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(57) Abstract

The present invention relates to a method employing a tissue–directed ^{13}C, ^{13}C–^{13}C, ^{13}C–^{15}N or ^{13}C–^{13}C–^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent in magnetic resonance imaging to enhance the contrast of a targeted site in a mammalian tissue.

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APPLICATION OF ^{13}C-^{13}C, ^{13}C-^{15}N, AND ^{13}C-^{13}C-^{15}N ISOTOPICALLY ENRICHED PROTEINS AS TISSUE-DIRECTED IMAGE-ENHANCEMENT REAGENTS FOR MAGNETIC RESONANCE IMAGING

This application is a continuation-in-part of application serial no. 08/878,022, filed 18 June 1997, and application serial no. 60/063,252, filed 24 October 1997.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for employing a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent in magnetic resonance imaging to enhance the contrast of a targeted site in a mammalian tissue. The method comprises the steps of (a) administering to mammalian tissue an amount effective to produce a detectable magnetic resonance imaging signal of a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula: T-L-R; wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion; and (b) when the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent has reached the targeted site in the mammalian tissue, performing magnetic resonance imaging at the targeted site employing one or more sensitivity-

enhancement techniques and selectivity techniques to detect the signal produced by the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent. The sensitivity-enhancement techniques comprise (i) using ^{1}H-detection of ^{13}C or ^{15}N in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, respectively, (ii) using flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C, or ^{15}N transverse nuclear relaxation rates, and (iii) using repeating sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing residue, Y is a water-soluble hydrophilic residue, and n is an integer ranging from about 2 to about 10,000. The selectivity techniques comprise suppressing signals from natural abundance ^{13}C and ^{15}N by employing heteronuclear editing including the use of combined pulsed field gradients heteronuclear coherence selection and phase-cycled H-X, H-X-Y, or H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N, and supressing signals from solvent H_2O by combined use of frequency selection excitation pulses with substantially no amplitude at the H_2O resonance frequency together with heteronuclear coherence selection with pulsed field gradients providing dephasing of homonuclear H_2O coherences. The present invention is also directed to a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent represented by the formula: T-L-R, wherein T, L, and R are as defined above.

Description of the Background

Many diagnostic and therapeutic medical procedures for visualizing internal organs for the early detection and treatment of many diseases require the administration of contrast enhancing agents to improve the quality of the procedure. Contrast-enhancing agents are used in Magnetic Resonance Imaging (MRI), Computerized Tomography (CT), and X-ray procedures. Computerized Tomography provides a more sophisticated visualization of tissues and organs than does conventional X-ray techniques. MRI provides a superior soft tissue differentiation than does Computerized Tomography. MRI procedures generally employ the nuclear magnetic resonance of hydrogen (^{1}H, usually of H_2O) or fluorine (^{19}F). The nuclear magnetic resonance sensitivity of ^{19}F is nearly equivalent to that of ^{1}H but the biological background of ^{19}F is negligible. The usefulness of a contrast enhancing agent for diagnostic *in vivo* imaging depends upon the tissue-specificity of the agent, the ability to obtain sufficient signal intensity from the tissue-localized agent, and the

requirement for a clear distinction of the signals from the imaging reagent from background MRI signals. In addition, the usefulness of the agent for medical imaging depends on the costs of preparing the agent, the ease of administering the agent, the toxicological and immunological properties of the agent, and the resistance to *in vivo* hydrolysis or decomposition of the agent.

In radioscintigraphy, a radioactive monoclonal antibody is typically injected into a patient for identifying and localizing a tumor, (reviewed in Bischof Delaloye, A. and Delaloye, B.: Tumor imaging with monoclonal antibodies. *Seminars in Nuclear Medicine* 25(2):144-164, 1995). In radioimaging with monoclonal antibodies, a chemically modified (chelate) form of a monoclonal antibody is typically prepared and stored as a relatively stable product. To be used clinically, however, the monoclonal antibody sample must be mixed with a radioactive metal, such as ^{99}Tc, then purified to remove excess, unbound radioactive metal, and then administered to a patient within 6 hours, (Eckelman, W. C., Paik, C. H., and Steigman, J.: Three approaches to radiolabeling antibodies with ^{99}Tc. *Nuc. Med. Biol.* 16: 171-176, 1989). The entire process is cumbersome and dangerous due to the many manipulations requiring use and disposal of radioactivity. There is some health risk and fear accompanying injection of radioactivity into the patient. For these reasons, diagnostic imaging methods using stable isotopes (e.g. ^{13}C or ^{15}N) will be of significant value in medicine. Moreover, the stable isotopes ^{13}C and ^{15}N are environmentally safe, and do not have high costs associated with their disposal.

Another example of imaging technology is the diagnosis of blood clots. Despite the frequency of pulmonary thromboembolism and its associated morbidity and mortality, diagnosis remains suboptimal. Similarly, noninvasive detection of both deep vein and cerebral thrombosis is currently difficult. Various radiolabeled proteins, such as antifibrin monoclonal antibodies (Rosebrough, S. F. and Hashmi, M.: Galactose-modified streptavidin-GC4 antifibrin monoclonal antibody conjugates: application for two-step thrombus/embolus imaging. *J. Pharm. Exp. Ther.* 276: 770-775, 1996), fibrin-binding domain fragment of fibronectin (Rosenthall. L. and LeclerC, J.: A new thrombus imaging agent. Human recombinant fibrin binding domain labeled with In-111. *Clin. Nucl. Med.* 20: 398-402, 1995), activated-platelet binding protein fragment (Muto, P., Lastoria, S., Varrella, P., et al.: Detecting deep venous thrombosis with technetium-99-labeled synthetic peptide P280. *J. Nucl. Med.* 36: 1384-1391, 1995) and (inactivated) tissue plasminogen activator (De Bruyn, V. H., Bergmann, S. R., Keyt, B. A. and Sobel, B. E.:

Visualization of thrombi in pulmonary arteries with radiolabeled, enzymatically inactivated tissue-plasminogen activator. *Circulation* 92: 1320-1325, 1995) have been utilized for imaging thrombi.

Tissue-targeted proton-detected ^{13}C MRI, using ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N coherence selection, as outlined in this patent application, has not been described in the existing literature. The use of non-targeted, ^{13}C-enriched contrast reagents, such as ^{13}C glucose, has been described previously for MRI and for metabolic studies (Shulman, R. G., Blamire, A. M., Rothman, D. L. and McCarthy, G. Nuclear magnetic imaging and spectroscopy of human brain function. *Proc. Natl. Acad. Sci. USA* 90: 3127-3133, 1993; Sonnewald, U., Gribbstad, I. S., Westergaard, N. Nilsen, G., Unsgard, G., Schousboe, A. and Peterson, S. B. Nuclear magnetic resonance spectroscopy: biochemical evaluation of brain function in vivo and in vitro. *Neurotox.* 15(3): 579-590, 1994). Non-targeted homonuclear ^{1}H-^{1}H double quantum NMR imaging experiments have also been described (Hurd, R. E. and Freeman, D. M. Metabolite specific proton magnetic resonance imaging. *Proc. Natl. Acad. Sci. U.S.A.* 86: 4402-4406, 1989), but do not provide an avenue for tissue-selective targeting. Proton-detected ^{13}C heteronuclear multiple quantum coherence (HMQC) NMR and direct ^{13}C detection methods not involving spatial imaging have been described for measuring sucrose concentrations in plants (Tse, T.Y., Spanswick, R. M., Jelininski, L.W., Quantitative evaluation of NMR and MRI methods to measure sucrose concentrations in plants. *Protoplasma* 194: 54-62), but have not been used for *in vivo* tissue-directed magnetic resonance imaging applications in humans or animals.

The feature of tissue-directed binding to a disease-indicating locus has been described for radioactive isotope-enriched reagents (see references above). Furthermore, reagents containing metals such as gadolinium, which enhance contrast in proton (i.e. H_2O) MRI, have been described for MRI contrast-enhancement of tumors, thrombosis, and pulmonary embolisms (Young, S. W., Qing, F., Harriman, A., Sessler, J. L., Dow, W. C., Mody, T. D., Hemmi, G. W., Hao, Y. and Miller, R. A. Gadolinium(III) texaphyrin: A tumor selective radiation enhancer that is detectable by MRI. *Proc. Natl. Acad. Sci. USA* 93: 6610-6615 ; Igarashi, N., Igarashi, S., Fujio, N. and Yoshida, A. Magnetic resonance imaging in the early diagnosis of cavernous sinus thrombosis. *Ophthalmologica* 209(5): 292-296, 1995; Williams, R. F., Siegle, R. L., Pierce, B. L. and Floyd, L. J. Analogs of synthetic melanin polymers for specific imaging applications. *Invest. Radiology* 29: S116-119, 1994; Orang-Khadivi,

K., Pierce, B. L., Ollom, C. M., Floyd, L. J., Siegle, R. L. and Williams, R. F. New magnetic resonance imaging techniques for the detection of breast cancer. *Breast Cancer Res. Treat.* 32(1): 119-135, 1994; Meaney, J. F. M., Weg, J. G., Chenevert, T. L., Stafford-Johnson, D., Hamilton, B. H. and Prince, M. R. Diagnosis of pulmonary embolism with magnetic resonance angiography. *The New England Journal of Medicine* 336: 1422-1427, 1997).

