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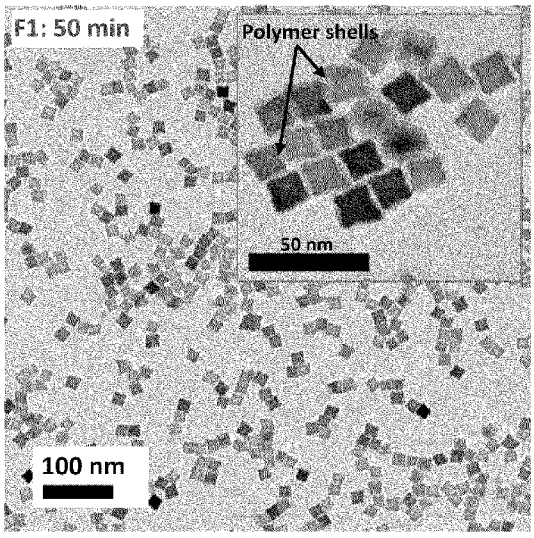

Fig. 3

(57) Abstract: It is described a continuous process for the production of ferrite nanoparticles having the surface functionalized with thermo-responsive polymeric moieties, useful in therapeutic and diagnostic applications in cancer therapy. An apparatus for carrying out the continuous process is also described.

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PROCESS FOR THE CONTINUOUS PRODUCTION OF POLYMER FUNCTIONALIZED FERRITE NANOPARTICLES

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FIELD OF THE INVENTION

The present invention relates to a continuous process for the production of ferrite nanoparticles having the surface functionalized with thermo-responsive polymeric moieties.

STATE OF THE ART

Within the domain of nanomedicine, magnetic nanoparticles (MNPs), especially those with an iron oxide-based composition approved by the FDA, have found great theranostic (therapeutic and diagnostic) applicability in cancer therapy. Thanks to their unique features such as biocompatibility, high saturation magnetization (Ms), less toxicity, and ability to generate heat when exposed to externally applied alternating magnetic field (AMF), they have been employed as contrast agents in magnetic resonance and particle imaging (MRI and MPI), biosensors, and as heat mediators in magnetic hyperthermia (MHT), a treatment modality that uses MNP generated heat to kill cancer cells at therapeutic temperatures (40-46 °C). In addition, MNPs can also be used as nanocarriers for targeted combined MHT and drug delivery applications. For this last role, the hydrophobic surface of the MNP can be engineered to bind hydrophilic and biocompatible stimuli sensitive (pH, heat, etc.) polymers containing covalently linked or encapsulated drug molecules. Given the heat generated by MNPs, during MHT thermoresponsive polymers (TR-polymers) are often preferred. In this case, the MNPs heat is used not only to directly damage cancer cells, but also to trigger deformations/cleavage on the grafted TR-polymer shell, which thereby enable the release of the linked drug only upon heat application. Another possibility of exploiting the heating properties of MNPs is disclosed in patent application WO 2011/147926 A2: in this case, the MNPs are embedded in a lipidic bilayer in the form of a vesicle enclosing a drug; upon exposure to an alternating magnetic field, the MNPs heat up and cause deformation and permeability change of the lipidic bilayer, and as a

consequence the release of the drug present in the vesicle cavity.

Because of the many possible mechanisms of action of nanoplatforms comprising MNPs, and of the synergistic anti-cancer effects that can be achieved with these combined nanoplatforms, their development has been the object of intense research activity in recent years.

A possible process for the production of MNPs is reported in the article "Fabrication of polystyrene-encapsulated magnetic iron oxide nanoparticles via batch and microfluidic-assisted production", C. Taddei *et al.*, Colloid Polym. Sci. 2019, 297 (6), 861–870. The process of this document is based on a nanoemulsion encapsulation technique; the present inventors have noted that this process has several drawbacks, namely, poor size distributions, big size of iron oxide nanoparticles (> 100 nm), clustering phenomena which result in multiple NPs encapsulated in a polymer shell (that can reduce their heating performance under applied alternating magnetic field), or the need to use a micro-fluidic device to aid the emulsion formation, which is however expensive and difficult to microfabricate and leads to limited flowrates of reagents and as a consequence low throughput.

Patent application WO 2013/150496 A1 describes the production of ferrite nanocrystals (meaning iron oxides and mixed oxides of Fe-Mn or Fe-Co) in cubic shape; despite their potential excellent properties, the NPs obtained according to this document have not found wide application because they are not soluble in aqueous media due to the coverage of their surface with a layer of hydrophobic ligands; besides, they tend to form aggregates, making their functionalization with a polymeric coating difficult.

Patent application WO 2018/138677 A1 describes a method for the production of magnetic nanoparticles coated with a thermo- or pH-responsive polymer, comprising providing a solution including magnetic nanoparticles functionalized at their surface with a polymerization initiator, and causing the radical polymerization of a monomer or co-monomers forming a thermo-polymer or pH-responsive copolymer on the surface of the magnetic nanoparticles. The particles produced with this method have suitable

properties for the intended application, in particular in the field of nanomedicine, and the therapeutic efficacy of the produced MNPs was demonstrated through in vitro and in vivo experiments done on skin and colorectal cancers in murine model at clinical MHT conditions (11 KA/m and 110 kHz). The drawback of the production process of this document is that it takes place in batch mode, with problems of batch-to-batch variability in terms of polymer shell thickness and morphology, long reaction times and most critically poor reaction scale-up possibilities; these problems have so far hindered a widespread application of the obtained MNPs in cancer therapy. WO 2018/138677 A1 also describes the relevance of the lower critical solution temperature (also referred to as LCST) of the polymeric shell of the MNPs: this is the temperature threshold that, when overcome, causes the contraction of the polymeric molecules of the shell, and thus a change in the capability of the shell to shield the inner part of the MNPs; when MNPs are loaded with a drug during their production, the heating of the magnetic core during hyperthermia treatment causes thus the shrinking of the shell and the release of the drug at the site reached by the MNPs. It is thus necessary that the LCST is above the physiological temperature (37 °C) to prevent premature leakage of the drug from the MNPs. The LCST varies depending on several parameters, among which the specific monomers the polymer shell is made of and its production process.

Patent application WO 2020/222133 A1 discloses a method for producing ferrite nanocrystals, which comprises the steps of providing a solution comprising a fatty acid, an aliphatic amine and an alcoholic solvent; adding to the solution at least one organometallic precursor compound comprising a metal selected from Fe, Mn, Co or Zn and an aromatic organic molecule obtaining a reaction mixture; transferring the reaction mixture to a sealed reactor, filling the reactor at a percentage between 20 and 70 vol.%; and heating the sealed reactor to a temperature between 160-240 °C for at least 3 hours. This process yields nanoparticles of cubic shape, having excellent properties in view of the intended application in nanomedicine, but again is a batch process suffering of the limits of low productivity indicated above for discontinuous processes.

Continuous processes are well-known in the chemical industry.

For instance, the book “Photochemical processes in continuous-flow reactors - From engineering principles to chemical applications”, 2017, editor Timothy Noël (Eindhoven University of Technology, The Netherlands), describes in chapter 1 tubular reactors for continuous flow photochemistry.

