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(54) **MRI CONTRAST AGENTS AND HIGH-THROUGHPUT SCREENING BY MRI**

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(57) **ABSTRACT**

The present invention provides an MRI contrast agent, comprising: MRI contrast agent particles, and oligonucleotides, attached to the particles.

29 Claims, 17 Drawing Sheets

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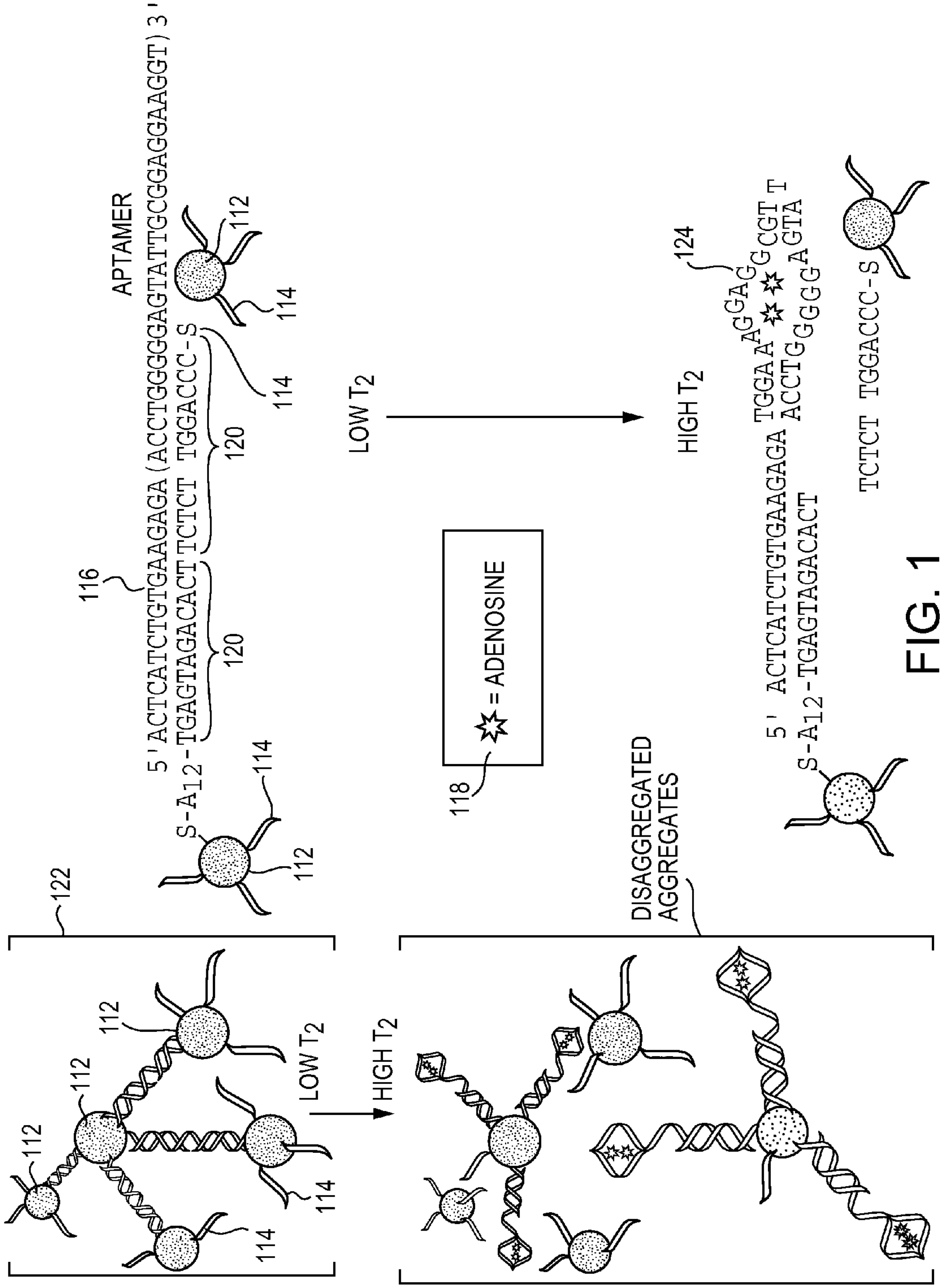
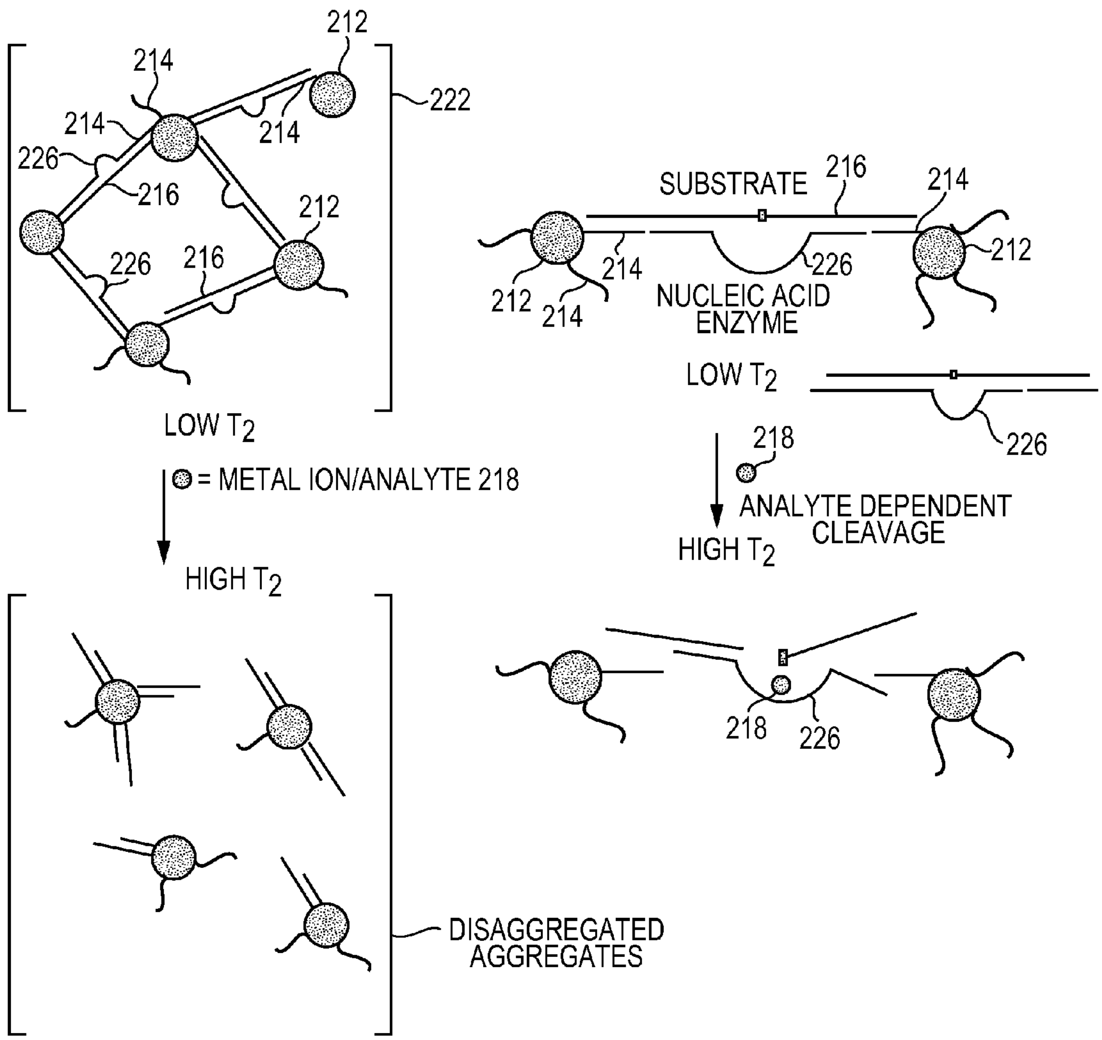


APTAMER
5' ACTCATCTGTGAAGAGA (ACCTGGGGGAGTATTGCGGAGGAAGGT) 3'
S-A12-TGAGTAGACACT TCTCT TGGACCC-S
116
120
120
112
114
122
118
= ADENOSINE
LOW T2
HIGH T2
DISAGGREGATED AGGREGATES
124
5' ACTCATCTGTGAAGAGA
S-A12-TGAGTAGACACT
TCTCT TGGACCC-S

FIG. 1



212
214
222
214
214
226
216
212
226
216
LOW T2
= METAL ION/ANALYTE 218
HIGH T2
DISAGGREGATED
AGGREGATES
SUBSTRATE
216
214
212
214
226
212
214
NUCLEIC ACID
ENZYME
LOW T2
226
218
ANALYTE DEPENDENT
CLEAVAGE
HIGH T2
218
226

FIG. 2

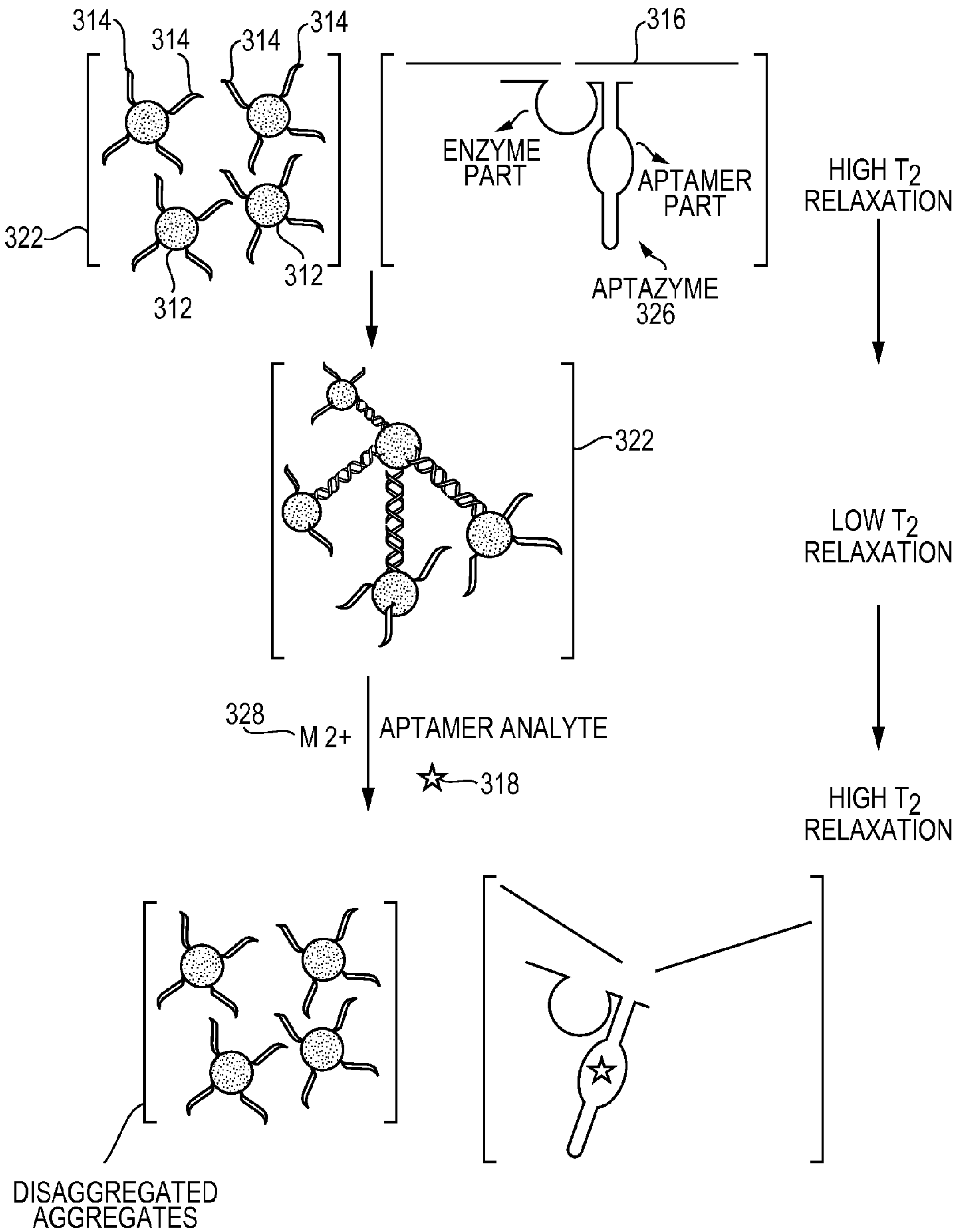
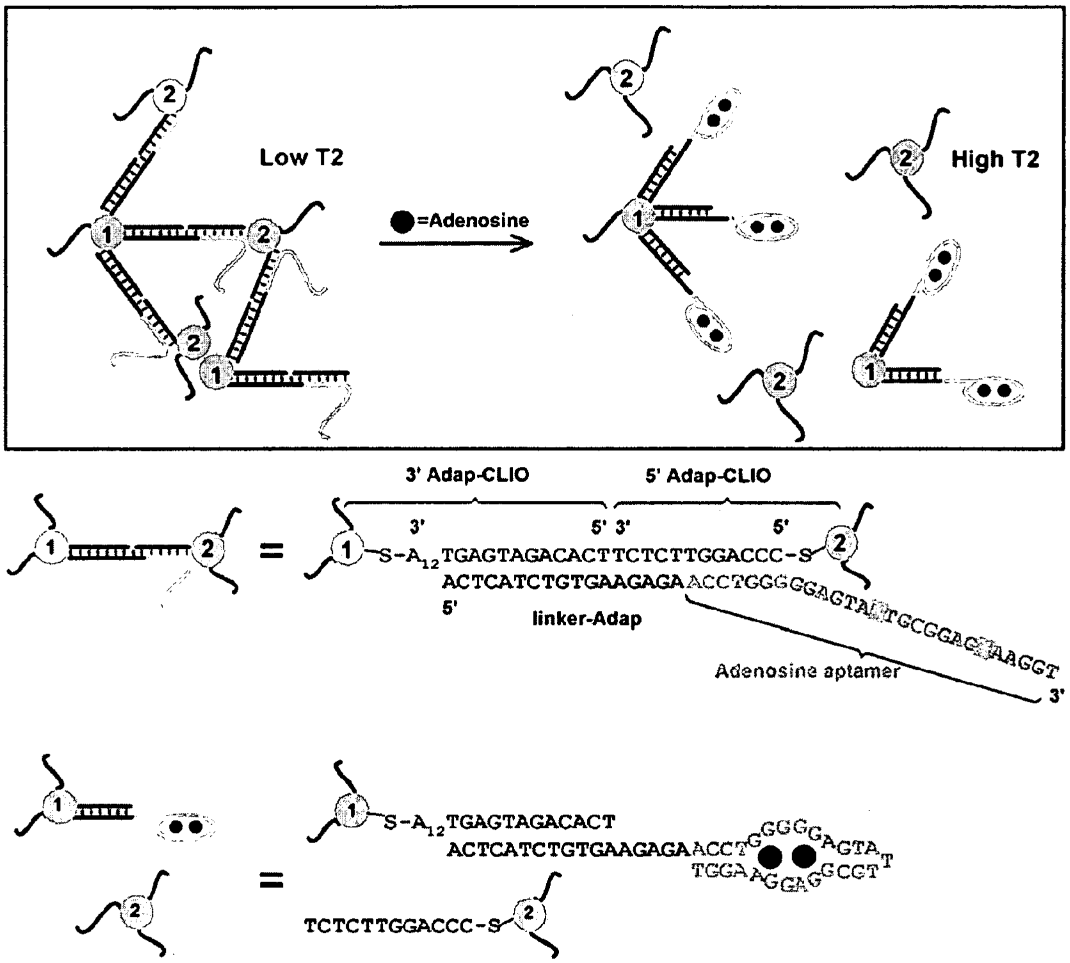

