Abstract: Methods and reagents for the installation of click chemistry handles on target proteins are provided, as well as modified proteins comprising click chemistry handles. Further, chimeric proteins, for example, bi-specific antibodies, that comprise two proteins conjugated via click chemistry, as well as methods for their generation and use are disclosed herein.

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USING SORTASES TO INSTALL CLICK CHEMISTRY HANDLES FOR PROTEIN LIGATION

RELATED APPLICATIONS


GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

[0003] Protein engineering is becoming a widely used tool in many areas of protein biochemistry. One engineering method is controlled protein ligation. Native chemical protein ligation relies on efficient preparation of synthetic peptide esters, which can be technically difficult to prepare for many proteins. Recombinant technologies can be used to generate protein-protein fusions, joining the C-terminus of one protein with the N-terminus of another protein. Intein-based protein ligation systems can also be used to join proteins. A prerequisite for this intein-mediated ligation method is that the target protein is expressed as a correctly folded fusion with the intein, which is often challenging. The difficulties of conventional native and recombinant ligation technologies significantly limit the application of protein ligation.

[0004] The transpeptidation reaction catalyzed by sortases has emerged as a general method for derivatizing proteins with various types of modifications. For conventional sortase modifications, target proteins are engineered to contain a sortase recognition motif (LPXT) near their C-termini. When incubated with synthetic peptides containing one or more N-terminal glycine residues and a recombinant sortase, these artificial sortase substrates undergo a transacylation reaction resulting in the exchange of residues C-terminal to the threonine residue with the synthetic oligoglycine peptide, resulting in the protein C-terminus being ligated to the N-terminus of the synthetic peptide.
SUMMARY OF THE INVENTION

[0005] Some aspects of this invention relate to sortase-mediated modification of proteins, in particular on the installation of reactive chemical groups, e.g., click chemistry handles, on protein sequences. Methods and reagents for the installation of reactive chemical groups on proteins are provided, as are modified proteins, e.g., proteins comprising a C-terminal or an N-terminal click chemistry handle. Further, methods to conjugate two proteins that are modified according to aspects of this invention are provided. Such methods are useful to dimerize monomeric proteins, and to generate chimeric proteins that combine the characteristics of heterologous single proteins, e.g., chimeric, bi-specific antibodies.

[0006] Some aspects of this invention provide methods, compositions, and reagents for the N-terminal or C-terminal addition of click chemistry handles to proteins using a sortase transacylation reaction. Some aspects of this invention provide methods for installing a click chemistry handle at or proximal to the C-terminus of a protein comprising a sortase recognition motif (e.g., LPXT) near the C-terminus. Some aspects of this invention provide methods for installing a click chemistry handle on the N-terminus of a protein comprising one or more N-terminal glycine residues.

[0007] For example, some embodiments provide a method of conjugating a target protein to a C-terminal click chemistry handle. In some embodiments, the method comprises providing the target protein with a C-terminal sortase recognition motif (e.g., LPXT); for example, as a C-terminal fusion. In some embodiments, the method further comprises contacting the target protein with an agent, for example, a peptide, a protein, or a compound, comprising 1-10 N-terminal glycine residues or an N-terminal alkylamine group, and the click chemistry handle. In some embodiments, the contacting is carried out in the presence of a sortase enzyme under conditions suitable for the sortase to transamidate the target protein and the peptide comprising the click chemistry handle, thus conjugating the target protein to the click-chemistry handle.

[0008] Some embodiments provide a method of conjugating a target protein to an N-terminal click chemistry handle is provided. In some embodiments, the method comprises providing the target protein with 1-10 N-terminal glycine residues or an N-terminal alkylamine group, for example, as an N-terminal fusion. In some embodiments, the method further comprises contacting the target protein with a peptide comprising a sortase recognition motif (e.g., LPXT), and the click chemistry handle. In some embodiments, the contacting is carried out in the presence of a sortase enzyme under conditions suitable for the
sortase to transamidate the target protein and the peptide, thus conjugating the target protein
to the click-chemistry handle.

[0009] Any chemical moiety can be installed on a protein using the methods described
herein. Of particular use according to some aspects of this invention are click chemistry
handles. Click chemistry handles are chemical moieties that provide a reactive group that can
partake in a click chemistry reaction. Click chemistry reactions and suitable chemical groups
for click chemistry reactions are well known to those of skill in the art, and include, but are
not limited to terminal alkynes, azides, strained alkynes, dienes, dieneophiles, alkoxyamines,
carbonyls, phosphines, hydrazides, thiols, and alkenes. For example, in some embodiments,
an azide and an alkyne are used in a click chemistry reaction.

[0010] Some aspects of this invention provide modified proteins, for example, proteins
comprising a C-terminal or an N-terminal click chemistry handle. Such proteins can be
conjugated to other molecules, for example, proteins, nucleic acids, polymers, lipids, or small
molecules, comprising a moiety that can react with the click chemistry handle of the protein.
In some embodiments, the modified protein comprises an antigen-binding domain, for
example, an antigen-binding domain of an antibody, e.g., a camelid antibody, a single-
domain antibody, a VHH domain, a nanobody, or an ScFv, or an antigen-binding fragment
thereof.

[0011] Some aspects of this invention provide methods for the conjugation, or ligation, of
two protein molecules via click chemistry. In some embodiments, a first click chemistry
handle is installed on the first protein, and a second click chemistry handle is installed on the
second protein, wherein the first click chemistry handle can form a covalent bond with the
second click chemistry handle. For example, some embodiments provide a method for post-
translationally conjugating two proteins to form a chimeric protein. In some embodiments,
the method comprises contacting a first protein conjugated to a first click-chemistry handle
with a second protein conjugated to a second click chemistry handle under conditions suitable
for the first click chemistry handle to react with the second click chemistry handle, thus
generating a chimeric protein comprising the two proteins linked via a covalent bond.

[0012] The methods provided herein allow for the generation of N-terminus to N-
terminus conjugation and of C-terminus to C-terminus conjugation of proteins, which cannot
be achieved by recombinant means (e.g., expression of protein fusions). For example, in
some embodiments, the first click chemistry handle is conjugated to the N-terminus of the
first protein, and the second click chemistry handle is conjugated to the N-terminus of the
second protein, and the chimeric protein is an N-terminus-to-N-terminus conjugation of the
two proteins. In other embodiments, the first click chemistry handle is conjugated to the C-terminus of the first protein and the second click chemistry handle is conjugated to the C-terminus of the second protein, and the chimeric protein is a C-terminus-to-C-terminus conjugation of the two proteins. In some embodiments, click handles are used to join C- and N-termini of a first and a second polypeptides, e.g., as an alternative to producing a fusion protein recombinantly. This is particularly useful, e.g., if a fusion protein is very large, toxic, hard to purify, encoded by nucleic acid sequences that are hard to clone, or to avoid cloning.

[0013] Some embodiments of this invention provide chimeric proteins, for example, chimeric proteins that have been generated by post-translational conjugation of the two proteins according to aspects of this invention. Some embodiments provide chimeric, bispecific antibodies, comprising two antigen-binding proteins, for example, single-domain antibodies, that are conjugated together via click chemistry. Some embodiments provide a bispecific, chimeric antibody comprises a first antibody or antigen-binding antibody fragment comprising a sortase recognition sequence, and a second antibody or antigen-binding antibody fragment comprising a sortase recognition sequence; and the first and the second antibody or antibody fragment are conjugated together via click chemistry.

[0014] It should be noted that the invention is not limited to the conjugation of antigen-binding proteins, but that any protein can be conjugated with any molecule which comprises a suitable click chemistry handle, or on which such a handle can be installed according to methods described herein or methods known to those of skill in the art. Accordingly, some embodiments provide chimeric proteins comprising a target protein with a sortase recognition motif (e.g., LPXT), and a second molecule conjugated to the protein via click chemistry. In some embodiments, the chimeric protein is generated by post-translationally installing a click chemistry handle on the target protein and contacting the target protein including the click chemistry handle with the second molecule, wherein the second molecule comprises a second click chemistry handle that can react with the click chemistry handle of the target protein to form a covalent bond.

[0015] Some embodiments provide modified proteins, for example, proteins comprising a sortase recognition motif (e.g., LPXT) and a click chemistry handle conjugated to the sortase recognition motif, for example, directly to one of the amino acids of the sortase recognition motif, or via a linker. In some embodiments, the modified protein comprises an antigen-binding domain, e.g., an antibody or an antigen-binding antibody fragment. Exemplary, modified proteins provided herein include, but are not limited to, a camelid antibody or antigen-binding fragment thereof, a VHH domain, a single-domain antibody, a nanobody, an
scFv, an affibody, an anticalin, a DARPin, or an adnectin. In some embodiments, the click chemistry handle is positioned at the C-terminus of the protein, while in other embodiments, the click chemistry handle is positioned at the N-terminus of the protein. In some embodiments, the click chemistry handle is selected from the group consisting of terminal alkyne, azide, strained alkyne, diene, dieneophile, alkoxyamine, carbonyl, phosphine, hydrazide, thiol, and alkene.

