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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present disclosure relates generally to methods for the generation of aptamers and photoaptamers having improved properties and the improved aptamers and photoaptamers generated thereby. In particular, the present disclosure describes slow off-rate aptamers that are highly specific to a target of interest. The disclosure describes the composition of these slow off-rate aptamers as well methods for their selection. Further the disclosure describes aptamer constructs with improved functionalities for detection methods. Further, the disclosure describes applications enabled by these improved aptamers.

BACKGROUND

[0002] The following description provides a summary of information relevant to the present disclosure and is not a concession that any of the information provided or publications referenced herein is prior art to the claimed invention.

[0003] The SELEX process is a method for the *in vitro* selection of nucleic acid molecules that are able to bind with high specificity to target molecules and is described in U.S. Patent No. 5,475,096 entitled "Nucleic Acid Ligands" and U.S. Patent No. 5,270,163 (see also WO 91/19813) entitled "Nucleic Acid Ligands". These patents, collectively referred to herein as the SELEX Patents, describe methods for making an aptamer to any desired target molecule.

[0004] The basic SELEX process has been modified to achieve a number of specific objectives. For example, U.S. Patent No. 5,707,796, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. Patent No. 5,580,737, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific aptamers able to discriminate between closely related molecules, termed Counter-SELEX. U.S. Patent No. 5,567,588, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. U.S. Patent No. 5,496,938, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved aptamers after SELEX has been performed. U.S. Patent No. 5,705,337, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," describes methods for covalently linking an aptamer to its target.

[0005] The SELEX process encompasses the identification of high-affinity aptamers containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in*

vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified aptamers containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5'- and 2'-positions of pyrimidines. U.S. Patent No. 5,580,737, *see supra,* describes highly specific aptamers containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe).

[0006] Further modifications of the SELEX process are described in U.S. Patent No. 5,763,177, U.S. Patent No. 6,001,577, and U.S. Patent No. 6,291,184, each of which is entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX; " see also, *e.g.,* U.S. Patent No. 6,458,539, entitled "Photoselection of Nucleic Acid Ligands". These patents, collectively referred to herein as "the PhotoSELEX Patents," describe various SELEX methods for selecting aptamers containing photoreactive functional groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. The resulting photoreactive aptamers are referred to as photocrosslinking aptamers or photoaptamers.

[0007] Although these SELEX and photoSELEX processes are useful, there is always a need for processes that lead to improved properties of aptamers generated from *in vitro* selection techniques. For example, a need exists for aptamers to target molecules with better binding affinities than those achieved with naturally occurring DNA or RNA nucleotides, as well as methods for producing such aptamers. For many applications, such as for example, *in vitro* assays, diagnostics, therapeutic, or imaging applications, it is of interest to produce aptamers with slow dissociation rates from the aptamer/target affinity complex. Several techniques have been proposed for producing such reagents (see, e.g., WO99/27133 and US2005/0003362). However, these selection processes do not discriminate between the selection of reagents that have fast association kinetics with the target (i.e., fast on-rates) and the selection of reagents that have slow dissociation kinetics with the target (i.e., slow off-rates). Thus, there is a need for novel processes and techniques that favor the selection of slow off-rate aptamers while inhibiting the selection of aptamers that simply have a fast association rate with the target.

[0008] US 2005/130,195 (Fujihara Tsuyoshi et al) (2005-06-16) describes "functional molecule" nucleic acids that incorporate modifications.

[0009] David J H et al., "Isolation of high-affinity GTP aptamers from partially structured RNA libraries" PNAS (2002) 99, 18:11616-11621 discloses high-affinity aptamers with a slow off-rate, using aptamers containing an engineered stem-loop.

[0010] Finally, there is a need for aptamer constructs that include different built -in functionalities. These functionalities may include tags for immobilization, labels for detection, means to promote or control separation, etc.

SUMMARY

[0011] The invention herein is as described by the claims.

[0012] The present disclosure describes novel aptamers, and methods to produce and use such aptamers. In particular, the disclosure describes slow off-rate (slow rate of dissociation) aptamers, slow off-rate aptamers containing C-5 modified pyrimidines, and processes for the selection of slow off-rate aptamers by dilution, by the addition of a competitor, or by a combination of both approaches. In addition, slow off-rate aptamers to various targets such as proteins and peptides are described. Slow off-rate aptamers with unique structural features and melting temperatures are also described. The disclosure also describes slow - off rate aptamers with photoreactive functional groups, aptamers that are refractory to the presence of poly-anionic materials, and a selection process for these aptamers, as well as aptamers constructed with a variety of other functionalities to improve their utility in various applications.

[0013] The present disclosure describes improved SELEX methods for generating aptamers that are capable of binding to target molecules. More specifically, the present disclosure describes methods for producing aptamers and/or photoaptamers having slower rates of dissociation from their respective target molecules than aptamers and photoaptamers obtained with previous SELEX methods. Generally, after contacting the candidate mixture with the target molecule and allowing the formation of nucleic acid-target complexes to occur, a slow off-rate enrichment process is introduced wherein nucleic acid-target complexes with fast dissociation rates will dissociate and not reform, while complexes with slow dissociation rates will remain intact. Methods for introducing a slow off-rate enrichment process include, but are not limited to, adding competitor molecules to the mixture of nucleic acids and target molecules, diluting the mixture of nucleic acids and target molecules, or a combination of both of these. The disclosure further describes aptamers and photoaptamers obtained using these methods.

[0014] The method comprises preparing a candidate mixture of nucleic acids; contacting the candidate mixture with a target molecule wherein nucleic acids with the highest relative affinities to the target molecule preferentially bind the target molecule, forming nucleic acid-target molecule complexes; introducing a slow off-rate enrichment process to induce the dissociation of nucleic acid-target molecule complexes with relatively fast dissociation rates; partitioning the remaining bound nucleic acid-target molecule complexes from free nucleic acids in the candidate mixture; and identifying the nucleic acids that were bound to the target molecule. The process may further include the iterative step of amplifying the nucleic acids that bind to the target molecule to yield a mixture of nucleic acids enriched with nucleic acids that bind to the target molecule yet produce nucleic acid-target molecule complexes having slow dissociation rates.

[0015] In an embodiment, the candidate mixture of nucleic acids includes nucleic acids containing modified nucleotide bases that may aid in the formation of modified nucleic acid-

target complexes having slow dissociation rates. Improved methods for performing SELEX with modified nucleotides, including nucleotides which contain photoactive or other functional groups, or nucleotides which contain placeholders for photoactive groups are disclosed in U.S. Application Serial No. 12/175,388, filed July 17, 2008 and entitled "Improved SELEX and PHOTOSELEX" which is being filed concurrently with the instant application. Placeholder nucleotides may also be used for the mid-SELEX or post-SELEX introduction of modified nucleotides that are not photoreactive.

[0016] The various methods and steps described herein can be used to generate an aptamer capable of either (1) binding to a target molecule or (2) binding to a target molecule and subsequently forming a covalent linkage with the target molecule upon irradiation with light in the UV or visible spectrum.

[0017] In another aspect, the various methods and steps described herein can be used to generate an aptamer capable of modifying the bioactivity of a target molecule through binding and/or crosslinking to the target molecule. An aptamer to a unique target molecule associated with or relevant to a specific disease process may be identified. This aptamer can be used as a diagnostic reagent, either *in vitro* or *in vivo.*

[0018] An aptamer to a target molecule associated with a disease state may be administered to an individual and used to treat the disease *in vivo.* The aptamers and photoaptamers identified herein can be used in any diagnostic, imaging, high throughput screening or target validation techniques or procedures or assays for which aptamers, oligonucleotides, antibodies and ligands, without limitation can be used. For example, aptamers and photoaptamers identified herein can be used according to the methods described in detail in the concurrently filed U.S. Application Serial No. 12/175,446, entitled "Multiplexed Analyses of Test Samples",

[0019] Previous aptamers that do not have the slow off-rate properties of the aptamers of the present invention have been used for a variety of purposes. In almost all such uses, slow off-rate aptamers will have improved performance relative to aptamers not selected to have slow off-rate properties.

[0020] The aptamer Macugen®, (See, e.g., U.S. Patent No. 6,168,778; U.S. Patent No. 6,051,698; U.S. Patent No. 6,426,335; and U.S. Patent No. 6,962,784; each of which is incorporated herein by reference in its entirety) has been approved for the treatment of macular degeneration, and functions due to its specific affinity for VEGF. Other aptamers have been studied and/or are in development for use as therapeutic agents. Aptamers not selected to have slow off-rate properties have also been used in many diagnostic and imaging applications (See, e.g., U.S. Patent No. 5,843,653; U.S. Patent No. 5,789,163; U.S. Patent No. 5,853,984; U.S. Patent No. 5,874,218; U.S. Patent No. 6,261,783; U.S. Patent No. 5,989,823; U.S. Patent No. 6,177,555; U.S. Patent No. 6,531,286; high-thorough put screening (See, e.g., U.S. Patent No. 6,329,145; U.S. Patent No. 6,670,132; U.S. Patent No. 7,258,980;
and in PCR kits (See, e.g., U.S. Patent No. 6,183,967; U.S. Patent No. 6,020,130; U.S. Patent No. 5,763,173; U.S. Patent No. 5,874,557; U.S. Patent No. 5,693,502). The slow-off rate

aptamers of this disclosure may be used in any diagnostic, therapeutic, imaging or any other use for which antibodies, aptamers and ligand binding pairs have been used.

[0021] The disclosure provides aptamers and photoaptamers identified by the improved methods disclosed herein, diagnostic kits that include such aptamers and photoaptamers, and therapeutic and diagnostic uses of such aptamers and photoaptamers. The novel, slow off-rate aptamers and photoaptamers identified using the described methods can be used in a variety of assays including, assays that use planar arrays, beads, and other types of solid supports. The assays may be used in a variety of contexts including in life science research applications, clinical diagnostic applications, (e.g., a diagnostic test for a disease, or a "wellness" test for preventative healthcare); ALONA and UPS assays, and *in vivo* imaging applications. For some applications, multiplexed assays employing the described aptamers and photoaptamers may be used.

[0022] The slow off-rate aptamers (or photoaptamers) described herein can be used as intravenous or oral contrast agents for CAT scans and other imaging applications. CAT scans are used in the diagnosis of muscle and bone disorders, locating blood clots, detecting internal bleeding, monitoring diseases such as cancer, etc. The slow off-rate aptamers may be labeled with a CAT scan detectable component, such as, for example, iodine, barium, or gastrograffin. In addition to carrying the detectable component, the aptamer may be designed to direct that component to a specific tissue or desired target. The aptamer may serve to concentrate or localize the detectable component and thus improve the signal to noise ratio by increasing available signal. Because the off-rate of the aptamer can be sufficiently slow, the duration of the scan can be increased, and the signal to noise ratio of the scan may be improved. The specificity of the aptamer for the target may also improve the signal to noise ratio in these imaging applications.

[0023] The slow off-rate aptamer labeled with a diamagnetic or paramagnetic material. In this embodiment, the labeled aptamer may be used to improve the performance of magnetic resonance imaging (MRI). MRI is particularly well suited to the imaging of small, selective areas and tissues with high water content or to monitoring blood flow. The specificity of the slow off-rate aptamers may improve the localization of the MRI reagent to a desired tissue section. Similarly, slow off-rate aptamers may be modified with materials such as fluorine, carbon11, oxygen15, or nitrogen13, for use in PET scans. In another the aptamers may be labeled with IR active materials that may be used for infrared imaging. It is also contemplated that slow off-rate aptamers may be labeled for use with other imaging modalities.

[0024] The slow off-rate aptamers may be used as a very sensitive and specific reagent for incorporation into a variety of *in vitro* diagnostic methods or kits. In some embodiments, the slow off-rate aptamers are used as substitutes for antibodies in a number of infectious, or other type of, disease detection methods where the aptamer to the target of interest includes either or both a detectable material and an immobilization or capture component. After the aptamer from the kit is mixed with a clinical specimen, a variety of assay formats may be utilized. The aptamer may include a detectable label, such as a fluorophore. The assay format may include

fluorescence quenching, hybridization methods, flow cytometry, mass spectroscopy, inhibition or competition methods, enzyme linked oligonucleotide assays, SPR, evanescent wave methods, etc. The aptamer may be provided in the kit in solution. The aptamer in the kit may be immobilized onto a solid support used in conjunction with the assay for testing the specimen. The solid support may be designed for the detection of one or more targets of interest. The kit may further include reagents to extract the target of interest, reagents for amplifying the aptamer, reagents for performing washing, detection reagents, etc.

[0025] The slow off-rate aptamers may be used in therapeutic imaging studies. During the development of new therapeutic compounds, it is often difficult to assess certain characteristics of the compound, such as, for example, biodistribution, the washout rate, bioavailability, *in vivo* drug/target interactions, etc. In many cases, if a suitable detectable material was used to modify the therapeutic compound, imaging studies could be used to assess all of these characteristics. Though direct modification of a therapeutic compound frequently inhibits its ability to interact with its target and thus reduces efficacy, an aptamer's small size and customizable specificity, render it potentially well-suited to react with a therapeutic compound (for example, an antibody or other protein-based therapeutic) while minimizing any undesirable effects on the compound's therapeutic efficacy. To assess such characteristics as biodistribution and the washout rate, the aptamer/therapeutic complex may survive for an extended period of time. These types of studies may be simplified in cases where the therapeutic compound is a slow off-rate aptamer. Aptamers used in therapeutic, imaging, and diagnostic applications may include various modifications, such as, for example, 2' fluoro and other modifications, to increase the stability of the aptamer upon exposure to various components that may be present in a test sample or in vivo, such as, for example, nucleases and other sample or bodily fluid components.

BRIEF DESCRIPTION OF THE FIGURES

[0026]

FIG. 1A illustrates an exemplary SELEX method and FIG. 1B illustrates an exemplary SELEX method which includes the step of incorporating a slow off-rate enrichment process or process.

FIG. illustrates representative aptamer template, primer, and complementary oligonucleotide sequences used in the disclosure. The oligonucleotides were prepared by standard solid-phase synthesis techniques. B=dT-biotin.

FIG. 3 illustrates histograms of dissociation rate constants for affinity aptamers selected without (A) and with (B) a slow off-rate enrichment process as described in Example 2.

FIGS. 4A and B shows oligonucleotides that were used to prepare the candidate mixtures or perform various steps in the selection process described in Examples 3 and 4. The oligonucleotides were prepared by standard solid-phase synthesis techniques. Two candidate mixture sequences were used in this example, designated 1 and 2. B = dT-biotin. BrdU (5-

bromo-dUTP), Anthraquinone (AQ), and psoralen (Psor) chromophores were purchased as phosphoramidites and added to the 5' terminus of the forward primer during synthesis. 4-azido-2-nitro-aniline (ANA) was prepared as a para-nitro-phenyl carbonate derivative and coupled to a 5' hexylamine phosphoramidite after synthesis. Two candidate mixture sequences were used in this example, designated 1 and 2. (A) Template 1 was used with candidate mixtures containing 5'- BrdU, AQ, and ANA, and (B) Template 2 was used with candidate mixtures containing 5'- Psor.

FIG. 5 illustrates chemical structures of the chromophores coupled to the 5' terminus of the forward primer as illustrated in FIGS. 4A and 4B.

FIG. 6 illustrates a PAGE analysis of crosslink activity of TIMP-3 5'ANA/BzdU enriched library using 5'-Fixed PhotoSELEX described in Example 3. The gel illustrates the separation of free aptamer (A_f), intramolecular crosslinked aptamer (A_f^*), and crosslinked protein:aptamer complexes (P:A).

FIG. 7 is a chart of over 500 targets for which slow off-rate aptamers have been identified.

FIGS 8A to 8D illustrate aptamer constructs that contain a variety of different and optional functionalities including immobilization tags, labels, photocrosslinking moieties, spacers, and releasable moieties.

FIGS. 9 A to 9F illustrate examples of aptamer constructs including a cleavable or releasable element, a tag (for example biotin), a spacer, and a label (for example Cy3).

FIG. 10 illustrates the aptamer and primer constructs described in the disclosure. Cy3 represents a Cyanine 3 dye, PC a photocleavable linker, ANA a photoreactive crosslinking group, $(AB)_2$ a pair of biotin residues separated by dA residues, and $(T)_8$ a poly dT linker. Primer constructs are complementary to the complete 3' fixed region of the aptamer constructs.

FIGS. 11 A to 11 C illustrates dose response curves for slow off-rate aptamers versus traditional aptamers for three different targets.

FIGS. 12 A and 12B illustrate performance curves for a slow off-rate aptamer where the target was a peptide.

FIG. 13 illustrates a plot of the measured melting temperature of a number of slow off-rate aptamers relative to the predicted melting temperature.

FIG. 14 describes the base modifications of nucleotides included in this disclosure. The R groups that may be used are described in addition to the linkers(X) that may be used between the nucleotide attachment point and the R group. The positions for modification of the nucleotides are also indicated.

FIG. 15 illustrates a plot used in the determination of the binding constant for an aptamer containing C- 5 modified pyrimidines.

DETAILED DESCRIPTION

[0027] The practice of the practice of the invention disclosed herein employs, unless otherwise indicated, conventional methods of chemistry, microbiology, molecular biology, and recombinant DNA techniques within the level of skill in the art. Such techniques are explained fully in the literature. See, *e.g.,* Sambrook, et al. Molecular Cloning: A Laboratory Manual (Current Edition); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., Current Edition); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., Current Edition); Transcription and Translation (B. Hames & S. Higgins, eds., Current Edition).

[0028] All publications, published patent documents, and patent applications cited in this specification are indicative of the level of skill in the art(s) to which the invention pertains. All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

[0029] As used in this specification, including the appended claims, the singular forms "a," "an," and "the" include plural references, unless the content clearly dictates otherwise, and are used interchangeably with "at least one" and "one or more." Thus, reference to "an aptamer" includes mixtures of aptamers, reference to "a probe" includes mixtures of probes, and the like.

[0030] As used herein, the term "about" represents an insignificant modification or variation of the numerical values such that the basic function of the item to which the numerical value relates is unchanged.

[0031] As used herein, the terms "comprises," "comprising," "includes," "including," "contains," "containing," and any variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, product-by-process, or composition of matter that comprises, includes, or contains an element or list of elements does not include only those elements but may include other elements not expressly listed or inherent to such process, method, product-by-process, or composition of matter.

[0032] As used herein, "nucleic acid ligand" "aptamer" and "clone" are used interchangeably to refer to a non-naturally occurring nucleic acid that has or may have a desirable action on a target molecule. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way that modifies or alters the target or the functional activity of the target, covalently attaching to the target (as in a suicide inhibitor), and facilitating the reaction between the target and another molecule. In one

embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure, other than a polynucleotide, that binds to the aptamer through a mechanism which is predominantly independent of Watson/Crick base pairing or triple helix binding, wherein the aptamer is not a nucleic acid having the known physiological function of being bound by the target molecule. Aptamers include nucleic acids that are identified from a candidate mixture of nucleic acids, the aptamer being a ligand of a given target, by the method comprising: (a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to other nucleic acids in the candidate mixture may be partitioned from the remainder of the candidate mixture; (b) partitioning the increased affinity and/ or slow - off rate nucleic acids from the remainder of the candidate mixture; and (c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids, whereby aptamers of the target molecule are identified. It is recognized that affinity interactions are a matter of degree; however, in this context, the "specific binding affinity" of an aptamer for its target means that the aptamer binds to its target generally with a much higher degree of affinity than it may binds to other, non-target, components in a mixture or sample. An "aptamer" or " nucleic acid ligand" is a set of copies of one type or species of nucleic acid molecule that has a particular nucleotide sequence. An aptamer can include any suitable number of nucleotides. "Aptamers" refers to more than one such set of molecules. Different aptamers may have either the same number or a different number of nucleotides. Aptamers may be DNA or RNA and maybe single stranded, double stranded, or contain double stranded regions.

[0033] As used herein, "slow off-rate" or "slow rate of dissociation" or "slow dissociation rate" refers to the time it takes for an aptamers/target complex to begin to dissociate. This can be expressed as a half life, $t_{1/2}$, or the point at which 50% of the aptamer/target complex has dissociated. The off-rate or dissociation rate of a slow off-rate aptamer, expressed as $t_{1/2}$ values, can be about ≥ 30 min., >_ 60 min., >_ 90 min., >_ 120 min. ≥ 150 min. ≥ 180 min. ≥ 210 and about ≥ 240 min.

[0034] A method for producing a synthetic library of nucleic acids comprises: 1) synthesizing the nucleic acids; 2) deprotecting the nucleic acids; 3) purifying the nucleic acids; and 4) analyzing the nucleic acids. In the synthesis step, a monomer mixture is prepared where the ratio of the various nucleotides in the mix is optimized to yield equal ratios of each nucleotide in the final product. One or more of the monomers in the mixture may comprise a modified nucleotide. Amidite protection groups are used in this procedure and in one embodiment, the monomer concentration is 0.1M. During synthesis, the five prime protecting group is retained in the product nucleic acid. Synthesis is conducted on a solid support (controlled pore glass, CPG) and at least about 80 cycles are completed to synthesis the final product.

[0035] After the synthesis process, the nucleic acid product is deprotected. A1.0 M aqueous lysine buffer, pH 9.0 is employed to cleave apurinic sites while the product is retained on the support (controlled pore glass, CPG). These cleaved truncated sequences are washed away with deionized (dI) water two times. 500 uL of dI water are added after the two washes in preparation for the deprotection step. This step involves the treatment with 1.0 mL of T-

butylamine: methanol: water, 1:1:2, for 5 hours 70°C, is followed by freezing, filtration, and evaporation to dryness. The nucleic acid product is purified based on the hydrophobicity of the protecting group on PRP-3 HPLC column (Hamilton). Appropriate column fractions are collected and pooled, desalted, and evaporated to dryness to remove the volatile elution buffers. The final product is washed with water by a centrifugation process and then re-suspended. Finally the resuspended material is treated to deprotect the final product. Final product is characterized by base composition, primer extension, and sequencing gel.

[0036] A candidate mixture of nucleic acids may also be produced by an enzymatic method using a solid phase. In one embodiment this method comprises of the same basic steps described above. In this case the goal is the synthesis of an antisense library and these libraries are produced with a 5' biotin modification. All remaining synthetic processes are as described above. Once the synthetic library is prepared, the nucleic acids maybe used in a primer extension mix containing one or more modified nucleotides to produce the final candidate mixture in a classic primer extension method.

[0037] Aptamers may be synthesized by the same chemistry that is used for the synthesis of a library. However, instead of a mixture of nucleotides, one nucleotide is introduced at each step in the synthesis to control the final sequence generated by routine methods. Modified nucleotides may be introduced into the synthesis process at the desired positions in the sequence. Other functionalities may be introduced as desired using known chemical modifications of nucleotides.

[0038] As used herein, "candidate mixture" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. Modified nucleotides, such as nucleotides with photoreactive groups or other modifications, can be incorporated into the candidate mixture. In addition, a SELEX process can be used to produce a candidate mixture, that is, a first SELEX process experiment can be used to produce a ligand-enriched mixture of nucleic acids that is used as the candidate mixture in a second SELEX process experiment. A candidate mixture can also comprise nucleic acids with one or more common structural motifs. As used herein, a candidate mixture is also sometimes referred to as a "pool" or a "library." For example, an "RNA pool" refers to a candidate mixture comprised of RNA.

[0039] Each nucleic acid in a candidate mixture may have fixed sequences on either side of a randomized region, to facilitate the amplification process. The nucleic acids in the candidate mixture of nucleic acids can each further comprise fixed regions or "tail" sequences at their 5' and 3' termini to prevent the formation of high molecular weight parasites during the amplification process.

[0040] As used herein, "nucleic acid," "oligonucleotide," and "polynucleotide" are used interchangeably to refer to a polymer of nucleotides of any length, and such nucleotides may

include deoxyribonucleotides, ribonucleotides, and/or analogs or chemically modified deoxyribonucleotides or ribonucleotides. The terms "polynucleotide," "oligonucleotide," and "nucleic acid" include double- or single-stranded molecules as well as triple-helical molecules.

[0041] If present, chemical modifications of a nucleotide can include, singly or in any combination, 2'-position sugar modifications, 5-position pyrimidine modifications (e.g., 5-(N-benzylcarboxyamide)-2'-deoxyuridine, 5-(N-isobutylcarboxyamide)-2'-deoxyuridine, 5-(N-[2-(1H-indole-3y1)ethyl]carboxyamide)-2'-deoxyuridine, 5-(N-[1-(3-trimethylamonium) propyl]carboxyamide)-2'-deoxyuridine chloride, 5-(N-naphthylcarboxyamide)-2'-deoxyuridine, or 5-(N-[1-(2,3-dihydroxypropyl)]carboxyamide)-2'-deoxyuridine), 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo- or 5-iodo-uracil, backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine, and the like. Modifications can also include 3' and 5' modifications, such as capping or pegylation. Other modifications can include substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and those with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), those with intercalators (*e.g.*, acridine, psoralen, etc.), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, and those with modified linkages (*e.g.*, alpha anomeric nucleic acids, etc.). Further, any of the hydroxyl groups ordinarily present in a sugar may be replaced by a phosphonate group or a phosphate group; protected by standard protecting groups; or activated to prepare additional linkages to additional nucleotides or to a solid support. The 5' and 3' terminal OH groups can be phosphorylated or substituted with amines, organic capping group moieties of from about 1 to about 20 carbon atoms, or organic capping group moieties of from about 1 to about 20 polyethylene glycol (PEG) polymers or other hydrophilic or hydrophobic biological or synthetic polymers. If present, a modification to the nucleotide structure may be imparted before or after assembly of a polymer. A sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0042] Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. As noted above, one or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalky, cycloalkenyl or aralkyl. Not all linkages in a polynucleotide need be identical. Substitution of analogous forms of sugars, purines, and pyrimidines can be advantageous in designing a final product, as can alternative backbone structures like a polyamide backbone, for example.

