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(54) **Title:** METHOD FOR THE TRANSFER OF NANOPARTICLES

(57) **Abstract:** The present invention relates to a method for transferring at least one nanoparticle into eukaryotic cells or tissue and the use of this method in therapeutic or diagnostic applications. The disclosure provides a method for transferring at least one nanoparticle into eukaryotic cells or tissue comprising the steps of adding at least one thiol group to one end of at least one oligonucleotide to obtain at least one thiolated oligonucleotide, binding of the at least one thiolated oligonucleotide to at least one nanoparticle to obtain at least one oligonucleotide-coated nanoparticle, forming a complex comprising the at least one oligonucleotide-coated nanoparticle by adding an encapsulating reagent to the at least one oligonucleotide-coated nanoparticle, and applying the complex to the eukaryotic cells or tissue. A major advantage of the present method according to the present disclosure is the efficiency, accumulation of the nanoparticles within the cells and the independency of the particle size.



Description

Title: Method for the transfer of nanoparticles

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Field of the Invention

[0001] The present invention relates to a method for transferring at least one nanoparticle
10 into cells or tissue and the use of this method in therapeutic or diagnostic applications.

Background of the invention

[0002] Nanoparticles have been widely used in a variety of disciplines such as in modern
15 natural, (bio)technical and medical science for several decades. The first therapeutic
nanoparticles were developed as carriers of anticancer and other pharmaceuticals
(Couvreur et al., J. Pharm. Sci., 1982, 71: 790-92). Later, nanoparticles have also been
used for the treatment of certain diseases, e. g. for preventing restenosis in patients
undergoing coronary angioplasty (Labhasetwar et al., Adv. Drug Del. Rev., 1997, 24: 63-
20 85).

[0003] Nanoparticles have been used for biomedical applications, like targeted delivery
of the nanoparticles to various cell types, bioimaging, biodetection, gene delivery, drug
delivery, in antisense technology and other therapeutic and diagnostic applications (Tiwari
25 et al., Nanomaterials, 2011, 1: 31-63). Nanoparticles have also been used in radiotherapy.

[0004] Radiotherapy, in combination with surgery and chemotherapy, is nowadays a
fundamental part of multimodal cancer treatment. Over 50% of all patients who develop
cancer will require radiotherapy at some time during their illness, depending on the
30 malignancy (Boyle and Levin, World cancer report 2008. International Agency for
Research on Cancer, WHO Press, Lyon Geneva, 2008).

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[0005] In radiotherapy, the tumor-control probability directly correlates with the amount of dose given to the tumor, yet is also limited by the damage to the surrounding normal tissue. Recent advances in radiation technologies, treatment planning and treatment delivery have resulted in increased sparing of normal tissue outside the clinical target volume. Yet normal-tissue reactions from high doses received by critical structures inside the target volume are still a major concern. Therefore there is a need for new approaches that can increase the therapeutic window. Beside radioprotection of the normal tissue, radiosensitization of the tumor tissue can be an option to improve radiation therapy.

[0006] One possibility for enhancing the radiosensitivity of tumor cells is the usage of materials with a high atomic number (high-Z). These materials are able to absorb more photon energy than low-Z materials, thereby generating more short-range Auger- and photoelectrons during irradiation and therefore should increase the damage to the DNA of tumor cells treated with such materials. Matsudaira et al. showed such an effect 1980 by using iodine ($Z=53$) contrast medium as radiosensitizer (*Radiat. Res.*, 1980, 84: 144-48). Santos Mello et al. (1983) were able to support this concept by showing significant growth suppression of 80% of tumours in mice after intratumoral injection of iodine contrast medium and subsequent x-ray irradiation (*Med. Phys.*, 1983, 10: 75-78).

[0007] Using gold instead of iodine is expected to further enhance this effect because of its higher Z number ($Z=79$). Hainfeld et al. (2004) showed a significant increase in one year survival (20% for x-rays alone vs. 50-86% for x-rays with gold nanoparticles (GNP)) of mice with tumors after 250 kV x-ray therapy using intravenous injected GNPs (1.9 nm diameter) as radiosensitizers (Hainfeld et al., *Phys. Med. Biol.*, 2004, 49: N309-15).

[0008] Accumulation of GNP in the tumor tissue is achieved by passive targeting caused by the enhanced permeability and retention (EPR) effect of the tumor vasculature compared to the vasculature in non-malignant tissue (Hainfeld et al., *Phys. Med. Biol.*, 2004, 49: N309-15; Wang et al., *Curr. Drug. Metab.*, 2010, 11: 129-41). The gaps in the former have a size of about 600 nm, whereas particles larger than 300 nm are eliminated by macrophages. So the optimal size for specific accumulation in the tumor tissue seems to be about 100-300 nm. Whereas very small particles (<30 nm) can also enter the tumor

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tissue by the EPR effect, they could also potentially leave passively. Yet this effect is expected to be reduced in the tumor tissue due to lack of draining lymphatic vessels (Wang et al., Curr. Drug. Metab., 2010, 11: 129-41).

5 [0009] Kircher et al. (Nat. Medicine, 2012, 18(5): 829-34) describe an *in vivo* molecular imaging technology using nanoparticles in order to delineate the margins of brain tumors in living mice both preoperatively and intraoperatively. Intravenous injection can lead to particle accumulation and retention of the nanoparticles by the tumours while the surrounding healthy tissue does not accumulate the nanoparticles.

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[0010] Monte Carlo simulations by Petersheim et al. (In World Congress on Medical Physics and Biomedical Engineering (Dössel and Schlegel, Eds.), 2009, pp. 809-12) showed a 2.5-fold local dose enhancement by GNP for kV radiation of tumor tissue. Additional theoretical calculations by others showed large effects through GNP at
15 kilovoltage energies and only small effects at megavoltage energies that are more often used in radiotherapy (Jones et al., Med. Phys., 2010, 37: 3809-16). Cho et al. calculate a dose enhancement factor (DEF) of 1.007 - 1.014 at 6 MV vs. 2.114 at 140 kV (7 mg gold/g tumor) (Phys. Med. Biol., 2005, 50: N163-73).

20 [0011] Interestingly, initial *in vitro* experiments showed larger than expected effects also in the megavoltage range. Chithrani et al. showed a DEF of 1.17 for 6 MV X-rays, 1.43 for 220 kV and 1.66 for 105 kV X-rays in HeLa cells (Radiat. Res., 2010, 173: 719-28).

[0012] All of the described applications require the efficient uptake of the nanoparticles.
25 Non-functionalized nanoparticles are taken up by passive endocytosis. This type of uptake, however, is particle size-dependent, not efficient and difficult to modify or influence in any way.

[0013] Chithrani et al. (Radiat. Res., 2010, 173: 719-28) disclose an *in vitro* study with
30 gold nanoparticles and show that cellular uptake depends on the size of the nanoparticles. The optimal size is a diameter of 50 nm. They also disclose that a higher concentration of nanoparticles in the cells leads to greater radiosensitization. This restricts the use of smaller

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or larger particles that may prove to be more efficient in sensitizing tumor cells. Smaller GNP are expected to show increased dose-modifying effects, as in larger particles the short-ranged Auger electrons are more likely to be trapped before leaving the GNP. As they can more readily escape from smaller particles, they are more likely to deposit their energy in the surrounding tissue. Hossain and colleagues confirmed this using Monte Carlo modelling, as they could show that the DMF inversely correlated with the particle size (Nanomaterials and interfaces, 2012, 116, 23047-23052).

[0014] A free choice of particle size is highly relevant for a lot of applications. Maximal dose-effect relations require optimization of nanoparticle uptake. Nanoparticles of a different diameter or higher amounts of nanoparticles can lead to stronger effects.

[0015] The size of the nanoparticles is also an important parameter for targetability. While larger nanoparticles (diameter greater than/equal to 30 nm) can enter cells but not the cell nucleus, smaller nanoparticles (diameter of about 5 nm) do cross the nuclear membrane and can therefore be targeted to the nucleus. Some applications require the uptake of nanoparticles into the nucleus.

[0016] Consequently, according to the methods currently used nanoparticle size seems to be the only way of influencing the efficiency of uptake of the nanoparticles, the amount of nanoparticles in the cells and thereby an effect of the nanoparticles.

[0017] Therefore, current methods of nanoparticle uptake are disadvantageous because they are inefficient and the uptake of the nanoparticles is size-dependent. There is an increasing need of improving the uptake of nanoparticles.

