Title: METHOD OF PREPARING MACROMOLECULAR CONTRAST AGENTS AND USES THEREOF

Abstract: Disclosed are methods of preparing a macromolecular conjugated ligand and a metal complex thereof. The metal complex is targeted for use as a contrast agent, for example, in MRI. The method of preparing a macromolecular conjugated ligand comprises: (a) providing a compound of formula (I) \[ R \begin{array}{c}
\text{CH}_2\text{COOPg}_n \\
\text{A}
\end{array} \tag{I} \]
wherein R, A, and Pg are as defined herein, (b) reacting the compound of formula (I) with a macromolecular compound (e.g., dendrimer) in an organic solvent medium which is substantially free of water to obtain a macromolecular conjugated compound, and (c) removing the carboxyl-protecting groups to obtain a carboxyl-deprotected macromolecular conjugated compound. The metal complex can be prepared by reacting the carboxyl-deprotected macromolecular conjugated compound with an ion (e.g., Gd(III)). Also disclosed are carboxyl-protected intermediate compounds.
METHOD OF PREPARING MACROMOLECULAR CONTRAST AGENTS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 60/864,503, filed November 6, 2006, which is incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] The development of contrast agents for magnetic resonance imaging (MRI) in clinical settings continues to receive great attention (Merbach et al., The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging. John Wiley & Sons: New York, 2001). Paramagnetic metal chelates, such as Gd(III)-diethylenetriaminepentaacetic acid (Gd(III)-DTPA) (Magnevist), Gd(III)-N,N,N"'-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane (Gd(III)-DOTA), and their analogs have proven to increase the relaxation rate of surrounding protons and have been widely used as MRI contrast agents (Lauffer, Chem. Rev., 1987, 87, 901-927 and Caravan et al., Chem. Rev., 1999, 99, 2293-2352). However, these low molecular weight agents have disadvantages such as rapid circulation and clearance rates in vivo, and relatively low molar relaxivity properties thus limiting time-dependent imaging studies or acquisition of highly resolved images of patients (Kobayashi et al., Adv. DrugDeliv. Rev., 2005, 2271-2286; Comblin et al., Coord. Chem. Rev., 1999, 186, 451-470; and Raymond et al., Bioconjugate Chem., 2005, 16, 3-8).

[0003] Attempts have been made to develop bifunctional chelates as imaging agents. Such attempts have led to the establishment of a library such as 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pentaacetic acid (1B4M-DTPA), N-[2-amin0-3-(4-isothiocyanatobenzyl)propyl]-czs-cyclohexyl-1,2-diamine- N,N',N"'-pentaacetic acid (CHX-A-DTPA), and 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N"'-tetraacetic acid (p-SCN-Bz-DOTA), that are potentially useful for forming Gd(III) complexes and thus MRI contrast agents (Brechbiel et al., Bioconjug. Chem., 1991, 2, 187-194 and Wu et al., Bioorg. Med. Chem. Lett., 1997, 5, 1925-1934). The bifunctional chelators permit, on the one hand, conjugation to biomolecules such as, antibodies and peptides, dendrimers, and other macromolecular structures (Milenic et al., Nature Reviews Drug Discovery, 2004, 3, 488-499), and chelation to metal ions, on the other hand.
The synthetic methods attempted in the past to prepare macromolecular conjugated bifunctional chelators have one or more drawbacks such as the use of large excess of reagents or the need to carry out extensive purification from impurities formed in the conjugation reaction.

Thus, there is a desire for an improved synthesis of macromolecular-based magnetic resonance agents.

**BRIEF SUMMARY OF THE INVENTION**

The invention provides a synthetic approach of a metal chelate based on a multidentate polyamino ligand having one or more carboxymethyl groups, such as 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pentaacetic acid (1B4M-DTPA), that is conjugated to a biomolecule (e.g., antibody or peptide) or dendrimer. In particular, the synthesis provides an improvement in overall efficiency of bifunctional chelator binding to poly-amine surface dendrimers by elimination of loss of the reagent to aqueous basic pH conditions, shorter reaction times, and a potential increase in loading efficiency of chelator onto the macromolecular structure. Specifically, the synthesis of a conjugate complex includes the following steps: (a) preparation of a protected multidentate polyamino ligand having one or more carboxymethyl groups, (b) conjugation to a macromolecular structure (e.g., dendrimer) in an organic solvent, (c) deprotection of the carboxylic acid groups, and (d) complexation with a metal (e.g., Gd(III)). Advantageously, elimination or delay of using an aqueous solvent helps improve the overall efficiency of conjugation and reduces possible contamination by spurious metals that could compromise the radiolabeling and/or metal complexation in step (d).
BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0007] Figure 1 illustrates a method of preparing protected 1B4M-DTPA compounds, in accordance with an embodiment of the invention. The reaction conditions are: (a) H₂, 10% Pd/C, EtOH, 96%; (b) glutaric anhydride, EtOAc, 82%; (c) EDCI, NHS, MeCN, 88%; (d) ClCSCl, EtOAc, 96%; and (e) N-maleimidocaproic acid, EDCI, CH₂Cl₂, 84%.

[0008] Figure 2 illustrates a method of preparing Gd-1B4M-DTPA functionalized PAMAM G4 dendrimers, in accordance with an embodiment of the invention. The reaction conditions are: (a) NHS-1B4M-DTPA (4), Et₃N, DMSO for 9a; (b) NCS-1B4M-DTPA (5), MeOH for 9b; (c) trifluoroacetic acid; and (d) Gd(OAc)₃·2H₂O, citrate buffer.

[0009] Figure 3 illustrates a method of preparing 2-methyl-6-(p-aminobenzyl- N-[5-oxopentanoic acid])diethylene- N,N,N’,N”-penta-tert-butylacetate methyl ester (7), which is a precursor to a compound in accordance with an embodiment of the invention.

[0010] Figure 4 illustrates a method of preparing 2-methyl-6-(p-methylisothiourethanebenzyl)diethylene- N,N,N’,N”-penta-tert-butylacetate (8), which is a precursor to a compound in accordance with an embodiment of the invention.

[0011] Figure 5A is a MALDFTOF mass spectrum of macromolecular conjugate ligand complex 11a, and Figure 5B is a MALDI/TOF mass spectrum of macromolecular conjugate ligand complex lib, in accordance with an embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

[0012] The present invention provides, in an embodiment, a method of preparing a macromolecular conjugated ligand comprising:

(a) providing a compound of formula (I)

\[ \text{R} \]

\[ \text{A} \]

\[ \text{m} \]

\[ \text{CH₂COOPg} \]

\[ \text{n} \]

\[ \text{(I)} \]

wherein
A is a polyamino group,
R is hydrogen, halo, alkyl, hydroxy, nitro, amino, alkyamino, dialkylamino, thiocyano, isothiocyano, carboxyl, carboxyalkyl, carboxyalkyloxy, amido, alkylamido, haloallylamido,
Pg is a carboxyl-protecting group,

n is 1 to 5, and m is 0 to 3,

(b) reacting the compound of formula (I) with a macromolecular compound in an organic solvent medium which is substantially free of water to obtain a carboxyl-protected macromolecular conjugated ligand, and

(c) removing the carboxyl-protecting group to obtain a macromolecular conjugated ligand.

