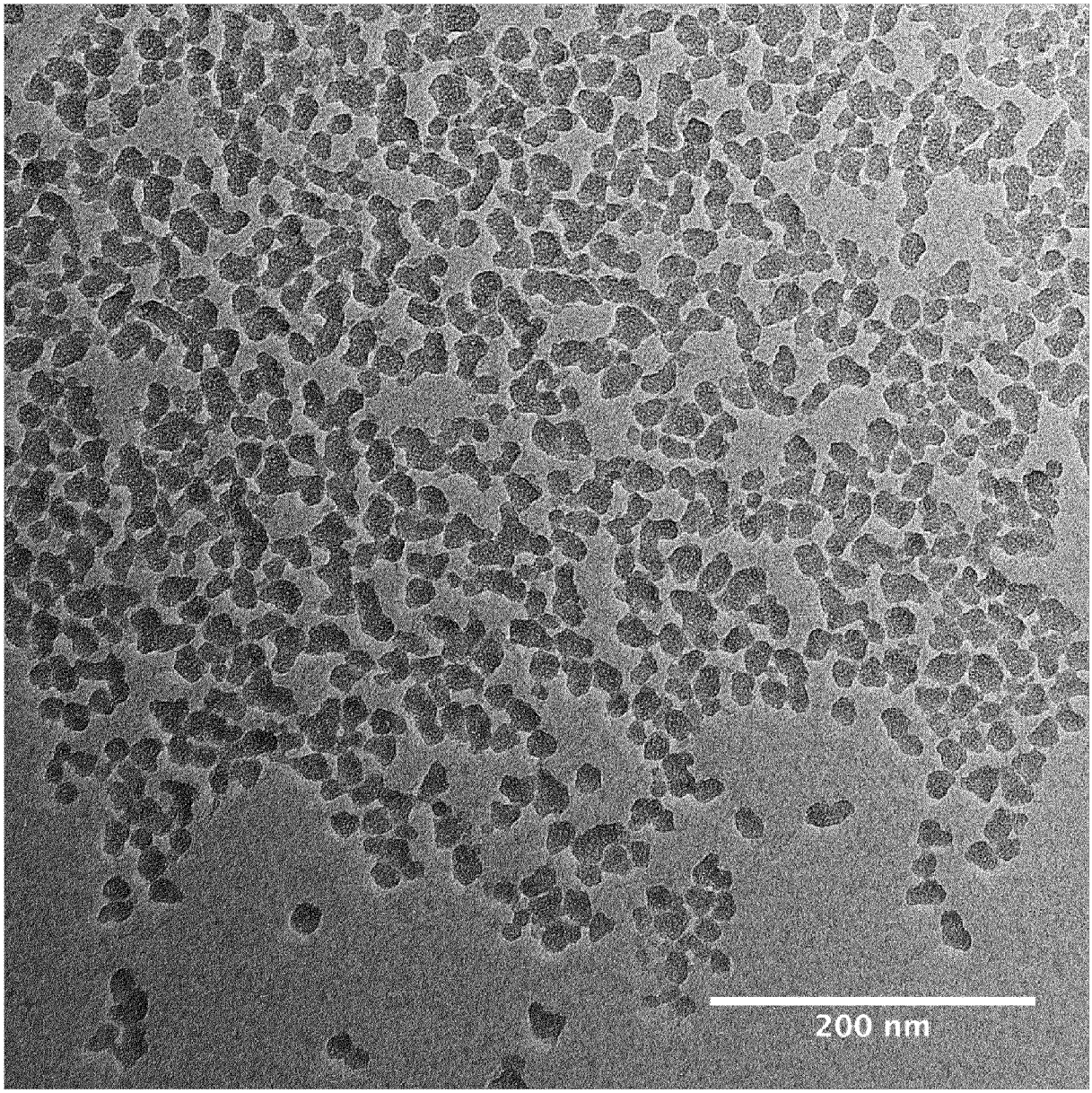


Figure 1d

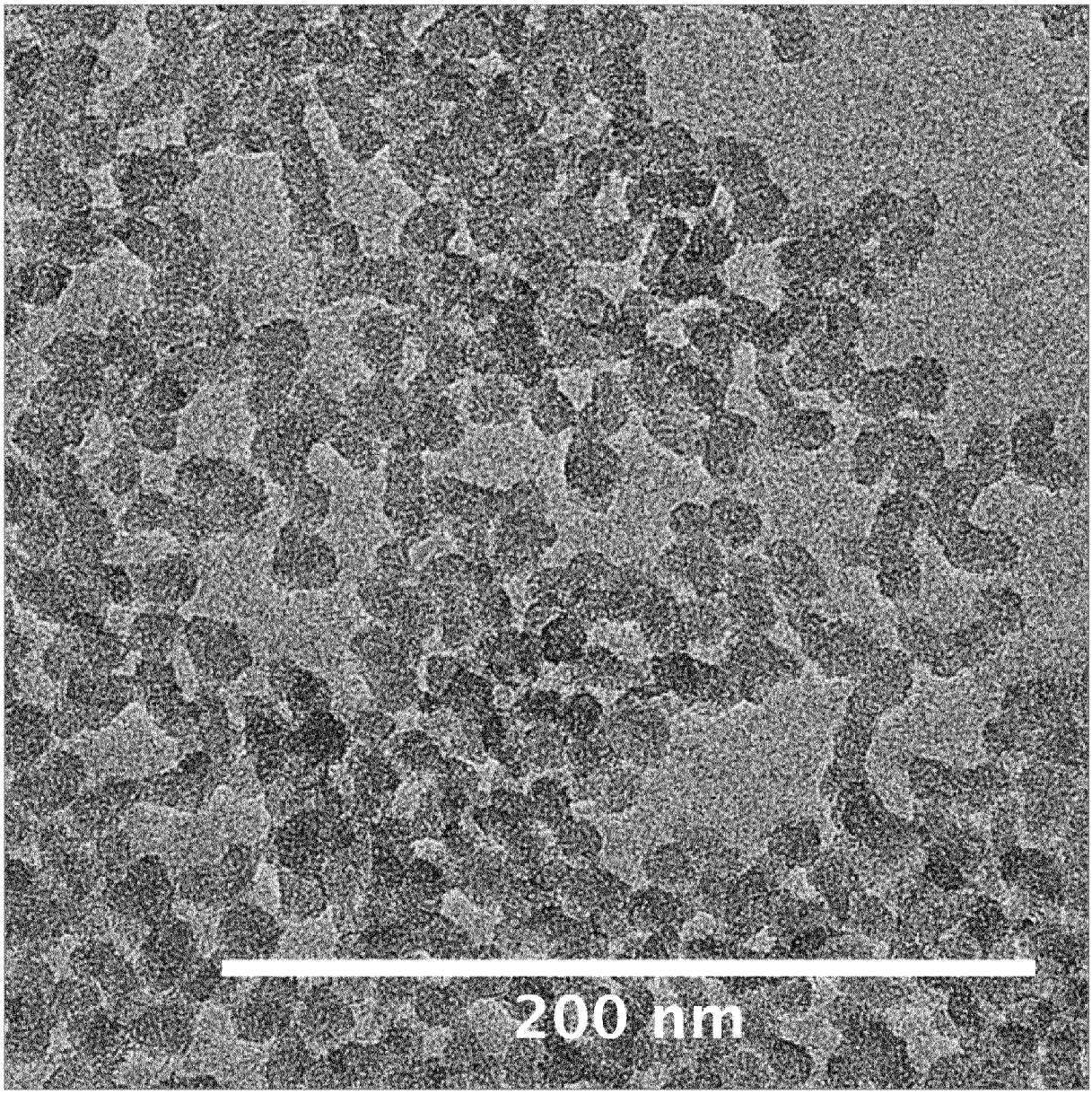


Figure 2

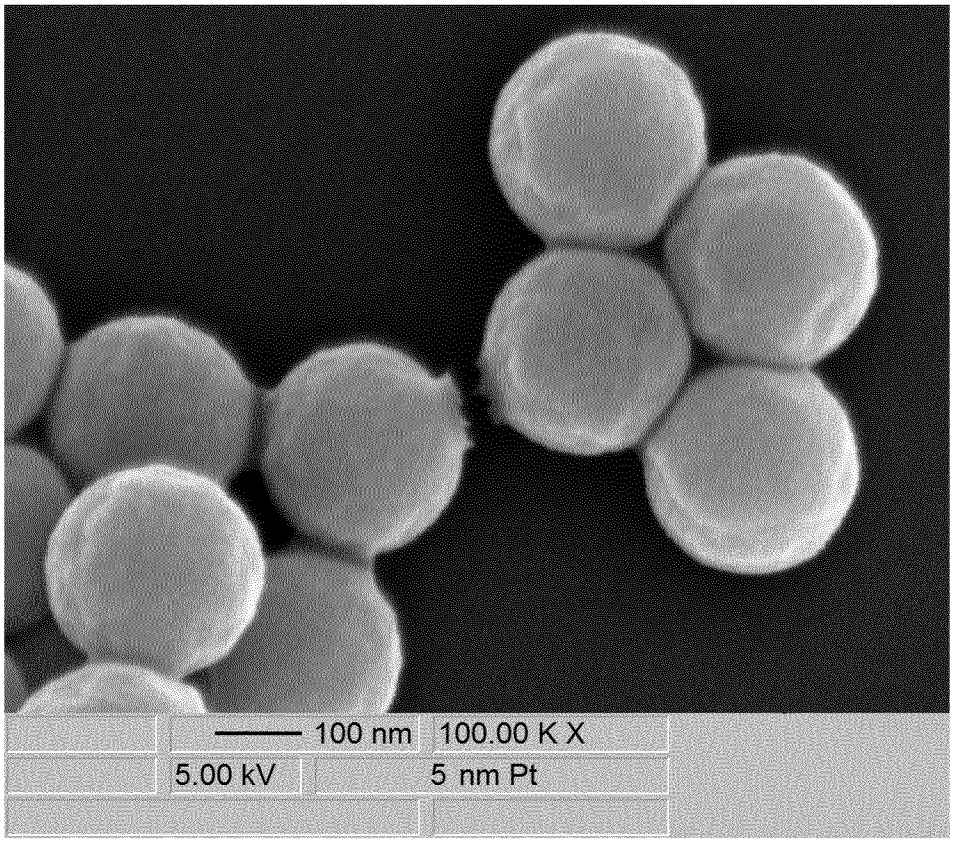


Figure 3

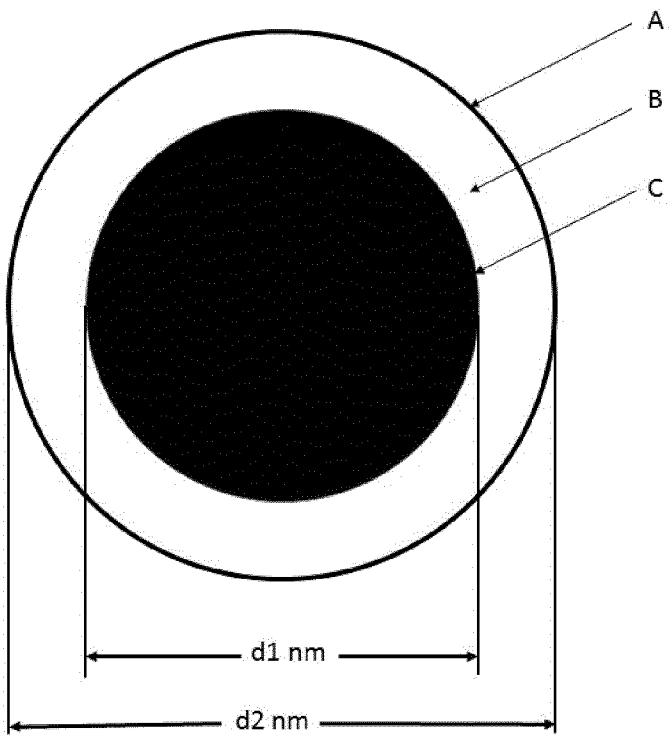


Figure 4a

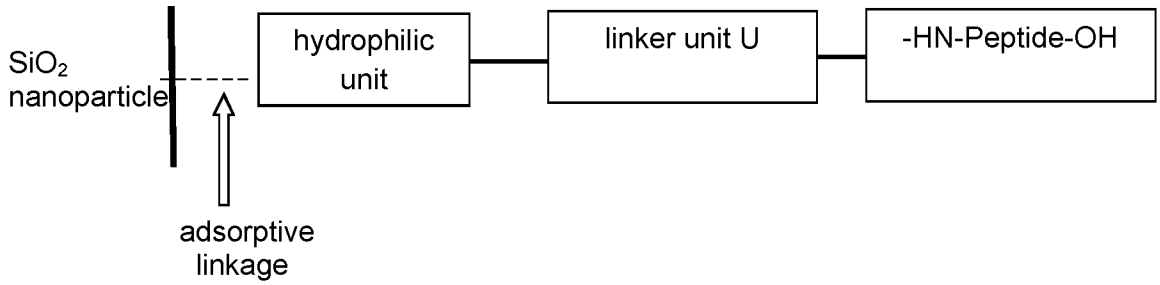


