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(54) Title: IMMUNOACTIVATING CONJUGATES COMPRISING NANOPARTICLES COATED WITH PEPTIDES

(57) Abstract: The present invention relates to an immunoactivating conjugate having colloidal stability in a medium comprising a metallic nanoparticle coated with a non-activating peptide which is ordered on the nanoparticle surface. It also relates to a pharmaceutical composition and to a process for the preparation thereof. The immunoactivating conjugates of the invention are used in the activation of the immune system, in particular the innate immune system, as well as for the modulation of immune responses, production of antibodies and detection of substances via antigen-antibody interactions.



Immunoactivating conjugates comprising nanoparticles coated with peptides

The present invention relates to an immunoactivating conjugate having colloidal stability in a medium. The immunoactivating conjugate comprises a
5 metallic nanoparticle coated with a non-activating peptide which is ordered on the nanoparticle surface. The present invention also relates to a pharmaceutical composition and to a process for the preparation of the said immunoactivating conjugate. These conjugates are used in the activation of the immune system, in particular the innate immune system, as well as for the
10 modulation of immune responses, production of antibodies and detection of substances via antigen-antibody interactions.

BACKGROUND ART

15 The immune system protects organisms from infection. Physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. However, if the innate response is not sufficient to eliminate the pathogens, vertebrates possess another layer of
20 protection, the adaptative immune system, which is triggered by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This adaptative response is then retained after the pathogen has been eliminated, in the form of immunological memory, and allows the adaptive immune system to mount faster and
25 stronger attacks each time the same pathogen is encountered.

The cells of the adaptive immune system are special types of leukocytes, called lymphocytes. B cells and T cells are the major types of lymphocytes and are derived from hematopoietic stem cells in the bone marrow. B cells are
30 involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response.

- The innate (or natural) immunity is made up of several components: physical barriers are the first line of defense against infection. The skin and mucous membranes provide a continuous surface which must be breached and back this up with mechanical protection through cilia and mucous. Physiological factors such as pH, temperature and oxygen tension limit microbial growth. The acid environment of the stomach combined with microbial competition from the commensal flora inhibits gut infection. Protein secretions into external body fluids such as lysozyme also help resist invasion. Soluble factors within the body such as complement, interferons and collectins and other "broadly specific" molecules such as C-reactive protein are of considerable importance in protection against infection. Phagocytic cells are critical in the defense against bacterial and simple eukaryotic pathogens. Macrophages and Polymorphonuclear leucocytes (PMN) can recognise bacterial and yeast cell walls through broadly specific receptors (usually for carbohydrate structures) and this recognition is greatly enhanced by activated complement (opsonin, as well as by specific antibody).
- It is well established that usually the administration of purified peptides alone is not sufficiently antigenic to elicit a strong immune response. The isolated antigen may be given together with helper substances called adjuvants. Within these adjuvants, the antigen is not modified but attracts several cells and molecules to produce a strong response. In addition, some adjuvants are able to prevent the biodegradation of the antigen.

- In other cases, in order to improve the immune response, the antigen may be in an aggregated state. Further, it has been observed that ordered-periodic antigen/epitope repetition on the surface of some substances, for example a

liposome or a virus-like particle (non-infectious empty virus capsides), also boost immune response.

In WO 2006/037979 the low immunogenicity of carbohydrates and peptide antigens is solved by attaching said carbohydrates and peptide antigens to nanoparticles including a metallic core. Here, the nanoparticles are therefore used as a drug delivery system for improving the immunogenicity of the antigens. Further, the peptides used are *per se* antigenic, that means that they are able to induce an immune response, although weak.

Levy R. et al (J. Am. Chem. Soc. 2004, vol. 126, pp. 10076-10084) describes the rational design of gold nanoparticles conjugated to a pentapeptide ligand. This article focuses on the synthesis but nothing is said regarding their use.

These references are silent on the distribution of the peptides on the surface of the nanoparticle. However, the large peptides used and the synthesis method used for the preparation of the nanoparticles would not lead to ordered structures. Here, the nanoparticles are therefore used as a drug delivery system for improving the immunogenicity of the antigens.

In the above mentioned cases, the peptides used are *per se* antigenic, that means that they are able to induce an immune response, although weak. The use of peptides *per se* shows limitations in some applications. One of them is their toxicity which may lead to inflammation. Another one is their difficult isolation from pathogens, what makes their obtaining costly, as well as the poor opportunities to rational design their physicochemical and biological properties.

Thus, despite the progress made, there is a need for providing new compounds and systems that take into account the capacity of the peptides to

act as adjuvants stimulating the innate immune response and offers more flexibility in their design.

SUMMARY OF THE INVENTION

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Inventors have found that non-activating peptides which, in themselves, do not trigger any activation of the immune system, even when they are in an aggregated state, are capable to activate the immune system, in particular the innate immune system, when ordered onto the surface of a metallic nanoparticle, thereby forming immunoactivating conjugates. Thus, these conjugates are able to achieve activation of the innate immune system, in particular the innate immune system despite their small size. The use of the immunoactivating conjugates of the invention offers the possibility to render molecules, otherwise undetectable, visible to the immune system.

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These facts may offer several advantages such as rational design of conjugates, low (or null) toxicity, low-cost and modifiable biodistribution, while avoiding the secondary effects of conventional activators of the innate immune system.

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Therefore, a first aspect of the present invention refers to an immunoactivating conjugate having colloidal stability in a medium comprising a gold, silver or platinum nanoparticle coated with at least 100 not linearly aligned molecules of a non-activating peptide which is ordered on the nanoparticle surface, the peptide comprising a terminal cysteine attached to the nanoparticle through its sulfur group, and wherein the peptide does not contain other free –SH groups and fulfills the following conditions:

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- a) the peptide is substantially unbranched;
- b) the peptide comprises from 2 to 50 amino acids; and
- c) the peptide comprises from 30 % to 80 % of hydrophobic amino acids;

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with the proviso that when the nanoparticle is a gold nanosphere, the peptide is not selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3).

The specific nanosphere AuNP-CLPFFD-NH₂ (SEQ ID NO: 1), was disclosed in "Nanoparticle-Mediated Local and Remote Manipulation of Protein Aggregation", Kogan et al, Nano Lett. 2006, vol. 6, pp.110-5, and "Gold nanoparticles for selective and remote heating of b-amyloid protein aggregates", Bastus et al, Materials Science and Engineering C 2007, vol. 27, pp. 1236-1240. These publications described that the application of local heat delivered by metallic nanoparticles selectively attached to their target can be used as a "molecular surgery" to safely remove toxic and clogging amyloid beta protein aggregates involved in the Alzheimer's disease.

The nanospheres AuNP-CLPFFD-NH₂ (SEQ ID NO: 1), AuNP-CDLPFF-NH₂ (SEQ ID NO: 2) and AuNP-CLPDFF-NH₂ (SEQ ID NO: 3) were disclosed in "How changes in the sequence of the peptide CLPFFD-NH₂ can modify the conjugation and stability of gold nanoparticles and their affinity for β -amyloid fibrils", Olmedo et al, Bioconjugate Chem 2008, vol. 19, pp. 1154-1163.

However, nothing is suggested in these references about the capacity to activate the innate immune system by non-activating peptides when ordered onto the surface of a metallic nanoparticle.

The stable colloidal immunoactivating conjugates as defined above may be administered to a vertebrate, including a mammal, including a human, in order to activate the immune system, in particular the innate immune system. Thus, another aspect of the present invention relates to pharmaceutical compositions comprising the immunoactivating conjugates as defined above together with pharmaceutically acceptable carriers.

The stable colloidal immunoactivating conjugates of the invention can be conveniently prepared by an appropriate conjugation method. Therefore, another aspect of the invention refers to a process for preparing the

conjugates as defined above comprising incubating gold, silver or platinum nanoparticles with an excess of a non-activating peptide in an aqueous solution, and removing the excess of the peptide by a mild purification technique.

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As previously described, the compositions of the invention are useful for activating the immune system, in particular the innate immune system. Thus, a further aspect of the invention relates to an immunoactivating conjugate as defined above for use as an activator of the immune system, in particular the innate immune system. Therefore, this aspect relates to the use of the immunoactivating conjugates as defined above as activators of the immune system, in particular as activators of the innate immune system. Alternatively, this aspect may also be formulated as a method for the activation of the immune system, in particular the innate immune system in a vertebrate, including a mammal, including a human, the method comprising administering to said vertebrate an effective amount of the previously defined conjugates of the invention, together with pharmaceutically acceptable carriers.

Another aspect of the invention relates to the immunoactivating conjugate as defined above for use in the production of antibodies. Therefore, this aspect relates to the use of the immunoactivating conjugates as defined above for the manufacture of a product for the production of antibodies. Alternatively, this aspect may also be formulated as a method for the production of antibodies in a vertebrate, including a mammal, including a human, the method comprising administering to said vertebrate an effective amount of the previously defined immunoactivating conjugates of the invention, together with pharmaceutically acceptable carriers.

Alternatively, this aspect may also be formulated as a process for the production of an antibody in a host vertebrate, including a mammal, including a human, comprising injecting into the host vertebrate an immunoactivating

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conjugate as defined above; and isolating a target conjugate-specific antibody.

Another aspect of the invention relates to the immunoactivating conjugate as defined above for use as immunomodulator, i.e. in the modulation of the immune response, in cases of allergy, implant rejection, autoimmune diseases and non-controlled cell proliferation. Thus, this aspect relates to the use of the immunoactivating conjugates as defined above for the manufacture of a product for use in the modulation of the immune response in cases of allergy, implant rejection, autoimmune diseases and non-controlled cell proliferation. Alternatively, this aspect may also be formulated as a method for the modulation of the immune response in cases of allergy, implant rejection, autoimmune diseases and non-controlled cell proliferation in a vertebrate, including a mammal, including a human, in need thereof, the method comprising administering to said vertebrate an effective amount of the previously defined immunoactivating conjugates of the invention, together with pharmaceutically acceptable carriers.

The particles of the invention may also be useful in the detection of substances in samples via antigen-antibody interactions. Therefore, a further aspect of the present invention relates to a process for detecting the presence of a substance in a biological sample comprising the following steps:

- a) producing an antibody by process as defined above, wherein said antibody specifically binds to the substance to be detected;
- b) contacting said antibody with the biological sample in conditions that allow the binding of the antibody to the substance to be detected; and
- c) detecting the formation of a complex antibody-substance.

These aspects of the present invention will be further described in the detailed description section that follows. Unless otherwise defined, all

technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skilled in the art to which this invention belongs. Methods and materials similar or equivalent to those described herein can be used in the practice of the present invention.

5 Throughout the description and claims the word "comprise" and its variations are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. Furthermore, the
10 present invention covers all possible combinations of particular and preferred embodiments described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 shows a schematic view of an immunoactivating conjugate according to the invention. In this case, the nanoparticle is a nanosphere.

FIG. 2 shows UV-Vis spectra, monitoring the red-shift of the surface plasmon resonance (SPR) band when the peptide coats the Au surface.

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FIG. 3 shows the ζ -potential drop as the nanoparticle surface is coated.

FIG. 4 shows a dynamic light scattering (DLS) study where a clear increase in the hydrodynamic size of the nanoparticles is observed after conjugation.

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FIG. 5 shows High Resolution Transmission Electron microscope (HRTEM) images of uncoated nanoparticles and immunoactivating conjugates.

FIG. 6 shows High-resolution XPS of Au4f, S2p and S2s spectral regions of
30 uncoated nanoparticles AuNP and immunoactivating conjugates.

FIG. 7 shows electrophoresis of uncoated nanoparticles (1), immunoactivating conjugates (2), and BSA fragments (3) in agarose gel.

