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(54) Title: A PROCESS FOR THE PREPARATION OF NANOPARTICLES FOR USE AS CONTRAST AGENTS IN THE MAGNETIC RESONANCE IMAGING

(57) Abstract: The present invention relates to a process for preparing nanoparticles of a polysaccharide crosslinked with a suitable crosslinking agent, inside which a gadolinium- or manganese-based contrast agent is geometrically confined; this process of preparation is based on the use of microfluidic devices and on the control of temperatures, flows and respective concentrations in the several steps of the process, so as to obtain a final product having nanometric size wherein the metal, thanks to its geometric confinement in the crosslinked polysaccharide lattice, has an increased relaxivity, useful for increasing the contrast of images in the magnetic resonance imaging, or in combination with other imaging diagnostic techniques or more in general in nuclear medicine.



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TITLE

**A PROCESS FOR THE PREPARATION OF NANOPARTICLES
FOR USE AS CONTRAST AGENTS
IN THE MAGNETIC RESONANCE IMAGING**

DESCRIPTIONField of the Invention

The present invention relates in general to the field of the synthesis of products having a biomedical application, and more precisely it refers to a process for the preparation of nanoparticles of polysaccharides, in particular of hyaluronic acid, crosslinked with a suitable crosslinking agent, wherein a gadolinium- or manganese-based contrast agent is geometrically confined, useful as contrast agent in magnetic resonance imaging.

State of the Art

The Magnetic Resonance Imaging (or MRI) represents by far the most widespread imaging technique that is used for several indications. It is in fact a non-invasive technique, now tested and well-defined, able to provide anatomical and functional three-dimensional images of every body part.

In this technique the intensity of the signal is proportional to the relaxation rate of water protons *in vivo* and can be increased by administering to the patient under examination, before proceeding to the scanning, a so-called contrast agent.

These contrast agents contain paramagnetic metal ions and strengthen the image contrast in MRI thus affecting the relaxation rates of the protons of water in the close proximity of the tissue, in which they are located. The ability of the contrast agents to effectively intensify the contrast of the images depends on their level of accumulation in the target site and on their relaxivity, which has two components, the longitudinal component r_1 and the transverse component r_2 .

Several contrast agents are known; among these, in approximately 30% of the cases, gadolinium-based contrast agents are used, and the contrast agents that are clinically much used in the MRI are polyamine carboxylates complexes of Gd^{3+} . In these complexes the cytotoxicity of the Gd^{3+} ion is neutralized by sequestration of the ion by chelation with ligands such as DTPA (Diethylenetriamine-N, N, N', N', N'-

pentaacetate), DOTA (1,4,7,10-tetraazacyclododecane -1,4,7,10-tetraacetate), and the like.

gadolinium, however, as most of the contrast agents used in clinical magnetic resonance, is characterized by a low relaxivity (r_1 in particular is equal to approximately 4 mM⁻¹s⁻¹), it has no specificity to the tissues and, in addition to that, it can be the cause of serious allergic phenomena and severe nephrotoxicity. Through the design of new ligands, in recent years attempts have been made to improve the performance of contrast agents containing gadolinium, focusing the research in particular on the improvement of relaxivity.

Recently, in the context of such research, new gadolinium-based contrast agents have been proposed in which the Gd³⁺ ion is geometrically confined within biopolymers, in order to improve their relaxivity. It is a real new strategy, alternative to the more traditional chemical synthesis of new ligands, for the definition of ever more efficient contrast agents, where chemical changes to the contrast agent are not proposed, but it is the geometric confinement of the metal ion that increases the relaxivity of the agent itself. In the scientific literature there are some examples of studies that are in this field of research, and offer porous, silicon-based constructs (Sethi et al., Contrast Media and Molecular Imaging, 2012; 7 (6): 501-508) or carbon nanotubes (Sethi et al., Inorganica Chimica Acta, 2012; 393: 165-172), in order to achieve the geometrical confinement of the Gd³⁺ ion and create new improved contrast agents. Both the above mentioned silicon-based constructs and the carbon nanotubes have shown problems of rigidity of the structure and of cytotoxicity; the biocompatibility of these systems is currently under discussion and these systems are far from a possible approval for therapeutic purposes. Furthermore, in the case of porous, silicon-based constructs the particles described are of micrometric size, while the carbon nanotubes do not have particulate form but tubular, which makes them strongly anisotropic materials.

As far as the Applicant is aware of, to date are not known methods for preparing new gadolinium- or manganese-based contrast agents, in which the metal ion is geometrically confined in an organic biopolymer in nanoparticle form, thus combining the enhanced relaxivity of the paramagnetic ion to the well-known properties of the nanoparticles when applied to the transport and release of active substances in the

human organism, and to the biocompatibility characteristics of organic biopolymers.

In recent years, the techniques for the preparation and manipulation of substances in microfluidic devices have led to a promising development of inorganic microparticles and nanoparticles obtained with such techniques, while little has been done in the field of synthesis of organic nanoparticles. From this point of view, examples of control of the precipitation of polymeric nanoparticles in microfluidic channels are known (see for instance Karnik et al., *Nanoletters*, 2008; 8 (9): 2906-2912), and such techniques have also been used for the encapsulation in polymer matrices of pharmaceutical and diagnostic agents, including contrast agents (see for example WO 2007/150030). None of these known techniques, however, was able to obtain a strict confinement of the paramagnetic ion in the polymer matrix that, as mentioned above, would instead be required for an improved magnetic resonance imaging in particular in the ability of the paramagnetic ion to intensify the contrast of images thanks to an increased relaxivity. In view of what said above, the need to have available a preparation process of a contrast agent of this type is still very much felt.

Summary of the invention

Now the Applicant has found a process for the microfluidic preparation of a novel contrast agent for the use in the magnetic resonance imaging, having increased relaxivity, thanks to the geometric confinement of gadolinium or manganese inside nanoparticles of crosslinked polysaccharides: as a matter of fact, this particular form of complexation influences the paramagnetic behaviour of the metal, just increasing its relaxivity. The present process of preparation allows a strict control of the nanoparticles features and of the metal ion confinement inside of them; moreover it shows a low polydispersity and a high efficiency in the encapsulation of the metal inside the nanoparticles, as well as an easy recovery of the particles obtained, without any long and expensive purification procedures, as it is on the contrary for the prior art processes. Furthermore, the efficiency of encapsulation, or better of confinement, of the metal inside the nanoparticles of the invention can be modulated, i.e. it may be dosed according to needs.

It is therefore subject of the present invention a process for the microfluidic preparation of nanoparticles of a polysaccharide crosslinked with a crosslinking agent,

inside which a gadolinium- or manganese-based contrast agent is geometrically confined, the essential characteristics of this process being defined in the first of the annexed claims.

The nanoparticles of crosslinked polysaccharide, inside of which a gadolinium- or manganese-based contrast agent is geometrically confined, and their use as contrast agent in the magnetic resonance imaging, as defined in the claims 12-15 here attached, are further subjects of the invention.

Further important characteristics of the process of preparation of the invention and of the above said nanoparticles are illustrated in the following detailed description.

Brief Description of the Figures

The Figures 1a) and 1b) respectively show two microfluidic devices having different geometry, described in details in the following, for the use in the process of the invention. In Figure 1b) it is also shown an enlarged detail of the device, at the point of confluence of the central channel with the two intermediate channels.

The Figures 2 and 3 show the trend of the size of nanoparticles obtained by the present process respectively at varying of the flow rate between the starting solution (indicated in the figure as "solvent") and the anti-solvent (with the flow rate of the starting solution containing the polysaccharide maintained constant at 30 and at 20 $\mu\text{L}/\text{min}$), and at varying of the concentration of NaCl added to the starting solution, under the conditions described in the following Example 1.

The Figures 4a) and 4b) are micrographs of the nanoparticles obtained by Scanning Electron Microscopy (SEM) under conditions of a flow rate solvent/anti-solvent equal to 1, indicated in the following Example 1.

The Figures 5a), 5b) and 5c) are micrographs of the nanoparticles obtained by Scanning Electron Microscopy (SEM) under conditions of flow rate solvent/anti-solvent equal to 0.3, indicated in the following Example 1.

The Figure 6 shows the distribution of intensity of the MRI signal recorded for the nanoparticles obtained by the present process using different concentrations of the crosslinking agent DVS.

The Figures 7a), 7b) and 7c) show the increase of relaxivity of the present nanoparticles containing gadolinium DTPA prepared in Examples 1-4 hereinafter

illustrated, with the increase of the concentration of the contrast agent, recorded with a 1.5 Tesla MRI scanner, in comparison with the same contrast agent as such, not encapsulated in nanoparticles.

Detailed description of the invention

5 The process for the preparation of nanoparticles of a crosslinked polysaccharide inside of which a gadolinium- or manganese-based contrast agent is geometrically confined according to the present invention is based on a known microfluidic technique, called "flow focusing", wherein a solution of a product in a solvent thereof, that is called here "starting solution", is injected in appropriate devices provided with microchannels
10 called "microfluidic devices" and, thanks to side flows of an anti-solvent for the product in question and to the control of the relative flow rate of the flows of solvent and anti-solvent, the solution can be channelled and its focused flow can be directed towards a specific outlet in the device. This allows controlling the precipitation of the product in solution promoting the nucleation of new particles rather than their growth, obtaining
15 the formation of particles of nanometric size of the above said product. By the term "anti-solvent" in the present invention is meant a solvent, wherein the product of interest is less soluble than in the "solvent" used for preparing the starting solution.

