



(43) International Publication Date
2 June 2016 (02.06.2016)

(10) International Publication Number
WO 2016/083343 A1

(51) International Patent Classification:

A61K 9/00 (2006.01) A61K 47/48 (2006.01)
A61K 41/00 (2006.01) A61K 51/12 (2006.01)
A61K 9/127 (2006.01)

(21) International Application Number:

PCT/EP2015/077446

(22) International Filing Date:

24 November 2015 (24.11.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

14306874.0 25 November 2014 (25.11.2014) EP

(71) Applicant: NANOBLOTIX [FR/FR]; 60 rue de Wattignies, 75012 Paris (FR).

(72) Inventors: GERMAIN, Matthieu; 3 avenue Marx Dormoy, Entrée 4, 94500 Champigny Sur Marne (FR). MEYRE, Marie-Edith; 65 boulevard Soult, 75012 Paris (FR). POTTIER, Agnès; 6 rue Sainte-Beuve, 75006 Paris (FR). LEVY, Laurent; 246 Boulevard Raspail, 75014 Paris (FR).

(74) Agent: CABINET BECKER ET ASSOCIES; 25, rue Louis le Grand, 75002 Paris (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: PHARMACEUTICAL COMPOSITION COMBINING AT LEAST TWO DISTINCT NANOPARTICLES AND A PHARMACEUTICAL COMPOUND, PREPARATION AND USES THEREOF

(57) Abstract: The present invention relates to a pharmaceutical composition comprising the combination of (i) at least two distinct biocompatible nanoparticles and (ii) at least one compound of interest, typically at least one pharmaceutical compound, to be administered to a subject in need of such at least one compound of interest, wherein the at least two distinct biocompatible nanoparticles potentiate the compound(s) of interest efficiency. The at least two biocompatible nanoparticles can be administered sequentially or simultaneously to the subject but are to be administered separately, typically with an interval of between more than about 5 minutes and about 72 hours, from the at least one compound of interest, preferably before the administration of the at least one compound of interest, to said subject. The longest dimension of the at least two biocompatible nanoparticles is typically between about 4 nm and about 500 nm. The absolute surface charge value of a first biocompatible nanoparticle is of at least |10 mV| and the absolute surface charge value of the second biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle.



WO 2016/083343 A1

PHARMACEUTICAL COMPOSITION COMBINING AT LEAST TWO DISTINCT NANOPARTICLES AND A PHARMACEUTICAL COMPOUND, PREPARATION AND USES THEREOF

5 **FIELD OF THE INVENTION**

The invention relates to a pharmaceutical composition comprising the combination of (i) at least two distinct biocompatible nanoparticles and (ii) at least one compound of interest, typically at least one pharmaceutical compound, to be administered to a subject in need of
10 such at least one compound of interest, wherein the at least two distinct biocompatible nanoparticles potentiate the compound(s) of interest efficiency.

The at least two biocompatible nanoparticles can be administered sequentially or simultaneously to the subject but are to be administered separately, typically with an interval of between more than about 5 minutes and about 72 hours, from the at least one compound of
15 interest, preferably before the administration of the at least one compound of interest, to said subject.

The longest dimension of the at least two biocompatible nanoparticles is typically between about 4 nm and about 500 nm. The absolute surface charge value of a first biocompatible nanoparticle is of at least 10 mV ($|10 \text{ mV}|$) and the absolute surface charge value of the
20 second biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle.

The combined, typically sequential, administration to the subject of the at least two distinct
25 biocompatible nanoparticles together with the at least one compound of interest maintains the pharmaceutical (i.e. therapeutic, prophylactic or diagnostic) benefit of said at least one compound of interest for a reduced toxicity thereof in said subject, or increases its pharmaceutical benefit for an equivalent or reduced toxicity, when compared to the pharmaceutical benefit and toxicity induced by said at least one compound when administered
30 at the standard pharmaceutical dose(s).

The pharmaceutical composition of the invention typically allows a reduction of at least 10% of the administered compound pharmaceutical dose when compared to the standard pharmaceutical dose of the at least one compound of interest while maintaining the same pharmaceutical benefit for an equivalent toxicity, preferably a reduced toxicity, for the

subject, or while increasing the pharmaceutical benefit for an equivalent or reduced toxicity for the subject.

BACKGROUND

5

In order to ensure safety and efficacy, therapeutic compounds are required to be selectively delivered to their target site at an optimal rate in the subject in need thereof.

Pharmacokinetics (pK) is a branch of pharmacology dedicated to the determination of the fate of substances administered externally to a living organism. This determination involves steps of measuring compound's concentrations in all major tissues over a long enough period of time, preferably until the compound's elimination. Pharmacokinetics is necessary to efficiently describe the compound's behavior *in vivo*, including the mechanisms of its absorption and distribution as well as its chemical changes in the organism. The pK profile in the blood can be fitted using various programs to obtain key pK parameters that quantitatively describe how the body handles the compound. Important parameters include maximum concentration (C_{\max}), half-life ($t_{1/2}$), clearance, area under curve (AUC), and mean resident time (MRT), i.e. the average time during which a compound stays in an organism. When a prolonged blood circulation of the compound formulation is observed, it is usually associated to an increased $t_{1/2}$, a reduced clearance, an increased AUC, and an increased MRT. pK data are often used in deciding the optimal dose and dose regimen for maintaining the desirable blood concentration in order to improve therapeutics' efficiency with minimal side effects. In addition, as well known by the skilled person, the blood concentration of a compound is correlated with both its efficacy and toxicity in most cases, typically for free drugs.

The physico-chemical properties of therapeutic as well as prophylactic compounds have an important impact on their pharmacokinetic and metabolic fate in the body. Therefore, selection of appropriate physico-chemical properties is key when designing such a compound. However, since the compound is not always endogenously provided by the organism itself and is usually externally administered, its biodistribution profile has to be optimized in order to fit with, and preferably optimize, the desired pharmacological action thereof.

Several approaches have been explored to optimize the delivery of a compound to its target site. A strategy is to design a therapeutic compound with stealth properties to prolong its blood half-life and consequently, to enhance its accumulation to the target site. One favorable

approach is the covalent attachment of polyethylene glycol (PEG) to the therapeutic compound that has proved to increase the *in vivo* half-life ($t_{1/2}$) of the circulating compound, the level of the *in vivo* half-life increase varying depending partly on the nature of the compound and on that of the coating. Also, drug carriers such as liposomes, emulsions or micelles have been developed to enhance therapeutic efficacy of drugs by modifying their biodistribution profile in the subject's body.

However, lack of selectivity in the biodistribution of the therapeutic compounds still remains a concern. So far, poor pharmacokinetics and high toxicity are important causes of failure in therapeutic compounds development.

As an example, in the context of cancer treatment, intentional inhibition of essential functions of the body in order to kill cancer cells results in toxicity in normal cells, and clinicians have to rely on differences in dose-response and therapeutic compounds distribution between tumors and normal tissues to find a possible therapeutic window. Of note, hepatotoxicity remains a major reason for drug withdrawal from pharmaceutical development and clinical use due to direct and indirect mechanisms of drug-induced cell injury in the liver.

An approach proposed for nanoparticulate compounds such as drug carriers [*Critical Reviews in Therapeutic Drug Carrier Systems* 11(1):31-59 1994] is to pre-inject a decoy carrier to decrease, saturate, or even inactivate the phagocytic capacity of the reticuloendothelial system (RES). Impairment or blockade may also be associated with decreased plasma levels of opsonic molecules. Intravenous administration of certain agents, such as alkyl esters of fatty acids, dextran sulfate, salts of rare earth elements (e.g. $GdCl_3$), drug carriers, either empty or encapsulating clodronate, prior to administration of test particles, has been demonstrated to induce moderate to dramatic reduction in kupffer cells uptake.

For instance, authors in "*Biomimetic amplification of nanoparticle homing to tumors*" [*PNAS* 2007], reported the role of RES in the clearance of their nanoparticles "CREKA-SPIO". Initial experiments showed that intravenous (IV) injected "CREKA-SPIO" nanoparticles did not effectively accumulate in MDA-MB-435 breast cancer xenografts. In contrast, a high concentration of particles was seen in RES tissues. By depleting RES macrophages in the liver with liposomal clodronate, they found a 5-fold prolongation of their particle's half-life. However, clodronate agent induces the apoptosis of macrophages from liver and spleen, and this is considered as globally detrimental as macrophages depletion increases the risks associated to immunosuppression and infection. In a second experiment, the authors tested liposomes coated with chelated Ni (II) as a potential decoy particle hypothesizing that iron

oxide and Ni (II) would attract similar plasma opsonins, and that Ni-liposomes could therefore deplete them in the systemic circulation. Indeed, intravenous (IV) injected Ni-liposomes, whether administered 5 minutes or 48 hours before the injection of CREKA-SPIO nanoparticles, allows a five-fold increase of the nanoparticles' blood half-life. However, high toxicity was observed causing deaths among tumor mice. Plain liposomes were also tested instead of Ni-liposomes. However, while reducing the toxicity when compared to said Ni-liposomes, plain liposomes were far less effective than them. Indeed, the blood half-life increase was only of a factor about 2.