United States patent no. 4,624,846 (*Goldenberg*) discloses a method for enhancing the target specificity of antibody localization. The method comprises injecting a second antibody specific to a labeled target-specific antibody to reduce the level of non-targeted circulating specific antibody and thereby increase the localization ratio. Specifically, the method comprises injecting a human subject parenterally with a marker-specific antibody labeled with a pharmacologically inert radioisotope, capable of detection using a photoscanning device, or with a paramagnetic conjugate, capable of detection with a magnetic resonance detector, and subsequently scanning with the device or detector to detect and locate the site of uptake of the labeled antibody by the tumor. The improvement provided by the method comprises injecting the subject parenterally, at a time after injection of the marker-specific antibody sufficient to permit maximum selective uptake by the tumor, and prior to scanning, with a second, non-labeled antibody specific against the marker-specific antibody, in an amount sufficient to decrease the level of circulating labeled marker-specific antibody or fragment by 10-85% within 2-72 hours. *Goldenberg* discloses the use of numerous antibodies labeled with radionuclei for detection by photoscanning devices and paramagnetic species for detection by a magnetic resonance detector. The method is said to be useful to help determine the location of a tumor which produces or is associated with a Cytoplasmic, intracellular, or cell-surface marker substance.

United States patent no. 5,236,694 (*Antich et al. '694*) discloses the use of ^{19}F labeled compounds in methods of NMR imaging and spectroscopy. The compounds comprise a ^{19}F-containing sensor moiety and a transport polymer, and may also comprise a spacer moiety to separate the sensor moiety and the transport polymer. Specifically, the method comprises administering to a living subject a ^{19}F labeled NMR agent comprising (a) a transport polymer selected from the group consisting of dextran polymers and aminodextrans, having a molecular weight between approximately 100 D and 500 kD, and antibodies and fragments thereof, and (b) a ^{19}F-containing sensor moiety selected from the group consisting of fluorinated

alkyls, fluorinated acetates, fluoroaniline, and fluoroalkyl phosphonates, in an amount effective to provide a detectable NMR signal. The signal produced by the ^{19}F labeled NMR agent in the subject is then detected.

United States patent no. 5,308,604 (*Sinn et al.*) discloses conjugates composed of a) at least one polyalcohol or a derivatized polyalcohol, b) at least one active agent, c) at least one linker, and d) a protein. The polyalcohols are compounds which are not recognized by the defense system of an organism as exogenous, such as sorbitol or derivatized sorbitol, with at least one OH group being replaced by ^{19}F, $C^{19}F_3$, mono- or poly-^{19}F-substituted C_1-C_4 alkyl, mono-, di-, tri-, tetra- or penta-^{19}F-substituted phenyl. The active agent is a compound which is able to emit a signal to an external scanning device and/or is able to have a direct or indirect therapeutic effect on tumor tissue, and preferably is a ^{19}F, ^{131}I, or ^{132}I labeled aromatic compound. The linker is a compound which may be used as a coupling member or spacing member between the protein and active agent. Examples of the linker, which are usually bifunctional, are 2,4-dichloropyrimidine, 4,4'-diisothiocyanoato-2,2'-stilbenedisulfoninc acid, and cyanuric chloride (2,4,6-trichloro-s-triazine). The protein is a compound which can be taken up by the tumor specifically or non-specifically, and is not recognized by the defense system of an organism as exogenous, such as autologous serum albumin. The conjugates are said to be suitable for providing a very sensitive method in nuclear medicine for the diagnosis of tumors and also offering methods for diagnosing tumors in X-ray diagnosis, computerized tomography, nuclear spin tomography, electron spin resonance spectroscopy, or electron microscopy. The application of ^{13}C or ^{15}N is not disclosed in this patent.

United States patent no. 5,401,493 (*Lohrmann et al.*) discloses organic compounds for diagnostic imaging which contain at least one aryl group which has been derivatized to contain at least one perfluoro-^1H,^1H-neopentyl moiety. The perfluoro-^1H,^1H-neopentyl groups produce a single magnetic resonance to provide a maximum signal to noise ratio. A preferred perfluoro-1H,1H-neopentyl group is 3,5-$(CF_3)_3C(CH_2)$-C_6H_3-. A lipid emulsion may also be provided as a carrier vehicle to deliver the derivatized analog to a mammalian recipient. The application of ^{13}C or ^{15}N is not disclosed.

United States patent no. 5,422,094 (*Antich et al.'094*) discloses an ^{19}F labeled NMR composition said to be useful in methods of NMR imaging and

spectroscopy comprising a ^{19}F-containing sensor moiety and an antibody and optionally a spacer moiety to separate the sensor moiety and the antibody. The sensor moiety comprises $-COCF_3$ or $-NHCOCF_3$ and produces a single ^{19}F NMR signal. The antibody reacts specifically with a particular antigen and is bound to the ^{19}F-containing sensor moiety. *Antich et al.'094* states that the spacer moiety can be used to isolate the ^{19}F atoms from the substrate thereby enhancing the NMR signal produced. *Antich et al.'094* states that the spacer moiety can be, for example, an alkyl hydrocarbon having a chain length of approximately 1-100 carbon atoms and containing an amino group, or alternatively, the spacer moiety can be selected from the group consisting of alkyl, alkoxy, aryl, and alkaryl hydrocarbons which contain an amino group, hydrazine, hydrazide, semicarbazide, and hydroxylamine. *Antich et al.'094* state that the spacer moiety can optionally include one or more ^{19}F atoms. The application of ^{13}C or ^{15}N is not disclosed in this patent.

SUMMARY OF THE INVENTION

The present invention pertains to a method for employing a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent in magnetic resonance imaging to enhance the contrast of a targeted site in a mammalian tissue which comprises the steps of:

(a) administering to mammalian tissue an amount effective to produce a detectable magnetic resonance imaging signal of a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion;

(b) when the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent has reached the targeted site in the mammalian tissue, performing magnetic resonance imaging at the targeted site employing one or more sensitivity-enhancement techniques and selectivity techniques to detect the

signal produced by the tissue-directed ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the sensitivity-enhancement techniques comprise:

(i) using ^{1}H-detection of ^{13}C or ^{15}N in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, respectively,

(ii) using flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C or ^{15}N transverse nuclear relaxation rates, and

(iii) using repeating sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing residue, Y is a water-soluble hydrophilic residue, and n is an integer ranging from about 2 to about 10,000;

and the selectivity techniques comprise:

(iv) suppressing signals from natural abundance ^{13}C and ^{15}N by employing heteronuclear editing including the use of combined pulsed field gradients heteronuclear coherence selection and phase-cycled H-X, H-X-Y, or H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N; and

(v) supressing signals from solvent H_2O by combined use of frequency selection excitation pulses with substantially no amplitude at the H_2O resonance frequency together with heteronuclear coherence selection with pulsed field gradients providing dephasing of homonuclear H_2O coherences.

The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent may be selected from the group consisting of intrinsically isotopically enriched protein reagents, proteins bound to isotopically enriched conjugates, and isotopically enriched proteins bound to a tissue-directed targeting group. The ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion is preferably ^{13}C-labeled polyethylene glycol. In one embodiment, the tissue-directed targeting group is an organic compound, peptide, or protein selected from the group consisting of polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, nucleic acid binding proteins, and specific protein binding proteins. In another embodiment, the tissue-directed targeting group is selected from the group consisting of tumor-specific antigen or receptor targeting groups, normal tissue-specific antigen or receptor targeting groups, and specific antigen or receptor targeting groups of infectious agents. The tissue-directed targeting group may also be selected from the group consisting of blood clot targeting groups, ß-amyloid plaque targeting groups of Alzheimer's disease, Congo red, and tumor-specific antigen targeting groups. The tissue-directed targeting group is preferably selected from the group consisting of antifibrin monoclonal antibodies,

fibrin-binding domain fragment of fibronectin, activated-platelet binding protein fragment, and inactivated tissue plasminogen activator. The linker group is preferably selected from the group consisting of *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, 2,4-dichloropyrimidine, 4,4'-diisothiocyanoato-2,2'-stilbenedisulfoninc acid, and cyanuric chloride.

The sensitivity-enhancement technique may also comprise the use of ^{1}H-detection of ^{13}C or ^{15}N, or both, in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, by coherence transfer pathways which begin with proton polarization, followed by transfer to directly bound ^{13}C using standard HSQC-INEPT or HMQC methods, frequency labeling of ^{1}H or ^{13}C in the presence of 3D magnetic field gradients to determine the spatial localization of the ^{13}C nuclei, followed by transfer back to ^{1}H for signal detection. The sensitivity-enhancement technique may also comprise the use of flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C or ^{15}N transverse nuclear relaxation rates, wherein the flexible polypeptide or polymeric chains are segments placed either at the N- or C-termini, or as engineered surface loops within the polypeptide chain, or as flexible ^{13}C-enriched conjugates attached to the protein. The sensitivity-enhancement technique may also comprise the use of repeating amino-acid sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing an amino acid residue selected from the group consisting of Alanine, Serine, Threonine, Leucine, Isoleucine, and Valine; Y is a water-soluble hydrophilic amino acid residue selected from the group consisting of Serine, Aspartic acid, Asparagine, and Glutamic acid; and n is an integer ranging from about 2 to about 10,000. The sensitivity-enhancement technique may further comprise the use of partial random ^{2}H-enrichment of the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents to lengthen nuclear transverse relaxation rates and enhance sensitivity. The sensitivity-enhancement technique may further comprise the use of heteronuclear single-quantum and heteronuclear multiple-quantum coherence generation and selection with pulsed-field gradients and phase cycling. The selectivity technique may also comprise suppressing signals from water and natural abundance ^{13}C and ^{15}N by employing heteronuclear editing, including the use of H-X, H-X-Y and H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N, including applying phase-cycled radiofrequency pulses or magnetic field gradients to the carbon-13 or nitrogen-15 that are cycled so as to select for magnetic transfer pathways H->X->H, H->X->Y->X->H, H->X->Y->Z->Y->X->H, H->X->Y->H, or H->X->Y->Z->H, that both begin and end on H nuclei and which involve

directly bonded X, X-Y or X-Y-Z atoms. The H-X, H-X-Y or H-X-Y-Z editing may further include frequency-selective saturation of water resonances and band-selective excitation of upfield aliphatic proton resonances. In one embodiment, the method for employing a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent is carried out *in vivo*; in another embodiment, the method is carried out *in vitro*. In one embodiment, the tissue-directed isotopically enriched protein reagent is a uniformly or selectively ^{13}C enriched reagent; in another embodiment, the tissue-directed isotopically enriched protein reagent is a uniformly or selectively ^{13}C-^{13}C enriched reagent; in still another embodiment, the tissue-directed isotopically enriched protein reagent is a uniformly or selectively ^{13}C-^{15}N enriched reagent; in yet another embodiment, the tissue-directed isotopically enriched protein reagent is a uniformly or selectively ^{13}C-^{13}C-^{15}N enriched reagent.