[0043] In one embodiment, the variable region of the aptamer includes nucleotides that include modified bases. Certain modified aptamers may be used in any of the described methods, devices, and kits. These modified nucleotides have been shown to produce novel aptamers that have very slow off-rates from their respective targets while maintaining high affinity to the target. In one embodiment, the C-5 position of the pyrimidine bases may be modified. Aptamers containing nucleotides with modified bases have a number of properties that are different than the properties of standard aptamers that include only naturally occurring nucleotides (i.e., unmodified nucleotides). In one embodiment, the method for modification of the nucleotides includes the use of an amide linkage. However, other suitable methods for modification may be used. It has been surprisingly observed that the structure of the identified slow off-rate aptamers does not appear to be entirely in accordance with the structure predicted by standard base pairing models. This observation is supported by the fact that the measured melting temperatures of the slow off-rate aptamers are not consistent with the melting temperatures predicted by the models, see Fig. 13. As shown, there appears to be no correlation between the measured and predicted melting temperatures of the slow off-rate aptamers. On average, the calculated melting temperature (Tm) is 6°C lower than the measured Tm. The measured melting temperatures indicate that slow off-rate aptamers including these modified nucleotides are more stable than may be predicted and potentially possess novel secondary structures. These modified aptamers also have different circular dichorism spectra than corresponding aptamers that include only unmodified nucleotides. In the case of many targets, slow off-rate aptamers to the target are more likely to be identified when modified nucleotides are used in the production of the initial library or candidate mixture.

[0044] Particular 5-position pyrimidine modifications include those described in U.S. Patents 5,719,273 and 5,945,527,as well as those illustrated in Fig. 14.

[0045] As used herein, "modified nucleic acid" refers to a nucleic acid sequence containing one or more modified nucleotides. In some embodiments it may be desirable that the modified nucleotides are compatible with the SELEX process.

[0046] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and/or it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. Polypeptides can be single chains or associated chains.

[0047] As used herein, "photoreactive nucleotide" means any modified nucleotide that is capable of photocrosslinking with a target, such as a protein, upon irradiation with certain wavelengths of light. For example, photoaptamers produced by the photoSELEX process can

include a photoreactive group selected from the following: 5-bromouracil (BrU), 5-iodouracil (IU), 5-bromovinyluracil, 5-iodovinyluracil, 5-azidouracil, 4-thiouracil, 5-bromocytosine, 5-iodocytosine, 5-bromovinylcytosine, 5-iodovinylcytosine, 5-azidocytosine, 8-azidoadenine, 8-bromoadenine, 8-iodoadenine, 8-azidoguanine, 8-bromoguanine, 8-iodoguanine, 8-azidohypoxanthine, 8-bromohypoxanthine, 8-iodohypoxanthine, 8-azidoxanthine, 8-bromoxanthine, 8-iodoxanthine, 5-bromodeoxyuridine, 8-bromo-2'-deoxyadenine, 5-iodo-2'-deoxyuracil, 5-iodo-2'-deoxycytosine, 5-[(4-azidophenacyl)thio]cytosine, 5-[(4-azidophenacyl)thio]uracil, 7-deaza-7-iodoadenine, 7-deaza-7-iodoguanine, 7-deaza-7-bromoadenine, and 7-deaza-7-bromoguanine. A "photoreactive pyrimidine" means any modified pyrimidine that is capable of photocrosslinking with a target upon irradiation of certain wavelengths. Exemplary photoreactive pyrimidines include 5-bromo-uracil (BrdU), 5-bromo-cytosine (BrdC), 5-iodo-uracil (IdU), and 5-iodo-cytosine (IdC). In various embodiments, the photoreactive functional group will absorb wavelengths of light that are not absorbed by the target or the non-modified portions of the oligonucleotide.

[0048] "SELEX" refers to a process that combines the selection of nucleic acids that interact with a target in a desirable manner (*e.g.*, binding to a protein) with the amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids that interact most strongly with the target from a pool that contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. The SELEX methodology is described in the SELEX Patents. In some embodiments of the SELEX process, aptamers that bind non-covalently to their targets are generated. In other embodiments of the SELEX process, aptamers that bind covalently to their targets are generated.

[0049] As used herein the term "amplification" or "amplifying" means any process or combination of process steps that increases the amount or number of copies of a molecule or class of molecules.

[0050] "SELEX target" or "target molecule" or "target" refers herein to any compound upon which a nucleic acid can act in a desirable manner. A SELEX target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, any portion or fragment of any of the foregoing, etc., without limitation. In one embodiment, a SELEX target does not include molecules that are known to bind nucleic acids, such as, for example, known nucleic acid binding proteins (e.g. transcription factors). Virtually any chemical or biological effector may be a suitable SELEX target. Molecules of any size can serve as SELEX targets. A target can also be modified in certain ways to enhance the likelihood or strength of an interaction between the target and the nucleic acid. A target can also include any minor variation of a particular compound or molecule, such as, in the case of a protein, for example, minor variations in amino acid sequence, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component, which does not substantially alter the identity of the molecule. A

"target molecule" or "target" is a set of copies of one type or species of molecule or multimolecular structure that is capable of binding to an aptamer. "Target molecules" or "targets" refer to more than one such set of molecules. Embodiments of the SELEX process in which the target is a peptide are described in U.S. Patent No. 6,376,190, entitled "Modified SELEX Processes Without Purified Protein,"
in its entirety. Figure 7 lists over 500 targets for which aptamers have been produced including a variety of slow off-rate aptamers.

[0051] As used herein, "competitor molecule" and "competitor" are used interchangeably to refer to any molecule that can form a non-specific complex with a non-target molecule. In this context, non - target molecules include free aptamers, where, for example, a competitor can be used to inhibit the aptamer from binding (re-binding), non-specifically, to another non-target molecule. A "competitor molecule" or "competitor" is a set of copies of one type or species of molecule. "Competitor molecules" or "competitors" refer to more than one such set of molecules. Competitor molecules include, but are not limited to oligonucleotides, polyanions (e.g., heparin, herring sperm DNA, salmon sperm DNA, tRNA, dextran sulfate, polydextran, abasic phosphodiester polymers, dNTPs, and pyrophosphate). In various embodiments, a combination of one or more competitor can be used.

[0052] As used herein, "non-specific complex" refers to a non-covalent association between two or more molecules other than an aptamer and its target molecule. A non-specific complex represents an interaction between classes of molecules. Non-specific complexes include complexes formed between an aptamer and a non-target molecule, a competitor and a non-target molecule, a competitor and a target molecule, and a target molecule and a non-target molecule.

[0053] As used herein, the term "slow off-rate enrichment process" refers to a process of altering the relative concentrations of certain components of a candidate mixture such that the relative concentration of aptamer affinity complexes having slow dissociation rates is increased relative to the concentration of aptamer affinity complexes having faster, less desirable dissociation rates. The slow off-rate enrichment process may be a solution-based slow off-rate enrichment process. A solution-based slow off-rate enrichment process takes place in solution, such that neither the target nor the nucleic acids forming the aptamer affinity complexes in the mixture are immobilized on a solid support during the slow off-rate enrichment process.

[0054] The slow - off rate enrichment process can include one or more steps, including the addition of and incubation with a competitor molecule, dilution of the mixture, or a combination of these (e.g., dilution of the mixture in the presence of a competitor molecule). Because the effect of an slow off-rate enrichment process generally depends upon the differing dissociation rates of different aptamer affinity complexes (i.e., aptamer affinity complexes formed between the target molecule and different nucleic acids in the candidate mixture), the duration of the slow off-rate enrichment process is selected so as to retain a high proportion of aptamer affinity complexes having slow dissociation rates while substantially reducing the number of aptamer affinity complexes having fast dissociation rates. The slow off-rate enrichment process

may be used in one or more cycles during the SELEX process. When dilution and the addition of a competitor are used in combination, they may be performed simultaneously or sequentially, in any order. The slow - off rate enrichment process can be used when the total target (protein) concentration in the mixture is low.

[0055] When the slow off-rate enrichment process includes dilution, the mixture can be diluted as much as is practical, keeping in mind that the aptamer retained nucleic acids are recovered for subsequent rounds in the SELEX process.

[0056] The slow off-rate enrichment process may include the use of a competitor as well as dilution, permitting the mixture to be diluted less than might be necessary without the use of a competitor.

[0057] In one method the slow off-rate enrichment process includes the addition of a competitor, and the competitor is a polyanion (e.g., heparin or dextran sulfate (dextran)). Heparin or dextran have been used in the identification of specific aptamers in prior SELEX selections. In such methods, however, heparin or dextran is present *during* the equilibration step in which the target and aptamer bind to form complexes. In such methods, as the concentration of heparin or dextran increases, the ratio of high affinity target/aptamer complexes to low affinity target/aptamer complexes increases. However, a high concentration of heparin or dextran can reduce the number of high affinity target/ aptamer complexes at equilibrium due to competition for target binding between the nucleic acid and the competitor. By contrast, the presently described methods add the competitor *after* the target/aptamer complexes have been allowed to form and therefor does not affect the number of complexes formed. Addition of competitor after equilibrium binding has occurred between target and aptamer creates a non-equilibrium state that evolves in time to a new equilibrium with fewer target/aptamer complexes. Trapping target/aptamer complexes before the new equilibrium has been reached enriches the sample for slow off-rate aptamers since fast off-rate complexes will dissociate first.

[0058] A polyanionic competitor (e.g., dextran sulfate or another polyanionic material) may be used in the slow off-rate enrichment process to facilitate the identification of an aptamer that is refractory to the presence of the polyanion. In this context, "polyanionic refractory aptamer" is an aptamer that is capable of forming an aptamer/target complex that is less likely to dissociate in the solution that also contains the polyanionic refractory material than an aptamer/target complex that includes a non-polyanionic refractory aptamer. In this manner, polyanionic refractory aptamers can be used in the performance of analytical methods to detect the presence or amount or concentration of a target in a sample, where the detection method includes the use of the polyanionic material (e.g. dextran sulfate) to which the aptamer is refractory.

[0059] Thus, a method for producing a polyanionic refractory aptamer is disclosed. In this method , after contacting a candidate mixture of nucleic acids with the target. The target and the nucleic acids in the candidate mixture are allowed to come to equilibrium. A polyanionic

competitor is introduced and allowed to incubate in the solution for a period of time sufficient to insure that most of the fast off rate aptamers in the candidate mixture dissociate from the target molecule. Also, aptamers in the candidate mixture that may dissociate in the presence of the polyanionic competitor will be released from the target molecule. The mixture is partitioned to isolate the high affinity, slow off-rate aptamers that have remained in association with the target molecule and to remove any uncomplexed materials from the solution. The aptamer can then be released from the target molecule and isolated. The isolated aptamer can also be amplified and additional rounds of selection applied to increase the overall performance of the selected aptamers. This process may also be used with a minimal incubation time if the selection of slow off-rate aptamers is not needed for a specific application.

[0060] Thus, a modified SELEX process is provided for the identification or production of aptamers having slow (long) off rates wherein the target molecule and candidate mixture are contacted and incubated together for a period of time sufficient for equilibrium binding between the target molecule and nucleic acids contained in the candidate mixture to occur. Following equilibrium binding an excess of competitor molecule, e.g., polyanion competitor, is added to the mixture and the mixture is incubated together with the excess of competitor molecule for a predetermined period of time. A significant proportion of aptamers having off rates that are less than this predetermined incubation period will dissociate from the target during the predetermined incubation period. Re-association of these "fast" off rate aptamers with the target is minimized because of the excess of competitor molecule which can non-specifically bind to the target and occupy target binding sites. A significant proportion of aptamers having longer off rates will remain complexed to the target during the predetermined incubation period. At the end of the incubation period, partitioning nucleic acid-target complexes from the remainder of the mixture allows for the separation of a population of slow off-rate aptamers from those having fast off rates. A dissociation step can be used to dissociate the slow off-rate aptamers from their target and allows for isolation, identification, sequencing, synthesis and amplification of slow off-rate aptamers (either of individual aptamers or of a group of slow off-rate aptamers) that have high affinity and specificity for the target molecule. As with conventional SELEX the aptamer sequences identified from one round of the modified SELEX process can be used in the synthesis of a new candidate mixture such that the steps of contacting, equilibrium binding, addition of competitor molecule, incubation with competitor molecule and partitioning of slow off-rate aptamers can be iterated/repeated as many times as desired.

[0061] The combination of allowing equilibrium binding of the candidate mixture with the target prior to addition of competitor, followed by the addition of an excess of competitor and incubation with the competitor for a predetermined period of time allows for the selection of a population of aptamers having off rates that are much greater than those previously achieved.

[0062] In order to achieve equilibrium binding, the candidate mixture may be incubated with the target for at least about 5 minutes, or at least about 15 minutes, about 30 minutes, about 45 minutes, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours or about 6 hours.

[0063] The predetermined incubation period of competitor molecule with the mixture of the candidate mixture and target molecule may be selected as desired, taking account of the factors such as the nature of the target and known off rates (if any) of known aptamers for the target. Predetermined incubation periods may be chosen from: at least about 5 minutes, at least about 10 minutes, at least about 20 minutes, at least about 30 minutes, at least 45 about minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 5 hours, at least about 6 hours.

[0064] A dilution may be used as an off rate enhancement process and incubation of the diluted candidate mixture, target molecule/aptamer complex may be undertaken for a predetermined period of time, which may be chosen from: at least about 5 minutes, at least about 10 minutes, at least about 20 minutes, at least about 30 minutes, at least about 45 minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 5 hours, at least about 6 hours.

[0065] Embodiments of the present disclosure are concerned with the identification, production, synthesis and use of slow off-rate aptamers. These are aptamers which have a rate of dissociation ($t_{1/2}$) from a non-covalent aptamer-target complex that is higher than that of aptamers normally obtained by conventional SELEX. For a mixture containing non-covalent complexes of aptamer and target, the $t_{1/2}$ represents the time taken for half of the aptamers to dissociate from the aptamer-target complexes. The $t_{1/2}$ of slow dissociation rate aptamers according to the present disclosure is chosen from one of: greater than or equal to about 30 minutes; between about 30 minutes and about 240 minutes; between about 30 minutes to about 60 minutes; between about 60 minutes to about 90 minutes, between about 90 minutes to about 120 minutes; between about 120 minutes to about 150 minutes; between about 150 minutes to about 180 minutes; between about 180 minutes to about 210 minutes; between about 210 minutes to about 240 minutes.

[0066] A characterizing feature of an aptamer identified by a SELEX procedure is its high affinity for its target. An aptamer will have a dissociation constant (k_d) for its target that is chosen from one of: less than about 1μM, less than about 100nM, less than about 10nM, less than about 1nM, less than about 100pM, less than about 10 pM, less than about 1pM.

[0067] "Tissue target" or "tissue" refers herein to a certain subset of the SELEX targets described above. According to this definition, tissues are macromolecules in a heterogeneous environment. As used herein, tissue refers to a single cell type, a collection of cell types, an aggregate of cells, or an aggregate of macromolecules. This differs from simpler SELEX targets that are typically isolated soluble molecules, such as proteins. In some embodiments, tissues are insoluble macromolecules that are orders of magnitude larger than simpler SELEX targets. Tissues are complex targets made up of numerous macromolecules, each macromolecule having numerous potential epitopes. The different macromolecules which comprise the numerous epitopes can be proteins, lipids, carbohydrates, etc., or combinations thereof. Tissues are generally a physical array of macromolecules that can be either fluid or

rigid, both in terms of structure and composition. Extracellular matrix is an example of a more rigid tissue, both structurally and compositionally, while a membrane bilayer is more fluid in structure and composition. Tissues are generally not soluble and remain in solid phase, and thus partitioning can be accomplished relatively easily. Tissue includes, but is not limited to, an aggregate of cells usually of a particular kind together with their intercellular substance that form one of the structural materials commonly used to denote the general cellular fabric of a given organ, *e.g.*, kidney tissue, brain tissue. The four general classes of tissues are epithelial tissue, connective tissue, nerve tissue and muscle tissue.

[0068] Examples of tissues which fall within this definition include, but are not limited to, heterogeneous aggregates of macromolecule such as fibrin clots which are a cellular; homogeneous or heterogeneous aggregates of cells; higher ordered structures containing cells which have a specific function, such as organs, tumors, lymph nodes, arteries, etc.; and individual cells. Tissues or cells can be in their natural environment, isolated, or in tissue culture. The tissue can be intact or modified. The modification can include numerous changes such as transformation, transfection, activation, and substructure isolation, e.g., cell membranes, cell nuclei, cell organelles, etc.

[0069] Sources of the tissue, cell or subcellular structures can be obtained from prokaryotes as well as eukaryotes. This includes human, animal, plant, bacterial, fungal, and viral structures.

[0070] As used herein, the term "labeling agent," "label," or "detectable moiety", or "detectable element" or "detectable component" refers to one or more reagents that can be used to detect a target molecule that is bound to an aptamer. A detectable moiety or label is capable of being detected directly or indirectly. In general, any reporter molecule that is detectable can be a label. Labels include, for example, (i) reporter molecules that can be detected directly by virtue of generating a signal, (ii) specific binding pair members that may be detected indirectly by subsequent binding to a cognate that contains a reporter molecule, (iii) mass tags detectable by mass spectrometry, (iv) oligonucleotide primers that can provide a template for amplification or ligation, and (v) a specific polynucleotide sequence or recognition sequence that can act as a ligand, such as, for example, a repressor protein, wherein in the latter two instances the oligonucleotide primer or repressor protein will have, or be capable of having, a reporter molecule, and so forth. The reporter molecule can be a catalyst, such as an enzyme, a polynucleotide coding for a catalyst, promoter, dye, fluorescent molecule, quantum dot, chemiluminescent molecule, coenzyme, enzyme substrate, radioactive group, a small organic molecule, amplifiable polynucleotide sequence, a particle such as latex or carbon particle, metal sol, crystallite, liposome, cell, etc., which may or may not be further labeled with a dye, catalyst or other detectable group, a mass tag that alters the weight of the molecule to which it is conjugated for mass spectrometry purposes, and the like. The label can be selected from electromagnetic or electrochemical materials. In one embodiment, the detectable label is a fluorescent dye. Other labels and labeling schemes will be evident to one skilled in the art based on the disclosure herein.

[0071] A detectable moiety (element or component) can include any of the reporter molecules

listed above and any other chemical or component that may be used in any manner to generate a detectable signal. The detectable moiety may be detected via a fluorescent signal, a chemiluminescent signal, or any other detectable signal that is dependent upon the identity of the moiety. In the case where the detectable moiety is an enzyme (for example, alkaline phosphatase), the signal may be generated in the presence of the enzyme substrate and any additional factors necessary for enzyme activity. In the case where the detectable moiety is an enzyme substrate, the signal may be generated in the presence of the enzyme and any additional factors necessary for enzyme activity. Suitable reagent configurations for attaching the detectable moiety to a target molecule include covalent attachment of the detectable moiety to the target molecule, non-covalent association of the detectable moiety with another labeling agent component that is covalently attached to the target molecule, and covalent attachment of the detectable moiety to a labeling agent component that is non-covalently associated with the target molecule. Universal protein stains (UPS) are described in detail in U.S. Patent Application Serial No. 10/504,696, filed August 12, 2004, entitled "Methods and Reagents for Detecting Target Binding by Nucleic Acid Ligands."

[0072] "Solid support" refers herein to any substrate having a surface to which molecules may be attached, directly or indirectly, through either covalent or non-covalent bonds. The substrate materials may be naturally occurring, synthetic, or a modification of a naturally occurring material. Solid support materials may include silicon, graphite, mirrored surfaces, laminates, ceramics, plastics (including polymers such as, *e.g.,* poly(vinyl chloride), cyclo-olefin copolymers, polyacrylamide, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), polytetrafluoroethylene (PTFE or Teflon®), nylon, poly(vinyl butyrate)), germanium, gallium arsenide, gold, silver, etc., either used by themselves or in conjunction with other materials. Additional rigid materials may be considered, such as glass, which includes silica and further includes, for example, glass that is available as Bioglass. Other materials that may be employed include porous materials, such as, for example, controlled pore glass beads. Any other materials known in the art that are capable of having one or more functional groups, such as any of an amino, carboxyl, thiol, or hydroxyl functional group, for example, incorporated on its surface, are also contemplated.

[0073] The solid support may take any of a variety of configurations ranging from simple to complex and can have any one of a number of shapes, including a strip, plate, disk, rod, particle, including bead, tube, well, and the like. The surface may be relatively planar (*e.g.,* a slide), spherical (*e.g.,* a bead), cylindrical (*e.g.,* a column), or grooved. Exemplary solid supports that may be used include microtitre wells, microscope slides, membranes, paramagnetic beads, charged paper, Langmuir-Blodgett films, silicon wafer chips, flow through chips, and microbeads.

[0074] As used herein, "partitioning" means any process whereby one or more components of a mixture are separated from other components of the mixture. For example, aptamers bound to target molecules can be partitioned from other nucleic acids that are not bound to target molecules and from non-target molecules. More broadly stated, partitioning allows for the separation of all the nucleic acids in a candidate mixture into at least two pools based on their

relative affinity and/or dissociation rate to the target molecule. Partitioning can be accomplished by various methods known in the art, including filtration, affinity chromatography, liquid-liquid partitioning, HPLC, etc. For example, nucleic acid-protein pairs can be bound to nitrocellulose filters while unbound nucleic acids are not. Columns that specifically retain nucleic acid-target complexes can also be used for partitioning. For example, oligonucleotides able to associate with a target molecule bound on a column allow the use of column chromatography for separating and isolating the highest affinity aptamers. Beads upon which target molecules are conjugated can also be used to partition aptamers in a mixture. If the beads are paramagnetic, the partitioning can be achieved through application of a magnetic field. Surface plasmon resonance technology can be used to partition nucleic acids in a mixture by immobilizing a target on a sensor chip and flowing the mixture over the chip, wherein those nucleic acids having affinity for the target can be bound to the target, and the remaining nucleic acids can be washed away. Liquid-liquid partitioning can be used as well as filtration gel retardation and density gradient centrifugation. Affinity tags on the target molecules can also be used to separate nucleic acid molecules bound to the tagged target from aptamers that are free in solution. For example, biotinylated target molecules, along with aptamers bound to them, can be sequestered from the solution of unbound nucleic acid sequences using streptavidin paramagnetic beads. Affinity tags can also be incorporated into the aptamer during preparation.

[0075] As used herein, "photoSELEX" is an acronym for Photochemical Systematic Evolution of Ligands by Exponential enrichment and refers to embodiments of the SELEX process in which photocrosslinking aptamers are generated. In one embodiment of the photoSELEX process, a photoreactive nucleotide activated by absorption of light is incorporated in place of a native base in either RNA- or in ssDNA-randomized oligonucleotide libraries, the nucleic acid target molecule mixture is irradiated causing some nucleic acids incorporated in nucleic acid-target molecule complexes to crosslink to the target molecule via the photoreactive functional groups, and the selection step is a selection for photocrosslinking activity. The photoSELEX process is described in great detail in the PhotoSELEX Patents.

[0076] As used herein, "photoaptamer," "photoreactive aptamer," and "photoreactive aptamer" are used interchangeably to refer to an aptamer that contains one or more photoreactive functional groups that can covalently bind to or "crosslink" with a target molecule. For example, a naturally occurring nucleic acid residue may be modified to include a chemical functional group that confers photoreactivity upon the nucleic acid residue upon exposure to a radiation source of an appropriate wavelength. In some embodiments, a photoreactive aptamer is identified initially. In other embodiments, an aptamer is first identified and is subsequently modified to incorporate one or more photoreactive functional groups, thereby generating a photoaptamer. In these embodiments, one or more photoreactive nucleic acid residues can be incorporated into an aptamer either by substituting a photoreactive nucleic acid residue in the place of one or more other nucleotides, such as one or more of the thymidine and/or cytidine nucleotides in the aptamer, for example, or by modifying one or more nucleic acid residues to include a photoreactive functional group.

[0077] Exemplary photoreactive functional groups that may be incorporated by a photoaptamer include 5-bromouracil, 5-iodouracil, 5-bromovinyluracil, 5-iodovinyluracil, 5-azidouracil, 4-thiouracil, 5-thiouracil, 4-thiocytosine, 5-bromocytosine, 5-iodocytosine, 5-bromovinylcytosine, 5-iodovinylcytosine, 5-azidocytosine, 8-azidoadenine, 8-bromoadenine, 8-iodoadenine, 8-aziodoguanine, 8-bromoguanine, 8-iodoguanine, 8-azidohypoxanthine, 8-bromohypoxanthine, 8-iodohypoxanthine, 8-azidoxanthine, 8-bromoxanthine, 8-iodoxanthine, 5-[(4-azidophenacyl)thio]cytosine, 5-[(4-azidophenacyl)thio]uracil, 7-deaza-7-iodoadenine, 7-deaza-7-iodoguanine, 7-deaza-7-bromoadenine, and 7-deaza-7-bromoguanine.

[0078] In addition to these exemplary nucleoside-based photoreactive functional groups, other photoreactive functional groups that can be added to a terminal end of an aptamer using an appropriate linker molecule can also be used. Such photoreactive functional groups include benzophenone, anthraquinone, 4-azido-2-nitro-aniline, psoralen, derivatives of any of these, and the like.

