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(54) Title: ARTICLES COMPRISING MAGNETIC MATERIAL AND BIOACTIVE AGENTS

(57) Abstract: The invention is directed to magnetoarticles comprising a superparamagnetic material core and a polymeric scaffold encapsulating the magnetic material. The magnetoarticles further comprise bioactive agents.

WO 2005/070471 A2

Articles Comprising Magnetic Material and Bioactive Agents

FIELD OF THE INVENTION

The present invention is directed to the field of delivery of bioactive agents via nanoarticles comprising magnetic material, and the use thereof in separation techniques, magnetic resonance imaging, and therapeutic treatments.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made with government support under a grant supported by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Chemotherapeutics are widely used in the treatment of cancer. While somewhat efficacious, the toxicity of the chemotherapeutics is severe and harmful, remission is often incomplete, and the eventual regrowth and spread of cancerous tissue is the norm. Efforts at localizing chemotherapeutics to the cancerous tissues, for instance through the attachment of chemotherapeutics to monoclonal antibodies which bind to receptors over-expressed on cancer cells, has thus far been a modest success.

To reduce side effects, drug molecules have been directly conjugated to monoclonal antibodies (mAb). These constructs are known as immunoconjugates. Despite improvement in biodistribution, immunoconjugates still struggle to deliver an effective dose to tumors and not to healthy tissue.

Hyperthermia, in which the temperature of cancerous tissue is raised, has demonstrated some efficacy in treating multiple types of cancer. One way that the temperature can be raised is through first localizing magnetic articles within a tumor, and subsequently heating the magnetic articles by subjecting them to an alternating magnetic field. Magnetic articles used for this type of therapeutic regimen may be made using several methods; one such method is described by Tan, et.al., in PCT application WO 01/88540 A1. It has been reported that certain magnetic particles can reach temperatures in excess of 150 °C. However, to effectively heat the tumor mass, small particles require a high level of accumulation that is very difficult to achieve.

Cancer cells are under intrinsic oxidative stress and as such are vulnerable to free radical-induced apoptosis. The use of free radicals / free radical-producing agents as a therapeutic for the treatment of cancer has been investigated. For instance, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2(-)$) are known to be involved in the cytotoxic

action of a number of drugs (Akiyama and Natori, *Cancer Sci.*, 2003, 94: 400-404; Pelicano et al., *J Biol Chem.*, 2003, 278: 37832-9.) A further example is calicheamicin, an enediyne antibiotic capable of binding DNA which, following activation, results in cleavage of the double strand of the oligopyrimide-oligopurine sections of DNA. Recently, an immunoconjugate of this drug, gemtuzumab ozogamicin, has been approved by the FDA for the treatment of acute myelogenous leukemia. Thus, drug formulations that produce free radicals have demonstrated clear therapeutic benefit. If properly targeted to the tumor, the potential side effects of such formulations may be minimized.

Thus, although all the above cancer treatment methods are somewhat efficacious, improved cancer treatments are urgently needed.

SUMMARY OF THE INVENTION

This invention is directed to discrete particles, preferably nanoparticles, comprising magnetic, preferably superparamagnetic, material and a polymeric scaffold outer layer. Preferably, the superparamagnetic material is selected from the iron oxides, such as magnetite, maghemite, and greigite. The magnetoarticles may further comprise bioactive agents (such as, for example, chemotherapeutics, cytotoxics, free radical-generating agents, other toxic agents and other therapeutic agents). The particles of the invention will be referred to herein as magnetic therapeutic nanoparticles, or MTNPs.

More particularly, the invention, in a first embodiment, is directed to MTNPs comprising a superparamagnetic material core encapsulated by a crosslinked polymeric scaffold and having least some of the bioactive agents bound to the polymer matrix.

In a second embodiment, the MTNPs comprise a superparamagnetic material core encapsulated by a polymer material, preferably selected from hydrophilic polycarboxylates. The superparamagnetic material is bound to the polymer matrix by coordination bonds with carboxylate moieties on the polymeric material. The polymer matrix further incorporates bioactive agent molecules, preferably chemotherapeutic molecules, within the matrix. Preferred chemotherapeutic molecules for this embodiment are comprised of platinum atoms, at least some of the platinum atoms forming coordination bonds with carboxylate moieties on the polymer material.

In a third embodiment of the invention, the MTNPs comprise a superparamagnetic core and a polymeric scaffold where the polymer scaffold itself is comprised of free radical-generating agents, the bonds of which break apart upon heating to release free radicals. In this embodiment, other bioactive agents may or may not also be attached to the MTNP.

The various MTNPs of the invention may further comprise recognition elements (REs) to facilitate targeting and/or delivery by binding to certain biomolecules found in pathogenic

tissue, such as certain cellular receptors that are overexpressed on the surface of some cancer cells. The articles may also optionally comprise polyethylene glycol (PEG)-based molecules. The PEG chains may be used to extend the circulation time of the article *in vivo*; they may also serve as linkers or tethers, with one end attached to the article scaffold and the other end functionalized with a recognition element or another nanoarticle.

At least four mechanisms may localize the magnetoarticles of the invention in a particular environment, such as pathogenic tissue. First, the article size is such that preferential tumor accumulation can occur through the well-documented enhanced permeability and retention (EPR) effect. Second, binding elements on the surface of the nanoarticles can bind to tumor-associated antigens. Several different recognition elements (REs) may be utilized, including specific small molecule or peptide ligands, antibodies or antibody fragments (*e.g.*, Fab and scFv fragments) and other proteins, including natural proteins and engineered proteins, as well as carbohydrates. Third, application of a localized magnetic field to a specific tissue may cause the retention of magnetic nanoarticles in this tissue. Fourth, application of an alternating magnetic field (AMF) of an appropriate strength and frequency may be directed to the pathogenic tissue and will heat the articles in the area of application, and less so systemically where the field is not applied, the heating causing the release of a bioactive agent included with the MTNP.

The MTNPs of the present invention may be used in the treatment of diseases such as cancer, inflammatory diseases, and infectious diseases. Several types of pathogenic tissues may be treated with the articles disclosed in the present invention containing appropriate bioactive agents. For instance, antibiotics may be incorporated for the treatment of fungal and bacterial infections, anti-inflammatory agents may be incorporated for the treatment of inflammatory diseases such as rheumatoid arthritis, and chemotherapeutic agents may be incorporated for the treatment of cancer.

In the case of chemotherapeutics, elevated temperature is known to increase the toxicity and anti-cancer potency of chemotherapeutics, so the localized heating generated by the magnetic material is expected to increase the potency of the released chemotherapeutic. The heating itself can result in the death of a portion of cancer cells in a tumor. Additionally, the heating of the tumor tissue is expected to aid in the permeation of the chemotherapeutic into the tumor, resulting in the killing of more cancer cells. Without an applied magnetic field AMF of sufficient strength and appropriate frequency range wherein the articles are heated, the articles of the invention will release the chemotherapeutic toxins to a lesser extent compared to articles subject to an alternating magnetic field. The toxicity of the chemotherapeutic both systemically and in certain organs, such as in the heart, liver, kidney,

and lung, can be substantially reduced by applying the alternating magnetic field locally to pathogenic tissue.

Advantageously, the location of the articles of the instant invention within the mammalian body may be determined using magnetic resonance imaging (MRI) of the superparamagnetic cores. In addition to the utility of enabling release of bioactive agents under the application of heat, preferably created by an AMF, and the utility of enhancing MRI images, the incorporation of iron oxides into the articles of the instant invention can be used advantageously in several additional ways. For instance, the superparamagnetic core allows for expedient purification of therapeutic agent-containing MTNPs from reactants after various reaction and fabrication steps. For instance, therapeutic-loaded MTNPs may be separated from unattached therapeutics by using a separation scheme wherein after the therapeutic incorporation procedure is completed, MTNPs are retained in a reaction vessel by a permanent magnetic field, while the solution (with therapeutic agents that are unassociated with the articles) is decanted off, siphoned off, or otherwise removed.

Thus, the invention is further directed to methods of using these MTNPs, as well as to methods of synthesizing the articles. The invention is also directed to a composition for treatment of pathogenic tissue comprising a plurality of MTNPs, each MTNP having a superparamagnetic material core and a polymeric outer layer and optionally, a bioactive agent and/or recognition elements, and to a therapeutic drug delivery system comprising such a MTNP-containing composition.

DETAILED DESCRIPTION OF THE INVENTION

The terms "a" and "an" mean "one or more" when used herein.

By "soluble" is meant, herein and in the appended claims, having a solubility in water of greater than 1 mg/mL, preferably greater than 10 mg/mL, and more preferably greater than 50 mg/mL.

Disclosed herein are MTNPs comprising magnetic material, a polymeric outer layer or scaffold encapsulating the magnetic material, and bioactive agents.

Preferred magnetic cores are superparamagnetic, which are magnetic only when a magnetic field is applied. Preferred superparamagnetic materials are iron oxides, such as magnetite, maghemite, and greigite.

The MTNP of the invention may be further comprised of targeting agents or recognition elements that bind to certain biomolecules found in pathogenic tissue, for instance cancerous tissue, such as certain cellular receptors that are overexpressed on the surface of some cancer cells, including growth factor receptors, or in the tumor vasculature, such as integrins or growth factor receptors. Targeting agents that can be used include, but

are not limited to, small molecules; vitamins, such as folate; peptides, such as those that target receptors; proteins, such as transferrin; and monoclonal antibodies and monoclonal antibody fragments. Unless otherwise specifically indicated, the terms "targeting agent" "recognition element" are used interchangeably herein.

While the MTNPs of the invention may be larger in size, they are preferably from about 5 nm to about 1000 nm, more preferably from about 10 nm to about 500 nm, in diameter. Because of their size and structure, the nanoarticles may circulate in the blood stream without being eliminated by the kidney or taken up by the reticulo-endothelial system system, and may localize in pathological tissue via passage through the pathological tissue's leaky vasculature; the incorporation of targeting agents can further increase article accumulation in tissue to be treated, as described below. Larger MTNPs may be better retained in a desired location in the body, for instance in pathological tissue, through the application of a high strength non-oscillating magnetic field.

Magnetorticles of the present invention can be made through the incorporation of bioactive agents, such as chemotherapeutic molecules or free radical-generating agents, with certain iron oxide articles. Iron oxides may be incorporated into the articles in several ways. Magnetic articles useful in the present invention include dextran-magnetite nanoparticles. These particles may be fabricated or may be purchased, for instance from companies such as Micromod Partikeltechnologie (Rostock, Germany). Magnetosomes from bacteria may also be used in the invention. In most magnetotactic bacteria, magnetosomes comprise membrane-bound crystals of magnetite, Fe_3O_4. Predominantly, members of the bacterial species *Magnetospirillum* form these highly ordered magnetite crystals to allow their orientation within the earth's magnetic field. The bacterial magnetosomes are characterized by narrow size distributions and uniform crystal habits, which are unknown from magnetite particles produced abiotically. Magnetosomes range in size from 35-100 nm.

Magnetic Colloid Formation: Magnetic cores, such as those comprised of magnetite or maghemite, can be formed using reverse microemulsions. For instance, Fe(II) is prepared by dissolving $FeSO_47H_2O$ in water and an Fe(III) solution is prepared by dissolving $FeCl_36H_2O$. These aqueous phases can then be combined in a reverse microemulsion. Addition of a base results in the formation of a magnetite colloid. This colloid can then be incorporated into a polymeric matrix as described below. Prior to incorporation into the polymer matrix, the magnetic colloid can be encapsulated in silica, and may be further functionalized via alkoxysilane derivatives to provide desired surface functionalities, such as acids and amines that can provide greater water solubility, covalent ligation to chemo-loaded

articles, and/or facilitate hydrogel incorporation. Silica encapsulation is discussed by Grasset, et.al. in *European Cells and Materials*, Vol. 3, Suppl. 2, 2002, pp. 110-113.