[0013] The method can further comprise step (d), wherein step (d) comprises reacting the macromolecular conjugated ligand with an ion (e.g., a metal or non-metal ion), which is optionally radioactive, to obtain a macromolecular conjugated ligand complex.

[0014] The polyamino group A is a polyallcyleneimine group and is cyclic (e.g., together with carboxymethyl groups, DOTA, 1,4,7-triazacyclononane-\(N,\text{iV}^7,\text{N''-triacetic acid (NOTA)}\)) or acyclic (e.g., together with carboxymethyl groups, DTPA). Examples of A, together with carboxyethyl groups, are described in WO 04/021996 and U.S. Patents 7,081,452, 5,434,287, 5,286,850, 5,246,692, and 5,124,471, which are incorporated herein by reference.

The number of carboxymethyl groups varies depending on the structure and number of amino moieties. For this reason, n is 1 to 5 (e.g., 1, 2, 3, 4, or 5). Typically for DOTA and DTPA moieties, n is 4 or 5, respectively. The backbones of the polyamino cyclic or acyclic ligand can be substituted with, for example, alkyl (e.g., methyl), cycloalkyl (e.g., cyclohexyl), or heterocycloalkyl (e.g., piperidinyl). The backbone substituents can be any suitable stereochemistry, such as cis or trans, (e.g., \(\text{p} \alpha\text{w}\)-cyclohexyl). In an embodiment, the polyamino group A together with \((-\text{CH}_2\text{COOPg})_n\) can be a compound of formula (II) or (III):
wherein $R^1$ is hydrogen, a $C_{i-2}$ alkyl group, or a point of attachment of $R^1$ and $R^2$ or $R^3$ and $R^4$ together form a $C_5$-$C_7$ cycloalkyl group.

[0015] In an embodiment of the invention, $-A-(CH_2COOPg)_n$ can be, for example, 6-methyl-diethylenetriamine pentaacetic acid (a), cyclohexyl-l,2-diamine- $N,N',N''$-pentaacetic acid (b), or 1,4,7,10-tetraazacyclododecane- $N,N',N''$-tetraacetic acid (c).

[0016] $R$ is selected from the group consisting of hydrogen, halo, alkyl, hydroxy, nitro, amino, alkyamino, dialkylamino, thiocyano, isothiocyano, carboxyl, carboxyalkyl, carboxyallcyloxy, amido, alkylamido, haloalkylamido, and...
Preferably, R is isothiocyanato (-NCS), or. The substituent R can be at any suitable position on the phenyl ring relative to the remainder of the molecule (e.g., ortho, meta, para). Preferably, R is para to the remainder of the molecule. The substituent m is 0 to 3 (e.g., 0, 1, 2, or 3). The value of m depends on the specific structure of formula (I) and can be varied for ease of conjugation to the macromolecular compound. Preferably m is 1.

[0017] In an embodiment of the invention, the compound of formula (I) is

[0018] In a specific embodiment, a compound of formula (I) includes a compound of formula (Ia), (Ib), (Ic), (Id), (Ie), and/or (If):
wherein $Pg$ is a carboxyl-protecting group.
In the compound of formula (I), (Ia), (Ib), (Ic), (Id), (Ie), and/or (If), the protecting group, Pg, is any suitable carboxylic acid protecting group. Typically Pg forms an ester with the carboxylate functional group. For example, suitable moieties for Pg include alkyl (e.g., methyl, \(^\text{-butyl}\), benzyl, 9-fluorenylmethyl, diphenylmethyl, silylalkyl, haloalkyl, and 1,1-dimethylallyl (DMA). Preferably, Pg is an alkyl, such as f-butyl.

The protecting group can be added to the -A-(CH\(_2\))\(_n\)COOPg\(_n\) group (e.g., DTPA) by any suitable method known in the art. For example, alkyl esters can be formed by Fisher esterification, addition of acid chloride and alcohol, addition of isobutylene and acid, or addition of diazomethane. Use of a coupling agent, such as dicyclohexylcarbodiimide (DCC) (Tetrahedron Letters, 1983, 24, 281) is a common method of forming protected carboxylic acids (e.g., 9-fluorenylmethyl esters, haloesters, 2-(trimethylsilyl)ethyl esters, benzyl esters, and diphenylmethyl esters). DMA esters can be formed using procedures known in the art. See, for example, Sedighi et al. Organic Letters, April 2006, 7(8), 1473-1475.

When necessary, the protecting group can be removed by any suitable method known in the art, which method typically includes addition of acid, base or an organometallic reagent (e.g., magnesium bromide), enzymatic hydrolysis (e.g., pig liver esterase), hydrogenolysis, reduction, or addition of dibutyltin oxide. Any suitable acid or base can be used, including trifluoroacetic acid, HCl, H\(_2\)SO\(_4\), H\(_3\)PO\(_4\), NaOH, KOH, or LiOH. For example, for the cleavage of 9-fluorenylmethyl, addition of a mild base (e.g., diethylamine, piperidine, or ammonia) is preferred. For hydrogenolysis, conditions such as addition of hydrogen gas in the presence of a catalyst (e.g., palladium on carbon) can be used. Silylalkyl esters can be cleaved, for example, with fluoride ion. Halooesters can be removed, for example, with Zn(O) dust or electrochemically. Diphenylmethyl esters can be cleaved, for example, with boron trifluoride. Additional protecting group and methods of addition and removal are discussed in T. W. Greene et al. Protective Groups in Organic Synthesis (2nd Ed.) J. Wiley and Sons, 1991, which is incorporated by reference.

The term "alkyl" means a straight-chain or branched alkyl substituent containing from, for example, 1 to about 12 carbon atoms, preferably from 1 to about 8 carbon atoms, more preferably from 1 to about 6 carbon atoms. Examples of such substituents include methyl, ethyl, propyl, isopropyl, \(-\text{butyl}, \text{sec-butyl}, \text{isobutyl}, \text{\textasciitilde butyl}, \text{pentyl}, \text{isoamyl}, \text{hexyl}, \text{octyl}, \text{dodecanyl}, \text{and the like, preferably} f\text{-butyl.}
The term "halo" as used herein, means a substituent selected from Group VIIA of the Periodic Table of Elements, such as, for example, fluorine, bromine, chlorine, and iodine. Preferably, the halo is bromine or iodine.

The term "haloalkyl," as used herein, means an alkyl substituent that is bonded to at least one halo as described herein. The alkyl group of haloalkyl is as described above.

The term "alkylamino" refers to a secondary amine substituent with one hydrogen and one alkyl group directly attached to a trivalent nitrogen atom. The term "dialkylamino" refers to a tertiary amine substituent with two of the same or different alkyl groups directly attached to a trivalent nitrogen atom. The alkyl group is described above.

The term "carboxyl" refers to the group -C(O)OH. The term "carboxyalkyl" refers to the group -R'C(O)OH that is connected to the compound through the alkyl R' group. The term "carboxyalkyloxy" refers to the group -OR'C(O)OH, in which the R' is an alkyl (e.g., \((CH_2)_n\) alkylene group, n is 1 to 12) group. The alkyl group is described above.