FIG. 8 shows UV-Vis spectra, monitoring the red-shift of the surface plasmon resonance (SPR) band when the BSA fragments coat the Au surface.

FIG. 9 shows the ζ -potential drop (A) and a dynamic light scattering (DLS) study (B) as the nanoparticle surface is coated with BSA fragments.

FIG. 10 shows the macrophage proinflammatory response towards the control (STARV), uncoated nanoparticles and conjugates measured by Real Time PCR. Schemas are not drawn to scale.

FIG. 11 shows macrophage thymidine incorporation assay after treatment with uncoated nanoparticles, unconjugated peptides or conjugates.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, the term “conjugate” (also referred to herein as NP-peptide) refers to a gold, silver or platinum nanoparticle which is bound to a non-activating peptide through a pseudo-covalent bond, like the one occurring between S and Au (45 kcal/mol). The term “immunoactivating conjugate” means that the conjugates of the invention are immunoactivators, i.e., substances capable of activating the immune system, in particular the innate immune system, thereby generating an immune response. The term “immunoactivator” includes immunogens and adjuvants. The term “immunogen” refers to the ability of a substance for inducing an immune response. For the purposes of the invention, the term “immune response” refers to the immunological response in a vertebrate, including a mammal (including human beings and animals) against an activator. It is intended that

the term can encompass other types of immune responses, including but not limited to humoral (i.e., antibody-mediated) and cellular immune responses.

As it will be shown in detail in the examples, the immunoactivating ability of the conjugates can be tested by measuring the pro-inflammatory response of bone marrow macrophages to the conjugates (e.g. cytokines production and blockage of the macrophage proliferation).

The immunoactivating conjugates of the invention have colloidal stability in a medium. This means that the conjugate of the invention when dispersed in another medium is able to resist aggregation (i.e. precipitation). Thus, the dispersion obtained exhibits a long shelf-life and has the appearance of a solution. In a preferred embodiment, the conjugates show colloidal stability in physiologic conditions, that is, the condition or state of the body or bodily functions comprising pH close to neutral (7) and high saline concentration.

In the case of the conjugates of the invention, the stabilization can be provided either by electrostatic, steric or electro-steric interaction. This kind of stabilization depends on the size, charge and structure of the conjugated molecule as well as on the characteristics of the medium, and can be monitorized by zeta potential measurements.

When highly dense packing is obtained, what is optimal for immunogenicity, steric repulsion is then limited and a terminal charge would be beneficial for electrostatic stabilization. This ratio of electrostatic vs. steric stabilization is related to the degree of packing and charges in a particular peptide. In the present invention as far as the conjugate is stable (easily determined by UV-Vis) the only concern is to have nanoparticles coated with at least 100 not linearly aligned molecules of a non-activating peptide which is ordered on the nanoparticle surface to produce the desired immune stimulation.

The immunoactivating conjugates of the invention comprise nanoparticles (also referred to herein as NPs) made of gold, silver or platinum. These metals show a high affinity towards sulfur groups (-SH), such as the sulfur group of the amino acid side chain of cysteine. Thus, a free SH group has a high tendency to react with the metallic nanoparticle to form a pseudo-covalent bond metal-S. The strong binding between the peptide and the nanoparticle is needed to avoid desorption of the molecule or avoid competence with other terminal groups.

For this reason and except for one of the terminal amino acids of the peptide, which is cysteine, the rest of the amino acids forming the peptide may not comprise free thiol groups. However, where said thiol groups are no longer present in its free form but as protected sulfur groups, they may be present in the peptide. The skilled in the art may easily determine in each case which protecting group is the most convenient (see for example "Protective Groups in Organic Synthesis", Greene T.W. and Wuts P.G.M, 3rd Edition, June 1999, Ed. John Wiley & Sons, Chapter 4 "Protection for the Thiol Group").

In a preferred embodiment of the invention, gold nanoparticles (AuNP) are used.

Thus, the immunoactivating conjugates of the invention are stable in the sense that they do not precipitate in a medium, and in the sense that the peptide does not detach from the nanoparticle in the working environment.

For the purposes of the invention, the term "nanoparticles" (NP) refers to particles of nanometric size which may have different shapes and sizes. The size and shape of the nanoparticles are important factors which will determine that the peptides coated onto their surface give rise to an ordered domain. As regards the shape of the nanoparticles defined herein, spheres and polyhedra comprising flat faces and straight edges are comprised in the scope of the invention. Examples of such polyhedra include, without limitation, cubes, prisms and rods. In a preferred embodiment, the

nanoparticles are spheres. In a more preferred embodiment, the nanoparticles are gold nanospheres.

The size of the nanoparticle must be such that non-specific detection, as well
5 as non-inflammatory elimination of large entities in the systemic system, is avoided. In the case of nanospheres, the diameter is comprised in the range from 3 to 100 nm, preferably in the range from 10 to 50 nm. In a more preferred embodiment, the nanospheres of the invention have a diameter of about 10 nm. In the case of cubes and prisms, the size is defined in terms of
10 the sphere, inscribed inside the cube or the prism, which has the maximum diameter possible. In both cases, the diameter of said sphere is comprised in the range from 3 to 100 nm, preferably in the range from 10 to 50 nm. In a more preferred embodiment, the inscribed sphere has a diameter of about 10 nm. Further, in the case of rods, the size is 100 nm length and 15 nm width,
15 preferably 45 nm length x 15 nm width. In the most preferred embodiment, the nanoparticles of the invention are gold nanospheres having a diameter from 10 to 50 nm.

As mentioned in the summary, the peptides of the invention are *per se* non-
20 activators of the innate immune system. The term "non-activating" means that they are not capable of activating the immune system, in particular the innate immune system, generating an immune response, even when they are in an aggregated state. In fact, when comparing the effect of simple aggregating a peptide and ordering it on a nanoparticle surface, it has been demonstrated
25 that the aggregate is unable to activate the immune system, whereas the peptide conjugated to the nanoparticles activates the innate immune system even at a 100 times lower concentration (Bastus et al, ACS Nano 2009, vol. 3, pp. 1335-1344).

30 In order to activate the immune system, in particular the innate immune system, it is not necessary that the metallic nanoparticle is totally coated with

the peptide. In fact, in order to achieve an activation of the immune system, in particular the innate immune system, the nanoparticle must be coated with at least 100 not linearly aligned molecules of the non-activating peptide. The sentence "100 not linearly aligned molecules" refers to the fact that the 100 molecules should not be disposed along a line (one dimension), but rather they must occupy a two dimensional space, for example they may be distributed in a square, or a circle area. It is important that the peptide is in a regular distribution.

10 This fact has the advantage that the remaining surface of the metallic nanoparticle may be coated with other substances, which may comprise other peptides or other substances with other properties or uses. In addition the conjugates of the invention may also be loaded with distinct complementary molecules.

15 The non-activating peptides of the invention are ordered on the nanoparticle surface and may comprise natural and/or synthetic amino acids, whenever they fulfill the requirements described herein. The peptide may be modified chemically at either end to endow it with properties that will facilitate its use, for example the C-terminal end may be optionally modified with a CONH₂ group. In general, as far as the coating molecule gives a molecular order on top of the particle the intense exacerbation of the immune system, in particular the innate immune system, will be achieved.

25 In the present invention, non activating peptides are understood at concentrations in which they are still soluble. Higher concentrations beyond the point of the point of solubility that leads to precipitates may activate non specifically the immune system by physical reasons, not chemical or biological. Thus, if peptides at over-saturant concentrations may activate the immune system, these concentrations are not of therapeutic interest and therefore not considered in this invention. Therefore, the non-activating

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peptides are invisible to the immune system at any concentration up to solubility saturation. However, these peptides, if properly ordered on the surface of the nanoparticle, will activate the immune system.

- 5 As already mentioned, for the purpose of close packing structures of ordered peptides attached to the nanoparticle, the peptide must comply with certain requirements, which are described in more detail below.

The physico-chemical characterization of the order of the peptide is not
10 straightforward, however knowledge in the profusely studied self assembled monolayers of organic molecules onto gold surfaces is a good guide to control order in the NP coating. In the present invention, the experimental technique that allows to differentiate the ordered conjugates of the invention from disordered conjugates is gel electrophoresis. Thus, while ordered and
15 disordered conjugates show similar behavior in the UV-Vis, Z-Potential, TEM, XPS and DLS measurements, the migration of the ordered conjugates in the gel occurs in a narrow band. On the contrary, the migration of the disordered conjugates results in a broad band (FIG. 7). In any case, the ordered and disordered conjugates can be easily differentiated because of their response
20 to bone marrow macrophages. Thus, as it will be shown in the examples, the ordered conjugates of the invention, which are immunoactivators, increase the cytokine production and stop the macrophage proliferation, whereas disordered conjugates do not (FIG. 10 and 11).

- 25 As the first requirement, the non-activating peptide must be substantially unbranched. The term "substantially unbranched" intends to include linear peptides, as well as branched peptides which do not significantly interfere with each other. The actual degree of branching which may be allowed without affecting the order in the conjugate will be function of the size and
30 shape of the particle, the overall length of the peptide, the branched peptides, the nature of the amino acids and their ability or tendency to interact with

each other and in general it may be determined by routine screening or computer modeling.

Secondly, the peptide comprises from 2 to 50 amino acids. In a preferred embodiment, the peptide comprises from 2 to 30 amino acids. In another preferred embodiment, the peptide comprises from 5 to 50 amino acids, more preferably from 5 to 30 amino acids. In a more preferred embodiment, the peptide comprises from 5 to 15 amino acids. In the most preferred embodiment, the peptide comprises 6 amino acids.

Regarding the molecular load on the nanoparticle, generally the number of peptides per NP in a condensed (highly packed) structure oscillates between about 0.2 to about 2 nm² occupied surface per single molecule. Generally, in the case of a nanosphere having a diameter of 8 nm the load would be around 100 molecules of peptides. The load depends on the size, shape of the nanoparticle and structure of the molecule, however, in all cases will range between those values (between 0.2 to 2 nm²).

A third aspect which is important to achieve the peptide ordered on the nanoparticles surface is that the peptide comprises from about 30% to about 80% of hydrophobic amino acids, preferably from 35% to 70%. Preferably hydrophobic amino acids are placed in the middle of the peptidic chain far from the nanoparticle surface. In the case that hydrophilic peptides are present, they are preferably disposed close to the metallic surface to maintain the order. Further, good results have also been obtained with peptides comprising a higher percentage of hydrophobic amino acids, in particular from about 80 to about 90%, so that peptides comprising from about 30% to about 90% also form part of the invention.

In general, the hydrophilic and hydrophobic properties of the amino acids may be determined by using the hydropathy index proposed by Kyte and Doolittle (Kyte J, Doolittle RF, J. Mol. Biol. 1982, vol. 157, pp. 105–32).

Based on this classification, the hydropathy index for natural amino acids is shown in table 1.

Table 1

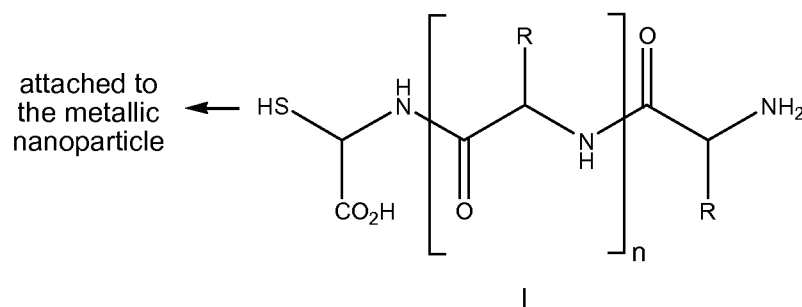
Amino acid	Hydropathy Index	Amino acid	Hydropathy Index	Amino acid	Hydropathy Index
R	-4.5	P	-1.6	M	1.9
K	-3.9	Y	-1.3	C	2.5
N	-3.5	W	-0.9	F	2.8
D	-3.5	S	-0.8	L	3.8
Q	-3.5	T	-0.7	V	4.2
E	-3.5	G	-0.4	I	4.5
H	-3.2	A	1.8		

- According to this index, the larger is the value, the more hydrophobic is the amino acid. Thus, the most hydrophobic amino acids are isoleucine (4.5) and valine (4.2), and the most hydrophilic ones are arginine (-4.5) and lysine (-3.9).
- The amino acids having a positive hydropathy index are considered as having hydrophobic properties for the purposes of the invention, whereas amino acids having a negative hydropathy index are considered as having hydrophilic properties. In general, peptides showing high values of hydropathy index promote a dense packing and peptides showing low values of hydropathy index help solubility and conjugation. Therefore, an experimental compromise has to be found as in the present invention.