Some embodiments of this invention provide kits comprising one or more reagents useful in carrying out methods provided herein. For example, in some embodiments, the invention provides a kit comprising a first peptide comprising 1-10 glycine residues or a terminal alkylamine conjugated to a first click chemistry handle, and a second peptide comprising a sortase recognition motif conjugated to a second click chemistry handle, wherein the click chemistry handle of the first and the second peptide can react. In some embodiments, the kit comprises a first peptide comprising 1-10 glycine residues or a terminal alkylamine conjugated to a first click chemistry handle, and a second peptide comprising 1-10 glycine residues or a terminal alkylamine conjugated to a second click chemistry handle, wherein the click chemistry handle of the first and the second peptide can react. In some embodiments, the kit comprises a first peptide comprising a sortase recognition motif conjugated to a first click chemistry handle, and a second peptide comprising a sortase recognition motif conjugated to a second click chemistry handle, wherein the click chemistry handle of the first and the second peptide are capable of reacting with each other. In some embodiments, the kit further comprises a sortase enzyme. In some embodiments, the kit further comprises instructions for use, a catalyst, for example, a metal catalyst, and/or a reaction buffer.

The above summary is intended to give an overview over some aspects of this invention, and is not to be construed to limit the invention in any way. Additional aspects, advantages, and embodiments of this invention are described herein, and further embodiments will be apparent to those of skill in the art based on the instant disclosure. The entire contents of all references cited above and herein are hereby incorporated by reference.

**BRIEF DESCRIPTION OF THE DRAWING**

Figure 1. Generation of C-C protein dimers and N-N protein dimers using sortases and click chemistry. In the upper panel, the term "LEPTGG" refers to a sortase recognition motif, for example, a recognition motif comprising an LPXT sequence, such as
LPETGG (SEQ ID NO: 1). Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0019] **Figure 2.** A) Schematic representation of the sortase-catalyzed transacylation reaction. B) Exemplary click chemistry handles and reactions suitable for the generation of conjugated proteins. C) Installation of C-terminal click handles A and B on Antibodies 1 and 2. D) Dimerization of Antibodies 1 and 2. Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0020] **Figure 3.** A) Exemplary additional functionalities that may be incorporated onto proteins using click chemistry. B) Synthesis of PEGylated bispecific antibodies and protein trimers.

[0021] **Figure 4.** Optimization of the click chemistry using N-terminally labeled ubiquitin analogues. A) Labeling of G3Ub-VME with the click-handles. B) Determination of the activity the formed constructs. UbVME monomers and dimmers were incubated with UCHL3. Labeling of the DUB results in a shift of molecular weight. Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0022] **Figure 5.** N-terminal sortagging using ubiquitin as a model protein. Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0023] **Figure 6.** Kinetics of the click chemistry N-N dimerization of azide-Ub and cyclooctyne-Ub.

[0024] **Figure 7.** Schematic of C-C dimerization of anti-p2M and anti-GFP antibodies. The term "LEPTGG" refers to a sortase recognition motif, for example, a recognition motif comprising an LPXT sequence, such as LPETGG (SEQ ID NO: 1). Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0025] **Figure 8.** Purification by size exclusion chromatography.

[0026] **Figure 9.** Sortagging of an anti-GFP nanobody.

[0027] **Figure 10.** Sortagging of interferon alpha and anti-GFP (anti-eGFP) nanobody. 37: C-terminal azide; 57: C-terminal cyclooctyne; 40: N-terminal cyclooctyne; 41: N-terminal azide; LPETGG: SEQ ID NO: 1.

[0028] **Figure 11.** Sortagging of INFA and anti-GFP. LPETGG: SEQ ID NO: 1.

[0029] **Figure 12.** Schematic overview of the approach. Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

[0030] **Figure 13.** Requirements for dimerization of ubiquitin. (A) Schematic approach. (B) Ubiquitin is sortagged with 1 or 2 for 3h and analyzed with LC/MS. (C, D) Dimerization of ubiquitin. Azido modified ubiquitin (2 nmol) is incubated with an equimolar amount of
cyclooctyne equipped ubiquitin in 13 μL H₂O. The dimer was resolved on a 15% SDS-PAGE and the proteins were detected by Coomassie (C) and (D) immunoblotting for ubiquitin. (E) Azido-ubiquitin (0.1 nmol) incubated with DIBAC-ubiquitin (0.1 nmol) for the indicated time was resolved on TRIS/Tricine gel, stained by Coomassie and the resulting protein was quantified by ImageJ. The relative amount of monomer and dimer per lane was determined as follows: relative amount of dimer = intensity of dimer/ total intensity; relative amount of monomer = intensity of monomer/ total intensity. (F) Labeling of UCHL3 with either ubiquitin or UbVME; left panel Coomassie stained gel, right panel immunoblotting for the his₆ tag. Sequences correspond, from top to bottom, to SEQ ID NOs XX-XX, respectively.

**Figure 14.** Synthesis of N-to-N fused proteins. (A) structures of the used N-terminal probes 1 and 2. (B, C) labeling of his-tagged UCHL3 with dimeric UbVME. (B) Coomassie brilliant blue stained tris-tricine gel. (C) Immunoblot using anti His antibody. Ub-UbVME*: ubiquitin-UbVME bound to a single UCHL3. UbVME₂*: dimeric UbVME bound to a single UCHL3 molecule. UbVME₂**: dimeric UbVME bound to two UCHL3 molecules.

**Figure 15.** C-to-C homodimeric antibodies. (A) Structures of the probes 3 and 4. (B) dimerization of anti GFP. (C) size exclusion experiment demonstrating that both anti GFPs bind GFP. Red line: anti GFP dimer, green line: GFP, light blue line: anti-GFP dimer + 2.5 μL GFP, dark blue line: anti GFP +10 μL GFP, black line: anti-GFP dimer + 20 μL GFP (excess).

**Figure 16.** (A) purification of anti GFP sortagged with probe 4. Coomassie brilliant blue stained gel (B) and mass spectrum (C) of purified anti-GFP labeled with 4. (D) dimerization of aGFP-3 and aGFP-4. aGFP-3 (2.5 μg, 0.17 nmol) in TRIS (50 mM, pH 7.4, 150 mM NaCl) was incubated with an equimolar amount of aGFP-4 for the indicated time at room temperature. The dimerized product was resolved from the monomer on a TRIS/Tricine SDS-PAGE. Proteins were visualized by fluorescent imaging (λₑₓ = 532, λₑₘ = 580, left panel) and Coomassie brilliant blue (middle panel) and quantified (right panel). The relative amount of monomer versus dimer was determined as described for ubiquitin. (E) Purification of anti-GFP dimer on a Superdex™ 75 10/30. (F) Analysis of the concentrated purified protein on a 15% SDS-PAGE.

**Figure 17.** Superdex™ 200 10/30 elution profile of monomer anti GFP-3 and anti GFP-4 incubated in the presence and absence of GFP.
Figure 18. The peaks eluting at 12.5 mL (1) and 15.5 mL (2) of anti GFP dimer incubated with 30 μL GFP were concentrated and loaded on a native page.

Figure 19. Dimerization and purification of fluorescent anti GFP-4 - VHH7-3 (A) and non-fluorescent anti GFP-4 - VHH7-5 (B).

Figure 20. (A) FACS staining of mouse lymph node cells with anti MHC II-anti GFP antibodies. Upper panels: Staining observed in wild type cells. Lower panels: staining of MHC class II deficient cells. (B) In vivo delivery of GFP. Mice were injected with 50 μg bispecific and either received directly intraperitoneally or after 1h intravenously 50 μg GFP. Stained cells were analyzed by flow cytometry.

Figure 21. FACS staining of mouse lymph node cells with anti MHC II-anti GFP antibodies. Upper panels: Staining observed in wild type cells. Lower panels: staining of MHC class II deficient cells.

Figure 22. Production of heterodimers of aGFP with VHH7, IL2, and IFNcc.