[0018] A wide variety of transfection methods are known which allow uptake of DNA or RNA into cells of different origin. Among the methods commonly known are DNA precipitation onto the cell surface with calcium phosphate or enclosure of the DNA in liposomes. Liposomes which are induced in connection with cell uptake are also called lipoplexes. They involve cationic lipids which form complexes (liposomes/lipoplexes) with the negatively charged nucleic acids. The lipoplexes fuse with the cell membrane so that

the nucleic acids are introduced into the cell (endocytic uptake). One example of a cationic lipid is Didodecyl dimethylammonium bromide (DDAB). Cationic polymers can also be used instead of cationic lipids. The resulting complexes are called polyplexes. Known polymers comprise Polyethyleneimine (PEI), Poly(2-dimethylamino-ethylmethacrylate) (PDMAEMA) or Poly(amido amine) (PAMAM). Polyethylene glycol (PEG) can be added as it helps the complexes to fuse with the cell membrane. More recent transfection methods comprise electroporation, microinjection or biolistics. All of these transfection methods can also be referred to as non-targeted methods. They do not involve binding of specific structures of the nanoparticles to specific structures on the cell membrane, for example.

[0019] Cebrian et al. (Acta Biomater., 2011, 7(10): 3645-55) use nanoparticles as transfection reagents. Only gold nanoparticles were coated with PEI and complexed with DNA. They achieve better transfection efficiencies with smaller Au-nanoparticle-PEI-DNA complexes. Thus, the authors conclude that transfection efficiency using Au-nanoparticle-PEI-DNA complexes is size-dependent.

[0020] Li et al. also report that cationic lipids or cationic polymers can be coupled to nanoparticles to be used as transfection reagents (Biomaterials, 2010, 31(7): 1850-57). They show that gold nanoparticles coated with a cationic lipid can enhance the transfection efficiency of cationic liposomes. Complexes are formed out of lipid-coated nanoparticles, DNA and liposomes but the DNA is not attached to the nanoparticles.

[0021] Several publications deal with the attachment of nucleotides to nanoparticles: For example, the patent application US 2007/0154891 A1 discloses a structure and method for forming single-stranded DNA segments/single-wall carbon nanotube complexes and a method of preparing single-stranded DNA segments. The method for forming single-stranded DNA segments/single-wall carbon nanotube complexes includes attaching single-stranded DNA segments to single-wall carbon nanotubes to form single-stranded DNA segment/single-wall carbon nanotube complexes, each of the single-stranded DNA segments having a same length of greater than 2,000 bases. This document also discloses the attachment of DNA to gold nanoparticles.

[0022] US 2010/0075314 A1 discloses stable bioconjugate-nanoparticle probes which are useful for detecting nucleic acids and other target analytes, e.g., proteins, and methods of preparing those probes. The document also discloses methods for preparing bioconjugate-nanoparticle probes, methods of detecting target analytes using the probes, and kits comprising the probes. They also suggest using thiolated nucleotides for attachment to nanoparticles.

[0023] The EP 2 511 231 A2 relates to a dimeric core-shell nanostructure comprising two nanoparticles in which a Raman active molecule is localized at an interparticle junction. The document also discloses a method for constructing the dimeric nanostructure and a method for detecting an analyte using the dimeric nanostructure. A kit for detecting an analyte comprising the dimeric nanostructure is also provided. The method for constructing the dimeric nanostructure also involves the attachment of thiolated DNA sequences to nanoparticles.

[0024] The patent US 6,495,324 B1 provides methods of detecting a nucleic acid. The methods comprise contacting the nucleic acid with one or more types of particles having oligonucleotides attached thereto (nanoparticle-oligonucleotide conjugates). In one embodiment of the method, the oligonucleotides are attached to nanoparticles and have sequences complementary to portions of the sequence of the nucleic acid. The invention further provides nanomaterials and nanostructures comprising nanoparticles and methods of nanofabrication utilizing the nanoparticles.

[0025] However, none of the documents above disclose methods of size-independent transfer of such nanoparticles or conjugates into cells or tissue.

[0026] Franzen et al. (US patent No. 7,332,586) disclose a nanoparticle delivery vehicle which comprises a nanoparticle, an active agent and a nuclear localization signal. They also disclose methods of modulating gene expression and protein expression using the nanoparticle vehicle. Depending on the size of the nanoparticle, the active agent is delivered to the cytoplasm or to the nucleus of the cell. The active agent can be any kind of therapeutic agent. Delivery into the cell is mediated by targeted endocytosis, e. g. receptor-

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mediated endocytosis. They do not disclose a method of delivery of nanoparticles into cells by means of non-targeted endocytosis.

[0027] The document KR 20100029062 discloses a delivery method for the delivery of bioactive substances, e. g. DNA, using a lipid-gold nanoparticle complex. DNA is attached to the gold nanoparticle by electrostatic interaction. They do not disclose a delivery method for nanoparticles.

[0028] Several publications deal with the encapsulation of nanoparticles: For example, the document WO 2012/001579 A1 deals with the synthesis of iron-oxide nanoparticles. The authors also disclose the encapsulation of nanoparticles with a carrier and decorating the nanoparticle with a targeting ligand.

[0029] The WO 2009/097480 A2 discloses liposomal delivery compositions comprising a nanostructure. They also disclose thiolation of nanostructures and encapsulation of nanostructures within liposomes.

[0030] The EP 1614414 A2 discloses a composition for use in the treatment of mammalian diseases. The composition comprises a core containing a biologically active copper compound, and a sheath. The sheath may be a glucose or liposome encapsulating the core.

[0031] None of the cited documents, however, provide an efficient method for size-independently transferring nanoparticles into cells or tissue.

Object of the Invention

[0032] The object of the present disclosure is the provision of an efficient method for transferring at least one nanoparticle into eukaryotic cells or tissue independent of its size.

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[0033] The present disclosure provides a method for transferring at least one nanoparticle into eukaryotic cells or tissue comprising the steps of adding at least one thiol group to the 5' end of at least one oligonucleotide so that at least one thiolated oligonucleotide is obtained, binding of the at least one thiolated oligonucleotide to at least one nanoparticle so that at least one oligonucleotide -coated nanoparticle is obtained, forming a complex comprising the at least one oligonucleotide-coated nanoparticle by adding an encapsulating reagent to the at least one oligonucleotide-coated nanoparticle, and applying the complex to the eukaryotic cells or tissue.

[0034] Use of the method in therapeutic or diagnostic applications is also provided.

Summary of the Figures

[0035] Fig. 1: Schematic illustration of the generation and application of DNA-coated metallic nanoparticles (MNPs) for the efficient uptake into cells

[0036] Fig. 2: Determination of potential cytotoxic effects of the GNP constructs on cell proliferation

[0037] Fig. 3: Fluorescence (A) and confocal (B) microscopy of HeLa cells treated with the different GNP constructs

[0038] Fig. 4: Quantitative analysis of samples detected using confocal microscopy

[0039] Fig. 5: Transmission electron microscopy images of GNP-DT-treated cells

[0040] Fig. 6: Clonogenic survival of HeLa cells after treatment with the different GNP constructs and subsequent X-ray irradiation

Detailed Description of the Invention and the Figures

[0041] The present disclosure provides a method for transferring at least one nanoparticle into eukaryotic cells or tissue.

[0042] An „oligonucleotide“ shall be understood within the meaning of the present disclosure as a single or double-stranded chain or polymer of nucleotides, preferably as individual structures making up DNA or RNA.

[0043] An active uptake into cells means within the present disclosure that nanoparticles are coated with or attached to DNA and subsequently encapsulated with a suitable reagent like a transfectant, e.g. for lipofection.

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[0044] The disclosure provides a method for transferring at least one nanoparticle into eukaryotic cells or tissue comprising the steps of adding at least one thiol group to at least one end of at least one oligonucleotide to obtain at least one thiolated oligonucleotide, binding of the at least one thiolated oligonucleotide to at least one nanoparticle to obtain at least one oligonucleotide-coated nanoparticle, forming a complex comprising the at least one oligonucleotide-coated nanoparticle by adding an encapsulating reagent to the at least one oligonucleotide-coated nanoparticle, and applying the complex to the eukaryotic cells or tissue.

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[0045] Thus, thiol-labeled is synonymous with thiolated.