The iron oxide colloids may also be formed following the procedure published by Hyeon, et al. (*J Am Chem Soc* (2001) **123** 12798-12801). Compared to other methods, this method allows careful control of the iron oxide surface chemistry. This method results in oleic acid coupled to the surface of the iron oxide with a carboxylate moiety forming coordination bonds with iron atoms on the surface of the iron oxide core. The hydrocarbon chain of the oleic acid is then exchanged for a water-soluble coating, for instance with hydrophilic polycarboxylates, to provide for aqueous solubility.

Article Scaffold Fabrication in Reverse Microemulsions: Components of the present invention can be fabricated using reverse microemulsions. In one embodiment, magnetic colloids are formed in the dispersed aqueous phase of a reverse microemulsion. Next, polymer scaffolds are formed around the magnetic core through the addition of hydrophilic building blocks to the reverse microemulsion containing the magnetic colloids. The hydrophilic building blocks are then polymerized, forming a magnetic colloid-containing hydrogel nanoarticle. The organic solvent and non-reactive surfactants are removed after polymerization to yield crosslinked, water-soluble, iron oxide-cored nanoscopic articles. Bioactive agents, if included in the final nanoarticle, may be incorporated either during or after hydrogel formation.

As used herein, the terms "nanoarticle scaffold", "hydrogel scaffold" and "scaffold" are used interchangeably and refer to the portion of the nanoarticle (the polymeric matrix structure) that incorporates the magnetic material.

Reverse microemulsions for magnetic colloid and scaffold fabrication are formed by combining aqueous buffer or water, building blocks, organic solvent, surfactants and initiators in the appropriate ratios to yield a stable phase of surfactant-stabilized aqueous nanodroplets dispersed in a continuous oil phase. Stable reverse microemulsion formulations can be found using known methods by those skilled in the art. They are discussed, for example, in *Microemulsion Systems*, edited by H. L. Rosano and M. Clausse, New York, N.Y., M. Dekker, 1987; and in *Handbook of Microemulsion Science and Technology*, edited by P. Kumar and K.L. Mittel, New York, N.Y., M. Dekker, 1999. In this invention, an aqueous phase with solubilized hydrophilic building blocks is added to an organic solvent containing one or more solubilized surfactants to form a reverse microemulsion.

The dispersed aqueous phase includes hydrophilic building blocks solubilized at about 1 to about 65 wt%, preferably about 5 to about 25 wt%, most preferably 10 to 20 wt%. While not wishing to be bound by theory, the use of high water-content hydrogel scaffolds

also may reduce immunogenicity in end uses, because there is less foreign surface for immune system components to recognize. The high water content also provides compliancy through a more flexible scaffold. Thus, when attaching to cell surface receptors, the articles are able to conform to the cell surface, allowing more surface receptors to be bound. Binding more receptors may allow the article to better function to localize in desired tissues and bind to desired cells. Additionally, while not wishing to be bound by theory, it is believed that article cell surface coverage can inhibit other cell signaling pathways.

Polymerization of the building blocks in the nanodroplets of the dispersed aqueous phase of the reverse microemulsion follows procedures known to those skilled in the art (see, for example, Odian G.G., *Principles of Polymerization, 3rd Ed.*, Wiley, New York, 1991; L.H. Sperling, *Introduction to Physical Polymer Science*, Chapter 1, pp. 1-21, John Wiley and Sons, New York, 1986; and R.B. Seymour and C.E. Carraher, *Polymer Chemistry*, Chapters 7-11, pp. 193-356, Dekker, New York, 1981). Polymerization has been performed in the dispersed phase of microemulsions and reverse microemulsions (for a review, see Antonietti, M.; and Basten, R., Macromol. *Chem. Phys.* 1995, 196, 441; for a study of the polymerization of a hydrophilic monomer in the dispersed aqueous phase of a reverse microemulsion, see Holtzscherer, C.; and Candau, F., *Colloids and Surfaces*, 1988, 29, 411). Such polymerization may yield articles in the 5 nm to 50 nm size range.

The size of the nanodroplets of the dispersed aqueous phase is determined by the relative amounts of water, surfactant and oil phases employed. Surfactants are utilized to stabilize the reverse microemulsion. These surfactants do not include crosslinkable moieties; they are not building blocks. Surfactants that may be used include commercially available surfactants such as Aerosol OT (AOT), polyethyleneoxy(n)nonylphenol (Igepal™, Rhodia Inc. Surfactants and Specialties, Cranbrook, NJ), sorbitan esters including sorbitan monooleate (Span® 80), sorbitan monolaurate (Span® 20), sorbitan monopalmitate (Span® 40), sorbitan monostearate (Span® 60), sorbitan trioleate (Span® 85), and sorbitan tristearate (Span® 65), which are available, for example, from Sigma (St Louis, MO). Sorbitan sesquioleate (Span® 83) is available from Aldrich Chemical Co., Inc. (Milwaukee, WI). Other surfactants that may be used include polyoxyethylenesorbitan (Tween®) compounds, including polyoxyethylenesorbitan monolaurate (Tween® 20 and Tween® 21), polyoxyethylenesorbitan monooleate (Tween® 80 and Tween® 80R), polyoxyethylenesorbitan monopalmitate (Tween® 40), polyoxyethylenesorbitan monostearate (Tween® 60 and Tween® 61), polyoxyethylenesorbitan trioleate (Tween® 85), and polyoxyethylenesorbitan tristearate (Tween® 65), which are available, for example, from Sigma (St Louis, MO). Other exemplary commercially available surfactants include polyethyleneoxy(40)-sorbitol hexaoleate ester (Atlas G-1086, ICI Specialties, Wilmington

DE), hexadecyltrimethylammonium bromide (CTAB, Aldrich), and linear alkylbenzene sulfonates (LAS, Ashland Chemical Co., Columbus, OH).

Other exemplary surfactants include fatty acid soaps, alkyl phosphates and dialkylphosphates, alkyl sulfates, alkyl sulfonates, primary amine salts, secondary amine salts, tertiary amine salts, quaternary amine salts, n-alkyl xanthates, n-alkyl ethoxylated sulfates, dialkyl sulfosuccinate salts, n-alkyl dimethyl betaines, n-alkyl phenyl polyoxyethylene ethers, n-alkyl polyoxyethylene ethers, sorbitan esters, polyethyleneoxy sorbitan esters, sorbitol esters and polyethyleneoxy sorbitol esters.

Other surfactants include lipids, such as phospholipids, glycolipids, cholesterol and cholesterol derivatives. Exemplary lipids include fatty acids or molecules comprising fatty acids, wherein the fatty acids include, for example, palmitate, oleate, laurate, myristate, stearate, arachidate, behenate, lignocerate, palmitoleate, linoleate, linolenate, and arachidonate, and salts thereof such as sodium salts. The fatty acids may be modified, for example, by conversion of the acid functionality to a sulfonate by a coupling reaction to a small molecule containing that moiety, or by other functional group conversions known to those skilled in the art.

Additionally, polyvinyl alcohol (PVA), polyvinylpirolidone (PVP), starch and their derivatives may find use as surfactants in the present invention.

Cationic lipids may be used as cosurfactants, such as cetyl trimethylammonium bromide/chloride (CTAB/CTAC), dioctadecyl dimethyl ammonium bromide/chloride (DODAB/DODAC), 1,2-diacyl-3-trimethylammonium propane (DOTAP), 1,2-diacyl-3-dimethyl ammonium propane (DODAP), [2,3-bis(oleoyl)propyl] trimethyl ammonium chloride (DOTMA), and [N-(N'-dimethylaminoethane)-carbamoyl]cholesterol, dioleoyl) (DC-Chol). Alcohols may also be used as cosurfactants, such as propanol, butanol, pentanol, hexanol, heptanol and octanol. Other alcohols with longer carbon chains may also be used.

Polymer Encapsulant Formation in Reverse Microemulsions: In one embodiment of the invention, a crosslinked polymeric scaffold that incorporates magnetic material is formed by crosslinking builiding blocks around iron oxide colloids in the dispersed aqueous phase of a reverse microemulsion. Hydrophilic building blocks with polymerizable groups are employed to form stable nanoarticle scaffolds. In this embodiment, preferred building blocks are comprised of carbohydrate or derivatized carbohydrate. For example, the carbohydrate region may be derived from simple sugars, such as N-acetylglucosamine, N-acetylgalctosamine, N-acetylneuraminic acid, neuraminic acid, galacturonic acid, glucuronic acid, ioduronic acid, glucose, ribose, arabinose, xylose, lyxose, allose, altrose, apiose, mannose, gulose, idose, galactose, fucose, fructose, fructofuranose, rhamnose,

arabinofuranose, and talose; a disaccharide, such as maltose, sucrose, lactose, or trehalose; a trisaccharide; a polysaccharide, such as cellulose, starch, glycogen, alginates, inulin, pullulan, dextran, dextran sulfate, chitosan, glycosaminoglycans, heparin, heparin sulfate, hyaluronates, tragacanth gums, xanthan, other carboxylic acid-containing carbohydrates, uronic acid-containing carbohydrates, lactulose, arabinogalactan, and their derivatives, and mixtures of any of these; or modified polysaccharides. Other representative carbohydrates include sorbitan, sorbitol, chitosan and glucosamine.

The carboxyl, amine and hydroxyl groups of the carbohydrates can be modified, or replaced, to include crosslinking groups, other functionalities, or combinations thereof.

Carbohydrate-based building blocks may be prepared from the carbohydrate precursor (e.g., sucrose, inulin, dextran, pullulan, etc.) by coupling technologies known in the art of bioorganic chemistry (see, for example, G Hermanson, *Bioconjugation Techniques*, Academic Press, San Diego, 1996, pp 27-40,155, 183-185, 615-617; and S. Hanesian, *Preparative Carbohydrate Chemistry*, Marcel Dekker, New York, 1997). For example, a crosslinkable group may be attached to a carbohydrate via the dropwise addition of acryloyl chloride to an amine-functionalized sugar. Amine-functionalized sugars can be prepared by the reaction of ethylene diamine (or other amines) with 1,1'-carbonyldiimidazole-activated sugars. Ester-linked reactive groups can be synthesized through the reaction of acrylic or methacrylic anhydrides with the hydroxyl group of a carbohydrate such as inulin in pyridine. Aldehyde- and ketone-functionalized carbohydrates can be obtained by selective reduction of the sugar backbone or addition of a carbonyl-containing moiety. Other reactions that introduce an amine on the carbohydrate may also be used, many of which are outlined in *Bioconjugation Techniques (supra)*.

Carbohydrate-based building blocks may also be prepared by the partial (or complete) functionalization of the carbohydrate with moieties that are known to polymerize under free radical conditions. For example, methacrylic esters may be placed on a carbohydrate at varying substitution levels by the reaction of the carbohydrate with methacrylic anhydride or glycidyl methacrylate (Vervoort, L.; Van den Mooter, G.; Augustijins, P.; Kinget, R. *International Journal of Pharmaceutics,* 1998, 172, 127-135).

Carbohydrate-based building blocks may also be prepared by chemoenzymatic methods (Martin, B. D. et. al., *Macromolecules*, 1992, 25, 7081), for example in which *Pseudomonas cepacia* catalyzes the transesterification of monosaccharides with vinyl acrylate in pyridine or by the direct addition of an acrylate (Piletsky, S., Andersson, H., Nicholls, *Macromolecules*, 1999, *32*, 633-636). Other functional groups may be present, as numerous derivatized carbohydrates are known to those familiar with the art of carbohydrate chemistry.

