APTAMER MEDICINAL CHEMISTRY

FIELD OF INVENTION

[0001] The invention relates generally to the field of nucleic acids and more particularly to aptamers useful as therapeutics, diagnostics and in research such as for target validation. The invention further relates to materials and methods for enhancing aptamers for use in therapeutics, diagnostics and research.

BACKGROUND OF THE INVENTION

[0002] Aptamers are nucleic acid molecules having specific binding affinity to molecules through interactions other than classic Watson-Crick base pairing.

[0003] Aptamers, like peptides generated by phage display or monoclonal antibodies ("mAbs"), are capable of specifically binding to selected targets and modulating the target's activity or binding interactions, *e.g.*, through binding aptamers may block their target's ability to function. Discovered by an *in vitro* selection process from pools of random sequence oligonucleotides, aptamers have been generated for over 130 proteins including growth factors, transcription factors, enzymes, immunoglobulins, and receptors. A typical aptamer is 10-15 kDa in size (20-45 nucleotides), binds its target with nanomolar to sub-nanomolar affinity, and discriminates against closely related targets (*e.g.*, aptamers will typically not bind other proteins from the same gene family). A series of structural studies have shown that aptamers are capable of using the same types of binding interactions (*e.g.*, hydrogen bonding, electrostatic complementarities, hydrophobic contacts, steric exclusion) that drive affinity and specificity in antibody-antigen complexes.

[0004] Aptamers have a number of desirable characteristics for use as therapeutics and diagnostics including high specificity and affinity, biological efficacy, and excellent pharmacokinetic properties. In addition, they offer specific competitive advantages over antibodies and other protein biologics, for example:

[0005] 1) Speed and control. Aptamers are produced by an entirely *in vitro* process, allowing for the rapid generation of initial leads, including therapeutic leads. *In vitro*

selection allows the specificity and affinity of the aptamer to be tightly controlled and allows the generation of leads, including leads against both toxic and non-immunogenic targets.

[0006] 2) Toxicity and Immunogenicity. Aptamers as a class have demonstrated therapeutically acceptable toxicity and lack of immunogenicity. Whereas the efficacy of many monoclonal antibodies can be severely limited by immune response to antibodies themselves, it is extremely difficult to elicit antibodies to aptamers most likely because aptamers cannot be presented by T-cells via the MHC and the immune response is generally trained not to recognize nucleic acid fragments.

[0007] 3) Administration. Whereas most currently approved antibody therapeutics are administered by intravenous infusion (typically over 2-4 hours), aptamers can be administered by subcutaneous injection (aptamer bioavailability via subcutaneous administration is >80% in monkey studies (Tucker *et al.*, J. Chromatography B. 732: 203-212, 1999)). With good solubility (>150 mg/mL) and comparatively low molecular weight (aptamer: 10-50 kDa; antibody: 150 kDa), a weekly dose of aptamer may be delivered by injection in a volume of less than 0.5 mL. In addition, the small size of aptamers allows them to penetrate into areas of conformational constrictions that do not allow for antibodies or antibody fragments to penetrate, presenting yet another advantage of aptamer-based therapeutics or prophylaxis.

[0008] 4) Scalability and cost. Therapeutic aptamers are chemically synthesized and consequently can be readily scaled as needed to meet production demand. Whereas difficulties in scaling production are currently limiting the availability of some biologics and the capital cost of a large-scale protein production plant is enormous, a single large-scale oligonucleotide synthesizer can produce upwards of 100 kg/year and requires a relatively modest initial investment. The current cost of goods for aptamer synthesis at the kilogram scale is estimated at $500/g, comparable to that for highly optimized antibodies. Continuing improvements in process development are expected to lower the cost of goods to < $100/g in five years.

[0009] 5) Stability. Therapeutic aptamers are chemically robust. They are intrinsically adapted to regain activity following exposure to factors such as heat and denaturants and can be stored for extended periods (>1 yr) at room temperature as lyophilized powders.

[0010] Furthermore, the aptamer discovery process readily permits lead modification, such as aptamer sequence optimization and the minimization of aptamer length [Conrad et al. 1996, Eaton et al. 1997]. Additionally, 2' modifications such as 2'-fluoro and 2'-O-Me may be utilized for stabilization against nucleases without compromising the aptamer binding interaction with the target. See e.g. Lin et a *Nucleic Acids Res.* 22, 5229-5234 (1994); Jellinek *et al.,Biochemistry* 1995, 34, 11363-1137; Lin *et al., Nucleic Acids Res.,* 1994, 22, 5229-5234; Kubik *et al., J Immunol.,*1997, 159(1), 259-267; and Pagratis *et al., Nat. Biotechnol.,* 1997, 1, 68-73.

[0011] However, there are only a few examples of the post-discovery introduction of chemical substitutions into aptamers with a view to increasing characteristics other than nuclease-resistance, such as target affinity. See, *e.g.*, Green *et al,. Chem. Biol.*,10, 683-695 (1995), Eaton *et al., Bioorg. Med. Chem.*, 5, 1087-1096 (1997), He *et al., J. Med. Chem.*, 41, 4224-4231 (1998), He *et a*l., *J. Med. Chem.*, 41, 2234-2242 (1998), Wang *et al., Biochemistry*, 32, 11285-11292 (1993), and Krawczyk *et al., Nucleosides and Nucleotides*, 14, 1109-1116 (1995).

[0012] Chemical substitutions have been incorporated into libraries of transcripts from which aptamers are discovered with the view towards selecting aptamers with various characteristics such as increased target affinity See, *e.g.*,. Latham *et al., Nucleic Acids Res.* ,22, 2817-2822 (1994), Vaish *et al.*, . *Biochemistry*, 42, 8842-8851 (2003), Saitoh *et al., Nucleic Acids Res. Suppl.*, 2, 215-216 (2002), Masud *et al., Bioorg. Med. Chem.*, 12, 1111-1120 (2004), King *et al., Biochemistry*, 41, 9696-9706 (2002), and Yang, X. and Gorenstein, D.G. *Curr. Drug Targets*, 5, 705-715 (2004). However, introduction of substitutions into libraries of transcripts *via* transcription is a "global" approach, in which all nucleotides of a given kind are simultaneously substituted. This "global" approach does not allow for the discovery of single substitutions which increase a desired aptamer characteristic, *e.g.* aptamer-target affinity, but may not be tolerated at other positions within the aptamer.

[0013] It would be beneficial to alter aptamer characteristics in addition to and/or other than nuclease resistance, *e.g.* to achieve particular therapeutic and/or diagnostic criteria, while minimizing the number of chemical substitutions required to do so. The present invention provides materials and methods to meet these and other needs.

SUMMARY OF THE INVENTION

[0014] The present invention provides methods and materials related to the identification or substitution of aptamers for use in the treatment of disease, for use in diagnostic applications and/or for use in research, *e.g.* target validation.

[0015] In one embodiment, a method of identifying a substituted aptamer that binds to a target, wherein the substituted aptamer has a higher binding affinity for the target than that of an aptamer identical to substituted aptamer except that it lacks the substitution is provided. One embodiment the method comprises the steps of: a) substituting a single nucleotide modified at a base, sugar or phosphate position or a residue for an unsubstituted nucleotide, and b) assaying the substituted aptamer for binding affinity to the target. In some embodiments, the substituting step of the method further comprises substituting at least two nucleotides modified at the same position *e.g.*, having the same chemical modification, for at least two unmodified nucleotides while in other embodiments embodiments the substituting step comprises substituting at least two nucleotides modified at different positions, *e.g.* having different chemical modifications, for at least two unmodified nucleotides. In some embodiments of all aspects of the invention an improvement of aptamer activity, e.g. in a functional assay such as cell based assay, rather than or in addition to an improvement in binding affinity is detected in the substituted and/or twice substituted aptamer. In some aspects of the method of the invention, the assaying step comprises assaying the aptamer for activity against the target in a functional assay, *e.g.* a cell based assay.

[0016] In some embodiments, the substituting step further comprises substituting at least three nucleotides substituted at the same position, *e.g.* having the same chemical modification, for at least three unmodified nucleotides, whereas in other embodiments at least two of the three nucleotides to be substituted are modified at different positions, *e.g.* have different chemical modifications. An aptamer identified by the method of this aspect of the invention is also provided.

[0017] The invention also provides a single stranded aptamer that specifically binds to a target, wherein the aptamer comprises a nucleotide sequence having a substituted nucleotide or residue selected to increase the binding affinity of the aptamer to the target relative to the

binding affinity of a second aptamer to the same target, the second aptamer having the same nucleotide sequence but lacking the substituted nucleotide, wherein the substituted nucleotide comprises a modification at a phosphate, sugar or base position. In another embodiment, the invention provides a single stranded aptamer comprising no more than four, no more than 3, no more than 2 or no more than one nucleotide or residue substitution wherein the substituted nucleotide comprises a chemical modification at a base, a sugar or phosphate position and wherein the single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the substitution.. In some embodiments of this aspect of the invention the substituted single stranded aptamer comprises higher activity against the aptamer target than that of a second aptamer lacking the substitution.

[0018] The invention also provides a method of identifying a substituted single stranded aptamer comprising the steps of: a) substituting a phosphorothioate or phosphorodithioate for a phosphate at an internucleotide linkage position in a single stranded aptamer; b) assaying the substituted single stranded aptamer for affinity to the target; and c) identifying the substituted single stranded aptamer having higher affinity for the target relative to that of a starting aptamer which is identical to the modified single stranded aptamer except that it lacks the phosphorothioate substitution. As used herein, a single stranded aptamer encompasses aptamers having stem loop structures. In some embodiments of the methods of the invention, the phosphorothioate substitution occurs at non-bridging position. The invention is intended to cover stereochemical mixtures of the aptamers of invention, including racemic mixtures, as well as substantially pure stereochemical mixtures, e.g. 95% or more of one diasteriomer. In some embodiments, the substituting step of the method of this aspect of the invention further comprises incorporating a phosphorothioate at the internucleotide linkage position by chemical synthesis. In some embodiments, the substituting step does not comprise incorporating the phosphorothioate substitution at the internucleotide linkage between the two nucleotides at the 3' or 5' end of the single stranded aptamer. In some embodiments, the substituting step comprises incorporating a single phosphorothioate substitution into the single stranded aptamer. In some embodiments the singly substituted phosphorothioate substituted single stranded aptamer comprises a binding affinity for the

target that is at least two fold, at least three fold, at least four fold or at least five fold higher than that of the starting aptamer.

[0019] In some embodiments of the method of this aspect of the invention further comprises the step of incorporating an additional substitution into the substituted single stranded aptamer to result in a twice substituted single stranded aptamer. In some embodiments the method further comprises the step of assaying the twice substituted single stranded aptamer for affinity to the target and identifying the twice substituted single stranded aptamer that has an affinity equal to or higher than that of the starting unsubstituted single stranded aptamer. While in other embodiments, the method of this aspect of the invention further comprises assaying the twice substituted single stranded aptamer for affinity to the target and identifying the twice substituted single stranded aptamer that has an affinity equal to or higher than that of the phosphorothioate substituted single stranded aptamer. In some embodiments, the additional substitution is an additional phosphorothioate substitution at a phosphate position different than that of the first phosphorothioate substitution. In some embodiments, wherein the twice substitute aptamer comprises at least two phosphorothioate substitutions at different nucleotide positions, the binding affinity for the target of the twice substituted aptamer is at least two fold, three fold, four fold or five fold higher than that of starting unsubstituted aptamer and/or the once modified aptamer.

[0020] In some embodiments, the additional substitution is selected from the group consisting of: substituting a nucleotide modified at a base position, substituting a nucleotide modified at sugar position, and substituting a nucleotide a modified at phosphate position. In some embodiments, the additional substitution is selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine, a 2'-deoxy-5-methyl nucleotide substitution for another nucleotide, a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide, a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide, and a 2-aminopurine substitution for a purine. In some embodiments, the additional substitution is selected from the group consisting of: a 2'-deoxy inosine or 2'-OMe inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine substitution for a cytidine, and a 2'-deoxy nucleotide substitution for a 2'-OMe

nucleotide. An aptamer identified by the methods of this aspect of the invention is also provided. In some embodiments, wherein the twice substitute aptamer comprises at least two different types of substitutions one of which is a phosphorothioate substitution, the binding affinity for the target of the twice substituted aptamer is at least two fold, three fold, four fold or five fold higher than that of starting unsubstituted aptamer and/or the once modified aptamer.

[0021] In another aspect of the invention, a method of identifying a substituted aptamer comprising the steps of: a) incorporating a substitution into a starting aptamer wherein the substitution is selected from the group consisting of: an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, a 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide; b) assaying the substituted aptamer for affinity to the target; and c) identifying the substituted aptamer having higher affinity for the target relative to that of the starting aptamer which is identical to the substituted aptamer except that it lacks the substituted nucleotide is provided. In some embodiments, the incorporating step of this method further comprises incorporating the substitution into the starting aptamer through chemical synthesis. In some embodiments of the method of this aspect of the invention the identified substituted aptamer is single stranded. In some embodiments, the method of this aspect of the invention comprises substituting an inosine for another nucleotide. In some embodiments of the method of the invention an inosine is substituted for another nucleotide, e.g. for a purine, while in some embodiments a 2'-deoxy nucleotide is substituted for a 2'-OMe nucleotide. In some embodiments, wherein an inosine is substituted for another nucleotide, *e.g.* for a purine, the binding affinity for the target of the substituted aptamer is at least two fold, three fold, 4 fold or 5 fold higher than that of the starting unsubstituted aptamer. In some embodiments, wherein a 2'-deoxy nucleotide is substituted for a 2'-OMe nucleotide the binding affinity for the target is at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 30 fold, at least 40 fold, at least 50 fold, at least 60 fold, at least 70 fold or at least 80 fold higher than that of starting unsubstituted aptamer.

