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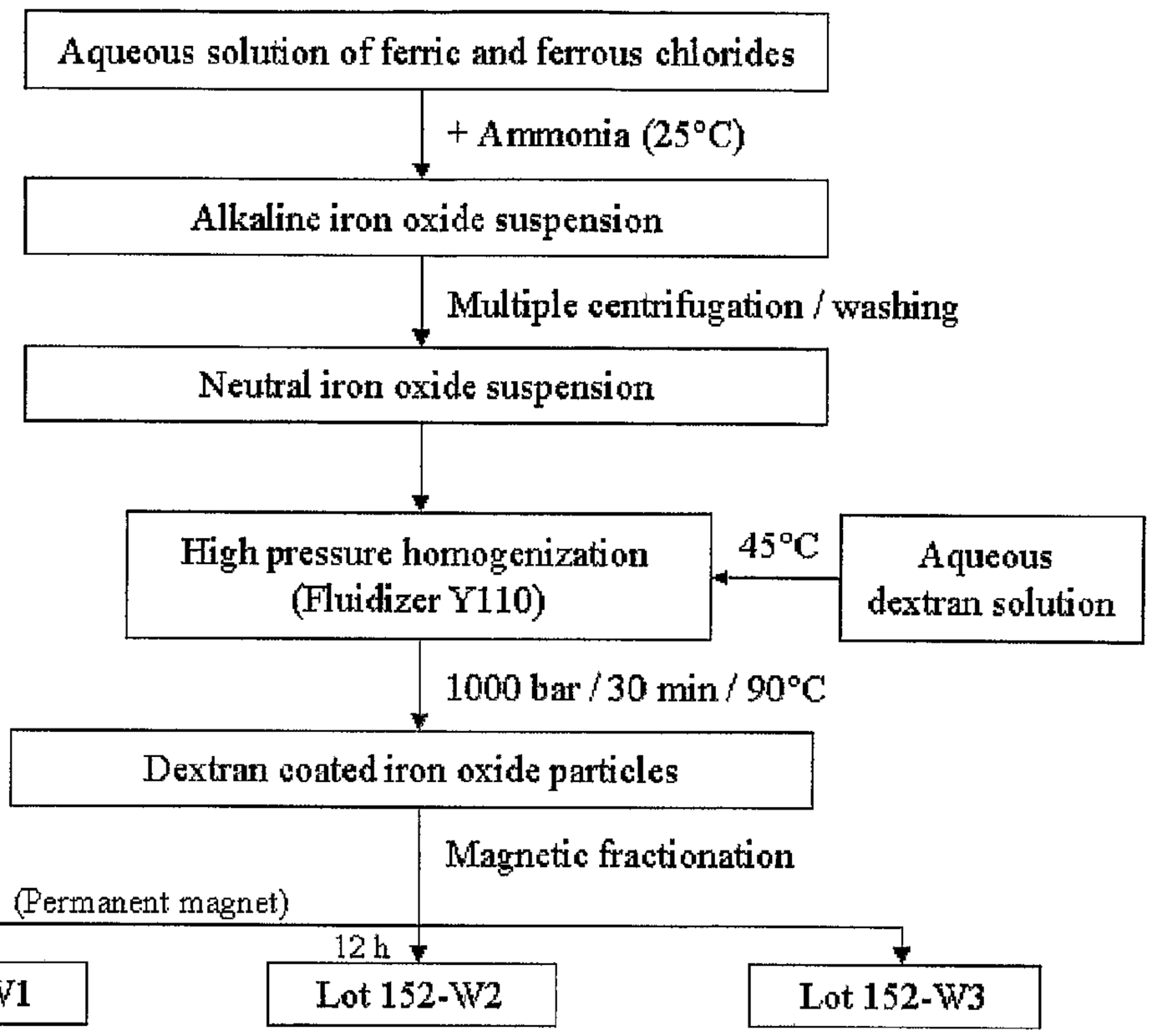
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(54) Titre : COMPOSITIONS DE NANOPARTICULES MAGNETIQUES ET PROCEDES RELATIFS A CES COMPOSITIONS

(54) Title: MAGNETIC NANOPARTICLE COMPOSITIONS, AND METHODS RELATED THERETO



(57) Abrégé/Abstract:
Disclosed are biocompatible magnetic nanoparticle compositions for various therapeutic or biological applications, and methods related thereto. Specifically, the present invention pertains to magnetic nanoparticle compositions prepared via high-pressure homogenization processes that include a turbulent flow zone. The methods of production may involve a two-step or a one-step process. The disclosed magnetic nanoparticle compositions may be useful in the treatment of the body, body part, tissue, cell, or body fluid of a subject for a variety of indications. The disclosed magnetic nanoparticle compositions may also be useful in the fixation, separation, transportation, marking or coding of targets, or energy transformation processes.

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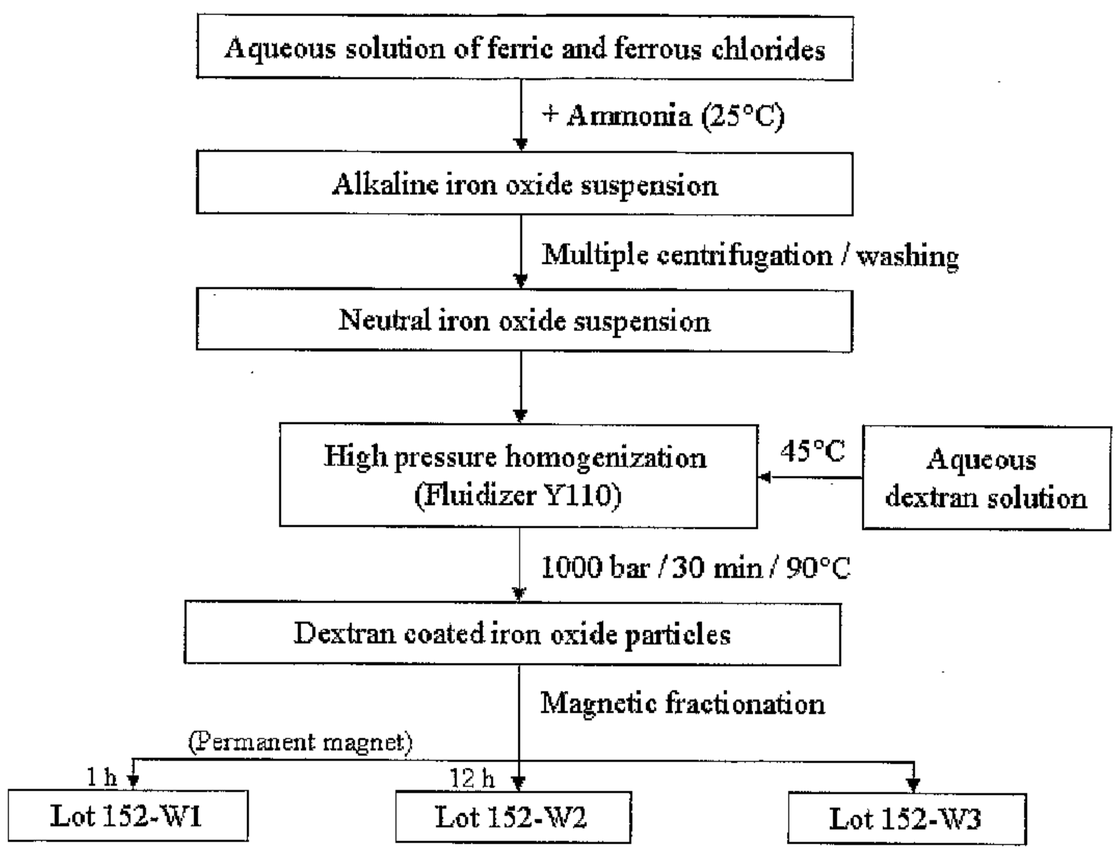
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(54) Title: MAGNETIC NANOPARTICLE COMPOSITIONS, AND METHODS RELATED THERETO



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MAGNETIC NANOPARTICLE COMPOSITIONS, AND METHODS RELATED THERETO

CROSS REFERENCE TO RELATED APPLICATION

[0001] This is a continuation-in-part application claiming the benefit of and priority to U.S. patent application serial number 10/360,561 filed on February 6, 2003, and German patent application number 103 33 631 filed on July 10, 2003, which are incorporated by reference.

TECHNICAL FIELD

[0002] The present invention relates generally to biocompatible magnetic nanoparticle compositions for various biological and therapeutic applications, and methods related thereto. Specifically, the present invention pertains to magnetic nanoparticle compositions prepared via high-pressure homogenization processes that include a turbulent flow zone.

BACKGROUND

[0003] A number of commercial applications for magnetic nanoparticles exist today. Examples include the isolation, fixation and cleaning of cells, parts of cells, nucleic acids, enzymes, antibodies, proteins or peptides; phagocyte diagnostics in cell biology; parts of diagnostic assays or therapeutic drug forms in clinical chemistry; contrast media, radionuclide or drug carrier in clinical diagnostics; and in bio- and technical chemistry as solid phase for the analysis of molecular recognition phenomena and hetero catalytic processes.

[0004] Various polymer coated metal oxide particles for biological applications have been disclosed since the mid-1980's. Examples include magnetizable nanoparticles below 200 nanometers (nm) that open new possibilities for transport and separation of cells, cell parts, bioactive molecules and radionuclides (*e.g.*, US 2003/0099954, Miltenyi; WO 01/17662 Zborowski; WO 02/43708, Alexiou); markers for contrast enhanced nuclear

magnetic imaging or diagnostic methods (*e.g.*, US 2003/0092029A1, Josephson; WO 01/74245, Johansson; and US 5,427,767, Kresse); and mechanical and thermal modifications of living cells (*e.g.*, DE 10020376A1, Koch; and US 6,541,039, Lesniak).

[0005] All such applications involve magnetizable metal oxide particles coated with biocompatible polymer layers and combined to form composite particles of 5 nm to 500 nm in diameter in a colloidal stable water-based suspension. The coating material is selected to eliminate any undesired interaction between the particles and the biological material, *i.e.* to make the particles biocompatible, so as to guarantee a sufficient tolerability of the coated particle with vital cells, influence the mechanism of metabolism of the particles in the living environment, or enable a selective binding at the cell surfaces, or enable the controlled release of enclosed substances. Due to their magnetic properties, the particles may align with applied magnetic fields and react to changes of said fields.

[0006] Various methods to produce these metal oxide, particularly as iron oxide, particles have been reported. Examples of these methods include sintering at high temperatures followed by mechanical grinding, clustering under vacuum conditions, and wet chemical synthesis from solution. The precipitation of iron oxide can be initiated under non-aqueous conditions (*e.g.*, US 4,677,027, Porath) and be continued under aqueous conditions (*e.g.*, US 5,160,725, Pilgrim), or it can be accomplished completely under aqueous conditions (*e.g.*, US 4,329,241, Massart). Due to toxicity concerns, an aqueous formulation is generally used for biological applications (*e.g.*, US 4,101,435, Hasegawa). Wet chemical synthesis can be preceded by a coating with polymer components (Core-shell-Method), or it can be performed in the presence of the polymer (One-pot-Method). For the core-shell-method, it is necessary to add stabilizing substances to the iron oxide, because these tend to aggregate in aqueous solutions, thereby limiting aggregation of the oxide particles. Amphiphilic substances (*e.g.*, WO 01/56546, Babincova) or additional nanoparticles with electrically

charged surfaces can be selected as stabilizers (*e.g.*, US 4,280,918, Homola). Surface-active substances used as stabilizers may influence and limit the functionality of said surface. Iron-containing magnetizable nanoparticle composites produced by the One-pot method are accepted for medical applications due to their physical and chemical properties as well as to their pharmacological stability. The One-pot method involves the coating of the polymer directly during the formation of the iron oxide to stabilize the formation process and the growth of the crystals from the solution. The most commonly used coating material is dextran in its various forms. Also used are other biocompatible carbohydrates, such as arabinogalactan, starch, glycosaminoglycan and proteins (*e.g.*, US 6,576,221, Kresse). One such method is the precipitation of Fe(II)-and Fe(III)-salts in the presence of dextran (*e.g.*, US 4,452,773, Molday). A modified method involves the use of ultrasound treatment followed by thermal treatment in the same apparatus (*e.g.*, US 4,827,945, Groman). The quality of this method is enhanced by magnetic classification (*e.g.*, WO 9007380, Miltenyi). Further encapsulation/coating may improve the biocompatibility of the nanoparticles due to the use of amphiphilic substances for stabilization. (*e.g.*, US 5,545,395 Tournier; and EP 0272091, Eley).

[0007] Suspensions of nanoparticles produced using the techniques described hereinabove typically contain particles with varying characteristics such as size. The non-homogeneous nature of the particle suspension degrades the performance of the suspension for many biological applications. Methods to improve the homogeneity of the particles are utilized for the production of dispersed aqueous systems that are intended for use as injectable fluids. Such homogenization methods include the rotor-stator- and high-pressure-methods. The use of liquid-jet- or liquid-slot-nozzle-high-pressure homogenization machines (*e.g.*, available from Microfluidics, a division of MFIC, Corp., Newton, MA) enables high

mechanical energy deposition, which is especially useful for the production of liposomes (*e.g.*, US 5,635,206, Ganter) or injectable active substances (*e.g.*, US 5,595,687, Raynolds).

[0008] High-pressure homogenization machines for the production of metal oxide nanoparticle compositions using controlled coalescence followed by drying in emulsion, in which the non-aqueous component contains an oxide as sol, have been reported in conjunction with industrial production of catalytic materials (*e.g.*, US 5,304,364, Costa) as well as electrographic pigment particles, ceramic powders, felt materials, spray layers, active substances carriers, and ion exchange resins (*e.g.*, US 5,580,692, Lofftus). An emulsion is the dispersion of multi-phase systems of two or more insoluble liquids. Emulsions consist of at least one continuous (outer) phase (*e.g.* water) and one isolated (dispersed or inner) phase (*e.g.*, oil). Emulsions are thermodynamically unstable. High-pressure homogenization (HPH) is often used for the preparation or stabilization of emulsions and suspensions in pharmaceutical, cosmetic, chemical and food industries. It is also known that nano-scale metal oxides can be prepared using high shear forces in a fluidizing apparatus but it concerns only plain oxides, *i.e.*, those without any coating (*e.g.*, US 5,417,956, Moser). For some applications, pressures up to 200 Mega Pa (MPa) or higher are used.

SUMMARY OF THE INVENTION

[0009] Various methods for producing magnetic nanoparticle compositions exist. However, a major problem has been the inability to produce biocompatible magnetic nanoparticle compositions having enhanced homogeneity, especially those having high metallic content and high magnetic mobility.

[0010] In view of the above, there is a need for biocompatible magnetic nanoparticle compositions that have enhanced homogeneity and comprise high metallic content nanoparticles possessing high magnetic mobility. There is also a need for methods for producing such compositions.

[0011] It is, therefore, an aspect of the present invention to provide biocompatible magnetic nanoparticle compositions having enhanced homogeneity.

[0012] It is also an aspect of the present invention to provide magnetic nanoparticle compositions comprising high metallic content nanoparticles that possess high magnetic mobility.

[0013] It is another aspect of the present invention to provide methods for producing magnetic nanoparticle compositions, particularly with the use of high-pressure homogenization processes that include a turbulent flow zone.

[0014] It is another aspect of the present invention to provide applications for biocompatible magnetic nanoparticle compositions comprising high metallic content nanoparticles that possess high magnetic mobility.

[0015] The present invention pertains to biocompatible magnetic nanoparticle compositions that possess high metallic content and exhibit enhanced homogeneity and high magnetic mobility, which contributes to higher heating rates. Higher heating rates are desirable for various therapeutic applications. These compositions are preferably prepared via high-pressure homogenization processes, and are useful for various therapeutic and biological applications.

[0016] In one embodiment, the magnetic nanoparticle composition comprises one or more metal-containing magnetic nanoparticles and a suitable medium for suspending the nanoparticles. The nanoparticles comprise a suitable biocompatible coating material. The magnetic nanoparticles possess a low-field magnetization when an external magnetic field is applied to the magnetic nanoparticles. The magnetic material of the nanoparticles exhibits a low-field magnetization greater than that of the material from which it was derived. The nanoparticles have at least an average 50 mass percent of metal and an average hydrodynamic diameter of less than 200 nm.

