COMPOSITIONS AND METHODS OF REDUCING CONVERSION OF PHOSPHOROTHIOATE BONDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/634,877, filed April 16, 2024; U.S. Provisional Application No. 63/646,600, filed May 13, 2024; and U.S. Provisional Application No. 63/719,075, filed November 11, 2024; each of which are incorporated by reference herein in their entireties.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The official copy of the Sequence Listing submitted concurrently with the specification, with a file name of "CX10-279WO1_ST26.xml," a creation date of April 15, 2025, and a size of 35,605 bytes, is part of the specification and is incorporated by reference herein in its entirety.

TECHNICAL FIELD

[0003] The present disclosure is directed to compositions and methods of reducing conversion, such as by oxidation, of phosphorothioate bonds during the synthesis and storage of oligonucleotides. In some related embodiments, the present disclosure is directed to compositions and methods of reducing conversion of thio-NTPs. In some embodiments, the methods or compositions of the present disclosure find use in reducing conversion of phosphorothioate to phosphodiester in an oligonucleotide or therapeutic oligonucleotide.

BACKGROUND

[0004] The use of therapeutic RNAs and other therapeutic oligonucleotides is expanding as new therapies are developed to treat a variety of target diseases and conditions. Therapeutic oligonucleotides include small interfering RNA (siRNA), single guide RNA for CRISPR complexes (sgRNA), messenger RNA (mRNA), micro RNA(miRNA), antisense RNA (asRNA), RNA aptamers, DNA aptamers, and antisense oligonucleotides (ASOs), among others.

[0005] Most therapeutic RNAs are designed with one or more nucleotide modifications to enhance stability, efficacy, specificity, and/or targeting of the oligonucleotide *in vivo*. Common modifications include modifications at the 2' or 3' position of the sugar moiety, modifications to the nucleobase, and modification to the nucleotide phosphate chain or internucleoside linkage (e.g., a phosporothioate bond). Of these, many siRNAs are currently designed with one or more phosphorothioate bonds, where one or more non-bridging oxygen atoms in the phosphate of the phosphodiester internucleoside linkage or bond are replaced with a sulfur atom.

[0006] Although phosphorothioate linkages have demonstrable advantages in therapeutic oligonucleotides, the sulfur atoms are susceptible to conversion, including by oxidation, to oxygen atoms during synthesis or storage of the oligonucleotide. Similarly, conversion of thio-nucleoside triphosphates (thio-NTPs) used during some synthesis methods may also contribute to product oligonucleotide in which the phosphorthioate linkage is replaced with a phosphodiester linkage.

[0007] The conversion of phosphorothioate linkages in therapeutic oligonucleotides leads to a recognized and undesirable impurity in the therapeutic. According to the European Medicines Agency's draft "Guideline on the Development and Manufacture of Oligonucleotides," replacement of one or more phosphorothioate bonds by a phosphodiester bond represents a Class II impurity (see, e.g., EMA, draft Guideline, EMA/CHMP/CVMP/QWP/262313/202424, July 17, 2024).

[0008] Therefore, methods of reducing conversion of phosphorothioate linkages in oligonucleotides and of thio-NTP substrates during synthesis and storage are desirable to enhance the purity of therapeutic oligonucleotides.

SUMMARY

[0009] In some embodiments, the present disclosure is directed to compositions and methods of reducing conversion, including by oxidation, of phosphorothioate bonds during the synthesis and/or storage of oligonucleotides. In some related embodiments, the present disclosure is directed to compositions and methods of reducing conversion of thio-NTPs. In some embodiments, the methods or compositions of the present disclosure find use in reducing phosphorothioate to phosphodiester impurities in an oligonucleotide or therapeutic oligonucleotide.

[0010] In some embodiments, the method or composition comprises an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the method or composition comprises a thio-NTP, that is a NTP or modified NTP comprising replacement of one or more non-bridging oxygen atoms with a sulfur atom in one or more phosphates in the phosphate chain of the NTP or modified NTP. In some embodiments, the method or composition comprises contacting the oligonucleotide comprising one or more phosphorothioate bonds or the thio-NTP with a sufficient amount of antioxidant or reducing agent to reduce the coversion of the phosphorothioate to the phosphodiester.

[0011] In some embodiments, the method comprises immobilization of one or more enzymes on a solid support. In some embodiments, the one or more enzymes are used to extend an oligonucleotide by addition of one or more NTPs or modified NTPs or thio-NTPs. In some embodiments, the one or more enzymes comprise a polymerase, a terminal nucleotidyl transferase (TnT), a terminal deoxynucleotidyl transferase (TdT), a phosphatase, an inorganic pyrophosphatase (iPP), an alkaline phosphatase (AP), a kinase, a ligase, or another enzyme. In some embodiments, the following

immobilization of an enzyme on a support medium, unreacted or remaining reactive groups on the support are neurtralized or capped with one or more quenching agents.

[0012] In some other embodiments, the method comprises immobilization of an oligonucleotide on a solid support. In some embodiments, one or more enzymes or reagents may be used to extend the oligonucleotide by addition of one or more NTPs or modified NTPs or thio-NTPs.

[0013] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises one or more of the following:

i) selecting a solid support for immobilization of one or more enzymes or an oligonucleotide;

ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support;

iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent;

iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition; and optionally

v) degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds;

such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0014] In some embodiments where the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises one or more enzymes, the method may result in reduced activity of one or more enzymes.

[0015] Therefore, in certain preferred embodiments of the method of reducing oxidation of a thio-NTP or an oligonucleotide where the method comprises one or more enzymes, the method further comprises reduced loss of activity or increased maintenance of activity of one or more enzymes.

[0016] In some embodiments, the method or composition comprises a support material or solid support. In some embodiments, the support material or solid support is used in a method of oligonucleotide synthesis. In some embodiments, the selecting of the support material or solid support comprises reduced oxidation of a thio-NTP or one or more phosphorothioate bonds in an oligonucleotide.

[0017] In some embodiments, an oligonucleotide or one or more enzymes are immobilized onto a solid support. In certain embodiments, the oligonucleotide or the enzyme is immobilized onto a solid support by covalent, ionic, electrostatic, or affinity attachment or adsorption to an ion, reactive chemical group, or other moiety.

[0018] In some embodiments, the present disclosure provides compositions or methods of quenching or capping unreacted reactive chemical groups on a resin or solid support, such that the unreacted chemical reactive groups are reduced or neutralized.

[0019] In some embodiments of the disclosure, an antioxidant or reducing agent is combined with or contacted with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0020] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises combining or contacting a thio-NTP or oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0021] In some embodiments of the disclosure, a buffer or another additive is combined with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In any of these embodiments, the buffer or another additive may comprise one or more additional reaction conditions, including temperature, pH, concentration, or another reaction condition described herein or know to a person of skill in the art.

[0022] In any of the embodiments described herein, one or more buffers, additives, or reaction conditions may be used in a method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0023] In some embodiments, the present disclosure is directed to compositions and methods of reducing oxidation using a degassing step or a sparging step.

[0024] In some embodiments, the degassing step removes oxygen from the system. Degassing the solutions and mixtures may prevent oxidation of thio-NTPs or oligonucleotides or both.

[0025] In some embodiments of the disclosure, a solution or mixture is degassed or sparged before, after, or continuously while being combined with at thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

DESCRIPTION OF THE INVENTION

[0026] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein, and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry, and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well-known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0027] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present disclosure, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the invention, as a whole.

[0028] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the present disclosure. The section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described. Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0029] As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes more than one polypeptide. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0030] It is to be understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of." It is to be further understood that where descriptions of various embodiments use the term "optional" or "optionally" the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not.

Abbreviations

[0031] The abbreviations used for the genetically encoding nucleosides are conventional and are as follows: adenosine (A); guanosine (G); cytidine (C); thymidine (T); and uridine (U). These abbreviations are also used interchangeably for nucleosides and nucleotides (nucleosides with one or more phosphate groups). Unless specifically delineated, the abbreviated nucleosides or nucleotides may be either ribonucleosides (or ribonucleotides) or 2'-deoxyribonucleosides (or 2'-deoxyribonucleotides). The nucleosides or nucleotides may also be modified at the 3' position. The nucleosides or nucleotides may be specified as being either ribonucleosides (or ribonucleotides) or 2'-

deoxyribonucleosides (or 2'-deoxyribonucleotides) on an individual basis or on an aggregate basis. When nucleic acid sequences are presented as a string of one-letter abbreviations, the sequences are presented in the 5' to 3' direction in accordance with common convention, and the phosphates are not indicated.

Definitions

[0032] In reference to the present disclosure, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise. Accordingly, the following terms are intended to have the following meanings.

[0033] "Protein," "polypeptide," and "peptide" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids, as well as polymers comprising D- and L-amino acids, and mixtures of D- and L-amino acids.

[0034] "Amino acids" are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single letter codes.

[0035] "NQP" as used herein refers to a modified NTP comprising a phosphate group at the 3' position of the sugar moiety, to form a nucleoside tetraphosphate or nucleoside quadriphosphate i.e., NQP. The NQP may serve as a NTP with a removable blocking group (phosphate) during methods of enzymatic oligonucleotide synthesis. The NQP may also comprise additional nucleotide modifications, as described herein.

[0036] "Thio-NTP" or "thio-NQP" as used herein refers to NTPs or NQPs where one or more non-bridging oxygen atoms are replaced with a sulfur atom. In some embodiments, the thio-NTP or thio-NQP may comprise additional modifications. In some embodiments, the thio-NTP comprises a thiophosphate at the alpha (α) phosphate, the beta (β) phosphate, or the gamma (γ) phosphate. In some embodiments, the thio-NTP comprises a mixture of S p and R p diastereomers. In some embodiments, the thio-NTP is a monothiophosphate. In some embodiments, the thio-NTP comprises a dithiophosphate, wherein two non-bridging oxygen atoms in a phosphate group are replaced with two sulfur atoms (e.g., nucleoside 5'-1-1-dithiotriphosphate). In some less common instances, the term thio-NTP may also comprise a dithio-NTP, wherein one non-bridging oxygen atom in two phosphate groups are replaced with two sulfur atoms (e.g., nucleoside 5'-1-2-dithiotriphosphate). In some other embodiments, the thio-NTP may comprise a thio-NTP, wherein three or more non-bridging oxygen

atoms in two or more phosphate groups are replaced by sulfur atoms. As used herein, "thio-NTP" and "thio-NQP" are intended to encompass and refer to all of these variations.

[0037] "Phosphorothioate internucleoside linkages" or "phosphorothioate bonds" as used herein refer to replacement of one or more non-bridging oxygen atoms with sulfur atoms in the phosphate of a typical phosphodiester bond between two nucleosides. The phosphorothioate bond may have one oxygen replaced with sulfur or may comprise a phosphorodithioate bond, where both non-bridging oxygen atoms are replaced with sulfur atoms.

[0038] "Polynucleotide," "oligonucleotide," and "nucleic acid'' are used interchangeably herein and refer to two or more nucleosides or nucleotides that are covalently linked together. The polynucleotide may be wholly comprised of ribonucleotides (i.e., RNA), wholly comprised of 2' deoxyribonucleotides (i.e., DNA), wholly comprised of other synthetic nucleotides or comprised of mixtures of synthetic, ribo- and/or 2' deoxyribonucleotides. The polynucleotides may also include modified nucleotides with substitutions, including 2' substitutions (e.g., 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, locked or constrained ethyl modifications, and others known to those skilled in the art). Nucleosides will be linked together via standard phosphodiester linkages or via one or more non-standard linkages, including but not limited to phosphorothioate linkages. The polynucleotide may be single-stranded or double-stranded or may include both single-stranded regions and double-stranded regions. Moreover, while a polynucleotide will typically be composed of the naturally occurring encoding nucleobases (i.e., adenine, guanine, uracil, thymine, and cytosine), it may include one or more modified and/or synthetic nucleobases, such as, for example, inosine, xanthine, hypoxanthine, etc. In some embodiments, such modified or synthetic nucleobases are nucleobases encoding amino-acid sequences. Nucleobases that are modified or synthetic may comprise any known or hypothetical or future discovered modification or structure that would be recognized by one of skill in the art as a modified or synthetic nucleobase. Similarly, the terms "polynucleotide," "oligonucleotide," and "nucleic acid'' are intended to comprise any modified or synthetic structure that is now known or discovered in the future that would be recognized by one of skill in the art as being or having the function of a "polynucleotide," "oligonucleotide," or "nucleic acid.'' An example of a modified or synthetic structure having the function of a "polynucleotide," "oligonucleotide," or "nucleic acid'' is PNA or peptide nucleic acid.

[0039] "Initiator oligonucleotide", "another initiator," or "oligo acceptor substrate" are used interchangeably herein and refer to any oligo or nucleotide chain or similar moiety with an exposed 3'-OH or equivalent thereof that may be used as a substrate for nucleoside addition or synthesis. In some embodiments, the initiator oligonucleotide may be single stranded. In yet other embodiments, the initiator oligonucleotide may be double stranded or partially doubled stranded. In some embodiments, the initiator oligonucleotide may comprise a nucleotide chain consisting of 1-10 nucleotides, 5-20 nucleotides, 15-50 nucleotides, 30-100 nucleotides, or greater than 100 nucleotides.

In some embodiments, the initiator oligonucleotide may comprise a chemical moiety that is not a nucleotide chain but contains a free -OH capable of being recognized as a substrate by a wild-type or engineered TnT, referred to herein as a "3'-OH equivalent". Exemplary initiator oligonucleotides are provided in the Examples.

[0040] "Removable blocking group" and "blocking group" and "terminator group" and "reversible terminating group" and "inhibitor group" and related variations of these terms are used interchangeably herein and refer to a chemical group that would hinder addition of a second NTP or other natural or modified NTP substrate to the 3' end of the growing oligonucleotide strand prior to removal of the removable blocking from the first round of addition. In some embodiments, the removable blocking group is selected from the group consisting of NTP-3'-O-NH_2, or NTP-3'-O-PO_3.

[0041] "Template-independent synthesis" refers to synthesis of an oligonucleotide or a polynucleotide without the use of template strand as a guide for synthesis of a complementary oligo or polynucleotide strand. Thus, template-independent synthesis refers to an iterative process, whereby, successive nucleotides are added to a growing oligo or nucleotide chain or acceptor substrate. Template-independent synthesis may be in a sequence defined manner or may be random, as is the case with the wild-type TnT (or TdT) in creating antigen receptor diversity. Processes for template-independent synthesis are further described herein.

[0042] "Enzymatic activity" refers to a property of an enzyme, which can be represented by specific activity (*e.g.*, product produced/time/weight protein) or percent conversion of the substrate to the product. Enzyme activity can be measured by any one of many standard assays, such as by monitoring changes in properties of substrates, cofactors, or products. In some embodiments, the amount of products generated can be measured by Liquid Chromatography-Mass Spectrometry (LC-MS), HPLC, capillary electrophoresis, or other methods, as known in the art. Comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein.

[0043] "Conversion" in context of enzymatic activity refers to the enzymatic conversion of the substrate(s) to the corresponding product(s). "Percent conversion" refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, the "enzymatic activity" or "activity" of an enzyme can be expressed as "percent conversion" of the substrate to the product.

[0044] "Conversion" in context of conversion of a thio-phosphate, such as in an oligonucleotide or thio-NTP, to the corresponding phosphodiester refers to replacement of the sulfur atom with an oxygen atom.

[0045] "Suitable reaction conditions" or "reaction conditions" refer to those conditions in a reaction solution (*e.g.*, ranges of enzyme loading, substrate loading, cofactor loading, temperature, pH, buffers,

co-solvents, etc.) under which an enzyme or reagent of the present disclosure is capable of converting one or more substrate compounds to a product. Exemplary "suitable reaction conditions" are provided in the present disclosure and illustrated by the Examples. In some cases, the term "reaction conditions" is also intended to encompass conditions where no reaction occurs, for example, during storage of an oligonucleotide or thio-NTP.

[0046] "Composition" refers to a mixture or combination of one or more substances, wherein each substance or component of the composition retains its individual properties.

[0047] Alkyl" refers to saturated hydrocarbon groups of from 1 to 18 carbon atoms inclusively, either straight chained or branched, more preferably from 1 to 8 carbon atoms inclusively, and most preferably 1 to 6 carbon atoms inclusively. An alkyl with a specified number of carbon atoms is denoted in parenthesis (e.g., $(C_1\text{-}C_6)$alkyl refers to an alkyl of 1 to 6 carbon atoms).

[0048] "Alkenyl" refers to hydrocarbon groups of from 2 to 12 carbon atoms inclusively, either straight or branched containing at least one double bond but optionally containing more than one double bond.

[0049] "Alkynyl" refers to hydrocarbon groups of from 2 to 12 carbon atoms inclusively, either straight or branched containing at least one triple bond but optionally containing more than one triple bond, and additionally optionally containing one or more double bonded moieties.

[0050] "Heteroalkyl, "heteroalkenyl," and heteroalkynyl," refer respectively, to alkyl, alkenyl and alkynyl as defined herein in which one or more of the carbon atoms are each independently replaced with the same or different heteroatoms or heteroatomic groups. Heteroatoms and/or heteroatomic groups which can replace the carbon atoms include, but are not limited to -O-, -S-, -S-O-, -NR^{γ}-, -PH-, -S(O)-, -S(O)2-, -S(O) NR^{γ}-, -$S(O)_2NR^{\gamma}$, and the like, including combinations thereof, where each R^{γ} is independently selected from hydrogen, alkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

[0051] "Amino" refers to the group -NH_2. Substituted amino refers to the group –NHR^{η}, $NR^{\eta}R^{\eta}$, and $NR^{\eta}R^{\eta}R^{\eta}$, where each R^{η} is independently selected from substituted or unsubstituted alkyl, cycloalkyl, cycloheteroalkyl, alkoxy, aryl, heteroaryl, heteroarylalkyl, acyl, alkoxycarbonyl, sulfanyl, sulfinyl, sulfonyl, and the like. Typical amino groups include, but are limited to, dimethylamino, diethylamino, trimethylammonium, triethylammonium, methylysulfonylamino, furanyl-oxy-sulfamino, and the like.

[0052] "Aminoalkyl" refers to an alkyl group in which one or more of the hydrogen atoms are replaced with one or more amino groups, including substituted amino groups.

[0053] "Aminocarbonyl" refers to -$C(O)NH_2$. Substituted aminocarbonyl refers to –$C(O)NR^{\eta}R^{\eta}$, where the amino group $NR^{\eta}R^{\eta}$ is as defined herein.

[0054] "Oxy" refers to a divalent group -O-, which may have various substituents to form different oxy groups, including ethers and esters.

[0055] "Alkoxy" or "alkyloxy" are used interchangeably herein to refer to the group $-OR^{\zeta}$, wherein R^{ζ} is an alkyl group, including optionally substituted alkyl groups.

[0056] "Carboxy" refers to -COOH.

[0057] "Carbonyl" refers to -C(O)-, which may have a variety of substituents to form different carbonyl groups including acids, acid halides, aldehydes, amides, esters, and ketones.

[0058] "Carboxyalkyl" refers to an alkyl in which one or more of the hydrogen atoms are replaced with one or more carboxy groups.

[0059] "Aminocarbonylalkyl" refers to an alkyl substituted with an aminocarbonyl group, as defined herein.

[0060] "Halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

[0061] "Haloalkyl" refers to an alkyl group in which one or more of the hydrogen atoms are replaced with a halogen. Thus, the term "haloalkyl" is meant to include monohaloalkyls, dihaloalkyls, trihaloalkyls, etc. up to perhaloalkyls. For example, the expression "(C_1 - C_2) haloalkyl" includes 1-fluoromethyl, difluoromethyl, trifluoromethyl, 1-fluoroethyl, 1,1-difluoroethyl, 1,2-difluoroethyl, 1,1,1 trifluoroethyl, perfluoroethyl, etc.

[0062] "Hydroxy" refers to -OH.

[0063] "Hydroxyalkyl" refers to an alkyl group in which in which one or more of the hydrogen atoms are replaced with one or more hydroxy groups.

[0064] "Thiol" or "sulfanyl" refers to –SH. Substituted thiol or sulfanyl refers to $-S-R^{\eta}$, where R^{η} is an alkyl, aryl, or other suitable substituent.

[0065] "Sulfonyl" refers to $-SO_2-$. Substituted sulfonyl refers to $-SO_2-R^{\eta}$, where R^{η} is an alkyl, aryl, or other suitable substituent.

[0066] "Alkylsulfonyl" refers to $-SO_2-R^{\zeta}$, where R^{ζ} is an alkyl, which can be optionally substituted. Typical alkylsulfonyl groups include, but are not limited to, methylsulfonyl, ethylsulfonyl, n-propylsulfonyl, and the like.

[0067] "Phosphate" as used herein refers to a functional group comprised of an orthophosphate ion (phosphorous atom covalently linked to four oxygen atoms). The orthophosphate ion is commonly found with one or more hydrogen atoms or organic groups.

[0068] "Phosphorylated" as used herein refers to the addition or presence of one of more phosphoryl groups (phosphorous atom covalently linked to the three oxygen atoms).

[0069] "Reaction" as used herein refers to a process in which one or more substances or compounds or substrates is converted into one or more different substances, compounds, or processes.

[0070] "Oxidation" as used herein refers to a type of reaction wherein, generally, a molecule loses electrons. In some embodiments, "oxidation" refers to the loss of sulfur atoms in a phosphorothioate bond or a thio-NTP via replacement with oxygen atoms. Thus, oxygen or another oxidizing agent may lead to oxidation of one or more phosphorothioate bonds or thio-NTPs. As described herein, oxidation may be measured as a percentage or ratio of oxidized species to non-oxidized species.

[0071] "Reduction" as used herein refers to a type of reaction wherein, generally, a molecule gains electrons. In some embodiments, a reducing agent or antioxidant prevents oxidation of a phosphorothioate bond or a thio-NTP. Herein, "reduce" or "reducing" may also be used in the ordinary context to mean a lowering of or decrease.

Phosphorothioate Bonds and Thio-NTPs in Oligonucleotide Synthesis

[0072] In some embodiments, the present disclosure is directed to compositions and methods of reducing conversion (e.g., by oxidation, etc.) of phosphorothioate bonds during the synthesis and storage of oligonucleotides. In some related embodiments, the present disclosure is directed to compositions and methods of reducing conversion of thio-NTPs. In some embodiments, the methods or compositions of the present disclosure find use in reducing phosphorothioate to phosphodiester impurities in an oligonucleotide or therapeutic oligonucleotide.

[0073] Many therapeutic oligonucleotides include one or more phosphorothioate internucleoside linkages or bonds to enhance stability or provide other beneficial effects *in vivo*, e.g., to reduce susceptibility to nucleases.

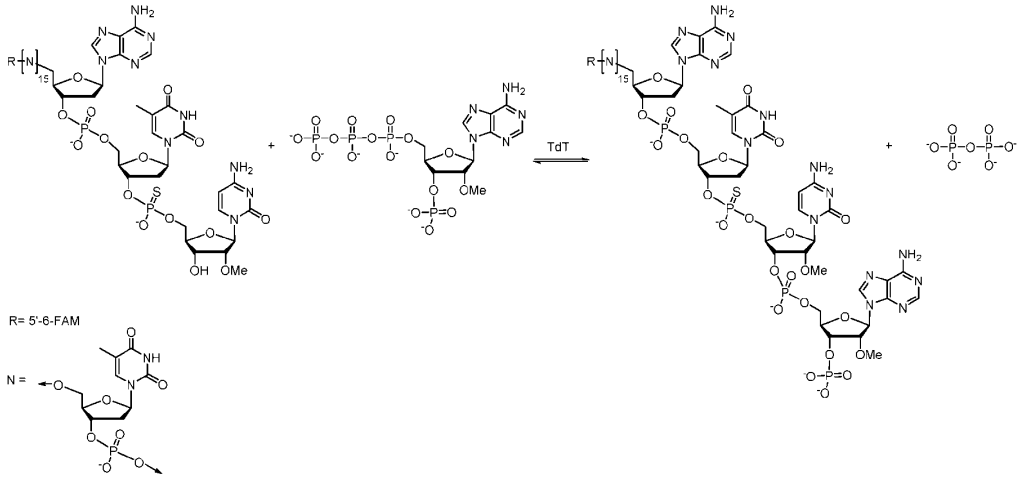
[0074] In traditional phosphoramidite chemistry, phosphorothioate bonds are typically installed on the nucleotide after addition to the growing oligonucleotide chain. In contrast, enzymatic methods of oligonucleotide synthesis may use a nucleoside triphosphate containing one or two sulfur atoms in place of non-bridging oxygen atoms at the alpha position of the phosphate chain (thio-NTP). In this case, the addition of the thio-NTP to the growing oligonucleotide chain creates the phosphorothioate or phosphorodithioate bond.

[0075] In some embodiments, the present disclosure provides compositions and methods of reducing conversion of phosphorothioate bonds during enzymatic oligonucleotide synthesis. In some embodiments, the enzymatic oligonucleotide synthesis comprises template independent oligonucleotide synthesis.

Enzymatic Oligonucleotide Synthesis

[0076] Enzymatic oligonucleotide synthesis is a promising alternative to traditional chemical oligonucleotide synthesis, with less environmental waste, among several advantages. A recent report

(PCT/US2023/076667) describes one method of template independent oligonucleotide synthesis using a modified terminal deoxynucleotidyl transferase (TdT) together with an inorganic phosphatase and an alkaline phosphatase. In some embodiments, the engineered TdT is referred to as a terminal nucleotidyl transferase or TnT. In one embodiment, an oligonucleotide or initiator substrate with an exposed 3'-OH allows addition of a defined modified NTP substrate (in this example, a NTP-3'-P), as depicted in **Scheme 1**, below.



Scheme 1

[0077] The TdT (or TnT) enzyme catalyzes the addition of the NTP-3'P, while an inorganic pyrophosphatase (iPP or iPPase) is used concurrently to degrade the pyrophosphate product and shift the reaction equilibrium toward the forward direction. After reaction of the NTP-3'-P (or NQP) with the 3'-OH of the oligonucleotide, the TdT is blocked from further reaction by the 3'-phosphate. The 3'-phosphate is then removed by an alkaline phosphatase, exposing the 3'-OH and allowing another round of addition. After each round of addition, the 3'-phosphate is removed from the NTP, and a new NTP-3'-P (NQP) is added to sequentially and efficiently create a defined oligonucleotide sequence. After synthesis of the defined oligonucleotide is complete, the oligonucleotide chain may be cleaved or released from the initiator.