 According to the present invention, by the terms "solvent" and "anti-solvent" single pure solvents are meant as well as mixtures of solvents wherein all the
20 components of the "solvent" or "anti-solvent" mixture have the characteristics defined above for the respective terms "solvent" or "anti-solvent".

 Thanks to the present process the Applicant has achieved the geometric confinement of the gadolinium- or Manganese-based contrast agent that is not just an encapsulation, as it is for the polymeric matrices in the state of the art. In the present
25 invention, by the expression "geometric confinement" is meant the dispersion of gadolinium or Manganese in the polysaccharide nanoparticles so as to interact with the crosslinked polysaccharide molecules creating a rigid structure wherein the metal ions are blocked in a certain position. It was observed that such rigidity of the nanostructure implies a reduced mobility also of the water molecules present in the tissues where the
30 nanostructure goes, thus causing a shortening of the relaxation times of the related protons and an increase in relaxivity of the MRI signal. Thanks to the geometric

confinement of gadolinium and manganese in the present nanoparticles, the relaxivity measured for the contrast agent of the invention may be of approximately 10-12 times higher than the relaxivity of the contrast agents as such, not formulated with the present nanoparticles.

5 The present process for the preparation of nanoparticles of a crosslinked polysaccharide inside of which is geometrically confined a gadolinium- or manganese-based contrast agent, comprises the following steps:

- i) preparation of a starting solution comprising said polysaccharide in a solvent thereof and a gadolinium- or manganese-based contrast agent,
 - 10 ii) injection of the solution coming from the step i) in a central channel of a microfluidic device comprising said central channel and at least a side channel wherein an anti-solvent of the polysaccharide is injected;
 - iii) addition of a crosslinking agent of said polysaccharide in said microfluidic device;
 - iv) precipitation of the nanoparticles of crosslinked polysaccharide containing the
 - 15 contrast agent at the point of confluence between said central channel and said at least a side channel of the microfluidic device,
- said process being characterized in that:
- the addition of said crosslinking agent of the polysaccharide in step iii) is carried out in said side channel containing the anti-solvent or, when carried out in said central
 - 20 channel, the temperature of said solution previously injected in the central channel is maintained at a constant value comprised between 5 and 23°C;
 - in step iv) the temperature inside the microfluidic device is brought to a value comprised between 25 and 40°C, and
 - the ratio between the volumetric flow of said solution in said central channel and the
 - 25 volumetric flow of said anti-solvent in said at least one side channel ranges between 0.001 and 3.

According to a preferred embodiment of the present process the crosslinking agent is added to the solution containing the polysaccharide and the contrast agent in the central channel of the microfluidic device at low temperature, in the range 5-23°C,

30 then raising the temperature in the range 25-40°C during precipitation. The temperature in the range 5-23°C, and preferably the temperature of 5°C, is maintained

for the time needed for the whole solution in the central channel to reach such value, then the temperature is brought to a constant value in the range 25-40°C, and preferably 37°C. In the present process the time needed for the whole solution to reach a same temperature value comprised in the range said above, is typically comprised
5 between 1 minute and 24 hours, mainly according to the amount of solution present. As experimentally observed and described in the following, in these conditions the effect of increased relaxivity of the MRI signal is surprisingly maximised albeit it is anyway greatly increased with respect to the use of the contrast agent as such, also when the nanoparticles are prepared according to an alternative embodiment where the
10 crosslinking agent is added to the anti-solvent in the side channel then, at a temperature in the range 25-40°C, and preferably at 37°C, the precipitation is carried out.

By the term "polysaccharide" in the present invention is meant a biocompatible polysaccharide soluble in water, such as hyaluronic acid, whereby "soluble in water" is
15 meant a product that can dissolve in water forming a homogeneous solution, at room temperature and room pressure, in the amounts defined for instance in the official European Pharmacopoeia, or a hydrophilic polysaccharide that, albeit not soluble in water, may dissolve in aqueous solutions of acids, such as chitosan. It is understood that in the term "polysaccharide" are moreover included polysaccharides derivatives as
20 long as they maintain the above said characteristics of the non-derivatized polysaccharide, and mixtures of such polysaccharides. Exemplary polysaccharides suitable for use in the present invention are dextran, chitosan, carboxy methyl cellulose, and glycosaminoglycans, such as hyaluronic acid. Derivatives of polysaccharides of possible use are for instance polysaccharides conjugated with
25 proteins or polysaccharides conjugated with polyethylene glycol.

Preferred polysaccharides are hyaluronic acid, possibly derivatized with thiol groups, and chitosan. A particularly preferred polysaccharide for carrying out the present invention is hyaluronic acid.

The solvent wherein the polysaccharide is dissolved in step i) of the process of
30 the invention is a polar solvent, it is preferably water or an aqueous mixture, more preferably it is water. Any skilled person in the art shall easily identify the solvent and

the dissolution conditions most appropriate for each selected polysaccharide.

Divinyl sulphone (DVS), polyethylene glycol vinyl sulphone (PEG-VS), glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) in combination with N-hydroxysuccinimide (NHS) are examples of crosslinking agents of the polysaccharide of possible use in step i) of the present process. A person of ordinary skills in the art shall easily identify further crosslinking agents equivalent to those mentioned above and substitutes for them with similar results, as well as they shall identify without any efforts the crosslinking conditions for the selected crosslinking agent, including the possible addition of agents to activate the crosslinking, also depending on the polysaccharide chosen.

According to a particular embodiment of the present process, in step i) DVS is used as crosslinking agent and hyaluronic acid as polysaccharide; it is known that this reaction can be carried out at high pH values, typically at pH of 11-13, following addition of NaOH in the presence of NaCl, so as to activate the hydroxy groups of the hyaluronic acid that link then to the vinyl groups of DVS. The product coming from the crosslinking between hyaluronic acid and DVS is a widely studied product and known since long time, whose biocompatibility was confirmed by histological evidences and has allowed this product to be also approved by the US Food and Drug Administration; also for these reasons this product is a preferred embodiment for use in the present process. The concentration of the DVS crosslinking agent in the starting solution is for instance comprised between 0.01 and 1 M, and is preferably comprised between 0.03 and 0.3 M, in this latter range of concentration the best results are obtained in terms of crosslinking of the hyaluronic acid in this kind of process.

In the starting solution in step i) of the present process the molar ratio between polysaccharide and metal of the contrast agent can be for instance comprised between 1:0.01 and 1:350, whereas optimum results are obtained for a ratio comprised between 1:5 and 1:150.

As the contrast agent for use in the process of the invention any contrast agents based on gadolinium or manganese can be used, selected for instance amongst gadolinium chloride, gadolinium DTPA (dimegluminic salt of gadopentetic acid), mangafodipir trisodium, and similar.

The temperature control inside the microfluidic device at the above said values can be for instance achieved by using a heating chamber or one or more resistances directly attached to the device. Any other heating system that allows a strict control of the temperature in this kind of device can be used and it is considered as comprised
5 within the scope of this invention. Preferably also the temperature of the fluids at the inlet and at the outlet of the microfluidic device are monitored and possibly controlled so that it is included in the ranges described above.

As concerns to the ratio between the volumetric flows of the solution and of the anti-solvent, in the present process an optimum value for this ratio is equal to about
10 0.3, obtained for instance by using a volumetric flow of 30 $\mu\text{L}/\text{minute}$ for the solution and a flow of 110 $\mu\text{L}/\text{minute}$ for the anti-solvent.

In the Figures 1a) and 1b) the magnified images are shown of two types of microfluidic device of possible use in the present process: the first in Figure 1a) is a cross flow device, wherein a central channel is intended for containing the starting
15 solution, and two side channels, crossing with the first channel, intended for containing the anti-solvent, possibly added with other components, such as surfactants, phosphate-buffered saline (PBS), compounds based on gadolinium or other metals, crosslinking agents; the second in Figure 1b) is a flow focusing device, provided with 5 channels, of which the two external side channels are intended for containing mineral
20 oil, the central channel contains the starting solution and the two intermediate channels the anti-solvent, possibly added also in this case with surfactants, crosslinking agents, or other additives based on saline solutions able to promote the chemical reaction or to delay or accelerate the times of extraction of the solvent allowing control of the solute nanoprecipitation and the confinement of the contrast medium. The device with 5
25 channels illustrated in Figure 1b) has an optimum configuration for carrying out the present process, and it is therefore preferred but, more in general, of possible use are flow focusing microfluidic devices provided with at least 3 channels, a central channel intended for containing the starting solution and two side channels for the anti-solvent. The diameter of the central channel in microfluidic devices of possible use in the
30 present process is preferably comprised between 80 and 200 μm .

Under the conditions of the present process, in particular thanks to the particular

controlled crosslinking conditions, the desired amount of gadolinium- or manganese-based contrast agent and of polysaccharide was surprisingly combined by immobilization of the contrast agent inside the crosslinking mesh of the polysaccharide, obtaining a final product having several advantages, besides the nanometric size
5 allowing excretion through the kidney. In particular, for the crosslinked hyaluronic acid, it was observed that in the present nanoparticles it maintains the advantageous features of hydrogel of the starting hyaluronic acid, in particular its biocompatibility, and the ability to store water. This latter characteristic, in particular, reduces greatly the mobility of the water molecules in proximity of the contrast agent, when it is arrived in
10 the target tissue, and the contrast of the signal due to the paramagnetic ion increases in a particularly significant amount. In other words, the present nanoparticles, thanks to the geometric confinement of the metal ion inside of them, having an effect of reducing the mobility also on the water molecules present in the surrounding tissues, are able to maximize the MRI signal. In this way the dosage of the contrast agent to be
15 administered to the patient shall be much lower than the average dosages used for the contrast agents known up to today for a same tissue under investigation.