Carbohydrates may be derivatized with allyl functionalities, including acrylates, methacrylates, acrylamides and methacrylamides to produce compounds such as inulin multi-methacrylate (IMMA). In a presently preferred embodiment, inulin with an average degree of polymerization (DOP) of about 10 to about 20 is used. The extent to which inulin is functionalized with methacrylate moieties, that is, the number of hydroxyl moieties on inulin that are converted to methacrylic esters to produce IMMA, is a statistical process governed by the concentrations and weight ratios of inulin and methacrylic anhydride starting material. The extent of functionalization may range from one methacrylate for every 1 to 100 monosaccharide repeat units, more preferably one methacrylate for every 3 to 20 monosaccharide repeat units. The number of monosaccharide repeat units in the IMMA may be from about 1 to about 100 or more, and is preferably from about 5 to about 50. The ester linkage to inulin may advantageously function as a site of degradation *in vivo*, allowing the article to degrade and be cleared from the body. Dextran multimethacrylamide and pullulan multimethacrylamide are additional preferred building blocks that may be prepared using methods similar to those for preparing IMMA. MTNP scaffolds may also be formed using inulin multibenzaldehyde or oxidized dextran, each of which may be synthesized by methods known in the art.

The carbohydrate structures are chosen in part for their hydrophilicity. Nano-articles that incorporate magnetic cores must possess highly hydrophilic scaffolds in order that high water solubility is maintained. MTNPs of the invention in one embodiment have a high water content for high water solubility. "High water content", as used herein, means an article comprised of about 65 to about 98 wt% water, more preferably about 75 to about 98 wt% water, and most preferably about 80 to 97 wt% water. Thus, the amount of breakdown products is less than articles with a higher polymer concentration. The high water content scaffolds also can reduce immunogenicity, because there are fewer surfaces for immune system components to interact with.

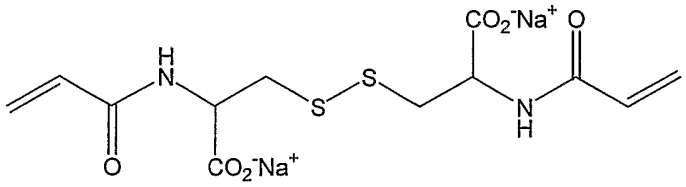
Besides carbohydrate-based building blocks, other examples of acrylate- or acrylamide-derivatized polymeric building blocks include polyethylene glycol-based molecules, such as polyethyleneglycol multiacrylates of molecular weights ranging from 200 to 10,000 daltons.

In one embodiment of the invention, to facilitate metabolism of the MTNP scaffold, degradable linkages are included within the crosslinked scaffold. Degradable linkages can be included through the use of polylactide, polyglycolide, poly(lactide-co-glycolide), polyphosphazine, polyposphate, polycarbonate, polyamino acid, polyanhydride, and polyorthoester – based building blocks, among others. Additionally, degradable linkages may be used to attach polymerizable moieties to carbohydrates. For instance, inulin multi-

methacrylate (IMMA) contains ester moieties that connect the inulin carbohydrate backbone to the alkyl chain that is formed upon free radical polymerization used to generate the scaffold of the present invention. Additionally, small molecule crosslinking agents containing similar hydrolyzable moieties as the polymers such as carbonates, esters, urethanes, orthoesters, amides, imides, imidoxy, hydrazides, thiocarbazides, and phosphates may be used as building blocks. To function as degradable components in the hydrogel scaffold, these building blocks must be functionalized with two or more polymerizable moieties. For example, polyglycolide diacrylate, polyorthoester diacrylate and acrylate-substituted polyphosphazine, acrylate-substituted polyamino acid, or acrylate-substituted polyphosphate polymers can be used as degradable building blocks.

Methacrylate or acrylamide moieties can be employed instead of acrylate moieties in the above examples. Similarly, small molecules containing a hydrolyzable segment and two or more acrylates, methacrylates, or acrylamides may be used. Such degradable polymers and small molecule building blocks may be functionalized with acrylate, methacrylate, acrylamide or similar moieties by methods known in the art.

Other agents can also be incorporated into the polymer matrix. These agents or "functional building blocks" have reactive groups, and such functional building blocks include, but are not limited to, N,N'-cystinebisacrylamide (CiBA), sodium acrylate (NaA), N-(3-aminopropyl)methacrylamide hydrochloride (APMA), N[ethylamino]-3-amino-propylmethacrylamide hydrochloride, polyethylene imine (PEI), polylysine, polyamido-acrylamide derivatives, and protamine sulfate. The composition of the nanoarticles can be manipulated using functional building blocks to produce articles with a desirable characteristic, such as charge (positive, negative or neutral) or degree of crosslinking. Additionally, functional building blocks may be chosen to achieve a desired content of certain functionalities in the article scaffold. Such functionalities can improve solubility and may also be used as points of attachment for REs or PEG chains. For instance, APMA may be used to introduce amines, sodium acrylate may be used to introduce carboxylates, and diacetone acrylamide (DAA) may be used to incorporate ketones. The disulfide linkage of the CiBA monomer, which has the following formula I, provides, after reduction, free thiols for linker attachment.



I

The MTNP scaffolds and the scaffold breakdown products of this invention are designed to be non-toxic and eliminated from the body. They may have degradable, preferably carbohydrate-based, polyamino acid-based, polyester-based, or PEG-based cores, with the rate of degradation controlled by the identity of the sugar, crosslink density, and other features. Thus, the articles can be metabolized in the body, preventing undesirable accumulation in the body.

Polymer Encapsulant Formation in Aqueous Solutions. In one preferred embodiment, polycarboxylate chains of a polymer scaffold are attached via coordination bonds of a fraction of the carboxylate moieties to iron atoms in the iron oxide colloid core. This attachment can be accomplished in an aqueous solution.

Incorporation of Bioactive Agent Molecules into Articles: The terms "bioactive agent" and "therapeutic agent" are used interchangeably herein and include, but are not limited to, chemotherapeutics, cytotoxics, free radical-generating agents, other drugs and toxic agents, and other therapeutic agents. A MTNP may comprise from one to up to 100 or more bioactive agents. A "plurality of bioactive agents" in a MTNP may all be the same bioactive agent or they may be two or more different bioactive agents; that is, the MTNP may comprise a plurality of one or more types of bioactive agents.

Drugs that may find use in the present invention include those that act on the peripheral nerves, adrenergic receptors, cholinergic receptors, nervous system, skeletal muscles, cardiovascular system, smooth muscles, blood circulatory system, synaptic sites, neuro-effector junctional sites, endocrine system, hormone systems, immunological system, reproductive system, skeletal system, autocoid systems, alimentary and excretory systems, histamine systems, respiratory system, reticuloendothelial system, skeletal system, skeletal muscles, smooth muscles, immunological system, reproductive system, cancerous tissues, and the like. The active drug that can be delivered for acting on these recipients includes, but is not limited to, anticonvulsants, analgesics, anti-parkinsons, anti-inflammatories, calcium antagonists, anesthetics, antimicrobials, antimalarials, antiparasitics, antihypertensives, antihistamines, antipyretics, alpha-adrenergic agonists, alpha-blockers, biocides, bactericides, bronchial dilators, beta-adrenergic blocking drugs, contraceptives, chemotherpeutics, cardiovascular drugs, calcium channel inhibitors, depressants, diagnostics, diuretics, electrolytes, enzymes, hypnotics, hormones, hypoglycemics, hyperglycemics, muscle contractants, muscle relaxants, neoplastics, glycoproteins, nucleoproteins, lipoproteins, ophthalmics, psychic energizers, sedatives, steroids,

sympathomimetics, parasympathomimetics, tranquilizers, urinary tract drugs, vaccines, vaginal drugs, vitamins, nonsteroidal anti-inflammatory drugs, angiotensin converting enzymes, polynucleotides, polypeptides, polysaccharides, and the like.

In a presently preferred embodiment, drugs that may be advantageously employed in the present invention include, but are not limited to, chemotherapeutics such as cisplatin, oxaliplatin, doxorubicin, paclitaxel, gemcitibine, vincristine, chlorambucil, topotecan, methotrexate, bortezomib, and any other FDA-approved chemotherapeutic, as well as molecules that may act as chemotherapeutics but that are not yet commercialized, and derivatives and analogues of all of the above chemotherapeutics.

For example, the chemotherapeutic doxorubicin may be attached to the scaffold through a EDC coupling reaction between the amine moiety on doxorubicin and a carboxylic acid moiety included in the hydrogel scaffold, for example by using sodium acrylate (NaA), malonate acrylamide (MalAc) or N,N'-cystinebisacrylamide (CiBA; synthesis described in PCT Publn. WO 03/101425) as a building block. In another embodiment, doxorubicin may be attached via an imine bond by reacting doxorubicin's amine moiety with an aldehyde moiety of the hydrogel scaffold. An aldehyde may be created by first using a carbohydrate-based building block to form the article, and then oxidizing the carbohydrate after the article is formed. In another embodiment, doxorubicin may be attached to the article matrix through its ketone moiety.

Carbohydrazide or other dihydrazide or di-amino-oxy functionalized structures may be used to link doxorubicin to a scaffold that contains an aldehyde or ketone through the formation of a hydrazone bond. An aldehyde or ketone may be incorporated into the scaffold through the use of a ketone-containing acrylate building block such as diacetone acrylamide (DAA). A hydrazone bond may favorably release the therapeutic compound under the mildly acidic physiological conditions encountered upon article endocytosis and entrance into lysosomes.

In another embodiment, nanoarticle scaffolds comprised of amino groups, for example through the inclusion of N-(3-aminopropyl)methacrylamide (APMA) or methacrylate-functionalized short peptide (prepared according to US patent 5,037,883) building blocks, may be used to covalently attach cyclosporins that contain carboxylate linkages. Cyclosporin drugs may find applications for pathologies that benefit from immunosuppression, such as inflammatory diseases, and for organ transplantation.

In another embodiment, nanoarticle scaffolds comprised of aldehyde or ketone groups (for example incorporated through the inclusion of DAA, levulinic acrylamide or oxidized carboxylates such as inulin or dextran building blocks) may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance calicheamicin, through the

use of a hydrazone coupling scheme. This coupling scheme results in a hydrazone bond that is hydrolytically labile, especially at low pH found in lysosomes (Bernstein I., et al., *Bioconjugate Chem.*, 2002, 13, 40-46).

In another embodiment, nanoarticle scaffolds comprised of acid or anhydride groups, for example incorporated through the inclusion of sodium acrylate or anhydride building blocks, may be used to covalently attach dexamethasone, through the use of an amide coupling scheme.

Nanoarticle scaffolds comprised of carboxylate groups (for example, incorporated through the inclusion of NaA, CiBA or MAlAc building blocks) may be used to covalently attach drugs or drug derivatives that contain an amine moiety, for instance peptide-modified camptothecin (Frigerio E., et al., *J. Controlled Release*, 2000, 65, 105-119) through an EDC-NHS coupling scheme. Carboxylate moieties can also be employed to form coordination bonds with platinum atoms found in bioactive platinum molecules.

In another embodiment, when the nanoarticle scaffolds are comprised of aldehyde or ketone groups (which may be incorporated through the use of DAA, levulinic acrylamide or oxidized carboxylates such as inulin or dextran), a drug or drug derivative possessing an amine, such as gemcitabine, may be incorporated through the use of a "Schiff base" coupling scheme. The imide bond formed from the attachment of gemcitabine to DAA can be cleaved in acidic media. During internalization, the drug is taken up by the cell, where it is exposed to the acidic environment of the lysosome, thereby releasing gemcitabine in its unmodified form.

