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(54) Title: TWIN FLUOROPHORE PEPTIDE NUCLEIC ACID HYBRIDIZATION PROBES

(57) Abstract: The invention provides a conjugate comprising a peptide nucleic acid molecule, wherein the peptide nucleic acid molecule is conjugated to two identical fluorophores through peptidic linkers. Upon binding to a complementary RNA or DNA sequence, the fluorescence of the construct is enhanced, allowing the detection of the binding event. The invention also relates to the identification of a RNA or DNA sequence in vitro or in vivo using such conjugate.



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TWIN FLUOROPHORE PEPTIDE NUCLEIC ACID HYBRIDIZATION PROBES

Cross-Reference to Related Applications

[0001] This application claims priority to U.S. Provisional Application No. 61/132,605, filed June 20, 2008, the entire disclosure of which is incorporated herein by reference.

Reference to Government Grant

[0002] The invention described herein was supported in part by the Government grant number U54 CA105008, awarded by the National Cancer Institute from the National Institutes of Health. The Federal Government may have certain rights in the invention.

Field of Invention

[0003] The invention relates to a conjugate comprising a peptide nucleic acid molecule, wherein each end of the peptide nucleic acid molecule is conjugated to the same fluorophore moiety through a peptidic linker. Upon binding to a complementary RNA or DNA sequence, the fluorescence of the construct is enhanced, allowing the detection of the binding event. The invention also relates to the identification of a RNA or DNA sequence in vitro or in vivo using such conjugate.

Background of Invention

[0004] DNA and RNA store the genetic information of an organism in their base sequences. In eukaryotes, genomic DNA is located primarily in the cell nucleus, with small amounts contained in mitochondria and chloroplasts. In prokaryotes, the DNA is held within an irregularly shaped body in the cytoplasm called the nucleoid. The genetic information in a genome is held within genes, and the complete set of this information in an organism is called its genotype. Upon activation of the translation machinery, the genetic information contained in the DNA material is translated into mRNA, which directs protein synthesis in the cytoplasm.

[0005] In recent years modern sequencing techniques have allowed scientists to determine the DNA sequence of genes of interest with minimal effort, and whole genomic sequencing has become a reality. Based on these developments, there has

been an increasing interest in determining how the information imprinted in the base sequence of nucleic acids controls specific cellular processes, and how changes in the base sequence of nucleic acids (by, for example, single-point mutation, insertion, deletion, or translocation, or a combination thereof), no matter how minimal, may dramatically alter cellular metabolism and contribute to disease, especially cancer.

[0006] Cancer is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, being present in all cells from birth. Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells, resulting in abnormal functions, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and ability to become established in diverse tissue environments. Tumor suppressor genes are inactivated in cancer cells, resulting in the loss of normal functions, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system. Such gross genetic abnormalities should be detected as soon as possible, in order to allow effective treatment. Generally speaking, the earlier the diagnosis of a cancer growth in a patient, the better the prospects for long-term survival of the patient.

[0007] For example, pancreatic cancer, a malignant neoplasm of the pancreas, is diagnosed in about forty-two thousand patients and responsible for about thirty-five thousand deaths in the US every year. The prognosis for this kind of cancer is generally regarded as poor: less than five percent of those diagnosed are still alive five years after diagnosis, because they are generally diagnosed at an advanced incurable stage. However, even before an enlarged mass may be seen by magnetic resonance imaging or computed tomography, early stage pancreatic intraepithelial neoplasia (PanIN-1) pancreas cells contain high levels of mRNAs copied from hyperactive cancer

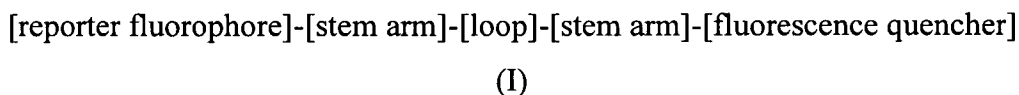
genes such as KRAS and HER2. However, PanIN-1 does not always progress. Later stage PanIN-3 pancreas cells, which do progress to frank cancer, contain high levels of mRNAs overproduced by activated CCND1 or BRCA2 oncogenes. Specific detection of PanIN-3 would enable resection of ductal pancreas cancer at a survivable stage. There is thus a need to identify a sensitive probe for such cancer-related genes and their products.

[0008] Identification of DNA or RNA sequences that may be involved in particular non-oncogenic or oncogenic biological processes require the use of nucleic acid probes. These probes are designed to bind (“hybridize”) to a specific DNA or RNA sequence within a mixture of closely related nucleic acid sequences. Nucleic acid probes must be specific, hybridizing with great selectivity with the nucleic acid of interest. Furthermore, the complex formed between the nucleic acid and the probe must be detectable at low concentrations.

[0009] As a consequence, different kinds of probes have been developed to determine DNA or RNA sequences in vitro and in vivo. Most nucleic acid probes contain the basic structure of DNA or RNA. However, DNA and RNA strands are notoriously sensitive to chemical and biochemical reagents, and undergo rapid and extensive modification or degradation in vivo. Furthermore, the highly charged backbone of DNA and RNA hampers the penetration of the probes in cells and tissues. In the hope of identifying an alternative to the DNA or RNA backbone, a new class of compounds, peptide nucleic acids (PNAs), was developed as antisense agents and nucleic acid probes. PNAs comprise nucleic acid mimics in which the sugar-phosphate backbone is replaced with a backbone based on amino acids. The PNA structure has improved nuclease stability and cell membrane permeability over DNA and RNA (Nielsen et al., 1991, Science 254, 1497-1500; Knorre et al., 1994, “Design and Targeted reactions of Oligonucleotide Derivatives”, CRC Press, Boca Raton, FL). PNAs generally exhibit sequence-specific binding to DNA and RNA with higher affinities and specificities than unmodified DNA, and they are resistant to nuclease and protease attack. Melting temperatures of their duplexes with DNA or RNA are much higher than any of the known DNA compounds, both modified and unmodified. The PNAs may be prepared inexpensively on a large scale using standard solution-phase or solid-phase peptide synthesis.

[0010] PNAs recognize DNA and RNA in a sequence specific manner and form complexes that can be characterized by biophysical methods. The binding motif is context dependent. PNAs containing both purine and pyrimidine bases afford a 1:1 heteroduplex with mismatch sensitivity comparable to that found in double-stranded (ds)DNA. On the other hand, homopyrimidine PNAs combine with complementary polypurine targets to form stoichiometric 2:1 complexes, where one strand of PNA binds to a strand of DNA to form a PNA-DNA duplex, and a second strand of the PNA binds the major groove of the PNA-DNA duplex through Hoogsteen-base pairing.

[0011] Binding of the probing tool to the nucleic acid sequence must be detectable at low concentrations. The ideal probing tool should include a reporter that is silent unless the probe is hybridized to its specific target. With this characteristic in mind, a novel kind of probe, called a molecular beacon, was developed (Tyagi et al., 1998, Nat. Biotechnol. 16:49-53; Tyagi & Kramer, 1996, Nat. Biotechnol. 14:303-308). A molecular beacon is a single-stranded oligonucleotide or equivalent (such as a PNA) that fluoresces only upon hybridization to its target nucleic acid. A molecular beacon comprises different domains, as shown schematically in (I):



The central domain (loop) contains the probe sequence, which binds to the target nucleic acid. The central domain is flanked by two complementary sequences (two stem arms; each stem arm is generally four to seven base pairs long). To the outer end of one stem arm is attached a reporter fluorophore, and to the outer end of the other stem arm is attached a fluorescence quencher. When the molecular beacon is free in solution, the two stem arms come together to form a double-stranded stem. In this conformation, the reporter fluorophore and the quencher are brought together in close proximity (less than about 10 nm), leading to quenching of any fluorescence derived from the fluorophore. When the central domain of the molecular beacon hybridizes to the nucleic acid, the stem arms separate, opening up the stem. This causes the reporter fluorophore to physically separate from the quencher, resulting in fluorescence. This unique on/off signal mechanism is commonly used in DNA and RNA probing

(Schofield et al., 1997, Appl. Environ. Microbiol. 63:1143-1147; Kim et al., 2008, Int. J. Clin. Exp. Pathol. 1, 105-116).

[0012] Probing of nucleic acid sequences using PNA-based molecular beacons has thus received a lot of attention lately. However, the synthesis and use of PNA-based molecular beacons still face considerable challenges. In one aspect, it would be useful to simplify the overall structure of PNA-based molecular beacon, making its design and synthesis more straightforward. For example, the need to incorporate stem arms in the molecular beacon increases the size and complexity of the molecule, and may further reduce its membrane permeability. In another aspect, a molecular beacon that shows greater increase in fluorescence once bound to the nucleic acid would improve the sensitivity associated with detection of the binding event. The present invention addresses these needs.

Summary of Invention

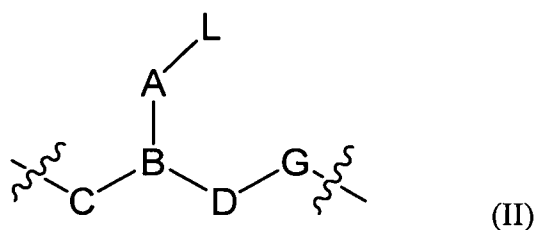
[0013] According to the present invention, a conjugate is provided comprising a peptide nucleic acid (PNA) oligomer conjugated at both ends, through a peptidic linker, to the same fluorophore, based on 4,5-benzo-1,1'-diethyl-3,3',3'-tetramethyl-indodicarbocyanine. When not hybridized to its specific complementary target sequence (i.e., unbound), the conjugate does not fluoresce. That results in negligible background fluorescence in the absence of a complementary target sequence. The conjugate binds to complementary DNA or RNA target sequences through the bases that are linked to a peptide backbone of the PNA, with the sequence of bases determining the target nucleic acid segment to which the oligomer binds. Upon hybridization of the conjugate to the nucleic acid, the two fluorophores undergo physical separation, and the conjugate is capable of fluorescing.

[0014] The conjugate of the invention differs from the PNA-based molecular beacons known in the art by lacking a quencher moiety and stem domains. Absence of these features simplifies synthesis of the conjugate of the invention by reducing its complexity. The presence of two fluorophore moieties in the conjugate of the invention results in a probe with a maximum fluorescence signal that is twice as intense as of a comparable molecular beacon known in the art, comprising a fluorophore and a quencher. This feature increases the sensitivity for detection of the binding of the

molecular beacon to the nucleic acid. The invention also relates to a method of detecting a nucleic acid sequence in vitro or in vivo using a conjugate of the invention.

[0015] The invention includes a conjugate comprising a peptide nucleic acid oligomer conjugated to two molecules of a [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore, wherein a first molecule of the fluorophore is conjugated to the oligomer through a first peptidic linker and a second molecule of the fluorophore is conjugated to the oligomer through a second peptidic linker.

[0016] In one embodiment of the invention, the oligomer comprises at least one subunit that is a peptide nucleic acid subunit of Formula (II):



wherein:

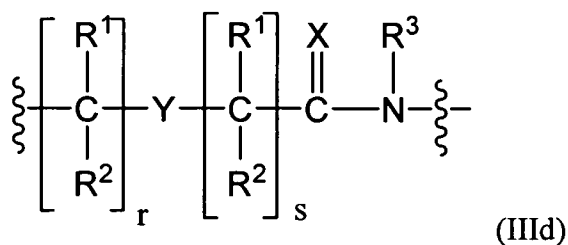
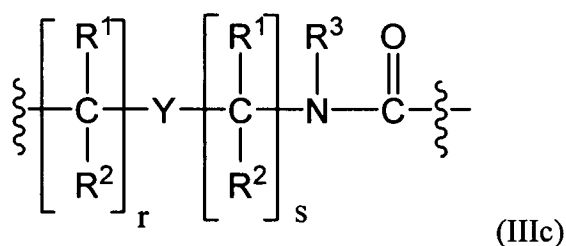
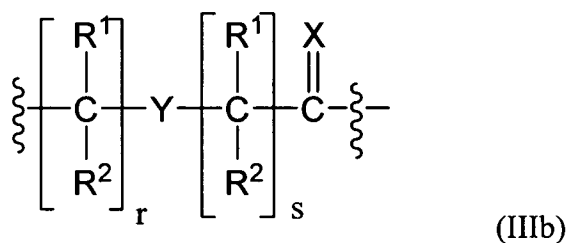
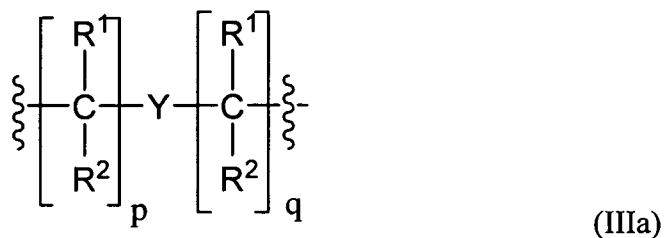
- L is one of the adenine, thymine, cytosine or guanine heterocyclic bases of the oligomer;
 - C is $(CR^6R^7)_y$ where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_2-C_6) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6) alkoxy, (C_1-C_6) alkylthio, NR^3R^4 and SR^5 , where each of R^3 and R^4 is independently selected from the group consisting of hydrogen, (C_1-C_4) alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C_1-C_4) alkyl, hydroxy, alkoxy, alkylthio and amino; and R^5 is hydrogen, (C_1-C_6) alkyl, hydroxy-, alkoxy-, or alkylthio-substituted (C_1-C_6) alkyl, or R^6 and R^7 taken together complete an alicyclic or heterocyclic system;
 - D is $(CR^6R^7)_z$, where R^6 and R^7 are as defined above;
- each of y and z is zero or an integer from 1 to 10, the sum y+z being greater than 2, and less than or equal to 10;

G is $-\text{NR}^3\text{C}(\text{O})-$, $-\text{NR}^3\text{C}(\text{S})-$, $-\text{NR}^3\text{S}(\text{O})-$ or $-\text{NR}^3\text{S}(\text{O})_2-$, in either orientation, where R^3 is as defined above;

each pair of A and B is selected such that:

(a) A is a group of Formula (IIIa), (IIIb) or (IIIc) and B is N or R^3N^+ ; or

(b) A is a group of Formula (IIId) and B is CH;



wherein:

X is O, S, Se, NR^3 , CH_2 or $\text{C}(\text{CH}_3)_2$;

Y is a single bond, O, S or NR^4 ;

each of p and q is zero or an integer from 1 to 5, the sum p+q being less than or equal to 10;

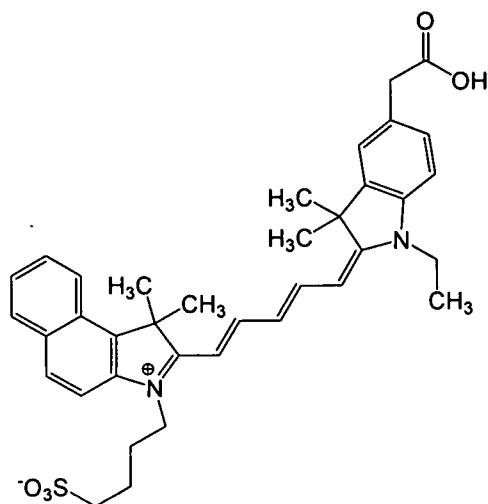
each of r and s is zero or an integer from 1 to 5, the sum $r+s$ being less than or equal to 10; and,

each R^1 and R^2 is independently selected from the group consisting of hydrogen, (C_1-C_4) alkyl which may be hydroxy- or alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen.

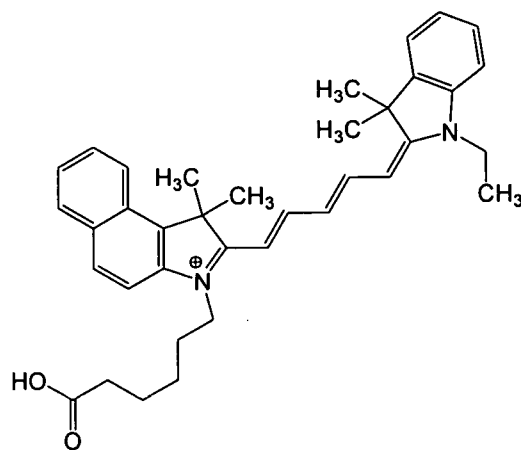
[0017] In another embodiment of the invention, A is $-CH_2C(O)-$; B is N, whereby B-A is $N-CH_2C(O)-$; C is $-CH_2CH_2-$; and D is $-CH_2-$. In yet another embodiment, all of the subunits of the oligomer are peptide nucleic acid subunits. In yet another embodiment, the oligomer has a subunit sequence such that the oligomer is capable of forming (i) a triplex with a double stranded DNA segment, or (ii) a duplex with a single stranded DNA segment or an mRNA segment.