The present process has moreover further advantages with respect to known processes. For instance, as explained above, this process allows the strict geometric confinement of the contrast agent, with a good efficiency with respect to the amounts of
20 the starting product used, as opposed to the known processes where a simple encapsulation of the agent is obtained, and even more with a low efficiency. Furthermore, with the present process nanoparticles having a low polydispersity index are obtained, which can be moreover directly used, without the need of further passages of recovery and purification, and consequent save of time and costs.

25 The nanoparticles obtainable by the present process can be used as contrast agents, in *in vivo* imaging magnetic resonance techniques, following administration, for instance by enteral or preferably parenteral route, in the human body: after a certain period of time when nanoparticles are internalized in the cells of the target tissues, thanks to their nanometric size, can be eliminated through the renal route, thus
30 reducing the nephrotoxicity risks linked to the use of the known contrast agents. The nanoparticles obtainable by the process of the invention have for instance an average

diameter that may vary between 20 nanometres and some micrometres. According to a preferred embodiment of the present process the nanoparticles with the best characteristics in terms of polydispersity have an average diameter of approximately 35 nanometres.

5 The nanoparticles of the present invention can be loaded with one or more pharmaceutically active agents able to carry a therapeutic function too, without interfering with the confinement characteristics of the gadolinium ion; in alternative or in addition to the loading of the present nanoparticles with active agents, the conjugation on the nanoparticles surface is also possible, or its coating by electrostatic charge, with
10 one or more active agents or with prodrugs thereof.

 The present nanoparticles can be moreover mixed with nutraceutical agents, i.e. with agents having a beneficial nutritional effect to the health of the organism, and they can be also decorated with polyethylene glycol (PEG) or similar known substance for prolonging the duration of stay of the nanoparticles in the blood circulation, or with
15 fluorescent compounds or peptides having diagnostic relevance, or still with ligands for the active targeting.

 The nanoparticles obtainable by the present process showed a high stability during time; for instance it was observed that their morphological characteristics and their diagnostic properties are maintained completely unaltered even after 1 year of
20 storing at the temperature of 4°C.

 The following examples are provided to give a non-limiting illustration of the present invention.

EXAMPLE 1

Microfluidic synthesis of nanoparticles of HA-DVS and contrast agent Gd

25 Hyaluronic acid (HA) was dissolved in 2.5 mL of deionized water to obtain an aqueous solution of concentration of 0.05% by weight of hyaluronic acid with respect to the total volume of the solution, then sodium chloride at concentration of 0.05 M was added as well as sodium hydroxide at concentration of 0.2 M as pH stabilizer. The pH value measured was of 12.3. Then Gd-DTPA was added in form of an aqueous
30 solution highly concentrated, to obtain a molar ratio Gd:HA of 150.

The so obtained solution was injected in the central channel of a microfluidic device of the flow-focusing type, as that illustrated in Figure 1b, provided with 5 channels; the central channel where the precipitation of the nanoparticles occurs had a diameter of 190 μm . The crosslinking agent divinyl sulphone (DVS) was then added bringing preventively the temperature of the solution already present in the central channel of the device at 5°C. When the solution of hyaluronic acid and Gd-DTPA has homogeneously reached the temperature of 5°C, divinyl sulphone (DVS) was added at a starting concentration of 80 mM.

In the intermediate channels of the device acetone was introduced as anti-solvent, added with Span 80 at a concentration of 0.5% by weight with respect to the total volume, flowing at a rate of 110 $\mu\text{L}/\text{minute}$. The flowing rate for the solution in the central channel was set at 30 $\mu\text{L}/\text{minute}$, so as to obtain a ratio between the volumetric rates of the solution and of the anti-solvent of approximately 0.3.

In the most external channels mineral oil was let in at the temperature of 40°C with a rate of 20 $\mu\text{L}/\text{minute}$ so that the temperature of the solution in the central channel resulted of approximately 37°C. The precipitated nanoparticles were collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

The experiment described above was repeated twice varying the concentration of sodium chloride that in a first case was of 0.1 M and in a second case was 0, i.e. in the absence of sodium chloride. The three experiments above were finally repeated at a concentration of the aqueous solution of hyaluronic acid of 0.1% by weight with respect to the total volume of the solution.

The experiments described above were moreover repeated by varying the ratio between the solvent and the anti-solvent rates, with rate's values in the central channel constantly maintained equal to 30 $\mu\text{L}/\text{minute}$ and at 20 $\mu\text{L}/\text{minute}$.

At the end of each experiment the nanoparticles have been collected, and their average size was measured as a function of the flow rate ratio between solvent and anti-solvent and as a function of the different concentration of sodium chloride, reported respectively in the Figures 2 and 3.

In the Figures 4a and 4b are illustrated the SEM images of the nanoparticles obtained with a flow rate ratio solvent/anti-solvent of 1 and different concentrations of sodium chloride, respectively of 0.05 and 0.1 M.

In the Figures 5a and 5b are shown the SEM images of the nanoparticles obtained with a flow rate ratio solvent/anti-solvent of 0.3 respectively obtained with gadolinium DTPA and concentrations of HA equal to 0.05%, NaOH 0.2 M and of NaCl equal to 0.05 M. In Figure 5c) is illustrated the SEM image of HA nanoparticles obtained under the same conditions described above, except for the fact that the crosslinking agent DVS is added in the side channels.

The experiments described above have been repeated also varying the concentration of Gd-DTPA between 0.005% and 0.1% weight/volume for the contrast agent, evaluating the variation in size of the nanoparticles obtained in this case too.

Moreover, a selection of nanoparticles with contrast agent has been prepared, decorated with polyethylene glycol or with fluorescent molecules, where the coating product of the nanoparticles was injected, instead of the mineral oil, in a side channel of the same microfluidic flow-focusing device used for the synthesis, so as to create a coating on the nanoparticle at the outlet of the device.

EXAMPLE 2

Microfluidic synthesis of HA-DVS nanoparticles and contrast agent Gd

Hyaluronic acid (HA) was dissolved in 2.5 mL of deionized water to obtain an aqueous solution of concentration of 0.05% by weight of hyaluronic acid with respect to the total volume of the solution, then sodium chloride at concentration of 0.05 M was added as well as sodium hydroxide at concentration of 0.2 M as pH stabilizer. The pH value measured was 12.3. Then Gd-DTPA was added in form of a highly concentrated aqueous solution, to obtain a molar ratio Gd:HA of 150 in the central channel of a microfluidic device.

The so obtained solution was injected in the central channel of a flow-focusing microfluidic device, as that illustrated in Figure 1a, provided with 3 channels; the central channel where the nanoparticles precipitation occurs had a diameter of 190 μm . In the intermediate channels of the device acetone was introduced as anti-solvent, thereafter approximately 200 mM of divinyl sulphone (DVS) were added. The temperature in the

channels was then brought to 35°C, and the precipitated nanoparticles were collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

EXAMPLE 3

5 Microfluidic synthesis of nanoparticles of HA thiolate - PEG-VS and contrast agent Gd

Hyaluronic acid thiolate was dissolved in 2,5 mL of deionized water to obtain an aqueous solution of concentration 0.05% by weight of hyaluronic acid thiolate with respect to the total volume of the solution. Then Gd-DTPA was added in the form of
10 highly concentrated aqueous solution, to obtain a molar ratio Gd:HA-thiolate of approximately 130 in the central channel of a microfluidic device.

The so obtained solution was injected into the central channel of a flow-focusing microfluidic device, as that illustrated in Figure 1b, provided with 5 channels; the central channel, where the nanoparticles precipitation occurred, had a diameter of 150 µm. In
15 the intermediate channels of the device acetone was introduced as anti-solvent and let to flow at a rate of 90 µL/min, then the temperature of the solution and of the anti-solvent was brought to 5°C, then PEG-VS was added at a molar ratio thiol/VS of 1.2 for the crosslinking reaction and PEGylation of the hyaluronic acid thiolate. The flow rate for the solution in the central channel was set at 25 µL/min, so as to achieve a ratio
20 between the volumetric rates of the solution and of the anti-solvent of approximately 0.3. In the two external channels there was mineral oil at a temperature of 40°C and flow rate of 20 µL/minute so that the temperature of the solution in the central channel was of approximately 37°C. The precipitated nanoparticles were collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

25 The preparation described above was repeated but adding the crosslinking agent PEG-VS to the anti-solvent in the intermediate channels of the device. The temperature in the channels was then brought to 37°C, and the nanoparticles precipitated were collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

30 EXAMPLE 4

Microfluidic synthesis of nanoparticles of Chitosan-Glutaraldehyde and contrast agent Gd

Chitosan was dissolved in 5 μ L of acetic acid and 2.5 mL of deionized water to yield an aqueous solution of concentration 0.1% by weight of chitosan with respect to the total volume of the solution. The measured pH value was approximately 4.5. Then Gd-DTPA was added in the form of highly concentrated aqueous solution, to obtain a molar ratio gadolinium:chitosan of about 200 in the central channel of a microfluidic device.