Patent applications US 2018/0339914 A1 and US 2021/0261431 A1 describe a continuous, in-flow process for producing metallic nanoparticles. In the method of these documents, it is provided a first flow stream of an aqueous salt solution comprising ions of a transition metal and a branched polymeric template that is not a dendrimer; providing a second flow stream comprising a reducing agent to reduce the ions of the transition metal to form metallic nanoparticles within the branched polymeric template; and separating the metallic nanoparticles from the branched polymeric template (using various possible purification methods). The process of these documents is about the *in-situ* synthesis of metallic nanocrystals in the presence of a preformed polymer and has no reference to the production of metallic oxide nanoparticles, nor to their surface modification with polymers for drug delivery and bio applications.

Although methods for the continuous production of nanoparticles are known in the field, the present inventors have found that the direct implementation of the process conditions of documents WO 2018/138677 A1 and WO 2020/222133 A1 in a known continuous reactor or process does not lead to useful results, or even leads to a complete failure of the preparation run, because the obtained MNPs tend to precipitate rather than forming a stable water suspension, the form that is necessary for the intended medical uses. This behavior is maybe due to the fact that the nanoparticles of interest in the present case are magnetic and give rise to aggregation problems that are not faced when working with normal metal nanoparticles.

It is thus still felt in the field the need of a continuous process for the large-scale production of magnetic nanoparticles having the surface functionalized with thermo-responsive polymeric moieties, for use in nanomedicine applications.

SUMMARY OF THE INVENTION

This and other objects are achieved with the present invention, that relates to a continuous process for the production of magnetic nanoparticles comprising a ferrite core functionalized on its surface with a thermo-responsive polymer, comprising the steps of:

a) preparing a solution of nanoparticles of: i) a ferrite pre-functionalized with an atom transfer radical polymerization initiator linker; ii) a mixture of at least two thermo-responsive oligomer molecules; iii) a catalyst of the reaction between said initiator linker and said thermo-responsive oligomer molecules; and iv) a radical reaction initiator; the solvent of the solution being selected from tetrahydrofuran, dimethyl sulfoxide, dimethylformamide, acetonitrile, methyl cyanide, methanol, ethanol, water, ethylene glycol, and mixtures thereof;

b) degassing the solution of step a);

c) feeding, as a continuous flow, the degassed solution obtained in step b) at a first end of a reaction chamber, while irradiating the solution flowing in the reaction chamber with UV radiation in the range 315 – 578 nm, causing the reaction between said linker and said thermo-responsive oligomer molecules thus obtaining magnetic nanoparticles consisting of a ferrite core functionalized with a thermo-responsive polymer; and

d) collecting the thus obtained magnetic nanoparticles at a second end of the reactor.

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1 shows a schematic representation of a preferred embodiment of apparatus for carrying out the process of the invention;
- Fig. 2 reproduces FT-IR spectra collected on magnetic nanoparticles produced with the process of the invention and precursors of the same;
- Fig. 3 is the reproduction of a transmission electron microscope (TEM) image of magnetic nanoparticles produced with the process of the invention;
- Fig. 4 is a graph showing the particle size distribution of the magnetic

nanoparticles produced with the process of the invention;

- Fig. 5 is a graph showing the dependence on temperature of the particle size of the magnetic nanoparticles produced with the process of the invention;
- Fig. 6 is a graph showing the values of specific absorption rates (SAR) of magnetic nanoparticles produced with the process of the invention, measured at two values of magnetic field frequency;
- Fig. 7 is a graph showing the increase of temperature, in three consecutive cycles, of magnetic nanoparticles produced with the process of the invention when irradiated with a magnetic field;
- Fig. 8 is a graph confirming the non-cytotoxicity of the magnetic nanoparticles produced with the process of the invention;
- Fig. 9 reproduces a picture showing the results of five tests of preparation of suspensions, one test carried out according to the invention and four tests carried out in comparative conditions.

DETAILED DESCRIPTION OF THE INVENTION

In the description of the invention that follows, the abbreviation MNPs (that stands for magnetic nanoparticles) refers to the final product of the process, that is, the complete particles with a polymer shell encasing the magnetic material core.

The inventors have devised a process that allows to produce magnetic nanoparticles consisting of a ferrite core functionalized on its surface with a thermo-responsive polymer, in a continuous way and thus with an increased rate compared to the batch processes of the prior art.

As said above, the inventors have tried to adopt in a continuous process and apparatus the conditions developed in the past (WO 2018/138677 A1 and WO 2020/222133 A1), changing one parameter/condition from the prior art at a time, but these attempts had no success. The present invention derives from the realization that to achieve an effective continuous process it was necessary to change simultaneously various parameters/conditions compared to those adopted in the cited prior art documents. The inventors have also noted that the continuous process of the invention

affords said magnetic nanoparticles with a higher degree of homogeneity as to particle size and properties.

The first passage of the inventions, a), consists in preparing a solution of precursors of the MNPs of the invention.

This solvent useful for the preparation of the solution is selected from tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetonitrile (ACN), methyl cyanide (MeCN), methanol (MeOH), ethanol (EtOH), water, ethylene glycol and mixtures thereof. Preferably a mixture of THF and DMSO is used; a particularly preferred solvent is a 10/90 v/v mixture THF/DMSO.

The first precursor of the final MNPs are pre-produced nanoparticles of a ferrite on the surface of which are present molecules of a compound adapted for chemically binding to oligomer and/or polymer compounds. Ferrites are a class of magnetic compounds consisting of a mixture of iron oxides and optionally of other metals selected among Fe Mn, Co and Zn, having a high degree of magnetic response. Preferred ferrites are iron (III) oxide (Fe_2O_3) and magnetite (Fe_3O_4).

The nanoparticles of ferrite, and in particular the preferred ferrites of Fe_2O_3 and Fe_3O_4 are preferably produced in the shape of nanocubes; in fact, previous works of research groups of one of the present applicants have demonstrated that these nanocubes have superior magnetic heat properties in terms of specific absorption rates (SAR) compared to other nanoparticles shapes, deriving the specific cubic shape and the control of size and monodispersion (i.e. narrow size distribution) afforded.

The starting ferrite nanocrystals, that form the core of the final nanoparticles, may be produced according to any method known in the literature. In particular, in the preferred case of cubic MNPs, these can be produced following the procedure described in WO 2013/150496 A1 and have preferably a transmission electron microscopy TEM edge-size between about 10 and 35 nm.

These metal oxide nanocrystals are superficially functionalized with linker molecules, having a moiety that electrostatically binds (through Van der Walls force, or similar coordination mechanisms) on the surface of the metal oxide core, and a

moiety adapted to react with the oligomers or polymers precursors of the thermoresponsive polymeric shell of the final MNPs. The linker may be selected among phosphonic acid ($PO(OH)_2$) based linkers, linkers with a terminal functional group such as COOH, OH^- and NH_2 and, preferably, catechol derivatives; catechol derivatives are compounds having a 1,2-dihydroxybenzene moiety modified by a radical that bears a functionality capable to react with a functional group in said oligomers or polymers precursors. A preferred class of catechols for use in the present invention are the derivatives of dopamine, that is, 4-(2-aminoethyl)benzen-1,2-diol.

This precursor, that is, the linker-coated ferrite nanocrystals, is present in solution at a concentration between 0.2 and 4.0 g/L of iron. This range is higher than the precursor/solvent weight range taught by the available prior art (WO 2018/138677 A1) and is the first parameter that must be modified along with other parameters described below to obtain a successful process.