[0022] In some embodiments, the method further comprises the steps of incorporating an additional substitution into the substituted aptamer, wherein the additional substitution is of a different type than the first substitution, to result in a twice substituted aptamer. In some embodiments the method further comprises assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to or greater than that of the starting unsubstituted single stranded aptamer. In some embodiments, the method further comprises assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to or greater than that of the substituted aptamer. In some embodiments of the method of this aspect of the invention, the additional substitution is selected from the group consisting of: substituting a nucleotide modified at a base position, substituting a nucleotide modified at a sugar position, and substituting a nucleotide a modified at phosphate position. In some embodiments, the additional substitution is selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine, 2'-deoxy- 5-methyl nucleotide substitution for a nucleotide, a 2'- deoxy nucleotide substitution for a 2'-OMe nucleotide, a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide, and a 2- aminopurine substitution for a purine and wherein the additional substitution is different from the first substitution. In some embodiments, the additional substitution is selected from the group consisting of: a 2-deoxy inosine or 2'-OMe inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine substitution for a cytidine, and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide. In some embodiments of the method of this aspect of the invention, the first substitution is 2'-deoxy substitution for 2'-OMe nucleotide and the additional substitution is an inosine substitution for another nucleotide.

[0023] In some embodiments of the method of this aspect of the invention the method further comprises incorporating an additional substitution into the substituted aptamer to result in a twice substituted aptamer wherein the additional substitution is of the same type as the first substitution but at a different nucleotide position. In some embodiments of this aspect of the invention, the method further comprises assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to

or greater than that of the starting unsubstituted single stranded aptamer and/or the substituted aptamer.

[0024] The invention also provides, a single stranded aptamer comprising no more than four, no more than three, no more than two or no more than one phosphorothioate backbone substitutions wherein the single stranded aptamer binds specifically to a target with a binding affinity for the target that is higher relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking a phosphorothioate modification.

[0025] In some embodiment, the aptamer of this aspect of the invention comprises an additional nucleotide substitution selected from the group consisting of: a substitution with a nucleotide modified at a base position, with a nucleotide modified at a sugar position and with a nucleotide modified at phosphate position wherein when the nucleotide substituted comprises a modification at the phosphate position it is not a phosphorothioate substitution. In some embodiments, the aptamer of this aspect of the invention comprises an additional nucleotide substitution selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine, a 2'-deoxy-5-methyl nucleotide substitution for another nucleotide, a 2'- deoxy nucleotide substitution for a 2'-OMe nucleotide, a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide, and a 2- aminopurine substitution for a purine.

[0026] In another embodiment, the aptamer of this aspect of the invention comprises a e stranded aptamer comprising a nucleotide substitution selected from the group consisting of : an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, an 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide, wherein the substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is higher relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the nucleotide substitution. In some embodiments, the aptamer of this aspect of the invention comprises at least two nucleotide substitutions or at least three nucleotide substitutions selected from the group consisting of : an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a

uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, an 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide, wherein the twice substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is higher relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the at least two nucleotide substitutions.. In some embodiments, the twice substituted single stranded aptamer of this aspect of the invention binds specifically to a target with a binding affinity for the target that is higher relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking one of the nucleotide substitutions. In some embodiments, the triple substituted single stranded aptamer of this aspect of the invention binds specifically to a target with a binding affinity for the target that is higher relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking at least one of the nucleotide substitutions. In a particular embodiment, the nucleotide substitution is the substitution of 2'-deoxy nucleotide for a 2'-OMe nucleotide. In some embodiments, the nucleotide substitution is the substitution of an inosine for a purine.

[0027] In another embodiment of the invention, an aptamer that specifically binds to a target, wherein the aptamer comprises a nucleotide sequence having a phosphorothioate modification of the phosphate back bone at a position selected to increase the binding affinity of the aptamer to the target relative to the binding affinity of a second aptamer to the same target, the second aptamer having the same nucleotide sequence but lacking the phosphorothioate modification. is provided.

[0028] In another embodiment, a method is provided of stabilizing an aptamer comprising the steps of: a) introducing stablilizing modifications into a starting aptamer to result in a modified aptamer wherein the starting aptamer has a predeterimined binding affinity for a target, and b) assaying modified aptamer for binding affinity to the target and where the binding affinity is less than that of the starting aptamer introducing a nucleotide substitution to result in a substituted aptamer wherein the nucleotide substitution results in the substituted aptamer having a binding affinity for the target greater than that of the modified aptamer. In some embodiments of the method of this aspect of the invention, the substituted aptamer comprises a binding affinity for the target substantially the same as that of the starting

aptamer. In some embodiments, the stabilizing modification is a modification selected from the group consisting of: to increase aptamer resistance to nuclease degradation, to increase base pair strength, to increase hydrolytic resistance and to increase resistance to thermal degradation. In a particular embodiment, the stabilizing modification is a modification to increase resistance to nucelease degradation. In a more particular embodiment, the stabilizing modification comprises substituting a 2'-OMe nucleotide for another nucleotide, particularly incorporating more than one 2'-OMe substitution for another nucleotide. In some embodiments, the substituting step comprises a substitution selected from the group consisting of: a substitution with a nucleotide modified at a base position, with a nucleotide modified at a sugar position and with a nucleotide modified at phosphate position. in particular embodiments, the substitution is selected from the group consisting of: an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, a 2-amino purine substitution for a purine, a phosphorothioate substituted nucleotide for an unsubstituted nucleotide, a phosphorodithioate substituted nucleotide for an unsubstituted nucleotide and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide. In a particular embodiment, the substitution is a phosphorothioate substituted nucleotide substituted for an unsubstituted nucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] Figure 1 is a schematic representation of the *in vitro* aptamer selection (SELEX™) process from pools of random sequence oligonucleotides.

[0030] Figure 2 is an illustration of a 40 kDa branched PEG.

[0031] Figure 3 is an illustration of a 40 kDa branched PEG attached to the 5'end of an aptamer.

[0032] Figure 4 is an illustration depicting various PEGylation strategies representing standard mono-PEGylation, multiple PEGylation, and oligomerization via PEGylation.

[0033] Figure 5 is a graph depicting the percent of the indicated aptamers bound (vertical axis) versus concentration of human IL-23.

DETAILED DESCRIPTION OF THE INVENTION

[0034] The details of one or more embodiments of the invention are set forth in the accompanying description below. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description. In the specification, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the case of conflict, the present Specification will control.

APTAMER DEVELOPMENT

[0035] An aptamer, also referred to herein as a nucleic acid ligand, comprises an isolated nucleic acid molecule having specific binding affinity to a molecule through interactions other than classic Watson-Crick base pairing. A suitable method for identifying an aptamer is with the process entitled "Systematic Evolution of Ligands by Exponential Enrichment" ("SELEX™") generally depicted in Figure 1 and described in more detail below. Once aptamers for use as leads, *e.g.* therapeutic and/or diagnostic leads and/or target validation, have been identified, the lead aptamers may be modified to achieve desired criteria. For example, a lead aptamer may be truncated to result in a shorter aptamer that retains or attains a useful binding affinity for the desired target, *e.g.* a target binding affinity equal to or better than that of the parent aptamer. The primary nucleotide sequence of a lead aptamer may be varied at one or several positions to e.g. enhance the binding affinity of the resulting aptamer for the target. A lead aptamer can also be chemically modified to achieve desired criteria. None or combinations of these modification techniques may be used to achieve the aptamer characteristics desired for a particular use.

THE SELEX™ METHOD

[0036] The SELEX™ process is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules and is described in, *e.g.*, U.S. patent application Ser. No. 07/536,428, filed Jun. 11, 1990, now abandoned, U.S. Pat. No.

5,475,096 entitled "Nucleic Acid Ligands", and U.S. Pat. No. 5,270,163 (see also WO 91/19813) entitled "Nucleic Acid Ligands". Each SELEX™-identified nucleic acid ligand, *i.e.*, each aptamer, is a specific ligand of a given target compound or molecule. The SELEX™ process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (*i.e.*, form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets.

[0037] SELEX™ relies as a starting point upon a large library or pool of single stranded oligonucleotides comprising at least one degenerate position. The method is typically used to sample approximately 10^{14} different oligonucleotide species but may be used to sample as many as about 10^{18} different oligonucleotide species. Within a nucleic acid library containing a large number of possible sequences and structures, there is a wide range of binding affinities for a given target. Those which have the higher affinity (lower dissociation constants) for the target are most likely to bind to the target. The library is mixed with the target under conditions favorable for binding and subjected to step-wise iterations of binding, partitioning and amplification. After the first iteration of partitioning, dissociation and amplification, a second nucleic acid mixture is generated, enriched with the higher binding affinity candidates. Additional rounds of selection progressively favor the best ligands. Using the same general selection scheme, virtually any desired criterion of binding affinity and selectivity can be achieved. Often the resulting nucleic acid mixture is predominantly composed of only one or a few sequences. These can then be cloned, sequenced and individually tested for binding affinity as pure ligands or aptamers

[0038] More specifically, the oligonucleotides in the starting library can be modified or unmodified DNA, RNA, or DNA/RNA hybrids. The intended aptamer use will often inform the choice of the starting oligonucleotide library composition. For example, aptamers suitable for use as therapeutics, are preferably inexpensive to synthesize, safe and stable *in vivo*. Stability in the presence of biological fluids may also be important for use of aptamers in diagnostic and target validation applications. Depending on the intended medical indication, wild-type RNA and DNA aptamers are typically not sufficiently stable for use *in vivo* because

of their susceptibility to degradation by nucleases. Resistance to nuclease degradation can be greatly increased by the incorporation of modified nucleotides into the aptamer.

[0039] The SELEX™ method encompasses the identification of high-affinity nucleic acid ligands 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 sugar and/or phosphate and/or base positions. SELEX™-identified nucleic acid ligands containing modified nucleotides are described, *e.g.*, in U.S. Patent No. 5,660,985, which describes oligonucleotides containing nucleotide derivatives chemically modified at the 2' position of ribose, 5 position of pyrimidines, and 8 position of purines, U.S. Patent No. 5,756,703 which describes oligonucleotides containing various 2'-modified pyrimidines, and U.S. Patent No. 5,580,737 which describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe) substituents.

[0040] Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, flexibility, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and/or fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Modifications to generate oligonucleotide populations which are resistant to nucleases can also include one or more substitute internucleotide linkages, altered sugars, altered bases, or combinations thereof. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, and unusual base-pairing combinations such as the isobases isocytidine and isoguanosine. Modifications can also include 3' and 5' modifications such as capping.

[0041] In one embodiment, oligonucleotides are provided in which the P(O)O group is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), P(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal") or 3'-amine (-NH-CH_2-CH_2-), wherein each R or R' is independently H or substituted or unsubstituted alkyl. Linkage groups can be attached to

adjacent nucleotides through an -O-, -N-, or -S- linkage. Not all linkages in the oligonucleotide are required to be identical.

[0042] In further embodiments, the oligonucleotides comprise modified sugar groups, for example, one or more of the hydroxyl groups is replaced with halogen, aliphatic groups, or functionalized as ethers or amines. In one embodiment, the 2'-position of the furanose residue is substituted by any of an O-methyl, O-alkyl, O-allyl, S-alkyl, S-allyl, or halo group. Methods of synthesis of 2'-modified sugars are described, *e.g.*, in Sproat, *et al.*, Nucl. Acid Res. 19:733-738 (1991); Cotten, *et al.*, Nucl. Acid Res. 19:2629-2635 (1991); and Hobbs, *et al.*, Biochemistry 12:5138-5145 (1973).

[0043] SELEX™ methods used to generate 2'-modified aptamers are described, *e.g.*, in U.S. Provisional Patent Application Serial No. 60/430,761, filed December 3, 2002, U.S. Provisional Patent Application Serial No. 60/487,474, filed July 15, 2003, U.S. Provisional Patent Application Serial No. 60/517,039, filed November 4, 2003, U.S. Patent Application No. 10/729,581, filed December 3, 2003, U.S. Patent Application No. 10/873,856, filed June 21, 2004, entitled "Method for *in vitro* Selection of 2'-O-Methyl Substituted Nucleic Acids", and U.S. Provisional Patent Application Serial No. 60/696,292, filed June 30, 2005, entitled "Improved Materials and Methods for the Generation of Fully 2'-Modified Containing Nucleic Acid Transcripts", each of which is herein incorporated by reference in its entirety.

[0044] In the disclosed method, pools of transcripts are generated using any combination of modified nucleotides in the transcription mixture, including for example, ribonucleotides (2'-OH), 2'-deoxyribonucleotides (2'-deoxy), 2'-F, and 2'-OMe nucleotides.

[0045] A transcription mixture containing 2'-OH A and G and 2'-OMe C and U is referred to as an "rRmY" mixture and aptamers selected therefrom are referred to as "rRmY" aptamers. A transcription mixture containing 2'-deoxy A and G and 2'-OMe U and C is referred to as a "dRmY" mixture and aptamers selected therefrom are referred to as "dRmY" aptamers. A transcription mixture containing 2'-OH G and 2'-OMe A, C, and U, is referred to as a "rGmH" mixture and aptamers selected therefrom are referred to as "rGmH" aptamers. A transcription mixture alternately containing 2'-OMe A, C, U and G and 2'-OMe A, U and C and 2'-F G is referred to as an "alternating mixture" and aptamers selected therefrom are

referred to as "alternating mixture" aptamers. A transcription mixture containing 2'-OMe A, U, C, and G, where up to 10% of the G's are ribonucleotides is referred to as a "r/mGmH" mixture and aptamers selected therefrom are referred to as "r/mGmH" aptamers. A transcription mixture containing 2'-F G and 2'-OMe A, U, and Cis referred to as a "fGmH" mixture and aptamers selected therefrom are referred to as "fGmH" aptamers. A transcription mixture containing 2'-deoxy G and 2'-OMe A, U, and C, is referred to as a "dGmH" mixture and aptamers selected therefrom are referred to as "dGmH" aptamers. A transcription mixture containing 2'-deoxy A, and 2'-OMe C, G and U is referred to as a "dAmB" mixture and aptamers selected therefrom are referred to as "dAmB" aptamers and a transcription mixture containing all 2'-OH nucleotides is referred to as a "rN" mixture and aptamers selected therefrom are referred to as "rN", "rRrY" or " RNA" aptamers. A transcription mixture containing 2'-OH adenosine triphosphate and guanosine triphosphate and 2'-deoxy cytidine triphosphate and thymidine triphosphate is referred to as a rRdY mixture and aptamers selected therefrom are reffered to as "rRdY' aptamers. A "mRmY" aptamer is an aptamer containing only 2'-OMe nucleotides except for the starting nucleotide which is 2'-hydroxy.

[0046] In those instances where an RNA library is to be used as the starting library it is typically generated by synthesizing a DNA library, optionally PCR amplifying, then transcribing the DNA library *in vitro* using T7 RNA polymerase or modified T7 RNA polymerases, and purifying the transcribed library.