[0078] A variety of modified NTPs may be used in this method, as may be envisioned by one of skill in the art, including use of thio-NTPs to synthesize oligonucleotides with phosphorothioate internucleoside linkages or bonds. Similarly, an initiator oligonucleotide comprising one or more phosphorothioate bonds may be used in this method.

[0079] While a specific enzymatic synthesis method has been described herein, and in the Examples, the methods and compositions of the present disclosure are not so limited. Indeed, the methods and compositions described herein find utility in a variety of enzymatic and non-enzymatic methods, as

well as during storage of thio-NTPs or oligonucleotides produced used any methods. The compositions and methods of the present disclosure find use in any suitable method, including those methods of enzymatic oligonucleotide synthesis using terminal nucleotidyl transferases (TdTs), Pol X polymerases, Poly(N) polymerases, Polμ polymerases, Polβ polymerases, Polλ polymerases, and Polθ polymerases, and others (see for example, U.S. Pat. 10059929, U.S. Pat. 10,760,063, U.S. Pat. 10,774,316, U.S. Pat. 11,390,858, U.S. Pat. 10,745,727, PCT/GB2020/050247, US20210164008A1, US20210407509, WO2016GB50301, WO17216472, WO18215803, WO20072715, WO20077227, WO21122539, WO2022029427, and WO21116270, from Molecular Assemblies, Nuclera, DNA Script, and others, each of which is specifically incorporated herein).

[0080] As described elsewhere (including U.S. Provisional patent application No. 63/646,600) and further herein, methods of enzymatic oligonucleotide synthesis may be accomplished using a variety of reaction conditions and configurations, including those with an immobilized oligonucleotide or one or more immobilized enzymes. Similarly, the synthesis reaction may occur as a batch method or using a variety of reservoirs, solid supports, columns, or other reaction processes or conditions. While the methods and compositions of the present disclosure have been described with reference to specific embodiments in the Examples, a person of skill in the art will recognize their applicability in all such methods and compositions.

Methods and Compositions for Reducing Conversion of Thio-NTPs and Phosphorothioate Oligonucleotides

[0081] In some embodiments, the present disclosure is directed to compositions and methods of reducing conversion (e.g., by oxidation, etc.) of phosphorothioate bonds during the synthesis and storage of oligonucleotides. In some related embodiments, the present disclosure is directed to compositions and methods of reducing conversion of thio-NTPs. In some embodiments, the methods or compositions of the present disclosure find use in reducing phosphorothioate to phosphodiester impurities in an oligonucleotide or therapeutic oligonucleotide.

[0082] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises one or more of the following:

i) selecting a solid support for immobilization of one or more enzymes or an oligonucleotide;

ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support;

iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent;

iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition; and

v) optionally degassing or sparging the solution or mixture comprising a thio-NTP or an

oligonucleotide comprising one or more phosphorothioate bonds;

such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0083] In some embodiments wherein the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises one or more enzymes, the method may result in reduced activity of one or more enzymes.

[0084] Therefore, in certain preferred embodiments of the method of reducing oxidation of a thio-NTP or an oligonucleotide where the method comprises one or more enzymes, the method further comprises reduced loss of activity or increased maintenance of activity of one or more enzymes.

[0085] Therefore, in some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises one or more of the following:

i) selecting a solid support for immobilization of one or more enzymes or an oligonucleotide;

ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support;

iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent;

iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition; and

v) optionally degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds;

such that a) oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced; or b) the loss of activity is reduced, or the maintenance of activity is increased for one or more enzymes; or c) both a) and b) are true.

Storage (Non-Reactive) Methods

[0086] In some embodiments, the present disclosure provides a method of storing a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, storage is a non-reactive method (e.g., no synthesis reactions). Thus, in some embodiments, the method of reducing coversion (e.g., oxidation) of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0087] In some embodiments, the method comprising storage of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds further comprises combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition, such that conversion, e.g., by oxidation, of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0088] In some embodiments, the method comprising storage of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds further comprises degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent; ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition; and iii) degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced. In some embodiments, the solution or mixture may be degassed or sparged prior to combining or contacting with the thio-NTP or oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the solution or mixture may be degassed or sparged continuously while combining or contacting with the thio-NTP or oligonucleotide comprising one or more phosphorothioate bonds.

Synthesis Methods

[0089] In some embodiments, the method comprises immobilization of one or more enzymes or an oligonucleotide on a solid support. In some embodiments where the method comprises an oligonucleotide immobilized on a solid support, one or more enzymes or reagents may be used to extend the oligonucleotide by addition of one or more NTPs or modified NTPs (or NQPs). In some embodiments where the method comprises one or more enzymes immobilized on a solid support, the one or more enzymes may be used to extend an oligonucleotide by addition of one or more NTPs or modified NTPs (or NQPs). In some embodiments, the method further comprises a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0090] In some embodiments wherein the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises one or more enzymes, the method may result in reduced activity of one or more enzymes.

[0091] Therefore, in certain preferred embodiments of the method of reducing oxidation of a thio-NTP or an oligonucleotide where the method comprises one or more enzymes, the method further comprises reduced loss of activity or increased maintenance of activity of one or more enzymes.

[0092] Thus, in some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0093] In some embodiments, the method comprising immobilization of one or more enzymes or an oligonucleotide on a solid support further comprises contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide; ii) contacting a first quenching agent and, optionally, a second quenching agent with the solid support to reduce or neutralize unreacted reactive groups on the solid support; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0094] In some embodiments, the method comprising immobilization of one or more enzymes or an oligonucleotide on a solid support further comprises combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide; ii) contacting a first quenching agent and, optionally, a second quenching agent with the solid support to reduce or neutralize unreacted reactive groups on the solid support; iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support; and iv) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0095] In some embodiments, the method comprising immobilization of one or more enzymes or an oligonucleotide on a solid support further comprises combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide; ii) contacting a first quenching agent and, optionally, a second quenching agent with the solid support to reduce or neutralize unreacted reactive groups on the solid support; iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support; iv) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent; and v) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0096] In some embodiments, the method comprising immobilization of one or more enzymes or an oligonucleotide on a solid support further comprises combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide; ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support; iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support; iv) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent; and v) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

[0097] In some embodiments, the method comprising immobilization of one or more enzymes or an oligonucleotide on a solid support further comprises degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. Thus, in some embodiments, the method of reducing oxidation a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises i) selecting a solid support for and immobilization of one or more enzymes or an oligonucleotide; ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support; iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more

phosphorothioate bonds with the solid support; iv) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent; v) combining or contacting the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds with a buffer, additive, or another reaction condition; and vi) degassing or sparging the solution or mixture comprising the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced.

Thio-NTPs

[0098] In some embodiments of the present disclosure, the methods or compositions comprise a NTP or modified NTP with one or more modifications to the 5'-phosphate chain. In some embodiments, the modification is replacement of one or more non-bridging oxygen atoms with a sulfur atom in one or more phosphates in the phosphate chain of the NTP or modified NTP. In some embodiments, the NTP with an additional modification may comprise a 3'-phosphate blocking group (NQP).

[0099] In the present disclosure, NTPs or NQPs where one or more non-bridging oxygen atoms are replaced with a sulfur atom are referred to collectively as "thio-NTPs" or "thio-NQPs." In some embodiments, the thio-NTP or thio-NQP may comprise additional modifications, as described herein. Similarly, it is to be understood that thio-NDPs and thio-NMPs may also be present when using the compositions and methods of the present disclosure.

[0100] In some embodiments, the thio-NTP comprises a thiophosphate at the alpha (α) phosphate. In some embodiments, the thio-NTP comprises a thiophosphate at the beta (β) phosphate. In some embodiments, the thio-NTP comprises a thiophosphate at the gamma (γ) phosphate.

[0101] In some embodiments, the thio-NTP comprises a monothiophosphate at the alpha (α), beta (β), or gamma (γ) phosphate. In some embodiments, the thio-NTP comprises a mixture of S p and R p diastereomers.

[0102] In some embodiments, the thio-NTP comprises a dithiophosphate, wherein two non-bridging oxygen atoms in a phosphate group are replaced with two sulfur atoms (e.g., nucleoside 5'-1-1-dithiotriphosphate). In some less common instances, the term dithio-NTP may also comprise a thio-NTP, wherein one non-bridging oxygen atom in two phosphate groups are replaced with two sulfur atoms (e.g., nucleoside 5'-1-2-dithiotriphosphate). In some other embodiments, the thio-NTP may comprise a thio-NTP, wherein three or more non-bridging oxygen atoms in two or more phosphate groups are replaced by sulfur atoms.

[0103] In some embodiments, a nucleoside 5'-thiotriphosphate is referred to as a S-NTP or a NTP-S. In some embodiments, a nucleoside-5'-1-thio(triphosphate) is referred to as a NTPαS or a αS-NTP. In

some embodiments, a nucleoside-5'-2-thio(triphosphate) is referred to as NTPβS or βs-NTP. In some embodiments, a nucleoside-5'-3-thio(triphosphate) is referred to as NTPγS or γs-NTP.

[0104] In some embodiments of the present disclosure, one or more thio-NTPs or thio-NQPs are used in a composition or a method of synthesis of an oligonucleotide. In embodiments where the thio-NTP or thio-NQP is a αS-NTP or αS-NQP, the extension of the oligonucleotide with the αS-NTP or αS-NQP results in formation of a phosphorothioate (or phosphorodithioate) internucleoside linkage or bond.

[0105] In some embodiments, two or more thio-NTPs or thio-NQPs are used in a composition or a method of synthesis of an oligonucleotide to form an oligonucleotide with two or more phosphorothioate (or phosphorodithioate) internucleoside linkages or bonds.

[0106] In some embodiments, a thio-NTP or thio-NQP exposed to oxygen, an oxidant, or another oxidizing reaction condition may oxidize to a NTP or NQP, wherein one or more non-bridging sulfur atoms are replaced with an oxygen atom.

[0107] In some embodiments where one or more thio-NTPs or thio-NQPs are used in a composition or a method of synthesis of an oligonucleotide, oxidation of the thio-NTPs or thio-NQPs may result in synthesis of an oxidized oligonucleotide.

Oligonucleotides

[0108] In some embodiments of the present disclosure, the methods or compositions comprise one or more oligonucleotides.

[0109] The oligonucleotide generally comprises two or more nucleotides that are covalently linked together. The oligonucleotide may be wholly comprised of ribonucleotides (i.e., RNA), wholly comprised of 2' deoxyribonucleotides (i.e., DNA), or comprised of mixtures of ribo- and 2' deoxyribonucleotides.

[0110] In some embodiments, the oligonucleotide may refer generally to an initiator oligonucleotide or another initiator or oligo acceptor substrate, capable of accepting a NTP or NQP during synthesis or extension of the oligonucleotide. In some embodiments, the oligonucleotide comprises an initiator oligonucleotide of 1-2 nucleotides, 2-4 nucleotides, 4-7 nucleotides, or 5-12 nucleotides.

[0111] In some embodiments, the oligonucleotide comprises 3-10 nucleotides, 5-15 nucleotides, 10-20 oligonucleotides, 12-32 nucleotides, 20 to 50 nucleotides, 30-100 nucleotides, 50-300 nucleotides, 200 to 700 nucleotides, or more.

[0112] In some embodiments, the oligonucleotide is a therapeutic RNA or another therapeutic oligonucleotide. In some embodiments, the oligonucleotide is a siRNA, a sgRNA, a mRNA, a

miRNA, an asRNA, a RNA aptamer, a DNA aptamer, an antisense oligonucleotide, or another therapeutic oligonucleotide.

[0113] In some embodiments, the oligonucleotide may be single-stranded or double-stranded or may include both single-stranded regions and double-stranded regions. In some embodiments, the oligonucleotide may be circularly permutated.

[0114] In some embodiments, the oligonucleotide comprises one or more modified nucleotides. In some embodiments, the modified nucleotide comprises one or more modifications to the sugar moiety, the nucleobase, or the internucleoside linkage or bond.

[0115] In some embodiments, the oligonucleotide comprises nucleosides linked together by standard phosphodiester linkages. In some embodiments, the oligonucleotide may comprise one or more non-standard linkages as further described herein, such as a phosphorothioate linkage.

[0116] In some embodiments, the oligonucleotide comprises nucleotides composed of the naturally occurring encoding nucleobases (i.e., adenine, guanine, uracil, thymine, and cytosine). In some embodiments, the oligonucleotide comprises nucleotides composed of one or more modified and/or synthetic nucleobases, such as, for example, inosine, xanthine, hypoxanthine, as further described herein.

[0117] In some embodiments, the present disclosure is directed to a composition or method of synthesis of an oligonucleotide. In some embodiments, the oligonucleotide is immobilized on a solid support. In some embodiments, the oligonucleotide is present in a solution or other mixture. In some embodiments, the composition or method of synthesis comprises one or more enzymes. In some embodiments, the composition or method of synthesis comprises one or more of the following, a solid support, a first quenching agent (and, optionally, a second quenching agent), an antioxidant or reducing agent, a buffer, another additive, another reaction condition, and degassing or sparging of a solution or mixture.

[0118] In some embodiments directed to a composition or method of synthesis of an oligonucleotide, oxygen, an oxidant, or another oxidizing reaction condition may be present in a solution or mixture comprising the oligonucleotide. In some embodiments, the oligonucleotide may be exposed to oxygen, an oxidant, or another oxidizing reaction condition that may oxidize the oligonucleotide, wherein one or more phosphorothioate internucleoside linkages are oxidized to one or more phosphodiester internucleoside linkages.

Nucleotide Modifications

[0119] In some embodiments of the present disclosure, the composition or method comprises one or more oligonucleotides or one or more NTPs or NQPs (including thio-NTPs or thio-NQPs). In some

embodiments, the oligonucleotide comprises one or more nucleotides. In any of the embodiments described herein, the nucleotides, NTPs, or NQPs may comprise additional modifications.

[0120] In some embodiments, the nucleotide, NTP, or NQP may comprise one or more modifications to the sugar. In some embodiments, the nucleotide, NTP, or NQP may comprise one or more modifications to the nucleobase. In some embodiments, the nucleotide, NTP, or NQP may comprise one or more modifications to the phosphate chain or one or more non-standard internucleoside linkages or bonds.

2' Modifications of Sugar Moiety

[0121] In some embodiments, the nucleotide, NTP, or NQP may comprise one or more modifications to the sugar at the 2' position. In some embodiments, the modification at the 2'- position comprises a halo (e.g., Cl, F, Br, etc.) or -O-alkyl or 2'-alkoxy (e.g., O-methyl, O-ethyl, etc.). In some embodiments, the modification at the 2'- position comprises an allyl, amino, azido, SH, CN, OCN, CF_3, OCF_3, SCH_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, and NH_2. In some embodiments, the modification at the 2'- position comprises a O-(C_1-C_{10})alkoxy, alkoxyalkyl, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, O-alkynyl, S-alkynyl, N-alkynyl, O-alkyl-O-alkyl, alkynyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C_1-C_{10} alkyl or C_1-C_{10} alkenyl and alkynyl. In some embodiments, the modification at the 2'- position comprises an alkaryl, aralkyl, O-alkaryl, and O-aralkyl. In some embodiments, the modification at the 2'- position comprises a phosphate.

[0122] In some embodiments the modification at the 2'- position comprises a 2'-O-R', or 2'-O-COR', where R' is an alkyl, alkyloxyalkyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, arylalkyl, or heteroarylalkyl. In some embodiments, R' is a C_1-C_4alkyl. In some embodiments, the modified 2'-position comprises a 2'-O-R', wherein in R' is alkyloxyalkyl, alkylamine, cyanoalkyl, or -C(O)-alkyl. In some embodiments, the modification at the 2'- position comprises a -O-R', wherein R' is $-CH_3$ or $-CH_2CH_3$ or $-CH_2CH_2OCH_3$. In some embodiments, the modification at the 2'- position comprises a 2'-O-(2-methoxyethyl), 2'-O-allyl, 2'-O-propargyl, 2'-O-ethylamine, 2'-O-cyanoethyl, -2'-O-amine, or 2'-O-acetate ester.

[0123] In some embodiments, the sugar may have other modifications at other positions, such as locked nucleotides or constrained ethyl nucleotides, as is known in the art. In some embodiments, "locked nucleoside" or "locked nucleotide" or "locked nucleic acid" (LNA) refers to a nucleoside, nucleotide, or nucleic acid, respectively, in which the ribose moiety is modified with a bridge connecting the 2' oxygen and 4' carbon. The LNA may be either in the C3'-endo (beta-D-LNA) or C2'-endo (alpha-L-LNA) conformation (see, e.g., Obika et al., Tetrahedron Letters, 1997, 38(50):8735–8738; Orum et al., Current Pharmaceutical Design, 2008, 14(11):1138–1142).

Typically, the bridge is a methylene bridge. LNA may confer additional stability to an oligonucleotide.

[0124] In some embodiments, the nucleotide, NTP, or NQP comprises a glycol nucleic acid (GNA) modification, wherein the sugar moiety of the NTP is replaced by propylene glycol. GNA may confer additional stability to an oligonucleotide.

Phosphate Chain and Internucleoside Linkages

[0125] In some embodiments, the nucleotide, NTP, or NQP comprises one or more modifications of the phosphate chain. In some embodiments, the modification is exchange of one or more oxygen atoms in one or more phosphates of the triphosphate chain with one or more sulfur atoms to form a thio-NTP or thio-NQP, as described above. In some embodiments, the nucleotide comprises a non-standard internucleoside linkage (e.g., a phosphorothioate bond, as described above).

[0126] In some embodiments, the nucleotide is present in an oligonucleotide. In some embodiments, the oligonucleotide comprises one or more non-standard or modified internucleoside linkages. In some embodiments, the oligonucleotide has 1%, 2%, 5%, 10% 20%, 30%, 40%, 50%, or 60% or more modified internucleoside linkages. In some embodiments, all of the internucleoside linkages are modified internucleoside linkages.

[0127] In some embodiments, the modified internucleoside linkage is a phosphorous containing modified internucleoside linkage. Exemplary phosphorous containing internucleoside linkages include, among others, phosphotriesters, alkylphosphonates (e.g., methyl phosphonate, ethyl phosphonate, etc.), phosphoramidates, phosphorothioate, and phosphorodithioate.

[0128] In some embodiments, the modified internucleoside linkage is a non-phosphorous containing internucleoside linkage. Exemplary non-phosphorous containing internucleoside linkages include, among others, methylenemethylimino (-CH_2-N(CH_3)-O-CH_2), thiodiester, thionocarbamate (-O-C(=O)(NH)-S-); siloxane (-O-SiH_2-O-); N,N'-dimethylhydrazine (-CH_2-N((CH_3)-N((CH_3)-); MMI (3'-CH_2-N(CH_3)-O-5'), amide-3 (3'-CH_2-C(=O)-N(H)-5'), amide-4 (3'-CH_2-N(H)-C(=O)-5'), formacetal (3'-O-CH_2-O-5'), methoxypropyl, and thioformacetal (3'-S-CH_2-O-5'). In some embodiments, the modified internucleoside linkage is amide linkage, such as those of glycine nucleosides or nucleoside b-amino acids (see, e.g., Banerjee et al., Bioconjugate Chem., 2015, 26, 8, 1737–1742).

[0129] In some embodiments, the modified internucleoside linkages provide for a chiral center. For example, a phosphorothioate or alkylphosphonate internucleoside linkage can be in the Rp or Sp stereomeric configuration. In some embodiments, the oligonucleotide has a mixture of stereoisomers in the internucleoside linkage. In some embodiments, the oligonucleotide has greater than 50% of the

internucleoside linkages as Rp or Sp configuration. In some embodiments, the oligonucleotide has at least 60%, 70%, 80%, 90%, or greater of Rp or Sp stereomeric configuration.

Modified Nucleobase

[0130] In some embodiments, the nucleobase of the nucleotide, NTP, or NQP is adenine, cytosine, guanine, thymine, uracil, xanthine, hypoxanthine, 2,6-diaminopurine, purine, 6,8 diaminopurine, 5-methylcytosine (m5C), 2-thiouridine, pseudouridine, dihydrouridine, inosine, or 7 methylguanosine (m7G).

[0131] In some embodiments, the nucleotide, NTP, or NQP comprises a modified nucleobase. In some embodiments, the modified nucleobase of the nucleoside substrate is 5-bromo-uracil, 5-iodo-uracil, 6 mCEPh-purine, 6-phenylpyrrolocytidine, N2-alkyl 8-oxoguanosine, difluorotoluene, difluorobenzene, dichlorobenzene, imidazole, or benzimidazole.

[0132] In some embodiments, the nucleotide, NTP, or NQP comprises a nucleobase that is, among others, 5-methylcytosine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 3 methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5- alkylcytidines, 5 alkyluridines, 5 halouridines, 6 azapyrimidines, 6- alkylpyrimidines, 5-propynyl-uracil, 2 thio 5 propynyl uracil, quesosine, 2 thiouridine, 4-thiouridine, 4-acetyltidine, 5 (carboxyhydroxymethyl)uridine, 5 carboxymethylaminomethyl-2-thiouridine, 5 carboxymethylaminomethyluridine, D galactosylqueosine, 1-methyladenosine, 1 methylinosine, 2,2 dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2 methylguanosine, N6-methyladenosine, 7 methylguanosine, 5 methoxyaminomethy1 2 thiouridine, 5-methylaminomethy luridine, 5 methylcarbon ylmethyluridine, 5 methyloxyuridine, 5-methyl-2-thiouridine, 2 methylthio N6 isopentenyladenosine, D mannosylqueosine, uridine-5-oxyacetic acid, 2 thiocytidine, N1-methyl-adenine, N6-methyl- adenine, 8'-azido-adenine, N,N dimethy1 adenosine, aminoally1 adenosine, 5′-methy1-urdine, pseudouridine, N1 methy1 pseudouridine, 5′ hydroxy methy1 uridine, 2′-thio-uridine, 4'-thio- uridine, hypoxanthine, xanthine, 5′-methyl-cytidine, 5′ hydroxy methyl cytidine, 6' thio guanine, or N7 methyl-guanine.

[0133] In some embodiments, the nucleotide, NTP, or NQP comprises a noncanonical nucleobase, a removable tag, a cleavable linker, or a radio, a photo, and/or a chemical sensor.

Conjugates

[0134] In some embodiments, the oligonucleotide, nucleotide, NTP, or NQP further comprises a conjugate moiety. In some embodiments, the conjugate moiety comprises a carbohydrate (e.g. GalNAc), lipid, sterol, drug substance, hormone, polymer (e.g., polyethylene glycol, etc.), protein, peptide, toxin (e.g. bacterial toxins, etc.), vitamin (e.g., folate, tocopherol, retinoic acid, etc.), or combinations thereof. In some embodiments, the conjugate moiety is used to affect the pharmacokinetics of the oligonucleotide and/or oligonucleotide cell targeting.

[0135] In some embodiments, the conjugate moiety can be attached to the 5'-terminal nucleotide, the 3'-terminal nucleotide, or in a polynucleotide or oligonucleotide an internal nucleotide. In some embodiments, the conjugate moiety is attached the 2'-position of the sugar moiety of a nucleoside, for example, to the 2'-OH. In some embodiments, the conjugate moiety is attached to the 3'-position of the sugar moiety of the nucleoside, for example, to the 3'-OH. In some embodiments, the conjugate moiety is attached to the nucleobase.

[0136] In some embodiments, the conjugate moiety is attached directly or attached using a linker.

3' Blocking Group

[0137] In some embodiments, the nucleotide may comprise a NTP with a 3′ blocking group (e.g., a blocking group on the 3′ position of the sugar of the nucleotide). A 3' blocking group, also known to those skilled in the art as an inhibitor or reversible terminating group, may include a variety of groups that prevent the TdT or another enzyme from adding an additional nucleotide to the oligonucleotide. The blocking group may comprise a charged molecule, large molecule and moiety, or other blocking group known to those skilled in the art. Appropriate 3' blocking groups comprise carbonitriles, phosphates, carbonates, carbamates, esters, ethers, borates, nitrates, sugars, phosphoramidates, phenylsulfenates, and sulfates. Other 3′ blocking groups are also known in the art, including 3′-O-amines and methylamines.

[0138] NTPs with a phosphate group at the 3′ position of the sugar (nucleoside tetraphosphates or pppNps) are otherwise known herein as "NQPs", and may comprise additional modifications to the nucleobase, sugar, and/or phosphate chain, as provided herein.

[0139] In some embodiments described herein, and in the Examples, a NTP with a 3'-phosphate blocking group may comprise a thio-NQP and find use in a method of enzymatic oligonucleotide synthesis.

Support Materials and Solid Supports

[0140] In some embodiments, the method or composition comprises a support material or solid support. In some embodiments, the support material or solid support is used in a method of oligonucleotide synthesis. In some embodiments, the method of oligonucleotide synthesis comprises a thio-NTP or an oligonucleotide comprising at least one phosphorothioate bond. In some embodiments, selection a suitable support material or solid support comprises reduced oxidation of a thio-NTP or one or more phosphorothioate bonds in an oligonucleotide.

Attachment types

[0141] In some embodiments, the polypeptide is immobilized on a support material. In some embodiments, the polypeptide is adsorbed on the support material. In some embodiments, the polypeptide is immobilized on the support material by covalent attachment. In some embodiments, the

polypeptide is immobilized on the support material by affinity interaction. In some embodiments, the polypeptide is immobilized on the support material by an anionic, ionic, ion exchange, or electrostatic attachment.

[0142] Various methods for conjugation and immobilization of enzymes to solid supports (e.g., resins, membranes, beads, glass, etc.) are known in the art (see e.g., Yi et al., Proc. Biochem., 2007, 42(5): 895-898; Martin et al., Appl. Microbiol. Biotechnol., 2007, 76(4): 843-85; Koszelewski et al., J. Mol. Cat. B: Enzymatic, 2010, 63:39-44; Truppo et al., Org. Proc. Res. Dev., published online: dx.doi.org/10.1021/op200157c; Hermanson, Bioconjugate Techniques, 2nd Ed., Academic Press, Cambridge, MA (2008); Mateo et al., Biotechnol. Prog., 2002, 18(3):629-34; and "Bioconjugation Protocols: Strategies and Methods," In Methods in Molecular Biology, Niemeyer (ed.), Humana Press, New York, NY (2004); the disclosures of each which are incorporated by reference herein).

