ALKALINE PHOSPHATASE IMMOBILIZATION

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. 63/718,923, filed November 11, 2024, all of which are incorporated by reference herein in their entireties.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The Sequence Listing concurrently submitted herewith as file name CX10-277WO1_ST26.xml, created on April 15, 2025, with a file size of 64,912 bytes, is part of the specification and is hereby incorporated by reference herein.

TECHNICAL FIELD

[0003] The present disclosure provides immobilized alkaline phosphatases, immobilized recombinant alkaline phosphatases and methods of making the immobilized alkaline phosphatases. The disclosure further provides methods of using the immobilized alkaline phosphatases or compositions thereof for cleaving substrates with phosphate monoester groups and analogs thereof.

BACKGROUND OF THE INVENTION

[0004] Alkaline phosphatases are widely distributed enzymes found in both prokaryotes and eukaryotes that catalyze the hydrolysis of phosphate monoesters, with an optimal activity at alkaline pH. In mammals, alkaline phosphatases are present in the intestine (i.e., intestinal alkaline phosphatase) and placenta (i.e., placental alkaline phosphatase). Phosphatases are widely used in molecular biological applications, for example for the removal of 5'-phosphate from polynucleotides or oligonucleotides for subsequent labeling with labeled ATP, reducing or preventing ligation of polynucleotides or oligonucleotides, and reducing susceptibility of polynucleotides or oligonucleotides to certain nucleases, e.g., l exonuclease.

[0005] Alkaline phosphatases are also found in many different types of bacteria, and some are sold commercially for molecular biological applications. Three classes of prokaryotic alkaline phosphatases are known and include PhoA, PhoD, and PhoX. These phosphatases differ in their structure, substrate specificity, and dependence on different metal ions for activity. PhoD and Pho X phosphatases are commonly found in marine and soil bacteria, while the PhoX phosphatases are also found in cyanobacteria. PhoA represents the classical phosphatase present in E. coli. Bacteria typically express at least one of the phosphatases of the three phosphatase classes.

SUMMARY OF THE INVENTION

[0006] The present invention provides immobilized alkaline phosphatases, as well as compositions and methods of utilizing these immobilized polypeptides. This summary is intended to introduce the subject matter of the present disclosure, but does not cover each and every embodiment, combination, or variation that is contemplated and described within the present disclosure. Further embodiments are contemplated and described by the disclosure of the detailed description, drawings, and claims.

[0007] These alkaline phosphatases are capable of hydrolysis of or removing a phosphate group from a substrate, including a nucleoside triphosphate comprising a phosphate at the 3' position of the sugar moiety (NTP-3'P), an oligonucleotide comprising a 3' terminal nucleotide comprising a phosphate at the 3' position of the sugar moiety; or an oligonucleotide comprising a 5'-terminal nucleotide comprising a phosphate at the 5'-position of the sugar moiety.

[0008] The alkaline phosphatases are useful in various reactions and methods, including methods of oligonucleotide synthesis. In some embodiments, these methods comprise a terminal nucleotidyl transferase or other polymerase adding nucleoside triphosphates with a 3'-O-removable blocking group to the 3'-OH end of a growing oligonucleotide or polynucleotide chain in a template-independent manner. In embodiments where the 3'-O-removable blocking group comprises a phosphate, the alkaline phosphatase acts to hydrolyze or to remove the blocking group. After removal of the 3' phosphate blocking group, additional rounds of NTP addition can be used to synthesize a polynucleotide with a defined sequence of bases without using a complementary template strand as a guide for NTP incorporation (template-independent synthesis).

[0009] In at least one embodiment, the present invention provides a method for preparing an immobilized alkaline phosphatase comprising:

contacting an epoxide-functionalized solid support and an alkaline phosphatase polypeptide in solution under suitable conditions for conjugating or immobilizing the alkaline phosphatase polypeptide to the solid support; and

adding an epoxide quenching reagent to the solution to deactivate or cap the epoxide groups on the solid support.

[0010] In at least one embodiment, the present invention provides a method for preparing an immobilized alkaline phosphatase comprising:

(a) preparing a solution comprising an epoxide functionalized solid support and an alkaline phosphatase polypeptide;

(b) allowing the solution to incubate at a temperature of about 20 C to about 60 C for about 1 h to about 6 h; and

(c) adding an epoxide quenching reagent to the solution of step (b).

[0011] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, L-cysteine methyl ester, N-acetyl-L-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0012] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the solution of step (a) is a buffered aqueous solution at a pH of about 6.5 to about 8.5 containing the alkaline phosphatase polypeptide at a concentration of about 50 mM to about 250 mM.

[0013] In some embodiments, the buffer in the aqueous solution is selected from borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), and the buffer concentration is from about 100 mM to about 1000 mM.

[0014] In at least one embodiment, the concentration of the epoxide quenching reagent in the solution is about 10 mM to about 3000 mM, or about 500 mM to about 5000 mM. In at least one embodiment, the pH of the epoxide quenching reagent in the solution is about 7, 8, or 9.

[0015] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the solution with the added quenching reagent, such as in step (c) above, is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0016] In some embodiments, the method further comprises removing the quenching reagent by washing the immobilized alkaline phosphatase on the solid support with a wash solution.

[0017] In some embodiments, the method further comprises treating the epoxide functionalized solid support and the alkaline phosphatase polypeptide with a second quenching reagent. In some embodiments, the treatment with the second epoxide quenching reagent is after completion of treatment with the quenching reagent. In at least one embodiment of the method of preparing an immobilized alkaline phosphate, the solid support is washed to remove the quenching reagent prior to adding the second quenching reagent. In at least one embodiment of the method, the second quenching reagent is different from the first quenching reagent.

[0018] In some embodiments, the second quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0019] In at least one embodiment of the method, the suitable conditions comprise incubating the solution with the quenching reagent at a temperature of about 20 °C to about 60 °C.

[0020] In at least one embodiment of the method, the solution for contacting the epoxide functionalized solid support with the alkaline phosphatase comprises a buffered aqueous solution at a

pH of about 6.5 to about 8.5 containing the alkaline phosphatase polypeptide at a concentration of about 1 mg/ml to about 50 mg/ml, 10 mg/ml to about 100 mg/ml, 100 mg/ml to about 1000 mg/ml, or about 2 mg/ml.

[0021] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises particles comprising polyacrylate, methacrylate, polymethacrylate, amino-epoxy polymethacrylate, phenolate, polystyrene, polysaccharide, silica, or controlled pore glass.

[0022] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0023] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0024] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support is a resin selected from 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), EMC7042/M (Sunresin), 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), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0025] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the immobilized alkaline phosphatase polypeptide has an activity of hydrolysis of a 3'-phosphate from

a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide.

[0026] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the alkaline phosphatase polypeptide comprises an amino acid sequence having at least 85% identity to a sequence of SEQ ID NO: 12 or SEQ ID NO: 16.

[0027] In another aspect, the present invention includes in at least one embodiment, a composition comprising an immobilized alkaline phosphatase prepared by method of the present disclosure.

[0028] In at least one embodiment the composition is prepared by the method of:

contacting an epoxide-functionalized solid support and an alkaline phosphatase polypeptide in solution under suitable conditions for conjugating or immobilizing the alkaline phosphatase polypeptide to the solid support; and

adding an epoxide quenching reagent to the solution to deactivate or cap the epoxide groups on the solid support.

[0029] In at least one embodiment the composition is prepared by the method of:

(a) preparing a solution comprising an epoxide functionalized solid support and an alkaline phosphatase polypeptide;

(b) allowing the solution to incubate at a temperature of about 20 C to about 60 C for about 1 h to about 6 h; and

(c) adding an epoxide quenching reagent to the solution of step (b).

[0030] In at least one embodiment, the method of preparing the composition additionally comprises a second epoxide quenching reagent.

[0031] In another aspect, the present invention also provides an immobilized alkaline phosphatase comprising an alkaline phosphatase and an epoxide-functionalized solid support, wherein the alkaline phosphatase polypeptide is attached to the solid support through a covalent linkage comprising a β-hydroxy-amino, β-hydroxy-ether, β-hydroxy-carboxyl, and/or β-hydroxy-thio, and wherein at least 99% of epoxide groups are quenched.

[0032] In at least one embodiment of the immobilized alkaline phosphatase, the quenched epoxide groups comprise a covalent linkage with a quenching compound, and optionally, a second quenching compound, selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0033] In at least one embodiment of the immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm

to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0034] In at least one embodiment of the immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0035] In at least one embodiment of the immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

[0036] In at least one embodiment of the immobilized alkaline phosphatase, the epoxide functionalized solid support is a resin selected from 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), EMC7042/M (Sunresin), EMC7042/S (Sunresin),, 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), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0037] In at least one embodiment of the immobilized alkaline phosphatase, the immobilized alkaline phosphatase is capable of hydrolysis of a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide during a method of enzymatic template-independent synthesis.

[0038] In at least one embodiment of the immobilized alkaline phosphatase, the alkaline phosphatase polypeptide has an activity of hydrolysis of a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide.

[0039] In at least one embodiment of the immobilized alkaline phosphatase, the engineered alkaline phosphatase polypeptide comprises an amino acid sequence having at least 85% identity to a sequence of SEQ ID NO: 12 or SEQ ID NO: 16.

[0040] In another aspect, the present invention provides a method of cleaving a phosphate group, comprising

contacting a substrate with a cleavable phosphate group in a reaction solution with an immobilized alkaline phosphatase described herein under reaction conditions effective for cleavage of the phosphate group by the immobilized alkaline phosphatase.

[0041] In some embodiments, the substrate with a cleavable phosphate group comprises an oligonucleotide comprising a 3'-terminal phosphate, an oligonucleotide comprising a 5'-terminal phosphate, an NTP, an NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, or 3'-phosphate NMP.

[0042] In some embodiments, the present invention also provides a method for hydrolysis of a blocking group during oligonucleotide synthesis comprising:

contacting an immobilized alkaline phosphatase of the present disclosure with a reaction solution comprising an oligonucleotide comprising a 3' terminal nucleotide with a 3' phosphate blocking group on the sugar moiety, and whereby the 3' phosphate blocking group is hydrolyzed,

allowing a further cycle of addition of a NTP with a '3 phosphate blocking group to the 3' terminal end of the oligonucleotide.

[0043] In at least one embodiment of the method for hydrolysis, the oligonucleotide substrate with the 3'-phosphate, or the oligonucleotide with the 5'-phosphate comprises one or more modified nucleosides, and/or one or more modified internucleoside linkages.

[0044] In some embodiments, the modified nucleoside on the oligonucleotides comprises a 2' modification on the sugar moiety. In some embodiments, the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl. In some embodiments of the method, the modified internucleoside linkage on the oligonucleotide comprises a phosphorothioate internucleoside linkage.

[0045] In at least one embodiment of the method for hydrolysis, the NTP, NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, and 3'-phosphate NMP comprises a modified nucleoside and/or modified phosphate group.

[0046] In at least one embodiment of the method for hydrolysis, the alkaline phosphatase polypeptide has an activity of hydrolysis of a 3'-phosphate on a nucleotide triphosphate (NTP), 3'-phosphate NDP, and 3'-phosphate NMP.

[0047] In at least one embodiment of the method for hydrolysis, the NTP, NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, or 3'-phosphate NMP substrate comprises a 2' modification on the sugar

moiety. In some embodiments, the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

[0048] In at least one embodiment of the method for hydrolysis, the NTP, NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, or 3'-phosphate NMP substrate comprises a locked nucleic acid group.

[0049] In at least one embodiment of the method for hydrolysis, the immobilized alkaline phosphatase comprises a packed solid support or resin in a column.

[0050] In at least one embodiment of the method for hydrolysis, the contacting comprises flowing the reaction solution through the packed solid support or resin in the column.

[0051] In at least one embodiment of the method for hydrolysis, the immobilized alkaline phosphatase on solid support or resin is dispersed or suspended in the reaction solution. In some embodiments, the reaction solution with the dispersed or suspended solid support or resin is agitated.

[0052] In at least one embodiment of the method for hydrolysis, the method further comprises separating the immobilized alkaline phosphatase on solid support or resin from the reaction solution.

BRIEF DESCRIPTION OF DRAWINGS

[0053] **FIG. 1** provides graphs summarizing immobilization of multiple variant alkaline phosphatases (SEQ ID NOs: 12 or 16) to the indicated solid support in a variety of buffers and pH conditions before washing. **FIG. 1A** and **FIG. 1B** summarize results from different replicates. For both graphs, the wt% loaded is indicated on the y-axis, the resin material and the buffer conditions are indicated on the x-axis. The resins evaluated were COV-7, HFA403/M, EPA403/M, EMC2042/M, and EP600. The resins range in average pore diameter from 500Å to 1500Å; the particle sizes of the solid supports range from 100 μm to 700 μm. Buffer conditions were 1 M NaPi, 50 mM NaPi pH 7.0, 1 M TEoA, 50 mM TEoA pH 7.8 and water.

[0054] **FIG. 2** provides graphs summarizing bonding of multiple variant alkaline phosphatases (SEQ ID NOs: 12 or 16) after washing the solid support. **FIG. 2A** and **FIG. 2B** summarize results from different wash conditions. For both graphs, the wt% washed off is indicated on the y-axis, the resin material and the buffer conditions are indicated on the x-axis.

[0055] **FIG. 3** provides graphs summarizing alkaline phosphatase immobilization on the indicated solid support through covalent bonding. **FIG. 3A** and **FIG. 3B** summarize the amount of alkaline phosphatase loaded on the indicated resin after washing. For both graphs, the wt% loaded is indicated on the y-axis, the resin material and the buffer conditions are indicated on the x-axis.

[0056] **FIG. 4** provides graphs summarizing the activity of the immobilized alkaline phosphatases on indicated resins after immobilization in the indicated buffer. For both **FIG. 4A** and **FIG. 4B**, the percent conversion of mAQP to mA (% conversion) is shown on the y-axis. The resin and the loading

buffer conditions are shown on the x-axis. **FIG. 4A** summarizes the results obtained with 0.25 mM $CoCl_2$. **FIG. 4B** summarizes the results obtained 0.5 mM $CoCl_2$.

[0057] FIG. 5 provides graphs summarizing the activity of the immobilized alkaline phosphatases on indicated resins after normalization for enzyme loading. For both **FIG. 5A** and **FIG. 5B**, the turnover number is shown on the y-axis. The resin and the loading buffer conditions are shown on the x-axis. **FIG. 5A** summarizes the results obtained with 0.25 mM $CoCl_2$. **FIG. 5B** summarizes the results obtained 0.5 mM $CoCl_2$.

[0058] FIG. 6 provides graphs summarizing immobilization of recombinant alkaline phosphatase to the indicated solid support in different loading conditions before and after washing. **FIG. 6A** summarizes results from different loading or immobilization conditions before washing. **FIG. 6B** summarizes results from different loading or immobilization conditions after washing. For both graphs, the wt% loaded is indicated on the y-axis and the resin material is indicated on the x-axis. The resins evaluated were EP600, EP400, CPG-N12, CPG-N16 and CPG-No cap.

[0059] FIG. 7 provides graphs summarizing the activity of immobilized recombinant alkaline phosphatases in batch style deblocking reactions. Deblocking reactions were performed in the presence or absence of thiosulfate in either MOPS or TEoA buffer. **FIG. 7A** summarizes conversion results at 5 minutes of deblocking reaction. **FIG. 7B** summarizes conversion results at 15 minutes of deblocking reaction. For both graphs, the % deblocked is indicated on the y-axis and the immobilized AP and reaction conditions are indicated on the x-axis. The immobilized APs were designated by the solid support resin they each contained; thus, the immobilized APs are indicated on the x-axis as EP600, EP400, CPG-N12, CPG-N16 and CPG-No cap. Activity is high for all evaluated immobilized alkaline phosphatases. CPG-No cap had the lowest loading and lowest activity. CPG-N12 generally had higher activity than CPG-N16, except for MOPS without thiosulfate.

[0060] FIG. 8 provides analysis of the percent of PS to PO conversion during batch style deblocking reactions with the immobilized recombinant alkaline phosphatases. Deblocking reactions were performed in the presence or absence of thiosulfate in either MOPS or TEoA buffer. **FIG. 8A** summarizes the percent of PS to PO conversion exhibited by acceptor oligonucleotides in deblocking reactions with the immobilized recombinant alkaline phosphatases under various reaction conditions. The % PS to PO conversion is indicated on the y-axis and the immobilized AP and reaction conditions utilized are indicated on the x-axis. The immobilized APs were designated by the solid support resin they each contained; thus, the immobilized APs are indicated as EP600, EP400, CPG-N12, CPG-N16 and CPG-No cap. Control reactions containing only the 8 merP without immobilized AP were also performed. **FIG. 8B** provides a graph comparing the amount of alkaline phosphatase immobilized on the solid support (wt%) (x-axis) and the amount of PS to PO conversion (%) on the y-axis under indicated reaction conditions (MOPS +/- thiosulphate, TEoA +/- thiosulphate).

[0061] MOPS and thiosulfate reduce the amount of PS to PO conversion observed. CPG-12 and CPG-No cap showed low PS to PO conversion across all evaluated conditions. EP400 exhibited higher PS to PO conversion than EP600 in most of the evaluated conditions.

[0062] **FIG. 9** provides charts summarizing immobilization of recombinant alkaline phosphatase to the indicated solid support before and after washing. **FIG. 9A** summarizes the amount of alkaline phosphatase associated with the indicated solid supports before washing. **FIG. 9B** summarizes the amount of alkaline phosphatase immobilized on the indicated solid support after washing. For both graphs, the wt% loaded is indicated on the y-axis and the solid support material is indicated on the x-axis. The solid supports evaluated were EP600, EP400, CPG-N12, CPG-N16 and CPG-No cap.

[0063] **FIG. 10** provides analysis of the activity of different immobilized alkaline phosphatases packed in a column. The different immobilized alkaline phosphatases are designated by the immobilized alkaline phosphatase's solid support and indicated on the x-axis of **FIG. 10A**, **FIG. 10B** and **FIG. 10C**. **FIG. 10A** summarizes the activity of the immobilized alkaline phosphatases on the oligonucleotides in the deblocking reaction. The percent of deblocked oligonucleotide (% deblocked) is indicated on the y-axis. The CPG-N12 immobilized alkaline phosphatase deblocked 100% of the oligonucleotides in the reaction. The EP600, EP400 and CPG-N16 immobilized alkaline phosphatases deblocked 97%-98% of the oligonucleotides in the reaction. **FIG. 10B** summarizes the amount of PS to PO conversion in the reaction oligonucleotides from reactions with each indicated immobilized alkaline phosphatase and the dAdAdAdAmC*mA*mGmA-3'P starting material in a no enzyme control. The amount of PS to PO conversion of oligonucleotide for each immobilized alkaline phosphatase was lower than that seen with the 8 merP starting material in the no enzyme control. **FIG. 10C** summarizes the amount of alkaline phosphatase found in each fraction collected from the deblocking column reactions for each immobilized AP. The amount of alkaline phosphatase that leached off the immobilized AP with both the EP400 and CPG-N16 solid supports was below the limit of quantification (LOQ) in all collected fractions. The CPG-N12 immobilized AP showed significant leaching in all fractions. High salt washing increased the amount of leached AP.

[0064] **FIG. 11A** and **FIG. 11B** present the amount of alkaline phosphatase associated with EP400 resin before washing (**FIG. 11A**) and after washing (**FIG. 11B**). The EP400 resin was loaded with an alkaline phosphatase target loading of either 1 wt% or 2 wt% and the immobilized alkaline phosphatases were washed at either pH7 or pH8. The target wt % and the wash pH for each immobilized alkaline phosphatase are indicated on the x-axis of **FIGs. 11A** and **11B**. The wt % loaded of each immobilized alkaline phosphatase is indicated on the y-axis of both figures. Washing at pH 7 and pH 8 removed similar amounts of non-covalently bound enzyme from the solid support. **FIG. 11C** depicts the amount of deblocking by each indicated immobilized alkaline phosphatase. The target wt % and the wash pH for each immobilized alkaline phosphatase are indicated on the x-axis.