[0017] In another embodiment, the magnetic nanoparticle composition is prepared via a two-step process wherein a preformed metal-containing magnetic material is processed through a turbulent flow zone in a first step, and the resulting improved magnetic material of the first step is utilized in combination with a biocompatible coating material to generate a magnetic nanoparticle composition, via a turbulent flow zone in a second step. In another embodiment, the magnetic nanoparticle composition is prepared via a two-step process wherein a metal-containing magnetic material is generated from a metal-containing solution, via a turbulent flow zone, in a first step, and the resulting magnetic material of the first step is utilized in combination with a biocompatible coating material to generate a magnetic nanoparticle composition, via a turbulent flow zone, in a second step. In another embodiment, the magnetic nanoparticle composition is prepared via a one-step process wherein a metal-containing magnetic material is generated from a metal-containing solution, and processed with a biocompatible coating material, via a turbulent flow zone, to form a magnetic nanoparticle composition. The resulting magnetic nanoparticles may be separated from the carrier medium by an external magnetic field, for example, via permanent magnets.

[0018] The biocompatible coating material may comprise a polymer, metal compound, transfection agent, or any combination thereof. The polymer may comprise a naturally occurring, synthetic, or semi-synthetic polymer. Preferably, the polymer is dextran or dextran that contains functional groups, *e.g.*, sulfoalkyl, aminoalkyl, epoxyalkyl and carboxyalkyl whereby the alkyl chain may be substituted with heteroatoms, *e.g.*, oxygen. The metal-containing magnetic material comprises a metal, metal oxide, metal oxide-hydrate, metal hydroxide, metallic alloy of two or more metals, or any combination thereof. The metal-containing magnetic material has ferro-, antiferro-, ferri-, antiferri- or superparamagnetic properties. Preferably, the metal-containing magnetic material comprises an oxide of iron, more preferably, the iron oxide is magnetite, hematite, maghemite, or any

combination thereof. The magnetic nanoparticle composition may also comprise sub-structures that may comprise ligands or chelators, or that may comprise one or more bioactive substances.

[0019] The present invention also pertains to applications for biocompatible magnetic nanoparticle compositions, namely therapeutic and biological applications. The therapeutic applications pertain to the treatment of the body, body part, tissue, cell, or body fluid of a subject for a variety of indications, including but not limited to, cancer of any type, such as bone marrow, lung, vascular, neuro, colon, ovarian, breast and prostate cancer, epitheleoid sarcomas, adverse angiogenesis, restenosis, amyloidosis, tuberculosis, multiple sclerosis, cardiovascular plaque, vascular plaque, obesity, malaria, and illnesses due to viruses, such as HIV and AIDS. The therapeutic compositions may be administered via injection, topical application, transdermal application, oral ingestion, rectal insertion, inhalation through the mouth or nose, or any combination thereof. The biological applications include fixation, separation, transportation, marking or coding of targets, or energy transformation processes. The magnetic nanoparticle compositions may also be used in the separation, purification, or any combination thereof of nucleic acids, nucleic acid derivatives, nucleic acid fragments, proteins, protein derivatives, protein fragments, or any combination thereof.

[0020] The above summary of the present invention is not intended to describe each illustrated embodiment or every implementation of the present invention. The figures and the detailed description that follow particularly exemplify these embodiments.

Brief Description of the Drawings

[0021] The invention may be more completely understood in consideration of the following detailed description of various embodiments of the invention in connection with the accompanying drawings, in which:

[0022] Figure 1 schematically illustrates a bioprobe configuration, according to an embodiment of the present invention;

[0023] Figure 2 graphically illustrates size plots of various samples, according to an embodiment of the present invention;

[0024] Figure 2a: Size Plot of Lot-No. 146-T (iron oxide, example 1);

[0025] Figure 2b: Size Plot of Lot-No. 152-W1, of dextran-coated iron oxide, first fraction (example 2);

[0026] Figure 2c: Size Plot of Lot-No. 152-W2, of dextran-coated iron oxide, second fraction (example 2);

[0027] Figure 3 graphically illustrates the zetapotential results for iron oxide of Lot-No. 146-T (example 1) and dextran-coated iron oxide of Lot-No. 152-W (example 2), according to an embodiment of the present invention;

[0028] Figure 4 illustrates a process flow chart for samples 152-W1, 152-W2 and 152-W3 (examples 1 and 2), according to an embodiment of the present invention;

[0029] Figure 5 graphically illustrates zetapotential-pH function for nanoparticles of type 152-W with different PEG-COOH densities of Lot-No. 202-G1 – 202-G4 (example 3), according to an embodiment of the present invention; and

[0030] Figure 6 graphically illustrates isoelectric point (IEP) of nanoparticles of type 152-W with different PEG-COOH densities, according to an embodiment of the present invention.

[0031] While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described. On the contrary, the intention is to cover

all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

[0032] The present invention pertains to biocompatible magnetic nanoparticle compositions that possess high metallic content and exhibit enhanced homogeneity and high magnetic mobility, which contributes to higher heating rates. Higher heating rates are desirable for various therapeutic applications. These compositions are preferably prepared via high-pressure homogenization processes, and are useful for various therapeutic and biological applications.

1. Definitions

[0033] The terms "targeted therapy system", "targeted nanotherapeutics", and "targeted therapy", as used herein, refer to the methods and devices that involve the targeted delivery of bioprobe compositions for the treatment of an indication, including those disclosed in U.S. Patent Applications US2003/0032995, US2003/0028071, 10/360,578, and 10/360561.

[0034] The term "hyperthermia", as used herein, refers to heating of tissue to temperatures between 40°C and 46°C.

[0035] The term "susceptor", as used herein, refers to a magnetic energy susceptive particle that, when exposed to an energy source, either heats or physically moves.

[0036] The term "magnetic nanoparticle composition", as used herein, refers to a composition comprising a susceptor that comprises a biocompatible coating and a suitable medium.

[0037] The term "ligand", as used herein, refers to a molecule or compound that attaches to a susceptor (or a coating on the susceptor) and targets and attaches to a biological

marker. A monoclonal antibody specific for Her-2 (an epidermal growth factor receptor protein) is an exemplary ligand.

[0038] The term "marker", as used herein, refers to an antigen or other substance to which the ligand is specific. Her-2 protein is an exemplary marker.

[0039] The term "target", as used herein, refers to the matter for which deactivation, rupture, disruption or destruction is desired, such as a diseased cell, a pathogen, or other undesirable matter. A marker may be attached to the target. Breast cancer cells are exemplary targets.

[0040] The term "bioprobe", as used herein, refers to a susceptor comprising a biocompatible coating and at least one sub-structure. The term "bioprobe compositions", as used herein, refers to a composition comprising a magnetic nanoparticle composition and at least one l sub-structure. The sub-structure, preferably a ligand, acts to guide the bioprobe to a target. The term "bioprobe system", as used herein, refers to a bioprobe specific to a target that is optionally identified via a marker.

[0041] The term "linker molecule", as used herein, refers to an agent that targets particular functional groups on a ligand and on a susceptor or a coating, and thus forms a covalent link between the ligand and the susceptor or the coating.

[0042] The term "indication", as used herein, refers to a medical condition, such as a disease. Breast cancer is an exemplary indication.

[0043] The term "energy source ", as used herein, refers to a device that is capable of delivering AMF energy to the bioprobe's susceptor.

[0044] The term "AMF" (an abbreviation for alternating magnetic field), as used herein, refers to a magnetic field that changes the direction of its field vector periodically, for example in a manner that is sinusoidal, triangular, or rectangular. The AMF may also be added to a static magnetic field, such that only the AMF component of the resulting magnetic

field vector changes direction. It will be appreciated that an alternating magnetic field is accompanied by an alternating electric field and is electromagnetic in nature.

[0045] It is to be understood that the singular forms of "a", "an", and "the", as used herein and in the appended claims, include plural reference unless the context clearly dictates otherwise.

2. Nanoparticle Compositions

[0046] The present invention pertains to magnetic nanoparticle compositions and methods for producing these compositions.

2.1. Magnetic Nanoparticle Compositions

[0047] One aspect of the present invention relates to magnetic nanoparticle compositions that comprise one or more metal-containing magnetic nanoparticles and a suitable medium for suspending the nanoparticles. The magnetic nanoparticles comprise a susceptor that comprises a suitable biocompatible coating material. The magnetic nanoparticles possess a low-field magnetization when an external magnetic field is applied to the magnetic nanoparticles. The magnetic material of the nanoparticles exhibits a low-field magnetization greater than that of the material from which it was derived. The magnetic nanoparticle compositions and a sub-structure, preferably a ligand, form the basis for the bioprobe compositions useful in the treatment of the body, body part, tissue, cell, or body fluid of a subject.

2.2. High Pressure Homogenization ("HPH") Process for Producing Magnetic Nanoparticle Compositions

[0048] Another aspect of the present invention relates to methods for producing biocompatible magnetic nanoparticles that may be used in the treatment of various indications and in various biological applications. These methods for synthesizing the nanoparticles utilize the "core-shell method" or the "one-pot method". The core-shell

methods disclosed herein employ a two-step process; in the first step, 1) for example, a magnetic metal oxide is formed (core) from either a magnetic preformed material or a suitable metal-containing precursor material, which is then 2) coated with a biocompatible polymer - the coating (shell).

[0049] The two-step process may be accomplished by first processing a preformed metal-containing magnetic material to yield a metal-containing magnetic material having improved magnetic properties, and then generating a magnetic nanoparticle composition using the material resulting from the first step and a biocompatible coating material wherein both steps are processed via a turbulent flow zone. The two-step process may also be accomplished by first generating a metal-containing magnetic material from a metal-containing solution, and then generating a magnetic nanoparticle composition using the material resulting from the first step and a biocompatible coating material, wherein both steps are processed via a turbulent flow zone.

[0050] The one-pot method disclosed herein employs a one-step process, in which a metal-containing magnetic material is generated from a metal-containing solution, and it is processed with a biocompatible coating material, via a turbulent flow zone, to yield a magnetic nanoparticle composition.

[0051] The preformed metal-containing magnetic material for use herein comprises a metal, metal oxide, metal oxide-hydrate, metal hydroxide, metal alkoxide, metallic alloy with another metal, or any combination thereof. The magnetic preformed material has ferro-, antiferro-, ferri-, antiferri-, or superparamagnetic properties. Preferably, the magnetic preformed material comprises an oxide of iron, more preferably, the iron oxide is magnetite, hematite, maghemite, or any combination thereof. The iron oxide may be doped with bi- or tri-valent metal ions. Preferably, the magnetic preformed material comprises a combination of Fe-(II) and Fe-(III), more preferably Fe-(II) and Fe-(III) in a molar ratio of 1:1 to 1:2.

[0052] The metal-containing solution suitable for use herein can be prepared from one or a combination of two or more different metals, different metal compounds, or different valences of a metal, alone or in combination. Preferably, the solution comprises a combination of Fe-(II) and Fe-(III) salts, more preferably Fe-(II) and Fe-(III) salts in a molar ratio of 1:1 to 1:2.

[0053] To produce the core from a metal-containing solution, for example, an iron oxide suspension is produced by precipitation of ferric and ferrous chlorides in the presence of ammonia. After precipitation, the particles are separated from the liquid and re-suspended. In the second step, these suspended iron oxide precipitates, or cores, are coated with a biocompatible polymer under high-pressure homogenization conditions. Preferably, homogenization occurs above 100 bar, more preferably above 1000 bar. High-pressure homogenization takes advantage of high shear forces that are generated when a large quantity of fluid is forced through a nozzle with a high pressure to produce a polymer-coated particle. The fluid that is forced through the nozzle contains the precipitated particles, to which a liquid containing dissolved biocompatible coating material, preferably a polymer, is added. The resulting mixture is then further processed in the homogenizer by repeated cycling through the nozzle. The resulting magnetic nanoparticles may be separated from the carrier medium by an external magnetic field, for example, via permanent magnets.

[0054] Preferably, the resulting magnetic nanoparticles form a stable aqueous colloid or a stable colloid in physiological solution.

[0055] The first and the second steps are preferably processed in a liquid carrier medium at a temperature in the range from about 40°C and the boiling point of the medium, more preferably in the range from about 75°C to about 95°C. Preferably, the liquid medium comprises water or an aqueous alkaline solution having an ammonia, sodium or potassium

hydroxide basis. Preferably, the components are processed at a flow rate in the range from about 20 ml/min to about 200 ml/min through a turbulent flow zone in each step.

[0056] The biocompatible coating material suitable for use herein comprises a polymer, metal compound, or any combination thereof. The polymeric coating materials suitable for use herein may be naturally occurring, synthetic, semi-synthetic, or any combination thereof. The synthetic polymer may comprise polyamine, polyimine, polyvinyl, polyol, polyether, polycarboxylic acid, polysilicic acid, polyacrylate, polysiloxane, polyalkylene glycol, parylene, polylactic acid, polyglycolic acid, or any derivative thereof, or any combination thereof.

[0057] In one embodiment, the polymer comprises a functional group, preferably, a reactive or an ionic group, or any combination thereof. In another embodiment, the polymer comprises a homo-polymer, a co-polymer, or a polymer-blend. In another embodiment, the polymer comprises a biological material, such as polysaccharides, preferably dextran, polyamino acids, proteins, lipids, fatty acids, heparin, heparin sulfate, chondroitin sulfate, chitin, chitosan, alginate, glycosaminoglycan, cellulose, starch, any derivative thereof, and any combination thereof. Suitable proteins include an extracellular matrix protein, proteoglycan, glycoprotein, albumin, peptide, and gelatin. In another embodiment, the polymer comprises a hydrogel polymer, a histidine-containing polymer, or a combination of a hydrogel polymer and a histidine-containing polymer. Preferably, the polymer for use herein is dextran or dextran that contains functional groups. The preferred functional groups are hydroxyl, oxyalkyl, and carboxyl functional groups.

[0058] The metal compounds (of the biocompatible coating material) suitable for use herein include hydroxyapatite, metal carboxylate, sulfonate, phosphate, ferrite, phosphonate, and oxides of Group IV elements of the Periodic Table of Elements. These materials may form a composite coating with the biological or synthetic polymers. Where the magnetic

particle is formed from a biocompatible magnetic material, the surface of the particle itself may operate as the biocompatible coating.

[0059] The nanoparticles of the resulting magnetic nanoparticle composition may also comprise sub-structures that may comprise ligands, chelators or a combination thereof, or a bioactive substance. The sub-structures may be bound covalently or by physical interaction to uncoated portion of susceptor **142,** to coating **144,** or to an uncoated portion of susceptor **142** and partially covered by coating **144**, or they may be intercalated with coating **144**. The ligands or chelators may comprise a peptide, protein, nucleic acid, enzyme, antibody, antibody fragment, or any combination thereof. The ligands suitable for use herein are presented in Table I. The preferred antibody for use herein is ING-1. The bioactive substance may comprise a pharmaceutical agent, including chemotherapeutic and hormone, peptide, lipid, biochemical factor, or any combination thereof.

[0060] The resulting magnetic nanoparticles may be subjected to an external magnetic field. In such a case, the initial susceptibility (the ratio of the nanoparticle magnetic moment to applied magnetic field near zero field, especially in the range between – 20 and +20 Oersted) of the resulting magnetic nanoparticles is larger than that of the magnetic material from which it was derived.

[0061] The resulting magnetic nanoparticles have at least an average 50 mass percent of metal and an average hydrodynamic diameter of less than 200 nm.

3. Applications

[0062] Another aspect of the present invention relates to applications for the magnetic nanoparticle compositions, which include targeted therapies and biological applications. The magnetic nanoparticles of the compositions of the present invention possess high metallic content and exhibit enhanced homogeneity and high magnetic mobility, which contribute to higher heating rates. Higher heating rates are desirable for various therapeutic applications.

