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(54) Title: BIODEGRADABLE MACROMOLECULAR MRI CONTRAST AGENTS AND METHODS OF PREPARATION AND USE THEREOF

(57) Abstract: Novel methods for preparing degradable macromolecular magnetic resonance imaging contrast agents are disclosed. Degradable macromolecular magnetic resonance imaging contrast agents of defined or controlled molecular weight distribution for use in various diagnostic procedures, and methods for synthesizing, using and degrading these agents, are disclosed. The macromolecular contrast agents disclosed in various aspects of this invention are degradable paramagnetic polydisulfide compounds which show prolonged plasma retention, and enhanced permeability and retention in various tissues, organs or tumors, but are still capable of being rapidly cleared from the body.



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BIODEGRADABLE MACROMOLECULAR MRI CONTRAST AGENTS AND METHODS OF PREPARATION AND USE THEREOF

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PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number 60/814,449, filed June 16, 2006, for "BIODEGRADABLE MACROMOLECULAR MRI CONTRAST AGENTS AND METHODS OF PREPARATION AND USE THEREOF."

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Work described herein was supported by National Institutes of Health Grants CA095873. The United States government may have certain rights in the invention.

15

TECHNICAL FIELD

This invention relates generally to biodegradable macromolecular contrast agents used in diagnostic imaging, and methods of synthesizing, purifying, using and degrading such compounds.

20

BACKGROUND

Magnetic resonance imaging (MRI) is a non-invasive method for medical diagnosis. Paramagnetic metal complexes are often used as contrast agents to enhance the image contrast between normal tissue and diseased tissue. Paramagnetic metal ions that are typically used in diagnostic procedures include manganese (Mn^{2+}), iron (Fe^{3+}), and gadolinium (Gd^{3+}). Chelates of Gd^{3+} are frequently used as MRI contrast agents because of their long electronic relaxation time and high magnetic moment. Gadolinium-based contrast agents including small molecular gadolinium complexes such as $Gd(III)(DTPA)$ [diethylenetriaminepentaacetate], $Gd(III)(DOTA)$ [1,4,7,10-tetraazadodecanetetraacetic acid], $Gd(DTPA-BMA)$ (diethylenetriaminepentaacetic bismethyl amide), and their derivatives are routinely used as contrast agents for MRI in clinical practice. These agents are extracellular contrast agents, extravasate rapidly into extracellular fluid space and have short tissue retention time due to their small sizes, leading to some disadvantages including short imaging time window and low signal to noise ratio. In order to prolong the circulation and retention of contrast agents, significant amounts of efforts have been

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made on the increase in the sizes of the contrast agents by either chemical conjugation of Gd(III) chelates to biomedical polymers [1] or incorporation into the backbone of polymers [2].

Macromolecular MRI contrast agents, sometimes referred to as long-circulating or blood pool agents, are particularly useful because of their prolonged retention time in the blood pool. For example, the half-life of albumin-(Gd-DTPA) conjugate is approximately 3 hours in the blood [3]. Gd-DTPA labeled dextran (MW ~ 75 kDa) has a long half-life of 6.1 hours as compared to 13 minutes of Gd-DTPA in rats [4]. Polymeric contrast agents also possess increased proton T₁ relaxivity resulting from long rotational time compared to small size molecules [5]. Because of the enhanced permeability and retention, these contrast agents accumulate effectively in solid tumors and have a potential in contrast enhancement in MR cancer imaging.

However, macromolecular contrast agents have potential toxicities related to slow excretion and long-term tissue Gd accumulation, thus hinders their further development. As a result of poor clearance rates, use of Gd macromolecular agents may result in Gd accumulation in the bone and other tissues, resulting in toxicity and adverse side-effects. (Gd-DTPA)-albumin conjugate, a prototype of macromolecular MRI contrast agent, showed high accumulation of Gd in the bone and liver consequential of its slow excretion [3], which increased possibility of cellular uptake of the agent through endocytosis and dissociation of Gd-DTPA complexes in the lysosome due to low pH and enzymatic degradation [6,7]. High long-term Gd tissue accumulation was also observed for other macromolecular Gd(III) complexes. It has been reported that the conjugation of Gd-DO3A to carboxymethyl hydroxylethyl starch resulted in a macromolecular agent (72 kDa) that had about 47% of injected dose detected in rat body seven days after the injection [8]. A Gd-DTPA polypropyleneimine dendrimer (generation 2) conjugate (7 kDa) resulted in the retention of 45% of injected dose in rats 14 days after injection [9]. High long-term *in vivo* accumulation of the contrast agents significantly increases the possibility of metabolic release of toxic Gd(III) ions from the chelates.

The inventors recently designed and developed biodegradable macromolecular MRI contrast agents based on polydisulfide Gd(III) complexes to facilitate excretion of Gd chelates via *in vivo* degradation of the macromolecular agents [10-12]. Disulfide bonds in the polymer backbone can be rapidly reduced by free plasma thiols, e.g., glutathione and cysteine, or by enzymatic degradation. It has been shown that (Gd-DTPA)-cystamine copolymers (GDCC), the first polydisulfide MRI contrast agent,

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produced more significant blood pool contrast enhancement in rats than a clinically available MRI contrast agent, Gd-(DTPA-BMA), and then cleared rapidly from the blood pool. GDCC exhibited minimal long-term tissue accumulation of Gd comparable to the clinically used Gd-(DTPA-BMA).

5 Functional groups around the disulfide bonds have been introduced to tune the degradation rate of the paramagnetic polydisulfide agents and to prepare biodegradable macromolecular agents with various pharmacokinetic properties. Two modified polydisulfide MRI contrast agents, (Gd-DTPA)-cystine copolymers (GDCCP) and (Gd-DTPA)-cystine diethyl ester copolymers (GDCEP), and preliminary results of these agents
10 in contrast enhanced tumor MR imaging have been reported in Ref. [11].

DISCLOSURE OF INVENTION

 The present invention provides, among other things, degradable macromolecular contrast agents with a defined or controlled molecular weight and/or molecular weight
15 distribution. In a preferred embodiment, the contrast agents comprise polydisulfide. In another preferred embodiment, these contrast agents are complexes with metals, e.g., Mn^{2+} , Fe^{3+} and Gd^{3+} . Examples of various contrast agents are disclosed in US Patent 6,982,324, Ref. [10], Ref. [11], or Ref. [12]. These contrast agents can be used in various medical procedures, e.g., diagnostic and treatment procedures. In one embodiment, these
20 contrast agents are used in magnetic resonance imaging. In an alternative embodiment, these contrast agents are used in X-ray computed tomography. In other embodiments, the degradable polymer can form chelates with radioactive metal ions for scintigraphy, positron emission tomography and radiotherapy. In another aspect of the invention, the contrast agent complexes include targeting molecules. The incorporation of targeting
25 molecules, including, but are not limited to, antibodies, antibody fragments, peptides, other proteins and other chemical entities results in macromolecular contrast agents with targeting ability.