[0046] The method of the present invention differs from the state of the art:

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[0047] First of all, US 2007/0154891 A1, US 2010/0075314 A1, EP 2 511 231 A2 and US 6,495,324 B1 do not even describe nor address the problem of transferring the complexes into cells or tissue. These documents therefore do not anticipate the present invention.

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[0048] Although the KR 20100029062 describes the transfer of DNA into cells using gold nanoparticles, the authors do not disclose the use of thiolated DNA attached to nanoparticles. Instead, DNA is attached to the gold nanoparticle by electrostatic interaction. The interaction between gold and thiol groups is much stronger than mere electrostatic interaction. The use of thiol-labeled DNA attached to gold nanoparticles results in an accumulation of nanoparticles within a cell, whereas passively transferred nanoparticles will not stay in a cell after its transfer. In addition, using thiolated DNA allows orientation control of the attached molecule because thiol groups have shown to assemble into well-ordered structures on gold surfaces. In addition, the purpose of KR

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20100029062 is the delivery of the DNA and not the delivery of nanoparticles. It was completely surprising that the presently disclosed method allows the efficient and size-independent uptake of nanoparticles into eukaryotic cells or tissue.

5 [0049] The WO 2012/001579 A1 does not disclose the attachment of thiolated nucleotides before encapsulation and does not address the problem of delivery of nanoparticles into cells or tissue.

[0050] And while WO 2009/097480 A2 discloses thiolation of nanostructures, it does not
10 disclose thiolation of nucleotides and attachment of thiolated nucleotides to nanostructures.

[0051] EP 1614414 A2 discloses the encapsulation of cores, but this document does not address thiolation of nucleotides or the use of nucleotides and attaching thiolated nucleotides to nanoparticles.

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[0052] The method according to the present invention provides a size-independent, active and efficient uptake of nanoparticles into eukaryotic cells or tissue by transfection. The transfection allows for targeted or non-targeted endocytosis. Transfection is known as a method for delivery of oligonucleotides or DNA. It was completely surprising to a person
20 skilled in the art that transfection can also be used for uptake of oligonucleotides which are attached to nanoparticles. Thereby, transfection provides a novel means for uptake of nanoparticles into cells or tissue.

[0053] Nanoparticles can have a diameter of about 1 to 1000 nm and may be metal
25 nanoparticles. It is intended that the metal is selected from the group comprising titanium, bismuth, titanium dioxide, tin, tin oxide, iron, iron(III)oxide, silver, nickel, gold, copper and aluminum.

[0054] Gold nanoparticles (GNP) are advantageous for several reasons. Gold is very well
30 tolerated in, e. g., human organisms and provides a biologically inert material. Gold nanoparticles can also be easily synthesized in various sizes and easily functionalized.

[0055] Functionalization of nanoparticles may either involve chemical functional groups or biological molecules. Functionalization is performed in order to render the nanoparticles more stable and increase their functionality and biocompatibility. Both, the properties of the nanoparticles and of the bound biological molecules have to be preserved. Surface functionalization is important in order to target the nanoparticles to specific areas so that the nanoparticles can selectively interact with the (e. g. biological) structures of interest.

[0056] Functionalization may also allow gene transfer or use of nanoparticles as transporters of biological molecules or chemical functional groups. Gene transfer may comprise the transfer of unmodified or modified oligonucleotides, e. g. of DNA.

[0057] Functionalization with chemical functional groups can prevent aggregation of the nanoparticles, even under unfavourable pH or ionic strength conditions. Thereby, the nanoparticles can be stabilized by electrostatic repulsion. Chemical functional groups can also allow attachment of biological molecules. Possible chemical functional groups can be chosen depending on the particle size and the solvent. Chemical functional groups comprise but are not restricted to mercaptocarboxylic acid or polyethylene glycol (PEG). Chemical functional groups may also comprise biotin, chemotherapeutics or fluorochromes.

[0058] In case the nanoparticles are functionalized with biological molecules the nanoparticles can be stabilized through passive adsorption of the biological molecules onto the surface of the nanoparticles by electrostatic and hydrophobic interactions between the nanoparticle and the biological molecule. Passive adsorption is advantageous because structure and function of the biological molecules can be mostly retained. Electrostatic interaction is an easy and stable way to functionalize the nanoparticles. For example, positively charged nanoparticles can bind to negatively charged nucleophilic biological molecules by ionic interactions. The biomolecules can also be thiolated. Thiolated biomolecules bind to gold nanoparticles, for example, via thiol-gold affinity interactions.

[0059] Biological molecules can also be attached to the nanoparticles via covalent bonds between the nanoparticle and the biological molecules or between chemical functional

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groups (attached to the nanoparticle) and the biological molecules. Biological molecules comprise but are not restricted to oligonucleotides, sugars, peptides or proteins. The proteins may comprise cell surface receptors or antibodies. Covalent binding may also comprise a covalent bond between nanoparticles and thiol groups. Thiolated nanoparticles
5 can in turn bind to, for example, oligonucleotides.

[0060] In particular, functionalization of nanoparticles may lead to binding of the following molecules to the nanoparticles: diagnostic molecules such as imaging molecules, therapeutic molecules such as chemotherapeutic molecules, radiotherapeutic molecules,
10 radiosensitizing molecules, molecules that bind to a peptide, a protein, a nucleic acid or any ligand for diagnostic or therapeutic applications. Any combinations of these molecules are also provided.

[0061] An oligonucleotide-coated nanoparticle according to the present disclosure
15 comprises a nanoparticle as described e.g. in paragraph 54, which is coated with at least one labeled oligonucleotide. The labeled (e.g. thiolated) oligonucleotide for coating the nanoparticle may be synthesized by commonly known techniques like for instance solid-phase synthesis or polymerase chain reaction (PCR). Polymerase chain reaction is an easy and effective way of thiolating oligonucleotides. Primers with thiol groups are used
20 together with an oligonucleotide template in the polymerase chain reaction for amplification and thiolation of the oligonucleotide, resp. the PCR product. Thus, it is intended that the thiol group is added to the at least one oligonucleotide by using thiolated oligonucleotides for generating the at least one thiolated oligonucleotide.

25 [0062] Using thiolated primers in a PCR allows to amplify a region of interest whether it is a coding region or a non-coding region. The PCR product (containing a thiol group) will then be added to at least one nanoparticle.

[0063] So unlike described in the paper by Jung et al. (Biosensors and Bioelectronics, 2010, 25: 1941–46), where “labeled” gold nanoparticles with thiol-labeled
30 oligonucleotides are used, the present disclosure uses the oligonucleotides first in a PCR to amplify a double-stranded DNA fragment (e. g., 300 or 1200 bp) and then labels the nanoparticles with the DNA product of the PCR. Thus, a different approach is disclosed,

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which allows for the functionalization of double-stranded DNA and offers additionally a second functional site for further functionalization like adding of thiols, biotin, fluochromes etc.

5 [0064] The encapsulating reagent may be a transfection reagent which may be based for instance on liposomes, cationic lipids or calcium phosphate. Liposomes are artificially-prepared vesicles composed of a lipid bilayer. Liposomes are made out of cationic lipids or cationic polymers and allow for efficient uptake of the nanoparticles. Basically, the invention is not limited to a specific transfection reagent.

10 [0065] It is further intended that a thiol group, biotin or fluorochemicals may modify the 3'-end of the thiolated oligonucleotide. A second thiol group may serve for nanoparticle crosslinking. Biotin or fluorochemicals may serve for indirect or direct labeling of the nanoparticles (biodetection). It is obvious for a person skilled in the art that the 3'-end can
15 be modified with any detectable compound.

[0066] The at least one thiolated oligonucleotide may be covalently bound to the at least one nanoparticle, wherein the transfer of a labeled nanoparticle is independent from the size of the oligonucleotide.

20 [0067] HeLa cells have been proven successful for treatment with nanoparticles. It is obvious for a person skilled in the art that the invention is not limited to eukaryotic cells.

[0068] It is a further object of the present invention to use the above described method in
25 therapeutic or diagnostic applications. Such applications comprise targeted delivery of nanoparticles into cells or tissue, imaging, gene delivery, drug delivery, and antisense technology.

[0069] The method of the present disclosure may also be used in tumor therapy, for
30 example in radiotherapy, targeting of chemotherapeutics or hyperthermia.

[0070] Use of the method of the present disclosure in radiotherapy might lead to an enhancement of the therapeutic index of radiotherapy. Materials with a high z number absorb more energy during radiation which means that more secondary electrons are generated compared with materials with a lower z number. These electrons can cause greater DNA damage to tumor tissue and thereby radiosensitization of tumor tissue.