3.1. Targeted Nanotherapeutics

[0063] The bioprobe compositions of the present invention are useful in the treatment of the body, body part, tissue, cell, or body fluid of a subject. The bioprobe compositions, as a component of targeted nanotherapeutic systems, may be used in the treatment of a variety of indications, including but not limited to, cancer of any type, such as bone marrow, lung, vascular, neuro, colon, ovarian, breast and prostate cancer, epitheleoid sarcomas, AIDS, adverse angiogenesis, restenosis, amyloidosis, tuberculosis, cardiovascular plaque, vascular plaque, obesity, malaria, and illnesses due to viruses, such as HIV.

[0064] The targeted therapy system, based on the principles of hyperthermia, comprises a bioprobe system in conjunction with an energy source to treat an indication. **Figure 1** discloses a bioprobe configuration according to an embodiment of the present invention. A bioprobe **190** comprises a magnetic energy susceptive particle **142**, also referred to as a susceptor, and at least one targeting ligand **140**, such as, but not limited to, an antibody, which may be located on an exterior portion of susceptor **142**. The susceptor **142** may comprise a biocompatible coating **144**. Coating material **144** may fully or partially coat susceptor **142**. Heat is generated in susceptor **142** when susceptor **142** is exposed to an energy source, such as AMF. Coating **144** may enhance the heating properties of bioprobe **190**, particularly if coating **144** has a high viscosity, for example, coating is a polymeric material. The coated susceptor is produced in accordance to the HPH processes and examples described herein. The targeting ligand **140** may be selected to seek out and attach to a target, such as a particular type of cell or disease matter. The targeting ligand **140** may be specific to a substance, such as an antigen, on the target, referred to as a marker.

[0065] The temperature to which susceptor **142** heats is dependent upon, *inter alia*, the magnetic properties of the material, characteristics of the magnetic field, and the cooling capacity of the target site. If bioprobe **190** contains a magnetite (Fe_3O_4) particle **142**, then a

diameter of susceptor **142** may be in the range of about 8 nm to about 80 nm, more specifically in the range of 10 nm and 40 nm. In this case, bioprobes **190** may be sufficiently small to evade the liver, and yet the magnetic particle **142** still retains a sufficient magnetic moment for heating in an applied AMF. Magnetite particles larger than about 8 nm generally tend to be ferrimagnetic and thus appropriate for disease treatment. If other elements, such as cobalt, are added to the magnetite, this size range can be smaller. This results directly from the fact that cobalt generally possesses a larger magnetic moment than magnetite, which contributes to the overall magnetic moment of cobalt-containing susceptor **142**. In general, the size of bioprobe **190** may be about 0.1 nm to about 200 nm, depending upon the disease indication and materials from which the bioprobe is composed.

[0066] The presence of coating **144** and the composition of the coating material may form an integral part of the energy loss, and thus the heat produced, by bioprobe **190**. In addition, coating **144** may serve additional purposes. Coating **144** may provide a biocompatible layer separating the magnetic material from proteins and enzymes as well as the immunologic defenses in a patient, thereby controlling the residence time of the nanoparticles in the blood or tissue fluids. This control of residence time allows one to select targeting ligands **140** that are best suited for a particular tissue type. In addition, coating **144** may serve to protect the patient from potentially toxic elements in susceptor **142**. The coating **144** may also serve to prevent particle aggregation, as bioprobes **190** may be suspended in a fluid. The coating **144** may preserve the integrity of contained metal oxide to prevent further oxidation or chemical change, and thus may preserve the structural and magnetic properties of the oxide. It may also be advantageous to coat bioprobe **190** with a biocompatible coating that is biodegradable or resorbable. In such an application, both the coating **144** and the susceptor **142** may be digested and absorbed by the body.

[0067] The coating **144** may also serve to facilitate transport of bioprobe **190** into a cell, a process known as transfection. Such coating materials, known as transfection agents, may include vectors, prions, polyaminoacids, cationic liposomes, amphiphiles, non-liposomal lipids, or any combination thereof. A suitable vector may be a plasmid, a virus, a phage, a viron, or a viral coat. The bioprobe coating may comprise a combination of transfection agents with organic and/or inorganic materials, such that the particular combination may be tailored for a particular type of a diseased cell and a specific location within a patient's body.

[0068] To ensure that bioprobe **190** selectively attaches to, or otherwise associates with, the target, an appropriate ligand **140** may be combined with bioprobe **190**. The association of a ligand or ligands with bioprobes **190** allows for targeting of cancer or disease markers on cells. It also allows for targeting biological matter in the patient The term ligand relates to compounds which may target molecules including, for example, proteins, peptides, glycans, antibodies, antibody fragments, saccharides, carbohydrates, cytokines, chemokines, lipids, nucleotides, lectins, receptors, steroids, neurotransmitters, imprinted polymers, Cluster Designation/Differentiation (CD) markers, and the like. Examples of protein ligands include cell surface proteins, membrane proteins, proteoglycans, glycoproteins, peptides, and the like. Example nucleotide ligands include complete nucleotides, complimentary nucleotides, and nucleotide fragments. Example lipid ligands include phospholipids, glycolipids, and the like. Ligand **140** may be covalently bonded to or physically interacted with susceptor **142** or coating **144**. Ligand **140** may be bound covalently or by physical interaction to an uncoated portion of susceptor **142**. Ligand **140** may be bound covalently or by physical interaction directly to an uncoated portion of susceptor **142** and partially covered by coating **144**. Ligand **140** may be bound covalently or by physical interaction to a coated portion of bioprobe **190**. Ligand **140** may be intercalated to the coated portion of bioprobe **190**. Covalent bonding may be achieved with a linker molecule. Examples of functional groups used in linking

reactions include amines, sulfhydryls, carbohydrates, carboxyls, hydroxyls, and the like. The linking agent may be a homobifunctional or heterobifunctional crosslinking reagent, such as carbodiimides, sulfo-N-hydroxy succinamide (NHS) ester linkers, and the like. The linking agent may also be an aldehyde crosslinking reagent, such as glutaraldehyde. The linking agent may be selected to link ligand **140** to susceptor **142** or coating **144** in a preferable orientation, specifically with the active region of the ligand **140** available for targeting. Physical interaction does not require that the linking molecule and ligand **140** be bound directly to susceptor **142** or to coating **144** by non-covalent means such as, for example, absorption, adsorption, or intercalation.

[0069] The targeting ligands, and their corresponding targets and markers for given indications are presented in Table I.

TABLE I. BIOPROBE SYSTEMS AND INDICATIONS

BIOPROBE SYSTEM			INDICATION
TARGET	**MARKER**	**LIGAND**	
Endothelial cells of growing blood vessels of metastatic cancer cells	Integrin vβ3	Ber EP4 antibody LM609 antibody Integrin antagonist	Metastatic breast cancer, metastatic colon carcinoma
Cancer cells	Unglycosylated DF3 antigen	Anti-DF3 antibody	Breast cancer
Cancer cells	Kallikreins	Anti-kallikrein antibody	Ovarian and prostate cancer
Cancer cells	ErbB2 (HER-2/neu)	Anti-ErbB2 antibody, and scFv (F5), IDM-1 (aka MDX-210) variants	Breast and ovarian cancers
Cancer cells	Prostate specific membrane antigen (PSMA)	MDX-070 and 7E11-C5.3 antibodies	Prostate cancer
MCF-7 breast cancer cells	43 Kd membrane associated glycoprotein	323/A3 antibody	Breast cancer
Receptor tyrosine kinases-- FLT1 FLK1	Vascular endothelial growth factor (VEGF) and VEGFB and placental growth factor receptors (PGFR)	Anti-FLT1 antibody Anti-FLK1 antibody, 2C3 antibody	Tumour angiogenesis Tumour angiogenesis
Metastatic cancer cells	CAR (coxsackie adenovirus cell-surface receptor)	Anti-CAR antibody	Metastatic prostate cancer
Vascular smooth muscle cells of	Urokinase type plasminogen	Urokinase type plasminogen activator	Cancer

BIOPROBE SYSTEM			INDICATION
TARGET	MARKER	LIGAND	
cancer cells	activator receptor (uPAR)	(uPA)	
Blood vessels of cancer cells	Plasminogen activator inhibitor 1(PAI-1)	Anti-PAI-1 antibody	Breast cancer
Epithelial ovarian tumour cells	Matrix metaloproteinase 9 (MMP-9)	Anti-MMP-9 antibody	Ovarian carcinomas with lymph node metastasis.
Cancer cells	Cyclin A	Anti-cyclin A antibody	Squamous cell carcinoma of the tongue
Cancer cells	Cyclin D	Anti-cyclin D(1,2,3) antibody	Malignant breast cancer, head and neck squamous cell carcinomas, mantle cell carcinomas, laryngeal squamous cell carcinomas
Kidney cortex tissue	Cyclin E	Anti-cyclin E antibody	Human renal cell carcinoma
Tumorigenic human breast epithelial cells	Cyclin E	Anti-cyclin E antibody	Breast cancer
Malignant epithelial bladder tissue	Cyclin E	Anti-cyclin E antibody	Transitional cell carcinoma of the urinary bladder
Cancer cells	Cdc 2	Anti-cdc 2 antibody	Breast cancer
Malignant epithelial bladder tissue	P27	Anti-phospho p27 antibody	Transitional cell carcinoma of the urinary bladder
Cancer cells	P73	Anti-p73 antibody	Lung carcinogenesis, bladder carcinogenesis, neuroblastoma, breast cancer
Cancer cells	Ras	Anti-ras antibody	Breast cancer
Cancer cells	c-myc	Anti C-myc antibody	Breast cancer
Cancer cells	c-fms	Anti-c-fms antibody	Breast cancer
Cancer cells	Hepatocyte growth factor receptor (HGFR)	Anti-HGFR antibody	Colorectal cancer
Cancer cells	c-met	Anti-c-met antibody	Gastric and colon cancers, hepatomas, ovarian cancer, skin cancer
Large granular lymphocyte (LGL) leukaemia cells	Apoptosis related factors: Fas FasL	Anti-CD95 (Fas) antibody	Leukaemia, prostate cancer
Cancer cells	Non-receptor protein tyrosine kinase V-Src and C-Src	Anti c-src-polyclonal antibody	Metastatic colorectal cancer, and late stage breast cancer
Cancer cell	CAR (coxsackie adenovirus cell-surface receptor)	Onyx-015 adenovirus	Lung, ovarian, other cancers
Cancer cell	Epidermal growth factor receptor (EGFR)	Molecule 225 antibody	Cancer
Cancer cells	D6 antigen	Anti-D6 antibody	Vascular tumours including Kaposi's sarcoma
Cancer cells	2C4 antigen	Anti-2C4 antibody	Breast, prostate, other cancers
Cancer cells	Cytokeratin epithelial marker and/or telomerase reverse transcriptase	S5A10-2 antibody	Non-small cell lung cancer
Cancer cells	Carcinoembryonic antigen (CEA)	MFE-23 scFv of anti-CEA antibody	Colorectal cancer
Cancer cells	Proliferating cell nuclear antigen	Anti-PCNA antibody	Breast cancer

BIOPROBE SYSTEM			INDICATION
TARGET	MARKER	LIGAND	
	(PCNA)		
Cancer cells	Neu 3, a membrane associated sialidase	Anti-neu 3 sialidase antibody	Colon cancer
Cancer cells	P13KC2 beta (cancer cell signal mediator)	Anti-P13KC2beta antibody	Lung cancer
Cancer cells	Guanylyl cyclase-C (GC-C) receptor	Anti-GC-C antibody	Esophageal or gastric cancer
Cancer cells	Transforming growth factor beta (TGFB) receptor	Anti-TGFB antibody	Breast cancer
Cancer cells	Platelet derived growth factor receptor (PDGFR) PDGFR-A (alpha) PDGFR-B (beta)	Anti-PDGF-A antibody Anti-PDGF-B antibody	Lung cancer Bone cancer
Cancer cells and blood vessels	Vascular endothelial growth factors VEGFR Angiopoietin	Tie1 Tie2	Cancer Cancer
Cancer cells	Mucin family of receptors	Anti-MUC-1 antibody, 12E antibody 3D antibody A5 antibody	Colorectal and ovarian carcinomas
Cancer cells	TAG-72	B72.3 antibody	Breast and lung cancers
Cancer cells	Human milk fat globule receptor	NCL-HMFG1 and NCL-HMFG2 antibodies	Breast, lung, colon, and prostate cancers
Methionine synthase and L-methylmalonyl-CoA mutase	Cobalamin receptor	B12 (riboflavin, and variants) cobalamin and variants such as adenosylcobalamin transcobalamin	Breast, lung, colon, sarcomatous thyroid or central nervous system malignancies cancer
Cancer cells	Glioma chloride channel	Scorpion toxin—chlorotoxin and chlorotoxin-like molecules	Gliomas
Cancer cells	40 kD glycoprotein antigen	NR-LU-10 antibody	Small cell lung cancer
CNS cells and tissue	Brain-specific chondroitin sulphate proteoglycan Brain enriched hyaluronan binding protein (BEHAB-aka brevican	Anti-BEHAB antibody	Gliomas
Cancer cells	Catenins Alpha catenin Beta catenin Gamma catenin	Anti-alpha catenin antibody Anti-beta catenin antibody Anti-gamma catenin antibody	Colorectal carcinoma, non-small cell lung cancer Breast cancer Thyroid cancer
Cancer cells	Interleukin (IL) receptors IL13 receptor	IL13-PE38 antibody	Kidney, brain, breast, and head and neck cancers, and Kaposi's sarcoma
Cancer cells	Mesothelin receptor	Anti-mesothelin antibody, and	Mesotheliomas Ovarian cancer and mesotheliomas

BIOPROBE SYSTEM			INDICATION
TARGET	MARKER	LIGAND	
		SS1(dsFv) variant	
Cancer cells	CD44 surface adhesion molecule	Anti-CD44 antibody	Prostate cancer
Cancer cells	EGFRvIII	Ua30:2 antibody L8A4 antibody DH8.3 antibody 81C6 antibody	Brain, colorectal, pancreatic, billary, liver cancers and soft tissue sarcomas.
Receptor tyrosine kinases FLT1	Vascular endothelial growth factor (VEGF) and VEGFB	Anti-FLT1 antibody	Atherosclerotic plaques
Smooth muscle cells in the lumen of blood vessels	Basic fibroblast growth factor receptor (bFGFR)	Anit-bFGF antibody	Restenosis
Vulnerable plaque	Oxidized low density lipoprotein (OxLDL)	Oxidation-specific antibodies (Ox-AB) MDA-2 antibody	Atherosclerosis and vascular disease
Vulnerable plaque	Malondialdehyde-modified LDL (MDA-LDL)	IK17 antibody	Atherosclerosis and vascular disease
M. Tuberculosis bacilli	APA-antigen	Anti-APA antibody	Tuberculosis
Retrovirus infected cells	TGFA (alpha)	Anti-TGFA antibody	HIV
Leukocytes	Alpha4 subunit of alpha4beta1-integrin (VLA-4) and alpha4beta7-integrin	Antegren	Multiple sclerosis
Receptor tyrosine kinases FLT1	Vascular endothelial growth factor (VEGF) and VEGFB	Anti-FLT1 antibody	Autoimmune joint destruction (arthritis, lupus, etc)
Plasmodium falciparum	Apical membrane antigen-1 (AMA-1)	Anti-AMA-1 antibody	Malaria
Cells of the immune system	CD30	AC10, HeFi1, and derivatives of AC10 and HeFi1	Immunological disorders other than cancer
Hepatitis C virus	Hepatitis C virus core protein	19D9D6 Monoclonal Antibody	Hepatitis C infection
Tumor vascular cells	Vascular endothelial growth factor (VEGF)	MV833 and HuMV833 antibodies	Cancer
Tumor cells	Cytokeratin	Anti-cytokeratin AE1/3 and anti-CAM5.2 antibodies	Epitheleoid sarcomas
Tumor cells	Thomsen Friedenreich (TF) antigen	M170, chimeric M170, MaB 170H.82R1808	Breast, Prostate, Ovarian, and Lung cancers
Tumor cells	CEA	HumaSpect™, Votumumab, Mab 88BV59	Colon and Ovarian cancers
Tumor cells	EFG-r	ABX-EGF	Colon, NSCLC, Prostate, and Renal cancers
Tumor cells	EGF-r	HuMax-EGFr	Head, Neck, Breast, Colon, Prostate, Lung, and Ovarian cancers
Tumor cells	EGF-r	TheraCIM™, h-R3	Head and Neck cancers
Tumor cells	CEA	KSB309™	Oral cavity, and Pharngial cancers
Tumor cells	CEA	4B5-H	Melanoma