Support materials

[0143] In some embodiments, the support material comprises a substrate or support medium, such as a solid substrate, a porous substrate, a membrane, or particles. In some embodiments, the support material comprises inorganic materials, such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, phenol/formaldehyde, and bentonite. In some embodiments, the support material comprises organic materials, such as cellulose (CMC, DEAE-cellulose), starch, activated carbon, polyacrylamide, polymethacrylate, polyacrylate, polystyrene, styrene, microporous styrene, crosslinked aliphatic polymer, and ion-exchange resins, such as Amberlite, Sephadex, and Dowex. In some embodiments, the support material comprises polymeric materials such as calcium-alginate, agar, k-carrageenin, polyacrylamide, and collagen. In some embodiments, the support material is a particle, a membrane, or a fiber. Types of membranes include, among others, nylon, cellulose, polysulfone, or polyacrylate.

[0144] In some embodiments, the support material comprises a polymethacrylate resin. Exemplary polymethacrylate resins include HFA (Resindion), HA (Resindion), BU (Resindion), EP (Resindion), EP/S (Resindion), EP403/M (Resindion), EP600 (Resindion), SP600 (Resindion), HFA403/S (Resindion), ECR8804F (Purolite), ECR8405F (Purolite), and EMC7042/M (Sunresin).

[0145] In some embodiments, the support material comprises a polyacrylic resin. Exemplary polyacrylic resins include IB-COV-2 (ChiralVision BV), IB-COV-6 (ChiralVision BV), IB-COV-7 (ChiralVision BV), IB-COV-8 (ChiralVision BV), IB-ANI-5 (ChiralVision BV), IB-ANI-7 (ChiralVision BV), IB-ANI-8 (ChiralVision BV), IB-ANI-10 (ChiralVision BV), IB-ANI-13 (ChiralVision BV), EMC7025 (Sunresin), EMC7014 (Sunresin), EMC7032 (Sunresin), EMC7120/M (Sunresin), Chelex 7350 (Sunresin), EMC7225/M (Sunresin), EMC7042/M (Sunresin), EA403/M (Resindion), and HA403/M (Resindion).

[0146] In some embodiments, the support material comprises a cellulose resin. Exemplary cellulose resins include IB-ANI-13 (ChiralVision BV) and IB-COV-10 (ChiralVision BV).

[0147] In some embodiments, the support material comprises a polystyrene, styrene, or macroporous styrene resin. Exemplary polystyrene, styrene, or macroporous styrene resins include IB-ANI-2 (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), and ECR1640 (Purolite).

[0148] In some embodiments, the support material comprises a controlled pore glass (CPG) resin. Exemplary CPGs include CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), and CPG-21 (LGC).

[0149] In some embodiments, the support material comprises silica. Exemplary silica resins include IB-SLC(500A)-MPTMS-P500DGE (ChiralVision), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision), IB-SLC(500A)-GPTMS (ChiralVision), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision), and IB-SLC(500A)-GPTMS-MTMS (ChiralVision).

[0150] In some embodiments, the support material comprises an affinity resin. Exemplary affinity resins include IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), and IB-His-2 Co(II) (ChiralVision).

[0151] In some embodiments, the support material comprises another organic or inorganic material. Additional exemplary support materials comprise A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

Particle sizes

[0152] In some embodiments, the solid support comprises a resin, bead, or particle with a uniform particle size. In some embodiments, the particle size is not uniform or comprises a mixture of one or more particle sizes. In some embodiments, the solid support comprises a particle size of about 80 μM, about 100 μM, about 150 μM, about 200 μM, about 250 μM, about 300 μM, about 350 μM, about 400 μM, about 450 μM, about 500 μM, about 550 μM, about 600 uM, about 650 uM, about 750 uM, about 850 uM, about 950 uM, or about 1000 uM. In some embodiments, the particle size comprises a range of sizes. Some exemplary particle size ranges include a particle size of about 80 μM to about 100 μM, about 100 μM to about 300 μM, about 200 μM to about 700 μM, about 300 μM to about 600 μM, and about 400 μM to about 600 μM.

Pore sizes

[0153] In some embodiments, the solid support comprises a resin, bead or particle that is porous. In some embodiments, the solid support comprises pores of size of about 200 angstroms, about 300 angstroms, about 400 angstroms, about 500 angstroms, about 600 angstroms, about 700 angstroms,

about 800 angstroms, about 900 angstroms, about 1000 angstroms, about 1100 angstroms, about 1200 angstroms, about 1300 angstroms, about 1400 angstroms, about 1500 angstroms, about 1600 angstroms, about 1750 angstroms, or about 2000 angstroms. In some embodiments, the pore size is not uniform or comprises a mixture of one or more pore sizes. In some embodiments, the pore size comprises a range of sizes. Some exemplary pore size ranges include a pore size of about 200 angstroms to about 500 angstroms, about 400 angstroms to about 700 angstroms, about 600 angstroms to about 1000 angstroms, about 1200 angstroms to about 1500 angstroms, and about 1000 angstroms to about 2000 angstroms.

Covalent attachments

[0154] In some embodiments, the solid support comprises a reactive chemical group. In some embodiments, the reactive chemical group facilitates the attachment or immobilization of a polypeptide. In some embodiments, the polypeptide is immobilized on the support material by covalent attachment to a reactive chemical group. Exemplary reactive chemical groups include sulfonic, epoxide, epoxy, amino-epoxy, iminodiacetate, amino, primary amine, secondary amine, quaternary amine, tertiary amine, NH2, octadecyl, butyl, high butyl, low butyl, and hydroxyethyl. In some embodiments, the reactive chemical group facilitates the attachment or immobilization of an oligonucleotide.

Linkers

[0155] In some embodiments, the reactive chemical group is attached to the resin via a linker. Any suitable linker may be used. Exemplary linkers include ethylamine, β-hydroxy-amino, β-hydroxy-ether, a β-hydroxy-carboxyl, β-hydroxy-thio, alkylene, alkenylene, alkynylene, heteroalkylene, heteroalkenylene, heteroalkynylene, cyclcoalkyl, heterocycloalkyl, arylene, or heteroarylene based linkers, and the like. In some embodiments the linker comprises a C_2-C_{20}alkylene or polyethylene linker. In some embodiments, the linker is a silane or silanol linker. In some embodiments, the linker is a poly(ethylene glycol) linker, such as (3-mercaptopropyl)trimethoxysilane, poly(ethylene glycol) diglycidyl ether, or (3-glycidyloxypropyl)trimethoxysilane. Various suitable linkers are known in the art.

[0156] In some embodiments, the solid support is selected to reduce oxidation of an oligonucleotide or a thio-NTP.

Enzymes

[0157] In some embodiments, the method comprises one or more enzymes. In some embodiments, the one or more enzymes are immobilized on a solid support. In some embodiments, the one or more enzymes are provided in a buffer or solution. In some embodiments, the one or more enzymes are contacted with or combined with a solid support.

[0158] In some embodiments, one enzyme is immobilized on a solid support. In some embodiments, two or more enzymes are co-immobilized on a solid support. In some embodiments, two or more enzymes are separately immobilized on one or more solid supports. In some embodiments, two or more enzymes are provided in buffer or solution. In some embodiments, two or more enzymes are separately provided in one or more buffers or solutions.

[0159] In some embodiments, one or more enzymes are contacted with or combined with a substrate. In some embodiments, one or more enzymes produce a product. In some embodiments, one or more enzymes produce a product that is a substrate for another enzyme or enzymes. In some embodiments, one or more enzymes are a cascade that produces a product from a substrate through a series of coupled enzymatic reactions. In some embodiments, the enzymes form a cycle, where a product is produced from a substrate through a series of iterative, repeating reactions.

[0160] In some embodiments, the enzymes synthesize an oligonucleotide through a series of extension and deblocking reactions using one or more blocked natural or modified NTPs or NQPs and a starter oligonucleotide or another initiator.

[0161] In some embodiments, the one or more enzymes comprise a polymerase, a terminal nucleotidyl transferase (TnT), a terminal deoxynucleotidyl transferase (TdT), a phosphatase, an inorganic pyrophosphatase, an alkaline phosphatase, a kinase, a ligase, or another enzyme. Any suitable enzyme may be used in the methods and compositions of the current disclosure. Exemplary suitable enzymes, among others, are disclosed in PCT/US2023/076667 and US 63/634,868, both of which are specifically incorporated, herein, in their entireties.

Quench and Quenching Agents

[0162] In another aspect, the present disclosure is directed to compositions for quenching and methods of quenching a solid support or resin, such that oxidation of an oligonucleotide or a thio-NTP contacted or combined with the solid support is reduced.

[0163] In some embodiments, one or more enzymes or polypeptides are immobilized onto a solid support. In certain embodiments, the enzyme or polypeptide is immobilized onto a solid support by covalent, ionic, electrostatic or affinity attachment or adsorption to an ion, reactive chemical group, or another moiety.

[0164] In some embodiments, one or more oligonucleotides are immobilized onto a solid support. In some embodiments, an oligonucleotide is immobilized onto a solid support by covalent, ionic, electrostatic or affinity attachment or adsorption to an ion, reactive chemical group, or other moiety.

[0165] In some embodiments, the solid support or resin comprising one or more polypeptides or enzymes immobilized by covalent, ionic, electrostatic or affinity attachment or adsorption to an ion,

reactive chemical group, or other moiety comprises additional ions, reactive chemical groups, moieties that are not reacted with or attached to a polypeptide.

[0166] In some embodiments, the solid support or resin comprising one or more oligonucleotides immobilized by covalent, ionic, electrostatic or affinity attachment or adsorption to an ion, reactive chemical group, or other moiety comprises additional ions, reactive chemical groups, or moieties that are not reacted with or attached to an oligonucleotide.

[0167] In some embodiments, the present disclosure provides compositions for quenching or methods of quenching or capping unreacted reactive chemical groups on a resin or solid support, such that the unreacted chemical reactive groups are reduced or neutralized.

[0168] In some embodiments, the method of capping or quenching a solid support comprises contacting a solid support with a first quenching agent such that unreacted chemical reactive groups are reduced or neutralized.

[0169] In some embodiments, the method of capping or quenching a solid support comprises i) contacting a solid support with a first quenching agent and ii) contacting the solid support with a second quenching agent, such that unreacted chemical reactive groups are reduced or neutralized.

[0170] In some embodiments, the method further comprises immobilizing at least one enzyme on the solid support. In some embodiments, the method of capping or quenching a solid support comprises i) immobilizing one or more enzymes onto a solid support and ii) contacting the solid support with a first quenching agent, such that unreacted chemical reactive groups are reduced or neutralized. In some embodiments, the method of capping or quenching a solid support comprises i) immobilizing one or more enzymes onto a solid support; ii) contacting the solid support with a first quenching agent; and iii) contacting the solid support with a second quenching agent, such that unreacted chemical reactive groups are reduced or neutralized.

[0171] In some embodiments, the method comprises or further comprises a step of contacting the solid support with a thio-NTP or an oligonucleotide, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support.; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0172] In some embodiments, the method comprises or further comprises a step of combining at least one enzyme with the solid support, such that oxidation of the oligonucleotide is reduced. In some embodiments, the method comprises i) immobilizing an oligonucleotide comprising one or more

phosphorothioate bonds on a solid support; ii) contacting the solid support with a first quenching agent, iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting at least one enzyme with the solid support, such that oxidation of phosphorothioate bonds to phosphodiester bonds in the oligonucleotide is reduced.

[0173] In some embodiments where the solid support comprises a silane or silanol linker attached to a reactive chemical group, the method may additionally comprise contacting the solid support with one or more capping agents prior to contacting the solid support with a first quenching agent. In some embodiments, the capping agent is methyltrimethoxysilane, N,O-bis(trimethylsilyl) acetamide, trimethylchlorosilane, or 1,1,1,3,3,3-hexamethyldisilazane.

Quench Agent

[0174] In some embodiments, the quenching agent is a reductant or antioxidant. In some embodiments, the quenching agent is an amino acid or amino acid analog or amino acid derivative. In some embodiments, the quenching agent comprises L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl. In some embodiments, the quench agent is in a solution comprising a high salt concentration. In some embodiments, the solution comprising a high salt concentration is 0.5 M, 1 M, 3 M, or 5 M sodium chloride or potassium chloride.

Quench Concentration

[0175] In some embodiments, the method of capping or quenching a solid support comprises a first quenching agent at a specific concentration. In some embodiments, the method of capping or quenching a solid support comprises a second quenching agent at a specific concentration. In some embodiments, the quenching agent comprises a concentration of about 1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 62.5 mM, 100 mM, 125 mM, 200 mM, 250 mM, 500 mM, 1 M, 3M, or 5 M.

Quench pH

[0176] In some embodiments, the method of capping or quenching a solid support comprises a first quenching agent at a specific pH. In some embodiments, the method of capping or quenching a solid support comprises a second quenching agent at a specific pH. In some embodiments, the quenching agent comprises a pH of about 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, or 9.5.

Quench Temperature

[0177] In some embodiments, the method of capping or quenching a solid support comprises a first quenching agent at a specific temperature. In some embodiments, the method of capping or quenching a solid support comprises a second quenching agent at a specific temperature. In some embodiments,

the quenching agent is contacted with the solid support at a temperature of about 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, or 60 °C.

Quench Specific Embodiments

[0178] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide methacrylate or polyacrylic solid support; ii) contacting the solid support with a first quenching agent selected from L-lysine, L-cysteine, ethanolamine, L-proline, L-alanine, and L-glycine; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 9 for the step of contacting the solid support with the first quenching agent. In some embodiments, the method additionally comprises a temperature of 25 °C, 40 °C, or 45 °C for the step of contacting the solid support with a first quenching agent. In some embodiments, the method additionally comprises a concentration of 1 M or 3 M for the first quenching agent. In some embodiments, the method additionally comprises sodium thiosulfate. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 10-fold or less that of another method (e.g., a reduction from 1% impurity to 0.1% impurity). In some embodiments, the activity of the TnT is maintained at 30%, 45%, 50%, or 80% conversion as compared to another method.

[0179] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent selected from L-proline, L-alanine, L-glycine, L-lysine, and L-cysteine; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 7, 8, or 9 for the step of contacting the solid support with the first quenching agent. In some embodiments, the method additionally comprises a temperature of 40 °C, 45 °C or 50 °C for the step of contacting the solid support with a first quenching agent. In some embodiments, the method additionally comprises a concentration of 1 M or 3 M for the first quenching agent. In some embodiments, the method additionally comprises sodium thiosulfate. In some embodiments, the activity of the AP is maintained at 60% or 90% conversion as compared to another method.

[0180] In a specific embodiment, the method comprises i) immobilizing an AP, a TnT, or a TnT and a iPP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent selected from sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, and β-mercaptoethanol; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more

phosphorothioate bonds with the solid support, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 5 or 9 for the step of contacting the solid support with the first quenching agent. In some embodiments, the method additionally comprises a temperature of 40 °C, 45 °C, or 50 °C for the step of contacting the solid support with a first quenching agent. In some embodiments, the method additionally comprises a concentration of 1 M or 3 M for the first quenching agent. In some embodiments, the method additionally comprises sodium thiosulfate. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 4-fold or less that of another method (e.g., a reduction from 0.4% impurity to 0.1% impurity). In some embodiments, the activity of the TnT or AP is maintained at 30%, 45%, 50%, or 80% conversion as compared to another method.

[0181] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide methacrylate or polyacrylic solid support; ii) contacting the solid support with a first quenching agent selected from L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, L-cysteine; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both with the solid support, such that oxidation of the thio-NTP or the oligonucleotide or both is reduced. In some embodiments, the method additionally comprises a pH of 9 for the step of contacting the solid support with the first quenching agent or the step of contacting the solid support with the second quenching agent or both. In some embodiments, the method additionally comprises a temperature of 25 °C, 40 °C, or 45 °C for the step of contacting the solid support with a first quenching agent or the step of contacting the solid support with the second quenching agent or both. In some embodiments, the method additionally comprises a concentration of 1 M or 3 M for the first quenching agent or the second quenching agent or both. In some embodiments, the method additionally comprises sodium thiosulfate. In some embodiments, the activity of the TnT is maintained at 30% or 50% or higher conversion as compared to another method.

[0182] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to silica or controlled pore glass solid support; ii) contacting the solid support with a first quenching agent selected from L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, L-cysteine; iii) optionally contacting the solid support with a second quenching agent selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, and TEoA-HCl; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both with the solid support, such that oxidation of the thio-NTP or the oligonucleotide or both is reduced. In some embodiments, the method additionally comprises a pH of 9 for the step of

contacting the solid support with the first quenching agent or the step of contacting the solid support with the second quenching agent or both. In some embodiments, the method additionally comprises a temperature of 25 °C, 40 °C, or 45 °C for the step of contacting the solid support with a first quenching agent or the step of contacting the solid support with the second quenching agent or both. In some embodiments, the method additionally comprises a concentration of 1 M or 3 M for the first quenching agent or the second quenching agent or both. In some embodiments, the method additionally comprises sodium thiosulfate. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 40-fold or less (e.g., a reduction from 4% impurity to 0.1% impurity) that of another method.

Antioxidants and Reducing Agents

[0183] In another aspect, the present disclosure is directed to compositions and methods of reducing oxidation using an antioxidant or reducing agent.

[0184] In some embodiments of the disclosure, an antioxidant or reducing agent is combined with or contacted with an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, an antioxidant or reducing agent is combined with or contacted with a thio-NTP. In some embodiments, the antioxidant or reducing agent is combined with both an oligonucleotide comprising one or more phosphorothioate bonds and a thio-NTP (as described herein at Example 30).

[0185] In any of the embodiments described herein, the antioxidant or the reducing agent may be used in a method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0186] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises combining or contacting a thio-NTP or oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0187] In some embodiments, an antioxidant or reducing agent is combined with or contacted with a thio-NTP or oligonucleotide during the synthesis of an oligonucleotide. In some embodiments, the method of synthesis comprises an immobilized oligonucleotide. In some embodiments, the method of synthesis comprises one or more immobilized enzymes, as described herein and in the Examples. In some embodiments, the oligonucleotide or the one or more enzymes are immobilized on a solid support. In some embodiments, the solid support is quenched with one or more quenching agents, as described herein.

[0188] Therefore, in some embodiments, the method further comprises a step of immobilizing an oligonucleotide on a solid support. In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more

phosphorothioate bonds on a solid support and ii) combining or contacting at least one enzyme with the solid support in the presence of one or more antioxidants or reducing agents, such that oxidation of the oligonucleotide is reduced.

[0189] Therefore, in some embodiments, the method further comprises a step of immobilizing one or more enzymes on a solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide or both comprises i) immobilizing one or more enzymes on a solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both with the solid support in the presence of one or more antioxidants or reducing agents, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0190] Therefore, in some embodiments, the method further comprises a step of contacting a first quenching agent and, optionally, contacting a second quenching agent with the solid support. In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more phosphorothioate bonds on a solid support; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting at least one enzyme with the solid support in the presence of one or more antioxidants or reducing agents, such that oxidation of the oligonucleotide is reduced.

[0191] Therefore, in some embodiments, the method further comprises a step of contacting a first quenching agent and, optionally, contacting a second quenching agent with the solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide or both comprises i) immobilizing one or more enzymes on a solid support; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both with the solid support in the presence of one or more antioxidants or reducing agents, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

Antioxidant or Reducing Agent

[0192] In some embodiments, the antioxidant or reducing agent comprises ascorbic acid, citric acid, formic acid, sodium thiosulfate, sodium metabisulfite, 2,6-dimethoxyphenol, catalase, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, or sodium thiophosphate.

[0193] In some embodiments, more than one antioxidant or reducing agent may be used. In some embodiments, more than one antioxidant or reducing agent may be combined with or contacted with a thio-NTP or an oligonucleotide at the same time. In some embodiments, more than one antioxidant or reducing agent may be combined with or contacted with a thio-NTP or an oligonucleotide at different

times. In some embodiments, an antioxidant or a reducing agent may be used together or combined with a buffer or with a first quenching agent or with a second quenching agent.

Reducing Agent Concentration

[0194] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both comprises an antioxidant or a reducing agent at a specific concentration. In some embodiments, the antioxidant or reducing agent comprises a concentration of about 50 μM, 80 μM, 100 μM, 400 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 3M, or 5 M.

Reducing Agent pH

[0195] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both comprises an antioxidant or a reducing agent at a specific pH. In some embodiments, the antioxidant or reducing agent comprises a pH of about 5, 5.5, 6, 6.5, 7, 7.5, 7.8, 8, 8.5, 9, or 9.5.

Reducing Agent Temperature

[0196] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds or both comprises an antioxidant or a reducing agent at a specific temperature. In some embodiments, the antioxidant or reducing agent is at a temperature of about 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, or 60 °C.

Reducing Agent Specific Embodiments

[0197] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more antioxidants or reducing agents, wherein the antioxidant or reducing agent comprises dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, or sodium thiophosphate, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 7, 7.8, or 8. In some embodiments, the method additionally comprises a temperature of 25 °C, 40 °C, or 45 °C. In some embodiments, the method additionally comprises an antioxidant or reducing agent concentration of 50 μM, 80 μM, 400 μM,1 mM, 2 mM, 10 mM, 50 mM, or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2-fold or less that of another method (e.g., a reduction from 10% impurity to 5% impurity). In some embodiments, the activity of the TnT is maintained at 85% or 97% conversion as compared to another method.

[0198] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more antioxidants or reducing agents, wherein the antioxidant or reducing agent comprises dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, sodium thiosulfate, or sodium thiophosphate, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 7, 7.8, or 8. In some embodiments, the method additionally comprises a temperature of 25 °C, 40 °C, or 45 °C. In some embodiments, the method additionally comprises an antioxidant or reducing agent concentration of 50 μM, 80 μM, 400 μM,1 mM, 2 mM, 10 mM, 50 mM, or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2 -fold or less that of another method (e.g., a reduction from 10% impurity to 5% impurity). In some embodiments, the activity of the TnT is maintained at 85% or 97% conversion as compared to another method.

[0199] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more antioxidants or reducing agents, wherein the antioxidant or reducing agent comprises ascorbic acid, citric acid, formic acid, sodium thiosulfate, sodium metabisulfite, 2,6-dimethoxyphenol, or catalase, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, or sodium thiophosphate, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 7, 7.8, or 8. In some embodiments, the method additionally comprises a temperature of 45 °C or 50 °C. In some embodiments, the method additionally comprises an antioxidant or reducing agent concentration of about 1 mM, 5 mM, 50 mM, or 100 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2.5-fold or less that of another method (e.g., a reduction from 10% impurity to 4% impurity). In some embodiments, the activity of the AP is maintained at 70% or 90% conversion as compared to another method.

[0200] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent, and optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more antioxidants or reducing agents, wherein the antioxidant or reducing agent is selected from ascorbic acid, citric acid, formic acid, sodium thiosulfate, sodium metabisulfite, 2,6-

dimethoxyphenol, or catalase, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 7, 7.8, or 8. In some embodiments, the method additionally comprises a temperature of 45 °C or 50 °C. In some embodiments, the method additionally comprises an antioxidant or reducing agent concentration of about 1 mM, 5 mM, 50 mM, or 100 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2.5-fold or less that of another method (e.g., a reduction from 10% impurity to 4% impurity). In some embodiments, the activity of the AP is maintained at 70% or 90% conversion as compared to another method.

Buffers and other Reaction Conditions

[0201] In another aspect, the present disclosure is directed to compositions and methods of reducing oxidation using buffers and other reaction conditions. Although the term reaction condition is used to generally refer to pH, temperature, concentration, additives, and other reaction conditions, a person of skill in the art will understand "reaction conditions" to encompass and include inert methods and compositions, where no reaction occurs.

[0202] In some embodiments of the disclosure, a buffer or another additive is combined with an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, a buffer or another additive is combined with or contacted with a thio-NTP. In some embodiments, the buffer or another additive is combined with both an oligonucleotide comprising one or more phosphorothioate bonds and a thio-NTP (as described herein at Example 30). In any of these embodiments, the buffer or another additive may comprise one or more additional reaction conditions, including temperature, pH, concentration, or another reaction condition described herein or know to a person of skill in the art.

[0203] In any of the embodiments described herein, one or more buffers, additives, or reaction conditions may be used in a method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0204] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises combining a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with a buffer or another additive, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0205] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds in the presence of one or more additional reaction conditions, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0206] In some embodiments, the buffer or another additive is combined with a thio-NTP or oligonucleotide during the synthesis of an oligonucleotide. In some embodiments, the method of

synthesis comprises an immobilized oligonucleotide. In some embodiments, the method of synthesis comprises one or more immobilized enzymes, as described herein and in the Examples. In some embodiments, the oligonucleotide or the one or more enzymes are immobilized on a solid support. In some embodiments, the solid support is quenched with one or more quenching agents, as described herein. In some embodiments, the method of synthesis additionally comprises one or more antioxidants or reducing agents.

[0207] Therefore, in some embodiments, the method further comprises a step of immobilizing an oligonucleotide on a solid support. In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more phosphorothioate bonds on a solid support and ii) combining or contacting at least one enzyme with the solid support in the presence of one or more buffers, additives, or reaction conditions, such that oxidation of the oligonucleotide is reduced.

[0208] Therefore, in some embodiments, the method further comprises a step of immobilizing one or more enzymes on a solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, additives, or reaction conditions, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0209] Therefore, in some embodiments, the method further comprises a step of contacting a first quenching agent, and optionally contacting a second quenching agent with the solid support. In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more phosphorothioate bonds on a solid support; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting at least one enzyme with the solid support in the presence of one or more buffers, additives, or reaction conditions, such that oxidation of the oligonucleotide is reduced.

[0210] Therefore, in some embodiments, the method further comprises a step of contacting a first quenching agent, and optionally contacting a second quenching agent with the solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, additives, or reaction conditions, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

Buffers or Additives

[0211] In some embodiments, the buffer comprises MOPS, TRIS, acetate, sodium acetate, sodium phosphate, triethanolamine (TEoA), or triethanolamine HCl (TEoAHCl). Any suitable buffer may be used, as understood by a person of skill in the art.

[0212] In some embodiments, the buffer comprises a divalent metal. In some embodiments, the divalent metal is magnesium, manganese, nickel, or cobalt.