The solution obtained was injected in the central channel of a flow-focusing microfluidic device, as that illustrated in Figure 1a, provided with 3 channels. The central channel, wherein the precipitation of the nanoparticles occurs, had a diameter of 100 μ m. In the side channels of the device was introduced ethanol as anti-solvent, then the temperature of the solution and of the anti-solvent was brought to 5°C, and glutaraldehyde 0.07% w/v was added to the solution in the central channel as crosslinking agent.

The temperature in the channels was then brought to 37°C, and the precipitated nanoparticles have been collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

The preparation described above was repeated by adding the crosslinking agent glutaraldehyde in the side channels of the microfluidic device containing the anti-solvent, and by adding the solution of chitosan and Gd-DTPA in the central channel of the same device. The temperature in the channels was then brought to 37°C, and the precipitated nanoparticles were collected in a Petri capsule containing 5 mL of anti-solvent and maintained under stirring overnight.

EXAMPLE 5

Purification and characterisation of the nanoparticles

Purification – The nanoparticles obtained in the experiments described above in Examples 1 and 2 have been purified to replace acetone with water by gradient dialysis for 5 hours. Then a further purification was carried out and the nanoparticles have been collected by means of ultracentrifugation at 20,000 rpm, at 4°C for 10 minutes.

Morphological characterization – After these purification treatments, about 100

microliters of the purified sample were deposited on a filter having a polycarbonate Isopore membrane (pores size 0.05 μm) by ultrafiltration. The so deposited particles were coated by a layer of 5 nm of Gold and Palladium and their morphology was investigated with a Field Emission Scanning Electron Microscope ULTRA PLUS (FE-
5 SEM Carl Zeiss, Germania).

In vitro and *in vivo* MRI – Hollow nanoparticles of HA-DVS and nanoparticles of HA-DVS containing gadolinium chloride or gadolinium DTPA as contrast agent at different concentrations were tested by MRI *in vitro*. The results obtained were compared with the known contrast agents Magnevist, gadolinium DTPA and
10 gadolinium chloride in water. Once vorticated the samples, the changes were evaluated for the relaxation times (t_1 and t_2) at two different values of the magnetic field, equal to 1.5 T and to 3 T in a magnetic resonance equipment for clinical use Philips Achieva using a Sense Head with a 8-elements coil and Relaxometer Minispec Bench Top (Brucker Corporation), by adding 300 μL of sample in a suitable vial. The
15 values of t_1 measured in this experiment have shown that, for a same contrast agent, it is enough a much lower concentration of agent to obtain a high relaxation time t_1 if the agent itself is confined in the nanoparticles of hyaluronic acid prepared with the process of the invention.

These measurements for different nanoparticles of the invention prepared with
20 different starting concentrations of DVS have shown the trend over time of the intensity of the signal measured at varying of the concentration of DVS (Figure 6).

In Figure 7a a graph is reported for example that shows the trend in relaxation times t_1 measured as a function of the concentration of contrast agent for gadolinium DTPA in the traditional form (-■-), for nanoparticles of HA-DVS with gadolinium DTPA prepared as described in Example 1 (-●-) and for nanoparticles of HA-DVS with
25 gadolinium DTPA prepared as described in Example 2 (-▲-), which show respectively a relaxivity r of $3.9 \text{ mM}^{-1} \text{ s}^{-1}$ (traditional form), $48.97 \text{ mM}^{-1} \text{ s}^{-1}$ (nanoparticles Ex. 1) and $14.09 \text{ mM}^{-1} \text{ s}^{-1}$ (nanoparticles Ex. 2). In the Figures 7b and 7c similar graphs are illustrated, showing the trend in the relaxation times t_1 measured as a function of the
30 concentration of contrast agent gadolinium DTPA, for the nanoparticles prepared in Examples 3 and 4, respectively, in comparison with the same contrast agent used in its

traditional form, not confined in the nanoparticles of the invention. Also from these graphs an evident increase in the relaxivity emerges when the contrast agent is confined in the nanoparticles prepared with the process of the invention; the increase is particularly significant when the nanoparticles are prepared by addition of the crosslinking agent in the central channel of the microfluidic device at low temperature.

A selection of the nanoparticles prepared as described above was inoculated *in vivo* in C57BL/6 mice and, following the sampling and analysis of the organs, a study was carried out of the acute and chronic toxicity, of the stability in blood, and of the accumulation of the nanoparticles in the main organs. Moreover, under anaesthesia, biodistribution tests were carried out of dynamic MRI, and it was observed as the nanoparticles of the invention containing a contrast agent were visible at lower concentration compared to the same contrast agent not confined in nanoparticles. This characteristic is due to the effect of amplification of the relaxivity for the confinement of the metal inside the polysaccharidic nanoparticle.

* * * * *

The present invention has been hereto described with reference to preferred embodiments thereof. It is understood that there may be other embodiments afferent to the same inventive core, all falling within the scope of protection of the claims set out below.

CLAIMS

1. A process for the preparation of nanoparticles of a cross-linked polysaccharide, inside said nanoparticles being geometrically confined a gadolinium-based or manganese-based contrast agent, said process comprising the following steps:
- 5 i) preparing a starting solution comprising said polysaccharide in a solvent thereof and a gadolinium-based or manganese-based contrast agent,
- ii) injecting the solution coming from step i) in a central channel of a microfluidic device comprising said central channel and at least one side channel wherein an anti-solvent of said polysaccharide is injected;
- 10 iii) adding a crosslinking agent of said polysaccharide in said microfluidic device;
- iv) precipitating nanoparticles of said cross-linked polysaccharide containing the contrast agent at the point of confluence of said central channel and said at least one side channel of the microfluidic device,
- said process being characterised in that:
- 15 - the addition of said crosslinking agent of the polysaccharide in step iii) is carried out in said side channel containing the anti-solvent or, when carried out in said central channel containing the solution, the temperature of said solution previously injected in the central channel is maintained at a constant value comprised between 5 and 23°C;
- in step iv) the temperature inside the microfluidic device is brought to a value
- 20 comprised between 25 and 40°C; and
- the ratio between the volumetric flow of said solution in said central channel and the volumetric flow of the anti-solvent in said at least one side channel ranges between 0.001 and 3.
2. The process according to claim 1, wherein said ratio between the volumetric flow
- 25 of said solution in said central channel and the volumetric flow of said anti-solvent in said at least one side channel is approximately 0.3.
3. The process according to any one of the preceding claims, wherein said crosslinking agent is added to the solution in said central channel of the microfluidic device at a value of temperature comprised between 5 and 23°C.
- 30 4. The process according to claim 3, wherein said value of temperature is 5°C.
5. The process according to any one of the preceding claims, wherein said

polysaccharide is a polysaccharide, which is soluble in water or hydrophilic, selected from the group consisting of dextran, chitosan, carboxymethylcellulose, glycosaminoglycans, including hyaluronic acid, derivatives and mixtures thereof.

6. The process according to claim 5, wherein said polysaccharide is hyaluronic acid.

5 7. The process according to any one of the preceding claims, wherein said cross-linking agent of the polysaccharide is selected from the group consisting of divinyl sulphone (DVS), polyethylene glycol vinyl sulphone, glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) in combination with N-hydroxysuccinimide (NHS).

10 8. The process according to claim 1, wherein said starting solution is an aqueous solution of hyaluronic acid and said cross-linking agent is divinyl sulphone (DVS).

9. The process according to claim 8, wherein said cross-linking agent is added to the solution of hyaluronic acid at high pH value by addition of NaOH, optionally in the presence of NaCl.

15 10. The process according to any one of the preceding claims, wherein the molar ratio between the polysaccharide and the metal in said contrast agent is comprised between 1:5 and 1:350.