The second precursor is a mixture of thermo-responsive oligomer molecules. Thermo-responsive oligomer molecules useful for the objects of the invention are N-isopropylacrylamide (NIPAM) derivatives, methacrylate derivatives such as methyl methacrylate (MMA) and butyl methacrylate, acrylamide derivatives and, preferably, ethylene glycol methyl ether methacrylate derivatives. The preferred oligomers of ethylene glycol methyl ether methacrylate are in particular diethylene glycol methyl ether methacrylate (DEGMEMA) and oligoethylene glycol methyl ether methacrylate (OEGMEMA) monomers with up to 15 ethylene glycol derived moiety units. The DEGMEMA/OEGMEMA molar ratio useful for the objects of the invention is in the range 50/50 to 75/25; this means that useful ratios are the two ends of the range and the intermediate ratios 55/45, 60/40, 65/35 and 70/30. This ratio is the second parameter that must be simultaneously changed compared to the teachings of WO 2018/138677 A1 to achieve a successful process; the reported ratios are calculated on the ethylene glycol monomeric units present in the oligomers, that are 2 in DEGMEMA and 3 to 15 in OEGMEMA. The inventors have found that adjusting the molar ratio of DEGMEMA/OEGMEMA in the range 50/50 to 75/25 allows producing a

stable aqueous suspension of MNPs, while adopting higher DEGMEMA/OEGMEMA ratios, such as the ratio 81/19 of WO 2018/138677 A1 (see Example 3 in connection with the information that the used OEGMEMA has a molecular weight of 500 Da), results in the precipitation of the MNPs. Following the teachings of the present invention, and adjusting the molar ratio DEGMEMA/OEGMEMA, it is possible to tune the LCST of the polymer from 25 to 60 °C. In the chosen experiment for a LCST of 43 °C, the two oligomers were present in the starting solution in volumes of 0.83 mL of DEGMEMA and 0.7 mL of OEGMEMA.

The third precursor is a catalyst of the polymerization reaction of the oligomers described above. This catalyst may be selected among amines, imines, or copper (I/II) halide based catalysts; examples of amines/imines are tris(2-pyridylmethyl)amine (TPMA), pentamethyldiethylenetriamine (PMDETA), hexamethyltriethylenetetramine (HMTETA), pyridineimine and 2,2′-bipyridine or their combinations; copper based catalysts may be CuBr, $CuBr_2$, or the preferred catalyst for this step, namely, copper tris(2-dimethylaminoethyl)amine bromide, generally indicated with the abbreviated formula [Cu(Me_6TREN)Br]Br. The catalyst is added to the solution with a concentration between 0.05 and 0.10 M.

Finally, the fourth precursor is a radical reaction initiator, that may be the same molecule as the linker present on the surface of the ferrite nanocrystals. Initiators useful for the objects of the invention are alkyl or aryl halides and pseudohalide derivatives; preferred initiators are ethyl α-bromophenylacetate, phenylethyl bromide, methyl 2-bromopropionate, methyl 2-bromoisobutyrate, ethyl chloroisobutyrate, and ethyl α-bromoisobutyrate; a particularly preferred radical reaction initiator is 2-bromo-N-[2-(3,4-dihydroxy-phenyl)-ethyl]-butyramide, a derivative of dopamine, also referred to in the following as DOPA-BiBA. This component is conveniently added to the solution at a concentration between 7×10^{-4} and 1×10^{-3} M. The amount of initiator is the third parameter that must be changed, compared to the teachings of WO 2018/138677 A1, to achieve an effective continuous process: the inventors have in fact observed that, adopting the conditions of the cited prior art in the continuous process, the result was

again the precipitation of the MNPs; in the present invention, the amount of initiator is increased of about 30% compared to the conditions described in WO 2018/138677 A1.

The solution is preferably prepared in separate steps, by preparing partial solutions that are then mixed in the amounts and ratios required to obtain the concentrations reported above for the different precursors.

In particular, it is preferable to prepare initially a first partial solution (in one of the cited solvents) containing the ferrite nanocrystals pre-coated with the linker, the oligomers and the radical initiator. This first partial solution is degassed from oxidizing gases, for instance through sonication and nitrogen purging for about 15 minutes before mixing with a second partial solution.

The second partial solution contains instead the catalyst.

These two partial solutions are mixed soon before the start of the process.

The second step of the process, b), the solution containing all the precursors (obtained for instance by mixing the two partial solutions above) is degassed for removing possible oxidizing gases. This passage takes place as indicated above for the first partial solution.

In the third step of the process, c), the degassed solution obtained in step b) is continuously fed at a first end of a reactor consisting in an elongated chamber with at least one wall transparent to UV radiation and irradiated with UV causing the polymerization of the thermo-responsive oligomer molecules.

Before feeding the reactant solution, the reactor chamber is preferably flushed for some minutes (e.g., 15 minutes) with the same solvent, previously degassed in the same way as described above, as the solvent of the reactant solution. This operation has the function of cleaning the reaction chamber and avoiding the presence of any impurities that could contaminate the final product or interfere with the polymerization reaction.

The wavelength range of the UV radiation useful for the objects of the invention is 315-578 nm, preferably about 365 nm.

The inventors have found that optimal reaction efficiency is obtained with a

residence time of the solution in the reactor chamber between 30 and 150 min.

The flowrate of the solution fed to the reaction chamber is regulated so as to obtain a residence time of the solution in the chamber in the range above, depending on the length of the chamber.

The reaction temperature is between 5 and 25 °C.

Finally, in the last step of the process, d), the magnetic nanoparticles coated with a polymeric shell (MNPs), useful in theranostic applications, are collected at a second end of the reactor. The inventors have found that the process of the invention simplifies the cleaning/washing or separation from by-products, as it does not require ultracentrifugation steps further shortening the process time compared to batch processes, and leading to an increased yield.

A possible apparatus for carrying out the process described above is described below.

The apparatus comprises four main elements, that are: i) an elongated reaction chamber with at least one wall transparent to UV radiation; ii) one or more UV lamps for irradiating the solution in the reaction chamber; iii) means connected to a first end of the elongated reaction chamber for pushing the degassed solution of step b) of the process into the chamber; and iv) a tank connected to a second end of the elongated reaction chamber for collecting the magnetic nanoparticles produced with the process.

The means for pushing the solution into the reaction chamber can be a pump or a syringe with automated control (namely, a syringe pump), for regulating the flowrate of the solution to a value such that the residence time of the solution inside the reaction chamber is between 30 and 150 min, that is the range of duration of the reaction.

In order to avoid the need of too long reaction chambers, given the residence times indicated above the automated means for pushing the solution into the chamber are preferably capable of delivering very low flowrates; commercial pumps or syringe pumps are available to this end with flowrates of 0.05 mL/min.

The reaction chamber may have any geometry that ensures a residence time of the reacting mixture inside the chamber at the selected flowrate. The chamber may be,

for instance, in the form of a channel obtained in a chip and closed with a flat slab or a tubular coil of a material transparent to UV.

Since the polymerization reaction is activated by UV irradiation, at least the wall of the reactor chamber facing the UV lamp(s) must be transparent to this radiation. Preferred materials for the production of this transparent wall are perfluoroalkoxy alkanes (PFA) and polytetrafluoroethylene (PTFE).