WO2005086639 relates to methods of administering a desired agent selectively to a target site in a subject, typically in the context of ultrasound or X-ray exposure, or in the context of magnetic resonance imaging (MRI), as well as in the context of therapy. The aim of the described method is to improve or maintain the efficiency of the agent of interest while reducing the total dose of agents concretely administered thanks to concomitant administration of a decoy inactive carrier.

The described invention employs a probability-based approach. A non-targeted inactive agent ("inactive carrier") is co-administered (i.e. "substantially simultaneously") with a targeted agent of interest (present in an "active composition") exhibiting similar physical features, in order to facilitate the evasion of the RES system by the targeted agent of interest thereby allowing an improved uptake of the agent of interest at the desired site. This approach results in a lower exposure of patients to the agent of interest and, as a consequence, in a lower per dosage cost of said agent of interest. The active composition and the decoy inactive carrier are administered within five minutes of each other, preferably within 2 minutes of each other, or even less. This approach relies on the presence of a large excess of untargeted "carrier" or "decoy" vehicles and on the probability that this decoy carrier in excess will compete with the targeted agent of interest for an uptake by the reticuloendothelial system when supplied in the presence of vehicles that are targeted to a desired location. The half-life of particles captured by RES is dose dependent, i.e. the circulating half-life of particles increases as the dosage increases. The slower clearance associated to higher dosages is thought to favor the maintaining of a total agents high concentration allowing a decrease of the dose of the agent of interest which is to be administered. In other words, an increased half-life of total agents due to a global higher dosage thereof should be beneficial to the targeted agents, according to the authors of WO2005086639. The requirement involved by this approach is that the active agent and the inactive one behave similarly with regard to their clearance characteristics in the RES, whatever their respective compositions.

In this approach, the quasi-concomitant injection of the inactive agent and of the active one is required to increase the global amount of agents present in the blood and consequently to prolong their blood half-life. Such strategy, which expressly relies on a probability-based approach, necessarily requires the association of the active agent with a targeting agent in order to achieve its successful accumulation on the target site by conferring said active agent an advantage over the inactive one. In addition, due to the quasi-concomitant injection, a specific design of the inactive carrier may be required depending on the intended use of the active composition.

WO2005/063305 relates to an assembly comprising a gas-filled microvesicle (with a size typically of at least 0.5 μm) and a component (with a size about below 100 nm) associated to said microvesicle. The resulting assembly is to be used as a pharmaceutically active component in diagnostically and/or therapeutically active formulations. The two components, i.e. the gas-filled microvesicle and the microvesicle associated component, are administered simultaneously typically for enhancing the imaging in the field of ultrasound contrast imaging, including targeted ultrasound imaging, ultrasound-mediated drug delivery and other imaging techniques.

As apparent from the prior art and despite of a long medical need, the improvement of compounds (including therapeutic, prophylactic as well as diagnostic compounds) which cannot be efficiently used in patients due to their unacceptable toxicity or to their unfavorable pharmacokinetics parameters remains a concern.

DETAILED DESCRIPTION

The present invention now allows optimization of the efficiency of a compound of interest (herein also simply identified as “the compound”), or of a combination of compounds of interest, whatever its (their) intended use in the context of therapy, prophylaxis or diagnostic. The composition herein described which is a combination of (i) at least two distinct biocompatible nanoparticles and of (ii) at least one compound of interest, typically at least one pharmaceutical compound (i.e. a therapeutic, prophylactic or diagnostic compound), optimize the at least one compound of interest pharmacokinetic parameters, and, as a consequence, now renders possible the development of therapeutic compounds which could not have been developed otherwise due for example to their unacceptable toxicity. Typically,

the biocompatible nanoparticle is not used as such as a pharmaceutical compound, i.e. as a therapeutic, prophylactic or diagnostic compound.

5 A typical composition of the invention (herein generally identified as “pharmaceutical composition”) is a composition comprising the combination of (i) at least two distinct biocompatible nanoparticles and (ii) at least one compound (“the compound of interest”), wherein the longest or largest dimension of each of the at least two biocompatible nanoparticles is typically between about 4 nm and about 500 nm, the absolute surface charge value of a first biocompatible nanoparticle (also herein identified as the “first” biocompatible
10 nanoparticle) is of at least 10 mV (≥ 10 mV) and the absolute surface charge value of a second biocompatible nanoparticle (also herein identified as the “second” biocompatible nanoparticle), or of any additional biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle.

Typically, the ratio between the (at least two distinct) biocompatible nanoparticles and
15 compounds of interest is between 0.1/1 and 1000/1 or 0.5/1 and 1000/1, preferably between 0.5/1 and 500/1, even more preferably between 0.5/1 and 300/1.

The terms “about” and “around” when associated to a value such as for example a nanoparticle’ size or a time interval indicates that a variation with the indicated value, which
20 would be recognized by the skilled person as small variation, does not substantially impact the properties of the subject-matter it is associated to and that said subject-matter remains in the spirit of the claimed invention.

A preferred objet of a the invention is a pharmaceutical composition comprising the
25 combination of (i) at least two distinct biocompatible nanoparticles and of (ii) at least one pharmaceutical compound, wherein the longest or largest dimension of each of the at least two biocompatible nanoparticles is between about 4 nm and about 500 nm, the absolute surface charge value of a first biocompatible nanoparticle is of at least 10 mV (≥ 10 mV) and the absolute surface charge value of a second biocompatible nanoparticle, or of any additional
30 biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle, said composition being for use for administering the at least two distinct biocompatible nanoparticles in a subject in need of the at least one pharmaceutical compound separately from the administration of said at least one pharmaceutical compound to said subject, typically with an interval of between more than 5

minutes and about 72 hours, and wherein the biocompatible nanoparticle is not used as such as a pharmaceutical compound.

5 The combined administration to the subject of the at least two distinct biocompatible nanoparticles and of the at least one compound of interest, through the composition of the invention, typically allows (maintains) the same pharmaceutical (i.e. therapeutic, prophylactic or diagnostic) benefit of the at least one compound for a reduced toxicity thereof for the subject, or increase the pharmaceutical benefit of the at least one compound for an equivalent or reduced toxicity thereof for the subject (preferably a reduced toxicity), when compared to
10 pharmaceutical benefit and toxicity induced by the standard pharmaceutical dose(s) of said at least one compound.

The pharmaceutical composition of the invention typically allows a reduction of at least 10%, preferably at least 15%, of the administered at least one pharmaceutical (i.e. therapeutic,
15 prophylactic or diagnostic) compound dose when compared to the standard pharmaceutical dose of said compound (i) while maintaining the same pharmaceutical benefit for an equivalent toxicity, preferably a reduced toxicity, for the subject or (ii) while increasing the pharmaceutical benefit for an equivalent or reduced toxicity for the subject.

20 As the shape of the particle can influence its “biocompatibility”, particles having a quite homogeneous shape are herein preferred. For pharmacokinetic reasons, nanoparticles being essentially spherical/round or ovoid in shape are thus preferred. Such a shape also favors the nanoparticle interaction with or uptake by cells. Spherical/round shape is particularly preferred.

25 In the spirit of the invention, the term “nanoparticle” refers to a product, in particular a synthetic product, with a size in the nanometer range, typically between about 1 nm and about 500 nm, preferably between about 4 nm and about 500 nm, between about 4 and about 400 nm, about 30 nm and about 300 nm, about 20 nm and about 300 nm, about 10 nm and about 300 nm, for example between about 4 nm and about 100 nm, for example between about 10
30 nm, 15 nm or 20 nm and about 100 nm, or between about 100 nm and about 500 nm, typically between about 100 nm and about 300 nm.

The terms “size of the nanoparticle”, “largest size of the nanoparticle” and “longest size of the nanoparticle” herein typically refer to the “longest or largest dimension of the nanoparticle” or “diameter of the nanoparticle” when spherical/round or ovoid in shape. Transmission Electron

Microscopy (TEM) or Cryo-TEM can be used to measure the size of the nanoparticle. As well, Dynamic Light Scattering (DLS) can be used to measure the hydrodynamic diameter of nanoparticles in solution. These two methods may further be used one after each other to compare the hydrodynamic diameter of a nanoparticle measured by DLS with the size of said nanoparticle measured by TEM or Cryo-TEM, in order to confirm said size. A preferred method is DLS (Ref. International Standard ISO22412 Particle Size Analysis – Dynamic Light Scattering, International Organisation for Standardisation (ISO) 2008).

To be usable in the context of the invention, the absolute electrostatic surface charge (also herein identified as “charge” or “surface charge”) of the “first” biocompatible nanoparticle is to be of at least, preferably higher than, $|10 \text{ mV}|$ (absolute value) and the surface charge value of the “second” biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has to have a difference of at least 10 mV with the absolute surface charge value of the “first” biocompatible nanoparticle. The surface charge of a nanoparticle is typically determined by zeta potential measurements in aqueous medium for a nanoparticles concentration between 0.2 and 10 g/L, for a pH between 6 and 8, and typically for electrolytes concentrations in the aqueous medium between 0.001 and 0.2 M, for example 0.01 M or 0.15 M.