[0213] In some embodiments, the buffer comprises or additionally comprises an additive, such as a surfactant or another additive. In some embodiments, the buffer comprises or additionally comprises Ecosurf EH, turgitol, or urea.

[0214] In some embodiments, the reaction conditions comprise a surfactant for stabilizing or enhancing the reaction. Surfactants can comprise non-ionic, cationic, anionic, and/or amphiphilic surfactants. Exemplary surfactants, include by way of example and not limitation, Ecosurf EH, turgitol, nonyl phenoxypolyethoxylethanol (NP40), TRITON™ X-100 polyethylene glycol *tert*-octylphenyl ether, polyoxyethylene-stearylamine, cetyltrimethylammonium bromide, sodium oleylamidosulfate, polyoxyethylene-sorbitanmonostearate, hexadecyldimethylamine, etc. Any surfactant that may stabilize or enhance the reaction may be employed. The concentration of the surfactant to be employed in the reaction may be generally from 0.1 to 50 mg/mL, particularly from 1 to 20 mg/mL.

[0215] In some embodiments, the reaction conditions include an antifoam agent, which aids in reducing or preventing formation of foam in the reaction solution, such as when the reaction solutions are mixed or sparged. Anti-foam agents include non-polar oils (e.g., minerals, silicones, etc.), polar oils (e.g., fatty acids, alkyl amines, alkyl amides, alkyl sulfates, etc.), and hydrophobic (e.g., treated silica, polypropylene, etc.), some of which also function as surfactants. Exemplary anti-foam agents include Y-30® (Dow Corning), poly-glycol copolymers, oxy/ethoxylated alcohols, and polydimethylsiloxanes. In some embodiments, the anti-foam can be present at about 0.001% (v/v) to about 5% (v/v), about 0.01% (v/v) to about 5% (v/v), about 0.1% (v/v) to about 5% (v/v), or about 0.1% (v/v) to about 2% (v/v). In some embodiments, the anti-foam agent can be present at about 0.001% (v/v), about 0.01% (v/v), about 0.1% (v/v), about 0.5% (v/v), about 1% (v/v), about 2% (v/v), about 3% (v/v), about 4% (v/v), or about 5% (v/v) or more as desirable to promote the reaction.

Buffer Concentration

[0216] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises buffer at a specific concentration. In some embodiments, the buffer comprises a concentration of about 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 2M, 3M, or 5 M.

Buffer pH

[0217] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises a specific pH. In some embodiments, the antioxidant or reducing agent comprises a pH of about 5, 5.5, 6, 6.5, 6.8, 7, 7.5, 7.9 7.8, 8, 8.5, 9, or 9.5.

Buffer Temperature

[0218] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises a specific temperature. In some embodiments, the buffer is at a temperature of about 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, or 60 °C.

Buffer Specific Embodiments

[0219] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPs, TRIS or TEoA-HCL, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 6.8, 7.9, or 9. In some embodiments the method additionally comprises sodium thiosulfate at a concentration of 2.5 mM or 5.0 mM. In some embodiments, the method additionally comprises buffer concentration of 25 mM, 137.5 mM, or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2.25-fold or less that of another method (e.g., a reduction from 3.5% impurity to 1.5% impurity). In some embodiments, the activity of the TnT is maintained at 85% or 95% conversion as compared to another method.

[0220] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 1.5-fold or less that of another method (e.g., a reduction from 15% impurity to 10% impurity). In some embodiments, the activity of the TnT is maintained at 85% or 95% conversion as compared to another method.

[0221] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPs, TRIS or TEoA-HCL, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises a pH of 6.8, 7.9, or 9. In some embodiments the method additionally comprises sodium thiosulfate at a concentration of 2.5 mM or 5.0 mM. In some embodiments, the method additionally comprises buffer concentration of 25 mM, 137.5 mM, or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 2.25-fold or less that of another method (e.g., a reduction from 3.5% impurity to 1.5% impurity). In some embodiments, the activity of the AP is maintained at 85% or 95% conversion as compared to another method.

[0222] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 1.4-fold or less that of another method (e.g., a reduction from 10% impurity to 7% impurity). In some embodiments, the activity of the AP enzyme is maintained at 85% or 98% conversion as compared to another method.

[0223] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP and a AP enzyme by covalent attachment to one or more epoxide solid supports; ii) contacting the one or more solid supports with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the one or more solid supports in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises a pH of 8 and a temperature of 40 °C or 50 °C. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to 1.4-fold or less that of another method (e.g., a reduction from 10% impurity to 7% impurity). In some embodiments, the activity of the TnT or AP enzyme is maintained at 85%, 95%, or 98% conversion as compared to another method.

Degassing or Sparging

[0224] In another aspect, the present disclosure is directed to compositions and methods of reducing oxidation using a degassing step or a sparging step.

[0225] In some embodiments, the method comprises one or more degassing or sparging steps. In some embodiments, the degassing step comprises a degasser configured to remove oxygen from the system. In some embodiments, the degassing step comprises a vacuum pump. In some embodiments, the sparging step comprises an in-line sparging system or the sparging of liquids in a reservoir. In some embodiments, the sparging step uses an inert gas. In some embodiments, the inert gas is helium, neon, argon, krypton, xenon, radon, nitrogen, or carbon dioxide.

[0226] In some embodiments, in-line degassing apparatuses, such as an in-line vacuum pump, may be included in the methods disclosed herein. Degassing the solutions and mixtures may prevent oxidation of thio-NTPs or oligonucleotides. In some embodiments, the systems optionally include one or more in-line degassers for degassing the buffers and/or solutions.

[0227] In some embodiments of the disclosure, a solution or mixture is degassed or sparged before, after, or continuously while being combined with an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, a solution or mixture is degassed or sparged before, after, or continuously while being combined with a thio-NTP. In some embodiments, a solution or mixture is degassed or sparged prior to or after or continuously while being combined with both an oligonucleotide comprising one or more phosphorothioate bonds and a thio-NTP.

[0228] In any of the embodiments described herein, one or more degassing or sparging steps may be used in a method of reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds.

[0229] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises degassing or sparging of a solution or mixture before, after, or continuously while combining the solution or mixture with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0230] In some embodiments, the degassing or sparging of a solution or mixture before, after, or continuously while combining the solution or mixture with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds occurs during the synthesis of an oligonucleotide. In some embodiments, the method of synthesis comprises an immobilized oligonucleotide. In some embodiments, the method of synthesis comprises one or more immobilized enzymes, as described herein and in the Examples. In some embodiments, the oligonucleotide or the one or more enzymes are immobilized on a solid support. In some embodiments, the solid support is quenched with one or more quenching agents, as described herein. In some embodiments, the method of synthesis

additionally comprises one or more antioxidants or reducing agents. In some embodiments, the method of synthesis additionally comprises one or more buffers, additives, or reaction conditions.

[0231] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises degassing or sparging of a solution or mixture before, after, or continuously while combining the solution or mixture with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the sparging of the solution of mixture is continuous during one or more steps of methods.

[0232] In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more phosphorothioate bonds on a solid support; ii) combining or contacting a solution comprising at least one enzyme with the solid support; and iii) degassing the solution comprising at least one enzyme, such that oxidation of phosphorothioate bonds to phosphodiester bonds in the oligonucleotide is reduced.

[0233] In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support; ii) combining or contacting a solution comprising an oligonucleotide comprising one or more phosphorothioate bonds with the solid support; and iii) degassing the solution comprising an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of phosphorothioate bonds to phosphodiester bonds in the oligonucleotide is reduced.

[0234] In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises degassing or sparging of a solution or mixture before, after, or continuously while combining the solution or mixture with a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0235] Therefore, in some embodiments, the method further comprises a step of immobilizing an oligonucleotide on a solid support. In some embodiments, the method of reducing oxidation of an oligonucleotide comprises i) immobilizing an oligonucleotide comprising one or more phosphorothioate bonds linkages on a solid support and ii) degassing or sparging of a solution or mixture before, after, or continuously while combining the solution or mixture with an oligonucleotide comprising one or more phosphorothioate bonds, such that oxidation of the oligonucleotide is reduced.

[0236] Therefore, in some embodiments, the method further comprises a step of immobilizing one or more enzymes on a solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support and ii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, additives, or reaction conditions,

and, optionally, degassing or sparging the buffer before, after, or continuously during this step, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

[0237] Therefore, in some embodiments, the method further comprises a step of contacting a first quenching agent, and optionally contacting a second quenching agent with the solid support. In some embodiments, the method of reducing oxidation of a thio-NTP or an oligonucleotide comprises i) immobilizing one or more enzymes on a solid support; ii) contacting the solid support with a first quenching agent; iii) optionally contacting the solid support with a second quenching agent; and iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, additives, or reaction conditions and, optionally, degassing or sparging the buffer before, after, or continuously during this step, such that oxidation of the thio-NTP or the oligonucleotide is reduced.

Degassing or Sparging Specific Embodiments

[0238] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM and, optionally, degassing or sparging the buffer before, after, or continuously during this step, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises sparging with nitrogen gas. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to less than 5% as compared to another method without sparging. In some embodiments, the activity of the TnT is maintained at greater than 97% conversion as compared to another method without sparging.

[0239] In a specific embodiment, the method comprises i) immobilizing an AP enzyme by covalent attachment to an epoxide solid support; ii) contacting the solid support with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM and, optionally, degassing or sparging the buffer before, after, or continuously during this step, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises sparging with nitrogen

gas. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to less than 5% as compared to another method without sparging.

[0240] In a specific embodiment, the method comprises i) immobilizing a TnT and, optionally, an iPP and a AP enzyme by covalent attachment to one or more epoxide solid supports; ii) contacting the one or more solid supports with a first quenching agent and, optionally, a second quenching agent; and iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with the one or more solid supports in the presence of one or more buffers, wherein the buffer comprises MOPS or TEoA-HCL at a concentration of 250 mM and, optionally, degassing or sparging the buffer before, after, or continuously during this step, such that oxidation of the thio-NTP or the oligonucleotide is reduced. In some embodiments, the method additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the method additionally comprises a pH or 8 and a temperature of 40 °C or 50 °C. In some embodiments, the method additionally comprises sparging with nitrogen gas. In some embodiments, the method additionally comprises reduction of the % phosphorothioate oxidation impurity to less than 2% or less than 1% or less than 0.3 % as compared to another method without sparging. In some embodiments, the activity of the TnT or AP enzyme is maintained at 97% or 99% conversion as compared to another method.

Compositions for Reducing Oxidation of Thio-NTPs and Phosphorothioate Oligonucleotides

[0241] In some embodiments, the present disclosure is directed to compositions for reducing oxidation of phosphorothioate bonds during the synthesis and storage of oligonucleotides. In some related embodiments, the present disclosure is directed to compositions for reducing oxidation of thio-NTPs. In some embodiments, the compositions of the present disclosure find use in reducing phosphorothioate to phosphodiester impurities in an oligonucleotide or therapeutic oligonucleotide.

[0242] In some embodiments, the composition for reducing oxidation of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprises one or more of the following:

i) A thio-NTP;

ii) An oligonucleotide comprising one or more phosphorothioate bonds;

iii) A solid support;

iv) A first quenching agent and, optionally, a second quenching agent;

v) An antioxidant or reducing agent; and

vi) A buffer, additive, or another reaction condition.

Storage (Non-reactive) Compositions

[0243] In some embodiments, the composition facilitates storage of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. Thus, in some embodiments, the composition comprises i) a thio-NTP and ii) an antioxidant or reducing agent. In some other embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds and ii) an antioxidant or reducing agent.

[0244] In some embodiments, the composition facilitating storage of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds further comprises a buffer, additive, or another reaction condition. Thus, in some embodiments, the composition comprises i) a thio-NTP; ii) an antioxidant or reducing agent; and iii) a buffer, additive, or another reaction condition. In some other embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) an antioxidant or reducing agent; and iii) a buffer, additive, or another reaction condition.

Synthesis Compositions

[0245] In some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds and ii) a solid support. In some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds and ii) a solid support.

[0246] In some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) one or more enzymes; and iii) a solid support. In some other embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) one or more enzymes; iii) a thio-NTP; and iv) a solid support.

[0247] In some embodiments, the composition further comprises a first quenching agent and, optionally, a second quenching agent. Thus, in some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; and iii) a first quenching agent and, optionally, a second quenching agent. In some other embodiments, the composition comprises i) one or more enzymes; ii) a solid support; and iii) a first quenching agent and, optionally, a second quenching agent.

[0248] In some embodiments, the composition further comprises a buffer, additive, or another reaction condition. Thus, in some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; and iii) a buffer, additive, or another reaction condition. In some other embodiments, the composition comprises i) one or more enzymes; ii) a solid support; and iii) a buffer, additive, or another reaction condition.

[0249] In some embodiments, the composition further comprises an antioxidant or reducing agent. Thus, in some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; and iii) an antioxidant or reducing agent. In some other embodiments, the composition comprises i) one or more enzymes; ii) a solid support; and iii) an antioxidant or reducing agent.

[0250] In some embodiments, the composition further comprises a buffer, additive, or another reaction condition. Thus, in some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; iii) an antioxidant or reducing agent; and iv) a buffer, additive, or another reaction condition. In some other embodiments, the composition comprises i) one or more enzymes; ii) a solid support; iii) an antioxidant or reducing agent; and iv) a buffer, additive, or another reaction condition.

[0251] In some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; iii) one or more immobilized enzymes; iv) an antioxidant or reducing agent; and v) a buffer, additive, or another reaction condition.

[0252] In some embodiments, the composition comprises i) an oligonucleotide comprising one or more phosphorothioate bonds; ii) a solid support; iii) one or more immobilized enzymes; iv) a thio-NTP; v) an antioxidant or reducing agent; and vi) a buffer, additive, or another reaction condition.

Compositions Specific Embodiments

[0253] In a specific embodiment, the composition comprises i) an AP or a TnT and, optionally, an iPP enzyme ii) an epoxide solid support; iii) a first quenching agent and, optionally, a second quenching agent; and iii) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the composition additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM.

[0254] In a specific embodiment, the composition comprises i) an epoxide solid support; iii) a first quenching agent of L-glycine, L-Lysine, or L-cysteine and, optionally, a second quenching agent; and iii) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the composition additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM.

[0255] In a specific embodiment, the composition comprises i) an epoxide solid support; ii) a first quenching agent of L-glycine, L-Lysine, or L-cysteine ; iii) a second quenching agent selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, and TEoA-HCl; and iv) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the

composition additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the composition additionally comprises a pH or 8 and a temperature of 40 °C or 50 °C.

[0256] In a specific embodiment, the composition comprises i) one or more enzymes selected from AP, TnT, and iPP; ii) a first quenching agent of L-cysteine; iii) a second quenching agent selected from L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, and TEoA-HCl; and iv) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds. In some embodiments, the composition additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the composition additionally comprises a pH of 7 or 9 and a temperature of 40 °C or 50 °C.

[0257] In a specific embodiment, the composition comprises i) one or more enzymes selected from AP, TnT, and iPP; ii) an epoxide solid support; iii) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds; and iv) MOPS or TEoA-HCL at a concentration of 250 mM. In some embodiments, the composition additionally comprises sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the composition additionally comprises a pH or 8 and a temperature of 40 °C or 50 °C.

[0258] In a specific embodiment, the composition comprises i) one or more enzymes selected from AP, TnT, and iPP; ii) an epoxide solid support; iii) a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds; and iv) sodium thiosulfate at a concentration of 50 mM or 250 mM. In some embodiments, the composition additionally comprises MOPS or TEoA-HCL at a concentration of 250 mM. In some embodiments, the composition additionally comprises a pH or 8 and a temperature of 40 °C or 50 °C.

EXAMPLES

[0259] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present disclosure. Indeed, there are various suitable sources for many of the reagents and equipment described below. It is not intended that the present disclosure be limited to any particular source for any reagent or equipment item.

[0260] In the experimental disclosure below, the following abbreviations apply: M (molar); mM (millimolar), μM and uM (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); ug and μg (micrograms); L and l (liter); ml and mL (milliliter); cm (centimeters); mm (millimeters); μM and μιη (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); psi and PSI (pounds per square inch);

°C (degrees Celsius); RT and rt (room temperature); CV (coefficient of variability); CAM and cam (chloramphenicol); PMBS (polymyxin B sulfate); IPTG (isopropyl β-D-l-thiogalactopyranoside); LB (lysogeny broth); TB (terrific broth); SFP (shake flask powder); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); nt (nucleotide; polynucleotide); aa (amino acid; polypeptide); E. coli W3110 (commonly used laboratory E. coli strain, available from the Coli Genetic Stock Center [CGSC], New Haven, CT); HTP (high throughput); HPLC (high pressure liquid chromatography); HPLC-UV (HPLC-Ultraviolet Visible Detector); 1H NMR (proton nuclear magnetic resonance spectroscopy); FIOPC (fold improvements over positive control); Sigma and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO; Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Microfluidics (Microfluidics, Westwood, MA); Life Technologies (Life Technologies, a part of Fisher Scientific, Waltham, MA); Amresco (Amresco, LLC, Solon, OH); Carbosynth (Carbosynth, Ltd., Berkshire, UK); Varian (Varian Medical Systems, Palo Alto, CA); Agilent (Agilent Technologies, Inc., Santa Clara, CA); Infors (Infors USA Inc., Annapolis Junction, MD); and Thermotron (Thermotron, Inc., Holland, MI).

Abbreviations for modified nucleotides	
Alias	**Modification**
3'P	3'-phosphorylation modification
mA	2'-methoxyadenosine
mC	2'-methoxycytidine
mG	2'-methoxyguanosine
mU	2'-methoxyuridine
fA	2'-fluoroadenosine
fC	2'-fluorocytidine
fG	2'-fluoroguanosine
fU	2'-fluorouridine
*	phosphorothioate linkage

Example 1

Shake Flask Expression and Purification of Enzymes

Shake Flask Expression

[0261] Selected cultures were plated onto LB agar plates with 1% glucose and 30 µg/ml chloramphenicol and grown overnight at 37 °C. A single colony from each culture was transferred to 5 ml of LB broth with 1% glucose and 30 µg/ml chloramphenicol. The cultures were grown for 20 h at 30 °C, 250 rpm, and subcultured at a dilution of approximately 1:50 into 250 ml of Terrific Broth with 30 µg/ml of chloramphenicol, to a final OD_{600} of about 0.05. The cultures were incubated for approximately 195 min at 30°C, 250 rpm, to an OD_{600} of about 0.6, and then induced with the addition of IPTG at a final concentration of 1mM. The induced cultures were incubated for 20 h at 30°C, 250 rpm. Following this incubation period, the cultures were centrifuged at 4000 rpm x 10 min. The

culture supernatant was discarded, and the pellets were resuspended in 35 ml of 20 mM triethanolamine, pH 7.5. This cell suspension was chilled in an ice bath and lysed using a Microfluidizer cell disruptor (Microfluidics M-110L). The crude lysate was pelleted by centrifugation (11,000 rpm for 60 min at 4 °C), and the supernatant was then filtered through a 0.2 μm PES membrane to further clarify the lysate.

Purification of Enzymes from Shake Flask Lysates

[0262] The clarified lysates were then purified using an AKTA Start purification system and a 5 mL HisTrap FF column (GE Healthcare) using the AC Step HiF setting (the run parameters are provided below in Table 1). The SF wash buffer comprised 50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 0.02% v/v Triton X-100 reagent.

Table 1. Purification Parameters

Parameter	Volume
Column volume	5 mL
Flow rate	8 mL/min
Pressure limit	0.3 MPa
Sample volume	35 mL
Equilibration volume	5 column volumes (CV) = 25mL
Wash Unbound volume	20 CV = 100 mLs
Elution	Isocratic (step)
Elution volume	5 CV = 25 mLs
Fraction volume	1.5 mLs
RE-equilibration volume	5 CV = 25 mLs

[0263] Elution fractions containing protein were identified by UV absorption (A280) and pooled, then dialyzed overnight in dialysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, and 50% glycerol) in a 3.5K Slide-A-Lyzer™ dialysis cassette (Thermo Fisher) for buffer exchange. iPP concentrations from the preparations were measured by absorption at 280 nm.

Example 2

High performance liquid chromatography (HPLC) analysis of nucleotides

[0264] The 5 to 20 μL crude reaction product is diluted to a final total nucleotide concentration of 100 μM nucleotide using 1 mM EDTA in RO water as diluent. 100 μL is transferred from the supernatant to a 96-well round bottom plate and then 10 μL is injected onto an Ultimate 3000 HPLC system using a PAL autosampler according to the method outlined in **Table 3.1**.

Table 2.1. Achiral HPLC Parameters (Ion Pairing Gradient)

Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Agilent Zorbax RR StableBond Aq, 150 x 3.0 mm x 3.5 µm
Guard Column	Agilent Zorbax StabeBond Aq, 5.0 x 3.0 mm, 1.8 µm
Mobile Phases	A: 50 mM sodium phosphate, 2 mM tetrabutylammonium bisulfate, pH 7.0 B: Acetonitrile C: Water
LC Gradient	- 0 – 4.0 min: 9.0 % B, 48.5 – 16.2 % C - 4.0 – 4.5 min: 9.0 % B, 16.2 – 48.5 % C - 4.5 – 5.5 min: 9.0 % B, 48.5 % C
Flow rate	1.2 mL/min
Run time	5.5 min
Column temperature	30 °C
Injection volume	10 µL
UV Detector	254 nm

Example 3

High performance liquid chromatography (HPLC) analysis of oligonucleotides

[0265] A 2 to 10 µL aliquot is removed from the reaction and diluted to a concentration of 25-50 µM oligonucleotide using 1 mM EDTA (pH 8) in RO water as diluent. A 10 µL portion from the diluted samples is then injected onto an Ultimate 3000 HPLC system using a PAL autosampler according to Method A outlined in Table 3.1, Method B outlined in Table 3.2, Method C as outlined in Table 3.3, or Method D as outlined in Table 3.4 as required. A 10 µL portion from the diluted samples is then injected onto Thermo Fisher Vanquish according to Method E outlined in Table 3.5 as required.

Table 3.1. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method A)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Agilent Advance Bio Amino Acid Analysis (AAA), 3.6x100 mm, 2.7 µm
Mobile Phases	A: 100 mM triethylamine acetate, pH 7.0 B: Acetonitrile
LC Gradient	- 0 – 1.0 min: 8.0% B - 1.0 – 8.0 min: 8.0 – 14.0% B - 8.0 – 9.0 min: 8.0% B
Flow rate	1.0 mL/min
Run time	9.0 min
Column temperature	65 °C
Injection volume	10 µL

Table 3.1. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method A)	
UV Detector	260 nm

Table 3.2. Oligonucleotide Analysis on AdvanceBio Amino Acid Analysis Column (Method B)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Agilent Advance Bio Amino Acid Analysis (AAA), 3.6x100 mm, 2.7 µm
Mobile Phases	A: 100 mM triethylamine acetate, pH 7.0 B: Acetonitrile
LC Gradient	- 0 – 1.0 min: 11.0% B - 1.0 – 5.0 min: 11.0 – 13.0% B - 5.0 – 7.0 min: 11.0% B
Flow rate	0.7 mL/min
Run time	7.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Table 3.3. Oligonucleotide Analysis on Oligonucleotide C18 Column (Method C)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler
Column	Waters ACQUITY Premier Oligonucleotide C18 Column, 2.1x100 mm, 1.7 µm
Mobile Phases	A: 100 mM hexafluoroisopropanol, 22.5 mM dibutylamine, 1% methanol in water B: 100 mM hexafluoroisopropanol , 22.5 mM dibutylamine, 95% methanol in water
LC Gradient	- 0 – 1.0 min: 5.0% B - 1.0 – 2.0 min: 5.0 – 45.0% B - 2.0 – 10.0 min: 45.0-65.0% B - 10.0-10.1 min: 65.0-5.0% B - 10.1-14.0 min: 5% B
Flow rate	0.25 mL/min
Run time	14.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Table 3.4. Oligonucleotide Analysis on Oligonucleotide C18 Column (Method D)	
Instrument	Ultimate 3000 HPLC System with a PAL autosampler

Table 3.4. Oligonucleotide Analysis on Oligonucleotide C18 Column (Method D)	
Column	Waters ACQUITY Premier Oligonucleotide C18 Column, 2.1x100 mm, 1.7 µm
Mobile Phases	A: 100 mM hexafluoroisopropanol, 22.5 mM dibutylamine, 1% methanol in water B: 100 mM hexafluoroisopropanol , 22.5 mM dibutylamine, 95% methanol in water
LC Gradient	- 0 – 1.0 min: 5.0% B - 1.0 – 2.0 min: 5.0 – 45.0% B - 2.0 – 8.0 min: 45.0-65.0% B - 8.0-8.1 min: 65.0-5.0% B - 8.1-10.0 min: 5% B
Flow rate	0.25 mL/min
Run time	10.0 min
Column temperature	65 °C
Injection volume	10 µL
UV Detector	260 nm

Table 3.5. Oligonucleotide Analysis on Oligonucleotide C18 Column (Method E)	
Instrument	Thermo Fisher Vanquish
Column	Waters XTerra MS C18 5 µm, 2.1 x 50 mm, with Phenomenex Guard
Mobile Phases	A: 15 mM dibutylamine and 25 mM hexafluoroisopropanol in water B: Methanol
LC Gradient	- 30 second equilibration hold at 10% B - 0 – 9.0 min: 10.0-45.0% B - 9.0 – 9.5 min: 45.0% B - 9.5-12.0 min: 10.0% B
Flow rate	0.6 mL/min
Run time	12.0 min
Column temperature	50.0 °C
Injection volume	10 µL
UV Detector	260 nm

Example 4

Liquid Chromatography Mass Spectrometry (LCMS) Analysis of Oligonucleotides and Nucleotides

[0266] Samples were pre-diluted to between 25 and 75 µM oligonucleotide in RO water and injected on an Agilent 6470B triple quadrupole LCMS system according to the method outlined in **Table 4.1** below.