[0079] A photoreactive functional group incorporated by a photoaptamer may be activated by any suitable method. In one embodiment, a photoaptamer containing a photoreactive functional group can be crosslinked to its target by exposing the photoaptamer and its bound target molecule to a source of electromagnetic radiation. Suitable types of electromagnetic radiation include ultraviolet light, visible light, X-rays, and gamma rays. Suitable radiation sources include sources that utilize either monochromatic light or filtered polychromatic light.

[0080] As used herein, the term "the affinity SELEX process" refers to embodiments of the SELEX process in which non-photocrosslinking aptamers to targets are generated. In some embodiments of the affinity SELEX process, the target is immobilized on a solid support either before or after the target is contacted with the candidate mixture of nucleic acids. The association of the target with the solid support allows nucleic acids in the candidate mixture that have bound and in the case where a slow - off rate enrichment process is used, stay bound to the target to be partitioned from the remainder of the candidate mixture. The term "bead affinity SELEX process" refers to particular embodiments of the affinity SELEX process where the target is immobilized on a bead, for example, before contact with the candidate mixture of nucleic acids. In some embodiments, the beads are paramagnetic beads. The term "filter affinity SELEX process" refers to embodiments where nucleic acid target complexes are partitioned from candidate mixture by virtue of their association with a filter, such as a nitrocellulose filter. This includes embodiments where the target and nucleic acids are initially contacted in solution, and contacted with the filter, and also includes embodiments where nucleic acids are contacted with target that is pre-immobilized on the filter. The term "plate affinity SELEX process" refers to embodiments where the target is immobilized on the surface of a plate, such as, for example, a multi-well microtiter plate. In some embodiments, the plate is comprised of polystyrene. In some embodiments, the target is attached to the plate in the plate affinity SELEX process through hydrophobic interactions.

[0081] The present disclosure describes improved SELEX methods for generating aptamers that are capable of binding to target molecules. More specifically, the present disclosure

describes methods for identifying aptamers and/or photoaptamers having slower rates of dissociation from their respective targeted molecules than aptamers obtained with previous SELEX methods. The disclosure further describes aptamers and/or photoaptamers obtained by using the methods described herein and methods of using the same.

[0082] A method is provided for identifying an aptamer having a slow rate of dissociation from its target molecule, the method comprising (a) preparing a candidate mixture of nucleic acid sequences; (b) contacting the candidate mixture with a target molecule wherein nucleic acids with the highest relative affinities to the target molecule preferentially bind the target molecule, forming nucleic acid-target molecule complexes; (c) applying an slow off-rate enrichment process to allow the dissociation of nucleic acid-target molecule complexes with relatively fast dissociation rates; (d) partitioning the remaining nucleic acid-target molecule complexes from both free nucleic acids and non - target molecules in the candidate mixture; and (e) identifying an aptamer to the target molecule. The process may further include the iterative step of amplifying the nucleic acids that bind to the target molecule to yield a mixture of nucleic acids enriched in sequences that are able to bind to the target molecule yet produce nucleic acid-target molecule complexes having slow dissociation rates. As defined above, the slow off-rate enrichment process can be selected from diluting the candidate mixture containing the nucleic acid-target molecule complexes, adding at least one competitor to the candidate mixture containing the nucleic acid-target molecule complexes, and diluting the candidate mixture containing the nucleic acid-target molecule complexes and adding at least one competitor to the candidate mixture containing the nucleic acid-target molecule complexes.

[0083] A method is provided for identifying an aptamer having a slow rate of dissociation from its target molecule, the method comprising: (a) preparing a candidate mixture of nucleic acids; (b) contacting the candidate mixture with a target molecule, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes; (c) incubating the candidate mixture and target molecule together for a period of time sufficient to achieve equilibrium binding; (d)adding an excess of at least one competitor molecule to the mixture of (c); (e) incubating the mixture of the candidate mixture, the nucleic acid-target molecule complexes and the competitor molecule from (d) for a predetermined period of time; (f) partitioning the nucleic acid-target molecule complexes from the candidate mixture; (g) dissociating the nucleic acid-target molecule complexes to generate free nucleic acids; (h) amplifying the free nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity, whereby an aptamer to the target molecule may be identified.

[0084] A method is provided for producing an aptamer having a slow rate of dissociation from its target molecule, the method comprising preparing or synthesizing an aptamer that includes a nucleic acid sequence identified by the following process comprising the steps of: (a) preparing a candidate mixture of nucleic acids; (b) contacting the candidate mixture with a target molecule, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic

acid-target molecule complexes; (c) incubating the candidate mixture and target molecule together for a period of time sufficient to achieve equilibrium binding; (d) adding an excess of at least one competitor molecule to the mixture of (c); (e) incubating the mixture of the candidate mixture, nucleic acid-target molecule complexes and the competitor molecule from (d) for a predetermined period of time; (f) partitioning the nucleic acid-target molecule complexes from the candidate mixture; (g) dissociating the nucleic acid-target molecule complexes to generate free nucleic acids; (h) amplifying the free nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity, whereby an aptamer to the target molecule is identified.

[0085] A method is provided of identifying an aptamer having a slow rate of dissociation from its target molecule, the method comprising: (a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture is chemically modified at the 5-position; (b) contacting the candidate mixture with a target molecule, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes; (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and (d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity, whereby an aptamer to the target molecule may be identified.

[0086] A method is provided for producing an aptamer having a slow rate of dissociation from its target molecule, said method comprising preparing or synthesizing an aptamer that includes a nucleic acid sequence identified by the following process: (a) preparing a candidate mixture of nucleic acids, wherein the candidate mixture comprises modified nucleic acids in which one, several or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture is chemically modified at the 5-position; (b) contacting the candidate mixture with a target molecule, wherein nucleic acids having an increased affinity to the target molecule relative to other nucleic acids in the candidate mixture bind the target molecule, forming nucleic acid-target molecule complexes; (c) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and (d) amplifying the increased affinity nucleic acids to yield a mixture of nucleic acids enriched in nucleic acid sequences that are capable of binding to the target molecule with increased affinity, whereby an aptamer to the target molecule is identified.

[0087] A non-covalent complex of an aptamer and its target is provided, wherein the rate of dissociation ($t_{1/2}$) of the aptamer from the target is chosen from one of: greater than or equal to about 30 minutes; between about 30 minutes and about 240 minutes; about 30 minutes to about 60 minutes; about 60 minutes to about 90 minutes; about 90 minutes to about 120 minutes; about 120 minutes to about 150 minutes; about 150 minutes to about 180 minutes; about 180 minutes to about 210 minutes; about 210 minutes to about 240 minutes.

[0088] A non-covalent complex of an aptamer and a target is provided, wherein the aptamer

has a K_d for the target of about 100nM or less, wherein the rate of dissociation ($t_{1/2}$) of the aptamer from the target is greater than or equal to about 30 minutes, and wherein one, several or all pyrimidines in the nucleic acid sequence of the aptamer are modified at the 5- position of the base,. The modifications may be selected from the group of compounds shown in FIG. 14, these modifications are referred to as "base modified nucleotide". Aptamers may be designed with any combination of the base modified pyrimidines desired.

[0089] Improved methods for performing SELEX with modified nucleotides, including nucleotides which contain photoactive groups or nucleotides which contain placeholders for photoactive groups are disclosed in U.S. Application Serial No. 12/175,388, entitled "Improved SELEX and PHOTOSELEX" which is being filed concurrently with the instant application
. In another embodiment, the candidate mixture of nucleic acid molecules includes nucleic acids containing modified nucleotide bases that may aid in the formation of modified nucleic acid-target complexes with relatively slow dissociation rates.

[0090] The various methods and steps described herein can be used to generate an aptamer capable of either (1) binding to a target molecule or (2) binding to a target molecule and subsequently forming a covalent linkage with the target molecule upon irradiation.

[0091] Aptamers identified according to the methods described herein are useful in a range of diagnostic and therapeutic methods. Slow off-rate aptamers will bind to the target for a longer duration. This is useful in diagnostic methods where the binding of an aptamer to the target may be used to detect the presence, absence, amount or quantity of the target molecule and a prolonged interaction of the aptamer and target facilitates such detection. A similar advantage may be afforded where slow off-rate aptamers are used in imaging methods, in vitro or in vivo. A prolonged interaction of aptamer and target also provides for improved therapeutic methods of treatment where the prolonged interaction may allow for an improved therapeutic effect, e.g. owing to the longer activation or inhibition of the target molecule or downstream signaling cascade.

[0092] Accordingly, , slow off-rate aptamers obtained, identified or produced by the described methods can be used in a variety of methods of medical treatment or method of diagnosis (*in vitro* or *in vivo*). Slow off-rate aptamers can be used in a method of treatment of disease. Slow off-rate aptamers can be used in a method for diagnosis of disease *in vivo.*

[0093] Slow off-rate aptamers can be used *in vitro* for the diagnosis of disease. A slow off-rate aptamer can be used in the manufacture of a therapeutic (e.g. pharmaceutical composition) or the manufacture of a diagnostic agent for use in a method of treatment or diagnosis of disease. Diagnostic or therapeutic applications of slow off-rate aptamers may involve a diagnostic or therapeutic outcome that depends on the specific and/or high affinity binding of the slow off-rate aptamer to its target. Slow off-rate aptamers may also be used in target validation and high throughput screening assays in the drug development process.

[0094] Slow off-rate aptamers are suitable reagents for molecular imaging *in vivo.* A slow off-

rate aptamer may be used *in vivo* to detect the presence of a pathology, disease process, or other condition in the body of an individual (e.g., a human or an animal), where the binding of the aptamer to its target indicates the presence of the disease process or other condition. For example, an aptamer to the VEGF receptor may be used *in vivo* to detect the presence of cancer in a particular area (e.g., a tissue, an organ, etc.) of the body of an individual, as the VEGF receptor is abundantly expressed within tumors and their neovasculature, or an aptamer to the EGF receptor may be used *in vivo* to detect the presence of cancer in a particular area (e.g., a tissue, an organ, etc.) of the body of an individual, as the EGF receptor is often expressed at high levels on tumor cells. That is, the molecular target will be the extracellular domain (ECD) of an induced receptor, as such targets are located outside of the cells and are accessible through the vasculature. Additionally, the ECDs tend to be localized at the site of pathology, even though some small fraction of the specific ECD may be shed through biological processes, including cell death.

[0095] The obvious candidates for molecular imaging, high affinity monoclonal antibodies, have not become the reagent of choice for this application. Molecular imaging reagents have precise requirements. They must have high binding activity for their intended target, and low binding activity for other targets in a human or animal. Slow off-rate aptamers have unique advantages that render them desirable for use in molecular imaging *in vivo.* On the one hand, they are selected to have slow dissociation rate constants, thus allowing residence *in vivo* on the intended target for a substantial length of time (at least about 30 minutes). On the other hand, slow off-rate aptamers are expected to have very fast clearance from the vasculature. Slow dissociation rate constants and fast clearance from the vasculature are two desired properties for molecular imaging *in vivo.* From a kinetic prospective, good *in vivo* molecular imaging reagents must stay localized at the site of the pathology while the free reagent concentration in the surrounding vasculature becomes low. This is a signal-to-noise constraint. Suitable signal-to-noise ratios may be obtained by accumulation of signal at the site of pathology in excess of the signal in the vasculature, or may be obtained by retention of a signal at the site of the pathology while the vasculature concentration is diminished.

[0096] Aptamers that do not have slow off-rate properties, of about the same molecular weight and net charge as slow off-rate aptamers, have been studied in animals and humans for more than a decade. Generally, it has been found that these aptamers clear from the vasculature quickly, usually by entering the kidney and/or the liver and then being further metabolized for excretion. Such aptamers show so-called "first pass" clearance unless high molecular weight adducts (such as, for example, PEG) are linked to the aptamers. Experiments have been done within a aptamer whose target is tenascin C, an extracellular protein (not an ECD) found at high concentrations in some tumors. In those experiments, the tenascin C-specific aptamer cleared quickly and was able to be retained at the site of the tumor because the extracellular local concentration of tenascin C is very high. Slow off-rate aptamers, by contrast, will maintain the fast clearance rate of aptamers but offer a kinetic advantage due to their slow dissociation rates, rendering them suitable for use with targets whose presence at the site of interest (e.g., the site of pathology) may be somewhat sparse (ECDs on tumors, for example).

[0097] Alternative reagents for molecular imaging do not share the two slow off-rate aptamer properties (i.e., slow dissociation rate and fast clearance from the body). Monoclonal antibodies often have high affinity and specificity, and may have slow dissociation rate constants; however, monoclonal antibodies have very slow clearance rates from the vasculature. Short peptides, identified through, for example, phage display, may have fast clearance but poor affinity and specificity and fast dissociation rates from their intended targets. Affibodies, a particular peptide version of an antibody mimetic, may have reasonable affinity and specificity and may have faster clearance than monoclonal antibodies, yet in order to achieve slow dissociation rates from their targets Affibodies are often made into dimers and higher order multimers, slowing their clearance at the same time that their dissociation rates are enhanced.

[0098] Slow off-rate aptamers may be used for molecular imaging *in vivo* with one or more low molecular weight adducts to both protect the slow off-rate aptamer from nucleases in the body and detect the intended target once bound by the slow off-rate aptamer. For example, slow off-rate aptamers may be attacked by nucleases in the blood, typically exonucleases (for DNA) that are easily blocked by using exonuclease refractive adducts at the 5' and 3' terminal positions of the slow off rate aptamer, or endonucleases (for RNA) that are easily blocked by incorporating endonuclease refractive pyrimidines (such as, for example, 2' fluoro nucleotides) in the slow off-rate aptamer. Detection of the slow off-rate aptamer-target complex may be achieved by attaching a detection moiety to the slow off-rate aptamer. The detection moiety for these purposes may include cages for radioactive molecules (e.g., technetium 99), clusters of iron for magnetic resonance detection, isotopes of fluorine for PET imaging, and the like. The modifications made to the slow off-rate aptamer to protect the integrity of the slow off-rate aptamer in the body and enable detection of the intended target should be designed such that they do not interfere with the slow off-rate aptamer's interaction with its target and do not cause the slow off-rate aptamer to clear too slowly from the vasculature.

[0099] Diagnostic or assay devices, e.g. columns, test strips or biochips, having one or more slow off-rate aptamers adhered to a solid surface of the device are also provided. The aptamer(s) may be positioned so as to be capable of binding target molecules that are contacted with the solid surface to form aptamer-target complexes that remain adhered to the surface of the device, thereby capturing the target and enabling detection and optionally quantitation of the target. An array of slow off-rate aptamers (which may be the same or different) may be provided on such a device.

[0100] Complexes including a slow off-rate aptamer and a target molecule are provided. A class of aptamers characterized by having high affinity for their corresponding target molecules and slow dissociation rates ($t_{1/2}$) from a non-covalent complex of the aptamer and target is

[0101] With reference to FIG. 1A, the basic SELEX process generally begins with the preparation of a candidate mixture of nucleic acids of differing sequence. The candidate mixture generally includes nucleic acid sequences that include two fixed regions (*i.e.,* each of the members of the candidate mixture contains the same sequences in the same location) and

a variable region. Typically, the fixed sequence regions are selected such that they assist in the amplification steps described below, or enhance the potential of a given structural arrangement of the nucleic acids in the candidate mixture. The variable region typically provides the target binding region of each nucleic acid in the candidate mixture, and this variable region can be completely randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent). The prepared candidate mixture is contacted with the selected target under conditions that are favorable for binding to occur between the target and members of the candidate mixture. Under these conditions, the interaction between the target and the nucleic acids of the candidate mixture generally forms nucleic acid-target pairs that have the strongest relative affinity between members of the pair. The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. The partitioning process is conducted in a manner that retains the maximum number of high affinity candidates. Those nucleic acids selected during partitioning as having a relatively high affinity to the target are amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively high affinity for the target. By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acid mixture to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a very small number of unique nucleic acids representing those nucleic acids from the original candidate mixture that have the highest affinity to the target molecule. However, this basic SELEX process does not select for aptamers that have slow off-rates from their targets.

[0102] The SELEX Patents and the PhotoSELEX Patents describe and elaborate on this process in great detail. These patents include descriptions of the various targets that can be used in the process; methods for the preparation of the initial candidate mixture; methods for partitioning nucleic acids within a candidate mixture; and methods for amplifying partitioned nucleic acids to generate enriched candidate mixtures. The SELEX Patents also describe aptamer solutions obtained to a number of different types of target molecules, including protein targets wherein the protein is and is not a nucleic acid binding protein.

[0103] With reference to FIG. 1B the modified SELEX process disclosed herein includes the introduction of a slow off-rate enrichment process following equilibration of the candidate mixture of nucleic acids with the target or targets and a partitioning step prior to subsequent steps in the SELEX process. Introduction of a slow off-rate enrichment process to the basic SELEX process provides a means for enrichment of aptamer affinity complexes with slow dissociation rates from a set of nucleic acid-target complexes that includes a variety of dissociation rates. Thus, the modified SELEX process provides a method for identifying aptamers that bind target molecules and, once bound, have relatively slow rates of dissociation (also referred to herein as "off-rates") from the target molecule.

[0104] As used herein "binding" generally refers to the formation of a non-covalent association between the ligand and the target, although such binding is not necessarily reversible. The

terms "nucleic acid-target complex" or "complex" or "affinity complex" are used to refer to the product of such non-covalent binding association.

[0105] The slow off-rate aptamers can be single- or double-stranded RNA or DNA oligonucleotides. The aptamers can contain non-standard or modified bases. Further, the aptamers can contain any type of modification. As used herein, a "modified base" may include a relatively simple modification to a natural nucleic acid residue, which modification confers a change in the physical properties of the nucleic acid residue. Such modifications include, but are not limited to, modifications at the 5-position of pyrimidines, substitution with hydrophobic groups, *e.g.,* benzyl, isobutyl, indole, or naphthyl or substitution with hydrophilic groups, e.g., quaternary amine or guanidinium, or more "neutral" groups, e.g., imidazole and the like. Additional modifications may be present in the ribose ring, e.g., 2'-position, such as 2'-amino (2'-NH_2) and 2'-fluoro (2'-F), or the phosphodiester backbone, e.g., phosphorothioates or methyl phosphonates.

[0106] A candidate mixture containing a randomized set of nucleic acid sequences containing modified nucleotide bases may be mixed with a quantity of the target molecule and allowed to establish binding equilibrium with the target molecule. Generally, only some of those nucleic acids that bind with high affinity to the target molecule will efficiently partition with the target.

[0107] The candidate mixture may include nucleic acid sequences having variable regions that include modified groups. The modified groups can be modified nucleotide bases. The variable region can contain fully or partially random sequences; it can also contain subportions of a fixed sequence that is incorporated within the variable region. The nucleotides within the fixed regions can also contain modified nucleotide bases, or they can contain the standard set of naturally occurring bases.

[0108] Amplification may occur after members of the test mixture have been partitioned, and it is the nucleic acid that is amplified. For example, amplifying RNA molecules can be carried out by a sequence of three reactions: making cDNA copies of selected RNAs, using the polymerase chain reaction to increase the copy number of each cDNA, and transcribing the cDNA copies to obtain RNA molecules having the same sequences as the selected RNAs. Any reaction or combination of reactions known in the art can be used as appropriate, including direct DNA replication, direct RNA amplification and the like, as will be recognized by those skilled in the art. The amplification method may result in the proportions of the amplified mixture being representative of the proportions of different sequences in the mixture prior to amplification. It is known that many modifications to nucleic acids are compatible with enzymatic amplification. Modifications that are not compatible with amplification can be made after each round of amplification, if necessary.

[0109] The nucleic acid candidate mixture can be modified in various ways to enhance the probability of the nucleic acids having facilitating properties or other desirable properties, particularly those that enhance the interaction between the nucleic acid and the target. Contemplated modifications include modifications that introduce other chemical groups that

have the correct charge, polarizability, hydrogen bonding, or electrostatic interaction to enhance the desired ligand-target interactions. The modifications that may enhance the binding properties, including the affinity and/or dissociation rates, of the nucleic acid, for example, include hydrophilic moieties, hydrophobic moieties, rigid structures, functional groups found in proteins such as imidazoles, primary alcohols, carboxylates, guanidinium groups, amino groups, thiols and the like. Modifications can also be used to increase the survival of aptamer-target complexes under stringent selection pressures that can be applied to produce slow off-rate aptamers to a wide range of targets. In one embodiment, Bz-dU(benzyl -dU) is used in the generation of the candidate mixtures used to produce slow off-rate aptamers, although other modified nucleotides are well suited to the production of such aptamers. Other modified nucleotides are shown in FIG. 14.

[0110] A modified nucleotide candidate mixtures for the purpose of this application is any RNA or DNA candidate mixtures that includes both naturally occurring and other than the naturally occurring nucleotides. Suitable modifications include modifications on every residue of the nucleic acid, on a single residue of the nucleic acid, on random residues, on all pyrimidines or all purines, on all occurrences of a specific base (*i.e.,* G, C, A, T or U) in the nucleic acid, or any other modification scheme that may be suitable for a particular application. It is recognized that modification is not a prerequisite for facilitating activity or binding ability of the aptamers. Aptamers may include modified dUTP and dCTP residues.

[0111] Candidate mixtures for slow off-rate aptamers may comprise a set of pyrimidines having a different modification at the C-5 base position. The C-5 modification may be introduced through an amide linkage, directly, or indirectly through another type of linkage. These candidate mixtures are used in a SELEX process to identify slow off-rate aptamers. This process may be also include the use of the slow off-rate enrichment process. Candidate mixtures may be produced enzymatically or synthetically.

[0112] As described above, the nucleotides can be modified in any number of ways, including modifications of the ribose and/or phosphate and/or base positions. Certain modifications are described in U.S. Patent No. 5,660,985 entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," U.S. Patent No. 5,428,149 entitled "Method for Palladium Catalyzed Carbon-Carbon Coupling and Products," U.S. Patent No. 5,580,972 entitled "Purine Nucleoside Modifications by Palladium Catalyzed Methods,". Modifications may be
those wherein another chemical group is attached to the 5-position of a pyrimidine, the 8-position of a purine, or the 2' position of a sugar. There is no limitation on the type of other chemical group that can be incorporated on the individual nucleotides. In some embodiments, the resulting modified nucleotide is amplifiable or can be modified subsequent to the amplification steps (*see, e.g.,* U.S. Patent No. 6,300,074 entitled "Systematic evolution of ligands by exponential enrichment: Chemi-SELEX".

[0113] Certain nucleotides may be modified such that a method for producing aptamers that bind and form a covalent crosslink to their target molecule upon photo-activation of the affinity complex. This method encompasses aptamers that bind, photocrosslink, and/or

photoinactivate target molecules. The aptamers may
contain photoreactive groups that are capable of photocrosslinking to the target molecule upon irradiation with light. In other methods , the aptamers are capable of bond formation with the target in the absence of irradiation.

[0114] A photoreactive group can be any chemical structure that contains a photochromophore and that is capable of photocrosslinking with a target molecule. Although referred to herein as a photoreactive groups, in some cases, as described below, irradiation is not necessary for covalent binding to occur between the aptamer and the target. Sometimes , the photoreactive group will absorb light of a wavelength that is not absorbed by the target or the non-modified portions of the oligonucleotide. Photoreactive groups include 5-halo-uridines, 5-halo-cytosines, 7-halo-adenosines, 2-nitro-5-azidobenzoyls, diazirines, aryl azides, fluorinated aryl azides, benzophenones, amino-benzophenones, psoralens, anthraquinones, etc.

[0115] The photoreactive groups generally form bonds with the target upon irradiation of the associated nucleic acid-target pair. In some cases, irradiation is not required for bond formation to occur. The photocrosslink that typically occurs will be the formation of a covalent bond between the associated aptamer and the target. However, a tight ionic interaction between the aptamer and target may also occur upon irradiation.

[0116] Photocrosslinking may occur due to exposure to electromagnetic radiation. Electromagnetic radiation includes ultraviolet light, visible light, X-ray, and gamma ray.

[0117] A limited selection of oligonucleotides using a SELEX method may be followed by selection using a photoSELEX method. The initial SELEX selection rounds are conducted with oligonucleotides containing photoreactive groups. After a number of SELEX rounds, photoSELEX is conducted to select oligonucleotides capable of binding the target molecule.

[0118] The production of an aptamer that includes a cleavable or releasable section (also described as an element or component) in the aptamer sequence is described. These additional components or elements are structural elements or components that introduce additional functionality into the aptamer and are thus functional elements or components. The aptamer is further produced with one or more of the following additional components (also described as a functional or structural element or component or moiety in any combination of these terms): a labeled or detectable component, a spacer component, and a specific binding tag or immobilization element or component.

[0119] As noted above, the present disclosure provides methods for identifying aptamers that bind target molecules and once bound have slow rates of dissociation or off-rates. The slow off-rates obtained with this method can exceed a half-life of about one hour and as much as about 240 minutes, that is, once a set of nucleic acid-target complexes is generated, half of the complexes in the set remain bound after one hour. Because the effect of a slow off-rate enrichment process depends upon the differing dissociation rates of aptamer affinity complexes, the duration of the slow off-rate enrichment process is chosen so as to retain a

high proportion of aptamer affinity complexes with slow dissociation rates while substantially reducing the number of aptamer affinity complexes with fast dissociation rates. For example, incubating the mixture for relatively longer periods of time after imposing the slow off-rate enrichment process will select for aptamers with longer dissociation rates than aptamers selected using slow off-rate enrichment process having shorter incubation periods.