The percent conversion (% conversion) is indicated on the y-axis. All samples showed at least 97% deblocking, with three samples showing complete deblocking.

[0065] **FIG. 12A** indicates the amount of alkaline phosphatase immobilized (wt % loaded, y-axis) on the indicated solid support for each replicate after washing. **FIG. 12B** depicts the amount of deblocking by each indicated immobilized alkaline phosphatase. The target wt % and the wash pH for each immobilized alkaline phosphatase are indicated on the x-axis. The percent conversion (% conversion) is indicated on the y-axis. **FIG. 12C** presents the amount of PS to PO conversion in the reaction oligonucleotides from reactions with each indicated immobilized alkaline phosphatase and the 8 merP starting material in a no enzyme control. **FIG. 12D** depicts the amount of deblocking by each indicated immobilized alkaline phosphatase upon reuse and at a higher flow rate. The target wt % and the wash pH for each immobilized alkaline phosphatase are indicated on the x-axis. The percent conversion (% conversion) is indicated on the y-axis. There is complete deblocking (conversion) on reuse at twice the original flowrate.

DETAILED DESCRIPTION OF THE INVENTION

[0066] The present disclosure provides immobilized alkaline phosphatase and compositions thereof, as well as methods of preparing the immobilized alkaline phosphate. The disclosure also provides methods of using of the immobilized alkaline phosphatase and compositions thereof for molecular biological, diagnostic, and other purposes, including synthesis of oligonucleotides.

Abbreviations and Definitions

[0067] 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.

[0068] As used herein, 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.

[0069] Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting. Thus, as used herein, the term "comprising" and its cognates are used in their inclusive sense (i.e., equivalent to the term "including" and its corresponding cognates).

[0070] It is to be further 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."

[0071] "About" means an acceptable error for a particular value. In some instances, "about" means within 0.05%, 0.5%, 1.0%, or 2.0%, of a given value range. In some instances, "about" means within 1, 2, 3, or 4 standard deviations of a given value.

[0072] "EC" number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0073] "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0074] "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0075] "Protein," "polypeptide," and "peptide" are used interchangeably 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 or phosphorylation).

[0076] "Amino acids" and "amino acid" 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. The abbreviations used for the genetically encoded amino acids are conventional and are as follows: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartate (Asp or D), cysteine (Cys or C), glutamate (Glu or E), glycine (Gly or G), glutamine (Gln or Q), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V). When the three-letter abbreviations are used, unless specifically preceded by an "L" or a "D" or clear from the context in which the abbreviation is used, the amino acid may be in either the L- or D-configuration about a-carbon (Ca). For example, whereas "Ala" designates alanine without specifying the configuration about the a-carbon, "D-Ala" and "L-Ala" designate D-alanine and L-alanine, respectively. When the one-letter abbreviations are used, upper case letters designate amino acids in the L-configuration about the a-carbon and lower case letters designate amino acids in the D-configuration about the a-carbon. For example, "A" designates L-alanine and "a" designates D-alanine. When polypeptide sequences are presented as a string of one-letter or three-letter abbreviations (or mixtures thereof), the sequences are presented in the amino (N) to carboxy (C) direction in accordance with common convention.

[0077] "Phosphatase" as used herein refers to enzymes that preferentially cleave phosphomonoester bonds, or analogs thereof, such as phosphorothioate. "Alkaline phosphatase" refers to a phosphatase that has activity at optimal pH of >7.

[0078] "Fusion protein," and "chimeric protein" and "chimera" refer to hybrid proteins created through the joining of two or more polynucleotides that originally encode separate proteins. In some embodiments, fusion proteins are created by recombinant technology.

[0079] "Polynucleotide," "nucleic acid," or "oligonucleotide" is used herein to denote a polymer comprising at least two nucleotides where the nucleotides are either deoxyribonucleotides or ribonucleotides or mixtures of deoxyribonucleotides and ribonucleotides. In some embodiments, the abbreviations used for genetically encoding nucleosides are conventional and are as follow: adenosine (A); guanosine (G); cytidine (C); thymidine (T); and uridine (U). Unless specifically delineated, the abbreviated nucleosides may be either ribonucleosides or 2'-deoxyribonucleosides. The nucleosides may be specified as being either ribonucleosides or 2'-deoxyribonucleosides on an individual basis or on an aggregate basis. When a polynucleotide, nucleic acid, or oligonucleotide 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. The term "DNA" refers to deoxyribonucleic acid. The term "RNA" refers to ribonucleic acid. The polynucleotide or nucleic acid may be single-stranded or double-stranded, or may include both single-stranded regions and double-stranded regions.

[0080] In some embodiments, the terms "polynucleotide," "nucleic acid" and "oligonucleotide" encompass polynucleotide or nucleic acid or oligonucleotide analogs or modified polynucleotide or nucleic acid or oligonucleotide, which include, among others, nucleosides linked together via internucleoside linkages other than standard phosphodiester linkages, such as non-standard linkages of phosphorothioates, amide linkages, etc.; nucleosides with modified and/or synthetic nucleobases, for example inosine, xanthine, hypoxanthine, etc.; nucleosides with modified sugar residues, such as 2'-O-alkyl, 2'-halo, 2,3-dideoxy, 2'-halo-2'-deoxy, □-D-ribo LNA, □-L-ribo-LNA (e.g., locked nucleic acids), etc.; and/or 5'-phosphate analogs, including, among others, phosphorothioate, phosphoacetate, phosphoramidate, monomethylphosphate, methylphosphonate, or phosphonocarboxylate.

[0081] "Duplex" and "ds" refer to a double-stranded nucleic acid (e.g., DNA or RNA) molecule comprised of two single-stranded polynucleotides that are complementary in their sequence (e.g., A pairs to T or U, C pairs to G), arranged in an antiparallel 5' to 3' orientation, and held together by hydrogen bonds between the nucleobases (e.g., adenine [A], guanine [G], cytosine [C], thymine [T], uridine [U]).

[0082] "Complementary" is used herein to describe the structural relationship between nucleotide bases that are capable of forming base pairs with one another. For example, a purine nucleotide base present on a polynucleotide that is complementary to a pyrimidine nucleotide base on a polynucleotide may base pair by forming hydrogen bonds with one another. Complementary

nucleotide bases can base pair via Watson/Crick base pairing or in any other manner than forms stable duplexes or other nucleic acid structures.

[0083] "Watson/Crick Base-Pairing" refers to a pattern of specific pairs of nucleobases and analogs that bind together through sequence-specific hydrogen-bonds, e.g. A pairs with T or U, and G pairs with C.

[0084] "Engineered," "recombinant," "non-naturally occurring," and "variant," when used with reference to a cell, a polynucleotide or a polypeptide refer to a material or a material corresponding to the natural or native form of the material that has been modified in a manner that would not otherwise exist in nature or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques.

[0085] "Wild-type" and "naturally-occurring" refer to the form found in nature. For example, a wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0086] "Coding sequence" and synonymously "encoding" refers to that part of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0087] "Percent (%) sequence identity" refers to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (Smith and Waterman, Adv. Appl. Math., 1981, 2:482), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, J. Mol. Biol., 1970, 48:443), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 1988, 85:2444), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining

percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms (see, e.g., Altschul et al., J. Mol. Biol., 1990, 215: 403-410; and Altschul et al., Nucleic Acids Res., 1977, 3389-3402). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length "W" in the query sequence, which either match or satisfy some positive-valued threshold score "T," when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (see Altschul et al, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters "M" (reward score for a pair of matching residues; always >0) and "N" (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity "X" from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see, e.g., Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA, 1989, 89:10915). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0088] "Reference sequence" refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, at least 100 residues in length or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (i.e., a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity.

[0089] "Comparison window" refers to a conceptual segment of contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence. In some embodiments, the comparison window is at least 15 to 20 contiguous nucleotides or amino acids and

wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. In some embodiments, the comparison window can be longer than 15-20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0090] "Corresponding to", "reference to," and "relative to" when used in the context of the numbering of a given amino acid or polynucleotide sequence refer to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of a recombinant phosphatase, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0091] "Amino acid difference" and "residue difference" refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The amino acid positions of amino acid differences generally are referred to herein as "Xn," where n refers to the corresponding position in the reference sequence upon which the residue difference is based. In some instances, herein, the specific amino acid residue difference at a position is indicated as "XnY" where "Xn" specified the corresponding residue and position of the reference polypeptide (as described above), and "Y" is the single letter identifier of the amino acid found in the engineered polypeptide (i.e., the different residue than in the reference polypeptide). In some instances (e.g., in the Tables in the Examples), the present disclosure also provides specific amino acid differences denoted by the conventional notation "AnB", where A is the single letter identifier of the residue in the reference sequence, "n" is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. In some embodiments, the amino acid difference, e.g., a substitution, is denoted by the abbreviation "nB," without the identifier for the residue in the reference sequence. In some instances, an amino acid residue difference or substitution may be a deletion and may be denoted by a "-" where appropriate.

[0092] "Functional fragment" and "biologically active fragment" are used interchangeably herein, to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full length recombinant phosphatase of the present invention) and that retains substantially all of the activity of the full-length polypeptide.

[0093] "Isolated polypeptide" refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it (e.g., protein, lipids, and polynucleotides). The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (e.g., host cell or in vitro synthesis). The recombinant phosphatase polypeptides may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the recombinant phosphatase polypeptides provided herein are isolated polypeptides.

[0094] "Substantially pure polypeptide" or "purified" refers to a composition in which the polypeptide species is the predominant species present (i.e., on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure phosphatase composition will comprise about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (i.e., contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated recombinant phosphatase polypeptides are substantially pure polypeptide compositions.

[0095] "Improved enzyme property" refers to a recombinant phosphatase polypeptide that exhibits an improvement in any enzyme property as compared to a reference phosphatase polypeptide, such as a wild-type phosphatase polypeptide or another recombinant phosphatase polypeptide. Improved properties include but are not limited to such properties as increased enzymatic activity, increased product yield, increased protein expression, increased thermoactivity, increased thermostability, increased stability, increased substrate specificity and/or affinity, increased substrate range, increased specific activity, increased resistance to substrate and/or end-product inhibition, increased chemical stability, improved solvent stability, increased solubility, and increased inhibitor resistance or tolerance. Exemplary improved properties are provided in the Examples.

[0096] "Codon optimized" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is more efficiently expressed in that organism. Although the genetic code is degenerate, in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding

regions of an organism's genome. In some embodiments, the polynucleotides encoding the phosphatase enzymes are codon optimized for optimal production from the host organism selected for expression.

[0097] "Control sequence" refers herein to include all components that are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present disclosure. Each control sequence may be native or foreign (e.g., heterologous) to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, leaders, polyadenylation sequences, propeptide sequences, promoter sequences, signal peptide sequences, initiation sequences, and transcription terminators. In some embodiments, the control sequences include a promoter, and transcriptional and translational stop signals.

[0098] "Operably linked" or "operatively linked" refers to a configuration in which a control sequence is appropriately placed (i.e., in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide of interest, and where appropriate, expression of the encoded polypeptide of interest.

[0099] "Promoter" or "promoter sequence" refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences that mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0100] "Suitable reaction conditions" or "suitable conditions" refers to those conditions in the enzymatic conversion reaction solution (e.g., ranges of enzyme loading, substrate loading, temperature, pH, buffers, co-solvents, etc.) under which a phosphatase polypeptide of the present disclosure is capable of cleaving a phosphomonoester bond. Exemplary "suitable reaction conditions" are provided herein (see, the Examples).

[0101] "Product" in the context of an enzymatic conversion process refers to the compound or molecule resulting from the action of the phosphatase polypeptide on the substrate.

[0102] "Culturing" refers to the growing of a population of cells under suitable conditions using any suitable medium (e.g., liquid, gel, or solid).

[0103] "Vector" is a recombinant construct for introducing a polynucleotide of interest into a cell. In some embodiments, the vector is an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polynucleotide or a polypeptide encoded in the polynucleotide. In some embodiments, an "expression vector" has a promoter

sequence operably linked to the polynucleotide (e.g., transgene) to drive expression in a host cell, and in some embodiments, also comprises a transcription terminator sequence.

[0104] "Expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0105] "Produces" refers to the production of proteins and/or other compounds by cells. It is intended that the term encompass any step involved in the production of polypeptides including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0106] "Heterologous" or "recombinant" refers to the relationship between two or more nucleic acid or polypeptide sequences (e.g., a promoter sequence, signal peptide, terminator sequence, etc.) that are derived from different sources and are not associated in nature.

[0107] "Host cell" and "host strain" refer to suitable hosts for expression vectors comprising a polynucleotide provided herein (e.g., a polynucleotide sequences encoding at least one recombinant phosphatase). In some embodiments, the host cells are prokaryotic or eukaryotic cells that have been transformed or transfected with vectors constructed using recombinant DNA techniques, and progeny thereof, as known in the art.

Immobilization of Engineered Alkaline Phosphatase Polypeptides

[0108] In one aspect, the present disclosure is directed to the preparation and use of immobilized alkaline phosphatase polypeptides. Generally, the immobilization process comprises contacting the engineered alkaline phosphatase polypeptide in solution with a solid support under conditions suitable for the formation of a covalent or non-covalent linkage between the polypeptide and a functional group on the solid support. The resulting attachment of alkaline phosphatase polypeptide to the support should allow the enzyme to retain its desired activity with a desired substrate. The immobilized alkaline phosphatase polypeptide can then be used in an enzymatic reaction, such as the hydrolysis of a phosphate blocking group at the '3 terminal nucleotide an oligonucleotide during oligonucleotide synthesis, while it is retained on a support that allows facile separation of the desired oligonucleotide reaction products.

[0109] In at least one embodiment, the immobilized alkaline phosphatase polypeptide is packed in a column allowing a flow-through of a substrate for the alkaline phosphatase, for example, during template-independent oligonucleotide synthesis, wherein oligonucleotide and NTP substrates are passed through the column, with the desired products collected at the column output. Such flow-through synthesis methods are disclosed in the Examples and elsewhere herein.

[0110] In some embodiments, the immobilized alkaline phosphatase is used in batch reaction, where the immobilized alkaline phosphatase on the support is mixed in the reaction solution in a vessel or container, such as with an impeller, with the phosphatase substrates until the desired level of cleavage of the phosphate is achieved. The immobilized alkaline phosphatase can then be separated out from the reaction solution, such as by filtration or centrifugation.

[0111] In some embodiments, the alkaline phosphatase can be immobilized non-covalently or covalently on various solid supports or support mediums (e.g., resins, membranes, beads, glass, etc.). In some embodiments, support medium, including solid supports, useful for immobilizing the alkaline phosphatase polypeptides include but are not limited to particles, beads, resins, or membranes, among others, of polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, polyacrylamide, polyamino acid or protein (e.g., gelatin), polysaccharide or cellulosic (e.g., agarose, dextran, chitosan, pectin, etc.), controlled pore glass, and silica (e.g., silicon dioxide).

[0112] In some embodiments, solid supports useful for immobilizing the engineered alkaline phosphatase polypeptides of the present invention include but are not limited to beads or resins comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups.

[0113] In some embodiments, the support medium or solid support is in the form of particles, such as beads, having a particle size range of about 1 μm to about 2000 μm, about 10 μm to about 1700 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. In some embodiments, the particles have a particle size of about 1 μm, about 10 μm, about 50 μm, about 100 μm, about 200 μm, about 300 μm, about 400 μm, about 500 μm, about 1000 μm, about 1500 μm, 1600 μm, 1700 μm, 1800 μm, 1900 μm, or 2000 μm. In some embodiments, the size of the particles are selected for solution flow properties, amount and density of functional groups, surface area, diffusion of substrates, and the like.

[0114] In some embodiments, the particles or resins for immobilization of alkaline phosphatase polypeptide comprises an average pore diameter or pore size of about 100 angstroms (Å) to about 2000 angstroms (Å), 150 angstroms (Å) to about 1800 angstroms (Å), 200 angstroms (Å) to about 1600 angstroms (Å), 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). In some embodiments, the particles comprise an average pore diameter or pore size of about 100 angstroms (Å), 150 angstroms (Å), 200 angstroms (Å), 250 angstroms (Å), 300 angstroms (Å), about 600 angstroms (Å), about 1000 angstroms (Å), about 1500 angstroms (Å), 1600 angstroms (Å), 1700 angstroms (Å), 1800 angstroms (Å), 1900 angstroms (Å), 2000 angstroms (Å), or greater.

[0115] A range of methods of enzyme immobilization are described in the art. These methods use a wide range of solid supports (e.g., resins, membranes, beads, glass, etc.) and result in enzymes that are bound either non-covalently or covalently. Immobilization methods disclosed in the art include, e.g.,

Yi et al., Proc. Biochem., 2007, 42(5):895-898; Martin et al., Appl. Microbiol. Biotechnol., 2007, 76(4): 843-851; 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.

[0116] A wide range of alternative solid supports useful for immobilizing enzymes are known in the art and it is contemplated that these also may be adapted for use with the alkaline phosphatase polypeptides.

[0117] In some embodiments, the support material includes, but are not limited to, EnginZyme (including, EziG-1, EziG-1, and EziG-3), chitosan beads, Eupergit C, and SEPABEADs (Mitsubishi) (including EC-EP, EC-HFA/S, EXA252, EXE119 and EXE120).

[0118] 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), and ECR8405F (Purolite).

[0119] 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).

[0120] 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).

[0121] 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).

[0122] 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).

[0123] 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).

[0124] 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).

[0125] 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).

[0126] Exemplary solid supports useful for immobilizing the engineered alkaline phosphatase polypeptides of the present invention include those disclosed for use as described in the Examples, and include but are not limited to the supports listed in **Table 1** below.