 The invention provides a method of preparing degradable macromolecular contrast agents in an aqueous medium. In a preferred embodiment, the reaction medium is a basic
30 aqueous solution. Use of aqueous solution as a medium for polymerization avoids polluting solvents, and also facilitates easy scale-up for manufacture.

 The invention provides a method of purifying degradable macromolecular ligands and contrast agents. In one embodiment, the ligands and contrast agents are purified by chromatography methods. In another embodiment, the contrast agents are purified by

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ultrafiltration. Biodegradable macromolecular MRI contrast agents comprising Gd can also be purified by raising pH of the contrast agent solutions to remove any residual free Gd(III) ions as Gd₂O₃ precipitates.

5 The invention also provides a method of fractionating degradable macromolecular contrast agents to provide contrast agents with narrower or desired molecular weight distributions. In one embodiment of the invention, contrast agents are fractionated by chromatography methods such as size exclusion chromatography.

10 The invention also provides a method of controlling the molecular weight and/or molecular weight distribution of degradable macromolecular contrasting agents. In one embodiment, the molecular weights of the contrasting agents are controlled by varying polymerization conditions, such as reaction temperature and/or feed ratios of polymerization reactants.

15 The invention provides a method for obtaining a magnetic resonance image of a tissue or organ of a mammal by administering one or more degradable macromolecular contrast agents and obtaining a magnetic resonance image. In several embodiments, the macromolecular contrast agents are capable of being degraded by both endogenous and exogenous compounds. In one embodiment, the macromolecular contrast agents are degraded by endogenous mercaptans and/or enzymes into small stable chelates.

20 The invention also provides a method to degrade or stimulate degradation of the macromolecular contrast agents by administering one or more disulfide bond reducing compounds or other compounds that stimulate the degradation of the macromolecular contrast agents. In one embodiment, exogenous mercaptans are delivered to the mammal. Another object of several embodiments of the current invention is to administer macromolecular contrast agents in conjunction with other agents. In various aspects, 25 physiologically acceptable agents, such as diluents and carriers, are also administered.

One aspect of the invention includes a method for obtaining a magnetic resonance image of a tissue or organ of a mammal by administering an effective amount of one or more macromolecular contrast agents to the mammal and obtaining a magnetic resonance image. In a preferred embodiment, one or more of the macromolecular contrast agents are 30 degraded by endogenous mercaptans and/or enzymes.

According to one aspect of the invention, one or more compounds that stimulate the degradation of said macromolecular contrast agent is also administered. In another aspect, one or more disulfide bond reducing compounds is also administered. The disulfide bond reducing compound is selected from the group consisting of one or more of

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the following: mercaptans, NADH, NADPH, hydrazines, phosphines, zinc, tin(II), sodium sulfide, performic acid, hydrogen peroxide.

5 Mercaptans used in various aspects of the present invention are selected from the group consisting of one or more of the following: cysteine and its derivatives, glutathione and its derivatives, cysteinylglycine and its derivatives, 2,3-dimercaptosuccinic acid and its derivatives, 2,3-dimercapto-1-propanesulfonic acid and its derivatives, 2-mercaptoethanol, penicillamine and its derivatives, mercaptoacetic acid and its derivatives, mercaptoanisole, 2-mercaptobenzoic acid and its derivatives, 4-mercaptobenzoic acid and its derivatives, 2-mercapto-5-benzimidazolesulfonic acid and
10 its derivatives, 2-mercaptobenzothiazole, 3-mercapto-iso-butyric acid, mercaptocyclohexane, 2-mercaptoethanesulfonic acid, 2-mercaptoethylamine, 2-mercaptoethylamine hydrochloride, 3-mercapto-1,2-propanediol, 3-mercapto-1-propanesulfonic acid, 3-mercapto-1-propanol, 2-mercaptopropionic acid, 3-mercaptopropionic acid, diethyldithiocarbamate, dithioerythritol, and dithioglycol.

15 According to one aspect of the invention, two or more of macromolecular contrast agents are administered simultaneously. In one embodiment, the macromolecular contrast formulation comprises a first macromolecular contrast agent and a second macromolecular contrast agent, wherein the second macromolecular contrast agent is administered after the administration of said first macromolecular contrast agent.

20 In another embodiment, at least one of the macromolecular contrast agents is administered in conjunction with one or more physiologically acceptable agents selected from the group consisting of: diluents, carriers, antibodies, antibody Fab' fragments, antibody F(ab')₂ fragments, and delivery systems.

25 In a preferred embodiment, at least one of the macromolecular contrast agents is administered in conjunction with one or more contrast agents selected from the group consisting of: paramagnetic metal complexes, radioactive metal complexes, therapeutic agents, proteins, DNA, RNA, drug delivery systems and gene delivery systems.

30 It is an object of several embodiments of the current invention to obtain a magnetic resonance image of healthy or tumorous tissues or organs, including but not limited to, liver, spleen, lung, heart, kidney, tumors, ovary, pancreas, biliary system, peritoneum, muscles, head, neck, esophagus, bone marrow, lymph node, lymph vessels, nervous system, brain, spinal cord, blood capillaries, stomach, small intestine, and large intestine.

It is an object of several embodiments of the present invention to provide a method to obtain a magnetic resonance image of magnetic resonance image of healthy or

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tumorous tissues or organs, the method comprising selecting a suitable molecular weight range for one or more contrasting agents. In one embodiment, contrast agents of high (preferably larger than 40 kDa) molecular weight provide more prolonged and significant contrast enhancement for cardiac and vasculature imaging. In another embodiment, contrast agents of low (preferably smaller than 40 kDa) molecular weight result in more significant enhancement in tumor tissues than high molecular weight agents.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 Effects of reaction temperatures on molecular weight distributions of polymer ligand DTPA-cystine copolymers, as also illustrated in the Table 1.

FIG. 2 Effect of feeding ratio of DTPA dianhydride to cystine on molecular weight distributions of copolymers, as also illustrated in Table 2.

FIG. 3 Comparison of purification of poly(GdDTPA-*co*-L-cystine) (GDCP) by PD-10 columns vs. ultrafiltration.

FIG. 4 Time signal intensity curve of major fractions of Gd-DTPA cystamine copolymers (GDCC) using AKTA P-920 FPLC, Superose 6 10/200 GL column and a Knauer RI detector.