Table 4.1. Oligonucleotide Analysis by LCMS (Method A)

Instrument	Agilent 6470B
Column	Waters XTerra MS C18 5 µm, 2.1 x 50 mm, with Phenomenex Guard
Mobile Phases	A: 15 mM dibutylamine and 25 mM hexafluoroisopropanol in water B: Methanol
LC Gradient	- 0 – 0.5 min: 10% B, 90% C - 0.5 - 10 min: 45% B, 55% C - 10 – 11.5 min: 45 – 75% B, 55 – 20% C - 11.5 – 11.9 min: 75% B, 20% C - 11.9 – 12 min: 75 – 10% B, 20 – 90% C - 12 – 14 min: 10% B, 90% C
Flow rate	0.6 mL/min
Run time	14 min
Column temperature	50 °C
Injection volume	6 µL
UV Detector	260 nm
MS-Mode	Negative
Scan Data	950-2050
Scan Time	400
Frag Voltage	200 V
Drying Gas	12 L/min, 275 °C
Sheath Gas	12 L/min, 350 °C
Nebulizer Pressure	35 psi
Cap Voltage	4000 V
Nozzle Voltage	2000 V

[0267] Samples were pre-diluted to between 25 and 100 µM nucleotide in RO water and injected on an Thermo LTQ XL LCMS system with a Thermo Fisher Vanquish UHPLC according to the method outlined in Table 4.2 below. Data was analyzed by extracting base peak chromatograms for targeted analytes.

Table 4.2. Nucleotide Analysis by LCMS (Method B)

Instrument	Thermo LTQ XL with Vanquish UHPLC
Column	Waters T3 3 µm, 2.1 x 100 mm, with Phenomenex C18 Guard
Mobile Phases	A: 6 mM dibutylamine and 20 mM ammonium acetate in water B: acetonitrile
LC Gradient	- 0 – 0.5 min: 100% A, 0% B - 0.5 - 8 min: 100-70% A, 0-30% B - 8 – 11 min: 70 – 10% A, 30-90% B - 11 – 12 min: 10% A, 90% B - 12 – 15 min: 100% A, 0% B

Flow rate	0.6 mL/min
Run time	15 min
Column temperature	30 °C
Injection volume	6 µL
UV Detector	260 nm
MS-Mode	Negative
Scan Data	325-625
Sheath Gas Flow Rate	20
Aux Gas Flow Rate	5
Sweep Gas Flow Rate	0
Spray Voltage	3.3 V
Capillary Temperature	275 °C
Capillary Voltage	-6.0 V
Tube Lens	-150 V
Divert	LC Flow diverted to waste ≤3.0 min and again ≥14.0 min

Example 5

Residual Alkaline Phosphatase Assay

[0268] Residual alkaline phosphatase (AP) is measured by reaction with *p*-nitrophenyl phosphate. Using a concentrated stock of the desired alkaline phosphatase variant to be measured, a 100 µL diluted sample containing 2.0 µM enzyme is prepared, using the same buffer as the analyte as diluent. 20 µL from the 2.0 µM stock is serially diluted by 2x across one row of a biorad 96-well PCR plate using analyte buffer as diluent, with a final volume of 10 µL in each well (final concentrations ranging from 2.0 to 0.001 µM enzyme). Then, 10 µL is removed from each sample to be analyzed and transferred to one well of the biorad 96-well PCR plate.

[0269] A reaction buffer stock is then prepared containing: *p*-nitrophenylphosphate (2.0 mM), $CoCl_2$ (1.0 mM), TEoA-HCl (50 mM, pH 7.8). 10 µL is transferred from the reaction stock to the wells containing analyte and serially diluted AP on the biorad 96-well PCR plate. The plate is heat sealed with an aluminum seal, mixed, and briefly centrifuged to collect the liquid in the bottom of each well. The plate is then heated at 50 °C for 10 minutes on a thermal cycler. Afterward, the reaction is quenched by diluting each well with 100 µL of 1.0 M NaOH. The samples are transferred to a clear flat-bottom 96-well plate for analysis by UV-Vis absorbance at 405 nm on a SpectraMax M2 plate reader. A linear least-squares fitting to the linear portion of the AP dose response curve from 0.0005 µM to 0.05 µM is used to measure the amount of AP in the analyte wells.

Example 6

Residual $CoCl_2$ Assay

[0270] A 10 mM $CoCl_2$ solution was prepared in the same buffer as the analyte to be assayed and serially diluted across 12 wells of a clear flat bottom plate so that each well had 50 μL and the last well was blank. A 100 μL aliquot was removed from each sample to be measured and diluted 2x across three wells in the same clear bottom plate so that each sample well contained 50 μL.

[0271] A working reagent stock was prepared using solutions A, B, and C from a Micro BCA™ protein assay kit purchased from Thermo Scientific according to the directions provided.

[0272] An equal volume of 50 μL was added from the BCA working reagent to each well in the clear bottom plate and mixed using a pipette. The plate was sealed and allowed to incubate at room temperature for 15 to 30 minutes before analyzing the absorbance at 562 nm on a SpectraMax M2 plate reader.

[0273] The absorbances of the serially diluted wells from the known 10 mM CoCl2 stock used as a calibration curve, with the linear portion of the curve fit with a linear least square fitting. The slope was then used to determine the concentration of $CoCl_2$ in each sample, accounting for dilution and averaged.

Example 7

General Enzyme Immobilization Procedure on Epoxide Functionalized Covalent Resins

[0274] The desired protein variant was first produced in shake flask and purified as described in Example 1.

[0275] The storage buffer from an aliquot of the desired protein stock was then exchanged for TEoA-HCl (0-500 mM, pH 7.0-8.0) or MOPS (0-500 mM, pH 7.0-8.0) by diluting 10x with TEoA-HCl or MOPS followed by concentration through a Sartirous VivaSpin 6 (10,000 MWCO) spin filter at 4000 rpm and 4 °C. The resulting small volume is then diluted another 2.5 x with TEoA-HCl (0-500 mM, pH 7.0-8.0) or MOPS (0-500 mM, pH 7.0-8.0) to give the original volume.

[0276] Between 2 and 50 mg epoxide functionalized resin was weighed out, either wet or dry, into a 96 well plate with a volume of 2 mL per well or into a 2 mL Eppendorf tube. For larger preparations, 0.5 to 1.0 g resin was weighed out into a 15 mL conical tube. The volume of protein stock containing the desired wt. % of protein vs resin mass was transferred to the 96 well plate or tube containing resin.

[0277] The samples were then incubated at 4-25 °C for 24 to 48 h using either 500 rpm agitation or on a tube rotator operating at 10 rpm. For smaller preparations utilizing epoxide functionalized covalent resins in 96 well plates or 2.0 mL Eppendorf tubes, the resin was washed three times with 300 μL wash volumes of TEoA-HCl (50 mM, pH 7.8) or MOPS (50 mM-250 mM, pH 7.0-8.0)

containing 500 mM NaCl, allowing the mixture to agitate at room temperature at 500 rpm for 5 minutes during each wash. Between each wash the tubes were briefly centrifuged to collect resin at the bottom. The resins were then washed with three times with 300 µL wash volumes of TEoA-HCl (50 mM, pH 7.8) or MOPS (50 mM-250 mM, pH 7.0-8.0), allowing the mixture to agitate at room temperature at 500 rpm for 5 minutes during each wash. Between each wash the tubes were briefly centrifuged to collect resin at the bottom. In the case of a larger preparation in a 15 mL conical tube, the resin was collected by vacuum filtration and then washed three times with 10 mL of TEoA-HCl (50 mM, pH 7.8) or MOPS (50 mM-250 mM, pH 7.0-8.0) containing 500 mM NaCl, followed by an additional three wash volumes of 10 mL TEoA-HCl (50 mM, pH 7.8) or MOPS (50 mM-250 mM, pH 7.0-8.0), allowing the resin to mix well on a rotator for 10 minutes at room temperature during each wash.

Example 8

Immobilization of Purified Alkaline Phosphatase on Epoxide Functionalized Resins and Enzymatic 3'-Dephosphorylation of an RNA Oligomer to Measure PS to PO Conversion

[0278] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0279] Epoxide functionalized covalent resins were weighed out in 28-32 mg quantities into a 96-well plate and enzyme immobilization was carried out according to the method described in Example 7. The AP stock was diluted to 2 mg/mL in water and in sodium phosphate (1 M, pH 7). Each resin sample contained 2.0 wt. % AP vs wet weight of resin.

[0280] After immobilization the samples were removed from the shaker and briefly centrifuged to collect the resin at the bottom of each well. After removing the supernatant, each resin was washed three times with 300 µL portions of TEoA-HCl (50 mM, pH 7.8) containing 500 mM NaCl and then three times with 300 µL portions of TEoA-HCl (50 mM, pH 7.8) as described in Example 7.

[0281] The activity of the immobilized AP was evaluated by first preparing a stock solution comprised of 8.7 mL of TEoA-HCl (50 mM, pH 7.8), 50 µL of a 100 mM $CoCl_2$ solution (0.5 mM), and 1.25 mL of a 2 mM solution of 5'- mC*mA*mGmAmAmAfGmU-3'P oligomer (0.25 mM). A 10 µL portion per 1 mg resin mass of the stock solution was added to each resin and the reaction tubes shook in an incubator set to 50 °C, 500 rpm for 90 minutes.

[0282] Following the 90-minute dephosphorylation reaction, a 20 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4. Method A. The degree of the PS to PO conversion was determined as the sum of area of all species containing at least one PS to PO conversion vs the total area in the LCMS chromatogram.

Table 8.1. PS to PO Conversion from AP Immobilized on Epoxide Functionalized Resins		
Resin	**Immobilization Buffer**	**% PO Impurity**
ChiralVision IB-COV-2	Water	++
	Sodium phosphate (1 M, pH 7)	++
ChiralVision IB-COV-7	Water	+++
	Sodium phosphate (1 M, pH 7)	++
SunResin EMC7032	Water	+++
	Sodium phosphate (1 M, pH 7)	++
Resindion, EP600/BB	Water	++
	Sodium phosphate (1 M, pH 7)	+
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" < 10%, "++" 10 to 20%, "+++" 20 to 30%		

Example 9

General Procedure for the Post-Immobilization Quench of Purified Alkaline Phosphatase on Covalent Resin

[0283] Alkaline phosphatase (AP) enzyme variants SEQ ID NO: 2 or SEQ ID NO: 4 were produced in shake flask and purified as described in Example 1.

[0284] Alkaline phosphatase was immobilized on covalent resin at 1.0-5.0 wt. % vs wet weight of resin according to Example 7.

[0285] After immobilization was complete, a 20-100 mg portion of epoxide functionalized resin loaded with 2.0 wt. % AP was weighed out into a 2 mL Eppendorf tube. A 50-300 mM aliquot of quench reagent dissolved in TEoA-HCl (50-250 mM, pH 6.0-9.0), MOPS (50-250 mM, pH 6.0-9.0) or pH adjusted RO water was added to each tube and the tubes shook at 600 rpm for 0.5-48 h at 4-60 °C. After incubation, the quench solution was removed, and the resin washed with three portions of TEoA-HCl (50 mM, pH 7.8) or MOPS (50-250 mM, pH 7.0-8.0). The wash solution was discarded, and the quenched resins stored at 4 °C until ready for use.

Example 10

Post-Immobilization Quench of Purified Alkaline Phosphatase on Covalent Resin and Enzymatic 3'-Dephosphorylation of RNA Oligomer

[0286] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 4 was produced in shake flask and purified as described in Example 1.

[0287] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 7.

[0288] The AP loaded epoxide functionalized resins were quenched according to the procedure described in Example 9.

[0289] After post-immobilization quenching of the epoxide functionalized resins loaded with 2.0 wt. % AP was complete, a 30-50 mg portion of resin was weighed out into 2 mL Eppendorf tubes.

[0290] A reaction solution comprised of 800 μM 5'-AAAAmC*mA*mGmA-3'P, residual mAQP, 1.0 mM $CoCl_2$, and 220 μM iPP in TEoA-HCl (50 mM, pH 7.8) was prepared. A 300-500 μL portion of this reaction solution was added to each of the 2 mL Eppendorf tubes containing epoxide functionalized resins loaded with 2.0 wt. % AP treated with a post-immobilization quench reagent. The reaction was then shaken at 45 °C, 600 rpm for 1 h. After the reaction was complete, a 5 μL aliquot of the reaction was diluted to 160 μL in 1 mM EDTA and analyzed via the HPLC method as described in Example 3, Method B.

Table 10.1. 3'P-Dephosphorylation Using AP Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Quench

Reagent	Concentration	% 5'-AAAA-mC*mA*mGmA
Sodium Thiosulfate	60 mM	+
L-cysteine	200 mM	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" >95%.		

Example 11

Post-Immobilization Quench of Purified AP variant on Covalent Resin and Enzymatic 3'-Dephosphorylation of RNA Oligomer

[0291] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0292] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 7.

[0293] The AP loaded epoxide functionalized resins were quenched according to the procedure described in Example 9.

[0294] After post-immobilization quenching of the epoxide functionalized resins loaded with 2.0 wt. % AP was complete, a 30-50 mg portion of resin was weighed out into 2 mL Eppendorf tubes.

[0295] A reaction solution comprised of 800 μM 5'-AAAAfGmUfGmU-3'P, residual mUQP, and 1.0 mM $CoCl_2$ in TEoA-HCl (50 mM, pH 7.8) was prepared. A 300-500 μL portion of this reaction

solution was added to each of the 2 mL Eppendorf tubes containing epoxide functionalized resins loaded with 2.0 wt. % AP treated with a post-immobilization quench reagent. The reaction was then shaken at 50 °C, 600 rpm for 1 h. After the reaction was complete, a 5 µL aliquot of the reaction was diluted to 160 µL in 1 mM EDTA and analyzed via the HPLC method as described in Example 3, Method A.

Table 11.1. 3'P-Dephosphorylation Using AP Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Quench

Reagent	Concentration	pH	% 5'-AAAAfGmUfGmU
Proline	3 M	9.0	+++
Imidazole	3 M	9.0	+
Alanine	3 M	9.0	++
TEoA-HCl	0.05 M	9.0	++
L-Glycine	3 M	9.0	+
L-Glycine	1.0 M	9.0	++
L-Glycine	0.3 M	9.0	++
L-Glycine	0.1 M	9.0	++
L-Glycine	0.04 M	9.0	++
L-Glycine	3 M	8.0	+++
L-Glycine	1.0 M	8.0	++
L-Glycine	0.3 M	8.0	++
L-Glycine	0.1 M	8.0	++
L-Glycine	0.04 M	8.0	+++
L-Glycine	3 M	7.0	+
L-Glycine	1.0 M	7.0	+
L-Glycine	0.3 M	7.0	+
L-Glycine	0.1 M	7.0	+
L-Glycine	0.04 M	7.0	+
L-Lysine	3 M	9.0	++
L-Lysine	1.0 M	9.0	++
L-Lysine	0.3 M	9.0	++
L-Lysine	0.1 M	9.0	++
L-Lysine	0.04 M	9.0	++
L-Lysine	3 M	8.0	+
L-Lysine	1.0 M	8.0	++
L-Lysine	0.3 M	8.0	+
L-Lysine	0.1 M	8.0	+
L-Lysine	0.04 M	8.0	+

Table 11.1. 3'P-Dephosphorylation Using AP Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Quench			
L-Lysine	3 M	7.0	+++
L-Lysine	1.0 M	7.0	++
L-Lysine	0.3 M	7.0	+++
L-Lysine	0.1 M	7.0	+++
L-Lysine	0.04 M	7.0	++
None	0 M	7.0	++
None	0 M	8.0	++
None	0 M	9.0	++
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0% to 60%, "++" 60% to 90%, "+++" >90%.			

Example 12

Effect of $Na_2S_2O_3$ on Immobilized Alkaline Phosphatase Activity

[0296] Alkaline phosphatase (AP) variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0297] Polymethacrylate epoxide resin (IB-COV-7) was purchased from ChiralVision, and 1.5 g was weighed out into a 15 mL conical tube. Immobilization of AP variant SEQ ID NO: 2 was carried out according to Example 10 with a target loading of .2.0 wt.% vs wet weight of resin.

[0298] The enzyme charged resin was weighed out in 25-30 mg quantities in a 1.0 ml 96-well half-deep plate. To each resin sample was added reaction buffer containing: 5'-AAAAmC*mA*mGmA-3'P (1 mM), $CoCl_2$ (1 mM), TEoA-HCl (50 mM, pH 7.8) and between 0 and 100 mM $Na_2S_2O_3$. The plate was incubated at 40 °C, 500 rpm, and sampled at 1 h. The % conversion to the 3'-dephosphorylated oligonucleotide product (5'-AAAAmC*mA*mGmA) was measured by HPLC according to Example 3, Method B.

Table 12.1. Immobilized AP Variant SEQ ID NO: 2 Activity Response to Sodium Thiosulfate		
Resin	**$Na_2S_2O_3$ (mM)**	**% 5'-AAAAmC*mA*mGmA**
IB-COV-7	100	+
IB-COV-7	20	+
IB-COV-7	10	++
IB-COV-7	5	++
IB-COV-7	2.5	++
IB-COV-7	1.25	++

Table 12.1. Immobilized AP Variant SEQ ID NO: 2 Activity Response to Sodium Thiosulfate		
IB-COV-7	0.63	++
IB-COV-7	0	++
% 5--AAAAmC*mA*mGmA oligo calculated as the area under curve (AUC) from the HPLC chromatogram belonging to the deblocked product divided by the sum of all oligonucleotide AUC in the chromatogram and defined as follows: "+" 70 to 85%, "++" 85 to 95%.		

Example 13

Effect of Reaction Additives (Antioxidants or Reducing Agents) on Immobilized AP with Respect to Activity and % PO Impurity

[0299] Alkaline phosphatase (AP) variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0300] Polymethacrylate resin decorated with epoxide residues (IB-COV-7) was purchased from ChiralVision and weighed out in 25-30 mg quantities in either a 1.0 ml 96-well half-deep plate or Eppendorf tubes. Immobilization of AP variant SEQ ID NO: 2 was carried out according to the general method described in Example 7, at a target loading of 2.0 wt% versus resin wet weight.

[0301] Immobilized AP variant SEQ ID NO: 2 was incubated for 1 h at 50 °C in the presence of buffer (TEoA-HCl, 50 mM, pH 7.8) which contained no additive (antioxidant or reducing agent) or one of the following additives (antioxidant or reducing agent): ascorbic acid (5 mM), citric acid (5 mM), formic acid (5 mM), sodium thiosulfate (5 mM), sodium metabisulfite (5 mM), 2,6-dimethoxyphenol (5 mM), or catalase (Sigma C3515, 5600 U/mL), at a volume of 10 mL per gram of resin and 50 °C with 500 rpm agitation. The resin was then washed with buffer (TEoA-HCl, 50 mM, pH 7.8) three times, 10 mL buffer per gram of resin each time.

[0302] To each resin sample was then added reaction buffer containing 3'-phos oligonucleotide (5'-AAAAmC*mA*mGmA-3'P, 500 μM), $CoCl_2$ (1.0 mM), TEoA-HCl (50 mM, pH 7.8) and either no additive (antioxidant or reducing agent) or one of the following additives (antioxidant or reducing agent): ascorbic acid (5 mM), citric acid (5 mM), formic acid (5 mM), sodium thiosulfate (5 mM), sodium metabisulfite (5 mM), 2,6-dimethoxyphenol (5 mM), or catalase (Sigma C3515, 5600 U/mL), at a volume of 10 mL per gram of resin and 50 °C with 500 rpm agitation.

[0303] The reaction was sampled at 0.5 h and the percent 3'-dephosphorylation of the oligonucleotide measured by HPLC according to Example 3, Method B. The reaction was allowed to continue for 18h, sampled again, and the % PO of all oligonucleotide measured by UV-LCMS according to Example 4, Method A.

Table 13.1. Immobilized AP Variant SEQ ID NO: 2 in the Presence of Additives to Prevent PO Impurities			
Resin	**Additive**	**% Deblocked Oligo**	**% PO Impurity**
IB-COV-7	Ascorbic Acid	+	+++
IB-COV-7	Citric Acid	+	+++
IB-COV-7	Formic Acid	++	+++
IB-COV-7	Sodium Thiosulfate	++	++++
IB-COV-7	Metabisulfite	++	+
IB-COV-7	2,6-dimethoxyphenol	++	++
IB-COV-7	Catalase	+++	+
IB-COV-7	None (Pos Control)	+++	++
% Deblocked oligo calculated as the area under curve (AUC) from the HPLC chromatogram belonging to the deblocked oligo product (5'-AAAAmC*mA*mGmA) divided by the sum of the substrate oligo and deblocked oligo AUCs and defined as follows: "+" 50 to 70%, "++" 70 to 90%, "+++" 90 to 100%. % PO impurity calculated from LCMS as the ratio of ion count belonging to all oligonucleotides containing at least one PO impurity to that of all oligonucleotide in the reaction product: "+" 10 to 20%, "++" 6 to 10%, "+++" 4 to 6%, "++++" <4%.			

Example 14

General Procedure for the Post-Immobilization Quench of Purified TnT Immobilized on Epoxide Functionalized Resins

[0304] TnT enzyme variant SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0305] The storage buffer for TnT was exchanged for TEoA-HCl (500 mM, pH 7.8) as described in Example 7.

[0306] Epoxide functionalized resins were weighed in 900-1100 mg quantities into 15 mL Falcon tubes. TnT was immobilized on epoxide functionalized resins at 5.0 wt. % vs wet weight of resin according to the method described in Example 7.

[0307] After immobilization was complete, a 20-100 mg portion of epoxide functionalized resin loaded with 5.0 wt. % TnT was weighed out into a 2 mL Eppendorf tube. A 0.5 mM-5 M aliquot of quench reagent dissolved in TEoA-HCl (50 mM) was added to each tube and the tubes shook at 600 rpm for 0.5-24 h at 25-60 °C. After incubation, the quench solution was removed, and the resin washed with three portions of either TEoA-HCl (50 mM, pH 7.8) or MOPS (250 mM, pH 8.0). The wash solution was discarded, and the quenched resins stored at 4 °C until ready for use.

Example 15

Post-Immobilization Quench of Purified TnT on Covalent Resin and Enzymatic Single Extension of an RNA Oligomer

[0308] TnT enzyme variant SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0309] Inorganic pyrophosphatase (iPP) enzyme variant SEQ ID NO: 6 was produced in shake flask and purified as described in Example 1.

[0310] The storage buffer for TnT was exchanged for TEoA-HCl (500 mM, pH 7.8) as described in Example 7.

[0311] Epoxide functionalized resins were weighed in 900-1100 mg quantities into 15 mL Falcon tubes. TnT was immobilized on epoxide functionalized resins at 5.0wt. % vs wet weight of resin according to the method described in Example 7.

[0312] The TnT loaded epoxide functionalized resins were quenched according to the procedure described in Example 14.

[0313] After post-immobilization quenching of the epoxide functionalized resins loaded with 5.0 wt. % TnT was complete, a 30-50 mg portion of resin was weighed out into 2 mL Eppendorf tubes.

[0314] A reaction solution comprised of 3.1 mL RO water, 175 µL 1 M TEoA-HCl (50 mM, pH 7.8), 35 µL of a 100 mM $CoCl_2$ solution (1.0 mM), 140 µL of a 20 mM solution of 5'-AAAAmC*mA*mG (800 µM), 42 µL of a 100 mM solution of mAQP (1.2 mM), and 15.9 µL of a 220 µM solution of iPP (1.0 µM) was prepared. A 300-500 µL portion of this reaction solution was added to each of the 2 mL Eppendorf tubes containing epoxide functionalized resins loaded with 5.0 wt. % TnT treated with a post-immobilization quench reagent. The reaction was then shaken at 50 °C, 600 rpm for 20 h. After the reaction was complete, a 5 µL aliquot of the reaction was diluted to 160 µL in 1 mM EDTA and analyzed via the HPLC method as described in Example 3, Method B.

Table 15.1. Single RNA Extension Using TnT Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Quench

Resin	Reagent	Amount	Quench Temp	Time	% 5'-AAAAmC*mA*mGmA-3'P
ChiralVision IB-COV-2	TEoA-HCl, pH 9.0	50 mM	50 °C	2 h	+++
ChiralVision IB-COV-2	Tris, pH 8.0	50 mM	50 °C	2 h	+++
ChiralVision IB-COV-7	TEoA-HCl, pH 9.0	50 mM	50 °C	2 h	+++
ChiralVision IB-COV-7	Tris, pH 8.0	50 mM	50 °C	2 h	+++
ChiralVision IB-COV-7	Ethanolamine	1 M	45 °C	5 h	+

Table 15.1. Single RNA Extension Using TnT Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Quench					
ChiralVision IB-COV-7	Ethanolamine	100 mM	45 °C	5 h	+
ChiralVision IB-COV-7	Ethanolamine	10 mM	45 °C	5 h	++
ChiralVision IB-COV-7	L-Lysine	100 mM	45 °C	5 h	+
ChiralVision IB-COV-7	L-Lysine	10 mM	45 °C	5 h	+
ChiralVision IB-COV-7	L-Lysine	1 mM	45 °C	5 h	+
ChiralVision IB-COV-7	L-Cysteine	200 mM	45 °C	5 h	+++
ChiralVision IB-COV-7	L-Cysteine	20 mM	45 °C	5 h	+++
ChiralVision IB-COV-7	L-Cysteine	2 mM	45 °C	5 h	+
ChiralVision IB-COV-7	Sodium Thiosulfate	500 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	250 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	125 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	62.5 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	31.3 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	15.6 mM	30 °C	18 h	+++
ChiralVision IB-COV-7	Sodium Thiosulfate	7.8 mM	30 °C	18 h	+++
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" < 30, "++" 30% to 50%, "+++" 50 to 95%.					

Example 16

General Procedure for the Post-Immobilization Quench of Purified TnT Co-immobilized with iPP on Epoxide Functionalized Resins

[0315] TnT enzyme variant SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0316] iPP enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0317] Epoxide functionalized resins were weighed in 10-1100 mg quantities into 15 mL Falcon tubes. TnT was co-immobilized at 5.0 wt. % vs wet weigh of resin on epoxide functionalized resins with 0.2 wt. % vs wet weight of resin iPP according to the method described in Example 7.

[0318] After immobilization was complete, a 20-100 mg portion of epoxide functionalized resin loaded with 5.0 wt. % TnT and 0.2 wt% iPP was weighed out into a 2 mL Eppendorf tube. A 0.5 mM-5 M aliquot of quench reagent dissolved in either TEoA-HCl (50 mM) or pH adjusted RO water was added to each tube and the tubes shook at 600 rpm for 0.5-24 h at 25-60 °C. After incubation, the quench solution was removed, and the resin washed with three portions of wash solution. The wash solution was discarded, and the quenched resins stored at 4 °C until ready for use.