The present invention is also directed to a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the use of ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, and ^{13}C-^{13}C-^{15}N isotopically enriched proteins as tissue-directed diagnostic reagents in magnetic resonance imaging. The specific applications of this technology include medical imaging of human and animal subjects. The invention employs tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N labeled protein molecules, prepared either by biosynthetic enrichment (intrinsically isotopically enriched protein reagents) or by covalent attachment of a protein to an isotopically-enriched conjugate, in combination with isotope-editing schemes implemented in novel MRI NMR pulse

sequences. Sensitivity enhancement, which is crucial to the success of such technology, is obtained by detecting ^{1}H nuclei bound to these ^{13}C or ^{15}N atoms, partial enrichment with ^{2}H isotopes, and by the use of dynamically flexible polypeptide or polymeric chains that will exhibit relatively long ^{1}H, ^{13}C and ^{15}N transverse nuclear relaxation rates. ^{13}C NMR signals arising from water and from the natural abundance of ^{13}C or ^{15}N in the body are suppressed by selecting for coherence transfer pathways involving ^{13}C-^{13}C and/or ^{13}C-^{15}N covalent bonds. These ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents are targeted to and capable of identifying, quantifying, and localizing disease-specific loci, such as tumors, through the use of magnetic resonance imaging NMR experiments incorporating heteronuclear coherence selection filters.

The principle technical issues addressed by the present invention are (i) tissue-selectivity of the MRI contrast enhancement reagent, (ii) high sensitivity in detecting the image enhancement reagent at the relatively low concentrations that will be achieved *in situ*, and (iii) selectivity for detecting the reagent over water and the natural abundance concentration of ^{13}C nuclei in biological systems. Processes which fail to adequately address these key issues do not provide a feasible approach for ^{13}C-based diagnostic MRI.

The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents of the present invention are targeted to and capable of binding selected loci in animals and humans, and thereby provide a means for detecting, identifying, localizing, and quantifying these loci by magnetic resonance imaging. By employing the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents of the present invention, it is possible to diagnose the presence and status of normal tissue and organ structure and to diagnose particular diseases related to these loci. The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents are partly or fully enriched in ^{13}C or ^{15}N, or both. Although the natural abundance of ^{13}C is relatively high, these ^{13}C-enriched reagents can be specifically probed to provide a signal in magnetic resonance imaging when localized *in vivo* using sensitivity-enhancement techniques and selectivity techniques to detect the signal produced by the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent.

As set out above, the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent of the present invention employed as a diagnostic contrasting agent in magnetic resonance imaging comprises a tissue-directed targeting group portion, which selectively binds to a normal or disease-related target in mammalian tissue (*in vivo* or *in vitro*), and a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein portion, which provides a magnetic resonance imaging signal. The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagent may be a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N intrinsically enriched protein reagent, a protein bound to a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched conjugate, or a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein bound to a tissue-directed targeting group. ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N intrinsically enriched protein reagents are reagents in which the tissue-directed targeting group portion itself is isotopically labeled and is therefore also the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion, which provides a magnetic resonance imaging signal. Proteins bound to ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched conjugates are reagents wherein the tissue-directed targeting group portion is unlabeled and is bound to a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched conjugate group. ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched proteins bound to a tissue-directed targeting group comprise a tissue-directed targeting group bound to a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein. other than a conjugate group. In general, the string of ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, ^{13}C-^{13}C-^{13}C, and ^{13}C-^{13}C-^{15}N atoms may be present in a uniformly isotope-enriched polypeptide, protein, polymer, or covalently-bound conjugate, or may be selectively enriched in the background of an otherwise unenriched polypeptide, protein, polymer, or covalently-bound conjugate. The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagent also includes glycoproteins, in which the ^{13}C-enrichment is in the carbohydrate portion that is covalently conjugated to an enriched, or unenriched, protein. The amount of tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent administered to mammalian tissue is an amount effective to produce a detectable magnetic resonance imaging signal when performing magnetic resonance imaging at the targeted site employing the sensitivity-enhancement techniques and selectivity techniques of the present invention.

The tissue-directed targeting groups in the diagnostic contrasting agents of the present invention are groups that preferentially bind to the site being targeted. The nature of the tissue-directed targeting group defines the diagnostic

application of the particular version of the invention. The tissue-directed targeting group may be any organic compound, peptide, or protein, that can bind to a specific target or locus, i. e., act as a ligand and bind to a receptor, in mammalian tissue, either *in vivo*, i.e., in an organism, or *in vitro*. The tissue-directed targeting group may be a polyclonal antibody, monoclonal antibody, single chain antibody, Fab fragment, nucleic acid binding protein, specific protein binding protein, or other tissue directed reagent. The tissue-directed targeting group includes (but is not limited to) tumor-specific antigen or cell-surface receptor targeting groups (e.g. epidermal growth factor receptor molecules of human breast tissue tumors), normal tissue-specific antigen or cell-surface receptor targeting groups (e.g. for image enhancement in *vivo* MRI imaging of abnormal heart muscle growth or vascular dilation in response to specific drug therapies), and specific antigens or surface targeting groups of infectious agents (e.g. the surface protein A molecules of infectious *Staphylococcal aureus* bacteria). Proteins and peptides for use as targeting groups can be isolated from natural sources, prepared by recombinant DNA technology, or prepared by chemical synthesis. It is particularly advantageous to use antibodies of high specificity, e.g., affinity-purified antibodies and/or monoclonal antibodies.

The ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion of the diagnostic contrasting agents of the present invention are groups that provide a magnetic resonance imaging signal. As used herein, the term "^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion" includes proteins, peptides, and polymers. Uniformly ^{13}C or ^{15}N, or both, enriched proteins with well-characterized tissue specificity can be produced biosynthetically in recombinant bacterial expression systems grown on minimal media using ^{13}C-glucose and ^{15}N-ammonia as the sole sources of metabolic carbon and nitrogen and purified using standard methods [for one of many examples of generalized expression and purification systems for production of high levels (i.e. 20-100 mg/ liter fermentation) of ^{13}C,^{15}N enriched proteins, see Jansson, M., Li, Y.-C., Jendeberg, L., Anderson, S., Montelione, G. T. and Nilsson, B. High level production of uniformly ^{15}N- and ^{13}C-enriched fusion proteins in *Escherichia coli*. *J. Biomol. NMR* 7, 131-141, 1996]. Alternatively, isotope-enriched synthetic peptides, small molecules, or polymers uniformly enriched in ^{13}C can be obtained from commercial sources, and covalently attached to the tissue-directed protein. The tissue-directed protein may be a naturally occurring isotope-enriched protein hormone, nucleic-acid binding protein, cytoskeletal binding protein, lipid-binding protein, carbohydrate-binding protein, monoclonal antibody (or F_{ab} antibody fragment), or other tissue-

specific protein directed to a cell surface or intracellular receptor molecule. The tissue-directed protein can either be biosynthetically-enriched in ^{13}C, ^{13}C-^{13}C and/or ^{13}C-^{15}N bonds or constructed with a covalently attached ^{13}C-^{13}C and/or ^{13}C-^{15}N protein, peptide, small molecule, or polymer.

The tissue-directed agent can be administered in sufficient quantities to saturate the available binding sites in biological subject by intravenous injection, or by injection directly into the tissue of interest. For certain applications involving diagnostic MRI of the digestive tract or lungs, the agent can be administered by ingestion or by aerosol. Immunoreactivity can be minimized using species specific protein as tissue-directed targets, and by creating sets of reagents with polypeptide or polymeric "tails" that have different sequences.

In a preferred embodiment, the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent is represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion.

The tissue-directed targeting group portion in the present invention is a group that preferentially binds to the site being targeted. The tissue-directed targeting group portion may be any organic compound, peptide, or protein, that can bind to a specific target or locus, i. e., act as a ligand and bind to a receptor. The tissue-directed targeting group portion may be a polyclonal antibody, monoclonal antibody, single chain antibody, or Fab fragment. The tissue-directed targeting group portion includes blood clot targeting groups, ß-amyloid plaque targeting groups of Alzheimer's disease, Congo red, and tumor-specific antigen targeting groups. Proteins and peptides for use as targeting groups can be isolated from natural sources, prepared by recombinant DNA technology, or prepared by chemical synthesis. It is particularly advantageous to use antibodies of high specificity, e.g., affinity-purified antibodies and/or monoclonal antibodies.