Typically, the “first” biocompatible nanoparticle of the present invention has an electrostatic surface charge of at least $|10 \text{ mV}|$, i.e. below -10 mV or above $+10 \text{ mV}$, for example below between -12 mV or -15 mV and -20 mV or above between $+12 \text{ mV}$ or $+15 \text{ mV}$ and $+20 \text{ mV}$, typically below -15 mV or above $+15 \text{ mV}$. The “second” biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has a (positive or negative) difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle, Preferably, the “first” biocompatible nanoparticle of the present invention has an absolute electronic surface charge value (“absolute surface charge value”) of more than 10 mV, said charge being even more preferably a negative charge.

The combined properties, size and surface charge of the at least two distinct biocompatible nanoparticles, allow for a short blood circulation of the least two distinct nanoparticles. Therefore, by sequentially administering at least two distinct biocompatible nanoparticles of the invention and the compound(s) of interest, no co-circulation or a limited co-circulation of the at least two distinct biocompatible nanoparticles and of the compound of interest is achieved. Therefore, the combined properties of the at least two distinct biocompatible nanoparticles, size and surface charge, permit the safe use of the compound(s) of interest

while allowing (maintaining) the same pharmaceutical (i.e. therapeutic, prophylactic or diagnostic) benefit of the at least one compound for a reduced toxicity thereof for the subject, or in other words while increasing the pharmaceutical benefit of the at least one compound for an equivalent or reduced toxicity thereof for the subject (preferably a reduced toxicity), when compared to pharmaceutical benefit and toxicity induced by the standard pharmaceutical dose(s) of said at least one compound.

The at least two biocompatible nanoparticles usable in the context of the invention can all be either organic or inorganic. A mixture of organic and inorganic nanoparticles can further be used as the at least two biocompatible particles.

When organic, the nanoparticles present in the pharmaceutical composition of the invention can be lipid-based nanoparticles (glycerolipid, phospholipid, sterol lipid, etc.), such as solid-lipid nanoparticles, protein-based nanoparticles also herein identified as “protein-nanoparticles” (albumin for instance), polymer-based nanoparticles (“polymeric nanoparticles”), co-polymer-based nanoparticles (“co-polymeric nanoparticles”), carbon-based nanoparticles, virus-like nanoparticles (for example viral vectors) or a mixture thereof.

The organic nanoparticles may further be nanospheres (plain nanoparticles) or nanocapsules (hollow nanoparticles) such as liposomes, gels, hydrogels, micelles, dendrimers, etc. A mixture of the herein described organic nanoparticles can also be used.

The polymer or co-polymer can be of natural or synthetic origin.

Examples of synthetic (artificial) and natural polymers or co-polymers usable in the context of the invention to prepare organic nanoparticles can be selected from polylactic acid (PLA), Poly (lactide-co-glycolic) acid (PLGA), Polyethyleneglycol (PEG), Polyglactin, Polylactide, Polyoxyethylene fatty acid esters, Polypropylene glycol, Polysorbate, Polyvinyl alcohol, Polyacrylamide, Polymethylmethacrylate, Polyalkylcyanoacrylate, Polylactate-co-glycolate, Poly(amido amine), Poly(ethyleneimine), alginate, cellulose and cellulose derivatives polymers, collagen, hyaluronic acid, polyglutamic acid (PGA), actin, polysaccharide, and gelatin.

When inorganic and when its longest dimension is typically below about 10 nm, for example below about 8 nm, below about 7 nm, typically comprised between about 7 nm and about 4 nm, for example below about 6 nm, below about 5 nm or below about 4 nm, the nanoparticle may be made of any inorganic material. The inorganic material may for example comprise

metallic element from period 3, 4, 5, 6 of the Mendeleev's periodic table, including the lanthanides. When the longest dimension of the nanoparticle is typically below about 10 nm, the nanoparticles may assemble in larger structures. Assembling of nanoparticles in larger structure may typically be triggered by interactions between nanoparticles and a biocompatible polymer(s), protein(s), etc. Larger structure may also be obtained by trapping the nanoparticles in a carrier, typically a plain carrier such as gelatin structure (also herein identified as "gelatin nanoparticle") or a hollow carrier such as liposome. After *in vivo* administration, those larger structures may further release the nanoparticle(s).

When inorganic and when the longest dimension of said nanoparticle is typically of at least 10 nm, typically between 10 and 500 nm, the nanoparticle may comprise at least one of, or may consist in (i) one or more divalent metallic elements selected for example from Mg, Ca, Ba and Sr, (ii) one or more trivalent metallic element selected for example from Fe and Al, and (iii) one or more tetravalent metallic element comprising Si.

In a particular embodiment, the inorganic material of the nanoparticle is selected from (i) one or more divalent metallic elements selected for example from Mg, Ca, Ba and Sr (ii) one or more trivalent metallic element selected for example from Fe and Al and (iii) one or more tetravalent metallic element comprising Si.

In a further particular embodiment, the inorganic material of the nanoparticle is selected from calcium carbonate (CaCO_3), magnesium carbonate (MgCO_3), magnesium hydroxide (Mg(OH)_2), iron hydroxide (Fe(OH)_2), iron oxyhydroxide (FeOOH), iron oxide (Fe_3O_4 or Fe_2O_3), aluminium oxide (Al_2O_3), aluminium hydroxide (Al(OH)_3), aluminium oxyhydroxide (AlOOH) and silicium oxide (SiO_2).

The nanoparticles used in the herein described compositions are to be biocompatible, i.e. compatible with living tissues. When required by their composition, the nanoparticles are thus to be coated with a biocompatible material to become usable. In a particular embodiment of the invention, the herein mentioned nanoparticle is thus covered with a biocompatible coating. The biocompatible material can be an agent allowing interaction with a biological target.

An agent forming a positive charge on the nanoparticle's surface can be for example selected from aminopropyltriethoxysilane or polylysine. An agent forming a negative charge on the nanoparticle surface can be for example selected from a phosphate (for example a polyphosphate, a metaphosphate, a pyrophosphate, etc.), a carboxylate (for example citrate or dicarboxylic acid, in particular succinic acid) or a sulphate.

In a particular embodiment, as long as the absolute charge of the “first” nanoparticle is of at least 10 mV ($|10 \text{ mV}|$) and the absolute surface charge value of a second biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value of the first biocompatible nanoparticle, the at least two distinct nanoparticles can be coated with a biocompatible material selected from an agent displaying a steric group. Such a group may be selected for example from polyethylene glycol (PEG); polyethylenoxide; polyvinylalcohol; polyacrylate; polyacrylamide (poly(N-isopropylacrylamide)); polycarbamide; a biopolymer; a polysaccharide such as dextran, xylan and cellulose; collagen; a switterionic compound such as polysulfobetain; etc.

The biocompatible coating may further comprise a labelling agent, typically an agent allowing the visualisation of a color using standard imaging equipment.

The combined administration of the at least two distinct biocompatible nanoparticles together with the at least one compound of interest maintains the pharmaceutical benefit (i.e. therapeutic, prophylactic or diagnostic benefit), typically the therapeutic benefit, of the compound of interest for a reduced toxicity, or increases the pharmaceutical benefit of the compound for an equivalent or reduced toxicity, for the subject, typically when said at least two nanoparticles are (simultaneously or separately) administered to the subject in need of the at least one compound of interest separately from said at least one compound of interest, preferably with an interval of between more than 5 minutes and about 72 hours when compared to pharmaceutical benefit and toxicity induced by the standard pharmaceutical, typically therapeutic, dose(s) of said at least one compound of interest in the absence of any particles.

In a particular embodiment, the combined administration of the at least two distinct biocompatible nanoparticles and of the at least one compound of interest allows a reduction of at least 10%, preferably at least 15%, of the administered compound therapeutic dose, typically when administered in the subject in need of the at least one compound of interest separately from said at least one compound of interest, typically with an interval of between more than 5 minutes and about 72 hours before or after, preferably before, said compound of interest, when compared to the standard therapeutic dose of said compound while maintaining the same therapeutic benefit for an equivalent toxicity or a reduced toxicity (preferably a reduced toxicity) of the compound(s) for the subject; or while increasing the therapeutic benefit for an equivalent or reduced toxicity of the compound(s) for the subject.

In a particular embodiment, nanoparticles are administered with several compounds of interest, typically two compounds of interest.

The nanoparticles are preferably cleared from the subject to whom it has been administered typically within 1 hour and 6 weeks, for example 4 weeks, within 1 hour and 1 month, for example between 1 hour and 3 weeks, or between 1 hour and 2 weeks, or between 1 hour and 1 week, following their administration to a subject in need of the compound of interest.