[0018] In one embodiment of the invention, the oligomer has 6 to 25 subunits. In another embodiment, the oligomer has 6 to 20 subunits. In yet another embodiment, the oligomer comprises $PNA[GCCATCAGCTCC]$. In yet another embodiment, the first peptidic linker is attached to the N-terminal of the oligomer. In yet another embodiment, the first peptidic linker is 2-(2-aminoethoxy)ethoxyacetic acid (known as Aeea). In yet another embodiment, the second peptidic linker is attached to the C-terminal of the oligomer. In one embodiment, the second peptidic linker is Aeea-Lys-Aeea.

[0019] In one embodiment, the fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII). In another embodiment, the fluorophore is 3-(5-carboxypentyl)-2-((1E,3E,5E)-5-(1-ethyl-3,3-dimethylindolin-2-ylidene)penta-1,3-dienyl)-1,1-dimethyl-1H-benzo[e]indolium (VIII).



(VII)



(VIII)

[0020] In one embodiment of the invention, the first peptidic linker is Aeea; the second peptidic linker is Aeea-Lys-Aeea; and the fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII). In another embodiment, the oligomer comprises PNA[GCCATCAGCTCC]. In yet another embodiment, the first peptidic linker is attached to the N-terminal of the oligomer and the second peptidic linker is attached to the C-terminal of the oligomer.

[0021] The invention also includes a method of assessing the presence of a specific nucleic acid sequence in a biological sample in vitro. The method comprises the steps of exposing the biological sample to an excitation wavelength; measuring fluorescence of the biological sample at an emission wavelength; contacting the biological sample with a conjugate comprising a peptide nucleic acid oligomer conjugated to two molecules of a [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore, wherein a first molecule of the fluorophore is conjugated to the oligomer through a first peptidic linker and a second molecule of the fluorophore is conjugated to the oligomer through a second peptidic linker, thereby providing a test sample; exposing the test sample to the excitation wavelength; and then measuring fluorescence of the test sample at the emission wavelength.

[0022] In one embodiment of the invention, the excitation wavelength is about 650 nm and the emission wavelength is about 680 nm. In another embodiment, the specific nucleic acid sequence comprises the DNA sequence 5'-AGT TGG AGC TGA TGG CGT AG-3' or the RNA sequence 5'-AGU UGG AGC UGA UGG CGU AG-3'.

In yet another embodiment, the first peptidic linker is Aeea; the second peptidic linker is Aeea-Lys-Aeea; and the fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid. In yet another embodiment, the first peptidic linker is attached to the N-terminal of the oligomer and the second peptidic linker is attached to the C-terminal of the oligomer. In yet another embodiment, the oligomer comprises PNA[GCCATCAGCTCC].

[0023] The invention also includes a method of assessing the presence of a specific nucleic acid sequence in a tissue in a subject in vivo. The method comprises the steps of: exposing the tissue to an excitation wavelength; measuring fluorescence of the tissue at an emission wavelength; administering to the subject a pharmaceutical composition comprising a conjugate, wherein the conjugate comprises a peptide nucleic acid oligomer conjugated to two identical [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophores, wherein a first molecule of the fluorophores is conjugated to the oligomer through a first peptidic linker and a second molecule of the fluorophores is conjugated to the oligomer through a second peptidic linker; thereby providing a modified tissue; exposing the modified tissue to the excitation wavelength; and then measuring fluorescence of the modified tissue at the emission wavelength.

[0024] In one embodiment of the invention, the excitation wavelength is about 650 nm and the emission wavelength is about 680 nm. In another embodiment, the specific nucleic acid sequence comprises the DNA sequence 5'-AGT TGG AGC TGA TGG CGT AG-3' or the RNA sequence 5'-AGU UGG AGC UGA UGG CGU AG-3'. In yet another embodiment, the first peptidic linker is Aeea; the second peptidic linker is Aeea-Lys-Aeea; and the fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid. In yet another embodiment, the first peptidic linker is attached to the N-terminal of the oligomer and the second peptidic linker is attached to the C-terminal of the oligomer. In yet another embodiment, the oligomer comprises PNA[GCCATCAGCTCC].

[0025] As envisioned in the present invention with respect to the disclosed compositions of matter and methods, in one aspect the embodiments of the invention comprise the components and/or steps disclosed therein. In another aspect, the embodiments of the invention consist essentially of the components and/or steps

disclosed therein. In yet another aspect, the embodiments of the invention consist of the components and/or steps disclosed therein.

Description of Figures

[0026] Figure 1 shows the UV-visible spectra of compound (IX) in aqueous buffer and 50% acetonitrile-aqueous buffer.

[0027] Figure 2 shows the UV-visible spectra of compound (IX), at a concentration of 1 μ M, in the absence and the presence of 1 μ M RNA complementary sense strand.

[0028] Figure 3 shows the UV-visible spectra of compound (X) in aqueous buffer and 50% acetonitrile-aqueous buffer.

[0029] Figure 4 shows the UV-visible spectra of compound (X), at a concentration of 1 μ M, in aqueous buffer and in the presence of 1 μ M RNA complementary sense strand in aqueous buffer.

[0030] Figure 5 shows the overlay of UV-visible spectra of compound (X), at a concentration of 1 μ M, in different solutions: (i) in aqueous buffer; (ii) with 1 μ M RNA complementary sense strand in aqueous buffer; (iii) in 50 % acetonitrile-aqueous buffer; (iv) with 1 μ M RNA complementary sense strand in 50 % acetonitrile-aqueous buffer.

[0031] Figure 6 shows the excitation and emission fluorescence spectra for compound (XI), at a concentration of 1 μ M, recorded in aqueous buffer and in the presence of 2 μ M RNA complementary sense strand in aqueous buffer. The excitation spectra were recorded with λ_{exc} of 500-680 nm and λ_{emiss} of 690 nm. The emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 665-720 nm. Trace (a) represents the excitation spectrum in aqueous buffer. Trace (b) represents the excitation spectrum in the presence of the RNA complementary sense strand. Trace (c) represents the emission spectrum in aqueous buffer. Trace (d) represents the emission spectrum in the presence of the RNA complementary sense strand.

[0032] Figure 7 shows the excitation and emission fluorescence spectra for compound (X), at a concentration of 1 μ M, recorded in aqueous buffer and in the

presence of the RNA complementary sense strand (2 μ M concentration in aqueous buffer). The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. The emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. Trace (a) represents the excitation spectrum in aqueous buffer. Trace (b) represents the excitation spectrum in the presence of the RNA complementary sense strand. Trace (c) represents the emission spectrum in aqueous buffer. Trace (d) represents the emission spectrum in the presence of the RNA complementary sense strand.

[0033] Figure 8 shows the excitation and emission fluorescence spectra for compound (X), at a concentration of 100 nM, recorded in aqueous buffer and in the presence of the RNA complementary sense strand (2 μ M concentration in aqueous buffer). The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. The emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. Trace (a) represents the excitation spectrum in aqueous buffer. Trace (b) represents the excitation spectrum in the presence of the RNA complementary sense strand. Trace (c) represents the emission spectrum in aqueous buffer. Trace (d) represents the emission spectrum in the presence of the RNA complementary sense strand.

[0034] Figure 9 represents the excitation and emission fluorescence spectra for the aqueous buffer and for compound (X) at a concentration of 1 μ M in aqueous buffer. The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. Trace (a) represents the excitation spectrum of aqueous buffer. Trace (b) represents the excitation spectrum of the compound in the aqueous buffer. Trace (c) represents the emission spectrum of the aqueous buffer. Trace (d) represents the emission spectrum of the compound in the aqueous buffer.

[0035] Figure 10 represents the fluorescence spectra for compound (X), at a concentration of 1 μ M, in aqueous buffer and in 50% acetonitrile/aqueous buffer. The excitation spectra were recorded with λ_{exc} of 550-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-720 nm. Trace (a) represents the excitation spectrum of the compound in aqueous buffer. Trace (b) represents the excitation spectrum of the compound in 50 % acetonitrile/aqueous buffer. Trace (c) represents the emission spectrum of the compound in aqueous buffer.

Trace (d) represents the emission spectrum of the compound in 50 % acetonitrile/aqueous buffer.

[0036] Figure 11, panel (a), shows the confocal near-infrared fluorescence microscopy of AsPC1 KRAS G12D pancreas cancer cells after 24 hour incubation with 500 nM of compound (X) and 24 hour efflux. Label (A2) indicates live cells, as stained by CellTracker™ Green, as contrasted with the black background. Label (A1) indicates the nuclei, as stained by Hoechst 33342. Label (A3) indicates cytoplasmic fluorescence of compound (X), as evidenced by Cy5.5 filters. Figure 11, panel (b), shows the confocal near-infrared fluorescence microscopy of AsPC1 KRAS G12D pancreas cancer cells after 24 hour incubation with uncoupled fluorophore (VII) carboxylic acid. No major cytoplasmic fluorescence, which would appear as light areas in the picture, was evidenced.

[0037] Figure 12 represents the whole body near-infrared fluorescence imaging of a mouse with SW480 KRAS G12D colon xenografts. The imaging was done 48 hours after administration of 18 nmol of compound (X). Excitation was performed at 615-665 nm, and emission was monitored at 680-800 nm, in a Maestro™ CRI all-optical in vivo imaging instrument (Woburn, MA).

Definitions

[0038] The definitions used in this application are for illustrative purposes and do not limit the scope used in the practice of the invention.

[0039] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well known and commonly employed in the art.

[0040] The articles “a” and “an” are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0041] As used herein, the term “about” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used.

[0042] As used herein, the term “PNA” refers to a peptide nucleic acid oligomer or polymer, wherein the term “oligomer” or “polymer” are interchangeably used. As used herein, the term “subunit”, as applied to a PNA oligomer, refers to the individual chemical unit contained in the PNA oligomer and comprising a heterocyclic base and a backbone unit. As used herein, an “end subunit” or “terminal subunit” of a PNA oligomer refers to a subunit that is located at one of the extremities of the PNA oligomer and that is thus coupled to only one other PNA subunit in the PNA oligomer. As used herein, an “internal subunit” of a PNA oligomer refers to a subunit that is not located at one of the extremities of the PNA oligomer and is thus coupled to two other PNA subunits in the PNA oligomer.

[0043] As used herein, the “N-terminal” or “N-terminus” of a PNA oligomer corresponds to the subunit that is located in the extremity of the PNA oligomer and contains a backbone primary or secondary amino group that is not involved in a covalent bond with another PNA subunit in the PNA oligomer. As used herein, the “C-terminal” or “C-terminus” of a PNA oligomer corresponds to the subunit that is located in the extremity of the PNA oligomer and is not the “N-terminal” or “N-terminus” subunit.

[0044] As used herein, the heterocycle bases present in a PNA oligomer are abbreviated as “A” (adenine), “G” (guanine), “C” (cytosine) and “T” (thymine). As used herein, a PNA subunit is referred to as PNA[X], wherein “X” is the heterocycle base. As a non-limiting example, PNA[A] corresponds to a PNA subunit containing adenine as the heterocyclic base. As another non-limiting example, PNA[AGC] corresponds to a PNA trimer formed of subunits containing A, G and C as heterocycle bases, wherein the subunit containing adenine occupies the N-terminal and the subunit containing cytosine occupies the C-terminal.

[0045] As used herein, the term “[4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore” refers to a compound which structure may derived from the structure of 4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine by replacing one or more hydrogen atoms with other atoms or

chemical groups, such as hydroxyl, carboxymethyl, 2-carboxy-1-ethyl, and 3-carboxyl-1-propyl, methylamino, 2-ethylamino and 3-propylamino.

[0046] As used herein, the terms “peptide,” “polypeptide” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise the sequence of a protein or peptide. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types.

“Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

[0047] A “nucleic acid” refers to a polynucleotide and includes polyribonucleotides and polydeoxyribonucleotides.

[0048] “Homologous”, as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, such as two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (e.g., 9 of 10), are matched or homologous, the two sequences are 90% homologous. By way of example, the DNA sequences 3'-ATTGCC-5' and 3'-TATGGC-5' are 50% homologous. As used herein, “homology” is used synonymously with “identity.”

[0049] “Substantially the same” amino acid sequence is defined herein as a sequence with at least 70%, preferably at least about 80%, more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least 99% homology to another amino acid sequence, as determined by the FASTA search method in accordance with Pearson & Lipman, 1988, Proc. Natl. Inst. Acad. Sci. USA 85: 2444-2448.

[0050] “Isolated” means altered or removed from the natural state through the actions of a human being. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein may exist in substantially purified form, or may exist in a non-native environment such as a host cell for example.

[0051] A “subject”, as used therein, can be a human or non-human animal. Non-human animals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals, as well as reptiles, birds and fish. Preferably, the subject is human.

[0052] “Applicator,” as the term is used herein, is used to identify any device including, but not limited to, a hypodermic syringe, a pipette, and the like, for administering the compounds and compositions used in the practice of the invention.

Detailed Description of Invention

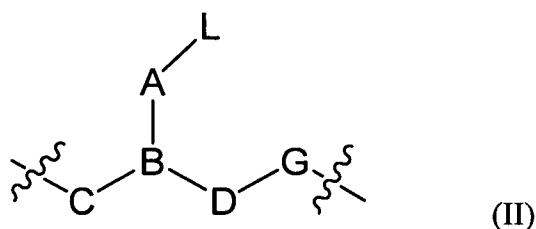
[0053] The present invention is based on the unexpected discovery that a conjugate comprising a peptide nucleic acid (PNA) oligomer conjugated at both ends, through a peptidic linker, to the same indodicarbocyanine fluorophore undergoes fluorescence self-quenching in its unbound form (i.e., in the absence of its specific complementary nucleic acid target sequence). Without wishing to be bound by any theory, the fluorophores appear to undergo intramolecular stacking in the unbound conjugate. Unlike molecular beacons known in the art, the conjugate of the invention does not require the presence of stem arm domains in its structure for proper self-quenching in the absence of its specific complementary nucleic acid target sequence. Once bound to its nucleic acid target, the conjugate of the invention fluoresces with

twice as much intensity as a molecular beacon comprising only one fluorophore of the same kind and a quencher.

Conjugates of the invention

[0054] The conjugate of the invention comprises a PNA oligomer. The PNA oligomer is a strand, analogous to a nucleic acid strand, comprising a sequence of naturally occurring or non-naturally occurring organic bases covalently linked by a backbone. Whereas in conventional nucleic acids the backbone consists of a series of ribosyl or deoxyribosyl moieties, the sugar backbone is replaced in PNAs by a backbone substantially comprising polyamide, polythioamide, polysulfonamide or polysulfonamide. Thus, the peptide nucleic acid may be viewed as a strand of bases covalently bound by linking moieties comprising amide, thioamide, sulfinamide or sulfonamide linkages. In one embodiment, the linking moieties in the backbone comprise N-ethylaminoglycine units. At least some of the bases are capable of hydrogen bonding with complementary bases of a target nucleic acids segment.

[0055] The PNA oligomer portion of the conjugates of the present invention comprise at least one peptide nucleic acid subunit of the Formula (II):



wherein:

- L is one of the adenine, thymine, cytosine or guanine heterocyclic bases of the oligomer;
- C is $(CR^6R^7)_y$ where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_2-C_6) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6) alkoxy, (C_1-C_6) alkylthio, NR^3R^4 and SR^5 , where each of R^3 and R^4 is independently selected from the group consisting of hydrogen, (C_1-C_4) alkyl, hydroxy- or

alkoxy- or alkylthio-substituted (C₁-C₄) alkyl, hydroxy, alkoxy, alkylthio and amino; and R⁵ is hydrogen, (C₁-C₆) alkyl, hydroxy-, alkoxy-, or alkylthio-substituted (C₁-C₆) alkyl, or R⁶ and R⁷ taken together complete an alicyclic or heterocyclic system;

D is (CR⁶R⁷)_z, where R⁶ and R⁷ are as defined above;

each of y and z is zero or an integer from 1 to 10, the sum y+z being greater than 2, and less than or equal to 10;

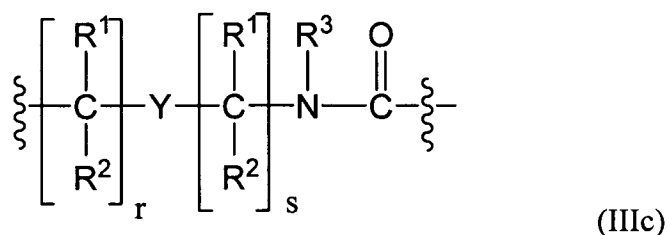
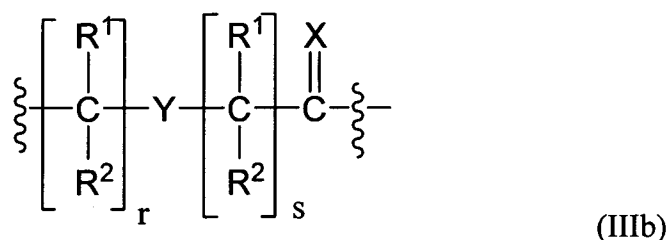
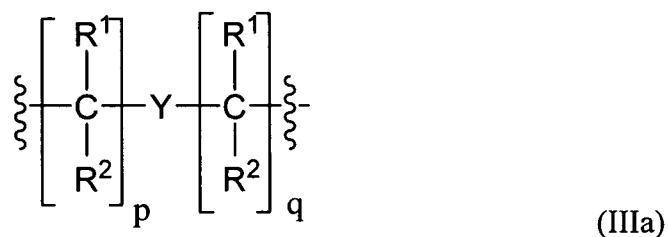
G is -NR³C(O)-, -NR³C(S)-, -NR³S(O)- or -NR³S(O)₂-, in either orientation, where R³ is as defined above;

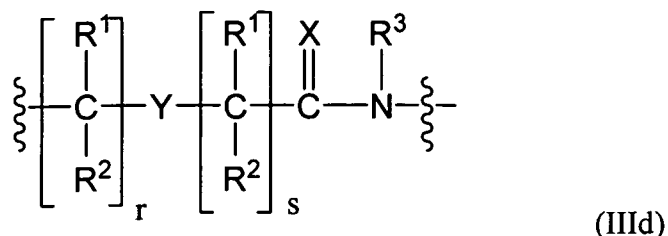
each pair of A and B is selected such that:

(a) A is a group of Formula (IIIa), (IIIb) or (IIIc) and B is N or R³N⁺;

or

(b) A is a group of Formula (IIId) and B is CH;





wherein:

X is O, S, Se, NR³, CH₂ or C(CH₃)₂;

Y is a single bond, O, S or NR⁴;

each of p and q is zero or an integer from 1 to 5, the sum p+q being less than or equal to 10;

each of r and s is zero or an integer from 1 to 5, the sum r+s being less than or equal to 10; and,

each R¹ and R² is independently selected from the group consisting of hydrogen, (C₁-C₄) alkyl which may be hydroxy- or alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen.