In a preferred embodiment, the tissue-directed targeting group portion is employed to diagnose for Alzheimer's disease. The chemical, Congo red, has been shown to bind to ß-amyloid plaques and a form of Congo red capable of chelating a radioactive metal has been prepared and used for imaging by radioscintigraphy. Alternatively, the protein, tissue plasminogen activator, can be used in a radiolabeled form as a diagnostic reagent to image ß-amyloid plaques. Still another potential imaging agent is ß-amyloid peptide, which can deposit into the plaque. Although ß-amyloid plaques are within the brain, they are also present in the small and medium-sized arteries serving the brain, and are uniquely associated with Alzheimer's disease. Thus, it may be possible to administer the imaging agent intravenously, not requiring it to pass the blood brain barrier. In each case, imaging of the ß-amyloid plaques would be done using stable isotope reagents.

In another preferred embodiment, the tissue-directed targeting group portion is employed to diagnose for blood clots. Despite the frequency of pulmonary thromboembolism and its associated morbidity and mortality, diagnosis remains suboptimal. Similarly, noninvasive detection of both deep vein and cerebral thrombosis is currently difficult. Various radiolabeled proteins, such as antifibrin monoclonal antibodies, fibrin-binding domain fragment of fibronectin, activated-platelet binding protein fragment and (inactivated) tissue plasminogen activator have been utilized for imaging thrombi. As above, any of these targeting agents can be prepared in the ^{13}C or ^{15}N -enriched form.

The ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion of the present invention is an isotopically enriched protein portion containing repeating ^{13}C or ^{15}N reporting groups which provide a magnetic resonance imaging signal. A preferred ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion is ^{13}C-labeled polyethylene glycol (PEG, also known as poly(ethylene oxide)), which can be prepared from ^{13}C labeled ethylene oxide, which is commercially available from Isotech Inc. In addition to its role as a carrier of the reporter atoms for the magnetic resonance signal, use of polyethylene glycol as the conjugation partner is advantageous in the present invention for its pharmacological properties. These properties include providing an extended circulating half-life in blood, as well as preventing or minimizing attack by antibodies or proteases in blood (Davis, S., Abuchowski, A., Park, Y. K. and Davis, F. F. Alteration of the circulating half life and antigenic properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol. *Clin. Exp. Immunol.* 46:

649-652, 1981). This description of polyethylene glycol does not rule out the use of other inert polymers, such as dextran, as the conjugation partner (Melton, R. G., Wiblin, C. N., Foster, R. L. and Sherwood, R. F. Covalent linkage of carboxypeptidase G2 to soluble dextrans-I. Properties of conjugates and effects on plasma resistance in mice. *Biochem. Pharmacol.* 36(1): 105-112, 1987). Furthermore, by localizing the signal generating element (^{13}C or ^{1}H attached to ^{13}C) to a repeating subunit, which is $-(CH_2-CH_2-O-)_n$ in polyethylene glycol, where n may range from 10 to 10,000 or more, the Magnetic Resonance Imaging instrument can be tuned to a specific and unique signal corresponding to the structure of polyethylene glycol. In this way, background signal from the natural abundance (ca. 1%) of ^{13}C in other biomolecules, such as fatty acids, may be screened out. Even further, the multiple copies of the signal generating element appended to each locus-binding molecule (i.e. with respect to both the multiple copies of isotope atom per polymer chain and the opportunity to append several polymer chains to each binding molecule) serves as a means for signal amplification. Another advantage is that the inherent flexibility of the long, thin polyethylene glycol or other hydrophilic polymer chain should provide extensive molecular motion, resulting in a much stronger signal than possible by a peptide or protein bound to its corresponding receptor. A preferred ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion is ^{13}C-labeled polyethylene glycol, represented by the formula $-(CH_2-CH_2-O-)_n$, where n may range from about 100 to about 100,000, preferably from about 1,000 to about 50,000, and more preferably from about 1,000 to about 10,000. Polyethylene glycol is also preferred because it is not immunoreactive in humans.

The linker group in the present invention is a group that connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion. The linker group is employed as coupling member or spacer between the tissue-directed targeting group portion and the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion. Linker groups are usually bifunctional compounds which use one functional group to enter into a chemical bond with the tissue-directed targeting group portion and use the second functional group to enter into a chemical bond with the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion. Examples of linkers are *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, for coupling a compound having a primary amino group with a compound having a thiol group, and ethylene glycobis (succinimidylsuccinate), for cross-linking two compounds having primary amino groups. Both of these cross-linkers, as well as a variety of other cross-linkers, are

available from Pierce (Rockford, IL). Other linker groups may be selected from the group consisting of 2,4-dichloropyrimidine, 4,4'-diisothiocyanoato-2,2'-stilbenedisulfoninc acid, and cyanuric chloride. The linker group is selectively attached to the tissue-directed targeting group portion at a site not directly involved in antigen-antibody or receptor-ligand binding, thereby allowing the tissue-directed targeting group portion to retain its binding function. Possible sites for attachment of the linker group to the tissue-directed targeting group portion include carbohydrate groups, amino groups, sulfhydryl groups, or combinations thereof.

As set out above, the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagents are administered to a mammal to produce a detectable magnetic resonance imaging signal when performing magnetic resonance imaging at the targeted site employing the sensitivity-enhancement techniques and selectivity techniques of the present invention. One novel enabling feature of this invention is the application of ^{1}H-detected ^{13}C and/or ^{15}N in these ^{13}C, ^{13}C-^{13}C and ^{13}C-^{15}N labeled proteins as a means of sensitivity-enhancement in diagnostic MRI. The gyromagnetic ratio (γ_H) of proton is approximately four times larger than that of ^{13}C (γ_C) and approximately 10 time larger than that of ^{15}N (γ_N). MRI experiments can be designed in which coherence transfer pathways begin with proton polarization, followed by transfer to directly bound ^{13}C using the HSQC-INEPT (Morris G. A. and Freeman, R., *J. Amer. Chem. Soc.* 101, 760 - 761, 1979; Morris, G., *J. Amer. Chem. Soc.* 102, 428 - 429, 1980; Bodenhausen, G. and Ruben, *Chem. Phys. Lett.* 69, 165 - 189, 1980) or HMQC (Maudsley, A. A. and Ernst, R. R., *Chem. Phys. Letts.* 50, 368 - 372, 1977; Bodenhausen G. and Freeman, R., *J. Amer. Chem. Soc.* 100, 320 - 321, 1978) methods, with frequency labeling of ^{1}H or ^{13}C in the presence of 3D magnetic field gradients to determine the spatial localization of these ^{13}C nuclei using standard proton magnetic resonance imaging (^{1}H MRI) methods (for a review of standard ^{1}H NMR MRI methods see Ernst, R. R., Bodenhausen, G., and Wokaun, A. *"Principles of Nuclear Magnetic Resonance in One and Two Dimensions"*, Claredon Press, Oxford, Chapt 10, pp. 539 - 596, 1987), followed by transfer back to ^{1}H for detection by the NMR probe.

Signal-to-noise ratios depend on the gyromagnetic ratios of excited (γ_{exc}) and observed (γ_{obs}) nuclei, and on the steady-state amplitude of polarization on the excited nucleus determined by the extent of recovery through longitudinal relaxation (T_1) between subsequent scans of the experiment separated by an interval

T (Ernst, R. R, Bodenhausen, G. and Wokaun, A. *"Principles of Nuclear Magnetic Resonance in One and Two Dimensions"*, Claredon Press, Oxford, 1987):

$$S/N \propto \gamma_{exc}(\gamma_{obs})^{3/2}\{1-\exp(-T/T_1^{exc})\}$$

The initial polarization is proportional to γ_{exc} and is modulated by the degree of relaxation due to T_1 during the recycle delay T, while the signal intensity of the observed nucleus is proportional to its gyromagnetic ratio γ_{obs}. The detector noise is approximately proportional to $(\gamma_{obs})^{1/2}$. Accordingly, under ideal conditions of perfect coherence transfer, the sensitivity enhancement of these ^1H-excite, ^1H-detected ^{13}C NMR experiments compared with ^{13}C-excite-^{13}C detect experiments is proportional to $(\gamma_H/\gamma_C)^{5/2}$ i.e. ~32-fold. Moreover, ^1H nuclei will generally exhibit much larger R_1 longitudinal relaxation rates than ^{13}C, providing significantly greater polarization recovery in the time period T between scans and higher sensitivity (Ernst, R. R, Bodenhausen, G. and Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Claredon Press, Oxford, 1987). Thus, these experiments generally provide more than 30-fold enhancement of signal intensity compared with direct MRI detection of ^{13}C.

