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(54) Title: METHOD FOR BINDING MOLECULAR AGENTS TO ANGIOGENIC BLOOD VESSELS

(57) Abstract:

METHOD FOR BINDING MOLECULAR AGENTS TO ANGIOGENIC BLOOD VESSELS

BACKGROUND

1. TECHNICAL FIELD

This disclosure relates to methods for determining the presence of angiogenic blood vessels using sequential administrations of contrast agents and imaging techniques, such as, for example, magnetic resonance imaging (MRI).

BACKGROUND OF RELATED ART

Tumor angiogenesis is the recruitment of new blood vessels by a growing tumor from existing neighboring vessels. This recruitment of new microvasculature is a central process in tumor growth and in the potential for aggressive spreading of the tumor through metastasis. All solid tumors require angiogenesis for growth and metastasis. Thus, the level of angiogenesis is thought to be an important parameter for the staging of tumors. Furthermore, new therapies are being developed which attack the process of angiogenesis for the purpose of attempting to control tumor growth and tumor spread by restricting or eliminating the tumor blood supply. It is therefore of clinical importance to be able to monitor angiogenesis in tumors in a noninvasive manner.

To assess angiogenic activity of tumors, two parameters are of primary importance: vascular volume and vascular permeability. High-field MRI has been used to assess tumor volume and tumor signal changes in animal models after treatment with tamoxifen, a type of antiangiogenic agent. By using an intravascular contrast agent, albumin-Gd-DTPA, tumor vascular volume and permeability were measured as well as spatial distribution of the neovasculature. In another study using a high polarizing field, tumor growth was followed by using a variety of NMR measurement pulse sequences that allowed the investigators to distinguish microvessels from larger vessels through blood oxygen level dependent effects. Permeability was assessed by

noting the time dependent changes in NMR signal when Gd-DTPA was administered to the animal.

At lower polarizing fields that are available at clinical sites, Gd-DTPA, an MRI contrast agent approved by the U.S. Food and Drug Administration has been used to estimate angiogenic activity of tumors. However, this contrast agent is not ideal for characterizing tumor vasculature because it does not bind to angiogenic vasculature and it rapidly migrates to the extravascular space before being excreted through the kidneys. The tumor NMR signal measurements become delicate, being based on the dynamics of contrast agent uptake and elimination. Staging of tumors by this approach has been difficult.

To avoid the delicate dynamic aspects of Gd-DTPA uptake measurements, others have used a macromolecular contrast agent, albumin--Gd-DTPA. In this instance, the elimination process does not play a role in the observed MR signals, so that a much simpler and more reliable signal analysis is possible. Thus, MR signals based on T_1 changes (proportional to agent concentration) have provided indications of tumor blood vessel leak rate and tumor blood volume. This then represents an effective imaging method for assessing tumor angiogenesis. There are however, several drawback to this approach. Permeability of tumor vasculature to such macromolecules is not high enough to produce large MR signal changes, thus limiting the sensitivity of this approach. The observable MR signal changes appear to be concentrated mainly at the rim of implanted tumors and a full volume assessment appears to be lacking. However, the most serious obstacle to implementation of this approach is that this macromolecular agent has associated immune reactions when injected and leads to substantial toxicities. Thus, at present, this contrast agent is unsuitable for clinical applications.

Therefore, it would be advantageous to have a quick and accurate method for assessing angiogenic activity of tumors which is based on a binding event and which avoids the immune response invoked by prior art methods.

SUMMARY

The present methods employ sequential injections of contrast agents for assessing angiogenic activity. Specifically, the methods involve administering a first dose of a first, macromolecular contrast agent and obtaining an image indicative of circulating blood concentration of the first contrast agent. A second, polymeric contrast agent is then administered and a second image is obtained. Because the polymeric contrast agent binds to the angiogenic microvasculature, a rise of MR signals in the second image reflects the angiogenic development of new blood vessels, such as, for example, at the periphery of a tumor.

The first, macromolecular contrast agent can be any biocompatible molecule containing a paramagnetic entity. Because the injection of the first contrast agent is intended only to show circulating blood concentration, there is no need for the first contrast agent to exhibit any binding to angiogenic blood vessels. The second, polymeric contrast agents used in accordance with this disclosure include a paramagnetic entity complexed with a substituted polymeric carrier molecule having a highly negative charge in an aqueous environment. The preferred substituted polymeric carriers have a length 5 to 500 times greater than their diameter, a net negative charge, and form a worm-like chain conformation with a long persistence length. In particularly useful embodiments, lanthanide complexes (e.g., gadolinium-diethylenetriamine pentaacetic acid complexes) are attached to the polymer backbone to create complex molecules which are introduced into a blood vessel of the subject. These complex molecules have been found to bind to angiogenic microvasculature. Accordingly, any increase in signal strength between the first image (taken after administration of the first contrast agent) and the second image (taken after administration of the second, polymeric contrast agent which binds to angiogenic blood vessels) gives a measure of the angiogenic activity in the area being imaged.

BRIEF DESCRIPTION OF THE DRAWINGS

While the novel features of the invention are set forth with particularity in the appended claims, the invention, both as to organization and content, will be better

understood and appreciated, along with other objects and features thereof, from the following detailed description taken in conjunction with the drawing, in which:

FIG. 1 is a reaction scheme for preparing highly conjugated polymers useful as the second contrast agent in accordance with one embodiment of this disclosure.

FIG. 2 is an illustration of the functioning of the present polymeric contrast agents in a subject.

FIG. 3 is an illustration of inter-strand and intra-strand cross-linking of polypeptides.

FIG. 4 an illustration of a highly substituted polypeptide useful as the polymeric contrast agent in the present methods.

FIG. 5 is a block diagram of an MRI system useful in the performance of the methods described herein.

FIG. 6 is a graphic representation of a pulse sequence performed by the MRI system of FIG. 5 to practice an embodiment of the methods described herein.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Methods for assessing angiogenic activity are accomplished in accordance with this disclosure by administering sequential doses of contrast agents. At least one dose administered includes a polymeric contrast agent that binds to angiogenic vessels. The use of a polymeric contrast agent that binds to the newly forming vasculature allows for the detection of angiogenic activity.

While the following disclosure is presented with respect to assessing the presence of angiogenic activity in tumors, it should be understood that the present methods are suitable for assessing angiogenic activity associated with any condition in which angiogenesis is present, such as, for example, myocardial angiogenesis.

A pre-injection baseline image is obtained to begin the present methods. Next, a dose of a first contrast agent is administered, and a second image is obtained. A

comparison of the second image to the baseline image provides an indication of the circulating blood concentration of the first contrast agent.

The first contrast agent can be any macromolecular molecule having an image producing entity associated therewith.