Table 1: Exemplary solid supports for immobilization of an alkaline phosphatase

Manufacturer	Product #	Mode	Type	Functional Group(s)	Low End Particle Size (µm)	High End Particle Size (µm)	Average Pore Diameter (Å)
ChiralVision	IB-COV-1	Covalent	Polyacrylic	Low epoxide, high butyl	150	600	
ChiralVision	IB-COV-2	Covalent	Polyacrylic	Epoxide, low butyl	150	600	
ChiralVision	IB-COV-6	Covalent	Polyacrylic	Epoxide, hydroxyethyl	300	710	
ChiralVision	IB-COV-7	Covalent	Polyacrylic	Epoxide	300	600	500
ChiralVision	IB-COV-8	Covalent	Polyacrylic	Epoxide	200	710	
ChiralVision	IB-COV-10	Covalent	Cellulose	Epoxide, anionic	80	120	
ChiralVision	IB-ANI-2	Anionic	Polystyrene	Tertiary Amine	300	700	
ChiralVision	IB-ANI-3	Anionic	Polystyrene	Quaternary Amine	400	1000	
ChiralVision	IB-ANI-5	Anionic	Polyacrylic	Primary, Secondary Amine	300	600	
ChiralVision	IB-ANI-7	Anionic	Polyacrylic	Primary, Secondary Amine	200	710	
ChiralVision	IB-ANI-8	Anionic	Polyacrylic	Primary Amine	200	710	
ChiralVision	IB-ANI-10	Anionic	Polyacrylic	Primary, Secondary Amine	150	600	

Table 1: Exemplary solid supports for immobilization of an alkaline phosphatase

Manufacturer	Product #	Mode	Type	Functional Group(s)	Low End Particle Size (µm)	High End Particle Size (µm)	Average Pore Diameter (Å)
ChiralVision	IB-ANI-13	Anionic	Cellulose	Primary, Secondary Amine	80	120	
Duolite	A-7 Freebase	IEX	Phenol/Formaldehyde	Amino			
Duolite	A568	IEX	Phenol/Formaldehyde	Tertiary Amine	150	850	400
Resindion	EC-HFA	Covalent	Methacrylic Polymer	Amino-epoxy	200	500	500
Resindion	EC-BU	Hydrophobic Adsorbent	Methacrylic Polymer	Butyl	200	500	500
Resindion	EC-HA	Ionic	Methacrylic Polymer	Hexamethylamino	200	500	500
Resindion	EC-EP/S	Covalent	Methacrylic Polymer	Epoxide	200	500	500
Resindion	EC-EP	Covalent	Methacrylic Polymer	Epoxide	200	500	500
Resindion	HFA 403/S	Covalent	Methacrylic Polymer	Amino-epoxy	200	500	667
Amberlite	XAD7HP	Adsorbent	Crosslinked Aliphatic Polymer	N/A	430	690	550
Amberlite	FPA51	IEX	Polystyrene	Tertiary Amine	490	690	
Purolite (Lifetech)	ECR8804F	Adsorption	Octadecyl Methacrylate	Octadecyl	150	300	400
Purolite (Lifetech)	ECR8405F	Covalent	Amino C6 Methacrylate	NH_2 (long spacer)	150	300	900
Purolite (Lifetech)	ECR1090F	Adsorption	Macroporous Styrene	N/A	150	300	1000
Purolite (Lifetech)	ECR1604	IEX	Styrene	Tertiary Amine	300	1200	
Purolite (Lifetech)	ECR1504	IEX	Styrene	Tertiary Amine	300	1200	
Purolite (Lifetech)	ECR1640	IEX	Styrene	Quaternary Amine	300	1200	
Sunresin	EMC7025	Covalent	Polyacrylic	Epoxide	150	300	403.25
Sunresin	EMC7014	Covalent	Polyacrylic	Epoxide	150	300	525
Sunresin	EMC7032	Covalent	Polyacrylic	Epoxide	150	300	315
Sunresin	EMC7120/M	Amine	Polyacrylic	Amino, C6 Spacer	300	700	320
Sunresin	EMC7225/M	Amine	Polyacrylic	Amino, C2 Spacer	300	700	620

Table 1: Exemplary solid supports for immobilization of an alkaline phosphatase

Manufacturer	Product #	Mode	Type	Functional Group(s)	Low End Particle Size (µm)	High End Particle Size (µm)	Average Pore Diameter (Å)
Sunresin	Chelex 7350	Affinity	Polyacrylic	Iminodiacetate	100	250	
Resindion - Relizyme	HA403/M	Amine	Polyacrylic	Amino	200	500	
Resindion - Relizyme	EA403/M	Amine	Polyacrylic	Amino	200	500	
Resindion - Relizyme	EP403/M	Covalent	Polymethacrylate	Epoxide	200	500	
Resindion - Relisorb	SP600	Adsorbent	Polymethacrylate	Sulfonic	100	200	1500
Resindion - Relisorb	EP600	Covalent	Polymethacrylate	Epoxide	100	200	1500
Resindion - Relizyme	HFA403/S	Covalent	Polymethacrylate	Amino - Epoxy	100	300	500
Sunresin	EMC7042S	Covalent	Polyacrylic	Epoxide	100	300	920

Covalent Attachment

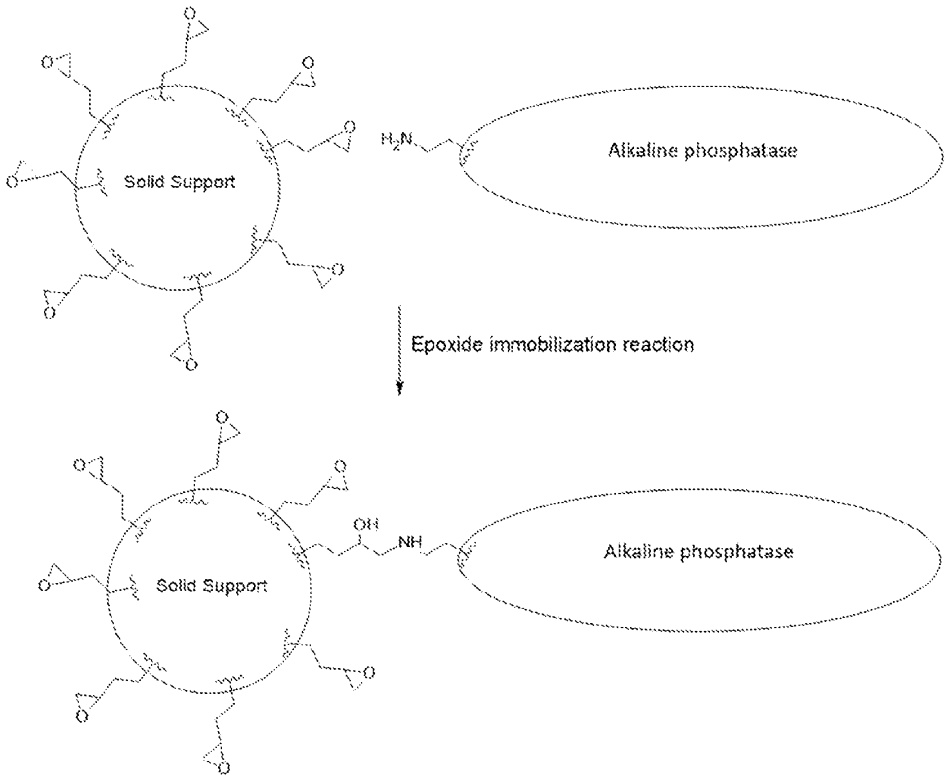
[0127] In some embodiments, the polypeptide is immobilized on the support material by covalent attachment to a reactive chemical group. Exemplary reactive chemical groups include sulfonic, epoxide, epoxy, amino-epoxy, iminodiacetate, amino, primary amine, secondary amine, quaternary amine, tertiary amine, NH_2, octadecyl, butyl, high butyl, low butyl, and hydroxyethyl. In some embodiments, the reactive chemical group facilitates the attachment or immobilization of an oligonucleotide.

[0128] In some embodiments, the alkaline phosphatase polypeptide comprises one or more amino acid residues reactive with the reactive chemical group on the support material. In some embodiments, the reactive amino acid is part of the amino acid sequence of the alkaline phosphatase, whether the alkaline phosphatase is naturally occurring or an engineered alkaline phosphatase. In some embodiments, reactive amino acids can be added into the alkaline phosphatase amino acid sequence by recombinant methods, such as insertion of cysteine, lysine, aspartic acid, tyrosine, serine, and/or threonine residues. In some embodiments, the alkaline phosphatase can be fused to polylysine residues (e.g., 2-12 or more lysine residues), for example, at the amino or carboxy terminus of the alkaline phosphatase.

[0129] In some embodiments, the reactive chemical is attached to the resin via a linker. Any suitable linker may be used. Exemplary linkers include ethylamine, alkylene, alkenylene, alkynylene, heteroalkylene, heteroalkenylene, heteroalkynylene, β-hydroxy-amino, β-hydroxy-ether, β-hydroxy-carboxyl, and/or β-hydroxy-thio cyclcoalkyl, heterocycloalkyl, arylene, or heteroarylene based

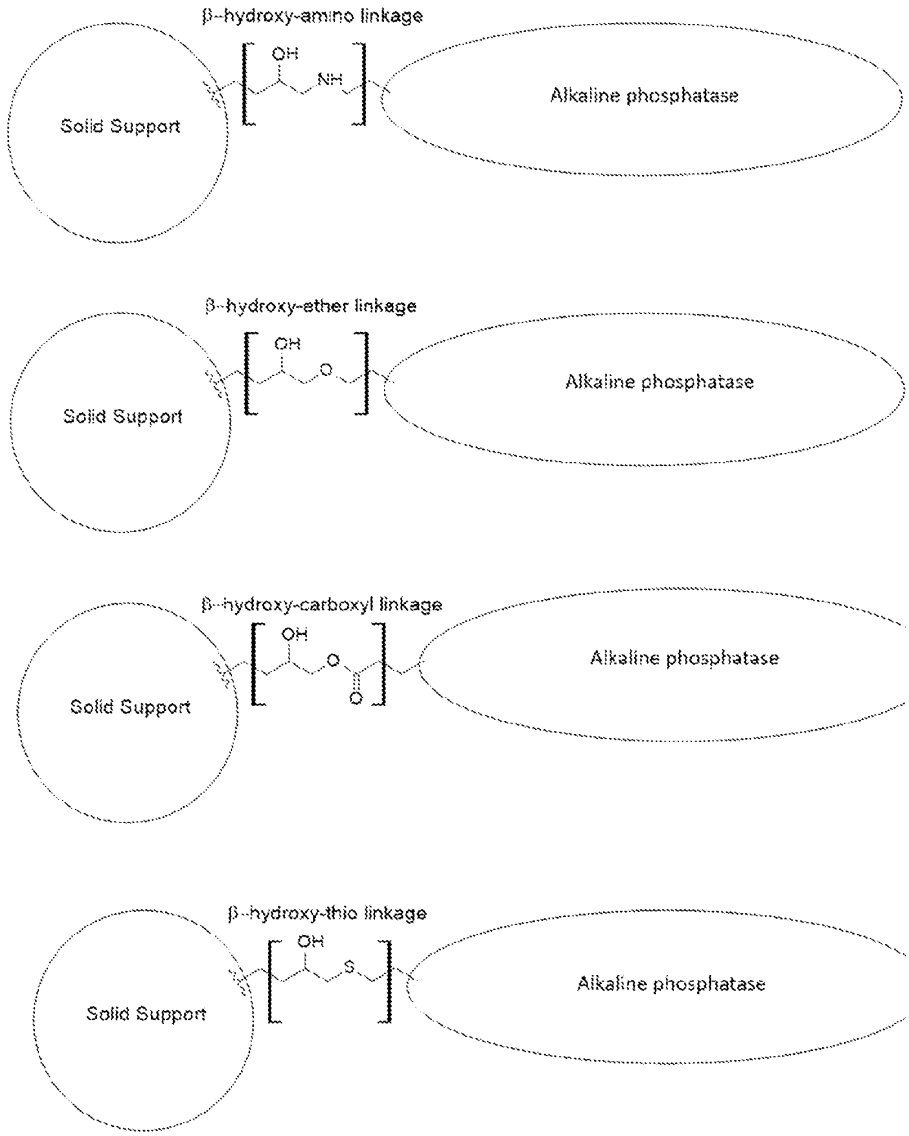
linkers, and the like. In some embodiments the linker comprises a C_2-C_{20}alkylene or polyethylene linker. In some embodiments, the linker is a silane or silanol linker. In some embodiments, the linker is a poly(ethylene glycol) linker, such as (3-mercaptopropyl)trimethoxysilane, poly(ethylene glycol) diglycidyl ether, or (3-glycidyloxypropyl)trimethoxysilane. Various suitable linkers are known in the art.

[0130] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the solid support is an epoxide functionalized solid support. In such an embodiment, the alkaline phosphatase polypeptide forms a covalent linkage with the solid support. A schematic illustration of immobilization reaction between epoxide-functionalized solid support and a primary amine bearing amino acid residue of an alkaline phosphatase polypeptide to form β-hydroxy amino linkage between solid support and protein as shown in Scheme 1 below.



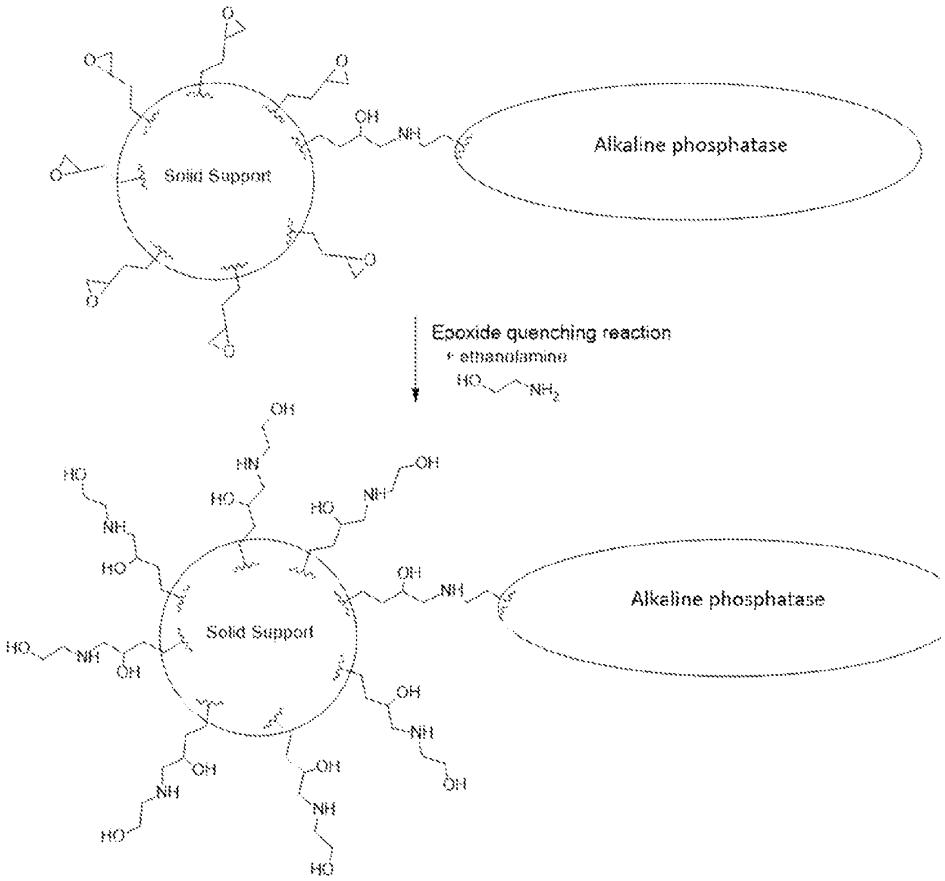
Scheme 1

[0131] The four exemplary β-hydroxy linkages that can form in the reaction between an epoxide functionalized solid support and an alkaline phosphatase protein are shown in **Scheme 2** below.



Scheme 2

[0132] Further, as described in the Examples and elsewhere herein, it has been found that an unexpected improvement in the performance of the immobilized alkaline phosphatase polypeptide occurs when immobilization on an epoxide functionalized solid support includes a step of quenching the excess epoxide functional group on the support with one or more quenching reagents. **Scheme 3** shows an exemplary quenching reaction that use ethanolamine to "quench" the remaining unreacted epoxide groups on a solid support following an epoxide immobilization reaction of alkaline phosphatase protein.



Scheme 3

[0133] Accordingly, in at least one embodiment, the present invention provides a method of preparing an immobilized alkaline phosphatase comprising:

contacting an epoxide-functionalized solid support and an alkaline phosphatase polypeptide in solution under suitable conditions for conjugating or immobilizing the alkaline phosphatase polypeptide to the solid support; and

adding an epoxide quenching reagent to the solution to deactivate or cap the epoxide groups on the solid support.

[0134] In at least one embodiment, a method for preparing an immobilized alkaline phosphatase comprises:

(a) preparing a solution comprising an epoxide functionalized solid support and an alkaline phosphatase polypeptide;

(b) allowing the solution to incubate at a temperature of about 4 C to about 60 C for about 4 h

to about 48 h; and

(c) adding an epoxide quenching reagent to the solution of step (b).

[0135] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, or TEoA-HCl, and any combinations thereof.

[0136] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide quenching reagent is 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, or TEoA-HCl, and any combinations thereof.

[0137] In some embodiments, the concentration of the epoxide quenching reagent used is about 10 mM to about 5000 mM, 500 mM to about 5000 mM, about 10 mM to about 3000 mM, about 100 mM to about 4000 mM, about 500 mM to about 3000 mM.

[0138] In some embodiments, the solution with the added quenching reagent is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0139] In some embodiments, as noted above, the reaction or incubation of the alkaline phosphatase polypeptide and the epoxide functionalized solid support is carried out at 20 °C to 60 °C.

[0140] In some embodiments, the method further comprises removing the quenching reagent by washing the immobilized alkaline phosphatase on the solid support.

[0141] In some embodiments, the wash solution has a pH of about 7, about 7.8, about 8, or about 9.

[0142] In some embodiments, the wash solution is selected from a solution of NaCl/ TEoA-HCL, TEoA-HCL, MOPS, MOPS/NaCl, and NaCl.

[0143] In some embodiments, the wash solution is selected from 500 mM NaCl/50 mM TEoA-HCL, 50mM TEoA-HCL, 250 mM MOPS, 250 mM MOPS/500 mM NaCl, and 500 mM NaCl.

[0144] In some embodiments, the wash solution is a high salt solution. Exemplary high salt solutions are provided in the Examples.

[0145] In some embodiments, the alkaline phosphatase polypeptide is provided at a concentration of about 0.1 g/L to about 100 g/L; about 1 g/L to about 90 g/L, about 2 g/L to about 80 g/L, 3 g/L to about 70 g/L, about 4 g/L to about 60 g/L, about 5 g/L to about 50 g/L, about 10 g/L to about 40 g/L, or about 20 g/L to about 30 g/L. In some embodiments, the alkaline phosphatase polypeptide is provided at a concentration of about 0.1 g/L, about 1 g/L, about 2 g/L, about 3 g/L, about 5 g/L, about

10 g/L, about 20 g/L, about 30 g/L, about 40 g/L, about 50 g/L, about 60 g/L, about 70 g/L. about 80 g/L, about 90 g/L or about 100 g/L.

[0146] In some embodiments of the method, the solution for reaction of the alkaline phosphatase polypeptide and the epoxide functionalized solid support is a buffered aqueous solution at a pH of about 6.5 to about 8.5. In some embodiments, the buffer in the aqueous solution is selected from borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris).

[0147] In some embodiments, the buffer concentration is from about 10 mM to about 2000 mM, 50 mM to about 1500 mM, about 100 mM to about 1000 mM, about 150 mM to about 800 mM, about 200 mM to about 600 mM, or about 300 to about 500 mM. In some embodiments, the buffer concentration is about 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 1000 mM, 1500 mM, or 2000 mM.

[0148] In least one embodiment of this method, the solution for contacting the alkaline phosphatase polypeptide and the epoxide functionalized solid support, such as in step (a) above, is a buffered aqueous solution at a pH of about 6.5 to about 9 containing the alkaline phosphatase polypeptide at a concentration of about 0.1 g/L to about 50 g/L; optionally, wherein the buffer in the aqueous solution is selected from borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), and the buffer concentration is from about 100 mM to about 1000 mM.

[0149] In some embodiments, a second epoxide quenching reagent is used. In some embodiments, the method further comprises adding a second quenching reagent to the solution of the alkaline phosphatase and epoxide functionalized solid support. In some embodiments, the second epoxide quenching reagent is different from the first epoxide quenching reagent. In some embodiments, the second epoxide quenching reagent is used after quenching or capping of the epoxide groups using the first quenching reagent. In some embodiments, the second quenching reagent is added after completion of the treatment with the quenching reagent, i.e., first quenching reagent. In some embodiments, the second quenching reagent is used after removal, e.g., washing, of the first quenching reagent from the immobilized alkaline phosphatase on the solid support.

[0150] In some embodiments, the second quenching reagent is reacted in a buffered aqueous solution at a pH of about 6.5 to about 8.5. In some embodiments, the solution comprises a buffer selected from borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris).

[0151] In some embodiments, the buffer concentration for use with the second quenching reagent is from about 10 mM to about 2000 mM, 50 mM to about 1500 mM, about 100 mM to about 1000 mM, about 150 mM to about 800 mM, about 200 mM to about 600 mM, or about 300 to about 500 mM. In some embodiments, the buffer concentration is about 10 mM, 50 mM, 100 mM, 150 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 1000 mM, 1500 mM, or 2000 mM.

[0152] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the second epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, or TEoA-HCl.

[0153] In some embodiments of the method of preparing an immobilized alkaline phosphatase, the second epoxide quenching reagent is 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, or N-acetyl-L-cysteine, β-mercaptoethanol, or TEoA-HCl, or any combinations thereof.