BIOPROBE SYSTEM			INDICATION
TARGET	MARKER	LIGAND	
Tumor cells	GD2 ganglioside	ABX-MA1	Melanoma, Neuroblastoma, NSCLC
Tumor cells	CTLA4; CD152	MDX-010	Melanoma
Tumor cells	GD2 ganglioside	TriGem, Mab-1A7	Melanoma
Tumor cells	CA125; MUC-16	ACA-125	Ovarian cancer
Tumor cells	Polymorphic epithelial mucin	R1549, Pemtumomab, MuHMFg1, HuHMFg1	Ovarian, Stomach, Breast, Lung, and Prostate cancers
Tumor cells	CA125	OvaRex™, Mab-B42.13, Ov	Ovarian cancer
Tumor cells		VB2-011, H-11 ScFv, Novo Mab-G2ScFv	Breast, Ovarian, and Colorectal cancers
Tumor cells	CEA	CEA-Cide, Labetuzumab	Breast, Colon, and Lung cancers
Tumor cells	VEGF	Avastin™, Bevacizumab, rhuMAb-VEGF	Breast, Colorectal, NSCLC, and Renal cancers
Tumor cells	LewisY Ag	SGN-15, cBR96	Breast, NSCLC, and Ovarian cancers
Tumor cells	HER2	OmniTag™, Pertuzumab, rhuMAb 2C4	Breast, Ovarian, Lung, and Prostate cancers
Tumor cells	MUC1	BrevaRex™, Mab AR20.5	Breast, Ovarian, and Multiple Myeloma cancer
Tumor cells	MUC1	Therex™, R1550, HuHMFG1	Breast, Ovarian, Pancreatic, and Gastric cancers
Tumor cells	Ep-CAM	ING-1	Breast, Lung, Prostate, and Pancreatic cancers
Tumor cells	αvβ3 integrin	Vitaxin™, huLM609	Solid tumors
Tumor cells	αvβ3 integrin	Mab-MEDI-522, huLM609	Advanced solid tumors

[0070] Antibodies can also be attached on the surface of magnetic nanoparticles, *e.g.*, by direct covalent binding of amino groups of the antibody molecule to carbodiimide activated carboxylic acid groups on the particle surface, by binding of biotinylated antibodies on streptavidin coated nanoparticles, and by binding of the antibodies on protein A or protein G coated nanoparticles. Nucleic acids can be immobilized on the surface of nanoparticles with covalently attached oligonucliotides.

[0071] In one embodiment, the targeting ligand is the ING-1 antibody. ING-1 (heMAb) is a high-affinity, human-engineered™ monoclonal antibody that recognizes a 40 kDa epithelial cell adhesion molecule (Ep-CAM - also known as 17-1A, EPG40, GA733-2, KSA and EGP). ING-1 was developed using human engineering, in which individual residues in the variable region of a murine antibody are replaced with the amino acid found in the

human framework, such that immunogenicity of the variable region is reduced. ING-1 is produced from Chinese hamster ovary (CHO) cells containing synthetic heavy and light chain genes encoding the modified variable regions linked to human IgG1 and kappa constant region cDNA, respectively.

[0072] *In vitro* studies have demonstrated that ING-1(heMAb) is active against a number of human tumor cell lines, and that ING-1(heMAb) has high affinity for adenocarcinomas of the breast, prostate, colon, NSC lung and pancreas.

[0073] The treatment of a patient with the bioprobe compositions of the present invention involves the administration of the composition to a subject, and administration of energy to the bioprobes in the composition, after a prescribed period of time for the bioprobe to locate and attach to a markered target, so as to destroy or inactivate the target or inhibit or destroy the vascularity of a tumor. The energy may be administered directly into a subject's body, body part, tissue, or body fluid (such as blood, blood plasma, blood serum, or bone marrow), or extracorporeally to a subject's body, organ or body fluid. The bioprobe composition may be administered via injection, topical application, transdermal application, oral ingestion, rectal insertion, inhalation through the mouth or nose, or any combination thereof. The targeted thermotherapy may be combined with at least one other treatment.

[0074] Exemplary energy forms useful herein include AMF, radio frequency, microwave, acoustic, or a combination thereof, and may be created using a variety of mechanisms. The preferred energy form is AMF.

3.2. Biological Applications

[0075] The magnetic nanoparticle compositions of the present invention are also useful for various biological applications, including, but not limited to, the fixation, separation, transportation, marking or coding of targets, or energy transformation processes. In one embodiment, the magnetic nanoparticles recognize, selectively fixate and controllably

release metal ions, via ligands and chelators. In another embodiment, the nanoparticles are used as radionuclide carriers, wherein the nanoparticles are doped with radioactive isotopes or the nanoparticles are processed to render them radioactive, *e.g.*, for use in radio-nuclide therapy. In another embodiment, the magnetic nanoparticles are used in the separation or purification of biomolecules, or any combination thereof. In such applications, the magnetic nanoparticle compositions may be used in the separation, purification, or any combination thereof of nucleic acids, nucleic acid fragments, nucleic acid derivatives, proteins, protein derivatives, protein fragments, or any combination thereof. In one embodiment, the magnetic nanoparticles bind to cells, and are used for the sorting and purification of cells. In another embodiment, the magnetic nanoparticles are used in analytical processes or diagnostic assays, for example, as contrast media.

Examples

[0076] Having generally described the invention, a more complete understanding thereof may be obtained by reference to the following examples that are provided for purposes of illustration only and do not limit the invention.

The examples described herein below utilize the following analysis techniques:

[0077] Photon correlation spectroscopy (PCS) is a technique used for measuring the random diffusion of particles to determine their size. PCS measurements lead to the determination of the average hydrodynamic diameter of the nanoparticles in suspension. The weighing of data analysis is carried out according to the signal intensity, the volume, and the number of the nanoparticles. There are three types of data analysis. The first, a monomodal analysis performs an analysis of only the cumulants. In the second, an analysis of the multimodal distribution is performed, resulting in 24 size classes on the X-axis. The Y scale of the plot yields the relative intensity of light scattered by particles in each size class. This is therefore known as an intensity plot. The third analysis method is known in the industry as

CONTIN mode. This method provides the distributions and detects traces of aggregates. Therefore, the CONTIN analysis has been selected as the standard analytical method herein for determination of the size distribution from PCS data.

[0078] The quality of the size distribution is characterized by the polydispersity index (PI). This value is calculated as the deviation between the measured autocorrelation function and the mathematically adapted correlation function. This value is below 0.2 for nanoparticles with a small size distribution, and between 0.2 and 0.5 for broad size distributions. PI values higher than 0.5 indicate that the measurement is not reliable and that the data cannot be analyzed.

[0079] The instrument utilized for conducting PCS is Zetasizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.), which is calibrated for the size range of 10 nm - 1500 nm.

[0080] Neutron Powder Diffractometry (NPD) is a method to validate the magnetic form and crystalline structure of the particles and is a diffraction technique that is analogous to X-ray powder diffraction, with notable differences. Because the incident radiation is composed of neutrons, which interact with the atomic nuclei of the sample, the result is a nuclear diffraction pattern that provides average distances between atomic nuclei within a crystal. Because the neutrons possess a magnetic moment, this technique yields information about the magnetic properties of materials not available from its electromagnetic counterparts.

[0081] Laser Doppler Velocimetry (LDV) is used for measuring the movement of particles in an electric field to determine the zetapotential of particles. Zetapotential measurement of iron oxide particles provides information about the charge load of the particles.

[0082] The instrument utilized for conducting zetapotential measurements is Zetasizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.), which is calibrated with the zetapotential standard DTS0050 (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.).

[0083] Examples 1 – 4 and 8 herein below provide a detailed description of the synthetic procedures for producing nanoparticles, methods used for characterizing the nanoparticles, methods for functionalizing the nanoparticles, and methods used for conjugating ChL6 and ING-1 antibodies (which are used only as an exemplary system) to produce the final product for *in vivo* use. The general approach for the synthesis of the nanoparticle compositions in these examples is the core-shell method, as follows:

[0084] First, the initial iron oxide suspension was produced from precipitation of ferric and ferrous chlorides in the presence of ammonia (example 1). Second, the iron oxide cores were coated with dextran under high-pressure homogenization conditions (example 2). Third, the nanoparticles were functionalized with carboxylic acid groups via a polyethylene glycol spacer (example 3). And, fourth, the ChL6 antibody (example 4) or ING-1 antibody (example 8) was covalently bound to the particle surface.

Example 1: Precipitation and analysis of iron oxide (Lot 146-T)

[0085] 54.1 g (0.2 mol) of $FeCl_3$ x 6 H_2O and 31.4 g (0.158 mol) of $FeCl_2$ x 4 H_2O were dissolved in 300 milliliter (ml) of de-ionized water. The solution of the iron salts was stirred with a mechanical stirrer at 500 revolutions per minute (rpm) in a 1 L two neck flask at room temperature. 220 ml of 25 % ammonium hydroxide was added to the solution with a peristaltic pump over a period of 30 minutes (min). After addition of the ammonium hydroxide, the stirring process was continued for 10 min. The iron oxide suspension was purified via centrifugation in two 500 ml centrifuge beakers at 1100 rpm for 10 min. After removal of the supernatant, the iron oxide pellet was re-suspended two times in 200 ml of de-ionized water. The re-suspension was centrifuged three more times for successive

neutralization of the iron oxide suspension. The iron oxide suspension was centrifuged again in two 500 ml beakers at 1100 rpm for 10 min. After removal of the supernatant, the iron oxide was re-suspended two times in 25 ml of de-ionized water and combined in one 500 ml beaker, followed by another centrifugation at 1100 rounds per minute (rpm) for 10 min.

[0086] The pellet was again re-suspended in 50 ml de-ionized water and centrifuged at 1100 rpm for 10 min. The supernatant was added to the product container. The process of re-suspension of the pellet in 50 ml of de-ionized water aliquot, centrifugation at 1100 rpm at 10 min, and addition of the obtained supernatants was repeated two more times to produce an overall yield of 200 ml iron oxide suspension. For final removal of larger aggregates, the iron oxide suspension was centrifuged at 700 rpm for 10 min. The supernatant was filtered through a glass-fiber filter (MN 85/90, Macherey-Nagel, Germany; size exclusion: greater than 600 nm). Finally, 200 ml iron oxide suspension of Lot 146-T, with a solids concentration of 45 milligrams per milliliter (mg/ml) was obtained (with a solid iron oxide yield of 9 g).

[0087] A representative plot of the size distribution obtained for Lot 146-T particles is shown in **Figure 2a.** The peak analysis by particle number is consistent with size determinations by transmission electron microscopy.

[0088] The PCS size measurement of the iron oxide suspension of Lot 146-T leads to a mean hydrodynamic diameter of 400 nm - 450 nm. The polydispersity index of 0.227 shows a monomodal size distribution. Thus, the individual iron oxide crystals have aggregated in suspension. This effect has implications for further processing, namely coating with dextran. Before the precipitates can be coated with dextran to yield the nominal nanoparticle that forms the bioprobe, the precipitated particles must be re-suspended and stabilized. Other characterization techniques must be employed to determine the nominal crystal size. For example, zetapotential measurement of iron oxide particles provides

information about the charge loaded to the particles. In order to make these particles biocompatible, it is essential that the charge of the particles is almost, and ideally, zero. The Zetasizer 3000 was combined with an autotitrator (available from Mettler-Toledo, Inc., Columbus, OH) to determine the zetapotential dependence on the solution pH. The nanoparticle suspension was first titrated with 0.1 M of sodium-hydroxide (NaOH) to increase the pH value to about 10. Then, the zetapotential - pH profile was measured during a titration with 0.1 M of hydrogen chloride (HCl) from pH = 9 to pH = 3.

[0089] The zetapotential - pH function of the iron oxide nanoparticles of Lot 146-T is shown in **Figure 3**. We observe a significant influence of the pH value on the zetapotential. In the basic to neutral pH range, the nanoparticles have strongly negative surface potentials in the range of -43 millivolt (mV) to -29 mV. After passing the isoelectric point at pH = 6.1, the nanoparticles show high positive surface potentials of +27 mV to +35 mV in the acidic pH range from 5 to 3.

[0090] The magnetic properties of Lot 146-T iron oxide nanoparticles were analyzed using a variety of standard magnetic techniques. Saturation magnetization (M_s), magnetic coercivity (H_c), and remanence ratio (ratio of magnetic remanence to saturation magnetization (M_r/M_s)) were measured. These magnetic measurements are indicators of the dominant magnetic properties of the particles, and can be used to determine the relative concentrations of magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3). These magnetic measurements and the determined magnetite content of the particles are presented in Table II.

TABLE II. PROPERTIES OF MAGNETIC NANOPARTICLES

Sample/ Lot ID	M_s (300 °K) (emu / mg-Fe)	H_c (Oe)	M_r/M_s	% Magnetite	T_b (Kelvin)	Category of magnetic properties
146-T	0.1085	< 5	0.0261	85.4	48	superparamagnetic

[0091] The standard value of saturation magnetization for pure magnetite is 0.1271 emu / mg-Fe, and for pure maghemite is 0.1087 electromagnetic unit (emu) / mg-Fe. The

remanence ratio provides a measure of magnetic domain character to the particles, on average, *i.e.*, single or multi-domain. The theoretical value for a magnetic nanoparticle that exhibits stable single-domain properties is $M_r/M_s = 0.5$. Superparamagnetic (SP) behavior is observed when the low-temperature remanence curves have a blocking temperature (T_b) distribution with "magnetic" particle sizes less than 30 nm.

[0092] Magnetite will display a Verwey transition near 100 °K on low-temperature remanence curves as the domains transition from blocked to unblocked behavior. On the other hand, maghemite has no Verwey transition. If magnetite particles show superparamagnetic behavior, then it is usually difficult to see the magnetic effects of the Verwey transition over the thermal unblocking effect of SP in this type of experiment. In this case, no Verwey transition was observed.

[0093] In preparation for Neutron Powder Diffractometry, the Lot 146-T nanoparticle suspensions were dried in a vacuum oven for several days. This is necessary to remove all traces of water, which, because of the scattering cross section of hydrogen, produces a high background to the data. For this study, measurements were conducted using the neutron powder diffractometer located at the Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD. The Si(531) monochromator was used because it provides the best resolution at high scattering angles, although longer data collection times are needed, often from 4 hour (h) to 24 h. This monochromator has a takeoff angle of 120^{o} and produces neutrons with wavelength 1.590 angstrom (Å). Diffraction intensities are approximately 30% of that obtained with the Cu(311) monochromator. With 7' collimation, perfectly Gaussian line shapes with widths full width at half maximum (FWHM) as small as 10' (delta d/d $= 8 \times 10^{-4}$, where d is the line width) are observed, but for most samples the intrinsic sample broadening exceeds the instrument resolution. Thus, use of

the Si(531) monochromator is restricted to those applications where highest resolution is clearly needed and where samples are available in sufficient quality and quantity.

[0094] Based on the measurements, we conclude that the mean crystal size of the nanoparticles is about 12 nm and that the sample consists mostly of magnetite, and that the mean magnetic moment of the octahedrally coordinated Fe atoms in the crystalline lattice is 4.4 Bohr magnetons, while that of the tetrahedrally coordinated atoms is 3.9 Bohr magnetons. This latter result is consistent with the conclusions arrived from the magnetic measurements, *i.e.*, that about 20% maghemite exists in the crystals.

[0095] The mean crystalline size obtained from NPD measurements also helps explain the lack of an observed Verwey transition in the magnetic properties, the low coercivity, and low remanence ratio. Together, these results demonstrate that the dominant SP behavior of the nanoparticles is due to their small size (<< 30 nm) and not to the composition consisting of a significant maghemite component.