[0056] As used herein, the term "subunits" refer to basic units that are chemically similar and that can form polymers. Repeating basic units form polymers referred to as "oligomers". The PNA oligomer portion of the conjugates of the present invention may comprise an oligomer in which substantially all subunits of the oligomer are subunits as described in Formula (II). The PNA oligomer may also comprise one or more subunits that are naturally occurring nucleotides or nucleotide analogs, as long as at least one subunit satisfies Formula (II). Thus, "PNA oligomers" as used herein may refer to a range of oligomers, from an oligomer comprising only one PNA subunit as defined in Formula (II), to an oligomer in which every subunit is a PNA subunit as defined in Formula (II). The amino acids that form the backbone may be identical or different.

[0057] Those subunits that are not PNA subunits comprise naturally occurring bases, sugars, and intersugar (backbone) linkages, as well as non-naturally occurring portions that function similarly to naturally occurring portions.

[0058] Sequences of oligomers are defined by reference to the L group (for PNA subunits) or nucleobase (for nucleotide subunits) at a given position. Thus, for a given oligomer, the nomenclature is modeled after traditional nucleotide nomenclature, identifying each PNA subunit by the identity of its L group such as the heterocycles adenine (A), thymine (T), guanine (G) and cytosine (C) and identifying nucleotides or nucleosides by these same heterocycle residing on the sugar backbone. The sequences are conveniently provided in traditional 5' to 3' or amino to carboxy orientation.

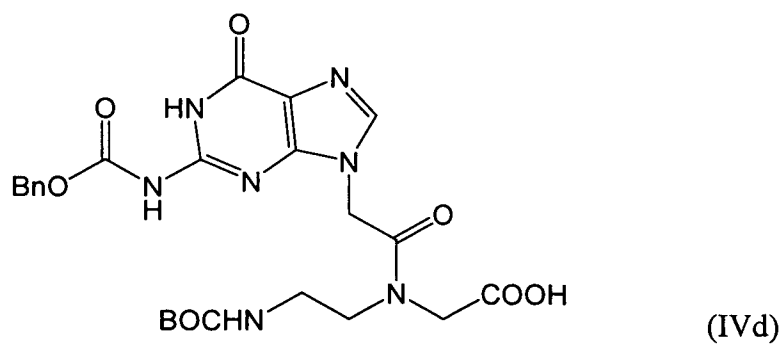
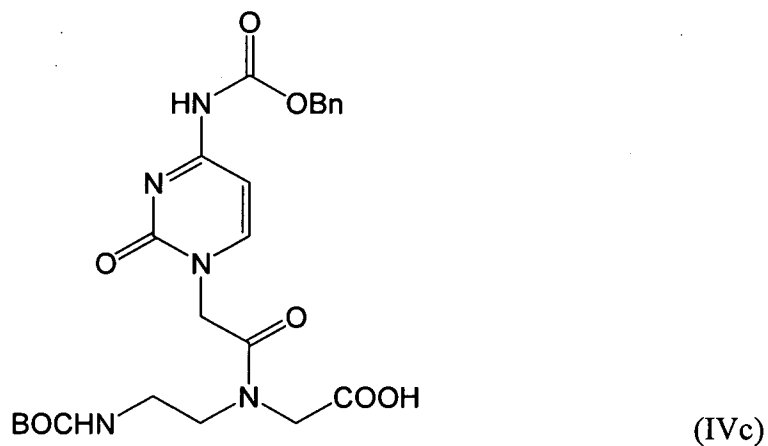
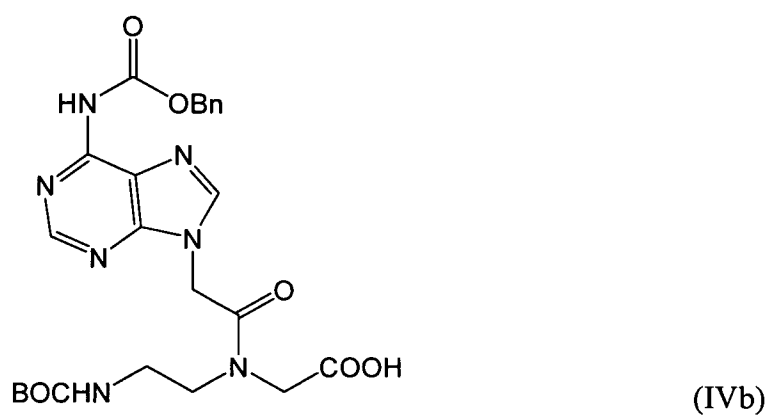
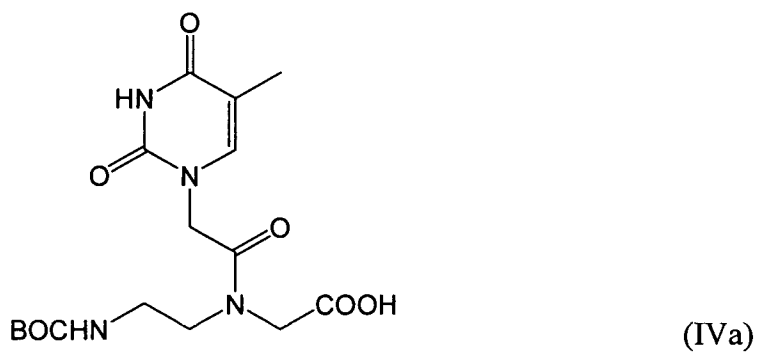
[0059] In one embodiment of the invention, the PNA oligomer portion of the inventive conjugate may range in size from about 6 to about 60 subunits in length. In another embodiment of the present invention, the PNA oligomers may range in size from about 6 to about 30 subunits in length. In yet another embodiment of the present invention, the oligomer may range in size from about 6 to about 25 subunits in length. In yet another embodiment, oligomers may range in size from about 6 to about 20 subunits in length. In yet another embodiment of the present invention, the oligomer may range in size from about 6 to about 15 subunits in length. In yet another embodiment, oligomers may range in size from about 6 to about 12 subunits in length.

[0060] Methods for the preparation of peptide nucleic acids are described in the following International Applications, the entire disclosures of which are incorporated herein by reference: International Patent Applications Nos. PCT/EP92/01219 (WO 92/20702), PCT/EP92/ 01220 (WO 92/20703), PCT/IB94/00142 (WO 94/25477), PCT/US94/06620 (WO 94/28720), PCT/US94/07319 (WO 95/01370), and PCT/US94/08465 (WO 95/03833).

[0061] Essentially, PNAs are synthesized by adaptation of solution or solid phase peptide synthesis procedures. The synthons are monomer amino acids or their activated derivatives, protected by standard protecting groups. The state of the art in PNA synthesis is extensively reviewed in PCT/US94/08465, from page 11, line 6 to page 23, line 7, which is specifically incorporated herein by reference.

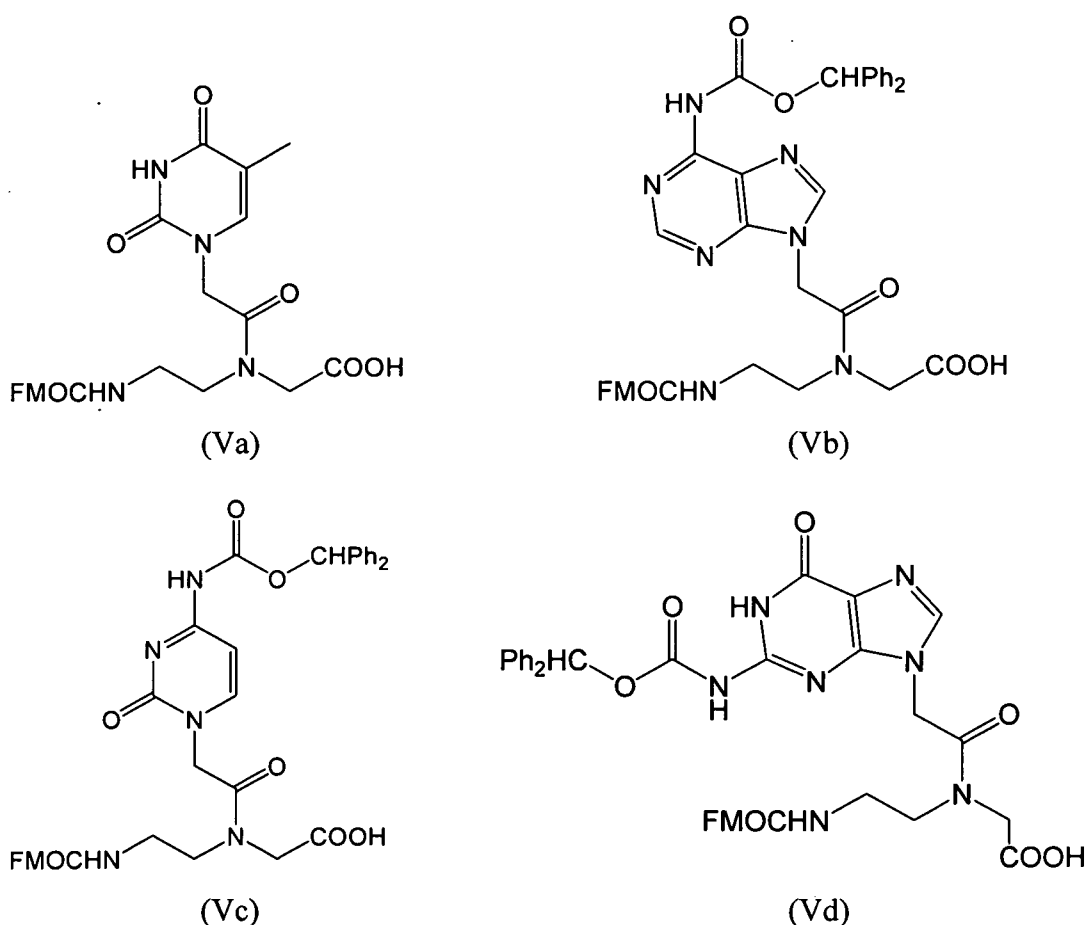
[0062] A PNA oligomer having the preferred backbone, that is, a backbone formed by N-ethylaminoglycine units, may be formed by linking the following commercially available BOC and Z-protected T, A, C, and G monomers (which are available from PerSeptive Biosystems, Framingham, MA): Compound (IVa), BOC-T-

OH; Compound (IVb), BOC-A(Z)-OH; Compound (IVc), BOC-C(Z)-OH; and Compound (IVd), BOC-G(Z)-OH:



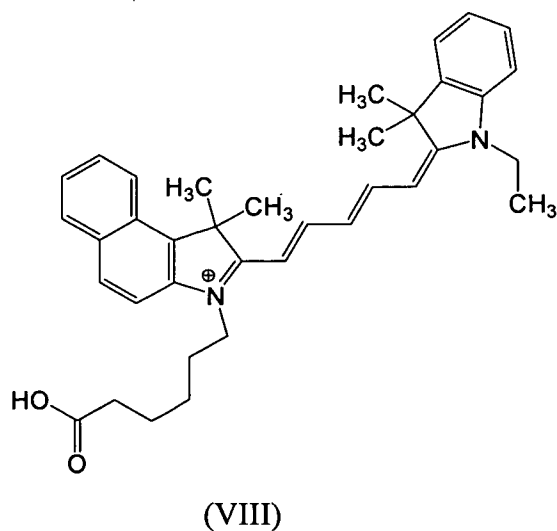
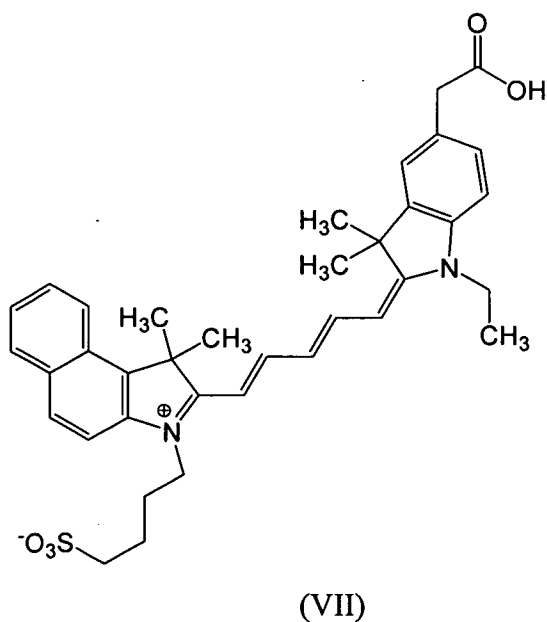
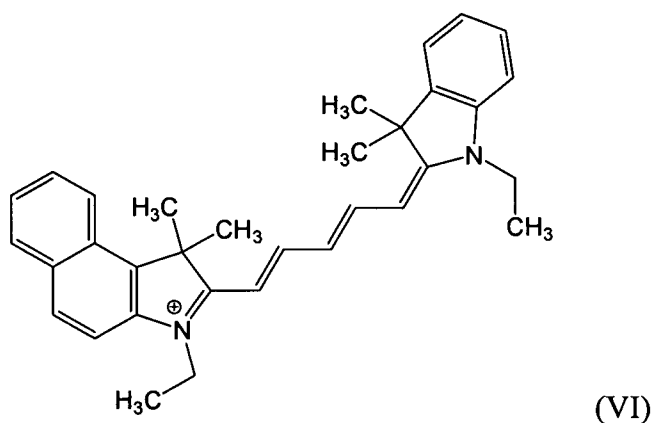
[0063] Methods for the solid-phase synthesis of peptide nucleic acids containing these monomers are described in Christensen et al., 1995, *J. Peptide Science* 3:175-183, the entire disclosure of which is incorporated herein by reference.

[0064] As an alternative to BOC chemistry, the PNA may be synthesized via Fmoc chemistry by linking the following commercially available Fmoc- and BhOC-protected T, A, C and G PNA monomers (available from PerSeptive Biosystems, Framingham, MA), where BhOC is benzhydryloxycarbonyl: Compound (Va), Fmoc-T-OH; Compound (Vb), Fmoc-A(BhOC)-OH; Compound (Vc), Fmoc (BhOC)-OH; and Compound (Vd), Fmoc-G(BhOC)-OH.



[0065] The conjugate of the invention comprises two identical units of a [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore. As used herein, a [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore refers to a compound which structure may be derived from the structure of Formula (VI) by replacing one or more hydrogen atoms with other atoms or chemical groups. Therefore, a fluorophore useful within the invention should not be construed to

be limited to the structure of Formula (VI). Rather, a fluorophore useful within the invention may include covalent modifications to the structure of Formula (VI) that improve the fluorescence of the compound, improve the physico-chemical properties of the compound or allow the compound to be covalently incorporated into the conjugate of the invention. Fluorophores useful within the invention include the compound of Formula (VII), named 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid, and the compound of Formula (VIII), named 3-(5-carboxypentyl)-2-((1E,3E,5E)-5-(1-ethyl-3,3-dimethylindolin-2-ylidene)penta-1,3-dienyl)-1,1-dimethyl-1H-benzo[e]indolium. A [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore may be purchased from commercial sources, or synthesized according to known methods, to be used in the preparation of the conjugates of the invention.