[0154] In at least one embodiment, the concentration of the second epoxide quenching reagent in the solution is about 10 mM to about 3000 mM, about 50 mM to about 2500 mM, about 100 mM to about 2500 mM, about 200 mM to about 2000 mM, or about 500 mM to about 1000 mM. In some embodiments, the concentration of second quenching reagent in the solution is about 10 mM, about 50 mM, about 100 mM, about 200 mM, about 500 mM, about 1000 mM, about 1500 mM, about 2000 mM, about 2500 mM, or about 3000 mM.

[0155] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the second epoxide quenching reagent is 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, or TEoA-HCl. In at least one embodiment, the concentration of the second epoxide quenching reagent in the solution is about 500 mM to about 5000 mM. In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the solution of step (c) is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

[0156] It is to be understood that the treatment of the support medium with quenching reagent following reaction of the alkaline phosphatase polypeptide with the epoxide functionalized support is not limited to two treatments. In some embodiments, the additional treatments of the support medium with the quenching reagent can be carried out multiple times to reduce the levels of epoxide groups on the support medium.

[0157] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises particles, beads, resins, or membranes, among others, comprised of polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, polyacrylamide, polyamino acid or protein (e.g., gelatin), polysaccharide or cellulosic (e.g., agarose, dextran, chitosan, pectin, etc.), controlled pore glass, or silica (e.g., silicon dioxide).

[0158] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises particles comprises polyacrylate, methacrylate, polymethacrylate, amino-epoxy polymethacrylate, phenolate, polystyrene, polysaccharide (e.g., cellulosic), silica, or controlled pore glass.

[0159] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises particles having a particle size range of about 1 μm to about 2000 μm, about 10 μm to about 1700 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. In some embodiments, the epoxide functionalized particles have a particle size of about 1 μm, about 10 μm, about 50 μm, about 100 μm, about 200 μm, about 300 μm, about 400 μm, about 500 μm, about 1000 μm, about 1500 μm, 1600 μm, 1700 μm, 1800 μm, 1900 μm, or 2000 μm. In some embodiments, the size of the particles are selected for solution flow properties, amount and density of functional groups, surface area, diffusion of substrates, and the like.

[0160] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

[0161] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises particles having an average pore diameter of about 100 angstroms (Å) to about 2000 angstroms (Å), 150 angstroms (Å) to about 1800 angstroms (Å), 200 angstroms (Å) to about 1600 angstroms (Å), 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). In some embodiments, the particles comprise an average pore diameter or pore size of about 100 angstroms (Å), 150 angstroms (Å), 200 angstroms (Å), 250 angstroms (Å), 300 angstroms (Å), about 600 angstroms (Å), about 1000 angstroms (Å), about 1500 angstroms (Å), 1600 angstroms (Å), 1700 angstroms (Å), 1800 angstroms (Å), 1900 angstroms (Å), 2000 angstroms (Å), or greater.

[0162] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

[0163] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the epoxide functionalized solid support is a resin selected from 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), EMC7042/M (Sunresin), EMC7042/S (Sunresin), 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), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0164] In various embodiments, the immobilized engineered alkaline phosphatase polypeptides can be prepared using alkaline phosphatase polypeptide in various forms, for example, such as an isolated preparation, as a substantially purified enzyme, whole cells transformed with gene(s) encoding the enzyme, and/or as cell extracts and/or lysates of such cells. In some embodiments, the final immobilized enzyme preparations can be lyophilized, spray-dried, precipitated, or be in the form of a crude paste, as further discussed below. In some embodiments, engineered alkaline phosphatase polypeptide is simultaneously purified and immobilized on a solid support.

[0165] In some embodiments of the methods of preparing the immobilized alkaline phosphatase as described herein, the alkaline phosphatase polypeptide may be added to the reaction mixture comprising the functionalized solid support in the form of a purified enzyme, partially purified enzyme, whole cells transformed with gene(s) encoding the enzyme, as cell extracts and/or lysates of such cells, and/or as an enzyme immobilized on a solid support. Whole cells transformed with gene(s) encoding the alkaline phosphatase enzyme or cell extracts, lysates thereof, and isolated enzymes may be employed in a variety of different forms, including solid (e.g., lyophilized, spray-dried, and the like) or semisolid (e.g., a crude paste). The cell extracts or cell lysates may be partially purified by precipitation (ammonium sulfate, polyethyleneimine, heat treatment or the like, followed by a desalting procedure prior to lyophilization (e.g., ultrafiltration, dialysis, etc.). Any of the enzyme preparations (including whole cell preparations) may be stabilized by crosslinking using known

crosslinking agents, such as, for example, glutaraldehyde or immobilization to a solid phase (e.g., Eupergit C, and the like).

[0166] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, a purified or substantially purified alkaline phosphatase polypeptide preparation will be used in the immobilization process and the immobilized enzyme retained as a slurry in an aqueous buffer. Such a resin slurry can then be used to pack a column for use in flow-through oligonucleotide synthesis process.

[0167] In at least one embodiment of the method of preparing an immobilized alkaline phosphatase, the immobilized alkaline phosphatase comprises between 0.5 wt%-5 wt% alkaline phosphatase polypeptide, 0.5 wt%- 3 wt% alkaline phosphatase polypeptide, 1 wt%-2.5 wt% alkaline phosphatase polypeptide or 1 wt% - 2 wt% alkaline phosphatase polypeptide.

[0168] In some embodiments of the method, the immobilized alkaline phosphatase on the support retains at least 50%, 60%, 70%, 75%, 80%, 85%, or 90% or greater phosphatase activity. In some embodiments, the % phosphatase activity retained on the support is determined by amount of phosphatase polypeptide activity on the support compared to amount of untreated alkaline phosphatase polypeptide.

[0169] In some embodiments of the method, the immobilized alkaline phosphatase on the support has leaching of phosphatase of less than 1%, 2%, 5%, 10%, 15%, 20%, 25% or 30% of measured activity on support. In some embodiments of the method, the immobilized alkaline phosphatase in after storage has less than 0.2% of the total wt% bound alkaline phosphatase leaching off the solid support.

[0170] In some embodiments of the method, at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of the total wt % bound alkaline phosphatase remains on the solid support.

[0171] In some embodiments, the immobilized alkaline phosphatase polypeptides described herein are provided in the form of kits. The immobilized enzymes in the kits may be present individually or as a plurality of immobilized enzymes. The kits can further include reagents for carrying out the enzymatic reactions, substrates for assessing the activity of enzymes, as well as reagents for detecting the products. The kits can also include reagent dispensers and instructions for use of the kits.

[0172] In some embodiments, the kits of the present invention include arrays comprising a plurality of different immobilized alkaline phosphatase polypeptides at different addressable position, wherein the different immobilized polypeptides are different variants of a reference sequence each having at least one different improved enzyme property. In some embodiments, a plurality of polypeptides immobilized on solid supports are configured on an array at various locations, addressable for robotic delivery of reagents, or by detection methods and/or instruments. The array can be used to test a variety of substrate compounds for conversion by the engineered alkaline phosphatase polypeptides.

Such arrays comprising a plurality of engineered polypeptides and methods of their use are known in the art (See e.g., WO2009/008908A2).

Compositions and Immobilized Recombinant Phosphatases

[0173] In a further aspect, the present disclosure provide a composition of an immobilized alkaline phosphatase disclosed herein. In some embodiments, the present disclosure provides a composition of immobilized alkaline phosphatase prepared by any of the methods described herein.

[0174] In some embodiments, the immobilized phosphatase is combined with other components and compounds to provide compositions and formulations comprising the immobilized alkaline phosphatase as appropriate for different applications and uses.

[0175] In some embodiments, the alkaline phosphatase is immobilized on a solid support comprising an ion-exchange resin, polyvinyl chloride, polyacrylamide, polyacrylate, polymethacrylate, polyethylene glycol, dextran, and/or agarose (e.g., cross-linked agarose).

[0176] In some embodiments, the solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

[0177] In some embodiments, the alkaline phosphatase is immobilized on a solid support comprising a medium selected from epoxide functionalized hydrophilic polyacrylate resin, polymethacrylate resin, epoxide functionalized polymethacrylate resin, amino-epoxy functionalized polymethacrylate resin, polyacrylic resin, epoxide functionalized polyacrylic resin, controlled pore glass (CPG) and epoxide functionalized controlled pore glass.

[0178] In some embodiments, the alkaline phosphatase is immobilized on a solid support which is an epoxide functionalized solid support or an amino-epoxide functionalized solid support.

[0179] In a preferred embodiment, the composition comprises an immobilized alkaline phosphatase wherein the solid support is a polyacrylate substrate, particularly a hydrophilic polyacrylate support where the alkaline phosphatase is covalently attached using epoxide groups on the polyacrylate.

[0180] In some embodiments, the solid support has an average pore diameter of about 250 angstroms (Å) to about 2000 (Å), about 250 angstroms (Å) to about 1500 Å, about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å). Other average pore sizes for the solid support include those described herein.

[0181] In some embodiments, the solid support has a particle size range of about 10 μm and 1500 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm. Other particles sizes for the solid support include those described herein.

[0182] In some embodiments of the immobilized alkaline phosphatase, the covalent linkage is selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

[0183] In some embodiments of the immobilized alkaline phosphatase, the covalent linkage comprises at least two types of covalent linkages selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

[0184] In some embodiments, the immobilized alkaline phosphatase comprises quenched epoxide group comprising a covalent linkage with a quenching reagent and, optionally, a second quenching compound, selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

[0185] In some embodiments, the immobilized alkaline phosphatase comprises quenched epoxide group comprising a covalent linkage with a quenching compound and, optionally, a second quenching compound, 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, TEoA-HCl, and any combinations thereof.

[0186] In some embodiments, the alkaline phosphatase is immobilized on a epoxide functionalized solid support comprising a resin selected from 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), EMC7042/M (Sunresin), EMC7042/S (Sunresin), 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), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH

(ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

[0187] In some embodiments, the immobilized alkaline phosphatase retains at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 96%, 97%, 98%, 99% or greater activity following attachment to the solid support.

[0188] In some embodiments, the immobilized alkaline phosphatase exhibits less than 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.25% or less of the total wt % bound alkaline phosphatase leaching off the solid support after at least 24 hr of storage at 4 °C.

[0189] In some embodiments, the immobilized alkaline phosphatase exhibits less than 5%, 4%, 3%, 2%, 1% or less of the total wt % bound alkaline phosphatase leaching off the solid support after wash at or above pH 7.00

[0190] In some embodiments, the immobilized alkaline phosphatase has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of total wt % bound alkaline phosphatase remaining attached to the solid support after a wash at or above pH 7.5.

[0191] In some embodiments, the immobilized alkaline phosphatase has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of the total wt % bound alkaline phosphatase remaining attached to the solid support after at least 24 hr of storage at 4 °C.

[0192] In some embodiments, the immobilized alkaline phosphatase has a total wt % bound alkaline phosphatase of at least 0.5 wt%.

[0193] In some embodiments, the immobilized alkaline phosphatase has a total wt% bound alkaline phosphatase of about 0.5 wt%, 1 wt%, 2 wt%, or 4 wt%.

[0194] In some embodiments, the immobilized alkaline phosphatase retains at least 85% of the original immobilized alkaline phosphatase activity after a first incubation with a substrate and a first wash.

[0195] In some embodiments, the immobilized alkaline phosphatase exhibits at least 1% conversion of mAQP to mA. In some embodiments, the immobilized alkaline phosphatase exhibits at least 1% conversion of mAQP to mA after 15 minutes.

[0196] In some embodiments, the immobilized alkaline phosphatase polypeptides retain their improved activity, and/or other improved properties. In such embodiments, the immobilized polypeptides can facilitate the biocatalytic conversion of the substrate compounds or other suitable substrates to the product and after the reaction is complete are easily retained (e.g., by retaining beads on which polypeptide is immobilized) and then reused or recycled in subsequent reactions. Such immobilized enzyme processes allow for further efficiency and cost reduction.

[0197] In some embodiments, the composition of an immobilized alkaline phosphatase comprises an alkaline phosphatase and a solid support wherein the alkaline phosphatase is attached to the solid support through a covalent linkage.

[0198] In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Thermoflexibacter*, *Pyrococcus*, *Thermotoga*, *Pseudothermotog*a, or *Bacillus*.

[0199] In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of T*hermoflexibacter*. In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Thermoflexibacter ruber*.

[0200] In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Pyrococcus*. In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Pyrococcus abyssi*, *Pyrococcus chitonophagus*, *Pyrococcus furiosus*, *Pyrococcus glycovorans*, *Pyrococcus horikoshii*, *Pyrococcus kodakaraensis*, *Pyrococcus kukulkanii*, *Pyrococcus woesei*, or Pyrococcus *yayanosii*.

[0201] In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Thermotoga*. In some embodiments, the recombinant phosphatase comprises an alkaline phosphatase of *Thermotoga caldifontis*, *Thermotoga elfii*, *Thermotoga hypogea*, *Thermotoga lettingae*, *Thermotoga maritima*, *Thermotoga naphthophila*, *Thermotoga neapolitana*, *Thermotoga petrophila*, *Thermotoga profunda*, *Thermotoga subterranea*, *Thermotoga thermarum*, *Pseudothermotoga caldifontis*, *Pseudothermotoga elfii*, *Pseudothermotoga hypogea*, *Pseudothermotoga hypogea*, *Pseudothermotoga lettingae*, *Pseudothermotoga profunda*, *Pseudothermotoga subterranea*, or *Pseudothermotoga thermarum*.

[0202] In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of bacillus. In some embodiments, the immobilized alkaline phosphatase comprises an alkaline phosphatase of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus siamensis*, *Bacillus mycoides*, *Bacillus thuringiensis*, *Bacillus pseudomycoides*, *Bacillus spizizenii*, *Bacillus anthracis*, *Bacillus paranthracis*, *Bacillus cereus*, *Bacillus mobilis*, *Bacillus toyonensis*, *Bacillus amyloliquefaciens*, *Bacillus pacificus*, *Bacillus pumilus*, *Bacillus velezensis*, *Bacillus paramycoides*, *Bacillus amyloliquefaciens*, *Bacillus albus*, *Bacillus sonorensis*, *Bacillus badius*, or *Bacillus thuringiensis*.

[0203] In some embodiments, the immobilized alkaline phosphatase is the alkaline phosphatase of *Thermoflexibacter ruber*, *Pyrococcus furiosus*, *Thermotoga maritima*, *Thermotoga sp. 50_64*, *Pseudothermotoga lettingae*, *Thermotoga neapolitana*, or *Bacillus licheniformis*.

[0204] In some embodiments, the immobilized alkaline phosphatase comprises an immobilized recombinant phosphatase comprising an N-terminal deletion of up to 5, 10, 25, 30, 40, 45, or 50 amino acids of the naturally occurring amino acid sequence of the phosphatase. In some

embodiments, the N-terminal deletion preserves the capability of the phosphatase to form an active dimer subunit quaternary structure, i.e., a multimer formed of two phosphatase monomers. In addition to activity, the presence of a dimer can be ascertained by known techniques, for example, molecular sieve chromatography.

[0205] In some embodiments, the alkaline phosphatase comprises an alkaline phosphatase provided as a fusion protein. In some embodiments, the alkaline phosphatase described herein can be fused to a variety of polypeptide sequences, such as, by way of example and not limitation, polypeptide tags that can be used for detection, purification, immobilization on a support medium, or fusion to another protein. In some embodiments, the fusion protein of the alkaline phosphatase comprises a glycine-histidine or histidine-tag (His-tag). In some embodiments, the fusion protein of the alkaline phosphatase comprises an epitope tag, such as c-myc, FLAG, V5, or hemagglutinin (HA). In some embodiments, the fusion protein of the alkaline phosphatase comprises a GST, SUMO, Strep, MBP, or GFP tag. In some embodiments, the alkaline phosphatase is fused to a polylysine, for example, for conjugation to a support medium via the amino group of the polylysine. In some embodiments, the polylysine is from 2-10 lysine units in length. In some embodiments, the fusion is to the amino (N-) terminus of alkaline phosphatase polypeptide. In some embodiments, the fusion is to the carboxy (C-) terminus of the alkaline phosphatase polypeptide. In some embodiments, the fusion is selected or designed to preserve the activity of the phosphatase.

[0206] In some embodiments, the immobilized alkaline phosphatase comprises the recombinant alkaline phosphatase selected from the group comprising SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18.

[0207] In some embodiments, the present disclosure further provides functional fragments or biologically active fragments of alkaline phosphatase described herein, where the fragments are immobilized on a solid substrate. Thus, for each and every embodiment herein of an alkaline phosphatase described herein, a functional fragment or biologically active fragment of the alkaline phosphatase is provided herewith. In some embodiments, a functional fragment or biologically active fragments of an alkaline phosphatase comprises at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the activity of the phosphatase polypeptide from which it was derived (i.e., the parent phosphatase). In some embodiments, functional fragments or biologically active fragments comprise at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the parent sequence of the phosphatase. In some embodiments, the functional fragment will be truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, less than 50 amino acids, less than 55 amino acids, less than 60 amino acids, less than 65 amino acids, or less than 70 amino acids.

[0208] In some embodiments, a functional fragment of a recombinant phosphatase herein comprises at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, or 99% of the parent sequence of the recombinant phosphatase. In some embodiments, the functional fragment will be truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, less than 50, less than 55, less than 60, less than 65, or less than 70 amino acids.

[0209] Chromatographic techniques for isolation/purification of the phosphatase polypeptides include, among others, reverse phase chromatography, high-performance liquid chromatography, ion-exchange chromatography, hydrophobic-interaction chromatography, size-exclusion chromatography, gel electrophoresis, and affinity chromatography. Methods of purifying active alkaline phosphatases are known in the art; any method of purifying an alkaline phosphatase may be used to obtain the alkaline phosphatase used in the immobilized alkaline phosphate compositions. Conditions for purifying the phosphatase depends, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., and will be apparent to those having skill in the art. In some embodiments, affinity techniques may be used to isolate the phosphatase. For affinity chromatography purification, an antibody that specifically binds the recombinant phosphatase polypeptide may be used. In some embodiments, an affinity tag, e.g., His-tag, can be introduced into the phosphatase for purposes of isolation/purification.

Uses of Immobilized Phosphatases

[0210] In another aspect, the present disclosure provides uses of the immobilized alkaline phosphatases for cleaving (i.e., hydrolysis) of a substrate having a cleavable phosphate groups, e.g., phosphate monoester or analog thereof.

[0211] In some embodiments, a method of cleaving a phosphate monoester or analog thereof, comprises contacting a substrate comprising a cleavable phosphate group, e.g., phosphate monoester or analog thereof, with an immobilized alkaline phosphatase described herein under suitable reaction conditions for cleaving of the phosphate group, such as the phosphate monoester or analog thereof, by the immobilized alkaline phosphatase. In some embodiments, the contacting the phosphatase substrate with the immobilized alkaline phosphatase is an in vitro reaction.

[0212] In some embodiments, the phosphatase substrate comprising a phosphate monoester or analog thereof is a phosphatase substrate used to measure phosphatase activity. Exemplary phosphatase substrates used to measure activity include, among others, dinitrophenyl phosphate (pNPP), 2,6-dichloro-4-nitrophenyl phosphate, 5-bromo-4-chloro-3-indolyl phosphate, dimethylacridinone (DDAO) phosphate, 4-methylumbelliferyl phosphate, and ELF 97 phosphate.

[0213] In some embodiments, the phosphatase substrate comprises NTP, NDP, NMP, 3'-P-NTP, 3'-P-NDP, 3'-P-NMP, or Np. In some embodiments, the phosphatase substrate comprises a modified NTP, NDP, NMP, 3'-P-NTP, 3'-P-NDP, 3'-P-NMP, or Np. In some embodiments, the modified NTP, NDP, NMP, 3'-P-NTP, 3'-P-NDP, 3'-P-NMP, or Np comprises a thiophosphate group, for

example, NTP-α-S, NDP- α -S, NMP- α -S, 3'-P-NTP- α-S, 3'-P-NDP- α-S, 3'-P-NMP- α-S, NpS, or any combinations thereof.