To achieve a complete reaction, the thickness of the wall of the chamber has maximum value of 1 mm in the direction perpendicular to the incident UV radiation; this condition ensures that the radiation extinction due to absorbance in the first layer of solution (that is, the layers closer to the UV lamp(s)) is low, and the radiation that reaches the layers of solution farther from the UV source is still intense enough to cause the polymerization reaction.

The elongated reaction chamber is preferably in the form of a tube having a convoluted shape, for instance in the form of a serpentine or, preferably, helically wound around a UV lamp; this latter configuration maximizes the surface of the reaction chamber directly exposed to the UV source, thus reaching the maximum possible efficiency of the apparatus.

In the preferred case of a tubular reaction chamber coiled around the UV source, the inner diameter of said chamber is preferably in the range 0.1-7.0 mm, more preferably of about 0.75 mm, while the wall of the reaction chamber has preferably a thickness in the range of 0.1 up to 1 mm, more preferably of about 0.85 mm.

Fig. 1 is a schematic representation of a preferred configuration of an apparatus of the kind described above.

The apparatus, 10, comprises a means 11 for pushing the degassed solution of step b) of the process into the reaction chamber 12. The means 11 is represented in the form of a syringe pump, while the reaction chamber is represented as a UV-transparent tubing, that in this preferred embodiment is coiled around a UV lamp 13; at the end of the reaction chamber opposite to the end connected to the syringe 11, there is the tank 14 for collecting the MNPs produced in the chamber 12. In a further

preferred embodiment, the apparatus also comprises a second coil wrapped around the system described above, in which a refrigerant fluid is circulated, to keep the system and the reacting mixture in the desired temperature range.

The invention will be further illustrated with the examples that follow.

INSTRUMENTS, METHODS AND EXPERIMENTAL CONDITIONS

The following products and tools were used to perform the examples reported below:

TEM: JEOL JEM 1400Plus electron microscope. This instrument is equipped with a LaB_6 crystal thermionic electron source having an acceleration voltage of 120 kV, and an Orius CCD Camera purchased from Gatan Company, USA.

DLS: Malvern ZetasizerNano series instrument.

FT-IR: Vertex 70 (Bruker) having an ATR Golden Gate accessory.

EXAMPLE 1

This example refers to the preparation of magnetic nanoparticles according to the continuous process of the invention.

For carrying out the test, the apparatus depicted in Fig. 1 was used; the reaction chamber had a total inner volume of 3.1 mL.

Iron oxide (Fe_3O_4) nanocubes customized with 2-bromo-N-[2-(3,4-dihydroxy-phenyl)-ethyl]-butyramide (DOPA-BiBA) were prepared following the procedure described in WO 2018/138677 A1. DOPA-BiBA is a catechol-based photo-atom transfer radical polymerization (ATRP) initiator, which triggers the polymerization reactions involved in the production of MNPs.

Next, to the DOPA-BiBA initiator functionalized nanocubes dispersed in 1.0 mL THF at a concentration of 4.0 g of iron per liter, 3.0 mL of DMSO was added obtaining a clear brown solution. To this mixture in 20 mL vial, an additional DMSO solution (4.0 mL) containing the monomers (827.0 µL of DEGMEMA and 692.0 µL of OEGMEMA) and 2.2 mg of free DOPA-BiBA initiator was added. Then, this mixture was sonicated for 30 seconds and purged with nitrogen through a needle placed on a septum for 15 minutes. In another separate 20.0 mL vial, a cocktail of the catalyst stock solution

containing CuBr2 (1.5 mg) and Me6TREN ligand (3.6 µL) in 11.25 mL of DMSO was prepared and similarly purged with nitrogen; the weight ratio between the sum of reactants and the solvent was 13.1. 1.0 mL of this catalyst solution was then injected into the first vial containing the initiator functionalized nanocubes. This reaction mixture was considered the stock solution for the test. While preparing and degassing the reactant mixture, a syringe loaded with the reaction solvent medium (10% v/v THF/DMSO) was mounted onto the syringe pump and charged into the photo-tubular reactor (with switched off UV-lamps), to flush the tube. This step takes about 15 min and ensures that the lamps remain cool before the polymerization starts. After nitrogen purging, the stock solution containing reactants and catalyst was then loaded into a 24 mL plastic syringe and mounted onto the syringe pump, replacing the one bearing the blank solvent. Before the start of the in-flow polymerization, the UV-lamps were switched on, and the flow rate of the pump operated at a flow rate of 0.07 mL/min, which is equivalent to an observed reaction time of 50 minutes.

After pumping the stock reaction mixture containing the nanocubes through the reactor for 50 minutes (the set reaction time), the emerging drops of viscous brown crude polymerization product (crude TR-Cubes) was collected in a glass vial. Upon complete processing of the 10.3 mL reactant mixture (i.e., total volume of solvents + reactants), the collected crude product in the receiving vial was quenched by air exposure and THF addition (5.0 mL) to dilute and thereby prevent any further polymerization. Subsequently, the obtained crude product was rinsed three times to precipitate the TR-cubes from the reaction mixture, by addition of 25.0 mL diethyl ether followed by centrifugation and 10.0 mL THF dissolution of the black gel-like pellet collected at the bottom of the falcon tube after removal of the solvent. After the final step of centrifugation, the MNPs were dried over nitrogen for 1 h before re-dispersing them in 10.0 mL Milli-Q water. The result of the procedure is shown in Fig. 9: the obtained product is the transparent suspension in the first test tube from right in the figure. Then the excess free polymer molecules were removed by centrifugal filtration (1500 rpm, 10 min) done twice and the sample concentrated to about 200 µL before

being dispersed in saline for further characterizations.

To confirm the obtainment of the polymer shell, comparative Fourier-transform infrared (FT-IR) measurements were carried out on the starting DOPA-BiBA functionalized nanocubes and on the final product. The two obtained spectra are reproduced in Fig. 2; in the figure, the abbreviation IONCs stands for “iron oxide nanocubes”, the definition “Initiator-IONCs” refers to the starting material of the preparation, while the definition “TR-Polymer IONCs” refers to the IONCs inside a shell of thermos-responsive polymer, that is, the final product.

The spectrum of the starting material (Fig. 2, lower graph) shows a broad peak at 3358 cm^{-1} ascribed to N-H stretching of dopamine. In the spectrum of the final product, reproduced in the upper part of Fig. 2, the presence of the sharp peaks at 1726 cm^{-1} (attributed to C=O groups) and 1115 cm^{-1} (C-O-C stretching vibrations) assignable to PEG chains confirms the successful formation of the polymer shell on the MNPs surface.

The process described above was repeated three times. The yield of the process, calculated as ratio of grams of iron in the recovered product over the grams of iron used in the process and averaged over the three runs, resulted 72.8 ± 3.8%. The product obtained in this Example is specimen 1.

EXAMPLE 2 (COMPARATIVE)

For comparative purposes, the batch procedure of WO 2020/222133 A1 was followed, using the same starting materials and solutions of Example 1. The only difference was that, due to higher efficiency and kinetics of the continuous process of Example 1, the reaction time was increased to 150 minutes. The result of the test is shown in Fig. 9 and is represented by the first test tube on the left in the picture; the MNPs are essentially completely precipitated and separated from the limpid solution above.

EXAMPLE 3 (COMPARATIVE)

For comparative purposes, the procedure of Example 1 was repeated adopting the process parameters of WO 2020/222133 A1, namely, a DEGMEMA/OEGMEMA

molar ratio equal to 81/19, higher than that of the present invention. The result of the test is shown in Fig. 9 and is represented by the second test tube from right in the picture; the MNPs are essentially completely precipitated and separated from the limpid solution above.