314
314
314
314
316
ENZYME PART
APTAMER PART
HIGH T_2 RELAXATION
322
312
312
APTAZYME 326
322
LOW T_2 RELAXATION
328
M 2+
APTAMER ANALYTE
318
HIGH T_2 RELAXATION
DISAGGREGATED AGGREGATES

FIG. 3



Low T2
=Adenosine
High T2
3' Adap-CLIO
5' Adap-CLIO
3'
5'
3'
5'
S-A12TGAGTAGACACTTCTCTTGGACCC-S
ACTCATCTGTGAAGAGAACCTGGGGGAGTA TGCGGAG AAGGT
5'
linker-Adap
Adenosine aptamer
3'
S-A12TGAGTAGACACT
ACTCATCTGTGAAGAGAACCTGGGGGAGTAT
TGGAAGGAGGCGT
TCTCTTGGACCC-S

FIGURE 4

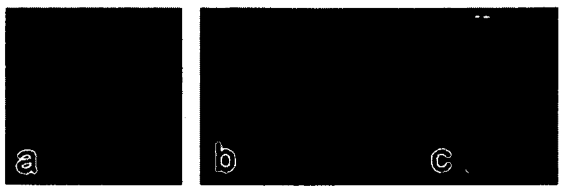


FIGURE 5

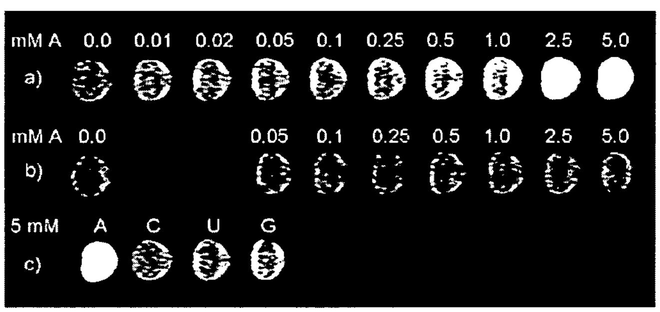


FIGURE 6

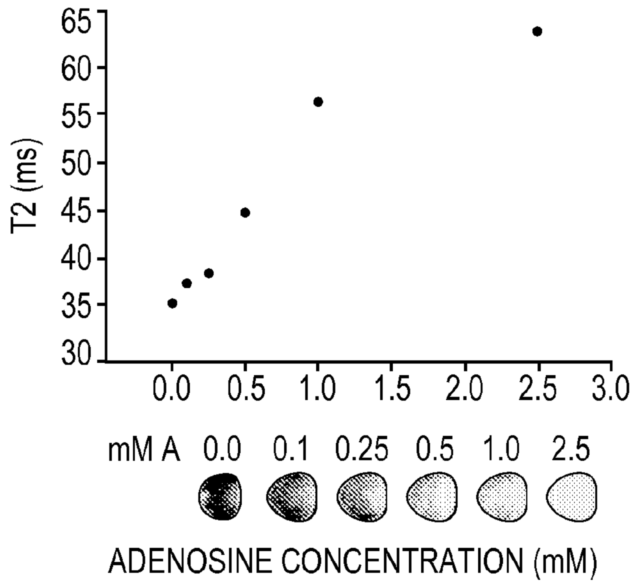

65
60
55
50
45
40
35
30
T2 (ms)
0.0 0.5 1.0 1.5 2.0 2.5 3.0
mM A 0.0 0.1 0.25 0.5 1.0 2.5
ADENOSINE CONCENTRATION (mM)

FIG. 7

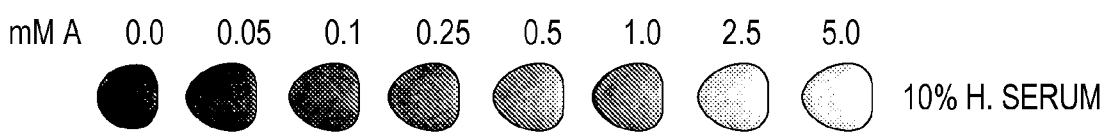

mM A 0.0 0.05 0.1 0.25 0.5 1.0 2.5 5.0
10% H. SERUM

FIG. 8

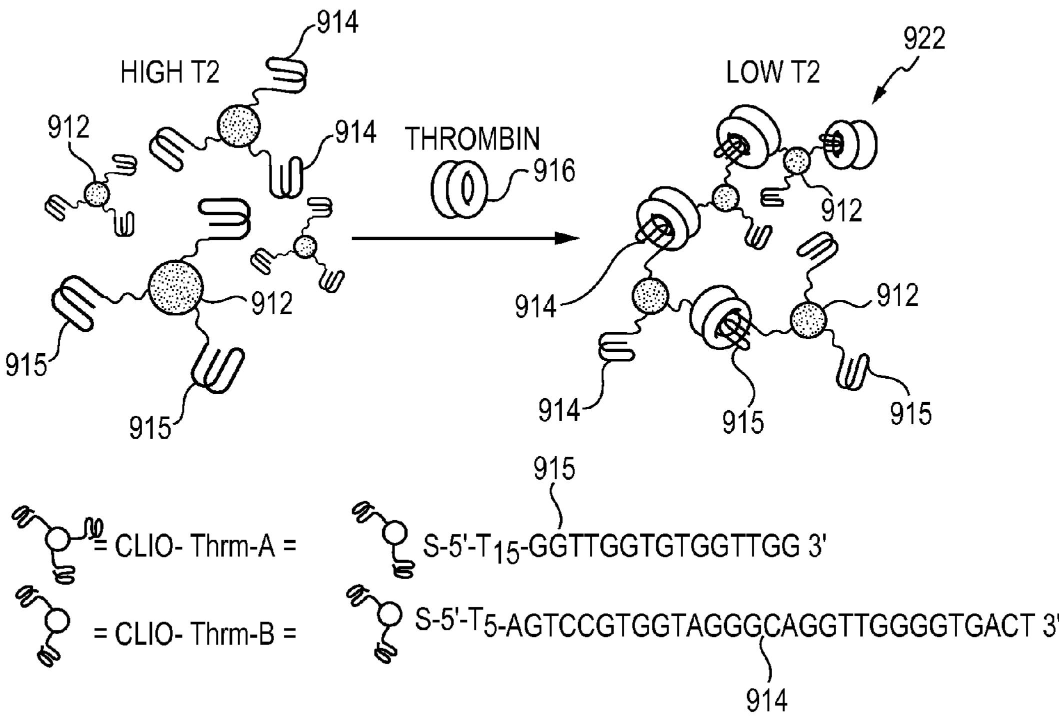

914
HIGH T2
912
914
THROMBIN
916
915
912
915
LOW T2
922
912
914
912
914
915
915
915
= CLIO- Thrm-A =
S-5'-T15-GGTTGGTGTGGTTGG 3'
= CLIO- Thrm-B =
S-5'-T5-AGTCCGTGGTAGGGCAGGTTGGGGTGACT 3'
914

FIG. 9

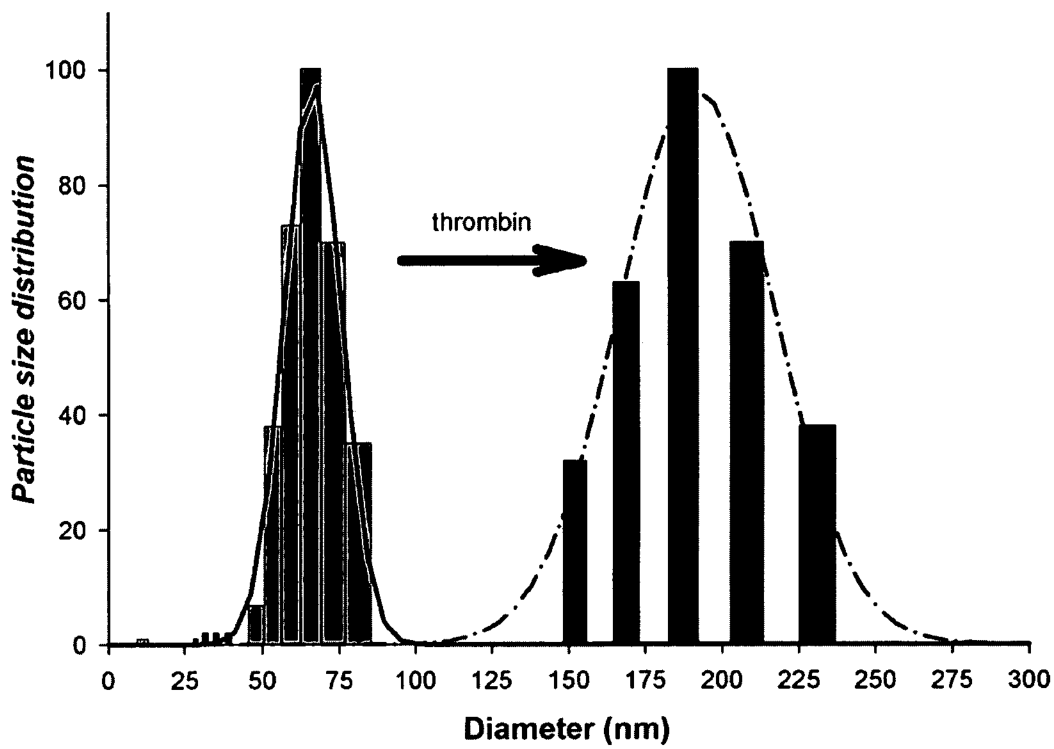

Particle size distribution
0
20
40
60
80
100
thrombin
0
25
50
75
100
125
150
175
200
225
250
275
300
Diameter (nm)

FIGURE 10

$$\text{CLIO-Thrm-A} + \text{CLIO-Thrm-B} \xrightarrow{\text{thrombin}} \text{CLIO-Thrm-A} + [\text{ thrombin }] + \text{CLIO-Thrm-B}$$

FIGURE 11

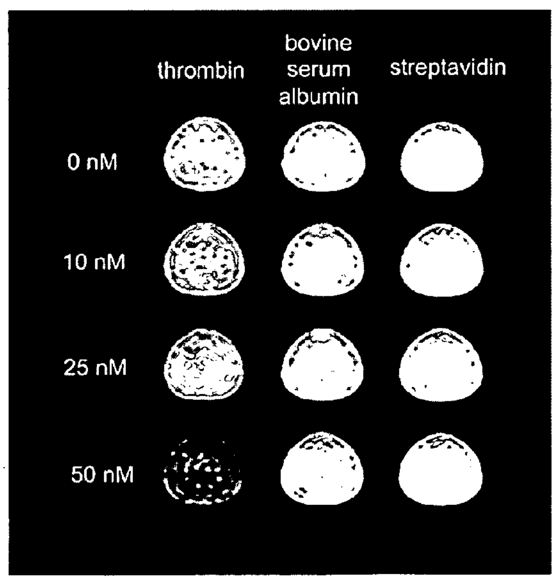


FIGURE 12

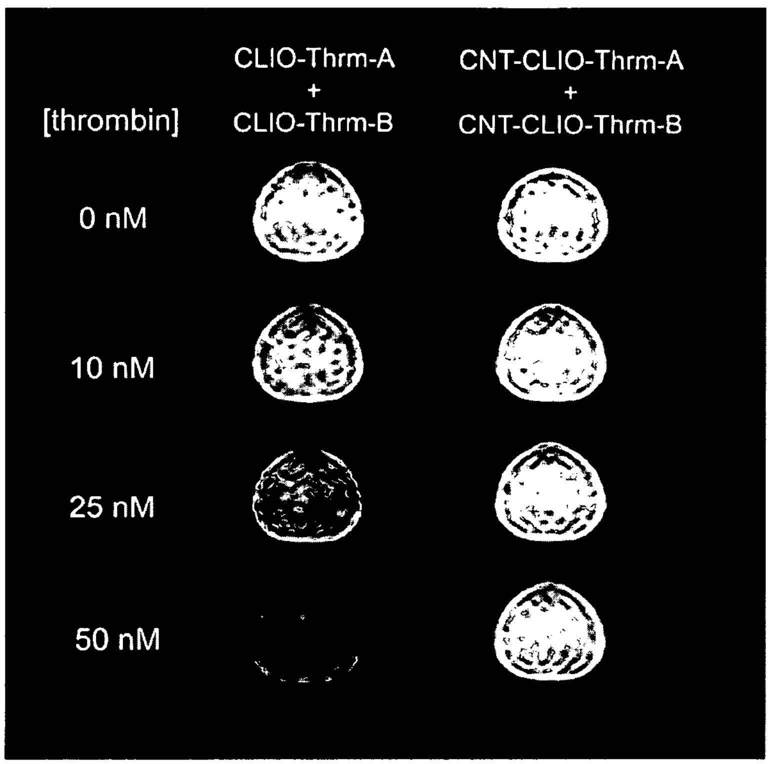

CLIO-Thrm-A
+
CLIO-Thrm-B
CNT-CLIO-Thrm-A
+
CNT-CLIO-Thrm-B
[thrombin]
0 nM
10 nM
25 nM
50 nM