In another embodiment, nanoarticle scaffolds comprised of carboxyl groups (for example, incorporated through the inclusion of CiBA, MalAc or NaA building blocks) may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance salicylic acid, through the use of an EDC-NHS coupling scheme. For instance, the hydroxyl group of salicylic acid will react with the carboxyl group of the CiBA, MalAc or NaA to form an ester link. Hydrolysis or the enzyme esterase will cleave the ester bond between salicylic acid and the carboxylic acid groups of CiBA or NaA, releasing salicylic acid in an unmodified form.

In another embodiment, a drug structure may be modified to facilitate attachment to a nanoarticle scaffold. For instance, the 2'-hydroxyl group of paclitaxel can be reacted with multiple linkers that enable the coupling to nanoarticle scaffolds. For example, the acid moiety of a resin-immobilized glycine linker can be attached to paclitaxel using a carbodiimide; the resulting compound can be cleaved at the site of the amine using 1% TFA, producing a free amine which can be conjugated with nanoarticles possessing carboxylates using an EDC coupling scheme.

In another embodiment, paclitaxel-2'-succinate (Deutsch H., et al., *J Med. Chem.*, 1989, 32, 788-792) conjugation to the nanoarticle is possible using a carbodiimide-mediated amide coupling. This coupling occurs between the paclitaxel-2'-succinate group and an amine group of the APMA component of the nanoarticle to form a labile ester.

In another embodiment, the nanoarticle can be directly coupled to paclitaxel by reacting the acid-functionalized (e.g., NaA) nanoarticle to the 2'-hydroxyl group of paclitaxel. This chemical pathway has been previously described using a poly(L-glutamic acid)-paclitaxel conjugate (Li H., et al., Cancer Res., 1998, 58, 2404-2409).

In another embodiment, nanoarticle scaffolds containing carboxylic acids, for example incorporated through the inclusion of NaA building blocks, may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance 5-flourouracil (5FU) (or derivatives) through the use of an amide-forming coupling reaction between an amine-functionalized 5FU derivative and the carboxylic acids located on the nanoarticle. The synthesis of 1-alkylcarbonyloxymethyl derivatives of 5FU has been previously described and those materials have been demonstrated to release 5FU in an unmodified form (Taylor H.E.; Sloan K.B., *Journal of Pharmaceutical Sciences*, 1998, 87, 15). The application of this synthetic route will yield the necessary amine-functionalized 5FU, whilst realizing a similar release profile.

Nanoarticle scaffolds comprised of carboxylate groups (for example incorporated through the inclusion of CiBA, MalAc and NaA building blocks) may be further used to covalently attach drugs or drug derivatives that contain a carboxylate moiety, for instance methotrexate, by first coupling the drug or drug derivative to boc-protected ethanolamine to form an ester, and then coupling to the nanoarticle through an EDC coupling scheme after deprotecting the modified drug. This ester conjugate is known to hydrolyze at low pH, releasing the drug in its original form (Wilson J.M., et al., *Biochem Biophys. Res. Commun.*, 1992, 184, 300-305; Ohkuma S., Poole B., *Proc. Natl. Acad. Sci. USA*, 1978, 75, 3327-3331). Such conditions of low pH are found in cellular lysosomes. These nanoarticles may find use in the treatment of multiple pathologies, including cancer and inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease.

In another embodiment, platinum is complexed to the nanoarticle polymer matrix via O,N-ligation, which is expected to yield a more stable compound. This can be accomplished preferentially for nanoarticles obtained by free-radical polymerization, and containing a combination of acid functions (such as from NaA) and amines (such as from APMA) or amide moieties (such as acrylamide), or building blocks carrying both types of functions such as CIBA, MalAc or methacryloylate-functionalized short peptides made according to US

5,037,883. Such moieties provide attachment points to generate a O,N-cis platinum nanoarticle conjugate, and also allow the possibility of targeting the nanoarticle.

In all embodiments, the bonds attaching the bioactive agents to the MTNP scaffold are heat-labile and are substantially disrupted at moderate temperatures, such that the bioactive agents are released from the articles upon application of moderate heat.

Platinum Incorporation into Polycarboxyate Scaffold. In a preferred embodiment an iron oxide colloid is encapsulated by a polymer-platinum complex. The polymer is preferably a polycarboxylate comprised of multiple carboxylate moieties. As described previously herein, the carboxylates can bind to surface iron atoms. In addition, carboxylates that are not bound to surface iron atoms can be used to form coordination bonds to platinum molecules.

As referred to herein, "platinum", typically used in the context of a platinum complex or compounds, refers to a platinum metal atom bound to one or more ligands. The platinum atom may carry a formal charge, such as in the case of platinum salts such as K_2PtCl_4, potassium tetrachloroplatinate, in which the platinum carries a formal charge of (-2), or may carry no formal charge, as in cisplatin, $PtCl_2(NH_3)_2$. The platinum metal atom may exist in various oxidation states, such as Pt(0), Pt(II), or Pt(IV). The platinum species can be in any coordination state, but is typically four-coordinate for Pt(II) complexes and six-coordinate for Pt(IV) complexes.

Presently preferred platinum chemotherapeutic agents are in the II^{nd} or IV^{th} oxidation state. In one preferred embodiment, platinum (II) compounds are incorporated into the article through carboxylate coordination bonds with the polymer scaffold and can, preferably, act as a crosslinking agent. Preferred platinum (II) compounds are of the general formula cis-$[PtX_2(NHR_1R_2)(NHR_3R_4)]$ where X is an anion, such as chloride or nitrate ion, where one or both of the anions coordinated with a particular platinum atom are displaced by carboxylate ligands in the process of incorporation into the nanoarticles of the invention. Each of R_1, R_2, R_3, and R_4 is independently selected from the group consisting of hydrogen, lower alkyl unsubstituted or substituted with a halo group or an alkyl group, lower alkenyl unsubstituted or substituted with a halo group or an alkyl group, lower cycloalkyl unsubstituted or substituted with a halo group or an alkyl group, and lower cycloalkenyl unsubstituted or substituted with a halo group or an alkyl group, or R_1 and R_2 together form an alkyl or an alkenyl bridge, or R_3 and R_4 together form an alkyl or alkenyl bridge.

In another embodiment of the invention, multinucleate platinum agents are incorporated into the nanoarticles. Such agents may form more stable networks by forming three or more coordination bonds with polyanion components.

Preferred scaffold polymers for platinum incorporation are comprised of multiple carboxylate moieties ("polycarboxylates"). The carboxylate moieties of this polymer can serve multiple purposes, first to form coordination bonds with platinum and with the magnetic colloids, as well as play an active role in favoring the nanoarticle localization and/or preferential uptake by cancer cells, or have intrinsic antiproliferation, antiangiogenic or general anticancer properties.

Polycarboxylate materials that may be used in the instant invention include carbohydrates with each chain comprised of multiple carboxylate moieties. Polycarboxylate materials that may be used in the instant invention include acrylic polymers with carboxylate side chains, such as polyacrylate and polymethacrylate. Polyethylene glycol can be attached to polycarboxylates to allow extended circulation, reduced binding by opsonization-promoting biomolecules and reduced reticulo-endothelial system uptake *in vivo*, as is well-described in the literature, and also serve as a tether for attaching targeting ligands or bioactive agent-containing nanoarticles to the MTNPs. Preferred copolymers for use in the instant invention are polyethylene glycol (PEG) – polycarboxylates, more preferably polyethylene glycol – poly(meth)acrylates, and most preferably polyethylene glycol – polymethacrylate (sodium salt) or polyethylene glycol – polyacrylate (sodium salt). For the PEG chain, a preferred molecular weight is in the range of 2000 to 10,000 Daltons. For the acrylic polymer, a preferred molecular weight is in the range of 2000 to 6000 Daltons.

Preferred carbohydrate polycarboxylates are inulin derivatives, polysialic acid, hyaluronic acid, and colominic acid. Pectins and alginates, both multi-acid carbohydrates that have been used for various surgical/device/delivery purposes, are avoided due to their high viscosity and lack of information on their use in circulating materials. Additional carbohydrates, particularly those with targeting or anti-cancer properties unto themselves, such as schizophyllan, also may be employed.

Inulin, consisting mainly of linear β–1,2 linked polyfructose with a glucopyranose unit at the reducing end, has been used extensively as an i.v. injection to assess kidney function. It can be readily modified with carboxylate groups through the use of cyclic anhydrides such as succinic anhydride and aconitic anhydride. Presently preferred functionalization levels include one acid group per saccharide repeat. Inulin with 10 repeats (that is, Degree of Polymerization (DP) = 10) to DP = 70 or higher may be used.

Polysialic acid (α-2,8 linkage) is very water soluble, behaving similarly to inulin. Polysialic acid is commercially available at a MW of 10,000 Da, which is a good size for constructing the nanoarticles of the instant invention. Advantages suggesting use are its natural occurrence in humans and its use as "nature's stealth" by bacteria.

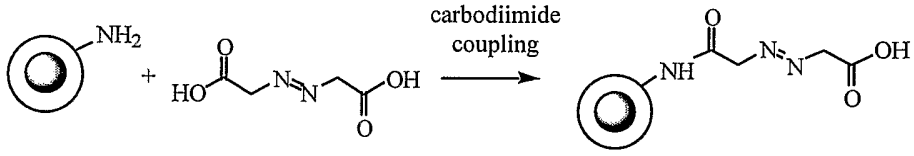
Hyaluronic acid, a β-1,4 linked D-glucuronic acid and β-1,3 linked N-acetyl-D-glucosamine polysaccharide, is commonly used for ophthalmic surgery and as an injectable into joints to ease osteoarthritis. Several types of cancer strains are characterized by an altered metabolism of hyaluronic acid, which can be used for advantageous purposes. In particular, some cancer cells overexpress the receptor CD-44 and some of its more active variants, which bind and in some cases rapidly internalize hyaluronic acid. Tumors in their invasive or metastasizing stages present increased expression of factors and receptors modifying their adhesion and motility abilities. One of such adhesion factors overexpressed in some cancerous phases is RHAMM, which also binds hyaluronic acid. Thus, the inclusion of hyaluronic acid in the MTNP scaffold may help localize the articles in these cancers. Furthermore, hyaluronan oligosaccharides have been shown to induce apoptosis and reduce tumor growth in vivo. Although not wishing to be bound by theory, we expect the degradation process of the nanoarticle to release such hyaluronan oligomers, which will compete with endogenous polymeric hyaluronans, leading to proapoptotic events. Finally, hyaluronans, especially shorter oligomers, have the potential to reverse multi-drug resistance, probably via interference with cell survival signaling pathways.

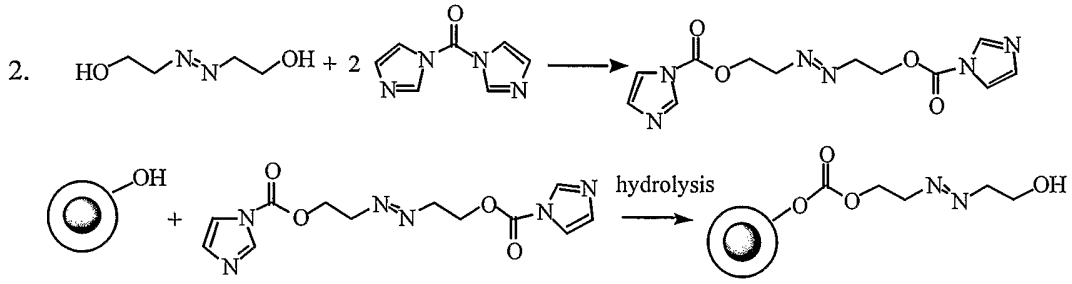
Additonal polycarboxylate polymers that may be used in the present invention include polyaminoacids where the amino acid chains are comprised of aspartic or glycolic acid residues.