The material constituting a nanoparticle (including its biocompatible coating when present) is important in determining the biopersistence of the nanoparticle. The nanoparticle may be regarded as biodegradable (when constituted for example by a biodegradable polymer such as PLGA or PLA), dissolvable (iron oxide for example) or non-biodegradable and non-dissolvable. Biodegradable and dissolvable nanoparticles facilitate rapid nanoparticle clearance from the subject.

Different molecules or agents can be used according to the present teaching as the at least one compound of interest, typically as the at least one pharmaceutical compound of interest, administered in combination with the at least two distinct biocompatible nanoparticles as described hereinabove. This compound may be a therapeutic, a prophylactic or a diagnostic compound as previously explained. It can be an organic compound or an inorganic compound.

Examples of organic compound usable as the compound of interest can be selected from a biological compound, an antibody, an oligonucleotide, a synthesized peptide, a small molecule targeted therapeutic, an oncolytic virus, a cytotoxic compound, and any corresponding prodrug or derivative thereof, etc.

In a particular embodiment, the compound of interest used in the context of the present invention is an organic compound preferably selected from a biological compound, a small molecule targeted therapeutic, an oncolytic virus, and a cytotoxic compound. In another particular embodiment, the compound of interest is selected from an antibody, an oligonucleotide, and a synthesized peptide.

A biological compound is for instance an antibody, an antibody drug conjugate, preferably a monoclonal antibody ("mAb"), such as infliximab, adalimumab, bevacizumab, rituximab,

trastuzumab, ranibizumab, cetuximab, panatimumab; a protein or a recombinant protein such as enbrel (etanercept) or interferon beta-1a; a peptide or a recombinant peptide such as insulin glargine or betaseron; a vaccine such as prevnar 13 or gardasil; a biosimilar such as epogin; an enzyme or a recombinant enzyme such as replagal or creon; etc.

- 5 An oligonucleotide is for instance an antisense oligonucleotide, an aptamer, such as mipomersen sodium or pursennid, etc.

A synthesized or artificial peptide is for instance glatiramer acetate or leuprolide acetate.

- An oncolytic virus is a therapeutically useful virus that selectively infects and damages cancerous tissues without causing harm to normal tissues. Oncolytic virus is for instance
10 selected from an adenovirus such as Onyx-015, a coxsackie virus such as Catavak, a herpes simplex virus such as talimogene laherparepvec, a measles virus such as MV-CEA, a newcastle disease virus, a parvovirus, a poliovirus, a reovirus, a Seneca valley virus, a retrovirus, a vaccinia, a vesicular stomatitis virus.

- A small molecule targeted therapeutic generally inhibits enzymatic domains on mutated,
15 overexpressed, or otherwise critical protein (potential target in the context of cancer treatment) within the malignant cells. Some therapeutics agents include those that target cell division (for example a aurora-kinase inhibitor or a cyclin-dependent-kinase inhibitor), as well as other biological mechanisms such as protein turnover and chromatin modification (for example a histone-deacetylase inhibitor). Small molecules targeted therapeutics are for
20 instance imatinib, rapamycin, gefitinib, erlotinib, sorafenib, sunitinib, nilotinib, dasatinib, lapatinib, bortezomib, atorvastatin, etc.

- A cytotoxic compound is for instance a DNA-modifying agent, such as an anthracycline (for example doxorubicine, daunorubicine, etc.), an alkylating agent (for example melphalan or temozolomide), as well as a drug interfering very precisely with defined physiological
25 mechanisms such as microtubule polymerization (for example taxol), or metabolite synthesis (for example methotrexate). An activable cytotoxic compound is typically used in the context of Photodynamic Therapy (for example photofrin), and is to be activated by an external source such as a laser source to produce its therapeutic effect. Other typical cytotoxic compounds are typically selected from chemotherapeutic agent as herein described or as
30 known by the skilled oncologist.

A prodrug (for instance capecitabine or irinotecan) is metabolized in its active form *in vivo* to produce its expected therapeutic effect.

Examples of inorganic compound usable as the at least one compound of interest can be selected from a transition metal coordination complex, a radiopharmaceutical compound, a nanoparticle, etc.

Transition metal coordination complexes offer potential advantages over the more common organic-based drugs, including a wide range of coordination numbers and geometries, accessible redox states, ‘tune-ability’ of the thermodynamics and kinetics of ligand substitution, as well as a wide structural diversity. Metal-based substances interact with cell molecular targets, affecting biochemical functions resulting in malignant cell destruction. Transition metal coordination complexes are typically cytotoxic agents (for instance, platinum coordination complexes: cisplatin, carboplatin, oxaloplatin, or ruthenium or gold coordination complexes) acting on DNA structures.

Radiopharmaceutical compounds emit radiations for diagnosis purposes or in order to selectively destroy malignant cells. Typical radiopharmaceuticals may contain for example strontium-89, thallium-201, technetium-99, samarium-83, etc.

Nanoparticle may be selected typically from a metal oxide nanoparticle (see WO 2009/147214 and WO 2007/118884 for example), a metallic nanoparticle (gold, platinum or silver nanoparticle for instance), a metal sulfide nanoparticle (Bi_2S_3 for instance), and any mixture thereof (for example a gold nanoparticle covered with hafnium oxide material). The nanoparticle is for example a nanoparticle which can be activated *via* an external source such as a electromagnetic radiation source, a ultrasound source, or a magnetic source, etc.

The at least one compound of interest, which is administered in combination with a biocompatible nanoparticle as described hereinabove (typically sequentially administered as herein described), may be encapsulated in a carrier or grafted (or bound) to such a carrier according to means known by the skilled person. A typical carrier is for example a liposome (such as DOXIL or ThermoDox which uses thermosensitive lipid), micelle, polymeric (or “polymer”) carrier, hydrogel, gel, co-polymeric carrier, protein carrier, inorganic carrier.

The pharmaceutical composition of the invention (defined by the combination of the compound of interest and of the at least two distinct nanoparticles) can be used in many fields, particularly in human or veterinary medicine. This composition is typically for use in an animal, preferably in a mammal (for example in the context of veterinary medicine), even more preferably in a human being whatever its age or sex.

The pharmaceutical compositions of the invention can be used in cardiovascular diseases, Central Nervous System (CNS) diseases, gastrointestinal diseases, genetic disorders, hematological disorders, hormonal disorders, immunology, infectious diseases, metabolic disorders, musculoskeletal disorders, oncology, respiratory diseases, toxicology, etc. In a preferred embodiment, the pharmaceutical composition is used in cardiovascular diseases, CNS diseases, oncology, infectious diseases, and metabolic disorders.

In the context of the present invention, the at least two distinct biocompatible nanoparticles on one side and the compound(s) (“compound(s) of interest”) on the other side are advantageously to be separately administered in a subject in need of said compound(s), typically with an interval of between more than 5 minutes and about 72 hours, typically between more than 5 minutes and about 24 hours, preferably between more than 5 minutes or 30 minutes and about 12 hours, in order to optimize the compound(s) pharmaceutical efficacy.

In the present invention, when the at least two distinct nanoparticles and the compound(s) (“compound(s) of interest”) are advantageously to be separately administered in a subject in need of said compound(s) as explained previously, typically with an interval of between more than 5 minutes and about 72 hours, the absolute surface charge value of the “first” biocompatible nanoparticle being advantageously of at least 10 mV ($|10 \text{ mV}|$) and the “second” biocompatible nanoparticle, or any additional biocompatible nanoparticle, having a difference of at least 10 mV with the absolute surface charge value of the “first” biocompatible nanoparticle.

In a particular embodiment of the present invention, when the at least two distinct nanoparticles and the compound(s) (“compound(s) of interest”) are separately administered in a subject in need of said compound with an interval of between more than 5 minutes and about 24 hours, the nanoparticles being preferably administered before the compound(s), the absolute surface charge value of the “first” biocompatible nanoparticle is advantageously of at least 15 mV ($|15 \text{ mV}|$).

Also herein described is a method for treating a subject suffering of a disease such as those herein mentioned, wherein said method comprises administering to said subject a pharmaceutical composition of the invention, typically administering at least two distinct

biocompatible nanoparticles and at least one compound of interest as herein described. Anyone of the at least two nanoparticles or at least one compound of interest can be administered first to the subject as long as the at least two biocompatible nanoparticles on one side and the at least one compound on the other side are administered between more than 5 minutes and about 72 hours from each other. Administration of any of said at least two nanoparticles or at least one compound of interest can be a single administration of each, repeated administrations of each, for example several consecutive administrations of each. The at least two distinct biocompatible nanoparticles may be administered once and the at least one compound of interest may be administered more than once and vice versa.

In a particular embodiment, the at least two distinct biocompatible nanoparticles are at least administered at the beginning of a protocol comprising several administrations of the at least one compound of interest, i.e. at least at the first administration of said at least one compound of interest and before or after the administration thereof.