FIGURE 13

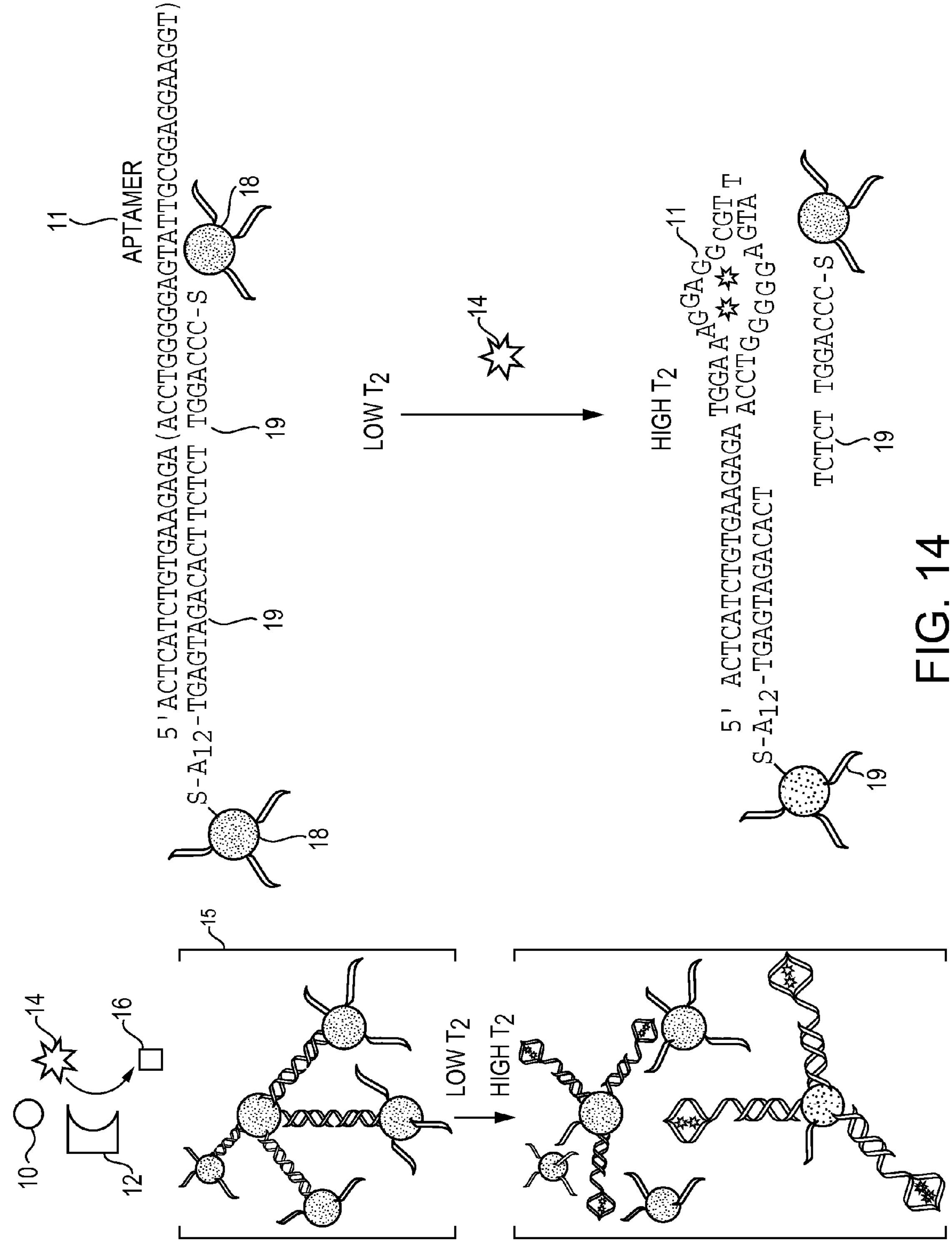
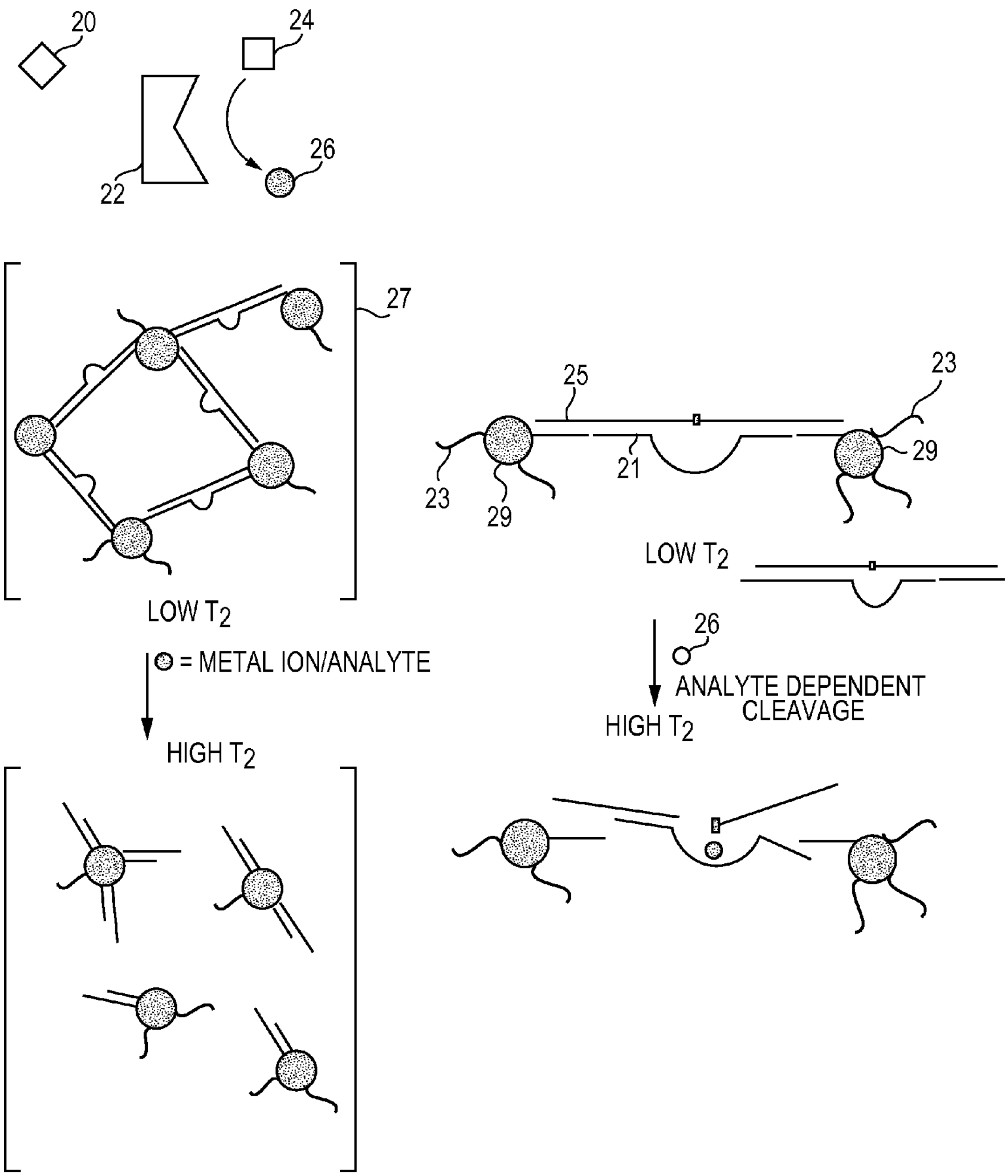
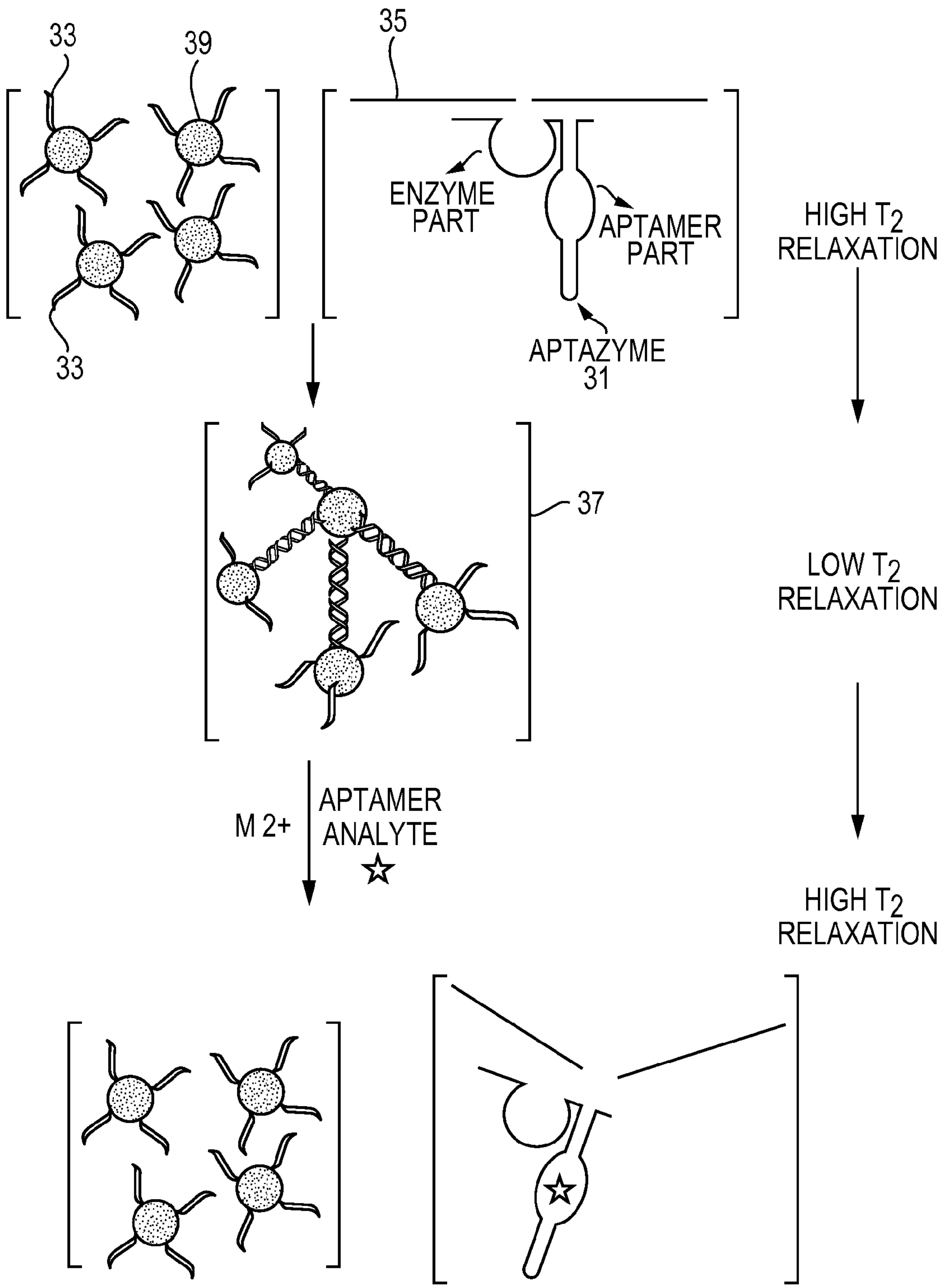

10
12
14
16
15
LOW T2
HIGH T2
11
APTAMER
5' ACTCATCTGTGAAGAGA (ACCTGGGGAGTATTGCGGAGGAAGGT)
S-A12-TGAGTAGACACT TCTCT TGGACCC-S
18
19
14
LOW T2
HIGH T2
5' ACTCATCTGTGAAGAGA TGGA
S-A12-TGAGTAGACACT ACCT
TCTCT TGGACCC-S
11
19

FIG. 14



20
24
22
26
27
25
23
21
29
LOW T_2
= METAL ION/ANALYTE
HIGH T_2
ANALYTE DEPENDENT CLEAVAGE

FIG. 15



33
39
35
33
ENZYME PART
APTAMER PART
APTAZYME 31
HIGH T_2 RELAXATION
37
LOW T_2 RELAXATION
M 2+
APTAMER ANALYTE ☆
HIGH T_2 RELAXATION