DEFINITIONS


The term "aliphatic," as used herein, includes both saturated and unsaturated, nonaromatic, straight chain (i.e., unbranched), branched, acyclic, and cyclic (i.e., carbocyclic) hydrocarbons, which are optionally substituted with one or more functional groups. As will be appreciated by one of ordinary skill in the art, "aliphatic" is intended herein to include, but is not limited to, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, and cycloalkynyl moieties. Thus, as used herein, the term "alkyl" includes straight, branched and cyclic alkyl groups. An analogous convention applies to other generic terms such as "alkenyl," "alkynyl," and the like. Furthermore, as used herein, the terms "alkyl," "alkenyl," "alkynyl," and the like
encompass both substituted and unsubstituted groups. In certain embodiments, as used herein, "aliphatic" is used to indicate those aliphatic groups (cyclic, acyclic, substituted, unsubstituted, branched or unbranched) having 1-20 carbon atoms (C<sub>1-20</sub> aliphatic). In certain embodiments, the aliphatic group has 1-10 carbon atoms (C<sub>1-10</sub> aliphatic). In certain embodiments, the aliphatic group has 1-6 carbon atoms (C<sub>1-6</sub> aliphatic). In certain embodiments, the aliphatic group has 1-5 carbon atoms (C<sub>1-5</sub> aliphatic). In certain embodiments, the aliphatic group has 1-4 carbon atoms (C<sub>1-4</sub> aliphatic). In certain embodiments, the aliphatic group has 1-3 carbon atoms (C<sub>1-3</sub> aliphatic). In certain embodiments, the aliphatic group has 1-2 carbon atoms (C<sub>1-2</sub> aliphatic). Aliphatic group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

[0042] The term "alkyl," as used herein, refers to saturated, straight- or branched-chain hydrocarbon radicals derived from a hydrocarbon moiety containing between one and twenty carbon atoms by removal of a single hydrogen atom. In some embodiments, the alkyl group employed in the invention contains 1-20 carbon atoms (C<sub>1-20</sub>alkyl). In another embodiment, the alkyl group employed contains 1-15 carbon atoms (Ci-isalkyl). In another embodiment, the alkyl group employed contains 1-10 carbon atoms (Q-ioalkyl). In another embodiment, the alkyl group employed contains 1-8 carbon atoms (C<sub>1-8</sub>alkyl). In another embodiment, the alkyl group employed contains 1-6 carbon atoms (C<sub>1-6</sub>alkyl). In another embodiment, the alkyl group employed contains 1-5 carbon atoms (Q-salkyl). In another embodiment, the alkyl group employed contains 1-4 carbon atoms (C<sub>1-4</sub>alkyl). In another embodiment, the alkyl group employed contains 1-3 carbon atoms (C<sub>1-3</sub>alkyl). In another embodiment, the alkyl group employed contains 1-2 carbon atoms (C<sub>1-2</sub>alkyl). Examples of alkyl radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, iso-butyl, sec-butyl, sec-pentyl, iso-pentyl, tert-butyl, n-pentyl, neopentyl, n-hexyl, sec-hexyl, n-heptyl, n-octyl, n-decyl, n-undecyl, dodecyl, and the like, which may bear one or more substituents. Alkyl group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. The term "alkylene," as used herein, refers to a biradical derived from an alkyl group, as defined herein, by removal of two hydrogen atoms. Alkylene groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted. Alkylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

[0043] The term "alkenyl," as used herein, denotes a monovalent group derived from a straight- or branched-chain hydrocarbon moiety having at least one carbon-carbon double
bond by the removal of a single hydrogen atom. In certain embodiments, the alkenyl group employed in the invention contains 2-20 carbon atoms (C_{2-20}alkenyl). In some embodiments, the alkenyl group employed in the invention contains 2-15 carbon atoms (C_{2-15}alkenyl). In another embodiment, the alkenyl group employed contains 2-10 carbon atoms (C_{2-10}alkenyl). In still other embodiments, the alkenyl group contains 2-8 carbon atoms (C_{2-8}alkenyl). In yet other embodiments, the alkenyl group contains 2-6 carbon atoms (C_{2-6}alkenyl). In still other embodiments, the alkenyl group contains 2-5 carbon atoms (C_{2-5}alkenyl). In yet other embodiments, the alkenyl group contains 2-4 carbon atoms (C_{2-4}alkenyl). In yet other embodiments, the alkenyl group contains 2-3 carbon atoms (C_{2-3}alkenyl). In yet other embodiments, the alkenyl group contains 2 carbon atoms (C_{2}alkenyl). Alkenyl groups include, for example, ethenyl, propenyl, butenyl, 1-methyl-2-butyl-1-yl, and the like, which may bear one or more substituents. Alkenyl group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. The term "alkenylene," as used herein, refers to a biradical derived from an alkenyl group, as defined herein, by removal of two hydrogen atoms. Alkenylene groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted. Alkenylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

[0044] The term "alkynyl," as used herein, refers to a monovalent group derived from a straight- or branched-chain hydrocarbon having at least one carbon-carbon triple bond by the removal of a single hydrogen atom. In certain embodiments, the alkynyl group employed in the invention contains 2-20 carbon atoms (C_{2-20}alkynyl). In some embodiments, the alkynyl group employed in the invention contains 2-15 carbon atoms (C_{2-15}alkynyl). In another embodiment, the alkynyl group employed contains 2-10 carbon atoms (C_{2-10}alkynyl). In still other embodiments, the alkynyl group contains 2-8 carbon atoms (C_{2-8}alkynyl). In still other embodiments, the alkynyl group contains 2-6 carbon atoms (C_{2-6}alkynyl). In still other embodiments, the alkynyl group contains 2-5 carbon atoms (C_{2-5}alkynyl). In still other embodiments, the alkynyl group contains 2-4 carbon atoms (C_{2-4}alkynyl). In still other embodiments, the alkynyl group contains 2-3 carbon atoms (C_{2-3}alkynyl). In still other embodiments, the alkynyl group contains 2 carbon atoms (C_{2}alkynyl). Representative alkynyl groups include, but are not limited to, ethynyl, 2-propynyl (propargyl), 1-propynyl, and the like, which may bear one or more substituents. Alkynyl group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. The term "alkynylene," as used herein, refers to a biradical derived from an
alkynylene group, as defined herein, by removal of two hydrogen atoms. Alkynylene groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted. Alkynylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

The term "carbocyclic" or "carbocyclvl" as used herein, refers to an as used herein, refers to a cyclic aliphatic group containing 3-10 carbon ring atoms (C3_iocarbocyclic). Carbocyclic group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

The term "heteroaliphatic," as used herein, refers to an aliphatic moiety, as defined herein, which includes both saturated and unsaturated, nonaromatic, straight chain (i.e., unbranched), branched, acyclic, cyclic (i.e., heterocyclic), or polycyclic hydrocarbons, which are optionally substituted with one or more functional groups, and that further contains one or more heteroatoms (e.g., oxygen, sulfur, nitrogen, phosphorus, or silicon atoms) between carbon atoms. In certain embodiments, heteroaliphatic moieties are substituted by independent replacement of one or more of the hydrogen atoms thereon with one or more substituents. As will be appreciated by one of ordinary skill in the art, "heteroaliphatic" is intended herein to include, but is not limited to, heteroalkyl, heteroalkenyl, heteroalkynyl, heterocycloalkyl, heterocycloalkenyl, and heterocycloalkynyl moieties. Thus, the term "heteroaliphatic" includes the terms "heteroalkyl," "heteroalkenyl," "heteroalkynyl," and the like. Furthermore, as used herein, the terms "heteroalkyl," "heteroalkenyl," "heteroalkynyl," and the like encompass both substituted and unsubstituted groups. In certain embodiments, as used herein, "heteroaliphatic" is used to indicate those heteroaliphatic groups (cyclic, acyclic, substituted, unsubstituted, branched or unbranched) having 1-20 carbon atoms and 1-6 heteroatoms (C1-2oheteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-10 carbon atoms and 1-4 heteroatoms (C1-oheteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-6 carbon atoms and 1-3 heteroatoms (C1-6_heteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-5 carbon atoms and 1-3 heteroatoms (C1-5sheteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-4 carbon atoms and 1-2 heteroatoms (C1-4heteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-3 carbon atoms and 1 heteroatom (C1-3sheteroaliphatic). In certain embodiments, the heteroaliphatic group contains 1-2 carbon atoms and 1 heteroatom (C1-2sheteroaliphatic). Heteroaliphatic group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.
The term "heteroalkyl," as used herein, refers to an alkyl moiety, as defined herein, which contain one or more heteroatoms (e.g., oxygen, sulfur, nitrogen, phosphorus, or silicon atoms) in between carbon atoms. In certain embodiments, the heteroalkyl group contains 1-20 carbon atoms and 1-6 heteroatoms (C_{1-20} heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-10 carbon atoms and 1-4 heteroatoms (C_{1-10} heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-6 carbon atoms and 1-3 heteroatoms (Ci-6 heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-5 carbon atoms and 1-3 heteroatoms (C_{1-5} heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-4 carbon atoms and 1-2 heteroatoms (C_{1-4} heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-3 carbon atoms and 1 heteroatom (C_{1-3} heteroalkyl). In certain embodiments, the heteroalkyl group contains 1-2 carbon atoms and 1 heteroatom (C_{1-2} heteroalkyl). The term "heteroalkylene," as used herein, refers to a biradical derived from an heteroalkyl group, as defined herein, by removal of two hydrogen atoms. Heteroalkylene groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted. Heteroalkylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

The term "heteroalkenyl," as used herein, refers to an alkenyl moiety, as defined herein, which further contains one or more heteroatoms (e.g., oxygen, sulfur, nitrogen, phosphorus, or silicon atoms) in between carbon atoms. In certain embodiments, the heteroalkenyl group contains 2-20 carbon atoms and 1-6 heteroatoms (C_{2-20} heteroalkenyl). In certain embodiments, the heteroalkenyl group contains 2-10 carbon atoms and 1-4 heteroatoms (C_{2-10} heteroalkenyl). In certain embodiments, the heteroalkenyl group contains 2-6 carbon atoms and 1-3 heteroatoms (C_{2-6} heteroalkenyl). In certain embodiments, the heteroalkenyl group contains 2-5 carbon atoms and 1-3 heteroatoms (C_{2-5} heteroalkenyl). In certain embodiments, the heteroalkenyl group contains 2-4 carbon atoms and 1-2 heteroatoms (C_{2-4} heteroalkenyl). In certain embodiments, the heteroalkenyl group contains 2-3 carbon atoms and 1 heteroatom (C_{2-3} heteroalkenyl). The term "heteroalkenylenyl," as used herein, refers to a biradical derived from an heteroalkenyl group, as defined herein, by removal of two hydrogen atoms. Heteroalkenylenyl groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted.