In another particular embodiment, the at least two distinct biocompatible nanoparticles are not administered at the beginning of a protocol comprising several administrations of the at least one compound of interest and are not administered before the second or third administration of said at least one compound of interest, and before or after the administration thereof.

In the context of these last two embodiments, the at least two distinct biocompatible nanoparticles can also be administered together (before or after as previously explained) with the at least one compound of interest during part or all of the subsequent administrations of said at least one compound of interest.

In a particular embodiment, the at least two distinct nanoparticles of the invention are administered to the subject before administration to said subject of the at least one compound of interest, typically between more than 5 minutes and about 72 hours before administration of said at least one compound of interest.

The at least two distinct biocompatible nanoparticles of the pharmaceutical composition of the invention can be administered by any route such as subcutaneous, intra dermic, oral route, nasal route (inhalation), intra venous (IV), intra-arterial, and/or intra peritoneal. A preferred route of administration is the intra venous route. The at least two distinct biocompatible nanoparticles of the pharmaceutical composition of the invention can be administered by different routes simultaneously or separately.

The compound(s) of interest of the pharmaceutical composition of the invention can be administered by different routes such as subcutaneous, intra venous (IV), intra-dermic, intra-arterial, airways (inhalation), intra peritoneal, intra muscular and/or oral route (*per os*).

- 5 The following examples illustrate the invention without limiting its scope.

BRIEF DESCRIPTION OF THE FIGURES

10

Figure 1: Schematic view of possible routes for therapeutic compounds removal from blood circulation depending on the compound's size (longest dimension).

Figure 2: Schematic representation of the treatments' schedule for the pharmaceutical composition comprising (i) the at least two biocompatible nanoparticles of example 2 and (ii)
15 the Dox-NP® in MDA-MB-231-lucD3H2LN xenografts.

Figure 3: Chemical formula of L-Glutamic acid, N-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA-lipid)

EXAMPLES

20

Example 1: Synthesis of liposomes as the “first” and/or “second” biocompatible nanoparticles.

Liposomes are prepared using the lipid film re-hydration method.

**Synthesis of “first” and/or “second” biocompatible nanoparticles with an absolute
25 surface charge value of at least 10 mV (|10 mV|):**

a) lipids were solubilized in chloroform. Chloroform was finally evaporated under a nitrogen flow. Re-hydration of the lipid film with HEPES 20 mM and NaCl 140 mM at pH 7.4 was performed at 60°C, so that the lipid concentration was 25 mM.

The following lipid composition was used to prepare charged liposomes: DPPC
30 (DiPalmitoylPhosphatidylCholine) 62% mol; HSPC (Hydrogenated Soybean PhosphatidylCholine) 20% mol; CHOL (Cholesterol) 16% mol; POPS (1-Palmitoyl-2-Oleoyl PhosphatidylSerine) 1% mol; DSPE-PEG (DiStearylPhosphatidylEthanolamine-[methoxy(PolyElthyleneGlycol)-2000]) 1% mol.

b) freeze-thaw cycles were then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C.

c) a thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) was used to calibrate the size of the liposomes under controlled temperature and pressure. In all cases, extrusion was performed at 60°C. First, 5 passages through a polyethersulfone (PES) 0.45µm pores-sized membrane were performed at a pressure of 5 bars, then 12 passages through a PES 0.22µm pores-sized membrane at 10 bars, and finally 12 passages through a 0.1µm polyvinylidene fluoride (PVDF) membrane at 15 bars.

Size distribution of the as-prepared liposomes was determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 90°C. The liposomes suspension was diluted 100 times in HEPES 20 mM and NaCl 140 mM at pH 7.4. Liposome size (i.e. hydrodynamic diameter) was equal to about 145 nm (distribution by intensity) with a polydispersity index (PDI) equal to about 0.1.

As understandable by the skilled person, the desired surface charge was obtained thanks to the selected lipidic composition, and its value was confirmed by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument).

The liposomes were diluted 100 times in a sodium chloride solution at 1mM and the pH of the resulting suspension was adjusted to pH 7.4. The liposomes surface charge was equal to about – 25 mV at pH 7.4, NaCl 1mM.

20 Synthesis of “second” and/or “first” biocompatible nanoparticles:

a) lipids were solubilized in chloroform. Chloroform was finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 was performed at 60°C, so that the lipid concentration was 50 mM.

25 The following lipid composition was used: DPPC (DiPalmitoylPhosphatidylCholine) 57% mol; HSPC (Hydrogenated Soybean PhosphatidylCholine) 21% mol; CHOL (Cholesterol) 16% mol; POPS (1-Palmitoyl-2-Oleoyl PhosphatidylSerine) 5% mol; DSPE-PEG (DiStearylPhosphatidylEthanolamine-[methoxy(PolyElthyleneGlycol)-2000]) 1% mol.

b) freeze-thaw cycles were then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C.

c) the liposomes solution was then ultra-sonicated with a probe at a power of 230W during 30s.

d) a thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) was used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion was performed at

60°C. First, 10 passages were applied through a 0.1µm pores size polyvinylidene fluoride (PVDF) membrane under a pressure of 10 bars, then 10 passages through a 0.08µm pores size polycarbonate (PC) membrane under a pressure of 15 bars, and finally 10 passages through a 0.05µm pores size PC membrane under a pressure of 20 bars.

- 5 e) the liposomes solution was then concentrated twice by membrane ultrafiltration on Vivaspin concentrators, with polyethylene sulfone (PES) membrane with a cut-off 300 KDa. Size distribution of the as-prepared liposomes was determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution was diluted 200 times in HEPES 25 mM and NaCl 150 mM
10 at pH 7.4. Liposomes size (i.e. hydrodynamic diameter) was equal to about 70 nm (distribution by intensity) with a polydispersity index (PdI) equal to about 0.1.

The surface charge of the liposomes was determined by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes solution was diluted 200 times in a sodium chloride solution at 1mM and the pH of the solution was adjusted to pH 7. The
15 liposomes surface charge was equal to about -40 mV at pH 7, NaCl 1mM.

Example 2: Synthesis of liposomes as the “first” and/or “second” biocompatible nanoparticles.

Liposomes are prepared using the lipid film re-hydration method.

- 20 - **Synthesis of “first” and/or “second” biocompatible nanoparticles with absolute surface charge value of at least 10 mV (|10 mV|):**

- a) lipids were solubilized in chloroform. Chloroform is finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 is performed at 60°C, so that the lipid concentration is 50
25 mM.

The following lipid composition is used: HSPC (Hydrogenated Soybean PhosphatidylCholine) 59% mol; CHOL (Cholesterol) 38% mol; DSPE-PEG (DiStearylPhosphatidylEthanolamine-[methoxy(PolyElthyleneGlycol)-2000]) 3% mol.

- b) freeze-thaw cycles were then performed 6 times, by successively plunging the sample into
30 liquid nitrogen and into a water bath regulated at 60°C.

c) the liposomes solution was then ultra-sonicated with a probe at a power of 230W during 30s.

d) a thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) was used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion was performed at

60°C. First, 10 passages were applied through a 0.1µm pores size polyvinylidene fluoride (PVDF) membrane under a pressure of 15 bars, then 18 passages through a 0.08µm pores size polycarbonate (PC) membrane under a pressure of 20 bars.

e) the liposomes solution was then concentrated twice by membrane ultrafiltration on

5 Vivaspin concentrators, with polyethylene sulfone (PES) membrane with a cut-off 300 KDa.

Size distribution of the as-prepared liposomes was determined by DLS using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution was diluted 200 times in HEPES 25 mM and NaCl 150 mM at pH 7.4. Liposomes size (i.e. hydrodynamic diameter) was equal to about 90 nm (distribution by intensity) with a
10 polydispersity index (Pdl) equal to about 0.1.

The surface charge of the liposomes was determined by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes solution was diluted 200 times in a sodium chloride solution at 1mM and the pH of the solution was adjusted to pH 7. The liposomes surface charge was equal to about -25 mV at pH 7, NaCl 1mM.

15 The final lipid concentration of the liposomes solution was measured by a colorimetric assay: phospholipase D cuts the phosphatidylcholine molecules, thus relieving the choline group which is going to form a blue pigment by reacting with the chromogenic substrate. Lipids concentration was found at 100mM.

- Synthesis of “second” and/or “first” biocompatible nanoparticles:

20 a) lipids were solubilized in chloroform. Chloroform was finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 was performed at 60°C, so that the lipid concentration is 50 mM.

The following lipid composition was used: DPPC (DiPalmitoylPhosphatidylCholine) 57%
25 mol; HSPC (Hydrogenated Soybean PhosphatidylCholine) 21% mol; CHOL (Cholesterol) 16% mol; POPS (1-Palmitoyl-2-Oleoyl PhosphatidylSerine) 5% mol; DSPE-PEG (DiStearylPhosphatidylEthanolamine-[methoxy(PolyElthyleneGlycol)-2000]) 1% mol.

b) freeze-thaw cycles were then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C.

