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(54) **MOLECULAR TARGETING AGENTS**

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(60) Provisional application No. 61/087,652, filed on Aug. 9, 2008, provisional application No. 61/155,288, filed on Feb. 25, 2009.

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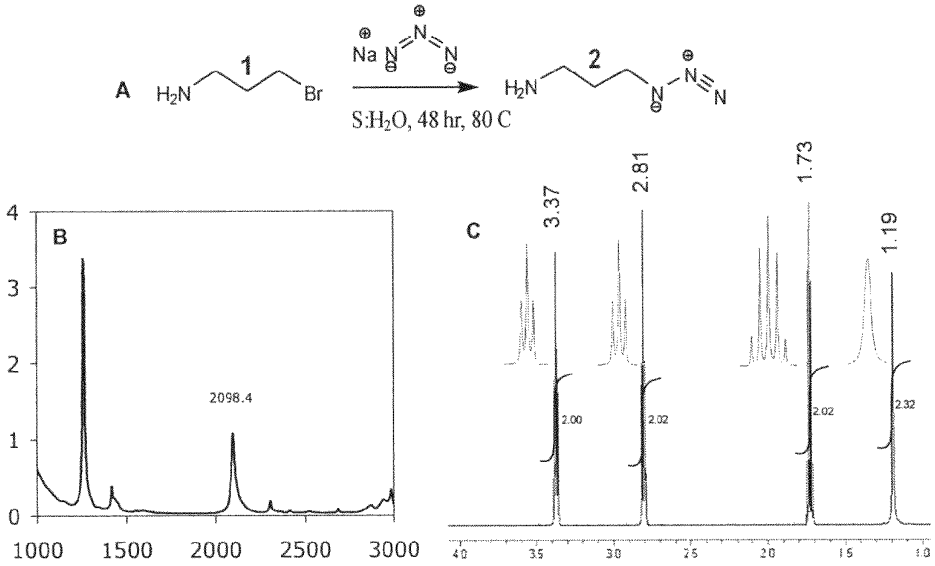
(52) **U.S. Cl.** ..... **514/44 R**; 536/23.1

(57) **ABSTRACT**

The present invention relates to aptamer conjugates comprising a metal chelating group and to methods of using these aptamers.




Figures 1A-1C

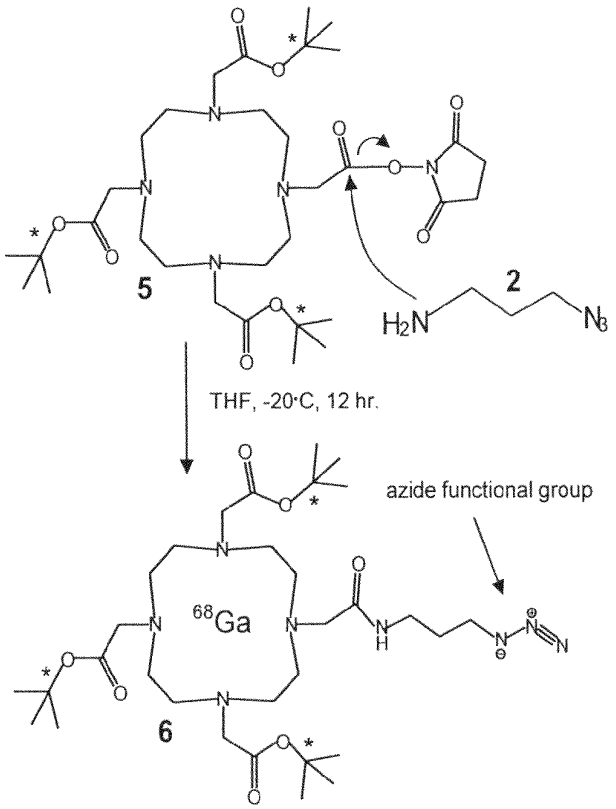



Figure 2

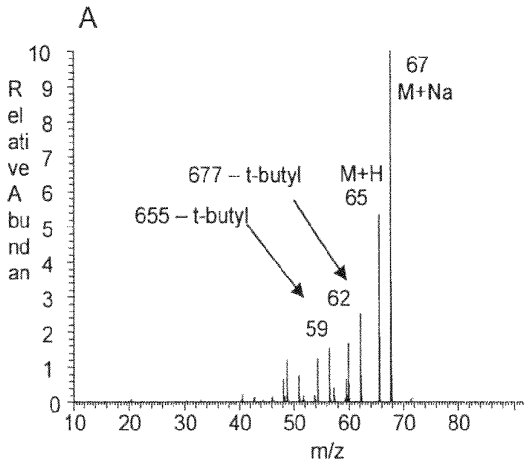



Fig. 3A

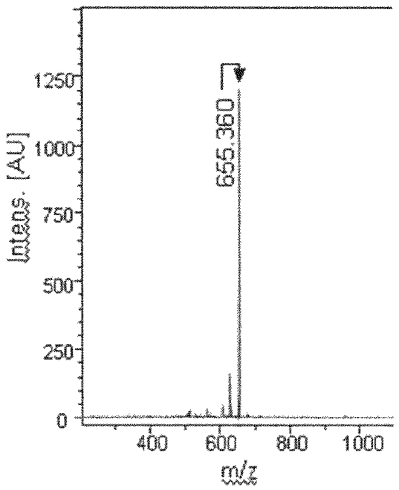



Fig. 3B

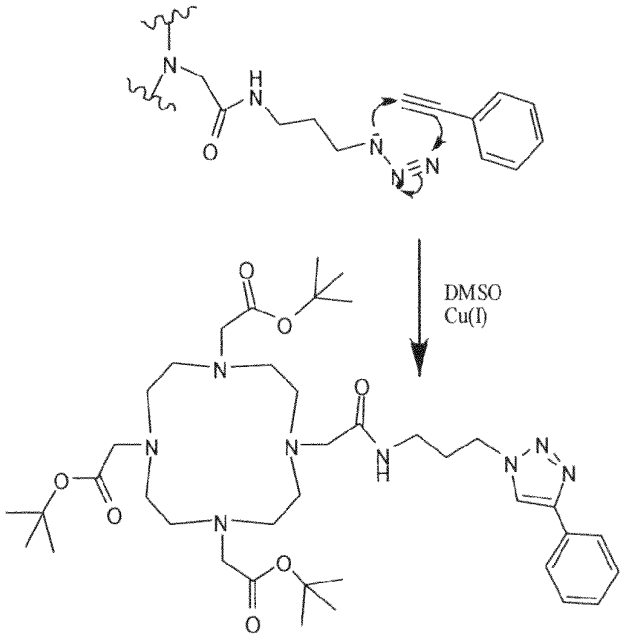



Figure 4

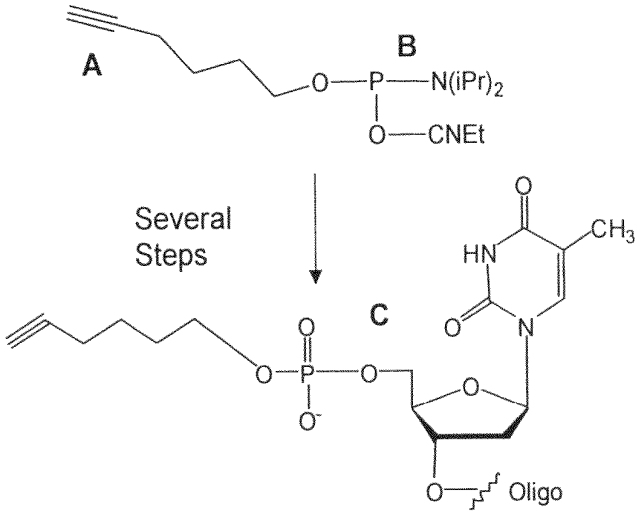



Figure 5

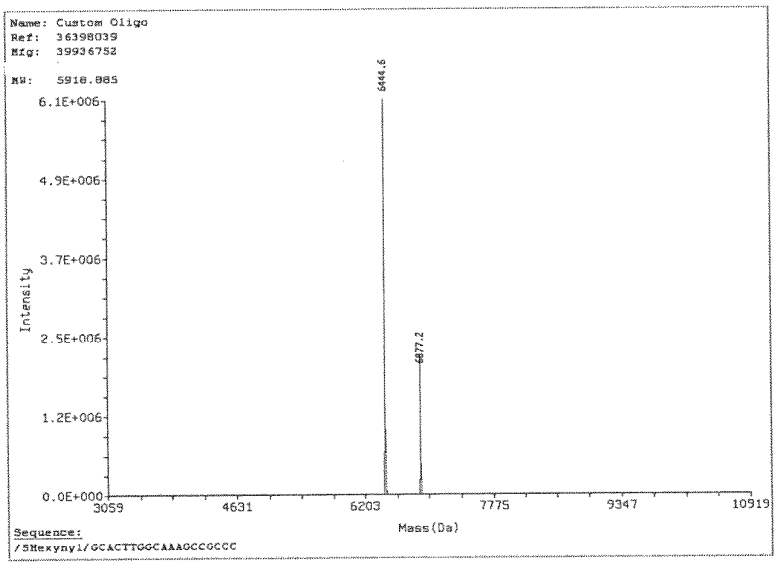



Figure 6

Fig. 7

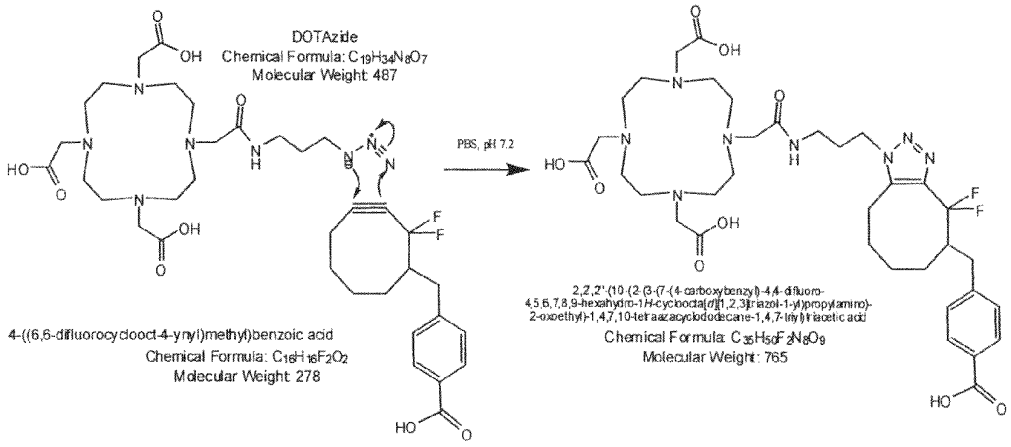
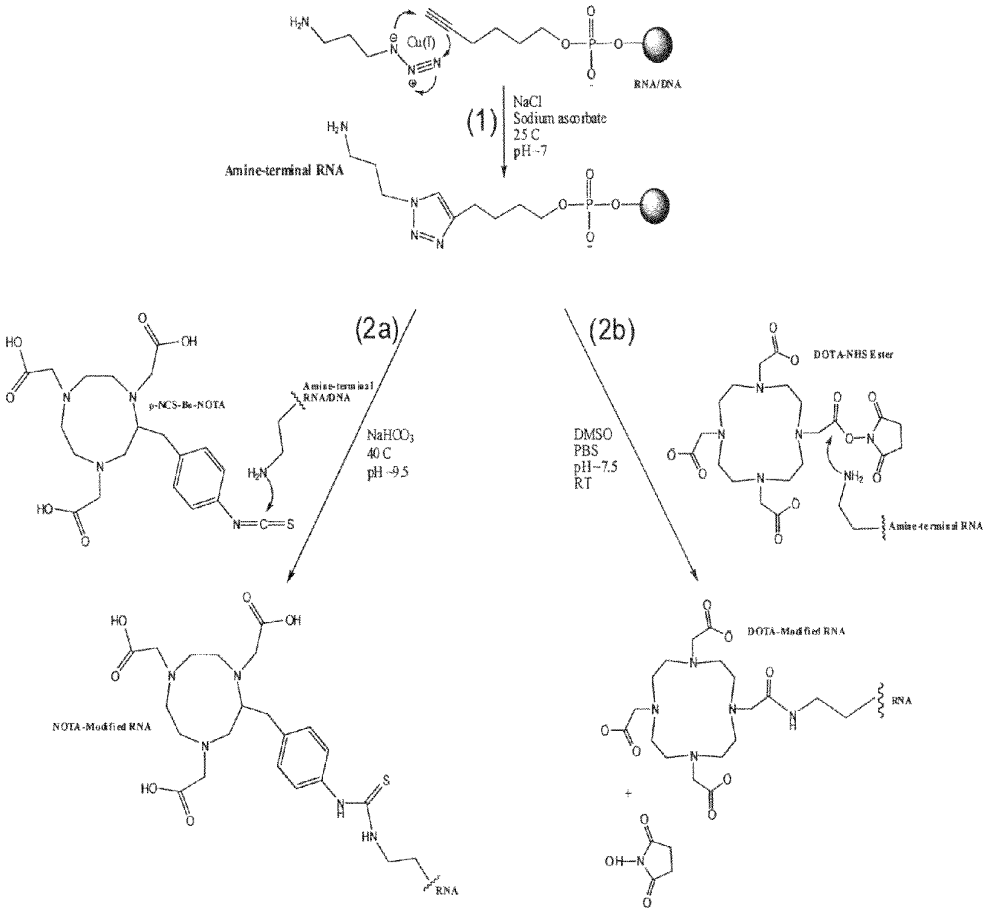

DOTAzide
Chemical Formula: C19H34N8O7
Molecular Weight: 487
PBS, pH 7.2
4-((6,6-difluorocyclooct-4-ynyl)methyl)benzoic acid
Chemical Formula: C16H16F2O2
Molecular Weight: 278
2,2',2''-(10-(2-(3-(7-(4-carboxybenzyl)-4,4-difluoro-4,5,6,7,8,9-hexahydro-1H-cycloocta[d][1,2,3]triazol-1-yl)propylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid
Chemical Formula: C35H50F2N8O9
Molecular Weight: 765

Fig. 8A


Cu(I)
RNA/DNA
(1)
NaCl
Sodium ascorbate
25 C
pH ~7
Amine-terminal RNA
(2a)
p-NCS-Bz-NOTA
Amine-terminal RNA/DNA
NaHCO3
40 C
pH ~9.5
NOTA-Modified RNA
(2b)
DMSO
PBS
pH~7.5
RT
DOTA-NHS Ester
Amine-terminal RNA
DOTA-Modified RNA
RNA

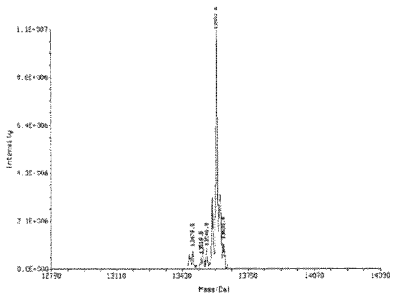


Fig. 8B

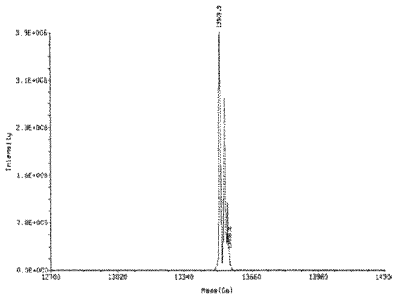


Fig. 8C

Fig.9A

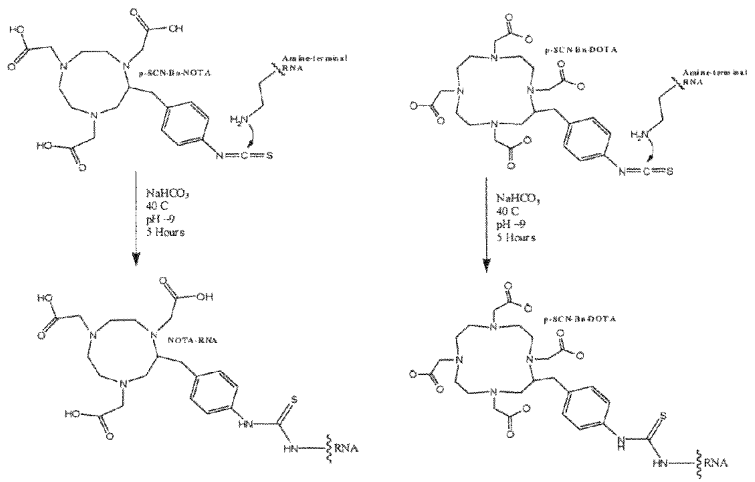



Fig. 9B

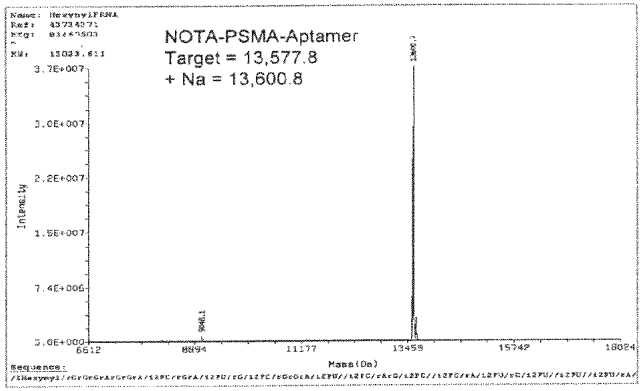
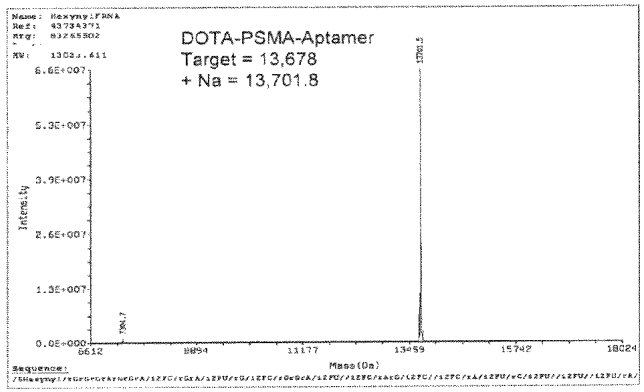
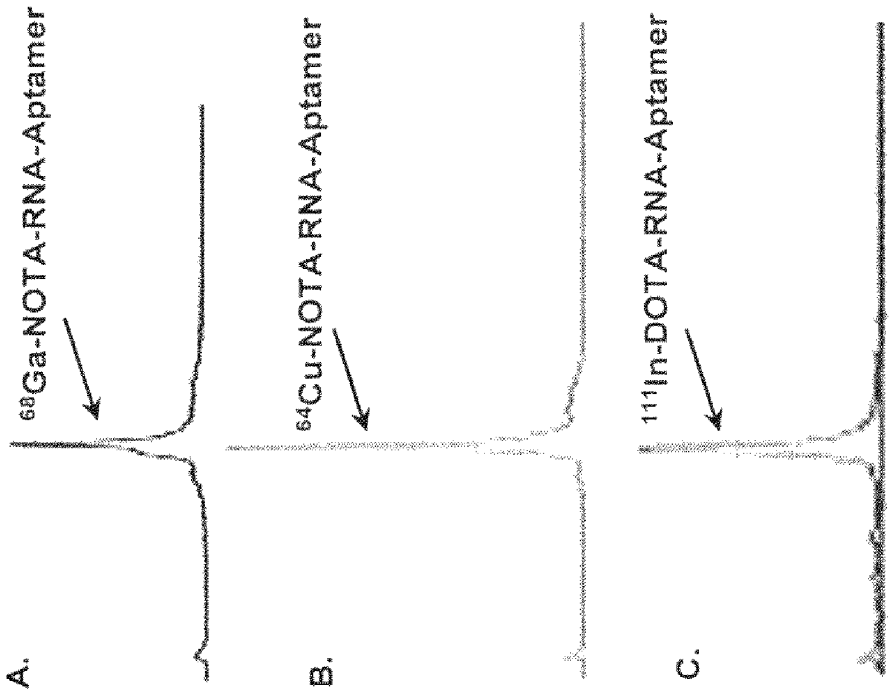
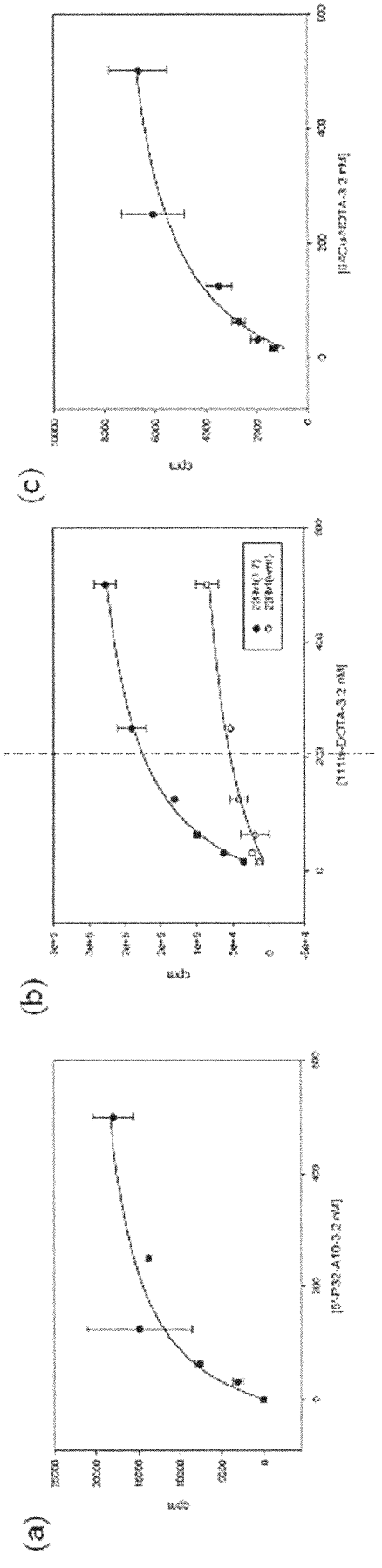