Figure 4b

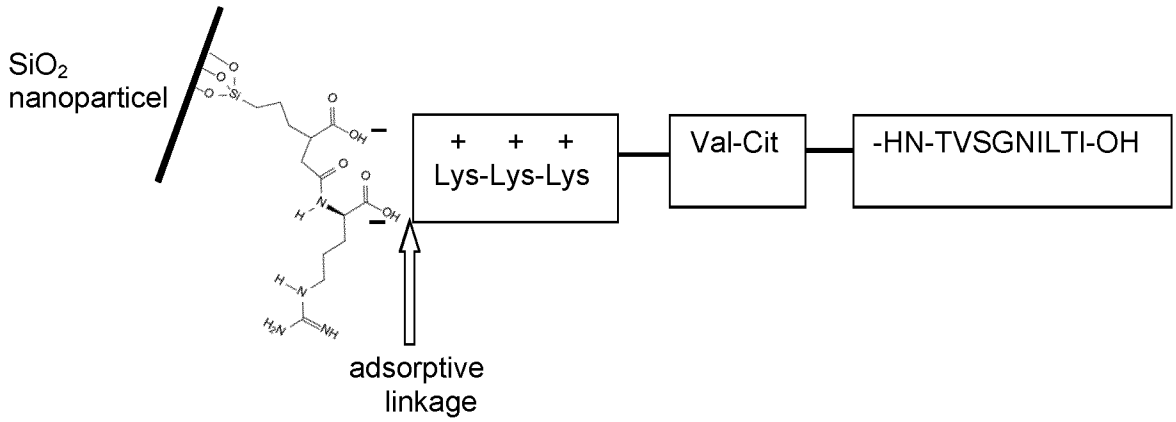


Figure 5a

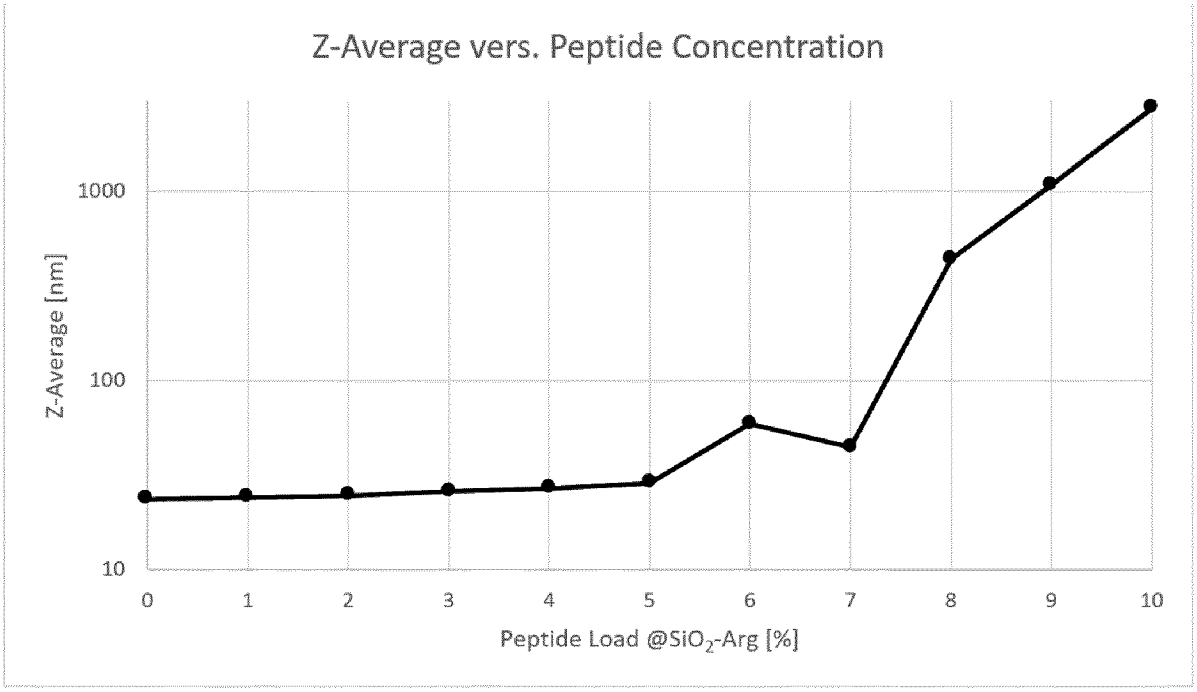


Figure 5b

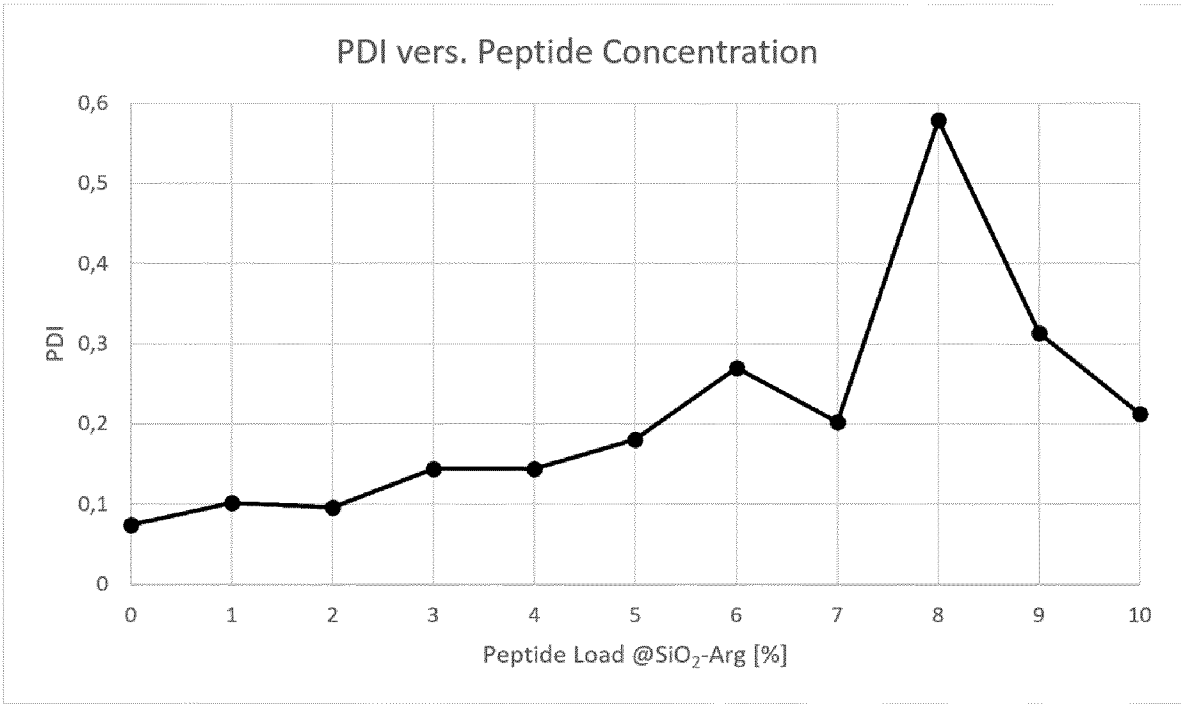


Figure 6a

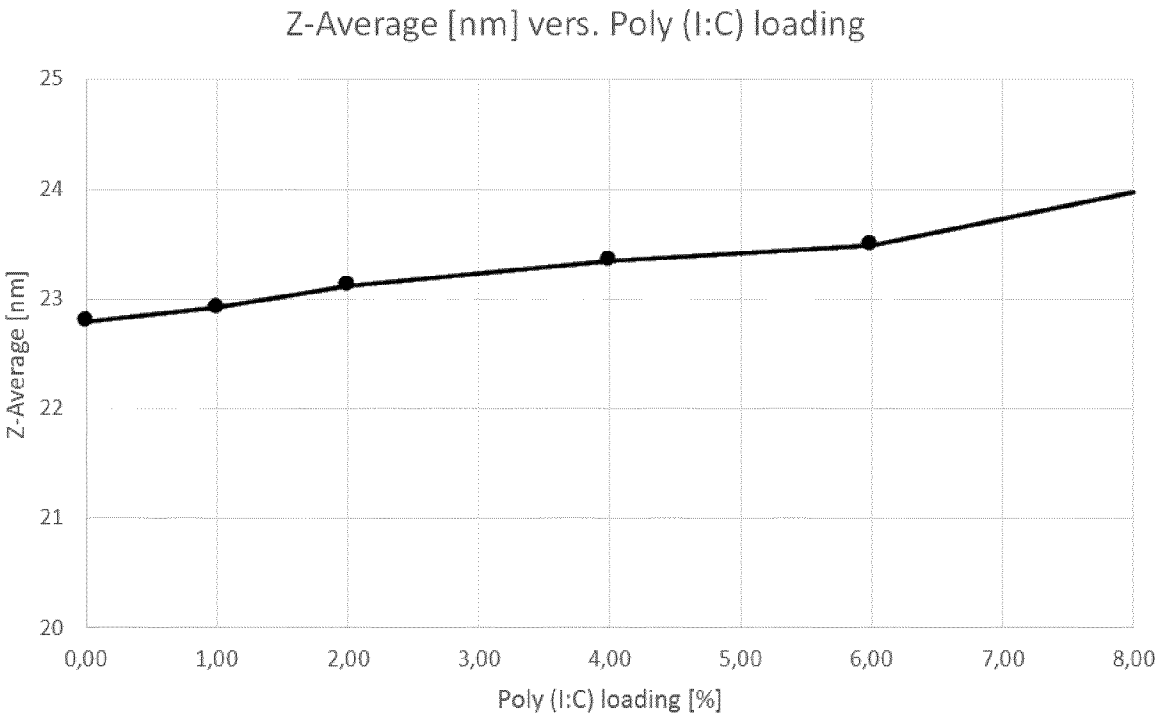


Figure 6b

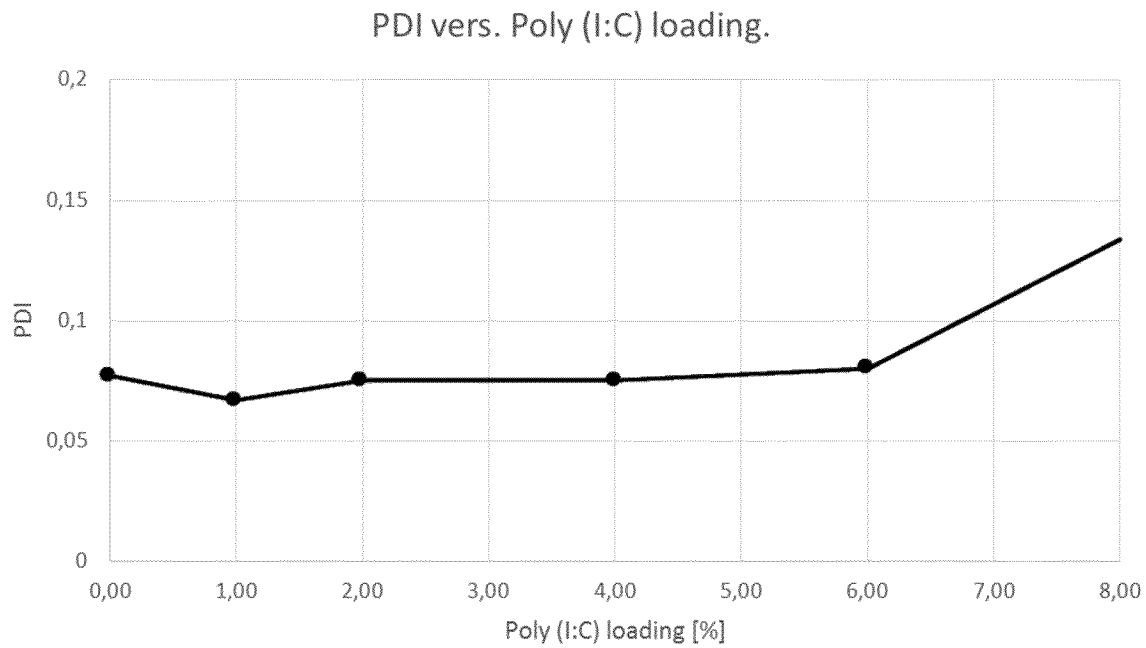