FIG. 16

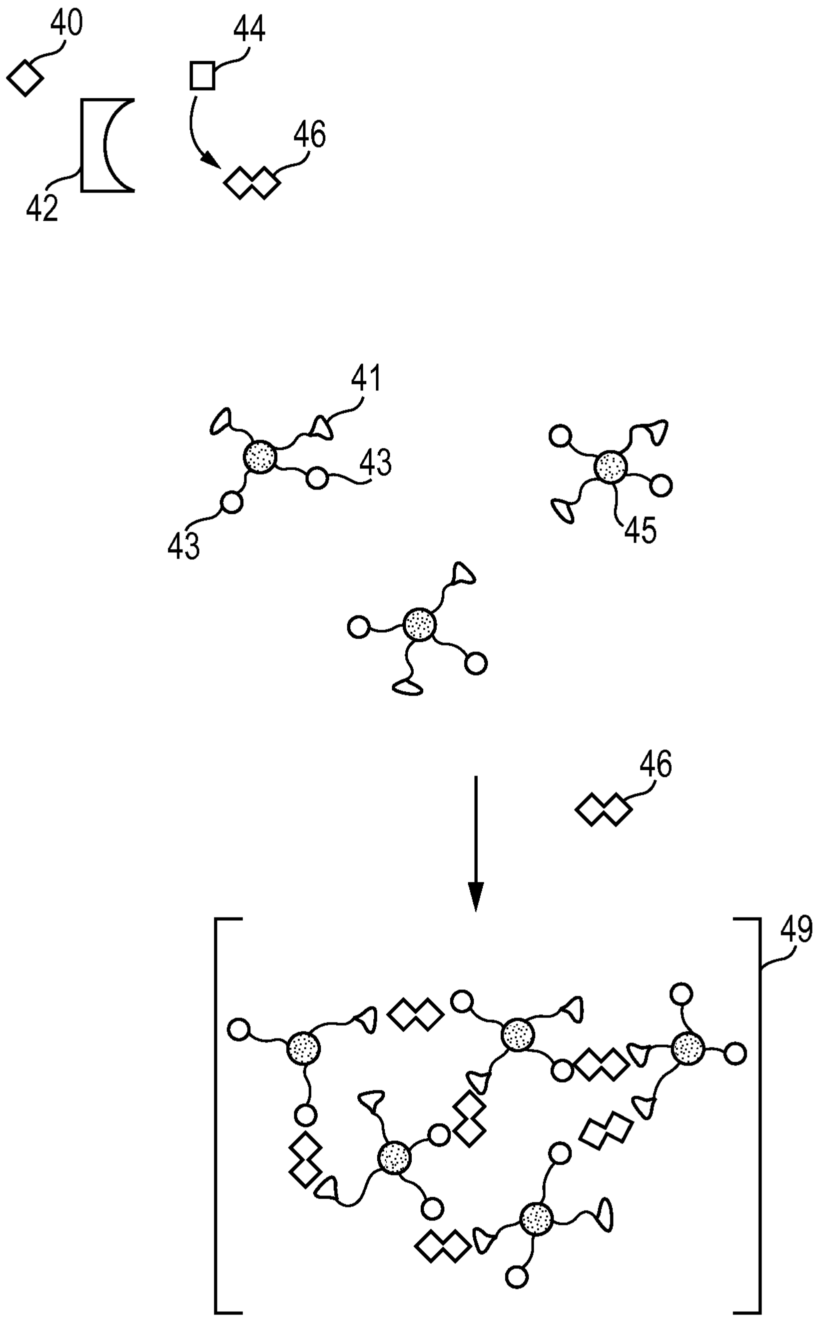


FIG. 17

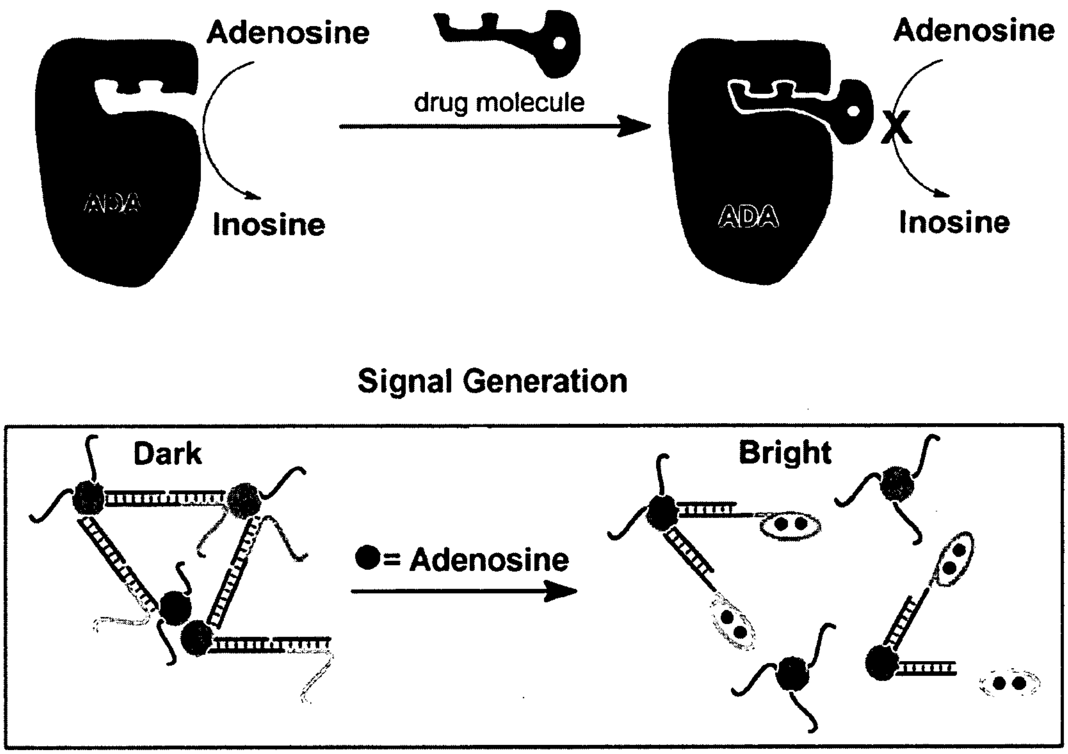

Adenosine
drug molecule
Inosine
ADA
Adenosine
X
Inosine
ADA
Signal Generation
Dark
= Adenosine
Bright

FIGURE 18

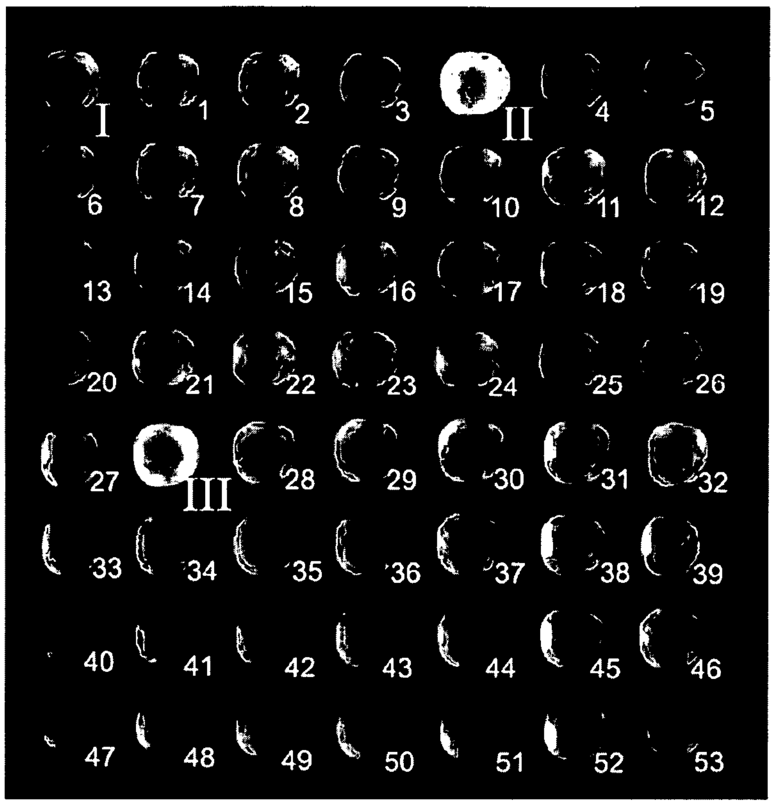

I
1
2
3
II
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
III
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53

FIGURE 19

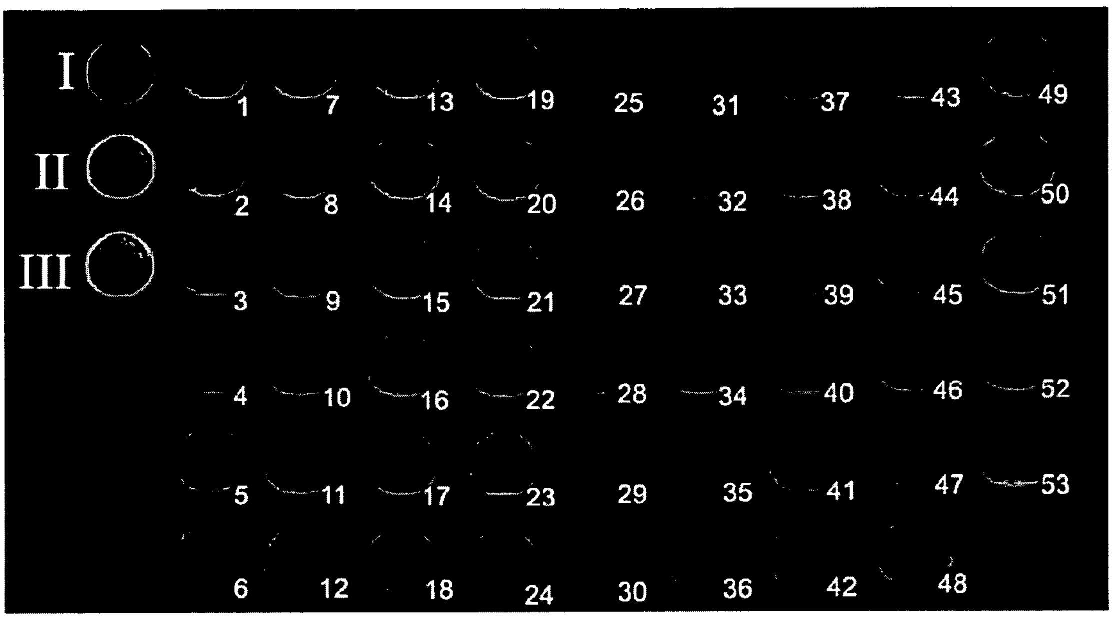

I
II
III
1
7
13
19
25
31
37
43
49
2
8
14
20
26
32
38
44
50
3
9
15
21
27
33
39
45
51
4
10
16
22
28
34
40
46
52
5
11
17
23
29
35
41
47
53
6
12
18
24
30
36
42
48

FIGURE 20

MRI CONTRAST AGENTS AND HIGH-THROUGHPUT SCREENING BY MRI

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Applications Nos. 60/953,193 entitled "MRI Contrast Agents" filed Jul. 31, 2007; and 61/020,659 entitled "High-throughput Screening by MRI" filed Jan. 11, 2008, both of which are incorporated by reference in their entirety.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The subject matter of this application may in part have been funded by the Department of Energy (DE-FG02-01ER63179) and the National Science Foundation (DMR-0117792, DMI-0328162 and CTS-0120978). The government may have certain rights in this invention.

BACKGROUND

Aptamers are single-stranded DNA or RNA molecules which can bind a variety of chemical and biological molecules with high affinity and selectivity. [1-4] They are isolated from a large random pool of DNA or RNA molecules using a combinatorial biology technique called systematic evolution of ligands by exponential enrichment (SELEX) [1,2]. They are often comparable to antibodies in their selective and sensitive binding to a broad range of molecules [5-8]. The major advantage of these molecules lies in the relative ease with which they can be selected for any target analyte and their stability against biodegradation and denaturation. Due to these properties aptamers are good candidates for making chemical and biological sensors in many fields such as medical diagnostics and environmental monitoring. Therefore, these aptamers have been converted into fluorescent [9-22], colorimetric [23-29] and electrochemical sensor [30-33].

For example, U.S. Publ. Pat. No. 20040175693 makes use of the discovery that the cleavage of a nucleic acid substrate by an aptazyme upon binding of an effector can be detected calorimetrically. In the presence of the effector, the substrate is cleaved and aggregated particles are dispersed, resulting in a color change. This system combines the benefit of elements that can recognize any molecule of choice with high sensitivity and ease-of-use provided by calorimetric detection.

While the above aptamer sensors have been widely explored in vitro (See, for example, U.S. Publ. Pat. No. 20030215810), their applications in vivo, particularly in humans, remain a significant challenge because of the difficulty light has in penetrating through skin and signal interference from cellular components.

Magnetic resonance imaging (MRI) is a powerful method for non-invasive three-dimensional imaging of cells and human bodies that is at the base of imaging techniques such as differential tensor imaging (DTI). One active area of research in this rapidly advancing field is development of novel MRI contrast agents, particularly smart agents that are responsive to small or biomolecular markers in cells or human bodies before cellular components or tissues display any MRI differences.

SUMMARY

In a first aspect, the invention provide an MRI contrast agent. The contrast agent comprises: (i) MRI contrast agent particles, and (ii) oligonucleotides, attached to the particles.

In a second aspect, the present invention provides a method of forming an MRI image of a sample with an MRI contrast agent. The contrast agent comprises: (i) MRI contrast agent particles, and (ii) oligonucleotides, attached to the particles. The method comprises: mixing the sample with the MRI contrast agent, and imaging the sample by MRI.

In a third aspect, the invention provides an MRI sensor system for screening a molecule against a test enzyme. The sensor system comprises: (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, wherein each oligonucleotide comprises an aptamer, and (iii) a test enzyme, wherein the test enzyme reacts with a corresponding substrate to form a product.

In a fourth aspect, the invention provides an MRI sensor system for screening a molecule against a test enzyme. The sensor system comprises: (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, (iii) bridges, hybridized to the oligonucleotides, and (iv) a test enzyme, wherein the particles, the oligonucleotides, and the bridges, together form aggregates, the bridges each comprise an aptamer that binds an effector, or the sensor system further comprises an assay enzyme and the bridges are cleaved by the assay enzyme in the presence of an effector, the test enzyme reacts with a corresponding substrate to form a product, and the corresponding substrate or the product is the effector.