The term "heteroalkynyl," as used herein, refers to an alkynyl moiety, as defined herein, which further contains one or more heteroatoms (e.g., oxygen, sulfur, nitrogen, phosphorus, or silicon atoms) in between carbon atoms. In certain embodiments, the heteroalkynyl group contains 2-20 carbon atoms and 1-6 heteroatoms (C_{2-20} heteroalkynyl).
In certain embodiments, the heteroalkynyl group contains 2-10 carbon atoms and 1-4 heteroatoms (C_{2-10} heteroalkynyl). In certain embodiments, the heteroalkynyl group contains 2-6 carbon atoms and 1-3 heteroatoms (C_{2-6} heteroalkynyl). In certain embodiments, the heteroalkynyl group contains 2-5 carbon atoms and 1-3 heteroatoms (C_{2-5} heteroalkynyl). In certain embodiments, the heteroalkynyl group contains 2-4 carbon atoms and 1-2 heteroatoms (C_{2-4} heteroalkynyl). In certain embodiments, the heteroalkynyl group contains 2-3 carbon atoms and 1 heteroatom (C_{2-3} heteroalkynyl). The term "heteroalkynylene," as used herein, refers to a biradical derived from an heteroalkynyl group, as defined herein, by removal of two hydrogen atoms. Heteroalkynylene groups may be cyclic or acyclic, branched or unbranched, substituted or unsubstituted.

The term "heterocyclic," "heterocycles," or "heterocyclyl," as used herein, refers to a cyclic heteroaliphatic group. A heterocyclic group refers to a non-aromatic, partially unsaturated or fully saturated, 3- to 10-membered ring system, which includes single rings of 3 to 8 atoms in size, and bi- and tri-cyclic ring systems which may include aromatic five- or six-membered aryl or heteroaryl groups fused to a non-aromatic ring. These heterocyclic rings include those having from one to three heteroatoms independently selected from oxygen, sulfur, and nitrogen, in which the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. In certain embodiments, the term heterocyclic refers to a non-aromatic 5-, 6-, or 7-membered ring or polycyclic group wherein at least one ring atom is a heteroatom selected from O, S, and N (wherein the nitrogen and sulfur heteroatoms may be optionally oxidized), and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms. Heterocyclyl groups include, but are not limited to, a bi- or tri-cyclic group, comprising fused five, six, or seven-membered rings having between one and three heteroatoms independently selected from the oxygen, sulfur, and nitrogen, wherein (i) each 5-membered ring has 0 to 2 double bonds, each 6-membered ring has 0 to 2 double bonds, and each 7-membered ring has 0 to 3 double bonds, (ii) the nitrogen and sulfur heteroatoms may be optionally oxidized, (iii) the nitrogen heteroatom may optionally be quaternized, and (iv) any of the above heterocyclic rings may be fused to an aryl or heteroaryl ring. Exemplary heterocycles include azacyclopropanyl, azacyclobutanyl, 1,3-diazatidinyl, piperidinyl, piperazinyl, azocanyl, thiaranyl, thietanyl, tetrahydrothiophenyl, dithiolanyl, thiacyclohexanyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropuranyl, dioxanyl, oxathiolanyl, morpholinyl, thioxanyl, tetrahydronaphthyl, and the like, which may bear one
or more substituents. Substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

[0051] The term "aryl," as used herein, refers to an aromatic mono- or polycyclic ring system having 3-20 ring atoms, of which all the ring atoms are carbon, and which may be substituted or unsubstituted. In certain embodiments of the present invention, "aryl" refers to a mono, bi, or tricyclic \( C_{4-20} \) aromatic ring system having one, two, or three aromatic rings which include, but are not limited to, phenyl, biphenyl, naphthyl, and the like, which may bear one or more substituents. Aryl substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. The term "arylene," as used herein refers to an aryl biradical derived from an aryl group, as defined herein, by removal of two hydrogen atoms. Arylene groups may be substituted or unsubstituted. Arylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. Additionally, arylene groups may be incorporated as a linker group into an alkenylene, alkyneylene, heteroalkylene, heteroalkenylene, or heteroalkynylene group, as defined herein.

[0052] The term "heteroaryl," as used herein, refers to an aromatic mono- or polycyclic ring system having 3-20 ring atoms, of which one ring atom is selected from S, O, and N; zero, one, or two ring atoms are additional heteroatoms independently selected from S, O, and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms. Exemplary heteroarylcs include, but are not limited to pyrrolyl, pyrazolyl, amidazolyl, pyridiny1, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, tetrazinyl, ppyrolizinyl, indolyl, quinolinyl, isoquinolinyl, benzimidazolyl, indazolyl, quinolinyl, isoquinolinyl, quinolizinyl, cinnolinyl, quinaazolynyl, phthalazinyl, naphthridinyl, quinoxaliny1, thiophenyl, thianaphthenyl, furanyl, benzofuranyl, benzothiazolyl, thiazolynyl, isothiazolyl, thiaziazolynyl, oxazolyl, isooxazolyl, oxadiaziolyl, and the like, which may bear one or more substituents. Heteroaryl substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety. The term "heteroarylene," as used herein, refers to a biradical derived from an heteroaryl group, as defined herein, by removal of two hydrogen atoms. Heteroarylene groups may be substituted or unsubstituted. Additionally, heteroarylene groups may be incorporated as a linker group into an alkenylene, alkenylene, alkynylene, heteroalkylene, heteroalkenylene, or heteroalkynylene group, as defined herein. Heteroarylene group substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.
The term "acyl," as used herein, is a subset of a substituted alkyl group, and refers to a group having the general formula \(-\text{C(=0)R}^A\), \(-\text{C(=0)OR}^A\), \(-\text{C(=0)-O-C(=0)R}^A\), \(-\text{C(=0)SR}^A\), \(-\text{C(=0)N(R}^A\text{)_2}\), \(-\text{C(=S)N(R}^A\text{)S(R}^A\text{)}\), \(-\text{C(=NR}^A\text{)R}^A\), \(-\text{C(=S)N(R}^A\text{)R}^A\), \(-\text{C(=S)N(R}^A\text{)S(R}^A\text{)}\), wherein \(R^A\) is hydrogen, halogen, substituted or unsubstituted hydroxyl; substituted or unsubstituted thiol; substituted or unsubstituted amino; acyl; optionally substituted aliphatic; optionally substituted heteroaliphatic; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted aryl, optionally substituted heteroaryl, aliphaticoxy, heteroaliphaticoxy, alkyloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticthioxy, heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroary thioxy, mono- or di- aliphaticamino, mono- or di- heteroaliphaticamino, mono- or di- alky lamino, mono- or di- heteroalkylamino, mono- or di- arylamino, or mono- or di- heteroaryl amino; or two \(R^A\) groups taken together form a 5- to 6- membered heterocyclic ring. Exemplary acyl groups include aldehydes (-CHO), carboxylic acids (-C02H), ketones, acyl halides, esters, amides, imines, carbonates, carbamates, and ureas. Acyl substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

The term "acylene," as used herein, is a subset of a substituted alkylene, substituted alkenylene, substituted alkynylene, substituted heteroalkylene, substituted heteroalkynylene, or substituted heteroalkynylene group, and refers to an acyl group having the general formulae: \(-\text{R}^\circ\text{-C(X}^1\text{-)}\text{-R}^0\), \(-\text{R}^\circ\text{-X}^2\text{-(C=X}^1\text{-)}\text{-R}^0\), or \(-\text{R}^\circ\text{-X}^2\text{-C(X}^1\text{-)}\text{X}^3\text{-R}^0\), where \(X^1\), \(X^2\), and \(X^3\) is, independently, oxygen, sulfur, or \(\text{NR}^f\), wherein \(R^f\) is hydrogen or optionally substituted aliphatic, and \(R^\circ\) is an optionally substituted alkylene, alkenylene, alky nylene, heteroalkylene, heteroalkynylene, or heteroalkynylene group, as defined herein. Exemplary acylene groups wherein \(R^\circ\) is alkylene includes \(-\text{(CH}_2\text{)}_\text{r}-0\text{(C=0)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-\text{NR}^f\text{(C=0)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-0\text{(C=NR}^f\text{)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-\text{S(C=S)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-\text{S(C=S)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-\text{S(C=0)-(CH}_2\text{)}_\text{r}\); \(-\text{(CH}_2\text{)}_\text{r}-\text{S(C=0)-(CH}_2\text{)}_\text{r}\); and the like, which may bear one or more substituents; and wherein each instance of \(T\) is, independently, an integer between 0 to 20. A cylene substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety.