A second enabling feature of this invention is the use of flexible polypeptide or polymeric chains for sensitivity enhancement. Transverse relaxation rates (R_2) determine the resonance linewidths and the rates of decay of magnetization during multiple-pulse coherence transfer pulse sequences. These R_2 rates increase as the rotational correlation time of internuclear vectors increase. For this reason, immobilized receptor-bound isotope-enriched proteins generally exhibit high transverse relaxation rates, that will be manifested as very broad resonance linewidths and corresponding poor signal intensity. This "transverse relaxation problem" severely limits the use of ^{13}C NMR in diagnostic MRI. In the embodiment of the current invention, the "transverse relaxation problem" is overcome using standard molecular biology methods to engineer into tissue-directed proteins of interest disordered polypeptide or polymeric segments containing ^{13}C, ^{15}N, and ^1H nuclei, or by creating conjugates with flexible ^{13}C, ^{13}N, ^1H enriched conjugates. These polypeptide segments are placed either at the N- or C-termini, or as engineered surface loops within the polypeptide chain. For example, in solution-state triple resonance (^{13}C, ^{15}N, ^1H) NMR experiments we observe large (~20-fold) signal enhancements for some of the residues in the disordered 14-residue N-terminal polypeptide tail of the Z-domain of staphylococcal protein A (Lyons, B. A. and

Montelione G. T., An HCCNH Triple-Resonance Experiment Using Carbon-13 Isotropic Mixing for Correlating Backbone Amide and Side-Chain Aliphatic Resonances in Isotopically Enriched Proteins, *J. Magn. Reson,* B101, 206 - 209, 1993; Lyons, B. A., Tashiro, M., Cedergren, L., Nilsson, B., Montelione, G. T., An Improved Strategy for Determining Resonance Assignments for Isotopically-Enriched Proteins and Its Application for an Engineered Domain of Staphylococcal Protein A, *Biochemistry*, 32, 7839 - 7845, 1993; Tashiro, M., Rios, C. B., Montelione, G. T., Classification of Amino Acid Spin Systems Using PFG HCC(CO)NH TOCSY With Constant-Time Aliphatic ^{13}C Frequency Labeling, *J. Biomol. NMR,* 6, 211 - 216, 1995; Tashiro, M. and Montelione, G. T., Structures of Bacterial Immunoglobin-Binding Domains and Their Complexes with Immunoglobulins, *Current Opin. Struct. Biol.* 5, 471 - 481, 1995; Jendeberg, L., Tashiro, M., Tejero, R., Lyons, B. A., Uhlén, M., Montelione, G. T., and Nilsson, B., The Mechanism of Binding Staphylococcal Protein A to Immunoglobulin G Does Not Involve Helix Unwinding, *Biochemistry* 35, 22 - 31, 1996) which is due to the longer R_2 relaxation rates that result from the relatively high mobility of this polypeptide tail. The enhanced flexibility of such disordered polypeptide segments provides significantly longer transverse relaxation rates, and greatly enhanced sensitivity for diagnostic MRI.

A third enabling feature of the invention is the use of repeating sequences of the form (X-Y)n where X represents a methyl, methylene, or isopropyl containing residue (e.g. an amino acid residue such as Alanine, Serine, Threonine, Leucine, Isoleucine, or Valine) and Y is a hydrophilic residue with high water solubility (e.g. an amino acid residue such as Serine, Aspartic Acid, Asparagine, Glutamic Acid, etc). The sensitivity for detecting the proton-carbon correlation of methyl resonances is enhanced by a factor of 3 for each Alanine, Serine, and Threonine in the sequence by the fact that the three protons of the methyl group always exhibit identical chemical shifts and their intensities sum together in the process of detection. The sensitivity for detecting proton-carbon correlation is enhanced by factor of 6 for each isopropyl methyl group of Leucine or Valine in the sequence as the two methyl groups on each of these residue types will have identical chemical shifts when occurring in a disordered region of the protein structure. Because these sequences can be engineered into "tails" or internal surface loops that can be designed to be highly disordered, the many methyl groups of the tissue-targeted protein will all have nearly identical ^{1}H and ^{13}C chemical shifts, providing a summed sensitivity enhancement factor of order 3n (e.g., for n=50-residue tail, an enhancement factor of ~150-fold is expected). In addition, the internal mobility of

such solvent-exposed methyl groups due to internal rotation about the C-C bond generally provides sharp resonance linewidths (and thus greatly enhanced sensitivity) even in very large protein systems.

The sensitivity-enhancement afforded by uniform ^{13}C-enrichment, use of MRI pulse sequences that initiate coherence transfer pathways from proton, rather than ^{13}C, polarization, sensitivity-enhancement due to proton, rather than ^{13}C detection, longer transverse relaxation rates observed for flexible "tails" (or engineered surface loops) of proteins, and detection of methyl resonance of amino acids with degenerate chemical shifts in the disordered polypeptide "tails" (or engineered surface loops) provides enhancement in sensitivity of 10^4-10^7 for these ^1H-detected ^{13}C MRI experiments compared to direct detection of ^{13}C atoms at natural abundance in an immobile receptor-bound molecule.

Although the approaches described above provide over 10,000-fold enhancement of the signal arising from the isotope-enriched tissue-directed reagent, the natural abundance of ^{13}C in biological systems (~1.1%) will result in significant background ^{13}C signal intensity from the high concentrations of unenriched proteins, lipids, and carbohydrates in the MRI sample. Although some of this background can be suppressed by using frequency-selective proton and carbon excitation pulses (see for example Kessler, H., Mronga, S., and Gemmecker, G., Multi-dimensional NMR Experiments Using Selective Pulses, *Magn. Reson. Chem.*, 29, 527 - 557, 1991; Kupce, E., Boyde, J., and Campbell, I. D., Short Selective Pulses for Biochemical Applications, *J. Magn. Reson.*, B106, 300 - 303, 1995) centered on the aliphatic methyl regions, background ^1H and ^{13}C signals also occur in these regions of the spectrum. For these reasons, the fourth requisite key feature of this invention is the use of H-X, H-X-Y and H-X-Y-Z filters in ^{13}C MRI, where X = ^{13}C (or ^{15}N) , Y = ^{13}C (or ^{15}N), and Z = ^{13}C (or ^{15}N), as a means of suppressing signal from background natural abundance ^{13}C and for suppressing the very strong MRI signal arising from H_2O. While various methods of heteronuclear editing are in general use in solution state heteronuclear NMR, the heteronuclear filters described here have not been described previously for diagnostic MRI.

The general concept of H-X-Y editing is to apply carbon-13 (or nitrogen-15) radiofrequency (RF) pulses and/or magnetic field gradients that are cycled so as to select for magnetic transfer pathways H->X->Y->X->H (out-and-back) or H->X->Y->H (straight through) that both begin and end on H nuclei (which

have the highest gyromagnetic ratio and thus provide the most initial polarization and highest sensitivity for detection) and which involve directly bonded X-Y atoms. The ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, ^{13}C-^{13}C-^{13}C, and ^{13}C-^{13}C-^{15}N filters involve ^{13}C atoms directly bonded to one another in the particular structure. In general, $(^{13}C)_n$ and $(^{13}C)_n$-^{15}N isotopically enriched molecules, where n=1 to 20, and their corresponding NMR filters, may be employed. Preferably, ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, ^{13}C-^{13}C-^{13}C, and ^{13}C-^{13}C-^{15}N isotopically enriched molecules, and their corresponding NMR filters, are employed. Without loss of generality, consider the specific case of a H-^{13}C-^{13}C moiety within an isotope-enriched tissue-directed protein (e.g. the H_3-$^{13}C\beta$-$^{13}C\alpha$ group of an Alanine residue). In this particular H-X-Y filter, the pathway requires that both X and Y be ^{13}C nuclei. This selection is achieved in either of two ways. By separately phase cycling RF pulses that create transverse magnetization states $C\beta_{x,y}$ (i.e. the ^{13}C pulse creating the coherence transfer from $H\beta$-$C\beta$ or a multiple-quantum $H\beta_{x,y}$-$C\beta_{x,y}$ state, or the corresponding pulse in the reverse pathway) and $C\alpha_{x,y}$ (i.e. the ^{13}C pulse creating the coherence transfer from $C\beta$-$C\alpha$ or a multiple-quantum $C\beta_{x,y}$-$C\alpha_{x,y}$ state, or the corresponding pulse in the reverse pathway) together with the phase of the detection, a family of MRI pulse sequences can be designed that select for a $H\beta_3$->$C\beta$->$C\alpha$->$C\beta$->$H\beta_3$ pathway and cancel magnetization that does not pass through these two sequentially-bonded carbon nuclei. In this case, the signal from solvent H_2O is also canceled by the phase cycling, but only in alternate scans. This results in a dynamic range problem in each scan for detecting the weak H signal of the agent in the presence of the strong signal of H_2O. This problem can be overcome by band-selective excitation of the upfield aliphatic proton resonances of interest (for methods of selective excitation see for example Kessler, H., Mronga, S., and Gemmecker, G., Multi-dimensional NMR Experiments Using Selective Pulses, *Magn. Reson. Chem.*, 29, 527 - 557, 1991; Kupce, E., Boyde, J., and Campbell, I. D., Short Selective Pulses for Biochemical Applications, *J. Magn. Reson.*, B106, 300 - 303, 1995). Alternatively, the fact that single quantum (SQ) and heteronuclear multiple quantum (HMQ) coherences evolve in exactly predictable ways in the presence of a pulsed field gradients (PFGs) (Hurd, R. E., Gradient-Enhanced Spectroscopy, *J. Magn. Reson.* 87, 422 - 428, 1990; Hurd, R. E. and John, B. K., Gradient-Enhanced Proton-Detected Heteronuclear Multiple-Quantum Coherence Spectroscopy, *J. Magn. Reson.*, 91, 648 - 653, 1991; Kay, L. E., Field Gradient Techniques in NMR Spectroscopy, *Curr. Opin. Struct. Biol.* 5, 674 - 681, 1995) allows the use of PFGs with precise amplitudes and phases for selection of ^{13}C-^{13}C HMQ coherences. This coherence selection process provides nearly complete suppression of the signal from H_2O even in a

single scan (Hurd, R. E., Gradient-Enhanced Spectroscopy, *J. Magn. Reson.* 87, 422 - 428, 1990; Hurd, R. E. and John, B. K., Gradient-Enhanced Proton-Detected Heteronuclear Multiple-Quantum Coherence Spectroscopy, *J. Magn. Reson.*, 91, 648 - 653, 1991; Li, Y.-C., Emerson, S. D., Saarien, T., and Montelione, G. T. Heteronuclear PFG NMR, *TAMU NMR Newsletter,* Aug. 6, 1997; Li, Y.-C. and Montelione, G. T. Solvent Saturation Transfer Effects in Pulsed-Field-Gradient Heteronuclear Single-Quantum Coherence (PFG-HSQC) Spectra of Polypeptides and Proteins, *J. Magn. Reson.*, B101, 315 - 319, 1993; Kay, L. E., Field Gradient Techniques in NMR Spectroscopy, *Curr. Opin. Struct. Biol.* 5, 674 - 681, 1995). The phase cycling and gradient coherence selection methods are independent of one another, and are implemented together in the single pulse sequence, providing cleaner and more complete selection of the desired ^{13}C-^{13}C HMQ coherences. These general methods of HMQ coherence selection have used extensively in the solution state NMR literature (for a review see Kay, L. E., Field Gradient Techniques in NMR Spectroscopy, *Curr. Opin. Struct. Biol.* 5, 674 - 681, 1995) with great success. The innovation of the present invention is to apply these specific ideas from solution state NMR in MRI pulse sequences for use in tissue-directed ^{13}C magnetic resonance imaging.