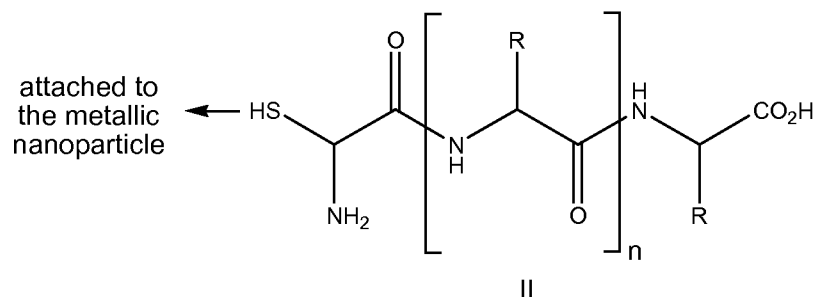
In the case of synthetic amino acids its hydrophilic and hydrophobic properties are also defined using the above cited method.

The presence of a cysteine and therefore strong binding to the nanoparticle surface in the peptide is crucial for achieving ordered domains. In one

embodiment of the invention, the terminal cysteine attached to the nanoparticle is located at the C-terminal region of the non-activating peptide. In this case, the peptide has the formula (I) depicted below, wherein each R group independently represents a side chain of a natural or synthetic amino acid, and n is a natural integer comprising values between 0 and 48. Preferably n is a natural integer comprising values between 3 and 48, more preferably between 3 and 28, even more preferably between 3 and 13.



In another embodiment of the invention, the terminal cysteine is located at the N-terminal region of the non-activating peptide, thus giving rise to a peptide of formula (II), wherein R and n have the meanings as described above.



Due to the high affinity of S to the metals Au, Ag or Pt, the conjugation is guaranteed. However, the conjugation can be optimized if the thiol is in the N-terminal region due to electrostatic interaction, i.e. positively amino N-terminal group in the vicinity of the thiol significantly accelerates thiol approach onto the nanoparticle surface. Moreover, this interaction may be additive to that of the N-terminal primary amine, since amino groups are also known to have a strong interaction with the metallic surfaces. Thus, in a preferred embodiment

the terminal cysteine attached to the nanoparticle is located at the N-terminal region of the non-activating peptide.

In a particular embodiment, the non-activating peptide to be attached to the nanoparticle surface may be an amino acid sequence, which is present in pathogens. Such amino acid sequence has to fulfil the conditions specified above and in addition preferably be present on the proteinic surface of the pathogen (virus, bacteria...) or the target molecular structure. In general, the size of the sequence has to be large enough to be selective against the pathogen and small enough to be easy to conjugate to the nanoparticle.

For the purposes of the invention, the nomenclature of the peptides used is such that the N-terminal residue is always placed on the left, while the C-terminal residue is placed on the right. Thus, in the examples of peptides mentioned above, the terminal cysteine is always placed on the N-terminal region. In addition, the peptides CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2), CLPDFF-NH₂ (SEQ ID NO: 3), CLLLLD-NH₂ (SEQ ID NO: 4), CPIVVD-NH₂ (SEQ ID NO: 5), and CFLLID-NH₂ (SEQ ID NO: 6) are modified in its C-terminal end, so that their terminal COOH function is modified to CONH₂.

The pharmaceutical compositions of the invention may be formulated as solid or liquid compositions. Such compositions will generally comprise a carrier 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 glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

In a preferred embodiment, the administration of the pharmaceutical composition is via intravenous, cutaneous or subcutaneous injection and the pharmaceutical composition is an aqueous solution which has suitable pH, isotonicity and stability.

In a preferred embodiment, the pharmaceutical composition is a vaccine. For the purpose of the present invention, the term "vaccine composition" is intended to mean a composition which can be administered to humans or to animals in order to induce a strong immune system response; this immune system response can result in a production of antibodies or simply in the activation of certain cells, in particular antigen-presenting cells, T lymphocytes and B lymphocytes. The vaccine composition can be a composition for prophylactic purposes or for therapeutic purposes, or both.

Ideally, a vaccine should be capable of stimulating antigen-specific B cells, cytotoxic T lymphocytes (CTLs) and helper T cells. B cell stimulation requires that the target antigen should bind with sufficiently high affinity to specific antigen receptors (surface Ig) on the B-cell surface and induce T and B memory cells.

As it will be shown in the examples, the immunoactivating conjugates of the invention promote the cytokine production, in particular the production of Interleukin-1 (IL-1). In the state of the art, it is well-known that the production of IL-1 is related to an adjuvancy effect desired for profilaxis and vaccination. Therefore, in another preferred embodiment, the pharmaceutical composition is an adjuvant. For the purpose of the present invention, the term "adjuvant" is intended to mean a substance that enhances, or potentiates the host's immune response to a vaccine antigen. Thus, it may be possible that an immunoactivating conjugate of the present invention, while activating the innate immune system, is not capable to generate an immune response strong enough by itself. In this case, the conjugate acting as an adjuvant is to be administered with another antigen.

The conjugates of the invention may be conveniently prepared by a process comprising incubating a gold, silver or platinum nanoparticle with an excess of a non-activating peptide in an aqueous solution, and removing the excess

of the peptide by a mild purification technique, such as dialysis or chemical destabilization (NaCl). These conditions are necessary in order to obtain a conjugate with a dense packing. If these conditions are not observed, random coatings and unstable conjugates are obtained. Aggressive purification
5 techniques such as centrifugation produces stress in the coating layer, which may spoil the order of the coating and/or lead to NP aggregation.

It also forms part of the invention an immunoactivating conjugate having colloidal stability in a medium comprising a gold, silver or platinum
10 nanoparticle coated with at least 100 not linearly aligned molecules of a non-activating peptide which is ordered on the nanoparticle surface, the peptide comprising a terminal cysteine attached to the nanoparticle through its sulfur group, and wherein the peptide does not contain other free –SH groups and fulfills the following conditions:

- 15 a) the peptide is substantially unbranched;
 - b) the peptide comprises from 2 to 50 amino acids; and
 - c) the peptide comprises from 30 % to 80 % of hydrophobic amino acids;
- with the proviso that when the nanoparticle is a gold nanosphere, the peptide is not selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1),
20 CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3);
obtainable by the process comprising incubating a gold, silver or platinum nanoparticle with an excess of the non-activating peptide in an aqueous solution, and removing the excess of the peptide by a mild purification technique.

25 In a preferred embodiment, the excess of peptide used is such that the ratio peptide: nanoparticle is 100:1.

Moreover, other parameters such as the time needed for conjugated may be
30 optimized. In fact, it is known that 80 to 90 percent of the nanoparticle surface is almost immediately coated with the peptide molecules in the initial few

minutes, leading to a random coating. However, to go from the 90% of coverage to the 100%, which implies going from the random coating to the ordered coating, needs more time.

- 5 Generally, the conjugation conditions have to be compatible with the chemical stability of the peptides, that is pH close to neutral and temperature between 4 to 40 °C, preferably 37°C.

10 Further, the medium used for conjugation may also help the conjugation process. To promote full coverage, the peptide molecules carrying a hydrophobic domain may present an extended conformation (at least during significant time). This may be achieved by using a medium containing a rather low electrolyte concentration, for instance 2.2 mM of sodium citrate in MilliQ-deionized water.

15 The uncoated metallic nanoparticles may be prepared by using synthesis protocols for nanoparticles that allow the simple and scalable production of monodisperse nanoparticles with control of size and shape. In particular the nanoparticles are prepared by rapid injection of a metallic salt selected from a salt of Au, Ag and Pt in a reducing agent, thus producing a temporally discrete homogeneous nucleation employed for the production of monodisperse metallic nanoparticles. The reducing agent may be, for example citrate at high temperature (classical Turkevitch method), sodium borohydride or a mixture of sodium borohydride and ascorbic acid, optionally
20 in the presence of Cetyl Trimethyl Ammonium Bromide (CTAB).

The formation of metallic nanoparticles may be observed by a change in colour in the reaction medium. Depending on the method used the nanoparticles obtained will have a different size and shape in the presence of
30 the right surfactants as CTAB.

The non-activating peptides may be isolated from pathogens by standard methods or may be synthesized by peptide synthesis technology well-known in the art. The peptides may be synthesized either chemically or recombinantly.

5

The immunoactivating conjugates of the invention may be used as activators of the innate immune system, in particular the innate immune system. In a preferred embodiment, the immunoactivating conjugates may be used as adjuvants. In a preferred embodiment, the conjugate used is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3), CLLLLD-NH₂ (SEQ ID NO: 4), CPIVVD-NH₂ (SEQ ID NO: 5), and CFLLID-NH₂ (SEQ ID NO: 6).

10

In another aspect of the invention, the conjugates are used in the production of antibodies in a vertebrate. The term "antibody" refers to a Y-shaped protein (known as immunoglobulin) on the surface of B cells that is secreted into the blood or lymph in response to an antigenic stimulus, such as an exogenous protein, bacterium, virus, parasite, tumor cell or transplanted organ, and that exhibits a specific binding activity for a target molecule called an "antigen".

15

20

The term "antigen" as used herein refers to a molecule which can initiate an immune response. Antigens can be any type of biologic molecule including, for example, viral antigens, bacterial antigens, fungal antigens, protozoa and other parasitic antigens, tumor antigens, antigens involved in autoimmune disease, allergy and graft rejection, and other miscellaneous antigens.

25

The term "antibody" includes monoclonal and polyclonal antibodies, either intact or fragments derived from them; and includes human antibodies, humanized antibodies and antibodies of non-human origin. The term "specific antibody" refers to an antibody generated against a specific antigen. The term "antibody-protein complex" refers to a complex formed by an antigen and its specific antibody.

30

The conjugates of the invention are also useful as immunomodulators in cases of allergy, implant rejection, autoimmune diseases and non-controlled cell proliferation. The term "immunomodulator" refers to the ability of a substance to modulate of the immune response in a vertebrate, including a mammal, including a human.

Allergies contemplated to be treated include all IgE and IgG allergies, hyper IgE syndrome, and dermatic conditions such as atopic dermatitis. It is also contemplated that the claimed methods can be used to treat transplant rejection, (graft vs. host disease) and implant reactions.

Autoimmune diseases can be classified as either non-organ-specific or organ-specific. Non-organ-specific autoimmune diseases include rheumatoid arthritis, gout and gouty arthritis, Systemic Lupus Erythematosus (SLE), Sjogren syndrome, scleroderma, polymyositis and dermatomyositis, ankylosing spondylitis, and rheumatic fever.

Organ-specific autoimmune diseases are known for virtually every organ, including insulin-dependent diabetes, thyroid diseases (Graves disease and Hashimoto thyroiditis), Addison disease, and some kidney and lung diseases including allergy and asthma, multiple sclerosis, myasthenia gravis, uveitis, psoriasis, forms of hepatitis and cirrhosis, celiac disease, inflammatory bowel disease, and some types of male and female infertility.