[0047] Starting with a mixture containing the starting library of nucleic acids, the SELEX™ method includes steps of: (a) contacting the mixture with the target under conditions favorable for binding; (b) partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules; (c) dissociating the nucleic acid-target complexes; (d) amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids; and (e) reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific, high affinity nucleic acid ligands to the target molecule. In those instances where RNA aptamers are being selected, the SELEX™ method further comprises the steps of: (i) reverse transcribing the nucleic acids dissociated from the nucleic acid-target complexes

before amplification in step (d); and (ii) transcribing the amplified nucleic acids from step (d) before restarting the process.

[0048] Cycles of selection and amplification are repeated until a desired goal is achieved. In the most general case, selection/amplification is continued until no significant improvement in binding strength is achieved on repetition of the cycle.

[0049] As part of the SELEX™ process, the sequences selected to bind to the target are then optionally minimized to determine the minimal sequence having the desired binding affinity. Random or directed mutagenesis of the selected sequence and/or the minimized sequences may, optionally, be performed to increase binding affinity or alternatively to determine which positions in the sequence are essential for binding activity. For instance, "doped reselections" may be used to explore the sequence requirements within an aptamer or minimized aptamer. Doped selections are SELEX™ *in vitro* selection iterations carried out with a synthetic, degenerate pool that has been designed based on the aptamer sequence of interest. The level of degeneracy usually varies from 70% to 85% wild type nucleotide. Neutral mutations or in some cases sequence changes can result in improvements in affinity. The composite sequence information can be used to identify a minimal binding motif and/or aid in optimization efforts.

APTAMER MEDICINAL CHEMISTRY

[0050] Aptamer medicinal chemistry is used to improve aptamer characteristics, to achieve particular, *e.g.*, therapeutic, criteria. Aptamer medicinal chemistry is performed following selection of the aptamer of interest and typically following the optional minimization and mutagenesis steps described above.

[0051] In one embodiment of the invention, aptamer medicinal chemistry uses a strategy in which sets of variant aptamers are chemically synthesized. These sets of variants typically differ from the parent aptamer by the substitution of a single nucleotide or other residue in place of a starting nucleotide or other residue. The substituted nucleotide or other residue differs from the one it is replacing by at least one chemical modification. In the context of a nucleotide, the chemical modification may occur at the nucleotide base, sugar or phosphate

positon. In the methods of the present invention, where the chemical modification is at a base position in a nucleotide the chemical modification does not result in the interconversion of one nucleotide for another within the group of A, U, G, C or within the group A, T, G, C.

[0052] Within the set of variant aptamers, the variant aptamers differ from each other by the location of the substituted nucleotide or other residue ('substituent"). These variants are then compared to each other and to the parent. Improvements in characteristics, particularly binding affinity, may be profound enough that the inclusion of a single substituent may be all that is necessary to achieve a particular therapeutic criterion.

[0053] Alternatively the information gleaned from the set of single variants may be used to design further sets of variants in which more than one substituted nucleotide or other residue is introduced simultaneously. In one design strategy, all of the single substituent variants are ranked based on the improvement conferred by the single substituent on therapeutic criteria, the top 4 are chosen and all possible double (6), triple (4) and quadruple (1) combinations of these 4 single substituent variants are synthesized and assayed. In a second design strategy, the best single substituent variant is considered to be the new parent and all possible double substituent variants that include this highest-ranked single substituent variant are synthesized and assayed. In another design strategy, single substituent variants in which the substitution did not significantly adversely affect binding affinity are combined with other single substitution variants, synthesized and assayed. Furthermore, single subsituent variants that do not significantly adversely effect binding affinity and/or those that increased binding affinity may be combined with a second type of substitution, synthesized and assayed. For example an inosine substitution may be combined with a substitution of a 2'-deoxy nucleotide for a 2'-OMe nucleotide to arrive at a variant having higher affinity relative to the unsubstitued starting aptamer or either singly substituted parent.

[0054] Other strategies may be used, and these strategies may be applied repeatedly such that the number of substituents is gradually increased while continuing to identify further-improved variants. For example, some substitution strategies use block substitutions. Particularly, the secondary structure of the aptamer may be predicted and based on predicted secondary structure blocks of nucleotides from the parent aptamer may be replaced with modified blocks of nucleotides. For example, where the predicted secondary structure

comprises a stem loop structure, the nucleotide blocks comprised in the predicted stem may be replaced with modified nucleotides (*e.g.* 2'-OMe nucleotides) as well as those completing the loop. Blocks that increase affinity are retained. Blocks that do not increase affinity may be further charcterized using the single substitution strategy within that block region.

[0055] In one embodiment, stabilizing substitutions, *e.g.* 2'-OMe substitutions for nuclease resistance, may be introduced into an aptamer that actually reduce the binding affinity of substituted aptamer relative to the unsubstituted starting aptamer. A second substitution, *e.g.* a phosphorothioate substitution, may be introduced into the substituted aptamer and assayed for binding affinity equivalent to or better than that of the unsubstituted starting aptamer.

[0056] Aptamer Medicinal Chemistry may be used particularly as a method to explore the local, rather than the global, introduction of substituents. Because aptamers are discovered within libraries that are generated by transcription, any substituents that are introduced during the SELEX™ process must be introduced globally. For example, if it is desired to introduce phosphorothioate linkages between nucleotides then they can only be introduced at every A (or every G, C, T, U etc.) (globally substituted). Aptamers which require phosphorothioates at some As (or some G, C, T, U etc.) (locally substituted) to achieve a desired thereapeutic criteria, but cannot tolerate it at other As, cannot be readily discovered by this process.

[0057] The types of substituent that can be utilized by the Aptamer Medicinal Chemistry process are not limited to nucleotides alone rather they are substituents that may be generated as solid-phase synthesis reagents and are capable of introduction into an oligomer synthesis scheme. Aptamer Medicinal Chemistry schemes may include substituents that introduce steric bulk, hydrophobicity, hydrophilicity, lipophilicity, lipophobicity, positive charge, negative charge, neutral charge, zwitterions, polarizability, nuclease-resistance, conformational rigidity, conformational flexibility, protein-binding characteristics, mass, etc. Aptamer Medicinal Chemistry schemes may include base-modifications, sugar-modifications or phosphodiester linkage-modifications.

[0058] When considering the kinds of substituents that are likely to be beneficial within the context of a therapeutic aptamer, it may be preferred to introduce substitutions that fall into one or more of the following categories:

(1) Substituents, that are naturally occurring, *e.g.*, 2'-deoxy, 2'-ribo, 2'-OMe purines or pyrimidines or 2'-deoxy-5-methyl cytidine.

(2) Substituents already part of an approved therapeutic, *e.g.*, phosphorothioate-linked oligonucleotides.

(3) Substituents that hydrolyze or degrade to one of the above two categories, *e.g.*, methylphosphonate-linked oligonucleotides.

[0059] The aptamers of the present invention can be synthesized using any oligonucleotide synthesis techniques known in the art including solid phase oligonucleotide synthesis techniques (see, *e.g.*, Froehler *et al.*, Nucl. Acid Res. 14:5399-5467 (1986) and Froehler *et al.*, Tet. Lett. 27:5575-5578 (1986)) and solution phase methods such as triester synthesis methods (see, *e.g.*, Sood *et al.*, Nucl. Acid Res. 4:2557 (1977) and Hirose *et al.*, Tet. Lett., 28:2449 (1978)).

MODULATION OF PHARMACOKINETICS AND BIODISTRIBUTION OF APTAMER THERAPEUTICS

[0060] It is important that the pharmacokinetic properties for all oligonucleotide-based therapeutics, including aptamers, be tailored to match the desired pharmaceutical application. While aptamers directed against extracellular targets do not suffer from difficulties associated with intracellular delivery (as is the case with antisense and RNAi-based therapeutics), such aptamers must still be able to be distributed to target organs and tissues, and remain in the body (unmodified) for a period of time consistent with the desired dosing regimen.

[0061] Thus, the present invention provides materials and methods to affect the pharmacokinetics of aptamer compositions, and, in particular, the ability to tune aptamer pharmacokinetics. The tunability of (*i.e.*, the ability to modulate) aptamer pharmacokinetics is achieved through conjugation of modifying moieties (*e.g.*, PEG polymers) to the aptamer and/or the incorporation of modified nucleotides (*e.g.*, 2'-fluoro or 2'-OMe) to alter the chemical composition of the nucleic acid. The ability to tune aptamer pharmacokinetics is

used in the improvement of existing therapeutic applications, or alternatively, in the development of new therapeutic applications. For example, in some therapeutic applications, *e.g.*, in anti-neoplastic or acute care settings where rapid drug clearance or turn-off may be desired, it is desirable to decrease the residence times of aptamers in the circulation. Alternatively, in other therapeutic applications, *e.g.*, maintenance therapies where systemic circulation of a therapeutic is desired, it may be desirable to increase the residence times of aptamers in circulation.

[0062] In addition, the tunability of aptamer pharmacokinetics is used to modify the biodistribution of an aptamer therapeutic in a subject. For example, in some therapeutic applications, it may be desirable to alter the biodistribution of an aptamer therapeutic in an effort to target a particular type of tissue or a specific organ (or set of organs). In these applications, the aptamer therapeutic preferentially accumulates in a specific tissue or organ(s). In other therapeutic applications, it may be desirable to target tissues displaying a cellular marker or a symptom associated with a given disease, cellular injury or other abnormal pathology, such that the aptamer therapeutic preferentially accumulates in the affected tissue. For example, as described in the provisional application United States Serial No. 60/550,790, filed on March 5, 2004, and entitled "Controlled Modulation of the Pharmacokinetics and Biodistribution of Aptamer Therapeutics", and in the non-provisional application United States Serial No. 11/075,648, filed on March 7, 2005, and entitled "Controlled Modulation of the Pharmacokinetics and Biodistribution of Aptamer Therapeutics", PEGylation of an aptamer therapeutic (*e.g.*, PEGylation with a 20 kDa PEG polymer) is used to target inflamed tissues, such that the PEGylated aptamer therapeutic preferentially accumulates in inflamed tissue.

[0063] To determine the pharmacokinetic and biodistribution profiles of aptamer therapeutics (*e.g.*, aptamer conjugates or aptamers having altered chemistries, such as modified nucleotides) a variety of parameters are monitored. Such parameters include, for example, the half-life ($t_{1/2}$), the plasma clearance (CL), the volume of distribution (Vss), the area under the concentration-time curve (AUC), maximum observed serum or plasma concentration (C_{max}), and the mean residence time (MRT) of an aptamer composition. As used herein, the term "AUC" refers to the area under the plot of the plasma concentration of

an aptamer therapeutic versus the time after aptamer administration. The AUC value is used to estimate the bioavailability (*i.e.*, the percentage of administered aptamer therapeutic in the circulation after aptamer administration) and/or total clearance (CL) (*i.e.*, the rate at which the aptamer therapeutic is removed from circulation) of a given aptamer therapeutic. The volume of distribution relates the plasma concentration of an aptamer therapeutic to the amount of aptamer present in the body. The larger the Vss, the more an aptamer is found outside of the plasma (*i.e.*, the more extravasation).

[0064] The present invention provides materials and methods to modulate, in a controlled manner, the pharmacokinetics and biodistribution of stabilized aptamer compositions *in vivo* by conjugating an aptamer to a modulating moiety such as a small molecule, peptide, or polymer terminal group, or by incorporating modified nucleotides into an aptamer. As described herein, conjugation of a modifying moiety and/or altering nucleotide(s) chemical composition alters fundamental aspects of aptamer residence time in circulation and distribution to tissues.

[0065] In addition to clearance by nucleases, oligonucleotide therapeutics are subject to elimination *via* renal filtration. As such, a nuclease-resistant oligonucleotide administered intravenously typically exhibits an *in vivo* half-life of <10 min, unless filtration can be blocked. This can be accomplished by either facilitating rapid distribution out of the blood stream into tissues or by increasing the apparent molecular weight of the oligonucleotide above the effective size cut-off for the glomerulus. Conjugation of small therapeutics to a PEG polymer (PEGylation), described below, can dramatically lengthen residence times of aptamers in circulation, thereby decreasing dosing frequency and enhancing effectiveness against vascular targets.

[0066] Aptamers can be conjugated to a variety of modifying moieties, such as high molecular weight polymers, *e.g.*, PEG; peptides, *e.g.*, Tat (a 13-amino acid fragment of the HIV Tat protein (Vives, *et al.* (1997), J. Biol. Chem. 272(25): 16010-7)), Ant (a 16-amino acid sequence derived from the third helix of the Drosophila antennapedia homeotic protein (Pietersz, *et al.* (2001), Vaccine 19(11-12): 1397-405)) and Arg_7 (a short, positively charged cell-permeating peptides composed of polyarginine (Arg_7) (Rothbard, *et al.* (2000), Nat. Med. 6(11): 1253-7; Rothbard, J *et al.* (2002), J. Med. Chem. 45(17): 3612-8)); and small

molecules, *e.g.*, lipophilic compounds such as cholesterol. Among the various conjugates described herein, *in vivo* properties of aptamers are altered most profoundly by complexation with PEG groups. For example, complexation of a mixed 2'F and 2'-OMe modified aptamer therapeutic with a 20 kDa PEG polymer hinders renal filtration and promotes aptamer distribution to both healthy and inflamed tissues. Furthermore, the 20 kDa PEG polymer-aptamer conjugate proves nearly as effective as a 40 kDa PEG polymer in preventing renal filtration of aptamers. While one effect of PEGylation is on aptamer clearance, the prolonged systemic exposure afforded by presence of the 20 kDa moiety also facilitates distribution of aptamer to tissues, particularly those of highly perfused organs and those at the site of inflammation. The aptamer-20 kDa PEG polymer conjugate directs aptamer distribution to the site of inflammation, such that the PEGylated aptamer preferentially accumulates in inflamed tissue. In some instances, the 20 kDa PEGylated aptamer conjugate is able to access the interior of cells, such as, for example, kidney cells.

[0067] Modified nucleotides can also be used to modulate the plasma clearance of aptamers. For example, an unconjugated aptamer which incorporates both 2'-F and 2'-OMe stabilizing chemistries, which is typical of current generation aptamers as it exhibits a high degree of nuclease stability *in vitro* and *in vivo,* displays rapid loss from plasma (*i.e.*, rapid plasma clearance) and a rapid distribution into tissues, primarily into the kidney, when compared to unmodified aptamer.