In a fifth aspect, the invention provides an MRI sensor system for screening a molecule against a test enzyme. The sensor system comprises: (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, (iii) first and second substrates, hybridized to the oligonucleotides, (iv) an assay enzyme, and (v) a test enzyme, wherein the first and second substrates are ligated by the assay enzyme in the presence of an effector, the test enzyme reacts with a corresponding substrate to form a product, and the corresponding substrate or the product is the effector.

In a sixth aspect, the invention provides a method for screening a molecule against a test enzyme, comprising: forming a mixture comprising the molecule and an MRI sensor system that comprises: (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, wherein each oligonucleotide comprises an aptamer, and (iii) a test enzyme, wherein the test enzyme reacts with a corresponding substrate to form a product; and imaging the mixture by MRI.

In a seventh aspect, the invention provides a method for screening a molecule against a test enzyme, comprising: forming a mixture comprising the molecule and an MRI sensor system that comprises (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, (iii) bridges, hybridized to the oligonucleotides, and (iv) a test enzyme, wherein the particles, the oligonucleotides, and the bridges, together form aggregates, the bridges each comprise an aptamer that binds an effector, or the sensor system further comprises an assay enzyme and the bridges are cleaved by the assay enzyme in the presence of an effector, the test enzyme reacts with a corresponding substrate to form a product, and the corresponding substrate or the product is the effector; and imaging the mixture by MRI.

In an eighth aspect, the invention provides a method for screening a molecule against a test enzyme, comprising: forming a mixture comprising the molecule and an MRI sensor system that comprises: (i) MRI contrast agent particles, (ii) oligonucleotides, attached to the particles, (iii) first and second substrates, hybridized to the oligonucleotides, (iv) an assay enzyme, and (v) a test enzyme, wherein the first and second substrates are ligated by the assay enzyme in the presence of an effector, the test enzyme reacts with a corresponding substrate to form a product, and the corresponding substrate or the product is the effector; and imaging the mixture by MRI.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates an example of an aptamer system. Figure discloses SEQ ID NOS 3, 4, 3, 1 and 2, respectively, in order of appearance.

FIG. **2** illustrates an example of a nucleic acid enzyme system.

FIG. **3** illustrates an example of an aptazyme system.

FIG. **4** illustrates an aptamer system for the detection of adenosine. Figure discloses SEQ ID NOS 4, 3, 1, 3 and 2, respectively, in order of appearance.

FIG. **5** illustrates the aptamer system of FIG. **4** in the presence of the linker (FIG. **5**C), with no linker (FIG. **5**A), and in the presence of a non-complementary sequence linker (FIG. **5**B).

FIG. **6** illustrates the MRI brightness of the aptamer system of FIG. **4** in the presence of adenosine and controls.

FIG. **7** illustrates the changes in T2 relaxation time (spin relaxation time) of the aptamer system of FIG. **4** in the presence of different concentrations of adenosine.

FIG. **8** illustrates the MRI brightness of the aptamer system of FIG. **4** in the presence of 10% human serum and different concentrations of adenosine.

FIG. **9** illustrates a multi-epitope effector system for the detection of thrombin. Figure discloses SEQ ID NOS 5-6, respectively, in order of appearance.

FIG. **10** illustrates the intensity weighted particle size distribution of CLIO nanoparticles with dynamic light scattering (DLS).

FIG. **11** illustrates the aggregation of the functionalized particles of the system of FIG. **9** in the presence of thrombin.

FIG. **12** illustrates the MRI brightness of the system of FIG. **9** in the presence of thrombin and controls.

FIG. **13** illustrates the MRI brightness of the system of FIG. **9** and of a control system with particles that do not bind to thrombin.

FIG. **14** illustrates the screening of molecules with an aptamer system. Figure discloses SEQ ID NOS 3, 4, 3, 1 and 2, respectively, in order of appearance.

FIG. **15** illustrates the screening of molecules with an assaying enzyme system.

FIG. **16** illustrates the screening of molecules with an assaying enzyme system, where the assaying enzyme is an aptazyme.

FIG. **17** illustrates the screening of molecules with a multi-epitope effector system.

FIG. **18** illustrates the screening of ADA inhibitors with an aptamer system.

FIG. **19** illustrates the MRI brightness of an aptamer system according to FIG. **18** in the presence of adenosine and controls, in a tris-acetate buffer.

FIG. **20** illustrates the MRI brightness of an aptamer system according to FIG. **18** in the presence of adenosine and controls, in a human serum buffer.

DEFINITIONS

An "effector" is a molecule that, when bound to an enzyme having an effector binding site, can enhance or inhibit enzyme catalysis, or when bound to an aptamer, causes a conformational change. An "effector binding site" may be "specific," that is, binding only one effector molecule in the presence of other effector molecules. An example of effector binding site specificity is when only Zn(II) ions bind in the presence of many other ions, such as Mn(II), Mg(II) or Pb(II). Alternatively, an effector binding site may be "partially" specific (binding only a class of molecules), or "non-specific" (having molecular promiscuity). Examples of effectors include metal ions, cancer antigens, anthrax, small pox, pollutants (such as nitrogen fertilizers, toxic molecules, etc.), cocaine, human immuno-deficiency virus (HIV) and adenosine.

An "aptamer" is an oligonucleotide or peptide nucleic acid (PNA) molecule that binds a specific effector such as a molecule or ion. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist. Aptamers can be combined with nucleic acid enzymes to provide aptazymes that are active in the presence of the effector. Less preferably, a protein aptamer [98-101] may be used, for example in the multi-binding site effector system.

A "nucleic acid enzyme" is an enzyme that principally contains nucleic acids, such as ribozymes (RNAzymes), deoxyribozymes (DNAzymes), and aptazymes. PNAs are also included. A nucleic acid enzyme usually requires a metal "co-factor" for efficient substrate cleavage and/or specific effector binding. Common co-factors include Mg(II) and Pb(II). In the case of a nucleic acid enzyme that catalyzes a reaction only in the presence of an ion (such as Mg(II)), the term co-factor and effector may be used interchangeably. In the case of an aptazyme that catalyzes a reaction only in the presence of an ion (such as Mg(II)), and a second ion or molecule, the is only referred to as a co-factor, while the second ion or molecule (for which the aptamer portion of the aptazyme has a binding site) is referred to as an effector.

"Polynucleotide", "oligonucleotide" and "oligonucleic acid" are used interchangeably, and refer to a nucleic acid sequence having at least two or more nucleotides. Polynucleotides may contain naturally-occurring nucleotides, unnatural nucleotides and/or modified nucleotides. PNA molecules are also embraced by this term.

"Base-pairing" or "hybridization" refers to the ability of a polynucleotide to form at least one hydrogen bond with another polynucleotide under low stringency conditions. The hydrogen bonds form between complementary bases of the polynucleotides. When each polynucleotide contains a sequential sequence of nucleotides that can form hydrogen bonds to each other, these sequential sequences are referred to as being complementary to each other.

"MRI contrast agent" refers to an agent that increase the contrast between different parts of a sample, by altering the relaxation times during MRI. MRI contrast agents are classified by the different changes in relaxation times after their addition to a sample.

A first class of MRI contrast agents, referred to as positive contrast agents or T1 contrast agents, causes a reduction in the spin-lattice relaxation time, or T1. The class of MRI contrast agents known as negative contrast agents or T2 contrast agents cause a decrease in the spin-spin relaxation time, or T2.

DETAILED DESCRIPTION

The present invention makes use of the discovery that the formation of aggregates of MRI contrast agent particles, or the disaggregation of these aggregates, will change the T1 and/or T2 relaxation time of nearby atoms during MRI. Therefore, by causing the aggregates to form or disaggregate in specific locations within a sample or a subject, contrast in an MRI image may be enhanced. By coupling the formation or disaggregation of the aggregates, to a sensor system for detecting an effector, the MRI contrast agents of the present invention may be used to provide increased contrast in an MRI image corresponding to the location(s) of the effector in the sample.

The active contrast agents of the present invention contain at least three parts: (i) MRI contrast agent particles; (ii) oligonucleotides, attached to the particles; and (iii) bridges, which bridge together the oligonucleotides, to form aggregates of the MRI contrast agent particles, or (iii') first and second substrates, that can form bridges. Optionally, (iv) enzymes may be included. The enzymes catalyze the cleavage of the bridges, or the formation of the bridge from the first and second substrates.

There are three major systems of MRI contrast agents of the present invention:

(1) The Aptamer System: this system uses as the bridge a molecule that comprises an aptamer, specific for an effector. The oligonucleotides attached to the MRI contrast agent particles are selected so that free ends of the oligonucleotides will hybridize with the aptamer: the aptamer hybridizes with two oligonucleotides attached to different particles, thereby bridging the particles together, to form aggregates. When the aptamer binds to the effector, it undergoes a conformational change, which prevents hybridization with the oligonucleotides. This causes the aggregate of particles to disaggregate.

(2) The Enzyme System: this system uses as the bridge a substrate for an enzyme. The oligonucleotides attached to the MRI contrast agent particles are selected so that free ends of the oligonucleotides will hybridize with the substrate. Also present is an enzyme, for example a nucleic acid enzyme or aptazyme (also called allosteric nucleic acid enzymes), which will cleave the substrate when an effector is present. When the enzyme binds to the effector, it cleaves the substrate. This causes the aggregate of MRI contrast agent particles to disaggregate. This system may also be formed using a ligase as the enzyme; here the enzyme will ligate pre-bridge components (first and second substrates) to form the bridge; this will cause the aggregates to form in the presence of the effector.

(3) The Multi-Epitope Effector System: this system uses the effector as the bridge. The oligonucleotides attached to the MRI contrast agent particles are selected to each include one of at least two different aptamers for the effector, each aptamer binding to different sites, or epitopes, on the effector. When the effector is present, the oligonucleotides will attach to it, thereby bridging the particles together and forming the aggregates.

These systems combine the benefit of elements that can recognize any molecule with high sensitivity, selectivity and ease-of-use, with an MRI contrast agent, thereby providing novel contrast agents for the detection of pathologies, such as tumors in humans, animals and excised tissue samples. For example, the effector can be an antigen on the surface of cells in a tumor, such as prostate specific membrane antigen (PSMA), extracellular proteins found in tumor matrix such as tenasin-C, growth factors such as basic fibroplastic growth factor and platelet derived growth factor (PDGF), nucleic acid binding proteins such as nuclear factor kB and transcription factor E2F, and peptides such as gonadotropin-releasing hormone. Aptamers have also been developed for a variety of protein targets [68], any of which can be the effector for the MRI contrast agents.

Aptamer System

The aptamer system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles; and

(iii) bridges, each comprising an aptamer for the effector.

FIG. **1** illustrates an aptamer system, featuring aptamers as the bridges. Particles **112** are attached to oligonucleotides **114**, and the aptamers **116** are hybridized to the oligonucleotides **114**. In the absence of the effector, for example adenosine **118**, the complementary portions **120** of the oligonucleotides **114** are hybridized to the aptamer **116**, thus resulting in the aggregation of the particles into aggregates **122**. If the effector is present, its binds to the aptamer to form an aptamer-effector complex **124**, which leads to structure switching of the aptamer, and the resulting dehybridization between the aptamer and the oligonucleotides leads to disaggregation of the aggregates. The presence of the effector thus results in disaggregation, causing the brightness change measured by MRI.

Accordingly, the presence of the effector can be detected by a change in brightness measured via MRI. Also, the concentration or the amount of the effector may be qualified by the amount of change in the brightness. For example, a low concentration of the effector will result in a small amount of cluster disaggregation, and thus a small change in brightness. On the other hand, a high concentration of the effector will result in a large amount of disaggregation and thus a large change in brightness.

The aptamer system can be used in an aptamer MRI sensor system for the high-throughput screening of enzyme inhibitors ("aptamer assaying system"). The aptamer assaying system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles;

(iii) bridges, comprising an aptamer;

(iv) test enzyme; and

(v) a corresponding substrate for the test enzyme.

FIG. **14** illustrates the screening of enzyme inhibitors by means of an aptamer assaying system. Molecule **10** is screened as an inhibitor of test enzyme **12**, which catalyzes the conversion of substrate **14** to product **16**. Whereas substrate **14** binds to aptamer **11**, product **16** does not.

Particles **18** are attached to oligonucleotides **19**, and the aptamer **11** is present in the mixture. If the molecule **10** is not effective in inhibiting test enzyme **12**, substrate **14** is transformed into product **16**, and the complementary portions of the oligonucleotides **19** are hybridized to the aptamer, thus resulting in the aggregation of the particles into aggregate **15**. If the molecule is effective as inhibitor of the test enzyme, then substrate **14** remains present and binds to the aptamer **11**, thereby inducing structure switching of the aptamer. The resulting dehybridization leads to the deaggregation of the clusters.

Alternatively, aptamer **11** may be chosen so that it binds to product **16** and not to substrate **14**. In this case, a molecule effective in inhibiting test enzyme **12** will lead to the formation of aggregates, and vice versa.

If the MRI contrast agent particles are T2 contrast agents, the aggregated state changes the magnetic relaxivity of nearby atoms, for instance protons in the vicinity. Conversely, the deaggregation of the clusters leads to an increase in the T2 relaxation time, which can be imaged as an increase in brightness via MRI. In addition, the deaggregation of the clusters can be used to produce an increase in T1 relaxation time which may also be imaged via MRI.

Accordingly, the efficacy of the molecule as an inhibitor can be detected by an increased brightness measured via MRI.

Enzyme System

The enzyme system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles;

(iii) bridges, each comprising a substrate for an enzyme, or

(iii) first and second substrates for the enzyme that will form bridges; and

(iv) enzymes, the enzymes responsive to an effector.

The enzyme cleaves the bridges when the effector is present, or forms the bridges from the first and second substrates, when the effector is present. Example enzymes include nucleic acid enzymes, aptazymes and protein enzymes.