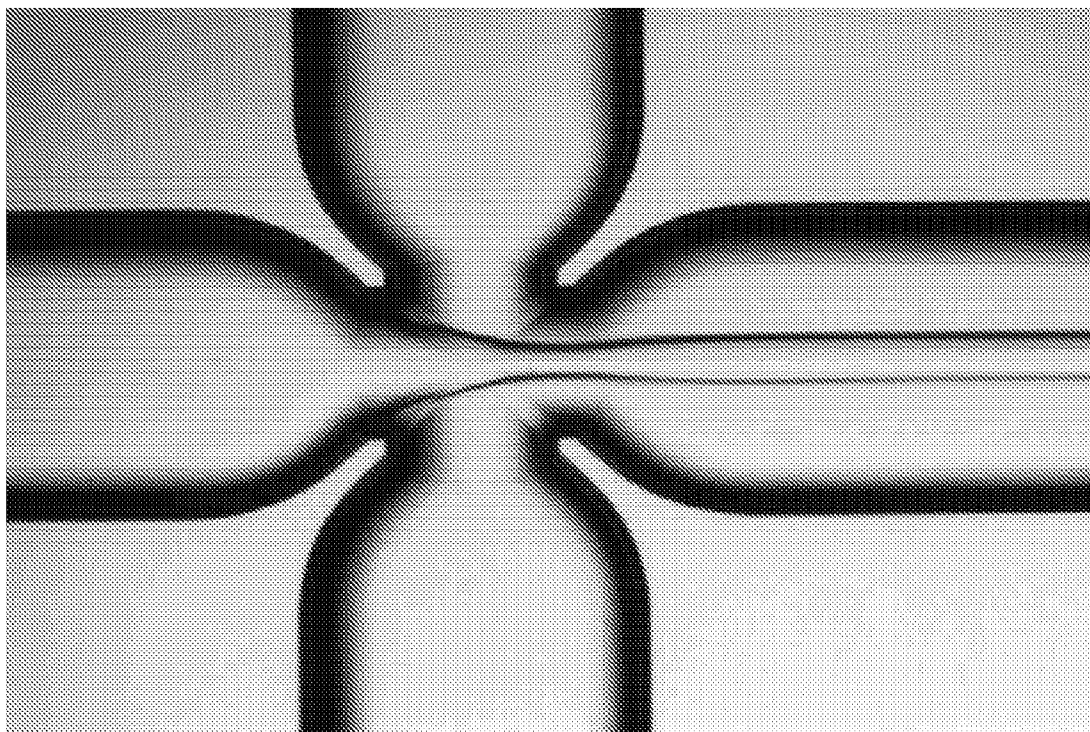
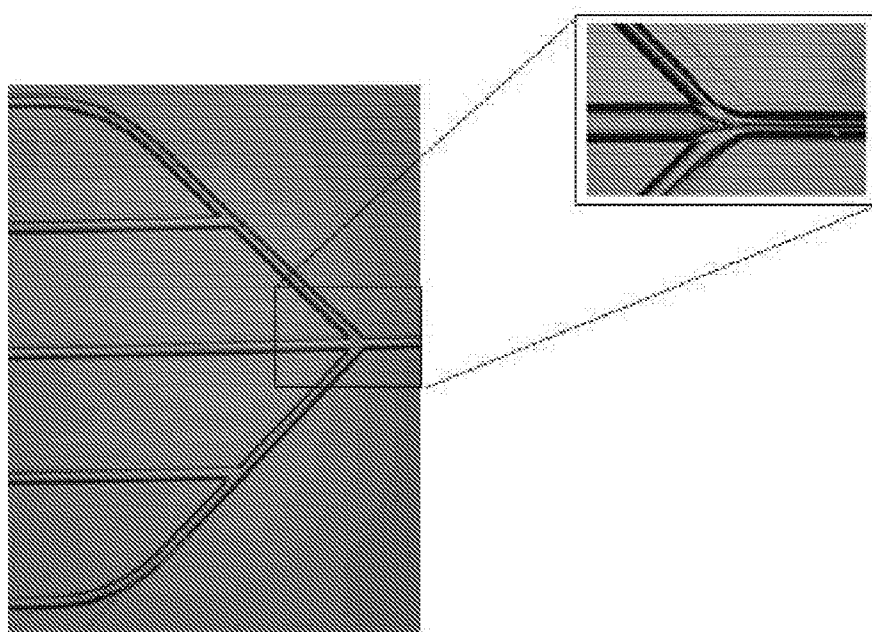
11. The process according to any one of the preceding claims, wherein said gadolinium-based or manganese-based contrast agent is selected from among
20 gadolinium chloride, gadolinium DTPA (gadopentetic acid dimeglumine salt) and mangafodipir trisodium.

12. Nanoparticles of a cross-linked polysaccharide inside said nanoparticles being geometrically confined a gadolinium-based or a manganese-based contrast agent, obtainable by the process of the claims 1-11.

25 13. The nanoparticles according to claim 12, being further loaded or conjugated on their surface with one or more pharmaceutically active and/or nutraceutical agents.

14. The nanoparticles according to claim 12 or 13, being further decorated on their surface with polyethylene glycol and/or with fluorescent molecules and/or ligands for active targeting.

30 15. Use of the nanoparticles as defined in claims 12-14, as contrast agent in magnetic resonance imaging, in diagnostic imaging and in nuclear medicine.

**FIG. 1 a)****FIG. 1 b)**

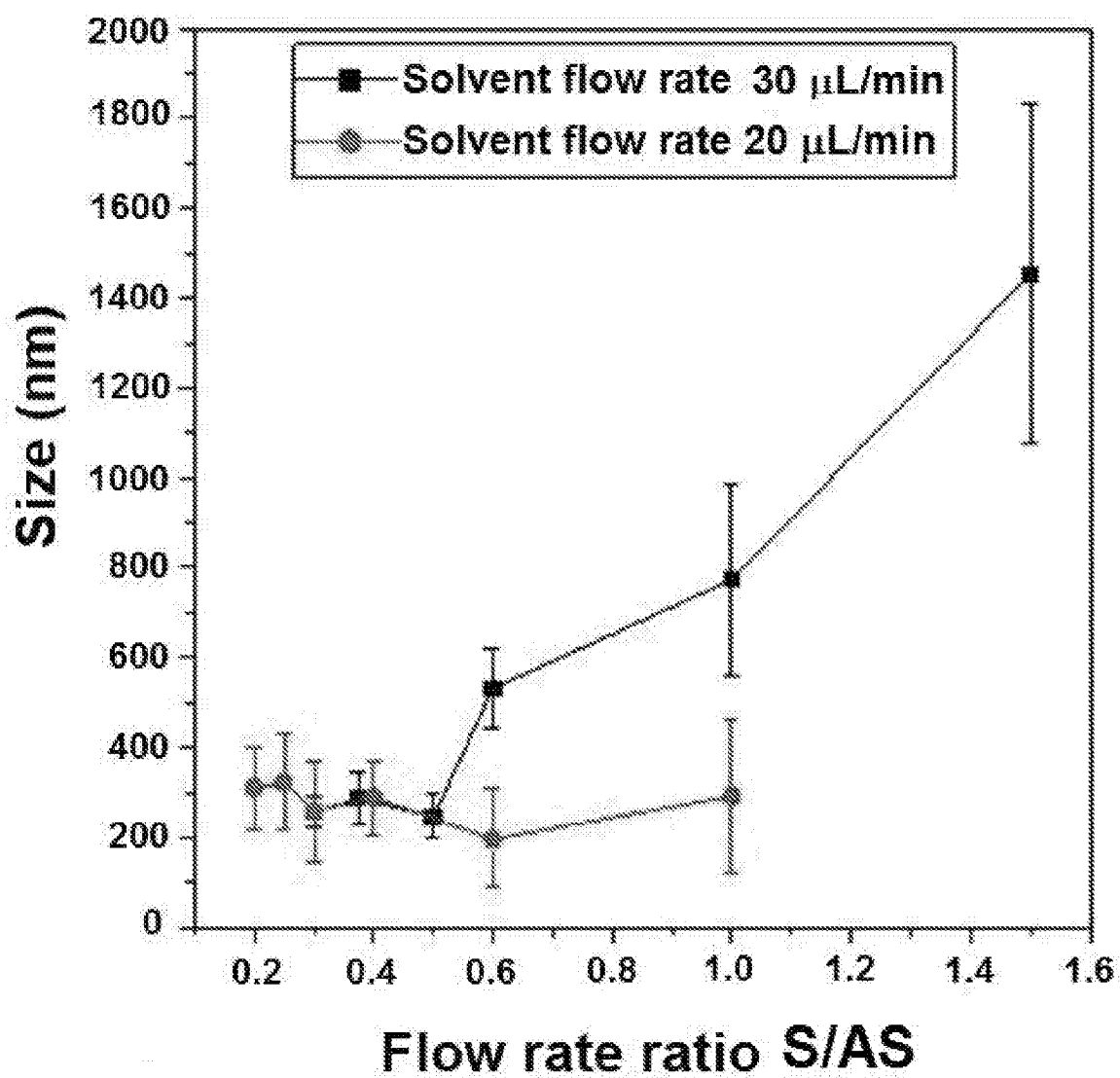
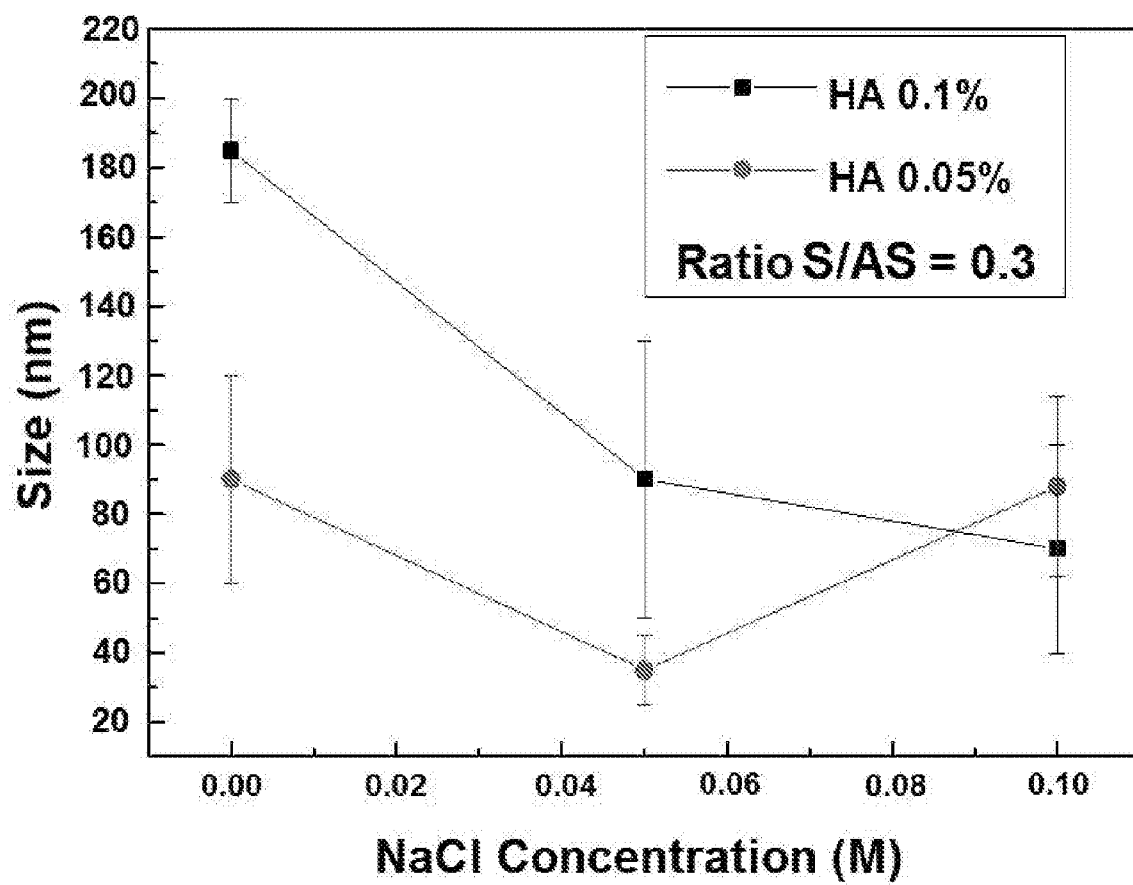
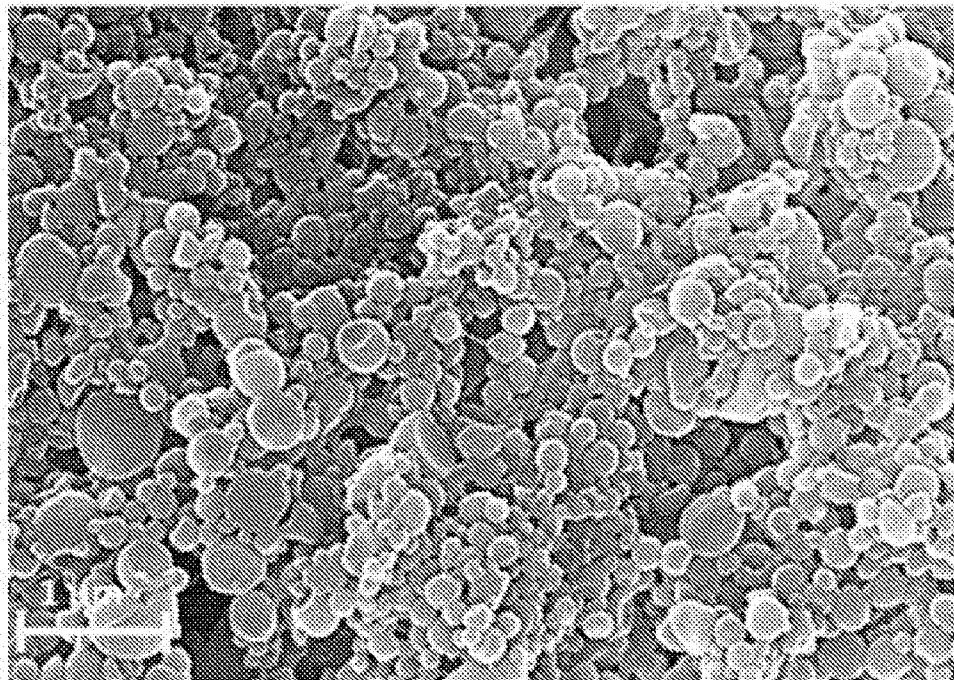