PEG-DERIVATIZED NUCLEIC ACIDS

[0068] As described above, derivatization of nucleic acids with high molecular weight non-immunogenic polymers has the potential to alter the pharmacokinetic and pharmacodynamic properties of nucleic acids making them more effective therapeutic agents. Favorable changes in activity can include increased resistance to degradation by nucleases, decreased filtration through the kidneys, decreased exposure to the immune system, and altered distribution of the therapeutic through the body.

[0069] The aptamer compositions of the invention may be derivatized with polyalkylene glycol ("PAG") moieties. Examples of PAG-derivatized nucleic acids are found in United States Patent Application Ser. No. 10/718,833, filed on November 21, 2003, which is herein

incorporated by reference in its entirety. Typical polymers used in the invention include polyethylene glycol ("PEG"), also known as polyethylene oxide ("PEO") and polypropylene glycol (including poly isopropylene glycol). Additionally, random or block copolymers of different alkylene oxides (*e.g.*, ethylene oxide and propylene oxide) can be used in many applications. In its most common form, a polyalkylene glycol, such as PEG, is a linear polymer terminated at each end with hydroxyl groups: $HO\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$. This polymer, alpha-, omega-dihydroxylpolyethylene glycol, can also be represented as HO-PEG-OH, where it is understood that the —PEG- symbol represents the following structural unit: $\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}$ where n typically ranges from about 4 to about 10,000.

[0070] As shown, the PEG molecule is di-functional and is sometimes referred to as "PEG diol." The terminal portions of the PEG molecule are relatively non-reactive hydroxyl moieties, the –OH groups, that can be activated, or converted to functional moieties, for attachment of the PEG to other compounds at reactive sites on the compound. Such activated PEG diols are referred to herein as bi-activated PEGs. For example, the terminal moieties of PEG diol have been functionalized as active carbonate ester for selective reaction with amino moieties by substitution of the relatively non-reactive hydroxyl moieties, -OH, with succinimidyl active ester moieties from N-hydroxy succinimide.

[0071] In many applications, it is desirable to cap the PEG molecule on one end with an essentially non-reactive moiety so that the PEG molecule is mono-functional (or mono-activated). In the case of protein therapeutics which generally display multiple reaction sites for activated PEGs, bi-functional activated PEGs lead to extensive cross-linking, yielding poorly functional aggregates. To generate mono-activated PEGs, one hydroxyl moiety on the terminus of the PEG diol molecule typically is substituted with non-reactive methoxy end moiety, $\text{-}OCH_3$. The other, un-capped terminus of the PEG molecule typically is converted to a reactive end moiety that can be activated for attachment at a reactive site on a surface or a molecule such as a protein.

[0072] PAGs are polymers which typically have the properties of solubility in water and in many organic solvents, lack of toxicity, and lack of immunogenicity. One use of PAGs is to covalently attach the polymer to insoluble molecules to make the resulting PAG-molecule

"conjugate" soluble. For example, it has been shown that the water-insoluble drug paclitaxel, when coupled to PEG, becomes water-soluble. Greenwald, *et al., J. Org. Chem.*, 60:331-336 (1995). PAG conjugates are often used not only to enhance solubility and stability but also to prolong the blood circulation half-life of molecules.

[0073] Polyalkylated compounds of the invention are typically between 5 and 80 kDa in size however any size can be used, the choice dependent on the aptamer and application. Other PAG compounds of the invention are between 10 and 80 kDa in size. Still other PAG compounds of the invention are between 10 and 60 kDa in size. For example, a PAG polymer may be at least 10, 20, 30, 40, 50, 60, or 80 kDa in size. Such polymers can be linear or branched. In some embodiments the polymers are PEG. In some embodiment the polymers are branched PEG. In still other embodiments the polymers are 40kDa branched PEG as depicted in Figure 2. In some embodiments the 40 kDa branched PEG is attached to the 5' end of the aptamer as depicted in Figure 3.

[0074] In contrast to biologically-expressed protein therapeutics, nucleic acid therapeutics are typically chemically synthesized from activated monomer nucleotides. PEG-nucleic acid conjugates may be prepared by incorporating the PEG using the same iterative monomer synthesis. For example, PEGs activated by conversion to a phosphoramidite form can be incorporated into solid-phase oligonucleotide synthesis. Alternatively, oligonucleotide synthesis can be completed with site-specific incorporation of a reactive PEG attachment site. Most commonly this has been accomplished by addition of a free primary amine at the 5'-terminus (incorporated using a modifier phosphoramidite in the last coupling step of solid phase synthesis). Using this approach, a reactive PEG (*e.g.*, one which is activated so that it will react and form a bond with an amine) is combined with the purified oligonucleotide and the coupling reaction is carried out in solution.

[0075] The ability of PEG conjugation to alter the biodistribution of a therapeutic is related to a number of factors including the apparent size (*e.g.*, as measured in terms of hydrodynamic radius) of the conjugate. Larger conjugates (>10kDa) are known to more effectively block filtration via the kidney and to consequently increase the serum half-life of small macromolecules (*e.g.*, peptides, antisense oligonucleotides). The ability of PEG conjugates to block filtration has been shown to increase with PEG size up to approximately

50 kDa (further increases have minimal beneficial effect as half life becomes defined by macrophage-mediated metabolism rather than elimination via the kidneys).

[0076] Production of high molecular weight PEGs (>10 kDa) can be difficult, inefficient, and expensive. As a route towards the synthesis of high molecular weight PEG-nucleic acid conjugates, previous work has been focused towards the generation of higher molecular weight activated PEGs. One method for generating such molecules involves the formation of a branched activated PEG in which two or more PEGs are attached to a central core carrying the activated group. The terminal portions of these higher molecular weight PEG molecules, *i.e.*, the relatively non-reactive hydroxyl (–OH) moieties, can be activated, or converted to functional moieties, for attachment of one or more of the PEGs to other compounds at reactive sites on the compound. Branched activated PEGs will have more than two termini, and in cases where two or more termini have been activated, such activated higher molecular weight PEG molecules are referred to herein as, multi-activated PEGs. In some cases, not all termini in a branch PEG molecule are activated. In cases where any two termini of a branch PEG molecule are activated, such PEG molecules are referred to as bi-activated PEGs. In some cases where only one terminus in a branch PEG molecule is activated, such PEG molecules are referred to as mono-activated. As an example of this approach, activated PEG prepared by the attachment of two monomethoxy PEGs to a lysine core which is subsequently activated for reaction has been described (Harris *et al.*, Nature, vol.2: 214-221, 2003).

[0077] The present invention provides another cost effective route to the synthesis of high molecular weight PEG-nucleic acid (preferably, aptamer) conjugates including multiply PEGylated nucleic acids. The present invention also encompasses PEG-linked multimeric oligonucleotides, *e.g.*, dimerized aptamers. The present invention also relates to high molecular weight compositions where a PEG stabilizing moiety is a linker which separates different portions of an aptamer, *e.g.*, the PEG is conjugated within a single aptamer sequence, such that the linear arrangement of the high molecular weight aptamer composition is, *e.g.*, nucleic acid – PEG – nucleic acid (– PEG — nucleic acid)$_n$ where n is greater than or equal to 1.

[0078] High molecular weight compositions of the invention include those having a molecular weight of at least 10 kDa. Compositions typically have a molecular weight

between 10 and 80 kDa in size. High molecular weight compositions of the invention are at least 10, 20, 30, 40, 50, 60, or 80 kDa in size.

[0079] A stabilizing moiety is a molecule, or portion of a molecule, which improves pharmacokinetic and pharmacodynamic properties of the high molecular weight aptamer compositions of the invention. In some cases, a stabilizing moiety is a molecule or portion of a molecule which brings two or more aptamers, or aptamer domains, into proximity, or provides decreased overall rotational freedom of the high molecular weight aptamer compositions of the invention. A stabilizing moiety can be a polyalkylene glycol, such a polyethylene glycol, which can be linear or branched, a homopolymer or a heteropolymer. Other stabilizing moieties include polymers such as peptide nucleic acids (PNA). Oligonucleotides can also be stabilizing moieties; such oligonucleotides can include modified nucleotides, and/or modified linkages, such as phosphorothioates. A stabilizing moiety can be an integral part of an aptamer composition, *i.e.*, it is covalently bonded to the aptamer.

[0080] Compositions of the invention include high molecular weight aptamer compositions in which two or more nucleic acid moieties are covalently conjugated to at least one polyalkylene glycol moiety. The polyalkylene glycol moieties serve as stabilizing moieties. In compositions where a polyalkylene glycol moiety is covalently bound at either end to an aptamer, such that the polyalkylene glycol joins the nucleic acid moieties together in one molecule, the polyalkylene glycol is said to be a linking moiety. In such compositions, the primary structure of the covalent molecule includes the linear arrangement nucleic acid-PAG-nucleic acid. One example is a composition having the primary structure nucleic acid-PEG-nucleic acid. Another example is a linear arrangement of: nucleic acid – PEG – nucleic acid – PEG — nucleic acid.

[0081] To produce the nucleic acid—PEG—nucleic acid conjugate, the nucleic acid is originally synthesized such that it bears a single reactive site (*e.g.*, it is mono-activated). In a preferred embodiment, this reactive site is an amino group introduced at the 5'-terminus by addition of a modifier phosphoramidite as the last step in solid phase synthesis of the oligonucleotide. Following deprotection and purification of the modified oligonucleotide, it is reconstituted at high concentration in a solution that minimizes spontaneous hydrolysis of the activated PEG. In a preferred embodiment, the concentration of oligonucleotide is 1 mM and

the reconstituted solution contains 200 mM $NaHCO_3$-buffer, pH 8.3. Synthesis of the conjugate is initiated by slow, step-wise addition of highly purified bi-functional PEG. In a preferred embodiment, the PEG diol is activated at both ends (bi-activated) by derivatization with succinimidyl propionate. Following reaction, the PEG-nucleic acid conjugate is purified by gel electrophoresis or liquid chromatography to separate fully-, partially-, and un-conjugated species. Multiple PAG molecules concatenated (*e.g.*, as random or block copolymers) or smaller PAG chains can be linked to achieve various lengths (or molecular weights). Non-PAG linkers can be used between PAG chains of varying lengths.

[0082] The 2'-OMe, 2'-fluoro and other modified nucleotide modifications stabilize the aptamer against nucleases and increase its half life *in vivo*. The 3'-3'-dT cap, or other nucleotide cap, abasic or amine group also increases exonuclease resistance. See, *e.g.*, U.S. Patents 5,674,685; 5,668,264; 6,207,816; and 6,229,002, each of which is incorporated by reference herein in its entirety.

PAG-DERIVATIZATION OF A REACTIVE NUCLEIC ACID

[0083] High molecular weight PAG-nucleic acid-PAG conjugates can be prepared by reaction of a mono-functional activated PEG with a nucleic acid containing more than one reactive site. In one embodiment, the nucleic acid is bi-reactive, or bi-activated, and contains two reactive sites: a 5'-amino group and a 3'-amino group introduced into the oligonucleotide through conventional phosphoramidite synthesis, for example: 3'-5'-di-PEGylation as illustrated in Figure 4. In alternative embodiments, reactive sites can be introduced at internal positions, using for example, the 5-position of pyrimidines, the 8-position of purines, or the 2'-position of ribose as sites for attachment of primary amines. In such embodiments, the nucleic acid can have several activated or reactive sites and is said to be multiply activated. Following synthesis and purification, the modified oligonucleotide is combined with the mono-activated PEG under conditions that promote selective reaction with the oligonucleotide reactive sites while minimizing spontaneous hydrolysis. In the preferred embodiment, monomethoxy-PEG is activated with succinimidyl propionate and the coupled reaction is carried out at pH 8.3. To drive synthesis of the bi-substituted PEG, stoichiometric excess PEG is provided relative to the oligonucleotide. Following reaction, the PEG-nucleic

acid conjugate is purified by gel electrophoresis or liquid chromatography to separate fully, partially, and un-conjugated species.

[0084] The linking domains can also have one or more polyalkylene glycol moieties attached thereto. Such PAGs can be of varying lengths and may be used in appropriate combinations to achieve the desired molecular weight of the composition.

[0085] The effect of a particular linker can be influenced by both its chemical composition and length. A linker that is too long, too short, or forms unfavorable steric and/or ionic interactions with the target will preclude the formation of complex between aptamer and the target. A linker, which is longer than necessary to span the distance between nucleic acids, may reduce binding stability by diminishing the effective concentration of the ligand. Thus, it is often necessary to optimize linker compositions and lengths in order to maximize the affinity of an aptamer to a target.

PHARMACEUTICAL COMPOSITIONS

[0086] The invention also includes pharmaceutical compositions containing modified aptamers of the invention. In some embodiments, the compositions are suitable for internal use and include an effective amount of a pharmacologically active aptamer of the invention, alone or in combination, with one or more pharmaceutically acceptable carriers. The compounds are especially useful in that they have very low, if any toxicity.

[0087] Compositions of the invention can be used to treat or prevent a pathology, such as a disease or disorder, or alleviate the symptoms of such disease or disorder in a patient

[0088] Compositions of the invention are useful for administration to a subject suffering from, or predisposed to, a disease or disorder which is related to or derived from a target to which the aptamers of the invention specifically bind. The method involves administering to the patient or subject an aptamer or a composition comprising aptamers that bind the target (*e.g.*, a protein) involved with the pathology, so that binding of the aptamer to the target alters the biological function of the target, thereby treating the pathology.

[0089] The patient or subject having a pathology, *i.e.,* the patient or subject treated by the methods of this invention, can be a vertebrate, more particularly a mammal, or more particularly a human.

[0090] In practice, the aptamers or their pharmaceutically acceptable salts, are administered in amounts which will be sufficient to exert their desired biological activity, *e.g.*, inhibiting the binding of the aptamer target to the target's receptor.

[0091] Therapeutic or pharmacological compositions of the present invention will generally comprise an effective amount of the aptamer, dissolved or dispersed in a pharmaceutically acceptable medium. Pharmaceutically acceptable media or carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the therapeutic compositions of the present invention.