The term "alkylamido" refers to substituents of the formula, -C(O)NR'R', or -NR'C(O)R", in which R' and R" are the same or different and each is a hydrogen or alkyl group, as described above. The term "haloalkylamido" is an alkylamido, in which one or more of the alkyl groups is substituted with a halo moiety as described above, such as for example, chlorine, bromine or iodine.

The term "silylalkyl" as used herein, means one, two, or three alkyl groups (the same or different) as defined herein, directly attached to a tetravalent silicon atom. Examples of such substituents include, for example, trimethylsilyl, methyl(dibutyl)silyl, tri-isopropylsilyl, and the like.

The compound of formula (I) (which includes compounds of formula (Ia), (Ib), (Ic), (Id), (Ie), and/or (If)) can be conjugated to any suitable macromolecular compound, e.g., biomolecule or a dendrimer. The term "biomolecule" refers to all natural and synthetic molecules that play a role in biological systems. A biomolecule includes a hormone, an amino acid, a peptide, a peptidomimetic, a protein, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), a lipid, an albumin, a polyclonal antibody, a receptor molecule, a receptor binding molecule, a hapten, a monoclonal antibody and an aptamer. Specific examples of biomolecules include insulins, prostaglandins, growth factors, liposomes and nucleic acids. An advantage of using biomolecules could be tissue targeting through specificity of delivery. Another advantage could be longer residence time.
Haptens such as hormones, steroids, enzymes and proteins are desirable in some applications because of their site specificity to tumors and/or various organs of the body. A preferred hapten for use in treating cellular disorders or various disease conditions is a monoclonal antibody. Methods of bonding a macrocyclic compound to a hapten are described in U.S. Patent No. 5,428,154, which are incorporated herein by reference.

Coupling of a compound of formula (I) to one or more macromolecular compounds can be accomplished by several known methods (see, for example, Krejcarek et al., Biochem. Biophys. Res. Commun., 1977, 30, 581; and Hnatowich et al., Science, 1983, 220, 613). For example, a reactive moiety present in the R substituent is coupled with a second reactive group located on the macromolecule. Typically, a nucleophilic group on the macromolecule is reacted with an electrophilic group on the compound of formula (I) to form a covalent bond between the two. Examples of nucleophilic groups include amines, anilines, alcohols, phenols, thiols and hydrazines.

The dendrimer refers to a synthetic, three dimensional molecule with branching parts. The branches are built up from monomers. The dendrimers are typically made by a nanoscale fabrication process compound suitable for conjugation to a compound of formula (I). Unlike classical polymers, dendrimers have a high degree of molecular uniformity, narrow molecular weight distribution, specific size and shape characteristics, and a highly-functionalized terminal surface. The manufacturing process is a series of repetitive steps starting with a central initiator core. Each subsequent growth step represents a new "generation" of polymer with a larger molecular diameter, twice the number of reactive surface sites, and approximately double the molecular weight of the preceding generation. Suitable dendrimers include polyamidoamine (PAMAM) dendrimers, polypropylenimine (PPI) dendrimers, and those described in the literature, such as U.S. Patents 4,289,872, 4,410,688, and 4,507,466, Tomalia et al., Polymer Journal, 1985, 17(1), 117-132, and Jiang et al., Nature, 1997, 388, 454-456. PAMAM dendrimers, which are preferred, represent a class of macromolecular architecture called "dense star" polymers. Suitable conjugation methods of a dendrimer and a compound of formula (I) are described herein and are also known in the art. See, for example, U.S. Patent 5,527,524, the entirety of which is incorporated herein.

When conjugating the compound of formula (I) to a dendrimer, typically an excess of compound compared to dendrimer is used. For example, typically at least a 2:1 (e.g., 2:2:1, 2:5:1, 2:8:1, 3:1, 4:1, 5:1) functional ratio of compound to dendrimer is used. For
most applications, a 2:1 ratio is preferred. A large excess of the compound of formula (I) is not required for step (b) of the inventive method, which can simplify the reaction monitoring process and purification step(s) since organic by-products and inorganic salts, which can be major components of the crude reaction solution, are minimized.

[0034] The conjugation reaction (i.e., step (b) of the inventive method) is carried out in a medium comprising at least one organic solvent that is free or substantially free of water, such as an alkane, an aromatic hydrocarbon, a haloalkane, an alcohol, an amide, an alkylester, a sulfoxide, a cycloalkane, a dialkylether, an alkyl aryl ether, a diarylether, and a cyclic ether. Specific organic solvents that are suitable include acetonitrile, dimethylformamide (DMF), benzene, methylene chloride, methanol, hexane, dimethylsulfoxide (DMSO), tetrahydrofuran (TEEF), furan, diphenyl ether, diethyl ether, methylethyl ether, and dioxane or any combination thereof. Preferred solvents include methanol and DMSO. The term "substantially free of water" means an organic solvent that contains less than 5% by wt. water (e.g., less than 4% by wt. water, less than 3% by wt. water, less than 2% by wt. water, less than 1% by wt. water, less than 0.5% by wt. water, less than 0.25% by wt. water).

[0035] If necessary, water can be used during steps of the inventive method other than the conjugation step. For example, to minimize any possible complications associated with removal of a high boiling point solvent (e.g., DMSO), crude conjugated compounds of formula (I) can be diluted with a first solvent (e.g., CH₂Cl₂) and then washed with water to remove the high boiling solvent. Reduction, elimination or delay of using an aqueous solvent in the synthesis helps improve the overall efficiency of conjugation.

[0036] The carboxyl-deprotected conjugated compound of formula (I) can be complexed to an ion, e.g., a metal ion or a non-metal ion, in which the ion is optionally radioactive. Typical metal ions for forming a complex of the invention include Ac, Bi, Pb, Y, Mn, Cr, Fe, Co, Ni, Tc, In, Ga, Cu, Re, a lanthanide (i.e., any element with atomic number 57 to 71 inclusive, such as Sm), and an actinide (i.e., any element with atomic number 89 to 103 inclusive). The metal ion is any metal ion that is suitable for the desired end use of the complex. For example, in proton magnetic resonance imaging, paramagnetic metal atoms such as gadolinium(III), manganese(II), manganese(III), chromium(III), iron(II), iron(III), cobalt(II), nickel(II), copper(II), praseodymium(III), neodymium(III), samarium(III), ytterbium(III), terbium(III), dysprosium(III), holmium(III), and erbium(III) (all are paramagnetic metal atoms with favorable electronic properties) are preferred as metals complexed by the carboxyl-deprotected conjugated compound. Gadolinium(III) is a further
preferred complexed metal due to the fact that it has high paramagnetism, low toxicity when complexed to a suitable ligand, and high lability of coordinated water. For use as x-ray contrast agents, the metal ion should be able to absorb an adequate amount of x-rays (i.e., radio-opaque), such as, for example, indium, yttrium, lead, bismuth, gadolinium, dysprosium, holmium and praseodymium.