FIG. 5 Three-dimensional maximum intensity projection MR images of mice before injection (a) and 2 (b), 5 (c), 10 (d), 15 (e), 30 (f) and 60 (g) min after injection of GDCP (A: 23 kDa, B: 43 kDa, C: 109 kDa) and GDGP (D: 21 kDa, E: 43 kDa, F: 108 kDa) at a dose of 0.1 mmol Gd/kg via a tail vein.

FIG. 6 Two-dimensional axial MR images of mice with human breast cancer (MB-231) (see arrows) before injection (a) and 2 (b), 5 (c), 10 (d), 15 (e), 30 (f) and 60 (g) min after injection of GDCP (A: 23 kDa, B: 43 kDa, C: 109 kDa) and GDGP (D: 21 kDa, E: 43 kDa, F: 108 kDa).

MODES FOR CARRYING OUT THE INVENTION

Traditionally, polydisulfide ligands are prepared in organic solvents, for example, polydisulfide ligands are obtained by condensation polymerization of DTPA dianhydride and disulfide-containing diamines in DMSO. Organic solvents are, in general, not environmentally friendly, especially when they are used in large scale manufacturing processes. In addition, it takes extra steps to remove the organic solvents prior to application of the polydisulfide agents.

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The present invention provides a method for preparing degradable macromolecular contrasting agents in an aqueous medium, thus offering advantages over traditional methods in less pollution and ease of scaling up. Preferably, the aqueous medium is a basic aqueous solution. Using this novel method, polydisulfide MRI contrast agents, with different chemical structures and molecular weights, have been made successfully. For example, *in vivo* MR imaging in mice showed that polydisulfide MRI contrast agents had long retention in blood pool compared to a clinically-used agent with small size. These polydisulfide contrast agents show great promises to diagnose tumor and other cardiovascular diseases.

The invention provides a method of purifying degradable macromolecular contrast agents. The contrast agents can be purified by various chromatography methods, such as HPLC (high performance liquid chromatography), GPC (gel permeation chromatograph), and SEC (size exclusion chromatography). In a particular embodiment, the contrast agents are purified by SEC equipped with a column packed with G-25 medium. The contrast agents can also be purified by various filtration methods, such as ultrafiltration using a filter with a desired molecular weight cut-off.

The present invention also provides a method to prepare biodegradable macromolecular MRI contrast agents with different molecular weights, and/or narrow molecular weight distribution for different clinical applications. Biodegradable macromolecular MRI contrast agents with various molecular weights can be readily controlled by varying the reaction conditions, for example, by varying the reaction temperature, or by varying the feed ratio of polymerization reactants. Methods to adjust reaction conditions are exemplified in the Examples herein. It is to be noted that other reaction conditions, such as pH of the reaction medium, reaction time, mixing speed of reactants, and other factors, can also affect the molecular weight and/or molecular weight distribution.

It has been demonstrated that high molecular weight biodegradable agents, for example, of 108 kDa, provide more prolonged and significant contrast enhancement for cardiac and vasculature imaging than the corresponding agents of low molecular weights. The polymer agents with low molecular weight, for example, of 23 kDa, resulted in more significant enhancement in the tumor tissues than high molecular weight agents. Therefore, the invention also provides a method to obtain a magnetic resonance image of healthy or tumorous tissues or organs, the method comprising selecting one or more contrast agents with a suitable molecular weight for a specific application.

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A macromolecular contrast agent may have a broader molecular weight distribution than desired. For example, in order to accurately assess the size effects of a paramagnetic polydisulfide on its pharmacokinetics and *in vivo* contrast enhancement, the paramagnetic polydisulfide with a narrower molecular weight distribution are prepared by
5 fractionation with size exclusion chromatography. It is to be noted other methods such as ultrafiltration or dialysis can also offer partial fractionation.

It will be appreciated that the structure, charge and/or size of a polydisulfide contrast agent can affect its degradation, pharmacokinetics and *in vivo* contrast enhancement. For example, polydisulfide Gd(III) complexes, (Gd-DTPA)-cystine
10 copolymers (GDGP), (Gd-DTPA)-glutathione (oxidized form) copolymers (GDGP) and (Gd-DTPA)-cystine diethyl ester copolymers (GDCEP) behaved differently in mice. The data and analysis with respect to degradation, pharmacokinetics and *in vivo* contrast enhancement for these polydisulfide contrasting agents are shown in FIGs. 5 and 6, and detailed in Ref. [12], the entire contents of which are incorporated herein by reference.
15 The effects of structure, size and/or charges of a polydisulfide contrast agent suggests that optimal polydisulfide Gd(III) complexes can be designed and prepared by structural optimization as safe and effective biodegradable macromolecular MRI contrast agents for various clinical applications. Further, contrast agents with different pharmacokinetic properties may be suitable for different applications. In general, agents with an acceptably
20 long blood circulation are more effective in contrast enhanced cardiovascular imaging and cancer imaging. Therefore, the invention also provides a method to obtain magnetic resonance images of healthy or tumorous tissues or organs, the method comprising selecting one or more contrasting agents with suitable properties of pharmacokinetic, degradation, and/or *in vivo* contrast enhancement for a specific application.

25 In another aspect, the present invention provides polydisulfide MRI contrast agents of defined or controlled molecular weight and/or molecular weight distribution. Polydisulfide MRI contrast agents of the present invention include, but are not limited to various contrast agents as disclosed in US Patent 6,982,324, Ref. 10, Ref. 11 or Ref. 12. In one embodiment of various contrast agents, the contrast agents are prepared in aqueous
30 medium. In another embodiment, the contrast agents of defined molecular weight and/or molecular weight distribution are prepared by varying reaction conditions. In another embodiment, the contrast agents of a narrow distribution range or a desired distribution range are prepared by purification or fractionation methods such as ultrafiltration, size exclusion, and dialysis.

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In another aspect, the invention provides a method for obtaining magnetic resonance images of a tissue or organ of a mammal by administering an effective amount of one or more macromolecular contrast agents to the mammal and obtaining magnetic resonance images. One skilled in the art will understand that many known methods of obtaining magnetic resonance images exist in the scientific and medical field. In a preferred embodiment, an MRI procedure is performed on a human subject. One skilled in the art will appreciate that a variety of tissues and organs may be examined using different aspects of this invention, including, but not limited to, liver, spleen, lung, esophagus, bone marrow, lymph node, lymph vessels, nervous system, brain, spinal cord, blood capillaries, stomach, small intestine, large intestine. One skilled in the art will appreciate that both normal tissues and abnormal tissues, such as tumors, can be examined.

Another aspect of this invention relates to a method of clearing metal complexes. Preferably, the clearance procedure is performed after the MRI procedure has been completed or substantially completed. In one embodiment, mercaptans, or other similar agents, are administered after the MRI procedure. In various embodiments, these agents facilitate the excretion process by cleaving the macromolecular backbone. Alternatively, or in addition, clearing occurs by removal of the paramagnetic metal complexes from the polymer carriers by cleavage of the disulfide bond. Several embodiments are particularly advantageous because the paramagnetic metal complexes released from the macromolecules can be cleared at a rate comparable to that of the small molecular contrast agents used clinically today.