The ability to suppress signals from natural abundance ^{13}C is critical to the success of this invention. For unenriched biomolecules with natural (1.1 %) ^{13}C abundance, a H-^{13}C-^{13}C filter provides a ~10,000-fold (0.01 x 0.01) suppression compared to sites with 100% ^{13}C enrichment. For a H-^{13}C-^{15}N filter, the ~0.4% natural abundance of ^{15}N provides a ~25,000-fold suppression of background signal relative to a 100% ^{13}C,^{15}N enriched site. Suppression of proton signal from H_2O, which has no ^{13}C atoms bound, is even larger. Proton H_2O signals are also suppressed in the conceived family of experiments by using proton frequency selective pulses (see for example Kessler, H., Mronga, S., and Gemmecker, G., Multi-dimensional NMR Experiments Using Selective Pulses, *Magn. Reson. Chem.*, 29, 527 - 557, 1991; Kupce, E., Boyde, J., and Campbell, I. D., Short Selective Pulses for Biochemical Applications, *J. Magn. Reson.*, B106, 300 - 303, 1995), centered on the aliphatic methyl resonances, which provide little or no excitation of the H_2O signal together with heteronuclear coherence selection with pulsed field gradients. For samples generating sufficient signal, triple H-X-Y-Z tuning is also possible. For H-^{13}C-^{13}C-^{13}C pathways, this provides a 10^6-fold suppression of background signal. Additional selectivity and background suppression can be obtained by preparing the isotope-labeled tissue-directed protein with random partial enrichment in ^{2}H isotopes

and using filters which select for ^{13}C nuclei bonded to ^{2}H nuclei, as has been demonstrated in solution state NMR (Kuslan, D. M. and LeMaster, D. M., Resolution and Sensitivity Enhancement of Heteronuclear Correlation for Methylene Resonances Via ^{2}H Enrichment and Decoupling *J. Biomol. NMR* 3, 701 - 708, 1993; Grzesiek, S., Anglister, J., Ren, H., and Bax, A. ^{13}C Line Narrowing by 2H Decoupling in 2H/13C/15N Enriched Proteins. Application to Triple Resonance 4D J Connectivity of Sequential Amides *J. Amer. Chem. Soc.* 115, 4369 - 4370, 1993; Yamazaki, T., Lee, W., Revington, M., Mattiello, D. L., Dahlquist, F. W., Arrowsmith, C. H., Kay, L. E. *J. Amer. Chem. Soc.* 116, 6464 - 6465, 1994).

These reagents and techniques would be used for diagnostic partial or whole body imaging by magnetic resonance techniques. They would allow enhanced spatial visualization of specific tissue types in MRI which exhibit specific types of molecules that are accessible to the isotope-enriched proteins. This technology would be applicable to the development of a wide range of MRI techniques for diagnosing human diseases.

In a specific embodiment, the invention is directed at a method for employing a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent in magnetic resonance imaging to enhance the contrast of a targeted site in a mammalian tissue which comprises the steps of:

(a) administering to mammalian tissue an amount effective to produce a detectable magnetic resonance imaging signal of a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion;

(b) when the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent has reached the targeted site in the mammalian tissue, performing magnetic resonance imaging at the targeted site employing one or more sensitivity-enhancement techniques and selectivity techniques to detect the

signal produced by the tissue-directed ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the sensitivity-enhancement techniques comprise:

(i) using ^{1}H-detection of ^{13}C or ^{15}N in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, respectively,

(ii) using flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C or ^{15}N transverse nuclear relaxation rates, and

(iii) using repeating sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing residue, Y is a water-soluble hydrophilic residue, and n is an integer ranging from about 2 to about 10,000;

and the selectivity techniques comprise:

(iv) suppressing signals from natural abundance ^{13}C and ^{15}N by employing heteronuclear editing including the use of combined pulsed field gradients heteronuclear coherence selection and phase-cycled H-X, H-X-Y, or H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N; and

(v) supressing signals from solvent H_2O by combined use of frequency selection excitation pulses with substantially no amplitude at the H_2O resonance frequency together with heteronuclear coherence selection with pulsed field gradients providing dephasing of homonuclear H_2O coherences.

In one specific embodiment, the sensitivity-enhancement technique of step (i) is employed; in another specific embodiment, the sensitivity-enhancement technique of step (ii) is employed; and in another specific embodiment, the sensitivity-enhancement technique of step (iii) is employed. In a preferred specific embodiment, the sensitivity-enhancement technique of steps (i) and (ii) are employed; in another preferred specific embodiment, the sensitivity-enhancement technique of steps (i) and (iii) are employed; and in another preferred specific embodiment, the sensitivity-enhancement technique of steps (ii) and (iii) are employed. In a more preferred specific embodiment, the sensitivity-enhancement technique of steps (i), (ii), and (iii) are employed together with the selectivity techniques of steps (iv) and (v). In a most preferred specific embodiment, the sensitivity-enhancement technique of any of the above embodiments is employed together with the selectivity techniques of steps (iv) and (v).

The present invention extends to methods for preparing the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagents. The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagents

may be synthesized using standard techniques and apparatus known to those skilled in the art. The ultimate tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagents are readily prepared using methods generally known in the chemical and biochemical arts. In general, the tissue-directed ^{13}C-enriched reagent can be synthesized by standard chemistry coupling reactions. Uniformly ^{13}C or ^{15}N, or both, enriched proteins with well-characterized tissue specificity can be produced biosynthetically in recombinant bacterial expression systems grown on minimal media using ^{13}C-glucose and ^{15}N-ammonia as the sole sources of metabolic carbon and nitrogen and purified using standard methods [for one of many examples of generalized expression and purification systems for production of high levels (i.e. 20-100 mg/ liter fermentation) of ^{13}C,^{15}N enriched proteins, see Jansson, M., Li, Y.-C., Jendeberg, L., Anderson, S., Montelione, G. T. and Nilsson, B. High level production of uniformly ^{15}N- and ^{13}C-enriched fusion proteins in *Escherichia coli*. *J. Biomol. NMR* 7, 131-141, 1996]. Alternatively, isotope-enriched synthetic peptides, small molecules, or polymers uniformly enriched in ^{13}C can be obtained from commercial sources, and covalently attached to the tissue-directed protein.

In general, the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent can be synthesized by standard chemistry coupling reactions. ^{13}C-Labeled polyethylene glycol can be prepared from the ^{13}C-labeled monomer, purified and activated, such as with but not limited to a protected thiol group (Woghiren, C., Sharma, B. and Stein, S. Protected thiol-polyethylene glycol: A new activated polymer for reversible protein modification. *Bioconj. Chem.* 4, 314-318, 1993). The activated, magnetic resonance responsive-enriched polyethylene glycol would then be reacted with the tissue-directed targeting group portion (protein, peptide or other organic molecule), using methods applicable to each particular product. For example, disulfide bond formation may be made by mixing the protected thiol-polyethylene glycol with a cysteine-containing protein or peptide. Otherwise, amino groups in the peptide or protein could be reacted at their primary amino groups (i.e. lysine side chains or the amino-terminus) using a heterobifunctional cross-linking agent with subsequent reaction to the thiol group of thiol-activated polyethylene glycol. Another approach would be to do a polymer extension reaction using a ^{13}C enriched monomer on a pre-existing polymer, such as a monomethyl ether of polyethylene glycol of 2 kDa or a protected/activated polyethylene glycol of 2kDa, as available from Shearwater Polymers Inc. (Huntsville, AL), and then proceed with the coupling to the tissue-directed targeting group portion /binding molecule.

Also, a ^{13}C-labeled copolymer having multiple attachment sites could be prepared (Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L. and Kohn, J. Copolymers of lysine and polyethylene glycol: A new family of functionalized drug carriers. *Bioconj. Chem.* 4, 54-62, 1993). In this way, multiple copies of the targeting/binding molecule, or a combination of different targeting and binding molecules can be linked to the same polymer molecule for achieving greater binding avidity or other special properties, such as enhanced cellular uptake after binding. Even further, the polyethylene glycol copolymer could be of especially high molecular weight for greatest signal generation, but designed to be unstable *in vivo*, so that it would eventually degrade into fragments small enough to be excreted. For example, individual 5,000-dalton subunits of ^{13}C-labeled polyethylene glycol could be covalently linked to one another in a linear and/or branched manner through ester bonds to form a compound having an average molecular weight in the hundreds of thousands or millions of daltons. This macromolecular polyethylene glycol could be covalently linked to one or multiple copies of the tissue-directed targeting group portion.

The tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N enriched protein reagents of the present invention may be used together with pharmaceutically acceptable carriers to provide pharmaceutical compositions which can be administered to mammals such as man in amounts effective to produce a detectable magnetic resonance imaging signal. The soluble carriers include lipid emulsions, liposomes, microparticles or microspheres. If the biological or pharmaceutical compound is water soluble a carrier is not required. Suitable carriers include propylene glycol-alcohol-water, isotonic water, sterile water for injection (USP), emulphor^{TM}-alcohol-water, cremophor-EL^{TM} or other suitable carriers known to those skilled in the art. Other suitable carriers include isotonic water, sterile water for injection (USP), alone or in combination with other solubilizing agents such as ethanol, propylene glycol, or other conventional solubilizing agents known to those skilled in the art.

Of course, the type of carrier will vary depending upon the mode of administration desired for the pharmaceutical composition as is conventional in the art. A preferred carrier is an isotonic aqueous solution of the inventive compound.

The compounds of the present invention can be administered to mammals, e.g., animals or humans, in amounts effective to produce a detectable magnetic resonance imaging signal. Since the activity of the compounds and the degree of the desired diagnostic effect vary, the dosage level of the compound employed will also vary. The actual dosage administered will also be determined by such generally recognized factors as the body weight of the patient and the individual hypersensitiveness of the particular patient.

The compounds of the present invention can be administered parenterally, in the form of sterile solutions or suspensions, such as intravenously, intramuscularly or subcutaneously in the carriers previously described.

For parental diagnostic administration, the compounds of the present invention may be incorporated into a sterile solution or suspension. These preparations should contain at least about 0.1% of the inventive compound, by weight, but this amount may be varied to between about 0.1% and about 50% of the inventive compound, by weight of the parental composition. The exact amount of the inventive compound present in such compositions is such that a suitable dosage level will be obtained.

The sterile solutions or suspensions may also include the following adjuvants: a sterile diluent, such as water for injection, saline solution, fixed oils, polyethylene glycol, glycerin, propylene glycol, or other synthetic solvent; antibacterial agents, such as benzyl alcohol or methyl paraben; antioxidants, such as ascorbic acid or sodium metabisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parental preparations may be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic.

It is especially advantageous to formulate the pharmaceutical compositions in dosage unit forms for ease of administration and uniformity of dosage. The term dosage unit forms as used herein refers to physically discrete units suitable for use as a unitary dosage, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier.

The present invention is further illustrated by the following examples which are not intended to limit the effective scope of the claims. All parts and percentages in the examples and throughout the specification and claims are by weight of the final composition unless otherwise specified.

Examples

Materials and Methods

Uniformly ^{13}C and/or ^{15}N enriched proteins can be prepared by standard methods described in the literature (see for example see Jansson, M., Li, Y.-C., Jendeberg, L., Anderson, S., Montelione, G. T. and Nilsson, B. High level production of uniformly ^{15}N- and ^{13}C-enriched fusion proteins in Escherichia coli. *J. Biomol. NMR* 7, 131-141, 1996 and Shimotakahara, S., Rios, C. B., Laity, J. H., Zimmerman, D. E., Scheraga, H. A., and Montelione, G. T. NMR Structural Analysis of an Analog of an Intermediate Formed in the Rate-Determining Step of One Pathway in the Oxidative Folding of Bovine Pancreatic Ribonuclease A: Automated Analysis of 1H, 13C, and 15N Resonance Assignments for Wild-Type and [C65S, C72S] Mutant Forms, Biochemistry 36, 6915 - 6929). ^{13}C, ^{15}N, ^{2}H - enriched proteins can also be prepared by methods which have been described in the literature (Kuslan, D. M. and LeMaster, D. M., Resolution and Sensitivity Enhancement of Heteronuclear Correlation for Methylene Resonances Via 2H Enrichment and Decoupling *J. Biomol. NMR* 3, 701 - 708, 1993; Grzesiek, S., Anglister, J., Ren, H., and Bax, A. ^{13}C Line Narrowing by 2H Decoupling in 2H/13C/15N Enriched Proteins. Application to Triple Resonance 4D J Connectivity of Sequential Amides *J. Amer. Chem. Soc.* 115, 4369 - 4370, 1993; Yamazaki, T., Lee, W., Revington, M., Mattiello, D. L., Dahlquist, F. W., Arrowsmith, C. H., Kay, L. E. *J. Amer. Chem. Soc.* 116, 6464 - 6465, 1994).

1. Inactivated tissue plasminogen activator-^{13}C-enriched polyethylene glycol conjugate.

Tissue plasminogen activator may be prepared from recombinant bacteria. After purification and renaturation, the protein would be inactivated with respect to its ability to activate plasminogen but not with respect to its ability to bind fibrin (De Bruyn, V. H., Bergmann, S. R., Keyt, B. A. and Sobel, B. E. Visualization of thrombi in pulmonary arteries with radiolabeled, enzymatically inactivated tissue-

plasminogen activator. *Circulation* 92(5): 1320-1325, 1995). The inactivated tissue plasminogen activator could be reacted with a bifunctional cross-linking reagent, such as sulfosuccinimidyl(4-iodoacetyl)aminobenzoate. After removal of excess cross-linking reagent, the protein derivative could then be reacted with a thiol-activated form of ^{13}C-enriched polyethylene glycol. The conjugate would then be purified, formulated and administered to the patient, in a manner known to those skilled in the art. This reagent could then provide a means of detecting blood clots and ß-amyloid plaques of Alzheimer's disease by Magnetic Resonance Imaging.

2. ß-amyloid peptide-^{13}C-enriched polyethylene glycol conjugate.

The amyloid-forming peptide of Alzheimer's disease is derived by enzymatic cleavage of the carboxy-terminal portion of the amyloid precursor protein (Sisodia, S. S. and Price, D. L. Role of the ß-amyloid protein in Alzheimer's disease. *FASEB J.* 9:368-370, 1995). The amyloid plaques increase in size by further deposition of ß-amyloid peptide and exogenous ß-amyloid peptide would deposit into pre-existing plaques (*Esler et al.*, A ß-deposition Inhibition Screen Using Synthetic Amyloid. Nature Biotechnology 15:258-263 (1997). ß-amyloid peptide would be chemically synthesized with an additional residue of cysteine at its amino-terminus. After purification, the desired conjugate could be prepared by mixing the peptide with a thiopyridine protected form of ^{13}C-enriched thiol-polyethylene glycol (see reference by Woghiren et al., above).

3. Fibrin-binding peptide-^{13}C-enriched polyethylene glycol conjugate.

For the detection of thrombi, it is possible to use a synthetic peptide corresponding in sequence to the fibrin-binding domain fragment of fibronectin, as previously described using a radiolabeled fibrin-binding peptide (see reference by Rosenthall and Leclerc, above). Fibrin-binding peptide would be chemically synthesized with an additional residue of cysteine at its amino- or carboxy-terminus. After purification, the desired conjugate could be prepared by mixing the peptide with a thiopyridine protected form of ^{13}C-enriched thiol-polyethylene glycol.

4. Antifibrin monoclonal antibody-^{13}C-enriched polyethylene glycol conjugate.

Monoclonal antibodies may be reacted with activated ^{13}C polyethylene glycol, in a manner so as not to interfere with the binding properties of

the antibody. Such conjugates of antibodies to fibrin may be used to image thrombi, such as with the GC4 antibody described above (see reference by Rosebrough and Hashmi, above).

5. Uniformly $^{13}C,^{15}N$-enriched epidermal growth factor (EGF).

These isotope-enrichment methods will be used to produce uniformly $^{13}C,^{15}N$-enriched epidermal growth factor (EGF) molecules engineered by standard molecular biology techniques to contain flexible N-terminal or C-terminal polypeptide tails containing the sequence $(Ala\text{-}Ser)_n$, with n=200. $^{13}C,^{15}N$-enriched EGF will then be used to visualize EGF-receptor-rich mammary tumors in human using the MRI methods outlined above.

6. Uniformly $^{13}C,^{15}N$-enriched IgG-binding domains of staphylococcal protein A.

These isotope-enrichment methods will be used to produce uniformly $^{13}C,^{15}N$-enriched IgG-binding domains of staphylococcal protein A engineered by standard molecular biology techniques to contain flexible N-terminal polypeptide tails containing the sequence $(Val\text{-}Thr)_n$, with n=200. These enriched proteins will then be non-covalently bound to human monoclonal antibodies directed to the EGF receptor. Bifunctional cross linking reagents will be then used to cross link the isotope-enriched IgG-binding domain to the monoclonal antibody. This $^{13}C,^{15}N$-enriched protein reagent will then be injected intravenously and used to visualize EGF-receptor-rich mammary tumors in humans using the MRI methods outlined above.

7. Uniformly $^{13}C,^{15}N$-enriched soy bean proteins

These isotope-enrichment methods will be used to produce uniformly $^{13}C,^{15}N$-enriched soy bean proteins engineered by standard molecular biology techniques to contain flexible N-terminal or C terminal polypeptide tails containing the sequence $(Val\text{-}Ser)_n$, with n=200. These enriched proteins will then be ingested by human subjects. This $^{13}C,^{15}N$-enriched protein reagent will then be used to visualize the structural and functional status of the digestive tract of the human subject.