Example 2: Synthesis and characterization of dextran-coated nanoparticles (Lot 152-W)

[0096] 21 g of dextran (molecular weight (MW) = 40.000 Daltons, available from Carl Roth, GmbH, Karlsruhe, Germany) was dissolved in 100 ml of de-ionized water. 75 ml of iron oxide suspension from Lot 146-T (concentration = 45 mg/ml) was homogenized in a high-pressure-homogenizer (Fluidizer Y110, Microfluidics, Inc., Newton, MA) at 500 bar for 7 min. This is a pressured, air driven plunger pump that drives the fluid through a 100 micrometer (μm) diamond gap in order to force a high shear stress. The pre-heated dextran solution (about 45 °C) was added to the suspension, warmed in the homogenizer to 90°C at 1000 bar and processed at 1000 bar for 30 min at a temperature between 87°C - 92°C. After cooling to room temperature, the nanoparticle suspension was transferred into a crystallizing dish (diameter: 12 centimeter (cm)) and placed on a permanent magnet plate for 1 hour. Then, the supernatant was transferred to a second crystallizing dish (diameter: 12 cm) and

placed on a permanent magnet plate for 16 hours. The pellet from the first crystallizing dish was re-suspended in 20 ml of isotonic saline to yield nanoparticle fraction of Lot 152-W1. After 16 hours of magnetic separation, the supernatant from the second crystallizing dish was removed and stored. The pellet was re-suspended in 20 ml of isotonic saline to yield nanoparticle fraction of Lot 152-W2.

[0097] The remaining supernatant was utilized to produce the nanoparticle fraction of Lot 152-W3 by separation of the nanoparticles in a high gradient magnetic field. Columns filled with coated iron grains were introduced into a permanent magnetic field. The supernatant was filled into the columns. The magnetic particles were retained in the column. The non-magnetic material, especially the excess dextran, was passed through the column. After washing the particles with isotonic saline, the column was removed from the magnetic field, releasing the nanoparticles from the column, and the nanoparticles were re-suspended in isotonic saline. The process flow chart for lots 152-W1, 152-W2 and 152-W3 is shown in **Figure 4**. All particle fractions were filtered through sterile filters (Rotilabo®, 0.22 μm, PES, P668.1; available from Carl Roth GmbH). The particle yield of the single fractions was determined by gravimetry. The iron concentration of all fractions was measured spectrophotometrically using the Spectroquant® -Kit (Merck & Co., Inc., Whitehouse Station, NJ; VWR International GmbH, Darmstadt, Germany). The properties of the magnetic nanoparticles are presented in Table III.

TABLE III. PROPERTIES OF MAGNETIC NANOPARTICLES

Lot ID.	Volume [ml]	Particle concentration [mg/ml]	Iron concentration [mg/ml]	Weight % (w/w) iron oxide	Particle yield [g]
152-W1	35	45	22	67	1.57
152-W2	20	34	17	69	0.68
152-W3	110	16	5	43	1.76

[0098] Size characterization: The hydrodynamic diameters of the nanoparticle fractions of lot 152-W were measured via PCS (see **Figure 2b** and **Figure 2c**). The resulting data is presented in Table IV.

TABLE IV. DIAMETER OF MAGNETIC NANOPARTICLES

Lot ID.	Mean diameter [nm] (Analysis by intensity)	Mean diameter [nm] (Analysis by volume)	Mean diameter [nm] (Analysis by number)
152-W1	109.1	79.3	63.8
152-W2	82.0	56.8	48.5
152-W3	50.4	22.2	16.6

[0099] The zetapotential measurement of dextran-coated iron oxide nanoparticles of Lot 152-W is shown in **Figure 3**. The comparison of the zetapotential – pH functions of the initial iron oxide particles of Lot 146-T and the corresponding dextran-coated particles shows a significant decrease over the entire pH range of 3 - 9. The isoelectric point of the iron oxide suspension at pH = 6.1 was shifted to 5.4 due to the dextran coating under HPH conditions.

Example 3: PEG-carboxylation of dextran-coated nanoparticles (Lot 202-G)

[00100] 6 ml of a suspension of dextran-coated nanoparticles of 152-W-type (example 2) with a particle concentration of 25 mg/ml in 0.1 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3) was treated with increasing amounts of carbodiimide activated 3,6-dioxaoctanedioic acid to yield the lots of nanoparticles 202-G1 – 202-G4 with various PEG-COOH densities, as presented in Table V.

TABLE V. THEORETICAL PEG-COOH DENSITY OF MAGNETIC NANOPARTICLES

Lot ID. of PEG-COOH particles	Amounts of EDC, and 3,6-dioxaoctanedioic acid [mg per 150 mg particle]	Theoretical PEG-COOH density [nmol/mg]
202-G1	18	625
202-G2	3.6	125
202-G3	0.72	25
202-G4	0.144	5

[00101] Equal amounts (as shown in Table V) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 3,6-dioxaoctanedioic acid were dissolved in 1.5 ml of 0.5 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3), incubated at 50°C for 10 min, and added to the particle suspension. After shaking the particle suspension for 2 hours at room temperature, the particles were washed twice with water after separation from the solution using a permanent magnet, resulting in a 5 ml of suspension of PEG-carboxylated nanoparticles with a concentration of about 20 mg/ml. The zetapotential – pH profile was determined for all lots of PEG-COOH modified nanoparticles by titration with 0.1 M NaOH to pH = 10, and then with 0.1 M HCl to pH = 3.

[00102] In general, the PEG-COOH modification leads to higher negative zetapotentials of the nanoparticles in the basic pH range and higher positive zetapotentials in the acidic pH range. From the zetapotential – pH functions of particles with increasing PEG-COOH densities, the range of saturation of the particle surface with functional groups can be derived by achieving constant zetapotentials at theoretical PEG-COOH densities greater than 125 nanomol (nmol)/mg. Thus, the nanoparticles of lots 202-G3 and 202-G4 show only low to medium coverage with PEG-COOH groups (as shown in **Figure 5**). The isoelectric point of the nanoparticles changed from pH = 5.9 for the particles 202-G4 with the lowest density of PEG-COOH on the surface to the acidic direction to pH = 4.8 for corresponding particles 202-G1 with the maximum saturation of the particle surface with PEG-COOH groups (as shown in **Figure 6**).

Example 4: Covalent binding of ChL6 antibody on the surface of dextran-coated nanoparticles (Lot 206-G)

[00103] 2.4 mg (12.6 μmol) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride and 4.8 mg (42 μmol) of N-hydroxysuccinimide were dissolved in 1.25 ml of 0.5 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3) and added to 5 ml of particle suspension containing PEG-COOH functionalized of lot 202-G2

(example 3) with a concentration of 20 mg/ml. After shaking the particle suspension for 2 hours, the particles were washed with 5 ml of phosphate buffered saline (pH=7.4) after separation from the solution using a permanent magnet, and suspended in 5 ml of phosphate buffered saline (pH=7.4). 1 mg of ChL6 antibody (obtained from UC-Davis) was dissolved in 1 ml of phosphate buffered saline (pH=7.4) and added to the particle suspension. After shaking the particle suspension for 4 hours at room temperature, the remaining active sites on the particle surface were blocked by addition of 2 ml of 0.3 M glycine in phosphate buffered saline (pH=7.4). After shaking the particle suspension for further 30 min at room temperature, the particles were washed three times, each time with 5 ml of phosphate buffered saline (pH=7.4) after separation from the solution with a permanent magnet, resulting in a 5 ml suspension having a particle concentration of 7 mg/ml. The average hydrodynamic diameter of the ChL6 coated nanoparticles (Lot 206-G) was determined by PCS to be in the range of 45 nm – 55 nm. The iron content in the particles was determined to be in the range of 56 % to 60 % (w/w).

Example 5: Synthesis of carboxy-dextran-coated nanoparticles (Lot 200-W)

[00104] Analogous to example 2, the iron oxide described in example 1 was coated with carboxydextran (available from Innovent e.V., Jena, Germany) instead of dextran to study the influence of carboxylic acid groups, which are already present in the initial dextran structure, on the integrity and density of the dextran shell. The resulting nanoparticles of Lot 200-W have a COOH density of 180 nmol/mg (determined via polyelectrolyte titration) and can be directly coated with antibodies according to the procedure described in example 4.

[00105] The examples 6 – 8 provide a detailed description of the synthetic procedures for the production of iron oxide nanoparticles by high pressure homogenization (HPH) (example 6), for the production of dextran coated nanoparticles from HPH-produced

iron oxide (example 7), for the functionalization of nanoparticles, and methods used for conjugating ING-1 antibody to produce the final product for *in vivo* application (example 8).

Example 6: Synthesis of iron oxide nanoparticles by high pressure homogenization (Lot 176-W)

[00106] 13.5 g of $FeCl_3*6H_2O$ and 7.85 g of $FeCl_2*4H_2O$ were dissolved in 250 ml of water. The temperature of this solution was increased to 80°C. The iron salt solution was filled into the reservoir of the Microfluidizer M-110Y and homogenized at a pressure of 1000 bar. 250 ml of 2 N sodium hydroxide solution was heated to 80°C and added to the iron salt solution while stirring. The homogenization was carried out at 90°C for 30 min. The resultant suspension was then cooled to room temperature. The iron oxide suspension was neutralized by successive centrifugation and washed with water to yield a stable aqueous iron oxide colloid. The average hydrodynamic diameter of the resulting nanoparticles was determined (via PCS) to be in the range of 200 nm – 500 nm.

Example 7: Synthesis and characterization of dextran-coated nanoparticles from HPH-produced iron oxide (Lot178-W)

[00107] 21 g of dextran (MW=40,000 D, Fluka) was dissolved in 100 ml of water and heated to 50°C. 200 ml of iron oxide suspension from Example 6 containing 2.6 g of iron oxide was placed in the Microfluidizer M-110Y and processed at 700 bar for 10 min. While stirring, the pre-heated dextran solution was added to the suspension, and the homogenization process was continued for an additional 40 min at a pressure of 1000 bar. The temperature during this process was maintained the range of 85°C – 90°C. The resultant suspension was cooled to room temperature. The excess dextran was removed by magnetic separation of the nanoparticles, and the nanoparticles were re-suspended in water. The average hydrodynamic diameter of the resulting nanoparticles was determined (via PCS) to be in the range of 20 nm – 80 nm. The iron content of the nanoparticles was determined to be in the range of 45 % to 55 % (w/w).

Example 8: ING-1 antibody binding on dextran coated nanoparticles (Lot 07304-G)

[00108] 6 g of dextran was dissolved in 20 ml of water. 22 ml of dextran coated nanoparticles of Lot 178-W (example 7) having a particle concentration of 46 mg/ml was added to the dextran solution. The particle suspension was heated to 100°C, with a rotation speed of 400 rpm for 1 hour. After cooling to room temperature, the magnetic particles were separated using a permanent magnet to remove the excess dextran from the supernatant. The particles were suspended in 20 ml of water to yield a particle concentration of 58 mg/ml.

[00109] 2.6 ml of this particle suspension was diluted with 2.4 ml of water to a particle concentration of 30 mg/ml. 3.6 mg (18.8 μmol) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride and 3.6 mg (20.2 μmol) of 3,6-dioxaoctanedioic acid were dissolved in 1.25 ml of 0.5 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3), incubated at 50°C for 10 min, and added to the particle suspension. After shaking the particle suspension for 2 hours at room temperature, the particles were washed twice with 5 ml of 0.1 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3) after separation from the solution using a permanent magnet, resulting in a 5 ml suspension of PEG-carboxylated nanoparticles with a concentration of 10 mg/ml.

[00110] 6 mg (31.4 μmol) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride and 12 mg (104 μmol) of N-hydroxysuccinimide were dissolved in 1.25 ml of 0.5 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3), and added to the particle suspension. After shaking the particle suspension for 2 hours, the particles were washed with 5 ml of phosphate buffered saline (pH=7.4) after separation from the solution using a permanent magnet, and suspended in 4 ml of phosphate buffered saline (pH=7.4). 500 μg of ING-1 antibody (obtained from XOMA Inc., Oakland, CA) was dissolved in 200 μl of phosphate buffered saline (pH=7.4), and added to the particle suspension. After shaking the

particle suspension for 4 hours at room temperature, remaining active sites on the particle surface were blocked by addition of 2 ml of 0.3 M glycine in phosphate buffered saline (pH=7.4). After shaking the particle suspension for further 30 min at room temperature, the particles were washed three times, each time with 5 ml of phosphate buffered saline (pH=7.4) after separation from the solution using a permanent magnet, resulting in a 5 ml suspension having a particle concentration of 8 mg/ml. The average hydrodynamic diameter of the ING-1 coated nanoparticles (Lot 07204-G) was determined by PCS to be in the range of 60 nm – 70 nm. The iron content in the nanoparticles was determined to be in the range of 58 % to 60 % (w/w).

Example 9: Synthesis of oligo-dT20 modified dextran-coated nanoparticles (Lot 208-G)

[00111] 6 mg (31.4 μmol) of 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride and 12 mg (104 μmol) of N-hydroxysuccinimide were dissolved in 1.25 ml of 0.5 M β-morpholino ethanesulfonic acid hydrate buffer (pH=6.3) and added to 5 ml of a suspension of PEG-carboxylated nanoparticles having a concentration of 10 mg/ml (from example 8). After shaking the particle suspension for 2 hours at room temperature, the particles were washed with 5 ml of imidazol buffer (pH=7.0) after separation from the solution using a permanent magnet, and suspended in 4 ml of imidazol buffer (pH=7.0). 200 nmol of oligo-dT20 C6 amino link (5') (available from Thermo Electron Corporation, Ulm, Germany) was dissolved in 200 μl of imidazol buffer (pH=7.0) and added to the particle suspension. After shaking the particle suspension for 4 hours at room temperature, the particles were washed three times, each time with 5 ml of 10mM Tris buffer (pH=7.5) and 500 mM of KCl for further binding of nucleic acids. The average hydrodynamic diameter of the oligo-dT20 coated nanoparticles (Lot 208-G) was determined by PCS to be in the range of 55 nm – 65 nm. The iron content in the nanoparticles was determined to be in the range of 58 % to 60 % (w/w).

Example 10: Calorimetric Measurements of Nanoparticle SAR Values

[00112] Equipment utilized:

- TIG 10/300 RF power supply with tank circuit (Huttinger Electronic Inc., Farmington, CT)
- RF coil (solenoid) having 8 turns, 0.75" ID, and 2.2" length (Triton BioSystems, Inc., Chelmsford, MA)
- UMI-4 temperature probe controller with FOT-M/2M temperature probe and charting software (Fiso, Inc., Quebec, Canada)
- TDS-3014 Oscilloscope (Tektronix, Inc., Beaverton, OR)
- Litz wire magnetic probe (Centre for Induction Technology, Inc., Auburn Hills, MI)

[00113] The apparatus was calibrated by setting the power supply at 20, 40, 60, 80, 90 and 100% of output voltage. At each setting, the peak magnetic field strength was measured at the center of the coil using the litz wire probe and oscilloscope. The field was also measured at small axial and radial offsets and it was determined the field was uniform within 10% throughout the planned 1 ml sample volume. The peak field strengths were charted versus the power supply's percent voltage output, and this curve allowed the presetting of field strength via the power supply's voltage readout. A calorimetric calibration was performed as well. This was accomplished by running de-ionized (DI) water samples immediately before SAR testing at each planned field strength, and following the same heating procedure. The temperature curves from the DI samples were subtracted from the SAR samples, thereby taking into account the heat losses (or additions) through the insulation.