[0092] The preparation of pharmaceutical or pharmacological compositions will be known to those of skill in the art in light of the present disclosure. Typically, such compositions may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection; as tablets or other solids for oral administration; as time release capsules; or in any other form currently used, including eye drops, creams, lotions, salves, inhalants and the like. The use of sterile formulations, such as saline-based washes, by surgeons, physicians or health care workers to treat a particular area in the operating field may also be particularly useful. Compositions may also be delivered via microdevice, microparticle or sponge.

[0093] Upon formulation, therapeutics will be administered in a manner compatible with the dosage formulation, and in such amount as is pharmacologically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

[0094] In this context, the quantity of active ingredient and volume of composition to be administered depends on the host animal to be treated. Precise amounts of active compound

required for administration depend on the judgment of the practitioner and are peculiar to each individual.

[0095] A minimal volume of a composition required to disperse the active compounds is typically utilized. Suitable regimes for administration are also variable, but would be typified by initially administering the compound and monitoring the results and then giving further controlled doses at further intervals.

[0096] The pharmaceutical compositions may be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure and/or buffers. In addition, they may also contain other therapeutically valuable substances. The compositions are prepared according to conventional mixing, granulating, or coating methods, and typically contain about 0.1% to 75%, preferably about 1% to 50%, of the active ingredient.

[0097] Liquid, particularly injectable compositions can, for example, be prepared by dissolving, dispersing, etc. The active compound is dissolved in or mixed with a pharmaceutically pure solvent such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form the injectable solution or suspension. Additionally, solid forms suitable for dissolving in liquid prior to injection can be formulated.

[0098] The compounds of the present invention can be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions.

[0099] Parenteral injectable administration is generally used for subcutaneous, intramuscular or intravenous injections and infusions. Additionally, one approach for parenteral administration employs the implantation of a slow-release or sustained-released systems, which assures that a constant level of dosage is maintained, according to U.S. Pat. No. 3,710,795, incorporated herein by reference.

[00100] Furthermore, preferred compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, inhalants, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in

that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen. Other preferred topical preparations include creams, ointments, lotions, aerosol sprays and gels, wherein the concentration of active ingredient would typically range from 0.01% to 15%, w/w or w/v.

[00101] For solid compositions, excipients include pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. The active compound defined above, may be also formulated as suppositories, using for example, polyalkylene glycols, for example, propylene glycol, as the carrier. In some embodiments, suppositories are advantageously prepared from fatty emulsions or suspensions.

[00102] The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, containing cholesterol, stearylamine or phosphatidylcholines. In some embodiments, a film of lipid components is hydrated with an aqueous solution of drug to a form lipid layer encapsulating the drug, as described in U.S. Pat. No. 5,262,564. For example, the aptamer molecules described herein can be provided as a complex with a lipophilic compound or non-immunogenic, high molecular weight compound constructed using methods known in the art. Additionally, liposomes may bear aptamers on their surface for targeting and carrying cytotoxic agents internally to mediate cell killing. An example of nucleic-acid associated complexes is provided in U.S. Patent No. 6,011,020.

[00103] The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropyl-methacrylamide-phenol, polyhydroxyethylaspanamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

[00104] If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and other substances such as for example, sodium acetate, and triethanolamine oleate.

[00105] The dosage regimen utilizing the aptamers is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular aptamer or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition.

[00106] Oral dosages of the present invention, when used for the indicated effects, will range between about 0.05 to 7500 mg/day orally. The compositions are preferably provided in the form of scored tablets containing 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 mg of active ingredient. Infused dosages, intranasal dosages and transdermal dosages will range between 0.05 to 7500 mg/day. Subcutaneous, intravenous and intraperitoneal dosages will range between 0.05 to 3800 mg/day. Effective plasma levels of the compounds of the present invention range from 0.002 mg/mL to 50 mg/mL. Compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily.

[00107] All publications and patent documents cited herein are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an admission that any is pertinent prior art, nor does it constitute any admission as to the contents or date of the same. The invention having now been described by way of written description, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples below are for purposes of illustration and not limitation of the claims that follow.

EXAMPLES

[00108] Three aptamers ARC979, ARC445 and ARC1172, each with high affinity and specificity to a different protein target, were identified and minimized according to the SELEX™ method. ARC979 (SEQ ID NO 1) is a 34 nucleotide aptamer to IL-23 containing 2'-deoxy purines and 2'-OMe pyrimidines ("dRmY" composition) with the following sequence:

5'-ACAGGCAAGUAAUUGGGGAGUGCGGGCGGGGUGU-3. ARC445 (SEQ ID NO 2) is a 23 nucleotide aptamer to IgE containing 2'-deoxy purines and 2'-OMe pyrimidines ("dRmY" composition) with the following sequence: 5'-AGCCUGGGGACCCAUGGGGGGCU-3'. ARC1172 (SEQ ID NO 3) is a 41 nucleotide DNA aptamer to von Willebrand Factor ("vWF") with the following sequence: 5'-GGCGTGCAGTGCCTTCGGCCGTGCGGTGCCTCCGTCACGCC-3'.

[00109] Aptamer Medicinal Chemistry was applied to ARC979, ARC445, and ARC1172 as described below resulting in derivative aptamers having superior characteristics relative to their respective parent or starting aptamer.

[00110] The binding affinities of the aptamers described herein to their respective targets were measured using a dot blot binding assay allowing determination of the aptamer target dissociation constant ((K_D). For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ-^{32}P ATP, combined with a dilution series of the target protein and incubated at room temperature for 30 minutes under the binding reaction conditions described below. The binding reactions were analyzed by nitrocellulose filtration using a Minifold I dot-blot, 96-well vacuum filtration manifold (Schleicher & Schuell, Keene, NH). A three-layer filtration medium was used, consisting (from bottom to top) of Protran nitrocellulose (Schleicher & Schuell), Hybond-P nylon (Amersham Biosciences) and GB002 gel blot paper (Schleicher & Schuell). RNA that is bound to protein is captured on the nitrocellulose filter, whereas the non-protein bound RNA is captured on the nylon filter. The gel blot paper was included simply as a supporting medium for the other filters. Following filtration, the filter layers were separated, dried and

exposed on a phosphor screen (Amersham Biosciences, Piscataway, NJ) and quantified using a Storm 860 Phosphorimager® blot imaging system (Amersham Biosciences).

EXAMPLE 1: INCREASED APTAMER TARGET AFFINITY BY PHOSPHOROTHIOATE SUBSTITUTION

EXAMPLE 1A: Phosphorothioate substitution in the anti-IL-23 aptamer ARC979

[00111] A set of ARC979 (SEQ ID NO 1) derivative aptamers introducing single phosphorothioate substitutions (Glen Research, Sterling, VA) were chemically synthesized by standard synthesis techniques. In each set of derivatives, single phosphorothioate substitutions were systematically introduced at each internucleotide linkage position resulting in each derivative within one set having an aptamer with a phosphorothioate substitution at a different internucleotide position. These derivatives were gel purified and assayed for IL-23 binding, using the dot blot assay described above under the following binding reaction conditions: 1X PBS (without Ca^{++} or Mg^{++}) plus 0.1 mg/mL BSA, for a 30 minute incubation at room temperature. For the ARC979 derivative binding, the fraction aptamer bound vs. full length human IL-23 concentration was used to calculate the K_D by fitting the following equation to the data:

Fraction aptamer bound = amplitude*([IL-23]/(K_D + [IL-23])) + background binding.

[00112] The inclusion of single phosphorothioate substitutions in ARC979 did not adversely affect binding affinity in most positions (*i.e.*, result in a significantly higher K_D value) when compared to the binding affinity of the starting aptamer ARC979. Point phosphorothioate substitutions in the internucleotide bond between two nucleotides at six positions (as shown in Table 1 and Figure 5) resulted in improved target binding affinity (*i.e.*, lower K_D values).

[00113] The nucleotide sequences for the ARC979 single phosphorothioate derivatives showing increased affinity as compared to the parent ARC979 are listed in Table 1. Unless noted otherwise, each of the sequences listed in Table 1 are in the 5'-3' direction. In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in

Table 1 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 1, where lacking, additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, "s" denotes an internucleotide phosphorothioate substitution.

TABLE 1: Sequences and K_Ds of selected ARC979 phosphorothioate substituted derivatives

SEQ ID NO	ARC#	Phosphoro-thioate interucleotide linkage position	Sequence (5' -> 3'), (3T = inv dT), (T=dT), (s=phosphorothioate), (mN = 2'-O Methyl containing residue)	K_D (nM)
1	ARC979	none	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdGmUdGmCdGdGdGmCdGdGdGdGmUdGmU	90
4	ARC1160	18, 19	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdG-s-dAdGmUdGmCdGdGdGmCdGdGdGdGdGmUdGmU	38
5	ARC1161	19, 20	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdA-s-dGmUdGmCdGdGdGmCdGdGdGdGdGmUdGmU	55
6	ARC1162	20, 21	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdG-s-mUdGmCdGdGdGmCdGdGdGdGmUdGmU	47
7	ARC1163	21-22	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdGmU-s-dGmCdGdGdGmCdGdGdGdGmUdGmU	49

8	ARC1164	22-23	dAmCdAdGdGmCdAdAdGmUd AdAmUmUdGdGdGdGdAdGm UdG-s-mCdGdGdGmCdGdGdGdGmUd GmU	79
9	ARC1165	26-27	dAmCdAdGdGmCdAdAdGmUd AdAmUmUdGdGdGdGdAdGm UdGmCdGdGdG-s-mCdGdGdGdGmUdGmU	55

[00114] EXAMPLE 1B: Phosphorothioate substitution in anti-vWF aptamers

A set of ARC1172 (SEQ ID NO 3) derivative aptamers introducing single phosphorothioate substitutions (Glen Research, Sterling, VA) were chemically synthesized using standard synthesis techniques. In each set of derivatives, single phosphorothioate substitutions were systematically introduced at each internucleotide linkage position resulting in each derivative set having an aptamer with a phosphorothioate substitution at a different internucleotide position. These derivatives were gel purified and assayed for vWF binding, using the dot blot assay described above under the following binding reaction conditions: 1X Dulbecco's PBS buffer which includes 0.1 mg/mL BSA, Ca^{++} and $Mg^{++}$and incubated with labeled aptamer for 30 minutes at 24°C. K_D values for the ARC1172 derivative aptamers were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00115] The systematic inclusion of single phosphorothioate linkages in the ARC1172 derivative aptamers did not adversely affect binding affinity in most positions and yielded one aptamer having an improved (*i.e.*, lower) K_D value as compared to the parent, ARC1172. As indicated in Table 2, a single phosphorothioate substitution between the G at position 21 and the T at position 22 resulted in a construct which showed measurable improvement in affinity (*i.e.*, lower K_D value) relative to the parent molecule, ARC1172.

[00116] In some embodiments, the invention comprises aptamers with γ–^{32}P nucleic acid sequences as described in Table 2 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 2 additionally comprise a 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 2 lack the indicated 3' cap (*e.g.*, a 3' inverted dT cap (3T)). Lower case letter "d" denotes 2'-deoxy modification and "s" denotes an internucleotide phosphorothioate substitution.

Table 2: Binding Affinity of ARC1172 and ARC1759

SEQ ID NO	ARC #	Phosphoro-thioate interucleotide linkage position	Sequence (5' -> 3'), phosphoramidite), (3T = inv dT), (T=dT), (s=phosphorothioate),	K_D (nM)
10	ARC1172 w/ 3T	------	dGdGdCdGTdGdCdAdGTdGdCdCTTdC dGdGdCdCdGTdGdCdGdGTdGdCdCTd CdCdGTdCdAdCdGdCdC-3T	2
11	ARC1759	21, 22	dGdGdCdGTdGdCdAdGTdGdCdCTTdC dGdGdCdCdG-s-TdGdCdGdGTdGdCdCTdCdCdGTdCdA dCdGdCdC-3T	0.7

[00117] Multiple modifications, including 2'-OMe stabilizing substitutions, were made in ARC1172 (SEQ ID NO 3), to arrive at ARC1361 (SEQ ID NO 12). ARC1361 (SEQ ID NO 12) served as the base sequence for introduction of single phosphorothioate phosphate backbone modifications that resulted in a set of nineteen phosphorothioate substituted ARC1361 derivatives each having a phosphorothioate substitution at a different position. As can been seen from Table 3 below, the multiple modifications introduced into ARC1172 to arrive at ARC1361 resulted in about a four fold reduction in binding affinity. A single phosphorothioate substitution increased the binding affinity of ARC1368 (SEQ ID NO 13) over four fold relative to the ARC1361 (SEQ ID NO 12) starting aptamer to roughly equal the binding affinity of the ARC1172 (SEQ ID NO 3) parent aptamer.

Table 3: Binding Affinity of ARC1172, ARC1361 and ARC1368

SEQ ID NO :	ARC #	Phosphoro-thioate internucleotide linkage positon	Sequence (5' -> 3'), (NH2 = 5'-hexylamine linker phosphoramidite), (3T = inv dT), (T=dT),	K_D (nM)

			(s=phosphorothioate), (mN = 2'-O Methyl containing residue), (PEG = polyethylene glycol), (dN=2'-deoxy residue)	
3	ARC1172	--	dGdGdCdGTdGdCdAdGTdGdCdCTTdCdGdGdCdCdGTdGdCdGdGTdGdCdCTdCdCdGTdCdAdCdGdCdC-3T	2
12	ARC1361	--	mGmCmGmUdGdCdAmGmUmGmCmCmUmUmCmGmGmCdCmGTmGdCdGdGTmGmCdCmUdCdCmGmUdCmAmCmGmC-3T	7.9
13	ARC1368	20, 21	mGmCmGmUdGdCdAmGmUmGmCmCmUmUmCmGmGmCdCmG-s-TmGdCdGdGTmGmCdCmUdCdCmGmUdCmAmCmGmC-3T	1.8

EXAMPLE 2: INCREASED APTAMER TARGET AFFINITY BY INOSINE SUBSTITUTION

EXAMPLE 2A: 2'-deoxy inosine substitution in the anti-IgE aptamer ARC1335

[00118] The effects of substituting purine residues with inosine on the binding affinity of ARC1335 (SEQ ID NO 14) derivatives as compared to the ARC1335 parent molecule was examined during the systematic replacement of 2'-deoxy purine residues with 2'-deoxy inosine (dI). ARC1335 is an ARC445 derivative containing 2'-OMe substitutions for 2'-deoxy purine residues at nucleotide positions 1, 2, 7, 10, 14, and 17 of ARC445. A set of aptamer derivatives replacing each 2'-deoxy guanosine one at a time with 2'-deoxy inosine was synthesized and tested for binding using the dot blot assay and the binding reaction

conditions previously described (Dulbecco's PBS (with Ca^{++} and Mg^{++}) plus 0.1 mg/mL BSA, room temperature for 30 minutes) to test whether 2'-deoxy inosine substitution improved affinity for human IgE and thus potency. K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00119] In the dI series of ARC1335 derivatives, substitutions adversely affected binding affinity at positions 6-9 (*i.e.*, increased K_D value) while they were tolerated from moderately to very well at positions 16-21 (*i.e.*, same or lower K_D value). As can be seen from Table 3 below, the results from the dI series yielded a number of constructs (ARC1562, ARC1564, and ARC1566) with nearly identical affinity (ARC1562 and ARC1564) and improved affinity (ARC1566) relative to the improved binding affinity conferred by both 2'-OMe substitutions as in ARC1335 (parent aptamer), and greatly improved affinity relative to the original aptamer ARC445 (SEQ ID NO 2).