[0120] The candidate mixture may be mixed with a quantity of the target molecule and allowed to establish binding equilibrium with the target molecule. Prior to partitioning the target bound nucleic acids from those free in solution, an slow off-rate enrichment process is imposed to enrich the bound population for slow dissociation rates. As noted above, slow off-rate enrichment process can be applied by the addition of a competitor molecule, by sample dilution, by a combination of sample dilution in the presence of a competitor molecule. Thus, the slow off-rate enrichment process may be applied by introducing competitor molecules into the mixture containing the nucleic acid-target complexes and incubating the mixture for some period of time before partitioning free from bound nucleic acids. The amount of competitor molecules is generally at least one order of magnitude higher than that of the nucleic acid molecules and may be two or more orders of magnitude higher. In another embodiment, the slow off-rate enrichment process is applied by diluting the sample mixture of nucleic acid-target complexes several fold (e.g. at least about one of 2x, 3x, 4x, 5x) in volume and incubating the mixture for some period of time before partitioning free from bound nucleic acids. The dilution volume is generally at least one order of magnitude higher, and may be about two or more orders of magnitude higher, than the original volume. A combination of both competitor molecules and dilution may be used to apply the slow off-rate enrichment process.

[0121] Candidate mixtures that have been shown to result in an increased frequency of slow dissociation aptamerscan be used to select a number of candidate aptamers. These aptamers are screened to identify slow dissociation rate aptamers.

[0122] A slow off-rate aptamer that includes a cleavable or releasable section in the fixed region of the aptamer may be produced. The aptamer can also be produced with one or more of the following additional components: a labeled component, a spacer component, and a specific binding tag. Any or all of these elements may be introduced into a single stranded aptamer. The element may be introduced at the 5' end of the aptamer. One or more of these elements can be included by creating a partially double stranded aptamer, where one strand contains the various elements desired as well as a sequence complementary to one of the fixed sequence sections of the second strand containing the variable target binding region.

[0123] A "releasable" or "cleavable" element or moiety or component refers to a functional group where certain bonds in the functional group can be broken to produce 2 separate components. The functional group can be cleaved by irradiating the functional group (photocleavable) at the appropriate wavelength or by treatment with the appropriate chemical or enzymatic reagents.

[0124] The releasable element may be a disulfide bond that can be treated with a reducing

agent to disrupt the bond. The releasable element allows an aptamer/target affinity complex that is attached to a solid support to be separated from the solid support, such as by elution of the complex. The releasable element may be stable to the conditions of the rest of the assay and may be releasable under conditions that will not disrupt the aptamer /target complex.

[0125] As disclosed herein, an aptamer can further comprise a "tag" or "immobilization component or element" or "specific binding component or element" which refers to a component that provides a means for attaching or immobilizing an aptamer (and any target molecule that is bound to it) to a solid support. A "tag" is a set of copies of one type or species of component that is capable of associating with a probe. "Tags" refers to more than one such set of components. The tag can be attached to or included in the aptamer by any suitable method. Generally, the tag allows the aptamer to associate, either directly or indirectly, with a probe or receptor that is attached to the solid support. The probe may be highly specific in its interaction with the tag and retain that association during all subsequent processing steps or procedures. A tag can enable the localization of an aptamer affinity complex (or optional covalent aptamer affinity complex) to a spatially defined address on a solid support. Different tags, therefore, can enable the localization of different aptamer covalent complexes to different spatially defined addresses on a solid support. A tag can be a polynucleotide, a polypeptide, a peptide nucleic acid, a locked nucleic acid, an oligosaccharide, a polysaccharide, an antibody, an affybody, an antibody mimic, a cell receptor, a ligand, a lipid, biotin, any fragment or derivative of these structures, any combination of the foregoing, or any other structure with which a probe (or linker molecule, as described below) can be designed or configured to bind or otherwise associate with specificity. Generally, a tag is configured such that it does not interact intramolecularly with either itself or the aptamer to which it is attached or of which it is a part. If SELEX is used to identify an aptamer, the tag may be added to the aptamer either pre- or post-SELEX. The tag is included on the 5'-end of the aptamer post-SELEX , or the tag is included on the 3'-end of the aptamer post-SELEX, or the tags may be included on both the 3' and 5' ends of the aptamers in a post-SELEX process.

[0126] As illustrated in FIG. 8D, a fluorescent dye (such as Cy3), the photocleavable and biotin moieties are all added to the end of the aptamer. Because of potential interactions between the photocleavable moiety and the dye, a spacer is inserted between these two moieties. All constructs can be synthesized using standard phosphoramidite chemistry. Representative aptamer constructs are shown in FIG. 9A through FIG. 9F. The functionality can be split between the 5' and 3' end or combined on either end. In addition to photocleavable moieties, other cleavable moieties can be used, including chemically or enzymatically cleavable moieties. A variety of spacer moieties can be used and one or more biotin moieties can be included. Tags (also referred to as immobilization or specific binding elements or components) other than biotin can also be incorporated. Suitable construction reagents include biotin phosphoramidite, PC Linker (Glen Research PN 10-4920-02); PC biotin phosphoramidite (Glen Research PN 10-4950-02); dSpacer CE phosphoramidite (Glen Research PN 10-1914-02); Cy3 phosphoramidite (Glen Research PN 10-5913-02); and Arm26-Ach Spacer Amidite (Fidelity Systems PN SP26Ach-05).

[0127] In one embodiment, base modifications of the nucleotides are used in the production of the variable region of the aptamer. These modified nucleotides have been shown to produce aptamers that have very slow off-rates their targets.

[0128] In the methods of the present disclosure the candidate mixture may comprise modified nucleic acids in which one, several (e.g. one of, or at least one of, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22,.23, 24, 25, 26, 27, 28, 29, 30) or all pyrimidines in at least one, or each, nucleic acid of the candidate mixture is chemically modified at the 5-position. Optionally, all C residues in the nucleic acids of the candidate mixture are chemically modified at the 5-position. Optionally, all T residues in the nucleic acids of the candidate mixture are chemically modified at the 5-position. Optionally, all U residues in the nucleic acids of the candidate mixture are chemically modified at the 5-position.

[0129] The slow off-rate aptamers may be mixed or exposed to a sample. The slow off-rate aptamer is allowed to react with, or bind to, its specific target in the sample to form a complex. A variety of methods may be used to detect either the target or the aptamer. The target may be detected in the complex or upon liberation from the complex. The aptamer may be detected in the complex or upon liberation from the complex. The aptamer/target complex may be used to isolate the specific target from other components in the test sample. Multiple aptamers may be used when a multiplexed assay for the detection of a variety of targets is desired.

[0130] The method of the instant disclosure is illustrated generally in Examples 1-7.

[0131] The method of the instant disclosure is illustrated generally in Examples 1-6. Example 1 describes the general affinity SELEX method using a candidate mixture comprised of modified nucleotides. Example 2 describes a photo SELEX method using a candidate mixture comprised of modified nucleotides and a 5'-terminal photoreactive group, and the improved SELEX method in which dilution is used to provide the slow off-rate enrichment process to the equilibrated aptamer:target mixture. Example 3 extends the method described in Example 2 by the addition of a competitor to the dilution step. Example 4 illustrates the effectiveness of the slow off-rate enrichment process. The average dissociation half-life value (t1/2) for aptamers using the modified nucleotides 5-benzyl-dUTP (BzdUTP), 5-isobutyl-dUTP (iBdUTP), or 5-tryptamino-dUTP selected in the absence of a slow off-rate enrichment process was 20 minutes with some aptamers having a t1//2 value of up to one hour. This is substantially longer than what has been previously described with natural bases or other modified nucleotides. The average for aptamers selected with a slow off-rate enrichment process was over 85 minutes. More specifically, with reference to Figure 3B, it can be seen that introduction of a slow - off rate enrichment process produced aptamers with $t_{1/2}$ values of about ≥ 30 min., >_ 60 min., ≥ 90 min., >_ 120 min. ≥ 150 min. ≥ 180 min. ≥ 210 and ≥ 240 min. These dissociation rates for aptamer:target complexes are unprecedented.

[0132] Example 5 describes the generation of slow off-rate aptamers using a NpdUTP candidate mixture.

[0133] Example 6 describes the generation of a slow off-rate aptamer to a peptide target.

EXAMPLES

[0134] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention as defined in the appended claims.

EXAMPLE 1. Incorporation of Modified Nucleotides in Nucleic Acid Libraries Leads to Higher Affinity Enriched Libraries in Affinity SELEX

A. Preparation of Candidate Mixtures

[0135] Candidate mixtures were prepared with dATP, dGTP, 5-methyl-dCTP (MedCTP) and either dTTP or one of three dUTP analogs: 5-Benzyl-dUTP (BzdUTP), 5-isobutyl-dUTP (iBdUTP), or 5-tryptamino-dUTP (TrpdUTP). Candidate mixtures were prepared by polymerase extension of a primer annealed to a biotinylated template (FIG. 2). For each candidate mixture composition, 4.8 nmol forward PCR primer and 4 nmol template were combined in 100 µL 1X KOD DNA Polymerase Buffer (Novagen), heated to 95°C for 8 minutes, and cooled on ice. Each 100 µL primer:template mixture was added to a 400 µL extension reaction containing 1X KOD DNA Polymerase Buffer, 0.125 U/ µL KOD DNA Polymerase, and 0.5 mM each dATP, MedCTP, dGTP, and dTTP or dUTP analog, and incubated 70°C, 30 minutes. Double-stranded product was captured via the template strand biotins by adding 1 mL streptavidin-coated magnetic beads (MagnaBind Streptavidin, Pierce, 5 mg/mL in 1M NaCl + 0.05% TWEEN-20) and incubating 25°C for 10 minutes with mixing. Beads were washed three times with 0.75 mL SB1T Buffer (40 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% TWEEN-20). The aptamer strand was eluted from the beads with 1.2 mL 20 mM NaOH, neutralized with 0.3 mL 80 mM HCl, and buffered with 15 µL 1 M HEPES, pH 7.5. Candidate mixtures were concentrated with a Centricon-30 to approximately 0.2 mL, and quantified by UV absorbance spectroscopy.

B. Immobilization of Target Proteins

[0136] Target proteins were purchased with poly His tags, such as, $(His)_6$ tags (R&D Systems) and immobilized on Co^{+2}-NTA paramagnetic beads (TALON, Invitrogen). Target proteins were diluted to 0.2 mg/mL in 0.5 mL B/W Buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0.01% TWEEN-20), and added to 0.5 mL TALON beads (pre-washed three times with B/W Buffer and resuspended to 10 mg/mL in B/W Buffer). The mixture was rotated for 30 minutes at 25°C and stored at 4°C until use. TALON beads coated with $(His)_6$ peptide were also

prepared and stored as above. Prior to use, beads were washed 3 times with B/W Buffer, once with SB1T, and resuspended in SB1T.

C. Aptamer Selection Scheme

[0137] Affinity selections were performed separately with each candidate mixture, comparing binding between target protein beads (signal, S) and $(His)_6$ beads (background, B). For each sample, a 0.5 µM candidate DNA mixture was prepared in 40 µL SB1T. 1 µL $(His)_6$-complement oligo (1 mM) (FIG. 2) was added to the DNA, along with 10 µL of a protein competitor mixture (0.1% HSA, 10 µM casein, and 10 µM prothrombin in SB1T).

[0138] Binding reactions were performed by adding 50 µL target protein-coated beads or $(His)_6$-coated beads (5 mg/mL in SB1T) to the DNA mixture and incubating 37°C for 15 minutes with mixing. The DNA solution was removed and the beads were washed 5 times at 37°C with SB1T containing 0.1 mg/mL herring sperm DNA (Sigma-Aldrich). Unless indicated, all washes were performed by re-suspending the beads in 100 µL wash solution, mixing for 30 seconds, separating the beads with a magnet, and removing the wash solution. Bound aptamers were eluted from the beads by adding 100 µL SB1T + 2 M Guanidine-HCl and incubating 37°C, 5 minutes with mixing. The aptamer eluate was transferred to a new tube after magnetic separation. After the first two selection rounds, the final two of five target beads washes were done for 5 minutes instead of 30 seconds.

[0139] Primer beads were prepared by immobilizing biotinylated reverse PCR primer to streptavidin-coated paramagnetic beads (MyOne-SA, Invitrogen). 5 mL MyOne-SA beads (10 mg/mL) were washed once with NaCIT (5 M NaCl, 0.01% TWEEN-20), and resuspended in 5 mL biotinylated reverse PCR primer (5 µM in NaCIT). The sample was incubated 25C, 15 minutes, washed twice with 5 mL NaCIT, resuspended in 12.5 mL NaCIT (4 mg/mL), and stored at 4°C.

[0140] 25 µL primer beads (4 mg/mL in NaCIT) were added to the 100 µL aptamer solution in Guanidine Buffer and incubated 50°C, 15 minutes with mixing. The aptamer solution was removed, and the beads were washed 5 times with SB1T. Aptamer was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 1 µL 0.5M Tris-HCl, pH 7.5.

D. Aptamer Amplification and Purification

[0141] Selected aptamer DNA was amplified and quantified by QPCR. 48 µL DNA was added to 12 µL QPCR Mix (5X KOD DNA Polymerase Buffer, 25 mM $MgCl_2$, 10 µM forward PCR primer, 10 µM biotinylated reverse PCR primer, 5X SYBR Green I, 0.125 U/ µL KOD DNA

Polymerase, and 1 mM each dATP, dCTP, dGTP, and dTTP) and thermal cycled in an ABI5700 QPCR instrument with the following protocol: 1 cycle of 99.9°C, 15 seconds, 55°C, 10 seconds, 70°C, 30 minutes; 30 cycles of 99.9°C, 15 seconds, 72°C, 1 minute. Quantification was done with the instrument software and the number of copies of DNA selected with target beads and $(His)_6$ beads were compared to determine signal/background ratios.

[0142] Following amplification, the PCR product was captured on MyOne-SA beads via the biotinylated antisense strand. 1.25 mL MyOne-SA beads (10 mg/mL) were washed twice with 0.5 mL 20 mM NaOH, once with 0.5 mL SB1T, resuspended in 2.5 mL 3 M NaCl, and stored at 4°C. 25 µL MyOne-SA beads (4 mg/mL in 3 M NaCl) were added to 50 µL double-stranded QPCR product and incubated 25°C, 5 minutes with mixing. The beads were washed once with SB1T, and the "sense" strand was eluted from the beads by adding 200 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. The eluted strand was discarded and the beads were washed 3 times with SB1T and once with 16 mM NaCl.

[0143] Aptamer sense strand was prepared with the appropriate nucleotide composition by primer extension from the immobilized antisense strand. The beads were resuspended in 20 µL primer extension reaction mix (1X KOD DNA Polymerase Buffer, 1.5 mM $MgCl_2$, 5 µM forward PCR primer, 0.125 U/ µL KOD DNA Polymerase, 0.5 mM each dATP, MedCTP, dGTP, and either dTTP or dUTP analog) and incubated 68°C, 30 minutes with mixing. The beads were washed 3 times with SB1T, and the aptamer strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 5 µL 0.1 M HEPES, pH 7.5.

E. Selection Stringency and Feedback

[0144] The relative target protein concentration of the selection step was lowered each round in response to the S/B ratio as follows, where signal S and background B are defined in Section C above:

if
$S/B < 10, [P](i+1) = [P]i$

if
$10 \leq S/B < 100, [P](i+1) = [P]i / 3.2$

if
$S/B \geq 100, [P](i+1) = [P]i / 10$

where [P] = protein concentration and *i* = current round number.

[0145] Target protein concentration was lowered by adjusting the mass of target protein beads (and $(His)_6$ beads for background determination) added to the selection step.

[0146] After each selection round, the convergence state of the enriched DNA mixture was determined. 5 µL double-stranded QPCR product was diluted to 200 µL with 4 mM $MgCl_2$ containing 1X SYBR Green I. Samples were overlaid with 75 µL silicon oil and analyzed for convergence using a C_0t analysis which measures the hybridization time for complex mixtures of double stranded oligonucleotides. The sample is thermal cycled with the following protocol: 3 cycles of 98°C, 1 minute, 85°C, 1 minute; 1 cycle of 93°C, 1 minute, 85°C, 15 minutes. During the 15 minutes at 85°C, fluorescent images are measured at 5-second intervals. The fluorescence intensity is plotted as a function of log (time) to evaluate the diversity of the sequences.

F. Measurement of Equilibrium Binding Constant (Kd)

[0147] Equilibrium binding constants of the enriched libraries were measured using TALON bead partitioning. DNA was renatured by heating to 95°C and slowly cooling to 37°C. Complexes were formed by mixing a low concentration of radiolabled DNA (~1×10^{-11} M) with a range of concentrations of target protein (1×10^{-7} M to 1×10^{-12} M final) in SB1 Buffer (described previously), and incubating at 37°C. A portion of each reaction was transferred to a nylon membrane and dried to determine total counts in each reaction. A small amount of 5 mg/mL MyOne TALON beads (Invitrogen) was added to the remainder of each reaction and mixed at 37°C for one minute. A portion was passed through a MultiScreen HV Plate (Millipore) under vacuum to separate protein-bound complexes from unbound DNA and washed with 100 µL SB1 Buffer. The nylon membranes and MultiScreen HV Plates were phosphorimaged and the amount of radioactivity in each sample quantified using a FUJI FLA-3000. The fraction of captured DNA was plotted as a function of protein concentration and a non-linear curve-fitting algorithm was used to extract equilibrium binding constants (K_d values) from the data. Table 1 shows the K_d values determined for each enriched candidate mixture to a set of targets. NT indicates that the enriched library for a particular base composition did not appear to have changed from the original candidate mixture, as determined by C_0t analysis (described previously), and was therefore Not Tested (NT).

[0148] Table 1 shows the equilibrium binding constants (K_d) for enriched pools to fifteen different protein targets and four different DNA libraries: naturally occurring bases (dT), benzyl (BzdU), isobutyl (iBdU) or tryptophan (TrpdU). An aptamer with a K_d of less than 1×10^{-8} is desirable. The use of modified bases in the SELEX process produces a significantly higher percentage of desirable high affinity aptamers. It was observed that only 2 of the 14 aptamers produced with the normal nucleotides have the desired slow dissociation rates. Slow off-rate aptamers produced with the modified nucleotides were identified 9 of 14, 7 of 14, and 14 of 14 for BzdUTP, iBdUTP, and TRPdUTP, respectively.

Table 1. Equilibrium binding constants (Kd) of the enriched libraries selected with different modified nucleotides, reported in units of molarity. NT = not tested.

Target Protein	dTTP	BzdUTP	iBdUTP	TrpdUTP
4-1BB	>1.0E-07	5.6E-09	>1.0E-07	3.9E-09
B7	>1.0E-07	1.1E-08	NT	7.2E-09
B7-2	>1.0E-07	NT	>1.0E-07	5.7E-09
CTLA-4	>1.0E-07	NT	NT	1.4E-09
E-Selectin	>1.0E-07	>1.0E-07	>1.0E-07	1.9E-09
Fractalkine	NT	>1.0E-07	NT	5.1E-11
GA733-1 protein	8.9E-09	2.8E-09	4.7E-09	4.5E-10
Gp130	>1.0E-07	5.9E-09	2.2E-08	1.2E-09
HMG-1	>1.0E-07	NT	2.2E-08	4.9E-09
IR	>1.0E-07	1.9E-09	1.2E-08	2.2E-10
OPG	3.7E-08	4.6E-09	9.5E-09	1.7E-10
PAI-1	>1.0E-07	3.7E-10	9.1E-10	4.3E-10
P-Cadherin	>1.0E-07	3.5E-09	5.2E-09	2.7E-09
sLeptin R	>1.0E-07	2.3E-09	NT	4.6E-10

EXAMPLE 2. Generation of PhotoAptamers using 5'-Fixed PhotoSELEX and Slow off-rate Enrichment Process by Dilution.

A. Preparation of Candidate Mixtures

[0149] Candidate mixtures containing dATP, dCTP, dGTP, and BzdUTP were prepared by polymerase extension of a primer annealed to a biotinylated template (FIG. 4A-B). For each template, four different forward primers were used, each possessing a unique chromophore at the 5' terminus (FIG. 5). For each candidate mixture, 11 nmol forward primer (with 5' chromophore) and 10 nmol template were combined in 250 µL Primer Extension Buffer (120 mM Tris-HCl, pH 7.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 7 mM $MgSO_4$, 0.1 mg/mL BSA, 0.1% Triton X-100), heated to 95°C for 5 minutes, and cooled on ice. 125 µL each primer:template mixture was added to a 1 mL extension reaction containing Primer Extension Buffer, 0.125 U/µL KOD DNA Polymerase, and 0.5 mM each dATP, dCTP, dGTP, and BzdUTP, and incubated 70°C, 30 minutes. Each 1 mL reaction was split into four 250 µL aliquots and chilled on ice. Double-stranded product was captured via the template strand biotins by adding 1 mL streptavidin-coated magnetic beads (MagnaBind-Streptavidin, Pierce, 5 mg/mL in 1M NaCl + 0.05% TWEEN-20) to each 250 µL aliquot and incubating 25°C for 60 minutes with mixing. Beads were washed three times with 0.5 mL SB17T Buffer (40 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% TWEEN-20). The aptamer strand was

eluted from the beads with 1 mL 20 mM NaOH, neutralized with 0.25 mL 80 mM HCl, and buffered with 10 µL 1 M HEPES, pH 7.5. Candidate mixtures were concentrated with a Centricon-30 to approximately 0.2 mL, and quantified by UV absorbance spectroscopy.

B. Preparation of Target Proteins

[0150] Untagged target proteins were biotinylated by covalent coupling of NHS-PEO4-biotin (Pierce) to lysines residues. Proteins (300 pmol in 50 µL) were exchanged into SB17T with a Sephadex G-25 microspin column. NHS-PE04-biotin was added to 1.5 mM and the reaction was incubated at 4°C for 16 hours. Unreacted NHS-PE04-biotin was removed with a Sephadex G-25 microspin column.

C. Aptamer Selection with Slow - off Rate Enrichment Process and Photocrosslinking

[0151] Selections were performed separately with each candidate mixture, comparing binding between samples with target protein (signal S) and samples without target protein (background B). The first three rounds were performed with selection for affinity (no photocrosslinking); the second and third included slow off-rate enrichment process. Rounds four through eight included both slow off-rate enrichment process and photocrosslinking.

[0152] For each sample, a 90 µL DNA mixture was prepared in SB17T with 10-20 pmoles candidate mixture (100 pmoles in the first round) and 100 pmoles reverse primer. Samples were heated to 95°C for 3 minutes and cooled to 37°C at a rate of 0.1C/second. Samples were combined with 10 µL protein competitor mixture (0.1% HSA, 10 µM casein, and 10 µM prothrombin in SB17T), added to 0.5 mg Dynal MyOne Streptavidin beads (pre-washed twice with 20 mM NaOH and once with SB17T), and incubated 37°C for 5 minutes with mixing. Beads were removed by magnetic separation.

[0153] Binding reactions were performed by adding 10 µL target protein (0.5 µM in SB17T) or SB17T to 40 µL DNA mixture and incubating 37°C for 30 minutes.

[0154] When slow off-rate enrichment process was employed, samples were diluted 20X by adding 950 µL SB17T (preheated to 37°C), and incubated at 37°C for 30 minutes prior to capturing complexes.

[0155] Complexes were captured on SA beads via protein biotins by adding 0.25 mg MyOne-SA beads (Invitrogen) and incubating 37°C for 15 minutes with mixing. Free DNA was removed by washing the beads five times with SB17T. Unless indicated, all washes were performed by resuspending the beads in 100 µL wash solution, mixing for 30 seconds at 25°C, separating the beads with a magnet, and removing the wash solution. The aptamer strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. 80

µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 1 µL 0.5 M Tris-HCl, pH 7.5.

[0156] When photo-selection was employed, the 50 µL binding reactions, (or 1 mL binding reactions after optional slow off-rate enrichment process by dilution) were irradiated from above with a high-pressure mercury lamp (Optical Associates, Inc. model 0131-0003-01, 500W, with 310 nm mirror set). Candidate mixtures possessing a BrdU chromophore were irradiated for 37 seconds, those possessing an ANA chromophore were irradiated for 60 seconds, and those possessing an AQ or psoralen chromophore were irradiated for 10 minutes. An additional filter (5 mm plate glass) was used for the ANA, AQ and psoralen chromophores to eliminate unnecessary, but potentially damaging wavelengths below 320 nm. Complexes were captured as above, and non-crosslinked DNA was removed by washing the beads once with 4 M guanidine-HCl + 0.05% TWEEN-20 at 50°C for 10 minutes, once with 20 mM NaOH at 25°C for 2 minutes, twice with SB17T, and once with 16 mM NaCl. Crosslinked DNA was not removed from the bead surface for the amplification steps.

D. Aptamer Amplification and Purification

[0157] Selected aptamer DNA was amplified and quantified by QPCR. 48 µL DNA was added to 12 µL QPCR Mix (5X KOD DNA Polymerase Buffer, 25 mM $MgCl_2$, 10 µM forward PCR primer, 10 µM biotinylated reverse PCR primer, 5X SYBR Green I, 0.125 U/ µL KOD DNA Polymerase, and 1 mM each dATP, dCTP, dGTP, and dTTP) and thermal cycled in an a Bio-Rad MyIQ QPCR instrument with the following protocol: 1 cycle of 99.9°C, 15 sec, 55°C, 10 sec, 68°C, 30 min, 30 cycles of 99.9°C, 15 seconds, 72°C, 1 minute. For photoSELEX rounds, the initial 30 min incubation is performed Quantification was done with the instrument software and the number of copies of DNA selected with and without target protein were compared to determine signal/background ratios.