FIG. **2** illustrates an enzyme system featuring a nucleic acid enzyme as the enzymes, and the bridges are substrates for the enzymes. The oligonucleotides **214**, attached to the particles **212**, are hybridized to the bridges (substrates) **216**. The nucleic acid enzymes **226** are also hybridized to the bridges **216**. The particles, the oligonucleotides, the bridges and the enzymes form aggregates **222**. If the effector **218** is absent, the nucleic acid enzymes are either inactive or show substantially reduced activity, resulting in little or no substrate cleavage, and thus the particles remain aggregated. If the effector is present, the enzymes are actived, and cleave the bridges, causing disaggregation of the aggregates because the link between the particles is broken. The presence of the effector thus results in disaggregation, causing the brightness change measured by MRI.

FIG. **3** illustrates an enzyme system featuring an aptazyme as the enzymes, and the bridges are substrates for the enzymes. The oligonucleotides **314**, attached to the particles **312**, are hybridized to the bridges (substrates) **316**. The aptazymes **326** are also hybridized to the bridges **316**. The particles, the oligonucleotides, the bridges and the aptazymes form aggregates **322**. If the effector **318** is absent (and/or any necessary co-factor **328**), the aptazymes are either inactive or show substantially reduced activity, resulting in little or little substrate cleavage, and thus the particles remain aggregated. If the effector is present (and any necessary co-factor), the enzymes are active and cleave the bridges, causing disaggregation of the aggregates because the link between the particles is broken. Accordingly, the presence of the effector causes a change in brightness measured by MRI.

The enzyme system can be used in an assaying enzyme MRI sensor system for the high-throughput screening of enzyme inhibitors ("assaying enzyme system"). The assaying enzyme system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles;

(iii) bridges, comprising a substrate for an assaying enzyme, or substrates for an assaying enzyme that will form bridges;

(iv) assaying enzyme, the enzyme having an effector binding site.

(v) test enzyme; and

(vi) a corresponding substrate for the test enzyme.

The assaying enzyme cleaves the bridges when the effector is present, or forms the bridges from substrates when the effector is present. Examples of assaying enzymes include nucleic acid enzymes, aptazymes and protein enzymes.

FIG. **15** illustrates the screening of inhibitors of a test enzyme by means of an assaying enzyme system. Molecule **20** is screened as an inhibitor of test enzyme **22**, which catalyzes the conversion of substrate **24** to product **26**. Product **26** is an effector of the assaying enzyme, whereas substrate **24** is not.

The complementary portions of polynucleotides **23** (the polynucleotides attached to the particles **29**) are hybridized to bridge **25** in the presence of a sample containing molecule **20**, substrate **24**, and enzyme **22**. The assaying enzyme **21** is also hybridized to the bridge **25**. The particles, the bridges, the substrates and the assaying enzymes thereby form aggregate **27**.

If molecule **20** is active as an inhibitor of the test enzyme **22**, substrate **24** is not converted into product **26**. As product **26** is absent, the assaying enzyme is either inactive or shows substantially reduced activity, resulting in little or no substrate cleavage, and thus the particles remain aggregated. If molecule **20** is inactive as an inhibitor of test enzyme **22**, product **26** is formed. Consequently, the assaying enzyme **21** is activated and cleaves the bridge **25**, causing deaggregation of the aggregate **27** because the link between the particles is broken by the cleavage of the bridge. The inactivity of molecule **20** thus results in deaggregation, causing the brightness change measured by MRI.

Alternatively, assaying enzyme **21** may be chosen so that substrate **24** is an effector of the assaying enzyme, whereas product **26** is not. In this case, a molecule effective in inhibiting test enzyme **20** will lead to the formation of aggregates.

FIG. **16** illustrates the screening of inhibitors of a test enzyme by means of the assaying enzyme system, where the assaying enzyme is an aptazyme and an oligonucleotide is the bridge. Molecule **30** is screened as an inhibitor of test enzyme **32**, which catalyzes the conversion of substrate **34** to product **36**. Product **36** is an effector of the assaying enzyme, whereas substrate **34** is not.

The complementary portions of the polynucleotide **33** (the polynucleotides attached to the particles **39**) are hybridized to bridge **35** in the presence of a sample containing molecule **30**, test enzyme **32** and the bridge. The assaying aptazyme **31** is also hybridized to the bridge **35**. The particles, the bridges, the substrates and the enzymes thus form aggregate **37**.

If molecule **30** is active as an inhibitor of the test enzyme **32**, substrate **34** is not converted into product **36**. In the absence of **36**, the assaying aptazyme is either inactive or shows substantially reduced activity, resulting in little or no substrate cleavage, and thus the particles remain aggregated. If molecule **30** is inactive as an inhibitor of test enzyme **32**, product **36** is formed. Consequently, the assaying aptazyme **31** is active and cleaves the bridge **35**, causing deaggregation of the aggregate **37** because the link between the particles is broken by the cleavage of the bridge. The inactivity of molecule **30** thus results in deaggregation, causing a change in MRI brightness.

Alternatively, assaying aptazyme **31** may be chosen so that substrate **34** is an effector of the assaying enzyme, whereas product **36** is not. In this case, a molecule effective in inhibiting test enzyme **30** will lead to the formation of aggregates.

Accordingly, the presence of the effector causes an increased brightness as measured by MRI. By including an aptamer recognizing a desired effector, MRI contrast agents for any desired effector can be easily made and used.

Multi-Epitope Effector System

The multi-epitope effector system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles, each oligonucleotide comprising one of at least two aptamers, wherein each of the at least two aptamers binds an effector at different binding sites.

FIG. **9** illustrates a multi-epitope effector system, featuring thrombin as the effector (which acts as the bridge), and CLIO-Thrm-A and CLIO-Thrm-B, two different aptamers of thrombin, as the oligonucleotides. The oligonucleotides, which are two different aptamers **914** and **915**, are attached to the particles **912**. If the effector **916** is absent, the particles and the oligonucleotides remain disaggregated. If the effector is present, it acts as a bridge, and the aptamers each attached to different sites (epitopes) of the effector, causing aggregation to form aggregates **922**. Accordingly, the presence of the effector causes a change in brightness measured by MRI.

The particles are attached to one of at least two aptamers. Each particle may be linked to each of the aptamers, resulting in one type of particle. Alternatively, a first subset of the particles is attached to first aptamers, and a second subset of the particles is attached to second aptamers, etc., resulting in more than one types of particle. Finally, a mixture of the two systems, as well as a random distribution between the two systems may also be used.

The multi-epitope effector system can be used in a multi-epitope MRI sensor system for the high-throughput screening of enzyme inhibitors ("multi-epitope effector assaying system"). The multi-epitope effector assaying system comprises:

(i) MRI contrast agent particles;

(ii) oligonucleotides, attached to the particles, wherein the oligonucleotides comprise a first aptamer that binds to a first binding site on an effector;

(iii) a second aptamer linked to at least one of the particles, wherein the second aptamer binds to a second binding site on the effector;

(iv) test enzyme; and

(v) a corresponding substrate for the test enzyme.

The particles are linked to the first aptamer and the second aptamer. Alternatively, a first subset of the particles is attached to the first aptamer, and a second subset of the particles is attached to the second aptamer.

FIG. **17** illustrates the screening of inhibitors of a test enzyme by means of the multi-epitope effector assaying system. Molecule **40** is screened as an inhibitor of test enzyme **42**, which catalyzes the conversion of substrate **44** to product **46**. Product **46** is an effector of the first aptamer **41** and second aptamer **43**, whereas substrate **44** is not.

If molecule **40** is an effective inhibitor of test enzyme **42**, no product **46** is formed and the particles **45** are dispersed in solution. If molecule **40** is not an inhibitor of the enzyme, product **46** is formed and the aptamers bind thereto, leading to the aggregation of the particles to form aggregate **49**. This will lead to a corresponding change in MRI brightness.

Alternatively, the first aptamer and the second aptamer may be chosen so that substrate **44** is an effector of the assaying enzyme, whereas product **46** is not. In this case, a molecule effective in inhibiting test enzyme **42** will lead to the formation of aggregates.

In vitro selection of aptamers, nucleic acid enzymes and aptazymes

Aptamers and aptazymes that bind a desired effector can be isolated by in vitro selection. In vitro selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification [42, 43]. DNAzymes and RNAzymes with maximized activities or novel catalytic abilities, as well as aptamers, can be obtained using, for example, the technique of systematic evolution of ligands by exponential enrichment (SELEX) [44].

In vitro selection is typically initiated with a large collection (pool) of randomized sequences, usually containing 10^{13}-10^{15} sequence variants. Chemical synthesis of a set of degenerated polynucleotides using standard phosphoramidite chemistry can be used to generate such randomized pools. The 3'-phosphoramidite compounds of the four nucleosides (adenosine, cytosine, guanine, thymidine) are premixed and used to synthesize the polynucleotides; randomness is generated by controlling the ratio of the four phosphoroamidites. Biases can also be achieved, as well as holding a phosphoramidite constant at a specific position. Other strategies for creating randomized DNA libraries include mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis [45, 46, 47]. If in vitro selection of RNA molecules is desired, randomized DNA libraries are first converted to an RNA library by in vitro transcription.

The randomized libraries are then screened for molecules possessing a desired function, such as binding an effector, and are isolated. Separation may be achieved using affinity column chromatography (using, for example, the effector), gel electrophoresis, or selective amplification of a tagged reaction intermediate. The selected molecules are amplified, using, for example, PCR for DNA, or isothermal amplification reaction for RNA. These selected, amplified molecules are then mutated (reintroducing diversity) using, for example, mutagenic PCR to attempt to select for molecules with yet higher activity. These three steps, selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences in vitro have extended the catalytic repertoire of RNA and DNA. Deoxyribozymes catalyze fewer types of reactions compared to ribozymes. The catalytic rate (k_{cat}) of most deoxyribozymes is comparable to that of ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency (k_{cat}/K_m) of nucleic acid enzymes even exceeds protein enzyme catalytic efficiency.

In vitro selection can be used to change the ion specificity or binding affinity of existing nucleic acid enzymes, or to obtain nucleic acid enzymes specific for desired substrates. For example, the Mg^{2+} concentration required for optimal hammerhead ribozyme activity has been lowered using in vitro selection to improve the enzyme performance under physiological conditions [48, 49].

Often nucleic acid enzymes developed for a specific effector by in vitro selection will have activity in the presence of other molecules. For example, 17E deoxyribozyme was developed by in vitro selection for activity in the presence of Zn^{2+}. However, the enzyme showed greater activity in the presence of Pb^{2+} than Zn^{2+}. Although produced in a process looking for Zn^{2+}-related activity, 17E may be used as a sensitive and selective sensor for Pb^{2+}. To produce nucleic acid enzymes with greater selectivity, a negative selection step may be introduced.

Other polynucleotide sequences are useful, including those described in U.S. Pat. No. 6,706,474 [50]. Representative aptazymes and methods for making aptazymes and attaching them to particles are described, for example, in U.S. Publ. Pat. No. 20040175693 [63].

MRI Contrast Agent Particles

Acceptable MRI contrast agent particles comprise paramagnetic materials, such as solid compounds of gadolinium, manganese or iron, or any other paramagnetic material. Examples include superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO), gadonanotubes (a gadolinium contrast agent trapped within a C_{60} or carbon nanotubes), Quantum dots coated or doped with a paramagnetic material [67], or silicates, phosphates, and carbonates of gadolinium, manganese or iron, as well as their complex oxides, or any other silicate, phosphate and carbonate that may be doped with gadolinium, manganese or iron. Furthermore, any existing MRI contrast agent based on paramagnetic compounds may be attached to the surface of a particle, such as alumina or silica, thereby transforming the particle in an MRI contrast agent particle.

In the case where the MRI contrast agent particles are T2 contrast agents, the aggregated state changes the magnetic relaxivity of nearby atoms, for instance protons in the vicinity. Conversely, the disaggregation of aggregates leads to an increase in the T2 relaxation time, which can be imaged as an increase in brightness by MRI. In addition, the disaggregation of the clusters can be used to produce an increase in T1 relaxation time which may also be imaged by MRI.

In order to attach polynucleotides to the particles, the particles, polynucleotides or both are first derivatized. For instance, if the particles are covered with a thin layer of gold, such as by sputtering, polynucleotides derivatized with alkanethiols at their 3'- or 5'-termini readily attach to gold surfaces [51]. A method of attaching 3' thiol DNA to gold surfaces can also be used to attach polynucleotides to the particles [52]. Alkanethiol-derivatized particles can be used to attach polynucleotides. Other functional groups for attaching polynucleotides to solid surfaces include phosphorothioates to attach polynucleotides to gold surfaces [53], as well as substituted alkylsiloxanes, aminoalkylsiloxanes and mercaptoaklylsiloxanes, for binding polynucleotides to oxides such as ceramics, or metals that form an oxide surface coat in air [54]. Polynucleotides terminating in a 5'-thionucleoside or a 3'-thionucleoside may also be used for attaching polynucleotides to solid surfaces. Some methods of attaching polynucleotides are presented in Table 1.

TABLE 1

Systems for attaching polynucleotides to particles

System	Reference
biotin-streptavidin	[55]
Carboxylic acids on aluminum	[56]
disulfides on gold	[57]
Carboxylic acids on oxides	[58, 59]
Carboxylic acids on platinum	[60]
aromatic ring compounds on platinum	[61]
silanes on oxides	[62]

Substrates for aptazymes and nucleic acid enzymes

The substrates for the aptazymes and the nucleic acid enzymes comprises three portions. The first and second portions of the substrate are separated by the third portion that is cleaved by the enzyme in the presence of the effector. When the substrate is a polynucleotide, it is usually modified by extension of the 3'- and 5'-ends by a number of bases which act as “sticky ends” for facilitating hybridization to complementary portions of oligonucleotides attached to the particles. Substrate modification allows complexes comprising substrate-linked particles to be formed without inhibiting the enzyme/substrate interaction. However, where the substrate contains regions not critical for interaction with the nucleic acid enzyme or aptazyme, modification may not be necessary.