Fig. 9C




A.
$^{68}$Ga-NOTA-RNA-Aptamer
B.
$^{64}$Cu-NOTA-RNA-Aptamer
C.
$^{111}$In-DOTA-RNA-Aptamer

Figs. 10A-10C




Figs. 11A-11C

Figs. 12A

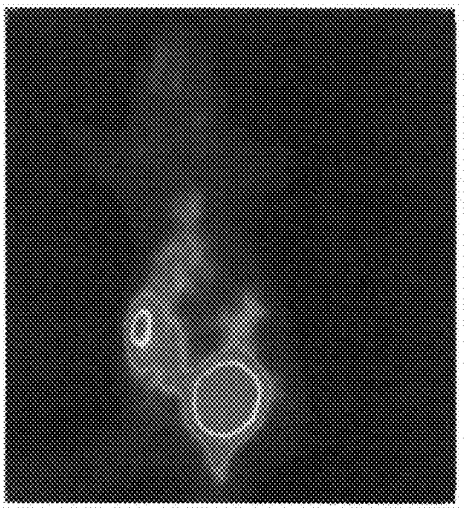
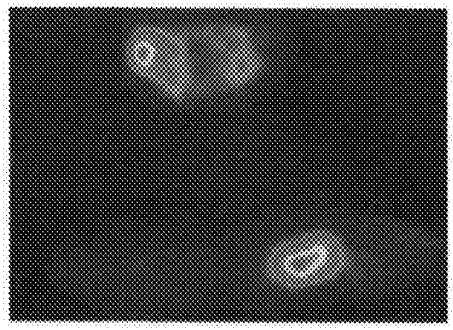
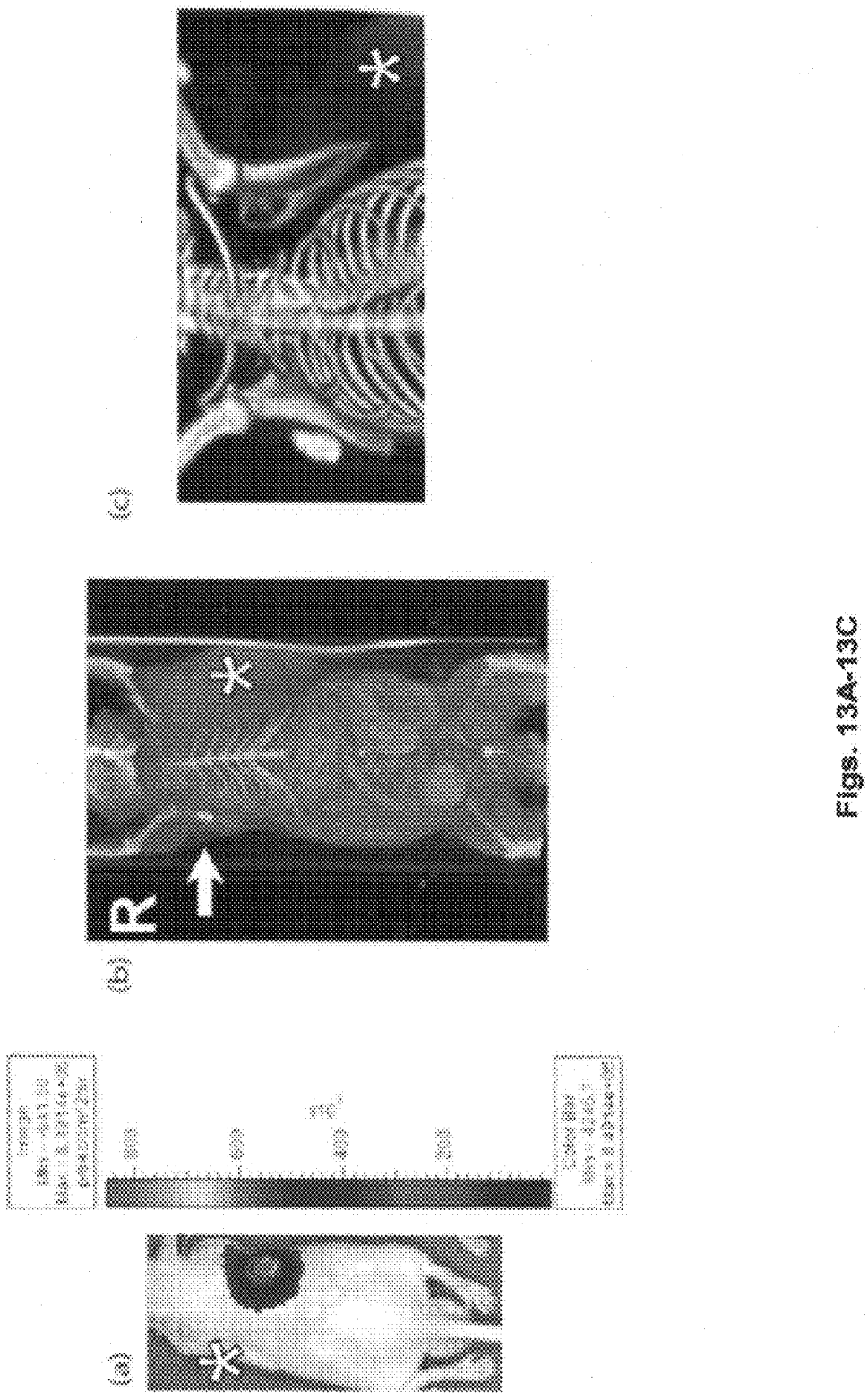


Fig. 12B






Figs. 13A-13C

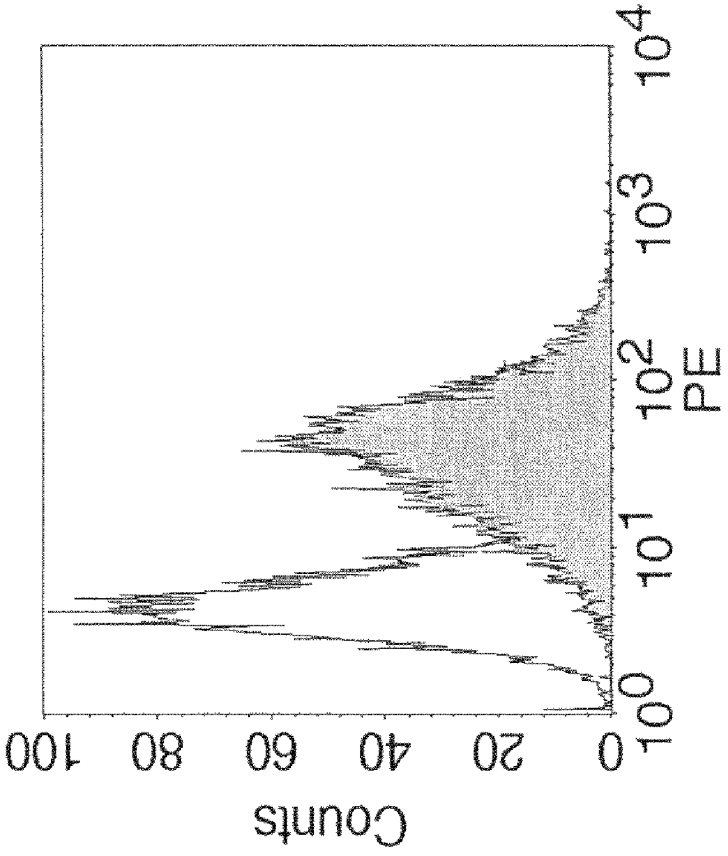



Figure 14

# MOLECULAR TARGETING AGENTS

## CROSS-REFERENCES TO RELATED APPLICATIONS

**[0001]** This application is a continuation-in-part of prior PCT Application No. PCT/US2009/053023 filed Aug. 6, 2009, which claims the benefit of U.S. Provisional Application No. 61/087,652 filed Aug. 9, 2008 and U.S. Provisional Application No. 61/155,288 filed Feb. 25, 2009.

## BACKGROUND OF THE INVENTION

**[0002]** Worldwide, cancer affects approximately 10 million people each year. Approximately 22 million people are living with cancer and almost 7 million people die worldwide from cancer each year. The most common cancers include cancers of the lung, breast, colon/rectum, stomach, liver, prostate, cervix, esophagus, and bladder. The elderly tend to be the highest population for new incidence, as more than 75% of all new cancer cases are diagnosed in people over the age of 60. With the aging population, incidence is expected to increase each year. Prostate cancer is the most common cancer in men and the second leading cause of cancer death in men, behind lung cancer. Approximately one in six men in the U.S. will contract prostate cancer in his lifetime. Approximately 80% of prostate cancers are diagnosed in men over 65 years of age, and, due to the lack of symptoms, 75% of first-time patients over 65 are diagnosed with advanced-stage prostate cancer. Worldwide, more than 680,000 men are diagnosed annually and over 29,000 men in the U.S. will die as a result of prostate cancer this year alone. Prostate cancer characteristically spreads to the bone. The therapeutic regimen for disease that is localized in the prostate bed (local disease) can be drastically different from the prescribed therapy for metastatic prostate cancer.

## SUMMARY OF THE INVENTION

**[0003]** Targeted molecular imaging and therapy provides precise delivery of diagnostic and therapeutic agents to the site of prostate cancer, which provides a critically-needed approach to improve outcomes for prostate cancer patients.

**[0004]** One embodiment the invention provides a conjugate of the invention, which is a ribonucleic acid (RNA) aptamer that is not more than 45 nucleotides in length comprising the nucleic acid sequence 5'-$n_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA-3' (SEQ ID NO:1) linked to one or more chelating groups, wherein each $n_x$ can be present or absent, wherein when present each $n_x$ represents any nucleotide, or a pharmaceutically acceptable salt thereof.

**[0005]** In one embodiment the invention provides a method for delivering a therapeutic metal or an imagable metal to a cell having a prostate specific membrane antigen (PMSA) receptor, comprising contacting the cell with a conjugate of the invention.

**[0006]** In one embodiment the invention provides a pharmaceutical composition comprising a conjugate of the invention and a pharmaceutically acceptable diluent or carrier.

**[0007]** In one embodiment the invention provides a method for treating a patient having cancer comprising administering a conjugate of the invention to the patient.

**[0008]** In one embodiment the invention provides a method for determining whether a patient has cancer (i.e., diagnosing a patient) comprising administering a conjugate of the invention that comprises an imagable metal to the patient and determining whether the patient has cancer. Imagable metals may be radionuclides for nuclear medicine imaging by positron emission tomography (PET) or single photon emission computed tomography (SPECT) or metals suitable for magnetic resonance imaging (MRI). These techniques may be combined with computed tomography to form dianostic imaging known as PET/CT, SPECT/CT, PET/MRI or other combination of imaging technique. Imaging techniques may be used for diagnosis or for measuring the response to therapy of any kind. For example, because certain conjugates of the invention are targeted to the prostate specific membrane antigen (PMSA) receptor and include an imagable metal, detection of a relatively higher level of the conjugate can be used to diagnose a patient as having prostate cancer or to determine the effectiveness of a therapeutic regimen of any type.

**[0009]** In one embodiment the invention also provides a conjugate of the invention for use in therapy.

**[0010]** In one embodiment the invention provides the use of a conjugate of the invention for treating cancer.

**[0011]** In one embodiment the invention provides a conjugate of the invention for use in the prophylactic or therapeutic treatment of cancer (e.g. a solid sarcoma, carcinoma, or prostate cancer).

**[0012]** The invention also provides in certain embodiments novel intermediates and processes disclosed herein that are useful for preparing conjugates of the invention synthetic processes described in the schemes and Examples herein.

**[0013]** In certain embodiments, the present invention provides a kit comprising the conjugate or the pharmaceutically acceptable salt thereof as described above, packaging material, and instructions for administering the conjugate or the pharmaceutically acceptable salt thereof to an animal to detect or treat prostate cancer. In certain embodiments, the kit further comprises an imageable metal or a therapeutic metal.

## BRIEF DESCRIPTION OF DRAWINGS

**[0014]** This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

**[0015]** FIGS. **1**A-**1**C Data confirming synthesis and purity of 1-amino-3-azidopropane (2): (A) Reaction of 1-amino-3-bromopropane (1) with sodium azide; (B) Infrared absorbance of 2098.4 $cm^{-1}$ (theoretical 2100 $cm^{-1}$) for the azide; (C) 1H NMR spectrum confirms structure of 1-amino-3-azido propane.

**[0016]** FIG. **2** illustrates a scheme for the preparation of DOTAzide. Synthesis of DOTAzide (6) from precursor DOTA-NHS (5) and 1-amino-3-azidopropane (2). Includes t-butyl ester protecting groups (*). Confirmed by ESI-MS and MALDI-TOF.

**[0017]** FIGS. **3**A-**3**B Confirmational analysis of the preparation of t-butyl protected DOTAzide (6): (A) Atomic mass determined by Electron Spray Ionization Mass Spectrometry=654.35; and (B) by LC-MS=655.36. Theoretical atomic mass 654.84. Minor peaks in A can be explained by ionization of t-butyl groups by analytical technique. No trace of starting material (5) could be detected using these techniques, indicating that the synthetic reaction proceeds to completion.

**[0018]** FIG. **4** illustrates a scheme for the preparation of DOTAzide conjugation to phenyl acetylene. DOTAzide conjugation to phenyl acetylene: (6) azide modified DOTAzide tether (DOTA ring not shown); (3) phenyl acetylene; and (7)

DOTAzide-phenyl acetylene conjugate showing triazole ring (*) and DOTA ring with t-butyl protecting groups and showing theoretical position of $^{68}$Ga. Molecular mass of construct (7) (without $^{68}$Ga) confirmed in our laboratory by MALDI-TOF at 758 and LC MS 756 (theoretical 757.7).

**[0019]** FIG. **5** illustrates the structure of the 5'-/5-hexynyl-phosphoramidite and 5'-/5-amino-phosphoramidite, bearing the terminal alkyne and amine functional groups that are available for conjugation to chelator groups. The alkyne function is reactive with azide functionalized chelator moieties such as DOTAzide by cycloaddition (click chemical reaction) to the azide functional group on the DOTAzide molecule. The 5'-amine terminus is reacted with amine-reactive functionalized chelators, such as isothiocyanato- or esterified chelators. The example shows the 5'-/5-hexynyl-phosphoramidite conjugated to the 5' end of a DNA oligo showing: (A) the alkyne functional group; (B) the reactive site on the phosphoramidite; and (C) phosphodiester linkage of post-synthetic 5' conjugation to DNA and RNA.

**[0020]** FIG. **6** is ESI-MS spectrum showing the mass peak for DOTA-conjugated 20mer DNA oligonucleotide sequence (SEQ ID NO:7), with an observed mass 6444.6 (theoretical 6442.9) and include Ca, and showing an impurity resulting from addition of tris-hydroxypropyltriazolyl copper stabilizer to the DOTA-conjugated oligo.

**[0021]** FIG. **7** shows the structure of difluoromethylene cyclooctyne modified phosphoramidite and Cu-catalyst-free click chemical reaction with DOTAzide.

**[0022]** FIGS. **8**A-**8**C: Schema of the preparations of DOTA- and NOTA-conjugated RNA aptamer A10-3.2. In this case, a two-step process was used starting with an alkyne modified RNA aptamer as starting material: (A) An amine terminal RNA (A1) was prepared by click chemical reaction of the 5'-alkyne modified RNA with 1-amino-3-azidopropane followed by desalting and careful removal of excess Cu using free DOTA; (B) NOTA-modified RNA (B1) was prepared by reacting the A1 with the p-NCS-Bn-NOTA; DOTA-modified RNA was prepared by reacting A1 with the active NHS ester of DOTA to afford B2; (C) Confirmation of the preparation of NOTA-RNA (C1, theoretical mass 13,510; observed 13,509. 5, 85% purity) and DOTA-RNA (C2, theoretical mass 13,13. 574.6; observed 13,575.5, 82% purity) by ESI mass spectrometry following HPLC purification. Soft ionization of ESI results in secondary peaks with mass values indicating association of sodium (Na) and small amounts of iron (Fe) with the macrocyclic rings. Na is not expected to interfere with radiolabeling, while Fe will need to be removed in subsequent preparations.

**[0023]** FIGS. **9**A-**9**C: Illustration of synthesis and confirmation of the preparation of NOTA and DOTA modified PSMA RNA aptamer by mass spectrometry. FIG. **9**A Schema of the preparations of DOTA- and NOTA-conjugated RNA aptamer A10-3.2 and FIGS. **9**B and **9**C confirmation by ESI mass spectrometry. In this case, an 5'-amine modified RNA aptamer is conjugated to p-SCN-Bn-NOTA and p-SCN-Bn-DOTA via an amine nucleophilic route in a single step. Purification is performed by dialysis spin filtration using a 10 k molecular weight cutoff filter. A 50 eq. excess of 2,2',2"-(2-(4-isothiocyanatobenzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (p-SCN-Bn-NOTA) (FIG. **9**B) or 2,2',2",2'"-(2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetate (p-SCN-Bn-DOTA) (FIG. **9**C) is added to a solution containing a 5'-amine modified PSMA-RNA aptamer in sodium bicarbonate at pH ~9 and reacted for 5 hours at 40° C., followed by purification by spin filter centrifugation (5×) in high purity water (molecular weight cutoff 10 kD), and confirmation of the product purity by electrospray ionization mass spectrometry.

**[0024]** FIGS. **10**A, **10**B, and **10**C: Demonstration of the radiochemical purity achievable by reacting imagable radiometals $^{64}$Cu (FIG. **10**B), $^{68}$Ga (FIG. **10**A) with a NOTA conjugated RNA aptamer and $^{111}$In (FIG. **10**C) with a DOTA conjugated RNA aptamer. For these representative figures, between 200 and 400 MBq of radioactivity was added to a buffer solution containing between 3.5 and 15 nmoles of NOTA- or DOTA-modified PSMA RNA aptamer and reacted for 15-60 minutes at 70° C. to 100° C. Final purification was performed by spin filter filtration to achieve radiochemical purity of >98%.

**[0025]** FIGS. **11**A-**11**C: Representative cell binding assays of the RNA aptamer radiolabeled with $^{111}$In and $^{64}$Cu when contacted with cells that express PSMA and cells that do not express PSMA. FIG. **11**A shows binding of the A10-3.2 aptamer labeled at the 5' end with 32P to 22Rv1(1.7) PSMA-positive prostate cancer cells with a KD of 106 nM. FIG. **11**B shows binding of 111In-DOTA-3.2 to 22Rv1(1.7) cells with a KD of 119 nM. The binding capacity (Bmax) to a PSMA negative cell line, 22Rv1(wmr), is much lower. FIG. **11**C shows binding of 64Cu-NOTA-3.2 to 22Rv1(1.7) cells with a KD of 131 nM. Addition of the chelator DOTA or NOTA to the A10-3.2 aptamer had minimal effect on its affinity for PSMA-expressing cells.

**[0026]** FIGS. **12**A and **12**B: PET imaging study of the biodistribution of [$^{68}$Ga]-NOTA-PSMA-Aptamer in a xenograft (subcutaneous, 22RV1 right flank) nude mouse model of prostate cancer (injected dose: 422 μCi (16 MBq) in 50 μL sterile isotonic saline, specific activity 11 MBq nmole$^{-1}$. A static image was acquired at 120 minutes, indicating a accumulation in the right flank tumor in this coronal slice with excellent tumor:background (8:1) conspicuity. Transaxial and Sagittal slices are at the center of the maximum intensity of the tumor indicated in the coronal slice of the image shown.