30 c) a thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) was used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion was performed at 60°C. First, 5 passages through a polyethersulfone (PES) 0.45µm pores-sized membrane were performed at a pressure of 5 bars, then 12 passages through a PES 0.22µm pores-sized

membrane at 10 bars, and finally 12 passages through a 0.1µm polyvinylidene fluoride (PVDF) membrane at 15 bars.

d) the liposomes solution was then concentrated twice by membrane ultrafiltration on Vivaspin concentrators, with polyethylene sulfone (PES) membrane with a cut-off 300 KDa.

5 Size distribution of the as-prepared liposomes was determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution was diluted 200 times in HEPES 25 mM and NaCl 150 mM at pH 7.4. Liposomes size (i.e. hydrodynamic diameter) was equal to about 145 nm (distribution by intensity) with a polydispersity index (PdI) equal to about 0.1.

10 The surface charge of the liposomes was determined by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes solution was diluted 200 times in a sodium chloride solution at 1mM and the pH of the solution was adjusted to pH 7. The liposomes surface charge was equal to about -40 mV at pH 7, NaCl 1mM.

The final lipid concentration of the liposomes solution was measured by a colorimetric assay:

15 phospholipase D cuts the phosphatidylcholine molecules, thus relieving the choline group which is going to form a blue pigment by reacting with the chromogenic substrate. Lipids concentration was found at 100mM.

Example 3: Method for the evaluation of the efficacy and toxicity of a pharmaceutical composition comprising the at least two biocompatible nanoparticles suspensions of example 2 and the Dox-NP® in MDA-MB-231-lucD3H2LN xenografts (cf. Figures 2)

20 The pharmaceutical composition comprising (i) the at least two distinct “first” and “second” biocompatible nanoparticles from example 2 and (ii) Dox-NP® (Liposomal Encapsulated Doxorubicin) as the therapeutic compound of interest, is administered in nude mice bearing MDA-MB-231-lucD3H2LN xenografted tumor in the following manner:

25 a) the Dox-NP® and the biocompatible nanoparticles from example 2 are administered by intravenous injection (IV) via lateral tail vein.

The Dox-NP® (Avanti Polar lipids – Liposomal formulation of 2 mg/ml doxorubicin HCl at pH 6.5-6.8, in 10mM histidine buffer, with 10% w/v sucrose) are injected without additional
30 dilution at the volume required to obtain 3mg/kg of injected doxorubicin.

The biocompatible nanoparticles suspensions from example 2 are used without any additional dilution.

b) four groups of mice are treated as illustrated on figure 2:

- Group 1: sterile glucose 5% (control (vehicle) group).

Mice are intravenously (IV) injected with a sterile glucose 5% solution on day 1, day 7 and day 14.

- Group 2: “First” and “second” biocompatible nanoparticles from example 2 (control group).

5 Mice are intravenously (IV) injected with the “first” and “second” biocompatible nanoparticles from example 2 (10 ml/kg) on day 1, day 7 and day 14. Each time (day), the injection of the first biocompatible nanoparticles is performed 4 hours before injection of the second biocompatible nanoparticles.

- Group 3: Dox-NP® (3mg/kg doxorubicin) (treatment group).

10 Mice are intravenously (IV) injected with Dox-NP® (3mg/kg doxorubicin) on day 1, day 7 and day 14.

- Group 4: pharmaceutical composition, i.e. the combination of (i) the at least two distinct (“first” and “second”) biocompatible nanoparticles from example 2 and of (ii) Dox-NP® (3mg/kg doxorubicin) (treatment group).

15 Mice are intravenously (IV) injected with the biocompatible nanoparticles from example 2 (10 ml/kg) and with the Dox-NP® (3mg/kg doxorubicin) on day 1, day 7 and day 14. Each time (day), the simultaneous injection of the “first” and “second” biocompatible nanoparticles from example 2 is performed 4 hours before the injection of Dox-NP® (3mg/kg doxorubicin).

- Group 5: pharmaceutical composition, i.e. the combination of (i) the at least two distinct
20 biocompatible nanoparticles from example 2 and of (ii) Dox-NP® (3mg/kg doxorubicin) (treatment group).

Mice are intravenously (IV) injected with the biocompatible nanoparticles from example 2 (10 ml/kg) and with the Dox-NP® (3mg/kg doxorubicin) on day 1, day 7 and day 14. Each time (day), the injection of the “first” and “second” biocompatible nanoparticles from
25 example 2 is performed 4 hours and 1 hours respectively before the injection of Dox-NP® (3mg/kg doxorubicin).

c) any clinical sign of toxicity is assessed after the administration of the pharmaceutical composition; and

d) the tumor volume is measured from two dimensional tumor volume measurements with a
30 digital caliper using the following formula:

$$\text{Tumor volume (mm}^3\text{)} = \frac{\text{length (mm)} \times (\text{width})^2 \text{ (mm}^2\text{)}}{2}$$

Example 4: Synthesis of liposomes as the “first” or “second” biocompatible nanoparticles

Liposomes are prepared using the lipid film re-hydration method:

- 5 a) Lipids are solubilized in chloroform. Chloroform is finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 is performed at 60°C, so that the lipid concentration is 50 mM.

10 The following lipid composition was used to prepare charged liposomes: DPPC (DiPalmitoylPhosphatidylCholine) 58% mol; HSPC (Hydrogenated Soybean PhosphatidylCholine) 21% mol; CHOL (Cholesterol) 16% mol; POPS (1-Palmitoyl-2-Oleoyl PhosphatidylSerine) 5% mol.

- 15 b) Freeze-thaw cycles are then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C. Ultra-sonication of the liposomes solution is performed during 30s every 3 freeze-thaw cycles and just before extrusion.

c) A thermobarrel extruder (LIPEX™ Extruder, Northern Lipids) is used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion is performed at 60°C. Ten passages are applied through a 0.1µm pores size polyvinylidene fluoride (PVDF) membrane under a pressure of 10 bars.

- 20 Size distribution of the as-prepared liposomes is determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution is diluted 200 times in HEPES 25 mM and NaCl 150 mM at pH 7.4. Liposomes size (i.e. hydrodynamic diameter) is equal to about 170 nm (distribution by intensity) with a polydispersity index (PdI) equal to about 0.2.

- 25 As understandable by the skilled person, the desired surface charge is obtained thanks to the selected lipid composition, and its value is confirmed by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes are diluted 200 times in a sodium chloride solution at 1mM and the pH of the solution is adjusted to pH 7. The liposomes surface charge is equal to about -40 mV at pH 7, NaCl 1mM.

- 30 The final lipid concentration of the liposomes solution is measured by a colorimetric assay (Bartlett method). The method is based on total phosphorus determination through an acidic digestion of phospholipid. The released inorganic phosphate is reacted with ammonium molybdate, the complex giving a strong blue color. Lipids concentration is equal to about 50 mM.

Example 5: Synthesis of liposomes as the “first” or “second” biocompatible nanoparticles

- 5 Liposomes are prepared using the lipid film re-hydration method:
- a) Lipids are solubilized in chloroform. Chloroform is finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 is performed at 60°C, so that the lipid concentration is 50 mM.
- 10 The following lipid composition was used to prepare the charged liposomes: DPPC (DiPalmitoylPhosphatidylCholine) 45.15% mol; CHOL (Cholesterol) 45.15% mol; DSPE-PEG (DiStearylPhosphatidylEthanolamine-[methoxy(PolyElthyleneGlycol)-2000]) 0.60% mol; L-Glutamic acid, N-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA-lipid) 9.10% mol. The SA-lipid brings COOH groups on the liposomes surface.
- 15 b) Freeze-thaw cycles are then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C.
- c) A thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) is used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion is performed at 60°C. Seven passages are applied through a 0.45µm pores size polyvinylidene fluoride (PVDF)
- 20 membrane under a pressure of 3 bars and ten passages through a 0.22µm pores size polyvinylidene fluoride (PVDF) membrane under a pressure of 10 bars. Size distribution of the as-prepared liposomes is determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution is diluted 200 times in HEPES 25 mM and NaCl 150 mM at pH 7.4. Liposomes size
- 25 (i.e. hydrodynamic diameter) is equal to about 230 nm (distribution by intensity) with a polydispersity index (PdI) equal to about 0.2.
- As understandable by the skilled person, the desired surface charge is obtained thanks to the selected lipid composition, and its value is confirmed by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes solution is diluted 200 times in a
- 30 sodium chloride solution at 1mM and the pH of the solution is adjusted to pH 7. The liposomes surface charge is equal to about -60 mV at pH 7, NaCl 1mM.
- The final lipid concentration of the liposomes solution is measured by a colorimetric assay (Bartlett method). The method is based on total phosphorus determination through an acidic digestion of phospholipid. The released inorganic phosphate is reacted with ammonium

molybdate and the complex giving a strong blue color. Lipids concentration is equal to about 50 mM.