Example 17

Effect of $Na_2S_2O_3$ on Immobilized TnT variant SEQ ID NO: 12 Activity

[0319] TnT enzyme variant SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0320] Inorganic pyrophosphatase variant SEQ ID NO: 8 was produced and purified, as described in Example 1.

[0321] Polymethacrylate affinity resin (IB-HIS-2) was purchased from ChiralVision, charged with $CoCl_2$, and 1.5 g was weighed out into a 15 mL conical tube. Co-immobilization using a target of 5.0 wt% TnT variant SEQ ID NO: 12 and 0.2 wt% iPP variant SEQ ID NO: 8 was carried out according to the method described in Example 7.

[0322] The enzyme charged resin was weighed out in 25-30 mg quantities in a 1.0 ml 96-well half-deep plate. To each resin sample was added reaction buffer containing: 5'-AAAAfGmUfG (1 mM), mAQP (1.5 mM), $CoCl_2$ (1 mM), TEoA-HCl (50 mM, pH 7.8) and between 0 and 100 mM $Na_2S_2O_3$. The plate was incubated at 40 °C, 500 rpm, and sampled at 1 h. The % conversion to the oligonucleotide product (5'-AAAAfGmUfGmA-3'P) was measured by HPLC according to Example 3, method A.

Table 17.1. Immobilized TnT variant SEQ ID NO: 12 Activity Response to Sodium Thiosulfate

Resin	$Na_2S_2O_3$ (mM)	% 5'-AAAAfGmUfGmA-3'P
IB-HIS-2-Co(II)	100	+++
IB-HIS-2-Co(II)	20	++
IB-HIS-2-Co(II)	10	++
IB-HIS-2-Co(II)	5	+
IB-HIS-2-Co(II)	2.5	+
IB-HIS-2-Co(II)	1.25	+
IB-HIS-2-Co(II)	0.63	+
IB-HIS-2-Co(II)	0	+
% 5'-AAAAfGmUfGmA-3'P oligo calculated as the area under curve (AUC) from the HPLC chromatogram belonging to the singly extended oligo product divided by the sum of all		

Table 17.1. Immobilized TnT variant SEQ ID NO: 12 Activity Response to Sodium Thiosulfate
oligonucleotide AUC in the chromatogram and defined as follows: "+" 50 to 70%, "++" 70 to 90%, "+++" 90 to 100%.

Example 18

Post-Immobilization Quenching of Co-immobilized TnT and iPP on Epoxide Functionalized Resins and Enzymatic Single Extension of an RNA Oligomer

[0323] TnT enzyme variant SEQ ID NO: 12 was produced in shake flask and purified as described in Example 1.

[0324] iPP enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0325] Epoxide functionalized resins were weighed in 900-1100 mg quantities into 15 mL Falcon tubes. TnT was co-immobilized at 5.0 wt. % vs wet weigh of resin on ChiralVision IB-COV-7 with 0.2 wt. % vs wet weight of resin iPP according to the method described in Example 7.

[0326] After immobilization was complete, 50 mg portions of TnT and iPP co-immobilized on a covalent resin were quenched according to the method described in Example 16.

[0327] Activity was evaluated by preparing a reaction solution comprised of 3.4 mL RO water, 190 µL of 1 M TEoA-HCl (50 mM, pH 7.8), 152 µL of a 20 mM stock solution of 5'-AAAAfGmUfG oligomer (800 µM), 45.6 µL of a 100 mM solution of mUQP (1.2 mM), and 38 µL of a 100 mM $CoCl_2$ solution (1.0 mM). A 500 µL portion of the reaction solution was then transferred to the tubes containing the resin, and each tube was incubated at 50°C, 600 rpm for 18 h.

[0328] After 18 h, a 10 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method A. The conversion of 5'-AAAAfGmUfG to 5'-AAAAfGmUfGmU-3'P was measured as the area of product vs the total area in the HPLC chromatogram.

Table 18.1. Single Extension of an RNA Oligomer with Co-immobilized TnT and iPP on Covalent Resin with a Post-Immobilization Quench			
Reagent	**Concentration**	**pH**	**% 5'-AAAAfGmUfGmU-3'P**
L-Glycine	3 M	9.0	+++
Proline	3 M	9.0	+++
Alanine	3 M	9.0	++
Imidazole	3 M	9.0	+
TEoA-HCl	3 M	9.0	+
None	0 M	7.8	++

Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0% to 45%, "++" 45% to 80%, "+++" >80%.

Example 19

Effect of Quenching Residual Epoxides on Silica Based Resins Post-immobilization

[0329] TnT enzyme variant SEQ ID NO: 14 was produced in shake flask and purified as described in Example 1.

[0330] Inorganic pyrophosphatase variant SEQ ID NO: 8 was produced and purified, as described in Example 1.

[0331] Epoxide functionalized resins based on silica (**Table 19.1**) or polymethacrylate resin purchased from ChiralVision (IB-COV-7) were weighed out in 25-30 mg quantities into 2 mL Eppendorf tubes and enzyme co-immobilization using a target of 5.0 wt% TnT variant SEQ ID NO: 14 of was carried out according to the method described in Example 7.

Table 19.1. Silica Based Epoxide Resin Chemistry

Resin	Epoxy Linker	Silanol Capping Agent
Silica A	(3-mercaptopropyl)trimethoxysilane and poly(ethylene glycol) diglycidyl ether (MN 500)	None
Silica B	(3-mercaptopropyl)trimethoxysilane and poly(ethylene glycol) diglycidyl ether (MN 1000)	None
Silica C	(3-glycidyloxypropyl)trimethoxysilane	None
Silica D	(3-mercaptopropyl)trimethoxysilane and poly(ethylene glycol) diglycidyl ether (MN 500)	methyltrimethoxysilane
Silica E	(3-mercaptopropyl)trimethoxysilane and Poly(ethylene glycol) diglycidyl ether (MN 1000)	methyltrimethoxysilane
Silica F	(3-glycidyloxypropyl)trimethoxysilane	methyltrimethoxysilane
CPG-19	(3-glycidyloxypropyl)trimethoxysilane	None
CPG-20	(3-glycidyloxypropyl)trimethoxysilane	N,O-bis(trimethylsilyl) acetamide
CPG-21	(3-glycidyloxypropyl)trimethoxysilane	trimethylchlorosilane and 1,1,1,3,3,3-hexamethyldisilazane

[0332] Resins were either used directly after completion of immobilization procedure (unquenched) or were subjected to a post-immobilization quench as described in Example 16.

[0333] The resin was then used to catalyze the addition of mAQP to mC*mA*mGmAmAmAfC according to the general procedure in Example 18. The reaction product after 18 h was analyzed by UV-LCMS according to Example 4, Method A to find the % PS to PO conversion (**Table 19.2**).

Table 19.2. Silica Based Epoxide Resins Compared: Effect on PS to PO With and Without Quenching

Resin	Quenched or Unquenched	% PO Impurity
CPG-19	Unquenched	+
CPG-20	Unquenched	+
CPG-21	Unquenched	+
Silica A	Unquenched	++
Silica B	Unquenched	++
Silica C	Unquenched	+
Silica D	Unquenched	+++
Silica E	Unquenched	+++
Silica F	Unquenched	+
CPG-19	Quenched	++
CPG-20	Quenched	++
CPG-21	Quenched	+++
Silica A	Quenched	+++
Silica B	Quenched	+++
Silica C	Quenched	+++
Silica D	Quenched	+++
Silica E	Quenched	+++
Silica F	Quenched	++

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" 1.0 to 2.5%. "++" 0.1 to 1.0%, "+++" 0 to 0.1%.

Using Elevated Temperature and/or Lower pH in Combination with Quench Agents

[0334] Resins were quenched after immobilization by incubating the resin in a solution of one of the following quench agents: L-glycine and NaCl (3M and 5M, respectively, pH 9), Gly-OBz (L-glycine benzyl ester, 1M, pH 9), Gly-OMe (L-glycine methyl ester, 1M, pH 9), Gly-OtBu (L-glycine *tert*-butyl ester, 1M, pH 9), L-cysteine (1M, pH 9), Cys-OMe (L-cysteine methyl ester, 1M, pH 9 or 5), N-Ac-Cys (N-Acetyl-L-cysteine, 1M, pH 9 or 5), Na2S2O3 (1M, pH 9 or 5), or BME (β-

mercaptoethanol, 1M, pH 5 or 9), at a volume of 10 mL per 1.0 g of resin, for 18h at either 25 °C or 40 °C.

[0335] The resin was then used to catalyze the addition of mAQP to mC*mA*mGmAmAmAfC according to the general procedure in Example 18. The reaction product after 18 h was analyzed by UV-LCMS according to Example 4, Method A to find the % PS to PO conversion (**Table 19.2**).

Table 19.3. Silica Based Epoxide Resins: Effect of pH and Temperature in Combination with Quench Agents

Resin	Quench Agent	Quench Temperature (°C)	Quench pH	% PO Impurity
CPG-20	N-Ac-Cys	40	5	++
CPG-20	N-Ac-Cys	40	9	++
CPG-20	$Na_2S_2O_3$	40	5	+
CPG-20	$Na_2S_2O_3$	40	9	+
CPG-20	Gly-OtBu	40	9	++
CPG-20	Gly-OMe	40	9	+++
CPG-20	Gly-OBz	40	9	+++
CPG-20	L-Glycine	40	9	+++
CPG-20	L-Glycine	25	9	+++
CPG-20	L-Cysteine	40	9	++
CPG-20	L-Cysteine	25	9	+++
CPG-20	Cys-OMe	40	5	+
CPG-20	Cys-OMe	40	9	++
CPG-20	BME	40	5	+
CPG-20	BME	40	9	+

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" 0.4 to 1.0%. "++" 0.1 to 0.4%, "+++" 0 to 0.1%.

Example 20

Effect of Quenching Residual Epoxides on Polymethacrylate Resin Post-Immobilization

[0336] TnT enzyme variant SEQ ID NO: 14 and SEQ ID NO: 16 was produced in shake flask and purified as described in Example 1.

[0337] Inorganic pyrophosphatase variant SEQ ID NO: 8 was produced and purified, as described in Example 1.

[0338] Polymethacrylate resin decorated with epoxide residues (IB-COV-7) was purchased from ChiralVision and weighed out in 25-30 mg quantities in either a 1.0 ml 96-well half-deep plate or Eppendorf tubes. Co-immobilization using a target of 5.0 wt% TnT of was carried out according to the method described in Example 7.

[0339] Resin with immobilized enzyme was either used directly after completion of immobilization procedure (unquenched) or quenched after immobilization by incubating the resin in a solution of one of the following quench agents: L-glycine and NaCl (3M and 5M, respectively, pH 9), Gly-OBz (L-glycine benzyl ester, 1M, pH 9), Gly-OMe (L-glycine methyl ester, 1M, pH 9), Gly-OtBu (L-glycine *tert*-butyl ester, 1M, pH 9), L-cysteine (1M, pH 9), Cys-OMe (L-cysteine methyl ester, 1M, pH 9 or 5), N-Ac-Cys (N-Acetyl-L-cysteine, 1M, pH 9 or 5), Na2S2O3 (1M, pH 9 or 5), or BME (β-mercaptoethanol, 1M, pH 5 or 9), at a volume of 10 mL per 1.0 g of resin, for 18h at either 25 °C or 40 °C.

[0340] The resin was then used to catalyze the addition of mAQP to mC*mA*mGmAmAmAfC according to the general procedure in Example 18. The reaction product after 18 h was analyzed by UV-LCMS according to Example 4, Method A to find the % PS to PO conversion (**Table 20.1**).

Table 20.1. Polymethacrylate Based Epoxide Resin: Effect of pH and Temperature in Combination with Quench Agents

Resin	Quench Agent	Quench Temperature (°C)	Quench pH	% PO Impurity
IB-COV-7	None	N/A	N/A	+
IB-COV-7	N-Ac-Cys	40	9	+++
IB-COV-7	N-Ac-Cys	40	5	++
IB-COV-7	$Na_2S_2O_3$	40	9	++++
IB-COV-7	$Na_2S_2O_3$	40	5	++++
IB-COV-7	Gly-OtBu	40	9	+++
IB-COV-7	Gly-OMe	40	9	++
IB-COV-7	Gly-OBz	40	9	++
IB-COV-7	L-Glycine	40	9	+++
IB-COV-7	L-Glycine	25	9	++
IB-COV-7	L-Cysteine	40	9	+++
IB-COV-7	Cys-OMe	40	9	+++
IB-COV-7	Cys-OMe	40	5	+++
IB-COV-7	BME	40	9	++++
IB-COV-7	BME	40	5	+

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the

Table 20.1. Polymethacrylate Based Epoxide Resin: Effect of pH and Temperature in Combination with Quench Agents				
Resin	**Quench Agent**	**Quench Temperature (°C)**	**Quench pH**	**% PO Impurity**
beginning of the reaction and is defined as follows: "+"4.0 to 10%. "++" 1.1 to 4.0%, "+++" 0.1 to 1.1%, "++++" 0 to 0.1%.				

Example 21

Immobilization of Purified TnT on Epoxide Functionalized Resins and Enzymatic Single Extension of an RNA Oligomer in the Presence of Sodium Thiosulfate

[0341] TnT enzyme variant SEQ ID NO: 10 was produced in shake flask and purified as described in Example 1.

[0342] Inorganic pyrophosphatase variant SEQ ID NO: 6 was produced and purified, as described in Example 1.

[0343] The storage buffer for TnT was exchanged for TEoA-HCl (500 mM, pH 7.8) as described in Example 7.

[0344] ChiralVision IB-COV-7 resin was weighed out in 25-30 mg quantities into 2 mL Eppendorf tubes and enzyme immobilization was carried out according to the method described in Example 7.

[0345] The activity of the immobilized TnT was evaluated by first preparing a stock solution comprised of 980 µL of RO water, 55 µL 1 M TEoA-HCl (50 mM, pH 7.8), 11 µL of a 100 mM $CoCl_2$ solution (1.0 mM), 44 µL of a 20 mM solution of 5'-AAAAmC*mA*mG oligomer (800 µM), 13.2 µL of a 100 mM solution of mAQP (1.2 mM), 5 µL of a 220 µM stock solution of iPP (1 µM), and additive (antioxidant or reducing agent). A 10 µL portion per 1 mg resin mass of the stock solution was added to each resin and the reaction tubes shook in an incubator set to 50 °C, 600 rpm. After 20 h, a 2.5 µL aliquot of each reaction was diluted to 25 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method B. The conversion of 5'-AAAAmC*mA*mG to 5'-AAAAmC*mA*mGmA-3'P was measured as the area of product vs the total area in the HPLC chromatogram.

Table 21.1. Single RNA Extension Using TnT Immobilized on Epoxide Functionalized Resins with Sodium Thiosulfate		
Additive	**Concentration**	**% 5'-AAAAmC*mA*mGmA-3'P**
Sodium Thiosulfate	800 mM	++
None	0 mM	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0% to 85%, "++" >85%		

[0346] After 20 h, a 2.5 µL aliquot of each reaction was diluted to 25 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method B. The total PS to PO conversion of 5'-AAAAmC*mA*mGmA-3'P was measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 21.2. PS to PO Conversion in a Single RNA Extension Using TnT Immobilized on Epoxide Functionalized Resins with Sodium Thiosulfate

Additive	Concentration	% PO Impurity
Sodium Thiosulfate	800 mM	++
None	0 mM	+

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" >20%, "++" 0% to 20%

Example 22

Co-immobilization of Purified TnT and iPP on Epoxide Functionalized Resins and Enzymatic Single Extension of an RNA Oligomer in the Presence of Reducing Additives (Antioxidant or Reducing Agent)

[0347] TnT variant SEQ ID NO: 14 was expressed and purified according to the procedure in Example 1.

[0348] Inorganic pyrophosphatase (iPP) variant SEQ ID NO: 8 was expressed and purified according to the procedure in Example 1.

[0349] ChiralVision IB-COV-7 was weighed out in 25-30 mg quantities into 2 mL Eppendorf tubes and enzyme immobilization was carried out according to the method described in Example 7.

[0350] After immobilization was complete, the co-immobilized TnT and iPP loaded resin was treated with a post-immobilization quench step according to the procedure in Example 16.

[0351] The activity of the co-immobilized TnT and iPP was evaluated by first preparing a 2x stock solution comprised of 6.68 mL of RO water, 820 µL 1 M TEoA-HCl (100 mM, pH 7.8), 164 µL of a 100 mM $CoCl_2$ solution (1.0 mM), 410 µL of a 20 mM solution of 5'-mG*mA*mCmUmUmUfC oligomer (1.0 mM), and 123 µL of a 100 mM solution of mAQP (1.5 mM). A 20 mM 2x stock solution of each additive (antioxidant or reducing agent) was prepared in RO water and serially diluted five-fold. A 20 mg portion of resin containing co-immobilized TnT and iPP was transferred to each well of a 96 well plate, followed by a 100 µL portion of the 2x reaction stock and a 100 µL portion of the serially diluted 2x additive (antioxidant or reducing agent) stock. The plate was sealed with a foil seal and heated to 50°C and shook at 600 rpm for 20 h.

[0352] After 20 h, a 10 μL aliquot of each reaction was diluted to 25 μM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method E. The conversion of 5'-mG*mA*mCmUmUmUfC to 5'-mG*mA*mCmUmUmUfCmA-3'P was measured as the area of product vs the total area in the HPLC chromatogram.

Table 22.1. Single RNA Extension Using Co-immobilized TnT and iPP on Epoxide Functionalized Resins with Reducing Additives

Additive	Concentration	% 5'-mG*mA*mCmUmUmUfCmA-3'P
Sodium Thiosulfate	10 mM	+++
Sodium Thiosulfate	2 mM	+++
Sodium Thiosulfate	0.4 mM	+++
Sodium Thiosulfate	0.08 mM	++
Dithiothreitol	10 mM	+
Dithiothreitol	2 mM	++
Dithiothreitol	0.4 mM	++
Dithiothreitol	0.08 mM	+++
Tris(2-carboxyethyl)phosphine-HCl	10 mM	+
Tris(2-carboxyethyl)phosphine-HCl	2 mM	+++
Tris(2-carboxyethyl)phosphine-HCl	0.4 mM	+++
Tris(2-carboxyethyl)phosphine-HCl	0.08 mM	++
Sodium Iodide	10 mM	+++
Sodium Iodide	2 mM	+++
Sodium Iodide	0.4 mM	+++
Sodium Iodide	0.08 mM	++
Diethyl Dithiophosphate	10 mM	+++
Diethyl Dithiophosphate	2 mM	+++
Diethyl Dithiophosphate	0.4 mM	+++
Diethyl Dithiophosphate	0.08 mM	++
Diethyl Thiophosphate Potassium Salt	10 mM	+++
Diethyl Thiophosphate Potassium Salt	2 mM	+++
Diethyl Thiophosphate Potassium Salt	0.4 mM	+++
Diethyl Thiophosphate Potassium Salt	0.08 mM	++
Sodium Thiophosphate	10 mM	+++
Sodium Thiophosphate	2 mM	+++

Table 22.1. Single RNA Extension Using Co-immobilized TnT and iPP on Epoxide Functionalized Resins with Reducing Additives		
Sodium Thiophosphate	0.4 mM	+++
Sodium Thiophosphate	0.08 mM	+++
None	0 mM	+++
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: “+” 0% to 85%, “++” 85% to 97%, "+++" >97%.		

[0353] After 20 h, a 10 µL aliquot of each reaction was diluted to 25 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method E. The total PS to PO conversion of 5’-mG*mA*mCmUmUmUfCmA-3’P was measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 22.2. PS to PO Conversion in a Single RNA Extension Using Co-immobilized TnT and iPP on Epoxide Functionalized Resins with Reducing Additives		
Additive	**Concentration**	**% PO Impurity**
Sodium Thiosulfate	10 mM	+++
Sodium Thiosulfate	2 mM	++
Sodium Thiosulfate	0.4 mM	++
Sodium Thiosulfate	0.08 mM	++
Dithiothreitol	10 mM	+++
Dithiothreitol	2 mM	++
Dithiothreitol	0.4 mM	++
Dithiothreitol	0.08 mM	++
Tris(2-carboxyethyl)phosphine-HCl	10 mM	+
Tris(2-carboxyethyl)phosphine-HCl	2 mM	++
Tris(2-carboxyethyl)phosphine-HCl	0.4 mM	++
Tris(2-carboxyethyl)phosphine-HCl	0.08 mM	++
Sodium Iodide	10 mM	++
Sodium Iodide	2 mM	++
Sodium Iodide	0.4 mM	++
Sodium Iodide	0.08 mM	++
Diethyl Dithiophosphate	10 mM	++
Diethyl Dithiophosphate	2 mM	++
Diethyl Dithiophosphate	0.4 mM	++
Diethyl Dithiophosphate	0.08 mM	++
Diethyl Thiophosphate Potassium Salt	10 mM	++
Diethyl Thiophosphate Potassium Salt	2 mM	++

Table 22.2. PS to PO Conversion in a Single RNA Extension Using Co-immobilized TnT and iPP on Epoxide Functionalized Resins with Reducing Additives		
Diethyl Thiophosphate Potassium Salt	0.4 mM	++
Diethyl Thiophosphate Potassium Salt	0.08 mM	++
Sodium Thiophosphate	10 mM	+++
Sodium Thiophosphate	2 mM	++
Sodium Thiophosphate	0.4 mM	++
Sodium Thiophosphate	0.08 mM	++
None	0 mM	+++
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: “+” >10%, "++" 5% to 10%, “+++” <5%.		

Example 23

Optimization of Reaction Components to Suppress PS to PO Conversion During a Single Extension of an RNA Oligomer

[0354] TnT variant SEQ ID NO: 14 was expressed and purified according to the procedure in Example 1.

[0355] Inorganic pyrophosphatase (iPP) variant SEQ ID NO: 8 was expressed and purified according to the procedure in Example 1.

[0356] ChiralVision IB-COV-7 was weighed out in 25-30 mg quantities into 2 mL Eppendorf tubes and enzyme immobilization was carried out according to the method described in Example 7.

[0357] After immobilization was complete, the co-immobilized TnT and iPP loaded resin was treated with a post-immobilization quench step according to the procedure in Example 16.

[0358] The activity of the co-immobilized TnT and iPP was evaluated by first preparing a 4x stock solution comprised of 10.3 mL of RO water, 480 µL of a 100 mM $CoCl_2$ solution (4.0 mM), 960 µL of a 20 mM solution of 5’-mG*mA*mCmUmUmUfC oligomer (1.6 mM), and 288 µL of a 100 mM solution of mAQP (2.4 mM). Buffer solutions were prepared at 2x concentrations with the specified pH, and 4x stock solutions of sodium thiosulfate were prepared in water. A 20 mg portion of resin containing co-immobilized TnT and iPP was transferred to each well of a 96 well plate, followed by a 50 µL portion of the 4x reaction stock, a 100 µL portion of the specified 2x buffer stock, and 50 µL of the 4x sodium thiosulfate reaction stock. For reactions ran in the absence of sodium thiosulfate, 50 µL of RO water was added. The plate was sealed with a foil seal and heated to 40°C and shook at 600 rpm for 18 h.

[0359] After 18 h, a 12 μL aliquot of each reaction was diluted to 50 μM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method C. The conversion of 5'-mG*mA*mCmUmUmUfC to 5'-mG*mA*mCmUmUmUfCmA-3'P was measured as the area of product vs the total area in the HPLC chromatogram.

Table 23.1. Effect of Reaction Component on Conversion of an RNA Oligomer with TnT and iPP Co-immobilized on Covalent Resin

Buffer	Buffer Conc.	pH	$Na_2S_2O_3$ Conc.	% 5'-mG*mA*mCmUmUmUfCmA-3'p
TEoA-HCl	250 mM	9.0	5.0 mM	++
TRIS	25 mM	6.8	5.0 mM	+++
TEoA-HCl	137.5 mM	9.0	5.0 mM	++
TEoA-HCl	25 mM	9.0	0.0 mM	++
MOPS	25 mM	6.8	5.0 mM	+++
TEoA-HCl	25 mM	9.0	5.0 mM	+++
TRIS	250 mM	9.0	5.0 mM	+
TEoA-HCl	250 mM	7.9	2.5 mM	+++
TRIS	137.5 mM	7.9	5.0 mM	+++
TRIS	25 mM	9.0	0.0 mM	++
MOPS	250 mM	6.8	5.0 mM	+++
TRIS	25 mM	9.0	5.0 mM	++
TRIS	250 mM	6.8	5.0 mM	+++
TEoA-HCl	250 mM	6.8	5.0 mM	+
TEoA-HCl	250 mM	9.0	0.0 mM	++
TEoA-HCl	25 mM	6.8	0.0 mM	+++
MOPS	25 mM	9.0	0.0 mM	+++
TRIS	25 mM	6.8	0.0 mM	+
TRIS	250 mM	9.0	0.0 mM	++
MOPS	25 mM	9.0	5.0 mM	+++
TEoA-HCl	250 mM	6.8	0.0 mM	+++
TEoA-HCl	25 mM	7.9	2.5 mM	+++
TRIS	137.5 mM	6.8	2.5 mM	+++
TRIS	250 mM	6.8	0.0 mM	+++
MOPS	250 mM	9.0	0.0 mM	+++
MOPS	25 mM	6.8	0.0 mM	+++
MOPS	250 mM	9.0	5.0 mM	+++
TRIS	137.5 mM	7.9	0.0 mM	+++

Table 23.1. Effect of Reaction Component on Conversion of an RNA Oligomer with TnT and iPP Co-immobilized on Covalent Resin				
TEoA-HCl	25 mM	6.8	5.0 mM	+++
MOPS	250 mM	6.8	0.0 mM	+
TRIS	137.5 mM	9.0	2.5 mM	++
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0-85%, "++" 85% to 95%, "+++" >95%.				