[0066] Each fluorophore is conjugated to the PNA oligomer through a peptidic linker. In one embodiment, the two peptidic linkers in the conjugate of the invention are identical. In another embodiment, the two peptidic linkers in the conjugate of the invention are not identical. The peptidic linker is a peptide of 1 to 10 amino acids. In one embodiment, the amino acids are selected from the group consisting of alanine, cysteine, cystine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine, ornithine, β -aminopropionic acid, γ -aminobutyric acid, δ -aminovaleric acid, ϵ -aminocaproic acid, (2-aminoethoxy)-acetic acid, 2-(2-aminoethoxy)ethoxyacetic acid (known as Aeea) and 2-(2-(2-aminoethoxy)ethoxy)ethoxyacetic acid. In another embodiment, at least one amino acid is Aeea. In yet another embodiment, the peptidic linker comprises a peptide of 1 to 8 amino acids. In yet another embodiment, the peptidic linker comprises a peptide of 1 to 5 amino acids. In yet another embodiment, the peptidic linker is Aeea. In yet another embodiment, the peptidic linker is a peptide of sequence Aeea-Lys-Aeea. In one embodiment, at least one of the amino acids of the peptidic linker is a D-amino acid. This has the effect of enhancing the conjugate's biological stability.

[0067] The peptidic linker and the fluorophore are connected through a covalent bond, and the nature of this covalent bond depends on the substituents contained in the peptidic linker and the fluorophore. In a non-limiting example, a free amino group in the peptidic linker, such as the ϵ -amino group of a lysine residue or the amino group of Aeea, may be coupled to an activated ester, such as the N-succinimidyl ester, of compound (VII) or compound (VIII), to form the corresponding amide. In another non-limiting example, compound (VII) or compound (VIII) may be converted to the corresponding acyl chloride, for example, by treatment with thionyl chloride; the acyl chloride may be reduced to the corresponding aldehyde, for example, by mild catalytic hydrogenation; the aldehyde may then be reacted with a free amino group in the peptidic linker, such as the ϵ -amino group of a lysine residue or the amino group of Aeea, in the presence of a reducing agent, such as sodium borohydride, and traces of acid, to form the corresponding substituted amine. Conditions for such transformations should be easily identified by those skilled in the art without undue experimentation.

[0068] The peptidic linker and the PNA oligomer are connected through a covalent bond, and the nature of this covalent bond depends on the substituents contained in the peptidic linker and the PNA oligomer. In one embodiment, the peptidic linker is covalently attached to the C-terminal subunit of the PNA oligomer. In another embodiment, the peptidic linker is covalently attached to the N-terminal subunit of the PNA oligomer. In yet another embodiment, the peptidic linker is covalently attached to an internal subunit of the PNA oligomer. In a non-limiting example, a carboxylic acid group or an activated carboxylic acid group on the C-terminal of the PNA oligomer is coupled with an amino group on the peptidic linker, such as the backbone amino group of the N-terminal amino acid of the peptidic linker, to form an amide bond. In another non-limiting example, an amino group on the N-terminal of the PNA oligomer is coupled with a carboxylic acid group or an activated carboxylic acid group on the peptidic linker, such as the backbone carboxylic acid group of the C-terminal amino acid of the peptidic linker, to form an amide bond. Conditions for such transformation should be easily identified by those skilled in the art without undue experimentation.

[0069] The synthesis of the conjugate of the invention may be performed in any order that is compatible with the substituents located on the PNA oligomer, peptidic group and fluorophore. In a non-limiting example, the PNA oligomer and the peptidic linker are synthesized separately by solution-phase or solid-phase methods, deprotected and then coupled to each other, and the two fluorophores are then coupled to the peptidic linker. In another non-limiting example, the PNA oligomer and the peptidic linker are synthesized using an “one-pot” approach, where the subunits are coupled to generate the PNA oligomer/peptidic linker molecule, using a known method such as solid-phase synthesis, and the PNA oligomer-peptidic linker molecule is then deprotected and derivatized with the fluorophore. In yet another non-limiting example, the peptidic linker is synthesized, deprotected and coupled to the fluorophore, and the resulting molecule is coupled to the PNA oligomer. The couplings used in these syntheses may result in mixtures of products, requiring purification by methods such as chromatography, extraction or crystallization, or a combination thereof, following procedures known to those skilled in the art.

[0070] Where Fmoc chemistry is used to synthesize the PNA oligomer, the PNA oligomer may be readily attached to the peptidic linker's amino or carboxy terminus. If it is desired to attach the PNA oligomer to an internal amino acid residue of the peptidic linker, an ϵ -(N-tBOC)-lysine residue would be included in the peptidic linker. After completion of peptide synthesis by Fmoc coupling, and cleaving of the terminal Fmoc group, the ϵ -(N-tBOC)-lysine is deprotected with acid, and can serve as the attachment site for tBOC coupling of a PNA oligomer.

[0071] According to one embodiment of the invention, the peptidic linker is first synthesized by any of the known peptide synthesis methods. While the PNA oligomer and peptidic linker may be synthesized separately and then covalently coupled using any of the known reagents suitable for coupling proteinaceous compounds, it is preferred that the peptidic linker is synthesized first, followed by synthesis of the PNA oligomer as an extension of the peptidic linker. The amino acids used to form the peptidic linker may comprise D- or L-amino acids, or a mixture of both. Different coupling chemistries may be used for the peptidic linker and PNA oligomer syntheses. For example, where BOC coupling is used for PNA oligomer synthesis and Fmoc coupling is used for peptidic linker synthesis, the protecting groups for the peptidic linker are chosen in such a way as to be compatible with BOC coupling and BOC deprotection. Thus, for Fmoc peptidic linker synthesis followed by BOC PNA oligomer synthesis, Fmoc amino-protected amino acids utilized in the peptidic linker synthesis would include appropriate blocking groups on the amino acid side chains. Such fully protected amino acids include, for example, Fmoc-Cys(MOB)-OH, wherein the native sulfhydryl group is protected by a methoxybenzyl group; Fmoc-Lys(Z)-OH, wherein the native ϵ -amino group is protected by a phenylmethoxycarbonyl group; and Fmoc-Ser(Bzl)-OH, wherein the native hydroxyl group is protected by a benzyl group. Other suitable side chain-protected Fmoc amino acids are known to those skilled in the art. Following the completion of the PNA oligomer synthesis onto the peptidic linker, the completed peptide/PNA oligomer conjugate is then finally deprotected and cleaved from its solid support, and reacted with the fluorophore. Alternatively, following the completion of the PNA oligomer synthesis onto the peptidic linker, the peptide/PNA oligomer conjugate is selectively

deprotected, reacted with the fluorophore, fully deprotected and cleaved from its solid support.

[0072] In one embodiment, the entire peptidic linker /PNA oligomer conjugate is synthesized by the same peptide synthesis chemistry. For example, it is possible to synthesize an entire peptidic linker/PNA oligomer conjugate via Fmoc chemistry originally designed for peptide synthesis. Fmoc-PNA subunits are commercially available (PerSeptive Biosystems, Framingham, Mass.).

[0073] The nucleic acid sequences targeted for PNA oligomer binding according to the practice of the present invention may comprise, for example, non-oncogenic, proto-oncogenic or oncogenic genomic DNA (through triplex formation) or mRNA (through duplex formation). In general, the PNA oligomer used in the practice of the present invention has a subunit sequence that is completely complementary to a selected portion of the target polynucleotide. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "subunit sequence complementary to" a target polynucleotide does not necessarily mean a sequence that has 100% complementarity with the target segment. In general, any PNA oligomer having sufficient complementarity to form a stable duplex or triplex with the target, i.e. an oligomer which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing PNA oligomer and the degree of complementarity with the target polynucleotide. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. One skilled in the art may readily determine the degree of mismatching that may be tolerated between any given PNA oligomer and the target sequence, based upon the melting temperature, and therefore the thermal stability, of the resulting duplex. Preferably, the thermal stability of hybrids formed by PNA oligomers is determined by way of melting, or strand dissociation, curves. The temperature of 50 % strand dissociation is taken as the melting temperature, which, in turn, provides a convenient measure of stability.

Methods of the invention

[0074] The binding of the conjugates of the invention to a RNA or DNA sequence may be assessed by monitoring the fluorescence of the system. Fluorescence is a kind of luminescence where the molecular absorption of a photon triggers the emission of a

photon with a longer wavelength. Fluorescence may be used as an imaging property, utilizing a fluorescence microscope, confocal laser scanning microscope or total internal reflection fluorescence microscope as a detector.

[0075] The majority of fluorescence detection techniques use fluorescence microscopes. These microscopes use high intensity light sources, usually mercury or xenon lamps, LEDs, or lasers, to excite fluorescence in the samples under observation. Optical filters then separate excitation light from emitted fluorescence, to be detected by eye, or with a (CCD) camera or other light detectors (photomultiplier tubes, spectrographs, etc). Much research is underway to improve the capabilities of such microscopes, the fluorescent probes used, and the applications they are applied to. Of particular note are confocal microscopes, which use a pinhole to achieve optical sectioning and afford a quantitative 3D view of the sample. Due to the limited penetration of radiation in tissues, fluorescence is especially useful in cell cultures or endoscopy techniques. Diffraction of light by body tissues limits the depth of disease zone identification to an average 2 cm.

[0076] Selectivity for a specific nucleic acid sequence is conferred by the PNA oligomer present in the conjugate, since the PNA oligomer or a portion thereof, hybridizes to the nucleic acid sequence of choice. The choice of PNA oligomer incorporated in the conjugate will thus ultimately help determine the specificity of the conjugate. By identifying the nucleic acid sequence of choice, one skilled in the art should be able to choose the desired PNA oligomer that binds the nucleic acid sequence of choice, based on the pairing of heterocyclic bases located on the nucleic acid sequence and the PNA oligomer.

[0077] The conjugates of the invention find utility in the probing of nucleic acids in cell cultures and in vivo. In this embodiment, the conjugate of the invention is added to a system containing the nucleic acid material of interest and fluorescence is monitored. The nucleic acid material may be free in solution or contained within a cell. When used to probe nucleic acid sequences in a cell culture, one skilled in the art should be able to vary the exposure time, the amount of conjugate and the final concentration of the conjugate to optimize the detection or imaging desired. Other experimental parameters may be varied to achieve the other effect, depending on the specific

experiment conducted, and identification of such parameters should involve minimal experimentation by those skilled in the art.

[0078] The conjugates of the invention also find utility in the probing of nucleic acids in organs and tissues in a subject in vivo. In this embodiment, the conjugate of the invention is formulated appropriately and administered to the subject where probing is to be performed, and fluorescence of the tissue of interest is monitored. For in vivo use, the conjugate of the invention may be formulated in a pharmaceutical composition, which may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like in addition to the conjugate of the invention. One preferred formulation, for intravenous or subcutaneous administration, consists of sterile normal saline. For slow release from subcutaneous or intramuscular depots, the conjugate may be combined with sterile ethanol, polyethylene glycol, e.g., PEG 400, or polyethyleneglycerol triricinoleate.

[0079] The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic exposure treatment is desired, and on the area to be exposed to the conjugate. Administration may be performed topically (including ophthalmically, vaginally, rectally, transdermally, and intranasally), orally, by inhalation, or parenterally, for example by intravenous infusion, drip or injection, or subcutaneous, intraperitoneal or intramuscular injection. Intravenous administration is utilized for rapid systemic distribution. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable. Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

[0080] Amounts of the conjugate of the invention to be administered to the subject depend, among other factors, on the administration route, weight of the subject,

area to be monitored, distribution of the conjugate in the subject, rate of clearance of the conjugate from the subject, possible toxicity of the conjugate, and sensitivity of the fluorescence assay used for detection. Such factors should be considered by those skilled in the art in the determining the dose to be given to the subject.

[0081] The pharmaceutical compositions of the invention may be dispensed to the subject under treatment with the help of an applicator. The applicator to be used may depend on the specific medical condition being treated, amount and physical status of the pharmaceutical composition, and choice of those skilled in the art.

[0082] The pharmaceutical compositions of the invention may be provided to the subject or the medical professional in charge of dispensing the composition to the subject, along with instructional material. The instructional material includes a publication, a recording, a diagram, or any other medium of expression, which can be used to communicate the usefulness of the composition and/or compound used in the practice of the invention in a kit. The instructional material of the kit may, for example, be affixed to a container that contains the compound and/or composition used in the practice of the invention or may be shipped together with a container that contains the compound and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the instructional material and the compound cooperatively. Delivery of the instructional material may be, for example, by physical delivery of the publication or other medium of expression communicating the usefulness of the kit, or may alternatively be achieved by electronic transmission, for example by means of a computer, such as by electronic mail, or download from a website.

Exemplification used in the practice of the invention

[0083] The invention is described hereafter with reference to the following examples. The 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 that become evident as a result of the teaching provided herein.

Materials

[0084] The aqueous buffer used in the experiments described herein was 0.14 M NaCl, 10 mM NaH₂PO₄, pH 7.4.

[0085] The PNA oligomer, PNA[GCCATCAGCTCC], was designed to be complementary to the sense KRAS 20-mer DNA sequence 5'-AGTTGGAGCTGATGGCGTAG-3' (SEQ ID NO:1), as shown below:

sense KRAS 20-mer DNA
(SEQ ID NO:1):

5' -AGT TGG AGC TGA TGG CGT AG-3'

KRAS D12 PNA:
molecular beacon

[3' - CC TCG ACT ACC G -5']
C-terminal N-terminal

[0086] The RNA complementary sense strand used in the experiment was a synthetic RNA oligomer that brackets the codon 12 target of KRAS mRNA: 5'-AGU UGG AGC UGA UGG CGU AG-3' (SEQ ID NO:2). The RNA complementary strand was synthesized by Dharmacon (Lafayette, CO).

[0087] The PNA oligomers may be synthesized according to the methods known in the literature: Amirkhanov et al., 2008, Biopolymers 89(12):1061-1076; Tian et al., 2007, Biochem. Soc. Trans. 35:72-76; Tian et al., 2007, J. Nucl. Med. 48(10):1699-1707; Chakrabarti et al., 2007, Cancer Biol. & Ther. 6(6):948-956; Chakrabarti et al., 2005, Nucleos. Nucleot. & Nucl. Acids 24(5-7):409-414; Amirkhanov & Wickstrom, 2005, Nucleos. Nucleot. & Nucl. Acids 24(5-7):423-426.

Methods

Comparative Example 1

Compound (IX):

Single-fluorophore-containing PNA oligomer

(compound VII)-Aeea-PNA[GCCATCAGCTCC]-Aeea-Lys-Aeea

[0088] As a control for the experiments described herein, the monolabeled fluorophore-containing conjugate (IX), comprising PNA oligomer PNA[GCCATCAGCTCC], was prepared:

(compound VII)-Aeea-PNA[GCCATCAGCTCC]-Aeea-Lys-Aeea (IX)

Compound (IX) comprises two peptidic linkers: the peptidic linker Aeea is coupled by an amide bond through its carboxylic acid group to the N-terminal amino group of the PNA oligomer, and the peptidic linker Aeea-Lys-Aeea is coupled by an amide bond through its N-terminal amino group to the C-terminal carboxylic acid group of the PNA oligomer. The N-terminal of the peptidic linker Aeea is coupled by an amide bond to the carboxylic acid group of the fluorophore 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII). The ϵ -amino group of the lysine residue of the peptidic linker Aeea-Lys-Aeea is underivatized.

Comparative Example 2

UV-visible spectra of compound (IX)

[0089] UV-visible spectra of compound (IX) were recorded in aqueous buffer and 50 % acetonitrile-aqueous buffer (Figure 1). In aqueous buffer, compound (IX) had absorption maxima at $\lambda_{\max} = 261$ nm (0.019), 616 nm shoulder (0.015), and 667 nm (0.037). The absorptivity ratios were $D_{665}/D_{616} = 2.47$, and $D_{665}/D_{260} = 1.95$. In 50 % acetonitrile/water, compound (IX) had absorption maxima at $\lambda_{\max} = 257$ nm (0.130), 616 nm shoulder (0.069), and 665 nm (0.174). The absorptivity ratios were $D_{665}/D_{616} = 2.52$, and $D_{665}/D_{260} = 1.34$. As shown in Figure 1, the superimposed spectra have similar shapes.

[0090] UV-visible spectra of compound (IX), at a concentration of 1 μ M in aqueous buffer, were recorded in aqueous buffer in the presence and absence of 1 μ M RNA complementary sense strand (Figure 2). In aqueous buffer, compound (IX) had absorption maxima at $\lambda_{\max} = 261$ nm (0.019), 616 nm shoulder (0.015), and 667 nm (0.037). The absorptivity ratios were $D_{665}/D_{616} = 2.47$, and $D_{665}/D_{260} = 1.95$. In aqueous buffer in the presence of 1 μ M RNA complementary sense strand, compound (IX) had absorption maxima at $\lambda_{\max} = 257$ nm (0.250), and 670 nm (0.071). As shown in Figure 2, the superimposed spectra have similar shapes.