[0158] When photo-selection was employed, a cDNA copy of the selected DNA was prepared by primer extension on the bead surface. Washed beads were resuspended in 20 µL cDNA extension mix (Primer Extension Buffer containing 5 µM reverse PCR primer, 0.5 mM each dATP, dCTP, dGTP, and dTTP, and 0.125 U/ µL KOD DNA Polymerase) and incubated 68°C for 30 minutes with mixing. The beads were washed 3 times with SB17T, and the aptamer strand was eluted by from the beads by adding 85 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 1 µL 0.5 M Tris-HCl, pH 7.5. The cDNA was amplified and quantified by QPCR as above for the 30 cycles of 99.9°C, 15 seconds, 72°C, 1 minute.

[0159] Following amplification, the PCR product was captured on MyOne-SA beads via the biotinylated antisense strand. 1.25 mL MyOne-SA beads (10 mg/mL) were washed twice with 0.5 mL 20 mM NaOH, once with 0.5 mL SB17T, resuspended in 1.25 mL 3 M NaCl + 0.05% Tween, and stored at 4°C. 25 µL MyOne-SA beads (10 mg/mL in 3 M NaClT) were added to

50 µL double-stranded QPCR product and incubated 25°C, 5 minutes with mixing. The beads were washed once with SB17T, and the "sense" strand was eluted from the beads by adding 200 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. The eluted strand was discarded and the beads were washed 3 times with SB17T and once with 16 mM NaCl.

[0160] Aptamer sense strand was prepared with the appropriate chromophore by primer extension from the immobilized antisense strand. The beads were resuspended in 20 µL primer extension reaction mixture (1X Primer Extension Buffer, 1.5 mM $MgCl_2$, 5 µM forward primer with appropriate 5' chromophore, 0.5 mM each dATP, dCTP, dGTP, and BzdUTP, and 0.125 U/µL KOD DNA Polymerase) and incubated 68°C, 30 minutes with mixing. The beads were washed 3 times with SB17T, and the aptamer strand was eluted from the beads by adding 85 µL 20 mM NaOH, and incubating 37°C, 1 minute with mixing. 80 µL aptamer eluate was transferred to a new tube after magnetic separation, neutralized with 20 µL 80 mM HCl, and buffered with 5 µL 0.1 M HEPES, pH 7.5.

E. Selection Stringency and Feedback

[0161] Target protein was adjusted at each round as described in Example 1. After each round of selection, the convergence state of the enriched pool was determined as described in Example 1.

F. Equilibrium Binding Constants of Enriched Libraries

[0162] The binding affinity was determined as described in Example 1 above, but with MyOne-SA capture beads. The following table, Table 2, summarizes the equilibrium binding constants (Kd) obtained using the photoSELEX protocol with slow off-rate enrichment process.

Table 2. Equilibrium binding constants (Kd) of the enriched libraries selected with different chromophores, reported in units of molarity. Measurements were not made on libraries that failed to converge (indicated with an x).

Target Protein	BrdU	AQ	ANA	Psor
β-catenin	2.7e0-8	3.6e-09	**1.1e-09**	1.6e-09
bFGF	3.1e-08	5.7e-10	7.1e-10	**5.1e-10**
CMP-SAS	×	**6.2e-09**	7.3e-09	4.9e-08
endostatin	1.3e-09	**8.7e-10**	8.8e-10	1.3e-09
IL-6	1.0e-09	5.4e-10	**4.0e-10**	×
myeloperoxidase	6.0e-10	2.8e-10	5.0e-10	**1.5e-10**
SDF-1β	8.1e-10	5.7e-10	**3.8e-10**	×
TIMP-1	**5.2e-09**	7.3e-09	8.9e-09	×
VEGF	7.2e-10	4.2e-09	**5.5e-10**	×

Target Protein	BrdU	AQ	ANA	Psor
vWF	2.6e-08	8.8e-09	**8.1e-09**	×

G. Crosslink Activity Assay

[0163] The crosslink yield of enriched libraries was determined by measuring the percent of DNA crosslinked to protein under conditions of saturating protein and light. Radiolabeled DNA (50 pM) was mixed with reverse primer (16 nM) in SB17T, heated to 95°C for 3 minutes, and cooled to 37°C at 0.1C/second. Target protein was added to the DNA mix to a final concentration of 10 nM and incubated at 37°C for 30 minutes. Control samples with no protein were simultaneously prepared. Samples were crosslinked with the chromophore-specific conditions described above, but with a saturating dose (6 minutes for BrdU, 10 minutes for ANA, and 30 minutes for AQ and Psor). Samples were analyzed by denaturing PAGE, FIG. 6, and quantified and the results are tabulated in Table 3.

Table 3. Crosslink yields of the enriched libraries selected with different chromophores, reported in units of percent of total DNA crosslinked to protein. Measurements were not made on libraries that failed to converge (indicated with an x).

Target Protein	BrdU	AQ	ANA	Psor
β-catenin	**15**	9	8	1
bFGF	4	9	**15**	4
CMP-SAS	×	3	**5**	2
Endostatin	2	1	**18**	3
IL-6	0	5	**9**	
Myeloperoxidase	4	**13**	9	8
SDF-1β	8	10	**17**	×
TIMP-1	1	**4**	2	×
VEGF	1	1	**4**	×
vWF	2	2	**7**	×

EXAMPLE 3. Generation of Slow - off-Rate Aptamers Using a Slow - off Rate Enrichment Process with a Competitor

A. Preparation of Candidate Mixtures

[0164] Candidate mixtures containing dATP, dCTP, dGTP, and BzdUTP were prepared by

polymerase extension of a primer annealed to a biotinylated template for 94 protein targets. 55 nmol forward primer (with 5' ANA chromophore) and 55 nmol template were combined in 0.5 mL Primer Extension Buffer (120 mM Tris-HCl, pH 7.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 7 mM $MgSO_4$, 0.1 mg/mL BSA, 0.1% Triton X-100), heated to 95°C for 5 minutes, 70°C for 5 minutes, 48°C for 5 minutes, and cooled on ice. The primer:template mixture was added to a 5.5 mL extension reaction containing Primer Extension Buffer, 0.125 U/µL KOD DNA Polymerase, and 0.5 mM each dATP, dCTP, dGTP, and BzdUTP, and incubated 70°C, 60 minutes. After completion of the extension reaction, the solution was chilled on ice. Double-stranded product was captured via the template strand biotins by adding 25 mL streptavidin-coated magnetic beads (MagnaBind-Streptavidin, Pierce, 5 mg/mL in 1 M NaCl + 0.05% TWEEN-20) to the primer extension product and incubating 25°C for 15 minutes with rotating. Beads were washed three times with 40 mL SB17T Buffer (40 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% TWEEN-20). The aptamer strand was eluted from the beads with 35.2 mL 20 mM NaOH for 5 minutes with shaking. The eluted strand was neutralized with 8.8 mL 80 mM HCl, and buffered with 400 µL 1 M HEPES, pH 7.3. Candidate mixtures were concentrated with a Centricon-30 to approximately 0.7 mL, and quantified by UV absorbance spectroscopy.

B. Preparation of Target Proteins

[0165] Untagged target proteins were biotinylated as described in Example 2.

C. Aptamer Selection with Slow off-rate Enrichment Process and Photocrosslinking

[0166] Selections were performed separately as described in Example 2, with the addition of 10 mM dextran sulfate as a competitor for aptamer rebinding during the slow off-rate enrichment process in rounds six through nine.

[0167] The slow - off rate enrichment process was employed in three different ways. In rounds two and three, samples were diluted 20X by adding 950 µL SB17T (preheated to 37°C), and incubated at 37°C for 30 minutes prior to capturing complexes. In rounds four and five, samples were diluted 20X by adding 950 µL SB17T (preheated to 37°C), and incubated at 37°C for 30 minutes prior to crosslinking. In rounds six and seven, samples were diluted 20X by adding 950 µL SB17T (preheated to 37°C). 50 µL of each diluted sample was diluted again by transferring to 950 µL SB17T + 10 mM 5000K dextran sulfate (preheated to 37°C) to give an overall 400X dilution, and incubated at 37°C for 60 minutes prior to crosslinking. In rounds eight and nine, samples were diluted 20X by adding 950 µL SB17T (preheated to 37°C), and 50 µL of each sample was diluted again by transferring to 950 µL SB17T (preheated to 37°C) to give 400X dilution. Finally 50 µL of each 400X diluted sample was diluted again by transferring to 950 µL SB17T + 10 mM 5000K dextran sulfate (preheated to 37°C) to give an overall 8000X dilution, and incubated at 37°C for 60 minutes prior to crosslinking. Complexes

were captured and washed as described in Example 2. When photo-crosslinking was employed, the 1 mL binding reactions after the slow off-rate enrichment process were irradiated from above with an array of 470 nm LEDs for 60 seconds prior to complex capture as in Example 2..

D. Aptamer Amplification and Purification

[0168] Amplification and purification were performed as in Example 2.

E. Selection Stringency and Feedback

[0169] Target protein was adjusted at each round as described in Example 1, except in rounds six and eight. In order to maximize signal after these large dilutions, the target protein was increased to 100 nM for rounds six and eight. After each round of selection, the convergence state of the enriched pool was determined as described in Example 1.

F. Dissociation Rate Constant Determination Protocol.

[0170] The rate constant for aptamer:protein complex dissociation (koff) was determined for each aptamer by measuring the fraction of pre-formed aptamer:protein complexes that remain bound after dilution as a function of time. Radiolabeled aptamer (50 pM) was equilibrated in SB17T-0.002 (SB17T with TWEEN-20 reduced to 0.002%) at 37°C with protein at a concentration 10X greater than the measured K_d value. Samples were diluted 100X with SB17T-0.002 at 37°C and aliquots were removed at various time points and partitioned to separate free aptamer from protein:aptamer complexes. Partitioning was accomplished by adding ZORBAX resin (Agilent) to the sample, capturing complexes on the resin, passing the sample through a DuraPore membrane under vacuum, and washing the resin with SB17T-0.002. For proteins not efficiently captured with ZORBAX resin, the assay was performed with biotinylated protein in SB17T and partitioning was accomplished by capturing complexes with Dyanal MyOne-SA beads. The amount of complex remaining at each time point was determined by quantifying the radiolabeled aptamer on the resin with a FUJI FLA-3000 phosphorimager. The fraction of complex was plotted as a function of time and the dissociation rate constant (koff) and dissociation half-life value ($t_{1/2}$) was determined by fitting the data to an analytic expression for bimolecular dissociation kinetics using non-linear regression.

G. Kinetic Properties of some Aptamers

[0171] The following table, Table 4, summarizes the dissociation half-life values ($t_{1/2}$) obtained

for aptamers selected against 10 targets using this protocol.

Table 4. Dissociation half-life values ($t_{1/2}$) of aptamers using the competitor slow off-rate enrichment step protocol.

Tareget Protein	t1/2 (min)
bFGF R	66
C3	164
catalase	58
FGF-17	91
group IB phospholipase A2	40
HB-EGF	49
HCC-4	143
IL-6 sRα	114
SAP	186
uPA	85

[0172] Example 4: The Slow off-rate Enrichment Process Increases the Dissociation Half-Life of Selected Aptamers.

[0173] Dissociation half-life values ($t_{1/2}$) were measured and plotted for 65 aptamers that were selected by either the affinity SELEX method described in Example 1 or photo SELEX methods described in U.S. Patent No. 6,458,539, entitled "Photoselection of Nucleic Acid Ligands" without a slow off-rate enrichment process (Fig. 3A). $t_{1/2}$ values were also measured and plotted for 72 aptamers that were selected by the slow off-rate enrichment process described in Example 2 with a slow off-rate enrichment process by dilution or dilution with competitor (Fig. 3B). The average $t_{1/2}$ value for aptamers using the modified nucleotides 5-benzyl-dUTP (BzdUTP), 5-isobutyl-dUTP (iBdUTP), or 5-tryptamino-dUTP selected in the absence of a slow off-rate enrichment process was 20 minutes with some aptamers having a $t_{1/2}$ value of up to one hour. This is substantially longer than what has been previously described with natural bases or other modified nucleotides. The average for aptamers selected with a slow off-rate enrichment process was over 85 minutes, with some aptamers having a $t_{1/2}$ value in excess of four hours.

Example 5: Generation of Aptamers From a NpdUTP Random Library

A. Preparation of Candidate Mixtures

[0174] Candidate mixtures containing dATP, dCTP, dGTP, and NpdUTP were prepared as

described in Example 3 but without the 5'-ANA photoreactive group.

B. Immobilization of Target Proteins

[0175] Target proteins contained a (His)6 tag and were captured with Co+2-NTA beads as described in Example 1.

C. Aptamer Selection with Slow off-rate Enrichment Process

[0176] Aptamer selection was performed as described in Example 3, but without photocrosslinking.

D. Aptamer Amplification and Purification

[0177] Amplification and purification were performed as described in Example 3.

E. Selection Stringency and Feedback

[0178] Selection stringency and feedback were performed as described in Example 3.

F. Aptamer Properties

[0179] The equilibrium binding constant (Kd) of four aptamers from this selection are listed in Table 5.

Table 5.

Equilibrium binding constants (Kd) of NpdUTP aptamers.	
Target Protein	**Kd (M)**
bFGF	1.1e-9
Endostatin	2.0e-10
TIMP-3	1.5e-10
VEGF	7.2e-10

EXAMPLE 6. Generation of Slow - off-Rate Aptamers for a Peptide Target Using a Slow off-rate Enrichment Process with a Competitor

A. Preparation of Candidate Mixtures

[0180] Candidate mixtures containing dATP, dCTP, dGTP, and BzdUTP were prepared by polymerase extension of a primer with a 5' ANA chromophore and purified as described in Example 3.

B. Aptamer Selection with Slow off-rate Enrichment Process and Photocrosslinking

[0181] Aptamer selection was performed as described in Example 3. with the 29 amino acid biotinylated target peptide SMAP29 (Sheep Myeloid Antibacterial Peptide MAP-29, Anaspec).

C. Aptamer Amplification and Purification

[0182] Amplification and purification were performed as described in Example 3.

D. Selection Stringency and Feedback

[0183] Selection stringency and feedback were performed as described in Example 3.

E. Aptamer Properties

[0184] The equilibrium binding constant (Kd) of an aptamer from this selection was 1.2e-8M (measured according to the protocol described in Example 1). The dissociation half-life (t1/2) of this aptamer was 69 minutes (measured according to the protocol described in Example 3). Results are shown in FIG. 12A and FIG. 12B.

EXAMPLE 7: AFFINITY BEAD HYBRID CAPTURE

[0185] Step 1: EXAMPLE 7: Protein Measurements in Test Samples Are Enabled By Aptamers with Slow Off-Rates.

[0186] Step 1: Preparation of Aptamer/Primer Mixtures and Test Samples Aptamers with a biotin Cy3 detection label (4 nM each) are mixed with a 3X excess of capture probe (oligonucleotide complementary to the 3' fixed region of the aptamer containing a biotin tag and photocleavable element) in 1X SB17T. and heated at 95°C for 4 minutes then 37°C for 13

minutes, and diluted 1:4 in 1x SB17T . 55 uL of aptamer/primer mix is added to a microtiter plate (Hybaid # AB-0407) and sealed with foil.. Test samples are prepared in a microtiter plate by mixing known concentrations of protein analytes in SB17T and diluting serially with SB17T.

Step 2: Sample Equilibration

[0187] 55 uL of aptamer/primer mix is added to 55 uL of test sample and incubated at 37°C for 15 minutes in a foil-sealed microtiter plate. The final concentration of each aptamer in the equilibration mixture is 0.5 nM. After equilibration, all subsequent steps of this method are performed at room temperature unless otherwise noted.

Step 3: Aptamer Capture and Free Protein Removal

[0188] A DuraPore filtration plate (Millipore HV cat# MAHVN4550) is washed once with 100 uL 1X SB17T by vacuum filtration, add 133.3 uL 7.5% Streptavidin-agarose resin (Pierce) is added to each well and washed twice with 200 uL 1X SB17T. 100 uL of equilibrated samples is transferred to the Durapore plate containing the Streptavidin-agarose resin and incubated on a thermomixer (Eppendorf) at 800 rpm for 5 minutes. The resin is washed once with 200 uL 1X SB17T + 100 uM biotin and once with 200 uL 1X SB17T .

Step 4: Protein Tagging with Biotin

[0189] 100 uL of 1.2 mM NHS-PEO4-biotin in SB17T, prepared immediately before use, is added to the resin with captured aptamer and aptamer:protein complexes and incubated on a thermomixer at 800 rpm for 20 minutes. The resin is washed five times with 200 uL 1X SB17T by vacuum filtration..

Step 5: Slow - off rate Enrichment Process & Photocleavage

[0190] The drip director is removed from underside of the DuraPore plate and the plate is placed over a 1 mL microtiter collection plate. The resin is washed once with 200 uL 1X SB17T by centrifugation at 1000 × g for 30 sec. 80 uL of 1X SB17T + 10 mM DxSO4 is added to the resin and irradiated with a BlackRay Mercury Lamp on a thermomixer at 800 rpm for 10 minutes. The DuraPore plate is transferred to a new 1mL deepwell plate and centrifuged at 1000 × g for 30 seconds to collect the photocleaved aptamer and protein:aptamer complexes.

Step 6: Protein Capture and Free Aptamer Removal

[0191] 50 uL of MyOne-streptavidin Cl paramagnetic beads (Invitrogen) (10 mg/mL in 1X SB17T) is added to a microtiter plate. The beads are separate with a magnet for 60 seconds and the supernatant is removed. 225 uL of photocleavage mixture is added to the beads and mixed for 5 minutes. The beads are washed four times with 200 uL 1X SB17T by separating the magnetic beads and replacing the wash buffer. The final wash buffer is removed.

Step 7: Aptamer Elution

[0192] 100 uL Sodium Phosphate Elution Buffer (10 mM Na_2HPO_4, pH 11)is added to the beads and mixed for 5 minutes. 90 uL of eluate is transferred to a microtiter plate and neutralized with 10 uL Sodium Phosphate Neutralization Buffer (10 mM NaH_2PO_4, pH 5).

Step 8: Aptamer Hybridization to Microarrays

[0193] DNA arrays are prepared with oligunucleotide capture probes comprised of the complementary sequence of the variable region of each aptamer immobilized on a custom microscope slide support. Multiple arrays (subarrays) exist on each slide, and subarrays are physically separated by affixing a gasket (Grace) for sample application. Arrays are pretreated with 100 uL Blocking Buffer and incubated for 15 minutes at 65°C on a thermomixer. 30 uL of high salt Hybridization Buffer is added to 90 uL of neutralized aptamer eluate in a microtiter plate, incubated at 95°C for 5 minutes in a thermalcylcer, and cooled to 65°C at 0.1°C/second. Blocking Buffer is removed from the arrays and 110 uL of aptamer sample is added to the arrays and incubate in a humid chamber at 65°C for 20 hours.

Step 9: Array Washing

[0194] Aptamer sample is removed from the arrays and the arrays are washed once with 200 uL of sodium phosphate Tween-20 wash buffer at 65°C, with the gasket in placce, and three times with 25 mL sodium phosphate, Tween-20 wash buffer at 65°C in a pap jar with the gasket removed. Arrays are dried with a nitrogen gun.

Step 10: Quantitate Signal On Arrays

[0195] Array slides are scanned on a TECAN LS300 Reloaded.in an appropriate channel for Cy3 detection and Cy3 signal on each array feature is quantified.

Results:

[0196] Apatmers specific to three different targets (bFGF, VEGF, and Myeloperoxidase) were produced using traditional SELEX methods and materials. A second set of aptamers specific to the same set of targets were made using 5- position modified nucleotides and selected for very slow off-rates for their respective targets. Aptamers made in the traditional process had measured off rates on the order of less than 5 minutes. Aptamers made with the modified nucleotides and using slow off-rate enrichment process during selection had off rates of greater than 20 minutes. Two sets of aptamers were made for each target by the two different methods for a total of 4 different aptamer populations for each target. The ability of these aptamer populations to measure analyte concentrations in test test samples was evaluated as described above over a range of target concentrations. Relative signal from the DNA chip detection was plotted against the input target concentration. See FIG. 11A to 11C. The response curve of the traditional aptamers is very flat and the sensitivity of the detection is fairly low. The sensitivity of detection of the respective targets with the slow off-rate aptamers is excellent. The data supports the need to use the slow - off aptamers for maximum analytic performance.

EXAMPLE 8. Generation of High Affinity BzdU Aptamers to Human Thrombin

A. Preparation of Candidate Mixture

[0197] A candidate mixture containing dATP, dCTP, dGTP, and BzdUTP was prepared by polymerase extension of a primer with a 5' ANA chromophore and purified as described in Example 3.

B. Preparation of Target Protein

[0198] Human thrombin was tagged with biotin as describe in Example 2.

C. Aptamer Selection with Slow Off Rate Enrichment and Photocrosslinking

[0199] Aptamer selection was performed as described in Example 3 with biotinylated human thrombin as the target.

D. Aptamer Amplification and Purification

[0200] Amplification and purification were performed as described in Example 3.

E. Selection Stringency and Feedback

[0201] Selection stringency and feedback were performed as described in Example 3.

F. Aptamer Properties

[0202] The equilibrium binding constant (Kd) of aptamer 2336-17 from this selection was 4.4e-11 M (measured according to the protocol described in Example 1) as demonstrated in FIG. 15.

[0203] Single-stranded DNA aptamers to human thrombin were selected from a library comprised of natural dA, dC, dG, and dT nucleotides (Bock, et al., 1992). The binding affinities of the aptamers had Kd values ranging from 2.5e-8 M to 2.0e-7 M. Using a similar protocol with a library comprised of natural dA, dC, dG, and modified 5-(1-pentynyl)-dUTP, aptamers were selected with Kd values ranging from 4e-7 M to 1e-6 M (Latham, et al., 1994). Example 7 describes the discovery of very high affinity aptamers to human thrombin selected from a library comprised of dA, dC, dG, and modified BzdU. The highest affinity aptamer from this library had a Kd value of 4.4e-11 M

[0204] Examples in cited publications and limitations related therewith are intended to be illustrative and not exclusive. Other limitations of the cited publications will become apparent to those of skill in the art upon a reading of the specification and a study of the drawings.

[0205] The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively.

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Ikke-kovalent kompleks af en aptamer og dens mål, hvor en, flere eller alle pyrimidiner i nukleinsyresekvensen af aptameren er modificeret i 5-positionen, hvor modifikationen omfatter substitution med benzyl, isobutyl, indol eller naphthyl, og hvor dissociationshastigheden ($t_{1/2}$) af aptameren fra målet er større end eller lig med 30 minutter, og hvor aptameren har en dissociationskonstant (K_d) for sit mål, der er på mindre end 1µM.

2. Ikke-kovalent kompleks af en aptamer og dens mål ifølge krav 1, hvor alle C-, alle T- eller alle U-rester i nukleinsyresekvensen af aptameren er kemisk modificeret i 5-positionen.

3. Ikke-kovalent kompleks af en aptamer og dens mål ifølge krav 1, hvori C-5-modifikationen af nukleotiderne anvender en amidbinding.

4. Ikke-kovalent kompleks af en aptamer og dens mål ifølge krav 1, hvor aptameren omfatter de modificerede nukleotider 5-benzyl-dUTP, 5-isobutyl-dUTP eller 5-tryptamino-dUTP.

5. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor dissociationshastigheden ($t_{1/2}$) af aptameren fra målet er valgt fra en af:

(i) mellem 30 minutter og 240 minutter;
(ii) 30 minutter til 60 minutter
(iii) 60 minutter til 90 minutter
(iv) 90 minutter til 120 minutter
(v) 120 minutter til 150 minutter
(vi) 150 minutter til 180 minutter
(vii) 180 minutter til 210 minutter
(viii) 210 minutter til 240 minutter.

6. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren har en dissociationskonstant (K_d) for dens mål, der er valgt blandt: mindre end 100 nM, mindre end 10 nM, mindre end 1 nM, mindre end 100 pM, mindre end 10 pM, mindre end 1 pM.

7. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren omfatter mindst én base-modificeret pyrimidin, der er uafhængigt valgt fra gruppen bestående af de base-modificerede pyrimidiner, der er vist i fig. 14.

8. Ikke-kovalent kompleks af en aptamer og dens mål ifølge krav 7, hvor den særlige 5-position pyrimidin modifikation omfatter en linker (X) vist i figur 14.

9. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor den kemiske modifikation i 5-stilling er valgt blandt 5-(N-benzylcarboxyamid)-2'-deoxyuridin, 5-(N-isobutylcarboxyamid)-2'-deoxyuridin, 5-(N-[2-(IH-indol-3yl)ethyl]carboxyamid)-2'-deoxyuridin og 5-(N- naphthylcarboxyamid)-2'-deoxyuridin, eller er uafhængigt valgt fra gruppen af kemiske modifikationer vist i fig. 14, forudsat at R er valgt blandt benzyl, isobutyl, indol eller naphthyl.

10. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren omfatter en sukkermodifikation, der er valgt fra gruppen bestående af 2'-O-methyl (2'-OMe), 2'-O-allyl, 2'-fluor (2'-F), 2'-amino (2'-NH2) og 2'-azido.

11. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren omfatter en epimerisk sukkerenhed, der er valgt fra gruppen bestående af arabinose, xyloser og lyxoser.

12. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren omfatter mindst én yderligere modifikation omfattende en rygradsmodifikation af phosphatenheden, der er valgt fra gruppen bestående af P(O)S ("thioat"), P(S)S ("dithioat"), P(O)(NR2) ("amidat"), P(O)R,

P(O)OR', CO og CH2, hvor R og R' er uafhængigt H, aryl, alkenyl, cycloalkyl, cycloalkenyl, aralkyl og C1-20-alkyl, eventuelt indeholdende en ether-(-O-)-binding.

13. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor aptameren har mindst én yderligere modificeret base valgt fra gruppen bestående af 5-bromo-1-uracilyl, 5-iodo-1-uracilyl, 5-bromovinyl-1-uracilyl, 5-iodovinyl-1-uracilyl, 5-azido-1-uracilyl, 4-thio-1-uracilyl, 4-thio-1-cytosinyl, 5-bromo-1-cytosinyl, 5-iodo-1-cytosinyl, 5-bromovinyl-1-cytosinyl, 5-iodovinyl-1-cytosinyl, 5-azido-1-cytosinyl, 8-azido-9-adeninyl, 8-bromo-9-adeninyl, 8-iodo-9-adeninyl, 8-azido-9-guaninyl, 8-bromo-9-guaninyl, 8-iodo-9-guaninyl, 8-azidohypoxanthinyl, 8-bromohypoxanthinyl, 8-iodohypoxanthinyl, 8-azido-9-xanthinyl, 8-bromo-9-xanthinyl, 8-iodo-9-xanthinyl, 5-[(4-azidophenacyl)thio]-1-cytosinyl, 5-[(4-azidophenacyl)thio]-1-uracilyl, 5-N-(benzylcarboxamido)-1-uracilyl, 5-(N-isobutylcarboxamido)]-1-uracilyl, 5-(N-tryptaminocarboxyamido)-1-uracilyl, 5-(N-[2-(1H-indole-3yl)ethyl]carboxamido)-1-uracilyl, 5-(N-[1-(3-trimethylammonium)propyl]carboxamido)-1-uracilylchlorid, 5-(N-naphthylmethylcarboxamido)-1-uracilyl, 5-(N-[1-(2,3-dihydroxypropyl)]carboxamido)-1-uracilyl, 7-deaza-7-iodo-9-adeninyl, 7-deaza-7-iodo-9-guaninyl, 7-deaza-7-bromo-9-adeninyl, 7-deaza-7-bromo-9-guaninyl, 1-isocytidinyl og 9-isoguaninyl.

14. Ikke-kovalent kompleks af en aptamer og dens mål ifølge et hvilket som helst af de foregående krav, hvor målet er et protein eller peptid.

15. Aptamer, hvor en, flere eller alle pyrimidiner i nukleinsyresekvensen af aptameren er modificeret i 5-positionen, hvor modifikationen omfatter substitution med benzyl, isobutyl, indol eller naphthyl, og hvor dissociationshastigheden ($t_{1/2}$) af aptameren fra målet er større end eller lig med 30 minutter, og hvor aptameren har en dissociationskonstant (K_d) for sit mål på mindre end 1 uM.

DRAWINGS

FIG. 1A

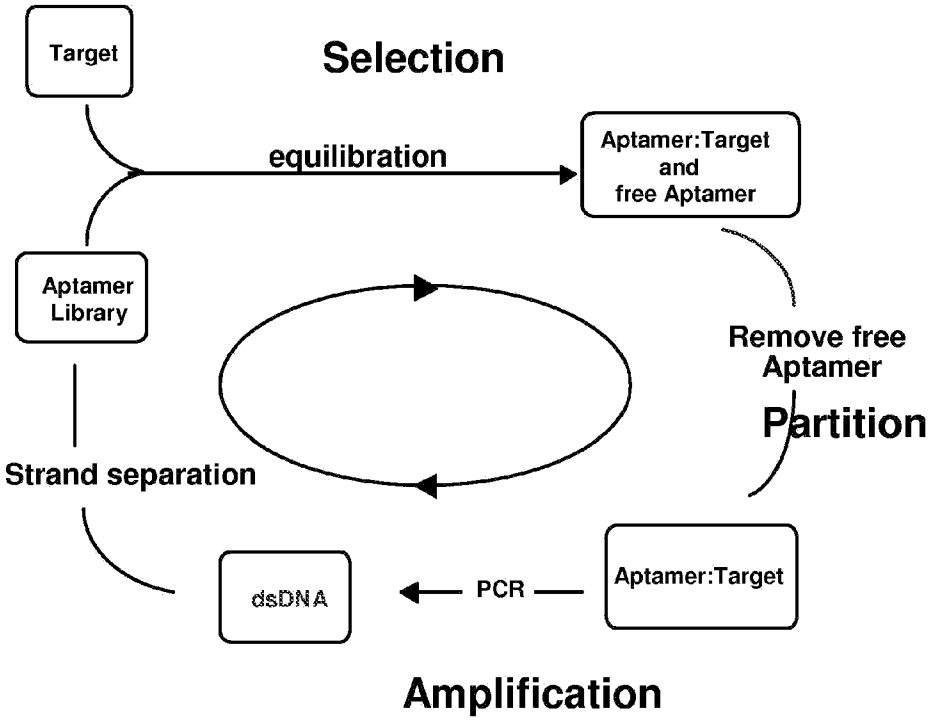
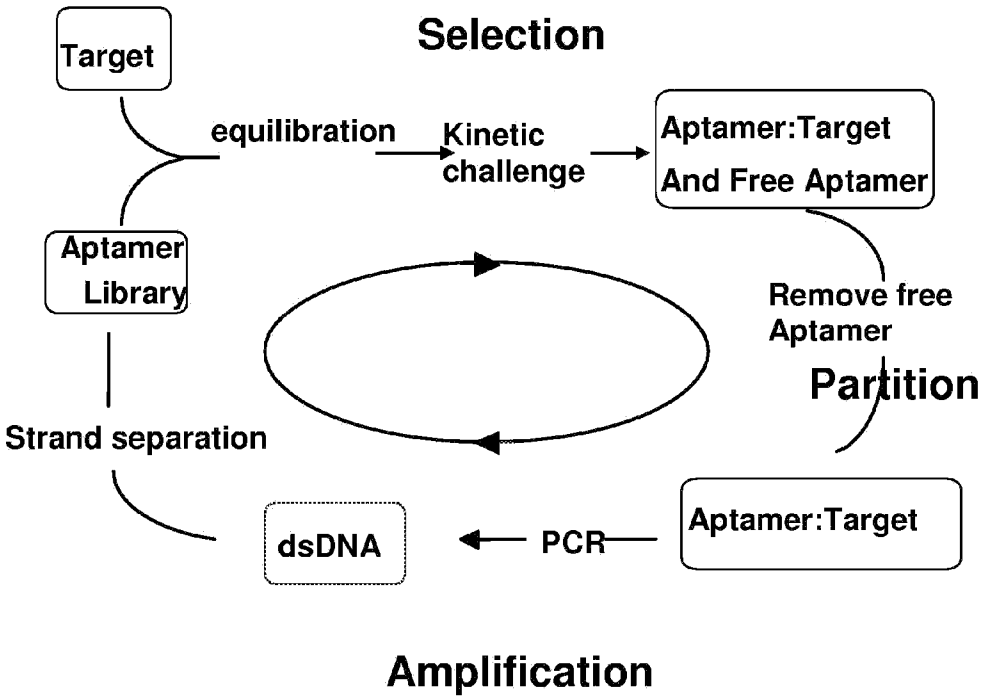


FIG. 1B


Selection
Target
equilibration
Kinetic challenge
Aptamer:Target And Free Aptamer
Aptamer Library
Remove free Aptamer
Partition
Strand separation
dsDNA
PCR
Aptamer:Target
Amplification

Template 1

5'-ABABGTCTTCTTGTCGTTTCGC-$(N)_{40}$-GGTGGAGTGTGGTGAGG–3'

(SEQ ID NO:1)

Forward PCR Primer 1

5'-ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:2)

Reverse PCR Primer 1

5'-ABABTTTTTTTTGTCTTCTTGTCGTTTCGC-3' (SEQ ID NO:3)

Template 2

5'-ABABCCGTCCTCCTCTCCGTC-$(N)_{40}$-GGGACACTGGGTGCAGG-3'

(SEQ ID NO:4)

Forward PCR Primer 2

5'-ATATATATCCTGCACCCAGTGTCCC-3' (SEQ ID NO:5)

Reverse PCR Primer 2

5'-ABABTTTTTTTTCCGTCCTCCTCTCCGTC-3' (SEQ ID NO:6)

$(His)_6$-complement Oligonucleotide

5'-GTCTTCTTGTCGTTTCGC-3' (SEQ ID NO:7)

FIG. 2

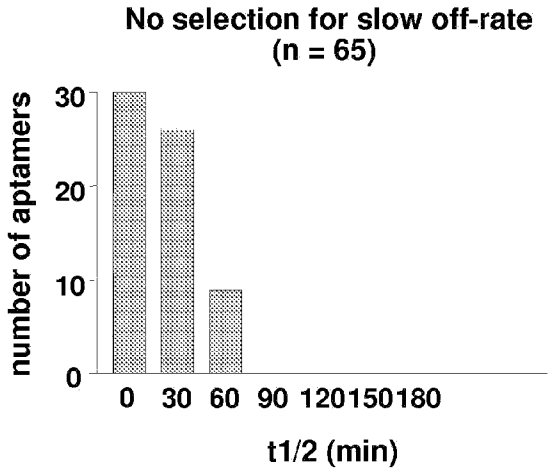

No selection for slow off-rate
(n = 65)
number of aptamers
30
20
10
0
0 30 60 90 120150180
t1/2 (min)

FIG. 3A

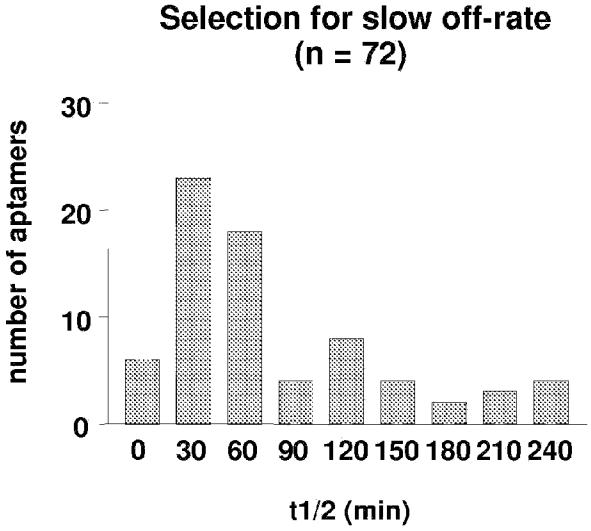

Selection for slow off-rate
(n = 72)
number of aptamers
30
20
10
0
0 30 60 90 120 150 180 210 240
t1/2 (min)

FIG. 3B

Template 1

5'-ABABCCCGCTCGTCGTCTG-$(N)_{40}$-CAGGCAGACGGTCACTC-3'

(SEQ ID NO:8)

Forward BrdU Primer 1

5'-BrdU **- ATATATATGAGTGACCGTCTGCCTG-3' (SEQ ID NO:9)**

Forward ANA Primer 1

5'- ANA **- ATATATATGAGTGACCGTCTGCCTG-3' (SEQ ID NO:10)**

Forward AQ Primer 1

5'- AQ **- ATATATATGAGTGACCGTCTGCCTG-3' (SEQ ID NO:11**

Forward Psor Primer 1

5'- Psor **- ATATATATGAGTGACCGTCTGCCTG-3' (SEQ ID NO:12)**

Forward PCR Primer 1

5'-ATATATATGAGTGACCGTCTGCCTG-3' (SEQ ID NO:13)

Reverse Primer 1

5'-TTTTTTTTCCCGCTCGTCGTCTG-3' (SEQ ID NO:14)

Reverse PCR Primer 1

5'-ABABTTTTTTTTCCCGCTCGTCGTCTG-3' (SEQ ID NO:15)

FIG. 4A

Template 2

5'-ABABGTGTCTGTCTGTGTCCTC-$(N)_{40}$-GGTGGAGTGTGGTGAGG-3'

(SEQ ID NO:16)

Forward BrdU Primer 2

5'- BrdU - ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:17)

Forward ANA Primer 2

5'- ANA - ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:18)

Forward AQ Primer 2

5'- AQ - ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:19)

Forward Psor Primer 2

5'- Psor - ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:20)

Forward PCR Primer 2

5'-ATATATATCCTCACCACACTCCACC-3' (SEQ ID NO:21)

Reverse Primer 2

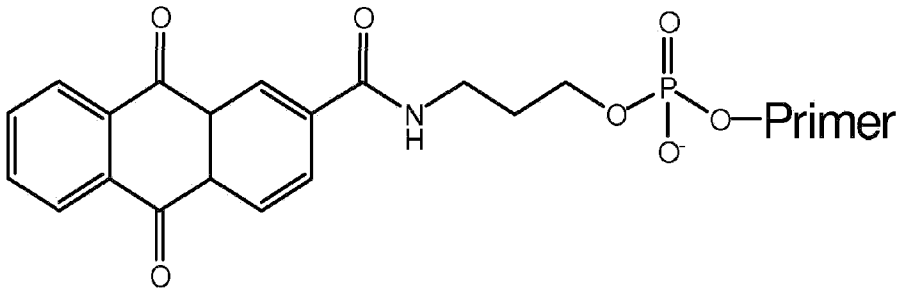
5'-TTTTTTTTGTGTCTGTCTGTGTCCTC-3' (SEQ ID NO:22)

Reverse PCR Primer 2

5'- ABABTTTTTTTTGTGTCTGTCTGTGTCCTC-3' (SEQ ID NO:23)

FIG. 4B

Anthraquinone (AQ)



Psoralen (Psor)

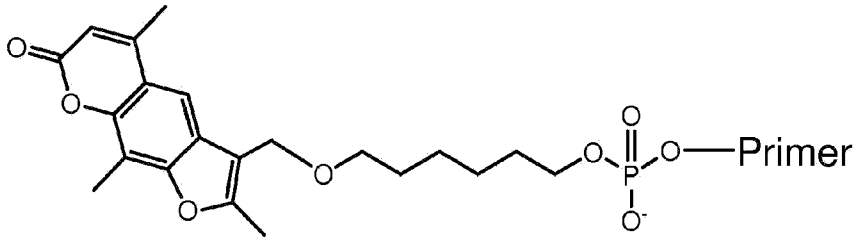
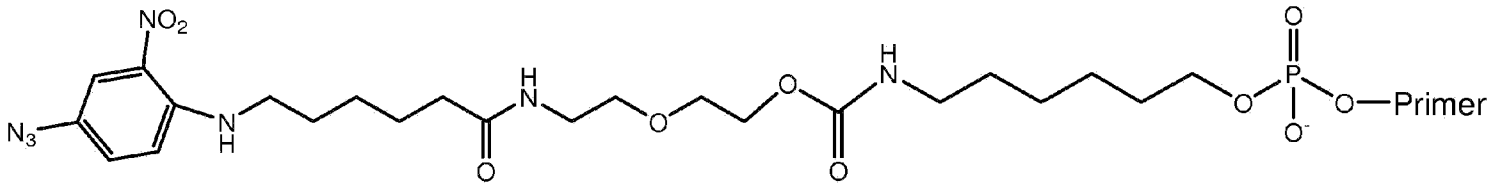


FIG. 5

FIG. 5 Cont'd

4-Azido-2-nitro-aniline (ANA)



DK/EP 3284832 T5

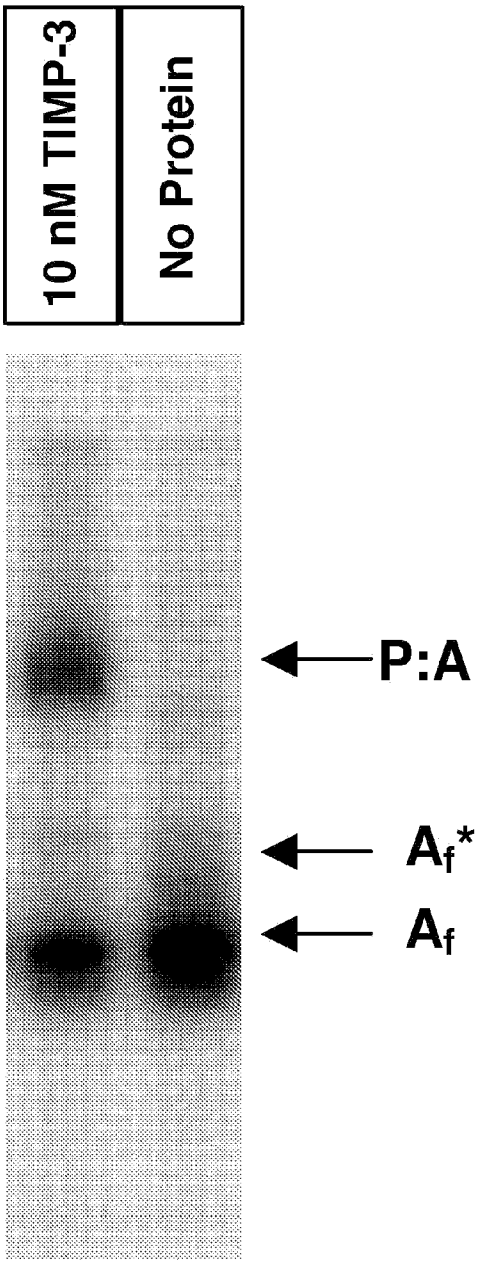


FIG. 6

FIG. 7

4-1BB	C4	Cytochrome P450 3A4	GAS1
4-1BB ligand	C4b	DAN	GASP-2
6Ckine	C5	DARPP-32	G-CSF-R
a1-Antichymotrypsin	C5a	DC-SIGN	GDF-9
a2-Antiplasmin	C5b, 6 Complex	DC-SIGNR	GDF-11
ACE2	C6	D-dimer	GDNF
Activated Protein C	C7	DEAD-box protein 19B	GFAP
Activin A	C8	Desmoglein-1	GFRa-1
Activin RIA	C9	DKK1	GFRa-2
Activin RIB	Cadherin E	DLL4	GFRa-3
ADAMTS-4	Cadherin-5	Dopa decarboxylase	GIB
ADAMTS-5	Calcineurin B a	DRG-1	GIIE
Aggrecan	Calpain I	DRR1	GITR
AIF1	Calpastatin	Dtk	Glucocorticoid receptor
ALCAM	Carbonic Anhydrase IV	EDA (A2)	Glutamate carboxypeptidase
ALK-1	Cardiotrophin-1	EDAR	Glutathione S-transferase Pi
Alkaline phosphatase, bone	Caspase-3 (pro)	EG-VEGF	Glypican 3
AMPM2	Catalase	eIF-5	gp130, soluble
Amyloid precursor protein	Cathepsin A	Elastase	GPC2
Angiogenin	Cathepsin B	EMAP-2	GPVI
Angiopoietin-1	Cathepsin D	ENA-78	Granulysin
Angiopoietin-2	Cathepsin G	Endostatin	Granzyme B
Angiopoietin-4	Cathepsin S	Eotaxin	Gro-a
ANGL3	Cathepsin V	Eotaxin-2	Gro-g
ANGL4	CCL28	Eotaxin-3	Growth hormone receptor
Apo A-I	CD5L	Ephrin-A4	GSK-3 beta
Apo B	CD22	Ephrin A5	GV
Apo E	CD23	Ephrin-B3	GX
Apo E2	CD30	Epithelial cell kinase	HAI-1
Apo E3	CD30 Ligand	EPO-R	Haptoglobin, Mixed type
Apo E4	CD36 ANTIGEN	ER	Hat1
APRIL	CD39	ERBB1	HB-EGF
AREG	CD97	ERBB2	HCC-1
ARGI1	CD109	ERBB3	HCC-4
ARSB	Ceruloplasmin	ERBB4	HDAC8
ART	CFC1	ERK-1	Hemopexin
Artemin	Chk1	ESAM	Heparin cofactor II
ASAH2	Ck-b-8-1	Factor B	HGF
ASAHL	CK-BB	Factor D	Histone H1.2
ATS1	CK-MM	Factor H	HIV-2 Rev
ATS13	CLF-1/CLC Complex	Factor I	HMG-1
Aurora kinase A	CMP-Sialic Acid Synthetase	Fas ligand, soluble	HO-2
Azurocidin	CNTF	Fas, soluble	HPLN1
B7	CNTFR alpha	FCG2A	HPV E7 Type 16
B7-2	CNTN2	FCG2B	HPV E7 Type18
BAFF	Coagulation Factor a-XIIa	FCG3B	HSP 60
BCAM	Coagulation Factor IX	FCGR1	HSP 70
b-Catenin	Coagulation Factor IXab	Ferritin	HSP 90a
Bcl-2	Coagulation Factor V	FGF-4	HSP 90b
BCMA	Coagulation Factor VII	FGF-5	HTRA2
BDNF	Coagulation Factor X	FGF-6	HVEM
b-Endorphin	Coagulation Factor Xa	FGF7	I11RA
bFGF	Coagulation Factor XI	FGF-8B	I12R2
bFGF-R	Coagulation Factor XIa	FGF9	I-309
BGLI3	Coagulation factor XIII	FGF-10	iC3b
b-Glucosidase	Collagen Type I	FGF-16	ICOS
BGN	COLEC12	FGF-17	IDE
BLC	COMMD7	FGF-18	IDS
BMP-7	Contactin-1	FGF-19	IDUA
BMP-14	Contactin-4	FGF-20	IFN-g
BMPER	COX-2	FGFR-2	IFN-g R1
BMPR1A	Cripto	Fibrinogen	IFN-lambda 1
BMP RII	CRIS3	Fibronectin	IFN-lambda 2
b-NGF	CRP	Flt-3	IgE
Bone proteoglycan II	CTACK	Flt-3 ligand	IGFBP-1
BPI	CTGF	Fractalkine/CX3CL-1	IGFBP-2
C1q	CTLA-4	FSH	IGFBP-3
C1r	CXCL16, soluble	FST	IGFBP-4
C2	Cystatin C	FYN	IGFBP-5
C3	Cystatin M	GA733-1 protein	IGFBP-6
C3a	CYTD	Galectin-2	IGFBP-7
C3adesArg	CYTF	Galectin-3	IGF-I
C3b	CYTN	Galectin-4	IGF-I sR
C3d	Cytochrome c	Galectin-7	IGF-II receptor

FIG. 7 cont'd

IgM
IL-1b
IL-1 R AcP
IL-1 R4
IL-1 sRI
IL-1F7
IL-1Rrp2
IL-2
IL-2 sRg
IL-4
IL-4 sR
IL-6
IL-6 sRa
IL-7
IL-7 R alpha
IL-8
IL-10
IL-10 Rb
IL-11
IL-12
IL-12 Rb1
IL-13
IL-13 Ra1
IL-15 Ra
IL-16
IL-17 sR
IL-17B
IL-17D
IL-17E
IL-17F
IL-18 Bpa
IL-18 Ra
IL-18 Rb
IL-19
IL-20
IL-21 sR
IL-22
IL-27
Inosine triphosphatase
IP-10
IR
I-TAC
JAM-B
JAM-C
Kallikrein 4
Kallikrein 5
Kallikrein 8
Kallikrein 11
Kallikrein 12
Kallikrein 13
Karyopherin-a2
Kininogen, HMW, Single Chain
Kininogen, HMW, Two Chain
KLH
KREM2
Ku70
Lactoferrin
LAG-1
Laminin
Layilin
LBP
LD78-beta
Leptin
Lipocalin 2
LKHA4
LRIG3
LRP8
LSAMP
Luteinizing hormone
LY86
LY9
Lymphotactin
Lymphotoxin b R

Lysozyme
LYVE1
Macrophage mannose receptor
MAPK14
MATN2
MATN3
MBL
MCP-1
MCP-2
MCP-3
MCP-4
M-CSF R
MDC
MEK1
MEPE
MER
Met
METAP1
MIA
MICA
Midkine
Mif4gd, Mouse
MIG
Miox, Rat
MIP-1a
MIP-1b
MIP-3a
MIP-3b
MK01
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-13
MMP-14
MMP-17
MOZ
MP112
MPIF-1
MSP R
Myeloperoxidase
Myosin regulatory light chain 2
NADPH-P450 Oxidoreductase
NANOG
NAP-2
NCAM-L1
NET4
NEUREGULIN-1
Neurotrophin-3
Neurotrophin-5
NG36
Nidogen
NKG2D
NKp30
NKp44
Noggin
Nogo Receptor
NovH
NPS-PLA2
NRP1
OLR1
ON
OPG
OSM
OX40 Ligand
PAFAH beta subunit
PAI-1
PAPP-A
PARC
Partner protein A
Partner protein B

P-Cadherin
PCNA
PDGF Rb
PDGF-AA
PDGF-BB
PD-L2
PDPK1
PECAM-1
Persephin
PF-4
PGRP-S
PIGR
PKB
PKC-A
PKC-B-II
PKC-D
PKC-Z
Plasmin
Plasminogen
PlGF
Prekallikrein
PRL
Properdin
Prostatic acid phosphatase
Protease nexin I
Protein C
Protein S
Prothrombin
PSA
PSA-ACT
P-Selectin
PSMA
PTHrP
PTN
PTP-1B
Rab GDP dissociation inhibitor beta
Rac1
RAD51
RANTES
RAP
RELT
Resistin
RET
RGMB
RGM-C
S100A4
SAP
SARP-2
sCD14
SCF sR
SCGF-alpha
SCGF-beta
SDF-1a
SDF-1b
Semaphorin 3A
sE-Selectin
SET9
sFRP-3
sICAM-2
sICAM-3
SIGIRR
Siglec-6
Siglec-7
Siglec-9
SLAMF8
sLeptin R
SLPI
sL-Selectin
SMAC
sn-1,2-Diacylglycerol Kinase
SOD
Soggy-1
Sonic Hedgehog