[0037] The carboxyl-deprotected conjugated compound of formula (I) also can be complexed with a radioactive ion, such as a radioactive metal ion, for use as a therapeutic agent (e.g., a radiopharmaceutical). Radioisotopes of any suitable ion are acceptable for forming metal or other ion complexes. For example, typical radioisotopes include isotopes of technetium, bismuth, lead, actinium, carbon, nitrogen, iodine, fluorine, oxygen, tellurium, helium, indium, gallium, copper, rhenium, yttrium, samarium and holmium. A radioactive isotope of yttrium is preferred. Specific examples of radionuclides suitable for complexing to a carboxyl-deprotected conjugated compound for various imaging techniques, including positron emission tomography and single photon emission computed spectroscopy, are, for example, ⁸⁶Y, ²¹⁵Bi, ²¹⁶Bi, ²¹²Bi, ²²⁵Ac, ¹⁷⁷Lu, ⁹⁹ᵐTc, ¹ⁱ¹In, ¹³¹⁴C, ¹³⁷⁹N, ¹²³⁸I, ¹⁸⁶Re, ¹⁸⁷F, ¹⁵⁰⁸O, ²⁰³¹Tl, ³He, ¹⁶⁶⁷Ho and ⁶⁷⁷Ga, preferably ⁸⁶Y, and ¹¹¹In.

[0038] To prepare metal complexes of the invention, the carboxyl-deprotected conjugated compound of formula (I) is complexed with an appropriate atom or ion, e.g., metal or metal ion. This can be accomplished by any methodology known in the art. For example, the metal can be added to water in the form of an oxide, halide, nitrate or acetate (e.g., yttrium acetate, bismuth iodide) and treated with an equimolar amount of the carboxyl-deprotected macromolecular conjugated compound of formula (I). The compound can be added as an aqueous solution or suspension. Dilute acid or base can be added (where appropriate) to maintain a suitable pH. Heating at temperatures as high as 100 °C for periods of up to 24 hours or more can be employed to facilitate complexation, depending on the metal, the compound, and their concentrations.

[0039] The macromolecular conjugated complexes prepared by the inventive method can be used for obtaining a diagnostic image of a host. In such methods, typically the host is administered a complex, in an amount effective to provide an image; and the host is exposed to an energy source, whereupon a diagnostic image of the host is obtained. The diagnostic image can be, for example, a magnetic resonance image (MRI), an x-ray contrast image, positron emission tomography (PET), single photon emission computed spectroscopy (SPECT) image, or the like.
The macromolecular conjugated complexes prepared by the inventive method can be used for treating a cellular disorder, such as cancer, in a mammal. The method comprises administering to the mammal (e.g., a human) a complex of the present invention in an amount effective to treat the cellular disorder, whereupon the cellular disorder is treated. A preferred complex comprises Pb or Y, in particular 90-Y.

The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

Materials and Methods: 2-Methyl-6-(p-nitrobenzyl)diethylene-\(N,N',N^{\prime},N^{\prime}\prime,N^{\prime\prime}\)-penta-tert-butylacetate (1) is prepared by modification of the previously described procedure (Brechbiel et al., J. Chromatogr., A, 1997, 771, 63-69). \(N\)-hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI), thiophosgene, glutaric anhydride, and peptide sequence grade trifluoroacetic acid are purchased from Aldrich/Sigma Chemical Company and are used as received. Generation 4 ethylenediamine core PAMAM dendrimers are obtained from Dendritech Inc (Midland, MI) as 15.35% w/w solution in MeOH. All experiments with moisture- and/or air-sensitive compounds are carried out under a dried \(N_2\) or Ar atmosphere. For column chromatography, Merck 60 Silica Gel is used (70-230 mesh). Thin-layer chromatography (TLC) is performed on silica gel 60 F-254 plates from EM Reagents. All water used is purified using a Hydro Ultrapure Water Purification system (Rockville, MD). Isoflurane are obtained from Abbott Laboratories. 3M™ Fluorinert™ Electronic Liquid FC-77 is used in placed of water to maintain the temperature of the mouse at 32±1 °C using a Polyscience Model 210 heating recirculator.

Proton and \(^{13}\)C NMR data are obtained using a Varian Gemini 300 MHz instrument and chemical shifts are reported in ppm on the \(\delta\) scale relative to TMS, TSP, or residual solvent. Proton chemical shifts are annotated as follows: ppm (multiplicity, coupling constant (Hz), integration). Low and high resolution mass spectra (HRMS) are obtained on a Waters’ LCT Premier time-of-flight mass spectrometer using an electrospray ionization (ESI/TOF/MS) in positive ion mode operated at a resolution of 10000. The electrospray capillary voltage was 3kV and the sample cone voltage is 60V. Desolvation temperature is 225 °C and the desolvation gas is nitrogen at 300 L/hr. Accurate masses are obtained using
the lock spray mode with Leu-Enkephalin as the external reference compound. Elemental analyses are performed by Desert Analytics (Tucson, Arizona) using combustion analysis method for C, H, N and S and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) method for determining the percentage of Gd. MALDI-TOF mass spectral data are obtained by the Scripps Center for Mass Spectrometry (La Jolla, CA).

[0044] Dendrimer conjugation and purity is assessed by size exclusion HPLC (SE-HPLC) using a Beckman System Gold (Fullerton, CA) equipped with model 126 solvent delivery module and a model 168 UV detector (λ 254 and 280 nm) controlled by 32 Karat software. Size exclusion chromatography is performed on a Tosohaa G2000SW or a G3000SW, 10 µm, 7.8mm x 30 cm column (Montgomeryville, PA) using phosphate buffered saline (IX PBS) solution as the eluent (0.5 mL/min).

EXAMPLE 1

[0045] This example demonstrates a method of synthesis of 2-methyl-6-(p-aminobenzyl)diethylene-N,N,N',N",N"-penta-tert-butylacetate (2). See Figure 1.

[0046] A solution of aryl nitro compound 1 (1.98 g, 2.41 mmol) in EtOH (30 mL) is treated with 10% Pd/C (0.2 g) and stirred under an H2 atmosphere overnight. The mixture is filtered on a glass frit through a pad of Celite 535 (Fluka) washing with EtOH (2 x 20 mL). The filtrate is evaporated at reduced pressure to give a pale, yellow oil. The residue is purified by flash chromatography on silica gel eluted with THF-hexanes 1:2 to 1:1 to afford amine 2 (1.81g, 95%) as a colorless oil.

[0047] 1H NMR (DMSO-d6) δ 6.91 (d, J = 8.2 Hz, 2 H), 6.53 (d, J = 8.5 Hz, 2 H), 3.40 (m, 10 H), 3.10-2.30 (complicated m, 8 H), 1.46 (m, 45 H), 0.98 (d, J = 6.3 Hz 3 H); HRMS: calcd for C43H73N4O10 [M + H+]: 793.5327, found 793.5349.

EXAMPLE 2

[0048] This example demonstrates a method of synthesis of 2-methyl-6-(p-aminobenzyl-N-[5-oxopentanoic acid])diethylene-N,N,N',N",N"-penta- tert-butylacetate (3). See Figure 1.

[0049] A solution of 2 (2.10 g, 2.65 mmol) in EtOAc (30 mL) is treated with glutaric anhydride (0.36 g, 3.18 mmol) and stirred at room temperature for 18 h. The solution is
evaporated at reduced pressure and the residue is chromatographed on silica gel eluting with EtOH-hexanes 1:4 to 1:1 to yield acid 3 as a colorless solid (1.98 g, 82%).

**EXAMPLE 3**

This example demonstrates a method of synthesis of 2-methyl-6-(p-aminobenzyl-N-[5-oxopentanoic acid])diethylene-\(N,N,N',N''-N''\)-penta-\(\text{tert}\)-butylacetate hydroxysuccinimidyl ester (4). See Figure 1.