**[0027]** FIGS. **13**A-**13**C: These figures demonstrate the in vivo affinity and specificity of the 111In-DOTA-PSMA-RNA aptamer to a PSMA-expressing prostate cancer tumor. Virtually no accumulation of the radiolabeled aptamer was observed in a xenograft PSMA-negative tumor, demonstrating specificity for PSMA. FIG. **13**A shows a bioluminescent imaging (BLI) experiment to confirm the presence of the PSMA-positive xenograft, which also expresses luciferase. The PSMA-positive tumor is on the animal's right side, while a PSMA-negative xenograft is on the animal's left side (asterisk). The animal is in the prone position. FIG. **13**B shows a fusion SPECT/CT image taken at 24 hours post injection of 1 mCi of 111In-DOTA-PSMA-RNA aptamer with the PSMA-positive tumor marked by the white arrow. The PSMA-negative tumor on the left of the animal is marked by an asterisk. The PSMA-positive tumor measured 3×3 mm, while the PSMA-negative tumor was much larger at approximately 1×2 cm. FIG. **13**C shows a close-up of a computerized reconstruction of the SPECT/CT image, clearly showing that our agent binds the PSMA-positive tumor on the right, but not the PSMA-negative tumor on the left (asterisk).

**[0028]** FIG. **14**. Flow cytometry was used to confirm expression of PSMA in the 22Rv1(1.7) cells used for in vitro binding experiments and in vivo xenograft experiments. The population of cells labeled with a fluorescent PE-conjugated

primary antibody against PSMA is shown in gray, compared with the population of unlabeled cells (unshaded).

## DETAILED DESCRIPTION OF THE INVENTION

**[0029]** The chemical structure of the invention can be described by conventions used commonly in the field of use as a molecular targeting agent that is composed of four parts: (1) an aptamer portion that is designed to bind to a cell surface receptor (e.g., PSMA); (2) a metal chelator that is designed to bind metals used for imaging and therapy (e.g., $^{68}$Ga for PET imaging, or stable Gd for MRI); (3) a linker that is a chemical entity that is used to connect the chelator and aptamer portions of the invention together; and (4) a radiometal or stable metal that is used for imaging or therapy using the aptamer as the targeting vector in vivo. The linker not only connects the chelator and aptamer portions together, but also can be used to optimize the in vivo characteristics of the entire molecular targeting agent (e.g, biodistribution and pharmacokinetics).

**[0030]** Molecular targeting refers to the development of ligands that are engineered to bind to a specific molecular target, e.g., a cell-surface receptor. Examples of targeting mechanisms (often referred to as "targeting vectors" or "targeting mechanisms") include monoclonal antibodies (mAbs), peptides, antisense DNA oligonucleotides (oligos), and more recently RNA aptamers (aptamers) and peptide-nucleic acid hybrids (PNAs). For in vivo imaging using metals and radiometals and for in vivo targeted radionuclide therapy applications, the addition of a chelator moiety to the targeting mechanisms results in a "bi-functional" ligand that enables the molecular targeting mechanism to bind a contrast-agent metal (e.g., gadolinium for magnetic resonance imaging, MRI) or radionuclide (e.g., gallium-68 (68Ga) for positron emission tomography, PET), or in some cases by connecting a light active moiety to the targeting vector for optical imaging or photodynamic therapy, while maintaining high affinity for the in vivo molecular target. A similar approach has been employed for specific targeting of therapeutic radionuclides (e.g., alpha and beta emitters) to the site of cancerous tissue, while sparing healthy cells in vivo. To be effective, a chelator-modified bifunctional ligand (bioconjugate) must maintain affinity for the molecular target, while also affording a highly stable metal-chelating complex with the contrast-agent metal or radionuclide.

**[0031]** One example of an effective chelator, which has demonstrated effectiveness in formation of extremely stable metal-chelator complexes, particularly with tri-valent metals and radionuclides, is the well-studied metal chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). DOTA derivatives have been synthesized for conjugation to mAbs and peptides. Recent developments of DOTA derivatives involve approaches for conjugation of peptides for radionuclide imaging and therapy in nuclear medicine. DOTA-modified peptides can be synthesized in solution or on a solid support, attaching the DOTA residue to a free amine or at a carboxy terminus of the resin-bound peptide. The usual synthetic approach involves using electrophilic/nucleophilic reactions with protected DOTA derivatives to prevent side reactions with the activated carboxylic acid groups of the DOTA ring. Examples of useful DOTA derivatives employed for these applications include protected and unprotected derivatives of the DOTA-tris-t-butyl ester, and the isothiocyanate functionalized p-NCS-Bz-DOTA. In alternative approaches, DOTA-derivatized amino acids or N-terminus reactions have been used to add the DOTA moiety at the conclusion of automated peptide synthesis. Because the active esters and other reactive chelator precursors described above are highly reactive (particularly in water), these reactions are generally carried out in organic solvents and the active precursors must be dissolved immediately before use to ensure that the precursors are chemically-available in the reactive form at the time of introduction of the targeting vector to which they are to be attached. Other examples of chelators that are useful for binding radiometals and metals for imaging and therapy include: NOTA and TETA.

**[0032]** A particularly promising new class of targeting mechanisms is modified ribonucleic acids (RNAs) that are selected to bind with high affinity to specific cell surface receptors that are overexpressed or amplified in specific cancer cell types in vivo. These RNAs are commonly referred to as aptamers and represent a new class of molecular targeting mechanism with ideal characteristics for delivery of radionuclides and other stable contrast metals for imaging and therapy of cancer and other diseases. However, facile post-synthesis modifications that include a chelator moiety are required to produce aptamers with bifunctional capability. The addition of a chelator can provide bifunctional aptamer-radionuclide bioconjugates, which are not only useful to enable further study of molecular targets and cellular pathways, but also have translational value as clinically viable molecular imaging agents for diagnosing and confirming diagnosis of disease, measuring response to therapy to evaluate therapeutic approaches, and monitoring of disease progression for treatment planning. Thus, preparation of bioconjugate bifunctional aptamers can lead to the development of radiopharmaceuticals for human use. Ideally, the chemical conditions for preparation of chelator-aptamer bioconjugates will be relatively mild, be possible in aqueous and mild organic solvent conditions and, importantly, be highly regioselective in character of conjugation reaction, such that side reactions can be circumvented to rapidly produce highly pure bifunctional ligands.

**[0033]** An embodiment of the invention described herein is an optimized RNA-based therapeutic reagent for the detection or treatment of prostate and other solid sarcomas and carcinomas. This reagent comprises four basic components, an RNA aptamer (a structural, synthetic RNA); a metal chelating group; a chemical linker to connect the chelator and RNA aptamer; and a radionuclide, stable metal that is used to image the location of the bioconjugate in vivo. In certain embodiments, the chelator can be a light active species that can be used for optical imaging, or photodynamic therapy of cancer. The aptamer portion of the reagent serves as a targeting moiety by binding specifically to a cell surface receptor (e.g., prostate specific membrane antigen; PSMA) expressed on cancer cells (e.g., prostate cancer cells).

### Aptamer Portion

**[0034]** Aptamers are single stranded oligonucleotides that can naturally fold into different 3-dimensional structures, which have the capability of binding specifically to biosurfaces, a target compound or a moiety. The term "conformational change" refers to the process by which a nucleic acid, such as an aptamer, adopts a different secondary or tertiary structure. The term "fold" may be substituted for conformational change.

**[0035]** Aptamers can be used as molecular targeting agents with applications in imaging and treatment of disease. Post-synthetic modifications can be employed to optimize the in

vivo stability and pharmacokinetics of these molecular constructs. One particularly attractive innovation is the introduction of oligonucleotides and RNA aptamers with post-synthetic modifications that enable radiolabeling of the targeting mechanisms for in vivo imaging by positron emission tomography (PET) and single photon emission computer tomography (SPECT). The same or similar molecular modifications can be made to enable radionuclide therapy, by employing a therapeutic radionuclide such as $^{90}Y$, $^{213}Bi$, $^{177}Lu$, $^{211}At$, $^{210}Po$, $^{223}Ra$, or other suitable radionuclide and using the RNA aptamer as the targeting vector in the same manner as for imaging applications.

**[0036]** Aptamers have advantages over more traditional affinity molecules such as antibodies in that they are very stable, can be easily synthesized, and can be chemically manipulated with relative ease. Aptamer synthesis is potentially far cheaper and reproducible than antibody-based diagnostic tests. Aptamers can be produced by solid phase chemical synthesis, an accurate and reproducible process with consistency among production batches. An aptamer can be produced in large quantities by polymerase chain reaction (PCR) and once the sequence is known, can be assembled from individual naturally-occurring nucleotides and/or synthetic nucleotides. Aptamers are typically stable to long-term storage at room temperature, and, if denatured, aptamers can easily be renatured, a feature not shared by antibodies. Furthermore, aptamers have the potential to measure concentrations of ligand in orders of magnitude lower (parts per trillion or even quadrillion) than those antibody-based diagnostic tests. These characteristics of aptamers make them attractive for diagnostic applications.

**[0037]** Aptamers are typically oligonucleotides that may be single stranded oligodeoxynucleotides, oligoribonucleotides, or modified oligodeoxynucleotide or oligoribonucleotides. The term "modified" encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleotides may also include 2' substituted sugars such as 2'-O-methyl-; 2-O-alkyl; 2-O-allyl; 2'-S-alkyl; 2'-S-allyl; 2'-fluoro-; 2'-halo or 2-azido-ribose, carbocyclic sugar analogues a-anomeric sugars; epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

**[0038]** Modified nucleotides are known in the art and include, by example and not by way of limitation, alkylated purines and/or pyrimidines; acylated purines and/or pyrimidines; or other heterocycles. These classes of pyrimidines and purines are known in the art and include, pseudoisocytosine; N4, N4-ethanocytosine; 8-hydroxy-N6-methyladenine; 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5-carboxymethylaminomethyl uracil; dihydrouracil; inosine; N6-isopentyl-adenine; 1-methyladenine; 1-methylpseudouracil; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; 3-methylcytosine; 5-methylcytosine; N6-methyladenine; 7-methylguanine; 5-methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; β-D-mannosylqueosine; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; 2-methylthio-N-6-isopentenyladenine; uracil-5-oxyacetic acid methyl ester; psueouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5-oxyacetic acid; queosine; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; and 2,6,-diaminopurine; methylpsuedouracil; 1-methylguanine; 1-methylcytosine.

**[0039]** The aptamers of the invention are synthesized using conventional phosphodiester linked nucleotides and synthesized using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may use alternative linking molecules. For example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'2; P(O)R'; P(O)OR6; CO; or CONR'2 wherein R is H (or a salt) or alkyl (1-12C) and R6 is alkyl (1-9C) is joined to adjacent nucleotides through —O— or —S—.

**[0040]** In certain embodiments of the present invention, the aptamer portion binds to Prostate-Specific Mediated Antigen (PSMA). A PSMA aptamer of 71 nucleotides (A10-Plk1) (Lupold et al., Cancer Res. 62(14):4029-33 (2002)) and a PSMA aptamer of 39 nucleotides (A10-3.2) have effective binding activity. In certain embodiments, additional modifications are made to the aptamer portion. Additional modifications to the aptamer portion include 2'O-methyl modification of the pyrimidines. In other embodiments, all of the nucleotides in the aptamer are 2'O-methyl modified. Alternatively, the pyrimidines, or all the nucleotides, may be modified with 2' fluoros (both pyrimidines and purines). Additional modifications to the nucleotides in the aptamer include large molecular weight conjugates like pegylation, lipid-based modifications (e.g., cholesterol) or nanoparticles (e.g., PEI or chitosan) to improve the pharmacokinetic/dynamic profile of the chimera.

**[0041]** Prostate-specific membrane antigen (PSMA) is a type-II transmembrane protein that comprises an intracellular part, a transmembrane part, and an extracellular domain that is expressed extracellularly on prostate cancer cells (and other solid tumors, such as renal cancer cells) and the endothelial cells of new blood vessels that supply most other solid tumors. However, it has also been shown to be present at low levels in the brain, kidneys (brush border of proximal tubes) and liver. One advantage of targeting PSMA is that it is a transmembrane protein, and is not secreted. The truncated PSMA aptamer can be used as a tool to target prostate cancer as well as the vasculature of all solid sarcomas and carcinomas. It has been previously shown that PSMA expression is elevated in malignant prostate disease as well as tumor vasculature.

**[0042]** In certain embodiments, modifications are introduced into the stem sequence in the aptamer. Different nucleotides can be used as long as the structure of the stem is retained.

**[0043]** Certain embodiments of the invention provide a conjugate of the invention, which is a conjugate comprising a nucleic acid aptamer that is not more than 45 nucleotides in length comprising the nucleic acid sequence 5'-$n_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA-3' (SEQ ID NO:1), linked to one or more chelating groups, wherein each $n_x$ can be present or absent, wherein when present each $n_x$ represents any nucleotide, or a pharmaceutically acceptable salt thereof. In certain embodiments, each of the $n_x$ nucleotides can be present or absent. In certain embodiments, the nucleic acid molecule includes a sufficient number of $n_x$ nucleotides so as to form the first, second and/or third stem structures. In certain embodiments the nucleic acid molecule is not more than

45 nucleotides in length, e.g., from 15-45 nucleotides in length, e.g., 39 nucleotides in length.

**[0044]** In certain embodiments nucleotides, $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a stem structure.

**[0045]** In certain embodiments, the nucleic acid molecule includes the nucleic acid sequence 5'-AUGCGGAUCAGC-CAUGUUUA-3' (SEQ ID NO:2).

**[0046]** In certain embodiments, the nucleic acid molecule includes the nucleic acid sequence 5'-$n_an_bn_cn_dn_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA$n_en_fn_gn_h$-3'(SEQ ID NO:3).

**[0047]** In certain embodiments, nucleotides $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a first stem structure and nucleotides $n_an_bn_cn_d$ and $n_en_fn_gn_h$ are present and hybridize to form a second stem structure.

**[0048]** In certain embodiments, the nucleic acid molecule includes the nucleic acid sequence 5'-GACGAUGCG-GAUCAGCCAUGUUUACGUC-3' (SEQ ID NO:4).

**[0049]** In certain embodiments, the nucleic acid molecule includes the nucleic acid sequence 5'-$n_{10}n_{11}n_{12}n_{13}n_{10}n_an_bn_cn_dn_1n_2n_3$CGGAUCAGC$n_4n_5n_6$G UUUA$n_en_fn_gn_hn_{15}n_{16}n_{17}n_{18}n_{19}$ $n_{20}n_{21}$-3' (SEQ ID NO:6). In certain embodiments, $n_{21}$ is U. In certain embodiments, $n_{21}$ is absent.

**[0050]** In certain embodiments, nucleotides $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a first stem structure, nucleotides $n_an_bn_cn_d$ and $n_en_fn_gn_h$ are present and hybridize to form a second stem structure, and nucleotides $n_{10}n_{11}n_{12}n_{13}n_{14}$ and $n_{15}n_{16}n_{17}n_{18}n_{19}$ are present and hybridize to form a third stem structure.

**[0051]** In certain embodiments, the nucleic acid molecule includes the nucleic acid sequence 5'-GGGAGGAC-GAUGCGGAUCAGCCAUGUUUACGUCACUCCU-3' (SEQ ID NO:5).

**[0052]** In certain embodiments, the nucleic acid molecule consists essentially of the nucleic acid sequence 5'-GGGAG-GACGAUGCGGAUCAGCCAUGUUUACGUCACUCCU-3' (SEQ ID NO:5).

**[0053]** In certain embodiments, the nucleic acid molecule consists of the nucleic acid sequence 5'-GGGAGGAC-GAUGCGGAUCAGCCAUGUUUACGUCACUCCU-3' (SEQ ID NO:5).

**[0054]** In certain embodiments, a molecular entity (such as polyethylene glycol PEG) is conjugated to the RNA at the 5'-end, the 3' end or at any other nucleic acid constituent on the aptamer. The molecular extension is added to confer some in vivo behavioral characteristic that improves the performance of the molecular targeting agent comprised by the chelator, linker, and aptamer. Examples of extensions include PEG groups with molecular weights ranging from 200 Da to 50,000 Da. Other possible extensions include the addition of peptide extensions comprised of amino acids, or other molecular structures that are designed to confer specific in vivo behavior.

**[0055]** In certain embodiments, the molecular extension is conjugated to the chelator that has been connected to the aptamers by the linker.

Metal Chelating Group

**[0056]** The disclosed compounds can be prepared using reagents that are readily available. A general procedure for the preparation of a polyamine polycarboxylic acid derivatives such as DOTA can be illustrated by preparation of 1,4,7,10, 13,16-hexaazacyclohexadecane-N,N',N'', N''', N'''', N''''', N''''''-hexaacetic acid derivatives in mg quantities as described in Deal et al., *J. Med. Chem.,* 1999, 42, 2988-2992. The hexaester 1,4,7,10,13,16-hexakis(tert-butoxycarbonyl-methyl)-1,4,7,10,13,16-hexaazacyclootadecane is prepared by reacting the free base ([18]aneN6 500 mg, 1 eq) in 25 mL anhydrous acetonitrile with the addition of 2 g sodium carbonate (10 eq) and 2.3 mL (8 eq) tert-butyl bromoacetate. The reaction is complete in about 5-10 days under inert atmosphere at room temperature. The desired product is purified by chromatographic means and solvents evaporated. The active ester (with a single useful leaving group) that can be used for conjugation to various molecular linkers (such as in the preparation of the DOTAzide compound) is then prepared by reacting the fully carboxylated species with 1 eq. N-hydroxy succinimide in dicyclohexyl carbodiimide (DCC) to afford an N-hydroxysuccinimide active ester, followed by purification by chromatographic methods, evaporation of solvents, and storage for use.

Linking Groups and Linkers

**[0057]** Chemistries that can be used to link the aptamers and metal chelating groups are known in the art, such as disulfide linkages, amino linkages, covalent linkages, etc. Additional linkages and modifications can be found on the world-wide-web at trilinkbiotech.com/products/oligo/oligo_modifications.asp. In certain embodiments, the present invention provides conjugates that comprise a nucleic acid molecule is operably linked to one or more metal chelating groups either directly (e.g., through a covalent bond) or through a linking group (i.e., a linker). The nature of the linker can be important, not only as a means to connect the chelator to the targeting vector, but also as a means to modify the pharmacokinetics and biodistribution of the bifunctional bioconjugate. It is also critical that the linker not interfere with the ability of the conjugate compound to function for its intended use, e.g., as a therapeutic or imaging agent. The metal chelating group or the linker can be linked to the compound at any synthetically feasible position on the compound that does not interfere with binding of the bioconjugate to the molecular target (e.g., PSMA).

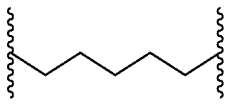
**[0058]** In the present invention, a linker is a molecular tether that connects the chelator group to the targeting mechanism or small molecule through any number of functional group combinations that afford facile conjugation chemistry. In certain embodiments, the linker connects an azide or alkynyl or cyclooctyne functional group to a macrocyclic polyamino ring or other molecular chelator entity such as ethylene diaamine tetraacetic acid that is intended to complex strongly a radioactive or stable metal for the purpose of delivery of the bound radionuclide or stable metal in vivo for imaging or therapy of disease, so as to reveal expression of a protein. In certain embodiments, the linker connects an azide or alkynyl functional group to a nucleic acid molecule. The azide functional group at the terminus of the linker is intended for applications in conjugation of the molecular construct described above to an alkyne-functionalized molecule, such as a peptide, oligonucleotide, or ribonucleic acid aptamer, or peptide-aptamer construct (PNA) by click chemical techniques as described in Sharpless et al., *Angew. Chem. Int. Ed.* 2001, 40, 2004-2021. In certain embodiments, the linker incorporates a primary amine, activated carboxy ester, or other reactive group, that can be used to conjugate the chelator or small molecule to the targeting vector for delivery of a radionuclide or other small molecule for imaging or therapy.

The conjugate is intended to deliver, e.g., via injection, the complete pharmacologic agent (including a radioactive or stable metal, chelator and targeting mechanism as a single combined-complete pharmaceutical agent) into the body of a mammal, e.g., human or other animal the radioactive or stable metals described above to a specific protein, mRNA, cell surface receptor, integrin, or other cellular or physiologically derived molecular construct or potentially some other molecular construct.