5 **Example 6: Synthesis of liposomes as the “first” or “second” biocompatible nanoparticles**

Liposomes are prepared using the lipid film re-hydration method:

- 10 a) Lipids are solubilized in chloroform. Chloroform is finally evaporated under a nitrogen flow to form a lipid film on the Pyrex tube walls. Re-hydration of the lipid film with HEPES 25 mM and NaCl 150 mM at pH 7.4 is performed at 60°C and the lipid concentration is 50 mM. The following lipid composition was used to prepare the charge liposomes: DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) 60 % mol, CHOL (Cholesterol) 35% mol; and Succinyl PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-succinyl) 5 % mol.
- 15 b) Freeze-thaw cycles are then performed 6 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 60°C. Ultra-sonication of the liposomes solution is performed during 30s, every 3 freeze-thaw cycles and just before extrusion.
- 20 c) A thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) is used to calibrate the size of the liposomes under controlled temperature and pressure. Extrusion is performed at 60°C. Twelve passages are applied through a 0.22µm pores size polyvinylidene fluoride (PVDF) membrane under a pressure of 12 bars.
- 25 d) Conjugation of p-aminophenyl-α-D-mannopyranoside (MAN) to Succinyl PE liposome: The succinyl PE liposome surface are modified with a mannose derived ligand p-aminophenyl-α-D-mannopyranoside (MAN), using carbodiimide coupling to develop mannose conjugated liposome. MAN is covalently coupled by its amino group to the carboxylic acid group of Succinyl PE, present on the surface of preformed Succinyl PE liposome. Briefly, to the preformed Succinyl PE liposome solution are added EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), (Succinyl PE/EDC 1:10 molar ratio) and N-hydroxysuccinimide (NHS) (NHS/EDC 1:2.5 molar ratio). The pH of the suspension is
- 30 then adjusted at 6 with NaOH 1M and the resulting suspension is stirred for 15 minutes at room temperature. Subsequently, the pH of the solution is adjusted at 7 with NaOH 1M and the aqueous MAN solution is added (Succinyl PE/MAN 1:2 molar ratio) to the solution. pH is readjusted at 7 using NaOH 1M and the suspension is stirred for 2 additional hours at room

temperature. Excessive unbound MAN, EDC and NHS molecules are removed by 3 steps of dialysis with dilution factor ($\times 500$; $\times 500$; $\times 500$) using a 50 KDa cellulose membrane.

Of note, due to possible dilution upon dialysis, the liposomes solution can be concentrated by centrifugation (typically a Sigma 3-15K centrifuge at 5°C; 1,200 rpm) using membrane ultrafiltration on Vivaspin concentrators with a polyethylene sulfone (PES) membrane and a cut-off 300 KDa.

Size distribution of the as-prepared liposomes is determined by dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern instrument) with a 633 nm HeNe laser at an angle of 173°C. The liposomes solution is diluted 200 times in HEPES 25 mM and NaCl 150 mM at pH 7.4. Liposomes size (i.e. hydrodynamic diameter) is about 230 nm (distribution by intensity) with a polydispersity index (PDI) around 0.2.

As understandable by the skilled person, the desired surface charge is obtained thanks to the selected lipid composition, and its value is confirmed by zeta potential measurement using a Zetasizer NanoZS (Malvern instrument). The liposomes solution is diluted 200 times in a sodium chloride solution at 1 mM and at pH 7. The liposomes surface charge is around -70 mV at NaCl 1 mM, pH 7.

The final lipid concentration of the liposomes solution is measured by a colorimetric assay (Bartlett method). The method is based on total phosphorus determination through an acidic digestion of phospholipid. The released inorganic phosphate is reacted with ammonium molybdate and the complex giving a strong blue color. Lipids concentration is equal to about 50 mM.

CLAIMS

1. A pharmaceutical composition comprising the combination of (i) at least two distinct biocompatible nanoparticles and of (ii) at least one pharmaceutical compound,
5 wherein the longest dimension of each of the at least two biocompatible nanoparticles is between about 4 nm and about 500 nm, the absolute surface charge value of a first biocompatible nanoparticle is of at least $|10 \text{ mV}|$ and the absolute surface charge value of a second biocompatible nanoparticle, or of any additional biocompatible nanoparticle, has a difference of at least 10 mV with the absolute surface charge value
10 of the first biocompatible nanoparticle, for use in a therapeutic, prophylactic or diagnostic method in a subject in need thereof, the therapeutic, prophylactic or diagnostic method comprising a step of administering the at least one pharmaceutical compound to the subject and a distinct step of administering the at least two distinct biocompatible nanoparticles, said at least two distinct biocompatible nanoparticles
15 being administered to the subject separately from the at least one pharmaceutical compound, typically between more than 5 minutes and about 72 hours before or after said at least one pharmaceutical compound, and wherein the biocompatible nanoparticle is not used as such as a pharmaceutical compound.
- 20 2. The pharmaceutical composition according to claim 1, wherein the at least two distinct biocompatible nanoparticles are to be administered separately in an additional distinct step, or simultaneously, in the subject in need of the pharmaceutical compound.
- 25 3. The pharmaceutical composition according to claim 1 or 2, wherein the first nanoparticle has an absolute surface charge value of more than $|10 \text{ mV}|$, said charge being a negative charge.
4. The pharmaceutical composition according to anyone of claim 1 to 3, wherein the at least two distinct nanoparticles are organic nanoparticles.
- 30 5. The pharmaceutical composition according to claim 4, wherein the at least two distinct nanoparticles are selected from lipid-based nanoparticles, protein-based nanoparticles, polymer-based nanoparticles, co-polymer-based nanoparticles, carbon-based nanoparticles, virus-like nanoparticles and any mixture thereof.

6. The pharmaceutical composition according to anyone of claims 1 to 3, wherein the at least two distinct nanoparticles are inorganic nanoparticles and the longest dimension of each of said nanoparticles is below about 7 nm.
- 5
7. The pharmaceutical composition according to anyone of claims 1 to 3, wherein the at least two nanoparticles are inorganic nanoparticles, the longest dimension of each of said nanoparticles is of at least 10 nm, and the inorganic material of each of the nanoparticles is selected from (i) one or more divalent metallic elements selected for example from Mg, Ca, Ba and Sr, (ii) one or more trivalent metallic elements selected for example from Fe and Al, and (iii) one or more tetravalent metallic elements comprising Si.
- 10
8. The pharmaceutical composition according to claim 7, wherein the inorganic material is selected from calcium carbonate (CaCO_3), magnesium carbonate (MgCO_3), magnesium hydroxide (Mg(OH)_2), iron hydroxide (Fe(OH)_2), iron oxyhydroxide (FeOOH), iron oxide (Fe_3O_4 or Fe_2O_3), aluminium oxide (Al_2O_3), aluminium hydroxide (Al(OH)_3), aluminium oxyhydroxide (AlOOH) and silicon oxide (SiO_2).
- 15
9. The pharmaceutical composition according to anyone of claims 1 to 8, wherein said pharmaceutical composition comprises both organic and inorganic nanoparticles.
- 20
10. The pharmaceutical composition according to anyone of claims 1 to 9, wherein the at least two biocompatible nanoparticles are administered separately or simultaneously in a subject in need of the at least one pharmaceutical compound, and between more than 5 minutes and about 72 hours before the pharmaceutical compound.
- 25
11. The pharmaceutical composition according to anyone of claims 1 to 10, wherein each of the at least two nanoparticles is further covered with a biocompatible coating.
- 30
12. The pharmaceutical composition according to anyone of claims 1 to 11, wherein the combined administration of the at least two biocompatible nanoparticles and of the at least one pharmaceutical compound maintains the therapeutic benefit of said at least one pharmaceutical compound for a reduced toxicity, or increases the therapeutic

benefit of said at least one pharmaceutical compound for an equivalent or reduced toxicity, for the subject, when compared to therapeutic benefit and toxicity induced by the standard therapeutic dose(s) of said at least one pharmaceutical compound.

- 5 13. The pharmaceutical composition according to anyone of claims 1 to 11, wherein the combined administration of the at least two biocompatible nanoparticles and of the at least one pharmaceutical compound allows a reduction of at least 10% of the administered at least one pharmaceutical compound therapeutic dose when compared to the standard therapeutic dose of said at least one pharmaceutical compound while
10 maintaining the same therapeutic benefit for an equivalent toxicity or a reduced toxicity for the subject or while increasing the therapeutic benefit for an equivalent or reduced toxicity for the subject.
14. The pharmaceutical composition according to anyone of claims 1 to 13, wherein the at
15 least two nanoparticles are cleared from the subject to whom it has been administered within one hour and six weeks after their administration to a subject in need of the at least one pharmaceutical compound referred to in claim 1.
15. The pharmaceutical composition according to anyone of claims 1 to 14, wherein the at
20 least one pharmaceutical compound is an organic compound preferably selected from a biological compound, a small molecule targeted therapeutic, an oncolytic virus and a cytotoxic compound.
16. The pharmaceutical composition according to claim 15, wherein the at least one
25 pharmaceutical compound is selected from an antibody, an oligonucleotide, and a synthesized peptide.
17. The pharmaceutical composition according to anyone of claims 1 to 16, wherein the at
30 least one pharmaceutical compound is an inorganic compound selected from a metallic nanoparticle, a metal oxide nanoparticle, a metal sulfide nanoparticle, and any mixture thereof.
18. The pharmaceutical composition according to anyone of claims 1 to 17, wherein the at least one pharmaceutical compound is encapsulated in, or bound to, a carrier.