The nature of the polymer backbone is not critical. Useful polymers include homo- and co-polymers of poly(amino acids), poly(vinyl amine), poly(4-aminostyrene), poly(acrylic acid), poly(methacrylic acid), poly(carboxynorbornene), and dextran. Preferably, the polymer is a polypeptide. The polypeptide can be an amino acid homopolymer or a copolymer of two or more amino acids. Preferably, the polypeptide is selected from the group consisting of polylysine, polyglutamic acid, polyaspartic acid, copolymers of lysine and either glutamic acid or aspartic acid.

Suitable imaging producing entities include paramagnetic entities and entities which undergo nuclear reaction resulting in release of detectable radiation. Non-limiting examples include ions which release alpha particles, gamma particles, beta particles, or positrons. Such image producing entities are known to those skilled in the art. Gamma emitters include, for example, ^{111}In and ^{153}Gd . Positron emitters include, for example, ^{89}Zr , which may be employed in positron emission tomography (PET) imaging. Preferred contrast agents include a polymeric backbone substituted with groups that can chelate a paramagnetic entity. Suitable paramagnetic ions include ions of transition and lanthanide metals (e.g., metals having atomic numbers of 6 to 9, 21-29, 42, 43, 44, or 57-71), in particular ions of Gd, Cr, V, Mn, Fe, Co, Ni, Cu, La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu, preferably Mn, Cr, Fe, Gd and Dy, most preferably Gd.

As is well known, a chelating agent is a compound containing donor atoms that can combine by coordinate bonding with a metal atom to form a cyclic structure called a chelation complex or chelate. Conventional metal chelating groups may be used which are well known to those skilled in the art, e.g., linear, cyclic and branched polyamino-polycarboxylic acids and phosphorus oxyacid equivalents, and other sulphur and/or nitrogen ligands known in the art. Suitable lanthanide ion chelating molecules include, but are not limited to diethylenetriaminepentaacetic acid (DTPA),

1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(2-propionic acid) (DOTMA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis[3-(4-carboxyl)-butanoic acid], 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(acetic acid-methyl amide), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonic acid), and p-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA). Ligands useful for chelating for other ions (such as, for example, Fe(III), Mn(II), Cu(II), etc.) include bis(thiosemicarbazone) and derivatives, porphyrins and derivatives, 2,3-bis(2-thioacetamido)propionates and derivatives, *N,N'*-bis(mercaptoacetyl)-2,3-diaminopropanoate, and bis(aminoethanethiol) and derivatives.

Particularly preferred chelating groups are DTPA and DOTA. Methods for metallating any chelating agents present are well-known. For example, metals can be incorporated into a chelant moiety by three general methods: direct incorporation, template synthesis and/or transmetallation.

Thus, for example, to achieve a MR active agent, a paramagnetic ion (such as, for example, gadolinium) can be incorporated into the chelating groups present on the polymer by dropwise addition of a gadolinium salt, such as, for example gadolinium chloride or gadolinium citrate. The dropwise addition of Gd continues until a slight indication of free Gd (not chelated by available DTPA groups) is noted (small aliquots of polymer solution added to 10 microMolar of arzenzo IIII in acetate buffer--free Gd turns the dye solution blue).

The first, macromolecular contrast agent preferably is of sufficient length to increase the time in which the product circulates in the blood. Clearance from the blood is rapid for short molecules, resulting in a short plasma lifetime. Plasma lifetime increases rapidly as the polymers increase in length. For example, where the polymer is a polypeptide, a plateau is reached for a molecular length of about 500 residues and little further change in lifetime occurs.

In order to perform one preferred embodiment of the invention, after a pre-injection baseline image is obtained, the first contrast agent is introduced into the subject by injecting the contrast agent intravenously. The dose of the polymeric contrast agent can be in the range of about 0.01 millimoles of image producing entity per kilogram to about 0.1 millimoles of image producing entity per kilogram. The subject is then imaged at one or more pre-selected tissue sites. This second image can advantageously be obtained anywhere from 15 seconds to 5 minutes after administration of the first contrast agent. A comparison of the second image to the baseline image provides an indication of the circulating blood concentration of the first contrast agent.

Once the images ascertaining the circulating blood level are obtained, the second, polymeric contrast agent is introduced into the subject by injecting the contrast agent intravenously.

Suitable polymeric contrast agents include an image producing entity complexed with a substituted polypeptide carrier molecule. The polymeric contrast agents have a length that is 5 to 500 times greater than their diameter, a net negative charge, and form a worm-like chain conformation with a long persistence length. The worm-like configuration of the complex molecule is achieved by attaching a sufficient number of steric hindrance molecules along the polypeptide chain to eliminate or reduce intra-chain ionic bonds as well to allow charge repulsion between chelating moieties to unfold and extend the polymer chain. The amount of substitutions (also referred to as the degree of conjugation) thus affects the configuration of the resulting complex, with a higher degree of conjugation providing a more consistent extended structure and better diagnosis. A degree of conjugation of above 90% is typically required for the proper polymer configuration to be realized in the case of a carrier molecule having a lysine homopolymer backbone. Lower degrees of conjugation can be tolerated for certain carrier molecules having an amino acid copolymer backbone, such as, for example, a backbone that is a copolymer containing lysine and either glutamic or aspartic acid.

The present carrier molecules include a polymer backbone that is substituted with steric hindrance molecules which facilitate the attachment of an image producing entity and which, due to their physical size, provide a physical restraint on polymer bending.

The nature of the polymer backbone is not critical, provided that the polymer has pendant groups which can be reacted with an activated SHM as described below to provide a polymer-SHM copolymer having an elongated structure. Suitable pendant groups which may be present on the polymer include, but are not limited to amine groups, carboxyl groups and hydroxyl groups. Useful polymers include homo- and co-polymers of poly(amino acids), poly(vinyl amine), poly(4-aminostyrene), poly(acrylic acid), poly(methacrylic acid), poly(carboxynorbornene), and dextran. Preferably, the polymer is a polypeptide. The polypeptide can be an amino acid homopolymer or a copolymer of two or more amino acids. Preferably, the polypeptide is selected from the group consisting of polylysine, polyglutamic acid, polyaspartic acid, copolymers of lysine and either glutamic acid or aspartic acid. Other polymers may be used provided that after reaction with the SHM, the resulting copolymer has an elongated structure characterized by a molecular length that is 5 to 500 times the cross-sectional diameter of the copolymer molecule and a net negative charge in an aqueous environment. In addition, the polymer preferably is of sufficient length to increase the time in which the product circulates in the blood. For polypeptides, the polymer backbone can advantageously be from 35 to 1500 amino acid residues long. Because the polymeric backbone is synthetic, the length can be tailored to provide desired residence times in the body. Clearance from the blood is rapid for short molecules, resulting in a short plasma lifetime. Plasma lifetime increases rapidly as the polymers increase in length. For example, where the polymer is a polypeptide, a plateau is reached for a molecular length of about 500 residues and little further change in lifetime occurs. Not only does the use of a synthetic polypeptide provide the ability to modify the polymer length so as to change the blood circulation times to smaller values, but the ability to modify the polymer length to probe small permeability modulations is also provided.