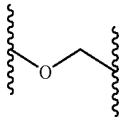
**[0059]** In certain embodiments, the linker is connected to the macrocyclic ring or linear chelator through an amide, thiourea bond, conjugation through Staudinger ligation, or any number of other conjugation strategies known in the art and is useful as molecular tethers designed to modify the pharmacokinetics and biodistribution of the final molecular composition of matter for the purpose of optimizing specific delivery of the radioactive metal or stable metal to a specific physiological location (such as a specific cancerous tumor or the location of volatile plaque in veins or arteries or blood vessels of the mammal, e.g., human (or in an animal) for research, imaging, therapy or other medically or research related reason, such as is described in Smith-Jones et al., Nuclear Medicine and Biology, 24:761-769, 1997 and Garrison et al., *Bioconjugate Chemistry*, September; 19(9):1803-12 (2008, Epub Aug. 20, 2008).

**[0060]** In certain embodiments, the linker includes (1) a repeating polyethylene glycol (PEG) tether of molecular weight from approximately 200 to 20,000 daltons; (2) an alkyl repeating tether of the form $(—CH_2—)_n$ where n can vary from 2 to about 1200; (3) an aromatic insertion, for example a benzyl group with appropriate functional terminus to allow for effective conjugation to the azide and the secondary amine on the macrocyclic ring; (4) an amino acid, deoxyribonucleic and ribonucleic acid or other insertion such as a glycine residue and (5) any combination of aromatic, alkyl, PEG, and amino acid or nucleic acid groups that constitutes an adequately stable or engineered cleavable sequence that is used to connect the chelator to the azide group for conjugation to a molecular targeting mechanism such as an RNA aptamer.

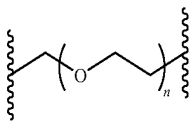
**[0061]** Exemplary linking groups include:



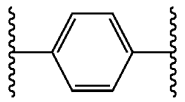
**[0062]** A: alkylene linkers



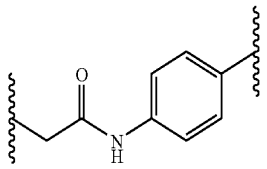

**[0063]** B: ether or ether/alkylene linkers



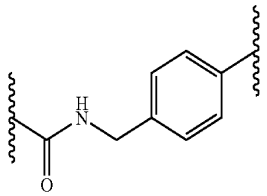

**[0064]** C: polyethylene glycol linkers



**[0065]** D: aromatic linkers




**[0066]** E: glycine/aromatic linkers




**[0067]** F: glycine/alkyl/aromatic linkers

**[0068]** and combinations of A-F or repeating units of A-F. Linkers can also consist of peptide-like amino acid sequences, PNA sequences in combination with or in addition to the specific linker sequences shown here.

**[0069]** In certain embodiments, the linking group includes an alkylene that can have from 2 to about 1200 carbon atoms, such as from 2 to about 50 carbon atoms, from 2 to about 20 carbon atoms, or even from about 2 to 5 carbon atoms. In certain embodiments, the linker comprises an alkylene or alkenylene group having or $(C_6\text{-}C_{10})$aryl; where the alkylene or alkenylene groups can be optionally interrupted by one or more heteroatom groups or an aromatic rings; where the heteroatom groups include $—NR^5—$, —O—, or —S—; each $R^5$ is hydrogen, $(C_1\text{-}C_6)$alkyl, $(C_6\text{-}C_{10})$aryl, substituted aryl, or absent; an amino acid, peptide, deoxyribonucleic or ribonucleic acid residue; or any combination of two or more of (a), (b), or (c); each X is independently $—(CH_2)_m—NR^1—$; n is 0, 1, 2, or 3; m is 2, or 3; each R' is independently $—(CH_2)_i—COOR^2$, where i is 0, 1, or 2; each $R^2$ is independently, hydrogen, or a protecting group for carboxyl groups; and each j is independently 1 or 2; or a pharmaceutically acceptable salt thereof.

**[0070]** The carbon atom content of various hydrocarbon-containing moieties is indicated by a prefix designating a lower and upper number of carbon atoms in the moiety, i.e., the prefix $C_{i\text{-}j}$ indicates a moiety of the integer "i" to the integer "j" carbon atoms, inclusive. Thus, for example, $C_{1\text{-}7}$alkyl refers to alkyl of one to seven carbon atoms, inclusive.

**[0071]** Specifically, $(C_1\text{-}C_6)$alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, 3-pentyl, or hexyl; $(C_3\text{-}C_6)$cycloalkyl can be cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl; $(C_3\text{-}C_6)$cycloalkyl$(C_1\text{-}C_6)$alkyl can be cyclopropylmethyl, cyclobutylmethyl, cyclopentylmethyl, cyclohexylmethyl, 2-cyclopropylethyl, 2-cyclobutyl-

ethyl, 2-cyclopentylethyl, or 2-cyclohexylethyl; ($C_1$-$C_6$) alkoxy can be methoxy, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, pentoxy, 3-pentoxy, or hexyloxy; $C_2$-$C_{20}$ alkenyl, can be an olefinically unsaturated branched or linear group having from two to twenty carbon atoms and at least one double bond. Typically, $C_2$-$C_{20}$ alkenyl groups include, but are not limited to, vinyl, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-butenyl, 2-butenyl, 1-pentenyl, 2-pentenyl, 1-hexenyl, 2-hexenyl, heptenyl, octenyl and the like; ($C_2$-$C_6$) alkynyl can be ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 1-hexynyl, 2-hexynyl, heptynyl, octynyl and the like; ($C_1$-$C_6$)alkanoyl can be acetyl, propanoyl or butanoyl; halo($C_1$-$C_6$)alkyl can be iodomethyl, bromomethyl, chloromethyl, fluoromethyl, trifluoromethyl, 2-chloroethyl, 2-fluoroethyl, 2,2,2-trifluoroethyl, or pentafluoroethyl; hydroxy($C_1$-$C_6$)alkyl can be hydroxymethyl, 1-hydroxyethyl, 2-hydroxyethyl, 1-hydroxypropyl, 2-hydroxypropyl, 3-hydroxypropyl, 1-hydroxybutyl, 4-hydroxybutyl, 1-hydroxypentyl, 5-hydroxypentyl, 1-hydroxyhexyl, or 6-hydroxyhexyl; ($C_1$-$C_6$)alkoxycarbonyl can be methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, isopropoxycarbonyl, butoxycarbonyl, pentoxycarbonyl, or hexyloxycarbonyl; ($C_1$-$C_6$)alkylthio can be methylthio, ethylthio, propylthio, isopropylthio, butylthio, isobutylthio, pentylthio, or hexylthio; ($C_2$-$C_6$)alkanoyloxy can be acetoxy, propanoyloxy, butanoyloxy, isobutanoyloxy, pentanoyloxy, or hexanoyloxy; aryl can be phenyl, indenyl, or naphthyl; and heteroaryl can be furyl, imidazolyl, triazolyl, triazinyl, oxazoyl, isoxazoyl, thiazolyl, isothiazoyl, pyrazolyl, pyrrolyl, pyrazinyl, tetrazolyl, pyridyl, (or its N-oxide), thienyl, pyrimidinyl (or its N-oxide), indolyl, isoquinolyl (or its N-oxide) or quinolyl (or its N-oxide). In certain embodiments, the linking group is ($C_3$)alkylene.

**[0072]** In certain embodiments, the linking group comprises an amino acid residue, peptide deoxyribonucleic or ribonucleic acid residue, or any combination thereof. In certain embodiments, the linking group is an amino acid residue or a peptide having from one to four amino acid groups in the chain. The term "amino acid", includes the residues of the natural amino acids (e.g. Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Hyl, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in Dextrorotary or Levorotary stereoisomeric forms, as well as unnatural amino acids (e.g. phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, and gamma-carboxyglutamate; hippuric acid, octahydroindole-2-carboxylic acid, statine, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, penicillamine, ornithine, citruline, alpha-methyl-alanine, para-benzoylphenylalanine, phenylglycine, propargyiglycine, sarcosine, and tert-butylglycine). The term also comprises natural and unnatural amino acids (Dextrorotary and Levorotary stereoisomers) bearing a conventional amino protecting group (e.g. acetyl or benzyloxycarbonyl), as well as natural and unnatural amino acids protected at the carboxy terminus (e.g. as a ($C_1$-$C_6$)alkyl, phenyl or benzyl ester or amide; or as an α-methylbenzyl amide). Other suitable amino and carboxy protecting groups are known to those skilled in the art (See for example, Greene, T. W.; Wutz, P.G.M., Protecting Groups In Organic Synthesis; second edition, 1991, New York, John Wiley & sons, Inc, and documents cited therein). An amino acid can be linked to the remainder of a compound of formula (I) through the carboxy terminus, the amino terminus, or through any other convenient point of attachment, such as, for example, through the sulfur of cysteine.

**[0073]** In certain embodiments, $R^2$ is a protecting group. In certain embodiments, each $R^2$ is independently hydrogen, —$C(CH_3)_3$, or —$CH_2Ph$ and each i is independently 0 or 1. In certain embodiments, each $R^2$ is hydrogen, or —$C(CH_3)_3$, and each i is 1. In certain embodiments, $R^2$ is —$C(CH_3)_3$, and each i is 1.

**[0074]** In certain embodiments, the alkylene in the linking group optionally comprises one or more aryl groups or a polyethylene glycol (PEG) polymer having a weight average molecular weight of about 200 to about 20,000, such as a molecular weight of about 200 to 1000. In certain embodiments, the PEG polymer has a weight average molecular weight of about 200 to about 500.

**[0075]** In certain embodiments, the alkylene groups can have from 2 to about 50 carbon atoms, such as from about 2 to 20 carbon atoms, or from 2 to about 10 carbon atoms. In certain embodiments, the alkylene group has from 2 to about 5 carbon atoms. In certain embodiments, the alkylene group may optionally contain an aryl group.

**[0076]** In another embodiment of the invention the linking group has a molecular weight of from about 20 daltons to about 400 daltons.

**[0077]** In another embodiment of the invention the linking group or linker has a length of about 5 angstroms to about 300 angstroms.

**[0078]** In another embodiment of the invention the linking group separates the nucleic acid molecule and the metal chelating group and a P(=$Y^1$) residue by about 5 angstroms to about 200 angstroms, inclusive, in length

**[0079]** In another embodiment of the invention the linking group is a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 2 to 25 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms is optionally replaced by (—O—), and wherein the chain is optionally substituted on carbon with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

**[0080]** In another embodiment of the invention the linking group is of the formula W-A wherein A is ($C_1$-$C_{24}$)alkyl, ($C_2$-$C_{24}$)alkenyl, ($C_2$-$C_{24}$)alkynyl, ($C_3$-$C_8$)cycloalkyl, ($C_6$-$C_{10}$)aryl or a combination thereof, wherein W is —N(R)C(=O)—, —C(=O)N(R)—, —OC(=O)—, —C(=O)O—, —O—, —S—, —S(O)—, —$S(O)_2$—, —N(R)—, —C(=O)—, —N(R)C=N(R)—N(R)—, —C(R)=N(R)—, —$S(O)_{M2}$—N(R)—, —N(R)—$S(O)_{M2}$—, or a direct bond; wherein each R is independently H or ($C_1$-$C_6$)alkyl. In certain embodiments, the linker is —$CH_2$—C(=O). In certain embodiments, each A is alkylene of 1 to 10 carbons.

**[0081]** In certain embodiments, each linker is a divalent radical formed from a peptide. In certain embodiments, each linker is a divalent radical formed from an amino acid. In another embodiment of the invention the linking group is a divalent radical formed from a peptide. In another embodiment of the invention the linking group is a divalent radical formed from an amino acid. In certain embodiments of the invention, the linking group is a divalent radical formed from poly-L-glutamic acid, poly-L-aspartic acid, poly-L-histidine, poly-L-ornithine, poly-L-serine, poly-L-threonine, poly-L-tyrosine, poly-L-leucine, poly-L-lysine-L-phenylalanine, poly-L-lysine or poly-L-lysine-L-tyrosine.

**[0082]** In certain embodiments, the linking group is of the formula W—$(CH_2)_n$ wherein, n is between about 1 and about 10; and W is —N(R)C(=O)—, —C(=O)N(R)—, —OC(=O)—, —C(=O)O—, —O—, —S—, —S(O)—, —S(O)$_2$—, —C(=O)—, —N(R)—, —N(R)C=N(R)—N(R)—, —C(R)=N(R)—, —S(O)$_{M2}$—N(R)—, —N(R)—S(O)$_{M2}$—, or a direct bond; wherein each R is independently H or ($C_1$-$C_6$)alkyl. In certain embodiments, each linker is methylene, ethylene, or propylene.

**[0083]** In another embodiment of the invention the linking group is methylene, ethylene, or propylene.

**[0084]** In another embodiment of the invention the linking group is attached to the phosphonate group through a carbon atom of the linker.

**[0085]** In certain embodiments, the linking group is $L_a$, wherein $L_a$ comprises (a) a repeating polyethylene glycol (PEG) polymer having an average molecular weight of about 200 to about 20,000; (b) an alkylene or alkenylene group having from 2 to about 1200 carbon atoms, or ($C_6$-$C_{10}$)aryl; where the alkylene or alkenylene groups can be optionally interrupted by one or more heteroatom groups or an aromatic rings; where the heteroatom groups include —$NR^5$—, —O—, or —S—; each $R^5$ is hydrogen, ($C_1$-$C_6$)alkyl, ($C_6$-$C_{10}$)aryl, substituted aryl, or absent; or (c) an amino acid, peptide, deoxyribonucleic or ribonucleic acid residue, or a combination thereof.

Biologically Active Conjugates of Aptamers and Metal Chelating Groups

**[0086]** In certain embodiments the present invention provides a conjugate comprising a nucleic acid aptamer linked to one or more metal chelating groups or a pharmaceutically acceptable salt thereof. The present invention employs these conjugates for targeted in vivo delivery of radionuclides and other metals and therapeutic entities such as described above for imaging and therapy of prostate cancer by targeting the PSMA protein expressed in prostate cancer, or by targeting PSMA for other cancers, which express this receptor in the neovasculature of early stage disease.

**[0087]** In addition, the biologically active conjugates, e.g., bifunctional aptamer-radionuclide bioconjugates described here, are useful for the study of molecular targets and cellular pathways, such as the development of neovasculature of cancerous tumors, but targeting PSMA. In their preferred form, the bifunctional bioconjugates can be prepared using mild or highly regioselective conjugation techniques or both mild or highly regioselective reaction conditions.

**[0088]** For in vivo imaging applications, a chelator is often added chemically to the aptamer to enable labeling of the resulting bifunctional ligand. Bifunctional refers to the characteristic that these chelator-modified aptamers bind a contrast metal or radionuclide strongly, while maintaining high affinity for the in vivo molecular target of interest (e.g., a cell surface receptor). These bifunctionals are often used a contrast-agent metal (e.g., gadolinium for magnetic resonance imaging, MRI) or radionuclide (e.g., gallium-68 ($^{68}$Ga) for positron emission tomography, PET).

**[0089]** An identical approach can be employed for specific targeting of therapeutic radionuclides (e.g., alpha and beta emitters) to the site of cancerous tissue, while sparing healthy cells in vivo. To be effective, the chelator-modified bifunctional ligand (the conjugate molecule) should maintain affinity for the molecular target, while also affording a highly stable metal-chelate complex with the contrast-agent metal or radionuclide.

**[0090]** In certain embodiments, one or more metals are chelated to the one or more chelating groups.

**[0091]** In certain embodiments the conjugate has formula I:

M-L-APT (I)

wherein:

**[0092]** M is a metal chelating group;

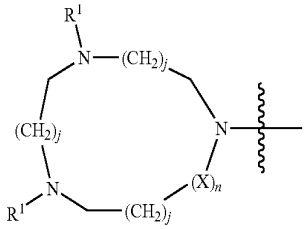
**[0093]** L is absent or is a linking group; and

**[0094]** APT is a nucleic acid aptamer that is not more than 45 nucleotides in length comprising the nucleic acid sequence 5'-$n_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA-3' (SEQ ID NO:1), wherein each $n_x$ can be present or absent, wherein when present each $n_x$ represents any nucleotide.

**[0095]** In certain embodiments the nucleic acid aptamer has a 5'-end and a 3'-end and is operably linked through the 5'-end or 3'-end to one or more metal chelating groups.

**[0096]** In certain embodiments the metal chelating group has the formula II:

(II)



**[0097]** wherein:

**[0098]** each X is independently —$(CH_2)_m$—$NR^1$—; m is 2 or 3; n is 0, 1, 2, or 3;

**[0099]** each $R^1$ is independently —$(CH_2)_i$—$COOR^2$;

**[0100]** each i is independently 0, 1, or 2;

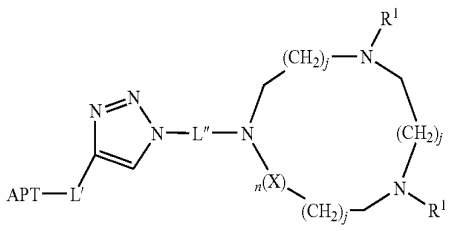
**[0101]** each $R^2$ is independently, hydrogen, or a protecting group for carboxyl groups; and

**[0102]** each j is independently 1 or 2;

**[0103]** or a salt thereof.

**[0104]** In certain embodiments the conjugate has formula III:

III



wherein:

**[0105]** APT is; a nucleic acid aptamer molecule having a 5'-end and a 3'-end wherein the aptamer molecule is operably linked to L' through the 5'-end or the 3'-end, and wherein the nucleic acid molecule is not more than 45 nucleotides in length comprising the nucleic acid sequence 5'-$n_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA-3' (SEQ ID NO:1),

wherein each $n_x$ can be present or absent, wherein when present each $n_x$ represents any nucleotide;

**[0106]** L' is a linking group;

**[0107]** L" is a linking group;

**[0108]** each X is independently $—(CH_2)_m—NR^1—$; m is 2 or 3; n is 0, 1, 2, or 3;

**[0109]** each $R^1$ is independently $—(C_{1-12})_i—COOR^2$,

**[0110]** each i is independently 0, 1, or 2;

**[0111]** each $R^2$ is independently, hydrogen, or a protecting group for carboxyl groups; and

**[0112]** each j is independently 1 or 2;

**[0113]** or a salt thereof.

**[0114]** In certain embodiments, L" is $-L_a-C(=O)—CH_2—$, wherein $L_a$ comprises a repeating polyethylene glycol (PEG) polymer having an average molecular weight of about 200 to about 20,000 (e.g., about 200 to about 1000); an alkylene or alkenylene group having from 2 to about 1200 carbon atoms, such as an alkylene group having from 2 to about 50 carbon atoms and optionally one or more aryl groups, or an alkylene group having from 2 to about 20 carbon atoms, or an alkylene group having from 2 to 5 carbon atoms (e.g., $(C_3)$alkylene), or $(C_6$-$C_{10})$aryl; where the alkylene or alkenylene groups can be optionally interrupted by one or more heteroatom groups or an aromatic rings; where the heteroatom groups include $—NR^5—$, —O—, or —S—; each $R^5$ is hydrogen, $(C_1$-$C_6)$alkyl, $(C_6$-$C_{10})$aryl, substituted aryl, or absent; or an amino acid, peptide, deoxyribonucleic or ribonucleic acid residue, or a combination thereof. In certain embodiments, $L_a$ is an amino acid residue or peptide having from one to four amino acid groups in the chain.

**[0115]** In certain embodiments, L' has a molecular weight of from about 20 daltons to about 400 daltons. In certain embodiments, L' has a length of about 5 angstroms to about 300 angstroms. In certain embodiments, L' is a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 2 to 25 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by (—O—), and wherein the chain is optionally substituted on carbon with one or more substituents selected from $(C_1$-$C_6)$alkoxy, $(C_3$-$C_6)$cycloalkyl, $(C_1$-$C_6)$alkanoyl, $(C_1$-$C_6)$alkanoyloxy, $(C_1$-$C_6)$alkoxycarbonyl, $(C_1$-$C_6)$alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy. In certain embodiments, L' is of the formula W-A wherein A is $(C_1$-$C_{24})$alkylene, $(C_2$-$C_{24})$alkenylene, $(C_2$-$C_{24})$alkynylene, $(C_3$-$C_8)$cycloalkylene, $(C_6$-$C_{10})$aryl or a combination thereof, wherein each W is $—N(R)C(=O)—$, $—C(=O)N(R)—$, $—OC(=O)—$, $—C(=O)O—$, —O—, —S—, —S(O)—, $—S(O)_2—$, $—N(R)—$, $—C(=O)—$, $—N(R)C=N(R)—N(R)—$, $—C(R)=N(R)—$, $—S(O)_{M2}—N(R)—$, $—N(R)—S(O)_{M2}—$, or a direct bond; wherein each R is independently H or alkyl of 1 to 10 carbons. In certain embodiments, A is alkylene of 1 to 10 carbons. In certain embodiments, L' is a divalent radical formed from an amino acid or a peptide. In certain embodiments, L' is a divalent radical formed from poly-L-glutamic acid, poly-L-aspartic acid, poly-L-histidine, poly-L-ornithine, poly-L-serine, poly-L-threonine, poly-L-tyrosine, poly-L-leucine, poly-L-lysine-L-phenylalanine, poly-L-lysine or poly-L-lysine-L-tyrosine.