Autoimmune processes may also be stimulated by viral infections including the HIV virus, may result from rejection of transplantation, and may accompany certain tumors, or be precipitated by exposure to some chemicals.

In another embodiment, the conjugates of the invention are used as immunomodulators in cases of non-controlled cell proliferation, such as in cases leading to cancer.

In a preferred embodiment, the immunoactivating conjugates used for any of the applications described above, i.e. activation of the innate immune system,

production of antibodies and modulation of the immune response in cases of allergy, implant rejection, autoimmune diseases and non-controlled cell proliferation are those wherein the peptide attached to the nanoparticle is selected from the group consisting of: CLPFFD-NH₂ (SEQ ID NO: 1),
5 CDLPFF-NH₂ (SEQ ID NO: 2), CLPDFF-NH₂ (SEQ ID NO: 3), CLLLLD-NH₂ (SEQ ID NO: 4), CPIVVD-NH₂ (SEQ ID NO: 5), and CFLLED-NH₂ (SEQ ID NO: 6). In a more preferred embodiment, the peptide attached to the nanoparticle is selected from the group consisting of: CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3). In an even
10 more preferred embodiment, the conjugate used is that wherein the peptide attached to the nanoparticle is CLPFFD-NH₂ (SEQ ID NO: 1).

In another preferred embodiment, the immunoactivating conjugates used for any of the applications described above are those wherein the nanoparticle is
15 a gold nanoparticle. In a more preferred embodiment, the nanoparticle is a nanosphere, preferably a gold nanosphere, and more preferably a gold nanosphere has a diameter from 10 to 50 nm.

In another preferred embodiment, the immunoactivating conjugates used for
20 any of the applications described above are those which exhibit colloidal stability in a physiological medium. In another preferred embodiment, the terminal cysteine comprised in the peptide and attached to the nanoparticle is located at the N-terminal region.

25 The immunoactivating conjugates of the invention may also be used for producing antibodies which are later on used for detecting the presence of substances in a biological sample. In this case, part of the peptidic structure of the substance to be detected must be isolated using conventional methods. This part of the sequence, if necessary, may be coupled with a cysteine in
30 order to have a terminal cysteine, used for the attachment to the metallic nanoparticle and a hydrophobic external sequence to maximize packing with

a charged amino acid at the end. The conjugate is obtained according to the process described above and is used for obtaining antibodies specific for the isolated region of the substance to be detected as already described. Finally the incubation of these antibodies in conditions that allow their binding to the substance to be detected; and detection of the bound antibody allows to obtain a measure of the amount of the substance to be detected.

In order to carry out the detection of this complex, detectable markers may be used, including metals, isotopes, radioisotopes, chromophores, fluorophores and the like.

EXAMPLES

The following examples are provided for illustrative means, and are not meant to be limiting of the present invention.

The following abbreviations have been used:

CTAB: Cetyl Trimethyl Ammonium Bromide

DIEA: N,N-diisopropylethylamine

DMF: N,N-dimethylformamide

Fmoc: 9-fluorenylmethoxycarbonyl

HBTU: 2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt: 1-hydroxybenzotriazole

PB: phosphate buffer

Pbf: 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl

PyBOP: benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate

TBTU: 2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate

tBu: tert-butyl

TEA: triethylamine

TFA: trifluoroacetic acid

TIS: triisopropylsilane

Characterization techniques

5

1) UV-Vis Spectroscopy

UV-Visible spectra were acquired with a Shimadzu UV-2400 spectrophotometer. 1 mL of nanoparticles or conjugates were placed in a cell, and spectral analysis was performed in the 300 nm to 800 nm range.

10

2) Dynamic light scattering (DLS) studies

For the DLS studies, a Malvern ZetaSizer Nano ZS instrument (Malvern Instruments, UK) operating at a light source wavelength of 532 nm and a fixed scattering angle of 173° was used to measure nanoparticles and conjugates size. These measurements were conducted with 1 cm optical path cell and precise control of temperature (25°C).

15

3) Zeta Potential

The zeta potential of nanoparticles and conjugates was determined using a Malvern ZetaSizer Analyzer (Malvern Instruments, UK). These measurements were performed with control of the pH (7.0).

20

4) Transmission Electron Microscopy (TEM)

Nanoparticles and conjugates were visualized using 80-keV TEM (JEOL 1010, Japan). Three 20 µL droplets of the sample were drop casted onto a piece of ultrathin formvar-coated 200-mesh copper grid (Ted Pella, Moncomp, Madrid, Spain) and left to dry in air. Cells incubated for 15 min, 30min, 1h, 2h, 6h, 12h and 24h were fixed with a glutaraldehyde 2.5% solution in PB 0.1 M for 1 h. Cells were then scraped, centrifuged - 4 °C, 2500 rpm, 10 min. and washed with PB 0.1 M. After staining with OsO₄ 1% for 1 h, cells were dehydrated in acetone at 4 °C and embedded in Spurr epoxy resin. After infiltration (60 °C, 48 h), 50 nm ultra-thin sections were made using an

25

30

ultramicrothom. Finally, cells were stained with uranyl acetate and lead citrate.

5) X-Ray Photoelectron Spectroscopy (XPS)

- 5 For XPS, 10 μ L of a solution of nanoparticles or conjugates was placed on a silicon nitride surface and analysed using PHI ESCA-5500 equipment. A monochromatic Al K_α X-ray source was used and the chamber was maintained below 2×10^{-9} Torr. Spectra were analysed using Multipak software.

10 6) Electrophoresis

Submarine gel electrophoresis was performed in 1.5% agarose gels (Molecular Biology grade, Roche) in buffer TEA 1X. The field strength was held constant at 120 V and the current was 97 mA.

15 Synthesis of the metallic nanoparticles

Gold nanospheres were synthesized using the following methods:

1) Citrate-reduction Method

- 20 The standard method, as described by Turkevich and Frens, is the reduction of hydrogen tetrachloroaurate at 100°C. The reduction of hydrogen tetrachloroaurate has been initiated by bringing a sodium citrate solution (150 mL, 2.2 mM) to boil in a three-neck round-bottom flask. When the solution started to boil, 1 mL of hydrogen tetrachloroaurate solution (23.4 mM) was added. The presence of a colloidal suspension was detected by the reflection of a laser beam from the particles. This method yielded AuNPs having a diameter of 10 nm.
- 25

Slightly variations of hydrogen tetrachloroaurate to citrate ratio yielded particles to 8 to 14 nm.

30

Thus, when carrying out the same procedure under identical conditions but injecting 1 mL of 11.7 mM HAuCl₄ into 150 mL of 2.2 mM sodium citrate (ratio

citrate:HAuCl₄ 6.9:1), gold nanoparticles having a diameter of 8 nm were obtained; and when injecting 1 mL of 46.8 mM HAuCl₄ into 150 ml of 2.2 mM sodium citrate (ratio citrate:HAuCl₄ 28.2:1), gold nanoparticles having a diameter of 14 nm were obtained.

5

2) Sodium borohydride-reduction Method

A 20 mL aqueous solution containing $2.5 \cdot 10^{-4}$ M HAuCl₄ and $2.5 \cdot 10^{-4}$ M trisodium citrate was prepared in a conical flask. Next, 0.6 mL of ice-cold, freshly prepared 0.1M NaBH₄ solution was added to the solution while stirring. The solution turned pink immediately after adding NaBH₄, indicating particle formation. This method yielded nanospheres having a diameter of 4nm.

10

3) Seeding-growth Method

15 i) Preparation of gold seeds

CTAB solution (5 mL, 0.20 M) was mixed with 5.0 mL of 0.00050M HAuCl₄. To the stirred solution, 0.60 mL of ice-cold 0.010 M NaBH₄ was added, which resulted in the formation of a brownish yellow solution. Vigorous stirring of the seed solution was continued for 2 min. After the solution was stirred, it was kept at 25 °C.

20

ii) Growth of gold nanoparticles.

CTAB (5 mL, 0.20 M) was added to 5.0 mL of 0.0010 M HAuCl₄, and after gentle mixing of the solution 70 µL of 0.0788 M ascorbic acid was added. Ascorbic acid as a mild reducing agent changes the growth solution from dark yellow to colorless. The final step was the addition of 12 µl of the seed solution to the growth solution at 27-30 °C. The color of the solution gradually changed within 10-20 min. The temperature of the growth medium was kept constant at 27-30 °C. This method yielded nanospheres having a diameter of 30 nm. Following this method, and increasing the HAuCl₄ concentration nanospheres having a diameter of 50 nm were obtained.

25

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Synthesis of the peptides

All peptides were characterised by MALDI-TOF mass spectrometry (Vogayer-DE RP MALDI-TOF, PE Biosystems with a N2 laser of 337 nm).

- 5 6 different peptides were synthesized: CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2), CLPDFF-NH₂ (SEQ ID NO: 3), CLLLLD-NH₂ (SEQ ID NO: 4), CPIVVD-NH₂ (SEQ ID NO: 5), and CFLLID-NH₂ (SEQ ID NO: 6).
- 10 All peptides were synthesised by solid phase synthesis using the Fmoc/tBu strategy and a multiple Advanced Chemtech 496 synthesiser. Rink amide resin, N α -Fmoc-protected amino acids (4eq.)/HBTU(4 eq.)/HOBT(4 eq.) and DIEA(8 eq.) were used. The Fmoc protecting group was cleaved by treatment with a solution of 25% piperidine in DMF (1 x 5 min, 1 x 15 min). Peptides
- 15 were cleaved from the resin by treatment with 95% TFA, 2.5% TIS, 2.5% water for 2h.

Synthesis of the immunoactivating conjugates

- 20 Conjugation process was performed incubating the gold nanospheres having a diameter of 8 nm as obtained above with addition of an excess of the N-terminal cysteine peptide in a 100:1 peptide:nanoparticle ratio in a 2,2 mM of sodium citrate aqueous solution for 30 min. Afterwards, three days of dialysis were carried out to remove the unbound peptide (1 mL conjugated specie vs.
- 25 5000 mL MQ water).

Thus, the following conjugates were obtained:

- Example 1: AuNP (8 nm)-CLPFFD-NH₂ (SEQ ID NO: 1)
Example 2: AuNP (8 nm)-CDLPFF-NH₂ (SEQ ID NO: 2)
30 Example 3: AuNP (8 nm)-CLPDFF-NH₂ (SEQ ID NO: 3)
Example 4: AuNP (8 nm)-CLLLLD-NH₂ (SEQ ID NO: 4)

Example 5: AuNP (8 nm)-CPIVVD-NH₂ (SEQ ID NO: 5)

Example 6: AuNP (8 nm)-CFLLED-NH₂ (SEQ ID NO: 6)

5 Comparative examples: synthesis of disordered, non-immunoactivating conjugates

1) Conjugates with peptides

Conjugation process was performed incubating the gold nanospheres having a diameter of 8 nm as obtained above with addition of an excess of the N-
10 terminal cysteine peptide in a 10:1 peptide:nanoparticle ratio in a 2,2 mM of sodium citrate aqueous solution for 30 min. Afterwards, centrifugation (5 min at 12.000 RPM (16.000 g)) was carried out to remove the unbound peptide.

Comparative example 1: AuNP (8 nm)-CLPFFD-NH₂ (SEQ ID NO: 1)
15 Comparative example 2: AuNP (8 nm)-CPIVVD-NH₂ (SEQ ID NO: 5)
Comparative example 3: AuNP (8 nm)-CFLLED-NH₂ (SEQ ID NO: 6)

2) Conjugate with BSA (AuNP-BSA)

As conjugation of the peptides to the nanoparticles generates a homogenous
20 distribution of peptides on the nanoparticles, as a control, a mixture of proteic residues obtained from the reduced digest of albumin (BSA) was also used in order to obtain a 100% peptidic random coat with respect to the nanoparticles coated by medium molecules.