EXAMPLE 4

This example refers to the morphological and polydispersity characterization of the MNPs produced according to the invention.

MNPs of specimen 1 were observed to be easily water dispersible and characterized by TEM; the obtained TEM picture is reproduced in Fig. 3, the inset representing a magnification of part of the picture. The MNPs show a clear cubic shape, with size of about 20 nm.

To highlight the thickness of the polymer shell, MNPs of specimen 1 were deposited on the TEM grid with a 1% uranyl acetate solution, that made the polymer shell on single nanocubes more visible: in these conditions, the polymer shell has a darker contrast.

The hydrodynamic size (Dh) of the MNPs of specimen 1 was further determined using dynamic light scattering (DLS) technique. The measurements were conducted at 25 °C using a Malvern ZetasizerNano series instrument and the scattered light was detected at an angle of 173°. 500 µL of MNPs sample in saline was pipetted into a disposable cuvette and triplicate readings were made following an equilibration time of 1 min per reading.

The results of this test are reported in the graph in Fig. 4.

In good agreement with TEM, the Dh weighted by intensity, volume and number were 85 ± 32, 62 ± 26 and 46 ± 14 nm respectively with monomodal peaks (curves 1, 2 and 3 respectively in Figure 4).

EXAMPLE 5

Next, the lower critical solution temperature (LCST) of the MNPs produced in Example 1 was determined. LCST is an important parameter that guarantees the release of any encapsulated drug by the shrinkage of the grafted polymer shell during

the heating of the magnetic core under AMF application.

The DLS technique was employed to measure the change in hydrodynamic size (Dh) of MNPs in solution as a function of temperature. The test was carried out on specimen 1 produced according to the invention. The results of the test are reproduced in the graph in Fig. 5: as is clear from the graph, the size of the MNPs remains essentially unchanged for temperatures below about 41-42 °C, and the LCST is of about 43 °C. This confirms that the MNPs of the invention remain stable, with the polymer chains in shrunk configuration at the physiological temperatures, thus forming a close shell capable of retaining the drugs loaded in the MNPs; at temperatures higher than the physiological ones, attainable by heating of the magnetic core of the MNPs upon application of a magnetic field, the polymer chains relax opening porosities in the shell, making possible the release of the embedded drug.

EXAMPLE 6 (COMPARATIVE)

The procedure of Example 1 was repeated adopting process parameters derived from WO 2020/222133 A1; in particular, 1.6 mg of free DOPA-BiBA initiator (lower than in Example 1) was used. The result of the test is shown in Fig. 9 and is represented by the second test tube from left in the picture; the MNPs are essentially completely precipitated and separated from the limpid solution above.

EXAMPLE 7 (COMPARATIVE)

The procedure of Example 1 was repeated adopting process parameters derived from WO 2020/222133 A1; in particular, a weight ratio between the sum of reactants and the solvent equal to 11.2 was used. The result of the test is shown in Fig. 9 and is represented by the central test tube in the picture; as can be seen in the figure, the MNPs are not dispersible in water.

EXAMPLE 8

The heat generation capability and sustained stability under AMF of MNPs of the invention were evaluated.

To this end, the magnetic heating losses of a part of specimen 1 was first performed by calorimetric measurements to determine the specific absorption rates

(SAR) values, which measures the heating efficiency of the magnetic nanoparticles. This SAR is defined as the power absorbed per mass of the heat mediator (expressed here as Watt per gram of iron, W/g Fe). For this measurement, clinically accepted MHT field conditions were used (frequency: 111 and 182 kHz; field: 24 kA/m) on a sample of specimen 1 dissolved in saline solution (0.9% NaCl). The SAR value of the MNPs of the invention is about 150 and 260 W/g Fe at frequency of 111 and 182 kHz, respectively (Fig. 6). These values are completely comparable to those reported for MNPs prepared by in-batch synthesis.

To evaluate the heating stability under repeated cycles of AMF exposure, the sample of specimen 1 was subjected to three cycles of magnetic hyperthermia of 30 minutes each, and the temperature was recorded during the hyperthermia cycle using an optical fiber probe while switching on and off the AMF. In detail, the sample of specimen 1 was diluted at 2.5 g/L [Fe] in saline solution and exposed to a clinically accepted MHT condition (field intensity of 15 kA/m, frequency 182 kHz). The results of the test are reproduced in Fig. 7. Within 30 minutes when the AMF was on, a therapeutic temperature of 46 °C was reached in few minutes and it was maintained constant until the MHT was switched off. The MHT cycle could be repeated for three times in a row with just 5 minutes in between without losing the ability of the MNPs of specimen 1 to reach the 46 °C temperature under the same AMF conditions. This suggests that the MNPs of the invention are stable and do not aggregate during heating, which would lead to a reduced or compromised heating performance.

EXAMPLE 9

This example is directed to verifying the biocompatibility of the MNPs prepared according to the invention; this is a necessary condition for the nanoparticles to be admitted as a possible clinical candidate to mediate cancer treatment.

An in vitro cytotoxicity study was carried out on U87 malignant Glioblastoma cell line cultured in 2D on a Petri dish, using Trypan blue viability assay. Briefly, the U87 cells were incubated with a sample of specimen 1, at a typical therapeutic MNPs dose of 1.5 g/L [Fe], for continuous exposure at 24 and 48 h. For comparison, the viability

of U87 cells to which just saline solution was added and cultured under the same conditions of the sample of the invention, was assessed. The results of the test are graphically shown in Fig. 8: for each couple of bars in the figure, the bar on the left refers to the control, while the bar on the right represents the results obtained with the sample of the invention. As can be seen in Fig. 8, the % of cell viability, corresponding to ca. 98 % with respect to the blank (cells in saline) revealed that the MNPs of specimen 1 were non-toxic to cells; by means of a t-test performed on these two groups (control and test sample), no statistically significant difference between the samples at p value = 0.05 was observed. Hence, it was here confirmed that the MNPs produced with the in-flow process of the invention are biocompatible at this concentration.

COMMENTS TO THE RESULTS

The results reported in examples 1, 4, 5, 8 and 9 confirm that the continuous process of the invention produces MNPs of similar characteristics to those obtained with the in-batch processes of the prior art, and suitable for use in theranostic applications.

The comparison between the results obtained in example 1 (invention) and example 2 (comparison) shows a yield of the process of the invention higher of about 40%, obtained with a duration of the process that is one third, compared to the yield and duration of the known in-batch process. This confirms that the in-flow process of the invention is suitable for producing MNPs in amounts, in the unit time, much higher than those possible with the processes of the prior art, making thus available the MNPs for a widespread use in theranostic application that was not possible with the known processes.

The comparison between the LCST values of MNPs produced in example 1 (invention) and in comparative examples 3, 6 and 7 demonstrates that the simple transfer of synthesis conditions known from in-batch processes of the prior art to a continuous process does not lead to a successful procedure, since stable aqueous suspensions of MNPs are not obtained, and rather the MNPs precipitate; the precipitation of the MNP would also be a problem during continuous flow production

because, on the long term, the precipitate could clog the channels of the in flow system.