In a preferred embodiment, the macromolecular compounds have a prolonged retention time in the blood pool, favorable accumulation in the solid tumor tissues, and are cleared rapidly after MRI. These macromolecular agents, and the methods described thereof, will be indispensable tools in a variety of medical procedures, including, but not limited to, angiography, plethysmography, lymphography, mammography, cancer diagnosis, and functional and dynamic MRI.

The present invention is further described in the following non-limiting examples, which are offered by way of illustration and are not intended to limit the invention in any manner.

EXAMPLES

Examples of preparation of DTPA-cystine copolymers, DTPA-cystine diethyl ester copolymers, and paramagnetic complexes GDCEP and GDCEP, degradation of GDCEP and GDCEP in rat plasma, MR imaging and Data Analysis, have been presented in Ref. [12] (Y. Zong, T. Ke, A.M. Mohs, J. Guo, D.L. Parker, Z.-R. Lu Effect of size and charge on in vivo MRI contrast enhancement of polydisulfide Gd(III) complexes. J. Controlled Rel. 2006, 112, 350-356), the entire contents of which are incorporated herein by this reference.

10 **EXAMPLE 1 Preparation of polydisulfide copolymers in aqueous phase Effects of reaction temperature on molecular weight distribution:**

Cystine (5 mmol, 1.200 g, >99%) was dissolved in 2 ml aqueous solution and the pH of the solution was adjusted with NaOH to 11 at room temperature. The reactions were then carried out at three different temperatures, -10°C (ice-NaCl bath), 0°C (ice water bath) and room temperature, respectively. Diethylenetriamine-N,N,N',N'',N''-pentaacetic acid dianhydride (DTPA-DA) (5 mmol, 1.787 g) was added in portions within 1 h with fast stirring. The pH of the reaction mixture was maintained at 11 with saturated NaOH aqueous solution. 30% more DTPA dianhydride was added in portions in 30 minutes at constant pH 11. Five minutes after adding the last portion of DTPA-DA, 10 % (by weight) of HCl was added to adjust pH of the reaction mixture to 7. The molecular weight distribution of the copolymers was analyzed by size exclusion chromatography (SEC) using AKTA™ FPLC system (Amersham Bioscience Corp., Piscataway, NJ) with a Superose 12™ column (Table 1). FIG. 1 shows the molecular weight distribution of DTPA cystine copolymers prepared at different temperature. It shows that the reaction temperature has significant impact on the molecular weight distribution. High reaction temperature increases the molecular weights of copolymers.

30 Table 1. The weight-average (Mw) and number-average (Mn) molecular weights of the biodegradable macromolecular ligands, DTPA-cystine copolymers prepared at different reaction temperature.

Reaction temperature	Mw (kDa)	Mn (kDa)
-10 °C	50	39
0 °C	40	33
room temperature (25 °C)	38	22

Effects of feed ratio of DTPA-DA to diamine on molecular weight distribution:

Cystine (5 mmol, 1.200 g) was dissolved in 2 ml water and the pH was adjusted with NaOH to 11 at room temperature. DTPA-DA of different molar ratios to cystine (0.9, 1.0, 1.1, 1.2 and 1.3) was added in portions within 1.5 h with fast stirring to the cystine solutions. The pH of the reaction mixtures was maintained at pH 11 with saturated NaOH aqueous solution. Five minutes after addition of the last portion of DTPA-DA, 10% (by weight) of HCl was added to adjust pH of the reaction mixture to 7. The molecular weight of the DTPA-cystine copolymers prepared at different molar ratios was analyzed by size exclusion chromatography with a Superose 12 column. See Table 2 and FIG. 2. High molar ratio (1.2 and 1.3) of DTPA dianhydride to cystine gives high molecular weight. The molecular weight of the copolymers was then decreased with decreasing molar ratios.

Table 2. The weight-average (Mw) and number-average (Mn) molecular weights of the biodegradable macromolecular ligands, DTPA-cystine copolymers prepared at different DTPA to cystine ratios.

[DTPA-dianhydride]/[cystine]	Mw (kDa)	Mn (kDa)
0.9	28	22
1.0	40	32
1.1	49	38
1.2	51	40
1.3	50	39

Copolymerization of DTPA-Cystine:

Cystine (10 mmol, 2.403 g) was dissolved in 5 ml of aqueous NaOH at pH 11 at room temperature and then the mixture was cooled in an ice water bath. DTPA dianhydride (10 mmol, 3.573 g) was then added in portions within 1 h, maintaining at pH 11 with NaOH aqueous solution. Five minutes after addition of the last portion of DTPA-DA, 10% (by weight) of HCl was added to adjust pH to 7 and the solution was dialyzed against deionized water using membrane with molecular weight cutoff of 6–8000 Da for 24 h. The copolymer solution was lyophilized giving 3.2 g colorless solid product (54%). The number (Mn) and weight (Mw) average molecular weights of the copolymers were 18 and 33 kDa as determined by SEC using AKTA FPLC system with a SuperoseTM 12 column. The system was calibrated with standard poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA).

Copolymerization of DTPA-Glutathione (oxidized form):

Glutathione (8 mmol, 4.901 g, oxidized form) was dissolved in 5 ml of aqueous NaOH at pH 11 at room temperature and then the mixture was cooled in an ice water bath. DTPA dianhydride (8 mmol, 2.850 g) was then added in portions within 1 h, maintaining at pH 11 with NaOH aqueous solution. Five minutes after addition of the last portion of DTPA-DA, 10 % (by weight) of HCl was added to adjust pH to 7 and the solution was dialyzed against deionized water using membrane with molecular weight cutoff of 6–8000 Da for 24 h. The copolymer solution was lyophilized giving 4.00 g colorless solid product (52%). The number (Mn) and weight (Mw) average molecular weights of the copolymers were 37 and 61 kDa as determined by SEC using AKTA FPLC system with a Superose™ 6 column.

Copolymerization of DTPA and Cystamine:

Cystamine hydrochloride (5 mmol, 1.126 g) was dissolved in 2 ml of de-ionized water at room temperature, and the pH was adjusted to 11 using saturated NaOH aqueous solution. Under fast stirring, DTPA dianhydride (6 mmol, 2.144 g) was then added in portions within 1 hour at room temperature, maintaining at pH 11 with NaOH aqueous solution. Five minutes after the addition of the last portion of DTPA-DA, 10% (by weight) of HCl was added to adjust pH to 7 and the solution was dialyzed against de-ionized water using membrane with molecular weight cutoff of 6–8000 Da for 24 h. The copolymer solution was lyophilized giving 1.46 g colorless solid product (50%). The number (Mn) and weight (Mw) average molecular weights of the copolymers were 35 and 42 Da as determined by size exclusion chromatography (SEC) using AKTA FPLC system with a Superose 6 column.