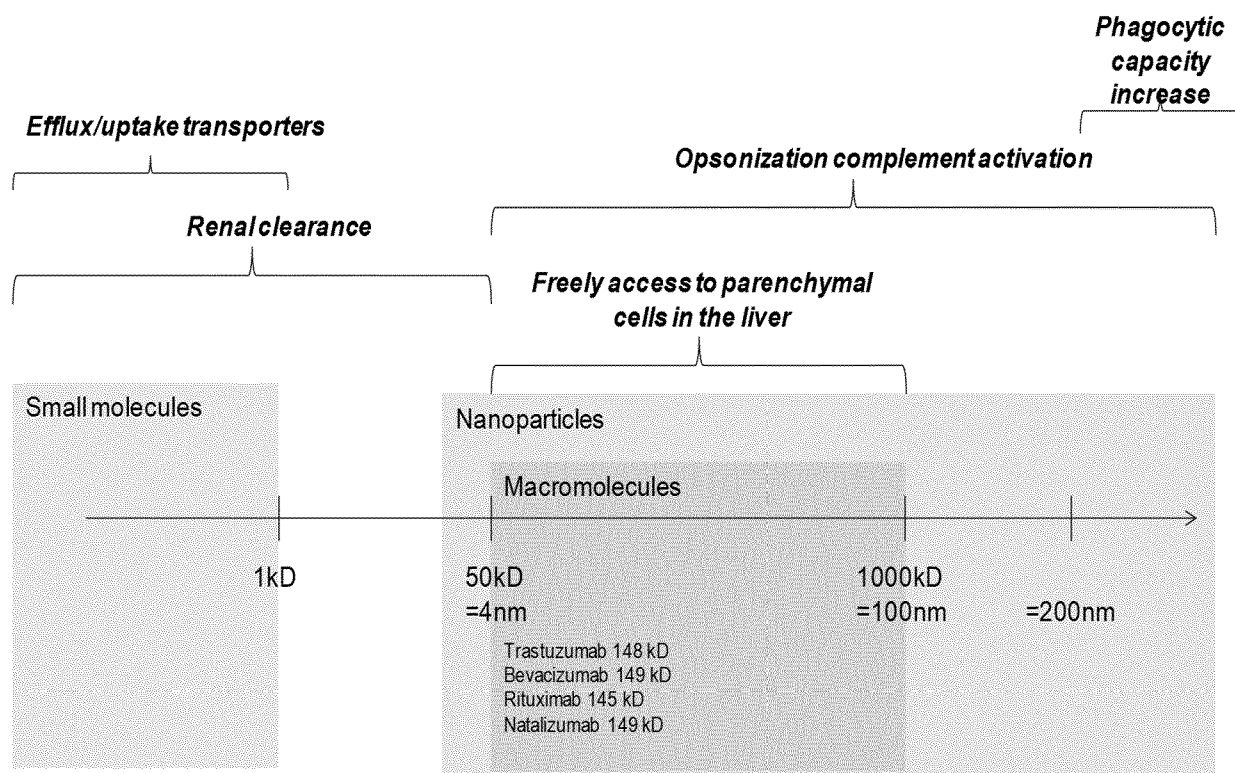


FIGURE 1

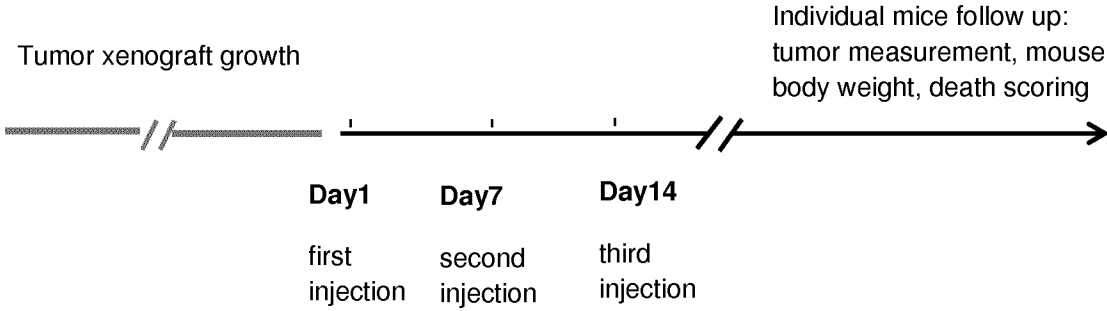


FIGURE 2

3/3

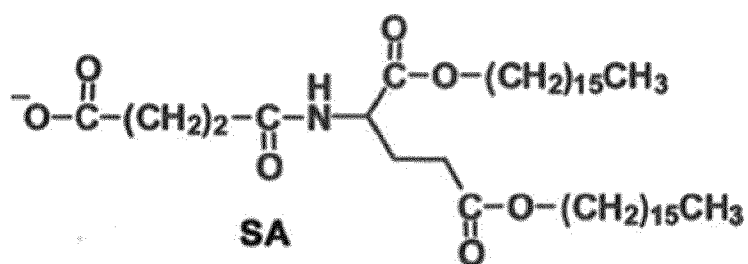


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/077446

A. CLASSIFICATION OF SUBJECT MATTER		
INV. A61K9/00	A61K41/00	A61K9/127 A61K47/48 A61K51/12
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/063305 A1 (BRACCO RESEARCH SA [CH]; SCHNEIDER MICHEL [CH]; BUSSAT PHILIPPE [FR];) 14 July 2005 (2005-07-14) page 21; claim 1; examples 20,21,22,24 pages 27-39	1-18
X	----- EP 2 000 150 A2 (TOTO LTD [JP]) 10 December 2008 (2008-12-10) paragraphs [0005], [0009], [0025] - [0028], [0043], [0067], [0068]; claims 1,9,10	1-18
X	----- WO 2012/104277 A2 (NANOBIOTIX [FR]; CENTRE NAT RECH SCIENT [FR]; POTTIER AGNES [FR]; LEVY) 9 August 2012 (2012-08-09) pages 1,4,10,; claims 1,9 pages 11,18,19 ----- -/-	1-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> 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 26 January 2016		Date of mailing of the international search report 03/02/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Kardas-Llorens, Eyüp

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/077446

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/104275 A2 (NANOBIOTIX [FR]; POTTIER AGNES [FR]; LEVY LAURENT [FR]; MEYRE MARIE-ED) 9 August 2012 (2012-08-09) page 18, line 19 - line 21; claim 1 -----	1-18
X	HE C ET AL: "Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 31, no. 13, 1 May 2010 (2010-05-01), pages 3657-3666, XP026946003, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2010.01.065 [retrieved on 2010-02-06] the whole document -----	1-18
A	"NCL Method PCC-2 Measuring Zeta Potential of Nanoparticles", , 1 November 2009 (2009-11-01), XP055171529, Retrieved from the Internet: URL: http://ncl.cancer.gov/NCL_Method_PCC-2 .pdf [retrieved on 2015-02-23] the whole document -----	1-18
X	WO 2014/057432 A2 (UNIV ROMA [IT]; UNI DEGLI STUDI CAMERINO [IT]) 17 April 2014 (2014-04-17) claims 12,14,15 -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/077446

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005063305	A1	14-07-2005	
		AU 2004308756 A1	14-07-2005
		CA 2547024 A1	14-07-2005
		CN 1897979 A	17-01-2007
		EP 1701745 A1	20-09-2006
		ES 2531732 T3	18-03-2015
		JP 5513708 B2	04-06-2014
		JP 2007515470 A	14-06-2007
		JP 2012051932 A	15-03-2012
		US 2007071685 A1	29-03-2007
		WO 2005063305 A1	14-07-2005
EP 2000150	A2	10-12-2008	
		EP 2000150 A2	10-12-2008
		US 2009130050 A1	21-05-2009
		US 2015352227 A1	10-12-2015
		WO 2007122956 A1	01-11-2007
WO 2012104277	A2	09-08-2012	
		EP 2670393 A2	11-12-2013
		JP 2014503575 A	13-02-2014
		US 2014227343 A1	14-08-2014
		WO 2012104277 A2	09-08-2012
WO 2012104275	A2	09-08-2012	
		AU 2012213538 A1	15-08-2013
		CA 2825564 A1	09-08-2012
		CN 103429227 A	04-12-2013
		EA 201391119 A1	30-01-2014
		EP 2670394 A2	11-12-2013
		JP 2014510040 A	24-04-2014
		KR 20140053843 A	08-05-2014
		US 2014056813 A1	27-02-2014
		WO 2012104275 A2	09-08-2012
WO 2014057432	A2	17-04-2014	NONE