To solution of acid 3 (2.20 g, 2.40 mmol) in MeCN (50 mL) is added EDCI (0.92 g, 4.80 mmol), and \(N\)-hydroxysuccinimide (0.41 g, 3.60 mmol). The mixture is stirred at room temperature for 18 h. Afterwards, the reaction solution is concentrated at reduced pressure, diluted with CH\(_2\)Cl\(_2\) (100 mL), and then washed successively with H\(_2\)O (2 x 100 mL), 5% w/v NaHCO\(_3\) (2 x 100 mL), and H\(_2\)O (2 x 100 mL). The organic layer is dried over anhydrous Na\(_2\)SO\(_4\) and evaporated to afford active ester 4 as a yellow solid (2.0 g, 83%).

**EXAMPLE 4**

This example demonstrates a method of synthesis of 2-methyl-6-(p-isothiocyanatobenzyl)diethylene-\(N,N,N',N''-N''\)-penta-\(\text{tert}\)-butylacetate (5). See Figure 1.

A solution of aniline 2 (8.10 g, 10.20 mmol) in EtOAc (30 mL) is treated with thiophosgene (1.52 g, 13.30 mmol) and stirred at room temperature for 4 h. The solution is evaporated under reduced pressure and dried under vacuum to afford 5 as a yellow solid (8.2 g, 98%).
EXAMPLE 5


[0058] To solution of aniline 2 (0.22 g, 0.28 mmol) in CH₂Cl₂ (10 mL) is added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.12 g, 0.63 mmol), N-ε-maleimidocaprio acid (0.06 g, 0.30 mmol). The mixture is stirred at room temperature for 18 h. Afterwards, the reaction solution is diluted with CH₂Cl₂ (50 mL), and then washed successively with H₂O (2 x 50 mL), 5% w/v NaHCO₃ (2 x 50 mL), and H₂O (2 x 50 mL). The organic layer is dried over anhydrous Na₂SO₄, evaporated, and chromatographed on silica gel eluting with EtOH-hexanes 1:4 to 1:1 to yield 6 as a colorless oil (0.23 g, 84%).

[0059] ¹H NMR (DMSO-d₆) δ 9.82 (s, 1H), 7.55 (d, J = 8.1 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 7.09 (s, 2H), 3.45 (m, 12H), 3.20-2.40 (complicated m, 8 H), 2.32 (t, J = 6.9 Hz, 2H), 1.80-1.20 (m, 51H), 1.00 (d, J = 6.3 Hz 3H); ES-MS: calcd for C₅₂H₈₄N₂O₃ [M + H⁺]: 986.6, found 986.6.

EXAMPLE 6

[0060] This example demonstrates a method of synthesis of 1B4M-DTPA functionalized dendrimer by active ester conjugation (10a) in accordance with an embodiment of the invention. See Figure 2.

[0061] A solution of amine-terminated G4-PAMAM dendrimer (1.34 g of a 15.35% w/w solution in MeOH, 0.01447 mmol) is evaporated in vacuo and washed with hexane (2 x 10 mL). The residue is dissolved in DMSO (15 mL) and 1B4M-DTPA derivative 4 (1.86 g, 1.85 mmol) is added. The mixture is stirred at room temperature for 24 h, diluted with CH₂Cl₂ (15 mL), and then treated with N-(2-aminoethyl)aminomethyl polystyrene (1.0 g, loading: ≥ 2.00 mmol/g) (NOVA BioChem). The resulting mixture is again stirred for 24 h, filtered, and the filtrate concentrated under reduced pressure to provide crude 9a.

Crade 9a is then treated with trifluoroacetic acid (20 mL) and stirred for 18 h. The solution is then evaporated, the resulting residue is washed with CH₂Cl₂ (2 x 30 mL), and then dissolved in H₂O (30 mL). The dendrimer solution is adjusted to pH = 5 with IN aqueous NaOH, dialyzed exhaustively with water using a Centriprep Ultracel YM-IO (MWCO 10000 Da) (Millipore), and lyophilized to give a 10a as a white solid (0.51 g, 89.6%).

MALDI/TOF/MS: m/z 39329, calcd for [G4(1B4M)₅₄(H₂O)₉, C₁₁₇₂,H₂₇₄₂,N₄₄₄,O₆₂₅] 39329; Anal. Calcd. for [G4(1 64M)₅₃(TFA)₉Na₄₁(H₂O)₂I₃]: C, 47.62; H, 7.18; N, 12.49. found: C, 47.55, 47.75; H, 6.50, 6.56; N, 12.77, 12.73; SE-HPLC: a single, symmetric peak with retention time at 12.4 min.

EXAMPLE 7

This example demonstrates a method of synthesis of 1B4M-DTPA functionalized dendrimer by isothiocyanate conjugation (10b). See Figure 2.

MeOH (15 mL) and 1B4M-DTPA derivative 5 (1.00g, 1.20 mmol) are added to a solution of amine-terminated G4-PAMAM dendrimer (0.87 g of a 15.35% w/w solution in MeOH, 0.00935 mmol). The mixture is stirred at room temperature for 24 h, evaporated, diluted with CH₂Cl₂ (15 mL), and then treated with N-(2-aminoethyl)-aminomethyl polystyrene (0.6 g, loading: ≥ 2.00 mmol/g). The resulting mixture is again stirred for 24 h, filtered, and the filtrate concentrated under the reduced pressure to afford crude 9b.

TLC: UV active long band starting from the origin on the silica/aluminum oxide-coated plate using CHCl₃-MeOH (9:1) as developing solvents; MALDI/TOF/MS: m/z 42014, calcd for [G4(1B4M- tert-butyl penta-ester)₃₃]: C₂₀₄₁H₃₅₅₂N₃₈₂O₄₅₄S₃₅ 41773.

The residue 9b is then treated with trifluoroacetic acid (10 mL) and stirred for 18 h. The solution is then evaporated and the resulting residue is washed by CH₂Cl₂ (2 x 30 mL) and dissolved in H₂O (30 mL). The dendrimer solution is adjusted to pH = 5 with IN aqueous NaOH, dialyzed exhaustively with water using Centriprep Ultracel YM-10 (MWCO 10000Da), and lyophilized to give 10b as a yellow solid (0.31g, 66.4%).

MALDI/TOF/MS: m/z 28403, calcd for [G4(1B4M)₂₅(H₂O)₉]: C₁₉₇₇,H₂₈₄,N₃₅₀,O₃₉₂,S₂₅ 28403; Anal. Calcd. for [G4(1B4M)₅₀(TFA)₉Na₂₅(H₂O)₉₀]: C, 48.07; H, 6.60; N, 14.17; S, 3.60. found: C, 48.93, 48.48; H, 6.65, 6.50; N, 14.51, 14.47; S, 1.06, 1.36; SE-HPLC: a single, symmetric peak with retention time at 12.7 min.
EXAMPLE 8

This example demonstrates a general procedure of Gd complexation to the dendrimer-lB4M conjugate.