Figure 7a

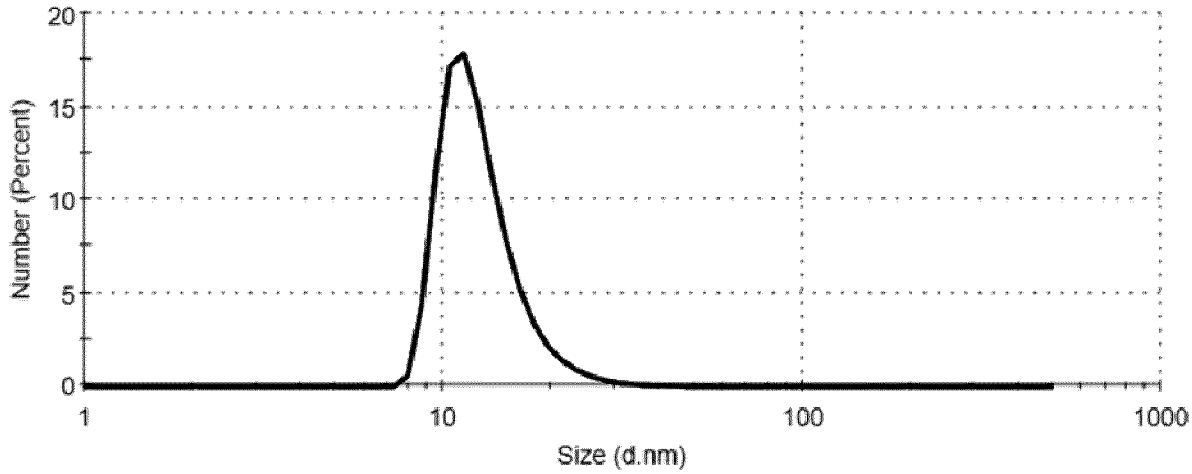


Figure 7b

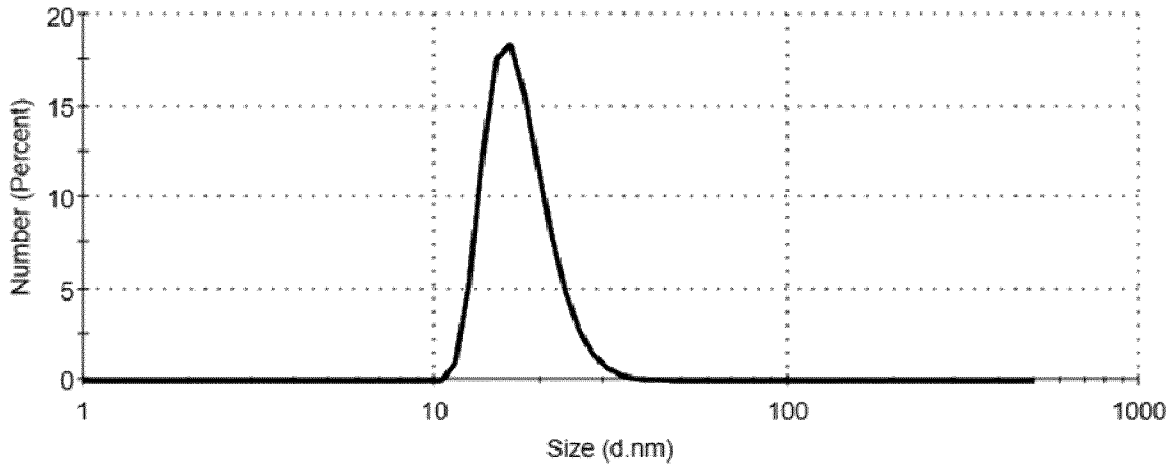


Figure 7c

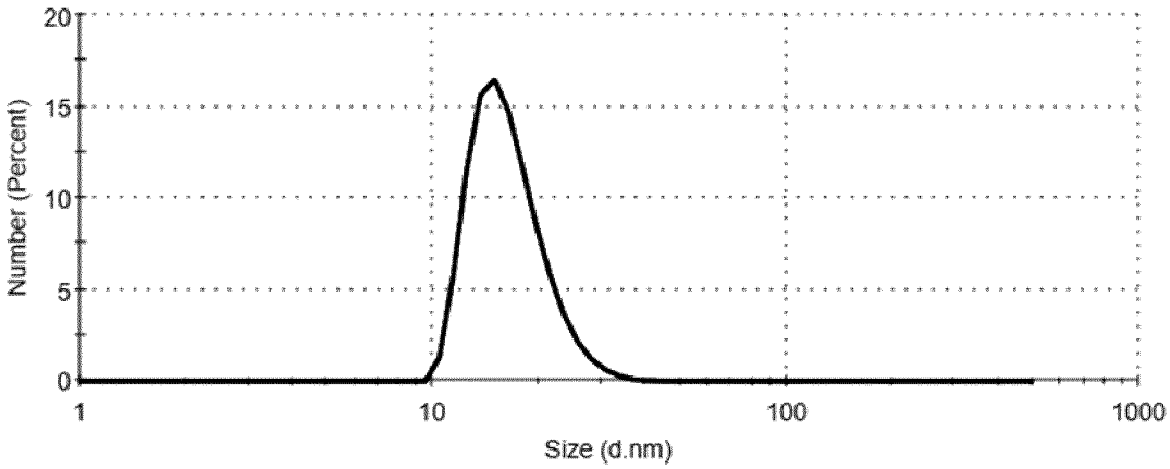


Figure 7d

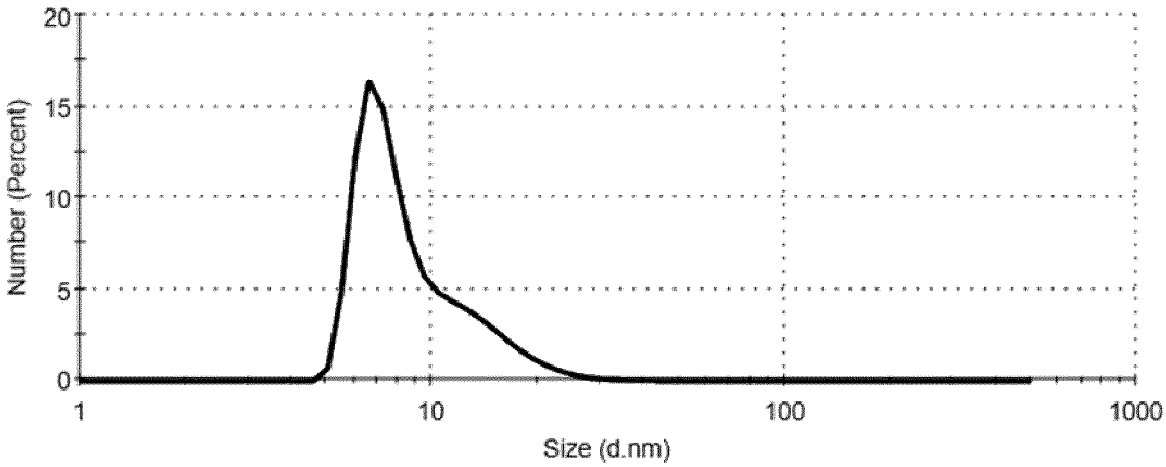


Figure 8

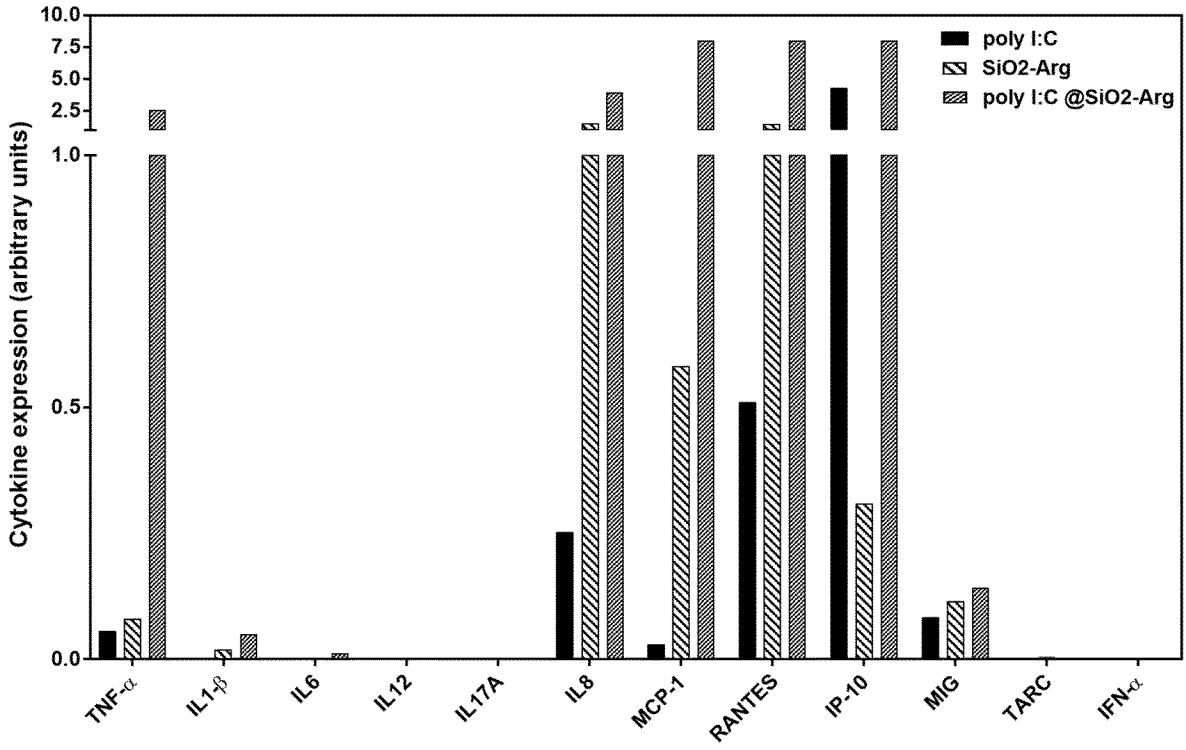