FIG. 2

**FIG. 3****FIG. 4 a)**

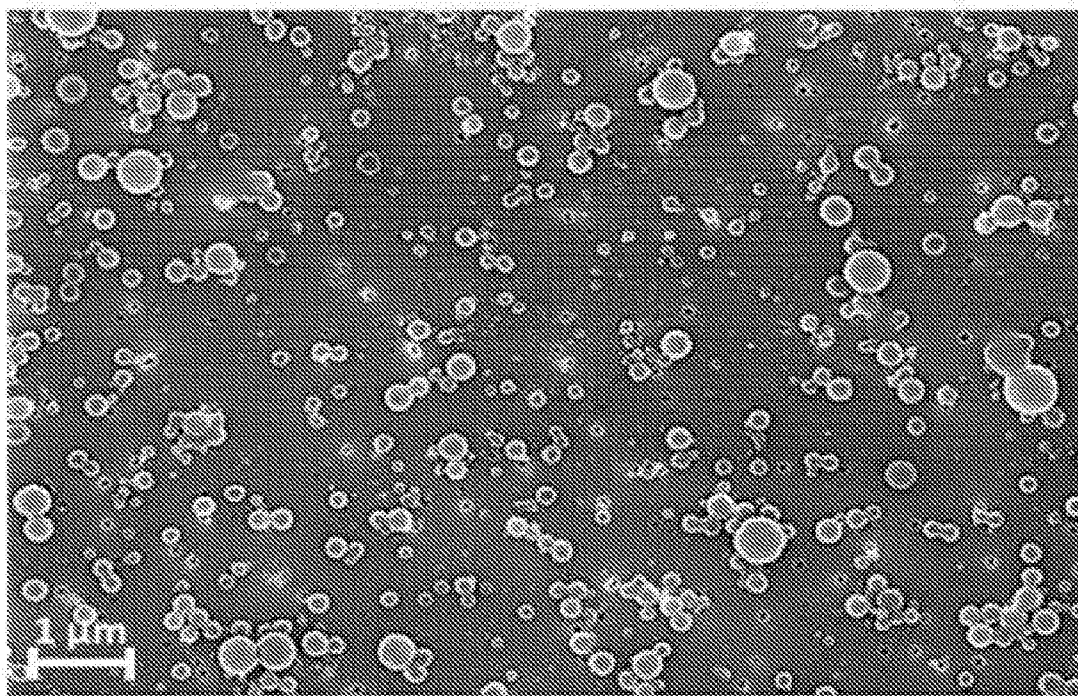


FIG. 4 b)