A preferred homopolymer is a lysine homopolymer.

Where a copolymer forms the backbone of the carrier molecule, the copolymer preferably contains lysine units and either glutamic acid units, aspartic acid units, or both. Glutamic and/or aspartic acid units may constitute from about 20 to about 60 percent of the copolymer. Preferably, the copolymer is a glutamic acid-lysine copolymer. Particularly useful copolymers have glu:lys ratios of about 1:4 to about 6:4. A high content of lysine is believed advantageous for imaging as it allows a high loading of the copolymer with paramagnetic ions. Without wishing to be bound by any theory, it is believed that the presence of glutamic acid residues in the copolymer backbone accomplishes two things. First, it is believed that the glutamic acid residues provide a stiffer initial copolymer backbone for the synthesis of the complete construct. Second, it is believed that the presence of glutamic acid residues in the copolymer promotes extension of the final polymer through charge repulsion.

At least a portion of the polymer backbone has steric hindrance molecules substituted thereon. The steric hindrance molecule ("SHM") can be any molecule that by its physical size enforces a elongated conformation by providing steric hindrance between neighboring steric hindrance molecules. Preferably the SHM is neutral in charge or presents negative charges in an aqueous environment along the polymer chain to assist in keeping the polymer backbone straight through coulombic repulsion.

In particularly useful embodiments, the SHM contains or chelates an imaging producing entity. Suitable imaging producing entities include paramagnetic entities, entities which undergo nuclear reaction resulting in release of detectable radiation. Non-limiting examples include ions which release alpha particles, gamma particles, beta particles, or positrons. Such image producing entities are known to those skilled in the art. Gamma emitters include, for example, ^{111}In and ^{153}Gd . Positron emitters include, for example, ^{89}Zr , which may be employed in positron emission tomography (PET) imaging.

Particularly preferred steric hindrance molecules are molecules that chelate with paramagnetic entities. As those skilled in the art will appreciate, paramagnetic entities include certain transition metals and lanthanide ions. Any molecule known to complex with paramagnetic entities and which is of sufficient size to provide steric

hindrance against polymer bending can be used as the SHM. Preferably, the SHM has a net negative charge. Suitable lanthanide ion chelating molecules include, but are not limited to diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(2-propionic acid) (DOTMA), 1,4,8,11-Tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis[3-(4-carboxyl)-butanoic acid], 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(acetic acid-methyl amide), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonic acid), and p-Isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA). Ligands useful for chelating for other ions (such as, for example, Fe(III), Mn(II), Cu(II), etc.) include Bis(thiosemicarbazone) and derivatives, Porphyrins and derivatives, 2,3-Bis(2-thioacetamido)propionates and derivatives, *N,N'*-bis(mercaptoacetyl)-2,3-diaminopropanoate, and Bis(aminoethanethiol) and derivatives.

To attach the SHM to the polymer, an activating group is provided on the SHM. The activating group present on the SHM can be any group which will react with a polymer. Suitable groups include, but are not limited to mixed carbonate carbonic anhydride groups, succinimidyl groups, amine groups and dicyclohexylcarbodiimide (DCC) groups. Those skilled in the art will readily envision reaction schemes for providing an activating group on any given SHM.

In one embodiment, the SHM is DTPA and the activating groups are mixed carbonate carbonic anhydride groups. In particularly useful embodiments, a substantially mono-activated SHM is provided. The term "activated" means that a functional group is present on the molecule which permits covalent bonding of the molecule to appropriate amino acids. By the term "substantially mono-activated" it is meant that about 90% or more of the steric hindrance molecules contain only a single activated site. Mono-activation is believed to more consistently result in high levels of conjugation. A typical reaction scheme for activating DTPA and reacting it with a polypeptide backbone is shown in Fig. 1. As seen therein, a monoanhydride-DTPA is first prepared. Specifically, a flask is charged with acetonitrile and DTPA.

Triethylamine is then added via syringe. The solution is warmed to 60°C. under a nitrogen atmosphere. The mixture is stirred until homogeneous. The clear solution is then cooled to -45°C. under nitrogen atmosphere and isobutyl chloroformate is slowly added to result in the mono-anhydride of DTPA. As those skilled in the art will appreciate, DTPA has five acid groups available for conversion to anhydride. However, since substantially mono-activated DTPA is desired, only one of these acid sites should be converted to anhydride. It has unexpectedly been found that the slow addition of the chloroformate while cooling below -40°C. accomplishes this result, i.e., that about 90% or more of the DTPA is a monoanhydride of DTPA.

The activated SHM is then reacted with the polymer backbone. The precise conditions for reacting the polymer with the activated SHM will depend upon a number of factors including the particular polymer chosen and the specific SHM used. Those skilled in the art will readily envision reaction schemes for any given pair of materials to produce the desired substituted polymer product.

In a particularly useful embodiment, for example, the monoanhydride-DTPA described above is simply added dropwise to an aqueous solution of polylysine under ambient atmospheric conditions.

In another example, where the reactive pendant groups on the polymer backbone are electrophilic groups (such as, for example, a carboxylic acid groups), the anhydride of DTPA described above can be reacted overnight with a diamine (in which the diamine is in large excess to the anhydride). Ethylene diamine is a suitable choice, giving in the end a DTPA linkage of the desired length to achieve proper steric hindrance against polymer chain bending. The product is separated from the diamine and from DTPA which was not reacted, by ion exchange chromatography. The product is substantially mono-amine DTPA. Where the substantially mono-activated steric hindrance molecule is the foregoing monoamine-DTPA, it can be linked to a carboxyl group containing polymer (such as, for example, poly-glutamic acid) by a carboxyl coupling method. The carboxy acid groups of the polymer are activated by a coupling reagent, such as, for example, 1 Ethyl-3-(3-Dimethylaminopropyl) carbodiimide Hydrochloride (EDC) (Pierce, Rockford, Ill.). The activated carboxy acid groups on

the polymer are then combined with the monoamine-DTPA to produce an amide linkage of the DTPA to the polymer backbone as a sidechain which acts as a steric hindrance straightening the polymer backbone.

The resulting polymer-steric hindrance molecule copolymer is then purified. During purification, the polymer-SHM copolymer is separated from the volatile solvents and other impurities. Any known techniques can be used to purify the polymer-SHM copolymers.

In a particularly useful embodiment, where a polypeptide backbone is used, a purification scheme is employed which does not result in complete drying of the polymer-SHM copolymer. It has unexpectedly been determined that excessive dryness affects the configuration of the copolymer and interferes with the determination of degree of conjugation.

A preferred purification scheme involves first exposing the reaction mixture to reduced pressure to remove impurities that are more volatile than water. Care should be taken not to remove all water from the reaction mixture during this step. The next step in this preferred purification scheme is to centrifuge the remaining reaction mixture. Soluble impurities remain in the supernatant fluid. The retentate from the centrifuge step is resuspended and subjected to dialysis. Optionally, ultrafiltration is performed on the dialyzed polymer. Techniques for these processes are within the purview of those skilled in the art.