[00120] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 4 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 4, where lacking, additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 4 lack the indicated 3' cap (*e.g.*, a 3' inverted dT cap (3T)). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, and "I" denotes an inosine substitution for guanosine.

Table 4: Sequences of ARC445 inosine derivatives and K_D summary

SEQ ID NO	ARC #	Location of 2'-deoxy inosine substitution	Sequence (5' -> 3'), (3T = inv dT), (T=dT), (mN = 2'-O Methyl containing residue) (dI = 2'- deoxy inosine containing residue)	K_D (nM)

2	ARC445	none	AdGmCmCmUdGdGdGdGdA mCmCmCdAmUdGdGdGdGd GdGmCmU	3
14	ARC1335	none	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	0.3
15	ARC1548	mG at position 2 of ARC1335 replaced with dI	mA**dI**mCmCmUdGmGdGdG mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	3.3
16	ARC1552	dG at position 6 of ARC1335 replaced with dI	mAmGmCmCmU**dI**mGdGdG mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	No binding
17	ARC1553	mG at position 7 of ARC1335 replaced with dI	mAmGmCmCmUdG**dI**dGdG mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	No binding
18	ARC1554	dG at position 8 of ARC1335 replaced with dI	mAmGmCmCmUdGmG**dI**dG mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	No binding
19	ARC1555	dG at position 9 of ARC1335 replaced with dI	mAmGmCmCmUdGmGdG**dI** mAmCmCmCmAmUdGmGd GdGdGdGmCmU-3T	No binding
20	ARC1562	dG at position 16 of ARC1335 replaced with dI	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmU**dI**mGdG dGdGdGmCmU-3T	0.6
21	ARC1563	mG at position 17 of ARC1335	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmUdG**dI**dG dGdGdGmCmU-3T	2.2

		replaced with dI		
22	ARC1564	dG at position 18 of AARC1335 replaced with dI	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmUdGmG**dI** dGdGdGmCmU-3T	0.5
23	ARC1565	dG at position 19 of ARC1335 replaced with dI	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmUdGmGd G**dI**dGdGmCmU-3T	1.4
24	ARC1566	dG at position 20 of ARC1335 replaced with dI	mAmGmCmCmUdGmGdGdG mAmCmCmCmAmUdGmGd GdG**dI**dGmCmU-3T	0.1

EXAMPLE 2B: 2'-deoxy inosine substitution in the anti-IL-23 aptamer ARC979

[00121] The effects of substituting purine residues, one at a time, with inosine on the binding affinity of ARC979 (SEQ ID NO 1) derivatives as compared to the ARC979 parent molecule was examined during the systematic replacement of 2'-deoxy purine residues with 2'-deoxy inosine (dI). A set of aptamer derivatives in which each guanosine of ARC979 was replaced one at a time with 2'-deoxy inosine and a set of aptamer derivatives in which each adenosine was replaced one at a time with 2'-deoxy inosine were synthesized and tested for binding. The dot blot binding assay previously described was used to characterize the relative binding affinity of the derivative aptamers synthesized and each was simultaneously compared in the same binding assay to the parent molecule ARC979. For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ–^{32}P ATP and were tested for direct binding to full length human IL-23 using a protein titration in the dot blot binding assay under the buffer conditions previously described (Dulbecco's PBS (with Mg^{++} and Ca^{++}) with 0.1 mg/ mL BSA, room

temperature for 30 minutes). K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00122] In the case of ARC979, systematic substitutions of 2'-deoxy guanosine with 2'-deoxy inosine did not adversely affect yet did not remarkably improve binding affinity. However, systematic substitutions of 2'-deoxy adenosine with 2'-deoxy inosine yielded two constructs, ARC1652 and ARC1654, with improved affinity (*i.e.*, lower K_D value) as compared to that of ARC979, as can be seen from the calculated K_D values in Table 5 below.

[00123] Unless otherwise indicated, sequences in Table 5 are shown in the 5' to 3' direction. In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 5 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 5 additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG, lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, "and "I" denotes an inosine substitution for adenosine.

Table 5: Sequences of ARC979/ARC1386 2'-deoxy inosine derivatives and K_D summary

SEQ ID NO	ARC #	Description	Sequence (5' -> 3'), (mN = 2'-O Methyl containing residue) (dI = 2'-deoxy inosine containing residue)	K_D (nM)	Parent (ARC979) K_D (nM)
25	ARC1648	ARC979 1st dA from the 5' end replaced by dI	**dI**mCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdGmUdGmCdGdGdGmCdGdGdGdGmUdGmU	12	13
26	ARC1649	ARC979 2nd dA replaced by dI	dAmC**dI**dGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdGmUdGmCdGdGdGmCdGdGdGdGmUdGmU	100,000	13
27	ARC1650	ARC979 3rd dA	dAmCdAdGdGmC**dI**dAdGmUdAdAmUmUdGdGdGdGdA	34	13

		replaced by dI	dGmUdGmCdGdGdGmCdGd GdGdGmUdGmU		
28	ARC1651	ARC979 4th dA replaced by dI	dAmCdAdGdGmCdA**dI**dGm UdAdAmUmUdGdGdGdGdA dGmUdGmCdGdGdGmCdGd GdGdGmUdGmU	100, 000	13
29	ARC1652	ARC979 5TH dA replaced by dI	dAmCdAdGdGmCdAdAdGm U**dI**dAmUmUdGdGdGdGdAd GmUdGmCdGdGdGmCdGdG dGdGmUdGmU	5.9	13
30	ARC1653	ARC979 6TH dA replaced by dI	dAmCdAdGdGmCdAdAdGm UdA**dI**mUmUdGdGdGdGdAd GmUdGmCdGdGdGmCdGdG dGdGmUdGmU	25	13
31	ARC1654	ARC979 7TH dA replaced by dI	dAmCdAdGdGmCdAdAdGm UdAdAmUmUdGdGdGdG**dI**d GmUdGmCdGdGdGmCdGdG dGdGmUdGmU	5.9	13

*30min RT incubation for K_D determination
*1X Dulbecco's PBS (with Ca^{++} and Mg^{++}) +0.1mg/mL BSA reaction buffer

EXAMPLE 2C: 2'-OMe inosine substitution in the anti-IL-23 aptamer ARC1386

[00124] ARC1386 (ARC979 with a 3'-inverted dT) was previously determined to tolerate 2'-OMe substitutions at particular positions. 2'-OMe residues in ARC1386 were systematically substituted with 2'-OMe Inosine residues (mI) (Glen Research, Sterling, VA) to result in a set of chemically synthesized derivative aptamers having an aptamer with a 2'-OMe inosine substitution at each different 2'-OMe residue. The effect of 2'-OMe inosine substitution on binding affinity was determined using the dot blot binding assay.

[00125] The dot blot binding assay previously described was used to characterize the relative potency of the aptamers synthesized as compared to the parent molecule, ARC1386. For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ-^{32}P ATP and were tested for direct binding to full length human IL-23 using a protein titration the dot blot binding assay in

Dulbecco's PBS (with Mg^{++} and Ca^{++}) with 0.1 mg/ mL BSA. K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00126] In two instances, single substitutions of 2'-OMe uridine with 2'-OMe inosine resulted in improved binding affinity (*i.e.*, lower K_D value) as compared to the parent molecule ARC1386. The parent aptamer was run each dot blot binding assay. The sequences and corresponding binding affinities of the constructs in which 2'-OMe inosine improved binding affinity relative to ARC1386 are listed in Table 6 below.

[00127] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 6 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 6 additionally comprise a 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 6 lack the indicated 3' cap (*e.g.*, a 3' inverted dT cap (3T)). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, and "I" denotes an inosine substitution.

Table 6: Sequences of ARC979 2'-OMe inosine derivatives and K_D summary

SEQ ID NO	ARC #	Location of 2'-OMe inosine substitution	Sequence (5' -> 3'), (3T = inv dT), (T=dT), (mN = 2'-O Methyl containing residue) (mI = 2'-OMe inosine containing residue) (d=2'-deoxy residue)	K_D (nM)	Parent (ARC1386) K_D (nM)
32	ARC1683	ARC1386 2nd mU replaced with mI	dAmCdAdGdGmCdAdAdG mUdAdA**mI**mUdGdGdGdG dAdGmUdGmCdGdGdGmC dGdGdGdGmUdGmU-3T	4.8	9.8
33	ARC1702	ARC1386 last mU replaced with mI	dAmCdAdGdGmCdAdAdG mUdAdAmUmUdGdGdGdG dAdGmUdGmCdGdGdGmC dGdGdGdGmUdG**mI**-3T	10	15

*30min RT incubation for K_D determination
*1X Dulbecco's PBS (with Ca^{++} and Mg^{++}) +0.1mg/mL BSA reaction buffer

EXAMPLE 3: INCREASED APTAMER TARGET AFFINITY BY 2'-DEOXY DIHYDROURIDINE SUBSTITUTION

[00128] The effects of systematically substituting 2'-OMe uridine residues with 2'-deoxy dihydrouridine (dhU) on the binding affinity of ARC1386 (ARC979 with a 3'-inverted dT) derivatives as compared to the parent ARC1386 molecule was examined. 2'-OMe uridine residues were systematically substituted with 2'-deoxy dihydrouridine (Glen Research, Sterling, VA) to result in a set of chemically synthesized derivative aptamers, each aptamer of the set having an 2'-deoxy dihydrouridine substituted at a different 2'-OMe uridine positon in ARC1386.

[00129] The dot blot binding assay previously described was used to characterize the relative potency of the majority of the aptamers synthesized as compared to the parent molecule, ARC1386. For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ–^{32}P ATP and were tested for direct binding to full human IL-23 using a protein titration the dot blot binding assay in Dulbecco's PBS (with Mg^{++} and Ca^{++}) with 0.1 mg/ mL BSA. K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00130] The sequences and corresponding binding affinities of the constructs in which 2'-deoxy dihydrouridine substitution improved binding affinity relative to ARC1386 are listed in Table 7 below. As seen in Table 7, single substitutions of 2'-OMe uridine for 2'-deoxy dihydrouridine improved binding affinity (*i.e.*, lower K_D value) as compared to the parent molecule ARC1386.

[00131] In some embodiments, the invention comprises aptamer with a nucleic acid sequence as described in Table 7 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 7 additionally comprise a 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 7 lack the indicated 3' cap (*e.g.*, a 3' inverted dT cap (3T)). Lower case

letters "m", "d", and "dh", denote 2'-OMe, 2'-deoxy, and dihydro2'-deoxy modifications respectively.

Table 7: Sequences of ARC979 2'-deoxy dihydrouridine derivatives and K_D summary

SEQ ID NO	ARC #	Location of dhU substitution position relative to the 5'end	Sequence (5' -> 3'), (3T = inv dT), (T=dT), (mN = 2'-OMe containing residue) (d=2'-deoxy containing residue) (dhU = 2'-deoxy dihydrouridine containing residue)	K_D (nM)	Parent (ARC 1386) Kd (nM)
34	ARC1713	ARC1386 32nd 2'-OMe uridine replaced with dhU	dAmCdAdGdGmCdAdAdGmUdAdAmUmUdGdGdGdGdAdGmUdGmCdGdGdGmCdGdGdGdG**dh**UdGmU-3T	2.2	3.7
35	ARC1709	ARC1386 10th2'-OMe uridine replaced with dhU	dAmCdAdGdGmCdAdAdG**dhU**dAdAmUmUdGdGdGdGdAdGmUdGmCdGdGdGmCdGdGdGdGmUdGmU-3T	2.8	3.7

*30min RT incubation for K_D determination
*1X Dulbecco's PBS (with Ca^{++} and Mg^{++}) +0.1mg/mL BSA reaction buffer

EXAMPLE 4: INCREASED APTAMER TARGET AFFINITY BY 2'-DEOXY SUBSTITUTION

[00132] A set of ARC445 derivatives was made by systematically substituting 2'-OMe pyrimidines with 2'-deoxy residues. The 2'-deoxy series was tested for binding to human IgE using the dot blot assay and the binding reaction conditions previously described (Dulbecco's PBS (with Ca^{++} and Mg^{++}) plus 0.1 mg/mL BSA, 0.1 mg/mL ssDNA, and 1 mg/mL tRNA at room temperature for 30 minutes). Introducing 2'-deoxy residues in place of stabilizing 2'-OMe residues improved the binding affinity relative to the parent molecule ARC445 in one

derivative. The sequence and corresponding K_D values of the construct in which 2'-deoxy substitutions improved binding affinity relative to ARC445 are listed Table 8 below.

[00133] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 8 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 8 additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively.