**[0116]** In certain embodiments, L' is of the formula $W—(CH_2)_n$ wherein, n is between about 1 and about 10; and W is $—N(R)C(=O)—$, $—C(=O)N(R)—$, $—OC(=O)—$, $—C(=O)O—$, —O—, —S—, —S(O)—, $—S(O)_2—$, $—C(=O)—$, $—N(R)—$, $—N(R)C=N(R)—N(R)—$, $—C(R)=N(R)—$, $—S(O)_2—N(R)—$, $—N(R)—S(O)_2—$, or a direct bond; wherein each R is independently H or $(C_1$-$C_6)$ alkyl. In certain embodiments, L' is methylene, ethylene, or propylene.

**[0117]** In certain embodiments, the aptamer portion is operably linked to its azido group or its alkynyl group by means of a linker prior to the formation of the conjugate compound. In certain embodiments the metal chelating group is linked to its azido group or its alkynyl group by means of a linker prior to the formation of the conjugate compound. The structure of each linker in the conjugate compound is not critical as long as it does not interfere with the intended function of the conjugate compound.

Metals to Chelate to Metal Chelating Group

**[0118]** In certain embodiments the metal chelating group is chelated to a metal. In certain embodiments, the metal is an imageable metal or a therapeutic metal. In certain embodiments the imageable metal is a radionuclide, such as an alpha or beta-emitting radionuclide. Exemplary radionuclides include gallium-68 (68Ga, half life $t_{1/2}$=68 min), yttrium-90 ($^{90}$Y, $t_{1/2}$=64 hours), lutetium-177 (177Lu, $t_{1/2}$=6.63 days), bismuth-213 ($^{213}$Bi, $t_{1/2}$=46 minutes), indium-111 ($^{111}$In, $t_{1/2}$=2.8 days); gallium-67 ($^{67}$Ga, $t_{1/2}$=3.3 days); copper-64 (64Cu, $t_{1/2}$=12.7 hours), actinium-225 ($^{225}$Ac, $t_{1/2}$=10 days), or radium-223 ($^{223}$Ra, $t_{1/2}$=11.4 days). Other radionuclides include: Cr-51, Ir-192, Sc-44, Co-55, Cu-61, Cu-67, Mn-51, Mn-52, Pb-203, Po-210, Phosphorus-32 and the like. In certain embodiments, the metal is a therapeutic metal.

Methods of Making Conjugates

**[0119]** Processes for preparing conjugates or for preparing intermediates useful for preparing conjugates are provided as further embodiments of the invention. Intermediates useful for preparing conjugates are also provided as further embodiments of the invention.

**[0120]** In certain embodiments, the present invention provides a method of covalently linking the nucleic acid aptamer as described above to the metal chelating group as described above by forming a reaction mixture of a corresponding aptamer comprising an alkynyl group with a corresponding metal chelating group comprising an azido group; or forming a reaction mixture of a corresponding aptamer comprising an azido group with a corresponding metal chelating group comprising an alkynyl group, wherein the reaction mixture is formed under conditions permitting a 1,3-dipolar cycloaddition reaction to occur between the azido and the alkynyl groups to covalently link the nucleic acid aptamer to the metal chelating group. In certain embodiments, the forming of the reaction mixture occurs at about room temperature. In certain embodiments, the reaction mixture further comprises an agent which catalyzes a 1,3-dipolar cycloaddition reaction. In certain embodiments, the agent is copper. In certain embodiments, the reaction mixture is devoid of copper.

**[0121]** In the preparation of molecular targeting agents for imaging and therapy, the aptamer is conjugated with a chelator for housing the radionuclide or metal used as the contrast agent for the imaging modality (PET, SPECT, MRI or other). Many methods have been applied to these conjugation techniques. For applications prior to the invention of RNA aptamers for molecular targeting, the chemical conditions could be relatively "harsh" (highly acidic or basic, high temperatures, organic solvents, etc.). For example, in the preparation of a

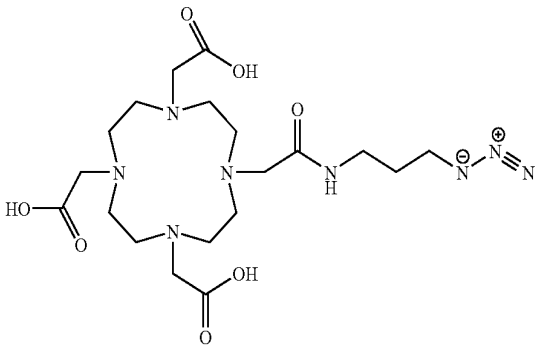
chelator modified peptides and antibodies, a deprotection step is employed where protecting groups (such as tertiary-butyl groups connected to the carboxylic acids of macrocyclic, polyamine, polycarboxylic acids that comprise the basic structure of many chelator groups) are removed from the chelator moiety to render the construct useful as a bifunctional.

**[0122]** The need for these approach arises from two problems: (1) in order for the conjugation reaction to be regioselective (add the chelator at the precise location necessary to maintain the targeting behavior of the aptamer, the carboxylic acid groups are protected by the presence of the protecting groups (e.g., the t-butyl groups); and (2) the t-butyl groups must be removed to preserve the binding affinity chelation behavior of the chelator group for the metal of interest. The conditions for the deprotection step (as an example of harsh conditions that peptides (amino acid constructs) and antibodies (larger amino acid sequences that peptides) are known in the art to generally be highly acidic (acid hydrolysis at pH conditions generally less than 2) or highly basic conditions (pH values generally greater than 9). These reaction are also generally slow (hours to days to completion), which is less than optimum for economical manufacturing. In addition, for RNA aptamers, these harsh conditions are intolerable and lead to unacceptable degradation of the RNA construct. Thus, an approach was needed for conjugation of chelators to RNA molecules that was rapid, regioselective, and could be applied under mild conditions in high chemical yield.

**[0123]** In certain embodiments, aptamers (such as the PSMA targeted RNA aptamer of the invention disclosed herein), peptides, antibodies, antibody fragments, PNAs, or other molecular targeting entity, are conjugated to a metal chelating group by means of "Click Chemistry." "Click Chemistry" is a term for a synthesis method developed by Nobel laureate chemist Karl Barry Sharpless (Scripps Research Institute) in 2001. It is a copper-catalyzed azide-alkyne reaction that makes it possible for certain chemical building blocks to "click" together in an irreversible linkage. Briefly, an azido group is operably linked to the aptamer and an alkynyl group is linked to the metal chelating group (or an alkynyl group is linked to the aptamer and an azido group is operably linked to the metal chelating group). The two components are combined under conditions amendable to Click Chemistry linkage. General methods for performing Click Chemistry are well known in the art. Kolb et al., *Angew. Chem. Int. Ed.* 40:2004-2021 (2001); Ju et al., US Patent Application Publication No. 2005/0032081; Kolb et al., US Patent Application Publication No. 2006/0263293; Kolb et al., US Patent Application Publication No. 2006/0269942. Alternative methods of "Click Chemistry" can be performed without the use of a copper catalyst. Sletten et al., Org. Lett. 10:3097-3099 (2008); Codelli et al., *J. Am. Chem. Soc.* 130: 11486-11493 (2008); Johnson et al., *Chem. Commun.* 2008, 3064-3066.

**[0124]** Prior to the current disclosure, despite numerous disclosures of chelator precursors with numerous descriptions functional groups for conjugation to targeting vectors such as peptides and antibodies, no mention is made to the need for or the synthesis of an azide of a chelator for conjugation to a molecular targeting agent. It is likely that the need for such a compound was not perceived given the chemical robustness of peptides, antibodies, antibody fragments, and PNAs. On the other hand, the advent of the use of RNA aptamers for molecular targeted created a need for innovation in preparing these new bifunctional bioconjugates. The click chemical approach seemed to provide the chemical conditional requirements for adding chelators to RNA. Unfortunately, no azide of a chemical chelator was available or had been described previously in the literature and would need to be conceived and carried forward to enable the reaction as a method for conjugating chelators to RNA aptamers.

**[0125]** To begin the development of the an azide modified chelator for click chemical conjugation to a molecular targeting vector, a synthetic route for the preparation of a prototype azide-functionalized chelator derivative and the popular chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, known as DOTA was used to develop an azide chelator as described earlier.



**[0126]** The resulting compound (termed DOTAzide) allows for the conjugation of the DOTA chelating moiety to nucleic acids, peptides, and other targeting vectors that have been modified with an alkyne-functional group. In one embodiment, the conjugation reaction is catalyzed by the addition of ascorbate or other suitable reducing agent, Cu(I) (or other suitable metal) catalyst and tris-hydroxypropyltriazolylamine (or other suitable redox stabilizer) as a Cu(I) stabilizer if required). The DOTAzide molecular construct is provides an azide-functionalized tether that allows for simple conjugation of the chelator to oligonucleotides and RNA aptamers by click chemistry methods. This allows for the preparation of radiolabeled aptamers for imaging and therapy of disease, particularly cancer through radiolabeling techniques. In one embodiment, the method employs the DOTAzide (or other azide modified chelator as described below) for copper-free click chemical conjugation using a cyclooctyne-modified functional group on the RNA or other targeting vector that is modified in such a way to enable fast click chemical conjugation that is promoted by ring strain. The cyclooctyne functional group enables the click chemical reaction using potential energy associated with ring strain at the carbon-carbon triple bond. The kinetics of the reaction is enhanced by addition of a difluorosubstitution at a carbon position that is adjacent to the carbon-carbon triple bond of the cyclooctyne functional group. This combination of ring strain and difluoro or other suitable electron withdrawing group promote the click reaction to be used as an efficient conjugation step for addition of the azide modified chelator in the absence of the copper catalyst. This is an important embodiment because the copper will need to be removed from the system prior to use as a pharmaceutical. Copper free click chemistry reduces the number of steps required for preparation of the bifunctional bioconjugate.

**[0127]** For example, positron emitting radionuclide gallium-68 (68Ga) can be labeled to an RNA aptamer that is targeted against a cell surface protein receptor (such as prostate specific membrane antigen, PSMA) for in vivo imaging of prostate cancer by PET. While DOTAzide (or 2,2',2"-(10-(2-(3-azidopropylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) is the first of the azide-modified chelators to be described, other chelator molecules can be prepared with the same azide functionality to enable the click chemical conjugation reaction to be used. Examples of popular chelators, described in one of many possible reactive forms that include the azide functional group for click chemistry include: 2,2',2"-(2-(2-(3-azidopropylamino)-2-oxoethyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid, referred to as NOTAzide); 10-(2-(3-azidopropylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclotridecane-1,4,7-tricarboxylic acid referred to as TETAzide; and 2,2',2", 2"',2""-(16-(2-(3-azidopropylamino)-2-oxoethyl)-1,4,7,10,13,16-hexaazacyclooctadecane-1,4,7,10,13-pentayl)pentaacetic acid, referred to as Hexazide). These compounds, referred to collectively as "Macrozides" allow radiolabeling with the most popular radiometals for research applications in targeted molecular imaging and therapy of peptide-, antibody- and nucleic-acid-based targeting molecules, such as aptamers. Representative metals used for radionuclide and metal imaging applications include, but are not limited to $Gd^{3+}$ and other lanthanides and transition metals for MRI, and radiometals $^{111}In$ $^{64}Cu$, $^{44}Sc$ and $^{68}Ga$ for nuclear imaging (e.g., PET, SPECT), as well as $^{90}Y$, $^{177}Lu$ and $^{213}Bi$ for radionuclide-based therapy of cancer. The PSMA aptamer described herein is effective in binding with high affinity to prostate cancer cells and expression of PSMA correlates with progression of disease, making it an excellent molecular target for in vivo imaging.

**[0128]** For DOTAzide preparation, high-yield synthesis of the bifunctional azide-modified chelator was accomplished via nucleophilic substitution of the N-hydroxysuccinimide group of precursor tris-1,4,7,10-tetraazacyclododecane-1,4,7-tris(t-butyl acetate)-10-succinimidyl acetate with 1.1 equivalents of 1-amino-3-azidopropane (THF, −20° C., 12 hours). The amino-azide precursor is prepared rapidly and in high yield with inexpensive-readily-available 1-amino-3-bromopropane by nucleophilic substitution of the bromo group with sodium azide salt (water, 80° C.). Straightforward purification and removal of t-butyl protecting groups is performed by reverse-phase HPLC and application of dilute hydrochloric acid to afford the water soluble azide derivative DOTAzide. The purified compound can be lyophilized for storage, is stable in saline solution at standard refrigeration temperatures, and appears stable at room temperature for extended periods of time (weeks). DOTAzide affords simple conjugation to alkyne-functionalized oligonucleotides and RNA aptamers via the Cu(I) catalyzed cyclo-addition of an azide and alkyne, popularized as so called "click" chemistry. The resulting triazole-linked bifunctional conjugate results in a stable chelator-oligonucleotide(aptamer) bifunctional—ligand that can be used for targeting in vivo delivery of radionuclides and other metals for imaging and therapy of cancer and other pathologies.

**[0129]** The regio-selective click reaction in the absence of the Cu(I) catalyst is preferential over click chemistry in the presence of Cu(I). For nuclear medicine applications, particularly in preparation of short-lived radiopharmaceuticals, streamlining the preparation and removing the metallic impurity for the synthetic approach is highly advantageous. The half-life of $^{68}Ga$ for example is 68 minutes. Thus, preparation time must be rapid.

**[0130]** Radiolabeling yields and percent incorporation of $^{68}Ga$ are compromised by the presence of non-radioactive metals—even small quantities can interfere. The right-strain energy of the smallest possible cyclic alkyne structure (cyclooctyne) promotes the click reaction. The inventors have developed a right-strain 5'-\5-cyclooctynyl phosphoramidite to enable Cu-free click chemical addition of chelators to nucleic acids (FIG. **7**). The inventors have been able to prepare a difluoromodified version of cyclooctyne and confirmed copper-free click chemical conjugation of DOTAzide to the molecule in high yield in less than 30 minutes at room temperature in a solution of water and methanol. It is important to note that the reaction can be undertaken in aqueous conditions with deprotected DOTAzide and RNA. Elimination of the need for Cu(I)-catalyst for aptamer-based imaging and therapeutic agents is advantageous because of the corresponding elimination of the possibility of metal contamination of drug product and because it would simplify the quality control and quality assurance testes that would be required for acceptance for clinical trials.

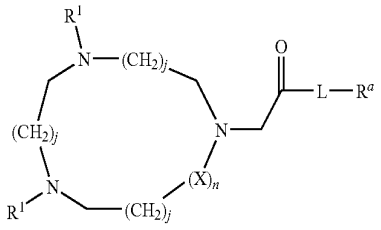
**[0131]** In certain embodiments, the complete bioconjugate consisting of chelator, aptamer, linker, and possibly another imaging molecular entity can be prepared by nucleophilic substitution or addition reactions that connect the reactive group of the aptamer, linkers, and chelators. For example, the inventors have shown that the active NHS ester of DOTA, and a para-isothiocyanoato-benzyl-functionalized NOTA can be conjugated to the aptamer using methods known in the art (FIGS. **8**A-**8**C).

Synthetic Intermediates

**[0132]** Specific synthetic intermediates can be used to prepare a conjugate of the invention that comprises a nucleic acid aptamer molecule linked to a reactive group (e.g. an alkynyl group, an azido group, an amine group, an isothiocyanato group, or an active ester of a carboxylic acid). In certain embodiments, the alkynyl group is linked to the 3' end. In certain embodiments, the alkynyl group is linked to the 5' end. In certain embodiments, the nucleic acid molecule is linked to an azido group. In certain embodiments, the azido group is linked to the 3' end. In certain embodiments, the azido group is linked to the 5' end. In certain embodiments, the nucleic acid is linked to an amine group. In certain embodiments, the amine group is linked to the 3' end. In certain embodiments, the amine group is linked to the 5' end. Other reactive groups include reactive groups for Staudinger ligations, isothiocyanato groups, active esters of carboxylic acids, and many other such reactive groups that are described and well-known to persons skilled in the art of the preparation of bifunctional biological molecules such as bifunctional RNA and DNA aptamers, peptides, antibodies, affibodies, and PNA's.

**[0133]** A synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula V:

V



**[0134]** wherein

**[0135]** L is a linking group:

**[0136]** each X is independently —$(CH_2)_m$—$NR^1$—; n is 0, 1, 2, or 3; m is 2, or 3;

**[0137]** each $R^1$ is independently —$(CH_2)_i$—$COOR^2$; each i is independently 0, 1, or 2;

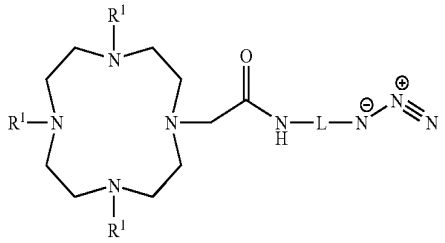
**[0138]** each $R^2$ is independently, hydrogen, or a protecting group for carboxyl groups;

**[0139]** each j is independently 1 or 2; and

**[0140]** $R_a$ is —C≡CH, or azido;

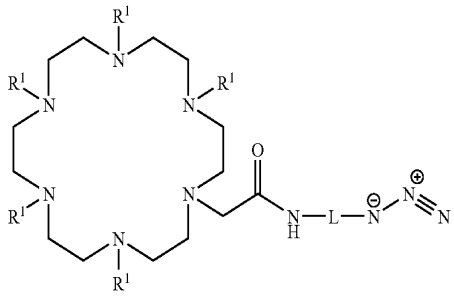
**[0141]** or a salt thereof.

**[0142]** Another synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula:



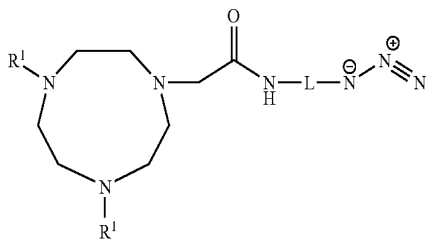
or a salt thereof.

**[0143]** Another synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula of the formula:



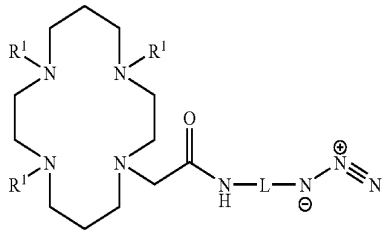
or a salt thereof.

**[0144]** Another synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula:



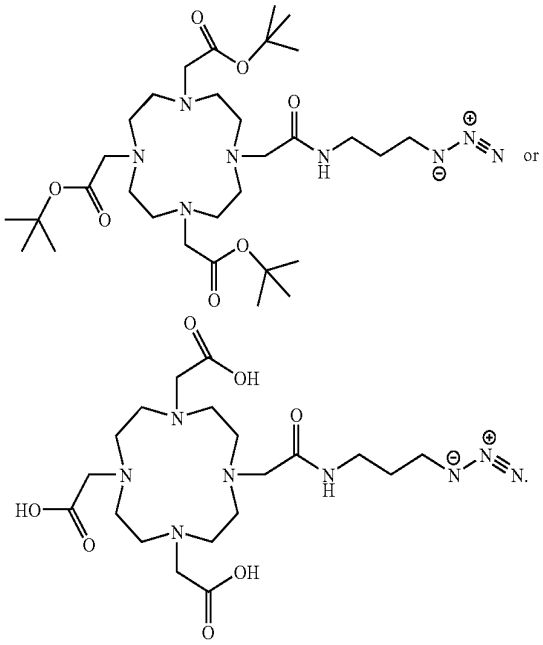
or a salt thereof.

**[0145]** Another synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula:



or a salt thereof.

**[0146]** Another synthetic intermediate that is useful for preparing a conjugate of the invention is a compound of formula:



Diseases and Conditions Amendable to the Methods of the Invention

**[0147]** Worldwide, cancer affects approximately 10 million people each year. Approximately 22 million people are living with cancer and almost 7 million people die worldwide from cancer each year. The most common cancers include cancers of the lung, breast, colon/rectum, stomach, liver, prostate, cervix, esophagus, and bladder. The elderly tend to be the highest population for new incidence, as more than 75% of all new cancer cases are diagnosed in people over the age of 60. With the aging population, incidence is expected to increase each year. Prostate cancer is the most common cancer in men and the second leading cause of cancer death in men, behind lung cancer. Approximately one in six U.S. men will contract prostate cancer in his lifetime. Approximately 80% of prostate cancers are diagnosed in men over 65 years of age, and, due to the lack of symptoms, 75% of first-time patients over 65 are diagnosed with Stage C or D, the two most advanced stages of prostate cancer. Worldwide, more than 680,000 men are diagnosed annually and over 28,000 U.S. men will die as

a result of prostate cancer this year alone. Prostate cancer characteristically spreads to the bone. The therapeutic regimen for disease that is localized in the prostate bed (local disease) can be drastically different from the prescribed therapy for metastatic prostate cancer. Targeted molecular imaging and therapy promise to provide precise delivery of diagnostic and therapeutic agents to the site of prostate cancer, providing a critically-needed approach to improve outcomes for prostate cancer patients.