[0214] In some embodiments, the phosphatase substrate comprises a polynucleotide or oligonucleotide with a 5'-P, 3'-P, 5'-P(S), 3'-P(S), or any suitable combinations thereof.

[0215] In some embodiments, the polynucleotide or oligonucleotide substrate is single stranded or double stranded. In some embodiments, the polynucleotide or oligonucleotide substrate is DNA, RNA, or a mixture of DNA and RNA. In some embodiments, the polynucleotide or oligonucleotide substrate comprises at least a modified nucleoside or internucleoside linkage, as described herein.

[0216] In some embodiments, phosphatase reaction is carried out at a suitable temperature and reaction time period. In some embodiments, the reaction temperature is from about 2° C to about 80° C. In some embodiments, the phosphatase reaction temperature is from 4 °C to 80 °C, 4 °C to 75 °C, 4 °C to 70 °C, 4 °C to 60 °C, 4 °C to 50 °C, or 10 °C to 40 °C. In some embodiments, the phosphatase reaction temperature is 2 °C, 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, or 80 °C. In some embodiments, the phosphatase reaction temperature can use different temperatures, for example a temperature at which double stranded polynucleotide or oligonucleotide substrates are single stranded and a lower temperature at which the phosphatase has greater stability.

[0217] In some embodiments, the suitable reaction conditions comprise a pH of ≥ 7. In some embodiments, the suitable reaction conditions comprise a pH of about 7-10. In some embodiments, the suitable reaction conditions comprise a pH of about 7.5-9, particularly pH 7.5-8. In some embodiments, the suitable reaction conditions comprise about pH 7. 7.5, 8, 8.5, 9, 9.5 or 10.

[0218] In some embodiments, the phosphatase reaction time can be a sufficient or suitable time for cleavage of phosphomonoester moiety or analog thereof. In some embodiments, the phosphatase reaction time is from 0.1-72 hr or longer. In some embodiments, the phosphatase reaction time is 0.1-72 hr, 0.5-65 hr, 1-50 hr, 2-48 hr, or 2-24 hr. In some embodiments, the phosphatase reaction time is 0.5, 1, 2, 4, 5, 12, 24, 48, or 72 hr or longer. In some embodiments, the reaction time is adjusted based on, among others, whether the acceptor oligonucleotide substrate is in solution or immobilized on a support medium, the nature of the substrate (e.g., nucleotide, oligonucleotide, phosphomonoester, phosphate monoester analog, etc.)

[0219] In some embodiments of the method, the recombinant phosphatase is immobilized on a support medium and the phosphatase substrate reacted with the immobilized recombinant phosphatase. In some embodiments, the immobilized recombinant phosphatase is retained in a chamber, for example a column or vessel, and the phosphatase substrate fed into the chamber, e.g., column or vessel, for reaction with the recombinant phosphatase.

[0220] In some embodiments, the phosphatase substrate is contacted with the immobilized alkaline phosphatase and separated from the immobilized alkaline phosphatase following the cleavage reaction. In some embodiments, the solution containing the phosphatase substrate and cleavage products can be recirculated through the immobilized recombinant phosphatase in the chamber to increase or complete the dephosphorylation reaction.

[0221] In some alternative embodiments, a polynucleotide or oligonucleotide substrate is bound to a support medium, and the polynucleotide or oligonucleotide bound to a support medium is contacted with the immobilized alkaline phosphatase. In some embodiments, the immobilized alkaline phosphatase is retained in a chamber, for example a column or a reaction vessel, and the polynucleotide or oligonucleotide substrate fed into the chamber, e.g., column or vessel, for reaction with the immobilized alkaline phosphatase.

[0222] In some embodiments of the method, the immobilized alkaline phosphatase comprises a packed solid support or resin in a column. In some embodiments, the contacting of the substrate with the immobilized alkaline phosphatase is by flowing a reaction solution comprising the substrate through the packed solid support or resin in the column.

[0223] In some embodiments, the immobilized alkaline phosphatase on a solid support or resin is dispersed or suspended in the reaction solution, such as in a chamber or vessel. In some embodiments, the reaction solution with the dispersed or suspended immobilized alkaline phosphatase is agitated, such as with an impeller.

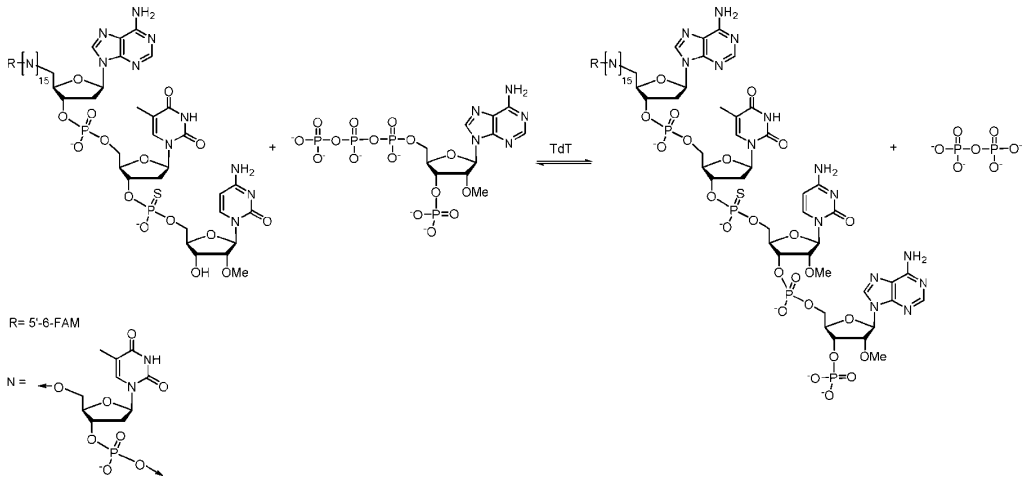
[0224] In some embodiments, the polynucleotide or oligonucleotide substrate in solution is separated from the immobilized alkaline phosphatase following dephosphorylation reaction. In some embodiments, the solution containing the polynucleotide or oligonucleotide substrate following separation from the immobilized alkaline phosphatase is recirculated through the chamber containing the immobilized alkaline phosphatase to increase or complete the dephosphorylation reaction.

[0225] In some embodiments, the immobilized alkaline phosphatase is used in a method for synthesis of an oligonucleotide, where the method comprises an aqueous liquid phase comprising substrates, e.g., NTPs and oligonucleotides, for reaction with the immobilized alkaline phosphatase. In some methods, the immobilized alkaline phosphatase is used in template dependent synthesis of an oligonucleotide. In some embodiments the immobilized alkaline phosphatase is used in template independent synthesis of an oligonucleotide.

[0226] In some embodiments, the method for synthesis of an oligonucleotide comprising an aqueous phase and an immobilized alkaline phosphatase further comprises a column solid support. In some embodiments, the method for synthesis of an oligonucleotide comprising an aqueous phase and an immobilized alkaline phosphatase further comprises a batch method with a solid support. In some embodiments, the oligo acceptor substrate and/or growing oligonucleotide chain are provided in an

aqueous phase. The phrases "oligo acceptor substrate", "acceptor substrate", "oligonucleotide substrate", "polynucleotide substrate" and "oligonucleotide or polynucleotide substrate" are used interchangeably herein. In some embodiments, the nucleotide triphosphate, the modified nucleotide triphosphate, or the NTP-3'-O-RBG are provided in an aqueous phase. In some embodiments, the method further comprises removing unreacted nucleotide triphosphates, modified nucleotide triphosphates, or NTP-3'-O-RBGs from the oligo acceptor substrate and/or growing oligonucleotide chain. In some embodiments of the described method for synthesis of an oligonucleotide, the oligo acceptor substrate and oligonucleotide are immobilized. In some embodiments, neither the oligo acceptor substrate nor the TdT or template-independent polymerase are immobilized.

[0227] In some embodiments, the alkaline phosphatase is used in a method of enzyme catalyzed template independent oligonucleotide synthesis, as depicted in **Scheme 4.**



Scheme 4

[0228] As depicted in **Scheme 4**, the terminal deoxynucleotidyl transferase (TdT) or terminal nucleotidyl transferase (TnT) is used to add an NTP comprising a phosphate at the 3' position of the sugar moiety to the end of the growing oligonucleotide substrate. The TnT catalyzes the addition, while an inorganic pyrophosphatase (not pictured) is used to degrade the resulting pyrophosphate to shift the reaction equilibrium toward the forward direction. The immobilized alkaline phosphate is used in a second step to remove the 3'-phosphate blocking group to allow addition of another NTP-3'P substrate.

[0229] The "NTP" substrates used are also called "NAP" substrates because they have a fourth ("Quad") phosphate at the 3' position. This 3'-phosphate is also referred to as a removable blocking group ("RBG").

[0230] The Nap's may also have an alpha-thio phosphate modification resulting in formation of phosphorothioate linkage in the extended oligonucleotide.

[0231] The NQPs may also have a 2'-modification to protect that position when used in synthesis ribo-oligonucleotides (e.g., siRNA oligos). **Table 3** below shows generic NQP structures with differing 2'-modifications.

Table 3	
2'-F-NQP	2'-F-α-thio-NQP
2'-OMe-NQP	2'-OMe-α-thio-NQP
2'-OEt-NQP	2'-OEt-α-thio-NQP
"Base" represents a standard nucleobase (e.g., A, C, T, G, U).	

[0232] In at least one embodiment of the immobilized alkaline phosphatase, the immobilized alkaline phosphatase is capable of hydrolysis of a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide during a method of enzymatic template-independent synthesis.

[0233] In at least one embodiment of the immobilized alkaline phosphatase, the alkaline phosphatase polypeptide has an hydrolysis activity on a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide.

[0234] In another aspect, the present invention also provides a method for hydrolysis of a blocking group during oligonucleotide synthesis comprising: contacting an immobilized alkaline phosphatase of the present disclosure with a reaction solution comprising an oligonucleotide comprising a 3' terminal nucleotide with a 3' phosphate blocking group on the sugar moiety, and whereby the 3' phosphate blocking group is hydrolyzed, allowing a further cycle of addition of a NTP with a 3' phosphate blocking group to the 3' terminal end of the oligonucleotide.

[0235] In at least one embodiment of the method for hydrolysis, the acceptor oligonucleotide is not immobilized or linked to a solid support.

[0236] As noted above, in at least one embodiment of the method for hydrolysis, the immobilized alkaline phosphatase comprises a packed resin in a column.

[0237] In at least one embodiment of the method for hydrolysis, said contacting comprises flowing the reaction solution through the packed resin in the column.

[0238] In at least one embodiment of the method for hydrolysis, the reaction solution comprises about 100 mM TEA buffer and is at about pH 7.8.

[0239] In at least one embodiment of the method for hydrolysis, the alkaline phosphatase polypeptide has an activity of hydrolysis of a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide.

[0240] In at least one embodiment of the method for hydrolysis, the donor nucleotide triphosphate (NTP) reagent comprises an α-thiophosphate group.

[0241] In at least one embodiment of the method for hydrolysis, the donor nucleotide triphosphate (NTP) reagent comprises a 2' modification; optionally wherein the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

[0242] In at least one embodiment of the method for hydrolysis, the donor nucleotide triphosphate (NTP) reagent comprises a locked nucleic acid group.

[0243] In at least one embodiment of the method for hydrolysis, the acceptor oligonucleotide has a length of at least 3 bp, at least 4 bp, at least 5 bp, at least 6 bp, and at least 7 bp. In at least one embodiment, the acceptor oligonucleotide has a length of between about 3 bp and 10 bp.

[0244] In at least one embodiment of the method for hydrolysis, the acceptor oligonucleotide comprises at least one ribonucleotide base.

[0245] In at least one embodiment of the method for hydrolysis, the acceptor oligonucleotide comprises at least one phosphorothioate linkage.

[0246] In at least one embodiment of the method for hydrolysis, the acceptor oligonucleotide comprises at least one locked nucleic acid (LNA) linkage.

[0247] In the embodiments provided herein and illustrated in the Examples, various ranges of suitable reaction conditions that can be used in the processes, include but are not limited to, substrate loading, co-substrate loading, pH, temperature, buffer, solvent system, cofactor, polypeptide loading, and reaction time. Further suitable reaction conditions for carrying out the process for biocatalytic conversion of substrate compounds to product compounds using an immobilized alkaline phosphatase described herein can be readily optimized in view of the guidance provided herein by routine experimentation that includes, but is not limited to, contacting the immobilized alkaline phosphatase polypeptide and one or more substrate compounds under experimental reaction conditions of concentration, pH, temperature, and solvent conditions, and detecting the product compound.

[0248] The oligonucleotide acceptor substrate may be any nucleotide chain or similar moiety with an exposed 3'-OH. In some embodiments, the oligonucleotide acceptor may be single stranded. In yet other embodiments, the oligonucleotide acceptor may be double stranded or partially doubled stranded. In some embodiments, the oligonucleotide acceptor 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 oligonucleotide acceptor may comprise a chemical moiety that is not a nucleotide chain but contains a free -OH capable of being recognized as a substrate by an immobilized alkaline phosphatase.

[0249] In some embodiments, the oligonucleotide acceptor may comprise a nucleotide chain consisting of three to seven nucleotides. In some embodiments, the oligonucleotide acceptor comprising three to seven nucleotides may comprise additional modifications, as described herein. In

at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one ribonucleotide base. In some embodiments, the oligonucleotide acceptor may comprise one or more additional modifications, such as a phosphorothioate linkage or a locked nucleic acid.

[0250] In some embodiments, the oligonucleotide acceptor may comprise a nucleotide chain consisting of three to seven nucleotides and a phosphate at the 5' end of the oligo acceptor substrate. In at least one embodiment of the method for synthesizing, the acceptor oligonucleotide has a length of at least 3 bp, at least 4 bp, at least 5 bp, at least 6 bp, and at least 7 bp. In at least one embodiment, the acceptor oligonucleotide has a length of between about 3 bp and 10 bp.

[0251] In some embodiments, the oligonucleotide acceptor may comprise one or more nucleotides with a 2' modification, as described herein. In some embodiments, the oligo acceptor substrate may comprise one or more nucleotides with a 2' modification selected from 2'-OH, 2'-H, 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R' (where R' is any alkyl or aryl), or another 2' atom or chemical group.

[0252] In at least one embodiment of the method, the immobilized alkaline phosphatase polypeptide has an activity of hydrolysis of a 3'-phosphate from a donor nucleotide triphosphate (NTP) reagent after addition of the donor NTP reagent to the 3'-OH end of an oligonucleotide. Accordingly, in at least one embodiment of the method of hydrolysis, the donor nucleotide triphosphate (NTP) reagent comprises a 2' modification; optionally wherein the 2' modification is selected from 2'-O-methyl, 2'-fluoro, or 2'-O-2-methoxyethyl, 2'-$OCH_2CH_2OCH_3$, 2'-CO_2R', wherein R' is an alkyl or aryl.

[0253] 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 a polynucleotide. Accordingly, in at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one locked nucleic acid (LNA) linkage.

[0254] In some embodiments, the nucleoside or nucleotide or NTP-RBG may comprise a glycol nucleic acid (GNA) modification, wherein the sugar moiety of the NTP is replaced by propylene glycol. GNA may confer additional stability to a polynucleotide.

[0255] In some embodiments, the 3'-phosphate group of the NQP may act as a removable blocking group or protecting group that may be selectively unblocked or removed to allow further

modifications, reactions, or incorporation of the NQP into a growing oligonucleotide chain during template-dependent or template-independent oligonucleotide synthesis.

[0256] In some embodiments, the oligo acceptor substrate comprises a nucleotide chain of repeating nucleotides. In other embodiments, the oligo acceptor substrate comprises a nucleotide chain of varied nucleotides that do not repeat. In some embodiments, the oligo acceptor substrate comprises a nucleotide chain with an odd number of nucleotides. In some embodiments, the oligo acceptor substrate comprises a nucleotide with an even number of nucleotides.

[0257] In some embodiments, the oligo acceptor substrate comprises one or more nucleotide sequences selected from the following 5'-6-FAM-T10mCmCmUfA, 5'-6-FAM-T11AmC*mA*mG, 5'-6-FAM-T11mU*fA*fA, 5'-6-FAM-T15mAmUmCmU, 5'-6-FAM-T16mC*mA*mGmA, 5'-6-FAM-T16mGmUmC*mC, 5'-6-FAM-T17*fA*fAfG, 5'-6-FAM-T21mGfUfAfC, 5'-6-FAM-T26mCfCmCfG, 5'-6-FAM-T26mUfGmUfC, 5'-6-FAM-T27fGmAfU, 5'-6-FAM-T31mAmAmAfG, 5'-6-FAM-T31mUfCmAfU, 5'-6-FAM-T36fCmAfUfC, 5'-6-FAM-T36fCmAmUmC, 5'-6-FAM-T36mC*mU*mAmC, 5'-6-FAM-T36mCfCmGfG, 5'-6-FAM-T41*fC*mAfA, 5'-6-FAM-T41mAfUfCfC, 5'-6-FAM-T41mAmUmCmU, 5'-6-FAM-T41mGfUmGfG, 5'-6-FAM-T46fAmUmUfG, 5'-6-FAM-T46mAfGmUfG, 5'-6-FAM-T46mUmCmUmU, 5'-6-FAM-T51fGmUfGmU, 5'-6-FAM-T51mU*fA*fAfG, 5'-6-FAM-T56mA*fC*mAfA, 5'-6-FAM-T9mAmAmAmUmCmU, 5'P-mUmCmU, mAfGmUfG, mAmAmAmAmUmCmU, mAmAmAmUmCmU, mAmAmUmCmU, mAmUmCmU, mC*mA*mGmA, mGmUmC*mC, mUmCmU, T8fCmAmUmC, T8mCfCmCfG, T8mCfCmGfG, T8mUfGmUfC, as further described in the Examples and the accompanying sequence listing. These embodiments are intended to be non-limiting. Any suitable oligo acceptor substrate finds use in the present invention.

[0258] In some embodiments, the NTP-3'-O-RBG substrate comprises a deoxyribonucleoside triphosphate with a 3'-O-RBG. In other embodiments, the NTP-3'-O-RBG substrate may comprise a ribonucleoside triphosphate with a 3'-O-RBG. In yet other embodiments, the NTP-3'-O-RBG substrate may comprise a synthetic nucleoside triphosphate with a 3'-O-RBG. In some embodiments, the NTP-3'-O-RBG substrate may comprise a sugar ring with a number of carbons that is not five. A non-limiting example of this is a threose nucleoside triphosphate.

[0259] A range of 3' removable blocking groups for the NTP-3'-O-RBG substrate useful in the present disclosure are known in the art and include but are not limited to, -O-NH_2, -O-NO_2, -O-PO_3. In some embodiments, the NTP-3'-O-RBG substrate with 3' removable blocking group can be selected from the group consisting of NTP-3'-O-NH_2, NTP-3'-O-NO_2, or NTP-3'-O-PO_3. In some embodiments, the NTP-3'-O-RBG substrate comprises another blocking group that would sterically hinder addition of a second NTP-3'-O-RBG substrate to the 3' end of the growing oligo acceptor substrate strand prior to removal of the removable blocking from the first round of addition.

[0260] In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a natural purine or pyrimidine base, such as adenine, guanine, cytosine, thymine, or uridine. In some embodiments, deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises an unnatural base analog such as inosine, xanthine, hypoxanthine, or another base analog, as is known in the art. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a base with modifications, as is known in the art. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises a 2' modification or substitution. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises substitution of an α phosphate oxygen for an α phosphorothioate group with a sulfur atom which allows for the creation of phosphorothioate linkages. In some embodiments, the deoxyribonucleoside triphosphate with a 3'-O-RBG or ribonucleoside triphosphate with a 3'-O-RBG further comprises substitution of two oxygens for sulfurs for the creation of phosphorodithioate linkages. Accordingly, in at least one embodiment of the method for synthesizing, the acceptor oligonucleotide comprises at least one phosphorothioate linkage.