Figure 9

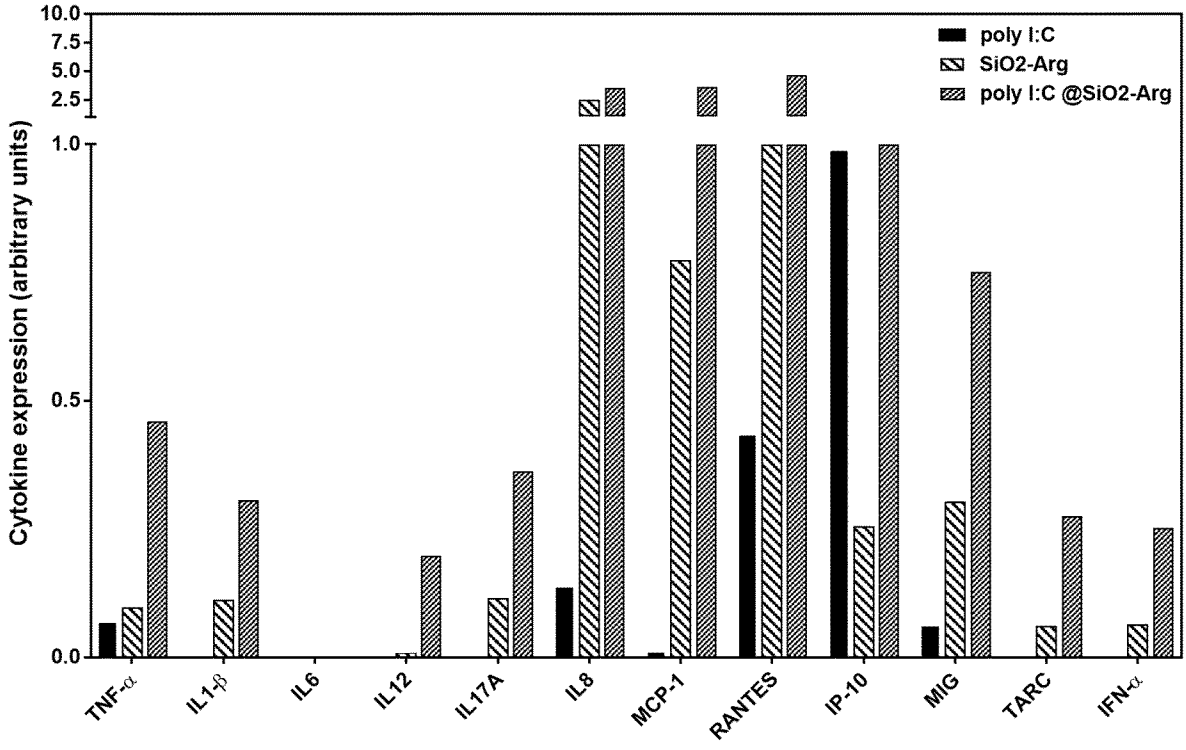


Figure 10a

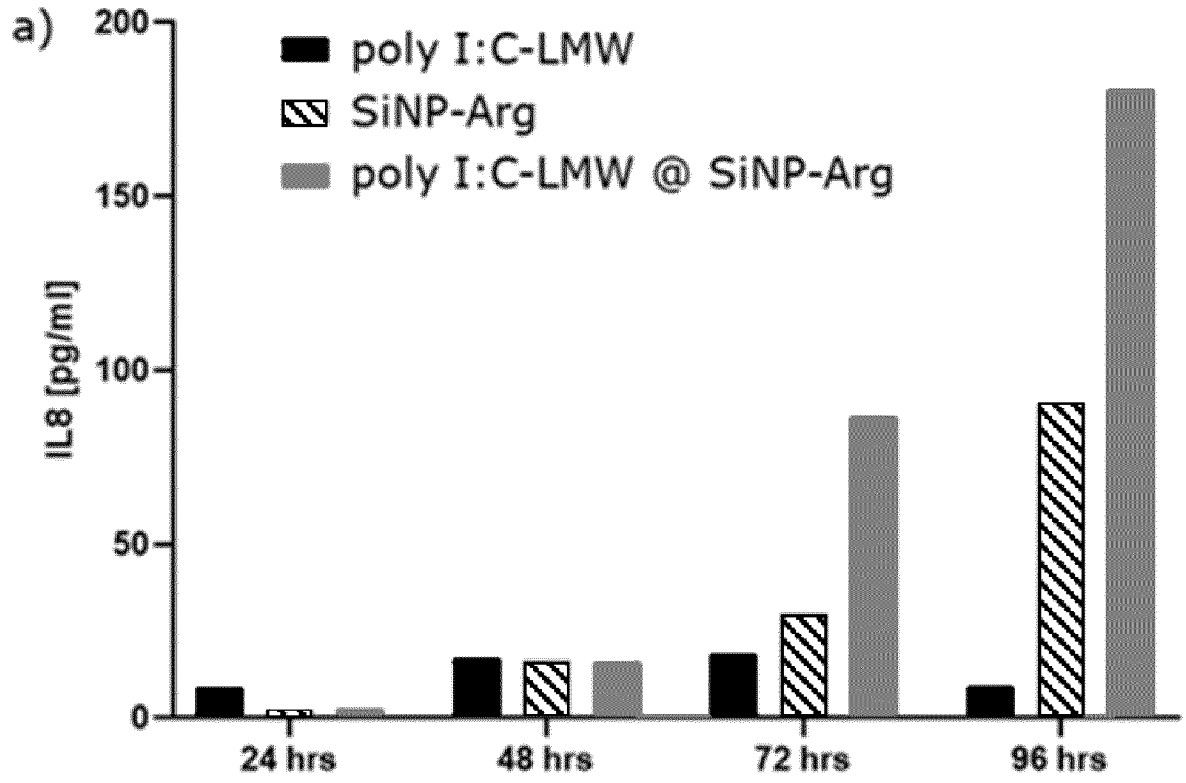


Figure 10b

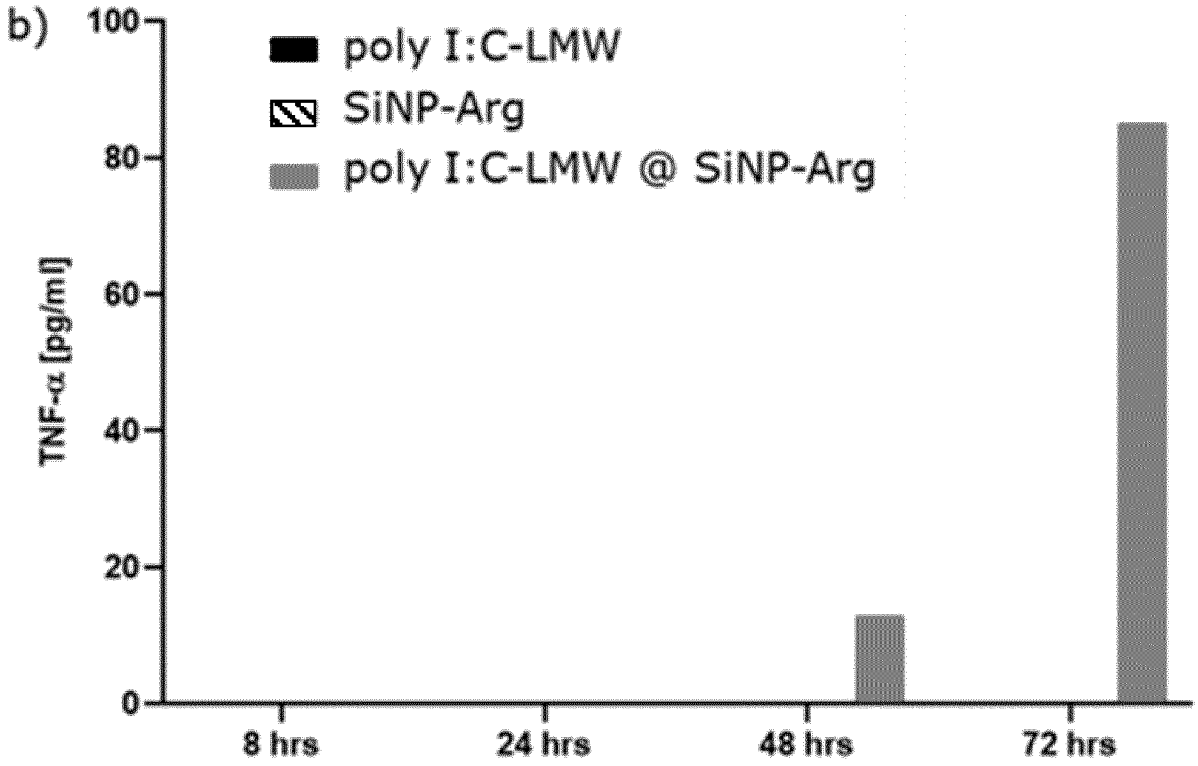


Figure 11a

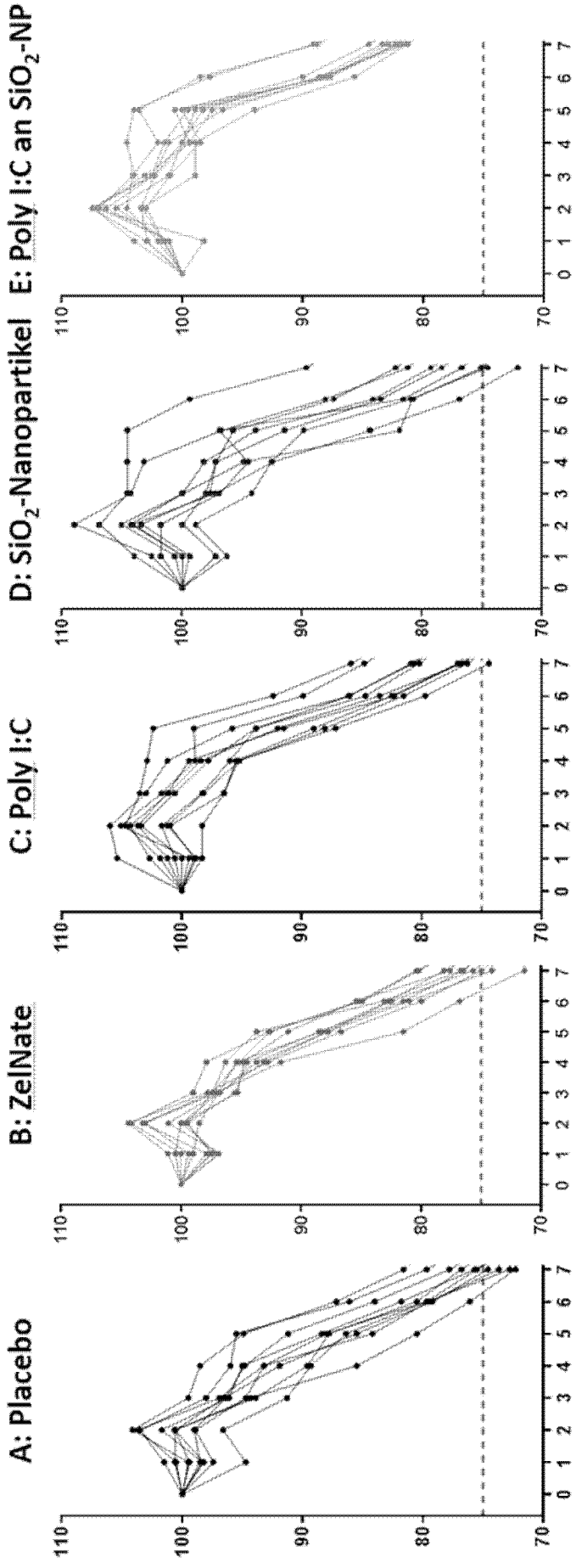

A: Placebo
B: ZelNate
C: Poly I:C