8. Uniformly $^{13}C,^{15}N$-enriched monoclonal antibodies to the human hepatitis C virus

These isotope-enrichment methods will be used to produce uniformly $^{13}C,^{15}N$-enriched monoclonal antibodies to the human hepatitis C virus, engineered by standard molecular biology techniques to contain flexible polypeptide tails containing the sequence $(Val\text{-}Ser)_n$, with n=200. These enriched proteins will then be injected intravenously and used to visualize the extent and locations of hepatitis C liver infection.

Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

While the invention has been particularly described in terms of specific embodiments, those skilled in the art will understand in view of the present disclosure that numerous variations and modifications upon the invention are now enabled, which variations and modifications are not to be regarded as a departure from the spirit and scope of the invention. Accordingly, the invention is to be broadly construed and limited only by the scope and spirit of the following claims.

We claim:

1. A method for employing a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent as a diagnostic contrasting agent in magnetic resonance imaging to enhance the contrast of a targeted site in a mammalian tissue which comprises the steps of:

(a) administering to mammalian tissue an amount effective to produce a detectable magnetic resonance imaging signal of a tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion;

(b) when the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent has reached the targeted site in the mammalian tissue, performing magnetic resonance imaging at the targeted site employing one or more sensitivity-enhancement techniques and selectivity techniques to detect the signal produced by the tissue-directed ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the sensitivity-enhancement techniques comprise:

(i) using ^{1}H-detection of ^{13}C or ^{15}N in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, respectively,

(ii) using flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C or ^{15}N transverse nuclear relaxation rates, and

(iii) using repeating sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing residue, Y is a water-soluble hydrophilic residue, and n is an integer ranging from about 2 to about 10,000;

and the selectivity techniques comprise:

(iv) suppressing signals from natural abundance ^{13}C and ^{15}N by employing heteronuclear editing including the use of combined pulsed field gradients heteronuclear coherence selection and phase-cycled H-X, H-X-Y, or H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N; and

(v) supressing signals from solvent H_2O by combined use of frequency selection excitation pulses with substantially no amplitude at the H_2O resonance frequency together with heteronuclear coherence selection with pulsed field gradients providing dephasing of homonuclear H_2O coherences.

2. The method according to claim 1, wherein the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent is selected from the group consisting of intrinsically isotopically enriched protein reagents, proteins bound to isotopically enriched conjugates, and isotopically enriched proteins bound to a tissue-directed targeting group.

3. The method according to claim 1, wherein the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion is ^{13}C-labeled polyethylene glycol.

4. The method according to claim 1, wherein the tissue-directed targeting group is an organic compound, peptide, or protein selected from the group consisting of polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, nucleic acid binding proteins, and specific protein binding proteins.

5. The method according to claim 1, wherein the tissue-directed targeting group is selected from the group consisting of tumor-specific antigen or receptor targeting groups, normal tissue-specific antigen or receptor targeting groups, and specific antigen or receptor targeting groups of infectious agents.

6. The method according to claim 1, wherein the tissue-directed targeting group is selected from the group consisting of blood clot targeting groups, ß-amyloid plaque targeting groups of Alzheimer's disease, Congo red, and tumor-specific antigen targeting groups.

7. The method according to claim 1, wherein the tissue-directed targeting group is selected from the group consisting of antifibrin monoclonal antibodies, fibrin-binding domain fragment of fibronectin, activated-platelet binding protein fragment, and inactivated tissue plasminogen activator.

8. The method according to claim 1, wherein the linker group is selected from the group consisting of *m*-maleimidobenzoyl-N-hydroxysuccinimide

ester, 2,4-dichloropyrimidine, 4,4'-diisothiocyanoato-2,2'-stilbenedisulfoninc acid, and cyanuric chloride.

9. The method according to claim 1, wherein the sensitivity-enhancement technique comprises the use of ^{1}H-detection of ^{13}C or ^{15}N, or both, in the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein, by coherence transfer pathways which begin with proton polarization, followed by transfer to directly bound ^{13}C using HSQC-INEPT or HMQC methods, frequency labeling of ^{1}H or ^{13}C in the presence of 3D magnetic field gradients to determine the spatial localization of the ^{13}C nuclei, followed by transfer back to ^{1}H for signal detection.

10. The method according to claim 1, wherein the sensitivity-enhancement technique comprises the use of flexible polypeptide or polymer chains that exhibit long ^{1}H, ^{13}C or ^{15}N transverse nuclear relaxation rates, wherein the flexible polypeptide or polymeric chains are segments placed either at the N- or C-termini, or as engineered surface loops within the polypeptide chain.

11. The method according to claim 1, wherein the sensitivity-enhancement technique comprises the use of repeating amino-acid sequences having the formula $(X\text{-}Y)_n$, wherein X is a methyl, methylene, or isopropyl group containing an amino acid residue selected from the group consisting of Alanine, Serine, Threonine, Leucine, Isoleucine, and Valine; Y is a water-soluble hydrophilic amino acid residue selected from the group consisting of Serine, Aspartic acid, Asparagine, and Glutamic acid; and n is an integer ranging from about 2 to about 10,000.

12. The method according to claim 1, wherein the sensitivity-enhancement technique further comprises the use of partial random ^{2}H-enrichment of the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagents to lengthen nuclear transverse relaxation rates and enhance sensitivity.

13. The method according to claim 1, wherein the sensitivity-enhancement technique further comprises the use of heteronuclear single-quantum and heteronuclear multiple-quantum coherence generation and selection with pulsed-field gradients and phase cycling.

14. The method according to claim 1, wherein the selectivity technique comprises suppressing signals from natural abundance ^{13}C and ^{15}N by employing heteronuclear editing, including the use of H-X, H-X-Y and H-X-Y-Z filters, wherein X = ^{13}C or ^{15}N, Y = ^{13}C or ^{15}N, and Z = ^{13}C or ^{15}N, including applying phase-cycled radiofrequency pulses or magnetic field gradients to the carbon-13 or nitrogen-15 that are cycled so as to select for magnetic transfer pathways

H->X->H,

H->X->Y->X->H,

H->X->Y->Z->Y->X->H,

H->X->Y->H, or

H->X->Y->Z->H,

that both begin and end on H nuclei and which involve directly bonded X, X-Y or X-Y-Z atoms.

15. The method according to claim 14, wherein the H-X, H-X-Y or H-X-Y-Z editing further includes frequency-selective saturation of water resonances and band-selective excitation of upfield aliphatic proton resonances.

16. The method according to claim 1, wherein the method is carried out *in vivo*.

17. The method according to claim 1, wherein the method is carried out *in vitro*.

18. A tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent, wherein the reagent may be represented by the formula:

T-L-R

wherein T is a tissue-directed targeting group portion, which selectively binds to a disease-related target in mammalian tissue, R is a ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion which provides a magnetic resonance imaging signal, and L is a linker group which connects the tissue-directed targeting group portion to the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion.

19 The isotopically enriched protein reagent according to claim 18, wherein the tissue-directed ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein reagent is selected from the group consisting of intrinsically isotopically enriched protein reagents, proteins bound to isotopically enriched conjugates, and isotopically enriched proteins bound to a tissue-directed targeting group.

20. The isotopically enriched protein reagent according to claim 18, wherein the ^{13}C, ^{13}C-^{13}C, ^{13}C-^{15}N, or ^{13}C-^{13}C-^{15}N isotopically enriched protein portion is ^{13}C-labeled polyethylene glycol.

21. The isotopically enriched protein reagent according to claim 18, wherein the tissue-directed targeting group is an organic compound, peptide, or protein selected from the group consisting of polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, nucleic acid binding proteins, and specific protein binding proteins.

22. The isotopically enriched protein reagent according to claim 18, wherein the tissue-directed targeting group is selected from the group consisting of tumor-specific antigen or receptor targeting groups, normal tissue-specific antigen or receptor targeting groups, and specific antigen or receptor targeting groups of infectious agents.

23. The isotopically enriched protein reagent according to claim 18, wherein the tissue-directed targeting group is selected from the group consisting of blood clot targeting groups, ß-amyloid plaque targeting groups of Alzheimer's disease, Congo red, and tumor-specific antigen targeting groups.

24. The isotopically enriched protein reagent according to claim 18, wherein the tissue-directed targeting group is selected from the group consisting of antifibrin monoclonal antibodies, fibrin-binding domain fragment of fibronectin, activated-platelet binding protein fragment, and inactivated tissue plasminogen activator.

25. The isotopically enriched protein reagent according to claim 23, wherein the tissue-directed targeting group is Congo red.

26. The isotopically enriched protein reagent according to claim 24, wherein the tissue-directed targeting group is inactivated tissue plasminogen activator.

27. The isotopically enriched protein reagent according to claim 23, wherein the tissue-directed targeting group is a ß-amyloid peptide of Alzheimer's disease.

28. The isotopically enriched protein reagent according to claim 18, wherein the linker group is selected from the group consisting of *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, 2,4-dichloropyrimidine, 4,4'-diisothiocyanoato-2,2'-stilbenedisulfoninc acid, and cyanuric chloride.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12568

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61B 5/055; A61K 39/42, 39/395
US CL :424/9.34, 9.3, 145.1, 158.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.34, 9.3, 145.1, 158.1; 514/2; 530/388.25, 389.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, MEDLINE, WPIDS
search terms: MRI, NMR, imaging, isotopes, targeting, amyloid, tpa, Alzheimer's, fibrin, carbon-13, nitrogen-15.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,308,604 A (SINN ET AL.) 03 May 1994, see column 7.	1-28
Y	US 5,094,848 A (BRIXNER) 10 March 1992, see columns 2-4, 10 and column 15, lines 46-49.	1-28
Y	US 5,593,658 A (BOGDANOV ET AL.) 14 January 1997. see columns 3, 6 and 9.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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