EXAMPLE 2 Preparation of paramagnetic polydisulfide copolymers complexes
Complexation of DTPA disulfide copolymers with Gd³⁺:

The paramagnetic complexes, (Gd-DTPA)-cystine copolymers (GDCP), (Gd-DTPA)-glutathione copolymers (GDGP) and (Gd-DTPA)-Cystamine (GDCC), were prepared by the complexation of DTPA-cystine copolymers (DCP), DTPA-glutathione copolymers (DGP) and DTPA-cystamine copolymers (DCC) with Gd³⁺, respectively. Briefly, 0.5 g of DCP or DGP or DCC was dissolved in de-ionized water. Xylenol orange will be added as the indicators of free Gd³⁺. Then a slight excess of Gd(OAc)₃ was added to the solution at pH 5.5-6.0 until the color was changed to red from orange. The pH was

then adjusted to 10 using 5 M of NaOH to precipitate the excess of Gd^{3+} as Gd_2O_3 . After centrifugation, the supernatant will be collected and dried to give GDCP or GDGP or GDCC.

5 **EXAMPLE 3 Purification of paramagnetic polydisulfide copolymers complexes**

Purification of paramagnetic polydisulfide copolymers complexes was further carried out by chromatographic methods or by ultrafiltration methods in the presence of citric acid to remove residual Gd^{3+} .

10 In the first purification method, free Gd^{3+} was removed by size exclusion chromatography with a column packed with G-25 medium. The column was eluted with pure water and polymer fractions were collected.

In a second method, the polymer solution was placed in an ultrafiltration chamber with a semipermeable membrane, MWCO = 10000 Da (protein). The solution was stirred under elevated pressure and diluted several times in the chamber. The concentrated
15 polymer solution was evaporated until dryness. The SEC profile from the polymer purified with the ultrafiltration was similar to that purified by the SEC (FIG. 3).

EXAMPLE 4 Fractionation of paramagnetic polydisulfide copolymers complexes

The fractionation of polydisulfide Gd-DTPA complexes were carried out by SEC
20 (Size exclusion chromatography) using AKTA™ FPLC system equipped with Hiload™ 16/60 Superdex™ 200 or Superose™ 6 XK50/100 prep grade column (Amersham Bioscience). Briefly, 50 mg of GDCP or GDGP was dissolved in water and loaded to the aforementioned columns. The flow phase is 0.02 M of Tris buffer at pH 7.4, and flow rate was 60 ml/hour for the Superdex™ 200 column and 5.0 ml/min for the Superose™ 6
25 column. The fractions were collected and their molecular weights were determined with SEC equipped with Superose™ 6 column (Table 3). Gd contents of polydisulfide contrast agents were determined using inductive coupling plasma-optical emission spectrum (ICP-OES).

30 **Table 3. GDCP and GDGP fractions with different molecular weight**

	GDCP			GDGP		
Mw (kDa)	109	43	23	108	43	21
Polydispersity Index	1.07	1.08	1.05	1.10	1.08	1.09
mmol Gd/g of polymer	0.90	0.82	0.88	0.63	0.63	0.62

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Procedure for fractionation of Gd-DTPA cystamine copolymers (GDCC) using AKTApriTM plus FPLC and SuperoseTM 6 prep grade XK 50/100 column is set forth as follows: (1) Before fractionation, wash pump of FPLC using Tris buffer (pH 7.5), then wash the column using same buffer for total 18 hr at 4.5 ml/min. (2) Prepare 2.7 ml Gd-DTPA cystamine copolymers solution at a concentration of 250 mg lyophilized polymer / ml DI water, then inject into the FPLC via a 0.2 μ L filter. Flow rate is set to 5.0 ml/min. (3) Discard first 700 ml eluent and collect fractions in the following 1200 ml. Each fraction contains 20 ml eluent.

The characterization of the fractions of Gd-DTPA cystamine copolymers is done with AKTA P-920 FPLC, Superose 6 10/200 GL column and a Knauer RI detector using PHPMA as standard. The flow rate is set to 0.5 ml/min. The characterization of major fractions is shown in FIG. 4. The number average, weight average molecular weight and polydispersion index (PDI) of some combined fractions and fractions are listed in Table 4.

Table 4. The number average, weight average molecular weight and polydispersion index (PDI) for major fractions.

Fraction(s)	6 and 7	8 and 9	10	11	12	13	14	15	17
Mn, KDa	225	192	163	152	136	127	116	102	84
Mw, KDa	234	198	169	158	141	131	120	106	89
Mw/Mn (PDI)	1.04	1.03	1.04	1.04	1.04	1.03	1.04	1.04	1.06
Fraction(s)	20	21	22	24	26	28	3031	32 and 33	34 and 35
Mn, KDa	66	60	55	47	38	34	28	22	19
Mw, KDa	69	63	58	49	41	36	30	24	20
Mw/Mn (PDI)	1.06	1.06	1.05	1.05	1.06	1.06	1.06	1.05	1.06

EXAMPLE 5 Application of Gd-containing polydisulfide MRI contrast agents in MR imaging

The *in vivo* contrast enhancement by GDGP and GDGP was investigated in female athymic nude mice (Charles River Lab) with human breast cancer (MB-231) using a Siemens Trio 3T scanner with a human wrist coil. The mice were anesthetized by the intramuscular administration of a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg). Contrast enhanced MR images of the mice were acquired using spin echo sequence before and at 2, 5, 10, 15, 30 and 60 minutes after injection of the contrast agents at a dose of 0.1 mmol-Gd/kg via a tail vein. The imaging parameters were 2.7 ms TE, 7.8 ms TR, 25 °C RF tip angle, 0.5 mm axial slice thickness.

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Three-dimensional maximum intensity project (MIP) MR images of mice before and at various time points after injection of GDCP and GDGP with different molecular weights are shown in FIG. 5. Strong contrast enhancement was observed in the liver, kidneys and blood in the heart and vasculature with all agents 2 min post-injection and the signal intensity gradually decreased thereafter. Both agents with highest MW can strongly enhanced blood vessel up to 30 minutes. Both the signal intensity and enhancement duration for GDCP and GDGP were increased with molecular weight. Gradual enhancement in the urinary bladder was observed for all compounds, indicating urinary clearance of the agents. High molecular biodegradable agents (108 kDa) provide more prolonged and significant contrast enhancement for cardiac and vasculature imaging than the corresponding agents of low molecular weights.