[0091] The experiments above indicated that the spectrum of compound (IX) in aqueous buffer does not change significantly in the presence of acetonitrile or RNA complementary sense strand. These results are consistent with the fact that the single

fluorophore in compound (IX) is incapable of intramolecular stacking, and thus its spectroscopic characteristics should not be significantly modified by addition of an apolar solvent to the solution or by hybridization of compound (IX) to a nucleic acid sequence.

Example 1

Compound (X):

Twin-fluorophore-containing PNA oligomer

(compound VII)-Aeea-PNA[GCCATCAGCTCC]-Aeea-Lys(ϵ -NH-compound VII)-Aeea

[0092] The title compound (X) is a conjugate comprising a PNA oligomer with a backbone formed by N-ethylaminoglycine units.

(compound VII)-Aeea-PNA[GCCATCAGCTCC]-Aeea-Lys(ϵ -NH-compound VII)-Aeea
(XI)

[0093] The PNA oligomer is PNA[GCCATCAGCTCC]. Compound (X) comprises two peptidic linkers: the peptidic linker Aeea is coupled by an amide bond through its C-terminal carboxylic acid to the N-terminal amino group of the PNA oligomer, and the peptidic linker Aeea-Lys-Aeea is coupled by an amide bond through its N-terminal amino group to the C-terminal carboxylic acid of the PNA oligomer. The N-terminal amino group of the peptidic linker Aeea is coupled by an amide bond to the carboxylic acid group of the fluorophore residue 4,5-benzo-1'-ethyl-3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII). The ϵ -amino group of the lysine residue of the peptidic linker Aeea-Lys-Aeea is coupled by an amide bond to the carboxylic acid group of the fluorophore residue 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII).

Example 2

UV-visible spectra of compound (X) in the absence of RNA

[0094] The UV-visible spectrum of compound (X) was recorded in aqueous buffer and 50% acetonitrile/aqueous buffer (Figure 3). The initial stock solution of compound (X) was in 50 % acetonitrile/aqueous buffer, and the assay solutions were

prepared by diluting 20 μL of the stock solution in 1 mL of aqueous buffer or 1 mL of 50 % acetonitrile/aqueous buffer.

[0095] In aqueous buffer, compound (X) had absorption maxima at $\lambda_{\text{max}} = 260$ nm (0.178), 358 nm (0.029), 616 nm (0.514) and 665 nm (0.207). The absorptivity ratios were $D_{665}/D_{616} = 0.40$, and $D_{665}/D_{260} = 1.16$. In 50 % acetonitrile/ aqueous buffer, compound (X) had absorption maxima at $\lambda_{\text{max}} = 259$ nm (0.245), 361 nm (0.045), 616 nm shoulder (0.374) and 665 nm (0.700). The absorptivity ratios were $D_{665}/D_{616} = 1.87$, and $D_{665}/D_{260} = 2.86$. As shown in Figure 3, compound (X) displayed a fairly different UV-visible spectrum in aqueous buffer and in 50% acetonitrile/aqueous buffer. This observation is consistent with the model where the two fluorophores of compound (X) undergo intramolecular stacking when the conjugate is unbound. The intramolecular stacking should be predominant in aqueous buffer and minimized in a lower polarity solvent such as 50 % acetonitrile/aqueous buffer.

Example 3

UV-visible spectra of compound (X) in the presence of RNA complementary sense strand

[0096] The spectrum of compound (X), at a concentration of 1 μM , was recorded in aqueous buffer in the presence and absence of the RNA complementary sense strand (Figure 4). The RNA complementary sense strand, if present, had a concentration of 1 μM in these experiments.

[0097] In aqueous buffer, compound (X) had absorption maxima at $\lambda_{\text{max}} = 262$ nm (0.098), 358 nm, 616 nm (0.158) and 672 nm (0.096). The absorptivity ratios were $D_{665}/D_{616} = 0.61$, and $D_{665}/D_{260} = 0.98$. In buffer with RNA, compound (X) had absorption maxima at $\lambda_{\text{max}} = 258$ nm (0.288), 358 nm, 621 nm shoulder (0.109) and 672 nm (0.202). The absorptivity ratio was $D_{674}/D_{616} = 1.94$.

[0098] UV-visible spectra were obtained for 1 μM compound (X) in aqueous buffer, 1 μM compound (X) with 1 μM RNA complementary sense strand in aqueous buffer, 1 μM compound (X) in 50 % acetonitrile-aqueous buffer, and 1 μM compound

(X) with 1 μ M RNA complementary sense strand in 50 % acetonitrile-aqueous buffer. These UV-visible spectra are superimposed for comparison in Figure 5.

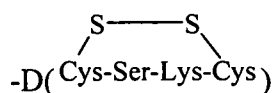
[0099] Figure 5 shows that compound (X) in aqueous buffer changed its spectrum upon addition of the RNA complementary sense strand, with its λ_{max} moving from 616 nm to 672 nm. Furthermore, the spectrum of compound (X) bound to the RNA complementary sense strand was remarkably similar to the spectrum of Compound (X) in 50 % acetonitrile-aqueous buffer, and the spectrum of compound (X) with the RNA complementary sense strand in aqueous buffer was similar to the spectrum of compound (X) with 1 μ M RNA complementary sense strand in 50 % acetonitrile-aqueous buffer. Taken together, these results are consistent with the model in which the two fluorophores of Compound (X) undergo intramolecular stacking in aqueous solution or in the absence of RNA. The UV-visible spectrum changes observed suggest that unstacking of the fluorophores was promoted by addition of RNA complementary sense strand (since the PNA oligomer hybridizes to the RNA complementary sense strand) and/or by addition of acetonitrile (since the apolar solvent destabilizes the hydrophobic stacking of the fluorophores).

Comparative Example 3

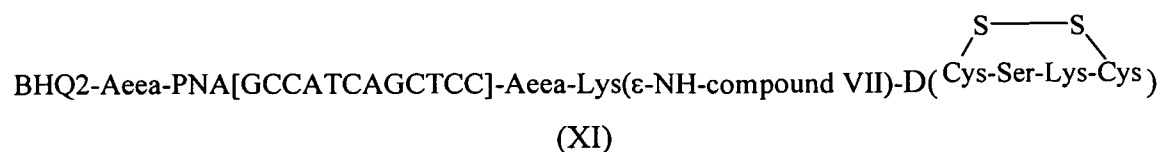
Compound (XI):

Fluorophore-quencher PNA oligomer conjugate

BHQ2-Aeea-PNA[GCCATCAGCTCC]-Aeea-Lys(ϵ -compound VII)-

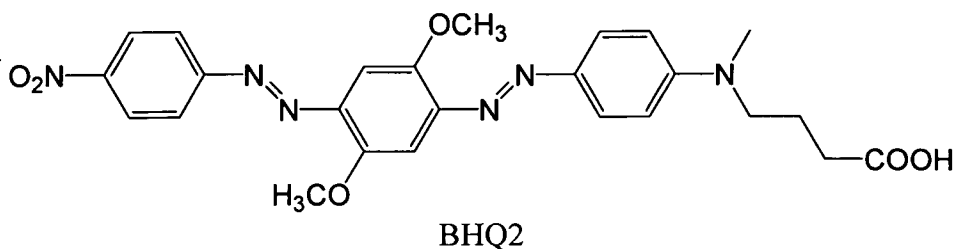


[00100] Compound (XI), a conjugate comprising the same PNA oligomer as compound (X), was prepared.



Compound (XI) comprises the fluorophore (VII) and the quencher BHQ2 (also known as 4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl) diazenyl)phenyl)diazenyl)

phenyl)methylamino)butanoic acid; Black Hole Quencher™-2, Biosearch Technologies, Novato, CA).



The fluorophore and the quencher are attached to opposite ends of the PNA oligomer by amide bonds: the fluorophore is attached to the Aeea linker that is connected to the N-terminal of the PNA oligomer, and the fluorophore (VII) is attached to the ϵ -amino group of lysine. The lysine residue is connected through its C-terminal to a cyclic tetrapeptide, wherein the side chains of the two cysteine residues are linked through a disulfide bridge.

[00101] Fluorescence spectra for compound (XI), at 1 μ M concentration in aqueous buffer, were recorded in the presence and absence of 2 μ M RNA complementary sense strand. The excitation spectra were recorded with λ_{exc} of 500-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 665-720 nm. Selected traces of the spectra are shown in Figure 6.

[00102] Figure 6 contains the superimposed emission spectra for compound (XI), hybridized to the RNA complementary sense strand or not. The fluorescence ratio measured at 680 nm for compound (XI) in the presence vs absence of RNA complementary sense strand was found to be 7.1.

Example 4

Excitation and emission fluorescence spectra for compound (X)

[00103] Fluorescence spectra for compound (X), at a concentration of 1 μ M in aqueous buffer, were recorded in the presence and absence of 2 μ M RNA complementary sense strand. The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. Selected traces of the spectra are shown in Figure 7.

[00104] Figure 7 contains the superimposed emission spectra for compound (X), at a concentration of 1 μ M, hybridized to the RNA complementary sense strand or not. The fluorescence ratio measured at 680 nm for compound (X) in the presence vs absence of RNA complementary sense strand was found to be 12.1. By comparison, the fluorescence ratio determined for the monofluorophore compound (XI) was 7.1 (Comparative Example 3). Therefore, Compound (X) showed a fluorescence ratio at 680 nm that was approximately 1.7 higher than compound (XI), consistent with the fact that the former has two fluorophores in its structure and the latter has one fluorophore in its structure.

[00105] Fluorescence spectra for compound (X), at a concentration of 100 nM in aqueous buffer, were recorded in the presence and absence of 2 μ M RNA complementary sense strand. The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. Selected traces of the spectra are shown in Figure 8.

[00106] Figure 8 contains the superimposed emission spectra for compound (X), at a concentration of 100 nM, hybridized to the RNA complementary sense strand or not. The fluorescence ratio measured at 680 nm for compound (X) in the presence vs absence of RNA complementary sense strand was found to be 102. Therefore, the twin fluorophore conjugate displayed a nearly 100-fold dynamic range at this concentration.

[00107] The excitation and emission fluorescence spectra for the aqueous buffer, in the absence of compound (X), were recorded. The excitation spectra were recorded with λ_{exc} of 640-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-690 nm. The resulting curves were superimposed with the corresponding spectra for compound (X) in aqueous buffer (Figure 9), showing that the fluorescence readings of the aqueous buffer were much lower than those for the compound of interest.

[00108] The excitation and emission fluorescence spectra of compound (X) in 50% acetonitrile/aqueous buffer were recorded, and plotted along with the corresponding spectra of compound (X) in aqueous buffer (Figure 10). The excitation spectra were recorded with λ_{exc} of 550-680 nm and λ_{emiss} of 690 nm. Emission spectra were recorded with λ_{exc} of 650 nm and λ_{emiss} of 660-720 nm. The fluorescence ratio

measured at 680 nm for compound (X) in 50% acetonitrile/aqueous buffer vs aqueous buffer was 21.2, and the fluorescence ratio measured at 670 nm for compound (X) in 50% acetonitrile/aqueous buffer vs aqueous buffer was 19.3. This results indicate that, under the conditions used in the Example, the compound showed a fluorescence intensity in 50 % acetonitrile/aqueous buffer (where the fluorophores were expected to be unstacked) at 680 nm that was approximately 20 times higher than in aqueous buffer (where the fluorophores were expected to be stacked). This observation is consistent with the fluorescence self-quenching expected from intramolecular stacking of the fluorophores.

[00109] The excitation and emission fluorescence spectra of compound (X) in 50 % acetonitrile/aqueous buffer had similar shape to those obtained for the compound in the presence of the RNA complementary sense strand (Figure 6). This observation is consistent with the model where the two fluorophores of compound (X), while stacked in aqueous buffer, undergo unstacking when hybridized to the RNA complementary sense strand or treated with an apolar solvent.

Example 5

In vitro experiments with compound (X)

[00110] AsPC1 KRAS G12D human pancreas cancer cells, maintained in wells of an 8-well chamber slide, were incubated with 500 nM of compound (X) in Opti-Mem low serum medium (Invitrogen, Carlsbad, CA) at 37°C for 24 hours. The medium with probe was removed, and replaced with fresh Opti-Mem. After 24 h efflux, the medium was replaced with fresh Opti-Mem. The live cells were viewed at 37 °C on a Zeiss 510 Meta laser scanning confocal 4-channel fluorescence microscope (shown in panel A of Figure 11, as a grayscale picture). CellTracker™ Green was used to stain live cells (marked with label A2). Hoechst 33342 was used to stain nuclei (the nuclei showed up as slightly lighter areas in the center of the live cells and were marked with label A1). Cy5.5 filters revealed cytoplasmic fluorescence of compound (X) as lighter spots in the cytoplasm of the cells - for the sake of clarity, some of these lighter spots were marked with label A3. The fluorophore (VII) as the uncoupled carboxylic acid showed little intracellular fluorescence (shown as panel B of Figure 11, as a grayscale picture).

Example 6**In vitro experiments with compound (X)**

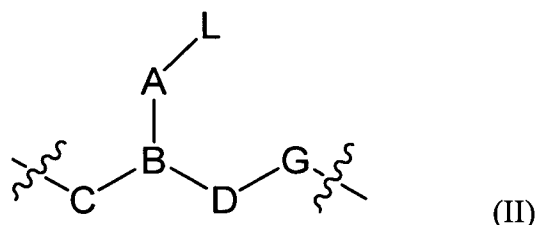
[00111] SW480 KRAS G12D colon cancer xenografts were implanted in a mouse model, and allowed to grow. The animal model was treated with 18 nmoles of compound (X). After 48 hours of the treatment, the animal was examined by whole body near-infrared fluorescence imaging, which allowed identification of the sites where the xenograft was uptaken (Figure 12). Excitation was performed at 615-665 nm, and emission was monitored at 680-800 nm, in a Maestro™ CRI all-optical in vivo imaging instrument (Woburn, MA).

[00112] The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. While the invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope used in the practice of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed:

1. A conjugate comprising a peptide nucleic acid oligomer conjugated to two molecules of a [4,5-benzo-1,1'-diethyl-3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore, wherein a first molecule of said fluorophore is conjugated to said oligomer through a first peptidic linker and a second molecule of said fluorophore is conjugated to said oligomer through a second peptidic linker.
2. The conjugate of claim 1, wherein said oligomer comprises at least one subunit that is a peptide nucleic acid subunit of Formula (II):



wherein:

- L is one of the adenine, thymine, cytosine or guanine heterocyclic bases of the oligomer;
- C is $(CR^6R^7)_y$ where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_2-C_6) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6) alkoxy, (C_1-C_6) alkylthio, NR^3R^4 and SR^5 , where each of R^3 and R^4 is independently selected from the group consisting of hydrogen, (C_1-C_4) alkyl, hydroxy- or alkoxy- or alkylthio-substituted (C_1-C_4) alkyl, hydroxy, alkoxy, alkylthio and amino; and R^5 is hydrogen, (C_1-C_6) alkyl, hydroxy-, alkoxy-, or alkylthio-substituted (C_1-C_6) alkyl, or R^6 and R^7 taken together complete an alicyclic or heterocyclic system;
- D is $(CR^6R^7)_z$, where R^6 and R^7 are as defined above;

each of y and z is zero or an integer from 1 to 10, the sum y+z being greater than 2, and less than or equal to 10;

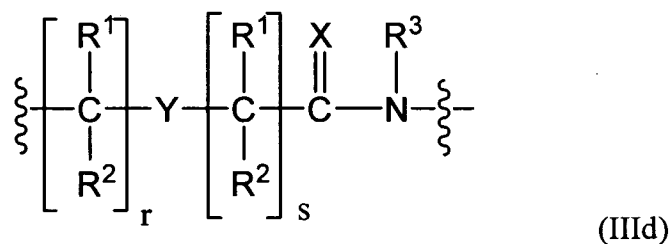
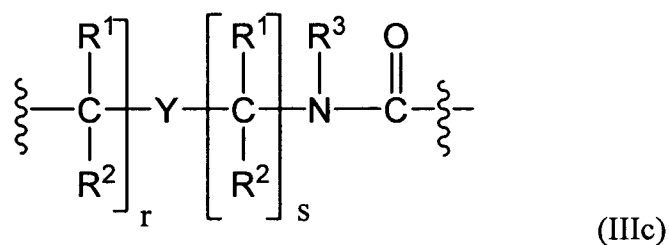
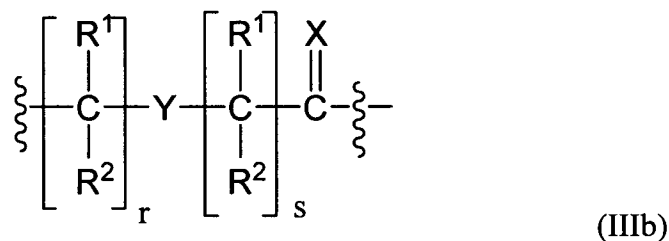
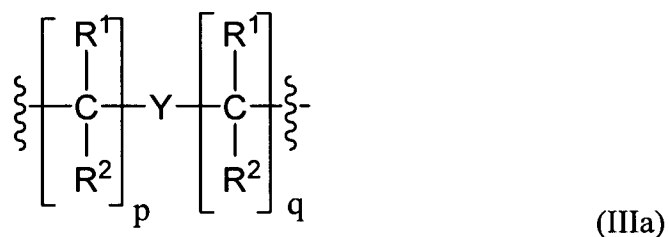
G is $-\text{NR}^3\text{C}(\text{O})-$, $-\text{NR}^3\text{C}(\text{S})-$, $-\text{NR}^3\text{S}(\text{O})-$ or $-\text{NR}^3\text{S}(\text{O})_2-$, in either orientation, where R^3 is as defined above;

each pair of A and B is selected such that:

(a) A is a group of Formula (IIIa), (IIIb) or (IIIc) and B is N or R^3N^+ ;

or

(b) A is a group of Formula (IIId) and B is CH_2 ;



wherein:

X is O, S, Se, NR^3 , CH_2 or $\text{C}(\text{CH}_3)_2$;

Y is a single bond, O, S or NR⁴;

each of p and q is zero or an integer from 1 to 5, the sum p+q being less than or equal to 10;

each of r and s is zero or an integer from 1 to 5, the sum r+s being less than or equal to 10; and,

each R¹ and R² is independently selected from the group consisting of hydrogen, (C₁-C₄) alkyl which may be hydroxy- or alkoxy- or alkylthio-substituted, hydroxy, alkoxy, alkylthio, amino and halogen.