D: SiO2-Nanopartikel
E: Poly I:C an SiO2-NP
110
100
90
80
70
0 1 2 3 4 5 6 7

Figure 11b

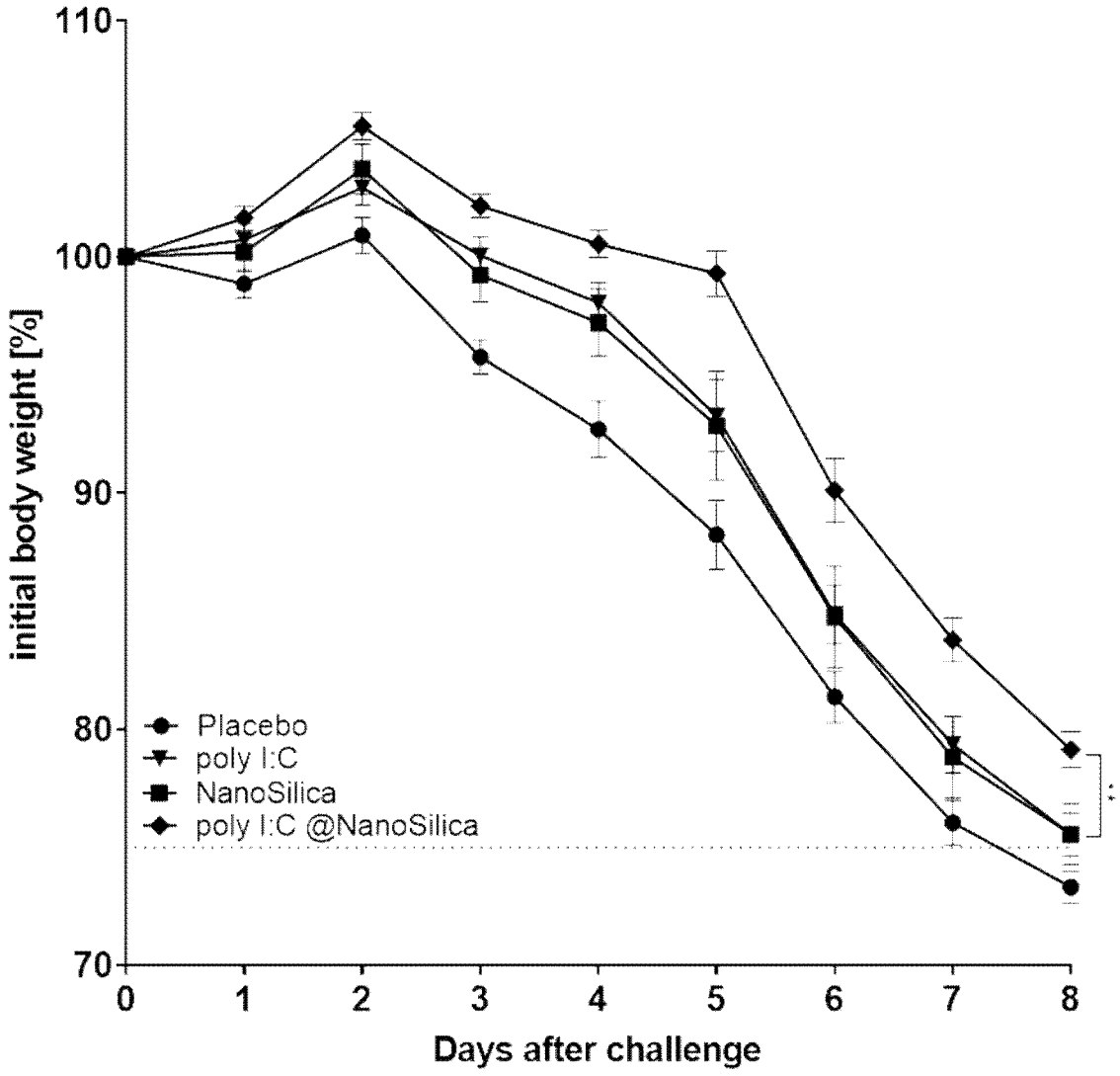

110
100
90
80
70
initial body weight [%]
0
1
2
3
4
5
6
7
8
Days after challenge
Placebo
poly I:C
NanoSilica
poly I:C @NanoSilica
**