[0360] After 18 h, a 12 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of 5'-mG*mA*mCmUmUmUfCmA-3'P was measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 23.2. Effect of Reaction Component on PS to PO Conversion of an RNA Oligomer with TnT and iPP Co-immobilized on Covalent Resin				
Buffer	**Buffer Concentration**	**pH**	**$Na_2S_2O_3$ Concentration**	**% PO Impurity**
TEoA-HCl	250 mM	9.0	5.0 mM	+++
TRIS	25 mM	6.8	5.0 mM	+
TEoA-HCl	137.5 mM	9.0	5.0 mM	+++
TEoA-HCl	25 mM	9.0	0.0 mM	+++
MOPS	25 mM	6.8	5.0 mM	++
TEoA-HCl	25 mM	9.0	5.0 mM	+++
TRIS	250 mM	9.0	5.0 mM	+++
TEoA-HCl	250 mM	7.9	2.5 mM	++
TRIS	137.5 mM	7.9	5.0 mM	++
TRIS	25 mM	9.0	0.0 mM	++
MOPS	250 mM	6.8	5.0 mM	+
TRIS	25 mM	9.0	5.0 mM	+++
TRIS	250 mM	6.8	5.0 mM	++
TEoA-HCl	250 mM	6.8	5.0 mM	+
TEoA-HCl	250 mM	9.0	0.0 mM	+++
TEoA-HCl	25 mM	6.8	0.0 mM	+
MOPS	25 mM	9.0	0.0 mM	++
TRIS	25 mM	6.8	0.0 mM	+
TRIS	250 mM	9.0	0.0 mM	+++
MOPS	25 mM	9.0	5.0 mM	++
TEoA-HCl	250 mM	6.8	0.0 mM	+

Table 23.2. Effect of Reaction Component on PS to PO Conversion of an RNA Oligomer with TnT and iPP Co-immobilized on Covalent Resin				
TEoA-HCl	25 mM	7.9	2.5 mM	++
TRIS	137.5 mM	6.8	2.5 mM	+
TRIS	250 mM	6.8	0.0 mM	+
MOPS	250 mM	9.0	0.0 mM	++
MOPS	25 mM	6.8	0.0 mM	+
TRIS	137.5 mM	7.9	0.0 mM	+
MOPS	250 mM	9.0	5.0 mM	++
TEoA-HCl	25 mM	6.8	5.0 mM	++
MOPS	250 mM	6.8	0.0 mM	++
TRIS	137.5 mM	9.0	2.5 mM	+++
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: “+” >3.5%, "++" 1.5% to 3.5%, “+++” <1.5%				

Example 24

Use of Sodium Thiosulfate as a Buffer Additive During Flow Reactions

[0361] TnT enzyme variant SEQ ID NO: R63bb was produced in shake flask and purified as described in Example 1.

[0362] Inorganic pyrophosphatase variant SEQ ID NO: 8 was produced and purified, as described in Example 1.

[0363] Polymethacrylate epoxide resins were purchased from ChiralVision (IB-COV-7), Resindion (HFA403M and EP403M), or Sunresin (EMC7042M). TnT enzyme variant SEQ ID NO: 14 and iPP enzyme variant SEQ ID NO: 8 were co-immobilized on each with a target loading of 5.0 wt.% and 0.2 wt.%, respectively, according to Example 7.

[0364] Each resin sample was quenched with a 3M L-glycine solution (also containing 5M NaCl, pH 9) at room temperature for 16h. Afterward the resin was washed three times with a 10 mL per gram of resin using a 250 mM MOPS (pH 8) solution.

[0365] A 340 mg sample of each resin was packed into a BioRad Econo Alpha column (manufacturer part # 12009463) which was wrapped in a resistive heating jacket set to 40 °C. Reaction buffer (7.0 mL) containing MOPS (250 mM, pH 8), NQP (0.75 mM, either fCQP or mAQP), $CoCl_2$ (1.0 mM), oligonucleotide (mC*mA*mGmAmAmAfG or mU*fA*mUmAmUfUmU), and $Na_2S_2O_3$ (0 or 50 mM) was recirculated across each column at a flow rate of 0.24 mL/min using a Masterflex L/S peristaltic pump.

[0366] A 5 µL sample was removed from each reservoir after 4h and the %PO impurity in the singly extended product measured by UV-LCMS as described in Example 4, Method A. The % conversion to singly extended product was measured by HPLC according to Example 3, Method D.

Table 24.1. Sodium Thiosulfate Buffer Additive Effect on %PO Impurity During Flow Experiment

Resin	$Na_2S_2O_3$ (mM)	Oligo Substrate	NQP	% Conversion	% PO Impurity
HFA403M	50	mC*mA*mGmAmAmAfG	mAQP	++	+
IB-COV-7	50	mC*mA*mGmAmAmAfG	mAQP	+	+
EP403M	50	mC*mA*mGmAmAmAfG	mAQP	+	+
EMC7042M	50	mC*mA*mGmAmAmAfG	mAQP	+	+
IB-COV-7	0	mU*fA*mUmAmUfUmU	fCQP	+	++

Percent singly extended oligo product calculated as the area under curve (AUC) from the HPLC chromatogram belonging to the deblocked product divided by the sum of all oligonucleotide AUC in the chromatogram and defined as follows: "+" 80 to 90%, "++" 90 to 100%. %PO impurity in the reaction product was measured by LCMS as the total ion count belonging to the %PO impurity in the product divided by the ion count sum belonging to all oligo in the product and defined as follows: "+" 0.5 to 1.5%, "++" 5.0 to 10.0 %.

Example 25

Iterative Extension and 3'P Dephosphorylation of an RNA Oligomer in a Batch Reactor with Reducing Additives (Antioxidant or Reducing Agent)

[0367] TnT variant SEQ ID NO: 14 was expressed and purified according to the procedure in Example 1.

[0368] Inorganic pyrophosphatase (iPP) variant SEQ ID NO: 8 was expressed and purified according to the procedure in Example 1.

[0369] Alkaline phosphatase (AP) variant SEQ ID NO: 2 was expressed and purified according to the procedure in Example 1.

[0370] TnT variant SEQ ID NO: 14 and iPP variant SEQ ID NO: 8 were co-immobilized on ChiralVision IB-COV-7 at 5.0 wt% and 0.2 wt% vs wet wight resin, respectively, according to the procedure outlined in Example 7.

[0371] Post-immobilization quench of co-immobilized TnT and iPP was completed as outlined in Example 16.

[0372] Alkaline phosphatase enzyme variant SEQ ID NO: 2 was immobilized at 2.0 wt% vs wet weight resin on ChiralVision IB-COV-7 according to the general procedure outlined in Example 7.

[0373] After immobilization was complete, AP immobilized on ChiralVision IB-COV-7 was quenched according to the procedure outlined in Example 9.

Extension Reaction: 5'-mC*mA*mGmAmAmAfG + mAQP => 5'-mC*mA*mGmAmAmAfGmA-3'P

[0374] The activity of the co-immobilized TnT and iPP was evaluated by first preparing a 4x stock solution comprised of 2.1 mL of RO water, 120 µL of a 100 mM $CoCl_2$ solution (4.0 mM), 600 µL of a 20 mM solution of 5'-mC*mA*mGmAmAmAfG oligomer (4.0 mM), and 180 µL of a 100 mM solution of mAQP (6 mM). Buffer solutions were prepared at 2x concentrations with the specified pH, and 4x stock solutions of serially diluted sodium thiosulfate were prepared in water.

[0375] A 30 mg portion of resin containing co-immobilized TnT and iPP was transferred to 2 mL Eppendorf tubes, followed by a 75 µL portion of the 4x reaction stock, a 150 µL portion of the specified 2x buffer stock, and 75 µL of the serially diluted 4x sodium thiosulfate reaction stock. For reactions ran in the absence of sodium thiosulfate, 75 µL of RO water was added. The reaction tubes were sealed and heated to 40°C and shook at 600 rpm for 18 h.

[0376] After 120 minutes elapsed, a 5 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method C. The conversion of 5'-mC*mA*mGmAmAmAfG to 5'-mC*mA*mGmAmAmAfGmA-3'P was measured as the area of product vs the total area in the HPLC chromatogram.

Table 25.1. Extension of an RNA Oligomer with Co-Immobilized TnT and iPP on Covalent Resin and Reducing Additives in a Batch Reactor

Buffer	Concentration	Additive Concentration	% 5'-mC*mA*mGmAmAmAfGmA-3'P
MOPS	250 mM	250 mM	+++
TEoA-HCl	50 mM	250 mM	++
MOPS	250 mM	50 mM	+++
TEoA-HCl	50 mM	50 mM	++
MOPS	250 mM	10 mM	++
TEoA-HCl	50 mM	10 mM	+
MOPS	250 mM	2 mM	++
TEoA-HCl	50 mM	2 mM	+
MOPS	250 mM	0.4 mM	++
TEoA-HCl	50 mM	0.4 mM	+
MOPS	250 mM	0.0 mM	++

Table 25.1. Extension of an RNA Oligomer with Co-Immobilized TnT and iPP on Covalent Resin and Reducing Additives in a Batch Reactor			
TEoA-HCl	50 mM	0.0 mM	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: “+” 0-85%, "++" 85-95%, “+++” >95%.			

[0377] After an additional 18 h of reaction time had elapsed, a 5 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of all RNA oligomer species were measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 25.2. PS to PO Conversion During Extension of an RNA Oligomer with Co-Immobilized TnT and iPP on Covalent Resins and Reducing Additives in a Batch Reactor			
Buffer	**Concentration**	**Additive Concentration**	**% PO Impurity**
MOPS	250 mM	250 mM	+++
TEoA-HCl	50 mM	250 mM	+++
MOPS	250 mM	50 mM	+++
TEoA-HCl	50 mM	50 mM	+++
MOPS	250 mM	10 mM	+
TEoA-HCl	50 mM	10 mM	+++
MOPS	250 mM	2 mM	+
TEoA-HCl	50 mM	2 mM	++
MOPS	250 mM	0.4 mM	+
TEoA-HCl	50 mM	0.4 mM	+
MOPS	250 mM	0.0 mM	+
TEoA-HCl	50 mM	0.0 mM	+
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: “+” >15%, "++"10-15%, “+++” <10%.			

[0378] Upon completion of the extension step, the reaction solution was transferred from the resin into a fresh 2 mL Eppendorf tube. No additional purification steps were completed prior to the 3’P dephosphorylation step.

3’P Dephosphorylation: 5’-mC*mA*mGmAmAmAfGmA-3’P => 5’-mC*mA*mGmAmAmAfGmA:

[0379] A portion of 30 mg AP immobilized on quenched IB-COV-7 was transferred to the crude 5’-mC*mA*mGmAmAmAfGmA-3’P oligomer solution obtained during the extension step. The tube

was sealed and briefly centrifuged to ensure the resin was completely submerged in the reaction mixture. The tube was then incubated at 50 °C, 600 rpm.

[0380] After 30 minutes had elapsed, a 5 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the HPLC method described in Example 3, Method C. The conversion of 5'-mC*mA*mGmAmAmAfGmA-3'P to 5'-mC*mA*mGmAmAmAfGmA was measured as the area of product vs the total area in the HPLC chromatogram.

Table 25.3. 3'P Dephosphorylation of an RNA Oligomer with AP immobilized on Covalent Resin and Reducing Additives in a Batch Reactor

Buffer	Concentration	Additive Concentration	% 5'-mC*mA*mGmAmAmAfGmA
MOPS	250 mM	250 mM	+++
TEoA-HCl	50 mM	250 mM	++
MOPS	250 mM	50 mM	+++
TEoA-HCl	50 mM	50 mM	++
MOPS	250 mM	10 mM	++
TEoA-HCl	50 mM	10 mM	++
MOPS	250 mM	2 mM	++
TEoA-HCl	50 mM	2 mM	++
MOPS	250 mM	0.4 mM	+
TEoA-HCl	50 mM	0.4 mM	+++
MOPS	250 mM	0.0 mM	+
TEoA-HCl	50 mM	0.0 mM	++

Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0-85%, "++" 85-98%, "+++" >98%.

[0381] After an additional 30 minutes of reaction time had elapsed, a 5 µL aliquot of each reaction was diluted to 50 µM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of all RNA oligomer species were measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 25.4. PS to PO Conversion During 3'P Dephosphorylation of an RNA Oligomer with AP immobilized on Covalent Resin and Reducing Additives in a Batch Reactor

Buffer	Concentration	Additive Concentration	% PO Impurity
MOPS	250 mM	250 mM	+++
TEoA-HCl	50 mM	250 mM	+++
MOPS	250 mM	50 mM	+++
TEoA-HCl	50 mM	50 mM	+++

Table 25.4. PS to PO Conversion During 3'P Dephosphorylation of an RNA Oligomer with AP immobilized on Covalent Resin and Reducing Additives in a Batch Reactor			
MOPS	250 mM	10 mM	++
TEoA-HCl	50 mM	10 mM	++
MOPS	250 mM	2 mM	++
TEoA-HCl	50 mM	2 mM	+
MOPS	250 mM	0.4 mM	++
TEoA-HCl	50 mM	0.4 mM	+
MOPS	250 mM	0.0 mM	++
TEoA-HCl	50 mM	0.0 mM	+
% PO impurity calculated from LCMS as the difference between the %PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" >10%, "++" 7% to 10%, "+++" <7%.			

Example 26

Single Extension of an RNA Oligomer in a Flow Reactor with Co-immobilized TnT and iPP on Covalent Resins with Continuous Reservoir Sparging

[0382] TnT enzyme variant SEQ ID NO: 16 was produced in shake flask and purified as described in Example 1.

[0383] Inorganic pyrophosphatase (iPP) enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0384] TnT variant SEQ ID NO: 16 and iPP variant SEQ ID NO: 8 were co-immobilized on ChiralVision IB-COV-7 at 5.0 wt% and 0.2 wt% vs wet wight resin, respectively, according to the procedure outlined in Example 7.

[0385] Post-immobilization quench of co-immobilized TnT and iPP was completed as outlined in Example 16.

[0386] To a 50 mL falcon tube was added 16.3 mL RO water, 180 µL of a 100 mM $CoCl_2$ stock solution (1.0 mM), 900 µL of a 1 M TEoA-HCl stock solution (50 mM), 450 µL of a 20 mM stock solution of 5'-mU*fA*mUmAmUfUmU oligomer (500 µM), and 135 µL of a 100 mM stock solution fCQP (750 µM). This solution was split into two equal portions in 15 mL falcon tubes.

[0387] Two Biorad EconoAlpha columns were packed with 450 mg of quenched IB-COV-7 resin containing co-immobilized TnT and iPP at 5.0 wt% and 0.2 wt% vs wet weight resin, respectively. Each column bed was heated to 40°C with a resistive heating jacket and maintained at this temperature throughout the duration of the experiment. Each column was washed with 20 column

volumes of 50 mM TEoA-HCl, pH 7.8, containing 0.5 M NaCl, followed by an additional wash with 20 column volumes of 50 mM TEoA-HCl, pH 7.8.

[0388] A stainless steel sparge tube with 0.2 μm pores was inserted into one 15 mL Falcon tube containing an equal volume portion of the reaction mix and the reservoir was continuously sparged with N_2 gas at a flow rate of 0.001 slpm for the duration of the extension reaction. The second equal volume portion of the reaction mix was not sparged with N_2 gas.

[0389] Each reservoir was recirculated through the columns heated to 40°C via peristaltic pumps set to a flow rate of 0.11 mL/min for 18 h until the reaction reached completion by the HPLC method described in Example 3, method D.

Table 26.1. Single Extension of an RNA Oligomer with Co-Immobilized TnT and iPP in a Flow Reactor with Continuous Sparging

Reservoir Conditions	% 5'- mU*fA*mUmAmUfUmUfC-3'P
Continuous Sparging	++
No Sparging	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" 0% to 97%, "++" >97%.	

[0390] After the reaction had reached completion, an aliquot of each reaction was diluted to 50 μM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of all RNA oligomer species were measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 26.2. PS to PO Conversion During a Single Extension of an RNA Oligomer with Co-Immobilized TnT and iPP in a Flow Reactor with Continuous Sparging

Reservoir Conditions	% PO Impurity
Continuous Sparging	++
No Sparging	+
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" >5%, "++" 0% to 5%.	

Example 27

3'P Dephosphorylation of an RNA Oligomer in a Flow Reactor with Immobilized AP on Covalent Resin with Continuous Reservoir Sparging

[0391] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0392] Alkaline phosphatase enzyme variant SEQ ID NO: 2 was immobilized at 2.0 wt% vs wet weight resin on ChiralVision IB-COV-7 according to the general procedure outlined in Example 7.

[0393] After immobilization was complete, immobilized AP was quenched according to the procedure outlined in Example 9.

[0394] A reaction solution comprised of 500 μM 5'- mU*fA*mUmAmUfUmUfC-3'P, residual fCQP, and 1.0 mM $CoCl_2$ in 50 mM TEoA-HCl, pH 7.8, was continuously sparged with N_2 gas at a flow rate of 0.001 slpm with a stainless steel sparge tube containing 0.2 μm pores throughout the duration of the reaction. An identical reaction solution was not sparged with N_2 gas.

[0395] Two BioRad Econo Alpha columns were packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. %. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. Each column was washed with 20 column volumes of 50 mM TEoA-HCl, pH 7.8, containing 0.5 M NaCl, followed by an additional 20 column volumes of 50 mM TEoA-HCl, pH 7.8.

[0396] Each reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 3, Method D. Dephosphorylation of residual fCQP was measured by the HPLC method as described in Example 2.

Table 27.1. 3'P Dephosphorylation of an RNA Oligomer with Immobilized AP in a Flow Reactor with Continuous Sparging

Reservoir Conditions	% 5'- mU*fA*mUmAmUfUmUfC
Continuous Sparging	+
No Sparging	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" > 98%.	

[0397] After the reaction had reached completion, an aliquot of each reaction was diluted to 50 μM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of all RNA oligomer species were measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 27.2. PS to PO Conversion During 3'P Dephosphorylation of an RNA Oligomer with Immobilized AP in a Flow Reactor with Continuous Sparging

Reservoir Conditions	% PO Impurity
Continuous Sparging	++
No Sparging	+

Table 27.2. PS to PO Conversion During 3'P Dephosphorylation of an RNA Oligomer with Immobilized AP in a Flow Reactor with Continuous Sparging
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" >5%, "++" 0% to 5%.

Example 28

Iterative Extension and 3'P Dephosphorylation in a Flow Reactor with Reducing Additives (Antioxidant or Reducing Agent) and Continuous Reservoir Sparging

[0398] TnT enzyme variant SEQ ID NO: 16 was produced in shake flask and purified as described in Example 1.

[0399] Inorganic pyrophosphatase (iPP) enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0400] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0401] TnT variant SEQ ID NO: 16 and iPP variant SEQ ID NO: 8 were co-immobilized on ChiralVision IB-COV-7 at 5.0 wt% and 0.2 wt% vs wet wight resin, respectively, according to the procedure outlined in Example 7.

[0402] Alkaline phosphatase enzyme variant SEQ ID NO: 2 was immobilized at 2.0 wt% vs wet weight resin on ChiralVision IB-COV-7 according to the general procedure outlined in Example 7.

[0403] Post-immobilization quenching of co-immobilized TnT and iPP was completed as outlined in Example 16.

[0404] Post-immobilization quenching of immobilized AP was completed as outlined in Example 9.

Extension Reaction: 5'-mC*mA*mGmAmAmAfG + mAQP => 5'-mC*mA*mGmAmAmAfGmA-3'P

[0405] To a 50 mL falcon tube was added 9.9 mL RO water, 180 µL of a 100 mM $CoCl_2$ stock solution (1.0 mM), 4.5 mL of a 1 M MOPS stock solution (250 mM, pH 8.0), 900 µL of a 1 M stock solution of sodium thiosulfate (50 mM), 1.8 mL of a 20 mM stock solution of 5'-mC*mA*mGmAmAmAfG oligomer (2.0 mM), and 720 µL of a 100 mM stock solution mAQP (3.0 mM). This solution was split into two equal portions in 15 mL falcon tubes.

[0406] Two Biorad EconoAlpha columns were packed with 450 mg of quenched IB-COV-7 resin containing co-immobilized TnT and iPP at 5.0 wt% and 0.2 wt% vs wet weight resin, respectively. Each column bed was heated to 40°C with a resistive heating jacket and maintained at this

temperature throughout the duration of the experiment. Each column was washed with 20 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional wash with 20 column volumes of 250 mM MOPS, pH 8.0.

[0407] A stainless steel sparge tube with 0.2 μm pores was inserted into one 15 mL Falcon tube containing an equal volume portion of the reaction mix and the reservoir was continuously sparged with N_2 gas at a flow rate of 0.001 slpm for the duration of the extension reaction. The second equal volume portion of the reaction mix was not sparged with N_2 gas.

[0408] Each reservoir was recirculated through the columns heated to 40°C via peristaltic pumps set to a flow rate of 0.11 mL/min for 18 h until the reaction reached completion by the HPLC method described in Example 3, method D.

[0409] Residual $CoCl_2$ concentration was determined using the procedure described in Example 6 and adjusted to 1.0 mM $CoCl_2$ prior to the 3'P dephosphorylation step. No additional modifications were made to the reservoir.

3'P Dephosphorylation: 5'-mC*mA*mGmAmAmAfGmA-3'P => 5'-mC*mA*mGmAmAmAfGmA

[0410] A reaction solution comprised of 2.0 mM 5'-mC*mA*mGmAmAmAfGmA-3'P, residual mAQP, 50 mM sodium thiosulfate, and 1.0 mM $CoCl_2$ in 250 mM MOPS, pH 8.0, was continuously sparged with N_2 gas at a flow rate of 0.001 slpm with a stainless steel sparge tube containing 0.2 μm pores throughout the duration of the reaction. An identical reaction solution was not sparged with N_2 gas.

[0411] Two BioRad Econo Alpha columns were packed with AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. %. The column bed was maintained at 50 °C via restive heating jacket throughout the duration of the experiment. Each column was washed with 20 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional 20 column volumes of 250 mM MOPS, pH 8.0.

[0412] Each reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.06 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel. The level of 3'P-dephosphorylation was measured via HPLC as described in Example 3, Method D. Dephosphorylation of residual mAQP was measured by the HPLC method as described in Example 2.

Table 28.1. Iterative Extension and 3'P Dephosphorylation of an RNA Oligomer with n a Flow Reactor with Continuous Sparging and Reducing Additives

Reservoir Conditions	% 5'-mC*mA*mGmAmAmAfGmA
Continuous Sparging	+
No Sparging	+

Table 28.1. Iterative Extension and 3'P Dephosphorylation of an RNA Oligomer with n a Flow Reactor with Continuous Sparging and Reducing Additives
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" > 97%.

[0413] After the reaction had reached completion, an aliquot of each reaction was diluted to 50 μM RNA oligomer concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method A. The total PS to PO conversion of all RNA oligomer species were measured as the area of PS to PO product vs the total area in the HPLC chromatogram.

Table 28.1. PS to PO Conversion in the Iterative Extension and 3'P Dephosphorylation of an RNA Oligomer in a Flow Reactor with Continuous Sparging and Reducing Additives	
Reservoir Conditions	**% PO Impurity**
Continuous Sparging	++
No Sparging	+
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all oligonucleotide at the end of the reaction and the % PO contained in all oligonucleotide at the beginning of the reaction and is defined as follows: "+" >2%, "++" 0% to 2%.	

Example 29

Suppressing PO Impurities of αS-NQPs in a Flow Reactor with Reducing Additives (Antioxidant or Reducing Agent) and Continuous Reservoir Sparging

[0414] TnT enzyme variant SEQ ID NO: 14 was produced in shake flask and purified as described in Example 1.

[0415] Inorganic pyrophosphatase (iPP) enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0416] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0417] TnT variant SEQ ID NO: 16 and iPP variant SEQ ID NO: 8 were co-immobilized on ChiralVision IB-COV-7 at 5.0 wt% and 0.2 wt% vs wet wight resin, respectively, according to the procedure outlined in Example 7.

[0418] Alkaline phosphatase enzyme variant SEQ ID NO: 2 was immobilized at 2.0 wt% vs wet weight resin on ChiralVision IB-COV-7 according to the general procedure outlined in Example 7.

[0419] Post-immobilization quenching of co-immobilized TnT and iPP was completed as outlined in Example 16.

[0420] Post-immobilization quenching of immobilized AP was completed as outlined in Example 9.

[0421] To a 50 mL falcon tube was added 11.7 mL RO water, 180 µL of a 100 mM $CoCl_2$ stock solution (1.0 mM), 4.5 mL of a 1 M MOPS stock solution (250 mM, pH 8.0), 900 µL of a 1 M stock solution of sodium thiosulfate (50 mM), and 720 µL of a 100 mM stock solution αS-mAQP (1.5 mM). This solution was split into two equal portions in 15 mL falcon tubes.

[0422] Two Biorad EconoAlpha columns were packed with 450 mg of quenched IB-COV-7 resin containing co-immobilized TnT and iPP at 5.0 wt% and 0.2 wt% vs wet weight resin, respectively. Each column bed was heated to 40°C with a resistive heating jacket and maintained at this temperature throughout the duration of the experiment. Each column was washed with 20 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional wash with 20 column volumes of 250 mM MOPS, pH 8.0.

[0423] A stainless steel sparge tube with 0.2 µm pores was inserted into one 15 mL Falcon tube containing an equal volume portion of the reaction mix and the reservoir was continuously sparged with N_2 gas at a flow rate of 0.001 slpm for the duration of the extension reaction. The second equal volume portion of the reaction mix was not sparged with N_2 gas.

[0424] Each reservoir was recirculated through the columns heated to 40°C via peristaltic pumps set to a flow rate of 0.11 mL/min for 23 h.

[0425] Residual $CoCl_2$ concentration was determined using the procedure described in Example 6.

[0426] After the reaction had reached completion, an aliquot of each reaction was diluted to 100 µM αS-NQP concentration in a 1 mM EDTA solution and analyzed according to the UV-LCMS method described in Example 4, Method B. The total PS to PO conversion of all nucleotide species were measured as the area of PS to PO product vs the total area in the UV-LCMS chromatogram.

Table 29.1. % PO Impurity in αS-NQP After Continuous Recirculation Through a Flow Reactor

Conditions	Time (min)	% PO Impurity
Continuous N_2 Sparging	23 h	++
No Sparging	23 h	+

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all nucleotide species at the end of the reaction and the % PO contained in all nucleotide species at the beginning of the reaction and is defined as follows: “+” >0.3%, "++" 0% to 0.3%.

Example 30

Iterative Extension and 3’P Dephosphorylation of αS-NQPs in a Flow Reactor with Reducing Additives (Antioxidant or Reducing Agent) and Continuous Reservoir Sparging

[0427] TnT enzyme variant SEQ ID NO: 16 was produced in shake flask and purified as described in Example 1.