It is to be noted that the conditions for an effective continuous process are a self-consistent set of parameters, different and not derivable from parameters known in the prior art; besides, comparative examples 3, 6 and 7 demonstrate that, starting from the conditions defined in the known batch processes, the skilled person would have not arrived at the present invention by simply changing a parameter at a time, since any attempt made by altering one single parameter of the known processes resulted in the precipitation of the MNPs; only the simultaneous modification of several parameters, as in the present invention, gives rise to the stable aqueous suspensions that are needed for the intended medical applications of the MNPs.

CLAIMS

1. Continuous process for the production of magnetic nanoparticles comprising an iron oxide core functionalized on its surface with a thermo-responsive polymer, comprising the steps of:
   a) preparing a solution of nanoparticles of: i) a ferrite pre-functionalized with an atom transfer radical polymerization initiator linker; ii) a mixture of at least two thermo-responsive oligomer molecules; iii) a catalyst of the reaction between said initiator linker and said thermo-responsive oligomer molecules; and iv) a radical reaction initiator; the solvent of the solution being selected from tetrahydrofuran, dimethyl sulfoxide, dimethylformamide, acetonitrile, methyl cyanide, methanol, ethanol, water, ethylene glycol, and mixtures thereof;
   b) degassing the solution of step a);
   c) feeding, as a continuous flow, the degassed solution obtained in step b) at a first end of a reaction chamber, while irradiating the solution flowing in the reaction chamber with UV radiation in the range 315 – 578 nm, causing the reaction between said linker and said thermo-responsive oligomer molecules thus obtaining magnetic nanoparticles consisting of a ferrite core functionalized with a thermo-responsive polymer; and
   d) collecting the thus obtained magnetic nanoparticles at a second end of the reactor.

2. Process according to claim 1, wherein the solvent of the solution of step a) is a 10/90 v/v mixture tetrahydrofuran/dimethyl sulfoxide.

3. Process according to any one of claims 1 or 2, wherein said ferrites are iron (III) oxide, Fe_2O_3, and magnetite, Fe_3O_4, have a transmission electron microscopy TEM edge-size between about 10 and 35 nm, and are present in the solution of

step a) at a concentration between 0.2 and 1.0 g/L of iron.

4. Process according to any one of the preceding claims, wherein said linker is selected among phosphonic acid ($PO(OH)_2$) based linkers, a linker with a terminal functional group COOH, a linker with a terminal functional group OH^-, a linker with a terminal functional group NH_2, and catechol derivatives.

5. Process according to any one of the preceding claims, wherein said at least two thermo-responsive oligomer molecules are selected among N-isopropylacrylamide derivatives, methacrylate derivatives, acrylamide derivatives, and ethylene glycol methyl ether methacrylate derivatives.

6. Process according to claim 5, wherein said ethylene glycol methyl ether methacrylate are diethylene glycol methyl ether methacrylate and oligoethylene glycol methyl ether methacrylate monomers with up to 15 ethylene moiety units, and the molar ration between diethylene glycol methyl ether methacrylate and oligoethylene glycol methyl ether methacrylate is in the range 50/50 to 75/25.

7. Process according to any one of the preceding claims, wherein said catalyst of the reaction between said initiator linker and said thermo-responsive oligomer molecules is selected among amines, imines, and copper (I/II) halide-based catalysts, and is added to the solution at a concentration between 0.05 and 0.10 M.

8. Process according to any one of the preceding claims, wherein said radical reaction initiator is selected among alkyl halides, aryl halides, and pseudohalide derivatives, and is added to the solution of step a) at a concentration between $5x10^{-4}$ and $1x10^{-3}$ M.