T1 weighted axial images of tumors were also acquired with human wrist coil using spin echo sequence with 10 ms TE, 400 ms TR, 90° RF tip angle, 2 mm axial slice thickness. T1 weighted tumor images showed strong enhancement by both GDCP and GDGP in the tumor periphery 2 minutes post-injection and up to 60 minutes (FIG. 6). The contrast enhancement in tumor center is weaker than in periphery. The polymer agents with low molecular weight (23 kDa) resulted in more significant enhancement in the tumor tissues than high molecular weight agents.

While this invention has been described in certain embodiments, the present invention can be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains and which fall within the limits of the appended claims.

All references, including publications, patents, and patent applications, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

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CLAIMS

What is claimed is

- 5 1. A method for preparing a biodegradable macromolecular contrast agent, the method comprising at least one step of polymerization in an aqueous medium, wherein the polymerization results in polydisulfides.
2. The method according to claim 1, wherein the aqueous medium is a
10 basic aqueous solution.
3. The method according to claim 1 or 4, comprising complexing the polydisulfides with Gd^{3+} .
- 15 4. A method for preparing a polydisulfide contrast agent, the method comprising:
selecting a reaction condition, wherein the reaction condition determines the molecular weight and/or molecular weight distribution of the contrast agent.
- 20 5. The method according to claim 4, wherein the reaction condition is reaction temperature.
6. The method according to claim 4, wherein the reaction condition is feed ratio of polymerization reactants.
- 25 7. The method according to claim 1, 3 or 4, comprising:
purifying the contrast agent by size exclusion chromatography, ultrafiltration and/or dialysis.
- 30 8. The method according to claim 1, 3 or 4, comprising:
fractionating the contrast agent by size exclusion chromatography.
9. The method according to claim 1, 3 or 4, comprising:
dissolving at least one reactant in a reaction medium,

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adjusting the pH of the reaction medium as needed,
adding at least one other reactant to the reaction medium,
stirring the reactants and the reaction medium for a period of time, and
adjusting the pH of the reaction medium as needed.

5

10. The method according to claim 3, further comprising:
precipitating excess of Gd^{3+} at pH = or > 10.

11. The method according to claim 1, 3, or 4, wherein the molecular weight
10 of the contrast agent is 40 to 200 kDa.

12. The method according to claim 1, 3, or 4, wherein the molecular weight
of the contrast agent is 5 to 40 kDa.

13. The method according to claim 9, further comprising:
15 lyophilizing resulting products of the reactants.

14. The method according to claim 9, further comprising:
purifying resulting products of the reactants by dialysis, chromatography and/or
20 ultrafiltration.

15. The method according to claim 9, further comprising:
fractionating the contrast agent using size exclusion chromatography.

16. A macromolecular contrast agent prepared by the method according to
25 claim 1 or claim 3 or claim 4, the contrast agent comprising a compound selected from
the group consisting of GDCC, GDCP, GDCEP, GDGP, compounds according to
Structures I through VII of US Patent 6,982, 324, and compounds according to Agents
1 through 11 of US Patent 6,982, 324, wherein the contrast agent has a controlled or
30 defined molecular weight and/or molecular weight distribution.

17. The macromolecular contrast agent of claim 16, comprising at least one
targeting molecule.

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18. The macromolecular contrast agent of claim 16, said contrast agent is able to provide prolonged and effective contrast enhancement for cardiac and vasculature imaging, and the molecular weight of said contrast agent is 40 to 200 kDa.

5 19. The macromolecular contrast agent of claim 16, said contrast agent is able to provide effective contrast enhancement for tumor tissue imaging, and the molecular weight of said contrast agent is 5 to 40 kDa.

10 20. A method of applying the contrast agent of claim 16 in a medical procedure, the method comprising:
administering the contrast agent of claim 16, or pharmaceutically acceptable salts thereof, or the contrast agent of claim 16 in conjunction with one or more physiologically acceptable agents, to a mammal.

15 21. The method of claim 20, wherein the medical procedure comprises magnetic resonance imaging, X-ray computed tomography, scintigraphy, positron emission tomography or radiotherapy.

20 22. The method of claim 20, wherein the contrast agent is degraded by endogenous agents.

25 23. The method of claim 20, wherein one or more disulfide reducing agents are administered to the mammal either simultaneously with the contrasting agent or sequentially.

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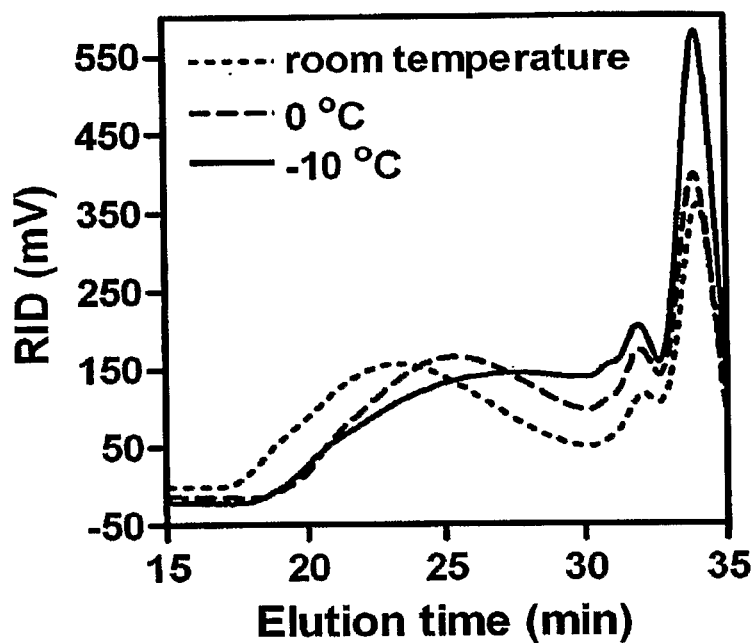


FIG. 1

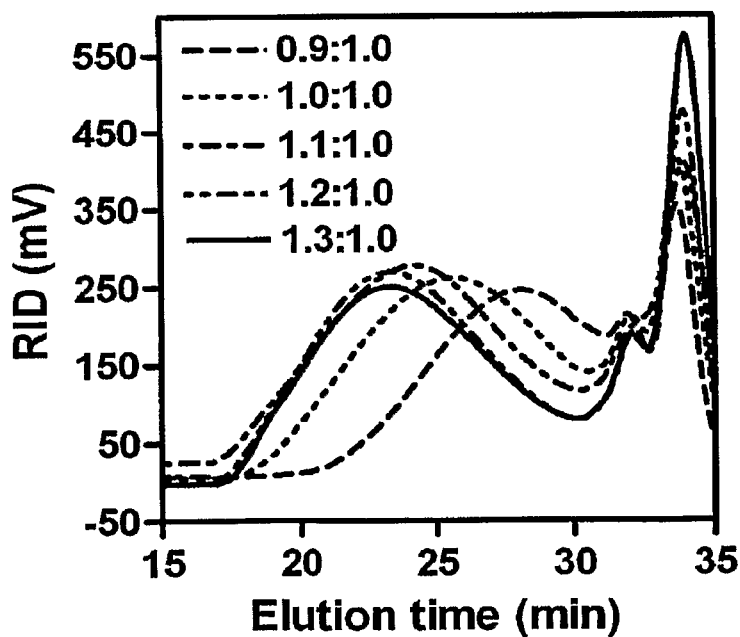


FIG. 2

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Comparison of the Purification of poly(GdDTPA-co-L-Cystine) by PD-10 Columns vs. Spin Cell