Gadolinium acetate (Gd(OAc)₃·XH₂O) is added to a solution of dendrimer-1B4M-DTPA (150 mg) in 0.3M citrate buffer (10 mL, pH = 4.5). The amount of gadolinium acetate used is calculated to be a 1.5 times molar excess based on the number of 1B4M units conjugated to the dendrimer. The solution is stirred at room temperature for 12 h and then dialyzed exhaustively with water using a Centriprep YM-10 (MWCO 10,000 Da) and monitored by SE-HPLC. The retentate is lyophilized, and the product is obtained as a yellow solid.

EXAMPLE 9

This example demonstrates a structural characterization of Gd-1B4M-DTPA functionalized dendrimer by active ester conjugation (lla) as described in Example 9. See Figures 2 and 5.

MALDI-TOF-MS: m/z 44326 (see Figure 5), calcd for [G₄(Gd-1B4M)3₉(H₂O)₂₅, C₆7₅H₅₀5Gd₃N₄0₆66]: 44318; Anal. Calcd for [04(164M)53Gd₃(C₆H₅O₂)2₁₂Na₄₅(H₂O)₂₁]: C, 44.35; H, 6.04; N, 11.16; Gd, 9.76. Found: C, 44.53, 44.18; H, 5.79, 5.76; N, 11.34, 11.29; Gd, 9.57, 10.00; SE-HPLC: a single, symmetric peak with retention time at 12.4 min.

EXAMPLE 10

This example demonstrates a structural characterization of Gd-1B4M-DTPA functionalized dendrimer by isothiocyanate conjugation (lib) as described in Example 9. See Figures 2 and 5.

MALDI-TOF-MS: m/z 43312 (see Figure 5), calcd for [G₄(Gd-1B4M)₄(H₂O)₇, C₇₈₅H₃₃₂Gd₄N₄₁₃O₄₅S₄₁]: 43318; Anal. Calcd for [04(164M)₅₇Gd₄(C₆H₅O₂)₃SN₄₅(H₂O)₇₇]: C, 41.08; H, 5.63; N, 11.57; S, 3.16; Gd, 11.14. Found: C, 41.23, 41.11; H, 5.48, 5.48; N, 11.18, 11.18; S, 2.19, 1.96; Gd, 12.13, 11.28; SE-HPLC: a single, symmetric peak with retention time at 12.7 min.
EXAMPLE 11

[0076] This example demonstrates a structural characterization of 2-methyl-6-(p-aminobenzyl-N-[5-oxopentanoic acid])diethylene-\(N,N,N',N'',N''''\)-penta-\textit{tert}-butylacetate methyl ester (7). See Figure 3.

[0077] To a solution of amine-terminated G4-PAMAM dendrimer (0.09 g of a 15.35% w/w solution in MeOH, 0.00094 mmol) are successively added MeOH (30 mL) and 1B4M-DTPA derivative 5 (0.12 g, 0.12 mmol) and \(\text{Et}_3\text{N}\) (0.02 mL, 0.12 mmol). The mixture is stirred at room temperature for 24 h and then concentrated under reduced pressure to afford the desired product 9a and by-product 7. Methyl ester 7 was isolated by preparative silica-coated TLC plate eluting with hexane/EtOAc (1:2) (25 mg, 25%).

\begin{eqnarray*}
^1\text{HNMR} & (\text{DMSO-}d_6) & \delta \, 9.88 \, (s, \, 1H), \, 7.55 \, (d, \, J = 8.1\, \text{Hz}, \, 2H), \, 7.19 \, (d, \, J = 6.9\, \text{Hz}, \, 2H), \, 3.69 \, (s, \, 3H), \, 3.42(m, \, 10H), \, 3.20-2.20 \, (\text{complicated} \, m, \, 12H), \, 1.90 \, (m, \, 2H), \, 1.45 \, (m, \, 45\, \text{H}), \, 1.00 \, (d, \, J = 6.9\, \text{Hz}, \, 3H); \, \text{ES-MS:} \, \text{calcd for} \, \text{C}_{48}\text{H}_{84}\text{N}_4\text{O}_13 \, [\text{M + H}^+]\, : \, 821.5800, \, \text{found} \, 821.5790. \end{eqnarray*}

EXAMPLE 12

[0079] This example demonstrates a structural characterization of 2-methyl-6-(p-methylisothiourethanebenzyl)diethylene-\(N,N',N'',N''''\)-penta-\textit{tert}-butylacetate (8). See Figure 4.

[0080] To a solution of amine-terminated G4-PAMAM dendrimer (0.44 g of a 15.35% w/w solution in MeOH, 0.0047 mmol) are successively added MeOH (15 mL) and 1B4M-DTPA derivative 4 (0.50g, 0.60 mmol) and \(\text{Et}_3\text{N}\) (0.09 mL, 0.60 mmol). The mixture is stirred at room temperature for 24 h, evaporated, diluted with CH\(_2\)Cl\(_2\) (15 mL), and then treated with \(N\)-(2-aminoethyl)-aminomethyl polystyrene (0.3 g, loading: \(\geq 2.00\, \text{mmol/g}\)). The resulting mixture is again stirred for 24 h, filtered, and the filtrate is concentrated under reduced pressure to afford crude desired product 9b and by-product 8. The methylisothiourethane 8 is isolated by flash chromatograph on aluminum oxide eluting with hexane/EtOAc (3:1:1:2) (40 mg, 7.7%).

\begin{eqnarray*}
^1\text{H NMR} & (\text{DMSO-}d_6) & \delta \, 11.20 \, (s, \, 1H), \, 7.30 \, (m, \, 4H), \, 4.00 \, (s, \, 3H), \, 3.80-3.00 \, (\text{complicated} \, m, \, 18H), \, 1.45 \, (s, \, 45\, \text{H}), \, 1.00 \, (d, \, J = 6.9\, \text{Hz}, \, 3H); \, \text{HRMS:} \, \text{calcd for} \, \text{C}_{44}\text{H}_{78}\text{N}_4\text{O}_n \, [\text{M + H}^+]\, : \, 867.5153, \, \text{found} \, 867.5164. \end{eqnarray*}
EXAMPLE 13


PAMAM dendrimer (10% solution in MeOH, Aldrich) (70 µmole) is added to a bicarbonate buffer solution (pH 8.5). The bifunctional chelate, p-SCN-IB4M-DTPA, (14 mmole) is added to dendrimer solution as a solid in portions during a course of a week. The mixture is stirred at room temperature for 14 days, while the pH is maintained at 8.5 by addition of 5% aqueous NaHCO3. On the 14th day, the reaction mixture is heated at 30 °C for another 24 h.

The reaction solution is transferred into a 250 mL Amicon diafiltration cell (Millipore) with a 10 kDa cut-off membrane (Millipore) and subjected to an exhaustive diafiltration with deionized water until no chelate is detected in the filtrate by SE-HPLC. The residual solution is lyophilized and the dendrimer-1B4M conjugate is obtained as an off-white solid (~90% yield based on dendrimer).

Compound 12 (precursor to G4-1134M62-Gd42): Anal. Calcd. for G4-(IB4M) 62Na138,8(HC03,35(H2O)0.3)
[(C622H1248N32O124)(C25H20N4O3S)62Na138(HCO3,3S(H2O)75): C, 45.93; H, 6.15; N, 13.09; S, 3.67. Found: C, 46.88, 46.49; H, 5.61, 5.60; N, 12.92, 12.88; S, 2.23, 2.60. MALDI-TOF (THAP): m/z for . SE-HPLC: R1 12.7 min.