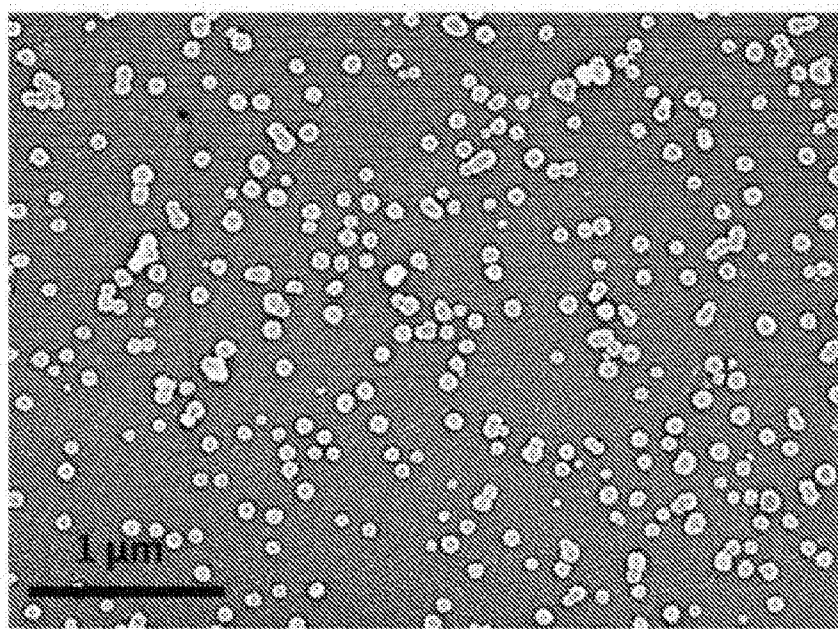
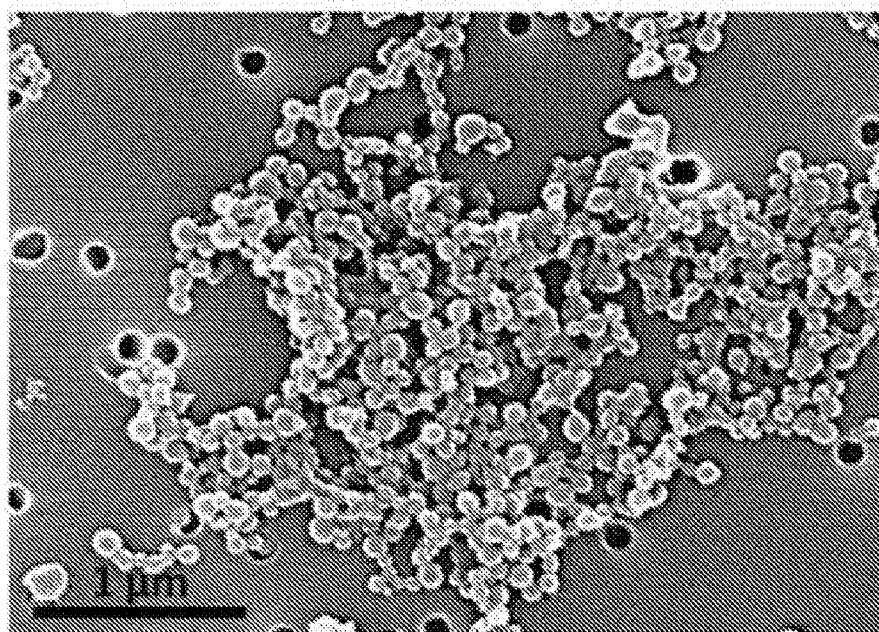
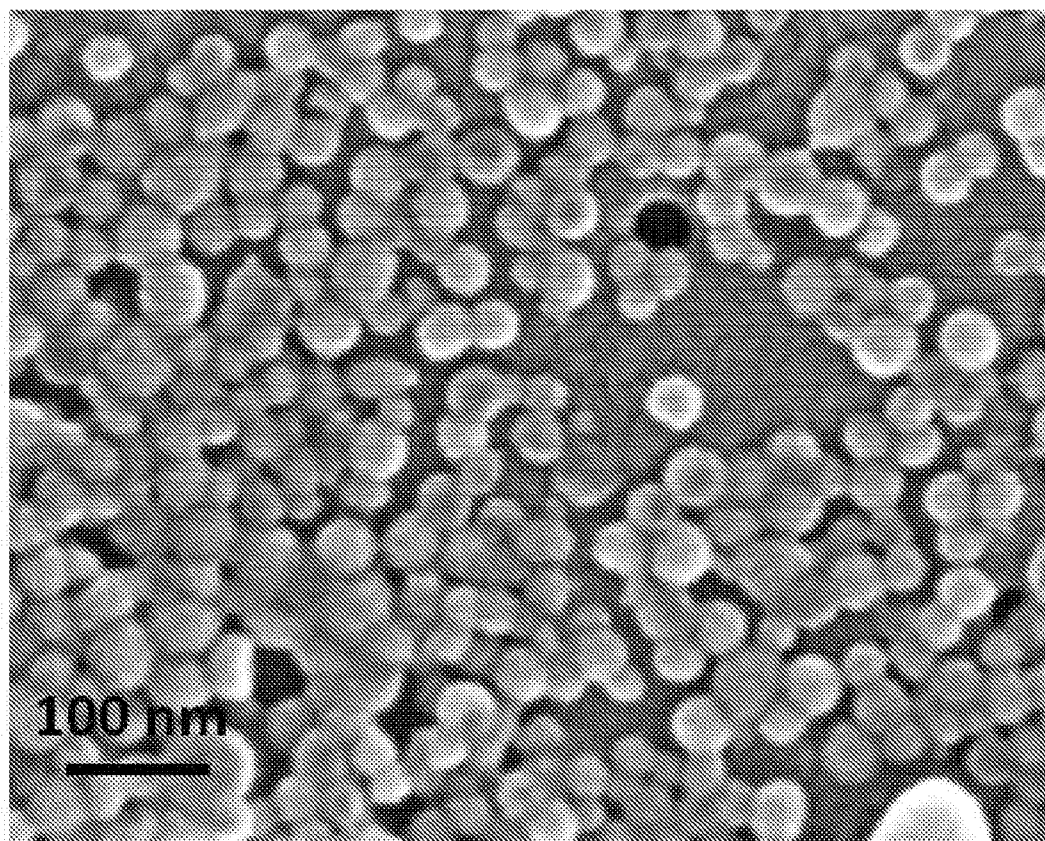
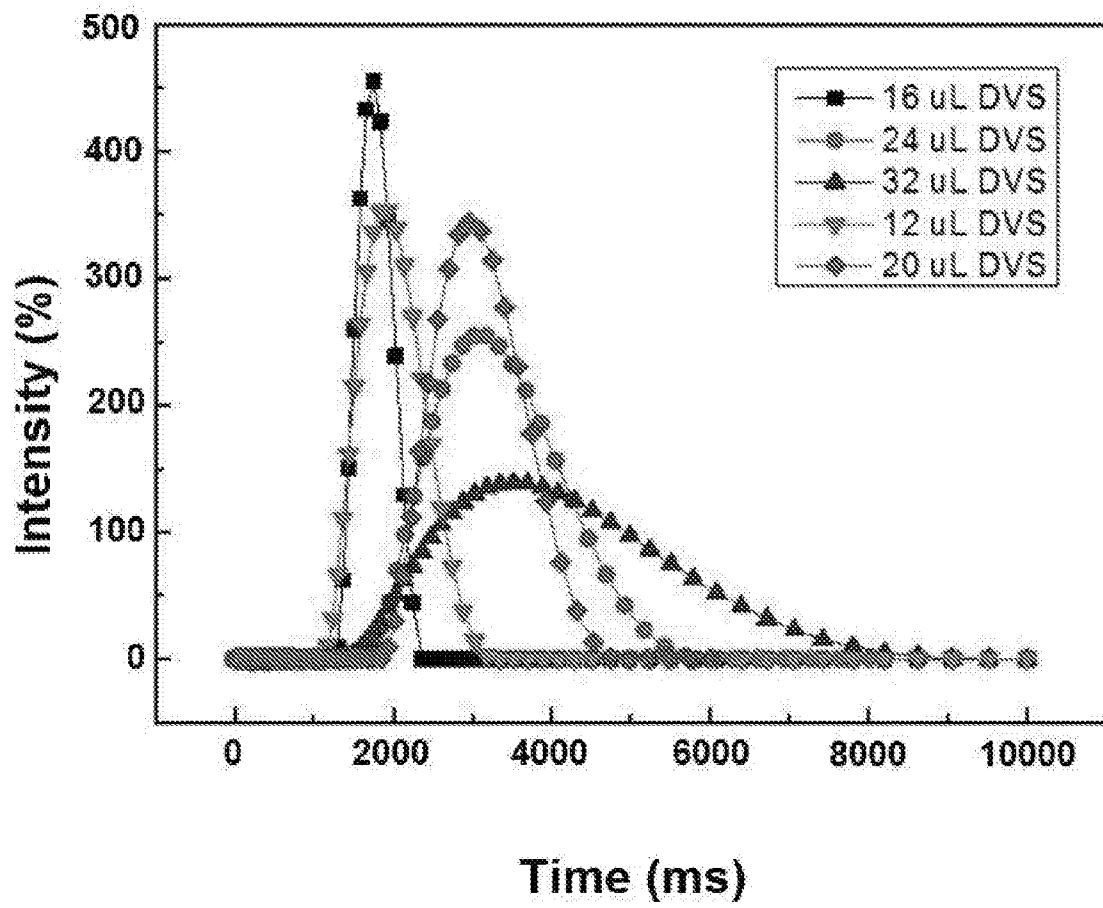


FIG. 5 a)

**FIG. 5 b)****FIG. 5 c)**

**FIG. 6**

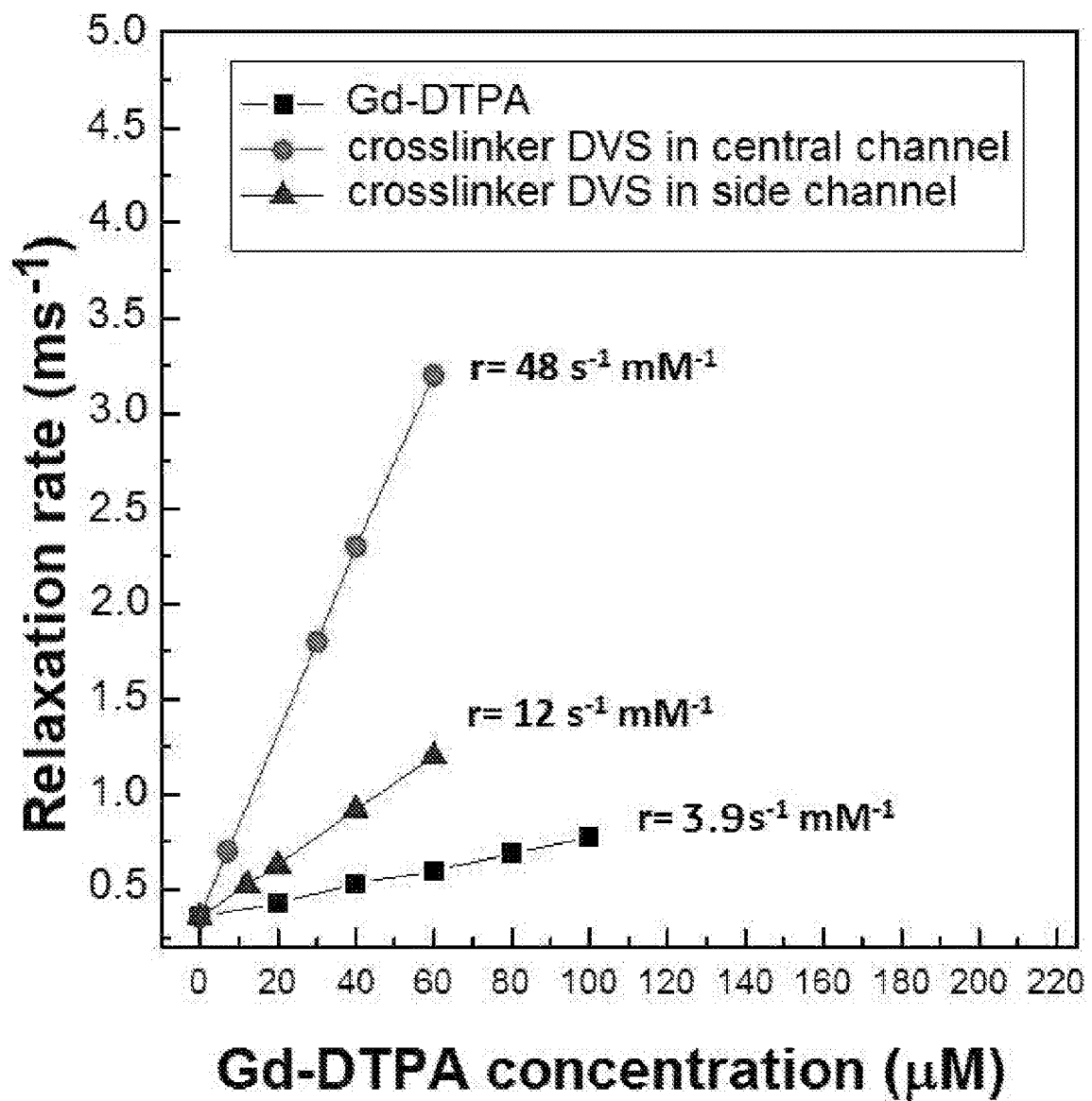


FIG. 7 a)