[00114] After thorough vortexing, 1 ml samples were dispensed into 8 mm glass test tubes. In some cases they were diluted with de-ionized water to keep the heating rate within easily measurable limits. (In all cases the sample size was maintained at 1 ml.) The test tube was vortexed, wrapped in ceramic felt insulation and placed in the solenoid coil. It was placed on a glass pedestal such that the liquid sample was axially and radially centered

within the coil. The temperature probe was immersed in the sample and the charting software started. At 10 seconds the RF was applied and at 60 seconds the RF was curtailed. After subtracting the DI calibration curve from the raw warm-up data, a characteristic heating curve resulted. The slope of this curve and the particle and iron concentrations were entered into the following formulae;

$$SAR_{particle}\ (W/gm) = 4180 * \text{slope}\ (^{o}C/sec) / \text{particle concentration}\ (mg/ml)$$

$$SAR_{iron}\ (W/gm) = 4180 * \text{slope}\ (^{o}C/sec) / \text{iron concentration}\ (mg/ml)$$

These SAR values were used to optimize the heating characteristics of the iron oxide nanoparticles. The SAR_{iron} values for Lots 146-T, 152-W1, 152-W2, and 178-W are presented in Table VI.

TABLE VI. SAR_{iron} VALUES FOR PARTICLES PRODUCED BY HPH PROCESS

Field	SAR_{iron} of Lot 146-T (W/gm_{iron})	A. SAR_{iron} of B. Lot 152-W1 (W/gm_{iron})	SAR_{iron} of Lot 152-W2 (W/gm_{iron})	SAR_{iron} of Lot 178-W (W/gm_{iron})
370	188	216	252	179
730	234	273	314	602
1080	211	287	321	1311
1300	151	301	358	2090
1670	271	NA	NA	NA

[00115] The HPH process (as illustrated via Examples 1-7) was used for enhancing the heating rates of coated nanoparticles, particularly the dextran-coated iron oxide nanoparticles, which exhibit heating when exposed to alternating magnetic field (AMF) amplitude of about 370 to about 500 Oe. First, a magnetic material having an Fe(II)/Fe(III)-ratio = 1.58:2.0 is selected. This material exhibits constant heating rates when exposed to an AMF amplitude of about 370 to about 1600 Oe (when produced via iron oxide precipitation

without HPH process). The iron oxide is cleaned, and HPH process is utilized to coat the nanoparticles with dextran. Due to this process, the heating rates of the nanoparticles are enhanced and occur when exposed to AMF amplitudes of about 370 to 500 W/g Fe with SAR values of 240 W/g Fe or greater.

[00116] The compositions of the present invention are applicable to human subjects (patient), as well as mammals, organ donors, cadavers and the like.

[00117] As noted above, the present invention pertains to biocompatible magnetic nanoparticle compositions prepared via high-pressure homogenization processes for various therapeutic and biological applications. The present invention should not be considered limited to the particular embodiments described above, but rather should be understood to cover all aspects of the invention as fairly set out in the appended claims. Various modifications, equivalent processes, as well as numerous structures to which the present invention may be applicable will be readily apparent to those skilled in the art to which the present invention is directed upon review of the present specification. The claims are intended to cover such modifications and devices.

We claim:

1. A method for preparing a magnetic nanoparticle composition, comprising:

a. generating a metal-containing magnetic material by processing a preformed metal-containing magnetic material through a turbulent flow zone in a first step, and generating a magnetic nanoparticle composition using the resulting improved magnetic material of the first step and a biocompatible coating material, via a turbulent flow zone, in a second step; or

b. generating a metal-containing magnetic material from a metal-containing solution via a turbulent flow zone in a first step, and generating a magnetic nanoparticle composition using the resulting magnetic material of the first step and a biocompatible coating material, via a turbulent flow zone, in a second step; or

c. forming a magnetic nanoparticle composition by generating a metal-containing magnetic material from a metal-containing solution and processing it with a biocompatible coating material, via a turbulent flow zone, in a single step.

2. A method according to claim 1, wherein in the first and the second steps are carried out in a liquid medium comprising water, or an aqueous alkaline solution having an ammonia sodium or potassium hydroxide basis.

3. A method according to claim 1, wherein the preformed metal-containing magnetic material comprises one or a combination of two or more of a) different metals, b) different metal compounds, c) metal with different valences, or d) any combination thereof.

4. A method according to claim 3, wherein the preformed metal-containing magnetic material comprises a combination of Fe-(II) and Fe-(III) in a molar ratio of 1:1 to 1:2.

5. A method according to claim 1, wherein the metal-containing solution is prepared from a metal, metal salt, metal alkoxyde, metal hydroxide, metal oxide, metal oxide-hydrate, metallic alloy of two or more metals, or any combination thereof.

6. A method according to claim 5, wherein the metal-containing solution comprises a combination of Fe-(II) and Fe-(III) salts in a molar ratio of 1:1 to 1:2.

7. A method according to claim 1, wherein the generated metal-containing magnetic material comprises a metal, metal salt, metal alkoxyde, metal hydroxide, metal oxide, metal oxide-hydrate, metallic alloy of two or more metals, or any combination thereof.

8. A method according to claim 7, wherein the metal oxide is iron oxide.

9. A method according to claim 8, wherein the iron oxide is magnetite, hematite, maghemite, or any combination thereof.

10. A method according to claim 8, wherein the iron oxide is doped with bi- or tri-valent metal ions.

11. A method according to claim 1, wherein the biocompatible coating material comprises a polymer, metal compound, transfection agent, or any combination thereof.

12. A method according to claim 11, wherein the polymer is naturally occurring, synthetic, or semi-synthetic.

13. A method according to claim 11, wherein the polymer comprises at least one reactive or ionic group, or any combination thereof.

14. A method according to claim 11, wherein the polymer comprises a homo-polymer, a co-polymer, or a polymer-blend.

15. A method according to claim 11, wherein the polymer comprises a biological material.

16. A method according to claim 15, wherein the polymer comprises a polysaccharide, polyamino acid, protein, lipid, fatty acid, heparin, heparin sulfate, chondroitin sulfate, chitin, chitosan, alginate, glycosaminoglycan, cellulose, starch, histidine-containing polymer, hydrogel polymer, any derivative thereof, or any combination thereof.

17. A method according to claim 16, wherein the polysaccharide is dextran.

18. A method according to claim 17, wherein the dextran comprises one or more functional groups.

19. A method according to claim 12, wherein the synthetic polymer comprises a polyvinyl compound, polyamine, polyimine, polyol, polyether, polycarboxylic acid, polysilicic acid, polyacrylate, polysiloxane, polyalkylene glycol, parylene, polylactic acid, polyglycolic acid, or any derivative thereof, or any combination thereof.

20. A method according to claim 2, wherein the first and the second steps are processed in a liquid carrier medium at a pressure above 100 bar.

21. A method according to claim 2, wherein the first and the second steps are processed in a liquid carrier medium at a pressure above 1000 bar.

22. A method according to claim 1, wherein the first and the second steps are processed in a liquid carrier medium at a temperature in the range from about 40°C and the boiling point of the medium.

23. A method according to claim 1, wherein the first and the second steps are processed in a liquid carrier medium at a temperature in the range from about 75°C to about 95°C.

24. A method according to claim 1, wherein the components are processed at a flow rate in the range from about 20 ml/min to about 200 ml/min through a turbulent flow zone in each step.

25. A method according to claim 2, wherein the resulting magnetic nanoparticles form a stable aqueous colloid.

26. A method according to claim 2, wherein the resulting magnetic nanoparticles form a stable colloid in physiological solution.

27. A method according to claim 2, wherein the resulting magnetic nanoparticles are separated from the carrier medium via an external magnetic field.

28. A method according to claim 27, wherein the resulting magnetic nanoparticles are separated with permanent magnets.

29. A magnetic nanoparticle composition, comprising:

a. at least one metal-containing magnetic nanoparticle possessing a low-field magnetization when an external magnetic field is applied to the at least one magnetic nanoparticle; and

b. a suitable medium for suspending the at least one nanoparticle,

wherein the at least one nanoparticle has a hydrodynamic diameter less than 200 nm, and contains at least 50 mass percent of metal, and

wherein the at least one metal-containing magnetic nanoparticle comprises a biocompatible coating material.

30. A magnetic nanoparticle composition according to claim 29, wherein the external magnetic field has an amplitude in the range from about 0 to about 400 Oersted.

31. A magnetic nanoparticle composition according to claim 29, wherein the nanoparticle composition has a PEG density in the range from about 2 μmol/g to about 250 μmol/g.

32. A magnetic nanoparticle composition according to claim 29, wherein the at least one magnetic nanoparticle comprises a) a metal, b) a metal oxide, c) a metal oxide-hydrate, d) a metal hydroxide, e) a metallic two or more metals, or f) any combination thereof.

33. A magnetic nanoparticle composition according to claim 32, wherein the at least one magnetic nanoparticle comprises an oxide of iron.

34. A magnetic nanoparticle composition according to claim 33, wherein the iron oxide is magnetite, hematite, maghemite, or any combination thereof.

35. A magnetic nanoparticle composition according to claim 33, wherein the iron oxide is doped with bi- or tri-valent metal ions.

36. A magnetic nanoparticle composition according to claim 29, wherein the at least one magnetic nanoparticle has ferro-, antiferro-, ferri-, antiferri- or superparamagnetic properties.

37. A magnetic nanoparticle composition according to claim 29, wherein the biocompatible coating material comprises a polymer.

38. A magnetic nanoparticle composition according to claim 37, wherein the polymer is naturally occurring, synthetic, or semi-synthetic.

39. A magnetic nanoparticle composition according to claim 37, wherein the polymer comprises a homo-polymer, a co-polymer, or a polymer-blend.

40. A magnetic nanoparticle composition according to claim 37, wherein the polymer comprises at least one reactive or ionic group, or any combination thereof.

41. A magnetic nanoparticle composition according to claim 37, wherein the polymer comprises a biological material.

42. A magnetic nanoparticle composition according to claim 37, wherein the polymer comprises a polysaccharide, polyamino acid, protein, lipid, fatty acid, heparin, heparin sulfate, chondroitin sulfate, chitin, chitosan, alginate, glycosaminoglycan, cellulose, starch, histidine-containing polymer, hydrogel polymer, any derivative thereof, or any combination thereof.

43. A magnetic nanoparticle composition according to claim 42, wherein the polysaccharide is dextran.

44. A magnetic nanoparticle composition according to claim 43, wherein the dextran comprises one or more functional groups.

45. A magnetic nanoparticle composition according to claim 38, wherein the synthetic polymer comprises a polyvinyl compound, polyamine, polyimine, polyol, polyether, polycarboxylic acid, polysilicic acid, polyacrylate, polysiloxane, polyalkylene glycol, parylene, polylactic acid, polyglycolic acid, or any derivative thereof, or any combination thereof.

46. A magnetic nanoparticle composition according to claim 29, wherein the magnetic nanoparticle composition comprises one or more sub-structures.

47. A magnetic nanoparticle composition according to claim 46, wherein the sub-structures comprise at least one ligand, chelator, or a combination thereof.

48. A magnetic nanoparticle composition according to claim 47, wherein the at least one ligand or chelator comprises a peptide, protein, nucleic acid, enzyme, antibody, antibody fragment, or any combination thereof.

49. A magnetic nanoparticle composition according to claim 48, wherein the antibody is ING-1.

50. A magnetic nanoparticle composition according to claim 46, wherein the sub-structures comprise a bioactive substance.

51. A magnetic nanoparticle composition according to claim 50, wherein the bioactive substance comprises a pharmaceutical agent, peptide, lipid, biochemical factor, or any combination thereof.

52. A magnetic nanoparticle composition produced according to the method of claim 1.

53. A magnetic nanoparticle composition according to claim 52, wherein the magnetic nanoparticle composition comprises one or more sub-structures.

54. A magnetic nanoparticle composition according to claim 29, wherein the composition is used in the fixation, separation, transportation, marking or coding of targets, or energy transformation processes.

55. A magnetic nanoparticle composition according to claim 54, wherein the composition is used in the separation or purification of biomolecules, or any combination thereof.

56. A therapeutic method according to claim 54, wherein the composition is used in the separation, purification, or any combination thereof of nucleic acids, nucleic acid derivatives, nucleic acid fragments, proteins, protein derivatives, protein fragments, or any combination thereof.

57. A magnetic nanoparticle composition according to claim 54, wherein the composition is used for the sorting and purification of cells.

58. A magnetic nanoparticle composition according to claim 54, wherein the composition is used in radionuclide therapy.

59. A magnetic nanoparticle composition according to claim 54, wherein the composition is used in analytical processes or diagnostic assays.

60. A magnetic nanoparticle composition according to claim 59, wherein the composition is used as contrast media.

61. A therapeutic method, comprising:

a. administering a magnetic nanoparticle composition according to claim 46 to at least a portion of the body, body part, tissue, cell, or body fluid of a subject comprising a target, and

b. administering energy to the magnetic nanoparticle composition combined with the target.

62. A therapeutic method according to claim 61, further comprising the step of applying an alternating magnetic field (AMF).

63. A therapeutic method according to claim 61, wherein the composition is administered via injection, topical application, transdermal application, orally ingestion, rectal insertion, inhalation through the mouth or nose, or any combination thereof.

64. A therapeutic method according to claim 61, wherein the method is utilized for the treatment of a cancer, AIDS, adverse angiogenesis, restenosis, amyloidosis, tuberculosis, multiple sclerosis, cardiovascular plaque, vascular plaque, obesity, malaria, illnesses due to viruses, or any combination thereof.

65. A therapeutic method according to claim 61, wherein the magnetic nanoparticles transport and release bioactive substances.

66. A therapeutic method, comprising:

a. administering a magnetic nanoparticle composition according to claim 53 to at least a portion of the body, body part, tissue, cell, or body fluid of a subject comprising a target, and

b. administering energy to the magnetic nanoparticle composition combined with the target.