The term "amino," as used herein, refers to a group of the formula \((-\text{NH}_2\)). A "substituted amino" refers either to a mono-substituted amine \((-\text{NHR}^b\)) of a disubstituted
amine (−NR$_2^h$), wherein the R$^h$ substituent is any substituent as described herein that results in the formation of a stable moiety (e.g., an amino protecting group; aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, amino, nitro, hydroxyl, thiol, halo, aliphaticamino, heteroaliphaticamino, alkylamino, heteroaalkylamino, arylamino, heteroarylamin, alkylaryl, arylalkyl, aliphaticoxy, heteroaliphaticoxy, alkyloxy, heteroalkyloxy, arlyloxy, heteroaryloxy, aliphaticthioxy, heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarythioxy, acyloxy, and the like, each of which may or may not be further substituted). In certain embodiments, the R$^h$ substituents of the di-substituted amino group (−NR$_2^h$) form a 5- to 6- membered heterocyclic ring.

[0056] The term "hydroxy" or "hydroxyl," as used herein, refers to a group of the formula (−OH). A "substituted hydroxyl" refers to a group of the formula (−OR$_1$), wherein R$_1$ can be any substituent which results in a stable moiety (e.g., a hydroxyl protecting group; aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, nitro, alkyaryl, arylalkyl, and the like, each of which may or may not be further substituted).

[0057] The term "thio" or "thiol," as used herein, refers to a group of the formula (−SH). A "substituted thiol" refers to a group of the formula (−SR$_2$), wherein R$_2$ can be any substituent that results in the formation of a stable moiety (e.g., a thiol protecting group; aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, sulfynyl, sulfonyl, cyano, nitro, alkyaryl, arylalkyl, and the like, each of which may or may not be further substituted).

[0058] The term "imino," as used herein, refers to a group of the formula (=NR$_1$), wherein R$_1$ corresponds to hydrogen or any substituent as described herein, that results in the formation of a stable moiety (for example, an amino protecting group; aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, amino, hydroxyl, alkyaryl, arylalkyl, and the like, each of which may or may not be further substituted).

[0059] The term "azide" or "azido," as used herein, refers to a group of the formula (−N$_3$).

[0060] The terms "halo" and "halogen," as used herein, refer to an atom selected from fluorine (fluoro, −F), chlorine (chloro, −Cl), bromine (bromo, −Br), and iodine (iodo, −I).

[0061] A "leaving group" is an art-understood term referring to a molecular fragment that departs with a pair of electrons in heterolytic bond cleavage, wherein the molecular fragment is an anion or neutral molecule. See, for example, Smith, March's Advanced Organic Chemistry 6th ed. (501-502). Exemplary leaving groups include, but are not limited to, halo (e.g., chloro, bromo, iodo) and activated substituted hydroxyl groups, e.g., of the
formula -OC(=0)SR \( \text{aa} \), -OC(=0)R \( \text{aa} \), -OC0 \( \text{a} \), -OC(=0)N(R\( \text{bb} \))\( _2 \), -OC(=NR\( \text{bb} \))R\( \text{aa} \), -OC(=NR\( \text{bb} \))OR\( \text{aa} \), -OC(=NR\( \text{bb} \))N(R\( \text{bb} \))\( _2 \), -OS(=0)R \( \text{aa} \), -OS0 \( \text{a} \), -OP(R\( \text{cc} \))\( _2 \), -OP(R\( \text{cc} \)), -OP(=0)\( \text{a} \), -OP(=0)(R\( \text{aa} \))\( _2 \), -OP(=0)(OR\( \text{aa} \))\( _2 \), -OP(=0)(N(R\( \text{bb} \))\( _2 \), or -OP(=0)(NR\( \text{bb} \))\( _2 \)

wherein R\( \text{aa} \) is optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl; R\( \text{bb} \) is hydrogen, an amino protecting group, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl; and R\( \text{cc} \) is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, or optionally substituted heteroaryl.

[0062] As used herein, the term Xaa refers to an amino acid for example, a standard amino acid of Table A, or a non-standard amino acid of table B. In some embodiments, the term Xaa refers to a compound e.g. of the formula:

\[
\begin{align*}
\text{alpha-amino acid} & \\
N & \quad \alpha \\
R' & \\
R^d & \\
\text{or} & \\
\text{beta-amino acid} & \\
N & \quad \beta \\
R' & \\
R^d & 
\end{align*}
\]

wherein each instance of R and R’ independently are selected from the group consisting of hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl, and optionally substituted heteroaryl; and R\( \text{d} \) is hydrogen or an amino protecting group. Amino acids encompassed by the above two formulae include, without limitation, natural alpha-amino acids such as D- and L-isomers of the 20 common naturally occurring alpha-amino acids found in polypeptides and proteins (e.g., A, R, N, C, D, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V, as depicted in Table A below, also referred to herein as standard amino acids), non-standard alpha-amino acids (examples of which are depicted in Table B below), and beta-amino acids (standard or non-standard, e.g., beta-alanine).

<table>
<thead>
<tr>
<th>Table A. Standard alpha–amino acids</th>
<th>R</th>
<th>R’</th>
</tr>
</thead>
<tbody>
<tr>
<td>L–Alanine (A)</td>
<td>–CH(_3)</td>
<td>–H</td>
</tr>
<tr>
<td>L–Arginine (R)</td>
<td>–CH(_2)CH(_2)CH(_2)=NHC(=NH)NH(_2)</td>
<td>–H</td>
</tr>
<tr>
<td>L–Asparagine (N)</td>
<td>–CH(_2)C(=O)NH(_2)</td>
<td>–H</td>
</tr>
<tr>
<td>L–Aspartic acid (D)</td>
<td>–CH(_2)CO(_2)H</td>
<td>–H</td>
</tr>
<tr>
<td>Table A. Standard alpha-amino acids</td>
<td>R</td>
<td>R’</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>L-Cysteine (C)</td>
<td>-CH₂SH</td>
<td>-H</td>
</tr>
<tr>
<td>L-Glutamic acid (E)</td>
<td>-CH₂CH₂C₀₂H</td>
<td>-H</td>
</tr>
<tr>
<td>L-Glutamine (Q)</td>
<td>-CH₂CH₂C(=0)NH₂</td>
<td>-H</td>
</tr>
<tr>
<td>Glycine (G)</td>
<td>-H</td>
<td>-H</td>
</tr>
<tr>
<td>L-Histidine (H)</td>
<td>-CH₂-2-(1H-imidazole)</td>
<td>-H</td>
</tr>
<tr>
<td>L-Isoleucine (I)</td>
<td>-sec-butyl</td>
<td>-H</td>
</tr>
<tr>
<td>L-Leucine (L)</td>
<td>-iso-butyl</td>
<td>-H</td>
</tr>
<tr>
<td>L-Lysine (K)</td>
<td>-CH₂CH₂CH₂CH₂NH₂</td>
<td>-H</td>
</tr>
<tr>
<td>L-Methionine (M)</td>
<td>-CH₂CH₂SCH₃</td>
<td>-H</td>
</tr>
<tr>
<td>L-Phenylalanine (F)</td>
<td>-CH₂Ph</td>
<td>-H</td>
</tr>
<tr>
<td>L-Proline (P)</td>
<td>-2-(pyrrolidine)</td>
<td>-H</td>
</tr>
<tr>
<td>L-Serine (S)</td>
<td>-CH₂OH</td>
<td>-H</td>
</tr>
<tr>
<td>L-Threonine (T)</td>
<td>-CH₂CH(OH)(CH₃)</td>
<td>-H</td>
</tr>
<tr>
<td>L-Tryptophan (W)</td>
<td>-CH₂3-(1H-indole)</td>
<td>-H</td>
</tr>
<tr>
<td>L-Tyrosine (Y)</td>
<td>-CH₂-(p-hydroxyphenyl)</td>
<td>-H</td>
</tr>
<tr>
<td>L-Valine (V)</td>
<td>-isopropyl</td>
<td>-H</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table B. Non-standard alpha-amino acids</th>
<th>R</th>
<th>R’</th>
</tr>
</thead>
<tbody>
<tr>
<td>D- Alanine</td>
<td>-H</td>
<td>-CH₃</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>-H</td>
<td>-CH₂CH₂CH₂-NHC(=NH)NH₂</td>
</tr>
<tr>
<td>D-Asparagine</td>
<td>-H</td>
<td>-CH₂C(=0)NH₂</td>
</tr>
<tr>
<td>D-Aspartic acid</td>
<td>-H</td>
<td>-CH₂C₀₂H</td>
</tr>
<tr>
<td>D-Cysteine</td>
<td>-H</td>
<td>-CH₂SH</td>
</tr>
<tr>
<td>D-Glutamic acid</td>
<td>-H</td>
<td>-CH₂CH₂C₀₂H</td>
</tr>
<tr>
<td>D-Glutamine</td>
<td>-H</td>
<td>-CH₂CH₂C(=0)NH₂</td>
</tr>
<tr>
<td>D-Histidine</td>
<td>-H</td>
<td>-CH₂2-(1H-imidazole)</td>
</tr>
<tr>
<td>D-Isoleucine</td>
<td>-H</td>
<td>-sec-butyl</td>
</tr>
<tr>
<td>D-Leucine</td>
<td>-H</td>
<td>-iso-butyl</td>
</tr>
<tr>
<td>D-Lysine</td>
<td>-H</td>
<td>-CH₂CH₂CH₂CH₂NH₂</td>
</tr>
<tr>
<td>Table B. Non-standard alpha–amino acids</td>
<td>R</td>
<td>R’</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>---------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>D–Methionine</td>
<td>–H</td>
<td>–CH₂CH₂SCH₃</td>
</tr>
<tr>
<td>D–Phenylalanine</td>
<td>–H</td>
<td>–CH₃Ph</td>
</tr>
<tr>
<td>D–Proline</td>
<td>–H</td>
<td>–2–(pyrrolidine)</td>
</tr>
<tr>
<td>D–Serine</td>
<td>–H</td>
<td>–CH₃OH</td>
</tr>
<tr>
<td>D–Threonine</td>
<td>–H</td>
<td>–CH₂CH(OH)(CH₃)</td>
</tr>
<tr>
<td>D–Tryptophan</td>
<td>–H</td>
<td>–CH₂–3–(1H–indole)</td>
</tr>
<tr>
<td>D–Tyrosine</td>
<td>–H</td>
<td>–CH₂–(p–hydroxyphenyl)</td>
</tr>
<tr>
<td>D–Valine</td>
<td>–H</td>
<td>–isopropyl</td>
</tr>
</tbody>
</table>