25 In detail, BSA (Cohn Fraction V, Fluka) was degraded with trypsin (trypsin from bovine pancreas, E.C. 3.4.21.4, Roche). BSA was incubated at 37 °C dissolved in Tris·HCl 100 mM at 150 µM, and trypsin was added at the final concentration of 35.8 mg/mL (3.94 U/mL, U: Chromozym Try as a substrate). After 24h at 37 °C, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was
30 obtained from Fluka at a final concentration of 10.5 mM was added and incubated during 1h 30 min at RT, in order to reduce disulphide bonds. This

solution was conjugated to gold nanospheres having a diameter of 8 nm with the same protocol used for the different peptides.

All experiments were made in strictly LPS-free conditions. All the conjugates were stable through all the experiments.

5

The conjugates obtained above were characterized by the following techniques: UV-Vis spectroscopy (UV-Vis), Zeta potential (Z-POT), dynamic light scattering (DLS), High Resolution Transmission Electron microscope (HRTEM), and Electrophoresis (Table 2 and FIG. 2-9).

10

Table 2

Product	UV-Vis	DLS	Z-POT
AuNP	518	17.90	-60
Example 1	526	22.43	-12
Example 5	523	20.60	-9
Example 6	525	20.06	-11
Comparative example 1	522	20.57	-66
Comparative example 2	520	19.15	-63.6
Comparative example 3	522	19.31	-43.8

As it can be seen in table 2, the ordered conjugates (examples 1, 5 and 6) show values of UV-Vis, DLS and Z-POT farther from the uncoated nanoparticles (AuNP) than the corresponding disordered conjugates comprising the same peptides (comparative examples 1, 2 and 3).

15

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FIG. 2 shows UV-Vis spectra (absorbance vs. wavelength), monitoring the red-shift of the surface plasmon resonance (SPR) band. The red-shift in the position of the SPR band verifies the conjugation process. In particular, FIG. 2A shows immunoactivating conjugates of examples 2 and 3; and FIG. 2B shows conjugates of examples 1, 5 and 6.

FIG. 3 shows the ζ -potential drop (intensity vs. Zeta potential) as the nanoparticle surface is coated. The nanoparticle surface charge varies from negative to neutral when conjugated to peptides. FIG. 3 shows immunoactivating conjugates of examples 2 and 3.

5

FIG. 4 shows a dynamic light scattering (DLS) study (volume vs. size) where a clear increase in the hydrodynamic size of the nanoparticles is observed after conjugation. The attachment of the peptides via the cysteine changes the dielectric environment of the gold nanoparticle producing an observable red-shift of the SPR of about 8-10 nm. FIG. 4 shows immunoactivating

10

FIG. 5 shows HRTEM images of uncoated nanoparticles (A) and conjugates of example 1 (B).

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FIG. 6 shows High-resolution XPS of Au4f, S2p and S2s spectral regions (C/s vs binding energy) of uncoated nanoparticles (AuNP, left) and conjugates of example 1 (right).

20

FIG. 7 shows electrophoresis of uncoated nanoparticles (1); conjugates of example 1 (2); and conjugates with BSA fragments (3) in agarose gel. Run was performed in 10 mM phosphate buffer pH 7.4 in 1.2 mM citrate at 120 V for 8 min. AuNPs presented aggregation in the running conditions in contrast with the conjugate of example 1. Ordered conjugates (example 1) migrated in a narrow band, while disordered conjugates (AuNP-BSA) migrated in a broad band.

25

FIG. 8 shows UV-Vis spectra (absorbance vs wavelength), monitoring the red-shift of the surface plasmon resonance (SPR) band when the BSA fragments coat the Au surface.

30

FIG. 9 shows the ζ -potential drop (intensity vs Zeta potential) (A) and a dynamic light scattering (DLS) study (volume vs size) (B) as the nanoparticle surface is coated with BSA fragments.

5 BIOLOGICAL TESTS

Bone marrow macrophages were used as an *in vitro* cells model of the innate immune system. Such cells proliferate in the presence of their specific growth factor M-CSF, while in the presence of pathogens, microbial substances, or
10 determined ligands, they stop proliferating and acquire effector functions, involving the production of pro-inflammatory cytokines and the induction of nitric oxide synthase (NOS2) in order to eliminate invaders. Thus, the potential pro-inflammatory response of macrophages to NPs conjugates and controls was studied analyzing i) cytokines production and ii) blockage of
15 macrophage proliferation.

Macrophage cultures

1) Cell Culture

20 Experiments were carried out with primary cultures of bone marrow-derived macrophages obtained from BALB/c mice (Harlan Ibérica, Barcelona, Spain) and cultured over 6 days at 37°C and 5% CO₂ incubators in Dulbecco's Modified Eagle's Medium DMEM, BioWhittaker-Cambrex, Emerainville, France), supplemented with 20% fetal-calf-serum (FCS) and 30% L-cell-
25 conditioned media. Exclusively for TLR experiments, C3H and C3H/HeJ mice were obtained from Charles River (Santa Perpetua de Mogoda, Spain). To render macrophages quiescent, they were deprived of Macrophage colony-stimulating factor (M-CSF) for 18 h before stimulation.

30 2) Proliferation Assays.

1,5x10⁵ cells were seeded in 24 well plates in DMEM plus 10% Fetal calf serum (FCS). After 24h of treatment with the indicated substances, cells were

pulsed with ^3H -Thymidine (1 $\mu\text{Ci}/\text{mL}$) (Amersham Pharmacia Biotech, Piscataway, NJ) for 6 h. Then cells were fixed with methanol (MeOH) 70% at 4°C overnight. Three washes of trichloroacetic acid (TCA) 10% were performed and cells were lysed with NaOH plus sodium dodecyl sulfate (SDS). ^3H incorporation was measured by standard liquid scintillation. Each point was performed in triplicate and the results are expressed as the mean $\pm$ S.D.

3) Limulus Lysate Assays.

The presence of LPS was determined using an LAL test with sensitivity limits of 0.03-0.1 endotoxin units (EU)/ml as indicated in manufacturer's instructions for E-Toxate (Sigma, St Louis, MI). Lipopolysaccharides (LPS) from 400 to 0.03 EU/ml was used as endotoxin standard to test our samples at the doses used in the rest of the experiments.

4) RNA extraction and real-time PCR.

Total RNA was extracted with the RNA Kit EZ-RNA (Biological Industries, Kibbutz beit haemek, Israel) as indicated. cDNA was obtained from 1 μg of total RNA using MMLV reverse transcriptase (Promega, Madison, WI). Primer Express software (Applied Biosystems) was used to design primer sequences for TNF- α , IL-1 β , IL-6, and NOS2. Specific primer pairs were as follows: CCTTGTTGCCTCCTCTTTT GC and TCAGTGATGTAGCGACAGCCTG for TNF- α , CCTGTGTTTTCTCCTTGCCT and CCTAATGTCCCCTTGAATCAA for IL-1 β , CAGAAGGAGTGGCTAAGGACCA and ACGCACT AGGTTTGCCGAGTAG for IL-6, GCCACCAACAATGGCAACA and GTACCGGATGAGCTGTG AATT for NOS2, ACTATTGGCAACGAGCGGTTC and AAGGAAGGCTGGAAAAGAGCC for β -actin, forward and reverse, respectively. Real-time PCR was carried out with a 2X SYBR Green PCR Master Mix using the ABI Prism 7900 detection system (Applied Biosystems, Foster city, CA). Thermal cycling conditions were as follows: 94°C 30s, 60°C 30s, and 72°C 30s for 35 cycles. Each sample was analyzed in triplicate.

Expression levels were normalized to β -actin. Relative values from a representative experiment based on three independent experiments are shown.

5 Cytokines production

Classical macrophage activation, typically triggered involves the RNA induction of proinflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 , as well as nitric oxide synthase (NOS2) in order to eliminate microorganisms. Briefly, macrophages were exposed to the conjugates of the invention for 6h
10 and then left for 24 h. As a positive control, Lipopolysaccharides LPS at subsaturant concentrations was used. Transcriptional induction of these molecules was analyzed by real-time PCR of extracted RNA under strictly LPS-free conditions. mRNA levels of $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 and NOS2 were measured in relation to β -actin of macrophages stimulated for 6 hours with
15 LPS (subsaturant doses, 10 ng/ml) in the presence of M-CSF.

In FIG. 10 (C/s vs binding energy) results showed that neither uncoated nanoparticles (AuNP) nor the unconjugated peptides (CLPFFD-NH₂ (SEQ ID NO: 1) (FIG. 10A), CLPDFF-NH₂ (SEQ ID NO: 3) (FIG. 10B)) activated any
20 pro-inflammatory response since both the cytokines and NOS2 levels were similar to the untreated starved control. However, an exacerbated cytokine and nitric oxide synthase induction, in comparison to low doses of LPS, was observed in the case of the conjugates of example 1 (AuNP-CLPFFD-NH₂ (SEQ ID NO: 1)) (FIG. 10A), and example 3 (AuNP-CLPDFF-NH₂ (SEQ ID
25 NO: 3)) (FIG. 8C) indicating a classical macrophage proinflammatory response.

Noticeable, the particular type of pro-inflammatory response seemed to be somehow dependent on the peptide sequence, as also happens in presence
30 of lipopolysaccharides from different bacterial species. By this way, although all conjugates induced the synthesis of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ typically implicated

in fever, osteolysis, leucopenia and hypotension, only the conjugate of example 1 (AuNP-CLPFFD-NH₂ (SEQ ID NO: 1)) was able to induce IL-6 that plays a role in inflammation mainly as an inducer of acute phase proteins.

5 Blockage of macrophage proliferation

Macrophage proliferation was assessed by radioactive thymidine incorporation. Starved macrophages (STARV) did not proliferate until the addition of their growth factor M-CSF. Then DNA synthesis was induced, what correlated with an increased number of cells. Accordingly with the pro-
10 inflammatory response induced by AuNP conjugates, macrophage proliferation in presence of M-CSF was not affected by either citrate stabilized AuNP or unconjugated peptides (CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2), CLPDFF-NH₂ (SEQ ID NO: 3)), whereas the corresponding conjugates completely blocked proliferation, underscoring the biological
15 relevance of conjugation.

FIG. 11 shows macrophage thymidine incorporation assay of uncoated nanoparticles, unconjugated peptides and immunoactivating conjugate of example 1 (FIG. 11A); immunoactivating conjugate of example 2 (FIG. 11B);
20 immunoactivating conjugate of example 3 (FIG. 11C); and conjugate with BSA (FIG. 11D). Resting macrophages were stimulated with 1200 U/ml of M-CSF in the presence of the indicated substances (1μM). After 24 hours, thymidine incorporation was measured.

25 Similar results were found in all cases, the ordered conjugates inhibit the macrophage proliferation while the unconjugated peptides, uncoated AuNP, or disordered conjugates did not. Further results are shown in table 3.