Kits

The invention also provides kits for producing an MRI contrast agent, or for producing an MRI image. The kit may comprise, in separate containers, each of the components of the MRI contrast agent. For example, the aptamer system could be supplied in a kit, with the MRI contrast agent particles, with the attached oligonucleotides, in a first container, and the bridges in a second container; optionally, a third container could contain solvent (such as sterile water). For the enzyme system supplied as a kit, for example, the MRI contrast agent particles, with the attached oligonucleotides, may be supplied in a first container, the bridges in a second container (or the first and second substrates supplied in one or two containers), and the enzymes supplied in a last container. For the multi-epitope effector system, for example, the MRI contrast agent particles, with the attached oligonucleotides, may be supplied in a first container, with a second container containing solvent (such as sterile water).

The MRI contrast agent may be ready to administer, being supplied in a unit dosage form, and in a form ready for administer, with or without a pharmaceutically acceptable carrier. For example, the MRI contrast agent may be supplied in a pre-measured syringe, in sterile form, already mixed with a pharmaceutically acceptable carrier, such as a saline solution. When a kit is supplied, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage of the active components.

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain one of more of the reagents, or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, etc.; ceramic, metal or any other material typically employed to hold similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, etc.

The kits may also contain other reagents and items. The reagents may include standard solutions containing known quantities of the effector, dilution and other buffers, pretreatment reagents, etc. Other items which may be provided as part of the kit include syringes, pipettes and containers.

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audiotape, etc. Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

EXAMPLES

The present invention is similar to sensor systems for detection of an analyte, which use the aggregation of disaggregation of particles to cause a color change, such as those described in WO2005/100602, and U.S. Publ. Pat. Nos. 20030215810, 20040175693, 20060166222 and 20070037171. The systems described in these references may be used as MRI contrast agents, if the particles used for color change are replaced with MRI contrast agent particles.

An aptazyme designed for the directed assembly of gold particles for calorimetric detection and quantification of adenosine may be used as a starting point, by replacing the gold particles with MRI contrast agent particles. By replacing the aptamer domain that recognizes adenosine in the exemplary adenosine biosensor with other aptamer domains recognizing pre-selected effectors, colorimetric sensors for any desired effector can be easily made and used. Furthermore, by replacing the catalytic core (the 8-17 motif in this case) with other catalytic cores, similar aptazymes may be engineered.

Example 1

SPIO nanoparticles were chosen as the contrast agents to functionalize by aptamers since it is efficient at dephasing the spins of neighboring water protons, leading to change in T2 [36, 37]. It has also been shown that oligonucleotide functionalized cross-linked dextran coated superparamagnetic iron oxide nanoparticles (CLIOs) form clusters when linked with a complementary sequence [38]. The CLIOs were combined with an adenosine aptamer as shown in FIG. **4**. The adenosine sensor comprised CLIO aggregates that are prepared using three components: CLIO functionalized with 3'- or 5'-thiol modified DNA, called 3'Adap-CLIO and 5'Adap-CLIO respectively and a linker DNA, called linker-Adap that can hybridize to both the 3'- and 5'Adap-CLIOs, leading to the formation of clusters.

One segment of the linker is the sequence for the adenosine aptamer. Seven bases of this aptamer are involved in the hybridization with the 5'Adap-CLIO. In the presence of adenosine the aptamer undergoes structure switching in order to form the adenosine binding pocket [17, 29, 39, 40], which results in disruption of base pairing interactions with 5'Adap-CLIO. The five remaining base pairs between the linker-Adap and the 5'Adap-CLIO are not enough to hold them together at room temperature leading to disassembly of clusters. The dispersed nanoparticles result in a higher T2 as compared to the clusters; and thus the adenosine induced disassembly can be monitored as an increase in T2 values and enhancement in brightness of T2 weighted MRI images.

A mutated, non-adenosine binding mutated linker was also prepared. The two nucleobases highlighted in grey in the adenosine aptamer segment of linker-Adap are the points of mutation (T→A, G→C) in the mutated linker sequence.

CLIO was synthesized according to literature procedures and functionalized with N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) which could be readily coupled to thiol modified DNA (3'Adap or 5'Adap) [38, 41]. Equimolar mixture of 3'Adap-CLIO and 5'Adap-CLIO (both at 100 μg Fe ml^{-1}) were incubated with the linker-Adap which lead to the formation of big clusters of nanoparticles that precipitate out of solution (FIG. **5**C). The same effect was not observed when no linker (FIG. **5**A) or a non-complementary sequence was used (FIG. **5**B).

In order to measure T2, lower concentrations of CLIO-DNA conjugates (~20 μg Fe ml^{-1}) were mixed with linker-Adap so that the sensor clusters do not precipitate out of solution. The prepared sensor was then aliquoted into the wells of a microplate, with progressively increasing amount of adenosine and a T2 weighted MRI image was obtained. An increase in adenosine concentration led to an increase in the brightness of the image, as illustrated in line a) of FIG. **6**. This increase was attributed to the increase in T2 due to disassembly of CLIO clusters into smaller particles. It is worth noting that even at 10 μM adenosine a detectable change in contrast was observed.

To ensure that the observed effect is solely due to specific binding of adenosine by the aptamer rather than other non-specific effects, the above non-adenosine binding mutated linker was used as a control. This mutated linker has been shown not to bind adenosine[17, 29] As was expected, no change in brightness was observed with increasing adenosine concentration, as illustrated in line b) of FIG. **6**. In a separate control aimed at investigating the selectivity of the system, the sensor was incubated with 5 mM of cytidine, uridine or guanosine. As seen in line c) of FIG. **6**, a significant change in contrast was not observed in any of these three cases.

Quantitative analysis was performed by measuring the T2 relaxation times of samples with varying adenosine concentration. A clear increase in T2 values was observed from 36 to 63 ms as the adenosine concentration increased from 0 to 2.5 Mm, as illustrated in FIG. **7**. To demonstrate utility and stability of the current system in vivo, the sensor was prepared in the presence of 10% human serum. The activity of the sensor is retained in the presence of serum. Upon addition of adenosine the contrast within the concentration gradient increases (FIG. **8**).

Experimental Section of Example 1

Materials: All DNA samples were purchased from Integrated DNA Technologies Inc. (Coralville, Iowa). The linker DNA molecules were purified by HPLC, whereas the thiol-modified DNA molecules were purified by the standard desalting method. Adenosine, cytidine, uridine and guanosine were purchased from Aldrich (St. Louis, Mo.). Cross-linked dextran coated superparamagnetic iron oxide nanoparticles (CLIO, 500 μg Fe ml-1) were synthesized and coupled to N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) according to literature procedure and purified with PD-10 column. [41] Thiol modified DNA (3'Adap and 5'Adap) was activated by incubating with eight equivalent of tris(2-carboxyethyl) phosphine hydrochloride (TCEP). Excess TCEP was removed by desalting using a SepPak C-18 cartridge. TCEP-activated thiol modified DNA (50 uM final concentration) was mixed with CLIO-SPDP (400 μg Fe ml-1) in 100 mM phosphate buffer pH 8.0 overnight. Excess DNA was removed by magnetic separation column (Miltenyi Biotec, Auburn, Calif.) from CLIO-DNA conjugates.

Aggregation of CLIO-DNA: 2 μl of 1 mM linker-Adap is added into 200 μl equimolar mixture of 3'Adap-CLIO and 5'Adap-CLIO (100 μg Fe ml-1) in 200 mM NaCl and 100 mM phospate buffer at pH 7.4. The solution was heated to 65° C. and cooled slowly to room temperature. The precipitation occurred within an hour. A non-complementary DNA was used as the linker for the control experiment.

Sensor preparation and MRI detection: 12 μl of 100 μM linker-Adap was added into 4 ml of 3'Adap-CLIO and 5'Adap-CLIO (20 μg Fe ml-1) in 300 mM NaCl and 25 mM tris-acetate buffer at pH 8.0. The solution was heated to 65° C. and cooled to room temperature overnight. 250 μl of sample was aliquoted into the wells of a microplate and varying amounts of adenosine was added in each well. The samples with serum were prepared by adding 10% human serum. Volume change was compensated by addition of distilled water.

T2 weighted MR images were obtained on a 4.7 T NMR instrument using a spin echo pulse sequence with variable echo time (TE=50-100 ms) and repetition time (TR) of 3000 ms. Relaxation times were measured on the same instrument with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence.

Example 2

Thrombin is a serine protease which plays a key role in procoagulant and anticoagulant functions. To demonstrate the aptamer functionalized CLIO nanoparticles for an analyte detection thrombin was chosen to be detected via MRI with the use of aptamers where we combined the CLIO nanoparticles with thrombin aptamers, Thrm-A which binds to the fibrinogen-recognition exosite of thrombin and Thrm-B which binds to the heparin-binding exosite of thrombin, as shown in FIG. **9** [69, 70]. The contrast agent designed for thrombin detection was composed of 1:1 mixture of Thrm-A and Thrm-B functionalized CLIO nanoparticles (CLIO-Thrm-A and CLIO-Thrm-B, respectively) in aqueous solution. In the presence of thrombin, aptamer sequences fold into G-quadruplex arrangement in order to bind to thrombin [70-72]. After attachment of the CLIO nanoparticles to thrombin molecule the disperse nanoparticles assembled into aggregates changing the magnetic relaxation properties of nearby water protons, reducing the T2 relaxation time. This event could be monitored as a decrease in brightness of T2-weighted MRI image of the solution via MRI.

To confirm that the aptamers functionalized nanoparticles bind to thrombin molecules, 1 μM thrombin was added into the 1:1 homogenous mixture of CLIO-Thrm-A and CLIO-Thrm-B (150 μg Fe ml^{-1}), which resulted in rapid precipitation in seconds (FIG. **11**). Similar behavior was not observed when BSA or streptavidin used as an effector. This result indicates that the precipitation of nanoparticles is due to the binding event of an effector and its aptamer. The particle size analysis also showed that upon addition of 50 nM thrombin into mixture of CLIO-Thrm-A and CLIO-Thrm-B (12 μg Fe ml^{-1}), the average diameter of CLIO nanoparticles immediately increased from 66.1±9.1 nm to 190.1±24.8. FIG. **10** shows the intensity weighted particle size distribution of CLIO nanoparticles with dynamic light scattering (DLS) which indicates that the nanoparticles were cross-linked by thrombin molecules therefore increasing the average diameter. At this CLIO nanoparticle concentration no precipitation of nanoparticles was observed. These results strongly suggest that the thrombin binding to aptamers on CLIO nanoparticles induces the assembly of nanoparticles.

This assembly decreased the T2 relaxation time of the neighboring water protons in the medium. Different thrombin concentrations from 0 to 50 nM were tested. A decrease in brightness of the MRI image of the samples was observed as the concentration of thrombin increased (FIG. **12**) which was attributed to decrease in T2 relaxation time. A noticeable change in contrast was observed at as low as 10 nM thrombin and a significant change was observed at 50 nM thrombin.

To ensure that the contrast is solely due to binding event but not any other artifact the system was tested with bovine serum albumin (BSA) and streptavadin. The MRI images obtained with these two analytes showed no difference in contrast as their concentrations increased from 0 to 50 nM. This result suggests that the change in contrast is due to thrombin but not any other effect. In order to check if the change in contrast is due to aptamer and effector binding but not the thrombin molecule itself, inactive variants of the thrombin aptamer that had random DNA sequences and were different in length, were also tested. To do so a 1:1 mixture of inactive DNA aptamers (CNT-Thrm-A and CNT-Thrm-B) functionalized CLIO nanoparticles (CNT-CLIO-Thrm-A and CNT-CLIO-Thrm-B) was prepared. The prepared samples were subjected to same procedure in preparing the CLIO-Thrm-A and CLIO-Thrm-B and then placed into wells of a microplate. Thrombin was added into both systems with an increasing concentration from 0 to 50 nM. The obtained MR images showed that there was change in brightness of the MRI images of samples with CLIO-Thrm-A and CLIO-Thrm-B but no change with CNT-CLIO-Thrm-A and CNT-CLIO-Thrm-B (FIG. **13**).

This result strongly suggests that the change in the MRI signal is due to the active aptamers but not any other non-specific interaction of DNA with thrombin. These two control experiments together strongly indicate that the change in MRI signal is solely due to the binding event of thrombin to the aptamers which then results in assembling CLIO nanoparticles into clusters decreasing the T2 relaxation time of the environment. The system demonstrated here is a strong potent aptamer-CLIO nanoparticle conjugate design for in vivo applications since (1) the CLIO nanoparticles are directly functionalized with aptamers, (2) the binding event is rapid, happening in seconds, (3) The required amount of CLIO nanoparticle for detection is as low as 12 μg Fe ml-1 (4) The MRI signal is due to binding of the aptamers to the target but not disassembly of the clustered CLIO nanoparticles into disperse ones, (5) The average diameter of CLIO nanoparticles is small enough to penetrate into tissue cells which can then carry on and bind to the target tissue, and last but not the least, (6) CLIO nanoparticles and DNA molecules are biocompatible and biodegradable in vivo. The system can be used for real target tissues for in vivo applications such as, prostate-membrane specific antigen (PMSA), which is an enzyme made on the surface of prostate cancer cells, can be targeted by PMSA functionalized CLIO nanoparticles and can be imaged by MRI.

Example 3

Screening of Inhibitors of Adenosine Deaminase (ADA)

ADA is an enzyme converting adenosine into inosine molecule. The screening of ADA inhibitors can be monitored with an aptamer system whereby an MRI signal is generated when a molecule inhibits the ADA.

For example, an aggregate of paramagnetic particles can be used to monitor the screening. To this end, a mixture including paramagnetic particles that are attached to oligonucleotides is prepared. A bridge including an aptamer that binds adenosine is also present in the mixture. If the tested molecule is not effective in inhibiting ADA, adenosine is transformed into inosine, and the complementary portions of the oligonucleotides are hybridized to the bridge. Aggregation of the particles into an aggregate follows, and an increase in MRI contrast is not observed.

If the molecule is effective as inhibitor of ADA, then adenosine remains present, and its binding to the aptamer leads to structure switching of the aptamer. The resulting dehybridization leads to deaggregation of the aggregate, generating an MRI signal (FIG. **18**).