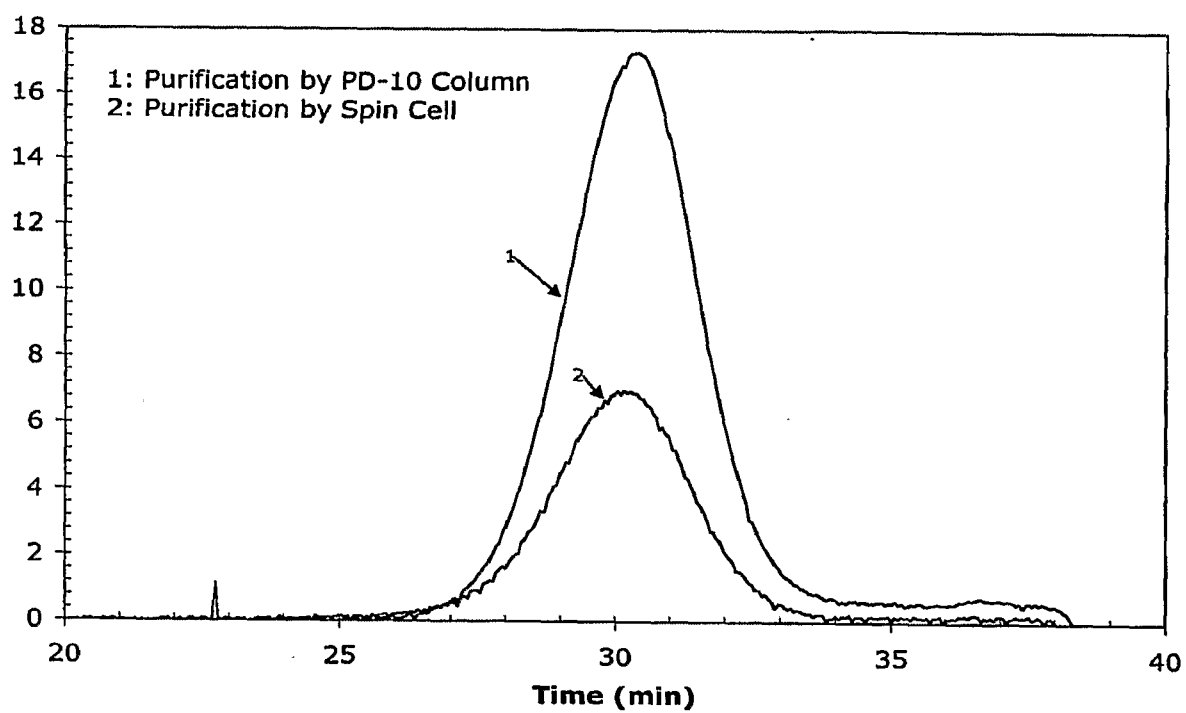
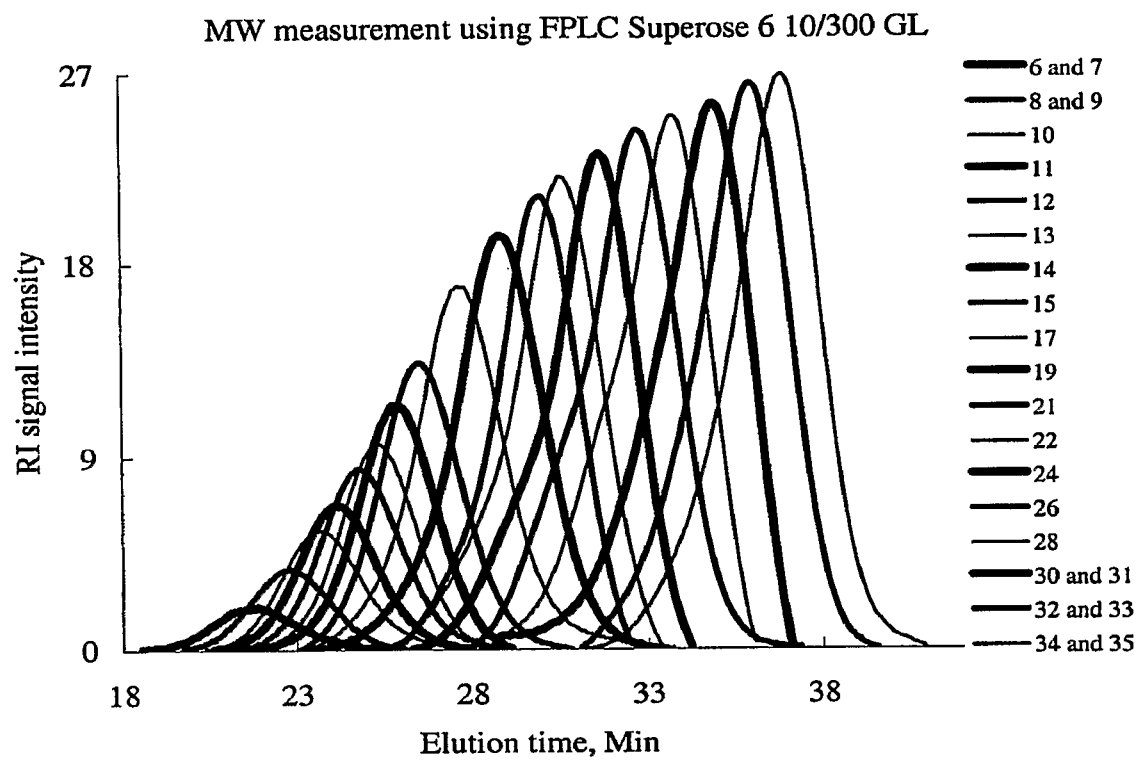