Figure 12

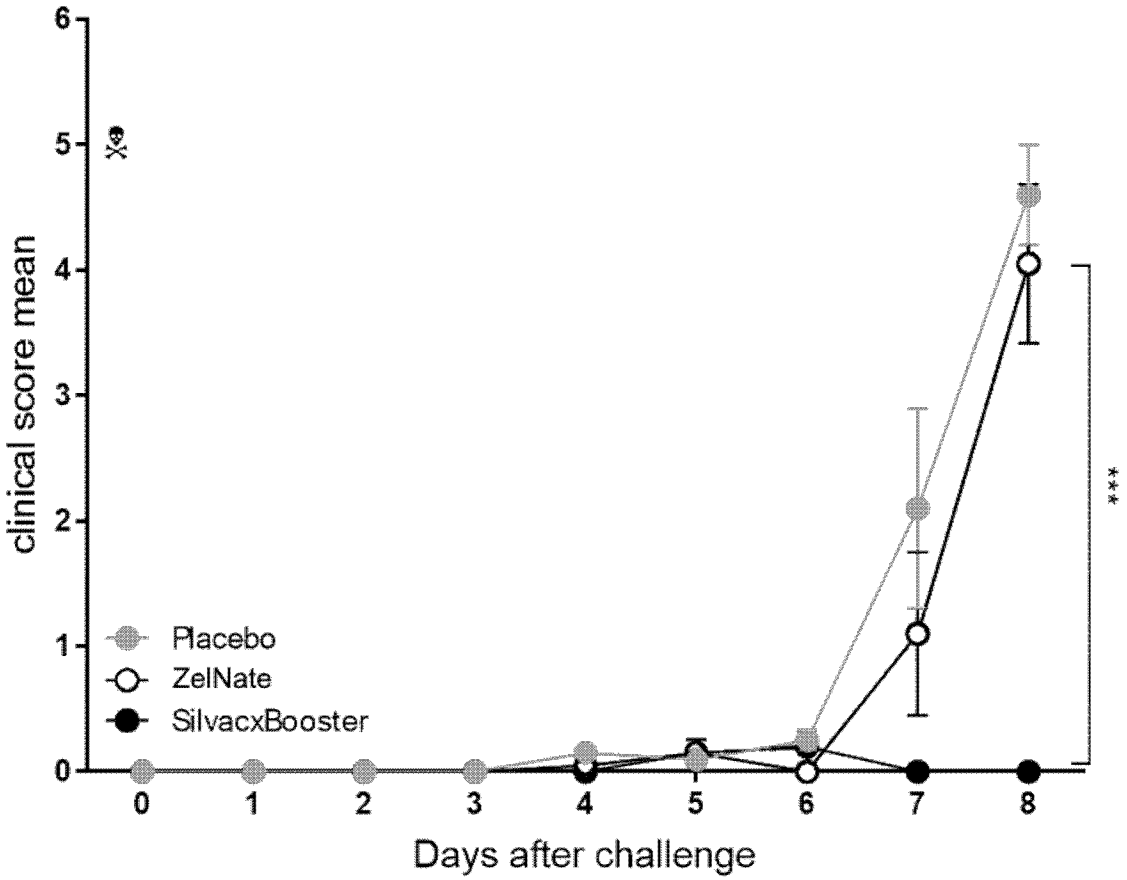

clinical score mean
Days after challenge
Placebo
ZelNate
SilvacxBooster
***

Figure 13

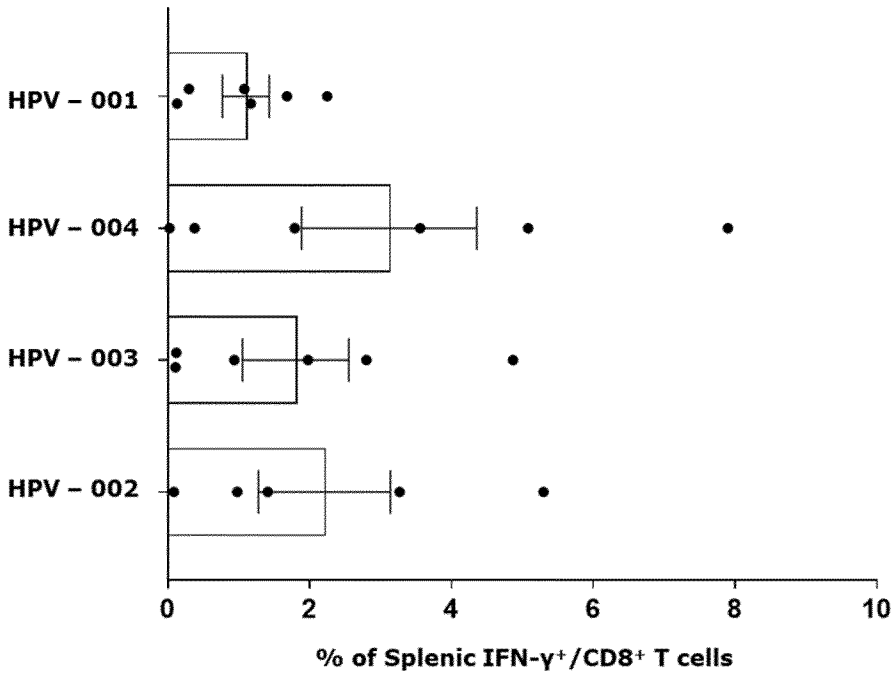

HPV – 001
HPV – 004
HPV – 003
HPV – 002
0
2
4
6
8
10
% of Splenic IFN-γ+/CD8+ T cells

Figure 14

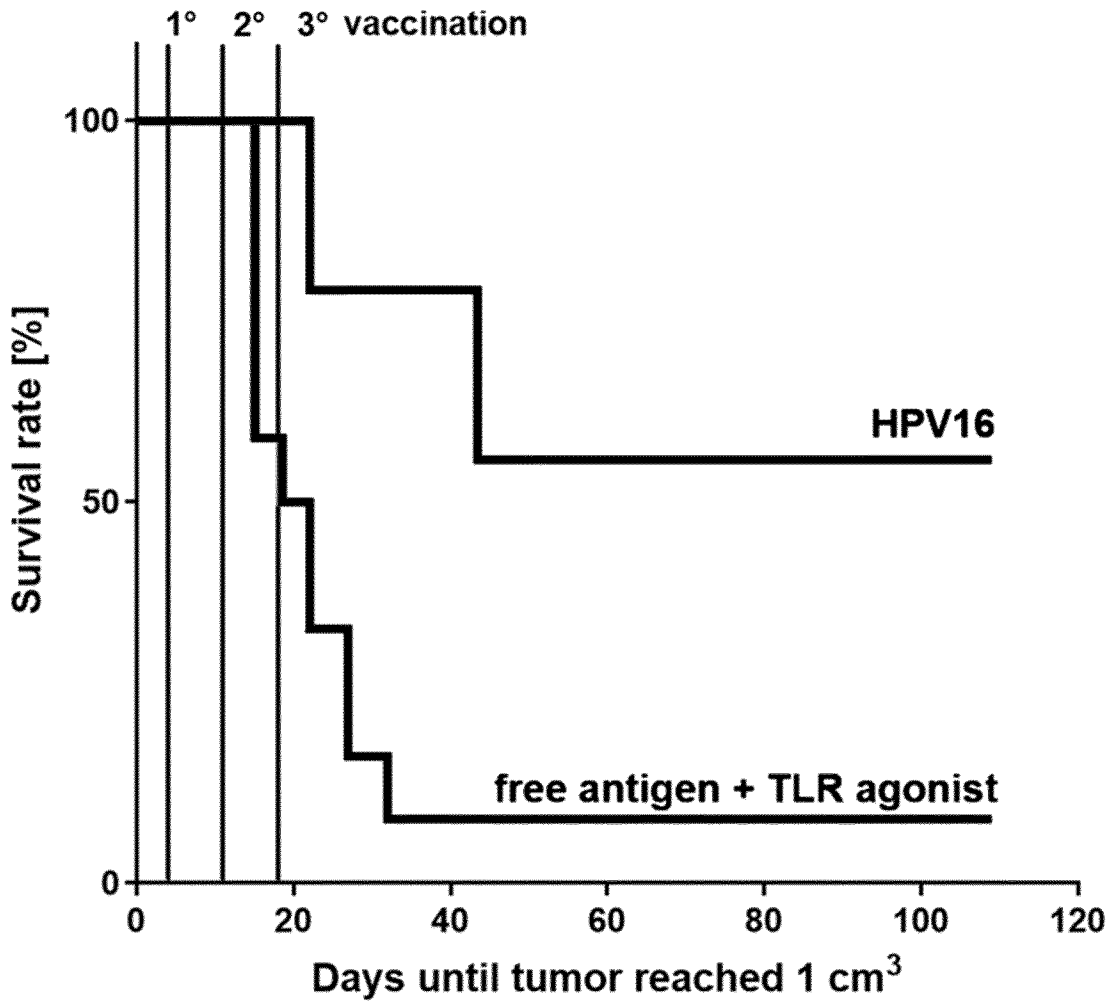

1° 2° 3° vaccination
100
50
0
Survival rate [%]
HPV16
free antigen + TLR agonist
0
20
40
60
80
100
120
Days until tumor reached 1 cm3