One or more PEG chains can be attached to all of the above polycarboxylate polymers to form PEG-polycarboxylate copolymers. For the PEG chains, preferred molecular weights are in the range of 2000 to 10,000 Daltons. For the polycarboxylates, preferred molecular weight is in the range of 1000 to 25,000 Daltons, preferably 2500 to 10,000 Daltons.

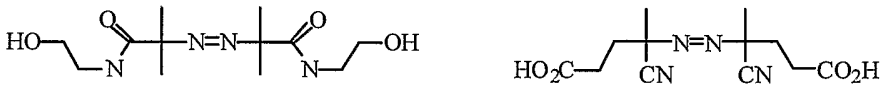
Radical-generating iron-oxide containing MTNPs: Polymer-coated magnetic nanoarticles that degrade to form radicals can be synthesized by two synthetic strategies. In the first strategy, radical-generating functionalities are attached to the polymeric matrix surrounding a MTNP (Reaction Scheme 1).

Reaction Scheme 1. Two synthetic routes which could be used to attach a radical generating species to a hydrogel coated nanoparticle.

1. 

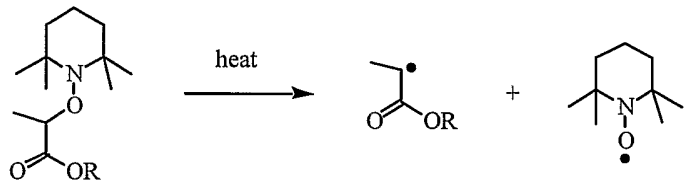


With the variety of temperature sensitivities that radical sources can display (see, e.g., Odian, G. *Principles of Polymerization* 2nd Edition, Wiley-Interscience, 1981, 196), a radical-producing structure that would be satisfactory for the temperature profile of the target product would be selected. Radical-generating functionalities, such as azo or peroxides, can be attached to the hydrogel through a variety of chemical bonds such as amides or carbonates. The exact strategy used would depend on the properties of the radical initiator being attached. For example, the use of peroxide radical sources would preclude the use of amine-containing hydrogels, because of the increased instability of those compounds in the presence of an amine. However, azo-containing compounds would be generally useful in either proposed attachment method. Two commercially available radical-generating azo compounds have been identified:



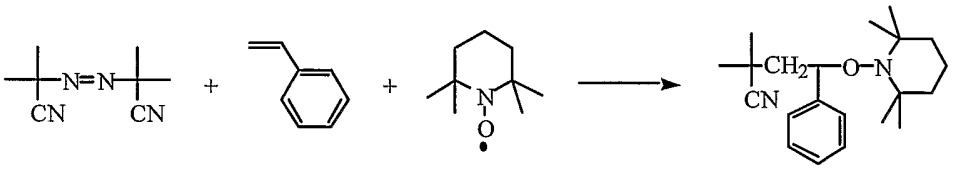
In the second strategy, the MTNP's polymeric scaffold itself degrades to form radical species. In this strategy, the unique reactivity of alkoxyamine compounds to generate radical species is used (for a general discussion of alkoxy amines, their thermal behavior, stability, and use in polymer chemistry, see Lizotte, J., "Synthesis and Characterization of Tailored Macromolecules via Stable Free Radical Polymerization Methodologies", *Dissertation Virginia Polytechnic Institute and State University*, 2003, Chapter 2) (Reaction Scheme 2).

Reaction Scheme 2. On heating, alkoxyamine compounds degrade to radical species. In this nonspecific example, the stable nitroxyl compound TEMPO is released.

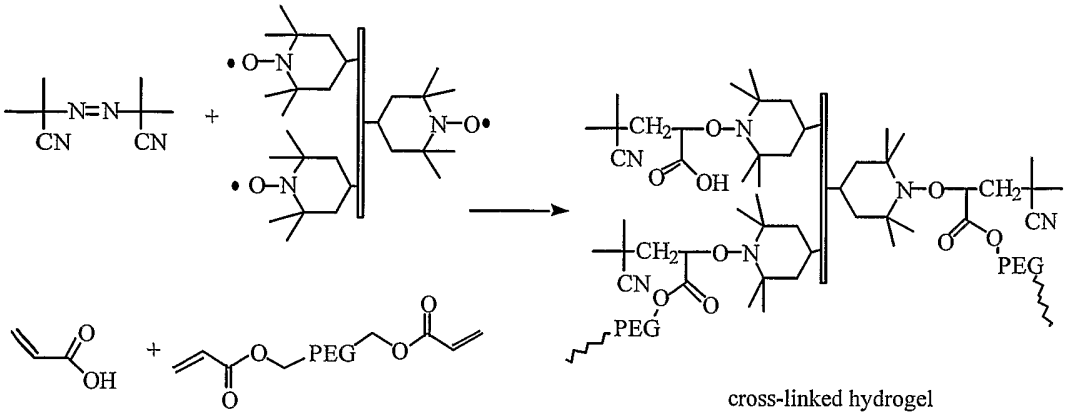


Magnetic colloids can be coated with an alkoxyamine-containing hydrogel by a strategy similar to that described above, using reverse microemulsions. In this procedure, a 2,2,6,6-tetramethyl-1-piperdinyloxy (TEMPO)-derivatized polysaccharide, radical initiator, acrylate-functionalized poly(ethylene glycol), and acrylic acid monomer are dissolved in the aqueous phase of the magnetic colloid-containing reverse microemulsion. Unlike typical polymerizations, the radical initiator would have a stoichiometry close to that of the total acrylate concentration of the monomers. On photolysis, the radical initiators initiate polymerization of the monomer species. However, the high concentration of the TEMPO nitroxyl (equal to that of the total acrylate concentration) would quickly react with the monomer-centered radical, making the stable alkoxy amine (Reaction Schemes 3 and 4).

Reaction Scheme 3. A representative reaction in which TEMPO is used to make a stable alkoxyamine (Hawker, C. et al., *Macromolecules*, 1996, 29, 5245). The alkoxyamine product, on heating, generates radical species, which will initiate a polymerization reaction.



Reaction Scheme 4. The reaction of a mixture containing a radical initiator, TEMPO-derivatized carbohydrate, acrylic acid, and PEG diacrylate. This reaction, if done in a reverse microemulsion containing a magnetic colloid, will make a hydrogel-coated nanoarticle.



The alkoxyamine-containing MTNPs, when heated, will degrade, releasing radicals.

Incorporation of Recognition Elements: MTNPs may be functionalized with recognition elements ("REs"). The REs can target a multitude of disease-associated biomolecules. Tumor-associated targets include folate receptors, transferrin receptors, erbB1, erbB2, erbB3, erbB4, CMET, CEA, EphA2, carcinoembryonic (CEA) antigen, mucin antigens, including Muc-1, cellular adhesion, of the cluster differentiation (CD) antigen family. Vascular targets associated with multiple pathologies, including cancer, include VEGFR-1, VEGFR-2, and integrins, including integrin αvβ3, and integrin αvβ1. Additional targets are extracellular proteins such as matrix metalloproteinases (MMPs), the collagen family, and fibrin.

The REs can be linked either directly or through a linker molecule to the nanoarticle. In a linker configuration, part or all of the REs are "displayed" at the end terminus of the tether. Therefore, in one application of the invention, the articles consist of REs displayed on a polymer scaffold. In another embodiment of the invention, the articles consist of an RE, such as a high affinity peptide, linked to the surface of the article core scaffold via a linker molecule, the linker comprising, in a preferred embodiment, polyethylene glycol (PEG). The PEG linker can be linear with reactive functionalities at both of the chain terminals; the PEG linker can also be multi-armed, for instance possessing three, four, five, six, eight arms or more, with two or more of the arms possessing reactive functionalities that can be used to attach the PEG to the nanoarticle scaffold and the RE to the PEG.

For each of these embodiments, it is possible to functionalize the articles with several coupling strategies, varying both the order of addition of the different components and the reactive chemical moieties used for the coupling.

The components may be attached to one another in the following sequences. The polymer scaffold is first reacted with a di-functional PEG-containing tether, followed by functionalization of the free terminus of a portion of the PEG chain with a RE. Alternatively, the RE is coupled first to the PEG-containing tether, followed by the attachment of the other PEG terminus to the scaffold.

Several combinations of reactive moieties can be chosen to attach the RE to the tether and to attach the tether to the nanoarticle scaffold. In using a series of orthogonal reaction sets, varying some of the scaffold building blocks and/or tethering arms, it is also possible to attach REs with different molecular structures that bind to different receptors, onto the same article scaffold in well-controlled proportions. Reactions using orthogonal reactive pairs can be done simultaneously or sequentially.

It is preferable to functionalize the articles in an aqueous system. The surfactants and the oil phase, residual from the synthesis of the polymer scaffold, can be removed through the use (singularly or in combination) of solvent washing, for instance using ethanol to solubilize the surfactant and oil while precipitating the articles; surfactant-adsorbing beads; dialysis; or the use of aqueous systems such as 4M urea. Methods for surfactant removal are known in the art.

The RE must contain a functionality that allows its attachment to the article. Preferentially, although not necessarily, this functionality is one member of a pair of chemoselective reagents selected to aid the coupling reaction (Lemieux, G., Bertozzi, C., *Trends in Biotechnology*, 1998, 16, 506-513). For example, when the article surface (and/or linkers grafted to its surface) displays a halo acetal, a peptide RE may be attached through a sulfhydryl moiety. A sulfhydryl moiety in the RE structure can be accomplished through inclusion of a cysteine residue.

Coupling is also possible between a primary amine on the article or the linker terminus and a carboxylic acid on the RE. A carboxylate in the peptide structure can be found either on its terminal amino acid, for linear peptides, or through the inclusion of aspartic or glutamic acid residues. The opposite configuration, where the carboxylic acid is on the article and a primary amine belongs to the peptide, is also easily accessible. Many polymerizable building blocks contain acidic moieties, which are accessible at the surface of the beads after their polymerization. As for poly(amino acid)-based REs, a primary amine function can be found either at its N-terminus (if it is linear) and/or via introduction of a lysine residue.

Another example of reactive chemical pairs consists of the coupling of a sulfhydryl with a halo acetal or maleimide moiety. The maleimide function can be easily introduced, either on a peptide, a linker, or the surface of the articles, by reacting other common

functionalities (such as carboxylic acids, amines, thiols or alcohols) with linkers through methods known to one of skill in the art, such as described for example by G. T. Hermanson in *Bioconjugate Techniques*, Academic Press Ed., 1996. In a preferred embodiment, the inclusion of CiBA or other disulfide-containing building blocks, in the scaffold facilitates the attachment of REs through thiol reactive moieties. After scaffold formation, reduction of the disulfide linkage in CiBA produces free thiols. Linker molecules containing groups that are reactive with thiol, such as bromoacetamide or maleimide, are added to the reduced therapeutic agent-containing article to attach the linker to the article scaffold. REs are then added, which react with the free terminus of the linker molecules to give RE-functionalized articles. Alternatively, the RE may be attached to one end of the linker molecule prior to attachment of the linker molecule to the reduced article.

Peptides can also be coupled to the article and/or the tether with a reaction between an amino-oxy function and an aldehyde or ketone moiety. The amino-oxy moiety (either on the articles or in the peptide) can be introduced, starting from other common functionalities (such as amines for example), by a series of transformations known to those skilled in the art. In the same way, aldehyde- or ketone-containing articles and aldehyde-containing peptides are readily synthesized by known methods.