[0428] Inorganic pyrophosphatase (iPP) enzyme variant SEQ ID NO: 8 was produced in shake flask and purified as described in Example 1.

[0429] Alkaline phosphatase (AP) enzyme variant SEQ ID NO: 2 was produced in shake flask and purified as described in Example 1.

[0430] TnT variant SEQ ID NO: 16 and iPP variant SEQ ID NO: 8 were co-immobilized on ChiralVision IB-COV-7 at 5.0 wt% and 0.2 wt% vs wet wight resin, respectively, according to the procedure outlined in Example 7.

[0431] Alkaline phosphatase enzyme variant SEQ ID NO: 2 was immobilized at 2.0 wt% vs wet weight resin on ChiralVision IB-COV-7 according to the general procedure outlined in Example 7.

[0432] Post-immobilization quenching of co-immobilized TnT and iPP was completed as outlined in Example 16.

[0433] Post-immobilization quenching of immobilized AP was completed as outlined in Example 9.

Extension Reaction: 5'-fGmUfCmUmAmG +αS-mAQP => 5'-fGmUfCmUmAmG*mA-3'P

[0434] To a 250 mL conical flask containing a 40 mL aqueous solution comprised of 250 mM MOPS buffer, pH 8.0, 620 μM 5'-fGmUfCmUmAmG oligomer, 1.0 mM $CoCl_2$, and 50 mM sodium thiosulfate was added 372 μL of a 100 mM stock solution of αS-mAQP (930 μM).

[0435] A Cytiva XK 16/20 column was packed with 2.0 g of quenched IB-COV-7 resin containing co-immobilized TnT and iPP at 5.0 wt% and 0.2 wt% vs wet weight resin, respectively. The resin bed was heated to 40°C with a recirculating water heater and maintained at this temperature throughout the duration of the experiment. The resin bed was washed with 40 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional wash with 20 column volumes of 250 mM MOPS, pH 8.0.

[0436] A stainless steel sparge tube with 0.2 μm pores was inserted into the reservoir containing the reaction mix and the reservoir was continuously sparged with N_2 gas at a flow rate of 0.001 slpm for the duration of the extension reaction.

[0437] The reservoir was recirculated through the columns heated to 40°C via peristaltic pumps set to a flow rate of 1.50 mL/min for 3.4 h until the reaction reached completion by the HPLC method described in Example 3, method D.

[0438] Upon completion of the extension reaction, the resin bed was washed with 250 mM MOPS buffer, pH 8.0, until the UV trace reached baseline to recover 5'-fGmUfCmUmAmG*mA-3'P oligomer. The wash fractions were combined with the initial reservoir and the resulting 55 mL

aqueous solution was carried forward into the 3'P dephosphorylation step without any additional purification.

Extension Reaction: 5'-fGmUfCmUmAmG*mA +αS-mAQP => 5'-fGmUfCmUmAmG*mA*mA-3'P

[0439] To a 250 mL conical flask containing a 56 mL aqueous solution comprised of 250 mM MOPS buffer, pH 8.0, 440 μM 5'-fGmUfCmUmAmG*mA oligomer, 1.0 mM $CoCl_2$, and 50 mM sodium thiosulfate was added 370 μL of a 100 mM stock solution of αS-mAQP (660 μM).

[0440] A Cytiva XK 16/20 column was packed with 2.0 g of quenched IB-COV-7 resin containing co-immobilized TnT and iPP at 5.0 wt% and 0.2 wt% vs wet weight resin, respectively. The resin bed was heated to 40°C with a recirculating water heater and maintained at this temperature throughout the duration of the experiment. The resin bed was washed with 40 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional wash with 20 column volumes of 250 mM MOPS, pH 8.0.

[0441] A stainless steel sparge tube with 0.2 μm pores was inserted into the reservoir containing the reaction mix and the reservoir was continuously sparged with N_2 gas at a flow rate of 0.001 slpm for the duration of the extension reaction.

[0442] The reservoir was recirculated through the columns heated to 40°C via peristaltic pumps set to a flow rate of 1.50 mL/min for 23 h until the reaction reached completion by the HPLC method described in Example 3, method D.

[0443] Upon completion of the extension reaction, the resin bed was washed with 250 mM MOPS buffer, pH 8.0, until the UV trace reached baseline to recover 5'-fGmUfCmUmAmG*mA-3'P oligomer. The wash fractions were combined with the initial reservoir and the resulting 105 mL aqueous solution was concentrated via a 1 KDa MWCO spin filter prior to the 3'P dephosphorylation step.

Table 30.1. % Conversion in the Iterative Extension of αS-NQPs in a Flow Reactor with Reducing Additives and Continuous Reservoir Sparging

Extended Product Oligomer Sequence	% Extended Product
5'-fGmUfCmUmAmG*mA-3'P	+
5'-fGmUfCmUmAmG*mA*mA-3'P	+
Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" > 99%.	

[0444] An aliquot of each 3'P dephosphorylated product was prepared as a 50 μM solution in 1 mM EDTA and the % PO impurity determined via the UV-LCMS method described in Example 4, Method B.

Table 30.2. % PO Impurity in the Iterative Extension of αS-NQPs in a Flow Reactor with Reducing Additives and Continuous Reservoir Sparging	
3'P Dephosphorylated Oligomer Sequence	**% PO Impurity**
5'-fGmUfCmUmAmG*mA	++
5'-fGmUfCmUmAmG*mA*mA	+
% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all nucleotide species at the end of the reaction and the % PO contained in all nucleotide species at the beginning of the reaction and is defined as follows: "+" 1.0% to 1.5%, "++" 0% to 1.0%.	

3'P Dephosphorylation: 5'-fGmUfCmUmAmG*mA-3'P => 5'-fGmUfCmUmAmG*mA

[0445] A 55 mL aqueous reaction solution comprised of 451 μM 5'-fGmUfCmUmAmG*mA-3'P oligomer, residual αS-mAQP, 50 mM sodium thiosulfate, and 1.0 mM $CoCl_2$ in 250 mM MOPS, pH 8.0, was continuously sparged with N_2 gas at a flow rate of 0.001 slpm with a stainless steel sparge tube containing 0.2 μm pores throughout the duration of the reaction.

[0446] A Cytiva XK 16/20 column was packed with 2.0 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. %. The resin bed was maintained at 30 °C via restive heating jacket throughout the duration of the experiment. The resin bed was washed with 40 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional 20 column volumes of 250 mM MOPS, pH 8.0.

[0447] The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.15 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel.

[0448] The level of 3'P-dephosphorylation was measured via HPLC as described in Example 3, Method D. Dephosphorylation of residual αS-mAQP was measured by the HPLC method as described in Example 2.

[0449] The level of residual AP in the reservoir was measured according to the assay protocol described in Example 5.

[0450] The $CoCl_2$ concentration was measured according to the procedure in Example 6 and adjusted to 1.0 mM $CoCl_2$ concentration prior to the next extension step.

3'P Dephosphorylation: 5'-fGmUfCmUmAmG*mA*mA-3'P => 5'-fGmUfCmUmAmG*mA*mA

[0451] A 21 mL aqueous reaction solution comprised of 1.17 mM 5'-fGmUfCmUmAmG*mA*mA-3'P oligomer, residual αS-mAQP, 50 mM sodium thiosulfate, and 1.0 mM $CoCl_2$ in 250 mM MOPS, pH 8.0, was continuously sparged with N_2 gas at a flow rate of 0.001 slpm with a stainless steel sparge tube containing 0.2 μm pores throughout the duration of the reaction.

[0452] A Cytiva XK 16/20 column was packed with 2.0 g AP immobilized on ChiralVision IB-COV-7 at 2.0 wt. %. The resin bed was maintained at 30 °C via restive heating jacket throughout the duration of the experiment. The resin bed was washed with 40 column volumes of 250 mM MOPS, pH 8.0, containing 0.5 M NaCl, followed by an additional 20 column volumes of 250 mM MOPS, pH 8.0.

[0453] The reaction solution was pumped through the packed resin bed using a peristaltic pump with a flow rate of 0.15 mL/min until the entire reaction solution had been pumped through the resin bed and collected in a separate vessel.

[0454] The level of 3'P-dephosphorylation was measured via HPLC as described in Example 3, Method D. Dephosphorylation of residual αS-mAQP was measured by the HPLC method as described in Example 2.

Table 30.3. % Conversion in the Iterative 3'P Dephosphorylation of αS-NQPs in a Flow Reactor with Reducing Additives and Continuous Reservoir Sparging

3'P Dephosphorylated Oligomer Sequence	% 3'P Dephosphorylated Product
5'-fGmUfCmUmAmG*mA	+
5'-fGmUfCmUmAmG*mA*mA	+

Conversion of oligonucleotide was determined as percent of total area under curve in the HPLC chromatogram and is defined as follows: "+" > 99%.

[0455] An aliquot of each 3'P dephosphorylated product was prepared as a 50 µM solution in 1 mM EDTA and the % PO impurity determined via the UV-LCMS method described in Example 4, Method A.

Table 30.4. % PO Impurity in the Iterative 3'P Dephosphorylation of αS-NQPs in a Flow Reactor with Reducing Additives and Continuous Reservoir Sparging

3'P Dephosphorylated Oligomer Sequence	% PO Impurity
5'-fGmUfCmUmAmG*mA	++
5'-fGmUfCmUmAmG*mA*mA	+

% PO impurity calculated from LCMS as the difference between the % PO impurity contained in all nucleotide species at the end of the reaction and the % PO contained in all nucleotide species at the beginning of the reaction and is defined as follows: "+" 1.0% to 1.5%, "++" 0% to 1.0%.

CLAIMS

What is claimed is:

1. A method of reducing conversion of phosphorothioate bonds comprising combining an oligonucleotide comprising one or more phosphorothioate bonds with one or more antioxidants or reducing agents, such that conversion of the one or more phosphorothioate bonds is reduced.

2. A method of reducing conversion of phosphorothioate bonds comprising combining an oligonucleotide comprising one or more phosphorothioate bonds with a buffer, such that conversion of the one or more phosphorothioate bonds is reduced.

3. A method of reducing conversion of thio-NTPs comprising combining a thio-NTP with one or more antioxidants or reducing agents, such that conversin of one or more sulfur atoms to one or more oxygen atoms in the thio-NTP is reduced.

4. A method of reducing conversion of thio-NTPs comprising combining a thio-NTP with a buffer, such that conversion of one or more sulfur atoms to one or more oxygen atoms in the thio-NTP is reduced.

5. A method of reducing conversion of an oligonucleotide comprising contacting a solid support with a first quenching agent and combining an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

6. A method of reducing conversion of an oligonucleotide or a thio-NTP comprising i) contacting a solid support with a first quenching agent and ii) contacting the solid support with a second quenching agent, such that conversion of the oligonucleotide is reduced.

7. The method of Claim 6, comprising the step of immobilizing at least one enzyme on the solid support prior to step i) or step ii).

8. A method of reducing conversion of an oligonucleotide comprising i) immobilizing an oligonucleotide on a solid support, ii) contacting the solid support with a first quenching agent, and iii) combining at least one enzyme with the solid support, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

9. A method of reducing conversion of an oligonucleotide comprising i) immobilizing one or more enzymes on a solid support, ii) contacting the solid support with a first quenching agent, and iii) combining an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

10. A method of reducing conversion of an oligonucleotide comprising i) immobilizing an oligonucleotide on a solid support and ii) combining at least one enzyme with the solid support in the presence of one or more antioxidants or reducing agents, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

11. A method of reducing conversion of an oligonucleotide comprising i) immobilizing one or more enzymes on a solid support and ii) combining an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of one or more antioxidants or reducing agents, such that conversion of bonds in the oligonucleotide is reduced.

12. A method of reducing conversion of an oligonucleotide comprising i) immobilizing an oligonucleotide on a solid support and ii) combining at least one enzyme with the solid support in the presence of a buffer, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

13. A method of reducing conversion of an oligonucleotide comprising i) immobilizing one or more enzymes on a solid support and ii) combining an oligonucleotide comprising one or more phosphorothioate bonds with the solid support in the presence of a buffer, such that oxidation of phosphorothioate bonds in the oligonucleotide is reduced.

14. A method of reducing conversion of an oligonucleotide comprising i) immobilizing an oligonucleotide on a solid support, ii) combining a solution comprising at least one enzyme with the solid support, and iii) degassing the solution comprising at least one enzyme, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

15. A method of reducing conversion of an oligonucleotide comprising i) immobilizing one or more enzymes on a solid support, ii) combining a solution comprising an oligonucleotide comprising one or more phosphorothioate bonds with the solid support, and iii) degassing the solution comprising an oligonucleotide comprising one or more phosphorothioate bonds, such that conversion of phosphorothioate bonds in the oligonucleotide is reduced.

16. A method of reducing conversion of a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds comprising one or more of the following:

i) selecting a solid support for immobilization of one or more enzymes or an oligonucleotide;

ii) contacting a first quenching agent and, optionally, a second quenching agent with a solid support to reduce or neutralize unreacted reactive groups on the solid support;

iii) combining or contacting a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds with an antioxidant or reducing agent;

iv) combining or contacting a thio-NTP or an oligonucleotide comprising one or more

phosphorothioate bonds with a buffer, additive, or another reaction condition; and

v) optionally degassing or sparging the solution or mixture comprising a thio-NTP or an oligonucleotide comprising one or more phosphorothioate bonds;

such that a) conversion of the thio-NTP or the oligonucleotide comprising one or more phosphorothioate bonds is reduced; or b) the loss of activity is reduced, or the maintenance of activity is increased for one or more enzymes; or c) both a) and b) are true.

17. The method of any one of Claims 1-13, additionally comprising a step of degassing a solution

18. The method of Claim 17, wherein the degassing occurs prior to combining the solution with the solid support.

19. The method of Claim 17, wherein the degassing occurs after combining the solution with the solid support.

20. The method of Claim 17, wherein the degassing occurs continuously during one or more steps of the method.

21. The method of any one of Claims 14, 15, and 17-20, wherein the degassing occurs in an in-line system.

22. The method of any one of Claims 14, 15, and 17-21, wherein the degassing uses a vacuum pump.

23. The method of any one of Claims 14, 15, and 17-21, wherein the degassing comprises sparging with an inert gas.

24. The method of Claim 23, wherein the inert gas comprises helium, neon, argon, krypton, xenon, radon, nitrogen, or carbon dioxide.

25. The method of any one of Claims 11, 13, and 15, additionally comprising a step of contacting the solid support with a first quenching agent prior to the step of combining at least one enzyme with the solid support.

26. The method of any one of Claims 10, 12, and 14, additionally comprising a step of contacting the solid support with a first quenching agent prior to the step of combining an oligonucleotide comprising one or more phosphorothioate bonds with the solid support.

27. The method of any one of Claims 5-9, 16, and 17-26, wherein the first quenching agent comprises L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole,

glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl.

28. The method of any one of Claims 5-9, 16, and 17-27, wherein the first quenching agent comprises a concentration of 1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 62.5 mM, 100 mM, 125 mM, 200 mM, 250 mM, 500 mM, 1 M, 2 M, 3M, 4 M, or 5 M.

29. The method of any one of Claims 5-9, 16, and 17-27, additionally comprising a step of contacting the solid support with a second quenching agent prior to the step of combining at least one enzyme or an oligonucleotide with the solid support.

30. The method of any one of Claims 5-9, 16, and 17-29, wherein the second quenching agent comprises L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl.

31. The method of any one of Claims 5-9, 16, and 17-30, wherein the second quenching agent comprises a concentration of 1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 62.5 mM, 100 mM, 125 mM, 200 mM, 250 mM, 500 mM, 800 mM, 1 M, 3M, or 5 M.

32. The method of any one of Claims 5-9, 16, and 17-31, wherein the contacting the solid support with a first or second quenching agent occurs at a temperature of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, or 50 °C.

33. The method of any one of Claims 5-9, 16, and 17-32, wherein the contacting the solid support with a first or second quenching agent occurs at a pH of 6.8, 7, 8, 9 or 10.

34. The method of any one of Claims 5 and 7-33, wherein the step of combining or contacting an oligonucleotide or at least one enzyme with the solid support occurs in the presence of one or more antioxidants or reducing agents.

35. The method of any one of Claims 1, 3, 10, 11, and 16-34, wherein the one or more antioxidants or reducing agents are selected from ascorbic acid, citric acid, formic acid, sodium thiosulfate, sodium metabisulfite, 2,6-dimethoxyphenol, catalase, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, and sodium thiophosphate.

36. The method of any one of Claims 1, 3, 10, 11, and 16-35, wherein the one or more antioxidants or reducing agents are at a concentration of 50 μM, 80 μM, 100 μM, 400 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 3M, or 5 M.

37. The method of any one of Claims 5 and 7-36, wherein the step of combining or contacting an oligonucleotide or at least one enzyme with the solid support occurs in the presence of a buffer.

38. The method of any one of Claims 2, 4, 5, and 7-37, wherein the buffer is selected from MOPS, TRIS, acetate, sodium acetate, sodium phosphate, triethanolamine (TEoA), and triethanolamine HCl (TEoAHCl).

39. The method of any one of Claims 2, 4, 5, and 7-38, wherein the buffer is at a concentration of 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 2M, 3M, or 5 M.

40. The method of any one of Claims 2, 4, 5, and 7-39, wherein the buffer is at a pH of 5, 5.5, 6, 6.5, 6.8, 7, 7.5, 7.9 7.8, 8, 8.5, 9, or 9.5.

41. The method of any one of Claims 2, 4, 5, and 7-40, wherein the buffer is at a temperature of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, or 60 °C .

42. The method of any one of Claims 5-41, wherein the solid support comprises polymethacrylate styrene/DVB copolymer, controlled pore glass, polyacrylic, or silica.

43. The method of Claim 42, wherein the solid support further comprises a particle size of about 80 μM, about 100 μM, about 150 μM, about 200 μM, about 250 μM, about 300 μM, about 350 μM, about 400 μM, about 450 μM, about 500 μM, about 550 μM, about 600 uM, about 650 uM, about 750 uM, about 850 uM, about 950 uM, or about 1000 uM.

44. The method of Claim 42 or 43, wherein the solid support further comprises pores.

45. The method of Claim 44, wherein the pores comprise a size of about 200 angstroms, about 300 angstroms, about 400 angstroms, about 500 angstroms, about 600 angstroms, about 700 angstroms, about 800 angstroms, about 900 angstroms, about 1000 angstroms, about 1100 angstroms, about 1200 angstroms, about 1300 angstroms, about 1400 angstroms, about 1500 angstroms, about 1600 angstroms, about 1750 angstroms, or about 2000 angstroms.

46. The method of any one of Claims 42-45, wherein the solid support further comprises a reactive group selected from epoxides, epoxys, octadecyls, aminoepoxides, amines, or glutaraldehyde.

47. The method of Claim 46, wherein the reactive group is attached to the solid support by a linker.

48. The method of any one of Claims 7-47, wherein the enzyme (or enzymes) comprises a terminal deoxynucleotidyl transferase, a polymerase, a phosphatase, an inorganic pyrophosphatase, an alkaline phosphatase, or a kinase.

49. The method of Claim 48, further comprising a step of addition of a natural or modified NTP with a 3' phosphate blocking group to the oligonucleotide.

50. The method of Claim 49, further comprising a step of deblocking the oligonucleotide by removal of the 3' phosphate from the terminal nucleotide or modified nucleotide in the oligonucleotide.

51. The method of Claim 48, further comprising iterative steps of extension of the oligonucleotide by addition of one natural or modified NTP with a 3' phosphate blocking group and deblocking of the oligonucleotide by removal of the 3' phosphate from the terminal nucleotide or modified nucleotide.

52. The method of any one of Claims 49-51, wherein the natural or modified NTP comprises a thio-phosphate or dio-thiophosphate.

53. The method of Claim 52, wherein the conversion of the natural or modified NTP comprising a thio-phosphate or di-thiophosphate is reduced at one or both of the sulfur positions.

54. The method of any one of Claims 1, 2, and 5-53, wherein the conversion of phosphorothioate internucleoside linkages to phosphodiester internucleoside linkages in the oligonucleotide is reduced by 0.5-fold, 0.6-fold, 0.7-fold, 0.8-fold, 0.9-fold, 1-fold, 1.1-fold, 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2.0-fold, 2.25-fold, 2.5 -fold, 3-fold, 5-fold, or more as compared to a reference method.

55. The method of any one of Claims 1, 2, and 5-54, wherein the conversion of phosphorothioate internucleoside linkages to phosphodiester internucleoside linkages in the oligonucleotide is reduced to 12%, 10%, 11%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less the total phosphorothioate linkages.

56. The method of any one of Claims 1, 2, and 5-55, wherein the oligonucleotide is DNA or RNA.

57. The method of any one of Claims 1, 2, and 5-56, wherein the oligonucleotide comprises 2-4, 3-7, 6-11, 10-20, 15-30, 25-125, 100-200 nucleotides or modified nucleotides.

58. A composition comprising an oligonucleotide and an antioxidant or reducing agent.

59. A composition comprising an oligonucleotide and a buffer.

60. A composition comprising an oligonucleotide and a solid support.

61. A composition comprising a thio-NTP and an antioxidant or reducing agent.

62. A composition comprising a thio-NTP and a buffer.

63. A composition comprising a thio-NTP and a solid support.

64. The composition of any one of Claims 58, 60, 62, and 63, further comprising an antioxidant or reducing agent.

65. The composition of any one of Claims 58, 60, 62, and 63, further comprising a buffer.

66. The composition of any one of Claims 58-60, further comprising a thio-NTP.

67. The composition of any one of Claims 58-66, further comprising at least one enzyme.

68. The composition of Claim 67, wherein the at least one enzyme comprises one or more of the following, a terminal deoxynucleotidyl transferase, a polymerase, a phosphatase, an inorganic pyrophosphatase, an alkaline phosphatase, or a kinase.

69. The composition of Claim 68, wherein the one or more enzymes is immobilized on the solid support.

70. The composition of Claim 59 or 60, wherein the oligonucleotide is immobilized on the solid support.

71. The composition of any one of Claims 60, 63, 69, and 70, wherein the solid support comprises polymethacrylate styrene/DVB copolymer, controlled pore glass, polyacrylic, or silica.

72. The composition of Claim 71, wherein the solid support further comprises a particle size of 80 μM, about 100 μM, about 150 μM, about 200 μM, about 250 μM, about 300 μM, about 350

μM, about 400 μM, about 450 μM, about 500 μM, about 550 μM, about 600 uM, about 650 uM, about 750 uM, about 850 uM, about 950 uM, or about 1000 uM.

73. The composition of Claim 71 or 72, wherein the solid support further comprises pores.

74. The composition of Claim 73, wherein the pores comprise a size of about 200 angstroms, about 300 angstroms, about 400 angstroms, about 500 angstroms, about 600 angstroms, about 700 angstroms, about 800 angstroms, about 900 angstroms, about 1000 angstroms, about 1100 angstroms, about 1200 angstroms, about 1300 angstroms, about 1400 angstroms, about 1500 angstroms, about 1600 angstroms, about 1750 angstroms, or about 2000 angstroms.

75. The composition of any one of Claims 69-74, wherein the solid support further comprises a reactive group selected from epoxides, epoxys, octadecyls, aminoepoxides, amines, or glutaraldehyde.

76. The composition of Claim 75, wherein the reactive group is attached to the solid support by a linker.

77. The composition of any one of Claims 60, 63, and 69-74, further comprising a first quenching agent.

78. The composition of Claim 77, wherein the first quenching agent comprises L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl.

79. The composition of Claim 77, wherein the first quenching agent comprises a concentration of 1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 62.5 mM, 100 mM, 125 mM, 200 mM, 250 mM, 500 mM, 1 M, 3M, or 5 M.

80. The composition of Claim 78 or 79, wherein the temperature is selected from 25C, 30C, 35C, 40C, 45C, or 50C.

81. The composition of any one of Claims 77-80, additionally comprising a pH selected from 6.8, 7, 8, 9 or 10.

82. The composition of any one of Claims 77-81, further comprising a second quenching agent.

83. The composition of Claim 82, wherein the second quenching agent comprises L-cysteine, L-lysine, ethanolamine, L-proline, L-alanine, L-glycine, imidazole, glucosamine, sodium thiosulfate, L-glycine benzyl ester, L-glycine methyl ester, L-glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl.

84. The composition of any one of Claims 77-83, wherein the second quenching agent comprises a concentration of1 μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 50 mM, 62.5 mM, 100 mM, 125 mM, 200 mM, 250 mM, 500 mM, 1 M, 3M, or 5 M.

85. The composition of any one of Claims 77-84, wherein the temperature is selected from 25C, 30C, 35C, 40C, 45C, or 50C.

86. The composition of any one of Claims 77-85, additionally comprising a pH selected from 6.8, 7, 8, 9 or 10.

87. The composition of any one of Claims 58, 61, 64, and 67-76, wherein the antioxidant or reducing agent comprises ascorbic acid, citric acid, formic acid, sodium thiosulfate, sodium metabisulfite, 2,6-dimethoxyphenol, catalase, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine-HCl, sodium iodide, diethyl dithiophosphate, diethyl thiophosphate potassium salt, and sodium thiophosphate.

88. The composition of Claim 87, wherein the concentration of the antioxidant or reducing agent comprises 50 μM, 80 μM, 100 μM, 400 μM, 500 μM, 1 mM, 2 mM, 5 mM, 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 3M, or 5 M .

89. The composition of Claim 59, 62, 65, and 67-76, wherein the buffer is selected from MOPS, TRIS, acetate, sodium acetate, sodium phosphate, triethanolamine (TEoA), and triethanolamine HCl (TEoAHCl).

90. The composition of Claim 89, wherein the buffer comprises a concentration of 10 mM, 30 mM, 50 mM, 100 mM, 250 mM, 500 mM, 800 mM, 1 M, 2M, 3M, or 5 M .

91. The composition of Claim 89 or 90, wherein the pH of the buffer is selected from 5, 5.5, 6, 6.5, 6.8, 7, 7.5, 7.9 7.8, 8, 8.5, 9, and 9.5.

92. The composition of any one of Claims 89-91, wherein the temperature of the buffer is selected from 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C .

93. The composition of one of Claims 89-92, wherein the buffer is degassed.

94. The composition of any one of Claims 58-93, further comprising a natural or modified NTP with a 3' phosphate blocking group.

95. The composition of Claim 94, wherein the natural or modified NTP comprises a thio-phosphate or dio-thiophosphate.