**R and R’ are equal to:**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-methyl-Alanine (Aib)</td>
<td>–CH₃,</td>
<td>–CH₃</td>
</tr>
<tr>
<td>α-methyl-Arginine</td>
<td>–CH₃,</td>
<td>–CH₂CH₂CH₂–NHC(=NH)NH₂</td>
</tr>
<tr>
<td>α-methyl-Asparagusine</td>
<td>–CH₃,</td>
<td>–CH₂C(=O)NH₂</td>
</tr>
<tr>
<td>α-methyl-Aspartic acid</td>
<td>–CH₃,</td>
<td>–CH₂CO₂H</td>
</tr>
<tr>
<td>α-methyl-Cysteine</td>
<td>–CH₃,</td>
<td>–CH₂SH</td>
</tr>
<tr>
<td>α-methyl-Glutamic acid</td>
<td>–CH₃,</td>
<td>–CH₂CH₂CO₂H</td>
</tr>
<tr>
<td>α-methyl-Glutamine</td>
<td>–CH₃,</td>
<td>–CH₂CH₂C(=O)NH₂</td>
</tr>
<tr>
<td>α-methyl-Histidine</td>
<td>–CH₃,</td>
<td>–CH₂–2–(1H–imidazole)</td>
</tr>
<tr>
<td>α-methyl-Isoleucine</td>
<td>–CH₃,</td>
<td>–sec–butyl</td>
</tr>
<tr>
<td>α-methyl-Leucine</td>
<td>–CH₃,</td>
<td>–iso–butyl</td>
</tr>
<tr>
<td>α-methyl-Lysine</td>
<td>–CH₃,</td>
<td>–CH₂CH₂CH₂CH₂CH₂NH₂</td>
</tr>
<tr>
<td>α-methyl-Methionine</td>
<td>–CH₃,</td>
<td>–CH₂CH₂SCH₃</td>
</tr>
<tr>
<td>α-methyl-Phenylalanine</td>
<td>–CH₃,</td>
<td>–CH₂Ph</td>
</tr>
<tr>
<td>α-methyl-Proline</td>
<td>–CH₃,</td>
<td>–2–(pyrrolidine)</td>
</tr>
<tr>
<td>α-methyl-Serine</td>
<td>–CH₃,</td>
<td>–CH₂OH</td>
</tr>
<tr>
<td>α-methyl-Threonine</td>
<td>–CH₃,</td>
<td>–CH₂CH(OH)(CH₃)</td>
</tr>
<tr>
<td>α-methyl-Tryptophan</td>
<td>–CH₃,</td>
<td>–CH₂–3–(1H–indole)</td>
</tr>
<tr>
<td>α-methyl-Tyrosine</td>
<td>–CH₃,</td>
<td>–CH₂–(p–hydroxyphenyl)</td>
</tr>
<tr>
<td>α-methyl-Valine</td>
<td>–CH₃,</td>
<td>–isopropyl</td>
</tr>
</tbody>
</table>
Table 1. Non-natural alpha-amino acids

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norleucine</td>
<td>–H, -CH₂CH₂CH₃</td>
<td></td>
</tr>
</tbody>
</table>

[0063] There are many known non-natural amino acids any of which may be included in the polypeptides of the present invention. See, for example, S. Hunt, *The Non-Protein Amino Acids: In Chemistry and Biochemistry of the Amino Acids*, edited by G. C. Barrett, Chapman and Hall, 1985. Some examples of non-natural amino acids are 4-hydroxyproline, desmosine, gamma-aminobutyric acid, beta-cyanoalanine, norvaline, 4-(E)-butenyl-4(R)-methyl-N-methyl-L-threonine, N-methyl-L-leucine, 1-amino-cyclopropanecarboxylic acid, 1-amino-2-phenyl-cyclopropanecarboxylic acid, 1-amino-cyclobutanecarboxylic acid, 4-amino-cyclooctenecarboxylic acid, 3-amino-cyclohexanecarboxylic acid, 4-piperidylacetic acid, 4-amino-1-methylpyrrole-2-carboxylic acid, 2,4-diaminobutyric acid, 2,3-diaminoproionic acid, 2,4-diaminobutyric acid, 2-aminoheptane-2,6-dioic acid, 4-(aminomethyl)benzoic acid, 4-aminobenzoic acid, *ortho*-, *meta*-, and *para*-substituted phenylalanines (*e.g.*, substituted with -C(=0)C₆H₅; -CF₃; -CN; -halo; -N0₂; -CH₃), disubstituted phenylalanines, substituted tyrosines (*e.g.*, further substituted with -C(=0)C₆H₅; -CF₃; -CN; -halo; -N0₂; -CH₃), and statine.

[0064] The term "click chemistry" refers to a chemical philosophy introduced by K. Barry Sharpless of The Scripps Research Institute, describing chemistry tailored to generate covalent bonds quickly and reliably by joining small units comprising reactive groups together. Click chemistry does not refer to a specific reaction, but to a concept including reactions that mimick reactions found in nature. In some embodiments, click chemistry reactions are modular, wide in scope, give high chemical yields, generate inoffensive byproducts, are stereospecific, exhibit a large thermodynamic driving force > 84 kJ/mol to favor a reaction with a single reaction product, and/or can be carried out under physiological conditions. A distinct exothermic reaction makes a reactant "spring loaded". In some embodiments, a click chemistry reaction exhibits high atom economy, can be carried out under simple reaction conditions, use readily available starting materials and reagents, uses no toxic solvents or use a solvent that is benign or easily removed (preferably water), and/or provides simple product isolation by non-chromatographic methods (crystallisation or distillation).
The term "click chemistry handle," as used herein, refers to a reactant, or a reactive group, that can partake in a click chemistry reaction. For example, a strained alkyne, e.g., a cyclooctyne, is a click chemistry handle, since it can partake in a strain-promoted cycloaddition (see, e.g., Table 1). In general, click chemistry reactions require at least two molecules comprising click chemistry handles that can react with each other. Such click chemistry handle pairs that are reactive with each other are sometimes referred to herein as partner click chemistry handles. For example, an azide is a partner click chemistry handle to a cyclooctyne or any other alkyne. Exemplary click chemistry handles suitable for use according to some aspects of this invention are described herein, for example, in Tables 1 and 2, and in Figure 2B. Other suitable click chemistry handles are known to those of skill in the art.