[0261] The substrate compound(s) in the reaction mixtures can be varied, taking into consideration, for example, the desired amount of product compound, the effect of each substrate concentration on the immobilized enzyme activity, stability of the immobilized enzyme under reaction conditions, and the percent conversion of each substrate to product. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.1 μM to 1 μM, 1 μM to 2 μM, 2 μM to 3 μM, 3 μM to 5 μM, 5 μM to 10 μM, or 10 μM to 50 μM,, or 50 μM to 100 μM, or 100 μM to 500 μM, or 500 μM to 1000 μM, or 1000 μM to 2000 μM, or 2000 μM to 5000 μM, or 5000 μM to 10000, or 10000 μM or greater. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.5 to about 25 g/L, 1 to about 25 g/L, 5 to about 25 g/L, about 10 to about 25 g/L, or 20 to about 25 g/L. In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each oligo acceptor substrate of at least about 0.5 g/L, at least about 1 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, or at least about 30 g/L, or even greater.

[0262] In some embodiments, the suitable reaction conditions comprise a substrate compound loading for each NTP-3'-O-RBG or natural or modified NTP substrate of at least about 1 μM to 5 μM, 5 μM to 10 μM, 10 μM to 25 μM, 25 μM to 50 μM, 50 μM to 100 μM, 100 μM to 200 μM, 200 μM to 300 μM, 300 μM to 500 μM, 400 μM to 600 μM, 700 μM to 900 μM, 800 μM to 1000 μM, 800 μM to 1200 μM, or 1000 μM to 1500 μM, or 1600 μM to 2500 μM, or 2500 μM to 5000 μM, or 6000 μM to 12000 μM . In some embodiments, the suitable reaction conditions comprise a substrate

compound loading for each NTP-3'-O-RBG or natural or modified NTP substrate of at least about 0.5 g/L, at least about 1 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, or at least about 30 g/L, or even greater.

[0263] In some embodiments, the improved activity of the immobilized alkaline phosphatase polypeptides disclosed herein provides for processes wherein higher percentage conversion can be achieved with lower concentrations of the engineered polypeptide. In some embodiments of the process, the suitable reaction conditions comprise an engineered polypeptide amount of about 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 20% (w/w), 30% (w/w), 40% (w/w), 50% (w/w), 75% (w/w), 100% (w/w) or more of substrate compound loading.

[0264] In some embodiments, the immobilized engineered polypeptide is present at a molar ratio of engineered polypeptide to substrate of about 50 to 1, 25 to 1, 10 to 1, 5 to 1, 1 to 1, 1 to 5, 1 to 10, 1 to 25 or 1 to 50, 1 to 100, 1 to 500, 1 to 1000, or 1 to 2000. In some embodiments, the immobilized engineered polypeptide is present at a molar ratio of engineered polypeptide to substrate from a range of about 50 to 1 to a range of about 1 to 2000.

[0265] In some embodiments, the immobilized engineered polypeptide is present at about 0.01 g/L to about 50 g/L; about 0.01 to about 0.1 g/L; about 0.05 g/L to about 50 g/L; about 0.1 g/L to about 40 g/L; about 1 g/L to about 40 g/L; about 2 g/L to about 40 g/L; about 5 g/L to about 40 g/L; about 5 g/L to about 30 g/L; about 0.1 g/L to about 10 g/L; about 0.5 g/L to about 10 g/L; about 1 g/L to about 10 g/L; about 0.1 g/L to about 5 g/L; about 0.5 g/L to about 5 g/L; or about 0.1 g/L to about 2 g/L.

[0266] In some embodiments, the immobilized alkaline phosphatase polypeptide is present at about 0.01 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L, 1, 2 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, or 50 g/L.

[0267] In some embodiments, the suitable reaction conditions comprise a divalent metal cofactor. In some embodiments, the divalent metal cofactor is cobalt. In some embodiments, the cobalt (II) chloride is present at concentrations of about 1 to 1200 μM; about 50 to 400 μM; about 100 to 300 μM; or about 200 to 600 μM; about 500 to 1000 μM. In some embodiments, the cobalt (II) chloride is present at concentrations of about 150 μM; about 200 μM; about 250 μM, about 500 μM; about 1000 μM; or about 1200 μM.

[0268] In some embodiments of the reaction, a phosphatase is used to degrade inorganic phosphate and shift the reaction equilibrium toward the oligo acceptor extension product. In some embodiments, the phosphatase is an *E. coli* pyrophosphatase. In some embodiments, the phosphatase is present at a concentrate on of about 0.0001 to 0.01 units/uL; about 0.001 to 0.005 units/uL; or about 0.002 to 0.003 units/uL. In some embodiments, the phosphatase is present at a concentration of about 0.001 units/uL; about 0.002 units/uL; or about 0.003 units/uL. In some embodiments, the phosphatase is from *Geobacillus zalihae*, *Geobacillus lituanicus*, *Methanococcus aeolicus*, or *Methanotorris igneus*.

In some embodiments, the phosphatase is present at a concentration of about 0.01 to 10 μM; about 0.01 to 0.1 μM; or about 0.1 to 1 μM; or about 0.1 to 10 μM. In some embodiments, the phosphatase is present at a concentration of about 0.05 μM; about 0.5 μM; or about 1 μM; or about 2 μM; or about 5 μM; or about 10 μM.

[0269] During the course of the reaction, the pH of the reaction mixture may change. The pH of the reaction mixture may be maintained at a desired pH or within a desired pH range. This may be done by the addition of an acid or a base, before and/or during the course of the reaction. Alternatively, the pH may be controlled by using a buffer. Accordingly, in some embodiments, the reaction condition comprises a buffer. Suitable buffers to maintain desired pH ranges are known in the art and include, by way of example and not limitation, borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), and the like. In some embodiments, the reaction conditions comprise water as a suitable solvent with no buffer present.

[0270] In some embodiments, the processes of the invention are carried out in a solvent. Suitable solvents include water, aqueous buffer solutions, organic solvents, polymeric solvents, and/or co-solvent systems, which generally comprise aqueous solvents, organic solvents and/or polymeric solvents. The aqueous solvent (water or aqueous co-solvent system) may be pH-buffered or unbuffered. In some embodiments, the processes using the immobilized engineered alkaline phosphatase polypeptides can be carried out in an aqueous co-solvent system comprising an organic solvent (e.g., ethanol, isopropanol (IPA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF) ethyl acetate, butyl acetate, 1-octanol, heptane, octane, methyl t butyl ether (MTBE), toluene, and the like), ionic or polar solvents (e.g., 1-ethyl 4 methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl 3 methylimidazolium hexafluorophosphate, glycerol, polyethylene glycols, and the like). In some embodiments, the co-solvent can be a polar solvent, such as a polyol, dimethylsulfoxide (DMSO), or lower alcohol. The non-aqueous co- solvent component of an aqueous co-solvent system may be miscible with the aqueous component, providing a single liquid phase, or may be partly miscible or immiscible with the aqueous component, providing two liquid phases. Exemplary aqueous co-solvent systems can comprise water and one or more co-solvents selected from an organic solvent, polar solvent, and polyol solvent. In general, the co-solvent component of an aqueous co-solvent system is chosen such that it does not adversely inactivate the immobilized alkaline phosphatase enzyme under the reaction conditions. Appropriate co-solvent systems can be readily identified by measuring the enzymatic activity of the specified immobilized alkaline phosphatase enzyme with a defined substrate of interest in the candidate solvent system, utilizing an enzyme activity assay, such as those described herein.

[0271] In some embodiments of the process, the suitable reaction conditions comprise an aqueous co-solvent, where the co-solvent comprises DMSO at about 1% to about 50% (v/v), about 1 to about 40%

(v/v), about 2% to about 40% (v/v), about 5% to about 30% (v/v), about 10% to about 30% (v/v), or about 10% to about 20% (v/v). In some embodiments of the process, the suitable reaction conditions can comprise an aqueous co-solvent comprising ethanol at about 1% (v/v), about 5% (v/v), about 10% (v/v), about 15% (v/v), about 20% (v/v), about 25% (v/v), about 30% (v/v), about 35% (v/v), about 40% (v/v), about 45% (v/v), or about 50% (v/v).

[0272] 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, nonyl phenoxypolyethoxylethanol (NP40), TRITON™ X-100 polyethylene glycol *tert*-octylphenyl ether, polyoxyethylene-stearylamine, cetyltrimethylammonium bromide, sodium oleylamidosulfate, polyoxyethylene-sorbitan monostearate, hexadecyl dimethylamine, 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.

[0273] 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.

[0274] The quantities of reactants used in the immobilized alkaline phosphatase reaction will generally vary depending on the quantities of product desired, and concomitantly the amount of substrates employed. Those having ordinary skill in the art will readily understand how to vary these quantities to tailor them to the desired level of productivity and scale of production.

[0275] In some embodiments, the order of addition of reactants is not critical. The reactants may be added together at the same time to a solvent (e.g., monophasic solvent, biphasic aqueous co-solvent system, and the like), or alternatively, some of the reactants may be added separately, and some together at different time points. For example, the cofactor, co-substrate, and substrate may be added first to the solvent.

[0276] The synthesis processes of the present invention are generally allowed to proceed until further conversion of substrate to product does not change significantly with reaction time (e.g., less than 10% of substrate being converted, or less than 5% of substrate being converted). In some

embodiments, the reaction is allowed to proceed until there is complete or near complete conversion of substrate to product. Transformation of substrate to product can be monitored using known methods by detecting substrate and/or product, with or without derivatization. Suitable analytical methods include gas chromatography, HPLC, MS, and the like.

EXAMPLES

[0277] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention. Indeed, there are various suitable sources for many of the reagents and equipment described below. It is not intended that the present invention be limited to any particular source for any reagent or equipment item.

[0278] In the experimental disclosure below, the following abbreviations apply: M (molar); mM (millimolar), μM and μM (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); ug and μg (micrograms); L and 1 (liter); ml and mL (milliliter); ul, μl, uL, μL (microliter); 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); 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

Abbreviations for modified nucleotides	
alias	**modification**
5'-FAM	5'-6-carboxyfluorescein modification
T{n}	poly-thymidine of length n
3'P	3'-phosphorylation modification
mA	2'-methoxyadenosine
mC	2'-methoxycytidine
mG	2'-methoxyguanosine

Abbreviations for modified nucleotides	
mU	2'-methoxyuridine
fA	2'-fluoroadenosine
fC	2'-fluorocytidine
fG	2'-fluoroguanosine
fU	2'-fluorouridine
rA	adenosine ribonucleotide
rC	cytidine ribonucleotide
rG	guanosine ribonucleotide
rU	uridine ribonucleotide
*	phosphorothioate linkage
5'-P	5'-phosphorylation modification
/sgna-A/	adenosine (S)-glycol nucleic acid
/sgna-C/	cytidine (S)-glycol nucleic acid
/sgna-G/	guanosine (S)-glycol nucleic acid
/sgna-T/	thymidine (S)-glycol nucleic acid
/sgna-U/	uridine (S)-glycol nucleic acid
+A	C2'-endo locked adenosine ribonucleotide
+C	C2'-endo locked cytidine ribonucleotide
+G	C2'-endo locked guanosine ribonucleotide
+T	C2'-endo locked thymidine ribonucleotide
+U	C2'-endo locked uridine ribonucleotide
(MOE)A	2'-O-(2-methoxyethyl)adenosine
(MOE)C	2'-O-(2-methoxyethyl)cytidine
(MOE)G	2'-O-(2-methoxyethyl)guanosine
(MOE)U	2'-O-(2-methoxyethyl)uridine
ddA	2',3'-dideoxyadenosine
ddC	2',3'-dideoxycytidine
ddG	2',3'-dideoxyguanosine
ddT	2',3'-dideoxythymidine
ddU	2',3'-dideoxyuridine

Example 1

Immobilization of Alkaline Phosphatase on Affinity Resin and Enzymatic 3'-Dephosphorylation of RNA Oligomer

[0279] A recombinant alkaline phosphatase (AP) was produced in shake flask and purified as described in U.S. Patent App. No. 18/485,855, filed October 12, 2023. The storage buffer in a 0.5 mL aliquot was exchanged for TEA HCl (20 mM, pH 7.8), giving a solution containing 7.1 g/L protein as measured by A280 on a nanodrop spectrophotometer.

[0280] Affinity resins from ChiralVision were weighed out in 2.5 to 3.0 mg quantities in Eppendorf tubes and charged with the appropriate amount of AP stock solution such that the amount of protein was 2.5 wt.% of the wet mass of resin in each tube. The immobilization was then carried out with a 24 h incubation according to U.S. Patent App. No. 18/485,855.

[0281] The samples were then diluted to 150 µL using TEA HCl (20 mM, pH 7.8) as diluent and incubated at 5 °C for 24 h with 500 rpm agitation. The A280 of the resulting supernatant solution was compared to that of a solution of AP that had been incubated under the same conditions but without resin present. The percent immobilization was then calculated as: $\left(1 - \frac{A280}{A280_{neg.\ ctrl.}}\right) \times 100$

[0282] To measure the 3'-dephosphorylation activity of the immobilized AP, a 1.8 mL reaction stock was prepared containing: TTTTTTTTTTTTTTTmAmG*mGmA-3'P (SEQ ID NO: 23, 99.9 µM), 5'-6-FAM-TTTTTTTTTTTTTTTmAmG*mGmA-3'P (SEQ ID NO: 24, 0.125 µM), CoCl2 (0.25 mM), and TEA HCl (20 mM, pH 7.8). 150 µL of reaction buffer was then transferred to each sample of resin and then briefly centrifuged to collect the resin in the bottom of the container. The reactions were then incubated at 50 °C with 500 rpm agitation.

[0283] After 0.5 h, a 2 µL aliquot was removed from the supernatant of each reaction mixture and transferred into 98 µL 1 mM EDTA quench solution. A subsequent 2 µL aliquot was removed from the quench solution and processed for CE analysis as described in U.S. Patent App. No. 18/485,855. The conversion of 5'-6-FAM-TTTTTTTTTTTTTTTmAmG*mGmA-3'P (SEQ ID NO: 24) to 5'-6-FAM-TTTTTTTTTTTTTTTmAmG*mGmA (SEQ ID NO: 26) was measured as the % fluorescence of product vs total fluorescence in the CE electropherogram.

Example 2

Immobilization of Alkaline Phosphatase on Affinity Resin and Enzymatic Dephosphorylation of ATP

[0284] The alkaline phosphatase (AP) was produced in shake flask and purified as described in U.S. Patent App. No. 18/485,855, filed October 12, 2023. The storage buffer in a 0.5 mL aliquot was exchanged for TEA HCl (20 mM, pH 7.8) as described in U.S. Patent App. No. 18/485,855, giving a solution containing 12.2 g/L protein as measured by A280 on a nanodrop spectrophotometer.

[0285] Affinity resins from ChiralVision were weighed out in 4.0 to 5.5 mg quantities in Eppendorf tubes and charged with the appropriate amount of AP stock solution such that the amount of protein was 5.0 wt.% of the wet mass of resin in each tube. The immobilization was then carried out with a 24 h incubation.

[0286] Each resin sample was washed according to the general procedure outline in U.S. Patent App. No. 18/485,855. To measure the dephosphorylation activity of the immobilized AP, a reaction stock was prepared containing: ATP (1 mM), CoCl2 (0.25 mM), and TEA HCl (20 mM, pH 7.8). The reaction stock was transferred to each resin sample (200 µL stock per mg resin). The reaction mixtures briefly centrifuged to collect the resin in the bottom of the container and then incubated at 50 °C with 500 rpm agitation.

[0287] After 1 h, a 5 µL aliquot was roved from each reaction supernatant and transferred to 115 µL 75% MeOH/milli-Q water quench solution in a 96 well round bottom plate. The plate was sealed, vortexed, and centrifuged at 4000 rpm, 4 °C, for 5 min. 100 µL was then transferred out of each well into fresh wells on a separate 96 well round bottom plate and analyzed by HPLC according to U.S. Patent App. No. 18/485,855. The percent conversion of ATP was measured as the area under curve (AUC) attributed to ATP vs the total AUC for the entire chromatogram.

Example 3

Immobilization of Alkaline Phosphatase on Epoxide Functionalized Resin

[0288] The alkaline phosphatase (AP) was produced in shake flask and purified as described in U.S. Patent App. No. 18/485,855. An aliquot of the purified AP in storage buffer was diluted into MOPS buffer (20 mM, pH 7.0) to give two stocks, with concentrations of 1.0 and 0.1 g/L.

[0289] Epoxide functionalized resins purchased from ChiralVision, IB-COV-2 and IB-COV-7, were weighed out in 19 to 26 mg quantities in 2.0 mL Eppendorf tubes and briefly centrifuged to collect the resin in the bottom. The 1.0 and 0.1 g/L stocks of AP in MOPS (20 mM, pH 7.0) were then added to the resin samples such that two samples received 2.5 wt. % protein vs wet weight of resin, and the other two received 0.25 wt. % protein vs wet weight of resin. These samples were incubated 48 h at 4 °C, with no agitation for the 0 to 24 h and then 10 rpm rotation for 24 to 48 h.

[0290] To measure the percent of AP that was immobilized, the supernatant was tested for residual enzymatic activity and compared to a sample of AP that had been subjected to the same immobilization conditions but with no resin present. First, 20 µL aliquots were transferred from the supernatant of each sample and diluted to 200 µL with MOPS (20 mM, pH 7.0) buffer. Three controls were prepared by serially diluting a 0.1 g/L AP stock into 200 µL MOPS (20 mM, pH 7.0) at 5, 20, and 40-fold dilutions.

[0291] In a separate 15 mL conical tube, 5.0 mL reaction buffer was prepared containing: p-nitrophenylphosphate (4 mM), CoCl2 (0.5 mM), and TEA HCl (40 mM, pH 7.8). Then, in a 96 well clear flat-bottom plate, 100 µL from the 10x dilution of each sample supernatant and each control was combined with 100 µL from the p-nitrophenylphosphate reaction buffer. The reaction was monitored at 405 nm in a plate reader at 25 °C for 10 min, acquiring data every 10 seconds. The ratio of the reaction rates to the negative control rate gave the percent of AP immobilized: $\left(1-\frac{Rate}{Rate_{neg.\ ctrl.}}\right)\times 100$.

Example 4

Iterative Enzymatic Extension of RNA Oligomer

[0292] The TdT enzyme variant was produced in shake flask and purified as described in U.S. Patent App. No. 18/485,855. The inorganic pyrophosphatase (IPP) was produced and purified, as described

in U.S. Patent App. No. 18/485,855. Alkaline phosphatase (AP) was produced and purified, as described in U.S. Patent App. No. 18/485,855. TdT and IPP were co-immobilized on ChiralVision IB-HIS-2 resin (0.5 g), pre-loaded with $CoCl_2$, at 2.5 and 0.2 wt. % vs wet weight of resin, respectively, according to U.S. Patent App. No. 18/485,855. AP was immobilized on a separate batch of Co(II)-loaded ChiralVision IB-HIS-2 resin (0.5 g) at 2.5 wt. % protein vs wet weight of resin, according to U.S. Patent App. No. 18/485,855.

[0293] For analysis of the reaction samples, capillary electrophoresis was performed using an ABI 3500xl Genetic Analyzer (ThermoFisher). Reactions (20 μL) were quenched by the addition of 60 μL of 35 mM aqueous EDTA. Reactions (1 μL) were quenched by the addition of 99 μL of 1 mM aqueous EDTA. Quenched reactions were diluted in water to 1.25 nM oligonucleotide, and a 2-μL aliquot of this solution was transferred to a new 96-well MicroAmp Optical PCR plate or 384-well MicroAmp Optical PCR plate containing 18 μL Hi-Di™ Formamide (ThermoFisher) containing an appropriate size standard (LIZ or Alexa633). The ABI3500xl was configured with POP6 polymer, 50 cm capillaries, and a 55 °C oven temperature. Pre-run settings were 18KV for 50 sec. Injection was 10KV for 2 sec, and the run settings were 19KV for 620 sec. FAM-labeled oligo substrates and products were identified by their sizes relative to the sizing ladder.