Table 8: Sequences of ARC445 2'-deoxy derivatives and K_D summary

SEQ ID NO	ARC #	Description of substitution position relative to the 5'end	Sequence (5' -> 3'), (mN = 2'-O Methyl containing residue) (d = 2'-deoxy containing residue)	Kd (nM)	Parent (ARC445) Kd (nM)
36	ARC608	ARC445 with dC in place of the sixth mC	dAdGmCmCmUdGdGdGdG dAmCmCmCdAmUdGdGd GdGdGdGdCmU	7.9	14.6

*30min RT incubation for K_D determination
*1X Dulbecco's PBS (with Ca^{++} and Mg^{++}) +0.1mg/mL BSA, 0.1 mg/mL ssDNA, and 1 mg/mL tRNA reaction buffer

EXAMPLE 5: INCREASED BINDING AFFINITY WITH COMBINED 2'-DEOXY INOSINE SUBSTITUTIONS IN ARC1335

[00134] A set of ARC1335 based derivatives were made by combining 2'-deoxy inosine substitutions at more than one position in ARC1335 that had previously been shown to tolerate inosine substitution (see, *e.g.* Example 2A above). The multiply substituted ARC1335 derivatives were tested for binding affinity to human IgE in the dot blot assay and the binding reaction conditions previously described (Dulbecco's PBS (with Ca^{++} and Mg^{++}) plus 0.1 mg/mL BSA, room temperature for 30 minutes). K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00135] As can be seen from Table 9 below, combining 2'-deoxy inosine substitutions at positions 14 and 20 resulted in improved binding affinity relative to ARC1335 (no inosine substitution), ARC1566 (single inosine substitution at position 20) and ARC1560 (single inosine substitution at position 14).

[00136] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 8 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 9, where lacking, additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 9 lack the indicated 3' cap (*e.g.*, a 3' inverted 2'-deoxy thymidine cap (3T)). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, and "I" denotes an inosine modification.

Table 9: Sequence and K_Ds related to combined inosine substitutions in ARC1335

SEQ ID NO	ARC #	Inosine Substitution description (relative to the 5' end)	Sequence	K_D (nM)
14	ARC1335	none	See Table 3 above	15
37	ARC1647	2'-deoxy inosine substitution at positions 20 and 14 of ARC1335	mAmGmCmCmUdGmGdGdGm AmCmCmCdImUdGmGdGdGdI dGmCmU-3T	1
24	ARC1566	2'-deoxy inosine substitution at position 20 of ARC1335	mAmGmCmCmUdGmGdGdGm AmCmCmCmAmUdGmGdGdG dIdGmCmU-3T	8
38	ARC1560	2'-deoxy inosine substitution at	mAmGmCmCmUdGmGdGdGm AmCmCmCdImUdGmGdGdGd GdGmCmU-3T	3

		position 14 of ARC1335		

EXAMPLE 6: INCREASED BINDING AFFINITY WITH COMBINED PHOSPHOROTHIOATE SUBSTITUTIONS IN ARC445

[00137] A set of ARC445 based derivatives was chemically synthesized by combining phosphorothioate substitutions at more than one position in ARC445 that had previously been determined to tolerate phosphorothioate substitution. The multiply substituted ARC445 derivatives were tested for binding affinity to human IgE in the dot blot assay and the binding reaction conditions previously described (Dulbecco's PBS (with Ca^{++} and Mg^{++}) plus 0.1 mg/mL BSA, room temperature for 30 minutes). K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00138] As can be seen from Table 9 below, combining phosphorothioate substitutions at some positions improved binding affinity relative to ARC445 and to some of the corresponding single substitution positions.

[00139] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 10 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 10, where lacking, additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 10 lack the indicated 3' cap (*e.g.*, a 3' inverted 2'-deoxy thymidine cap (3T)). Lower case letters "m", and "d" denote 2'-OMe and 2'-deoxy modifications respectively, and "s" denotes a phosphorothioate substitution.

TABLE 10: Sequence and K_Ds related to combined phosphorothioate substitutions in ARC445

SEQ ID NO	ARC#	Phosphorothioate substitution position description	Sequence	K_D (nM)
2	ARC445	none	See Table 3 above	12. 6
39	ARC588	ARC445 with Phosphorothioate between positions 20&21	dAdGmCmCmUdGdGdGd GdAmCmCmCdAmUdGd GdGdGdG-s-dGmCmU	5.3
40	ARC584	ARC445 with Phosphorothioate between positions 16&17	dAdGmCmCmUdGdGdGd GdAmCmCmCdAmUdG-s-dGdGdGdGdGmCmU	6.9
41	ARC702	ARC445 with Phosphorothioate between positions 16&17, 20&21	dAdGmCmCmUdGdGdGd GdAmCmCmCdAmUdG-s-dGdGdGdG-s-dGmCmU	2.6
42	ARC768	ARC445 with Phosphorothioate between positions 13 &14, 16&17, 20&21	dAdGmCmCmUdGdGdGd GdAmCmCmC-s-dAmUdG-s-dGdGdGdG-s-dGmCmU	3.5
43	ARC767	ARC445 with Phosphorothioate between positions 12 &13, 16&17, 20&21	dAdGmCmCmUdGdGdGdGd AmCmC-s-mCdAmUdG-s-dGdGdGdG-s-dGmCmU	2.7

EXAMPLE 7: INCREASED BINDING AFFINITY WITH COMBINED 2-AMINO PURINE SUBSTITUTIONS IN ARC1335

[00140] A set of ARC1335 single substitution derivatives having a 2-amino purine substitution at each different A or G was synthesized. Combinations of 2-amino purine substitutions were synthesized combining positions at which the 2-amino purine substitutions were tolerated. The ARC1335 derivatives having 2-amino purine substitutions at multiple positions were tested for binding affinity to human IgE in the dot blot assay and the binding

reaction conditions previously described (Dulbecco's PBS (with Ca^{++} and Mg^{++}) plus 0.1 mg/mL BSA, room temperature for 30 minutes). K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00141] As can be seen from Table 11 below, individual 2-amino purine substitutions at position 1, 8 and 14 each resulted in a decrease in binding affinity (*i.e.*, higher K_D value) over the starting aptamer, ARC1335 (no 2'-amino purine substitution). However, the combination of 2'-amino purine substitutions at position 8 and 14 resulted in an increase in binding affinity (*i.e.*, lower K_D value) compared to the individual substitutions at position 8 and 14, resulting in binding affinity identical to that of ARC1335. Furthermore, the combination of 2'-amino purine substitutions at positions 1, 8, and 14 resulted in increased binding affinity (*i.e.*, lower K_D value) compared to the individual substitutions at positions 1, 8, and 14, and remarkably improved binding affinity over that of the starting aptamer ARC1335.

[00142] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 11 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 11, where lacking, additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). In other embodiments, the nucleic acid sequences described in Table 11 lack the indicated 3' cap (*e.g.*, a 3' inverted 2'-deoxy thymidine cap (3T)). Lower case letters "m", and "d" denote 2-O-methyl, and 2'-deoxy modifications respectively, "mAP" denotes a 2'-OMe-2-amino purine substitution, and "dAP" denotes a 2'-deoxy-2-amino purine substitution.

Table 11: Sequence and Kds related to combined 2-amino purine substitutions in ARC445

SEQ ID NO	ARC #	Amino purine substitution	Sequence	Kd (nM)

		description		
14	1335	none		2.8
44	2040	mAP substituted at the 1st nucleotide position of ARC1335	mAPmGmCmCmUdGmGdGdGmAmCmCmCmAmUdGmGdGdGdGdGmCmU-3T	18.7
45	2044	dAP substituted at the 8th nucleotide position of ARC1335	mAmGmCmCmUdGmGdAPdGmAmCmCmCmAmUdGmGdGdGdGdGmCmU-3T	3.9
46	2047	mAP substituted at nucleotide position 14 of ARC1335	mAmGmCmCmUdGmGdGdGmAmCmCmCmAPmUdGmGdGdGdGdGmCmU-3T	4.8
47	2360	dAP substituted at nucleotide positions 8 and a mAP at nucleotide position 14 of ARC1335	mAmGmCmCmUdGmGdAPdGmAmCmCmCmAPmUdGmGdGdGdGdGmCmU-3T	2.7
48	2361	mAP substituted at nucleotide positions, 1, and 14 and dAP at position 8	mAPmGmCmCmUdGmGdAPdGmAmCmCmCmAPmUdGmGdGdGdGdGmCmU-3T	0.8

		in ARC1335		

EXAMPLE 8: INCREASED APTAMER TARGET AFFINITY BY COMBINED 2'-DEOXY-5-METHYL CYTIDINE SUBSTITUTION

[00143] A series of ARC445 derivatives was designed and synthesized in which 2'-OMe cytidines were systematically replaced with 2'-deoxy-5-methyl cytidine (5mC) residues (Glen Research, Sterling, VA). Each aptamer of the resulting set comprised a 5mC substitution at a different 2'-OMe position in ARC445. Based on the data generated from this series, a second set of ARC445 derivatives was designed in which combinations of 5mC residues were substituted at more than one position where individual 5mC substitutions were shown to have little or no adverse affect on binding affinity.

[00144] These 5mC series were tested for binding to human IgE using the dot blot assay previously described. For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ-^{32}P ATP and were tested for direct binding to human IgE using a protein titration in Dulbecco's PBS (with Mg^{++} and Ca^{++}) with 0.1 mg/ mL BSA, 0.1 mg/mL ssDNA, and 1 mg/mL tRNA. K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00145] As seen from Table 12 below, substituting 2'-OMe cytidine with individual 5mC substitutions at positions 3, 11, and 22 resulted in little to no change in binding affinity. However, double and triple combinations of these individual 5mC substitutions resulted in an improvement in binding affinity (*i.e.*, lower K_D value).

[00146] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 12 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 12 additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). Lower case letters "m",

and "d" denote 2'-OMe, and 2'-deoxy modifications respectively, and "5m" denotes a 2'-deoxy-5-methyl modification.

Table 12: Sequences of ARC445 2'-deoxy-5-methyl- cytidine derivatives and K_D summary

SEQ ID NO	ARC #	Description of substitution positon relative to 5' end	Sequence (5' -> 3'), (mN = 2'-OMe containing residue) (d = 2'-deoxy containing residue), 5m = 2'-deoxy-5 methyl containing residue)	Kd (nM)	Parent (ARC 445) Kd (nM)
49	ARC597	ARC445 with 5mC in place of the first mC (position 3 of ARC445)	dAdG**5mC**mCmCmUdGdGdGd GdAmCmCmCdAmUdGdG dGdGdGdGmCmU	21.5	20
50	ARC599	ARC445 with 5mC in place of the third mC (position 11 of ARC445)	dAdGmCmCmCmUdGdGdGdG dA**5mC**mCmCmCdAmUdGdGd GdGdGdGmCmU	20.5	20
51	ARC602	ARC445 with 5mC in place of the sixth mC (position 22 of ARC445)	dAdGmCmCmCmUdGdGdGdG dAmCmCmCmCdAmUdGdGd GdGdGdG**5mC**mCmU	26	20
52	ARC751	ARC445 with 5mC in place of the first and sixth mC	dAdG**5mC**mCmCmCmUdGdGdGd GdAmCmCmCmCdAmUdGdG dGdGdGdG**5mC**mCmU	15	20
53	ARC753	ARC445 with 5mC in place of the 3rd and 6th mC	dAdGmCmCmCmUdGdGdGdG dA**5mC**mCmCmCdAmUdGdGd GdGdGdG**5mC**mCmU	15	20

54	ARC756	ARC445 with 5mC in place of the 1st,3rd and 6th mC	dAdG**5mC**mCmCmUdGdGdGd GdA**5mC**mCmCmCdAmUdGd GdGdGdGdG**5mC**mU	14	20

*30min RT incubation for K_D determination
*1X Dulbecco's PBS (with Ca^{++} and Mg^{++}) +0.1mg/mL BSA, 0.1 mg/mL ssDNA, 1 mg/mL tRNA reaction buffer

EXAMPLE 9: INCREASED APTAMER TARGET AFFINITY BY COMBINED 2'-DEOXY-5-METHYL CYTIDINE SUBSTITUTION AND 2'- DEOXY CYTIDINE

[00147] A series of ARC445 derivatives was designed and synthesized in which combinations of the 2'-deoxy-5-methyl cytidine (5mC) substitutions at positions 3, 11, and 22 of ARC445 (described above in Example 8) were combined with one or more substitutions of 2'-deoxy cytidine residues (dC) for 2'-OMe cytidine residues at positions where the dC substitutions were previously determined to be well tolerated (positions 12 and 22 of ARC445, see Example 4).

[00148] These derivatives were tested for binding to human IgE using the dot blot assay previously described. For K_D determination, chemically synthesized aptamers were purified using denaturing polyacrylamide gel electrophoresis, 5'end labeled with γ–^{32}P ATP and were tested for direct binding to human IgE using a protein titration in Dulbecco's PBS (with Mg^{++} and Ca^{++}) with 0.1 mg/ mL BSA, 0.1 mg/mL ssDNA, and 1 mg/mL tRNA. K_D values were calculated by fitting the equation y= (max/(1+K/protein))+yint using KaleidaGraph (KaleidaGraph v. 3.51, Synergy Software).

[00149] As can be seen from Table 13 below, various combinations of one or more 2'-deoxy-5-methyl-cytidine substitutions at positions 3, 11, and 22 of ARC445, combined with one or more 2'-deoxy cytidine substitutions at positions 12 or 22 or ARC445 did not adversely affect binding affinity, and resulted in several constructs in which binding affinity was moderately improved (*i.e.*, lower K_D value.)

[00150] In some embodiments, the invention comprises aptamers with a nucleic acid sequences as described in Table 13 below. In some embodiments, the nucleic acid sequences of the aptamers described in Table 13 additionally comprise a 3' cap (*e.g.*, an inverted dT cap (3T)), and/or 5' amine (NH_2) modification to facilitate chemical coupling, and/or conjugation to a high molecular weight, non-immunogenic compound (*e.g.*, PEG). Lower case letters "m", and "d" denote 2'-OMe, and 2'-deoxy modifications respectively, and "5m" denotes a 2'-deoxy-5-methyl cytidine modification, and "dC" denotes a 2'-deoxy cytidine modification.