3. The conjugate of claim 2, wherein:

A is -CH₂C(O)-;

B is N, whereby B-A is N-CH₂C(O)-;

C is -CH₂CH₂-; and,

D is -CH₂-.

4. The conjugate of claim 2, wherein all of the subunits of said oligomer are peptide nucleic acid subunits.

5. The conjugate of claim 3, wherein all of the subunits of said oligomer are peptide nucleic acid subunits.

6. The conjugate of claim 4, wherein said oligomer has a subunit sequence such that said oligomer is capable of forming (i) a triplex with a double stranded DNA segment, or (ii) a duplex with a single stranded DNA segment or an mRNA segment.

7. The conjugate of claim 2, wherein said oligomer has 6 to 25 subunits.

8. The conjugate of claim 7, wherein said oligomer has 6 to 20 subunits.

9. The conjugate of claim 8, wherein said oligomer comprises

PNA[GCCATCAGCTCC].

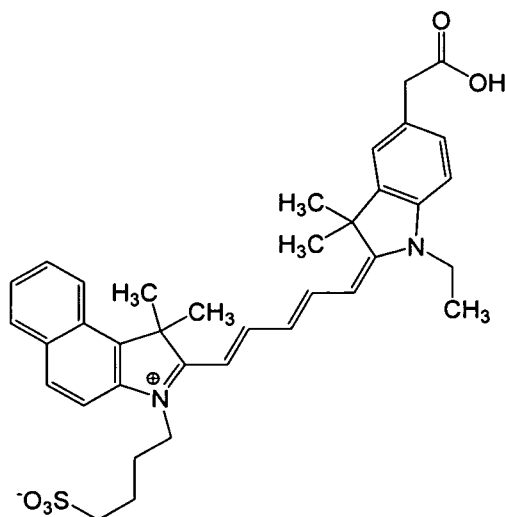
10. The conjugate of claim 2, wherein said first peptidic linker is attached to the N-terminal of said oligomer.

11. The conjugate of claim 2, wherein said first peptidic linker is Aeea [2-(2-aminoethoxy)ethoxyacetic acid].

12. The conjugate of claim 2, wherein said second peptidic linker is attached to the C-terminal of said oligomer.

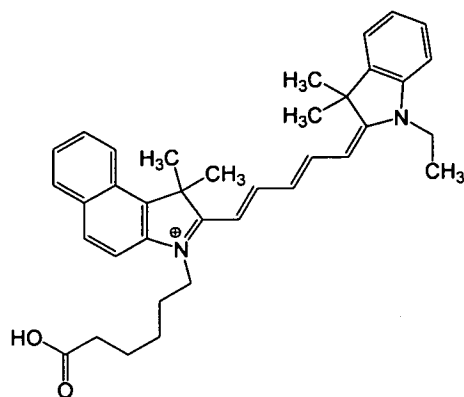
13. The conjugate of claim 2, wherein said second peptidic linker is Aeea-Lys-Aeea.

14. The conjugate of claim 2, wherein said fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid (VII):



(VII).

15. The conjugate of claim 2, wherein said fluorophore is 3-(5-carboxypentyl)-2-((1E,3E,5E)-5-(1-ethyl-3,3-dimethylindolin-2-ylidene)penta-1,3-dienyl)-1,1-dimethyl-1H-benzo[e]indolium (VIII):



(VIII).

16. The conjugate of claim 5, wherein:
 said first peptidic linker is Aeea;
 said second peptidic linker is Aeea-Lys-Aeea; and
 said fluorophore is 4,5-benzo-1'-ethyl-3,3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanine-5'-acetic acid (VII).
17. The conjugate of claim 16, wherein said oligomer comprises PNA[GCCATCAGCTCC].
18. The conjugate of claim 17, wherein said first peptidic linker is attached to the N-terminal of said oligomer and said second peptidic linker is attached to the C-terminal of said oligomer.
19. A method of assessing the presence of a specific nucleic acid sequence in a biological sample in vitro, said method comprising the steps of:
 exposing said biological sample to an excitation wavelength;
 measuring fluorescence of said biological sample at an emission wavelength;
 contacting said biological sample with a conjugate comprising a peptide nucleic acid oligomer conjugated to two molecules of a [4,5-benzo-1,1'-diethyl-3,3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophore, wherein a first molecule of said fluorophore is conjugated to said oligomer through a first peptidic linker and a second molecule of said fluorophore is conjugated to said oligomer through a second peptidic linker, thereby providing a test sample;
 exposing said test sample to said excitation wavelength; and

measuring fluorescence of said test sample at said emission wavelength.

20. The method of claim 19, wherein said excitation wavelength is about 650 nm and said emission wavelength is about 680 nm.

21. The method of claim 19, wherein said specific nucleic acid sequence comprises the DNA sequence 5'-AGT TGG AGC TGA TGG CGT AG-3' or the RNA sequence 5'-AGU UGG AGC UGA UGG CGU AG-3'.

22. The method of claim 19, wherein:
said first peptidic linker is Aeea;
said second peptidic linker is Aeea-Lys-Aeea; and,
said fluorophore is 4,5-benzo-1'-ethyl-3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid.

23. The method of claim 22, wherein said first peptidic linker is attached to the N-terminal of said oligomer and said second peptidic linker is attached to the C-terminal of said oligomer.

24. The method of claim 23, said oligomer comprises
PNA[GCCATCAGCTCC].

25. A method of assessing the presence of a specific nucleic acid sequence in a tissue in a subject in vivo, said method comprising the steps of:
exposing said tissue to an excitation wavelength;
measuring fluorescence of said tissue at an emission wavelength;
administering to said subject a pharmaceutical composition comprising a conjugate, said conjugate comprising a peptide nucleic acid oligomer conjugated to two identical [4,5-benzo-1,1'-diethyl-3,3',3'-tetramethyl-indodicarbocyanine]-based fluorophores, wherein a first molecule of said fluorophores is conjugated to said oligomer through a first peptidic linker and a second molecule of said fluorophores is conjugated to said oligomer through a second peptidic linker; thereby providing a modified tissue;

exposing said modified tissue to said excitation wavelength; and,
measuring fluorescence of said modified tissue at said emission wavelength.

26. The method of claim 25, wherein said excitation wavelength is about 650 nm and said emission wavelength is about 680 nm.

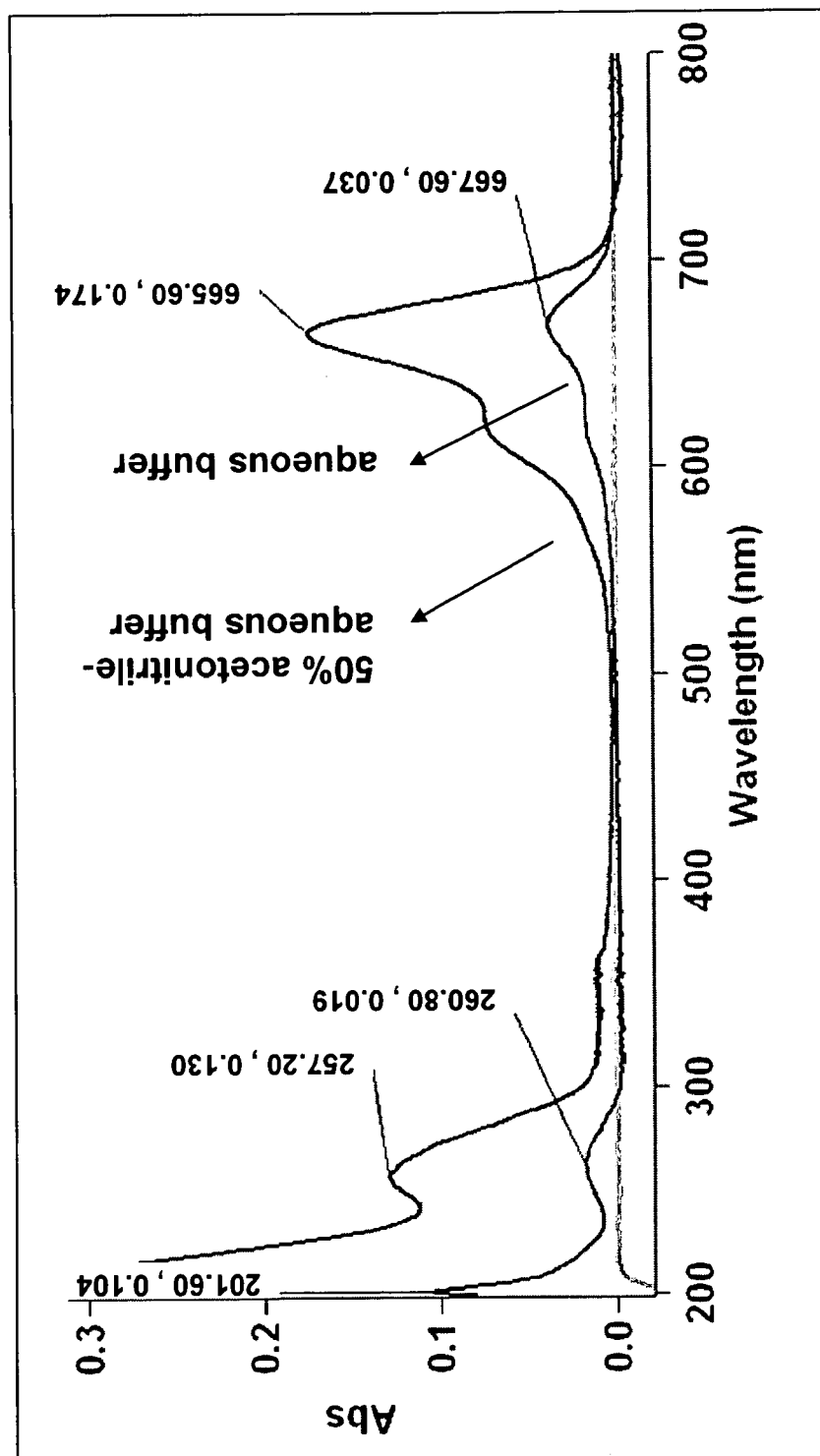
27. The method of claim 25, wherein said specific nucleic acid sequence comprises the DNA sequence 5'-AGT TGG AGC TGA TGG CGT AG-3' or the RNA sequence 5'-AGU UGG AGC UGA UGG CGU AG-3'.

28. The method of claim 25, wherein:
said first peptidic linker is Aeea;
said second peptidic linker is Aeea-Lys-Aeea; and,
said fluorophore is 4,5-benzo-1'-ethyl-3,3',3'-tetramethyl-1-(4-sulfobutyl)-indodicarbocyanin-5'-acetic acid.

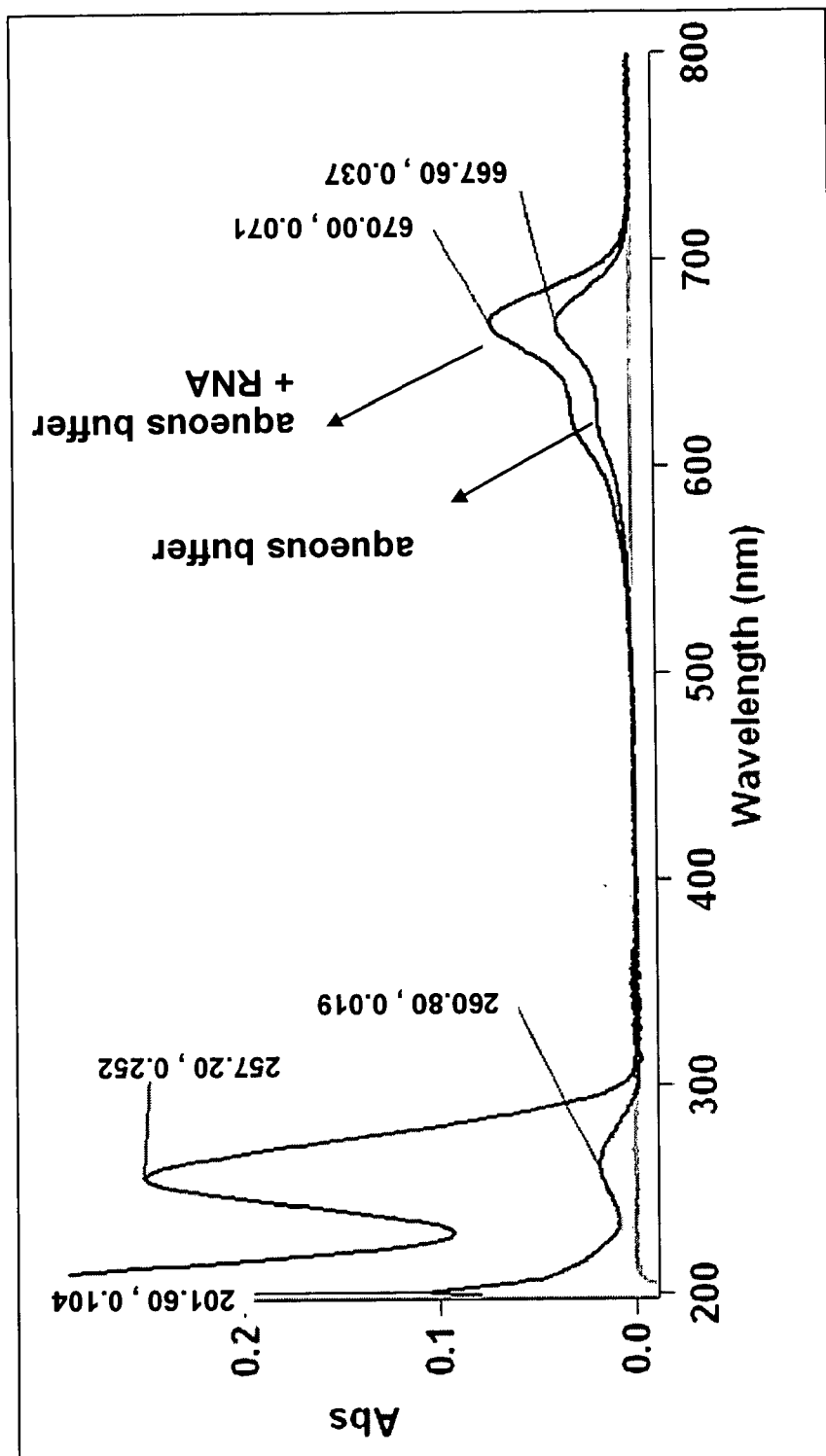
29. The method of claim 28, wherein said first peptidic linker is attached to the N-terminal of said oligomer and said second peptidic linker is attached to the C-terminal of said oligomer.