First Extension: 5'-6-FAM-T10fCmUfCmA + mATP-3'P to 5'-6-FAM-T10fCmUfCmAmA-3'P

[0294] To a 2.0 mL Eppendorf tube was added 23 mg IB-HIS-2 resin, charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). The tube was briefly centrifuged to collect the resin in the bottom. In a separate Eppendorf tube, 450 μL reaction master mix was prepared containing: mATP-3'P (150 μM), 5'-6-FAM-T10fCmUfCmA (100 μM), $CoCl_2$ (0.25 mM), MOPS (20 mM, pH 8.0). The reaction was then incubated at 50 °C for 1.5 h at 500 rpm.

[0295] After 1.5 h, the supernatant was transferred into a separate Eppendorf tube containing a fresh 23 mg of IB-HIS-2 resin, charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). The reaction was incubated for a further 1.5 h at 50 °C with 500 rpm agitation.

[0296] Afterward, a 2 μL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to pellet residual resin particulate. The supernatant was then transferred into a new 2.0 mL Eppendorf tube and the general AP catalyzed dephosphorylation procedure outlined below was carried out.

Second Extension: 5'-6-FAM-T10fCmUfCmAmA + fGTP-3'P to 5'-6-FAM-T10fCmUfCmAmAfG-3'P

[0297] To a 2.0 mL Eppendorf tube was added 15 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). In a separate 2.0 mL Eppendorf tube, 100 μL from the de-phosphorylated product of the first extension was diluted with 1.5 μL fGTP-3'P (20 mM),

5 μL $CoCl_2$ (10 mM), 20 μL TEA·HCl (200 mM, pH 7.8), and 174 μL milli-Q water. The reaction solution was then transferred onto the resin and incubated at 50 °C for 1.5 h with 500 rpm agitation.

[0298] Afterward, a 2 μL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to pellet residual resin particulate. The supernatant was then transferred into a new 2.0 mL Eppendorf tube and the general AP catalyzed dephosphorylation procedure outlined below was carried out.

Third Extension: 5'-6-FAM-T10fCmUfCmAmAfG + mUTP-3'P to 5'-6-FAM-T10fCmUfCmAmAfGmU-3'P

[0299] To a 2.0 mL Eppendorf tube was added 15 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). In a separate tube, 100 μL from the de-phosphorylated product of the second extension was diluted with 7.5 μL mUTP-3'P (2 mM), 5 μL TEA·HCl (20 mM, pH 7.8), and 38 μL milli-Q water. The reaction solution was then transferred to the tube containing resin and incubated at 50 °C with 500 rpm agitation.

[0300] After 1.5 h, the supernatant was separated from the resin and transferred to an Eppendorf tube containing a fresh 15 mg of IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). To this mixture was also added an additional 1.9 μL $CoCl_2$ (10 mM). The reaction was incubated at 50 °C with 500 rpm agitation for an additional 1.5 h.

[0301] Afterward, a 2 μL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to pellet residual resin particulate. The supernatant was then transferred into a new 2.0 mL Eppendorf tube and the general AP catalyzed dephosphorylation procedure outlined below was carried out.

Fourth Extension: 5'-6-FAM-T10fCmUfCmAmAfGmU + fGTP-3'P to 5'-6-FAM-T10fCmUfCmAmAfGmUfG-3'P

[0302] To a 2.0 mL Eppendorf tube was added 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). In a separate tube, 75 μL from the de-phosphorylated product of the third extension was diluted with 4.0 μL fGTP-3'P (2 mM). The reaction solution was then transferred to the tube containing resin and incubated at 50 °C with 500 rpm for 1.5 h.

[0303] Afterward, a 2 μL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to pellet residual resin particulate. The supernatant was then transferred into a new 2.0 mL Eppendorf tube and the general AP catalyzed dephosphorylation procedure outlined below was carried out.

Fifth Extension: 5'-6-FAM-T10fCmUfCmAmAfGmUfG + mUTP-3'P to 5'-6-FAM-T10fCmUfCmAmAfGmUfGmU-3'P

[0304] To a 2.0 mL Eppendorf tube was added 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). In a separate tube, 75 µL from the de-phosphorylated product of the fourth extension was diluted with 4.0 µL mUTP-3'P (2 mM). The reaction solution was then transferred to the tube containing resin and incubated at 50 °C with 500 rpm for 1.5 h.

[0305] Afterward, another 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22) weighed out into a separate 2.0 mL Eppendorf tube. The reaction mixture was separated from the used resin and added to the tube containing fresh resin along with 1.0 µL $CoCl_2$ (10 mM). The reaction was then incubated for 1.5 h at 50 °C with 500 rpm agitation.

[0306] Afterward, another 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22) was weighed out into a separate 2.0 mL Eppendorf tube. The reaction mixture was separated from the used resin and added to the tube containing fresh resin along with 4.0 µL mUTP-3'P (2 mM). The reaction was then incubated for another 1.5 h at 50 °C with 500 rpm agitation.

[0307] Afterward, another 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22) was weighed out into a separate 2.0 mL Eppendorf tube. The reaction mixture was separated from the used resin and added to the tube containing fresh resin and incubated for another 1.5 h at 50 °C with 500 rpm agitation.

[0308] Afterward, a 2 µL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to pellet residual resin particulate.

Sixth Extension: 5'-6-FAM-T10fCmUfCmAmAfGmUfGmU + fCTP-3'P => 5'-6-FAM-T10fCmUfCmAmAfGmUfGmUfC-3'P

[0309] To a 2.0 mL Eppendorf tube was added 8 mg IB-HIS-2 resin charged with 2.5 wt. % TdT (SEQ ID NO: 20) and 0.2 wt. % IPP (SEQ ID NO: 22). In a separate tube, 75 µL from the de-phosphorylated product of the fifth extension was diluted with 4.0 µL fCTP-3'P (2 mM). The reaction solution was then transferred to the tube containing resin and incubated at 50 °C with 500 rpm for 1.5 h.

[0310] Afterward, a 2 µL aliquot was removed for CE analysis as described above, while the reaction sample was allowed to cool in a 4 °C fridge for 10 minutes. Then, the supernatant was separated from the resin and placed in a 1.5 mL conical Eppendorf tube and centrifuged for 3 min at 14,000 rpm to

pellet residual resin particulate. The supernatant was then transferred into a new 2.0 mL Eppendorf tube and the general AP catalyzed dephosphorylation procedure outlined below was carried out.

General AP Catalyzed 3'-Dephosphorylation of RNA Oligomer

[0311] The supernatant was removed and added to a 2.0 mL Eppendorf tube containing IB-HIS-2 resin charged with 2.5 wt. % AP, 0.05 mg resin per µL reaction volume was used. The sample was then incubated at 50 °C for 20 minutes.

[0312] Afterward, a 2 µL aliquot was removed for CE analysis as described above. Meanwhile, the supernatant was separated from the resin, and transferred into a fresh Eppendorf tube. An equal volume of 25:24:1 chloroform:phenol:isoamyl alcohol was then added and the sample sealed and vortexed. After briefly centrifuging, the top aqueous layer was collected and transferred to a fresh sample tube. Then, an equal volume of 49:1 chloroform:isoamyl alcohol was added and the sample vortexed. After briefly centrifuging, the bottom organic layer was removed and discarded. The 49:1 chloroform:isoamyl alcohol extraction procedure was then repeated two more times. After the third time, the aqueous top layer was removed and transferred to a fresh 2.0 mL Eppendorf tube.

[0313] The aqueous layer containing de-phosphorylated oligo product was then placed in a vaccufuge for 15 minutes to remove residual organic solvents. The total volume lost was typically around 50 µL and was accounted for when adding reagents during subsequent extensions.

Example 5

Immobilization of Alkaline Phosphatase Variants on IB-COV-7 Resin

[0314] Variant Alkaline phosphatases (Aps, SEQ ID NOs 12 or 16) were immobilized onto IB-COV-7 resin (ChiralVision, Netherlands) using the development scale (6.6 x 50 mm) BioRad columns as described in U.S. Provisional Application No. 63/634,868 filed on April 16, 2024.

[0315] Prior mixing with the resin the buffer exchange was performed for all three alkaline phosphatase variants from storage buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.1 mM EDTA, and 50% glycerol) into 20 mM MOPS (pH 7). The enzyme was diluted 10-fold in the 20 mM MOPS (pH 7) and concentrated using the 10,000 MWCO spin filter. 3 mg of each enzyme variant was mixed with 150 mg of the IB-COV7 resin and incubated overnight at 4 °C. After overnight incubation, the resin was washed 3 times with 1 mL of 50 mM triethanolamine (pH 7.8). The washed resin was loaded onto BioRad columns and heated at 50 °C. The loaded resin was washed with 50 mM triethanolamine (pH 7.8) at 0.7 ml/min. Wash fractions were collected in volume of 420 µl and the p-nitrophenol phosphate assay activity test was performed as described below. % enzyme detected in wash fractions was calculated as ratio between enzyme detected in wash fraction and total bound enzyme and multiplied by 100.

Example 6

Activity of Alkaline Phosphatase with p-Nitrophenol Phosphate

[0316] Alkaline phosphatases (APs) were assayed for activity with p-nitrophenol phosphate to determine the amount of unbound enzyme to the resin as described in the U.S. Provisional Application No. 63/634,868 filed on April 16, 2024.

[0317] Reactions were performed in 96-well format 200 µL BioRad PCR plates. Standard curves were prepared for each enzyme variant by mixing 35 µL alkaline phosphatase of a known concentration (0.8 µM- 0.8 nM) and 35 µL of 2 mM p-nitrophenol phosphate in 50 mM triethanolamine buffer (pH 7.8) with 500 µM Cobalt (II) Chloride The reactions were set up as follows: (i) p-nitrophenol phosphate was aliquoted into each well of the 96-well plates (ii) AP solution was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a peelable aluminum seal and incubated in a thermocycler at 50 °C for 15 minutes then held at 4 °C until the reaction was quenched. The reactions were quenched by the addition of 35 µL of 2N NaOH, vortexed and centrifuged. Quenched reaction (50 µL) was transferred to half area UV star plates and the amount of produced p-nitrophenol was measured by absorption at 405 nm. For the wash fractions in the Example 5 above, the reactions included 35 µL of the wash fraction and 35 µL of p-nitrophenol phosphate. The amount of enzyme (nmol) in wash fractions was calculated based on a standard curve for each variant.

Example 7

Dephosphorylation Activity of Immobilized AP with 3'-phos-mATP

[0318] Immobilized alkaline phosphatases (APs) were assayed for activity with 3'-phosphate-mATP and species decomposition was analyzed by HPLC as described in U.S. Provisional Application No. 63/634,868 filed on April 16, 2024.

[0319] Reactions were performed in flow using the immobilized enzyme. 10 mL of solution containing 1 mM mATP-3'P, 50 mM triethanolamine (pH 7.8), and 500 µM cobalt (II) chloride was run through the column at the rate of 0.07 mL/min for 2 hours at 50 °C. 1 mL fractions were collected and the HPLC analysis from Example 12 was performed.

[0320] Activity relative to a reference variant (Activity FIOP) was calculated as the 1/ percent mATP-3'P of the variant compared with the 1/percent mATP-3'P observed by the reaction with the immobilized reference alkaline phosphatase.

Example 8

Immobilization of AP on Epoxide Resins

[0321] Approximately 22 mg of epoxide resins were loaded with a target of 2.0 wt% recombinant alkaline phosphatase (SEQ ID NOs: 12 or 16) in a 96-well half-deep (25 °C, 20h) in different buffers. The epoxide functionalized resins were EP403/M (Resindion-Relizyme™), EP600 (Resindion-

Relisorb™), HFA403/M (Resindion-Relizyme™) and EMC7042/M* (Sunresin). EP403/M (Resindion-Relizyme™) is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 200 µm to 500 µm. EP600 (Resindion-Relisorb™) is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 100 µm to 200 µm and an average pore diameter of 1500Å. HFA403/M (Resindion-Relizyme™) is an amino-epoxy functionalized polymethacrylate resin material with a particle size ranging from 200 µm to 500 µm and an average pore diameter of 500Å. EMC7042/M* (Sunresin) is an epoxide functionalized polyacrylic resin material with a particle size ranging from 300 µm to 700 µm and an average pore diameter of about 1000Å. The buffers were 1M NaPi, 50mM NaPi pH 7.0, 1M TEoA, 50mM TEoA pH 7.8 and water. Immobilization was measured by absorbance at A280 in the supernatant versus negative control with no resin. The resins were washed 3x with 0.5 M NaCl/50 mM TEoA-HCl and 3x with 50 mM TEoA-HCl and enzyme in each wash was measured by A280. Results from a series of experiments are presented in **FIGs. 1A** and **1B.**

Example 9

Covalent Bonding of AP on Epoxide Resins

[0322] Approximately 22 mg of epoxide resins were loaded with a target of 2.0 wt% recombinant alkaline phosphatase (SEQ ID NOs: 12 or 16) in a 96-well half-deep (25 °C, 20h) in different buffers as described above herein. The epoxide functionalized resins were EP403/M (Resindion-Relizyme™), EP600 (Resindion-Relisorb™), HFA403/M (Resindion-Relizyme™) and EMC7042/M* (Sunresin). After loading, the resins were washed either by transfer to a filter plate, adding wash buffer, shaking for 5' and collection by centrifugation (A) or the resins were washed by pipetting out the supernatant, adding wash buffer, shaking for 5' and pipetting out the wash buffer (B). The wash material was evaluated for the presence of alkaline phosphatase enzyme by A280 absorbance. It is noted that under wash condition B, some of the EP600 resin, which is very fine, ended up in the wash supernatants leading to a higher A280. Results obtained with wash condition A from one experiment are summarized in **FIG. 2A**; results obtained with wash condition B from one experiment are summarized in **FIG. 2B**. HFA403/M (Resindion-Relizyme™) showed the most ionically bound enzyme. The % wt of enzyme loaded on each resin and buffer condition after washing are summarized in **FIGs. 3A** and **3B.**

Example 10

Evaluation of Immobilized Alkaline Phosphatase Activity

[0323] Immobilized alkaline phosphatases (SEQ ID NOs: 12 or 16) were prepared as described above herein. Reaction mixtures comprising 10 wt % resin (10 mg loaded resin/100 µl reaction volume), 1 mM mAQP, 50 mM TEoA-HCl, pH 7.8 and either 0.25 mM $CoCl_2$ or 0.5 mM $CoCl_2$ were prepared. The reactions were incubated for 15 minutes at 50 °C. The reaction mixtures were

centrifuged at 500 rpm. The percent conversion of mAQP to mA was evaluated. Results from reactions with 0.25 mM $CoCl_2$ are summarized in **FIG. 4A.** Results from reactions with 0.5 mM $CoCl_2$ are summarized in **FIG. 4B.** Enzyme normalized results are presented in **FIG. 5A** and **FIG. 5B**. Alkaline phosphatases immobilized to a solid support in a 1M NaPi showed a high enzyme activity for each resin and recombinant alkaline phosphatase evaluated.

Example 11

Immobilization of AP on Epoxide Functionalized Solid Supports

[0324] Epoxide functionalized beads were loaded with a target of 2.0 wt% recombinant alkaline phosphatase (SEQ ID NO: 12) in either 250 mM MOPS pH 8.0 or 50 mM TEoA pH 7.8 overnight on a rotator at room temperature. The epoxide functionalized supports were EP600 (Resindion-Relisorb™), EP400, CPG-N12 (LGC™), CPG-N16 (LGC™) or CPG-NoCap (LGC™). EP400 is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 100 µm to 200 µm and an average pore diameter slightly smaller than EP600. EP600 (Resindion-Relisorb™) is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 100 µm to 200 µm and an average pore diameter of 1500Å. Controlled pore glass (CPG) were obtained from LGC™. The CPG core differs from the EP400 and EP600 cores. The CPG beads were epoxide modified with different chain lengths and cappings. CPG-N12 is Gly 1000Å N12. CPG-N16 is Gly 1000Å N16. CPG-no cap is Gly 1000Å NO-Bis Capped. CPG-N16 and CP6-no cap clumped and floated on top of the solution. CPG-N12 exhibited less clumping and floating. After the load incubation, immobilization was evaluated. After the load incubation, the solid supports were washed 3 times with 0.5 M NaCl buffer and 3 times with no NaCl buffer. Immobilization was evaluated after washing. Results from one series of immobilization experiments are shown in **FIG. 6A** (before washing) and **FIG. 6B** (after washing).

Activity of Immobilized AP on Epoxide Functionalized Solid Supports

[0325] The activity of immobilized APs (SEQ ID NOs: 12 or 16) comprising a EP600 (Resindion-Relisorb™), EP400, CPG-N12 (LGC™), CPG-N16 (LGC™) or CPG-NoCap (LGC™) solid support were evaluated. Immobilized AP resin (30 mg) was incubated with either 300 µl standard deblocking reaction buffer or with 300 µl PS to PO minimizing reaction buffer. The standard deblocking reaction buffer was 0.25 mM mC*mA*mGmAmAmAfGmA-3'P, 0.5 mM $CoCl_2$, and 50mM TEoA pH 7.8 with or without 20 mM thiosulfate. The PS to PO minimizing reaction buffer was 0.25 mMmC*mA*mGmAmAmAfGmA-3'P, 0.5 mM $CoCl_2$, and 250 mM MOPS pH 8.0 with or without 20 mM thiosulfate. Reaction mixtures were incubated at 50 °C with shaking. Aliquots were removed and evaluated at 5 minutes, 15 minutes and 60 minutes. The conversion results at 5 minutes are presented in **FIG. 7A**; the conversion results at 15 minutes are presented in **FIG. 7B**. In some instances, the enzyme activity and the measured enzyme loading did not correlate. The 60 minutes

conversion results are not shown. The percent PS to PO conversion from the batch reactions was also evaluated and results are summarized in **FIG. 8A** and **FIG. 8B**.

Example 12

Immobilization of AP on Epoxide Functionalized Solid Supports for Flow

[0326] Epoxide functionalized supports: EP600 (Resindion-Relisorb™), EP400, CPG-N12 (LGC™), CPG-N16 (LGC™) and CPG-NoCap (LGC™) were pre-wet prior to weighing. EP400 is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 100 µm to 200 µm and an average pore diameter slightly smaller than EP600. EP600 (Resindion-Relisorb™) is an epoxide functionalized polymethacrylate resin material with a particle size ranging from 100 µm to 200 µm and an average pore diameter of 1500Å. Controlled pore glass (CPG) were obtained from LGC™. The CPG core differs from the EP400 and EP600 cores. The CPG beads were epoxide modified with different chain lengths and cappings. CPG-N12 is Gly 1000Å N12. CPG-N16 is Gly 1000Å N16. CPG-no cap is Gly 1000Å NO-Bis Capped. The epoxide functionalized supports were incubated with 2 mg/mL recombinant AP (SEQ ID NO: 12) in water on a rotator overnight at room temperature. The target load was 2.0 wt% recombinant AP (SEQ ID NO: 12). CPG-N16 and CP6-no cap clumped and floated on top of the solution. After the load incubation, immobilization was evaluated. After the load incubation, the solid supports were washed 3 times with 250 mM MOPS pH 8.0 + 0.5 M NaCl buffer and 3 times with 250 mM MOPS pH 8.0 no NaCl. Immobilization was evaluated after washing. Results from one series of immobilization experiments are shown in **FIG. 9A** (before washing) and **FIG. 9B** (after washing).