The resulting RE-functionalized, bioactive agent-containing articles may be used immediately, may be stored as a liquid solution, or may be lyophilized for long-term storage.

The REs may be any small or large molecular structure that provides the desired binding interaction(s) with the cell surface receptors of the targeted molecule. The number of recognition element moieties per article can range from 2 to about 1000, preferably from 2 to 500, and most preferably from 2 to 100. The articles may optionally further be comprised of more than one type of RE. As used herein, a RE "type" is defined as a specific molecular structure.

In one embodiment REs are comprised of peptides. Peptides used as REs according to this invention will generally possess dissociation constants between 10^{-4} and 10^{-9} M or lower. Such REs may be comprised of known peptide ligands. For instance, Phoenix Peptides' peptide ligand-receptor library (http://www.phoenixpeptide.com/Peptidelibrarylist.htm) contains thousands of known peptide ligands to receptors of potential therapeutic value. The peptides may be natural peptides such as, for example, lactams, dalargin and other enkaphalins, endorphins, angiotensin II, gonadotropin releasing hormone, melanocyte-stimulating hormone, thrombin receptor fragment, myelin, and antigenic peptides. Peptide building blocks useful in this invention may be discovered via high throughput screening of peptide libraries (e.g. phage display libraries or libraries of linear sequences displayed on beads) to a protein of interest. Such

screening methods are known in the art (for example, see C.F. Barbas, D. R. Burton, J. K. Scott, G. J. Silverman, *Phage Display*, 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The high affinity peptides may be comprised of naturally-occurring amino acids, modified amino acids or completely synthetic amino acids. The length of the recognition portion of the peptide can vary from about 3 to about 100 amino acids. Preferably, the recognition portion of the peptide ranges from about 3 to about 15 amino acids, and more preferably from 3 to 10 amino acids. Shorter sequences are preferred because peptides of less than 15 amino acids may be less immunogenic compared to longer peptide sequences. Small peptides have the additional advantage that their libraries can be rapidly screened. Also, they may be more easily synthesized using solid-state techniques.

Particular peptides of interest are comprised of the amino acid sequence YCPIWKFPDEECY, or other sequences found in Greene, et.al., *J. Biol. Chem.*, 2002, 277(31), 28330-28339, that bind to erbB1; peptides comprised of the amino acid sequence CdFCDGFdYACYMDV, where dF and dY representing the D isomer of the amino acid residues or other sequences delineated in Murali, *J. Med. Chem.*, 2001, 44, 2565 - 2574, as REs; peptides disclosed in PCT WO 01/74849 that bind to CEA; and peptides comprised of the amino acid sequence ATWLPPR, as described in Demangel, et.al., *EMBO J.*, 2000, 19(7), 1525-1533.

REs may be comprised of a variety of other molecular structures, including vitamins such as folate, growth factors such as EGF, proteins such as transferrin, antibodies, antibody fragments, lectins, nucleic acids, and other receptor ligands. Humanized or fully human antibodies, and humanized or fully human antibody fragments are preferred for use in the present invention.

Additionally, it will be possible to design other non-protein compounds to be employed as the binding moiety, using techniques known to those working in the area of drug design. Such methods include, but are not limited to, self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics computer programs, all of which are well described in the scientific literature. See, Rein et al., *Computer-Assisted Modeling of Receptor-Ligand Interactions*, Alan Liss, New York (1989). Preparation of non-protein compounds and moieties will depend on their structure and other characteristics and may normally be achieved by standard chemical synthesis techniques. See, for example, *Methods in Carbohydrate Chemistry, Vols. I-VII*; Analysis and Preparation of Sugars, Whistler et al., Eds., Academic Press, Inc., Orlando (1962), the disclosures of which are incorporated herein by reference.

The use of multiple RE molecules of the same molecular structure or of different molecular structure to make up the article can increase the avidity of the article. As used in

the present invention, "high affinity" means a binding of a single RE to a single target molecule with a binding constant stronger than 10^{-4} M, while "avidity" means the binding of two or more such RE units to two or more target molecules on a cell or molecular complex. Use of two different REs to two different target molecules on the surface of a cell may have an advantage to select diseased tissue over normal tissue.

Pharmaceutical Compositions. For the herein-described uses, the MTNPs of the invention are provided as pharmaceutical preparations. The articles can be administered by injection (subcutaneous, intravenous, intramuscular, intradermal, intraperitoneal, intracerebral, or parenteral), with intraveneous injection being a preferred route. The articles may also be suitable for nasal, pulmonary, vaginal, ocular delivery and oral administration. A pharmaceutical preparation of a MTNP may be administered alone or in combination with pharmaceutically acceptable carriers, in either single or multiple doses. Suitable pharmaceutical carriers include inert solid diluents or fillers, sterile aqueous solution and various organic solvents. The pharmaceutical compositions formed by combining a nanoarticle of the present invention and the pharmaceutically acceptable carriers are then easily administered in a variety of dosage forms such as injectable solutions.

For parenteral administration, solutions of the nanoarticle in aqueous propylene glycol or in sterile aqueous solution may be employed. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic using, for example, saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy use with a syringe exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars such as mannitol or dextrose or sodium chloride.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents and the. like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the nanoarticle components, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Reagents and starting materials in some embodiments can be obtained commercially from chemical distributors such as Sigma-Aldrich (St Louise, MO and Milwaukee, WI), Kodak (Rochester, NY), Fisher (Pittsburgh, PA), Pierce Chemical Company (Rockford, IL), Carbomer Inc. (Westborough, MA), Radcure (Smyrna, GA), and Polysciences (Niles, IL). PEG compounds may be purchased through companies such as NOF America Corporation (White Plains, NY), and Nektar (Birmingham, AL). Peptides to be used as REs can be purchased from many sources, one being Bachem (King of Prussia, PA). Proteins may be obtained from sources such as Calbiochem (San Diego, CA).

Uses of MTNPs. The MTNPs of the present invention may be used for targeted bioactive agent delivery to either an in vivo or an in vitro environment. A plurality of bioactive agent-containing MTNPs are administered to the environment to be treated, and heat sufficient for release of bioactive agent from the MTNPs is applied to the environment. The bioactive agents are incorporated into the MTNP through heat-labile covalent or coordination linkages that are substantially disrupted at moderate temperatures (that is, temperatures above body temperature but lower than several hundred °C), causing the bioactive agents to be released from the article. Preferably, the MTNP is capable of releasing at least about 50% and preferably substantially all of its bioactive agent payload upon the application of heat, which in the locality of the MTNP is greater than 42 °C for longer than 10 minutes, and where the heat is preferably generated from within the iron oxide core through the application of an AMF.

Under the influence of a source of heat, the temperature of the MTNP is raised, resulting in an acceleration of release of bioactive agent molecules from the article and/or, where the nanoarticle includes free radical-generating components, the generation of free radicals. In a preferred embodiment, the source of heat is an applied magnetic field, most preferably an alternating magnetic field, the field being generated external to the environment in which the MTNPs have been administered. Additionally, as elevated temperature is known to increase the toxicity and anti-cancer potency of chemotherapeutics, the localized heating generated by the magnetic material can increase the potency of the released chemotherapeutic. This heating is expected to result in the death of at least a portion of cancer cells in a tumor. Additionally, the heating of the surrounding tissue is expected to aid in the diffusion of the chemotherapeutic or free radicals into the tumor, resulting in more complete cancer cell destruction. Without the application of the applied magnetic field, the articles of the invention will release the bioactive agents to a lesser degree and in a less controllable manner. Thus, with AMF application, the toxicity of the bioactive agent both systemically and in certain organs, such as the kidney, is reduced.

Utilizing the MTNPs of the present invention, it is not necessary to rely on heating as the sole and primary mechanism for tumor destruction. The superparamagnetic material needs to generate only enough heat to release the bioactive molecules, which are localized to the radius of the article itself. It is not required, although it will be of additional benefit, to heat up the surrounding tumor tissue.

The superparamagnetic particles used in the present invention are of a size smaller than a magnetic domain (1-100 nm). These subdomain superparamagnetic particles produce substantially more heat, especially at low amplitudes of alternating magnetic field (AMF). When suitable, physiologically acceptable frequencies and field strength combinations are used for the AMF, no interaction is observed between the human body and the field; hence tolerable low power absorption is obtained. The frequency of magnetic field used to heat the superparamagnetic material-containing MTNPs should be greater than that sufficient to cause any appreciable neuromuscular response, and less than that capable of causing any detrimental eddy current heating or dielectric heating of healthy tissue. Frequencies of around 50-200 kHz and a magnetic field strenght of around 50-100 kA/m is well-suited for human application (Jordan et al. (1999) *Journal of Magnetism and Magnetic Materials* **201**, 413-419); mice can tolerate substantially higher frequencies (greater than 1 MHz). Instrumentation is available that would allow the application of hyperthermia treatment in both animals and humans. Commercial source include Comdel, Inc. (Gloucester, MA), Bell Electrons NW, Inc., and Kandel Electronics. For frequencies suitable for both mice and humans, a frequency range of 20-450 kHz and output wattage of 500 W is adequate.

A key to efficient heating of an environment, such as a tumor, is to produce soluble particles that selectively locate to the tumor. At least three targeting mechanisms localize the MTNPs of the invention at the tumor site. First, the article size will be designed such that preferential tumor accumulation occurs through the well-documented enhanced permeability and retention (EPR) effect. Second, targeting (i.e., recognition) elements on the surface of the nanoarticles will help localize the articles to the tumor site by binding tumor-associated antigens. Several different targeting agents or REs may be utilized, including specific small molecule or peptide ligands, as well as antibodies or antibody fragments (*e.g.*, scFv). Finally, application of a localized magnetic field (which may be a substantially constant magnetic field or an alternating magnetic field) at the tumor site can be used to cause the articles to accumulate at the magnetized tumor site. This in turn can result in localized release of the bioactive agent. The magnetic field will also lead to heating within the cancer tissue, killing some tumor cells by this mechanism and, perhaps more importantly, increasing the vulnerability of the tumor cells to the released cytotoxic payload.

In a further aspect of the present invention, a MTNP as described herein can be utilized in an imaging method comprising administering to a subject (which can be a human or an animal) an amount of MTNPs, said amount being effective as a MR contrast or image-brightening agent, and imaging the subject using a magnetic resonance device.

In addition to the utility of enabling release of therapeutic agents under the application of an AMF and the utility of enhancing MRI images, the incorporation of iron oxides into the articles of the instant invention can be used advantageously in several additional ways. For instance, the superparamagnetic core allows for expedient purification of bioactive agent-containing MTNPs from reactants after various reaction and fabrication steps. For instance, agent-loaded particles may be separated from unattached bioactive agents by using a separation scheme wherein after the agent incorporation procedure is completed, the MTNPs are retained in a reaction vessel by a permanent magnetic field, while the solution that is unassociated with the articles is decanted off, siphoned off, or otherwise removed. Advantageously, the location of the MTNPs of the instant invention within the mammalian body may be determined using magnetic resonance imaging (MRI) of the superparamagnetic cores.

The following non-limiting examples are provided to further describe how the invention may be practiced.