The resulting product can then be characterized using any technique known to those skilled in the art, such as, for example, high performance liquid chromatography (HPLC).

Once the polymer-SHM copolymer is obtained, an image producing entity is incorporated into the conjugated polymer. Thus, for example, to achieve a MR active agent, a paramagnetic ion (such as, for example, gadolinium) can be incorporated into the product polymer chelating DTPA groups by dropwise addition of a gadolinium salt (such as, for example, gadolinium chloride or gadolinium citrate) into a solution of the polymer. The dropwise addition of Gd continues until a slight indication of free

Gd (not chelated by available DTPA groups) is noted (small aliquots of polymer solution added to 10 microMolar of arzenzo III in acetate buffer--free Gd turns the dye solution blue). The Gd-loaded highly conjugated polymer is then ready for introduction into a blood vessel of the subject.

In certain embodiments, the conjugated polymer can also be used for drug delivery. It is also contemplated that a therapeutic agent can be attached at a few sites along the substituted polymer chain. The therapeutic entity can be attached to the conjugated polymer using techniques known to those skilled in the art. It is also contemplated that, the polymer backbone can be highly conjugated with a non-therapeutic SHM which chelates an imaging agent and a therapeutic agent can be bound to the SHM at a few sites along the substituted polymer chain, rather than being bound directly to the polymer backbone.

In certain embodiments, the conjugated polymer can also have a targeting moiety attached thereto. It is contemplated that a targeting moiety can be attached at a few sites along the substituted polymer chain. The targeting moiety can be attached to the conjugated polymer using techniques known to those skilled in the art. It is also contemplated that targeting moieties can be bound to the SHM at a few sites along the substituted polymer chain, rather than being bound directly to the polymer backbone.

Figure 2 is a schematic cross section of a small tumor blood vessel 10. Solid linear objects 11 represent the agents in the blood circulation giving the bulk blood volume signal in any MR voxel. The dashed linear objects 12 represent the probes bound to the endothelial cell surfaces in the luminal space of the vessel. While not wishing to be bound by any theory, it is believed that the polymeric contrast agent molecules have dipoles for binding to dipoles present on integrins associated with endothelial cells.

An elongated, worm-like conformation having a net negative charge in an aqueous environment results in greater uptake than other conformations, such as folded, or globular conformations. Conformation may be measured by a persistence length of the molecule. This may be determined by light scattering.

Conformation is a result of intra-chain charge interaction, and rigidity of the molecule. As those skilled in the art will appreciate, many polypeptides tend to fold into tight random coils due to the relatively free rotation around each peptide bond. Also, if each polypeptide is composed of opposite charge amino acids, then intra-chain charge interaction as shown by bond 21 in FIG. 3. Inter-chain charge interaction between chains may also occur as shown by bond 23 of FIG. 3. If there is significant intra-chain charge interactions, the polypeptide-based molecules may assume a globular, or folded, conformation.

The conformation attained by the present polymeric contrast agents is that of a worm-like shape being essentially a stretched out, extended chain with little folding. A measure of the "straightness" of a molecule is a persistence length. Persistence length is related to a radius of gyration, measured by light scattering experiments. A folded polypeptide such as poly-L-lysine (PLL) with little or no substitution, has a low persistence length of about 10 Angstroms ($\text{\AA}$), and is not suitable for binding to angiogenic blood vessels. Therefore, the present polymeric contrast agents preferably have a persistence lengths of 100-600 $\text{\AA}$.

It is sometimes difficult to measure the persistence length of certain molecules by light scattering to determine their conformation because of the effects of contaminant particles in the test solutions. However, it was found that by measuring the magnetic resonance (MR) T_1 relaxation of a paramagnetic entity attached to the carrier, one could infer the conformation of the molecules of interest. This is performed by attaching paramagnetic ions, such as gadolinium, to the chelators along the polymer chain

When the carrier molecule is in an elongated conformation, the chelator/MR active entity is free to rotate about its attachment point to the main chain, allowing a long T_1 relaxation time of the surrounding water protons which are the source of the MR signal.

When the carrier molecule is in a globular or highly folded conformation, steric hindrance, and molecular crowding causes interaction with the chelator/MR active entity restricting rotation about its bond to the main chain. Thus, the chelator/MR

active entity moves only with the general slow motion of the carrier molecule. This produces a short T_1 relaxation time.

A high relaxivity is associated with a molecule which folds upon itself into a globular conformation, such as albumen, at about $15 \text{ sec.}^{-1} \text{ milliMolar}^{-1}$ ($\text{sec.}^{-1} \text{ mM}^{-1}$). A low relaxivity is associated with an elongated molecule such as highly substituted Gd-DTPA PLL^h in which the Gd can rotate rapidly, having a relaxivity of about $8 \text{ sec.}^{-1} \text{ mM}^{-1}$. The optimum conformation of the present invention is associated with a relaxivity of $7\text{-}8 \text{ sec.}^{-1} \text{ mM}^{-1}$. When the relaxivity of a peptide agent was high, the uptake coefficient of such an agent was invariably low, evidently due to the absence of the reptation mechanism.

Since many in-vivo chemical entities have a negative charge, molecules introduced into the subject can advantageously have a net negative charge to reduce agglutination and to allow for stable long circulation in the blood plasma. It is known that negatively charged dextran molecules undergo glomerular filtration at a much slower rate than equivalent dextran molecules of positive charge or neutral charge.

The high net negative charge is also desirable since it also assists in the polypeptide-based molecules to retaining their elongated, worm-like conformation.

In FIG. 4 a polymeric contrast agent having a plurality of side chains substituting the hydrogen atoms is shown. The polymeric contrast agent is comprised of a plurality of amino acids 31, each linked end to end through a polypeptide bond. A plurality of side residues 33 are attached which cause steric hindrances and repulsion to straighten the copolymer chain.

FIG. 4 also shows that the length of the polymeric contrast agent should be significantly longer than its diameter by approximately 5 to 500 times. This causes the polymeric contrast agent and any attached chemical entities to bind to the endothelia of capillary walls as discussed above.

It may be that in some applications, long blood circulation times would be undesirable. The present methods/materials provide the ability to reliably make short polymers of the desired worm-like conformation which allows the possible tailoring

of blood circulation time to certain target levels. Blood circulation time is directly dependent on polymer chain length. The signal response is fast and the clearance from the blood circulation is rapid for shorter polymer lengths, both of which may be desirable in certain clinical screening procedures.

The polymeric contrast agent molecules used in accordance with certain embodiments of the present disclosure do not normally accumulate in other organs such as muscle, kidney or liver. Therefore, the present agents are particularly well suited for imaging of angiogenic blood vessels compared to over other imaging agents that are based on globular proteins or coiled polymers, which tend to show accumulation in liver and kidneys of animal models.