Figure 15a

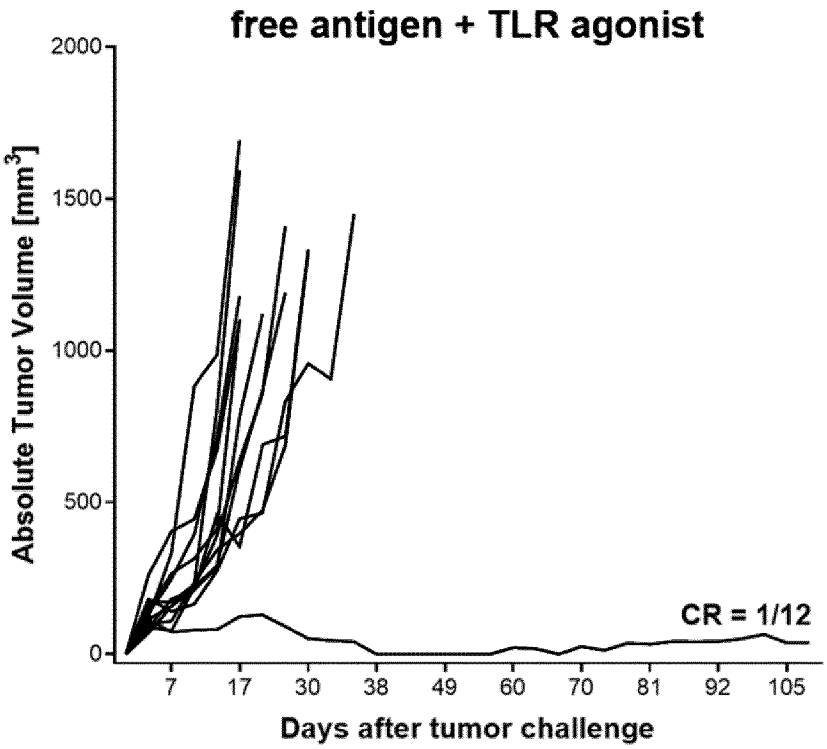


Figure 15b

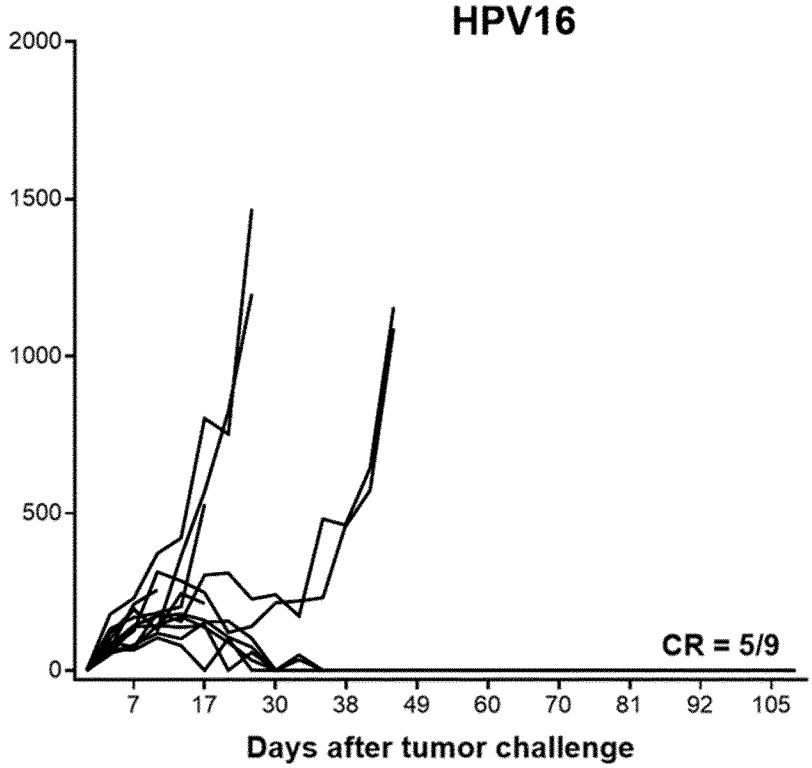


Figure 16

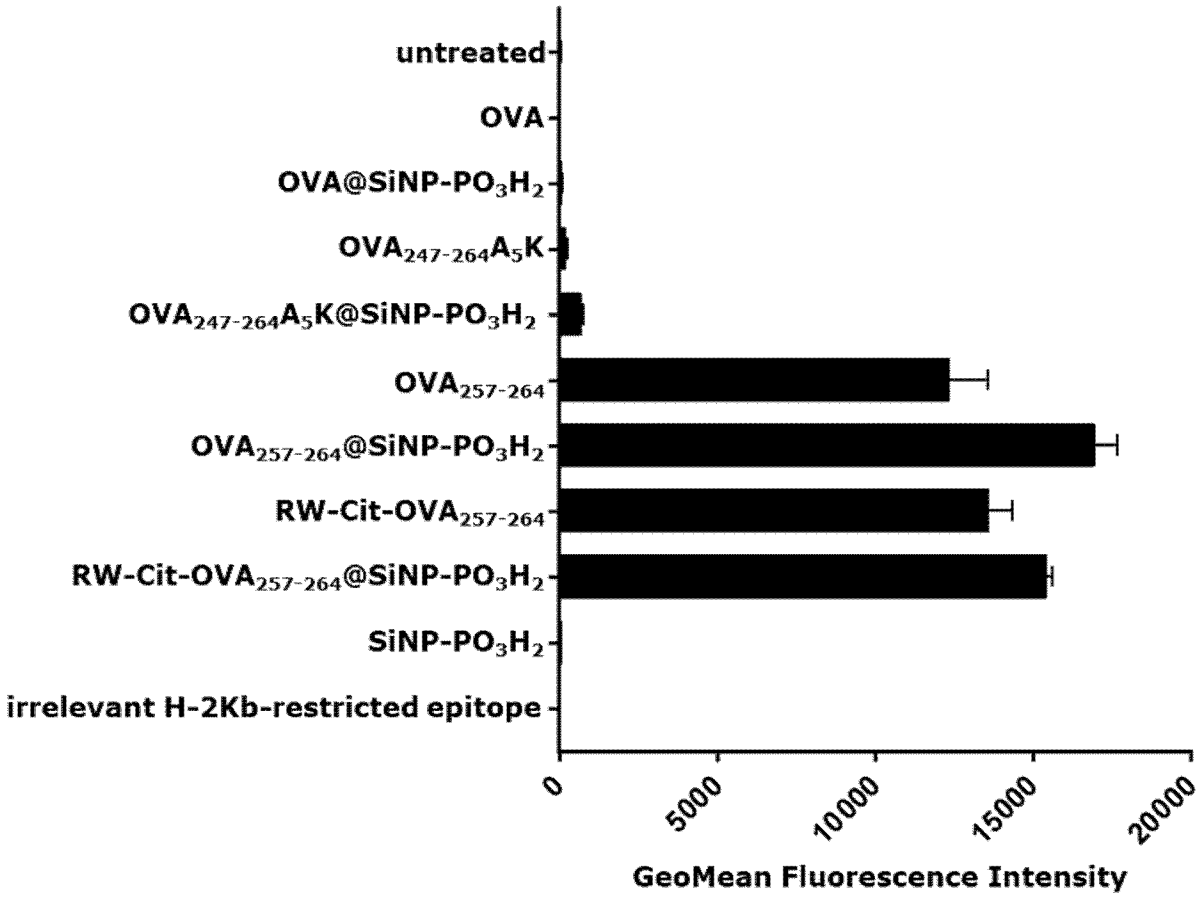

untreated
OVA
OVA@SiNP-PO3H2
OVA247-264A5K
OVA247-264A5K@SiNP-PO3H2
OVA257-264
OVA257-264@SiNP-PO3H2
RW-Cit-OVA257-264
RW-Cit-OVA257-264@SiNP-PO3H2
SiNP-PO3H2
irrelevant H-2Kb-restricted epitope
0
5000
10000
15000
20000
GeoMean Fluorescence Intensity