Table 3

<u>Product</u>	<u>Inhibition of the macrophage proliferation</u>
AuNP	NO
Example 1	SI
Comparative example 1	NO
Example 5	SI
Comparative example 2	NO
Example 6	SI
Comparative example 3	NO

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CLAIMS

1. An immunoactivating conjugate having colloidal stability in a medium comprising a gold, silver or platinum nanoparticle coated with at least 100 not
5 linearly aligned molecules of a non-activating peptide which is ordered on the nanoparticle surface, the peptide comprising a terminal cysteine attached to the nanoparticle through its sulfur group, and wherein the peptide does not contain other free –SH groups and fulfills the following conditions:
- a) the peptide is substantially unbranched;
 - 10 b) the peptide comprises from 2 to 50 amino acids; and
 - c) the peptide comprises from 30% to 80% of hydrophobic amino acids;
- with the proviso that when the nanoparticle is a gold nanosphere, the peptide is not selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3).
- 15
2. The immunoactivating conjugate according to claim 1, wherein the peptide comprises from 5 to 15 amino acids.
3. The immunoactivating conjugate according to any of the claims 1-2,
20 wherein the nanoparticle is a gold nanoparticle.
4. The immunoactivating conjugate according to any of the claims 1-3, wherein the nanoparticle is a nanosphere having a diameter from 10 to 50 nm.
- 25
5. The immunoactivating conjugate according to any of the claims 1-4, which has colloidal stability in a physiological medium.
6. The immunoactivating conjugate according to any of the claims 1-5,
30 wherein the terminal cysteine attached to the nanoparticle is located at the N-terminal region.

7. The immunoactivating conjugate according to claim 1, wherein the peptide attached to the nanoparticle is CLLLLD-NH₂ (SEQ ID NO: 4) or CFLLED-NH₂ (SEQ ID NO: 6).

5

8. The immunoactivating conjugate according to claim 1, wherein the peptide attached to the nanoparticle is CPIVVD-NH₂ (SEQ ID NO: 5).

9. A pharmaceutical composition comprising an immunoactivating conjugate as defined in any of the claims 1-6 together with pharmaceutically acceptable carriers.

10

10. The composition according to claim 9, which is administered by intravenous, subcutaneous or intramuscular injection.

15

11. The composition according to any of the claims 9-10, which is a vaccine or an adjuvant.

20

12. A process for preparing the immunoactivating conjugate as defined in any of the claims 1-6 comprising incubating a gold, silver or platinum nanoparticle with an excess of a non-activating peptide in an aqueous solution, and removing the excess of the peptide by a mild purification technique.

25

13. The process according to claim 12, wherein the technique for removing the excess of the peptide is dialysis.

30

14. An immunoactivating conjugate as defined in any of the claims 1-6, including the conjugate wherein the nanoparticle is a gold nanosphere and the peptide is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFD-NH₂ (SEQ ID NO: 3), for use as an activator of the innate immune system.

15. The immunoactivating conjugate according to claim 14, for use as an adjuvant.
- 5 16. The immunoactivating conjugate for use as an adjuvant according to claim 15, wherein the peptide attached to the nanoparticle is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3), and CPIVVD-NH₂ (SEQ ID NO: 5).
- 10 17. The immunoactivating conjugate for use as an adjuvant according to claim 15, wherein the peptide attached to the nanoparticle is selected from CLLLLD-NH₂ (SEQ ID NO: 4) or CFLLID-NH₂ (SEQ ID NO: 6).
- 15 18. An immunoactivating conjugate as defined in any of the claims 1-6, including the immunoactivating conjugate wherein the nanoparticle is a gold nanosphere and the peptide is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3), for use in the production of antibodies.
- 20 19. An immunoactivating conjugate as defined in any of the claims 1-6, including the conjugate wherein the nanoparticle is a gold nanosphere and the peptide is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1), CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3), for use as immunomodulator in cases of allergy, implant rejection, autoimmune
- 25 diseases and non-controlled cell proliferation.
20. Process for the production of an antibody in a host vertebrate, including a mammal, including a human, comprising injecting into the host vertebrate an immunoactivating conjugate as defined in any of the claims 1-6 including the
- 30 conjugate wherein the nanoparticle is a gold nanosphere and the peptide is selected from the group consisting of CLPFFD-NH₂ (SEQ ID NO: 1),

CDLPFF-NH₂ (SEQ ID NO: 2) and CLPDFF-NH₂ (SEQ ID NO: 3); and isolating the target conjugate-specific antibody.

21. Process for detecting the presence of a substance in a biological sample comprising the following steps:

- a) producing an antibody by process as defined in claim 20, wherein said antibody specifically binds to the substance to be detected;
- b) contacting said antibody with the biological sample in conditions that allow the binding of the antibody to the substance to be detected; and
- c) detecting the formation of a complex antibody-substance.

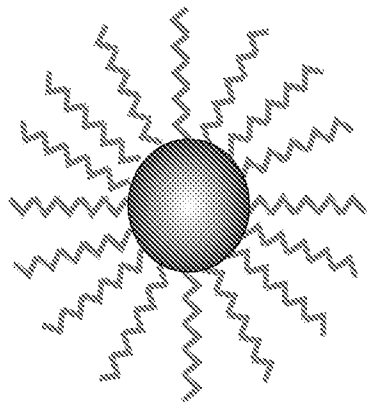


FIG.1

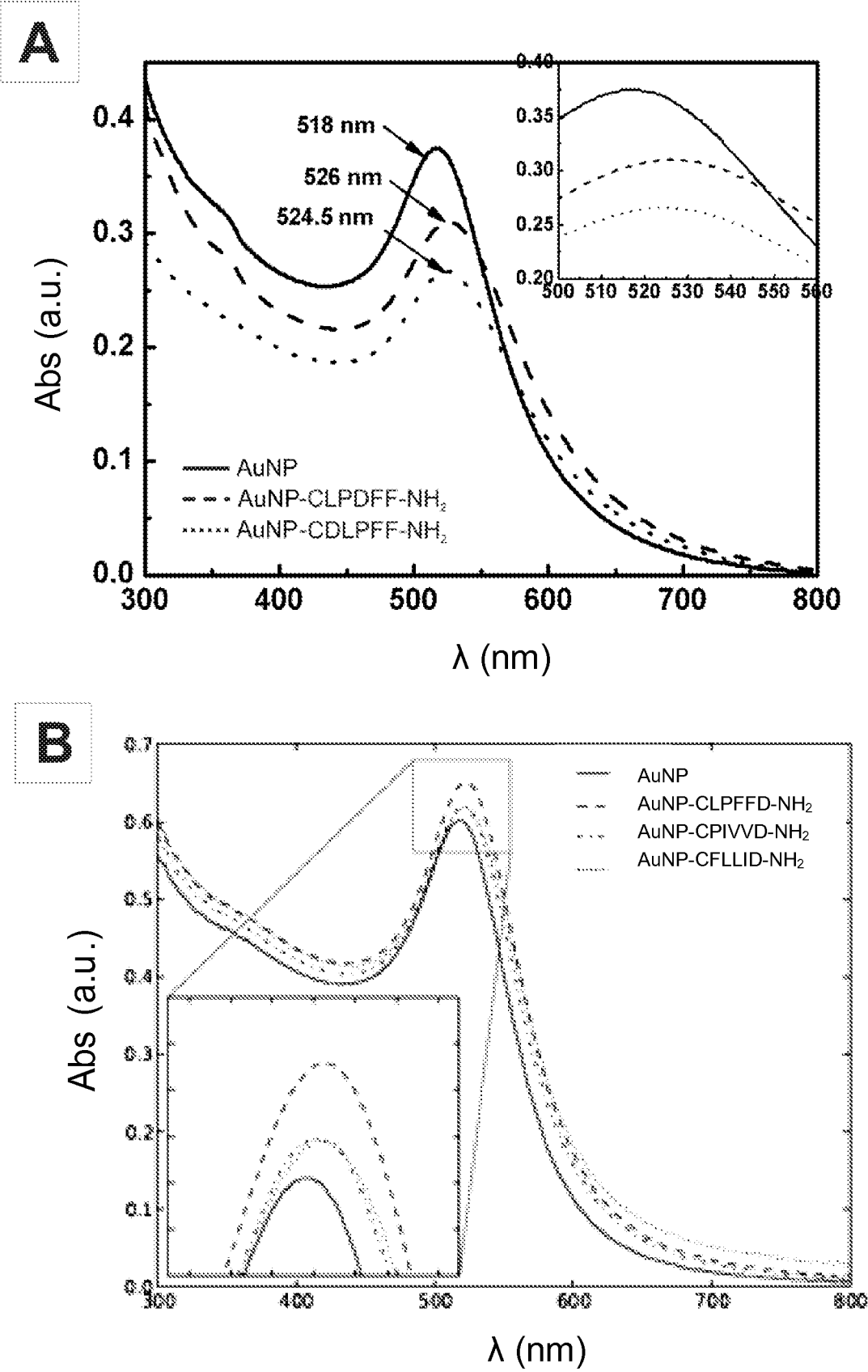


FIG.2

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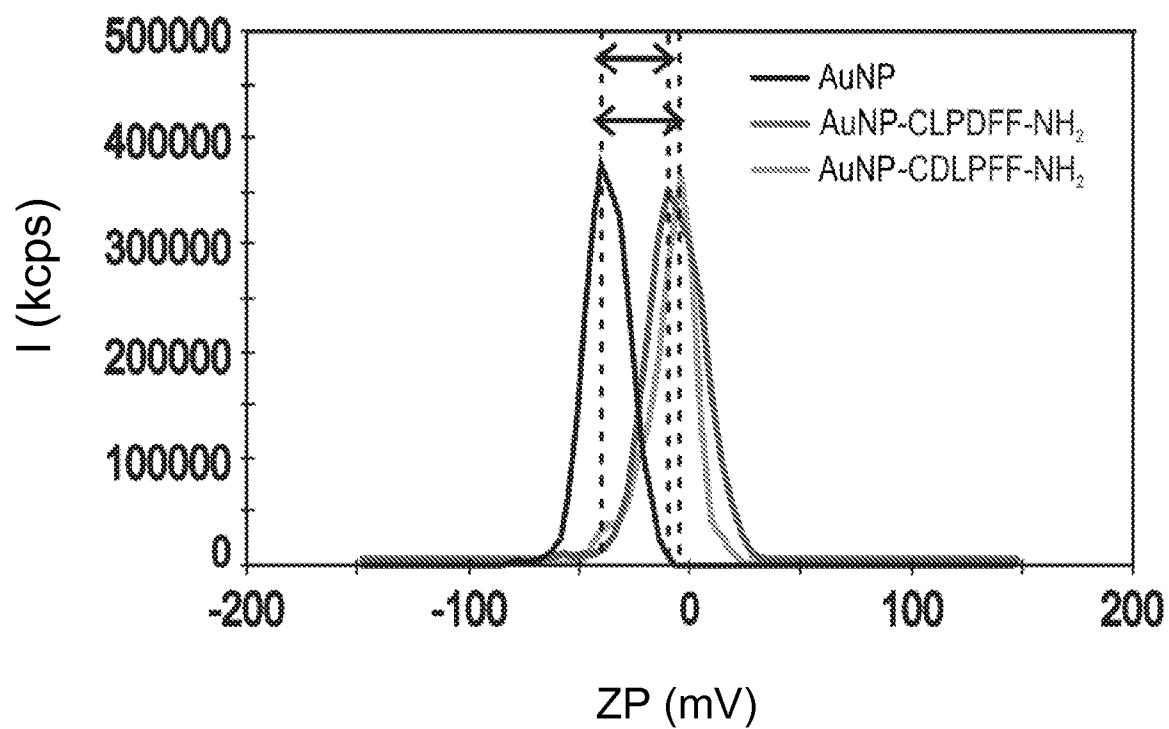