After injection of the first contrast agent and obtaining an image, the second, polymeric contrast agent is introduced into the subject by injecting the contrast agent intravenously. The dose of the polymeric contrast agent can be in the range of about 0.01 millimoles of image producing entity per kilogram to about 0. millimoles of image producing entity per kilogram. Preferably, the dose of the second contrast agent is the same as the dose of the first contrast agent on a image producing entity per kilogram basis and the dosages are normalized to take into account any differences in relaxivity between the first and second agents. The subject is then imaged at the same area of tissue previously imaged. The subject is preferably imaged within about 1 to 5 minutes after injection. This time period allows the polymeric contrast agent time to bind to new vascularization, but insufficient time for a significant amount of the polymeric contrast agent to pass completely through angiogenic blood vessels into the surrounding tissue.

Techniques for MR imaging are known to those skilled in the art. In a particularly useful method, images are obtained beginning immediately after injection and at certain timed intervals. Preferably, the timed intervals are shortly after injection (within 10 minutes) and up to 1 hour post injection. For highest sensitivity of permeability, an image at 24 hours may also be acquired. To determine blood volume, imaging should take place within 10 minutes of contrast agent injection.

FIG. 4 shows the major components of a preferred MRI system which can be used in practicing the invention. Operation of the system is controlled from an operator console 100 which includes a keyboard and control panel 102 and a display 104. Console 100 communicates through a link 116 with a separate computer system 107 that enables an operator to control the production and display of images on the screen of display 104. Computer system 107 includes a number of modules which communicate with each other through a backplane 120. These include an image processor module 106, a central processing unit (CPU) module 108 and a memory module 113, known in the art as a frame buffer for storing image data arrays. Computer system 107 is linked to a disk storage 111 and a tape drive 112 for storage of image data and programs, and communicates with a separate system control 122 through a high speed serial link 115.

System control 122 includes a set of modules connected together by a backplane 118. These include a CPU module 119 and a pulse generator module 121 which is coupled to operator console 100 through a serial link 125. Through link 125, system control 122 receives commands from the operator which determine the scan sequence that is to be performed.

Pulse generator module 121 operates the system components to carry out the desired scan sequence, and produces data which determine the timing, strength and shape of the RF pulses to be produced, and the timing and length of the data acquisition window. Pulse generator module 121 is coupled to a set of gradient amplifiers 127, to determine the timing and shape of the gradient pulses to be produced during the scan. Pulse generator module 121 also receives patient data from a physiological acquisition controller 129 that receives signals from a number of different sensors attached to the patient, such as electrocardiogram (ECG) signals from electrodes or respiratory signals from a bellows. Pulse generator module 121 is also coupled to a scan room interface circuit 133 which receives signals from various sensors associated with the condition of the patient and the magnet system. Through scan room interface circuit 133, a patient positioning system 134 receives commands to move the patient to the desired position for the scan.

Gradient amplifier system 127 that receives gradient waveforms from pulse generator module 121 is comprised of G_x , G_y and G_z amplifiers. Each gradient amplifier excites a corresponding gradient coil in an assembly 139 to produce the magnetic field gradients used for position encoding acquired signals. Gradient coil assembly 139 forms part of a magnet assembly 141 which includes a polarizing magnet 140 and a whole-body RF coil 152. A transceiver module 150 in system control 122 produces pulses which are amplified by an RF amplifier 151 and coupled to RF coil 152 by a transmit/receive switch 154. The resulting signals radiated by the excited nuclei in the patient may be sensed by the same RF coil 152 and coupled through transmit/receive switch 154 to a preamplifier 153. The amplified NMR signals are demodulated, filtered, and digitized in the receiver section of the transceiver 150. Transmit/receive switch 154 is controlled by a signal from pulse generator module 121 to electrically connect RF amplifier 151 to coil 152 during the transmit mode and to connect preamplifier 153 to coil 152 during the receive mode. Transmit/receive switch 154 also enables a separate RF coil (for example, a head coil or surface coil) to be used in either the transmit or receive mode.

The NMR signals picked up by RF coil 152 are digitized by transceiver module 150 and transferred to a memory module 160 in system control 122. When the scan is completed and an entire array of data has been acquired in memory module 160, an array processor 161 operates to Fourier transform the data into an array of image data. These image data are conveyed through serial link 115 to computer system 107 where they are stored in disk storage 111. In response to commands received from operator console 100, these image data may be archived on tape drive 112, or may be further processed by image processor 106 and conveyed to operator console 100 for presentation on display 104.

The polymeric contrast agent molecules do not bind to the endothelial cells of normal blood vessels. Thus if angiogenic blood vessels are present, their presence may be detected by an increase of signal in the tissue being examined over that to be expected from blood volume effects alone in that tissue. Thus, the present methods provide clear direct signals of the quantity of interest – namely, angiogenesis.

Although the present methods can be used with a number of different pulse sequences, a preferred embodiment employs a fast 3D (three dimensional) RF (radio frequency) phase spoiled gradient recalled echo pulse sequence, depicted in FIG. 5, to acquire the NMR image data. The pulse sequence "3dfgre" available on the General Electric 1.5 Tesla MR scanner sold by General Electric Company, Milwaukee, Wis., under the trademark "SIGNA" with revision level 5.5 system software is used.

As shown in FIG. 5, an RF excitation pulse 220 having a flip angle of from 40° to 60° is produced in the presence of a slab select gradient pulse 222 to produce transverse magnetization in the three-dimensional (3D) volume of interest as taught in Edelstein et al. U.S. Pat. No. 4,431,968 assigned to the instant assignee. This is followed by a slice encoding gradient pulse 224 directed along the z axis and a phase encoding gradient pulse 226 directed along the y axis. A readout gradient pulse 228 directed along the x axis follows, and a partial echo (60%) NMR signal 230 is acquired and digitized as described above. After the acquisition, rewinder gradient pulses 232 and 234 rephase the magnetization before the pulse sequence is repeated as taught in Glover et al. U.S. Pat. No. 4,665,365 assigned to the instant assignee. As is well known in the art, the pulse sequence is repeated and the respective slice and phase encoding gradient pulses 224 and 226 are stepped through a series of values to sample the 3D k-space.

The acquired 3D k-space data set is Fourier transformed along all three axes and a magnitude image is produced in which the brightness of each image pixel indicates the NMR signal strength from each corresponding voxel in the 3D volume of interest.

An initial signal is then compared with the signal enhancement observed at selected times, preferably a short time after injection (within 10 minutes) and then at several time points up to 60 minutes post injection. The initial image after injection (within 10 minutes) provides a measure of blood volume or microvascular density, for each pixel of the image. Subsequent images then establish binding to the capillaries, again on a pixel by pixel basis. Maps of blood volume and of capillary binding may then be generated and displayed as an image or overlaid on the MR image directly. Both anatomical and physiological features will then be displayed simultaneously, giving

radiologists not only the level of capillary binding as an average quantity but also its activity as a function of position--a very desirable feature for staging and prognosis.