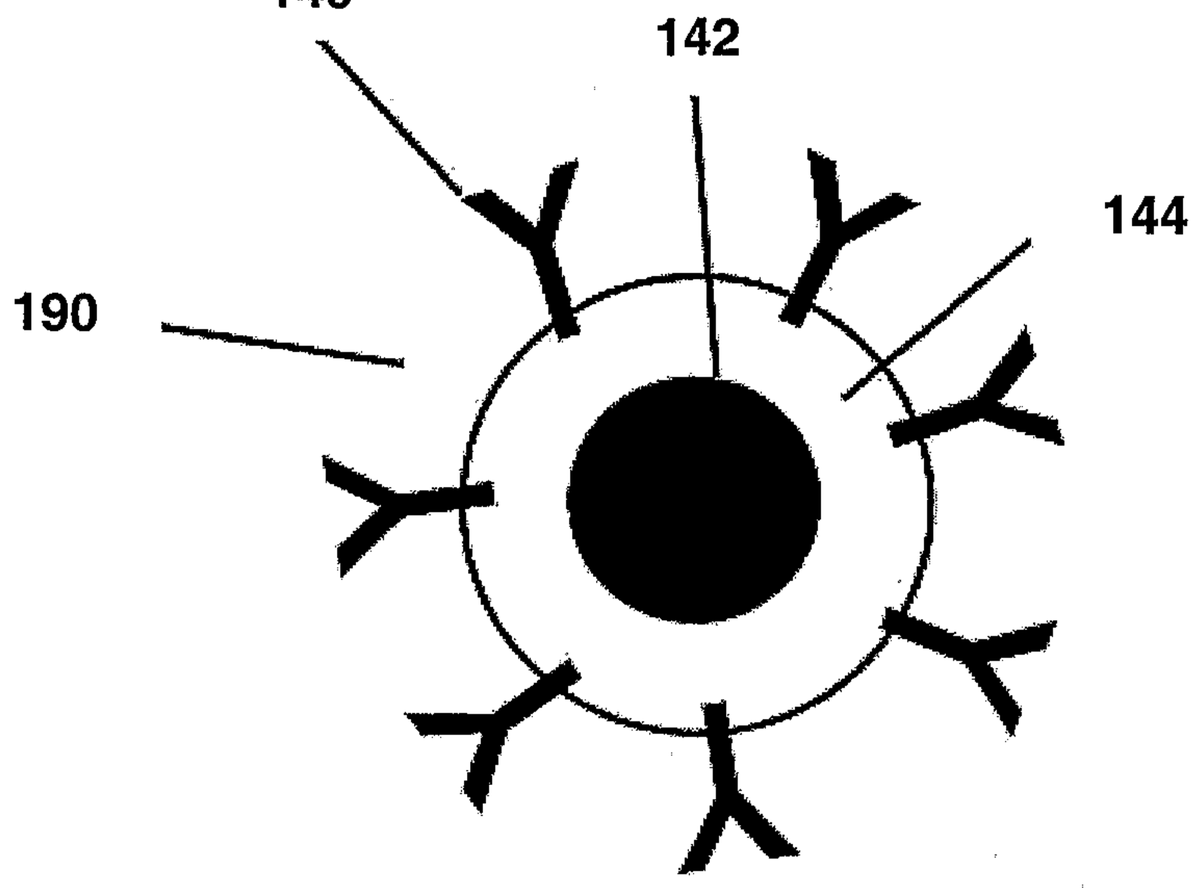


Figure 1

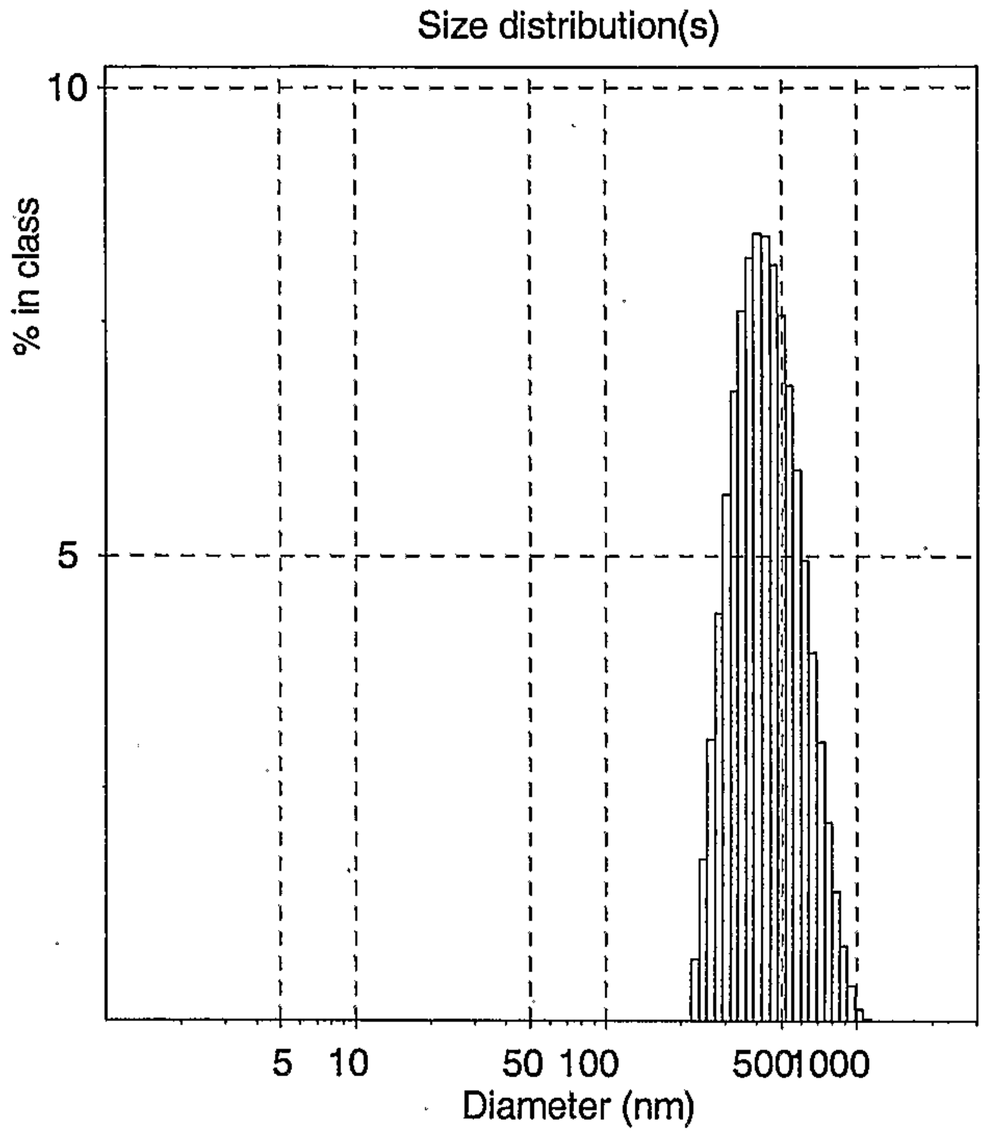


Figure 2a:

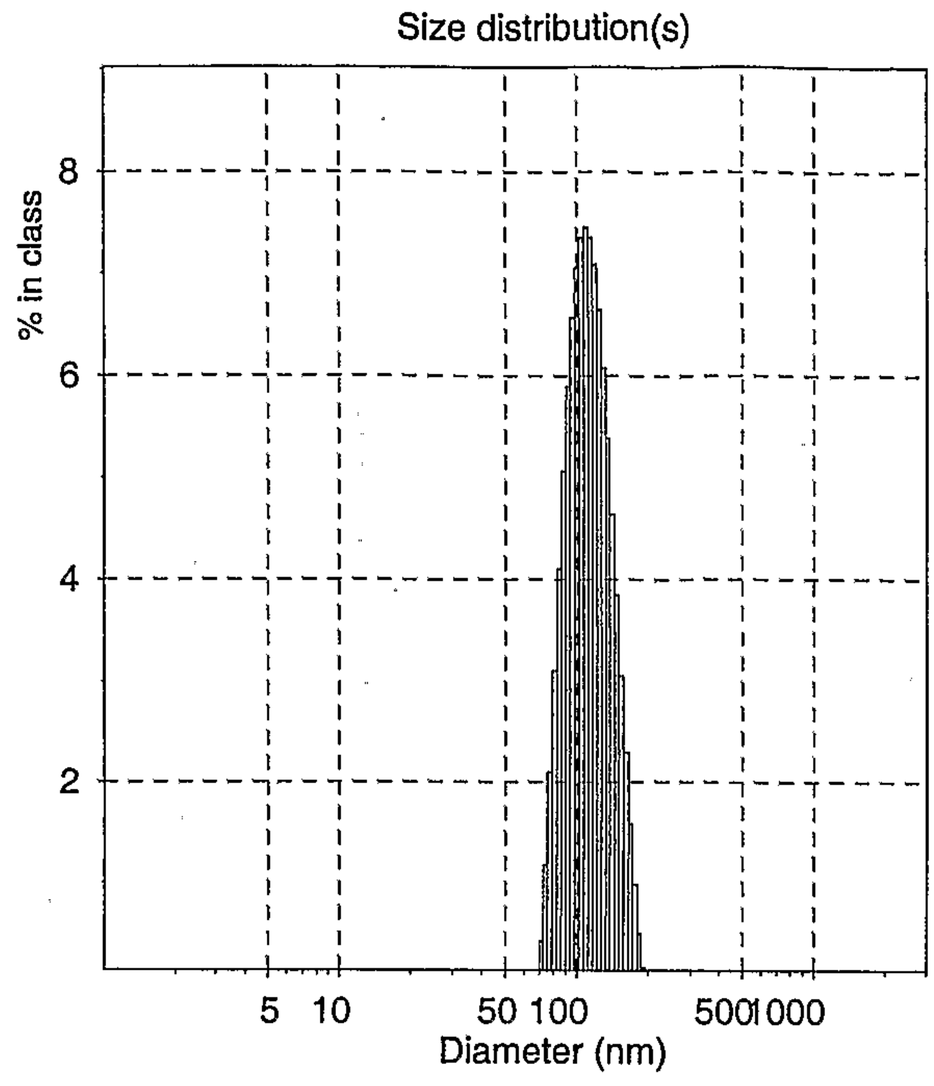


Figure 2b

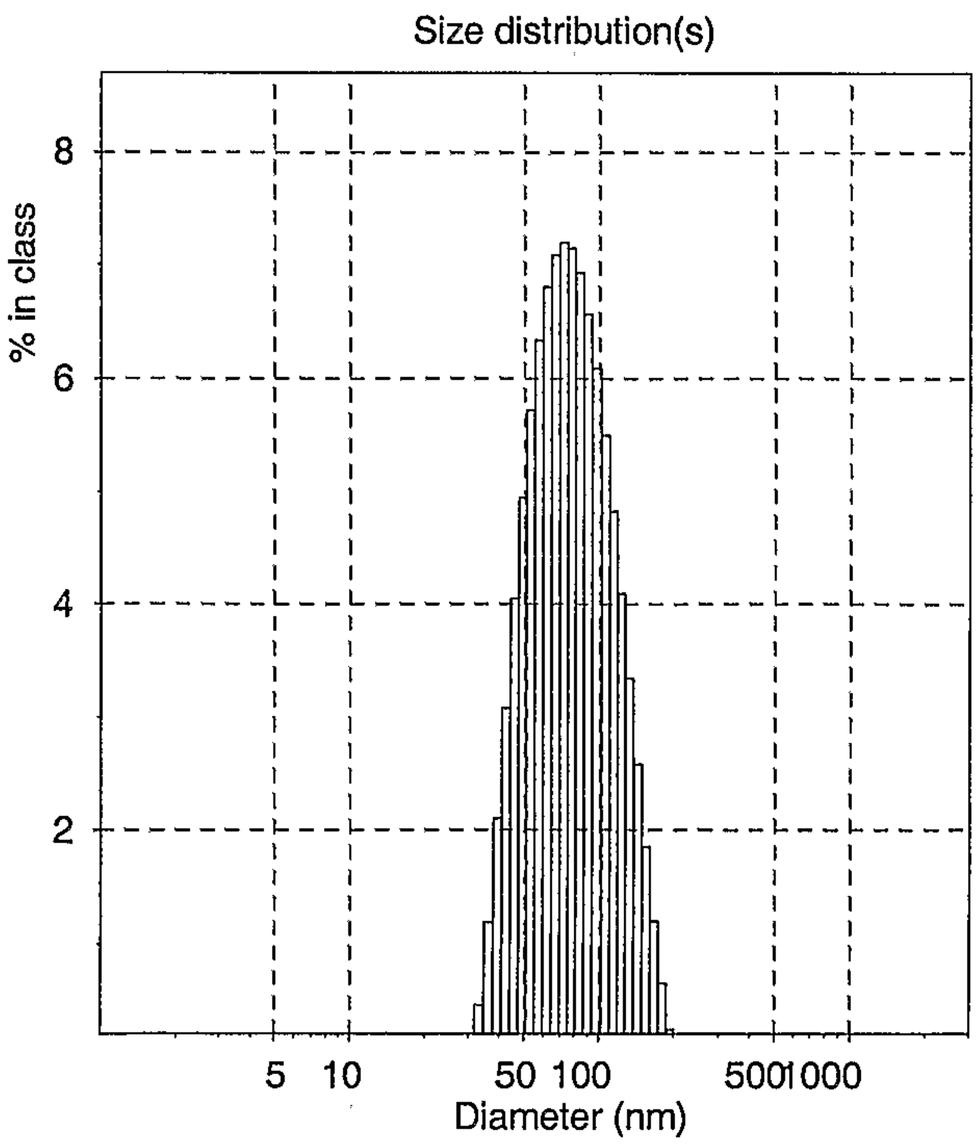


Figure 2c

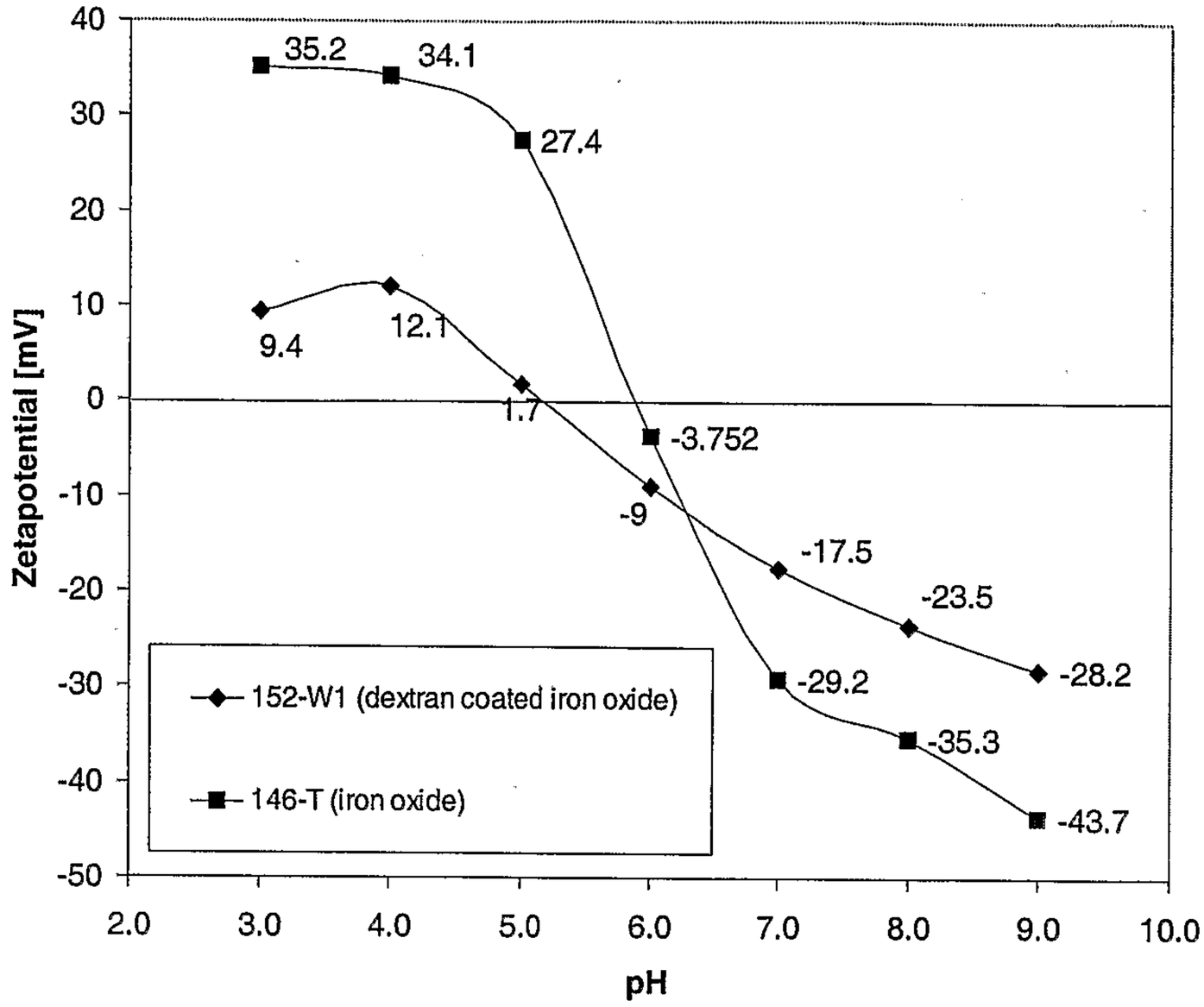

Zetapotential [mV]
pH
40
30
20
10
0
-10
-20
-30
-40
-50
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
35.2
34.1
27.4
-3.752
-29.2
-35.3
-43.7
9.4
12.1
1.7
-9
-17.5
-23.5
-28.2
152-W1 (dextran coated iron oxide)
146-T (iron oxide)