[0071] Imaging shall be understood as a technique or process used to create images of cells, tissues or even a whole organism or parts and functions thereof for clinical purposes (medical procedures seeking to reveal, diagnose or examine disease) or medical science (including the study of normal anatomy and physiology). Although imaging of removed organs and tissues can be performed for medical reasons, such procedures are not usually referred to as medical imaging, but rather are a part of pathology. Such in vitro or in situ techniques are well known to a person skilled in the art.

Examples

[0072] The invention will now be described on the basis of examples and figures. It will be understood that the embodiments and aspects of the invention described herein are only examples and do not limit the protective scope of the claims in any way. The invention is defined by the claims and their equivalents. It will be understood that features of one aspect or embodiment of the invention can be combined with a feature of a different aspect or aspects and/or embodiments of the invention.

Example 1: Schematic illustration of the generation and application of DNA-coated metallic nanoparticles (MNPs)

[0073] Figure 1 shows a schematic illustration of the generation and application of DNA-coated metallic nanoparticles (MNPs) for the efficient uptake into cells as an example of the presently disclosed method A. shows the thiolation and amplification of a DNA template by polymerase chain reaction (PCR) using a thiolated primer. Addition of the PCR product (the thiolated DNA) to metallic nanoparticles (i. e. labeling of the MNPs) like gold nanoparticles leads to DNA-coated MNPs. B. shows the addition of a transfection

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reagent to the DNA-coated MNPs which are then complexed into the transfection reagent. C. shows the transfection of the complex into a target cell and the release of the complex in the target cell. Elements are not drawn to scale.

5 Example 2: DNA amplification for synthesis of thiolated DNA

[0074] A DNA fragment with a total length of 0.3 kb was amplified by PCR using a 5'-thiol-labeled forward primer (ACAGGATGACTTTACCGCTCG) and an unmodified reverse primer (GCTTTGGCAATTCCCAGG; all Metabion GmbH, Martinsried, Germany). As a template, a non-coding fragment from the GSS gene (nm_000178; start: bp 365, stop: bp 665) was chosen. To ensure that the to be transfected PCR product will not result into an active gene product, a non-coding sequence was chosen as an amplicon. In addition, this fragment did not contain any promoter/transcription-initiation sequences. The product was amplified using the following conditions: 0.5 μ M primer mix (forward/ reverse primer), 200 μ g/l template, 0.2 mM dNTPs, 10 U Tfi DNA Polymerase, 20 μ l 5xTfi PCR Rxn Buffer, 1.5 mM $MgCl_2$ and adjusted with H_2O to 100 μ l (2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, 75 s at 72°C, finished with 10 min at 72°C). After amplification, the product was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and the product was checked by agarose-gel electrophoresis. The DNA concentration was measured using a Tecan Infinite M200 spectrometer with a NanoQuant plate (Tecan, Group Ltd., Männedorf, Switzerland).

Example 3: Oligonucleotide labeling of nanoparticles to obtain oligonucleotide-coated nanoparticles

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[0075] Citrate-stabilized GNP (size: 7.5 +/- 2.6 nm) in water were obtained from Particular GmbH (Hannover, Germany). To coat the GNP with the thiol-labeled PCR product, 10 μ l of the GNP solution (about 13 nM) and 0.5 μ g PCR product were mixed and incubated for 20 min at room temperature. To determine labeling efficiency, a colorimetric assay by Jung et al. (Biosensors and Bioelectronics, 2010, 25: 1941-46) was used (color shift by salt-induced aggregation of unlabeled GNP). To avoid the availability of unlabeled GNP, the latter was saturated with DNA. For unlabeled GNP, a change from brown/red to

light blue was observed caused by NaCl-induced GNP aggregation. No changes in color were observed for the DNA-labeled GNP suggesting an efficient labeling of GNP with DNA (not shown).

5 Example 4: Transfection of cells with nanoparticles

[0076] In general, HeLa cells (human cervix carcinoma cells) were grown in DMEM (Dulbecco's modified Eagle's medium, Biochrom AG, Berlin, Germany) containing 10% FBS (Biochrom AG). Cells were cultivated at 37°C in a humidified incubator with 95%
10 air/ 5% CO₂. For transfection, HeLa cells were cultivated for at least 2 hours in a 24 well plate and subsequently treated with H₂O (control), pure GNP (GNP), pure GNPs with transfection reagent (GNP-T) or DNA-labeled GNPs with transfection reagent (GNP-DT). Transfection was performed according to the manufacturer's protocol for 24 well plates using 2 µl metafectene (Biontex). For the control and pure GNPs group H₂O or GNP stock
15 solution was filled up with DMEM to 60 µl and added to the wells. (Mock-)Transfected cells were incubated for 16 h in a humidified incubator (37°C, 95% air/ 5% CO₂) and thereafter medium was replaced with fresh medium.

Example 5: Analysis of short term cytotoxicity

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[0077] To determine the cytotoxicity of the different GNP constructs, a proliferation (MTT; 48 h) assay was performed. HeLa cells were seeded in 24 well plates, per group 1*10⁵ cells in 500 µl complete medium per well. After transfection, cells were harvested and reseeded into a 96 well plate (8 wells per group, 5*10³ cells per well in 100 µl
25 complete medium). After 48h (including transfection), 20 µl MTT (5 mg/ml, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and incubated for 3 h at 37°C. Viable cells reduce the yellow MTT to a non-hydrosoluble purple formazan. Thereafter 100 µl of 10% SDS (Sodium dodecyl sulfate)/ 10 mM HCl in PBS was added to each well and plates incubated o/n at 37°C to allow dissolving of the
30 formazan. The next day, the absorbance at 590 nm (reference 690 nm) was quantified using a spectrophotometer (Tecan Infinite M200). Results are shown as percentage signal of the untreated control cells in table 1.

Treatment	Proliferation (vs PBS)
PBS	100%
GNP	95.1% $\pm$ 3.5%
GNP-T	82.2% $\pm$ 6.0%
GNP-DT	76.1% $\pm$ 7.9%

Table 1: Determination of potential short-term cytotoxic effects of the GNP constructs on cell proliferation.

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[0078] Figure 2 shows the determination of the potential cytotoxic effects of the GNP constructs on cell proliferation. Compared to untreated controls (PBS) negligible toxicity was observed using pure GNP (95.1% $\pm$ 3.5% vs PBS (= 100%)). The use of transfection resulted in increased toxicity, even without DNA (GNP-T: 82.2% $\pm$ 6.0%). Labelling of the GNP with DNA and subsequent transfection increased toxicity compared to untreated controls (GNP-DT: 76.1% $\pm$ 7.9%).

[0079] In summary, a low (PBS and GNP) to moderate (GNP-T and GNP-DT) proliferation-inhibiting effect on HeLa cells was observed. The addition of transfection (irrespective of the use of GNP) resulted in a significant increase in toxicity ($p_{\text{GNP vs GNP-T}}$: 0.03; $p_{\text{GNP vs GNP-DT}}$: 0.04; $p_{\text{GNP-T vs GNP-DT}}$: 0.21, ns). Data are presented as mean $\pm$ SD (n=3). *: $p < 0.05$.

Example 6: Efficiency of GNP uptake

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[0080] To determine the efficiency of the uptake of the different GNP constructs, fluorescence, confocal and transmission electron microscopy were performed.

[0081] Using fluorescence microscopy (Fig. 3A), after treatment with PBS or GNP, no fluorescence signal was observed in the HeLa cells. For the GNP-T-treated cells, a sporadic and weak red focal cellular staining could be detected in the cells, whereas a very

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strong, focal signal (primarily perinuclear) was observed in the DNA-labeled GNP-treated samples (GNP-DT). Nuclei were counterstained with DAPI (blue; magnification 40x10).

[0082] For confocal microscopy, cells were grown for another 24 or 48 hours at 37°C and subsequently fixed with 3% formaldehyde in PBS for 10 min (after treatment). Cells were washed with PBST/ 1% BSA (PBS/ 0,2% Triton X-100 /1% bovine serum albumin) and embedded with Mowiol-DABCO mounting medium. Samples were analyzed using a Leica TCS SP5X confocal laser microscope (Leica, Wetzlar, Germany). As suggested in the literature (He et al., Anal. Chem., 2008, 80: 5951-57), GNP can be excited by laser light with a wavelength of 520 nm and thereby detected using a confocal microscope. Use of higher wavelengths (in confocal; 570 nm) showed increased emission intensities and was therefore used. Emission was detected between 580 and 660nm. Analysis was performed using the Leica LAS AF (Leica Application Suite Advanced Fluorescence; Leica) software package.