**[0148]** In certain embodiments of the present invention, a human patient or mammalian research subject (such as a research rat or mouse or a larger animal such as a primate) has prostate cancer. In certain embodiments of the present invention, a human patient or mammalian research subject (such as a research rat or mouse or a larger animal such as a primate) has a form of cancer in which the transmembrane type II protein prostate specific membrane antigen is present at high concentrations in the cells of that cancer or other condition which is amenable to imaging or therapy using the composition of matter of the invention. In certain embodiments, PSMA is expressed at high concentrations in diseased tissue of the patient or research subject and the physician or researcher would like to image or treat the diseased tissue using targeted molecular imaging or therapy. As used herein, "targeted molecular imaging and targeted molecular therapy" refers to administration to the patient nucleic acid material (such as an RNA aptamer) that is designed to bind specifically with high affinity to an in vivo molecular target (such as PSMA). In so targeting the diseased tissue or otherwise targeting the tissue of interest, the administered nucleic acid material of the present invention delivers a radionuclide. Thus, the phrase "condition amenable to imaging or therapy of the invention" embraces conditions such as genetic diseases (i.e., a disease condition that is attributable to one or more gene defects), acquired pathologies (i.e., a pathological condition that is not attributable to an inborn defect), cancers, neurodegenerative diseases, e.g., trinucleotide repeat disorders, and prophylactic processes (i.e., prevention of a disease or of an undesired medical condition).

**[0149]** A condition amenable to targeted molecular imaging or therapy can be a genetic disorder or an acquired pathology that is manifested by abnormal cell proliferation, e.g., cancer. According to this embodiment, the instant invention is useful for targeted delivery of radionuclides, metals, or liposomes, nanoparticles, for the purpose of imaging or delivering a cytotoxic dose of radiation or other form of therapeutic dose to the tissue of interest (e.g., prostate cancer tumors or lesions of prostate cancer). The present invention can be used to treat a solid sarcoma or carcinoma, such as is prostate cancer.

Detection and Imaging Conjugates and Methods

**[0150]** The present invention provides methods for detecting PSMA in a sample or in vivo. For example, one can contact a sample with an aptamer as described herein or the composition as described herein to form bound PSMA, and detecting the presence or the quantity of bound PSMA. Alternatively, aptamers or compositions can be administered in vivo to a patient (e.g. injected intravenously using a syringe, cathetor, or other medical device). In certain embodiments, the bound PSMA is detected by means of PCR, nuclear magnetic resonance, fluorescent capillary electrophoresis, lateral flow devices, colorimetry, chemiluminescence, fluorescence, western blots, microarrays, ELISA, radioHPLC, single photon emission computed tomography (SPECT), or positron emission tomography (PET).

**[0151]** In one embodiment for in vivo imaging of prostate cancer, the present invention involves the administration of the invention as a radiopharmaceutical for imaging the location of prostate cancer cells in humans or research mammalian subjects such as rats, mice, dogs, cats, and primates that have been genetically engineered to contract or possess prostate cancer cells in vivo. In this embodiment, the invention consists of (1) an aptamer that is designed to bind with high affinity to PSMA; (2) a radionuclide for PET or SPECT imaging; (3) a chelator that binds the radionuclide tightly; and (4) and a linker that connects the chelator and aptamer together. In certain embodiments, other molecular entities are added at various positions on the structure to impart improved pharmacokinetic or biodistribution properties to the invention that improve the overall imaging or therapeutic characteristics of the invention. For example, a polyethylene glycol molecular entity can be added to the 3'-end of an aptamer in which the 5'-end has been modified to include the chelator and radionuclide, with the purpose of the PEG to slow the pharmacokinetics of the aptamer as a means of improving the overall imaging or therapeutic efficacy of the complete molecular structure.

**[0152]** In one embodiment of the present invention, the method also involves contacting the sample with at least one aptamer to form a hybridized nucleic acid and detecting the hybridized nucleic acid. In one embodiment, the detection is by amplification. "Amplifying" utilizes methods such as the polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction, LCR), strand displacement amplification, nucleic acid sequence-based amplification, and amplification methods based on the use of Q-beta replicase. These methods are well known and widely practiced in the art. Reagents and hardware for conducting PCR are commercially available. In one embodiment of the present invention, at least one type of aptamer is immobilized on a solid surface.

**[0153]** The methods of the present invention can be used to detect the presence of PSMA in a sample.

**[0154]** According to the methods of the present invention, the amplification of PSMA present in a sample may be carried out by any means known to the art. Examples of suitable amplification techniques include, but are not limited to, polymerase chain reaction (including, for RNA amplification, reverse-transcriptase polymerase chain reaction), ligase chain reaction, strand displacement amplification, transcription-based amplification, self-sustained sequence replication (or "3SR"), the Qβ replicase system, nucleic acid sequence-based amplification (or "NASBA"), the repair chain reaction (or "RCR"), and boomerang DNA amplification (or "BDA").

**[0155]** The bases incorporated into the amplification product may be natural or modified bases (modified before or after amplification), and the bases may be selected to optimize subsequent electrochemical detection steps.

**[0156]** Polymerase chain reaction (PCR) may be carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188. In general, PCR involves, first, treating a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) with one oligonucleotide primer for each strand of the specific sequence to be detected under hybridizing conditions so that an extension product of each primer is synthesized that is complementary to each nucleic acid strand, with the primers

sufficiently complementary to each strand of the specific sequence to hybridize therewith so that the extension product synthesized from each primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and then treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present. These steps are cyclically repeated until the desired degree of amplification is obtained. Detection of the amplified sequence may be carried out by adding to the reaction product an oligonucleotide probe capable of hybridizing to the reaction product (e.g., an oligonucleotide probe of the present invention), the probe carrying a detectable label, and then detecting the label in accordance with known techniques. Where the nucleic acid to be amplified is RNA, amplification may be carried out by initial conversion to DNA by reverse transcriptase in accordance with known techniques.

**[0157]** Strand displacement amplification (SDA) may be carried out in accordance with known techniques. For example, SDA may be carried out with a single amplification primer or a pair of amplification primers, with exponential amplification being achieved with the latter. In general, SDA amplification primers comprise, in the 5' to 3' direction, a flanking sequence (the DNA sequence of which is noncritical), a restriction site for the restriction enzyme employed in the reaction, and an oligonucleotide sequence (e.g., an oligonucleotide probe of the present invention) that hybridizes to the target sequence to be amplified and/or detected. The flanking sequence, which serves to facilitate binding of the restriction enzyme to the recognition site and provides a DNA polymerase priming site after the restriction site has been nicked, is about 15 to 20 nucleotides in length in one embodiment. The restriction site is functional in the SDA reaction. The oligonucleotide probe portion is about 13 to 15 nucleotides in length in one embodiment of the invention.

**[0158]** Ligase chain reaction (LCR) is also carried out in accordance with known techniques. In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denaturing (e.g., separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence of a heat stable ligase so that each pair of oligonucleotide probes is ligated together, then separating the reaction product, and then cyclically repeating the process until the sequence has been amplified to the desired degree. Detection may then be carried out in like manner as described above with respect to PCR.

**[0159]** Diagnostic techniques that are useful in the methods of the invention include, but are not limited to direct DNA sequencing, pulsed-field gel electrophoresis (PFGE) analysis, allele-specific oligonucleotide (ASO), dot blot analysis and denaturing gradient gel electrophoresis, and are well known to the artisan.

**[0160]** The sample may be contacted with the aptamer in any suitable manner known to those skilled in the art. For example, the sample may be solubilized in solution, and contacted with the aptamer by solubilizing the aptamer in solution with the sample under conditions that permit binding. Suitable conditions are well known to those skilled in the art. Alternatively, the sample may be solubilized in solution with the aptamer immobilized on a solid support, whereby the sample may be contacted with the aptamer by immersing the solid support having the aptamer immobilized thereon in the solution containing the sample.

**[0161]** In certain embodiments, for small animal imaging of the efficacy of the aptamer for in vivo imaging, a suitable amount of the RNA aptamer, in the range of 1-100 nmoles is conjugated to a chelator by methods described here or by methods that are well known to those who are skilled in the art. The bifunctional chelator modified PSMA-targeted aptamer can then be radiolabeled with radionuclides that are useful for imaging and therapy by methods that are well known to those that are skilled in the art. In an exemplary embodiment, a reactive derivative of NOTA can be conjugated to a 5'-hexynyl-RNA aptamer by a two step process as described below by first adding 1-amino-3-azidopropane to the hexynyl functionalized aptamer to obtain a primary amine reactive function on the aptamer. Following purification by HPLC methods, the amine modified RNA aptamer can be conjugated to a reactive NOTA derivative (2,2',2"-(2-(4-isothiocyanatobenzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid) to form the bifunctional as described in Example 3. The final purification is performed by size-exclusion and HPLC techniques that are well known to those that are skilled in the art. Following purification, the NOTA-PSMA-Aptamer can be radiolabeled with a radionuclide that is suitable for imaging by PET or SPECT or other imaging technique. In an exemplary form, the NOTA-PSMA-Aptamer is radiolabeled with $^{68}$Ga for PET, by dissolving the NOTA-PSMA-Aptamer in an appropriate solution and reacting the chelator modified aptamer with $^{68}$Ga under conditions that are well-known to those that are skilled in the art. The final radiolabeled [$^{68}$Ga]-NOTA-PSMA-Aptamer is then purified by size exclusion and precipitation methods that are well known to those that are skilled in the art and finally dissolved in a solution suitable for venous injection of the obtained radiopharmaceutical.

**[0162]** For small animal imaging, the final purified radiopharmaceutical is dissolved in a small volume (100-500 mL) of sterile isotonic saline or other suitable solution for injection. A small animal model such as a nude mouse is prepared according to methods that are well known to those that are skilled in the art and as exemplified in section "tumor implantation and monitoring tumor growth" presented here. Such an animal is anesthetized and injected with the solution containing the radiolabeled aptamer by methods that are well known to measure the accumulation of radiolabeled aptamer in the tumor over a time period ranging from a few minutes to as long as 4 days or longer. The time needed for accumulation depends on the pharmacokinetics of the radiolabeled tracer. An image of the accumulation is obtained by use of a specialized camera system known as a tomograph.

Kits Containing Agents of the Invention

**[0163]** The present invention provides kits comprising the conjugates described above, or the pharmaceutically acceptable salt thereof, packaging material, and instructions for administering the conjugate or the pharmaceutically acceptable salt thereof to an animal to detect or treat prostate cancer. In certain embodiments, the kit further comprises an imageable metal or a therapeutic metal. In certain embodiments, the imageable metal is a radionuclide. In certain embodiments,

the radionuclide is an alpha or beta-emitting radionuclide. In certain embodiments, the metal is Gallium-68 ($^{68}$Ga).

Dosages, Formulations and Routes of Administration of the Agents of the Invention

**[0164]** In certain embodiments, the agents of the invention are administered so as to result in a reduction in at least one symptom associated with a disease. The amount administered will vary depending on various factors including, but not limited to, the composition chosen, the particular disease, the weight, the physical condition, and the age of the mammal, and whether prevention or treatment is to be achieved. Such factors can be readily determined by the clinician employing animal models or other test systems, which are well known to the art.

**[0165]** In certain embodiments, the present invention is a pharmaceutical composition comprising a conjugate as described above and a pharmaceutically acceptable carrier. Pharmaceutical formulations, dosages and routes of administration for nucleic acids are generally known in the art.

**[0166]** The present invention envisions treating a disease, for example, cancer, in a mammal by the administration of an agent, e.g., a nucleic acid composition, an expression vector, or a viral particle of the invention. Administration of the therapeutic agents in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated.

**[0167]** One or more suitable unit dosage forms having the therapeutic agent(s) of the invention, which, as discussed below, may optionally be formulated for sustained release (for example using microencapsulation), can be administered by a variety of routes including parenteral, including by intravenous and intramuscular routes, as well as by direct injection into the diseased tissue. For example, the therapeutic agent may be directly injected into the cancer. In another example, the therapeutic agent may be introduced intramuscularly for viruses that traffic back to affected neurons from muscle, such as AAV, lentivirus and adenovirus. In another example, the therapeutic agent may be injected intravenously. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

**[0168]** When the therapeutic agents of the invention are prepared for administration, in certain embodiments, they are combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations include from 0.1 to 99.9% by weight of the formulation. A "pharmaceutically acceptable" is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a powder or as granules, as a solution, a suspension or an emulsion.

**[0169]** Pharmaceutical formulations containing the therapeutic agents of the invention can be prepared by procedures known in the art using well known and readily available ingredients. The therapeutic agents of the invention can also be formulated as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

**[0170]** The pharmaceutical formulations of the therapeutic agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

**[0171]** Thus, the therapeutic agent may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

**[0172]** It will be appreciated that the unit content of active ingredient or ingredients contained in an individual aerosol dose of each dosage form need not in itself constitute an effective amount for treating the particular indication or disease since the necessary effective amount can be reached by administration of a plurality of dosage units. Moreover, the effective amount may be achieved using less than the dose in the dosage form, either individually, or in a series of administrations.

**[0173]** The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0, saline solutions, and water.

General Terminology

**[0174]** "Synthetic" aptamers are those prepared by chemical synthesis. The aptamers may also be produced by recombinant nucleic acid methods. "Recombinant nucleic molecule" is a combination of nucleic sequences that are joined together using recombinant nucleic technology and procedures used to join together nucleic sequences known in the art.

**[0175]** As used herein, the term "nucleic acid" and "polynucleotide" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base that is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as

well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues.

**[0176]** Deoxyribonucleic acid (DNA) in the majority of organisms is the genetic material while ribonucleic acid (RNA) is involved in the transfer of information contained within DNA into proteins. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

**[0177]** The terms "nucleic acid," "nucleic acid molecule," "nucleic acid fragment," "nucleic acid sequence or segment," or "polynucleotide" may also be used interchangeably with gene, cDNA, DNA and RNA encoded by a gene, e.g., genomic DNA, and even synthetic DNA sequences. The term also includes sequences that include any of the known base analogs of DNA and RNA.

**[0178]** By "fragment" or "portion" is meant a full length or less than full length of the nucleotide sequence.

**[0179]** A "variant" of a molecule is a sequence that is substantially similar to the sequence of the native molecule. For nucleotide sequences, variants include those sequences that, because of the degeneracy of the genetic code, encode the identical amino acid sequence of the native protein. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis that encode the native protein, as well as those that encode a polypeptide having amino acid substitutions. Generally, nucleotide sequence variants of the invention will have in at least one embodiment 40%, 50%, 60%, to 70%, e.g., 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81%-84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98%, sequence identity to the native (endogenous) nucleotide sequence.

**[0180]** The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Genes include coding sequences and/or the regulatory sequences required for their expression. For example, gene refers to a nucleic acid fragment that expresses mRNA, functional RNA, or a specific protein, including its regulatory sequences. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. In addition, a "gene" or a "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. The term "intron" refers to a DNA sequence present in a given gene which is not translated into protein and is generally found between exons.

**[0181]** "Naturally occurring," "native" or "wild type" is used to describe an object that can be found in nature as distinct from being artificially produced. For example, a nucleotide sequence present in an organism (including a virus), which can be isolated from a source in nature and which has not been intentionally modified in the laboratory, is naturally occurring. Furthermore, "wild-type" refers to the normal gene, or organism found in nature without any known mutation.

**[0182]** "Homology" refers to the percent identity between two polynucleotides or two polypeptide sequences. Two DNA or polypeptide sequences are "homologous" to each other when the sequences exhibit at least about 75% to 85% (including 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, and 85%), at least about 90%, or at least about 95% to 99% (including 95%, 96%, 97%, 98%, 99%) contiguous sequence identity over a defined length of the sequences.

**[0183]** The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence," (b) "comparison window," (c) "sequence identity," (d) "percentage of sequence identity," and (e) "substantial identity."

**[0184]** (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full length cDNA or gene sequence, or the complete cDNA or gene sequence.

**[0185]** (b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

**[0186]** Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm.

**[0187]** Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed, using the default parameters.

**[0188]** Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (see the World Wide Web at ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative

scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

**[0189]** In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, less than about 0.01, or even less than about 0.001.

**[0190]** To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. When using BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=−4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. See the World Wide Web at ncbi.nlm.nih.gov. Alignment may also be performed manually by visual inspection.

**[0191]** For purposes of the present invention, comparison of nucleotide sequences for determination of percent sequence identity to the sequences disclosed herein is made using the BlastN program (version 1.4.7 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by a BLAST program.

**[0192]** (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid sequences makes reference to a specified percentage of residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window, as measured by sequence comparison algorithms or by visual inspection. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

**[0193]** (d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

**[0194]** (e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%; at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%; at least 90%, 91%, 92%, 93%, or 94%; or even at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters.

**[0195]** Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions (see below). Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point ($T_m$) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C. to about 20° C., depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

**[0196]** (e)(ii) For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

**[0197]** As noted above, another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that

sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

**[0198]** "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched nucleic acid. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the $T_m$ can be approximated from the equation of Meinkoth and Wahl: $T_m$ 81.5° C.+16.6 (log M)+0.41 (% GC)-0.61 (% form)-500/L. M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. $T_m$, is reduced by about 1° C. for each 1% of mismatching; thus, $T_m$, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the $T_m$ can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point ($T_m$) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C. lower than the thermal melting point ($T_m$); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10° C. lower than the thermal melting point ($T_m$); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C. lower than the thermal melting point ($T_m$). Using the equation, hybridization and wash compositions, and desired T, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T of less than 45° C. (aqueous solution) or 32° C. (formamide solution), the SSC concentration is increased so that a higher temperature can be used. Generally, highly stringent hybridization and wash conditions are selected to be about 5° C. lower than the thermal melting point ($T_m$) for the specific sequence at a defined ionic strength and pH.

**[0199]** An example of highly stringent wash conditions is 0.15 M NaCl at 72° C. for about 15 minutes. An example of stringent wash conditions is a 0.2×SSC wash at 65° C. for 15 minutes. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1×SSC at 45° C. for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6×SSC at 40° C. for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, such as about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30° C. and at least about 60° C. for long probes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2× (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

**[0200]** Very stringent conditions are selected to be equal to the $T_m$, for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1× to 2×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C.

**[0201]** "Operably-linked" molecules refers to the association of moieties to form a single molecule such that the function of one is not affected by the other, e.g., an arrangement of elements wherein the components so described are configured so as to perform their usual function. For example, an aptamer is said to be "operably linked to" or "associated with" a metal chelating group if the aptamer and metal chelating group are situated such that the aptamer still recognizes its target and the metal chelating group still operates as an imageable group. "Operably-linked" in reference to an azido group or an alkynyl group means that the group is affixed to a molecule or surface in such a way as to permit the azido or alkynyl group to undergo a 1,3-dipolar cycloaddition with an alkynyl or azido group, respectively, on a different molecule or surface, as applicable.

**[0202]** The terms "isolated and/or purified" refer to in vitro isolation of a nucleic acid, e.g., a DNA or RNA molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and in certain embodiments, is substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell, e.g., in a vector or plasmid.

**[0203]** The nucleic acid molecules of the invention include double-stranded interfering RNA molecules, which are also useful to inhibit expression of a target gene.

**[0204]** As used herein, the term "recombinant nucleic acid," e.g., "recombinant DNA sequence or segment" refers to a nucleic acid, e.g., to RNA, that has been derived or isolated from any appropriate cellular source, that may be subsequently chemically altered in vitro, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome that has not been transformed with exogenous DNA. An example of preselected RNA "derived" from a source would be a RNA sequence that is identified as a useful fragment within a given organism, and which is then

chemically synthesized in essentially pure form. An example of such RNA "isolated" from a source would be a useful RNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

**[0205]** Nucleic acid molecules having base substitutions (i.e., variants) are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the nucleic acid molecule.