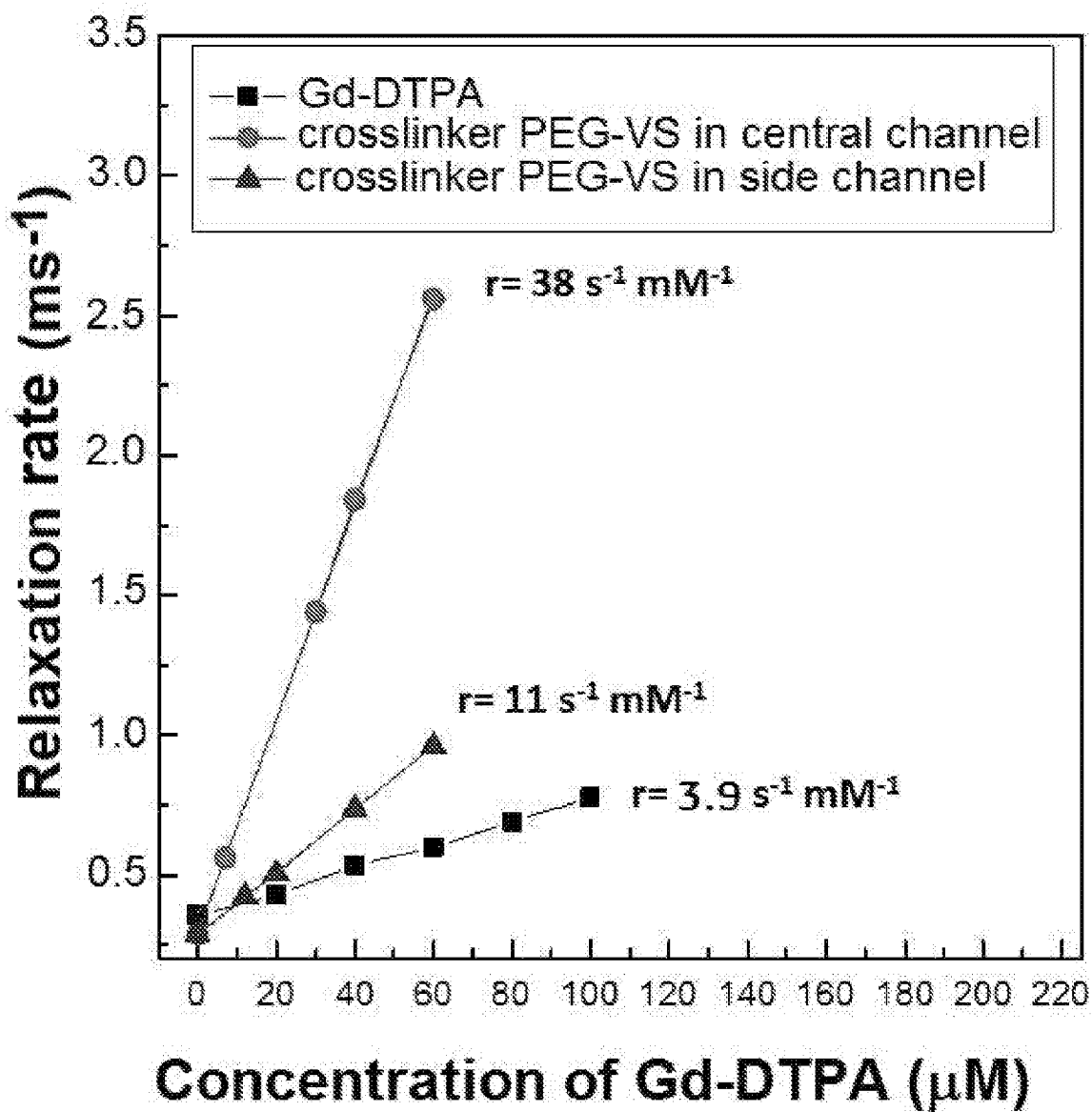


FIG. 7 b)

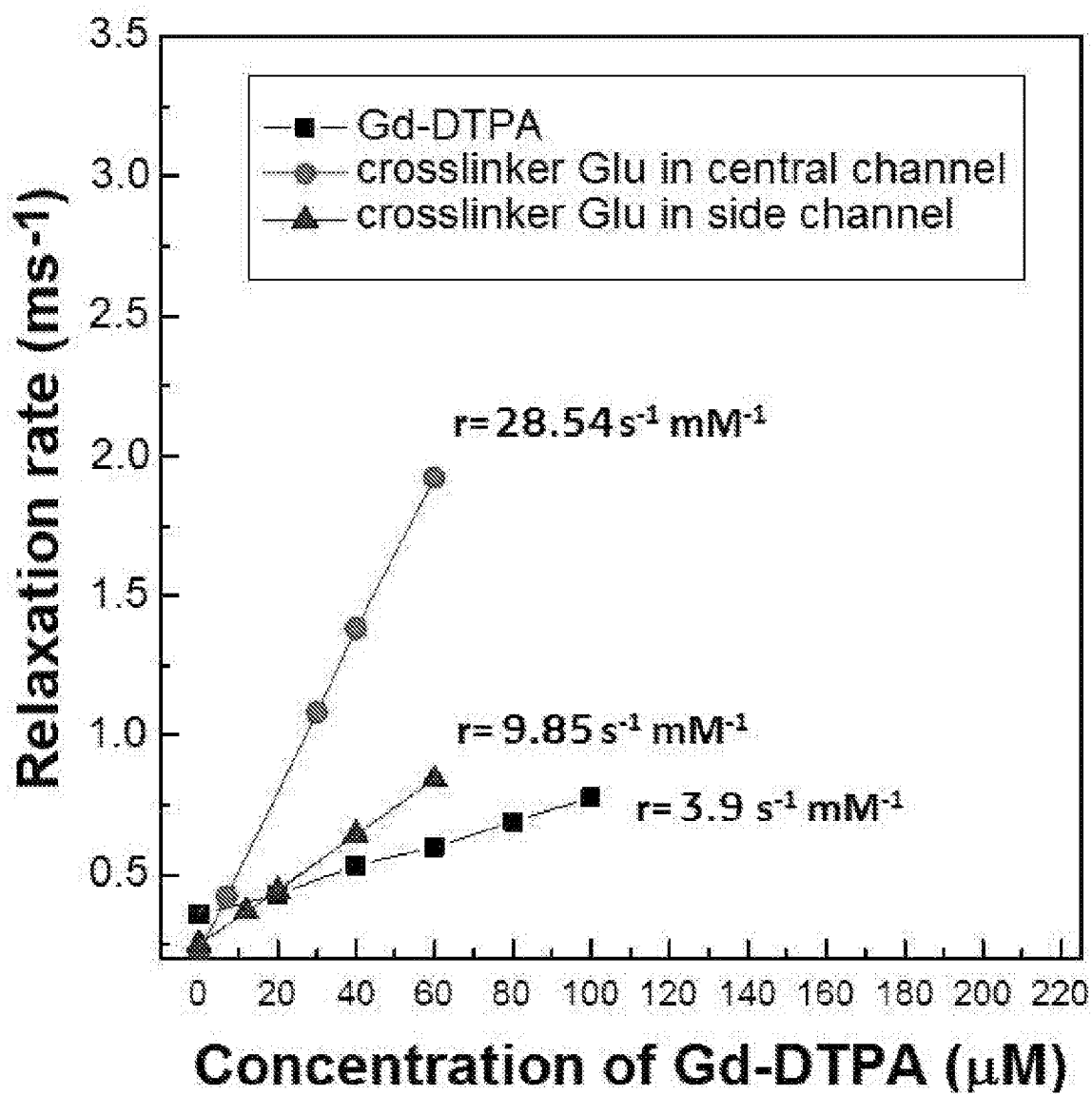


FIG. 7 c)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/057200

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/18
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, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/150030 A2 (MASSACHUSETTS INST TECHNOLOGY [US]; BRIGHAM & WOMENS HOSPITAL [US]; KA) 27 December 2007 (2007-12-27) paragraphs [0005], [0136], [0111] -----	1-15
A	US 2011/158901 A1 (SANTRA SWADESHMUKUL [US]) 30 June 2011 (2011-06-30) -----	1-15
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A	WO 2013/040295 A2 (UNIV SOUTH FLORIDA [US]; SANCHEZ-RAMOS JUAN [US]; SAVA VASYL [US]; SON) 21 March 2013 (2013-03-21) -----	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

9 March 2017

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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