[0083] Using confocal microscopy, a similar picture could be observed compared with fluorescence: Figure 3B show representative confocal images of HeLa cells treated with the respective GNP constructs. Focal signal intensity of cells treated with GNP-DT was significantly stronger compared to any of the other group ($p < 0.001$; native images; Fig. 3B). Enhancing the detected confocal signal revealed that using the GNP-T and GNP-DT constructs resulted in a clear primarily cytoplasmic focal localisation of the GNP in the cell, with some few foci visible within the nucleus. Enhancing the signal further reveals an intermediate fine (compared to GNP-DT) focal signal in GNP-T-treated cells. In addition, the GNP-DT foci seemed to be less in numbers than the GNP-T ones, yet the former were of significantly higher intensity. For the samples analyzed by confocal microscopy, it should be noted that the lack of laser-induced bleaching (as observed with autofluorescence or fluorochromes) suggests that the signal was indeed generated by GNP, as this signal was shown to be not bleachable (He et al., Anal. Chem., 2008, 80: 5951-57).

[0084] Figure 4 shows that the quantitative analysis of samples detected using confocal microscopy revealed a similar picture. Signal intensity of GNP-DT-treated cells was significantly higher than that of any other group ($*p < 0.001$).

[0085] An increased cellular accumulation of the gold nanoparticles leads to a more intensive signal intensity. Cells after transfection of DNA-coated gold nanoparticles (DNA has a length of 300 bp (GNP-DT300) or 1100 bp (GNP-DT1100) show a significantly higher signal intensity and a significantly higher percentage of positive cells compared with untreated cells (Ko.), cells after treatment with untreated gold nanoparticles (GNP) as well as cells after transfection of untreated gold nanoparticles (GNP-T). The DNA-coated gold nanoparticles are taken up by the cells more efficiently compared with the other groups.

[0086] In addition to the fluorescence and confocal microscopy, transmission electron microscopy (TEM) was performed to provide further qualitative information. After transfection, cells were washed with PBS and subsequently fixed with 3% glutardialdehyde (in PBS) for at least 12 hours. After fixation, cells were embedded in 25 µl of 10% gelatine in PBS and allowed to harden at 4°C followed by a second fixation in 3% glutardialdehyde for 30 min and a final wash with PBS. Cell pellets were then drained using Osmium (Plano GmbH, Wetzlar, Germany), Na-cacodylat, ethanol and acetone (Carl Roth GmbH&Co. KG, Karlsruhe, Germany), embedded in Epon (all components by SERVA Electrophoresis GmbH, Heidelberg, Germany) and cut into 60 - 90 nm slices. For TEM observation, the slices were bedded on formvar covered copper or nickel grids (Plano GmbH), followed by contrasting with uranyl acetate (SERVA Electrophoresis GmbH) or lead citrate (Merck KGaA, Darmstadt, Germany) (Glauert, Fixation, dehydration and embedding of biological specimens. North-Holland Publishing Company Amsterdam, 1975). Some slides were treated with R-Gent SE-EM silver-enhancement reagent (Biotrend, Köln, Germany) to enhance the signal of small GNP. Pictures were taken using a Zeiss EM 10 (Carl Zeiss AG, Oberkochen, Germany) with a Olympus Megaview -G2 camera (Olympus GmbH, Hamburg, Germany).

[0087] Figure 5 shows transmission electron microscopy images of GNP-DT-treated cells. Here, a local accumulation of GNP can be seen corresponding with the focal signal in cells analysed using fluorescent of confocal microscopy. At the chosen fixation timepoint (16 h after transfection), GNP were primarily found as clusters in the cytoplasm,

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especially located in lysosomes. Of note, the GNP remained visible as single particles within their expected size range of about 10 nm (with the exception of some aggregates). The data correlated well with the obtained fluorescence and confocal microscopy data.

[0088] Overall, using the novel approach, a significantly higher cellular uptake efficiency of the DNA-labeled GNP constructs using transfection could be obtained compared to the inefficient uptake of the unmodified GNP (with or without transfection), thereby showing both the inefficiency of the 10 nm unmodified GNP as well the lack of a size-dependency of the current approach (GNP-DT).

Example 7: Radiomodulating effects of the GNP constructs

[0089] In order to determine the effect of the different GNP constructs on clonogenic survival after irradiation and thereby potentially radiomodulating effects of the different treatments, the colony formation assay was performed.

[0090] After transfection, cells were harvested, transferred into Eppendorf tubes and centrifuged for 1 min at 200xg. Because of its common application, and therefore higher clinical relevance, a linear accelerator (6 MV X-rays) was chosen as radiation source (Synergy, Elekta AB, Stockholm, Sweden) at a dose rate of 6.67 Gy/min with a dose of 0-8 Gy for each group. Cells were seeded at their respective density (200-5000 cells/T25) in triplicates in T25 cell culture flasks in 4.1 ml complete medium and incubated for 2 weeks in an humidified incubator with 95% air/ 5% CO₂ at 37°C. Thereafter cells were fixed with methanol/ acetic acid and stained with crystal violet as described previously (Liu et al., Int. J. Radiat. Oncol. Biol. Phys., 2013, 85: 1127-33). Colonies (>50 cells) were scored and the surviving fraction (SF) was fitted using the linear-quadratic model: $\ln(SF) = -(\alpha D + \beta D^2)$, using the non-linear regression tool of SigmaPlot11.0 (Systat Software GmbH, Erkrath, Germany).

[0091] Figure 6 shows the clonogenic survival of HeLa cells after treatment with the different GNP constructs and subsequent X-ray irradiation. Between the PBS, GNP and GNP-T-treated cells no significant difference in clonogenic survival after irradiation was

observed. This suggests that the amount of GNP transferred into the cells by these methods (also see Fig. 3) was not sufficient to result in any significant radiomodulating effects when using 6 MV X-rays. Compared to the GNP and GNP-T-treated HeLa cells, a significant decrease in the surviving fraction (SF) could be observed for the GNP-DT group after irradiation (2-8 Gy: p_{vsGNP} : 0.005, $p_{vsGNP-T}$: 0.004), whereas a strong trend (p : 0.054) could be seen vs PBS-treated cells. This is in line with an increased accumulation of GNP after treatment compared to the other groups (Fig. 3; GNP-DT). Over the dose range of 4-8 Gy, significant dose-modifying factors were obtained (D4Gy: 1.25 ± 0.14 ($p=0.039$), D6Gy: 1.11 ± 0.06 ($p=0.028$) and D8Gy: 1.07 ± 0.04 ($p=0.032$)), thereby showing proof-of-principle of this approach. Data are presented as mean $\pm$ SD ($n=3$).

[0092] Summarizing the results shown in the figures, by binding unmodified 10 nm GNP to DNA using thiol groups in the DNA synthesis primer and using the DNA as a vehicle, it was surprisingly possible to transfect this complex (GNP-DT) efficiently into HeLa cells (Fig. 3 to 5). In contrast, using unmodified GNP resulted in no significant uptake, as was expected after extrapolation of the results of Chithrani and colleagues (Radiat. Res., 2010, 173: 719-28). Interestingly, the addition of transfection reagents to the unmodified GNP alone (GNP-T) did also result in an increased uptake of the GNP, yet at a significantly lower level than seen using the GNP-DT construct (Fig. 3B). Therefore, it seems that GNP are able to build complexes with the used transfection reagent without the need of DNA, yet with significantly reduced efficiency.

[0093] When determining the form and the localization of the two transfected GNP constructs (GNP-T and GNP-DT) in the cells, in general a focal and primarily cytoplasmic accumulation of the GNP was observed. These foci were finer and less intense in the GNP-T- than the larger and high-intensity foci observed in the GNP-DT-treated cells (Fig. 3B, bottom row). The TEM images (Fig. 5) seem to confirm this hypothesis. The complexing seems to be more efficient for GNP-DNA than for GNP alone (GNP-T).