The terms "protein," "peptide" and "polypeptide" are used interchangeably herein, and refer to a polymer of amino acid residues linked together by peptide (amide) bonds. The terms refer to a protein, peptide, or polypeptide of any size, structure, or function. Typically, a protein, peptide, or polypeptide will be at least three amino acids long. A protein, peptide, or polypeptide may refer to an individual protein or a collection of proteins. One or more of the amino acids in a protein, peptide, or polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A protein, peptide, or polypeptide may also be a single molecule or may be a multi-molecular complex. A protein, peptide, or polypeptide may be just a fragment of a naturally occurring protein or peptide. A protein, peptide, or polypeptide may be naturally occurring, recombinant, or synthetic, or any combination thereof.

The term "conjugated" or "conjugation" refers to an association of two molecules, for example, two proteins, with one another in a way that they are linked by a direct or indirect covalent or non-covalent interaction. In the context of conjugation via click chemistry, the conjugation is via a covalent bond formed by the reaction of the click chemistry handles. In certain embodiments, the association is covalent, and the entities are said to be "conjugated" to one another. In some embodiments, a protein is post-translationally conjugated to another molecule, for example, a second protein, by forming a covalent bond between the protein and the other molecule after the protein has been translated, and, in some embodiments, after the protein has been isolated. In some embodiments, the post-translational conjugation of the protein and the second molecule, for
example, the second protein, is effected via installing a click chemistry handle on the protein, and a second click chemistry handle, which can react to the first click chemistry handle, on the second molecule, and carrying out a click chemistry reaction in which the click chemistry handles react and form a covalent bond between the protein and the second molecule, thus generating a chimeric protein. In some embodiments, two proteins are conjugated at their respective C-termini, generating a C-C conjugated chimeric protein. In some embodiments, two proteins are conjugated at their respective N-termini, generating an N-N conjugated chimeric protein.

[0068] As used herein, a "detectable label" refers to a moiety that has at least one element, isotope, or functional group incorporated into the moiety which enables detection of the molecule, e.g., a protein or polypeptide, or other entity, to which the label is attached. Labels can be directly attached (i.e., via a bond) or can be attached by a tether (such as, for example, an optionally substituted alkyene; an optionally substituted alkenylene; an optionally substituted alkyynylene; an optionally substituted heteroalkylene; an optionally substituted heteroalkenylene; an optionally substituted arene; an optionally substituted arenylene or an optionally substituted acylene, or any combination thereof, which can make up a tether). It will be appreciated that the label may be attached to or incorporated into a molecule, for example, a protein, polypeptide, or other entity, at any position.

[0069] In general, a label can fall into any one (or more) of five classes: a) a label which contains isotopic moieties, which may be radioactive or heavy isotopes, including, but not limited to, $^2$H, $^3$H, $^{13}$C, $^{14}$C, $^{15}$N, $^{18}$F, $^{31}$P, $^{32}$P, $^{35}$S, $^{67}$Ga, $^{76}$Br, $^{99m}$Tc (Tc-99m), $^{111}$In, $^{123}$I, $^{125}$I, $^{13}$I, $^{157}$Gd, $^{169}$Yb, and $^{186}$Re; b) a label which contains an immune moiety, which may be antibodies or antigens, which may be bound to enzymes (e.g., such as horseradish peroxidase); c) a label which is a colored, luminescent, phosphorescent, or fluorescent moieties (e.g., such as the fluorescent label fluoresceinisothiocyanat (FITC); d) a label which has one or more photo affinity moieties; and e) a label which is a ligand for one or more known binding partners (e.g., biotin-streptavidin, FK506-FKBP). In certain embodiments, a label comprises a radioactive isotope, preferably an isotope which emits detectable particles, such as β particles. In certain embodiments, the label comprises a fluorescent moiety. In certain embodiments, the label is the fluorescent label fluoresceinisothiocyanat (FITC). In certain embodiments, the label comprises a ligand moiety with one or more known binding partners. In certain embodiments, the label comprises biotin. In some embodiments, a label is a fluorescent polypeptide (e.g., GFP or a derivative thereof such as enhanced GFP (EGFP)).
or a luciferase (e.g., a firefly, Renilla, or Gaussia luciferase). It will be appreciated that, in certain embodiments, a label may react with a suitable substrate (e.g., a luciferin) to generate a detectable signal. Non-limiting examples of fluorescent proteins include GFP and derivatives thereof, proteins comprising chromophores that emit light of different colors such as red, yellow, and cyan fluorescent proteins, etc. Exemplary fluorescent proteins include, e.g., Sirius, Azurite, EBFP2, TagBFP, mTurquoise, ECFP, Cerulean, TagCFP, mTFPl, mUkG1, mAG1, AcGFPl, TagGFP2, EGFP, mWasabi, EmGFP, TagYFP, EYFP, Topaz, SYFP2, Venus, Citrine, mKO, mK02, mOrange, mOrange2, TagRFP, TagRFP-T, mStrawberry, mRuby, mCherry, mRaspberry, mKate2, mPlum, mNeptune, T-Sapphire, mAmetrine, mKeima. See, e.g., Chalfie, M. and Kain, SR (eds.) Green fluorescent protein: properties, applications, and protocols (Methods of biochemical analysis, v. 47). Wiley-Interscience, Hoboken, N.J., 2006, and/or Chudakov, DM, et al, Physiol Rev. 90(3): 1103-63, 2010 for discussion of GFP and numerous other fluorescent or luminescent proteins. In some embodiments, a label comprises a dark quencher, e.g., a substance that absorbs excitation energy from a fluorophore and dissipates the energy as heat.

[0070] The term "antibody", as used herein, refers to a glycoprotein belonging to the immunoglobulin superfamily. The terms antibody and immunoglobulin are used interchangeably. With some exceptions, mammalian antibodies are typically made of basic structural units each with two large heavy chains and two small light chains. There are several different types of antibody heavy chains, and several different kinds of antibodies, which are grouped into different isotypes based on which heavy chain they possess. Five different antibody isotypes are known in mammals, IgG, IgA, IgE, IgD, and IgM, which perform different roles, and help direct the appropriate immune response for each different type of foreign object they encounter. In some embodiments, an antibody is an IgG antibody, e.g., an antibody of the IgGl, 2, 3, or 4 human subclass. Antibodies from non-mammalian species (e.g., from birds, reptiles, amphibia) are also within the scope of the term, e.g., IgY antibodies.

[0071] Only part of an antibody is involved in the binding of the antigen, and antigen-binding antibody fragments, their preparation and use, are well known to those of skill in the art. As is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in
antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab') fragment (or F(ab')2 fragment), retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford) In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of nonspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762, and 5,859,205.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (HAMA) responses when administered to humans.
Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab’), Fab, Fv, and Fd fragments; antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences. In some embodiments, the present invention provides so-called single chain antibodies (e.g., ScFv), (single) domain antibodies, and other antibodies, which in some embodiments are intracellular antibodies. Domain antibodies, camelid and camelized antibodies and fragments thereof, for example, VHH domains, or nanobodies, such as those described in patents and published patent applications of Ablynx NV and Domantis are also encompassed in the term antibody. The term “antigen-binding antibody fragment,” as used herein, refers to a fragment of an antibody that comprises the paratope, or a fragment of the antibody that binds to the antigen the antibody binds to, with similar specificity and affinity as the intact antibody.

Antibodies, e.g., fully human monoclonal antibodies, may be identified using phage display (or other display methods such as yeast display, ribosome display, bacterial display). Display libraries, e.g., phage display libraries, are available (and/or can be generated by one of ordinary skill in the art) that can be screened to identify an antibody that binds to an antigen of interest, e.g., using panning. See, e.g., Sidhu, S. (ed.) Phage Display in Biotechnology and Drug Discovery (Drug Discovery Series; CRC Press; 1st ed., 2005; Aitken, R. (ed.) Antibody Phage Display: Methods and Protocols (Methods in Molecular Biology) Humana Press; 2nd ed., 2009. In some embodiments, a monoclonal antibody is produced using recombinant methods in suitable host cells, e.g., prokaryotic or eukaryotic host cells. In some embodiments microbial host cells (e.g., bacteria, fungi) are used. Nucleic acids encoding antibodies or portions thereof may be isolated and their sequence determined. Such nucleic acid sequences may be inserted into suitable vectors (e.g., plasmids) and, e.g., introduced into host cells for expression. In some embodiments insect cells are used. In some embodiments mammalian cells, e.g., human cells, are used. In some embodiments, an antibody is secreted by host cells that produce it and may be isolated, e.g., from culture medium. Methods for production and purification of recombinant proteins are well known to those of ordinary skill in the art.
The term "chimeric antibody," as used herein, refers to an antibody, or an antigen-binding antibody fragment, conjugated to another molecule, for example, to a second antibody, or antigen-binding antibody fragment. Any antibody or antigen-binding antibody fragment, or antigen-binding protein domain can be used to generate a chimeric antibody according to aspects of this invention. In some embodiments, a chimeric antibody comprises two conjugated antibodies, or antibody fragments, or one antibody conjugated to an antibody fragment, wherein the antigen-binding domains of the conjugated molecules bind different antigens or different epitopes of the same antigen. Such chimeric antibodies are referred to herein as "bi-specific," since they bind two different antigens/epitopes.