EXAMPLES

Example 1. Formation of maghemite nanoparticles

Iron pentacarbonyl (0.74 mL) was added to a mixture of oleic acid (4.9 g) in octyl ether (28 mL) under a slow flow of N_2 gas at 100 °C. After 10 min, the solution was heated to 290 °C over one hour and held at this temperature until it turned black. After a further 30 min, heating was discontinued and the mixture was allowed to return to room temperature. Trimethylamine oxide (1.26g) was added to the mixture and heated to 130 °C (over approx. 20min), and held at this temperature for two hours. After heating to 290 °C over the next hour, and maintaining at this temperature for one hour, heating was discontinued. The product was recovered by precipitation with wash alcohol and centrifugation. Precipitation was repeated twice by dissolving the product in a minimum of hexanes (to which a few drops of oleic acid had been added), filtration through a 0.2 μm membrane filter, followed by addition of alcohol and subsequent centrifugation. Very fine suspensions of particles can be more easily isolated by placing the hexanes/alcohol solution in a beaker on a strong magnetic source, such as a NdFeB magnet (typically 8-12 KGauss), followed by decanting and/or pipetting the bulk solution for the solid precipitate. Transmission electron microscopy demonstrates the nanoparticles are of a narrow size distribution arround ~10nm.

Example 2. Exchange of oleic acid capping groups for tetraalkylammonium hydroxide

Oleic acid-stabilized iron oxide particles, from Example 1, were stirred in 20% aq. tetraethylammonium hydroxide (~10 mg particles/mL base solution) until the black solid dissolved. Mild heating (~50 °C) was applied as necessary to speed dissolution. The particles can be concentrated by spin filtration (such as with a Millipore Amicon-4 centrifugal filtration device), and washed with DI water to remove excess tetra-alkylammonium salts. The particles can alternately be concentrated and washed by magnetic separation methods. Thus, inert-polymer coated metallic beads (such as parylene coated steel beads, PTFE coated iron beads, etc) are added to the cooled solution and the vessel is transferred to a strong magnetic source such as a NdFeB magnet (typically 8-12 KGauss). A pair of magnets separated by a distance corresponding to the width of the container holding the nanoparticles solution is optimal. After the solution becomes transparent (with the nanoparticles coated onto the included beads), the bulk solution is removed. Removal of the container from the magnetic source releases the particles into solution which can then be diluted and the process of magnetic concentration and purification repeated as desired.

Example 3. Exchange of oleic acid capping groups for tetraalkylammonium hydroxide

Oleic acid-stabilized iron oxide particles, from Example 1, were stirred in water (~5 mg/mL) at 50 °C. A tetra-alkylammonium hydroxide solution (e.g. 20% aq tetraethylammonium hydroxide) was added drop-wise until the black solid started to dissolve to give a dark brown solution. Further hydroxide addition was made if the particles had not completely dissolved after 10 min. The final solution was approximately pH 12. The solution was cooled to room temperature and filtered (0.2 μm membrane). The particles were isolated and purified by the techniques described in Example 2.

Example 4. Hyaluronic Acid reaction with coated iron oxide particles

To a 1% wt/vol solution of tetraalkylammonium hydroxide coated iron oxide particles from Example 2 or 3, hyaluronic acid (10,000 MW, 10 wt. equiv.) was added and the mixture agitated. A dark-brown precipitate formed rapidly. This material could be dissolved by sonicating for several minutes, however, upon standing, a suspension reformed.

Example 5. PEG-Hyaluronic Acid reaction with coated iron oxide particles

To a 1% wt/vol solution of tetraalkylammonium hydroxide coated iron oxide particles from Example 2 or 3, PEG-Hyaluronic acid (PEG=5000 MW, HA=10,000 MW, 1 wt. equiv.) was added and the mixture agitated. A dark-brown precipitate formed rapidly. This material could not be re-dissolved in aqueous solution.

Example 6. Polyethylene oxide (PEO)-polymethylmethacrylate (PMMA) block copolymer-coated iron oxide particles

To a 1% wt/vol solution of tetraalkylammonium hydroxide-coated iron oxide particles from Example 2 or 3, PEO(7.8K)-*b* -PMAA(2K) (1 wt. equiv.) was added and the mixture agitated for several days. The mixture was filtered (0.2 μm membrane) and purified by spin filtration or magnetically concentrated and purified as in the above example the resulting MTNPs.

Example 7. Incorporation of platin into polyethylene oxide-polymethylmethacrylate block copolymer-coated iron oxide MTNPs

To a solution of purified PEO(7.8K)-*b*-PMAA(2K)-coated iron oxide nanoparticles from Example 6, at approx. 10 mg/mL, was added 2.5 mg/mL of *cis*-diamminoplatinum(II) nitrate (from a stock solution of 1-10% wt/vol). The reaction was agitated at room temperature for 1 day, after which the platinated PEO(7.8K)-*b*-PMAA(2K)-coated iron oxide MTNPs were

separated and purified by the magnetic techniques described above. Analysis by ICP indicated 4-5% wt incorporation of Pt.

Example 8. Direct synthesis of platinated polyethylene oxide-polymethyl-methacrylate block copolymer-coated iron oxide MTNPs

To a solution of the tetraalkylammonium hydroxide-coated iron oxide particles from Example 2 or 3 (10 mg/mL) was added PEO(7.8K)-*b* -PMAA(2K) (1 wt. equiv.), and the mixture was agitated at 40 °C overnight, followed by addition of *cis*-diamminoplatinum(II) nitrate (2.5 mg per 10mg of iron oxide particles from a stock solution of 1-10% wt/vol platin). After 24 hr, the platinated PEO(7.8K)-*b*-PMAA(2K)-coated iron oxide MTNPs were separated and purified by the magnetic techniques described above. Analysis by ICP indicated 4-6% wt incorporation of Pt

Example 9. Preparation of Silica-Coated Magnetite Colloids in Microemulsion

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_47H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared by dissolving 0.4055g $FeCl_36H_2O$ in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 1 hr to form a microemulsion. In another container, 100μL of NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 1 hr to form a NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH microemulsion was added dropwise to the Fe microemulsion with vigorous mechanical stirring for 1 hr to form magnetite nanoparticles.

50μL of tetraethylorthosilicate (TEOS) was then added to the magnetite nanoarticles solution and mechanically stirred for additional 24 hrs. Acetone was added to the colloidal microemulsion to precipitate the silica-coated nanoarticles. The nanoarticles were washed with acetone and ethanol several times, then dissolved in water and lyophilized to obtain magnetite nanoparticles in powder form.

Example 10. Preparation of Inulin Magnetite MTNPs in Microemulsion: Coating Crosslinked by Free Radical Polymerization

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_47H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared

by dissolving 0.4055g $FeCl_3$6H2O in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 30 minutes to form a Fe microemulsion. In another container, 100μL of NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 30 minutes to form NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH microemulsion was added dropwise to the Fe microemulsion with vigorous mechanical stirred for 1 hr to form magnetite nanoarticles.

166μL of monomers solution comprised of 25% inulin multi-methacrylate (IMMA), 2% cystine bisacrylamide (CiBA) and 1% sodium acrylate (NaA) in 10mM sodium phosphate buffer at pH7.2 was added to the magnetite nanoarticles solution, followed by 3μL of sodium persulfate (50mg/mL water) and 3μL of TEMED (5% solution). The solution was degassed using a water pump aspirator and mechanically stirred for 2 hrs to form a crosslinked scaffolding comprised of derivitized inulin. Ethanol was added to the microemulsion to precipitate the nanoarticles. The articles were then dissolved in water and purified using ion exchange BioBeads SM-2 for 2 hrs. The articles were filtered and lyophilized to obtain magnetite nanoarticles in powder form.

Example 11. Incorporation of Doxorubicin and Recognition Elements

Reduction of CiBA-Containing Nanoarticles: Dissolve 1.0 g nanoarticles as prepared in Example 10 in 6.16 mL PBS at 65 mg/mL. Add 268 mg of DTT (FW 154; 24-fold excess per mole of CiBA) into the nanoarticle solution. Agitate the reaction for 2 hours at room temperature. Pass the nanoarticle solution through three FPLC desalting columns to remove excess DTT, using PBS as buffer. Collect the nanoarticle fractions and concentrate to a total of 10 mL of buffer (50 mg/mL of nanoarticles) using Amicon Ultra-15, MWCO 50k centrifugal filters (2.5 mL per filter) spun at 4000 rpm.

Linker Attachment: Add 233.6 mg of PEG_{400}DBA (FW 641.86; 2.5-fold excess per mole of thiol) to the nanoarticle solution. Add 81.6 mg N-(ε-maleimidocaproic acid) hydrazide (EMCH) (FW 225.24; 2.5-fold excess per mole of thiol) 5 minutes after PEG_{400}DBA addition to the nanoarticle solution. Agitate the reaction for 2 hrs after this step. Remove unreacted linkers by centrifuging in Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute the nanoarticle retentate in 2.5 mL of 0.1 M PBS, pH 7.2 and repeat the centrifugation. Re-dissolve the retentate in each tube in 2.5 mL of 0.1 M PBS, pH 7.2.

Ligand Attachment: Add 42.08 mg of the peptide RGDdFC (FW 578; 0.5 equivalent of thiol) to the PEG_{400}DBA-nanoarticles (any peptide or ligand with a free thiol, readily incorporated via a cysteine residue, can be attached to the nanoarticle through reaction with a PEG multibromoacetate linker). Take a 10 μL aliquot at t = 0 and t = 1hr after peptide addition for HPLC analysis. Agitate the reaction for 1 hr. Cap unreacted bromoacetamide with 17.6 mg of cysteine (FW 121.16; 1 equivalent of thiol) to each solution. Agitate the reaction for 10 minutes. Remove unreacted ligands by centrifuging each nanoarticle solution in Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute each nanoarticle retentate in 2 mL of 0.1 M sodium phosphate buffer, pH 5, and repeat the centrifugation. Re-dissolve the retentate in 2.5 mL of 0.1 M sodium phosphate buffer, pH 5.

Doxorubicin attachment: Add 41.6 mg of doxorubicin (FW 579.99; 1 theoretical equivalent per 2 moles of thiol) to the above nanoarticle solution (at 50 mg NP/mL (5 mL buffer)), first pre-dissolving doxorubicin in 8 mL of de-ionized water at 5.2 mg/mL. React at 37°C for 20 h. Remove unreacted doxorubicin using Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute the nanoarticle retentate in 2.5 mL de-ionized water and repeat the centrifugation. Re-dissolve the retentate in 50 mL de-ionized water (5 mg/mL for lyophilization). Lyophilize the doxorubicin-containing MTNP solution overnight.

Example 12:

Add 5 fold excess 5/8 arm-PEG_{2000}BA linker to reduced CiBA-containing nanoarticles (prepared as in Example 11) in 0.1M PBS, 1.2 g. Allow to react 2 hours at room temperature with agitation. Remove unreacted linker on FPLC with three 26/10 desalting columns in-line, equilibrated and run with 0.1M PBS pH 7.2.

Example 13: Preparation of Carbohydrate-Coated Magnetite MTNPs: Coating Crosslinked by Michael-type addition reaction

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_4 7H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared by dissolving 0.4055g $FeCl_3$6H2O in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 1 hr to form an Fe microemulsion. In another container, 100μL of

NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 1 hr to form a NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH microemulsion was added dropwise to the Fe microemulsion with vigorous mechanical stirred for 1 hr to form magnetite nanoparticles.

150uL of 30%IMMA in 10mM sodium phosphate buffer at pH7.2 was added to the magnetite nanoarticles solution, followed by 5.7μL of PEG_{3400}dithiol. The solution was mechanically stirred for 24 hrs. Ethanol was added to the microemulsion to precipitate the nanoparticles. The articles were then dissolved in water and purify using ion exchange BioBeads SM-2 for 2 hrs. The articles were filtered and lyophilized to obtain iron oxide-containing nanoarticles in powder form.