Figure 17

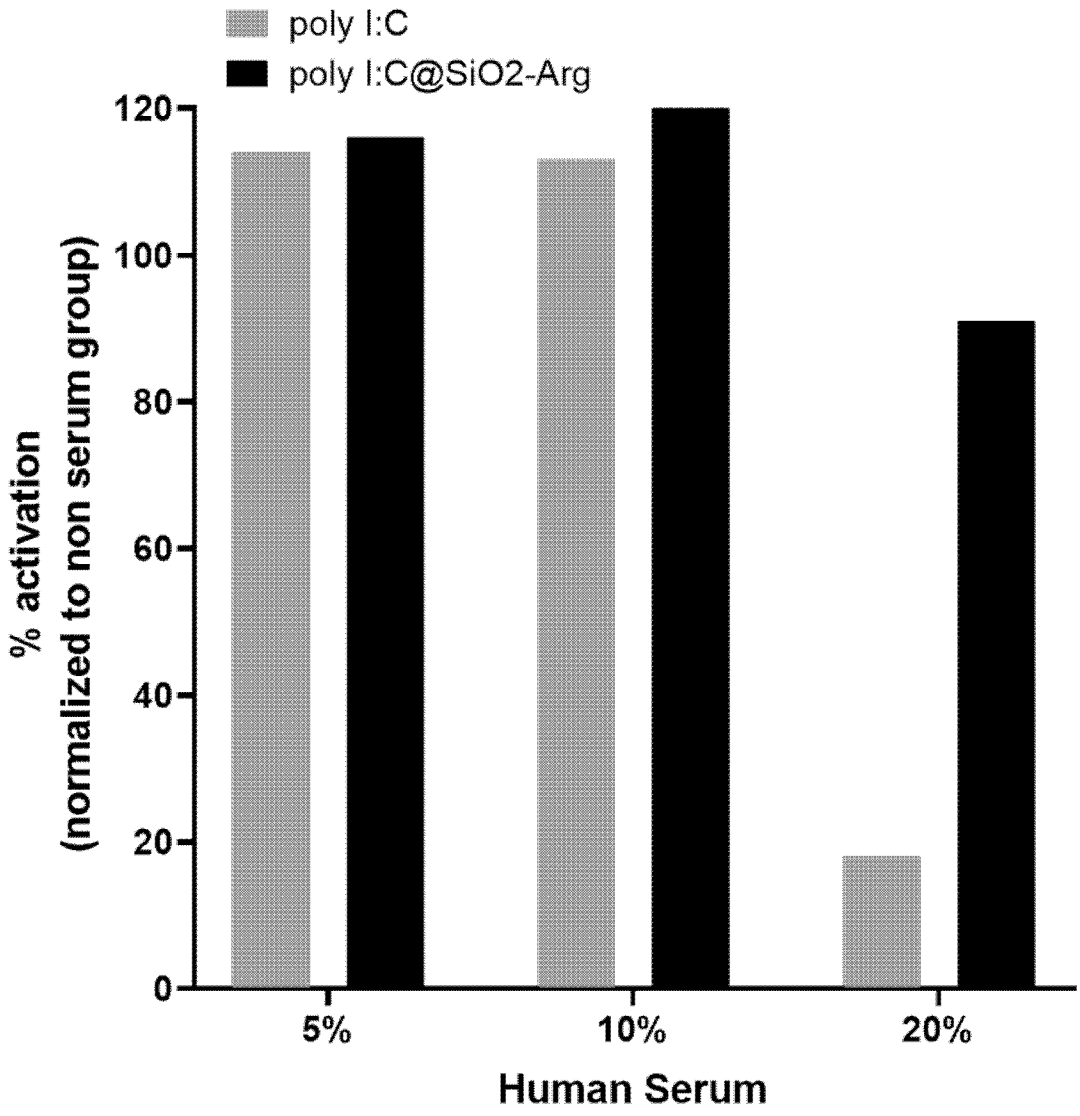
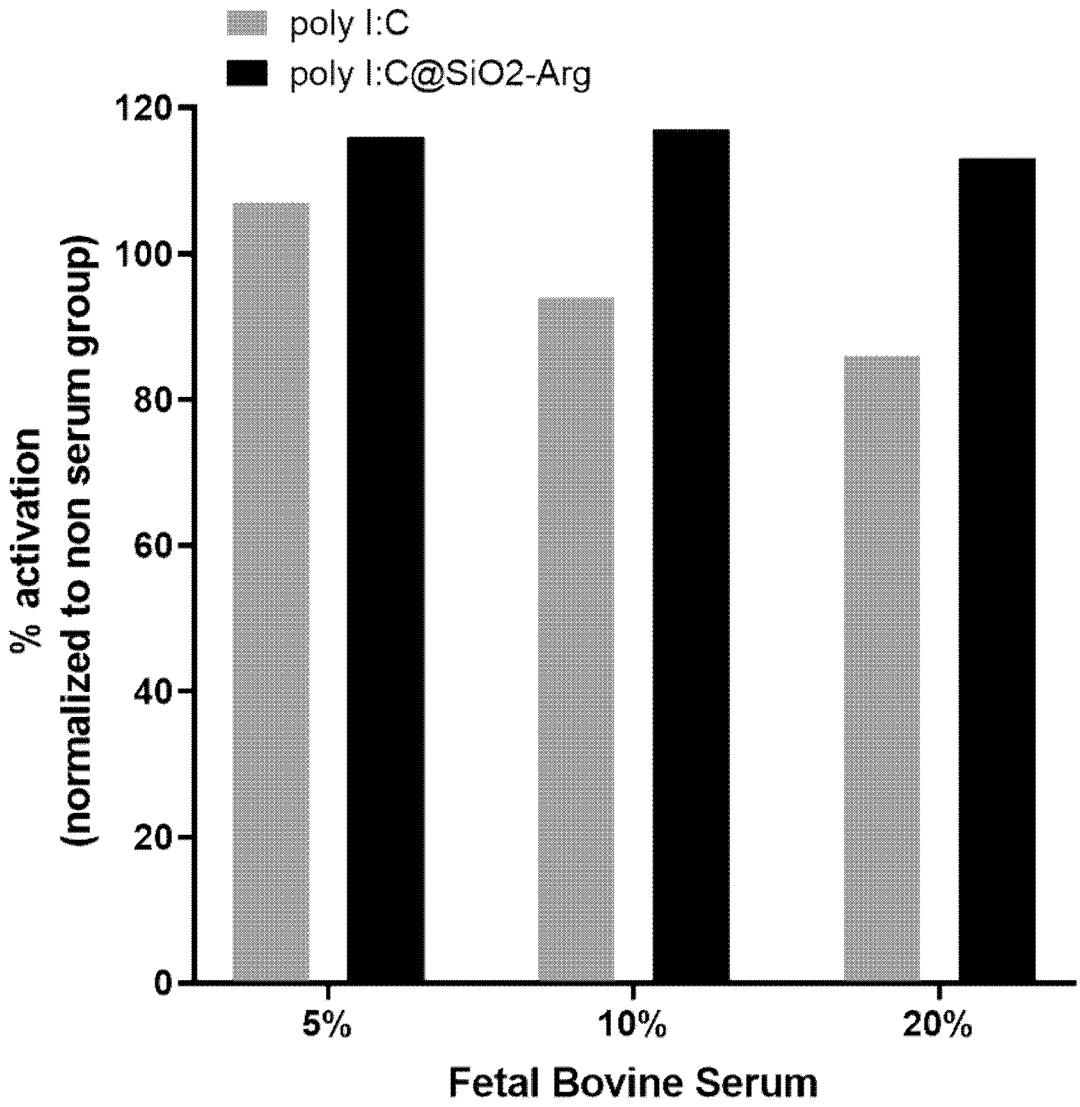

poly I:C
poly I:C@SiO2-Arg
120
100
80
60
40
20
0
% activation
(normalized to non serum group)
5%
10%
20%
Human Serum

Figure 18


poly I:C
poly I:C@SiO2-Arg
120
100
80
60
40
20
0
% activation
(normalized to non serum group)
5%
10%
20%
Fetal Bovine Serum

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/062751

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/69 A61P35/00 A61K39/395
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, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2016/090603 A1 (CARNES ERIC CHRISTOPHER [US] ET AL) 31 March 2016 (2016-03-31) figure 1B example 4; tables 2,3 ----- -/--	1-9,12, 17 10,11, 13-16

[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
1 July 2021	12/07/2021

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	Dullaart, Anwyn

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEONG SOO A. AN ET AL: "Analysis of SiO2 nanoparticles binding proteins in rat blood and brain homogenate", INTERNATIONAL JOURNAL OF NANOMEDICINE, vol. 9, no. Suppl.2, 15 December 2014 (2014-12-15), pages 207-215, XP055749347, ISSN: 1176-9114, DOI: 10.2147/IJN.S58203 abstract page 208, right-hand column tables 1-3 figures 1-2 page 214, left-hand column, paragraph Conclusion	1-17
X	DE 10 2011 018499 A1 (EMC MICROCOLLECTIONS GMBH [DE]) 25 October 2012 (2012-10-25) examples figures	1-17
X	WO 2019/097226 A1 (N4 PHARMA UK LTD [GB]) 23 May 2019 (2019-05-23) examples 2,3	1-17
X	ES 2 366 841 A1 (CONSEJO SUPERIOR INVESTIGACION [ES]; UNIV VALENCIA POLITECNICA ET AL.) 26 October 2011 (2011-10-26) figure 1; examples	1-17
X	WO 2006/017476 A2 (UNIV NEW YORK STATE RES FOUND [US]; PRASAD PARAS N [US] ET AL.) 16 February 2006 (2006-02-16) examples figures	1-17
X	SHAKIBA SHAHABI ET AL: "Utilizing the protein corona around silica nanoparticles for dual drug loading and release", NANOSCALE, vol. 7, no. 39, 2015, pages 16251-16265, XP055749759, ISSN: 2040-3364, DOI: 10.1039/C5NR04726A abstract figures; table 1 page 16264, left-hand column, paragraph Conclusions	1-17
X	WO 00/56288 A1 (INST NEUE MAT GEMEIN GMBH [DE] ET AL.) 28 September 2000 (2000-09-28) examples claims	1-17

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHRISTINA GRAF ET AL: "Surface Functionalization of Silica Nanoparticles Supports Colloidal Stability in Physiological Media and Facilitates Internalization in Cells", LANGMUIR, vol. 28, no. 20, 22 May 2012 (2012-05-22), pages 7598-7613, XP055749340, ISSN: 0743-7463, DOI: 10.1021/la204913t	17
Y	abstract figures; tables	1-16

X	WANG HANQI ET AL: "Amino acid-based anti-fouling functionalization of silica nanoparticles using divinyl sulfone", ACTA BIOMATERIALIA, vol. 40, 23 March 2016 (2016-03-23), pages 273-281, XP029652904, ISSN: 1742-7061, DOI: 10.1016/J.ACTBIO.2016.03.035	17
Y	abstract page 276 figures; tables	1-16

X	AGUS SETIABUDI ET AL: "Microscopic Observation of Solid-Liquid Reaction: A Novel Laboratory Approach to Teaching Rate of Reaction", INDONESIAN JOURNAL OF CHEMISTRY, vol. 17, no. 1, April 2017 (2017-04), page 119, XP055749820, ISSN: 1411-9420, DOI: 10.22146/ijc.23642	17
Y	abstract figures; tables page 261, right-hand column	1-16

X	SEONG SOO A. AN ET AL: "Surface treatment of silica nanoparticles for stable and charge-controlled colloidal silica", INTERNATIONAL JOURNAL OF NANOMEDICINE, December 2014 (2014-12), page 29, XP055261799, DOI: 10.2147/IJN.S57922	17
Y	abstract figure 6	1-16

Y	WO 2017/223085 A2 (UNIV MICHIGAN REGENTS [US]) 28 December 2017 (2017-12-28) examples	1-16

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2021/062751

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/062751

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016090603 A1	31-03-2016	US 2016090603 A1	31-03-2016
		US 2018028686 A1	01-02-2018
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		CN 111601771 A	28-08-2020
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		DE 19912502 A1	21-09-2000
		EP 1162955 A1	19-12-2001
		WO 0056288 A1	28-09-2000
WO 2017223085 A2	28-12-2017	CA 3028721 A1	28-12-2017
		EP 3471778 A2	24-04-2019
		US 2021106538 A1	15-04-2021
		WO 2017223085 A2	28-12-2017