EXAMPLE 1

A tightly coiled polymeric agent is injected at a dose of 0.025 mmole Gd/kg. The change in signal in the tumor periphery is noted to be 28% immediately after injection. An identical dose of a negatively charged linear polymer is injected at a later time and the change in signal in the same tumor region is noted to be 70% right after the injection. Thus the excess signal is 42% and nearly twice as large as the signal associated with the coiled agent in the same tumor.

The excess signal is saturable. In a comparison of responses in different animals to injection with linear and coiled agents there is a clear statistical difference(76 +/- 14% for the linear polymers, and 30 +/- 10 % for the coiled polymers). At a higher dose the difference is relatively smaller because the bulk signal dominates the saturated binding signal. Saturation is a hallmark of binding: There are only so many binding sites and when these are occupied there is no longer any further increase in MR signal. The free flowing blood volume signal can continue to increase without bound.

The excess signal is related to angiogenesis. In an animal tumor model (rat mammary adenocarcinoma) sequential passage of tumors from animal to animal sometimes results in a gradual loss of tumor growth potential. In the final stages of this progression, tumors fail to grow, reach a small size of 2 to 5 mm and then are seen to regress. In the case of dormant, small regressing tumors only the bulk blood signal is seen after agent injection and both coiled and linear polymer signals are the same within experimental error (30%+/-10% vs 29%+/-8%).

Coiled polymer agents fail to show any excess binding in growing tumors. The present polymeric contrast agents having an elongated configuration and a negative charge in an aqueous environment exhibit binding. In the present experiments, the polymer is on the average some 600 monomers long. Since each residue is labeled with Gd there is a 600 fold amplification of each binding site rendering the binding

events visible in MR imaging experiments. Thus the present results indicate that the structure present polymeric contrast agents having an elongated configuration and a negative charge in an aqueous environment may mimic some ligand whose receptor resides on the new endothelium cells involved in angiogenesis.

The excess signal is observed within several minutes after injection so that any signal due to penetration of agent from the blood to the interior of the tumor is not a factor in these experiments. Penetration effects typically require more than 10 - 15 minutes to be noticeable above the bulk blood signal.

While specific embodiments of the invention have been illustrated and described herein, it is realized that modifications and changes will occur to those skilled in the art. Thus, for example, the order of administration of the two contrast agents can be reversed - - i.e., the polymeric contrast agent which binds to the endothelial cells can be administered first, and the macromolecular contrast agent can be administered in a second injection. A relative change in signal strength indicative of angiogenic activity will be observed between the images taken after administration of each agent. As another example, a polymeric contrast agent may be used for each of the sequential injections. Again, a relative change in signal strength indicative of angiogenic activity will be observed between the images taken after each administration of contrast agent. It is therefore to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit and scope of the invention.

WHAT WE CLAIM IS:

1. A method for assessing the presence of angiogenic blood vessels, the method comprising:

intravenously administering a first macromolecular contrast agent to a subject;

obtaining a first image of tissue of the subject;

intravenously administering a second macromolecular contrast agent to a subject;

obtaining a second image of tissue of the subject; and

comparing the first and second images to assess the presence of angiogenic blood vessels, wherein a localized image enhancement indicates the presence of angiogenic blood vessels,

wherein at least one of the first or second macromolecular contrast agents is a polymeric contrast agent capable of binding to angiogenic blood vessels and having a length that is 5 to 500 times greater than its diameter.

2. A method as in claim 1 wherein the step of intravenously administering a second macromolecular contrast agent comprises administering a polymeric contrast agent capable of binding to angiogenic blood vessels and having a length that is 5 to 500 times greater than its diameter.

3. A method as in claim 1 wherein the step of intravenously administering a first macromolecular contrast agent comprises administering a polymeric contrast agent capable of binding to angiogenic blood vessels and having a length that is 5 to 500 times greater than its diameter.

4. A method as in claim 1 wherein the step of intravenously administering a first macromolecular contrast agent and the step of intravenously administering a second macromolecular contrast agent each comprises administering a polymeric contrast agent capable of binding to angiogenic blood vessels and having a length that is 5 to 500 times greater than its diameter.

5. A method for assessing the presence of angiogenic blood vessels, the method comprising:

intravenously administering a macromolecular contrast agent to a subject;

obtaining a first image of tissue of the subject reflecting blood circulation levels of the first contrast agent;

intravenously administering a second, polymeric contrast agent to a subject, the second contrast agent being prepared by reacting a substantially mono-activated steric hindrance molecule with a polymer, to provide a polymer-steric hindrance molecule copolymer having an elongated structure and having a degree of conjugation of 90% or greater and loading the polymer-steric hindrance molecule copolymer with an image producing entity, being capable of binding to angiogenic blood vessels and having a length that is 5 to 500 times greater than the diameter of the second contrast agent;

obtaining a second image of tissue of the subject; and

comparing the first and second images to assess the presence of angiogenic blood vessels, wherein a localized image enhancement indicates the presence of angiogenic blood vessels.

6. A method as in claim 5 wherein the polymeric contrast agent is prepared by reacting a substantially mono-activated diethylenetriamine pentaacetic acid with a polypeptide.

7. A method as in claim 5 wherein the polymeric contrast agent is prepared by loading the polymer-steric hindrance molecule copolymer with an image producing entity comprises contacting the polymer-steric hindrance molecule copolymer with a solution containing gadolinium ions.

8. A method as in any of claims 1-5 wherein the polymeric contrast agent comprises a backbone formed from a polypeptide selected from the group consisting of polylysine,

polyglutamic acid, polyaspartic acid and copolymers of lysine and either glutamic acid or aspartic acid.

9. A method as in any of claims 1-5 wherein the polymeric contrast agent comprises administering a contrast agent having a polymer backbone having covalently bound thereto at least one member from the group consisting of diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrakis(2-propionic acid) (DOTMA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecane -1,4,7,10-tetrakis(3-(4-carboxyl)-butanoic acid), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(acetic acid-methyl amide), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonic acid), and p-isothiocyanatobenzyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (p-SCN-Bz-DOTA), bis(thiosemicarbazone), derivatives of bis(thiosemicarbazone), porphyrins, derivatives of porphyrins, 2,3-bis(2-thioacetamido)propionates, derivatives of 2,3-bis(2-thioacetamido)propionates, *N,N'*-bis(mercaptoacetyl)-2,3-diaminopropanoate, bis(aminoethanethiol) and derivatives of bis(aminoethanethiol).

10. A method as in any of claims 1-5 wherein the polymeric contrast agent comprises a polypeptide backbone having a length of 35 to 1500 amino acid residues.

11. A method as in any of claims 1-5 wherein the polymeric contrast agent has a diameter in the range of 20 Angstroms to 50 Angstroms.

12. A method as in any of claims 1-5 wherein the polymeric contrast agent comprises one or more paramagnetic entities chelated to a polymeric backbone.

13. A method as in any of claims 1-5 wherein the polymeric contrast agent comprises one or more gadolinium ions chelated to a polymeric backbone.