Example 14:

Combine 1.0 mL of a 2.0 M $FeSO_4$ in 2 M HCl with 4.0 mL of a 1.0M $FeCl_3$ in 2 M HCl and stir with a magnetic stir bar. Add 50 mL of a 0.7 M NH_3 solution dropwise to the stirring solution. Allow the magnetite to settle and decant some of the liquid before centrifuging for 1 minute at 1000 rpm. Add 10 mL of 20% tetraethylammonium hydroxide to the precipitate and resuspend the magnetite. Use an aspirator vacuum to remove excess ammonia from the solution. Pour off some of the liquid and pour the magnetite-covered stir bar into a weigh boat. Using a strong magnet under the weigh boat to attract the magnetite, remove the stir bar and any excess liquid. Let the magnetite dry over the weekend in the hood. A ferrofluid solution of the magnetite was prepared containing 100mg/mL of deionized water.

Aqueous monomers solution was prepared containing 30 wt% IMMA, 4wt% CiBA and 2 wt% NaA in deionized water. The oil phase was prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. Thermal initiator, sodium persulfate (NaPS), solution was prepared containing 250mg/mL of deionized water. N,N,N',N'-Tetramethylethylenediamine (TEMED) was used as purchased.

1mL of aqueous ferrofluid was mixed with 1mL of aqueous monomers. The combined solution was then added dropwise to the oil phase with mechanical stirring. 50μL of NaPS 250mg/mL solution and 12.5μL of TEMED were added to the resulting microemulsion while stirring. The microemulsion was then transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution was placed on the shaker for 18 hrs. Ethanol was then added to the microemulsion to precipitate the nanoparticles. The particles were dissolved in water and purify using ion exchange (BioBeads SM-2) for 2 hrs. The particles were filtered and lyophilized to obtain polymer-coated magnetite nanoparticles.

Example 15:

A 0.27 M bis(2-ethylhexyl)sulfosuccinate sodium salt (Aerosol OT or AOT) solution was prepared by dissolving 12g AOT in 10mL isooctane. An aliquot of ultra-pure water was purged for one hour with N_2 gas. A stock solution of 1 M Fe(II) was prepared by dissolving 0.278g $FeSO_4.7H_2O$ in 1mL of the nitrogen purged water. Similarly, a stock solution of 1.5 M Fe(III) was prepared by dissolving 0.4055g $FeCl_3.6\ H_2O$ in 1mL of the nitrogen purged water. In a glass container, 25uL of the 1 M Fe(II) solution and 25uL of the 1.5 M Fe(III) solution were added to a 5mL aliquot of the AOT solution under a nitrogen atmosphere, and the resulting Fe/AOT mixture was magnetically stirred for 1 hr to form a Fe/AOT solution. In another container, 100μL NH_4OH (28-30 wt %) was added to another 5mL aliquot of the AOT solution, and the resulting NH_4OH/AOT mixture was magnetically stirred for 1 hr to form a NH_4OH /AOT solution. In absence of magnetic field, the NH_4OH/AOT solution was added dropwise to the Fe/AOT solution with vigorous mechanical stirring for 1 hr. 50μL of tetraethylorthosilicate (TEOS) was then added to the resulting brown solution and mechanical stirring was continued for an additional 24 hrs to give a magnetite microemulsion.

Aqueous monomers solution was prepared containing 25 wt% IMMA, 2 wt% CiBA and 1 wt% NaA in 10mM sodium phosphate pH 7.2 buffer. The oil phase was prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. Sodium persulfate (NaPS) thermal initiator solution was prepared containing 250mg/mL of deionized water. N,N,N',N'-tetramethylethylenediamine (TEMED) was used as purchased.

In a glass container, 0.5mL of aqueous monomer solution was added dropwise to 10mL of oil phase while stirring. 12.5μL of NaPS 250mg/mL solution and 5μL of TEMED were added to the microemulsion while stirring. With the magnetic stirrer removed, the monomer microemulsion was mixed with the magnetite microemulsion. The combined microemulsion was transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution was placed on the shaker for 18 hrs. Acetone was used to precipitate the nanoparticles. The nanoparticles were washed with acetone and ethanol several times with each solvent, then dissolved in water and lyophilized to obtain polymer-coated magnetite nanoparticles.

Example 16: Attachment of 4,4'-azobis(4-cyanovaleric acid) (ACVA) to amine-containing MTNPs

One gram of magnetic cored nanoarticles having a polymer scaffold composition of 25/2/1 IMMA/CiBA/APMA is dispersed in 50 mL of 0.2 M pH=7.5 HEPES buffer. N-hydroxysuccinimide (NHS, 0.109 g) and ACVA (0.133 g) are dissolved in a second 50 mL

quantity of the same buffer. To the solution containing the NHS and ACVA is added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 0.182 g). After stirring the NHS/ACVA/EDC solution at room temperature for 10 minutes with a mechanical stirrer, the magnetic nanoarticles are added over the course of 1 minute. One hour later, with the aqueous mixture being continuously agitated by a mechanical stirrer over that interval, a second quantity of EDC (0.182 g) is added. One hour later, a third quantity of EDC (0.182 g) is added. After one more hour, the particles are isolated by centrifugation or magnetic separation. Excess reagents and side products are removed from the particles by three re-suspension/isolation cycles, and the nanoarticle products are isolated by lyophilization.

Example 17: TEMPO-functionalized inulin

The product (1.0 g) of the reaction of 4-hydroxy-2,2,6,6-tetramethyl-piperdinyloxy, free radical (4-hydroxy-TEMPO) with 1,1'-carbonyldiimidazole (CDI) is added to an anhydrous DMSO (25 mL) solution containing inulin (DP=20, 1.22 g). The resulting mixture is stirred under a nitrogen atmosphere for two days, and is then poured into toluene (400 mL) with rapid stirring. The precipitated product is dried under vacuum, dissolved in de-ionized water, and dialyzed in a 500 MW cutoff dialysis membrane against de-ionized water. The product is isolated after lyophilization.

Example 18: Preparation of magnetic colloids

Combine 1.0 mL of a 2.0 M $FeSO_4$ in 2 M HCl with 4.0 mL of a 1.0M $FeCl_3$ in 2 M HCl and stir with a magnetic stir bar. Add 50 mL of a 0.7 M NH_3 solution dropwise to the stirring solution. Allow the magnetite to settle and decant some of the liquid before centrifuging for 1 minute at 1000 rpm. Add 10 mL of 20% tetraethylammonium hydroxide to the precipitate and re-suspend the magnetite. Use an aspirator vacuum to remove excess ammonia from the solution. Pour off some of the liquid and pour the magnetite-covered stir bar into a weigh boat. Using a strong magnet under the weigh boat to attract the magnetite, remove the stir bar and any excess liquid. Let the magnetite dry for two days in a fume hood.

Example 19: Synthesis of radical generating polymer-coated MTNPs

An oil phase is prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. An aqueous phase is prepared by adding TEMPO-functionalized inulin (0.491 g) from Example **17**, poly(ethylene glycol) diacrylate (formula weight 575, 0.100 g), sodium acrylate (0.022 g), and ACVA (0.162 g) to 2.4 mL deionized water. Two mL of the resulting solution is added to 100 mg of the dry magnetite colloids from Example **18**. After thorough mixing, the aqueous phase is then

added dropwise to the oil phase with mechanical stirring. The microemulsion is transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution is placed on a shaker for 2 hrs while being exposed to UV light. Ethanol is added to the microemulsion to precipitate the nanoparticles. The particles are dissolved in water and purified using ion exchange (BioBeads SM-2) for 2 hrs. The nanoarticles are filtered and lyophilized to obtain radical generating MTNPs.

Example 20. Pt release from platinated polyethylene oxide – polymethylmethacrylate block copolymer coated maghemite MTNPs.

A solution (~10mg/mL) of MTNPs such as prepared in Example 8 were heated to 80 °C for 30 min. The low molecular weight species were separated from the articles with a centrifugal membrane filtration device with a nominal molecular weight cut-off of 5000. Platinum release was monitored by an increase in absorbance at 300 nm and by %Pt determined by ICP. Compared to a sample stored at room temperature, heating at 80 °C increases Pt release at least ~2-5-fold over this 30 min period.

Example 21. Effect on in vitro toxicity by heat treatment of platin-containing polyethylene oxide-polymethylmethacrylate block copolymer-coated iron oxide MTNP.

A platin-containing MTNP (prepared as in Example 7) was heated at 80 °C for 30 min and cooled to room temperature, prior to incubating with an A-498 renal cell cancer line. Compared to a control of an identical, but non-heated MTNP, the heated sample demonstrated thirty times the potency in cell proliferation inhibition as measured a sulforhodamine B assay.

WHAT IS CLAIMED IS:

1. A magnetic therapeutic nanoparticle (MTNP) comprising a plurality of bioactive agents, a core of superparamagnetic material, and a polymeric scaffold encapsulant.

2. An MTNP according to claim 1 wherein the superparamagnetic material is selected from the iron oxides.

3. An MTNP according to claim 1 or 2 wherein the polymeric scaffold comprises crosslinked building blocks, at least some of which building blocks are carbohydrate-based monomers or polymers, and at least some of the bioactive agents are bound to the polymeric scaffold by coordination bonds or covalent bonds.

4. An MTNP according to claim 3 wherein the bioactive agent is a chemotherapeutic agent.

5. An MTNP according to claim 1 or 2 wherein the polymeric scaffold comprises polycarboxylates, the superparamagnetic material is bound to the polymeric scaffold by coordination bonds, and at least some of the bioactive agents are bound to the polymeric scaffold by coordination bonds.

6. An MTNP according to claim 5 wherein the bioactive agent comprises platinum.

7. An MTNP according to any of claims 1 to 6 wherein the polymeric scaffold further comprises one or more functional building blocks selected to introduce a desired characteristic or functionality into the scaffold.

8. An MTNP according to claim 7 wherein the functional building blocks are selected from the group consisting of N,N'-cystinebisacrylamide (CiBA), sodium acrylate (NaA), N-(3-aminopropyl)methacrylamide hydrochloride (APMA), N[ethylamino]-3-amino-propylmethacrylamide hydrochloride, polyethylene imine (PEI), polylysine, polyamido-acrylamide derivatives, and protamine sulfate, and mixtures thereof.

9. An MTNP according to any of claims 1 to 8 wherein the building blocks further comprise small molecule crosslinking agents.

10. An MTNP according to 9 wherein the crosslinking agent is comprised of platinum.

11. An MTNP according to any of claims 1 to 10 which further comprises one or more recognition elements covalently attached to the polymeric scaffold, the recognition elements having binding affinity to biomolecular structures expressed on certain cells or in certain tissues.

12. An MTNP according to any of claims 1 to 11 which further comprises at least one polyethylene glycol molecule covalently attached to the polymeric matrix.

13. A method of magnetic resonance image (MRI) enhancement in a subject, the method comprising:

administering to the subject an effective amount of an MRI contrast agent comprising MTNPs of any of claims 1 to 12; and

imaging the subject using a magnetic resonance device.

14. A method of purification of MTNPs from reactants, the method comprising:

applying a permanent magnetic field to a reaction vessel having a solution containing MTNPs of any of claims 1 to 12 and reactants unassociated with the MTNPs; and

removing the solution with reactants from the reaction vessel;

to give reactant-free MTNPs retained in the reaction vessel.

15. A method of delivering a bioactive agent to an environment, the method comprising:

administering to the environment a plurality of MTNPs of any of claims 1 to 12; and

applying to the environment heat sufficient for release of bioactive agent from the MTNPs into the environment.

16. A method according to claim 15 wherein the heat is applied by means of an alternating magnetic field generated external to the environment, with the alternating magnetic field causing the MTNP to heat up and release the bioactive agent from the MTNPs.

17. A method according to claim 15 or 16 comprising, after administering the MTNPs and prior to applying heat, the additional step of concentrating the magnetoarticles in the environment by applying a substantially constant magnetic field to the environment.