[0094] In addition two different lengths of the DNA fragment were tested (0.3 and 1.1 kb; the latter not shown) to test their effects, yet due to the fixed DNA-transfection reagent

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ratio this would lead to differences in the DNA-GNP stoichiometry (amount of GNP/ kb DNA) between the two fragments and thereby potentially differences in their radiosensitization efficiency. After treatment of cells with the two different DNA fragments (both GNP-DT), surprisingly, no significant differences in the optical accumulation as
5 observed by confocal microscopy was observed. It should be noted, that the fluorescence signal of the foci may be saturated at high densities and thereby do not reflect the differences in DNA-GNP stoichiometry between the two fragments. It was shown, however, that both fragments led to a significantly enhanced uptake of GNP compared with controls.

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[0095] In Figure 6, the radiosensitizing effect of the GNP-DT-treated groups was clearly shown in a clonogenic survival assay. Over the whole dose range, the GNP-DT-treated cells were significantly more sensitive to irradiation with 6 MV X-rays than the other groups.

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[0096] Thus, the figures illustrate the method of the present disclosure and show that the method achieves efficient and active uptake of oligonucleotide-coated nanoparticles into cells independently of the particle size.

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Claims

- 5 1. A method for transferring at least one nanoparticle into cells or tissue comprising the following steps:
- adding at least one thiol group to the 3' or 5' end of at least one oligonucleotide to obtain at least one thiolated oligonucleotide,
 - binding of the at least one thiolated oligonucleotide to at least one
10 nanoparticle to obtain at least one oligonucleotide-coated nanoparticle,
 - forming a complex comprising the at least one oligonucleotide-coated nanoparticle by adding an encapsulating reagent to the at least one oligonucleotide-coated nanoparticle, and
 - applying the complex to the cells or tissue.
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2. The method of claim 1, wherein the at least one nanoparticle is a metal nanoparticle.
- 20 3. The method of claim 2, wherein the metal is selected from the group comprising bismuth, titanium, titanium dioxide, tin, tin oxide, iron, iron(III)oxide, silver, nickel, gold, copper and aluminum.
4. The method of any of the preceding claims, wherein the cells are eukaryotic cells.
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5. The method of any of the preceding claims, wherein the at least one nanoparticle is functionalized with at least one chemical functional group, at least one biological molecule, at least one diagnostic molecule such as an imaging molecule, at least one therapeutic molecule such as a chemotherapeutic molecule, at least one
30 radiotherapeutic molecule, at least one radiosensitizing molecule, at least one molecule that binds to a peptide, a protein, a nucleic acid or any ligand for diagnostic or therapeutic applications or any combination thereof.

6. The method of claim 4, wherein the chemical functional group is selected from the group comprising mercaptocarboxylic acid, polyethylene glycol (PEG), biotin, chemotherapeutics and fluorochromes.

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7. The method of claim 4, wherein the biological molecule is selected from the group comprising oligonucleotides, sugars, peptides and proteins.

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8. The method of claim 6, wherein the proteins are selected from the group comprising cell surface receptors and antibodies.

9. The method of any of the preceding claims, wherein the thiolated oligonucleotide is synthesized by solid-phase synthesis or polymerase chain reaction (PCR).

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10. The method of claim 8, wherein primers with thiol groups are used together with an oligonucleotide template in the PCR for amplification and thiolation of the oligonucleotide.

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11. The method of any of the preceding claims, wherein the encapsulating reagent is a transfection reagent based on liposomes, cationic lipids or calcium phosphate.

12. The method of any of the preceding claims, wherein the 3' end of the at least one thiolated oligonucleotide is modified.

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13. The method of claim 11, wherein modification comprises modification by a thiol group, biotin or fluorochromes.

14. The method of any of the preceding claims, wherein the thiolated oligonucleotide bound to the at least one nanoparticle comprises a coding or non-coding region.

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15. The method of any of the preceding claims, wherein the at least one thiolated oligonucleotide is covalently bound to the at least one nanoparticle.

16. A use of the method according to any of the preceding claims in therapeutic or diagnostic applications.

- 5 17. The use according to claim 15, wherein the therapeutic or diagnostic application is selected from the group comprising targeted delivery of the nanoparticles into cells or tissue, imaging, gene delivery, drug delivery, antisense technology, radiotherapy, targeting of chemotherapeutics and hyperthermia.