14. A method as in claim 13 wherein the polymeric contrast agent is administered at a dose in the range of about 0.01 mmoles Gd/Kg to about 0.1 mmoles Gd/Kg.

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FIG. 1

Synthetic Scheme for Gd(DTPA) Polyllysine

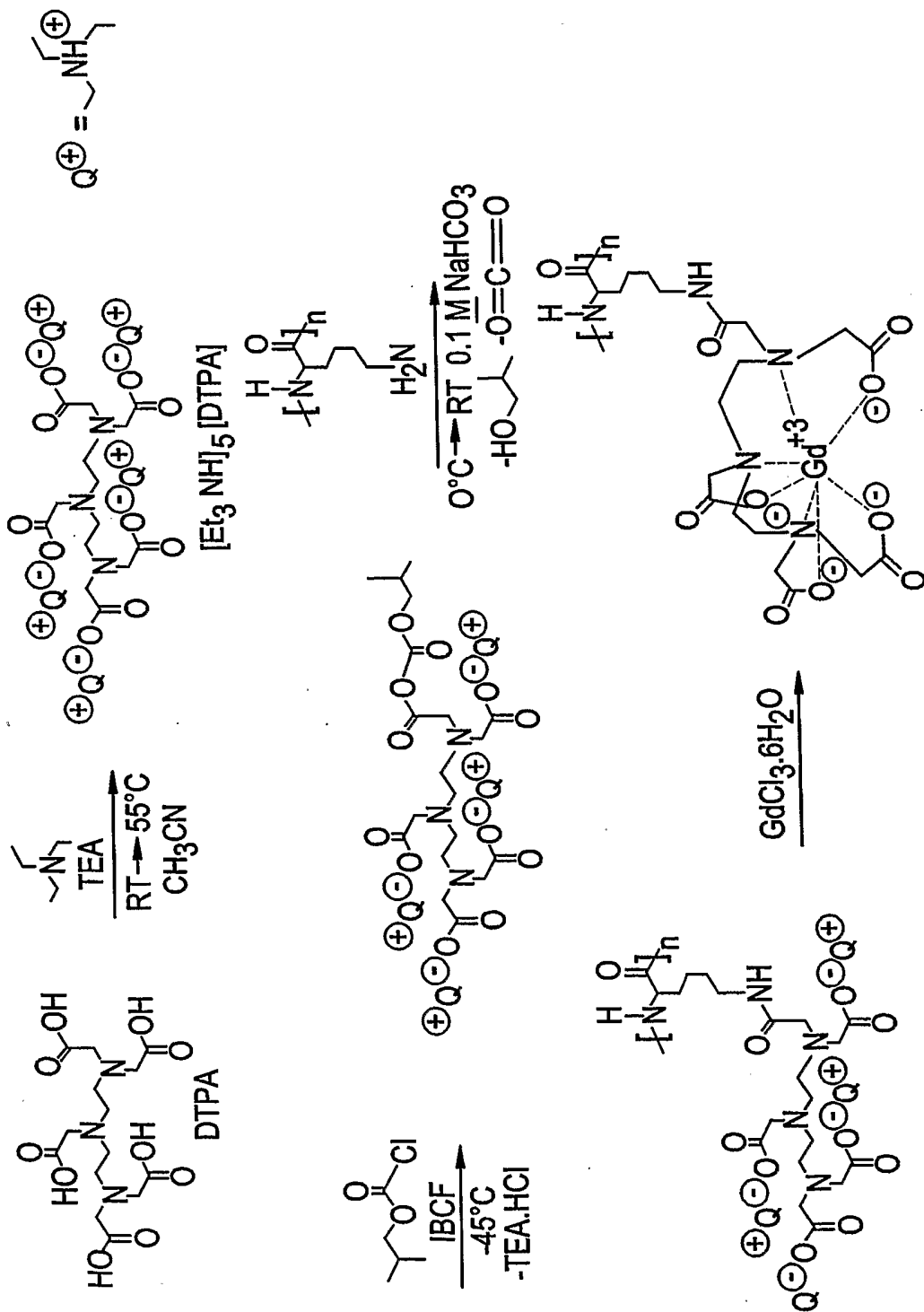


FIG. 2

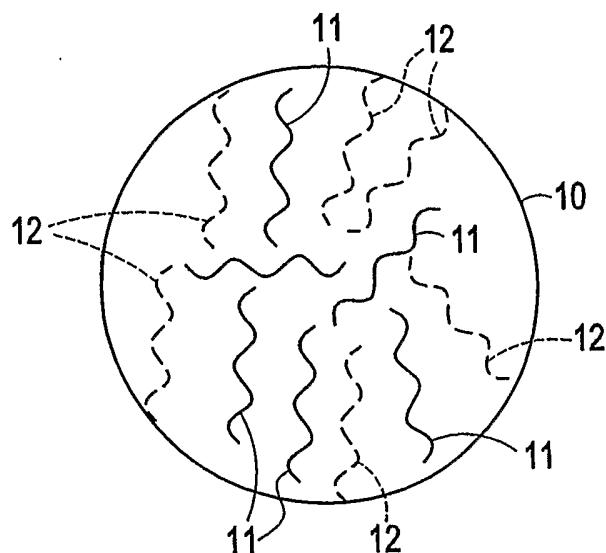


FIG. 3

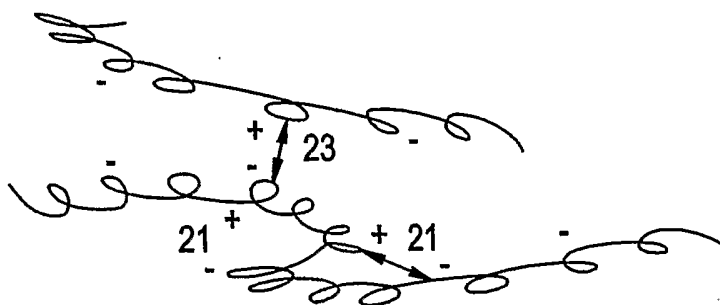


FIG. 4

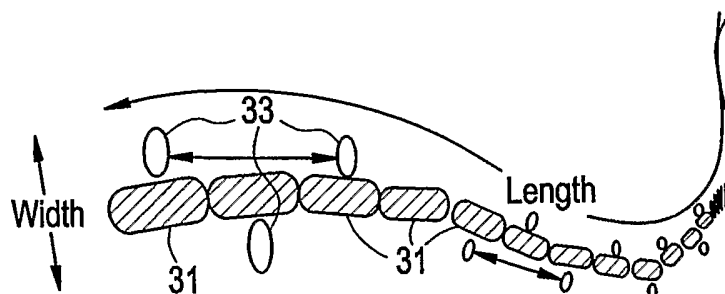


FIG. 5

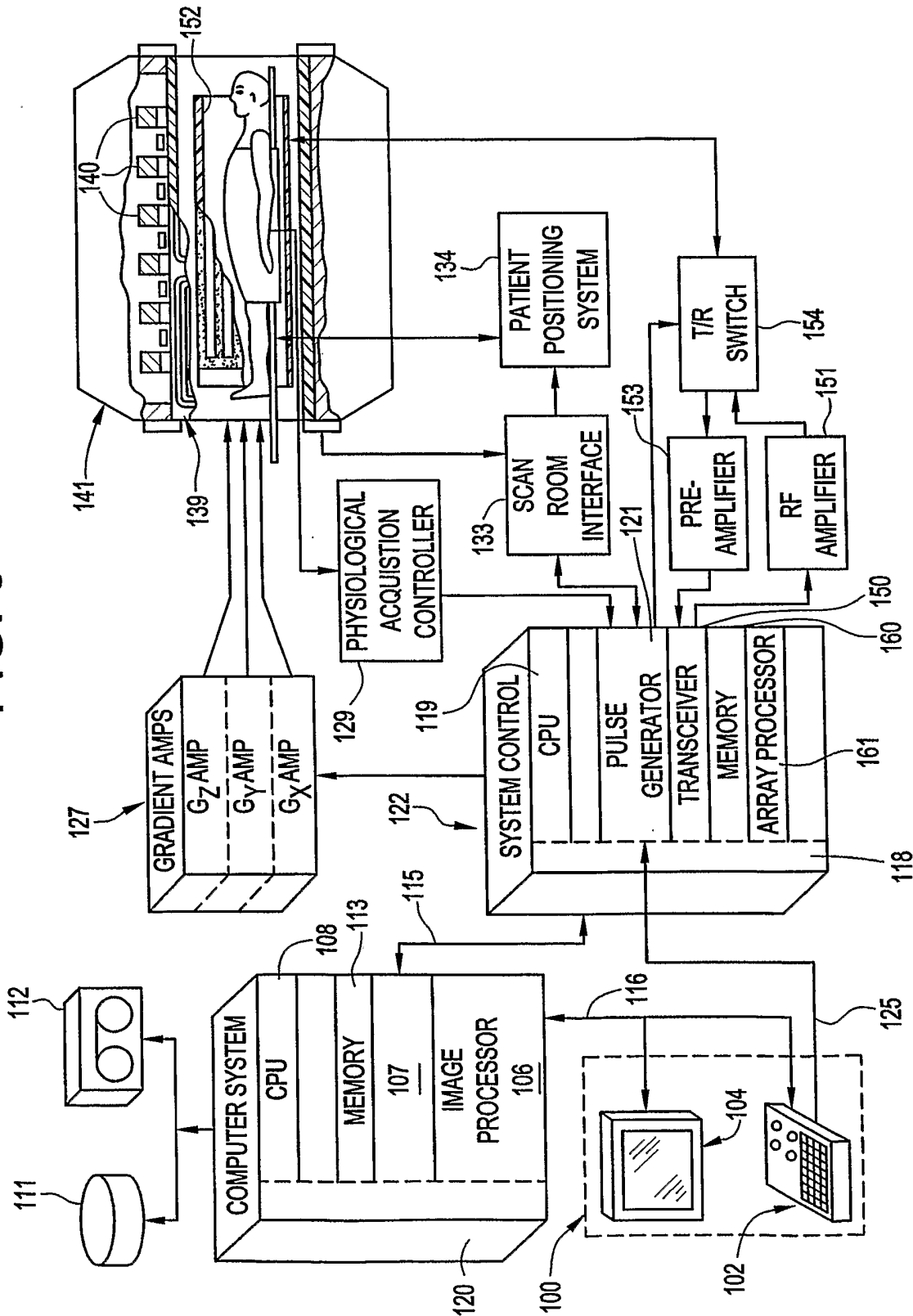
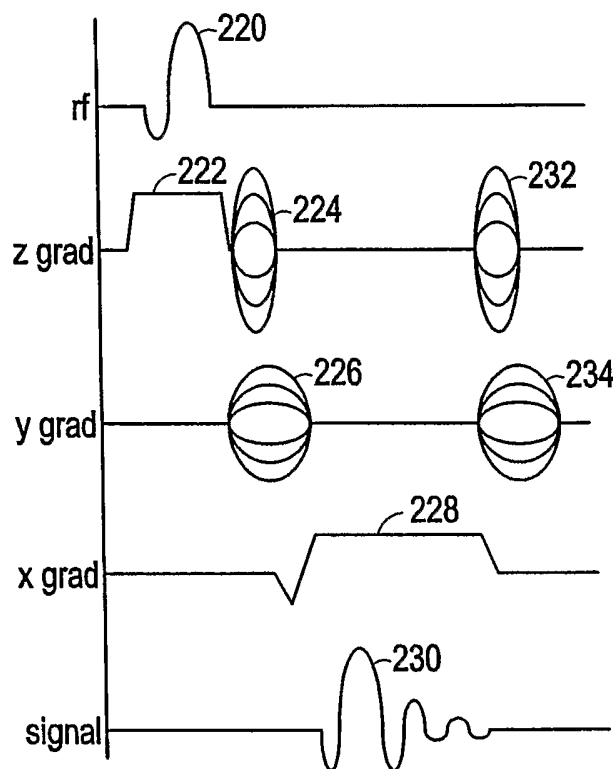


FIG. 6



PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13ter.1(c) and Rule 39)

Applicant's or agent's file reference RD29109	IMPORTANT DECLARATION	Date of mailing(day/month/year) 26/08/2003
International application No. PCT/US 03/ 17814	International filing date(day/month/year) 06/06/2003	(Earliest) Priority date(day/month/year) 31/07/2002
International Patent Classification (IPC) or both national classification and IPC		A61B5/055 A61B6/00 A61K49/06
Applicant GENERAL ELECTRIC COMPANY		

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below

1. ☒ The subject matter of the international application relates to:
 - a. ☐ scientific theories.
 - b. ☐ mathematical theories
 - c. ☐ plant varieties.
 - d. ☐ animal varieties.
 - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
 - f. ☐ schemes, rules or methods of doing business.
 - g. ☐ schemes, rules or methods of performing purely mental acts.
 - h. ☐ schemes, rules or methods of playing games.
 - i. ☒ methods for treatment of the human body by surgery or therapy.
 - j. ☒ methods for treatment of the animal body by surgery or therapy.
 - k. ☒ diagnostic methods practised on the human or animal body.
 - l. ☐ mere presentations of information.
 - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.

2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

☐ the description
 ☐ the claims
 ☐ the drawings

3. ☐ The failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions prevents a meaningful search from being carried out:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

4. Further comments: see further information continued from PCT/ISA/203

Name and mailing address of the International Searching Authority  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Johannes Van Brummelen
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 203

A meaningful search is not possible on the basis of all claims because all claims are directed to a method for treatment of the human or animal body by surgery (intravenously administering a contrast agent) and to a diagnostic method practised on the human or animal body (use of contrast agent requires the presence of medical staff) - Rule 39.1(iv) PCT.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.