FIGS. **19** and **20** feature MRI images of the screening of 50 molecules as ADA inhibitors. The screening was monitored by incubating with aggregates that included CLIO nanoparticles attached to polynucleotides and a bridge comprising an ADA-binding aptamer. The screening of FIG. **19** was carried out in the presence of tris-acetate buffer at pH 8.0. The screening of FIG. **20** was performed in human serum.

Sample I included ADA and adenosine but none of the screened molecules. Sample II included adenosine but no ADA, thereby resulting in an increase in MRI brightness when the aggregates were added.

Sample III included ADA, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), a known ADA inhibitor, and adenosine. As expected, the EHNA of sample III inhibited the conversion of adenosine into inosine, and the aggregates underwent deaggregation, resulting in a bright MRI image. By contrast, control molecules 1 to 49, listed in Table 2, none of which was known to inhibit ADA, did not induce deaggregation or an increase in MRI signal.

TABLE 2

	Compounds tested as ADA inhibitors
1	Nitrilotriacetic acid
2	PIPES
3	Boric acid
4	MES-Na Salt
5	Vanillin
6	Thiourea
7	Formic acid
8	2,4,6 Trihydroxyacetophenone monohydrate
9	Sucrose
10	TRIS
11	L-leucine
12	N-Lauroyl sarcosine
13	Deoxycholic acid
14	Taurocholic acid
15	L-tyrosine
16	L-histidine
17	L-lysine dihydrochloride
18	L-valine
19	L-tryptophan
20	L-glutamine
21	L-arginine
22	L-Aspartic acid
23	L-methionine
24	L-serine
25	L-cysteine
26	L-Asparagine
27	L-isoleucine
28	D-xylose
29	(−)-Ephedrine hydrochloride
30	Citric acid
31	meso-2,3-dimercaptosuccinic acid
32	Thiosemicarbazide
33	Tetrabutylammonium fluoride hydrate
34	Uracil
35	Imidazole
36	2,4-Dihydroxylbenzaldehyde
37	Cytidine
38	Ethylene diamine dihydrochloride
39	Ethylene diamine tetraacetic acid
40	Dextose
41	Propyl gallate
42	L-arginineamide
43	Inosine
44	d-Biotin
45	HEPES
46	Phenol
47	5-amino-1-pentanol
48	Cystamine dihydrochloride
49	(−)-Riboflavin
50	BLANK
51	BLANK
52	BLANK
53	BLANK

Experimental

DNA samples were purchased from Integrated DNA Technologies Inc. (Coralville, Iowa). The linker DNA was purified by HPLC and the thiol-modified DNA molecules were purified by the standard desalting method. Adenosine, EHNA and ADA were purchased from Sigma-Aldrich (St. Louis, Mo.). Cross-linked, dextran coated superparamagnetic iron oxide nanoparticles (CLIO, 500 μg Fe ml^{-1}) were prepared and coupled to N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and purified with a PD-10 column (Amersham; Piscataway, N.J.). The thiol modified oligos, 3' Adap (5'TCACAGATGAGT-A12-SH 3'(SEQ ID NO: 1)) and 5' Adap (5'SH-CCCAGGTTCTCT 3'(SEQ ID NO: 2)) were incubated with eight equivalents of tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in order to reduce the disulfide bond on thiol modifications. Excess TCEP was removed by desalting using a Sep-Pak C-18 catridge (Waters; Milford, Mass.). TCEP-activated thiol modified DNA (50 μM final concentration) was mixed with CLIO-SPDP (400 μg Fe ml^{-1}) in 100 mM phosphate buffer at pH 8.0 overnight. Excess DNA was removed by magnetic separation column (Miltenyi Biotec; Auburn, Calif.) from the CLIO-DNA conjugates.

Aptamer-functionalized CLIO nanoparticle aggregates were prepared by adding 27 μl of 100 μM linker-Adap to 8 ml of 3' Adap-CLIO and 5'Adap-CLIO (10 μg Fe ml^{-1}) in 300 mM NaCl and 25 mM tris-acetate buffer at pH 8.0. The resulting solution was heated to 65° C. and cooled to room temperature overnight.

The ADA solution was prepared by diluting 75 μl of an ADA stock solution in 6 ml of 300 mM NaCl and 25 mM tris-acetate buffer at pH 8.0, obtaining a final ADA concentration of 500 nM. The molecules to be tested were dissolved in water to a 5 mM final concentration. 50 μl of ADA solution were placed into the wells of a microplate and 4 μl of the solution of a molecule were added to the ADA solution and left to incubate for 30 minutes. 20 μl of 50 mM adenosine were then added to each well and the resulting solutions were incubated for 90 minutes. 150 μl of nanoparticle suspension were then added to each well. After incubating for 60 minutes, MRI images were taken. The samples in serum were prepared by adding 20 μl human serum to 50 μl of the ADA solution before aliquoting into the wells of microplate, and then incubating with the molecules. Volume differences in samples with no ADA were compensated by the addition of 300 mM NaCl and 25 mM tris-acetate buffer.

T2-weighted MRI images were obtained on a 4.7 T NMR instrument using a spin echo pulse sequence with variable echo time (TE=20−100 ms) and a repetition time (TR) of 2000 ms. Relaxation times were measured on the same instrument with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence.

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What is claimed is:

1. An MRI contrast agent, comprising:

(i) MRI contrast agent particles that are biocompatible and biodegradable in vivo,

(ii) oligonucleotides, attached to the particles, wherein the oligonucleotides have one end attached to the particles and a second free end; and

(iii) an aptamer comprising an oligonucleotide sequence that is complementary to the free end of two oligonucleotides attached to different particles, wherein the aptamer is hybridized to the free end of the two oligonucleotides thereby forming a bridge between the particles, wherein the particles, the oligonucleotides, and the aptamer, together form aggregates in absence of the effector and wherein the aptamer, specific for an effector, undergoes a conformational change when bound to the effector, causing the particles to disaggregate.

2. The MRI contrast agent of claim 1, wherein the MRI contrast agent particles comprise gadolinium, manganese, iron, superparamagnetic iron oxide (SPIO), oral SPIO, ultrasmall superparamagnetic iron oxide (USPIO), paramagnetic fullerene pipes, fullerene nanoparticles, nanotubes, gadonanotubes, quantum dots coated or doped with a paramagnetic material, paramagnetic liposomes, a paramagnetic material or perfluorocarbon particles.

3. The MRI contrast agent of claim 1, wherein the effector is adenosine or a metal ion.

4. An MRI contrast agent, comprising:

MRI contrast agent particles that are biocompatible and biodegradable in vivo;

oligonucleotides, attached to the particles, wherein the oligonucleotides have one end attached to the particles and a second free end; and

a nucleic acid enzyme specific for an effector, comprising

(a) an oligonucleotide substrate strand comprising an oligonucleotide sequence that is complementary to the free end of two oligonucleotides attached to different particles, wherein the oligonucleotide substrate strand is hybridized to the free end of the two oligonucleotides thereby forming a bridge between the particles, and

(b) an oligonucleotide enzyme strand which is hybridized to the oligonucleotide substrate strand,

wherein the particles, the oligonucleotides, the oligonucleotide substrate strand and the oligonucleotide enzyme strand, together form aggregates in absence of the effector; and

wherein the oligonucleotide enzyme strand cleaves the oligonucleotide substrate strand in the presence of the effector, causing the particles to disaggregate.

5. The MRI contrast agent of claim 4, wherein the oligonucleotide substrate strand comprises:

first and second portions, hybridized to the free end of the two oligonucleotides attached to the different particles, and

wherein the first and second portions are cleaved by the oligonucleotide enzyme strand in the presence of the effector.

6. A method of forming an MRI image of a sample, comprising:

mixing the sample with the MRI contrast agent of claim 1; and

imaging the sample by MRI.

7. An MRI sensor system for screening a molecule against a test enzyme, comprising:

the MRI contrast agent of claim 1, and

a test enzyme,

wherein the test enzyme reacts with a corresponding substrate to form a product, and wherein the substrate binds to the aptamer but the product does not or wherein the substrate does not bind to the aptamer but the product does.

8. An MRI sensor system for screening a molecule against a test enzyme, comprising:

the MRI contrast agent of claim 4, and

a test enzyme that catalyzes conversion to the effector,

wherein the bridge is cleaved in the presence of the effector.

9. An MRI sensor system for screening a molecule against a test enzyme, comprising:

the MRI contrast agent of claim 4, and

a test enzyme that catalyzes conversion of a corresponding substrate to a product, wherein the corresponding substrate is the effector,

wherein the bridge is cleaved in the presence of the corresponding substrate.

10. An MRI sensor system for screening a molecule against a test enzyme, comprising:

the MRI contrast agent of claim 5

an assay enzyme, and

a test enzyme,

wherein the first and second portions are ligated by the assay enzyme in the presence of an effector,

the test enzyme reacts with a corresponding substrate to form a product, and the corresponding substrate or the product is the effector.

11. The MRI sensor system of claim 7, wherein the MRI contrast agent particles comprise a paramagnetic material.

12. The MRI sensor system of claim 7, wherein the MRI contrast agent particles comprise at least one member selected from the group consisting of gadolinium, manganese, iron, SPIO, oral SPIO, USPIO, paramagnetic fullerene pipes, fullerene nanoparticles, nanotubes, gadonanotubes, quantum dots coated or doped with a paramagnetic material, paramagnetic liposomes and perfluorocarbon particles.

13. The MRI sensor system of claim 8, wherein the MRI contrast agent particles comprise a paramagnetic material.

14. A method of producing an MRI image in vivo, comprising:

administering the MRI contrast agent of claim 1 to a subject; and

imaging the subject by MRI.

15. A method of producing an MRI image in vivo, comprising:

administering the MRI contrast agent of claim 4 to a subject; and

imaging the subject by MRI.

16. An MRI contrast agent, comprising:

MRI contrast agent particles that are biocompatible and biodegradable in vivo;

oligonucleotides, attached to the particles, wherein the oligonucleotides have one end attached to the particles and a second free end; and

an aptazyme specific for an effector and a co-factor, comprising

(a) an oligonucleotide substrate strand comprising an oligonucleotide sequence that is complementary to the free end of two oligonucleotides attached to different particles wherein the oligonucleotide substrate strand is hybridized to the free end of the two oligonucleotides thereby forming a bridge between the particles, and

(b) an oligonucleotide enzyme strand hybridized to the oligonucleotide substrate strand, wherein the oligonucleotide enzyme strand comprises

(i) an aptamer portion comprising a binding site for the effector, and

(ii) an enzyme portion comprising a binding site for the co-factor when hybridized to the oligonucleotide substrate strand,

wherein the particles, the oligonucleotides, the oligonucleotide substrate strand and the oligonucleotide enzyme strand, together form aggregates in absence of the effector and the co-factor; and

wherein the oligonucleotide enzyme strand cleaves the oligonucleotide substrate strand in the presence of the effector and the co-factor, causing the particles to disaggregate.

17. A method of producing an MRI image in vivo, comprising:

administering the MRI contrast agent of claim **16** to a subject; and

imaging the subject by MRI.

18. The MRI contrast agent of claim **1**, wherein the effector is a cancer antigen, growth factor, nucleic acid binding protein, anthrax, small pox, a pollutant, cocaine, or human immuno-deficiency virus (HIV).

19. The MRI contrast agent of claim **4**, wherein the effector is a cancer antigen, growth factor, nucleic acid binding protein, anthrax, small pox, a pollutant, cocaine, adenosine, a metal ion, or human immuno-deficiency virus (HIV).

20. The MRI contrast agent of claim **16**, wherein the effector is a cancer antigen, growth factor, nucleic acid binding protein, anthrax, small pox, a pollutant, cocaine, adenosine, or human immuno-deficiency virus (HIV).

21. The MRI contrast agent of claim **1**, wherein the effector is prostate specific membrane antigen (PSMA), tenasin-C, basic fibroplastic growth factor, platelet derived growth factor (PDGF), nuclear factor kB, transcription factor E2F, or gonadotropin-releasing hormone.

22. The MRI contrast agent of claim **4**, wherein the effector is prostate specific membrane antigen (PSMA), tenasin-C, basic fibroplastic growth factor, platelet derived growth factor (PDGF), nuclear factor kB, transcription factor E2F, or gonadotropin-releasing hormone.

23. The MRI contrast agent of claim **16**, wherein the effector is prostate specific membrane antigen (PSMA), tenasin-C, basic fibroplastic growth factor, platelet derived growth factor (PDGF), nuclear factor kB, transcription factor E2F, or gonadotropin-releasing hormone.

24. The MRI contrast agent of claim **4**, wherein the MRI contrast agent particles comprise gadolinium, manganese, iron, superparamagnetic iron oxide (SPIO), oral SPIO, ultrasmall superparamagnetic iron oxide (USPIO), paramagnetic fullerene pipes, fullerene nanoparticles, nanotubes, gadonanotubes, quantum dots coated or doped with a paramagnetic material, paramagnetic liposomes, a paramagnetic material or perfluorocarbon particles.

25. The MRI contrast agent of claim **16**, wherein the MRI contrast agent particles comprise gadolinium, manganese, iron, superparamagnetic iron oxide (SPIO), oral SPIO, ultrasmall superparamagnetic iron oxide (USPIO), paramagnetic fullerene pipes, fullerene nanoparticles, nanotubes, gadonanotubes, quantum dots coated or doped with a paramagnetic material, paramagnetic liposomes, a paramagnetic material or perfluorocarbon particles.

26. The MRI contrast agent of claim **1**, wherein the MRI contrast agent particles comprise a paramagnetic material.

27. The MRI contrast agent of claim **4**, wherein the MRI contrast agent particles comprise a paramagnetic material.

28. The MRI contrast agent of claim **16**, wherein the MRI contrast agent particles comprise a paramagnetic material.

29. The MRI contrast agent of claim **1**, wherein the effector is thrombin.

* * * * *