**[0206]** A "control" cell, tissue, sample, or subject is a cell, tissue, sample, or subject of the same type as a test cell, tissue, sample, or subject. The control may, for example, be examined at precisely or nearly the same time the test cell, tissue, sample, or subject is examined. The control may also, for example, be examined at a time distant from the time at which the test cell, tissue, sample, or subject is examined, and the results of the examination of the control may be recorded so that the recorded results may be compared with results obtained by examination of a test cell, tissue, sample, or subject. The control may also be obtained from another source or similar source other than the test group or a test subject, where the test sample is obtained from a subject suspected of having a disease or disorder for which the test is being performed.

**[0207]** A "test" cell, tissue, sample, or subject is one being examined.

**[0208]** The term "affected cell" refers to a cell of a subject afflicted with a disease or disorder, which affected cell has an altered phenotype relative to a subject not afflicted with a disease or disorder. Cells or tissue are "affected" by a disease or disorder if the cells or tissue have an altered phenotype relative to the same cells or tissue in a subject not afflicted with a disease or disorder. A disease or disorder is "alleviated" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is reduced.

**[0209]** A "pathoindicative" cell, tissue, or sample is one which, when present, is an indication that the animal in which the cell, tissue, or sample is located (or from which the tissue was obtained) is afflicted with a disease or disorder. By way of example, the presence of one or more breast cells in a lung tissue of an animal is an indication that the animal is afflicted with metastatic breast cancer.

**[0210]** The terms "cell," "cell line," and "cell culture" may be used interchangeably.

**[0211]** As used herein, a "derivative" of a compound refers to a chemical compound that may be produced from another compound of similar structure in one or more steps, as in replacement of H by an alkyl, acyl, amino, or other chemically synthesized group.

**[0212]** The use of the word "detect" and its grammatical variants is meant to refer to measurement of the species without quantification, whereas use of the word "determine" or "measure" with their grammatical variants are meant to refer to measurement of the species with quantification. The terms "detect" and "identify" are used interchangeably herein.

**[0213]** As used herein, a "detectable marker" or is an atom or a molecule that permits the specific detection of a compound comprising the marker in the presence of similar compounds without a marker. Detectable markers include, but are not limited to, radioactive isotopes, antigenic determinants, enzymes, nucleic acids available for hybridization, chromophores, fluorophores, chemiluminescent molecules, electrochemically detectable molecules, and molecules that provide for altered fluorescence-polarization or altered light-scattering.

**[0214]** A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

**[0215]** A "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

**[0216]** A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

**[0217]** An "effective amount" or "therapeutically effective amount" of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. For example, an effective amount of an SIP receptor antagonist is an amount that decreases the cell signaling activity of the SIP receptor.

**[0218]** As used herein, an "instructional material" or "instructions" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the composition of the invention for its designated use. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the composition or be shipped together with a container which contains the composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the composition be used cooperatively by the recipient.

**[0219]** As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

**[0220]** As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

**[0221]** A "sample," as used herein, refers to a biological sample from a subject, including, but not limited to, normal tissue samples, diseased tissue samples, biopsies, blood, saliva, feces, semen, tears, and urine. A sample can also be any other source of material obtained from a subject who contains cells, tissues, or fluid of interest. A sample can also be obtained from cell or tissue culture.

**[0222]** The term "standard," as used herein, refers to something used for comparison. For example, it can be a known standard agent or compound which is administered or added

to a control sample and used for comparing results when measuring said compound in a test sample. Standard can also refer to an "internal standard," such as an agent or compound which is added at known amounts to a sample and is useful in determining such things as purification or recovery rates when a sample is processed or subjected to purification or extraction procedures before a marker of interest is measured.

**[0223]** A "subject" of analysis, diagnosis, or treatment is an animal. Such animals include mammals, such as a human.

**[0224]** The term "treating" includes prophylaxis of the specific disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition or preventing or eliminating said symptoms. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

**[0225]** A "functional" molecule is a molecule in a form in which it exhibits a property by which it is characterized. By way of example, a functional enzyme is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

**[0226]** The term "inhibit" refers to the ability of a disclosed compound to reduce or impede a described function. In certain embodiments, inhibition is by at least 10%, by at least 25%, by at least 50%, or even by at least 75%.

**[0227]** The term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

**[0228]** The term "parenteral" means not through the alimentary canal but by some other route such as subcutaneous, intramuscular, intraspinal, or intravenous.

**[0229]** "Halo" or "halogen" refers to fluoro, chloro, bromo, or iodo. Alkyl, alkoxy, alkenyl, alkynyl, etc. denote both straight and branched radical or linking groups; but reference to an individual radical such as "propyl" embraces only the straight chain radical or linking group, a branched chain isomer such as "isopropyl" being specifically referred to. Aryl denotes a phenyl radical or linking group, or an ortho-fused bicyclic carbocyclic radical or linking group having about nine to ten ring atoms in which at least one ring is aromatic. Heteroaryl encompasses a radical or linking group, attached via a ring carbon of a monocyclic aromatic ring containing five or six ring atoms consisting of carbon and one to four heteroatoms each selected from non-peroxide oxygen, sulfur, and N(X) wherein X is absent or is H, O, ($C_1$-$C_4$)alkyl, phenyl or benzyl, as well as a radical of an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived therefrom, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto.

**[0230]** The term "Aryl" denotes a phenyl radical or linking group, or an ortho-fused bicyclic carbocyclic radical or linking group, having about nine to ten ring atoms in which at least one ring is aromatic. "Aralkyl or arylalkyl" denotes an alkyl group substituted with an aryl group such as benzyl.

**[0231]** "Linker" or "link" refers to a chemical moiety comprising a covalent bond or a chain or group of atoms that covalently attaches the azide group to the chelation portion of the molecule. Linkers include moieties such as: repeating units of alkylene (—$(CH_2)_n$—), aryl (—$(C_6H_4)$—), alkyloxy (e.g., polyethylenoxy, PEG, polymethyleneoxy) and alkylamino; and diacid esters including succinate, succinamide, diglycolate, malonate, and caproamide.

**[0232]** The term "Het" is a 4-16 membered saturated or unsaturated monocyclic, bicyclic, or tricyclic ring system having 1, 2, 3, or 4 heteroatoms, such as oxygen (—O—), sulfur (—S—), oxygenated sulfur such as sulfinyl (S═O) and sulfonyl ($S(=O)_2$), or nitrogen, or an N-oxide thereof. Het includes "heteroaryl", which encompasses a radical attached via a ring carbon of a monocyclic aromatic ring containing five or six ring atoms consisting of carbon and 1, 2, 3, or 4 heteroatoms, such as non-peroxide oxygen (—O—), sulfur (—S—), oxygenated sulfur such as sulfinyl (S═O) and sulfonyl ($S(=O)_2$), or nitrogen N(X) wherein X is absent or is H, O, ($C_{1-4}$) alkyl, phenyl or benzyl, as well as a radical or linking group, of an ortho-fused bicyclic heterocycle of about eight to ten ring atoms derived there from, particularly a benz-derivative or one derived by fusing a propylene, trimethylene, or tetramethylene diradical thereto. When heteroaryl is an ortho-fused benz-derivative it can be attached via any atom in an aromatic ring (e.g. an atom of the benz-ring).

**[0233]** The term "partially unsaturated", for example, a $C_{1-7}$alkylene which is optionally partially unsaturated, means the named substituent or linking group has one or more unsaturations, such as one or more double bonds, one or more triple bonds, or both.

**[0234]** The term "optional" or "optionally" mean that the subsequently described term, event or condition may be but need not be present or occur, and that the description includes instances where the term, event or condition is present or occurs and instances in which it does not. For example, "optionally substituted" means that the named substituent may be present, but need not be present, and the description includes situations where the named substituent is included and situations where the named substituent is not included.

**[0235]** The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples, but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

## Example 1

### PSMA Aptamers Target PSMA-Expressing Prostate Cancers

**[0236]** The ability of the PSMA aptamers to bind the surface of prostate cancer cells expressing PSMA (LNCaP and 22Rv1 clone 1.7) was tested. A PSMA-negative prostate cancer cell line (PC-3) was used as a control for specificity. The surface expression of PSMA was verified using flow cytometry. To determine whether the PSMA aptamer can bind the surface of cells expressing PSMA, $^{32}$P-labeled PSMA aptamers were incubated with either LNCaP or PC-3 cells. Binding of the PSMA aptamers was specific for cells expressing PSMA.

**[0237]** Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich Co., all restriction enzymes were obtained from New England BioLabs, Inc. (NEB), and all cell culture products were purchased from GIBCO BRL/Life Technologies, a division of Invitrogen™ Corp. Antibodies

were purchased from the following manufacturers: Plk1 (cat #33-1700; Zymed®/Invitrogen™, Carlsbad, Calif.); Erkl K-23 (sc-94; Santa Cruz, Calif.); PSMA (cat# M20454M; Biodesign®, Saco, Me.); β-actin_(cat# A5441; Sigma-Aldrich Inc.); HRP-labeled rabbit anti-mouse IgG secondary antibody (cat# 61-6420 Zymed®/Invitrogen™, Carlsbad, Calif.).

**[0238]** Cell culture: Normal human foreskin fibroblasts cells were maintained at 37° C. and 5% $CO_2$ in DMEM supplemented with 10% FBS. Prostate carcinoma cell lines LNCaP (ATCC no. CRL-1740) were maintained in Ham's F12-K medium supplemented with 10% FBS. PC-3 and 22Rv1(1.7) luciferase expressing cells (obtained from Dr. Michael Henry, U Iowa) were grown in RPMI 1640 medium (GIBCO®) supplemented with 10% FBS (Hyclone), 1 mM non-essential amino acids (GIBCO®), and 100 μg/mL G-418.

**[0239]** $^{32}P$ Binding Assays: PC-3 PSMA-negative or LNCaP and 22Rv1(1.7) PSMA-positive prostate cancer cell lines were used for these experiments. 50,000 PC-3 or LNCaP cells (500 cells/A) in DPBS (including calcium and magnesium) were blocked with 100 μg/mL tRNA and poly (I:C) for 15 min. Blocked cells were then incubated at 37° C. for 30 min with 500,000 cpms of γ-$^{32}P$ end-labeled PSMA aptamers or in block solution. Cells were then washed profusely with DPBS (including calcium and magnesium) and bound/internalized RNAs measured by scintillation counter. % Aptamer Bound was calculated based on input counts. This experiment was performed in triplicate. For determining the relative affinity of the PSMA aptamers, LNCaP cells were fixed in 1% formaldehyde in PBS for 20 min at RT. Fixed cells were washed several times after which cells were diluted and blocked as mentioned above. Cells were then incubated with serial dilutions of γ-$^{32}P$ end-labeled RNAs ranging from 2 nM to 0 nM at 37° C. for 10 min. Bound RNAs were determined by filter binding assay as described in McNamara et al., 2008.

**[0240]** PSMA Cell-Surface Expression: PSMA cell-surface expression was determined by Flow cytometry and/or immunoblotting using antibodies specific to human PSMA. Flow cytometry: HeLa, PC-3, and LNCaP cells were trypsinized, washed three times in phosphate buffered saline (PBS), and counted using a hemocytometer. 200,000 cells ($1\times10^6$ cells/mL) were resuspended in 500 μl of PBS+4% fetal bovine serum (FBS) and incubated at room temperature (RT) for 20 min. Cells were then pelleted and resuspended in 100 μL of PBS+4% FBS containing 20 μg/mL of primary antibody against PSMA (anti-PSMA 3C6: Northwest Biotherapeutics) or 20 μg/mL of isotype-specific control antibody. After a 40 min incubation at RT cells were washed three times with 500 μL of PBS+4% FBS and incubated with a 1:500 dilution of secondary antibody (anti-mouse IgG-APC) in PBS+4% FBS for 30 min at RT. Cells were washed as detailed above, fixed with 400 μL of PBS+1% formaldehyde, and analyzed by Flow cytometry. Immunoblots: HeLa, PC-3, and LNCaP cells were collected as described above. Cell pellets were resuspended in 1×RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% NP-40) containing 1× protease and phosphatase inhibitor cocktails (Sigma) and incubated on ice for 20 min. Cells were then pelleted and 25 μg of total protein from the supernatants were resolved on a 7.5% SDS-PAGE gel. PSMA was detected using an antibody specific to human PSMA (anti-PSMA 3C6; Northwest Biotherapeutics).

**[0241]** Cell-Surface Binding of Aptamers: PC-3 or LNCaP cells were trypsinized, washed twice with 500 μL PBS, and fixed in 400 μL of FIX solution (PBS+1% formaldehyde) for 20 min at RT. After washing cells to remove any residual trace of formaldehyde, cell pellets were resuspended in 1× Binding Buffer (IXBB) (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCJ_2$, 0.01% BSA) and incubated at 37° C. for 20 min. Cells were then pelleted and resuspended in 50 μL of 1xBB (pre-warmed at 37° C.) containing either 400 nM FAM-G labeled PSMA aptamer. Concentrations of FAM-G labeled PSMA aptamer for the relative affinity measurements varied from 0 to 4 μM. Cells were incubated with the RNA for 40 min at 37° C., washed three times with 500 μL of 1xBB pre-warmed at 37° C., and finally resuspended in 400 μL of FIX solution pre-warmed at 37° C. Cells were then assayed using Flow cytometry as detailed above and the relative cell surface binding affinities of the PSMA aptamer was determined.

## Example 2

### Azides for Molecular Targeting

**[0242]** Chemical reagents for reactions presented in this example were ACS grade or better. DOTAzide precursor 1,4,7,10-Tetraazacyclododecane-1,4,7-tris(t-butyl acetate)-10-succinimidyl acetate (DOTA-NHS) of 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) was purchased from Macrocyclics, Inc. (Dallas, Tex., USA, catalog numbers: M140 and B270). 1-amino-3-bromopropane of sufficient purity was purchased from Alfa Aesar (Ward Hill, Mass. USA, stock number: B23254 or L12962). Custom oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa, USA). Alkyne-modified 5'-/5-hexynyl-phosphoramidite (6-hexyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite) was obtained from Glen Research (Sterling, Va. USA; catalog number 10-1908-xx). Copper catalyst was prepared using 99.99+% metals-basis anhydrous copper sulfate ($CuSO_4$) obtained from Sigma-Aldrich, Inc. (Milwaukee, Wis. USA, catalog number: 451657-50G). Water soluble Cu(I) stabilizing ligand tris-hydroxypropyltriazolyl amine (TriL) was employed as described by Graham et al. (Graham, D., et al., *J. Am. Chem. Soc., Perk Trans* 1, 1998, Volume 6, 1132-1138.). Other chemicals and reagents were obtained from either Fisher Scientific (Pittsburg, Pa. USA) or Sigma-Aldrich Chemicals, Inc. (Milwaukee, Wis., USA) and used without further modification. All aqueous experiments and reagents were conducted and prepared using double-distilled deionized 18 Mf water obtained using a model Synthesis Milli-Q Quantam® EX ultra-water purification system, equipped with Ultrapurex Organex (cat. QTUOOEX) and Q-gard® 2 (cat. QGARDOOD2) purification columns (Millipore, Billerica, Mass. USA).

**[0243]** Chemical reactions were conducted under high-purity argon or ultra-pure nitrogen gas unless otherwise stated. Synthesis of DOTAzide and 1-amino-3-azidopropane precursor was conducted in glass under argon. Glass reaction vessels were cleaned by washing with dilute hydrochloric acid (HCl), followed by water, 10 M sodium hydroxide (NaOH), a second water rinse, and final cleansing with 100% ethyl alcohol (EtOH) and finally acetone. After cleaning, glass reaction vessels were oven dried for at least 30 minutes at 80° C. Click chemical reactions were conducted in plastic 0.5 and 1.5 mL snap-cap v-vials, used without further treatments (USA Scientific, Ocala, Fla. USA) under ultra-high-purity nitrogen.

Gravimetric measurements were performed using a model AT261 Deltarange digital balance with a readability of 10 μg (Mettler-Toledo, Toledo, Ohio USA). Mass analysis of low-molecular weight precursors and high-molecular weight oligonucleotides and final bioconjugates were conducted by electrospray ionization (ESI) and MALDI-TOF mass spectrometry. Presence of azide functional groups was confirmed infrared spectrometry. Chemical structure ID NMR data were collected using a Unity Inova 600 MHz NMR spectrometer (Varian, Palo Alto, Calif. USA). NMR analysis was carried out using 7 in, 5 mm ID thin-wall glass NMR tubes (Wilmad Lab Glass, Buena, N.J. USA) in 99.99+% chloroform-d (Sigma-Aldrich). Spectra were collected using a 6 kHz sweep width, centered at 4.5 ppm, with a 2 s acquisition time and 1 s delay. The number of scans was scaled to reasonably achieve a signal to noise ratio of approximately 64:1. Spectra were evaluated qualitatively using the software program NUTS (Acorn NMR, Inc., Livermore, Calif. USA). Retention-time identification and purification of bioconjugates was performed using high performance liquid chromatography using model Agilent 1100 series (Agilent, Santa Clara, Calif. USA) or UltiMate 3000 HPLCs (Dionex, Sunnyvale, Calif. USA). Final purification of desired bioconjugates was carried out by HPLC using a 50×4.6 mm, column Clarity 5μ Oligo RP (cat. 00B-4442-E0, Phenomenex, Torrance, Calif. USA). Desalting of bioconjugate solutions was carried out using Illustra™ G-25 "NAPS" single-use size exclusion columns (Sephadex G-25, in gravity flow mode, using purified water as the mobile phase buffer (cat. 17-0853-02, maximum capacity 1 mg $mL^{-1}$ DNA, GE Healthcare Bio-Sciences Corp, Piscataway, N.J. USA).

**[0244]** 1-amino-3-azidopropane: Precursor 1-amino-3-azidopropane was prepared by reaction of 10% equivalent excess sodium azide ($NaN_3$, 5.39 g dissolved in 10 mL water) with 1-amino-3-bromopropane (8.39 g dissolved in 10 mL water). Reactants were combined by slowly adding $NaN_3$ solution for a total volume of 20 mL and mixed continuously under Ar at 80° C. for 48 hours (under argon) to apparent completion as revealed by iodine TLC staining, employing a mobile phase of 10% methyl alcohol (MeOH) in chloroform ($CHCl_3$). Following the reaction period, the solution was cooled to <10° C. in an ice bath, transferred to a separatory funnel and purification of the desired 1-amino-3-azidopropane product was accomplished by solvent extraction in 30 mL diethyl ether (prepared with dissolution of 0.7 g, 5 pellets NaOH immediately prior to extraction). The extraction was conducted a total of three times (cooling the solution to 10° C. each time), followed by drying over magnesium sulfate, glass wool filtration and careful in vacuo removal of solvents at 40° C. Remaining diethyl ether solvent was removed by final evaporation using a gentle stream of high purity argon to yield 1.5 g of pure product. $^1H$ NMR observed δ 1.19 (s, 2.32H); 1.73 (quintet, 2.0H); 2.81 (triplet, 2.0H); 3.37 (triplet, 2.0H). IR (neat, $cm^{-1}$) observed 2102; theoretical 2100 ($N_3$). LC-MS observed mass M+H 101.2; theoretical 100.12. Although perhaps not necessary for subsequent steps, the final purification step to remove remaining diethyl ether makes interpretation of $^1H$ NMR straightforward (FIG. **1**A-**1**C).

**[0245]** DOTAzide: 2,2',2"-(10-(2-(3-azidopropylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7,-triyl)triacetate was prepared as shown in (FIG. **2**): To a clean 100-mL triple-port glass reaction vessel was added 446 mg (0.67 mmol) DOTA-NHS. The DOTA-NHS reactant was dissolved in approximately 50 mL tetrahydrofuran (THF) to visually-apparent dissolution at room temperature (a few minutes). In a separate 2-mL glass vial, 79 mg (0.79 mmol, 1.2 eq. relative to DOTA-NHS) 1-amino-3-azidopropane was added to approximately 1 mL THF. The solution of 1-amino-3-azidopropane was then added dropwise to the DOTA-NHS solution (while stirring) over a period of about 1 minute. The reaction mixture was then placed in a −20° C. freezer and allowed to react overnight (15 hours). Following the reaction period, the solvent was removed in vacuo and used without further purification for initial click chemistry experiments. Confirmatory mass analysis LC-MS=655.36; ESI-MS=654.35; theoretical 654.84. No trace of DOTA-NHS starting material observed. Minor peaks associated with ionization of t-butyl protecting groups could be identified. Deprotection was carried out by dissolution of dried product in 10 mL DMSO and addition of 5 mL 2 M HCl. The solution was heated at 80° C. overnight, cooled and analyzed without further purification yielding a single-observable mass peak (LC-MS) 485.5 M+H; theoretical 484. (See FIGS. **3**A-**3**B). Function of the prepared DOTAzide for click chemical conjugation to alkyne modified molecular structures was confirmed by performing the click chemical reaction using the prepared DOTAzide and phenyl acetylene (FIG. **4**).