Activity of Immobilized AP

[0327] The activity of immobilized APs (SEQ ID NOs: 12 or 16) comprising a EP600 (Resindion-Relisorb™), EP400, CPG-N12 (LGC™), CPG-N16 (LGC™) or CPG-NoCap (LGC™) solid support packed in a column were evaluated. Immobilized AP resin (450 mg) was packed into a column. A 1.8 ml reaction solution containing 0.25 mM dAdAdAdAmC*mA*mGmA-3'P, 0.5 mM $CoCl_2$, 20 mM thiophosphate and 250 mM MOPS pH 8.0 was prepared. The reaction buffer was flowed over the immobilized AP column at 50 °C at a flow rate of 0.5 ml/min. The column was washed with two 1 ml no salt buffer washes followed by two 1 ml high salt buffer washes. Fractions were collected. The first fraction was approximately 0.3 ml (volume of column) and the second fraction was approximately 1.8 ml (volume of reaction solution). Wash fractions were collected in approximately 1 ml aliquots. The oligonucleotide in the reaction solution fraction (fraction 2) from each column was evaluated for deblocking (conversion from dAdAdAdAmC*mA*mGmA-3'P to dAdAdAdAmC*mA*mGmA) and the amount of PS to PO conversion. The collected fractions, including the wash fractions, were evaluated for alkaline phosphatase. The CPG-NoCap column had lots of channeling which may have impacted the deblocking percent. While not being limited by mechanism, hydrophobicity of the CPG-NoCap beads may have contributed to the channeling.

Results from one such experiment are shown in **FIG. 10A** and **FIG. 10B**. The amount of leached AP in each fraction is summarized in **FIG. 10C**.

Example 13

Solid Support Enzyme Load Evaluation

[0328] Immobilized alkaline phosphatase comprising the EP600 resin typically does not leach alkaline phosphatase. Immobilized alkaline phosphatase comprising the EP400 resin occasionally leaches alkaline phosphatase. At the same target loading, EP400 shows a greater amount of alkaline phosphatase immobilization than EP600. Immobilized alkaline phosphatase comprising a recombinant alkaline phosphatase covalently attached to a solid support comprising EP600 at different weight percent loadings (1%, 2% and 4%) showed similar activity levels (data not shown).

[0329] Recombinant AP (SEQ ID NO: 12) was immobilized on EP400 at 1 wt% and 2 wt% target loadings. After the load incubation, the immobilized alkaline phosphatase resin samples had approximately 95% target loading immobilized. Resins with approximately 95% target loading and 1.9 wt% AP were washed with pH 7 or pH 8 buffer to remove non-covalently bound enzyme (**FIG. 11A**). After washing, the resins were at 0.82 wt% or 1.8 wt% alkaline phosphatase (**FIG. 11B**). The immobilized APs were packed into columns. The resin was washed with 250 mM MOPS + 0.5 M NaCl at either pH 7.0 or pH 8.0. Fractions were collected and the alkaline phosphatase in each fraction was measured by PNPP assay. The packed resin was washed further with 250 mM MOPS at either pH 7.0 or pH 8.0. Fractions were collected and the alkaline phosphatase in each fraction was measured by PNPP assay. (Data not shown).

[0330] The immobilized AP columns contained 450 mg resin each. Deblocking reaction mixture containing 1 mM dAdAdAdAmC*mA*mGmA-3'P, 0.5 mM $CoCl_2$, 50 mM sodium thiosulfate and 250 mM MOPS, pH8 was prepared. Reaction buffer (2 ml) was added to each column at a 0.5 ml/min flow rate; columns were at 50 °C. The amount of deblocked oligonucleotide (conversion of dAdAdAdAmC*mA*mGmA-3'P to dAdAdAdAmC*mA*mGmA) in the reaction fraction was evaluated. Results from one such experiment are shown in **FIG. 11C**. Fractions were collected and the AP in each fraction was measured by PNPP assay; results are shown in **FIG. 11D.**

Example 14

Evaluation of Immobilized Alkaline Phosphatases for Multiple Uses

[0331] EP400 and CPG19 solid supports were prepared and incubated with recombinant alkaline phosphatase (SEQ ID NO: 12) in replicate. The replicates were prepared with two separate enzyme solutions. The resulting immobilized alkaline phosphates were washed. The wt% loaded for each solid support and replicate number are summarized in **FIG. 12A**. 450 mg of each immobilized alkaline phosphatase were used to pack development scale columns. A reaction mixture of 1 mM dAdAdAdAmC*mA*mGmA-3'P, 0.5 mM $CoCl_2$, 50 mM sodium thiosulfate, and 250 mM MOPS,

pH 8.0 was prepared. Reaction mixture (2 ml) was added to each column at 50 °C and a 0.1 ml/min flowrate. The amount of deblocked oligonucleotide was evaluated for each immobilized AP (**FIG. 12B**). The PS to PO conversion of the oligonucleotides from each immobilized AP was evaluated (**FIG. 12C).** The columns were washed, and fractions collected. The amount of leached AP in each fraction was evaluated (data not shown). The columns were stored overnight at 4 °C. A second deblocking reaction was performed by adding 2 ml reaction mixture to each column at 50 °C and a 0.2 ml/min flowrate. The amount of deblocked oligonucleotide was evaluated for each immobilized AP (**FIG. 12D**). The PS to PO conversion of the oligonucleotides from each immobilized AP was evaluated and no increase in PS to PO conversion was observed**.**

Example 15

General Procedure for Post-Immobilization Treatment of Alkaline Phosphatase on Covalent Resin

[0332] Alkaline phosphatase (AP) (SEQ ID NO: 16) was produced in shake flask and purified as described in Example 1.

[0333] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 3.

[0334] After immobilization was complete, a 20-100 mg portion of epoxide functionalized resin loaded with 2.0 wt. % AP (SEQ ID NO: 16) was weighed out into a 2 mL Eppendorf tube. A 50-300 mM aliquot of quench reagent dissolved in TEAHCl (50 mM) was added to each tube and the tubes shook at 600 rpm for 0.5-3 h at 25-60 °C. After incubation, the quench solution was removed, and the resin washed with three portions of TEAHCl (50 mM, pH 7.8). The wash solution was discarded, and the quenched resins stored at 4 °C until ready for use.

Example 16

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

[0335] Alkaline phosphatase (AP) (SEQ ID NO: 16) was produced in shake flask and purified as described in Example 1.

[0336] Alkaline phosphatase was immobilized on ChiralVision IB-COV-7 resin at 2.0 wt. % vs wet weight of resin according to Example 3.

[0337] The AP loaded epoxide functionalized resins were quenched according to the procedure described in Example 15.

[0338] After post-immobilization treatment 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.

[0339] A reaction solution comprised of 800 μM 5'-AAAAmC*mA*mGmA-3'P, residual mATP-3'P, 1.0 mM $CoCl_2$, and 220 μM iPP in TEAHCl (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 4, Method B.

Table 16.1. 3'P-Dephosphorylation Using AP Immobilized on Epoxide Functionalized Resins with a Post-Immobilization Treatment

Reagent	Concentration	Percent 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%.		

[0340] While the invention has been described with reference to the specific embodiments, various changes can be made and equivalents can be substituted to adapt to a particular situation, material, composition of matter, process, process step or steps, thereby achieving benefits of the invention without departing from the scope of what is claimed.

[0341] For all purposes in the United States of America, each and every publication and patent document cited in this disclosure is incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute an admission as to its contents or date.

CLAIMS

What is claimed is:

1. A method for preparing an immobilized alkaline phosphatase (AP), comprising:

contacting an epoxide-functionalized solid support and an alkaline phosphatase polypeptide in solution under suitable conditions for conjugating or immobilizing the alkaline phosphatase polypeptide to the solid support; and

adding an epoxide quenching reagent to the solution to deactivate or cap the epoxide groups on the solid support.

2. The method of claim 1, wherein the epoxide quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

3. The method of claim 1, wherein the epoxide quenching reagent is 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, TEoA-HCl, and any combinations thereof.

4. The method of any one of claims 1-3, wherein the concentration of the epoxide quenching reagent is about 10 mM to about 3000 mM.

5. The method of any one of claims 1-4, wherein the solution with the added quenching reagent is allowed to incubate until at least 90%, at least 95%, at least 99%, or at least 99.9% of epoxide functional groups are quenched.

6. The method of any one of claims 1-5, further comprising removing the quenching reagent by washing the immobilized alkaline phosphatase on the solid support with a wash solution.

7. The method of claim 6, wherein the wash solution has a pH of about 7, about 7.8, about 8, or about 9.

8. The method of claim 7, wherein the wash solution is selected from a solution of NaCl/ TEoA-HCL, TEoA-HCL, MOPS, MOPS/NaCl, and NaCl.

9. The method of claim 7 or 8, wherein the wash solution is selected from 500 mM NaCl/50 mM TEoA-HCL, 50mM TEoA-HCL, 250 mM MOPS, 250 mM MOPS/500 mM NaCl, and 500 mM NaCl.

10. The method of any one of claims 6-9, wherein the wash solution is a high salt solution.

11. The method of any one of claims 1-10, further comprising treating the epoxide functionalized solid support and an alkaline phosphatase polypeptide with a second quenching reagent.

12. The method of claim 11, wherein the treatment with the second epoxide quenching reagent is after completion of treatment with the quenching reagent.

13. The method of claim 12, wherein the solid support is washed to remove the quenching reagent prior to adding the second quenching reagent.

14. The method of any one of claims 11-13, wherein the second quenching reagent is different from the first quenching reagent.

15. The method of any one of claims 11-14, wherein the second quenching reagent is selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

16. The method of any one of claims 11-14, wherein the second epoxide quenching reagent is 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, TEoA-HCl, and any combinations thereof.

17. The method of any one of claims 1-16, wherein the suitable conditions comprise incubating solution with the quenching reagent at a temperature of about 20 °C to about 60 °C.

18. The method of any one of claims 1-17, wherein the solution is a buffered aqueous solution at a pH of about 6.5 to about 8.5 containing the alkaline phosphatase polypeptide at a concentration of about 1 mg/ml to about 50 mg/ml, 10 mg/ml to about 100 mg/ml, 100 mg/ml to about 1000 mg/ml, or about 2 mg/ml.

19. The method of claim 18, wherein the buffer in the aqueous solution is selected from borate, phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), acetate, triethanolamine (TEoA), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris).

20. The method of claim 19, wherein the buffer concentration is from about 10 mM to about 1.5 M.

21. The method of any one of claims 1-20, wherein the epoxide functionalized solid support comprises particles comprising polyacrylate, methacrylate, polymethacrylate, amino-epoxy polymethacrylate, phenolate, polystyrene, polysaccharide, silica, or controlled pore glass.

22. The method of any one of claims 1-21, wherein the epoxide functionalized solid support comprises polymer particles having a particle size range of about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

23. The method of any one of claims 1-22, wherein the epoxide functionalized solid support comprises polymer particles having an average pore diameter of about 250 angstroms (Å) to about 1500 (Å), about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

24. The method of any one of claims 1-23, wherein the epoxide functionalized solid support is a resin selected from 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), EMC7042/M (Sunresin), EMC7042/S (Sunresin), 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), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

25. The method of any one of claims 1-24, wherein the immobilized alkaline phosphatase comprises between 0.5 wt%-5 wt% alkaline phosphatase polypeptide, 0.5 wt%- 3 wt% alkaline phosphatase polypeptide, 1 wt%-2.5 wt% alkaline phosphatase polypeptide or 1 wt% - 2 wt% alkaline phosphatase polypeptide.

26. The method of any one of claims 1-25, wherein after storage less than 0.2% of the total wt% bound alkaline phosphatase leaches off the solid support.

27. The immobilized alkaline phosphatase of any one of claims 1-26, wherein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of the total wt % bound alkaline phosphatase remains on the solid support.

28. An immobilized alkaline phosphatase prepared by the method of any one of claims 1-27.

29. An immobilized alkaline phosphatase (AP) comprising an alkaline phosphatase and a solid support wherein the alkaline phosphatase is attached to the solid support through a covalent linkage.

30. The immobilized alkaline phosphatase of claim 29 wherein the alkaline phosphatase is an alkaline phosphatase selected from the group comprising an alkaline phosphatase of *Thermoflexibacter, Pyrococcus, Thermotoga, Pseudothermotoga*, or *Bacillus* and fragments thereof.

31. The immobilized alkaline phosphatase of claim 28 or 29, wherein the alkaline phosphatase is an alkaline phosphatase of *Thermoflexibacter ruber*, *Pyrococcus furiosus*, *Thermotoga maritima*, *Thermotoga sp. 50_64*, *Pseudothermotoga lettingae*, *Thermotoga neapolitana*, or *Bacillus licheniformis* or a fragment thereof.

32. The immobilized alkaline phosphatase of any one of claims 29-31, wherein the alkaline phosphatase is a recombinant alkaline phosphatase or a fragment thereof.

33. The immobilized alkaline phosphatase of any one of claims 29-32, wherein the recombinant alkaline phosphatase is selected from the group comprising SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18.

34. The immobilized alkaline phosphatase of any one of claims 29-33, wherein the solid support comprises an ion-exchange resin, polyvinyl chloride, polyacrylamide, polyacrylate, polymethacrylate, polyethylene glycol, dextran, and/or agarose (e.g., cross-linked agarose).

35. The immobilized alkaline phosphatase of any one of claims 29-34, wherein the solid support comprises a medium selected from the group comprising epoxide functionalized hydrophilic polyacrylate resin, polymethacrylate resin, epoxide functionalized polymethacrylate resin, amino-epoxy functionalized polymethacrylate resin, polyacrylic resin, epoxide functionalized polyacrylic resin, controlled pore glass (CPG) and epoxide functionalized controlled pore glass.

36. The immobilized alkaline phosphatase of any one of claims 29-35, wherein the solid support is an epoxide functionalized solid support or an amino-epoxide functionalized solid support.

37. The immobilized alkaline phosphatase of any one of claims 29-36, wherein the solid support has an average pore diameter of about 250 angstroms (Å) to about 2000 (Å), about 250 angstroms (Å) to about 1500 Å, about 300 angstroms (Å) to about 1000 (Å), or about 300 angstroms (Å) to about 600 (Å).

38. The immobilized alkaline phosphatase of any one of claims 29-37, wherein the solid support has a particle size range of about 10 μm and 1500 μm, about 50 μm to about 1500 μm, about 100 μm to about 1000 μm, about 200 μm to about 700 μm, or about 200 μm to about 500 μm.

39. The immobilized alkaline phosphatase of any one of claims 29-38, wherein the solid support comprises polymer particles, wherein the particles comprise a polymer type selected from polyacrylic, methacrylic, polymethacrylic, phenolic, polystyrene, and cellulosic.

40. The immobilized alkaline phosphatase of any one of claims 29-39, wherein the covalent linkage is selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

41. The immobilized alkaline phosphatase of any one of claims 29-40, comprising at least two types of covalent linkages selected from the group consisting of a β-hydroxy-amino linkage, a β-hydroxy-ether linkage, a β-hydroxy-carboxyl linkage and a β-hydroxy-thio linkage.

42. The immobilized alkaline phosphatase of any one of claims 29-41, wherein the quenched epoxide groups comprise a covalent linkage with a quenching reagent and, optionally, a second quenching compound, selected from cysteine, lysine, ethanolamine, proline, alanine, glycine, imidazole, glucosamine, sodium thiosulfate, glycine benzyl ester, glycine methyl ester, glycine tert-butyl ester, cysteine methyl ester, N-acetyl-cysteine, β-mercaptoethanol, TEoA-HCl, and any combinations thereof.

43. The immobilized alkaline phosphatase of any one of claims 29-41, wherein the quenched epoxide groups comprise a covalent linkage with a quenching compound and, optionally, a second quenching compound, 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, TEoA-HCl, and any combinations thereof.

44. The immobilized alkaline phosphatase of any one of claims **Error! Reference source not found.**-43, wherein the epoxide functionalized solid support is a resin selected from 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), EMC7042/M (Sunresin), EMC7042/S (Sunresin), 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), EA403/M (Resindion), HA403/M (Resindion), IB-ANI-13 (ChiralVision BV), IB-COV-10 (ChiralVision BV), (ChiralVision BV), IB-ANI-3 (ChiralVision BV), FPA51 (Amberlite), ECR1090F (Purolite), ECR1604 (Purolite), ECR1504 (Purolite), ECR1640 (Purolite), CPG-N12 (LGC), CPG-N16 (LGC), CPG-NO cap (LGC), CPG-19 (LGC), CPG-20 (LGC), CPG-21 (LGC), IB-SLC(500A)-MPTMS-P500DGE (ChiralVision BV), IB-SLC(500A)-MPTMS-P1000DGE (ChiralVision BV), IB-SLC(500A)-GPTMS (ChiralVision BV), IB-SLC(500A)-MPTMS-P500DGE-MTMS (ChiralVision

BV), IB-SLC(500A)-MPTMS-P1000DGE-MTMS (ChiralVision BV), IB-SLC(500A)-GPTMS-MTMS (ChiralVision BV), IB-His-2 COOH (ChiralVision), IB-His-7 COOH (ChiralVision), IB-His-8 COOH (ChiralVision), IB-His-2 Co(II) (ChiralVision), A568 (Duolite), A-7 Freebase (Duolite), and AD7HP (Amberlite).

45. The immobilized alkaline phosphatase of any one of claims 29-44, wherein the immobilized alkaline phosphatase retains at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 96%, 97%, 98%, 99% or greater activity following attachment to the solid support.

46. The immobilized alkaline phosphatase of any one of claims 29-45, wherein less than 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.25% or less of the total wt % bound alkaline phosphatase leaches off the solid support after at least 24 hr of storage at 4 °C.

47. The immobilized alkaline phosphatase of any one of claims 29-46, wherein less than 5%, 4%, 3%, 2%, 1% or less of the total wt % bound alkaline phosphatase leaches off the solid support after wash at or above pH 7.00

48. The immobilized alkaline phosphatase of any one of claims 29-47, wherein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of total wt % bound alkaline phosphatase remains attached to the solid support after a wash at or above pH 7.5.

49. The immobilized alkaline phosphatase of any one of claims 29-48, wherein at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of the total wt % bound alkaline phosphatase remains attached to the solid support after at least 24 hr of storage at 4 °C.

50. The immobilized alkaline phosphatase of any one of claims 29-49, wherein the total wt % bound alkaline phosphatase is at least 0.5 wt%.

51. The immobilized alkaline phosphatase of any one of claims 29-50, wherein the total wt% bound alkaline phosphatase is about 0.5 wt%, 1 wt%, 2 wt%, or 4 wt%.

52. The immobilized alkaline phosphatase of any one of claims 29-51, wherein the immobilized AP retains at least 85% of the original immobilized alkaline phosphatase activity after a first incubation with a substrate and a first wash.

53. The immobilized alkaline phosphatase of any one of claims 29-52, wherein immobilized alkaline phosphatase exhibits at least 1% conversion of mAQP to mA.

54. The immobilized alkaline phosphatase of any one of claims 29-53, wherein immobilized alkaline phosphatase exhibits at least 1% conversion of mAQP to mA after 15 minutes.

55. A kit comprising an immobilized alkaline phosphatase of any one of claims 28-54.

56. A method for cleaving a phosphate substrate comprising:
contacting a substrate with a cleavable phosphate group with an immobilized alkaline

phosphatase of any one of claims 28-54 under reaction conditions effective for cleavage of the phosphate group by the immobilized alkaline phosphatase.

57. The method of claim 56, wherein the substrate with a cleavable phosphate group comprises an oligonucleotide comprising a 3'-terminal phosphate, an oligonucleotide comprising a 5'-terminal phosphate, an NTP, an NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, or 3'-phosphate NMP.

58. The method of claim 57, wherein the oligonucleotide substrate with the 3'-phosphate or the oligonucleotide with the 5'-phosphate comprises one or more modified nucleosides, and/or one or more modified internucleoside linkages.

59. The method of claim 58, wherein the modified internucleoside linkage comprise a phosphorothioate internucleoside linkage.

60. The method of claim 57, wherein the NTP, NDP, NMP, 3'-phosphate NTP, 3'-phosphate NDP, and 3'-phosphate NMP comprises a modified nucleoside and/or modified phosphate group.

61. The method of claim 60, wherein the modified phosphate group comprises an alpha-thiophosphate group.