SPINT2
Spondin-1
sRAGE
sRANKL
sTie-1
sTie-2
sTREM-1
STX1a
Sulfotransferase, Nod Factor
suPAR
TACI
TARC
tau
TBP
TECK
Tenascin
Testican-2
TF
TFPI
TGF-b1
TGF-b2
TGF-b RIII
Thrombin
Thyroxine-Binding Globulin
TIG2
TIMP-1
TIMP-2
TIMP-3
TNF sR-I
TNF sR-II
TNFSF15
TNFSF18
Tom34
Topoisomerase I
tPA
Tpo
TRAIL
TRAIL R2
TRAIL R4
Transferrin
TrATPase
TrkB
TrkC
Troponin I
Troponin T
Trypsin
Trypsin 2
TSLP
TSLP R
TSP2
TSP4
TWEAK
UBC9
Ubiquitin+1
ULBP-1
ULBP-2
ULBP-3
uPA
URB
VCAM-1
VEGF
VEGF sR2
VEGF sR3
VEGF-C
VEGF-D
Vitronectin
vWF
WFKN2
WIF-1
WISP-3
XEDAR
Yes

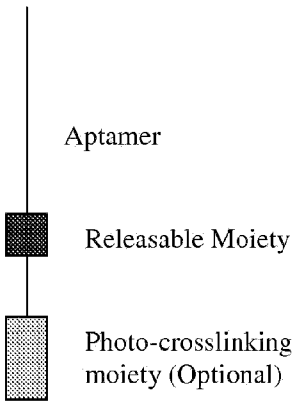


FIG. 8A

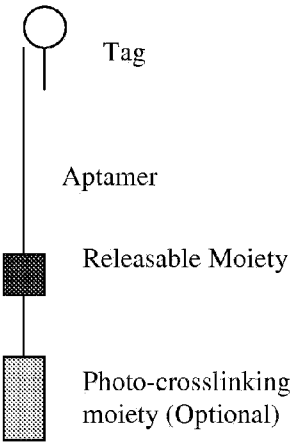


FIG. 8B

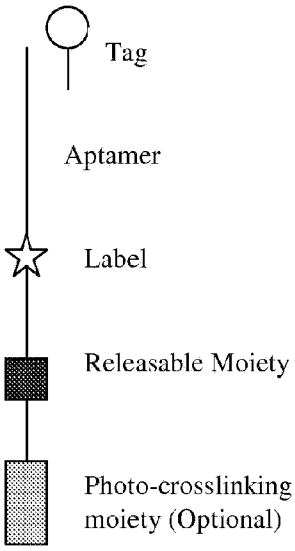


FIG. 8C

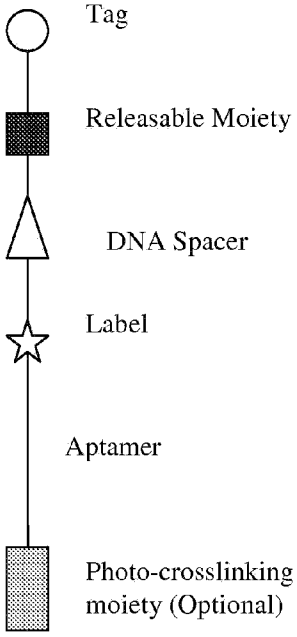
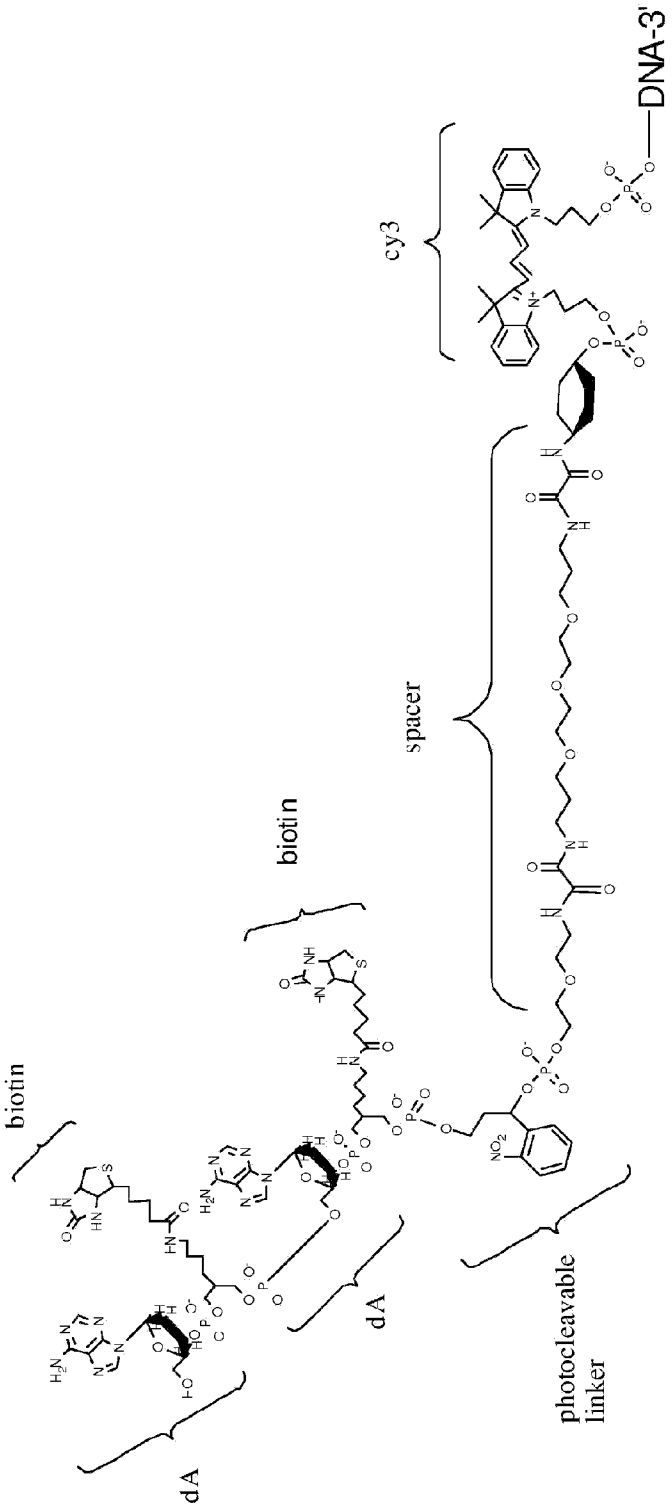


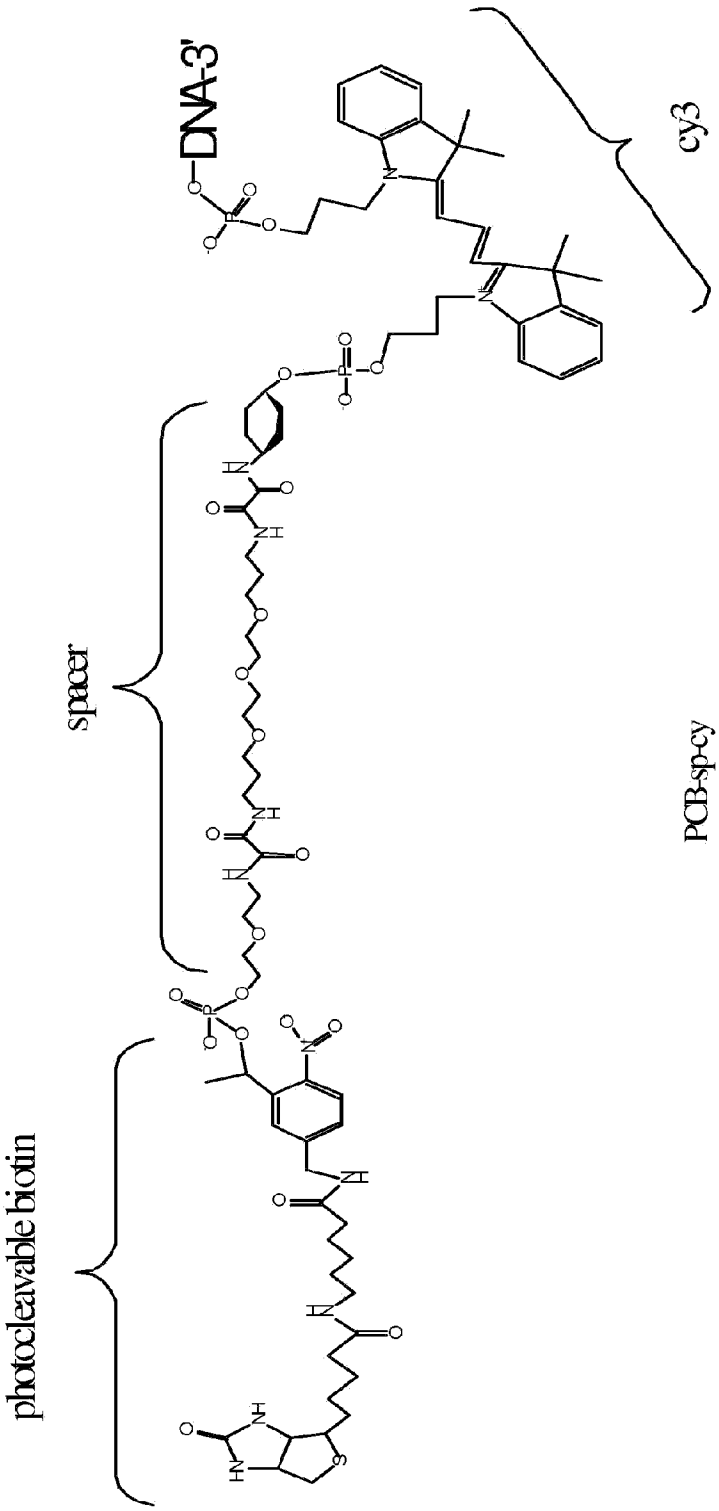
FIG. 8D



Molecular Weight: 2900.69

(AB)2-P-sp-cy

FIG. 9A



PCB-sp-cy

Molecular Weight: 1729.78

FIG. 9B

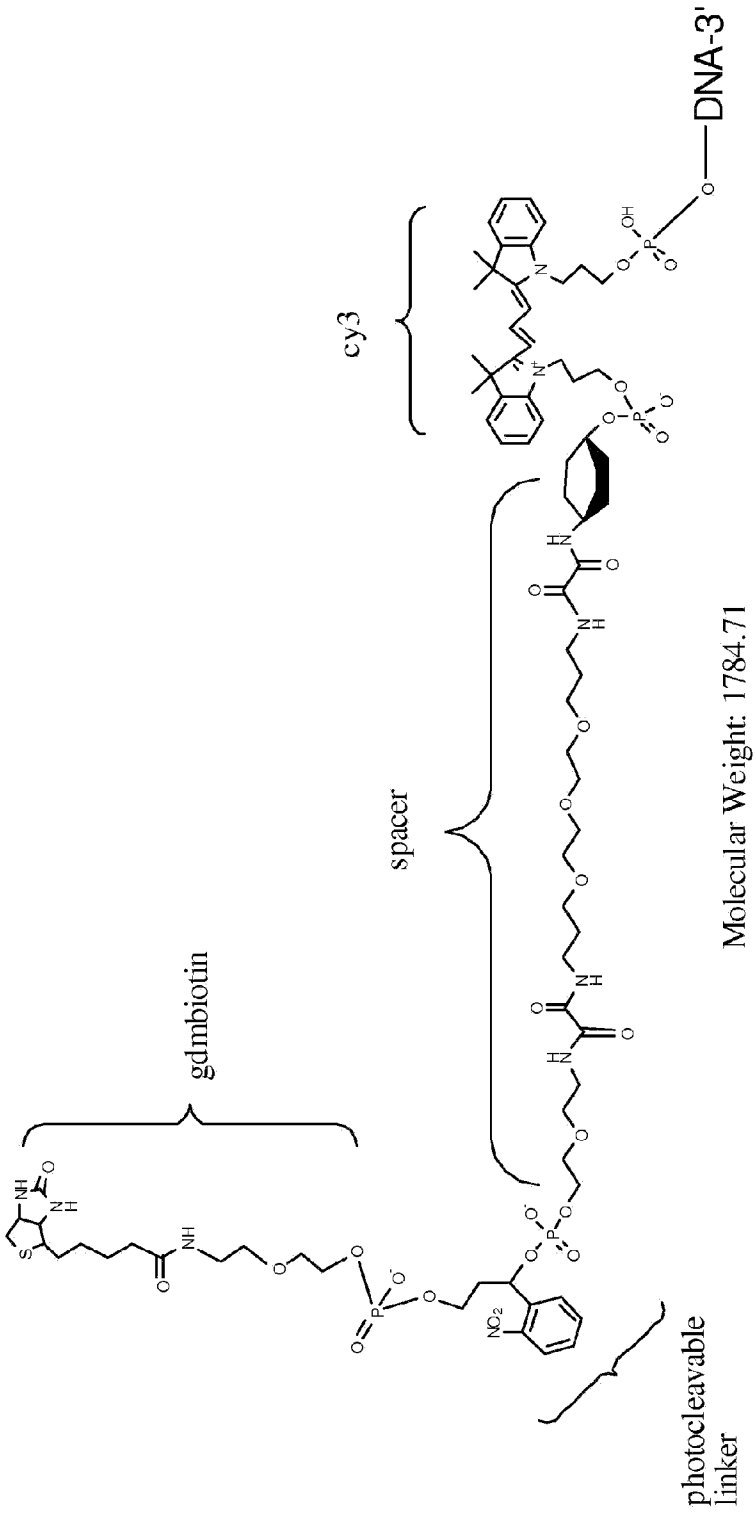
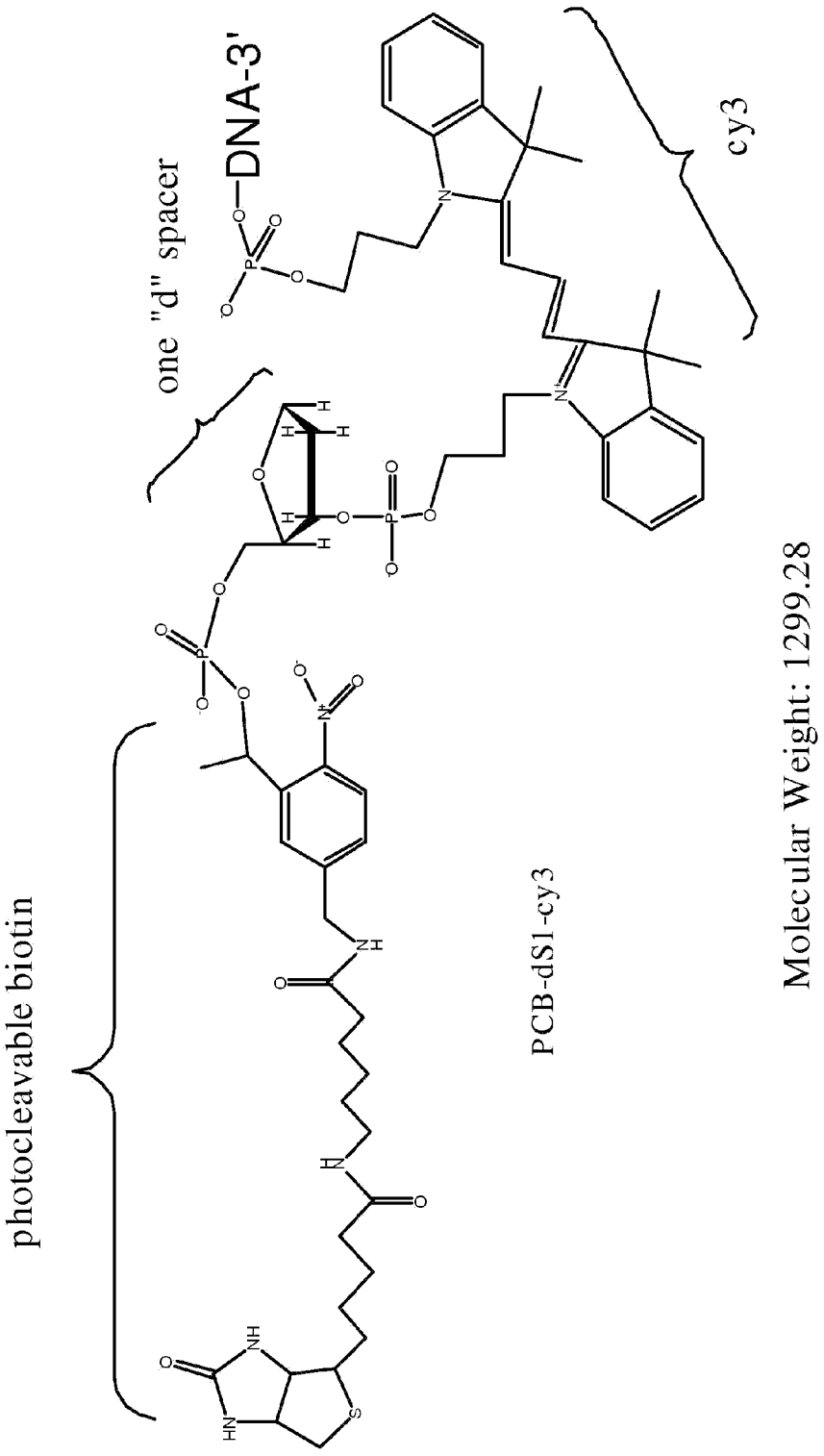

gdmbiotin
spacer
cy3
DNA-3'
photocleavable linker
Molecular Weight: 1784.71
GdmB-P-sp-cy

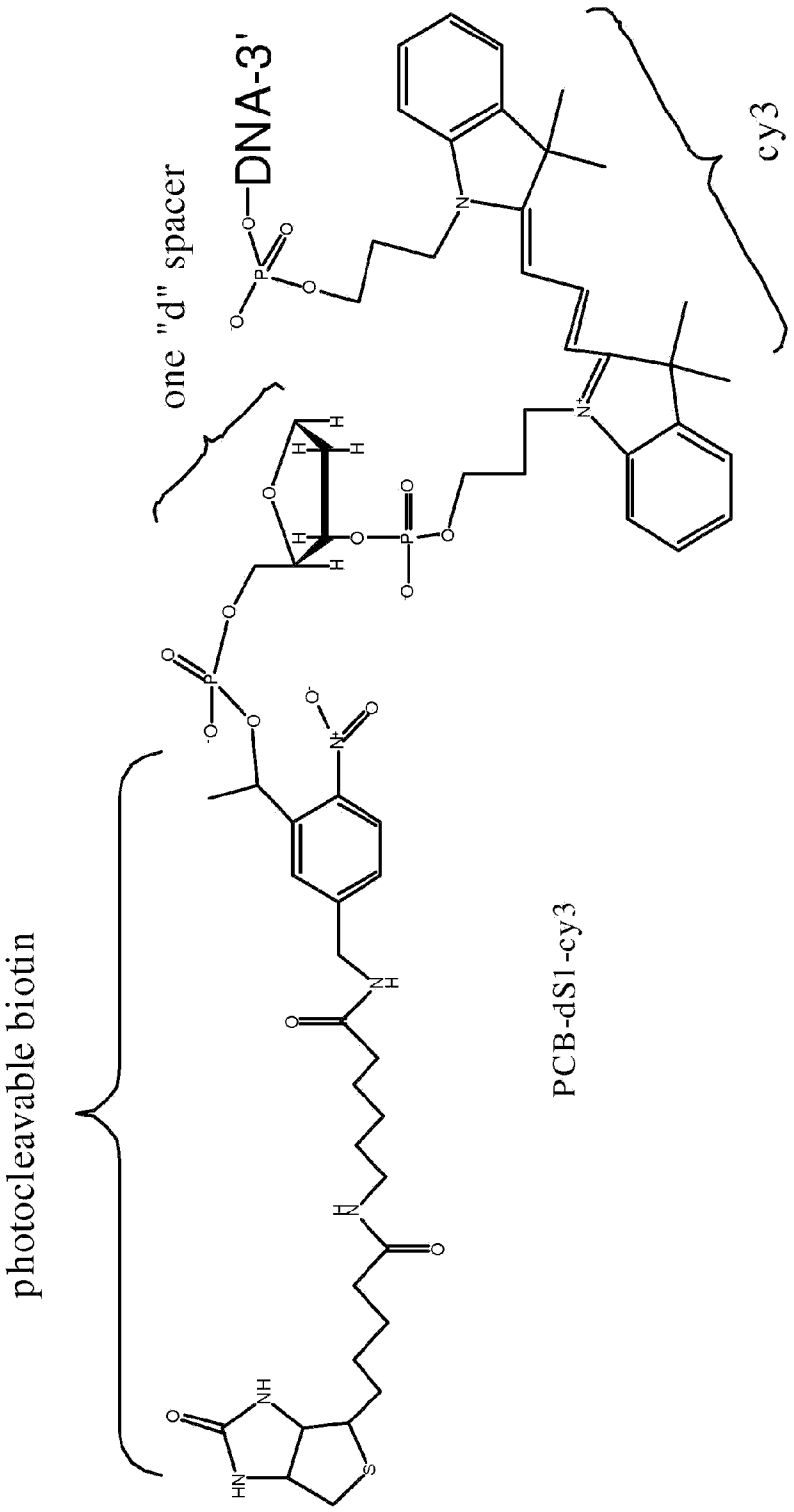
FIG. 9C



PCB-dS1-cy3

Molecular Weight: 1299.28

FIG. 9D



PCB-dS1-cy3

Molecular Weight: 1299.28

FIG. 9E

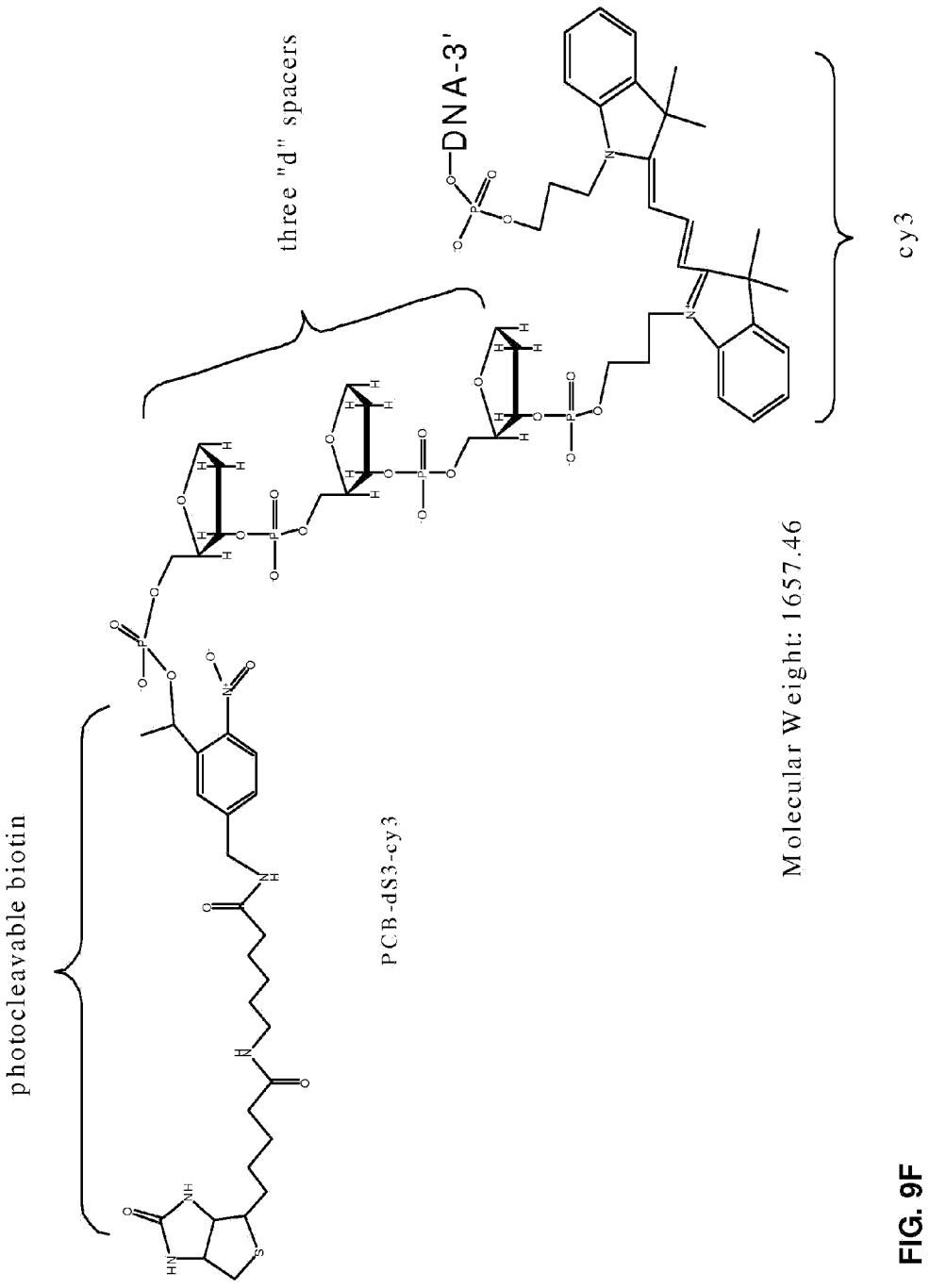

photocleavable biotin
three "d" spacers
—DNA-3'
cy3
PCB-dS3-cy3
Molecular Weight: 1657.46

FIG. 9F

FIG. 10

A 5' - CY3 – APTAMER – 3'

B 5' - $(AB)_2$ – $(T)_8$ – PC - PRIMER – 3'