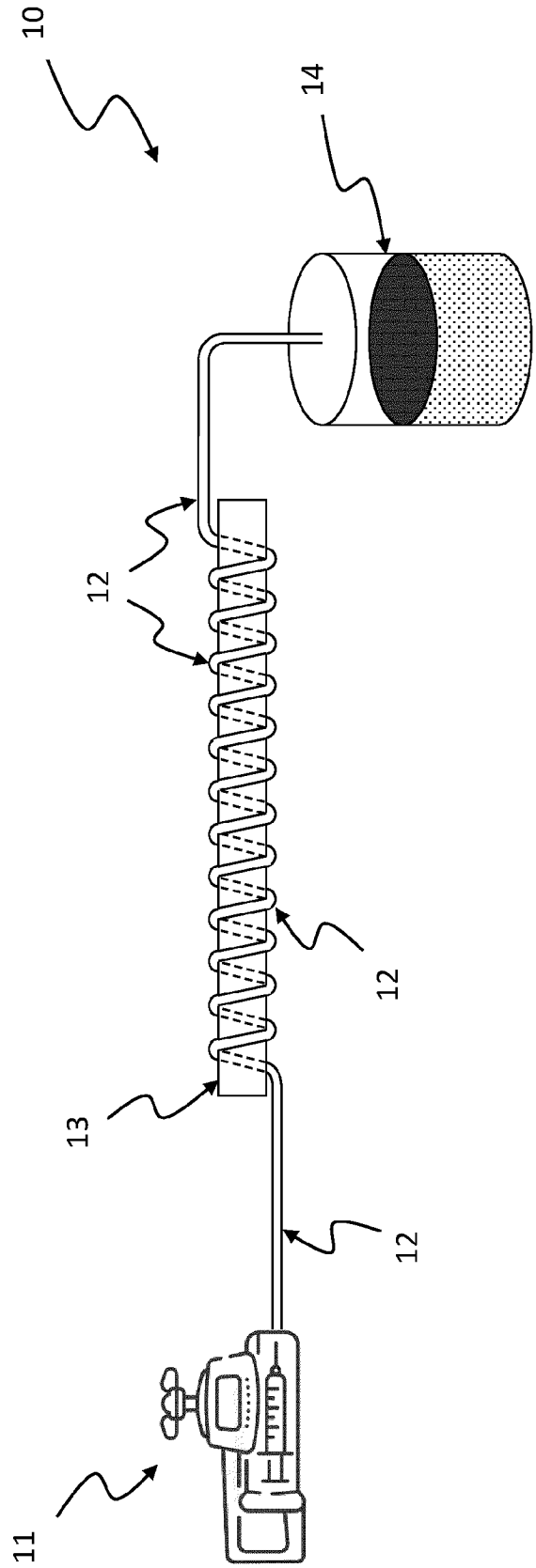

Fig. 1

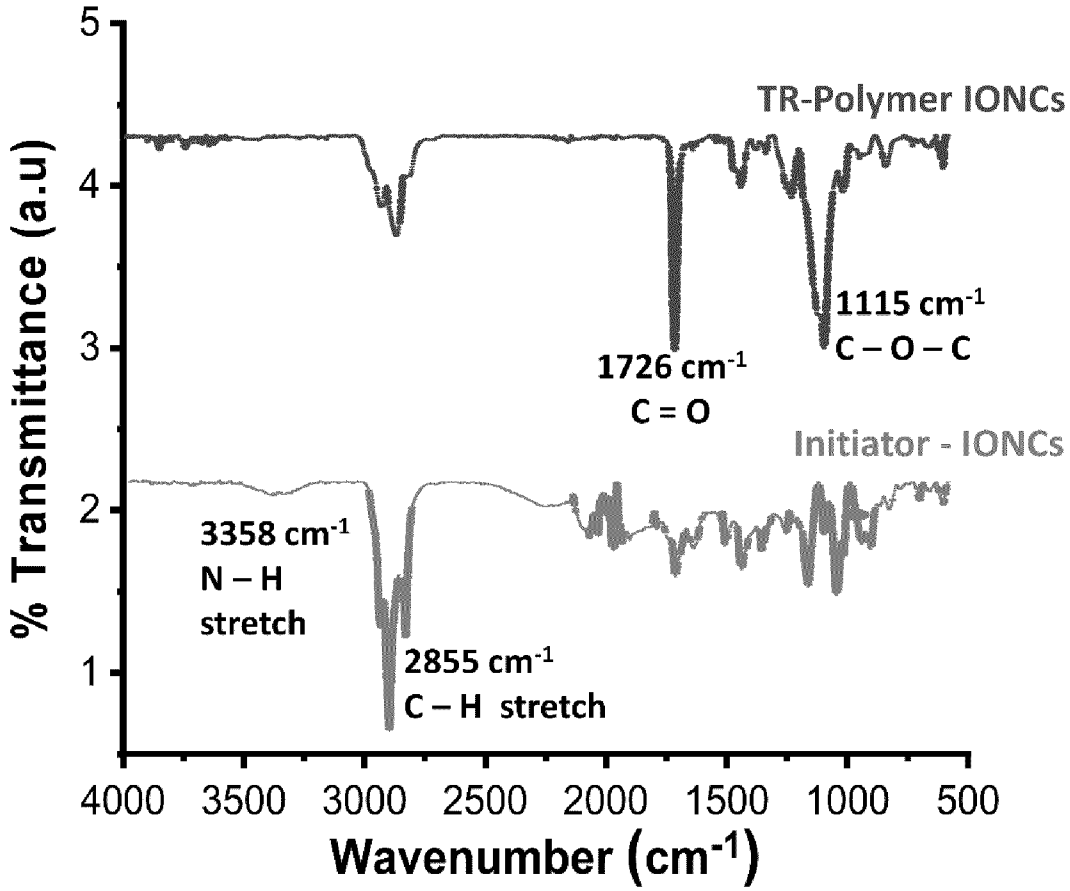
TR-Polymer IONCs
Initiator - IONCs
1726 cm-1
C = O
1115 cm-1
C – O – C
3358 cm-1
N – H
stretch
2855 cm-1
C – H stretch
% Transmittance (a.u)
Wavenumber (cm-1)
5
4
3
2
1
4000
3500
3000
2500
2000
1500
1000
500

Fig. 2

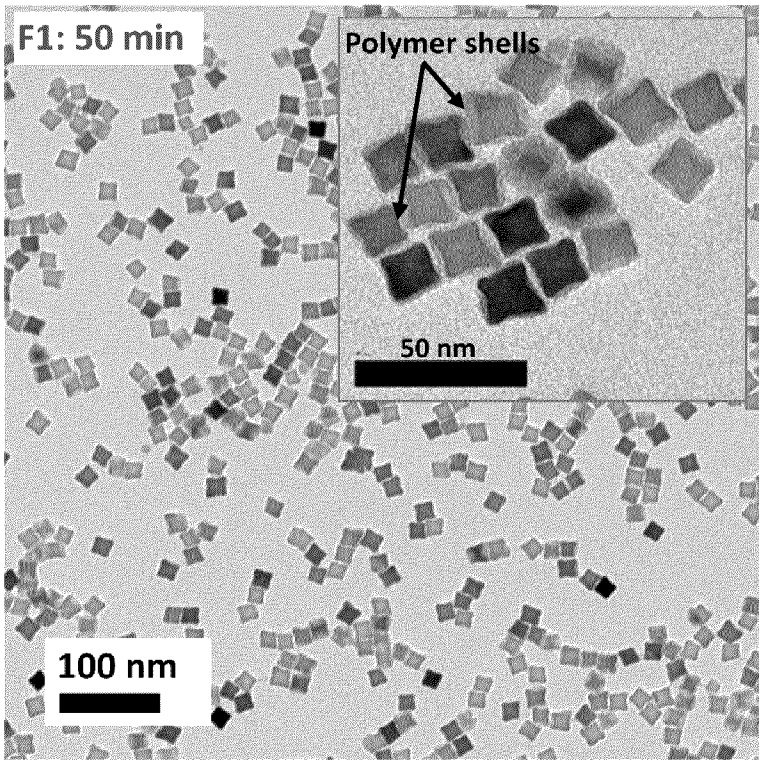
F1: 50 min
Polymer shells
50 nm
100 nm

Fig. 3

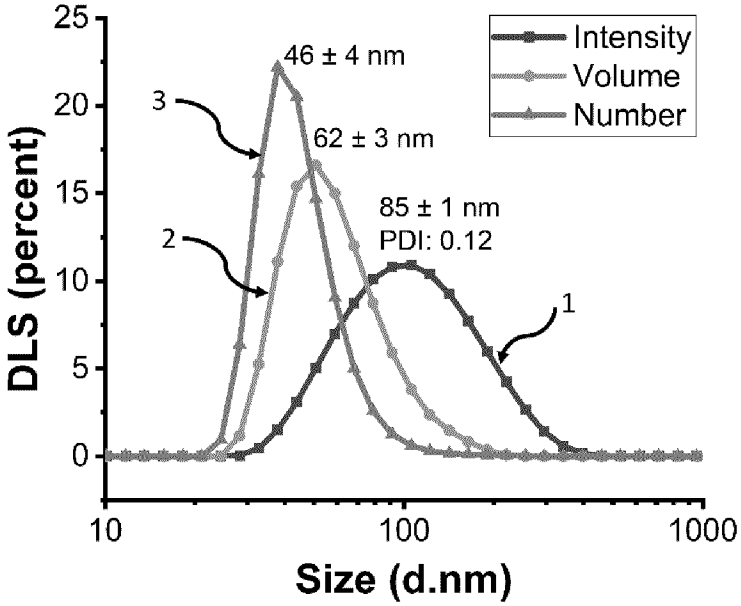
DLS (percent)
Size (d.nm)
Intensity
Volume
Number
46 ± 4 nm
62 ± 3 nm
85 ± 1 nm
PDI: 0.12
1
2
3
0
5
10
15
20
25
10
100
1000

Fig. 4

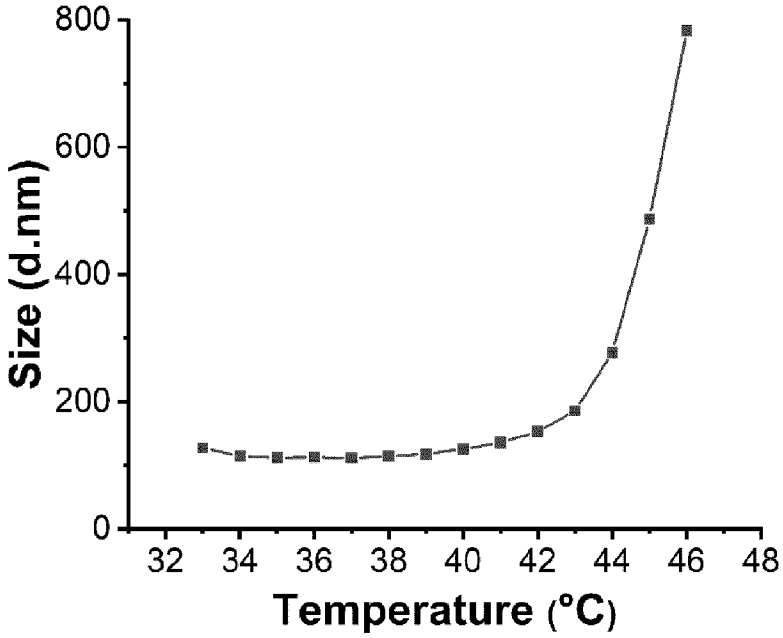
800
600
400
200
0
Size (d.nm)
32
34
36
38
40
42
44
46
48
Temperature (°C)