**[0246]** Conjugation by Click Chemistry: A stock solution of a 20mer DNA alkyne-functionalized (via 5' conjugation of 5'-/5-hexynyl-phosphoramidite (6-hexyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite, FIG. **5**) oligonucleotide (2.5 nmol $\mu L^{-1}$ in water) was prepared from purified-dried oligonucleotide obtained from the manufacturer. For experimental preparations, 10 μL aliquots were transferred to 500 μL plastic snap-cap v-vials and stored at −20° C. A solution of deprotected DOTAzide was prepared in a concentration of 25 nmol $\mu L^{-1}$ in water. To achieve the best results, the reactants were combined in the following way: A frozen aliquot of 25 nmol alkyne-modified oligo was removed from the −20° C. freezer and allowed to thaw slowly. To a solution of 100 μL 0.2 M sodium chloride (NaCl) was added 25 nmol (1 μL stock solution) DOTAzide, followed by 1 μL of a 1 μL calcium chloride ($CaCl_2$) solution (1 μmol $Ca^{2+}$). The $Ca^{2+}$ is added to interfere with $Cu^{1+}$ complexation by the DOTAzide ring, potentially inhibiting $Cu^{1+}$ catalytical activity required for the click cycloaddition reaction. The DOTAzide/$Ca^{2+}$ mixture was incubated for 1 hour at 80° C. in a hot water bath, removed and allowed to cool for 15 minutes. During the cooling period, a reagent solution was prepared: To a solution of 100 μL 0.2 M NaCl was added in order, 16 μL TriL solution (30 nmol $\mu L^{-1}$ in 0.2 M NaCl); 1 μL sodium ascorbate (NaAsc, 2.5 μmmol $\mu L^{-1}$ in 0.2 M NaCl); 2.5 μL $CuSO_4$ solution (0.1 M in 0.2 M NaCl). The reagent solution was degassed by application of a gentle stream of ultra-high-purity N2 for 5 min. and capped tightly. The cooled solution of DOTAzide/$Ca^{2+}$ was combined with the thawed oligo aliquot and degassed five minutes. The degassed solutions were then combined and allowed to react for 30 minutes at room temperature with continuous application of a gentle stream of $N_2$. Preparation was confirmed by ESI mass spectrometry and included a slight impurity of known composition that can be removed easily by preparatory HPLC, size exclusion, or other chromatographic methods (FIG. **6**). FIG. **7** shows the structure of difluoromethylene cyclooctyne modified phosphoramidite and Cu-catalyst-free click chemical reaction with DOTAzide.

Example 3

Conjugation an Amine Modified RNA Aptamer to a Reactive NOTA Derivative (2,2',2"-(2-(4-isothiocyanatobenzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid)

**[0247]** Chemical reagents for reactions presented in this example were ACS grade or better. DOTAzide precursor 1,4,7,10-Tetraazacyclododecane-1,4,7-tris(t-butyl acetate)-10-succinimidyl acetate (DOTA-NHS) of 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) was purchased from Macrocyclics, Inc. (Dallas, Tex., USA, catalog numbers: M140 and B270). 1-amino-3-bromopropane of sufficient purity was purchased from Alfa Aesar (Ward Hill, Mass. USA, stock number: B23254 or L12962). Custom oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa, USA). Alkyne-modified 5'-/5-hexynyl-PSMA-A10-3.2 RNA aptamer was custom ordered from Integrated DNA Technologies, Inc. (Iowa City, Iowa). Copper catalyst was prepared using 99.99+% metals-basis anhydrous copper sulfate ($CuSO_4$) obtained from Sigma-Aldrich, Inc. (Milwaukee, Wis. USA, catalog number: 451657-50G). Water soluble Cu(I) stabilizing ligand tris-hydroxypropyltriazolyl amine (TriL) was employed as described by Graham et al. (Graham, D., et al., *J. Am. Chem. Soc., Perk Trans* 1, 1998, Volume 6, 1132-1138.). Other chemicals and reagents were obtained from either Fisher Scientific (Pittsburg, Pa. USA) or Sigma-Aldrich Chemicals, Inc. (Milwaukee, Wis., USA) and used without further modification. All aqueous experiments and reagents were conducted and prepared using double-distilled deionized 18 MΩ water obtained using a model Synthesis Milli-Q Quantam® EX ultra-water purification system, equipped with Ultrapurex Organex (cat. QTUOOEX) and Q-gard® 2 (cat. QGARDOOD2) purification columns (Millipore, Billerica, Mass. USA).

**[0248]** Chemical reactions were conducted under high-purity argon or ultra-pure nitrogen gas unless otherwise stated. Synthesis of 1-amino-3-azidopropane precursor was conducted in as described in Example 2 above. Click chemical reactions were conducted in plastic 0.5 and 1.5 mL snap-cap v-vials, used without further treatments (USA Scientific, Ocala, Fla. USA) under ultra-high-purity nitrogen. Gravimetric measurements were performed using a model AT261 Deltarange digital balance with a readability of 10 μg (Mettler-Toledo, Toledo, Ohio USA). Mass analysis of low-molecular weight precursors and high-molecular weight oligonucleotides and final bioconjugates were conducted by electrospray ionization (ESI) and MALDI-TOF mass spectrometry. Presence of azide functional groups was confirmed infrared spectrometry. Chemical structure $^1$H 1D NMR data were collected using a Unity Inova 600 MHz NMR spectrometer (Varian, Palo Alto, Calif. USA). NMR analysis was carried out using 7 in, 5 mm ID thin-wall glass NMR tubes (Wilmad Lab Glass, Buena, N.J. USA) in 99.99+% chloroform-d (Sigma-Aldrich). Spectra were collected using a 6 kHz sweep width, centered at 4.5 ppm, with a 2 s acquisition time and 1 s delay. The number of scans was scaled to reasonably achieve a signal to noise ratio of approximately 64:1. Spectra were evaluated qualitatively using the software program NUTS (Acorn NMR, Inc., Livermore, Calif. USA). Retention-time identification and purification of bioconjugates was performed using high performance liquid chromatography using model Agilent 1100 series (Agilent, Santa Clara, Calif. USA) or UltiMate 3000 HPLC's (Dionex, Sunnyvale, Calif. USA). Final purification of desired bioconjugates was carried out by HPLC using a 50×4.6 mm, column Clarity 5μ Oligo RP (cat. 00B-4442-E0, Phenomenex, Torrance, Calif. USA). Desalting of bioconjugate solutions was carried out using Illustra™ G-25 "NAPS" single-use size exclusion columns (Sephadex G-25, in gravity flow mode, using purified water as the mobile phase buffer (cat. 17-0853-02, maximum capacity 1 mg $mL^{-1}$ DNA, GE Healthcare Bio-Sciences Corp, Piscataway, N.J. USA).

**[0249]** Amine-modified RNA aptamer: 100 nmoles of 5'-/5-hexynyl-PSMA-A10-3.2 RNA was dissolved in 100 μL of deionized-distilled water. To this solution was added 2000 nmoles 1-amino-3-azidopropane (described above) and the solution was allowed to mix gently several minutes. Meanwhile, in a separate reaction vessel, a reagent solution was prepared containing 100 μL DMSO, 200 μL 0.2 M NaCl, 100 μmoles sodium ascorbate, 14 μmoles triazolyl ligand Cu(I) stabilizer, and 4 μmoles $CuSO_4$. The solution was bubbled gently for several minutes with high purity argon at which time reagent solution was added to the solution containing the RNA and 1-amino-3-azidopropane and the combined solutions were allowed to react under argon for 35 minutes at room temperature. To this solution was added 100 μL of 620 nmole μL-1 DOTA as a scavenger of free Cu in solution. The solution was heated to 65° C. and allowed to incubate with gentle mixing for several minutes and slowly cooled to room temperature. The solutions were transferred to 10,000 MWCO Ambicon spin filters and diluted to 3.5 mL, followed by centrifugation at 3000 rpm until the unfiltered fraction containing the desired product was approximately 250 μL. To this solution was added a second 100 μL of 620 nmole μL-1 DOTA as a scavenger of free Cu in solution and the mixture was allowed to stand at room temperature for 10 minutes, followed by repeated centrifugation using the Ambicon spin filter to a final volume of <500 μL which was subsequently desalted on a NAPS size-exclusion column as described earlier. The final 1 mL eluate of the desalting step was analyzed for RNA concentration by spectrophotometry (Nanodrop, Nanodrop Technologies, Wilmington, Del.). The final solution was then lyophilized overnight and dissolved readily in 75 μL of 0.2 M NaCl. To this solution was added 10 μL 62 nmole $\mu L^{-1}$ DOTA and the solution was heated to 65° C. and allowed to cool slowly. These solutions were purified by RP-HPLC as described above to obtain 5 mL solutions that were subsequently concentrated using 10,000 MWCO Ambicon spin filters as described above to a final volume of <500 mL followed by a final desalting step by NAPS size exclusion chromatography as described above. The solution was freeze dried overnight and removed from the lyophilizer and stored at −20 for five days. A NOTA conjugate of the obtained amine-modified RNA was afforded by addition of the 1460 nmoles of 2,2',2"-(2-(4-isothiocyanatobenzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (NOTA-NCS) in a solution of 50 μL 1 M NaHCO3 at pH 9.5, 40° C., for 5 hours. The solution was then allowed to cool and desalted as described above (NAPS). Samples were lyophilized and stored at −20° C. for three days, redissolved in 75 mL of purified water and purified by RP-HPLC (4%-20% CH3CN (B); 100 mM triethylamine acetate/5% CH3CN (A), 45 minutes, retention time of conjugate 28.1 minutes, collected manually. Final fractions were lyophilized and dissolved in 25 μL pure water. Nanodrop measurements revealed a overall

synthetic yield of 22% and overall purity of 90% with no trace of Cu or Fe contaminants that could interfere with radiolabeling.

**[0250]** Schema of the preparations of DOTA- and NOTA-conjugated RNA aptamer A10-3.2 are provided in FIGS. **8**A-**8**C. Illustration of synthesis and confirmation of the preparation of NOTA and DOTA modified PSMA RNA aptamer by mass spectrometry is shown in FIGS. **9**A-**9**C.

**[0251]** FIGS. **10**A-**10**C show the radiochemical purity achievable by reacting imagable radiometals $^{64}$Cu (FIG. **10**B), $^{68}$Ga (FIG. **10**A) with a NOTA conjugated RNA aptamer and $^{111}$In (FIG. **10**C) with a DOTA conjugated RNA aptamer.

**[0252]** Results of representative cell binding assays of the RNA aptamer radiolabeled with $^{111}$In and $^{64}$Cu when contacted with cells that express PSMA and cells that do not express PSMA are provided in FIGS. **11**A-**11**C. These assays demonstrated that the RNA aptamer binding affinity to PSMA expressing cells was preserved when modified to include a chelator, linker, and radionuclide for imaging or therapy. Chemical conjugation of the NOTA or DOTA chelator to the A 10-3.2 aptamer had minimal effect on binding affinity, and $^{111}$In-DOTA- and $^{64}$Cu-NOTA-labeled A10-3.2 bound with comparable affinity to PSMA-expressing prostate cancer cells.

## Example 4

### In Vivo Imaging of NOTA-Conjugated PSMA-Targeted RNA Aptamer A10-3.2 by Positron Emission Tomography

**[0253]** To evaluate the effectiveness of the [$^{68}$Ga]-NOTA-PSMA-Aptamer described above for imaging prostate cancer by PET, imaging studies were carried out to examine the characteristics of the $^{68}$Ga image quality, and test the ability of the radiolabeled aptamer to accumulate in a flank xenograft model of prostate cancer in the mouse. For these experiments, Athymic nude male mice (nu/nu) 6-10 weeks old were obtained from Harlan Sprague Dawley, Inc. and maintained in a sterile environment according to guidelines established by the US Department of Agriculture and the American Association for Accreditation of Laboratory Animal Care (AAALAC). Xenograft tumors were induced by subcutaneous injection of 22Rv1(1.7) cells as described above and tumor growth was monitored by BLI as described above. For imaging, animals were fasted for 12 hours with ready access to water and preinjected with 100 μL of isotonic saline 30 minutes prior to radiopharmaceutical injection.

**[0254]** Radiolabeling was conducted by elution of the Eckert-Zeigler generator (740 MBq) in 10 mL 0.1 M HCl directly to a post-elution purification cation exchange column. Metal impurities and minor breakthrough of parent $^{68}$Ge were removed by passing 1 mL 80% acetone-0.5 M HCl over the column and discarding to waste. Purified $^{68}$Ga was eluted in 98% acetone-0.05 M HCl directly to a solution (2.5 mL sodium acetate/2.5 mL acetic acid) containing 15 nmole NOTA-conjugated PSMA aptamer and reacted for 20 minutes at 60° C. Final purification of the radiolabeled species was conducted using a StrataX C18 RP column to remove unlabeled $^{68}$Ga. The animal was anesthetized according to approved protocols at 4% isofluorane (maintained at 1.5% through the imaging procedure). The anesthetized animal was fixed prone to the bed holder, kept at a constant temperature of 23° C. for the entire procedure. The final purified [$^{68}$Ga]-PSMA-A10-3.2-RNA aptamer was injected by retroorbital injection of 422 μCi (16 MBq) [$^{68}$Ga]-NOTA-PSMA-Aptamer, with a specific activity of 11 MBq nmole$^{-1}$ NOTA-RNA Aptamer A10-3.2 in 50 μL of isotonic saline, followed by a 120 minute accumulation period.

**[0255]** At 120 minutes post-injection, a 15 min. static scan was undertaken and the animal was revived and recaged according to approved protocols. Bladder accumulation was rapid and obvious, based on examination a preliminary 15 minute scan performed at 60 minutes post-injection and previous studies using $^{32}$P-labeled A10-3.2 PSMA-aptamers (data not shown). Examination of the 120 minute scan demonstrates excellent conspicuity of the xenograft tumor (maximum intensity coronal slice of the right flank shown here, FIG. **12**A-**12**B). These images demonstrate excellent [$^{68}$Ga]-NOTA-PSMA-Aptamer accumulation in the xenograft tumor in sufficient quantity to enable PET imaging of the location of the PSMA expressing tumor.

## Example 5

### In Vivo Imaging of DOTA-Conjugated PSMA-Targeted RNA Aptamer A10-3.2 by Single Photon Emission Computed Tomography

**[0256]** To evaluate the effectiveness of the [$^{111}$In]-DOTA-PSMA-Aptamer described above for imaging prostate cancer by PET, imaging studies were carried out to examine the characteristics of the $^{111}$In image quality, and test the ability of the radiolabeled aptamer to accumulate in a xenograft model of prostate cancer in the mouse. For these experiments, Athymic nude male mice (nu/nu) 6-10 weeks old were obtained from Harlan Sprague Dawley, Inc. and maintained in a sterile environment according to guidelines established by the US Department of Agriculture and the American Association for Accreditation of Laboratory Animal Care (AAALAC). Xenograft tumors were induced by subcutaneous injection of 22Rv1(1.7) cells as described above and tumor growth was monitored by BLI as described above. For imaging, animals were fasted for 12 hours with ready access to water and preinjected with 100 μL of isotonic saline 30 minutes prior to radiopharmaceutical injection.

**[0257]** Radiolabeling was conducted by adding $^{111}$In dissolved in acetate buffer directly to a solution (2.5 mL sodium acetate/2.5 mL acetic acid) containing 3.8 nmole DOTA-conjugated PSMA aptamer and reacted for 30 minutes at 100° C. Final purification of the radiolabeled species was conducted by spin-filter dialysis to remove free $^{111}$In. Radiochemical purity of >98% was achieved for these experiments. The animal was anesthetized according to approved protocols at 4% isofluorane (maintained at 1.5% through the imaging procedure). The anesthetized animal was fixed prone to the bed holder, kept at a constant temperature of 23° C. for the entire procedure. The final purified [$^{111}$In]-PSMA-A10-3.2-RNA aptamer was injected by tail vein injection of 1000 μCui (37 MBq) [$^{111}$In]-DOTA-PSMA-Aptamer, with a specific activity of 37 MBq nmol$^{-1}$ DOTA-RNA Aptamer A 10-3.2 in 50 μL of isotonic saline, followed by a four hour accumulation period.

**[0258]** At four hours post-injection, a 60 min. static scan was undertaken and the animal was revived and recaged according to approved protocols. A second scan was performed at 24 hours post injection. Examination of the 24 hour scan demonstrates excellent tumor targeting affinity and exceptional specificity for the PSMA positive xenograft tumor, while no apparent accumulation was observed in a PSMA negative tumor present on the opposite shoulder of the

animal. FIG. **13**A-**13**C show views of a SPECT/CT imaging study of the biodistribution of [$^{111}$In]-DOTA-PSMA-Aptamer in a xenograft (subcutaneous, 22RV1 right flank) nude mouse model of prostate cancer (injected dose: 622 μCi, 17 MBq) in 100 μL phophate buffered saline (approximately 1 nmole, specific activity 17 MBq nmole$^{-1}$). Image was taken at 24 hours post injection. No apparent accumulation is observed in a large PSMA negative tumor injected in the left shoulder area. The PSMA negative tumors were approximately 40 times the mass of the PSMA positive tumor, which demonstrates excellent accumulation and specificity of the radiolabeled PSMA-Aptamer for a prostate cancer tumor. These images demonstrate excellent [$^{111}$In]-DOTA-PSMA-Aptamer accumulation in the xenograft tumor in sufficient quantity to enable SPECT or SPECT/CT imaging of the location of the PSMA expressing tumor.

**[0259]** Flow cytometry was used to confirm expression of PSMA in the 22Rv1(1.7) cells used for in vitro binding experiments and in vivo xenograft experiments (FIG. **14**). The population of cells labeled with a fluorescent PE-conjugated primary antibody against PSMA is shown in gray, compared with the population of unlabeled cells (unshaded).

**[0260]** Although the foregoing specification and examples fully disclose and enable the present invention, they are not intended to limit the scope of the invention, which is defined by the claims appended hereto.

**[0261]** All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

**[0262]** The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

**[0263]** Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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What is claimed is:

**1**. A conjugate comprising a nucleic acid aptamer that is not more than 45 nucleotides in length comprising the nucleic acid sequence 5'-$n_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA-3' (SEQ ID NO:1), linked to one or more chelating groups, wherein each $n_x$ can be present or absent, wherein when present each $n_x$ represents any nucleotide.

**2**. The conjugate of claim **1**, wherein nucleotides $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a stem structure.

**3**. The conjugate of claim **1**, wherein the nucleic acid aptamer molecule comprises the nucleic acid sequence 5'-AUGCGGAUCAGCCAUGUUUA-3' (SEQ ID NO:2).

**4**. The conjugate of claim **1**, wherein the nucleic acid aptamer molecule comprises the nucleic acid sequence 5'-$n_an_bn_cn_dn_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA$n_en_fn_gn_h$-3' (SEQ ID NO:3).

**5**. The conjugate of claim **4**, wherein nucleotides $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a first stem structure and nucleotides $n_an_bn_cn_d$ and $n_en_fn_gn_h$ are present and hybridize to form a second stem structure.

**6**. The conjugate of claim **1**, wherein the nucleic acid aptamer molecule comprises the nucleic acid sequence 5'-GACGAUGCGGAUCAGCCAUGUUUACGUC-3' (SEQ ID NO:4).

**7**. The conjugate of claim **1**, wherein the nucleic acid aptamer molecule comprises the nucleic acid sequence 5'-$n_{10}n_{11}n_{12}n_{13}n_{14}n_an_bn_cn_dn_1n_2n_3$CGGAUCAGC$n_4n_5n_6$GUUUA$n_en_fn_gn_hn_{15}n_{16}n_{17}n_{18}n_{19}n_{20}n_{21}$-3' (SEQ ID NO:6).

**8**. The conjugate of claim **7**, wherein nucleotides $n_1n_2n_3$ and $n_4n_5n_6$ are present and hybridize to form a first stem structure, nucleotides $n_an_bn_cn_d$ and $n_en_fn_gn_h$ are present and hybridize to form a second stem structure, and nucleotides $n_{10}n_{11}n_{12}n_{13}n_{14}$ and $n_{16}n_{17}n_{18}n_{19}n_{20}$ are present and hybridize to form a third stem structure.

**9**. The conjugate of claim **1**, wherein the nucleic acid aptamer molecule comprises the nucleic acid sequence aptamer A10-3.2 (5'-GGGAGGACGAUGCGGAUCAGC-CAUGUUUACGUCACUCCU-3' (SEQ ID NO:5)).

**10**. The conjugate of claim **1**, wherein a linker links the aptamer to the one or more chelating groups.

**11**. A pharmaceutical composition comprising the conjugate of claim **1** and a pharmaceutically acceptable carrier.

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