FIG.3

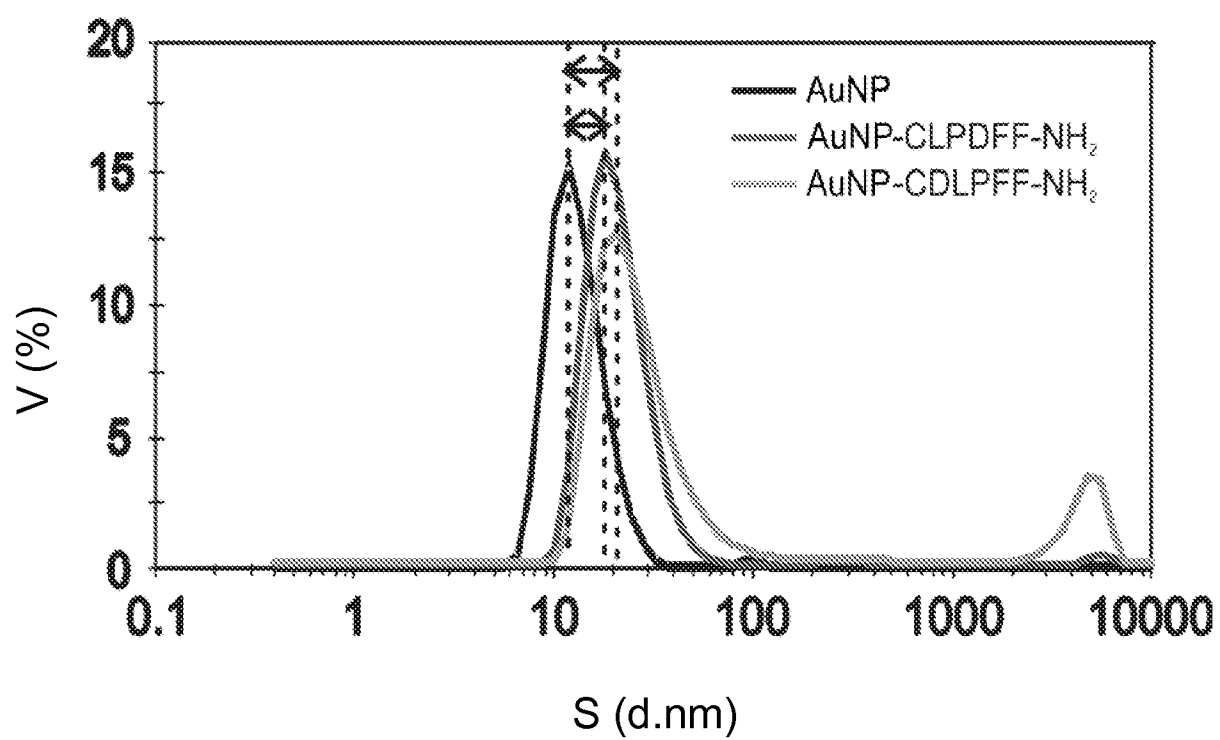


FIG.4

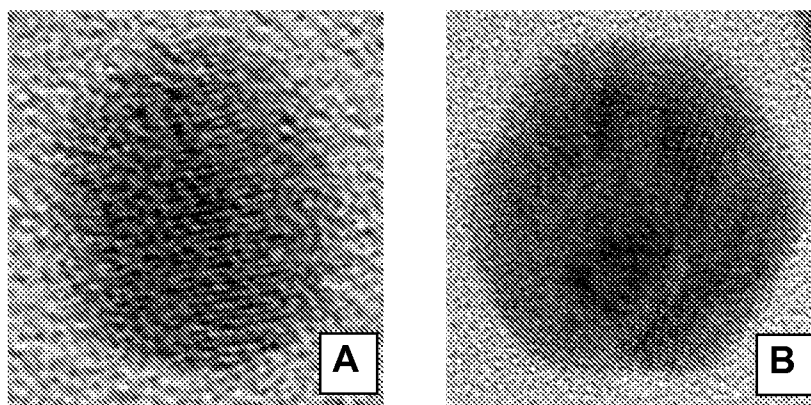


FIG. 5

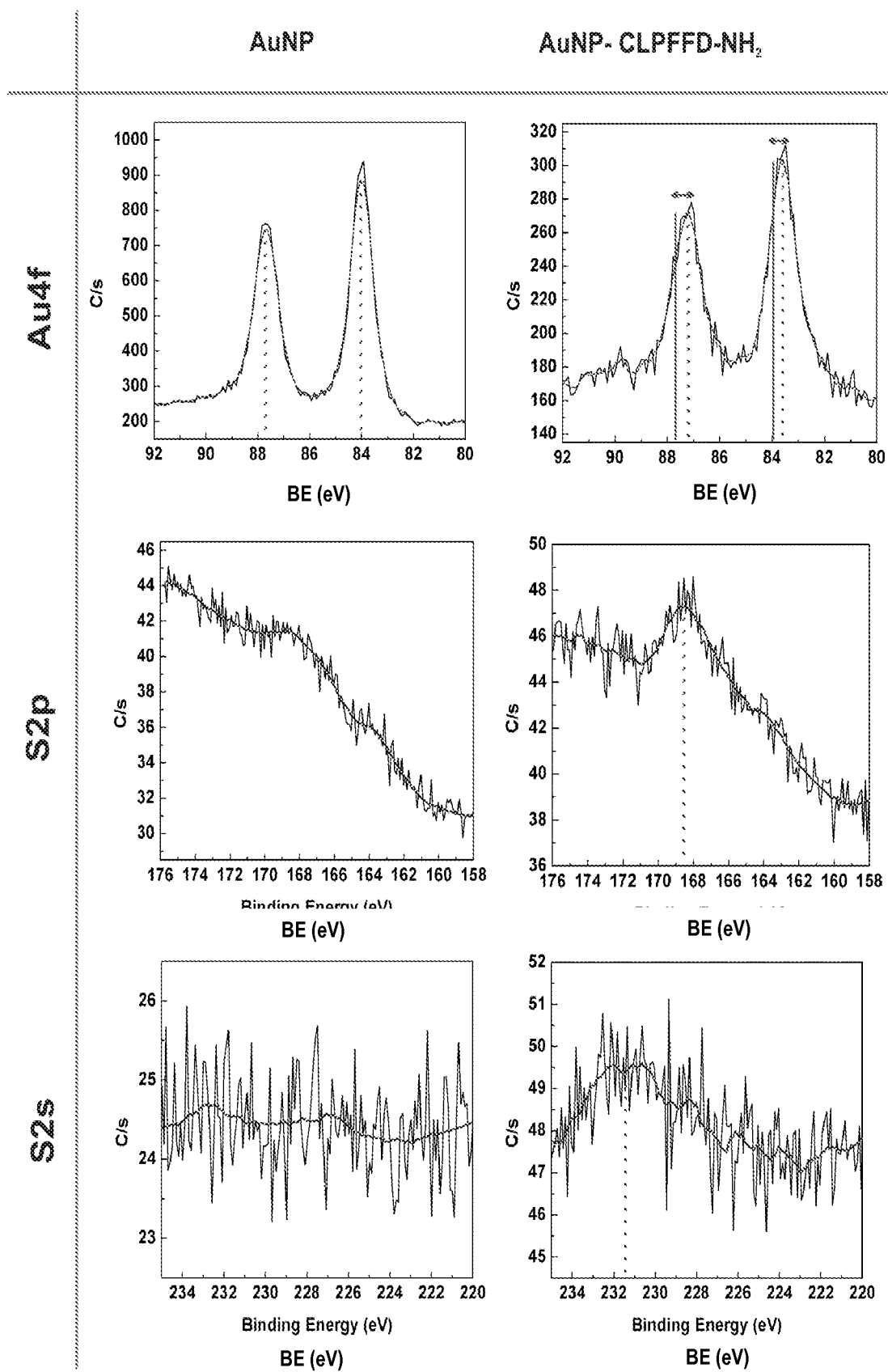


FIG. 6

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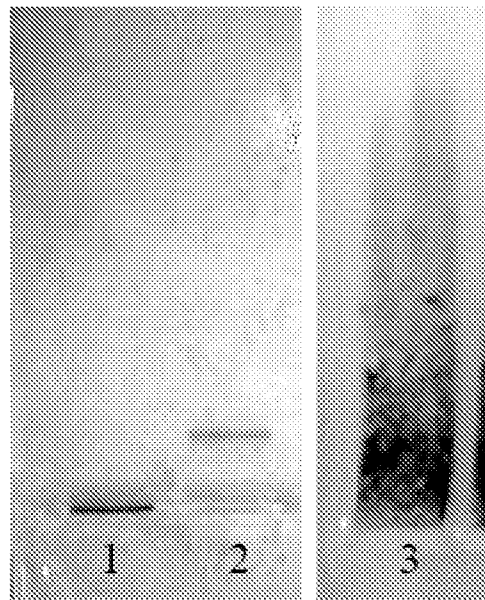