FIG. 3

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**FIG. 4**

4/4

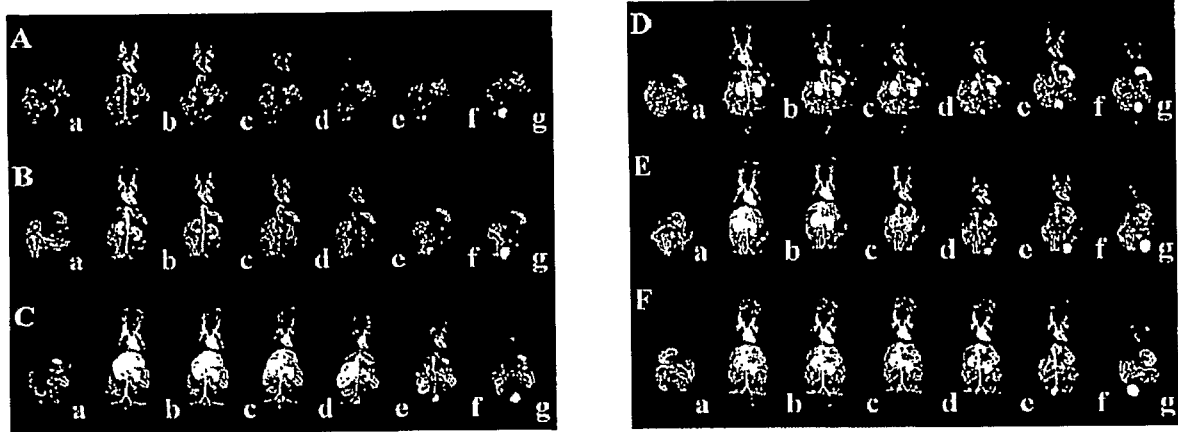


FIG. 5

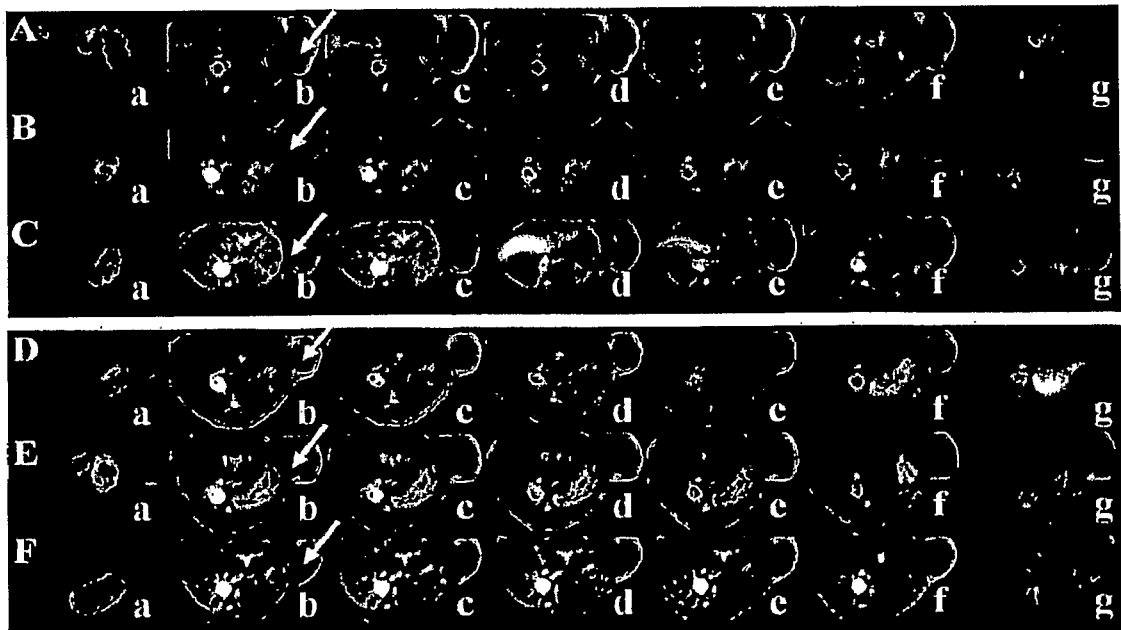


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/14223

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61B 5/055(2006.01) A61B 5/00(2006.01) USPC: 424/9.3,9.322,9.323,9.36 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/9.3,9.322,9.323,9.36 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,982,324 B1 (LU et al) 06 January 2006 (06.01.2006), see entire document.	16-23
X	ZONG et al. Contrast enhanced MRI with New Biodegradable Macromolecular Gd(III) Complexes in Tumor-Bearing Mice. Magnetic Resonance in Medicine. Published online 29 March 2005, Vol. 53, pages 835-842. See entire document.	1, 2, 4-6, 16 and 19-23
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Y		1, 2, 4-6 and 16-23
Y	WANG et al. Pharmacokinetics and Tissue Retention of (Gd-DTPA)-Cystamine Copolymers, A Biodegradable Macromolecular Magnetic Resonance Imaging Contrast Agent. Pharmaceutical Research. April 2005, Vol. 22, No. 4, pages 596-602. See entire document.	1, 2, 4-6 and 16-23
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 12 October 2007 (12.10.2007)	Date of mailing of the international search report 25 OCT 2007	
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Leah Schlientz <i>Helicia D. Roberts</i> Telephone No. 571-272-1600 <i>for</i>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/14223

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 3 and 7-15
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 3 depends on a claim that does not precede it. Claims 7-15 depend on claim 3.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
 - ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
 - ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US07/14223

Continuation of B. FIELDS SEARCHED Item 3:
EAST, MEDLINE
search terms: biodegradable, polydisulfide. MRI, gadolinium

专利名称(译)	可生物降解的大分子MRI造影剂及其制备和使用方法		
公开(公告)号	EP2037808A1	公开(公告)日	2009-03-25
申请号	EP2007809652	申请日	2007-06-13
[标]申请(专利权)人(译)	犹他大学研究基金会		
申请(专利权)人(译)	UTAH研究基金会大学		
当前申请(专利权)人(译)	UTAH研究基金会大学		
[标]发明人	ZONG YUDA LU ZHENG RONG MOHS AARON FENG YI		
发明人	ZONG, YUDA LU, ZHENG-RONG MOHS, AARON FENG, YI		
IPC分类号	A61B5/055 A61B5/00		
CPC分类号	G01R33/5601 A61K49/128		
优先权	60/814449 2006-06-16 US		
外部链接	Espacenet		

摘要(译)

公开了制备可降解大分子磁共振成像造影剂的新方法。公开了用于各种诊断程序的具有限定或控制的分子量分布的可降解的大分子磁共振成像造影剂，以及合成，使用和降解这些试剂的方法。在本发明的各个方面中公开的大分子造影剂是可降解的顺磁性聚二硫化物化合物，其显示出延长的血浆保留，并且在各种组织，器官或肿瘤中具有增强的渗透性和保留性，但仍然能够从体内快速清除。