C 5' - ANA – PC – CY3 – APTAMER – 3'

D 5' - $(AB)_2$ – $(T)_8$ – PRIMER – 3'

FIG. 11A

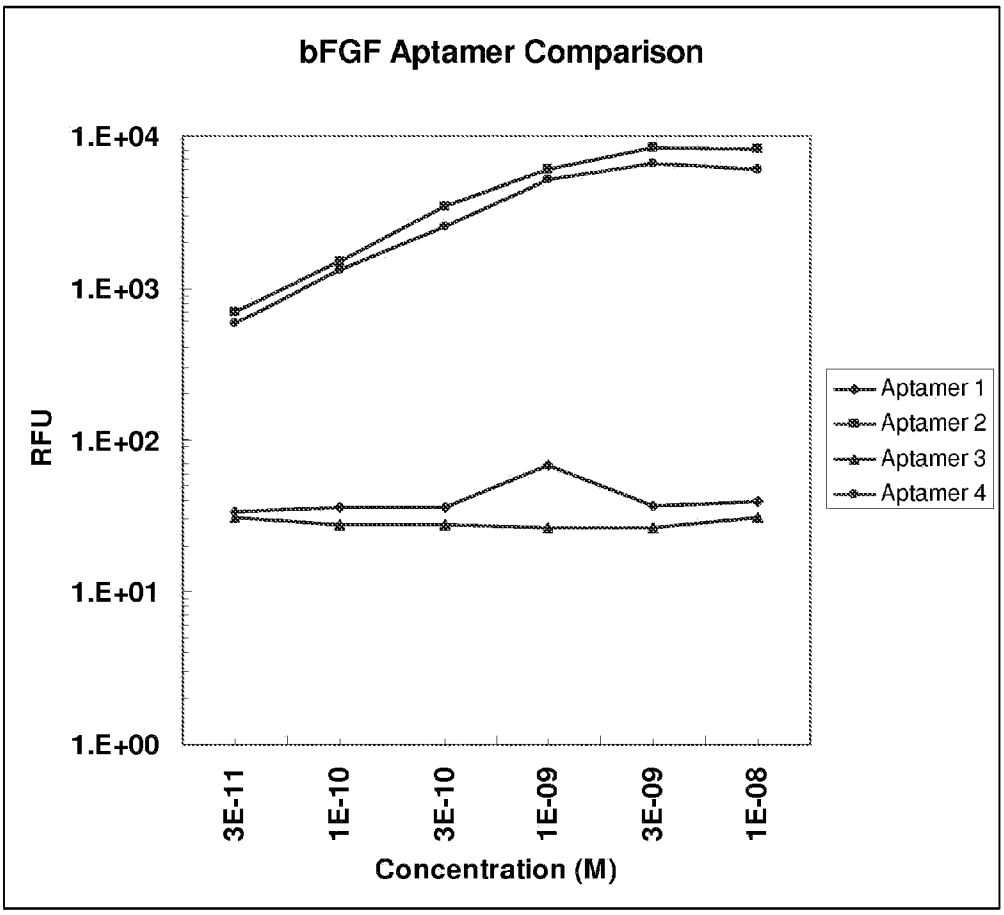

bFGF Aptamer Comparison
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
RFU
3E-11
1E-10
3E-10
1E-09
3E-09
1E-08
Concentration (M)
Aptamer 1
Aptamer 2
Aptamer 3
Aptamer 4

FIG. 11B

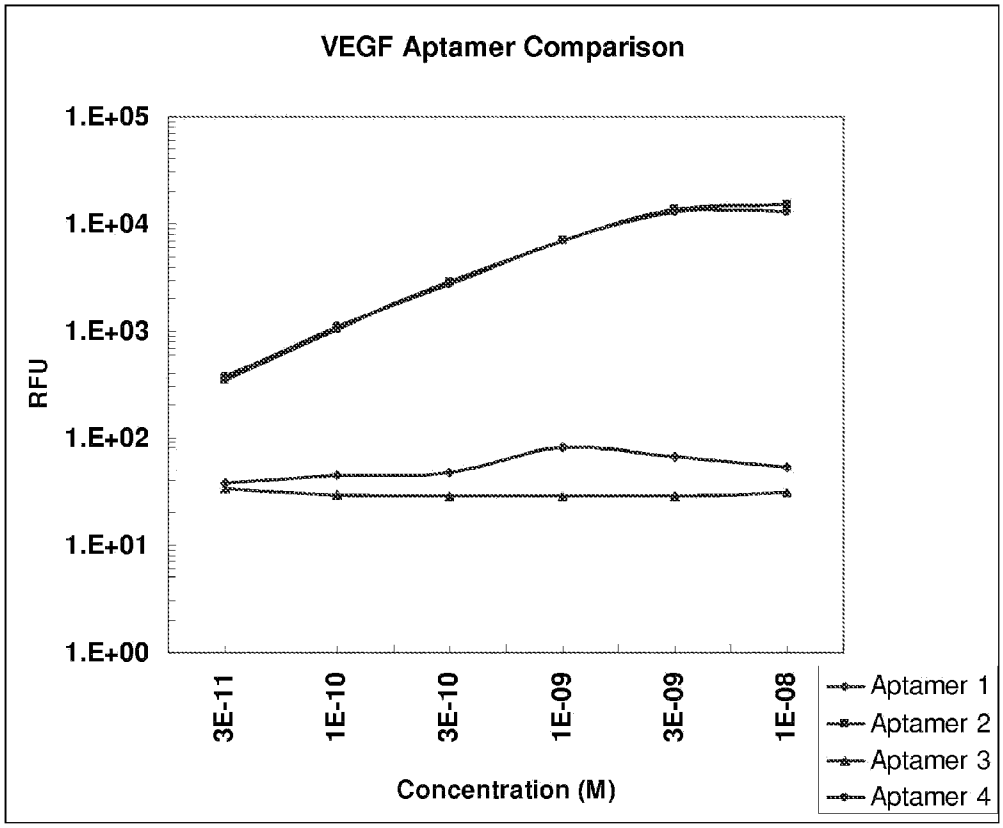

VEGF Aptamer Comparison
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
RFU
3E-11
1E-10
3E-10
1E-09
3E-09
1E-08
Concentration (M)
Aptamer 1
Aptamer 2
Aptamer 3
Aptamer 4

FIG. 11C

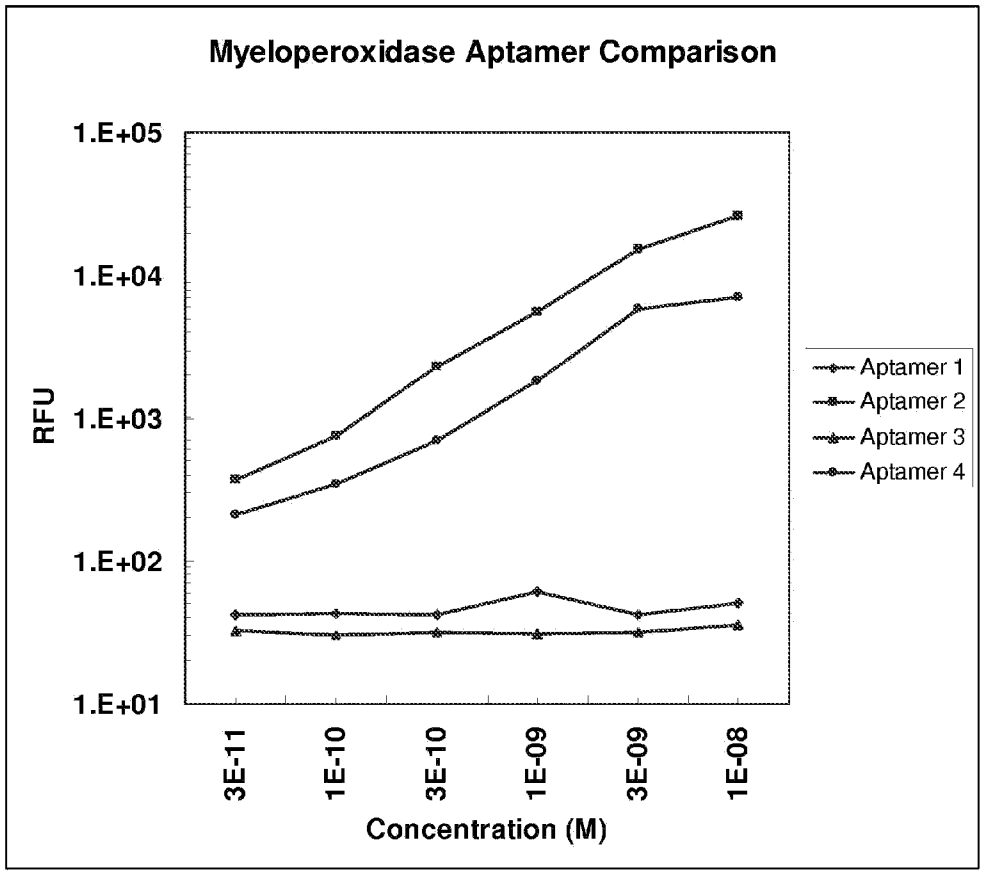

Myeloperoxidase Aptamer Comparison
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
RFU
3E-11
1E-10
3E-10
1E-09
3E-09
1E-08
Concentration (M)
Aptamer 1
Aptamer 2
Aptamer 3
Aptamer 4

FIG. 12A

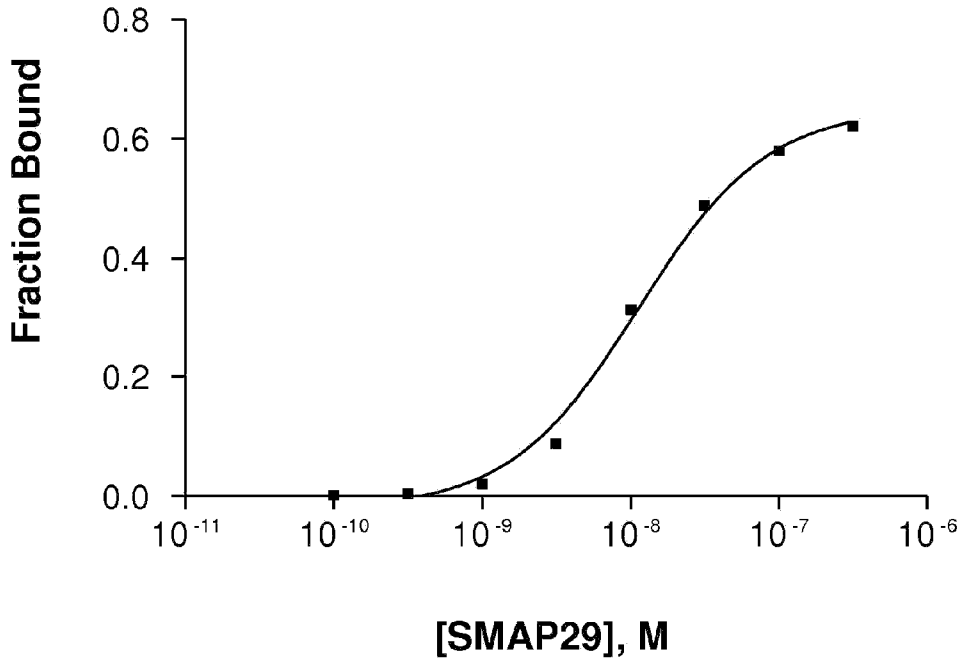

Fraction Bound
0.8
0.6
0.4
0.2
0.0
10-11
10-10
10-9
10-8
10-7
10-6
[SMAP29], M

FIG. 12B

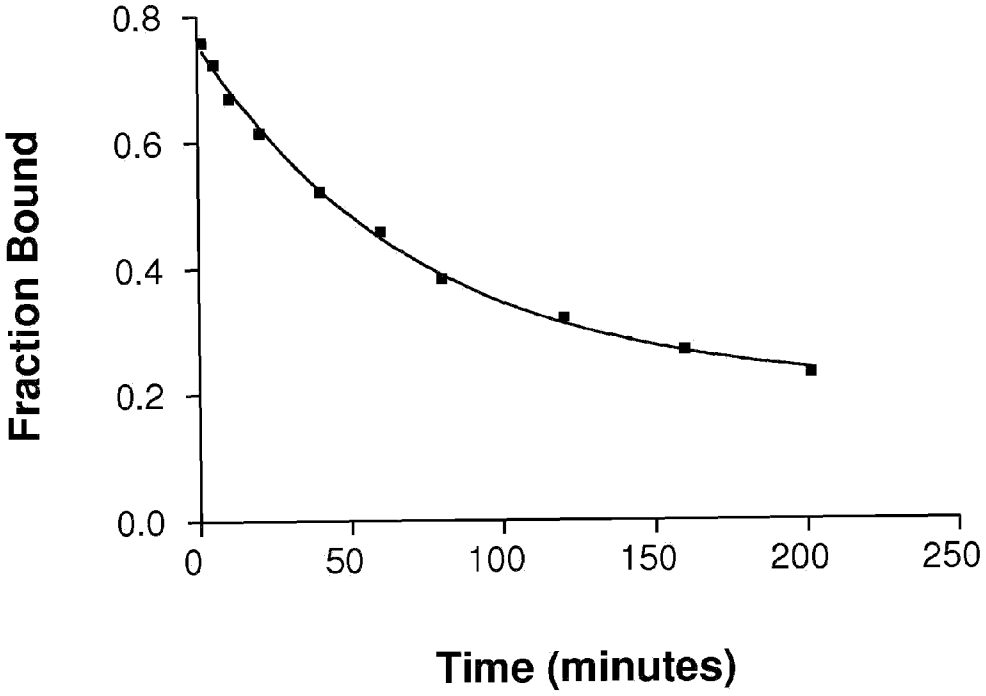


FIG. 13

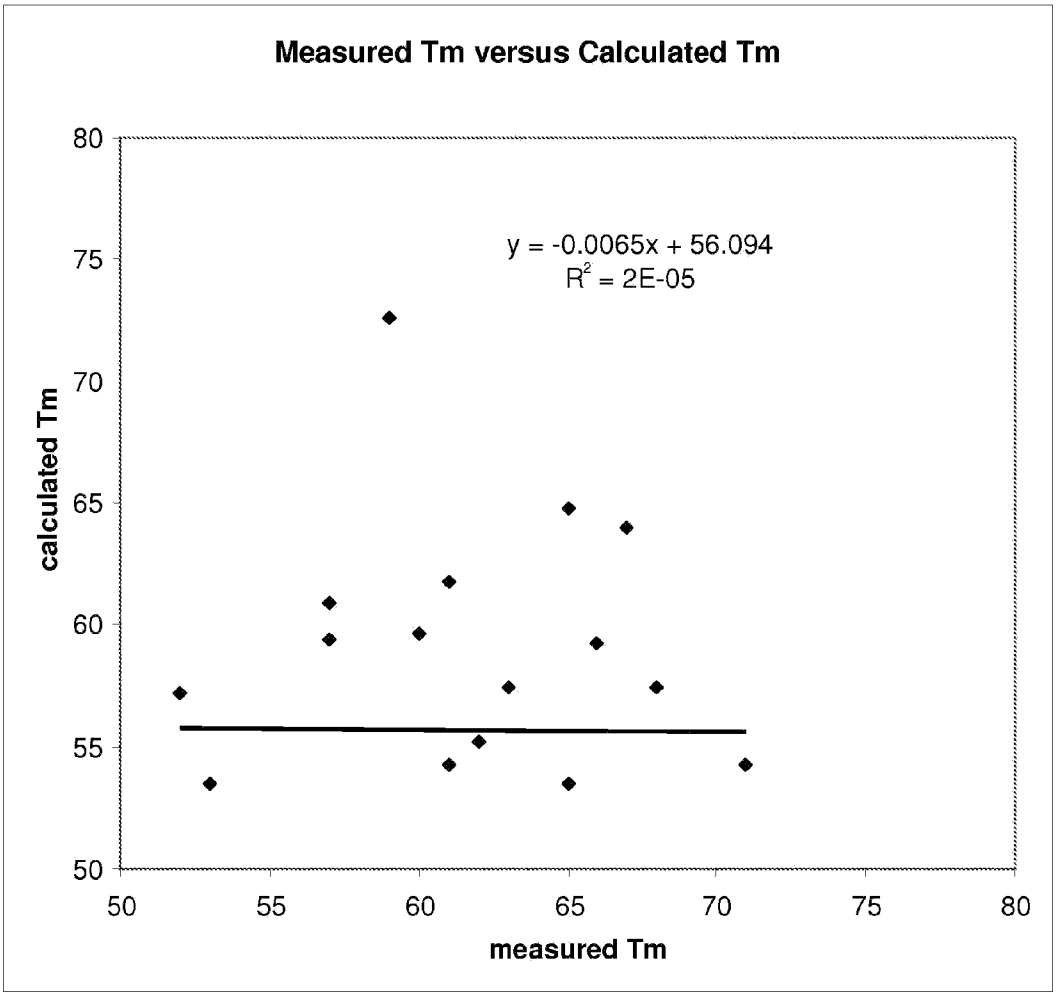

Measured Tm versus Calculated Tm
y = -0.0065x + 56.094
R2 = 2E-05
calculated Tm
measured Tm
80
75
70
65
60
55
50
50
55
60
65
70
75
80

FIG. 14

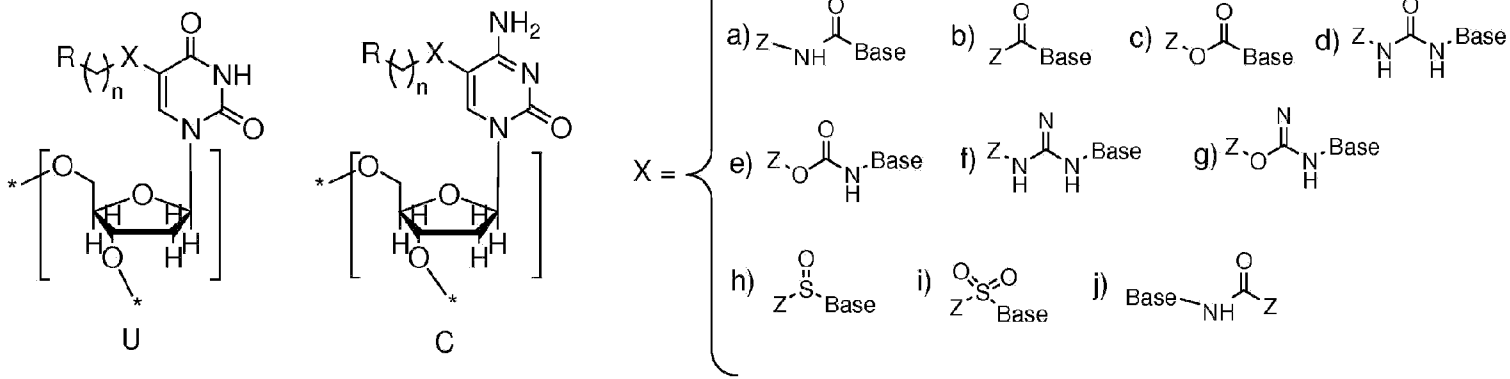

U
C
X =
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
Base = Uridine (U) or Cytidine (C) (attachment is to the 5-position)
Z = R plus (CH2)n connecting group, where n=0-3

FIG. 14 Cont'd

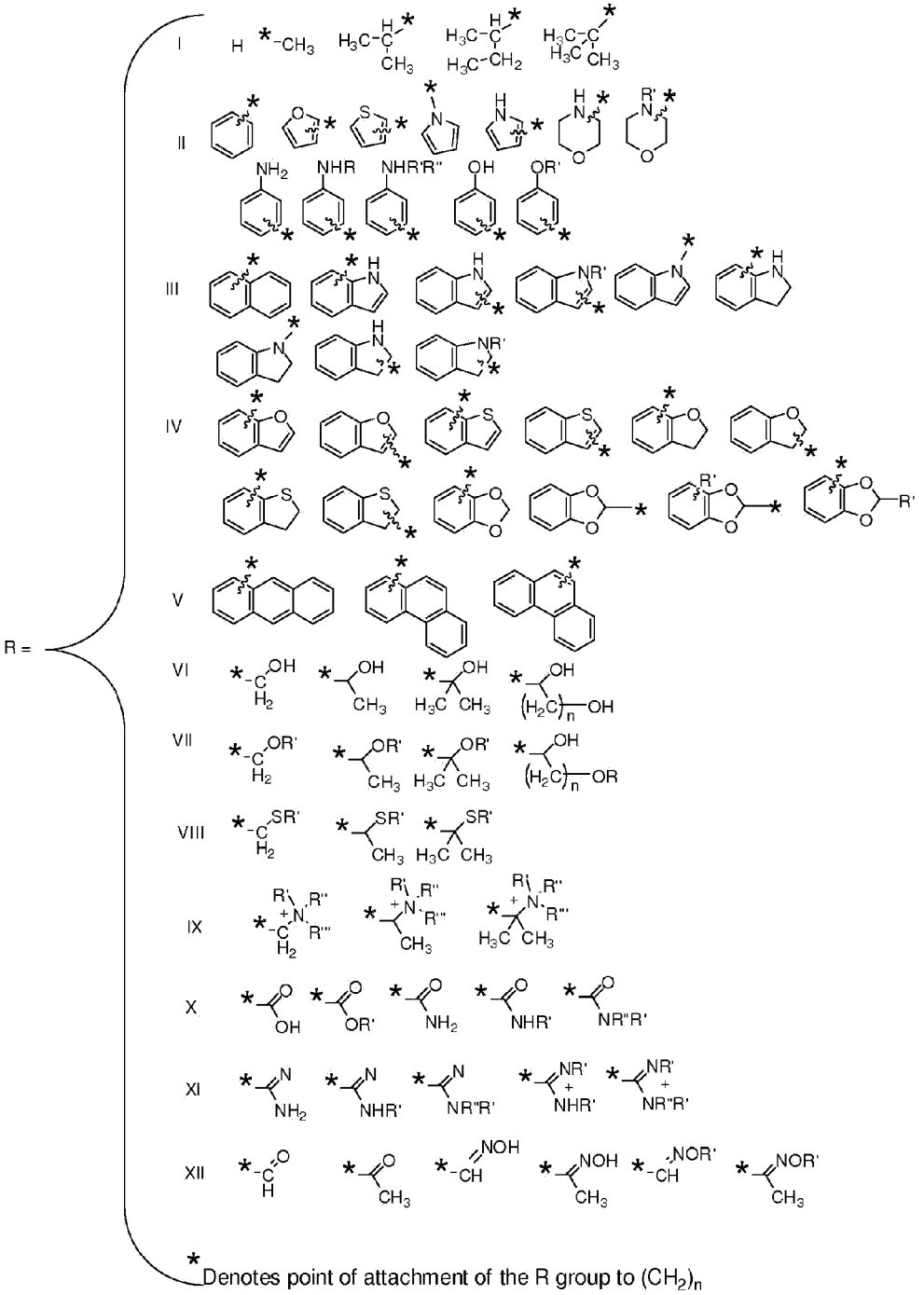
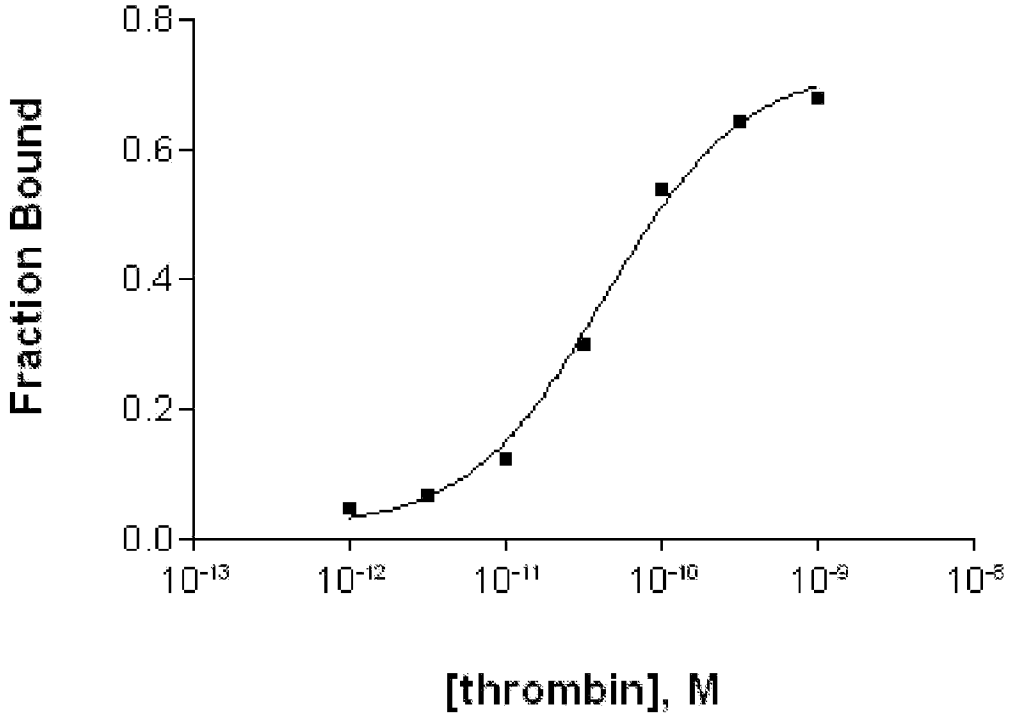

R =
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
* Denotes point of attachment of the R group to $(CH_2)_n$

FIG. 15


Fraction Bound
0.8
0.6
0.4
0.2
0.0
10-13
10-12
10-11
10-10
10-9
10-8
[thrombin], M

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.