Table 13: Sequences of ARC445 2'-deoxy-5-methyl-cytidine and 2'-deoxy cytidine derivatives and K_D summary

SEQ ID NO	ARC #	Description of substitution positon relative to 5' end	Sequence (5' -> 3'), (mN = 2'-OMe containing residue) (d = 2'-deoxy containing residue), 5m = 2'-deoxy-5 methyl containing residue)	K_D (nM)	Parent (ARC445) K_D (nM)
49	ARC597	ARC445 with 5mC in place of the first mC (position 3 of ARC445)	dAdG**5m**CmCmUdGdGdGd GdAmCmCmCdAmUdGdG dGdGdGdGmCmU	16	8.5
50	ARC599	ARC445 with 5mC in place of the third mC (position 11 of ARC445)	dAdGmCmCmUdGdGdGdG dA**5m**CmCmCdAmUdGdGd GdGdGdGmCmU	10.4	8.5
51	ARC602	ARC445 with 5mC in place of the sixth mC (position 22)	dAdGmCmCmUdGdGdGdG dAmCmCmCdAmUdGdGd GdGdGdG**5m**CmU	9	8.5
55	ARC914	ARC445 with 5mC in place of the first	dAdG**5m**CmCmUdGdGdGd GdAmCmCmCdAmUdGdG dGdGdGdGdCmU	11.9	27

		mC (position 3) and dC in place of the 6th mC (position 22)			
56	ARC915	ARC445 with 5mC in place of the 3rd mC position 11) and dC in place of the 6th mC (position 22)	dAdGmCmCmUdGdGdGdG dA**5mC**mCmCdAmUdGdGd GdGdGdG**d**CmU	16.9	27
57	ARC916	ARC445 with 5mC in place of the first mC (position 3) and dC in place of the 4th mC (position 12)	dAdG**5mC**mCmUdGdGdGd GdAmC**d**CmCdAmUdGdGd GdGdGdGmCmU	13.7	27
58	ARC918	ARC445 with 5mC in place of the 6th mC (position 22) and dC in place of the 4th mC (position 12)	dAdGmCmCmUdGdGdGdG dAmC**d**CmCdAmUdGdGdG dGdGdG**5mC**mU	14.6	27
59	ARC920	ARC445 with 5mC in place of the 1st and 6th mC (position 3 and 22) and dC in place of the 4th mC (position 12)	dAdG**5mC**mCmUdGdGdGd GdAmC**d**CmCdAmUdGdGd GdGdGdG**5mC**mU	12.8	27

60	ARC921	ARC445 with 5-Me-dC in place of the 3rd and 6th mC (position 11 and 22) and dC in place of the 4th mC (position 12)	dAdGmCmCmUdGdGdGdG dA**5mC**dCmCdAmUdGdGd GdGdGdG**5mC**mU	8.5	13
61	ARC925	ARC445 with 5mC in place of the 1st mC (position 3) and dC in place of the 4th and 6th mC (position 12 and 22)	dAdG**5mC**mCmUdGdGdGd GdAmCdCmCdAmUdGdGd GdGdGdGdCmU	8.1	13
62	ARC926	ARC445 with 5mCin place of the 3rd mC (position 11) and dC in place of the 4th and 6th mC (position 12 and 22)	dAdGmCmCmUdGdGdGdG dA**5mC**dCmCdAmUdGdGd GdGdGdGdCmU	5.5	13

[00151] The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the description and examples above are for purposes of illustration and not limitation of the following claims.

What is claimed is:

1) A method of identifying a substituted single stranded aptamer comprising the steps of:
   a) substituting a phosphorothioate or phosphorodithioate for a phosphate at an internucleotide linkage position in a single stranded aptamer;
   b) assaying the substituted single stranded aptamer for affinity to the target; and
   c) identifying the substituted single stranded aptamer having increased affinity for the target relative to that of a starting aptamer which is identical to the modified single stranded aptamer except that it lacks the phosphorothioate substitution.

2) The method of claim 1, wherein the substituting step further comprises incorporating a phosphorothioate at the internucleotide linkage position by chemical synthesis.

3) The method of claim 2, wherein the substituting step does not comprise incorporating the phosphorothioate substitution at the internucleotide linkage between the two nucleotides at the 3' or 5' end of the single stranded aptamer.

4) The method of claim 2, wherein the substituting step comprises incorporating a single phosphorothioate substitution into the single stranded aptamer.

5) The method of claim 4, wherein the substituted single stranded aptamer comprises a binding affinity for the target that is at least two fold higher than that of the starting aptamer.

6) A substituted single stranded aptamer identified by the method of claim 5.

7) The method of claim 4, further comprising the step of incorporating an additional substitution into the substituted single stranded aptamer to result in a twice substituted single stranded aptamer.

8) The method of claim 7, further comprising assaying the twice substituted single stranded aptamer for affinity to the target and identifying the twice substituted single stranded aptamer that has an affinity equal to or higher than that of the starting unsubstituted single stranded aptamer.

9) The method of claim 7, further comprising assaying the twice substituted single stranded aptamer for affinity to the target and identifying the twice substituted single stranded aptamer that has an affinity equal to or higher than that of the phosphorothioate substituted single stranded aptamer.

10) A twice substituted single stranded aptamer identified by the method of claim 9.

11) The method of claim 7, wherein the additional substitution is an additional phosphorothioate substitution at a phosphate position different than that of the first phosphorothioate substitution.

12) The method of claim 7, wherein the additional substitution is selected from the group consisting of : substituting a nucleotide modified at a base position, substituting a nucleotide modified at a sugar position, and substituting a nucleotide a modified at phosphate position.

13) The method of claim 12, wherein the additional substitution is selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine; a 2'-deoxy-5-methyl nucleotide substitution for another nucleotide; a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide; a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide; and a 2- aminopurine substitution for a purine.

14) The method of claim 13, wherein the additional substitution is selected from the group consisting of: a 2'-deoxy inosine or 2'-OMe inosine substitution for another nucleotide; a 2'-deoxy dihydrouridine substitution for a uridine; a 2'-deoxy-5-methyl

cytidine substitution for a cytidine; and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide.

15) A method of identifying a substituted aptamer comprising the steps of:

a) incorporating a substitution into a starting aptamer wherein the substitution is selected from the group consisting of: an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, a 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide

b) assaying the substituted aptamer for affinity to the target; and

c) identifying the substituted aptamer having increased affinity for the target relative to that of the starting aptamer which is identical to the substituted aptamer except that it lacks the substituted nucleotide.

16) The method of claim 15, wherein the incorporating step further comprises incorporating the substitution into the starting aptamer through chemical synthesis.

17) The method of claim 16, wherein the identified substituted aptamer is single stranded.

18) The method of claim 15, wherein the method further comprises the steps of incorporating an additional substitution into the substituted aptamer, wherein the additional substitution is of a different type than the first substitution, to result in a twice substituted aptamer.

19) The method of claim 18, further comprising assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to or higher than that of the starting unsubstituted single stranded aptamer.

20) The method of claim 18, further comprising assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to or higher than that of the substituted aptamer.

21) The method of claim 20, wherein the additional substitution is selected from the group consisting of : substituting a nucleotide modified at a base position, substituting a nucleotide modified at a sugar position, and substituting a nucleotide a modified at phosphate position.

22) The method of claim 21, wherein the additional substitution is selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine, 2'-deoxy-5-methyl nucleotide substitution for a nucleotide, a 2'- deoxy nucleotide substitution for a 2'-OMe nucleotide, a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide, and a 2- aminopurine substitution for a purine and wherein the additional substitution is different from the first substitution.

23) The method of claim 23, wherein the additional substitution is selected from the group consisting of: a 2-deoxy inosine or 2'-OMe inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine substitution for a cytidine, and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide.

24) The method of claim 15, further comprising incorporating an additional substitution into the substituted aptamer to result in a twice substituted aptamer wherein the additional substitution is of the same type as the first substitution but at a different nucleotide position.

25) The method of claim 24, wherein the method further comprises assaying the twice substituted aptamer for affinity to the target and identifying the twice substituted aptamer that has an affinity equal to or higher than that of the starting unsubstituted single stranded aptamer and/or the substituted aptamer.

26) A twice substituted aptamer identified by the method of claim 24.

27) The method of claim 15, wherein the incorporating step further comprises substituting an inosine for another nucleotide.

28) The method of claim 27, wherein the inosine substituted aptamer comprise a binding affinity at least two fold higher than that of the starting unsubstituted aptamer.

29) The method of claim 15, wherein the incorporating step comprises substituting a 2'-deoxy nucleotide for a 2'-OMe nucleotide.

30) The method of claim 29, wherein the 2'-deoxy substituted aptamer comprise a binding affinity at least two fold higher than that of starting unsubstituted aptamer.

31) The method of claim 20, wherein the first substitution is 2'-deoxy substitution for 2'-OMe nucleotide and the additional substitution is an inosine substitution.

32) The method of claim 12, wherein the twice substituted single stranded phosphorothioate aptamer comprises a binding affinity that is at least 2 fold higher than the starting unsubstituted aptamer and/or the substituted aptamer.

33) A single stranded aptamer comprising no more than one phosphorothioate backbone substitution wherein the single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the phosphorothioate substitution.

34) The single stranded aptamer of claim 33, comprising no more than one additional phosphorothioate substitution.

35) The singled stranded aptamer of claim 34 comprising no more than 2 additional phosphorothioate substitutions.

36) The single stranded aptamer of claim 35, comprising no more than 3 additional phosphorothioate substitutions.

37) The single stranded aptamer of claim 33, further comprising an additional nucleotide substitution selected from the group consisting of : a substitution with a nucleotide modified at a base position, a substitution with a nucleotide modified at a sugar position and a substitution with a nucleotide modified at phosphate position wherein when the nucleotide substituted comprises a modification at the phosphate position it is not a phosphorothioate substitution.

38) The single stranded aptamer of claim 37, wherein the additional nucleotide substitution is selected from the group consisting of: a phosphorodithioate substitution at a phosphate position, an inosine substitution for another nucleotide; a 2'-deoxy dihydro uridine substitution for a uridine, a 2'-deoxy-5-methyl nucleotide substitution for another nucleotide, a 2'- deoxy nucleotide substitution for a 2'-OMe nucleotide, a 2'-OMe nucleotide substitution for a 2'-deoxy nucleotide, and a 2- aminopurine substitution for a purine.

39) A single stranded aptamer comprising a nucleotide substitution selected from the group consisting of : an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, an 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide, wherein the substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the nucleotide substitution.

40) The single stranded aptamer of claim 39, comprising at least two nucleotide substitutions selected from the group consisting of: an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, an 2- amino purine substitution for a purine and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide, wherein the twice substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the at least two nucleotide substitutions.

41) The single stranded aptamer of claim 39, comprising at least three nucleotide substitutions selected from the group consisting of: an inosine substitution for another nucleotide, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, an 2- amino purine substitution for a purine and a 2'-deoxy

nucleotide substitution for a 2'-OMe nucleotide, wherein the triple substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the at least three nucleotide substitutions.

42) The single stranded aptamer of claim 40, wherein twice substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking one of the nucleotide substitutions.

43) The single stranded aptamer of claim 41, wherein the triple substituted single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking at least one of the nucleotide substitutions.

44) The single stranded aptamer of claim 39, wherein the nucleotide substitution is the substitution of 2'-deoxy nucleotide for a 2'-OMe nucleotide.

45) The single stranded aptamer of claim 39, wherein the nucleotide substitution is the substitution of an inosine for a purine.

46) An aptamer that specifically binds to a target, wherein the aptamer comprises a nucleotide sequence having a phosphorothioate modification of the phosphate back bone at a position selected to increase the binding affinity of the aptamer to the target relative to the binding affinity of a second aptamer to the same target, the of: an inosine substitution for a purine, a 2'-deoxy dihydrouridine substitution for a uridine, a 2'-deoxy-5-methyl cytidine for a cytidine, a 2-amino purine substitution for a purine, a second aptamer having the same nucleotide sequence but lacking the phosphorothioate modification.

47) A method of stabilizing an aptamer comprising the steps of:

a) introducing stablilizing modifications into a starting aptamer to result in a modified aptamer wherein the starting aptamer has a predeterimined binding affinity for a target,

b) assaying modified aptamer for binding affinity to the target and where the binding affinity is less than that of the starting aptamer introducing a nucleotide substitution to result in a substituted aptamer wherein the nucleotide substitution results in the substituted aptamer having a binding affinity for the target higher than that of the modified aptamer.

48) The method of claim 47, herein the substituted aptamer comprises a binding affinity for the target substantially the same as that of the starting aptamer.

49) The method of claim 47, wherein the stabilizing modification is a modification to increase aptamer resistance to nuclease resistance.

50) The method of claim 49, wherein the stabilizing modification comprises substituting a 2'-OMe nucleotide for another nucleotide.

51) The method of claim 50, wherein the introducing step comprises introducing more than one 2'-OMe substitution for another nucleotide.

52) The method of claim 51, wherein the substituting step comprises a substitution selected from the group consisting of: a substitution with a nucleotide modified at a base position, with a nucleotide modified at a sugar position and with a nucleotide modified at phosphate position.

53) The method of claim 52, where in the substitution is selected from the group consisting phosphorothioate substituted nucleotide for an unsubstituted nucleotide, a phosphorodithioate substituted nucleotide for an unsubstituted nucleotide and a 2'-deoxy nucleotide substitution for a 2'-OMe nucleotide

54) The method of claim 53, wherein the substitution is a phosphorothioate substituted nucleotide for an unsubstituted nucleotide.

55) A method of identifying a substituted aptamer that binds to a target, wherein the substituted aptamer has a higher binding affinity for the target than that of an identical aptamer but for the substitution, comprising the steps of

a) substituting a single nucleotide modified at a base, sugar or phosphate position for an unsubstituted nucleotide, and

b) assaying the substituted aptamer for binding affinity to the target.

56) The method of claim 55, wherein the substituting step further comprises substituting at least two nucleotides modified at the same position for at least two unmodified nucleotides.

57) The method of claim 56, wherein the substituting step further comprises substituting at least three nucleotides substituted at the same position for at least three unmodified nucleotides.

58) The method of claim 55, where the substituting step further comprises substituting at least two nucleotides modified at different positions for at least two unmodified nucleotides.

59) The method of claim 58, where the substituting step further comprises substituting at least three nucleotides wherein at least two of the nucleotides to be substituted are modified at different positions for at least three unmodified nucleotides.

60) An aptamer that specifically binds to a target, wherein the aptamer comprises a nucleotide sequence having a substituted nucleotide selected to increase the binding affinity of the aptamer to the target relative to the binding affinity of a second aptamer to the same target, the second aptamer having the same nucleotide sequence but lacking the substituted nucleotide, wherein the substituted nucleotide comprises a modification at a phosphate, sugar or base position.

61) A single stranded aptamer comprising no more than one nucleotide substitution wherein the substituted nucleotide comprises a chemical modification at a base, a sugar or phosphate position and wherein the single stranded aptamer binds specifically to a target with a binding affinity for the target that is increased relative to the binding affinity for the target of a second single stranded aptamer identical to the first but lacking the substitution.