A slight excess O1Gd(OAc)3 (0.1 mmol in excess) in 0.3 M citrate buffer (pH = 4.5) is added to a solution of 12 in the same buffer. The solution is stirred at room temperature for 15 h and then transferred into a 250 mL diafiltration cell with a 10 kDa cut-off membrane. The solution is subjected to an exhaustive diafiltration with deionized water and monitored by SE-HPLC. The retentate is lyophilized, and the product is obtained as an off-white solid (87% yield based on dendrimer 12). Low yield is attributed to the formation of aggregate losses and during the diafiltration process.

Compound G4-IB4M 62Gd42: Anal. Calcd. for 04-(164M) 62Gd42Na125(C6H7O7)4(H2O)0.3
[(C622H1248N32O124)(C25H20N4O3S)62Gd42Na125(C6H7O7)4(H2O)0.3]: C, 41.29; H, 5.44; N, 11.45; S, 3.21; Gd, 11.02. Found: C, 41.20, 42.41; H, 4.83, 4.63; N, 12.22, 11.87; S, 2.09, 2.05; Gd, 11.33, 10.99. MALDI-TOF (THAP): m/z for . SE-HPLC R1 11.5, 12.6 min.
EXAMPLE 14

This example demonstrates certain properties of the macromolecular conjugated metal complexes in accordance with an embodiment of the invention.

Solutions of compounds 11a and lib (0.25 - 1.0 mM) in 1X PBS (300 µL volume) are prepared along with a corresponding set from the G4-IB4M ψ0-Gd₄₂ (Example 13) prepared by aqueous chemistry for comparison purposes. Measurements are obtained at ~22 °C using a 3-Tesla clinical scanner (Signa Excite, General Electric Medical System, Waukesha, WI) equipped with a rectangular single loop receiver coil (84 x 126 x 6 mm). Images of the solutions using an 8-echo 2D-spin echo (2D-SE) sequence are acquired with repetition times of 167, 300, 617, 1250, 2500 and 5000 ms at echo time of 9.2 ms. Ti and T₂ maps are calculated using ImageJ MRI Analysis plug-in (http://rsb.info.nih.gov/ij/plugins/mri-analysis.html). Ti and T₂ relaxivities, R₁ and R₂, are determined from the slopes of the plot of relaxation rates, R₁ = 1/T₁ and R₂ = 1/T₂, vs [Gd]. The results are summarized in Table 1.

Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of Chelates[a]</th>
<th>Saturation Percentage</th>
<th>T₁ relaxivity mM⁻¹s⁻¹[b]</th>
<th>T₂ relaxivity mM⁻¹s⁻¹[c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>53</td>
<td>83</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10b</td>
<td>50</td>
<td>78</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11a</td>
<td>50</td>
<td>78</td>
<td>12.2 ± 0.6</td>
<td>24.9 ± 0.5</td>
</tr>
<tr>
<td>11b</td>
<td>57</td>
<td>89</td>
<td>14.2 ± 1.4</td>
<td>34.5 ± 2.1</td>
</tr>
<tr>
<td>G4-IB4Mψ0-Gd₄₂</td>
<td>60</td>
<td>94</td>
<td>13.9 ± 1.6</td>
<td>33.6 ± 3.1</td>
</tr>
</tbody>
</table>

[a]Reported values are the average values as calculated from the within 0.6% of the elemental analyses (C, H, N, S, and Gd) results which can be ± 10 of the mean value of the reported # of chelates, and ± 5 of the #Gd.
[b]Ti molar relaxivity values obtained from phantom measurements. Errors are reported as standard deviations.
[c]T₂ molar relaxivity values obtained from phantom measurements. Errors are reported as standard deviations.

ND = not determined.

EXAMPLE 15

This example demonstrates dynamic contrast-enhanced MR angiography imaging experiments.
[0091] All procedures are performed in accordance with the National Institutes of Health guidelines on the use of animals in research and are approved by the Animal Care and Use Committee of the National Cancer Institute.

[0092] Normal 6-8 weeks old athymic nu/nu mice (Charles Rivers Laboratories) are imaged in pairs to increase throughput on a 3-Tesla clinical scanner (Philips Intera 3.0T, Philips Medical System, Best, The Netherlands) using a parallel receiver coil array compromised of two modified Alderman-Grant resonators (38 mm OD x 75cm) and equipped with a multi-channel animal support and monitoring system. Mice (n = 4 per agent evaluated) are anesthetized with 2.5% Isoflurane (Abbott Laboratories, NJ) in O₂ delivered using a Summit Anesthesia Solutions vaporizer (Bend, OR) at a O₂ flow rate of 1.0 L/min. Respiration rate is kept at 25-30 respirations per min and monitored using a Biopac System MPI 50 (Biopac Inc., Goleta, CA). 3MTM FluorinertTM Electronic Liquid FC-77 is used in place of water to maintain the temperature of the mouse at 32 ± 1 °C using a Polyscience Model 210 heating recirculator while the mouse body temperature is monitored using FOT-M fiber optic temperature sensors (Fiso Technologies Inc., San Jose, CA) with a UMI-8 Universal Multichannel Instrument (Fiso Technologies Inc.) Prior to contrast agent injection, a Ti map is obtained by using a 3D-fast spoiled gradient echo image (3D-fSPGR) sequence at two different flip angles (repetition time/echo time 8.8/1.9 ms; flip angles 8° and 24°; bandwidth 31.25 Hz; matrix size 512x128x40; voxel resolution 156x156x600 μm; 4 excitations; scan time 4 min 29 sec). A 100 μL total volume consisting 50 μL of 12 mM Gd (dose of 0.03 mmole Gd/kg mouse) and 50 μL of IX PBS is injected in the tail vein of each mouse at a rate of 150 μL/min through 30-gauge needles attached to Tygon tubing (0.010 in id x 10 m length) using dual 1.0 cc syringes in a Harvard Apparatus PHD2000 (Holliston, MA) syringe pump. Dynamic MR angiography images are obtained immediately after injection by repeating the 3D-fSPGR sequence at the higher flip angle every 5 minutes for 1 h.

[0093] The dynamic 3D images are processed using ImageJ (http://rsb.info.nih.gov/ij/plugins/mri-analysis.html). The baseline pre-contrast 3D images are subtracted from each of the post-contrast 3D images and a maximum intensity projection (MIP) is calculated of the resulting 3D images. The resulting MIP images are subjectively compared by a board certified radiologist (PC) and an MR physicist (MB) for image clarity and for opacification of vessels and organs.
Time curves for clearance from the blood are measured from an ROI that is drawn over the jugular vein using ImageJ (http://rsb.info.nih.gov/ij/) and exported for analysis to Igor Pro (Wavemetrics, Inc., Lake Oswego, OR). The clearance rates are determined by fitting the decay curves to a single exponential decay function with the baseline fixed to zero and clearance pseudo-first order rate constants are calculated (Table 2). Results are averaged for all animals in each group (n ≥ 4). The statistical analysis of the differences between clearance rates and relaxivity values among the three agents is assessed with a student’s t-test using an Excel spreadsheet (Microsoft, Redmond, WA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$10^3 k_{ob}$, 1/min$^a$</th>
<th>half life, min$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>11a</td>
<td>8.012 ± 0.766</td>
<td>87.10 ± 8.15</td>
</tr>
<tr>
<td>11b</td>
<td>11.885 ± 2.594</td>
<td>60.29 ± 11.47</td>
</tr>
<tr>
<td>G4-1B4M$<em>{60}$-Gd$</em>{42}$</td>
<td>16.295 ± 1.994</td>
<td>43.07 ± 5.40</td>
</tr>
</tbody>
</table>

$^a$Calculated pseudo-first order rate constant according to the equation $[\text{Gc}^2]_i = [\text{Gd}]_o e^{-\chi t}$ as measured from the R1 map of the jugular vein. Errors are reported as standard deviations.