FIG. 7

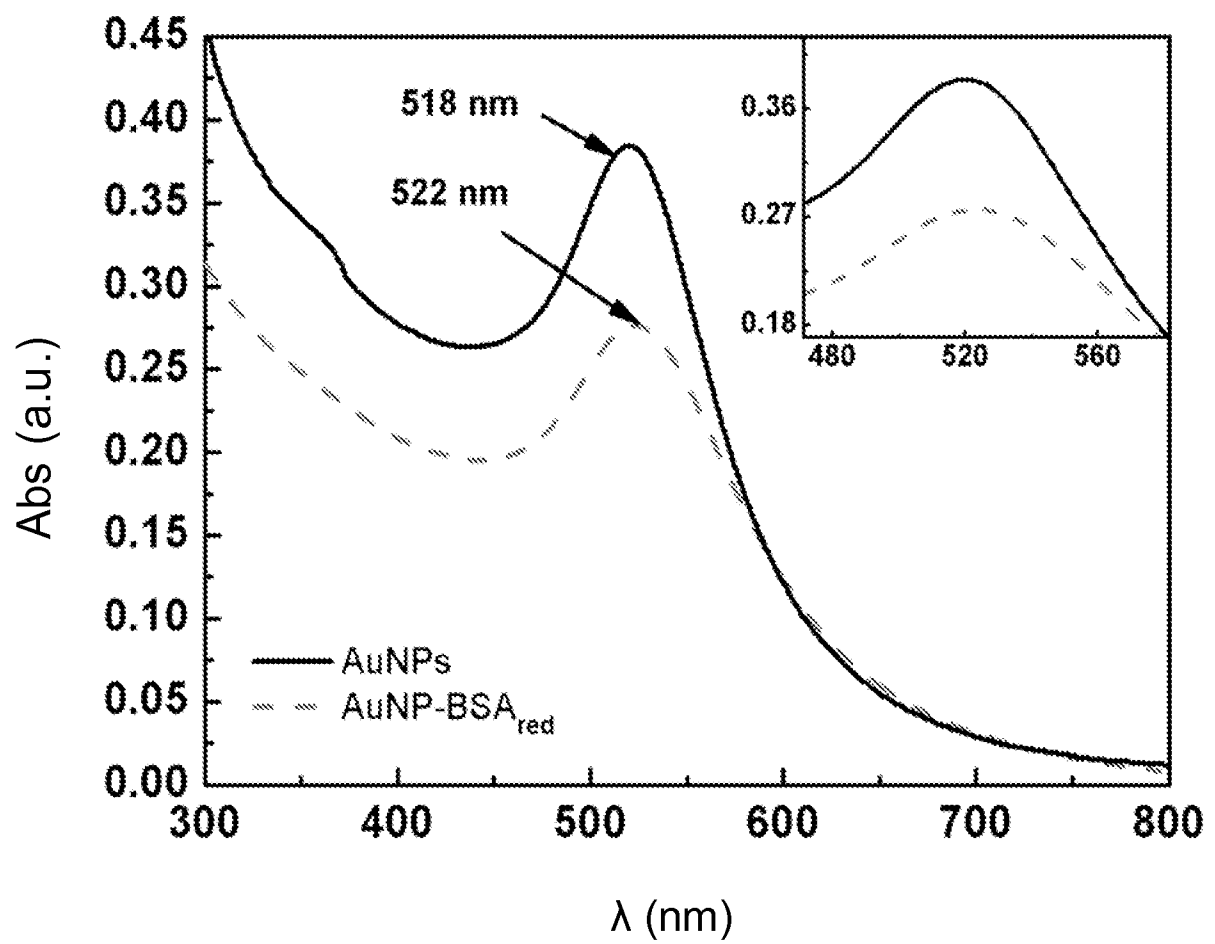


FIG.8

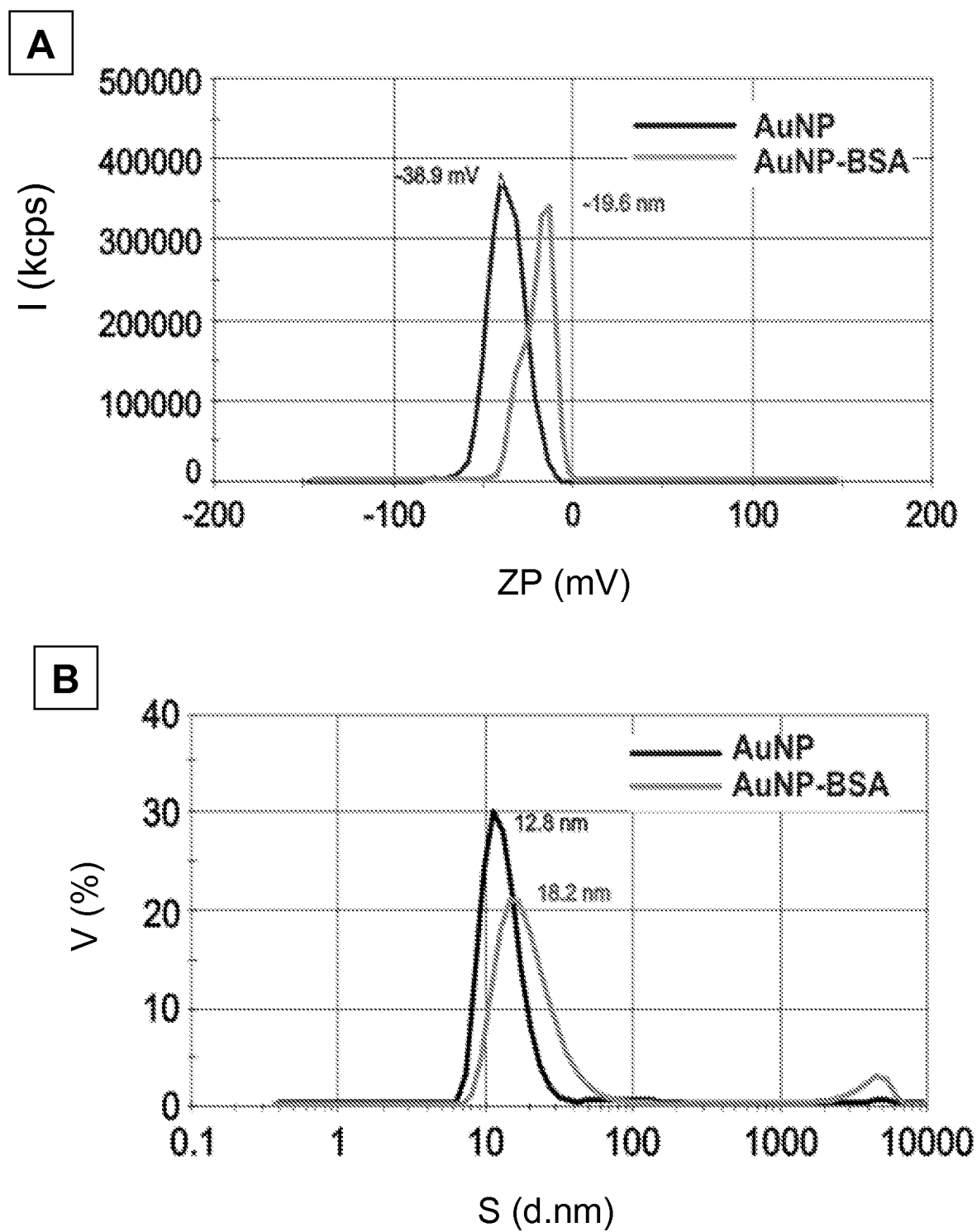


FIG. 9

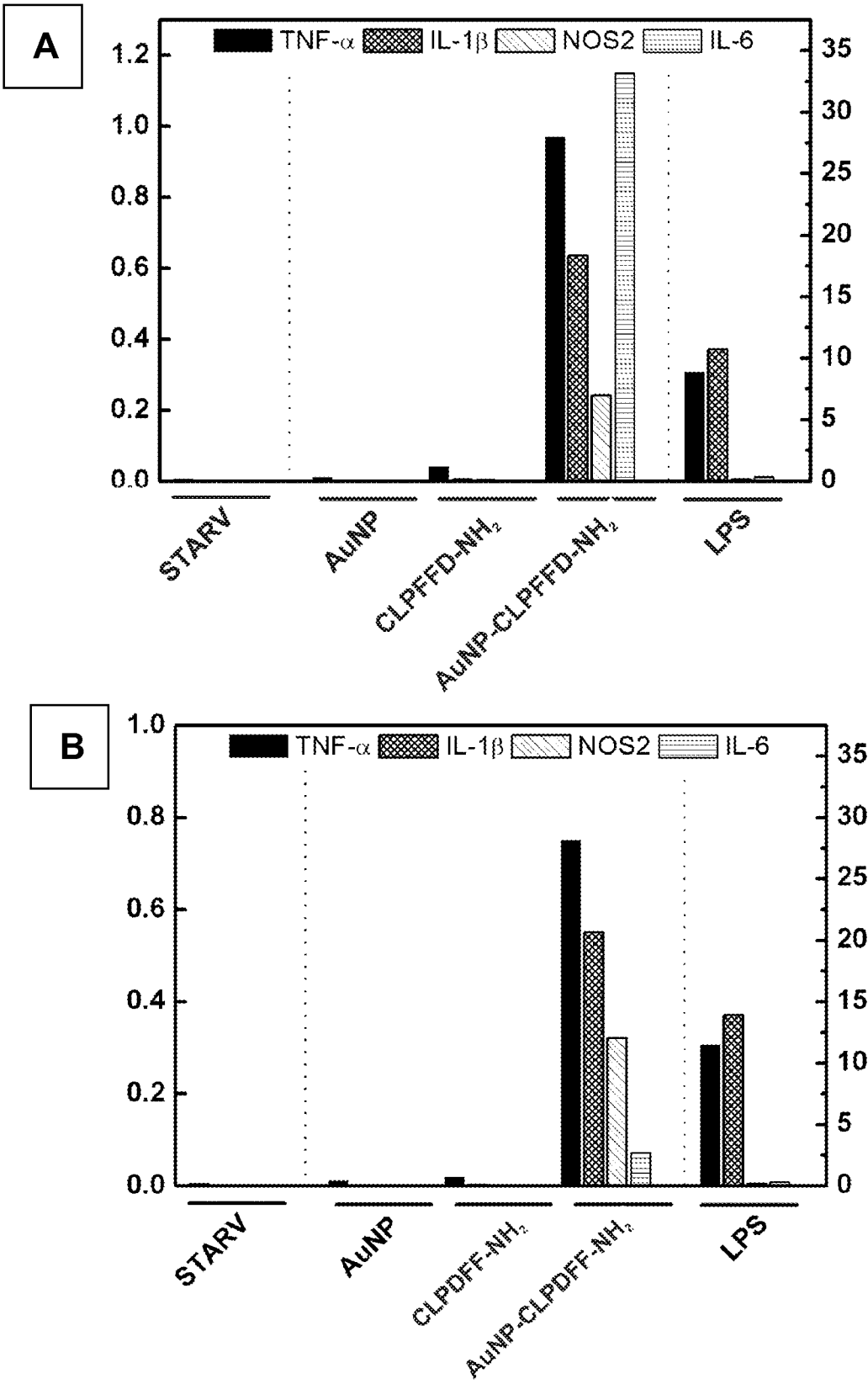


FIG. 10

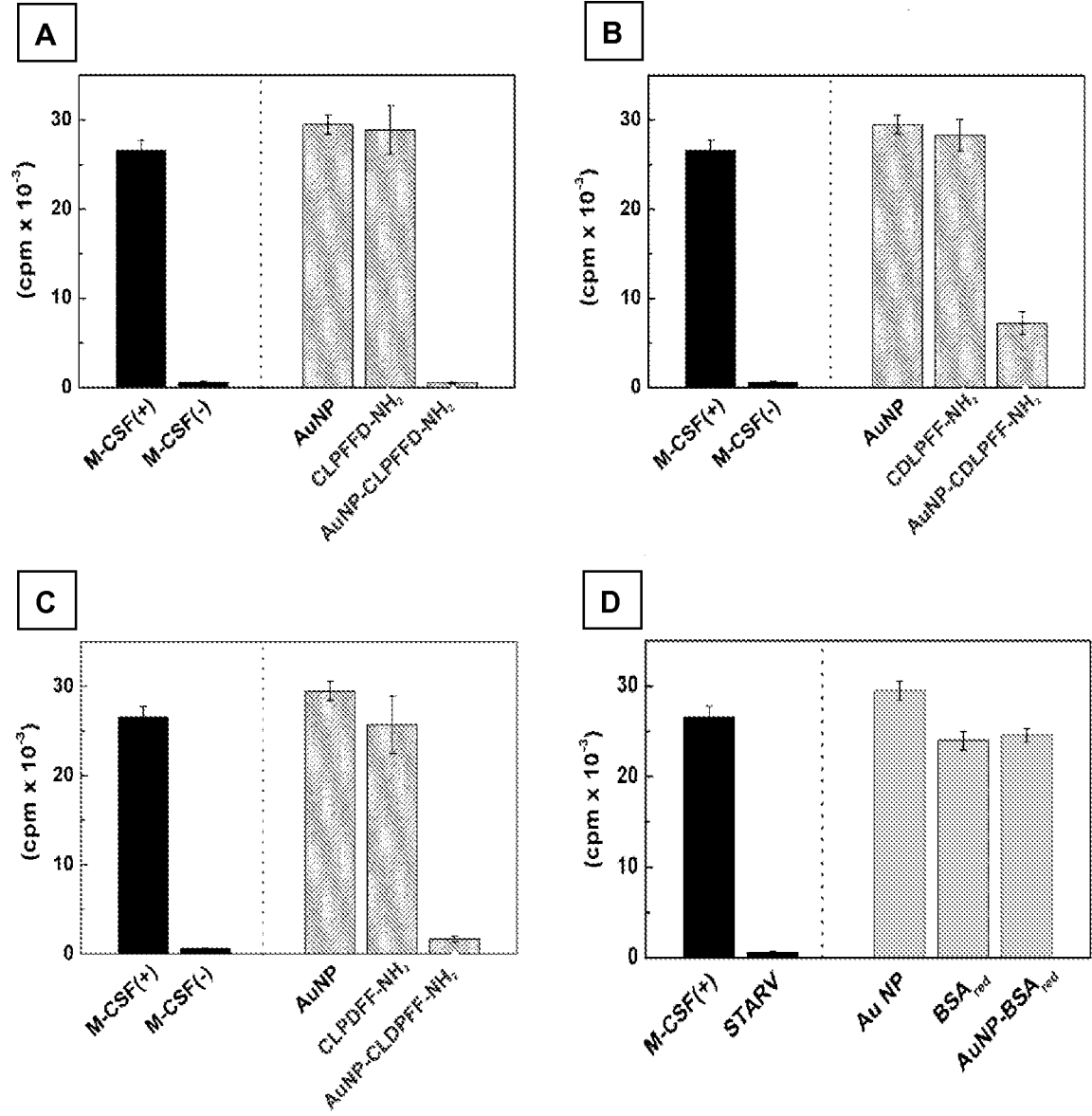


FIG. 11