Fig. 5

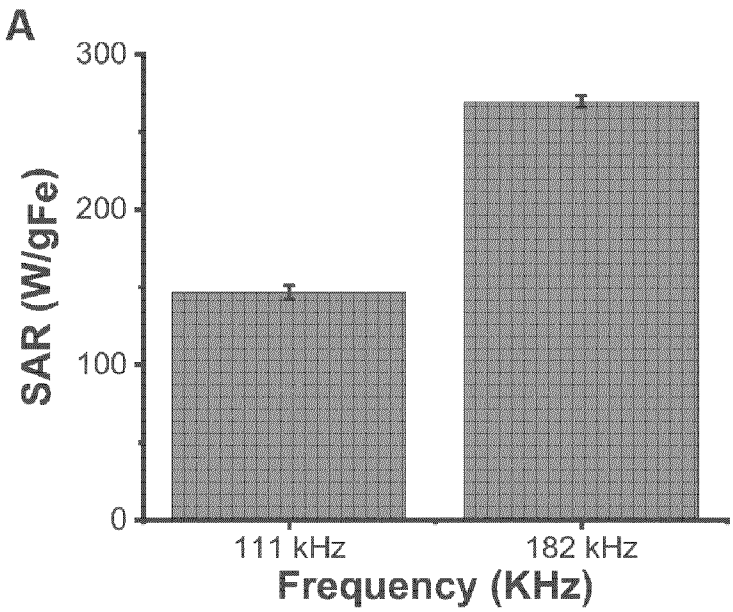
A
300
200
100
0
SAR (W/gFe)
111 kHz
182 kHz
Frequency (KHz)

Fig. 6

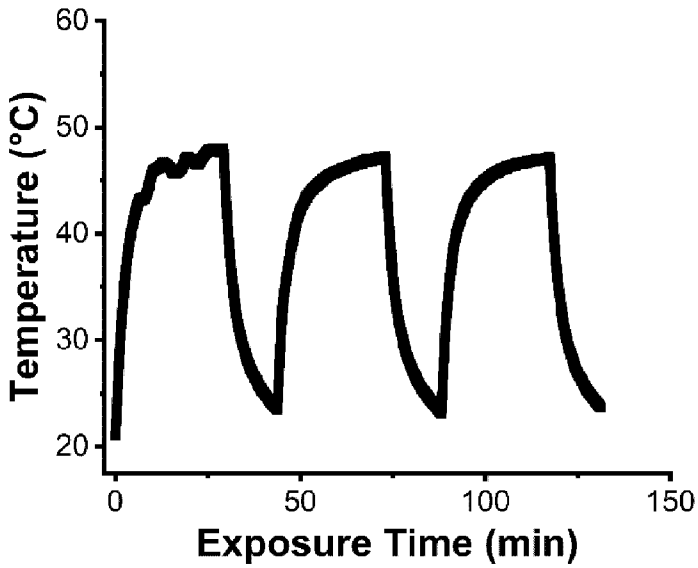
Temperature (°C)
60
50
40
30
20
0
50
100
150
Exposure Time (min)

Fig. 7

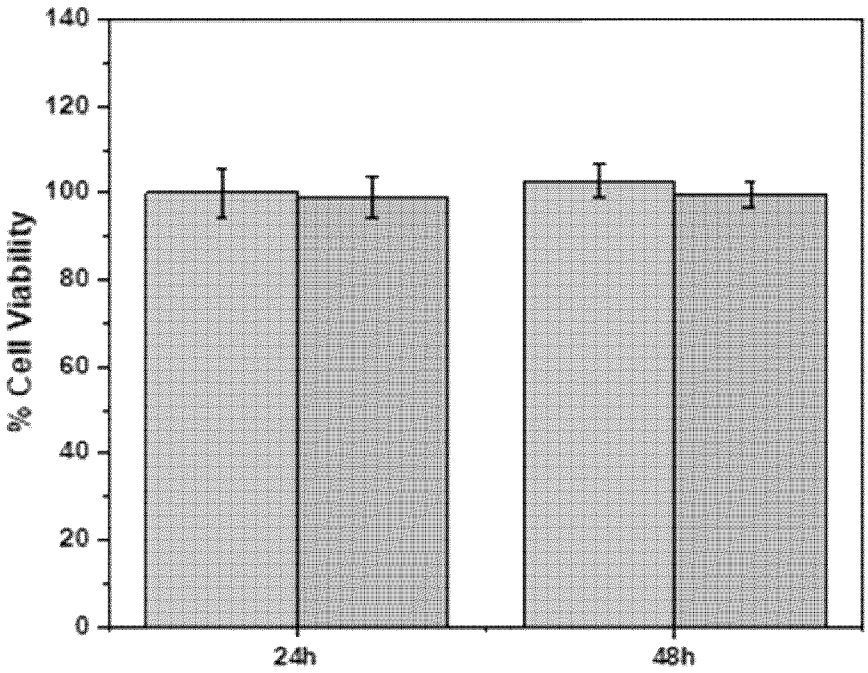
% Cell Viability
140
120
100
80
60
40
20
0
24h
48h

Fig. 8

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/066274

A. CLASSIFICATION OF SUBJECT MATTER
INV. H01F1/00 A61K49/18 A61K41/00
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)
H01F B82Y A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/138677 A1 (FONDAZIONE ST ITALIANO TECNOLOGIA [IT]; UNIV DEGLI STUDI GENOVA [IT]) 2 August 2018 (2018-08-02) cited in the application page 5, line 26 - page 6, line 1 page 6, lines 3-6; claims 1,3,12; examples 1,3 page 6, lines 13-16 page 7, lines 21-23 page 8, lines 4-16	1-8
A	US 2018/339914 A1 (CURRY MICHAEL L [US] ET AL) 29 November 2018 (2018-11-29) cited in the application paragraphs [0010], [0040], [0049]; figure 1	1-8

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 2024	27/09/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Primus, Jean-Louis

3

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/066274

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2020/222133 A1 (FONDAZIONE ST ITALIANO TECNOLOGIA [IT]) 5 November 2020 (2020-11-05) cited in the application example 9	1-8
A	WO 2011/147926 A2 (REIMHULT ERIK [AT]; AMSTAD ESTHER [CH]; TEXTOR MARCUS [CH]) 1 December 2011 (2011-12-01) cited in the application example 6	1-8

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/066274

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018138677 A1	02-08-2018	EP 3573662 A1	04-12-2019
		WO 2018138677 A1	02-08-2018
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		US 2021261431 A1	26-08-2021
WO 2020222133 A1	05-11-2020	EP 3962863 A1	09-03-2022
		US 2022227639 A1	21-07-2022
		WO 2020222133 A1	05-11-2020
WO 2011147926 A2	01-12-2011	EP 2575896 A2	10-04-2013
		WO 2011147926 A2	01-12-2011