$^b$Calculated half life from the first order rate constant $t_{1/2} = \frac{0.693}{k_{bs}}$. Errors are reported as standard deviations.

All references, including publications, patent applications, and patents, 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 use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to," unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or...
otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.
CLAIM(S):

1. A method of preparing a macromolecular conjugated ligand comprising:

   (a) providing a compound of formula (I)

   \[
   R \quad \begin{array}{c}
   \text{m} \\
   \text{A} \quad \left( \text{CH}_3\text{COOP}_g \right)_n
   \end{array}
   \]

   (I),

   wherein

   A is a polyamino group,

   R is hydrogen, halo, allyl, hydroxy, nitro, amino, alkylamino, dialkylamino, thiocyanato, isothiocyanato, carboxyl, carboxyalkyl, carboxyalkyloxy, amido, alkylamido,

   haloalcylamido,

   Pg is a carboxyl-protecting group,

   n is 1 to 5, and m is 0 to 3,

   (b) reacting the compound of formula (I) with a macromolecular compound in an organic solvent medium which is substantially free of water to obtain a carboxyl-protected macromolecular conjugated ligand, and

   (c) removing the carboxyl-protecting group to obtain a macromolecular conjugated ligand.

2. The method of claim 1, further comprising step (d), wherein step (d) comprises reacting the macromolecular conjugated ligand with an ion, which is optionally radioactive, to obtain a macromolecular conjugated ligand complex.
3. The method of claim 1 or 2, wherein the polyamino group is a cyclic or acyclic polyalkyleneimine group, optionally substituted with one or more alkyl or cycloalkyl groups.

4. The method of any one of claims 1-3, wherein \(-A-(\text{CH}_2\text{COOPg})_n\) is a compound of the formula (II) or (III):

\[
\begin{align*}
\text{(II)} & \quad \text{(III)} \\
\end{align*}
\]

wherein \(R^1-R^3\) is hydrogen, a \(C_1-C_2\) alkyl group, or a point of attachment of

\[
\begin{array}{c}
\text{Ph} \\
\end{array}
\]

wherein \(R^1\) and \(R^2\) or \(R^1\) and \(R^3\) together form a \(C_5-C_7\) cycloalkyl group.

5. The method of claim 4, wherein \(-A-(\text{CH}_2\text{COOPg})_n\) of the compound of formula (I) is selected from the following compounds a, b, and c:

\[
\begin{align*}
a & \quad b & \quad c \\
\end{align*}
\]

6. The method of any one of claims 1-5, wherein the compound of formula (I) is
7. The method of any one of claims 2-6, wherein the ion is a metal ion.

8. The method of any one of claims 1-7, wherein Pg is selected from the group consisting of alkyl, benzyl, 9-fluorenylmethyl, diphenylmethyl, silylalkyl, haloalkyl, and 1,1-dimethylallyl (DMA).

9. The method of any one of claims 1-8, wherein the macromolecular compound is a biomolecule.

10. The method of claims 1-8, wherein the macromolecule is a dendrimer.

11. The method of claim 10, wherein the dendrimer is polyamidoamine (PAMAM).

12. The method of claim 11, wherein the PAMAM is of generation 4 (G4).

13. The method of any one of claims 1-12, wherein Pg is allcyl.

14. The method of claim 13, wherein the allcyl is f-butyl.
15. The method of any one of claims 1-14, wherein the organic solvent medium comprises one or more organic solvents selected from the group consisting of alkanes, nitriles, aromatic hydrocarbons, haloalkanes, alcohols, amides, alkylesters, sulfoxides, cycloalkanes, dialkylethers, alkyaryl ethers, diarylethers, and cyclic ethers, and any combination thereof.

16. The method of any one of claims 1-15, wherein the organic solvent medium is selected from the group consisting of acetonitrile, dimethylformamide (DMF), benzene, methylene chloride, methanol, hexane, dimethylsulfoxide (DMSO), tetrahydrofuran (THF), furan, diphenyl ether, diethyl ether, methylethyl ether, and dioxane, and any combination thereof.

17. The method of any one of claims 1-16, wherein the organic solvent is methanol or DMSO.

18. The method of any one of claims 1-17, wherein the organic solvent is DMSO.

19. The method of any one of claims 1-18, wherein the ion is selected from the group consisting of Ac, Bi, Pb, Y, Mn, Cr, Fe, Co, Ni, Tc, In, Ga, Cu, Re, a lanthanide, and an actinide.

20. The method of any one of claims 1-19, wherein the ion is Gd(III), $^{111}$In, $^{86}$Y, or a lanthanide.

21. The method of any one of claims 1-20, wherein in step (c), the carboxyl-protecting group is removed by the addition of an acid.

22. The method of claim 21, wherein the acid is trifluoroacetic acid, HCl, H$_2$SO$_4$, or H$_3$PO$_4$.

23. The method of any one of claims 1-22, wherein R is NCS.
24. The method of any one of claims 1-22, wherein R is

\[
\begin{align*}
\text{R} & = \text{a carboxyl-protecting group.}
\end{align*}
\]

25. The method of any one of claims 1-22, wherein R is

\[
\begin{align*}
\text{R} & = \text{a carboxyl-protecting group.}
\end{align*}
\]

26. A compound of formula (Ia):

\[
\begin{align*}
\text{(Ia),}
\end{align*}
\]

wherein Pg is a carboxyl-protecting group.
27. A compound of formula (Ib):

![Diagram of compound (Ib)]

wherein \( \text{Pg} \) is a carboxyl-protecting group.

28. A compound of formula (Ic):

![Diagram of compound (Ic)]

wherein \( \text{Pg} \) is a carboxyl-protecting group.
29. A compound of formula (Id):

![Diagram](image1)

wherein Pg is a carboxyl-protecting group.

30. A compound of formula (Ie):

![Diagram](image2)

wherein Pg is a carboxyl-protecting group.

31. A compound of formula (If):

![Diagram](image3)

wherein Pg is a carboxyl-protecting group.
32. The compound of any one of claims 26-31, wherein Pg is selected from the group consisting of alkyl, benzyl, 9-fluorenylmethyl, diphenylmethyl, silylalkyl, haloalkyl, and 1,1-dimethylallyl (DMA).

33. The compound of any one of claims 26-32, wherein Pg is alkyl.

34. The compound of claim 33, wherein the alkyl is t-butyl.
Figure 1

1 \rightarrow a \rightarrow 2 \rightarrow b \rightarrow 3
1 \quad 2
\quad e

\rightarrow d \rightarrow 4
\quad 5

\quad 6

\rightarrow c \rightarrow 3