Figure 3

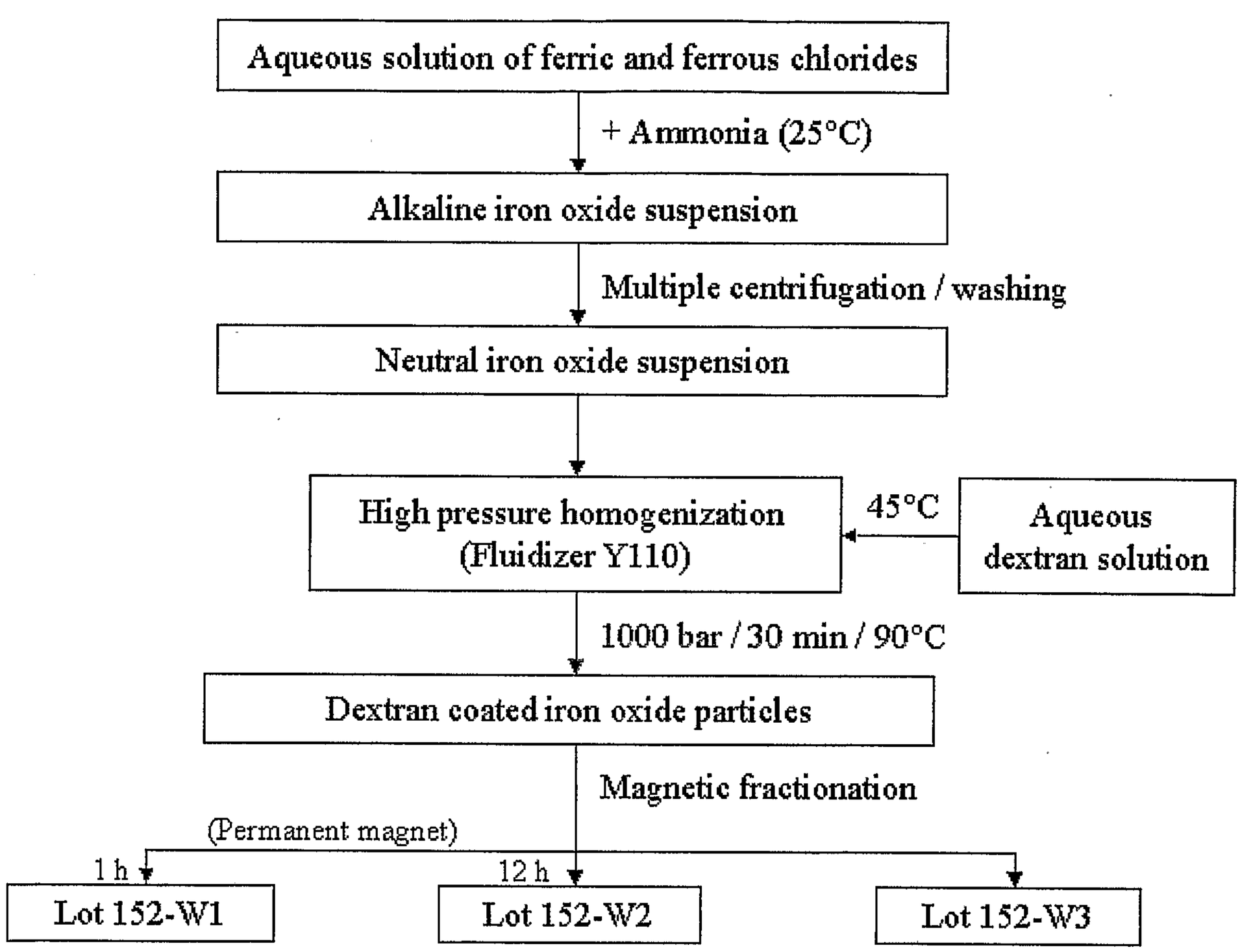

Aqueous solution of ferric and ferrous chlorides
+ Ammonia (25°C)
Alkaline iron oxide suspension
Multiple centrifugation / washing
Neutral iron oxide suspension
High pressure homogenization (Fluidizer Y110)
45°C
Aqueous dextran solution
1000 bar / 30 min / 90°C
Dextran coated iron oxide particles
Magnetic fractionation
(Permanent magnet)
1 h
12 h
Lot 152-W1
Lot 152-W2
Lot 152-W3

Figure 4

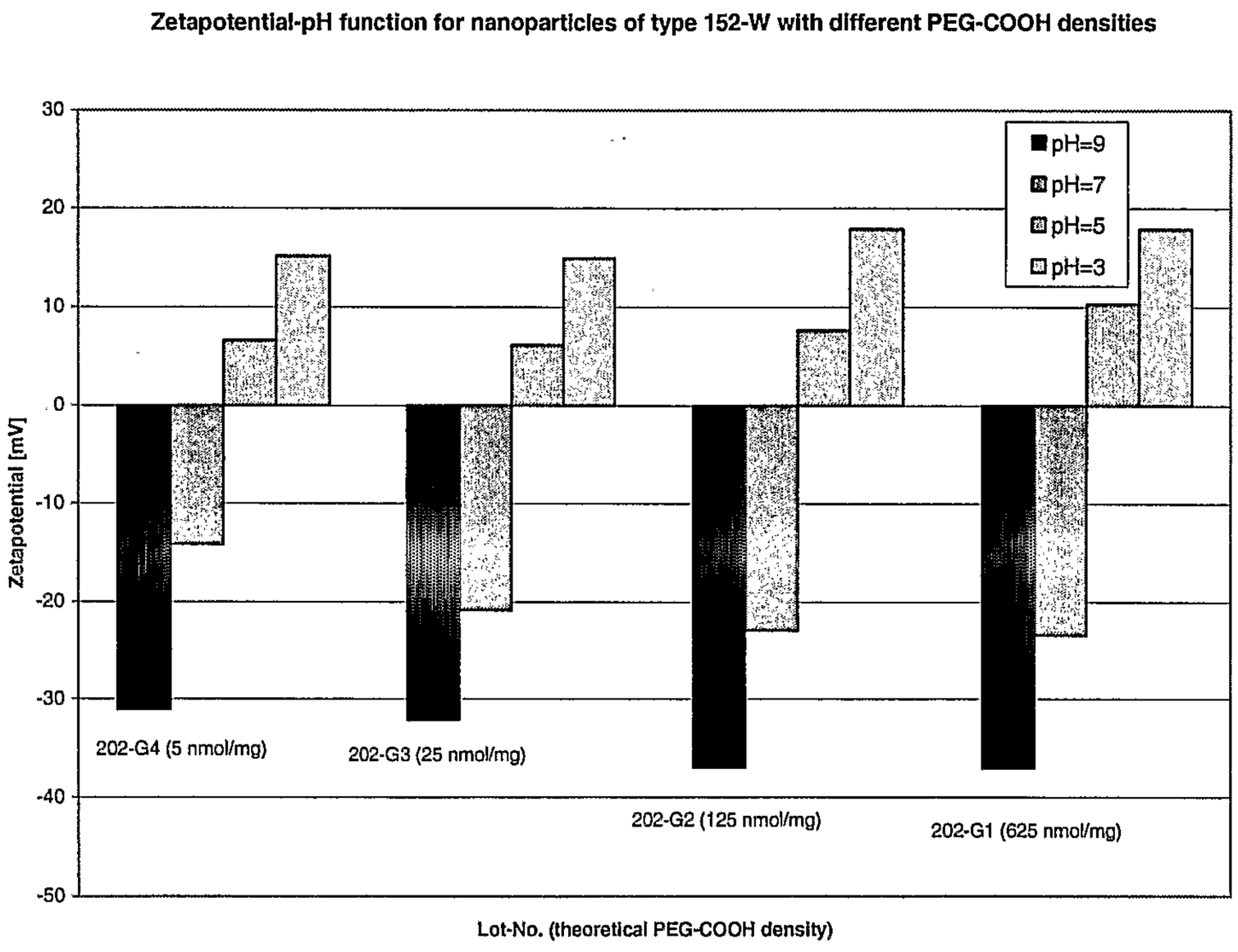

Zetapotential-pH function for nanoparticles of type 152-W with different PEG-COOH densities
30
20
10
0
-10
-20
-30
-40
-50
Zetapotential [mV]
pH=9
pH=7
pH=5
pH=3
202-G4 (5 nmol/mg)
202-G3 (25 nmol/mg)
202-G2 (125 nmol/mg)
202-G1 (625 nmol/mg)
Lot-No. (theoretical PEG-COOH density)

Figure 5

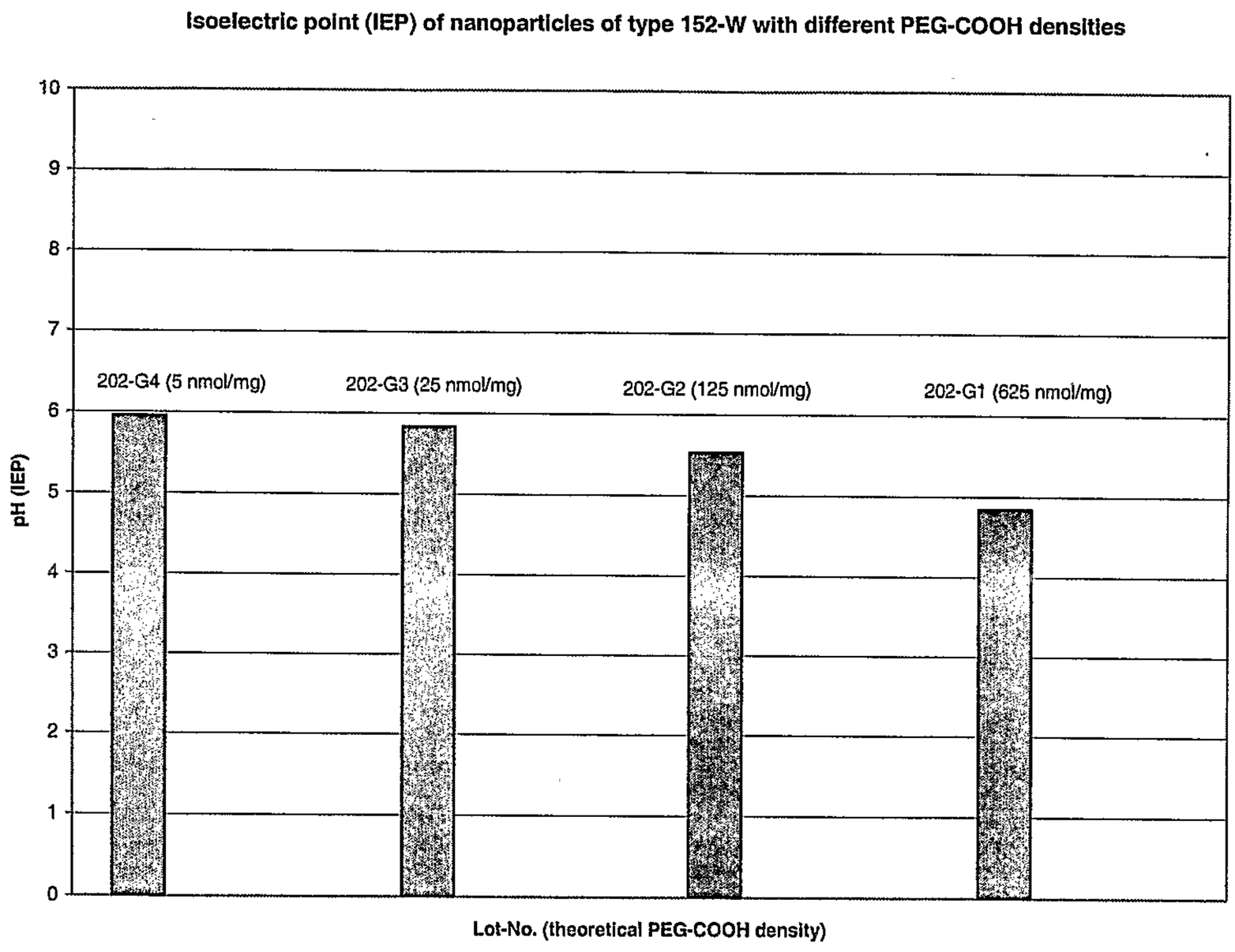
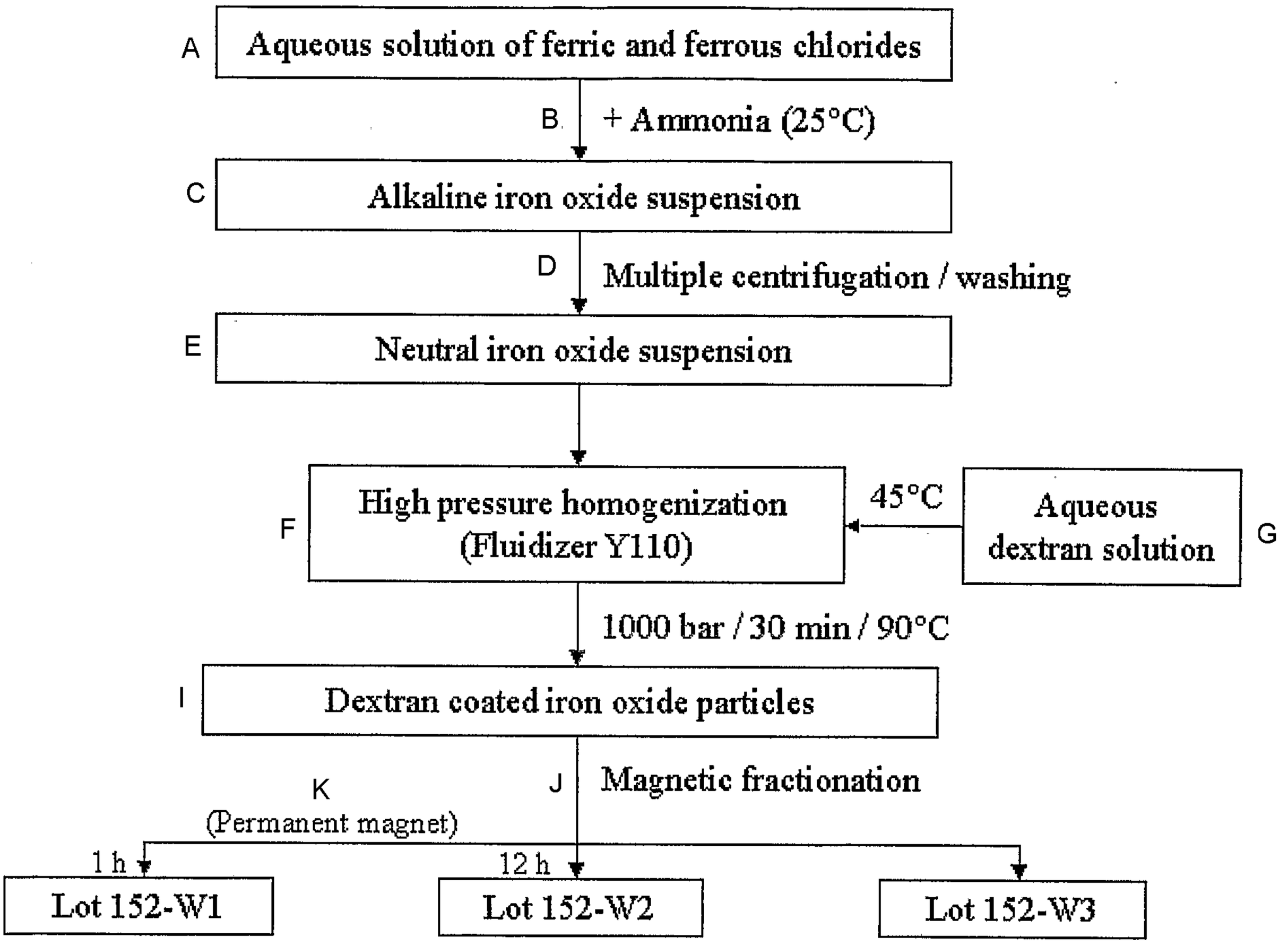

Isoelectric point (IEP) of nanoparticles of type 152-W with different PEG-COOH densities
10
9
8
7
6
5
4
3
2
1
0
pH (IEP)
202-G4 (5 nmol/mg)
202-G3 (25 nmol/mg)
202-G2 (125 nmol/mg)
202-G1 (625 nmol/mg)
Lot-No. (theoretical PEG-COOH density)

Figure 6



A...SOLUTION AQUEUSE DE CHLORURES FERRIQUES ET FERREUX
B...+ AMMONIAQUE (25 °C)
C...SUSPENSION D'OXYDE DE FER ALCALIN
D...CENTRIFUGATION MULTIPLE/LAVAGE
E...SUSPENSION D'OXYDE DE FER NEUTRE
F...HOMOGÉNÉISATION HAUTE PRESSION (FLUIDIFIANT Y110)
G...SOLUTION DE DEXTRAN AQUEUSE
I...PARTICULES D'OXYDE DE FER ENROBÉES DE DEXTRAN
J...FRACTIONNEMENT MAGNÉTIQUE
K...(AIMANT PERMANENT)