Figure 1

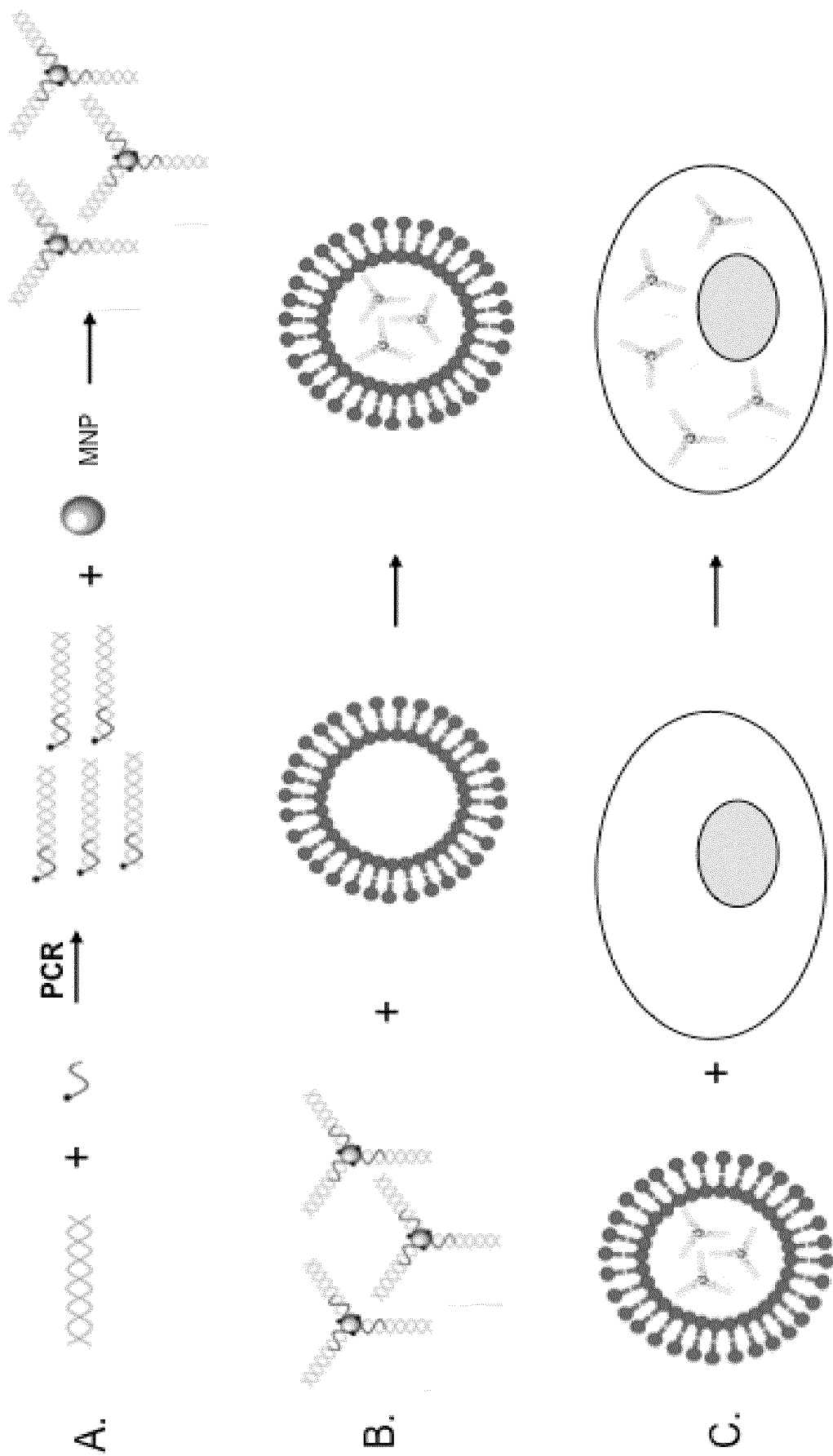


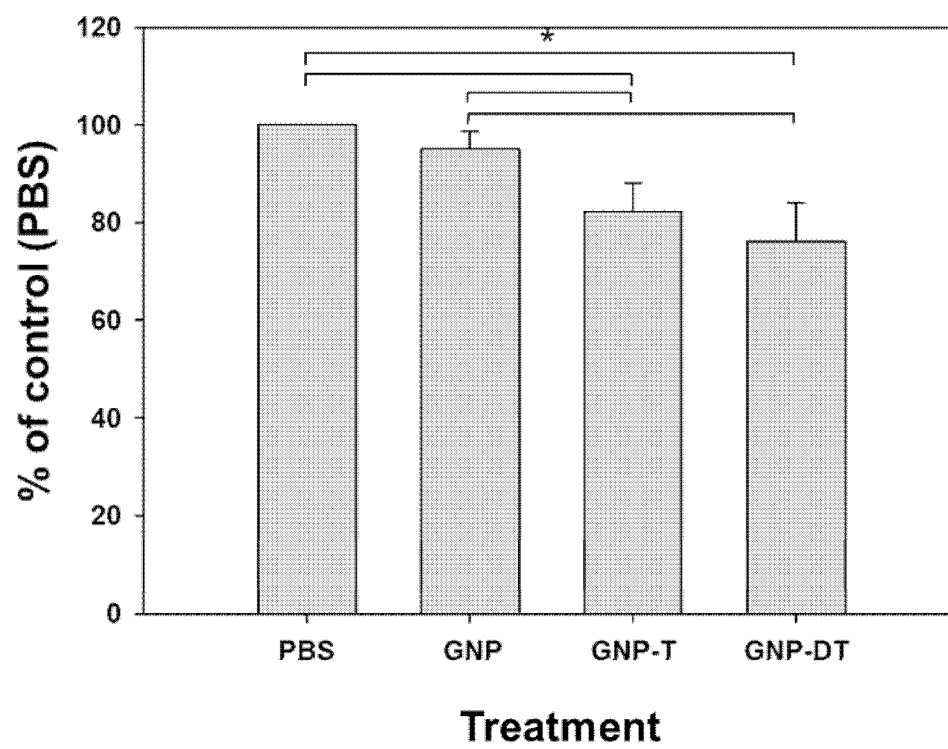
Figure 2

Figure 3

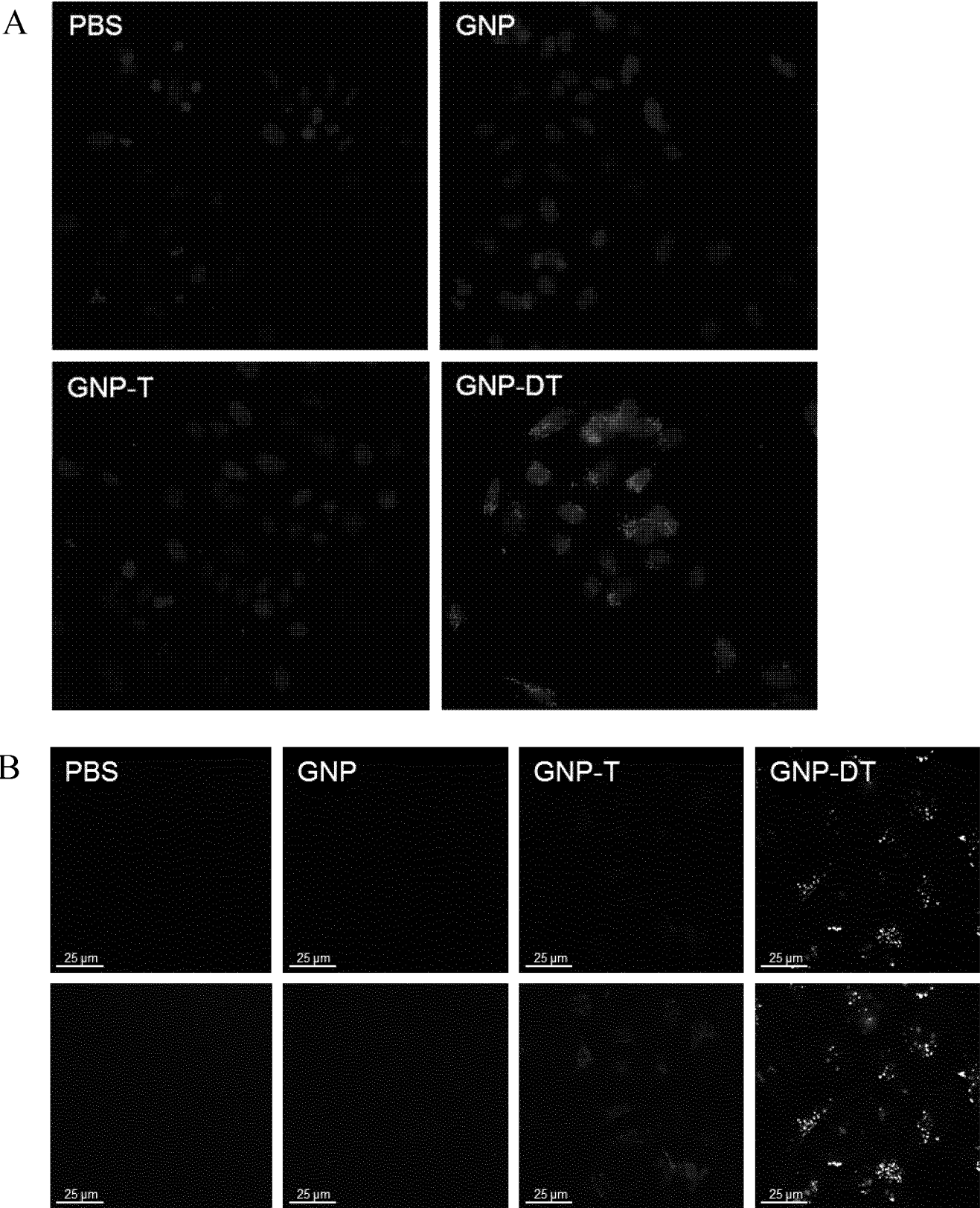


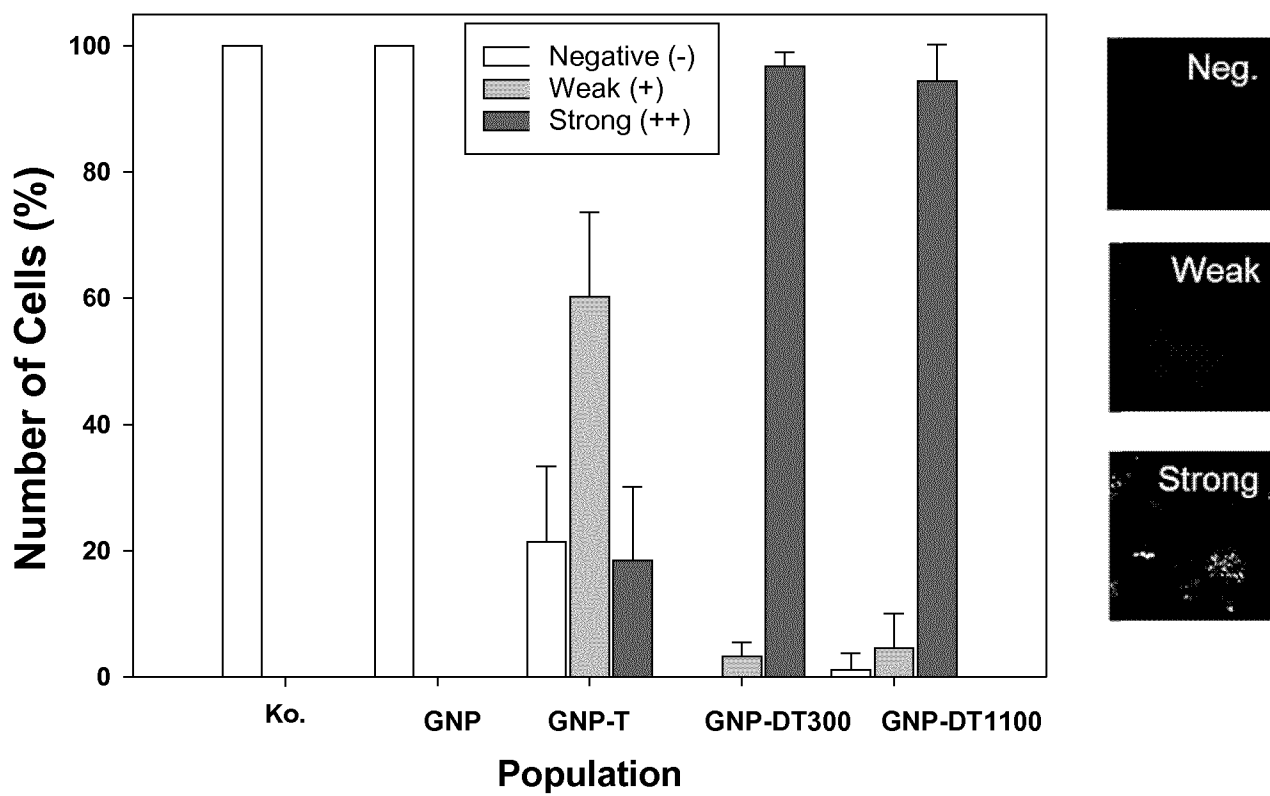
Figure 4

Figure 5

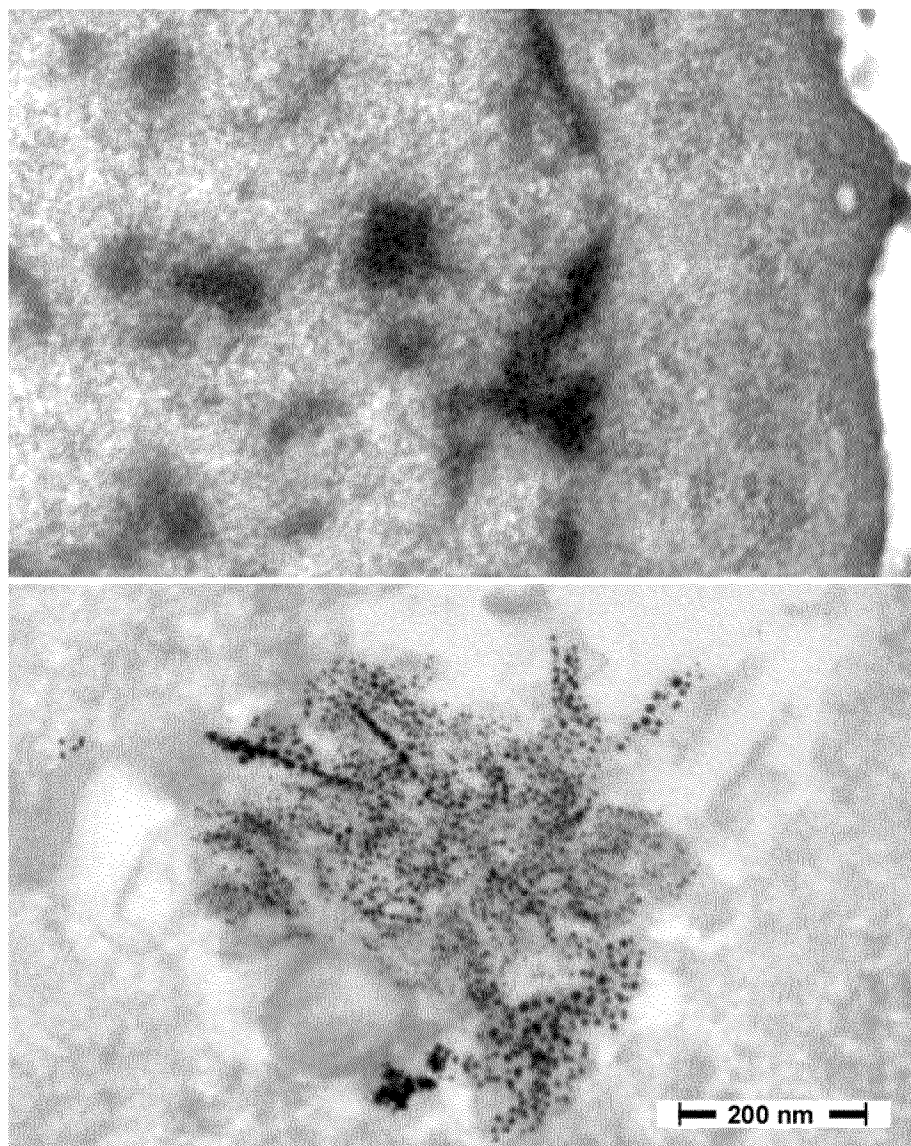
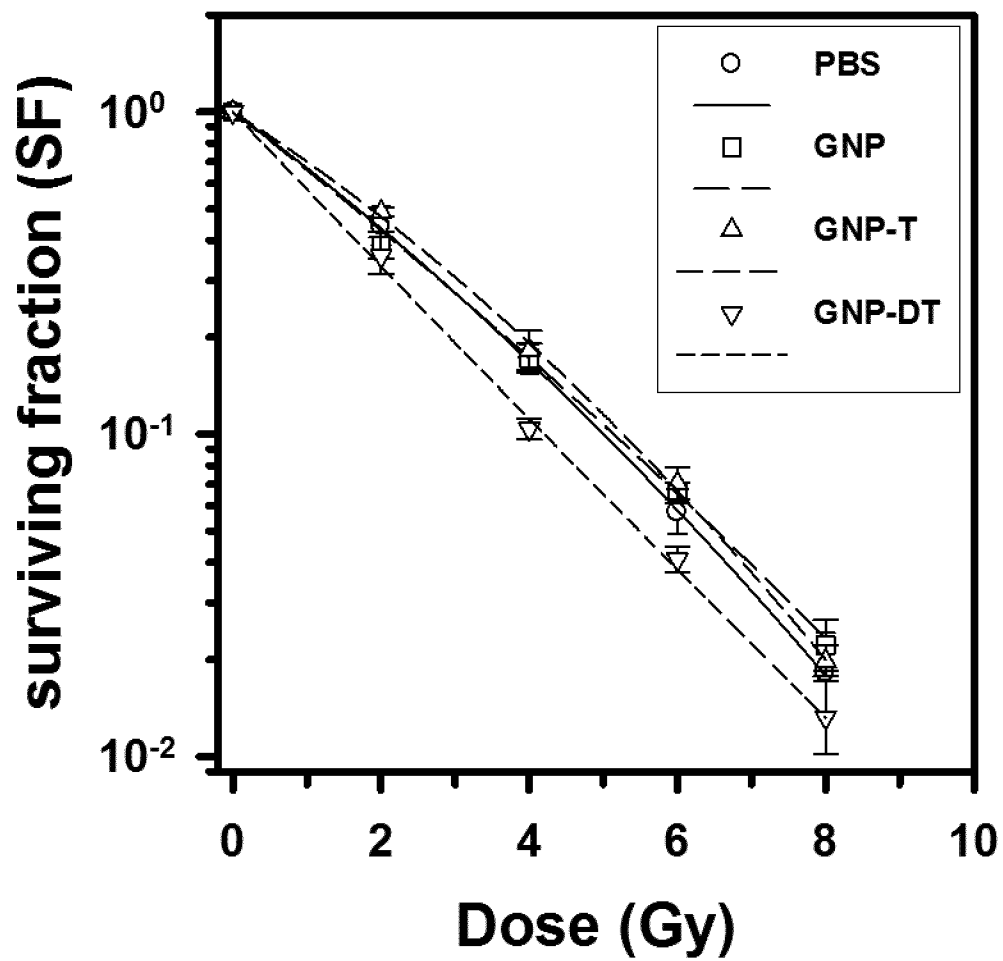


Figure 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068002

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/88
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)
C12N

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

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

EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KONG WON HO ET AL: "Multimerized siRNA Cross-linked by Gold Nanoparticles", BIOCONJUGATE CHEMISTRY, vol. 22, no. 10, October 2011 (2011-10), pages 1962-1969, XP002717281, page 1962 abstract page 1963, paragraph 1 - paragraph 5 page 1964; figure 1 page 1964, paragraph 2 page 1966; figure 4 page 1968, paragraph 3 - paragraph 4 ----- -/-	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

3 December 2013

Date of mailing of the international search report

13/12/2013

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/068002

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI D ET AL: "The enhancement of transfection efficiency of cationic liposomes by didodecyldimethylammonium bromide coated gold nanoparticles", BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 31, no. 7, 1 March 2010 (2010-03-01), pages 1850-1857, XP026827546, ISSN: 0142-9612 [retrieved on 2009-11-28] cited in the application page 1850 abstract Scheme 1; page 1851 page 1853; figure 3 -----	1
A	EP 2 087 912 A1 (UNIV TOKYO [JP]) 12 August 2009 (2009-08-12) page 5, paragraph 16 - paragraph 17 page 8, paragraph 44 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/068002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
EP 2087912	A1	12-08-2009	EP 2087912 A1	12-08-2009
			JP 5271715 B2	21-08-2013
			US 2010121043 A1	13-05-2010
			WO 2008062909 A1	29-05-2008