30. The method of claim 29, said oligomer comprises
PNA[GCCATCAGCTCC].

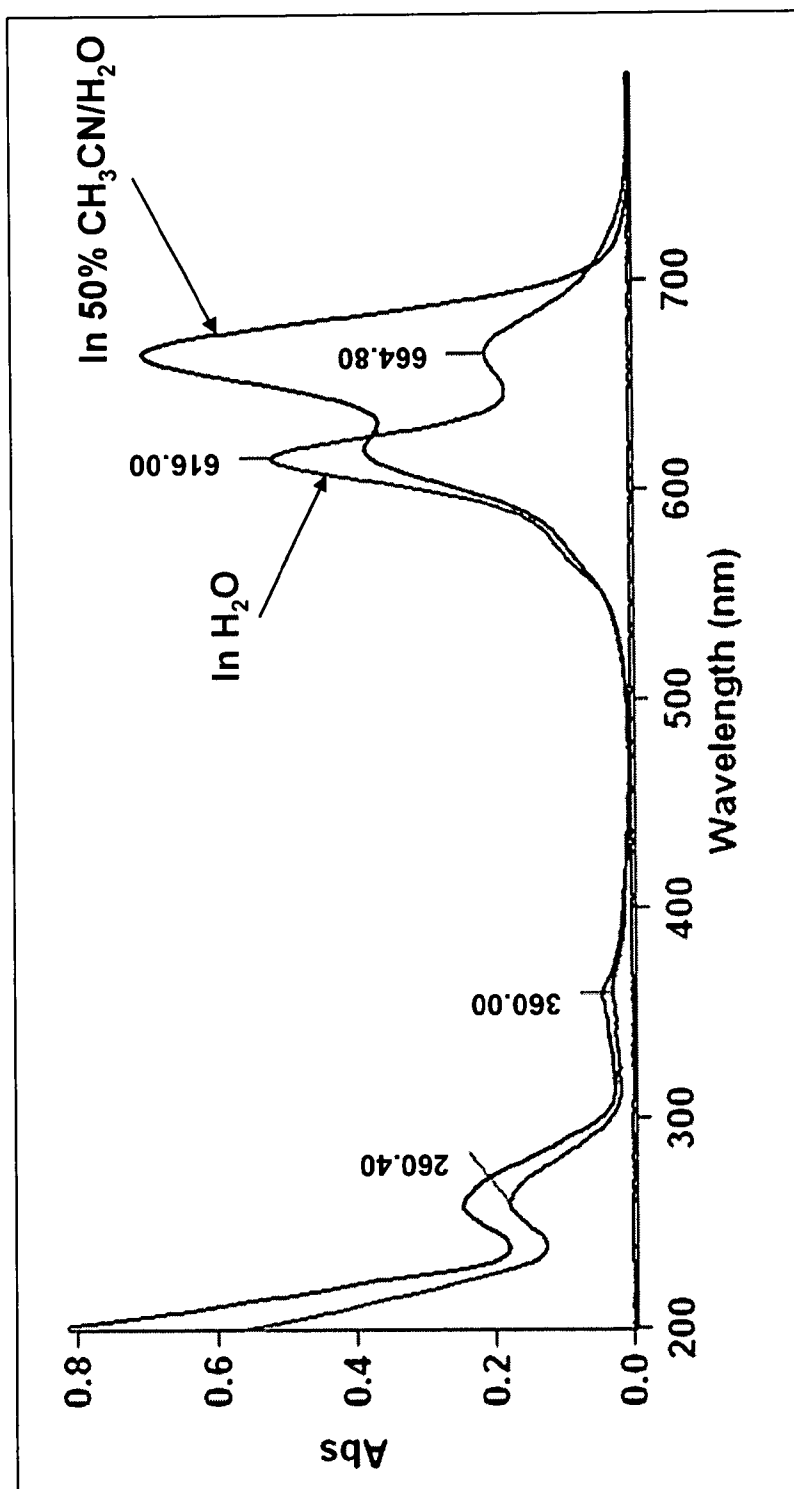
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**FIG. 1**

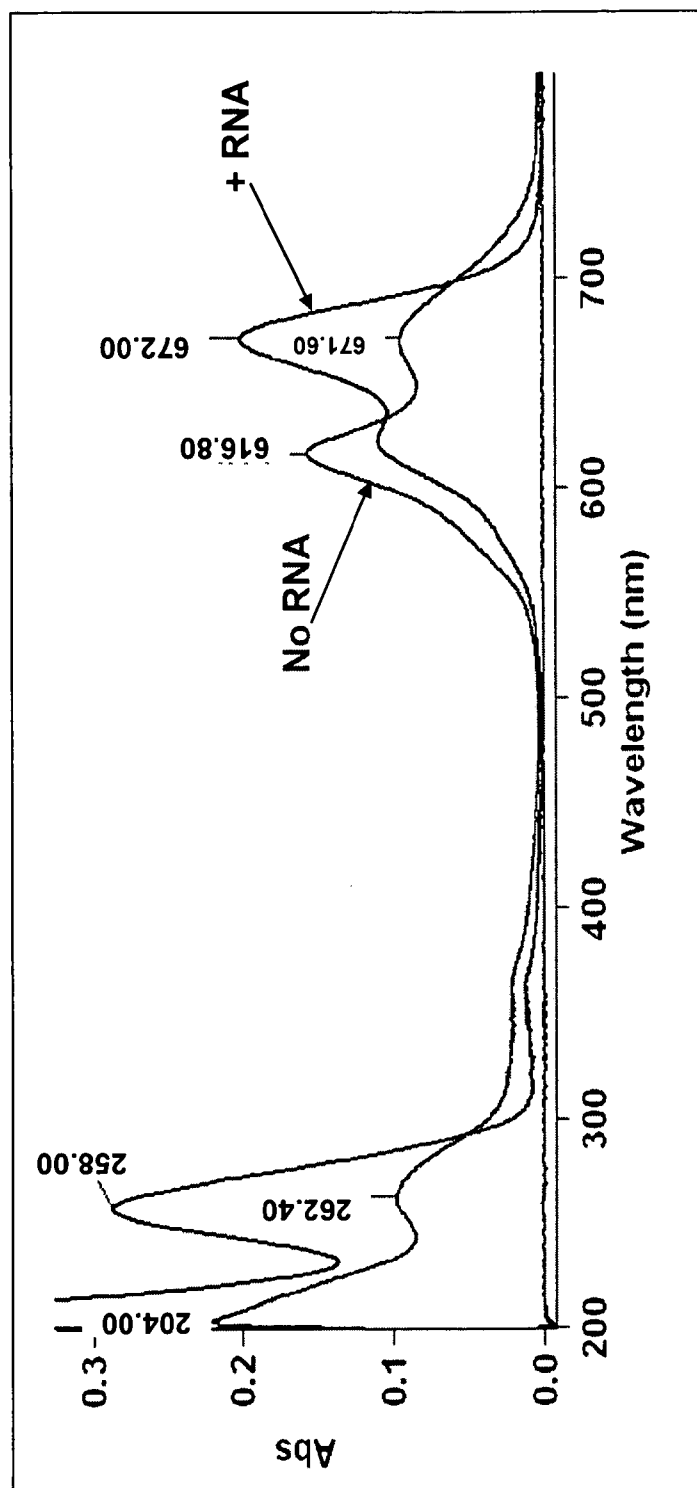
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**FIG. 2**

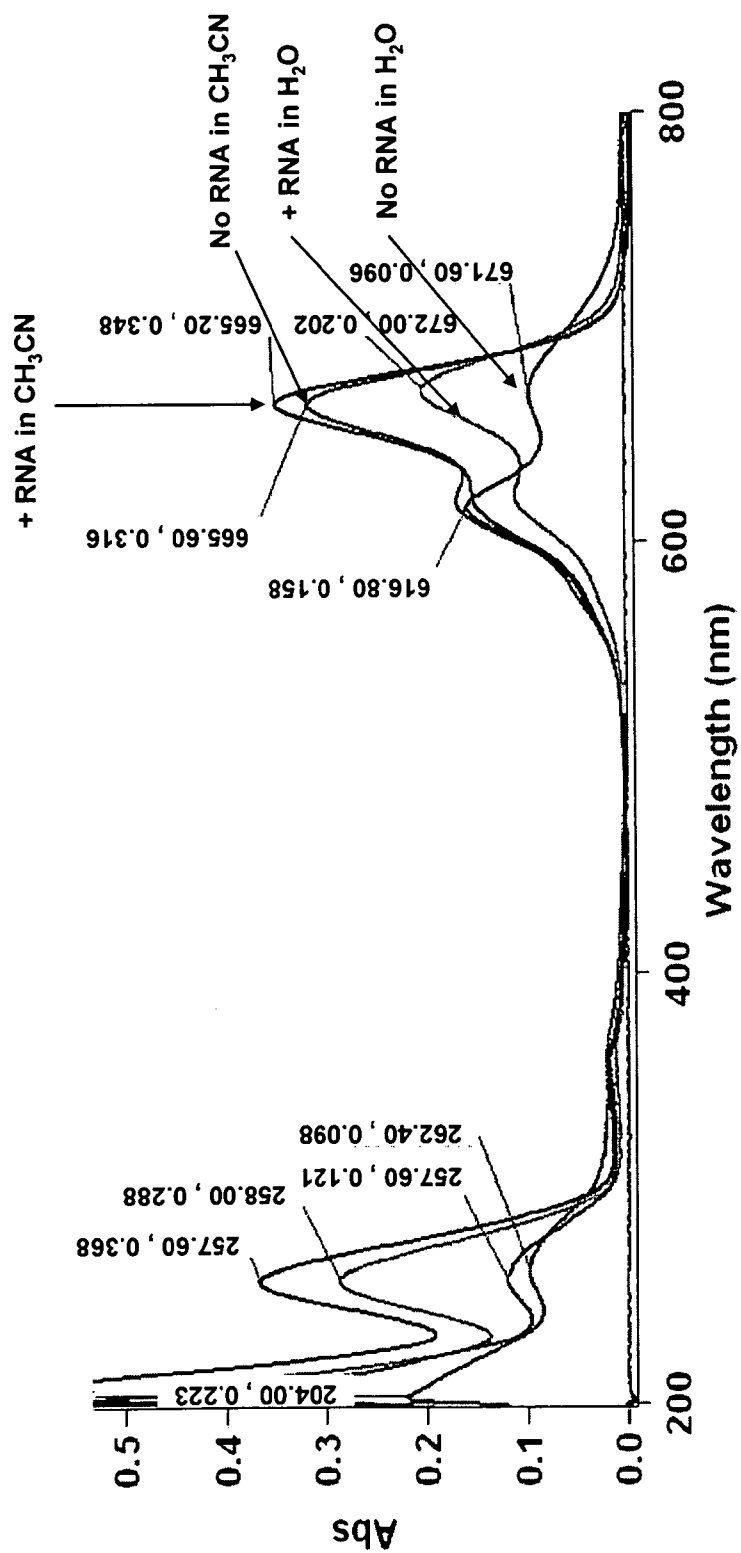
3/12

**FIG. 3**

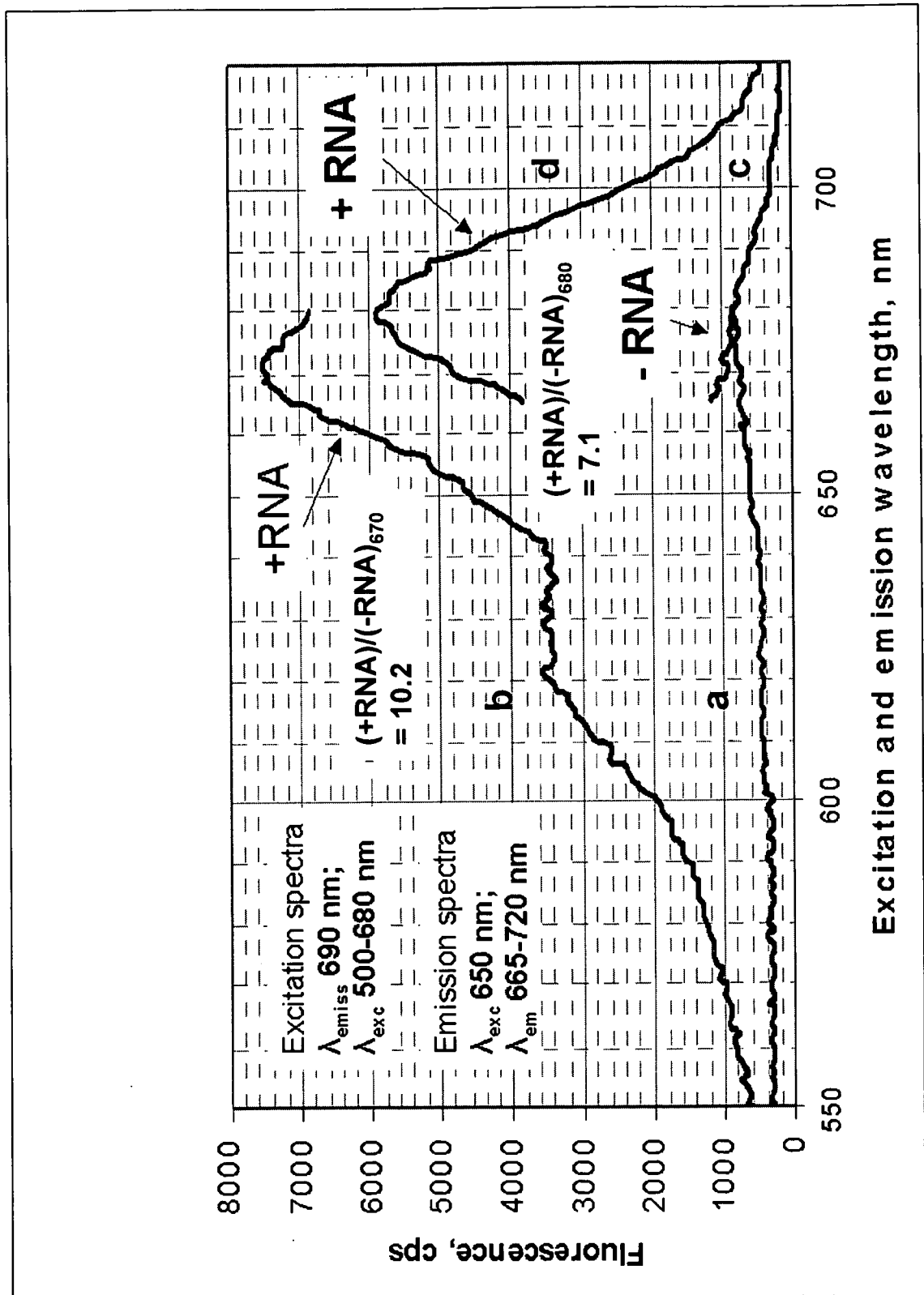
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**FIG. 4**

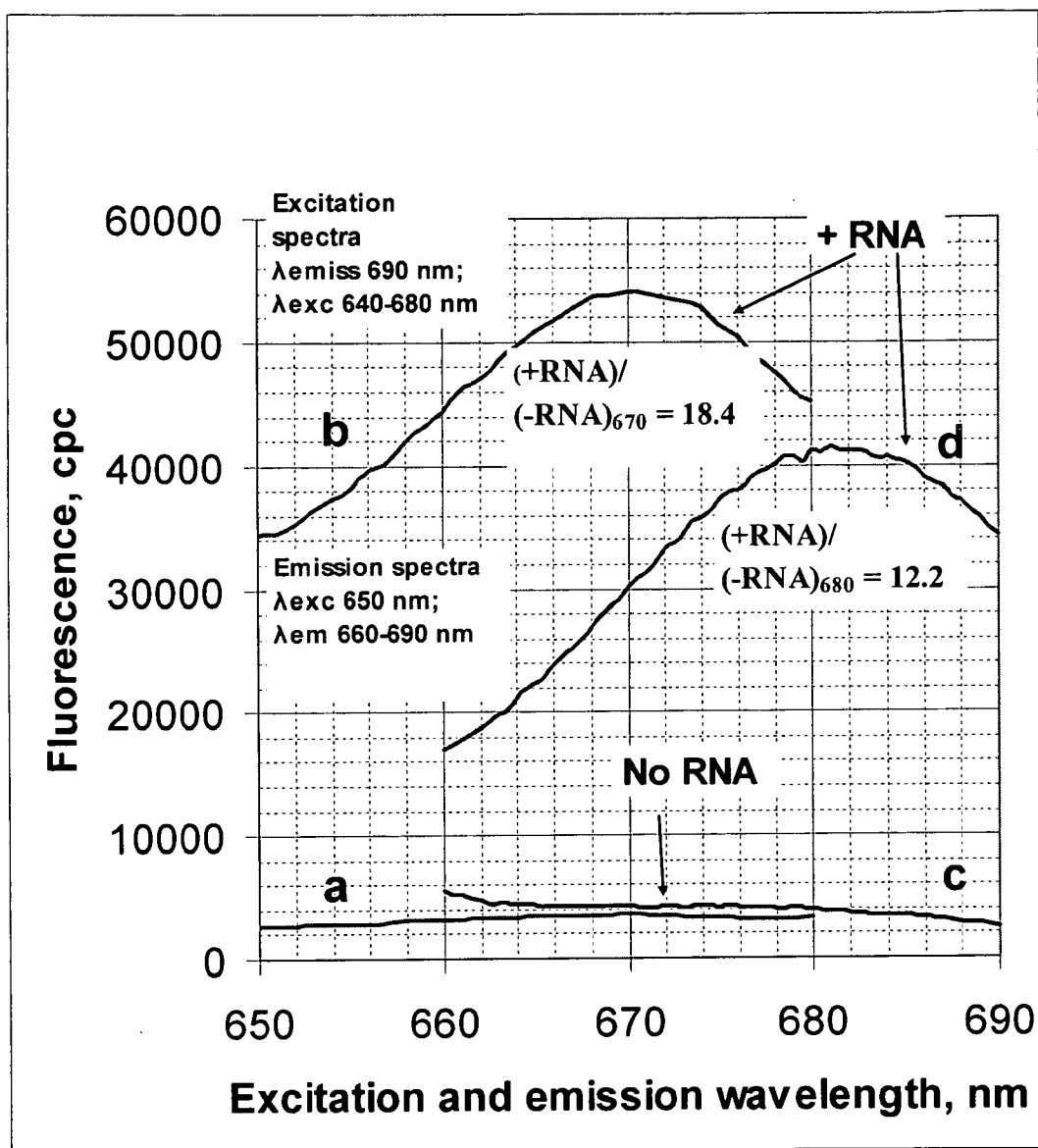
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**FIG. 5**

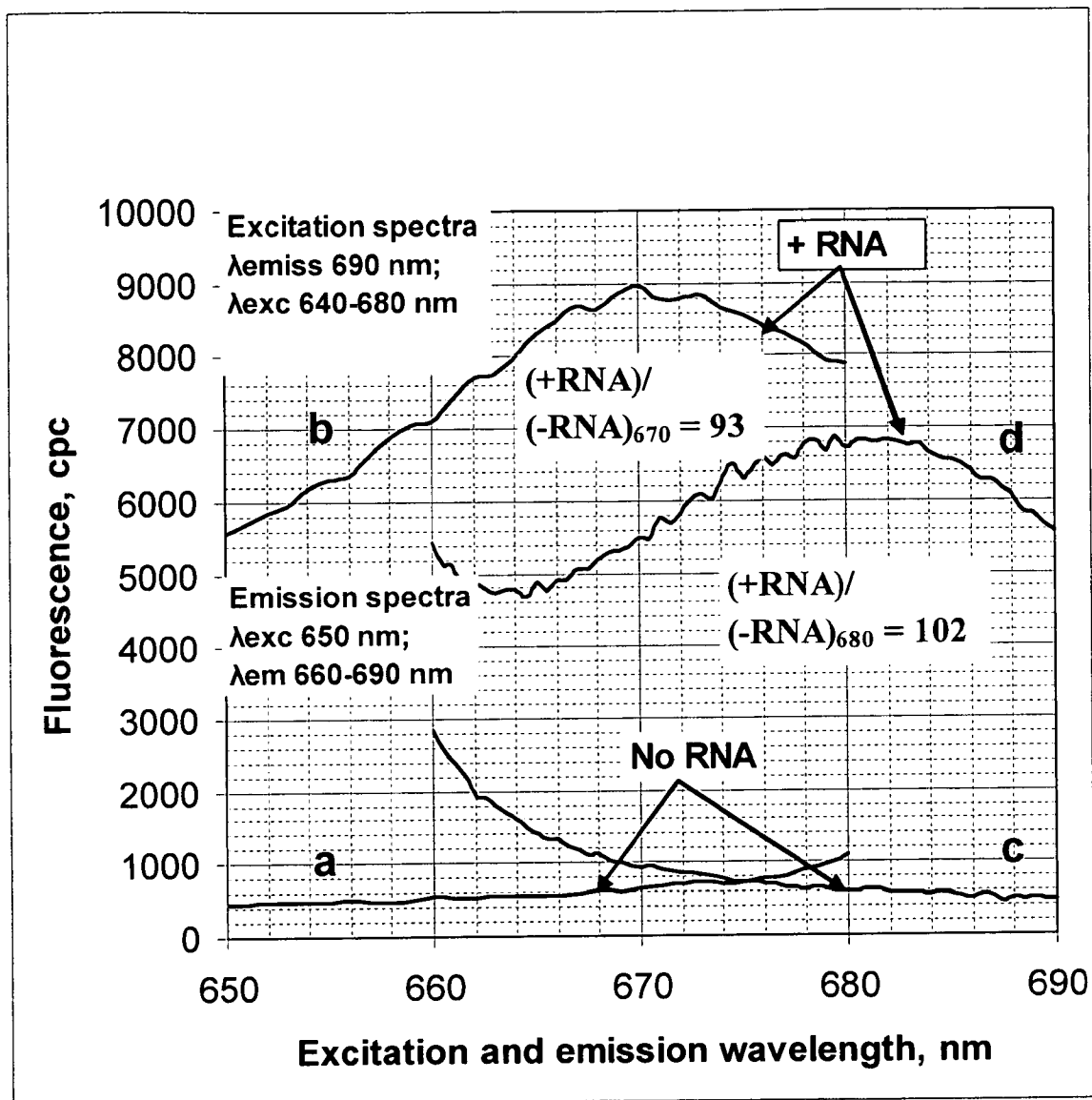
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**FIG. 6**

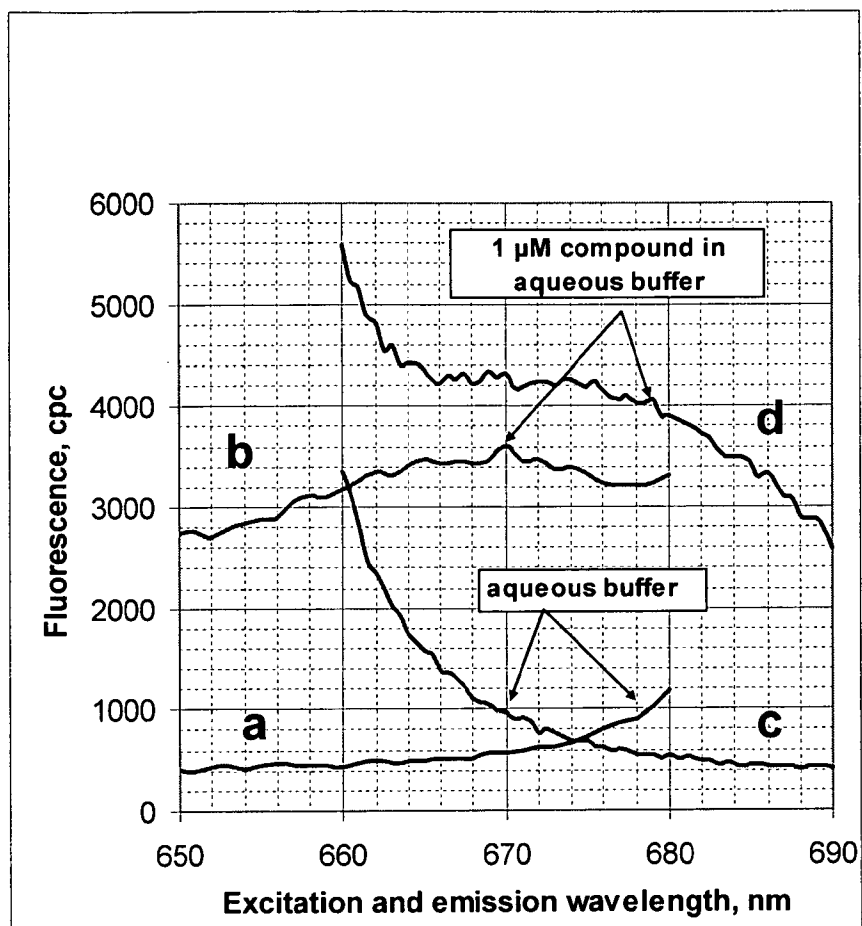
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**FIG. 7**

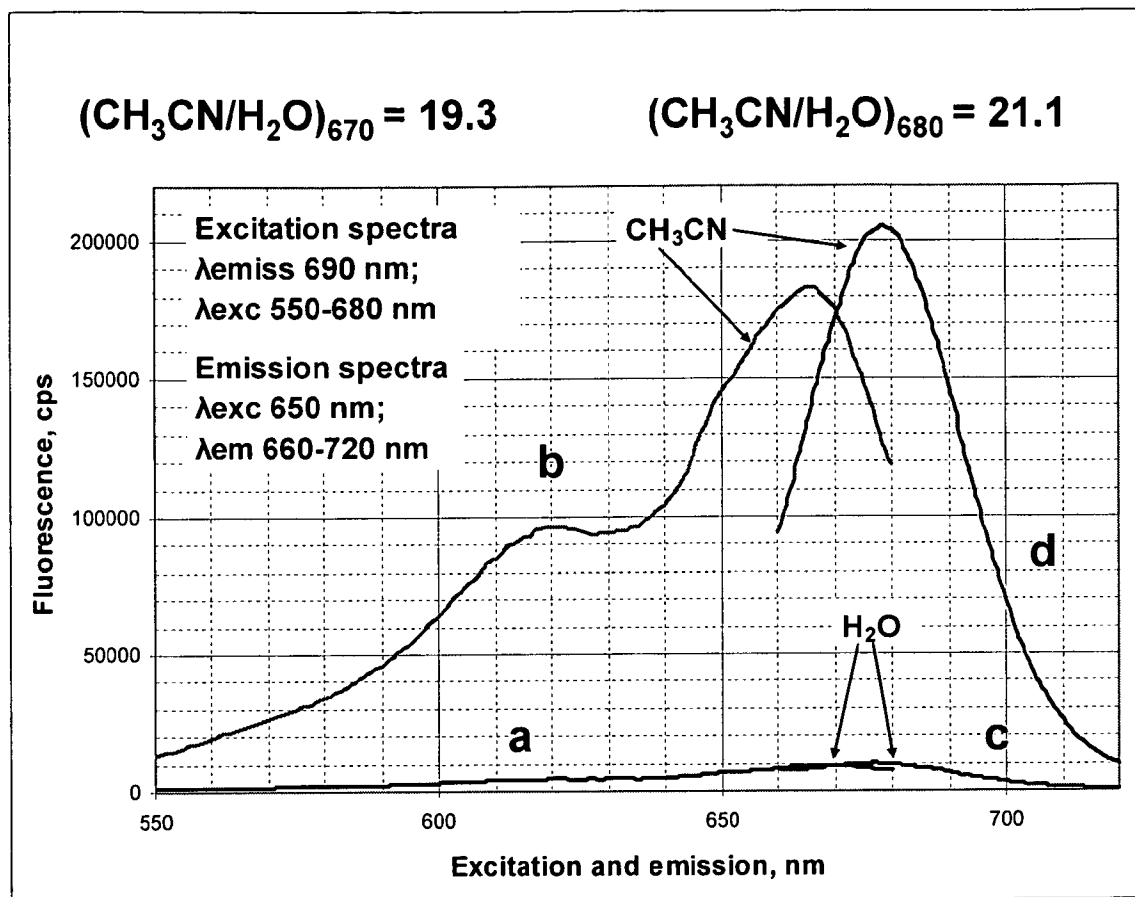
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**FIG. 8**

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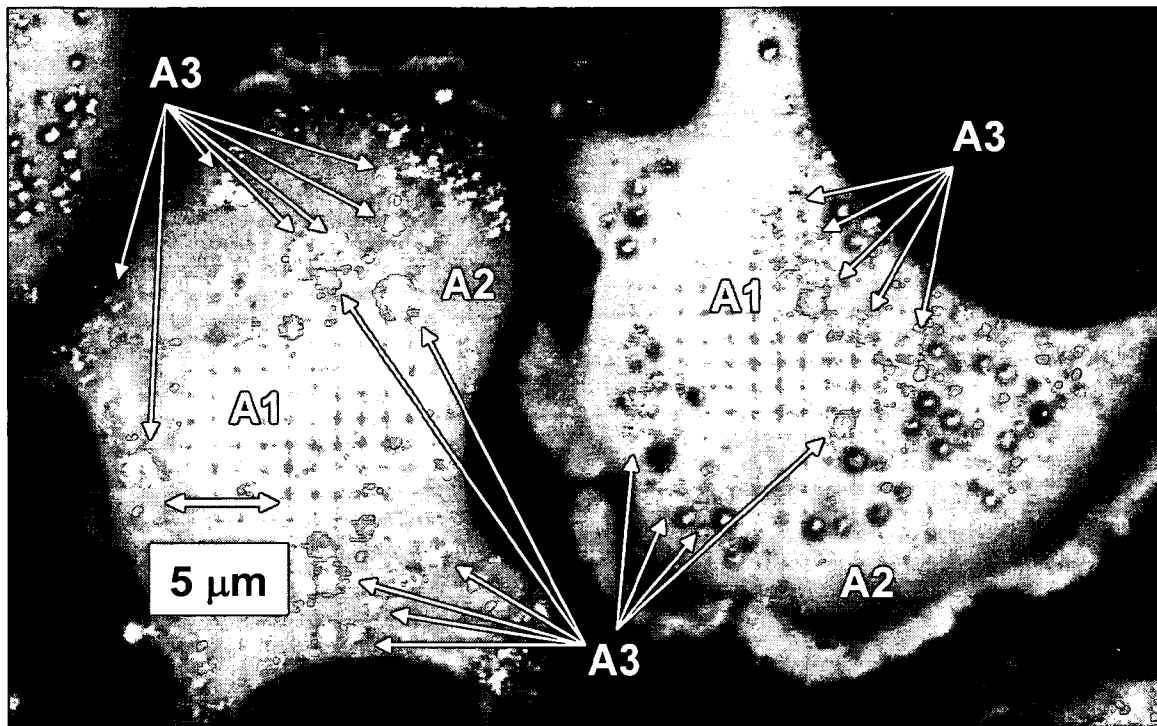
**FIG. 9**

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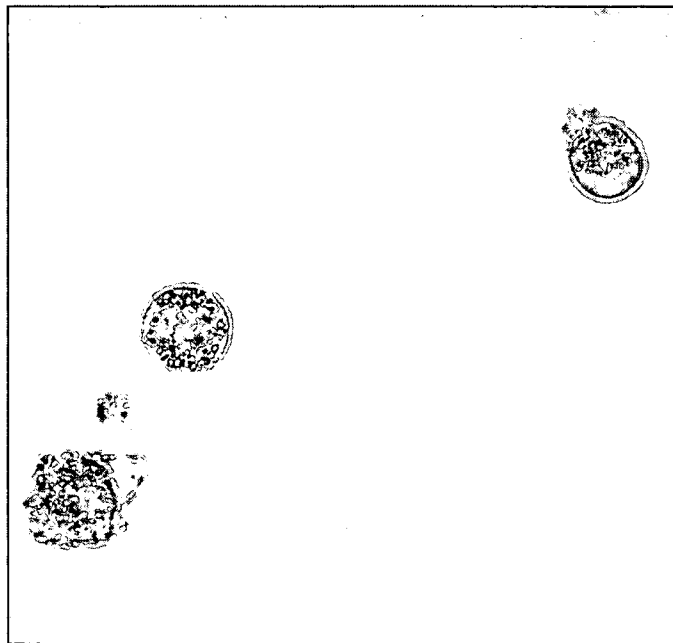
**FIG. 10**

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(A)



(B)

**FIG. 11**

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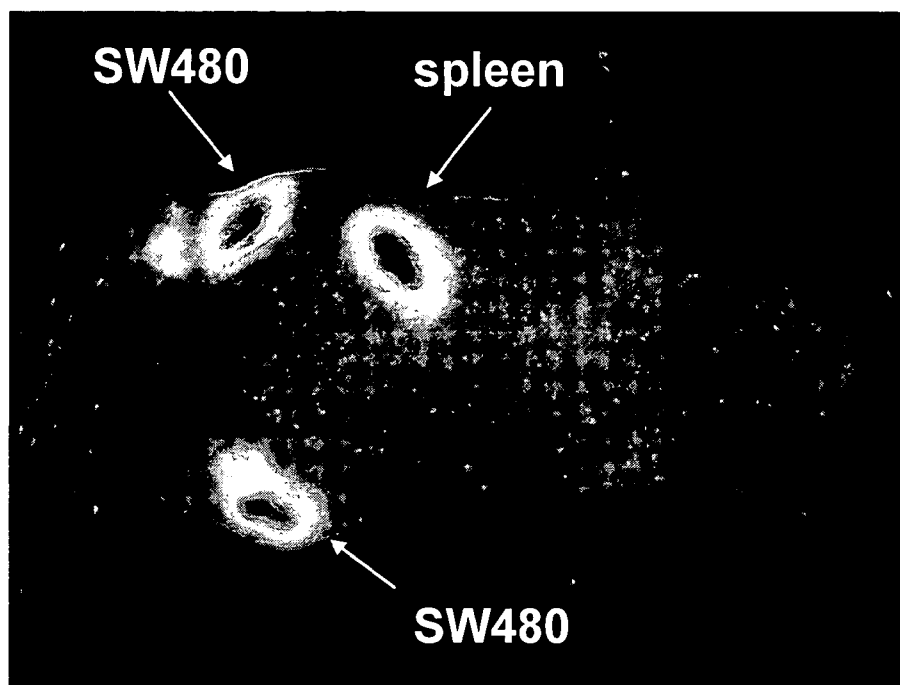


FIG. 12