The term "linker," as used herein, refers to a chemical group or molecule covalently linked to a molecule, for example, a protein, and a chemical group or moiety, for example, a click chemistry handle. In some embodiments, the linker is positioned between, or flanked by, two groups, molecules, or moieties and connected to each one via a covalent bond, thus connecting the two. In some embodiments, the linker is an amino acid or a plurality of amino acids. In some embodiments, the linker is an organic molecule, group, or chemical moiety.

The term "sortagging," as used herein, refers to the process of adding a tag, for example, a click chemistry handle, onto a target molecule, for example, a target protein. It should be noted that the term is not limited to click chemistry handles, but also refers to processes in which other tags are added. Examples of suitable tags include, but are not limited to, amino acids, peptides, proteins, nucleic acids, polynucleotides, sugars, carbohydrates, polymers, lipids, fatty acids, and small molecules. Other suitable tags will be apparent to those of skill in the art and the invention is not limited in this aspect. In some embodiments, a tag comprises a sequence useful for purifying, expressing, solubilizing, and/or detecting a polypeptide. In some embodiments, a tag can serve multiple functions. A tag is often relatively small, e.g., ranging from a few amino acids up to about 100 amino acids long. In some embodiments a tag is more than 100 amino acids long, e.g., up to about 500 amino acids long, or more. In some embodiments, a tag comprises an HA, TAP, Myc, 6XHis, Flag, or GST tag, to name few examples. In some embodiments a tag comprises a solubility-enhancing tag (e.g., a SUMO tag, NUS A tag, SNUT tag, or a monomeric mutant of the Ocr protein of bacteriophage T7). See, e.g., Esposito D and Chatterjee DK. Curr Opin Biotechnol.; 17(4):353-8 (2006). In some embodiments, a tag is cleavable, so that it can be removed, e.g., by a protease. In some embodiments, this is achieved by including a protease cleavage site in the tag, e.g., adjacent or linked to a functional portion of the tag. Exemplary
proteases include, e.g., thrombin, TEV protease, Factor Xa, PreScission protease, etc. In some embodiments, a "self-cleaving" tag is used. See, e.g., PCT/US05/05763.

**DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS**

[0080] Standard genetic approaches allow for the production of protein composites by fusion of polypeptides in head-to-tail fashion. Some applications, however, would benefit from constructions that are genetically impossible, such as the site-specific linkage of proteins via their N- or C-termini, when a remaining free terminus is required for biological activity.

Chimeric proteins, e.g., genetic fusions with fluorescent proteins are widely used to visualize their (sub)cellular localization in situ and in vivo (Lippincott-Schwartz J, Patterson GH (2003) Development and Use of Fluorescent Protein Markers in Living Cells. Science 300:87-91; the entire contents of which are incorporated herein by reference). For example, co-expression of two orthogonally labeled chimeras allows for the study of protein co-localization and dynamics of receptor dimerization. Moreover, protein fusions have been used to evaluate the biological relevance of otherwise transient protein complexes. Fusion or crosslinking of two or more of the interacting proteins can stabilize protein complexes, and has been used to explore signaling and (hetero)dimerization of G-protein coupled receptors (Seifert R, Wenzel-Seifert K, Kobilka BK (1999) GPCR-G fusion proteins: molecular analysis of receptor-G-protein coupling. Trends Pharmacol Sci 20:383-389; and Han Y, Moreira IS, Urizar E, Weinstein H, Javitch JA (2009) Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. Nat Meth 5:688-695; the entire contents of each of which are incorporated herein by reference), chemokines and cytokines (Leong SR et al. (1997) IL-8 single-chain homodimers and heterodimers: interactions with chemokine receptors CXCR1, CXCR2, and DARC. Protein Sci 6:609-617; Nassar MW et al. (2009) Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. J Immunol 183:3425-3432; and Drury LJ et al. (2011) Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. P Natl Acad Sci USA 108:17655-17660; the entire contents of each of which are incorporated herein by reference).

Besides being useful biochemical tools, chimeric proteins are also promising as treatment options for cancer, autoimmune diseases, lysosomal storages diseases and brain disorders (Boado RJ et al. (2008) Genetic Engineering, Expression, and Activity of a Chimeric Monoclonal Antibody- Avidin Fusion Protein for Receptor-Mediated Delivery of

[0081] The production and purification of fusion proteins remains a biotechnological challenge. To obtain an active product, both domains of the chimera must adopt the native fold, without modification of residues and regions that are required for activity. The standard method to produce fusion proteins is by genetic fusion of the open reading frames of the two proteins or protein fragments. Partly folded proteins and defective folding products are commonly observed in fusion proteins.

Standard sortase ligation approaches do not allow to yield protein-protein fusions that are genetically impossible (N-terminus to N-terminus; C-terminus to C-terminus), although such unnatural liaisons would have great appeal for the construction of bispecific antibodies or their fragments. Some aspects of this invention relate to the recognition that in order to accomplish such fusions, one has to resort to chemical ligation methods. Early chemical conjugation strategies relied on non-specific crosslinking via amines or sulfhydryls (Kim JS, Raines RT (1995) Dibromobimane as a fluorescent crosslinking reagent. Analytical Biochemistry 225:174-176; the entire contents of which are incorporated herein by reference). The lack of control over the site and stoichiometry of modification results in the formation of a heterogeneous product, limiting the usefulness of this approach. The rise of bioorthogonal chemistries combined with site-specific mutagenesis, native chemical ligation, intein-based ligation, and amber suppressor pyrrolysine tRNA technology has enabled the synthesis of non-natural protein fusions, as applied to the production of bivalent and multivalent antibodies (Schellinger JG et al. (2012) A general chemical synthesis platform for crosslinking multivalent single chain variable fragments. Org Biomol Chem 10:1521-1526; Natarajan A et al. (2007) Construction of di-scFv through a trivalent alkyne-azide 1,3-dipolar cycloaddition. Chem Commun:695-697; and Xiao J, Hamilton BS, Tolbert TJ (2010) Synthesis of N-Terminally Linked Protein and Peptide Dimers by Native Chemical Ligation. Bioconjug Chem 21:1943-1947; the entire contents of each of which are incorporated herein by reference). Structural analogs of ubiquitin dimers were prepared by a combination of intein-based ligation, site-specific mutation and copper-catalyzed click chemistry (Weikart...

Nonetheless, the synthesis of bispecifics would benefit from a method that is orthogonal to the published methods and that allows easy access to modified native protein, as well as enables efficient non-natural conjugation of protein termini. Moreover, the availability of orthogonal methods allows for the synthesis of protein structures of even greater complexity (e.g., heterotrimers and higher order complexes). Disclosed herein are reagents and methods related to a versatile approach that allows the conjugation of proteins at their N- or C-terminus to other entities, including, but not limited to, other proteins. Some of the conjugation strategies described herein comprise the addition of click chemistry handles to a protein using a sortase-catalyzed transpeptidation reaction. The resulting modified proteins can then be conjugated to a molecule that also comprises a reactive click chemistry handle.

Some aspects of this invention relate to the recognition that the sortase transacylation reaction allows for the facile installation of all kinds of substituents at the C-terminus of a suitably modified protein. The sole requirement for a successful transacylation reaction is the presence of a suitably exposed sortase recognition motif, e.g., an LPXT or LPXTG (SEQ ID NO: 2) motif, in the target protein. The design of nucleophiles that can be used in a sortase catalyzed reaction is likewise straight-forward: a short run (e.g., 1-10) of glycine residues, or even an alkylamine suffices to allow the reaction to proceed. The key advantages of using a sortase transacylation strategy to modify a target protein are the ease of synthesis, and execution of the reaction on native proteins under physiological conditions.

Some aspects of this invention relate to the recognition that the nucleophiles that are used in the sortase reaction can be modified to include any number of modifications:
biotin, detectable labels (e.g., fluorophores), fatty acids, nucleic acids, lipids, radioisotopes, carbohydrates or even proteins with a suitably exposed N-terminal stretch of glycine residues. Further, some aspects of this invention provide that nucleophiles can be used in a sortase reaction that comprise reactive chemical moieties, for example, moieties, or "handles", suitable for a click chemistry reaction, e.g., a copper-free click chemistry reaction. Such nucleophiles, e.g., peptides comprising 1-10 glycine residues (e.g., GGG), or any compound (e.g. a peptide) comprising an alkylamine group, and a click chemistry handle, can be employed to install a C-terminal click chemistry handle on a target protein comprising a C-terminal sortase recognition motif. The sortase recognition motif does not have to be positioned at the very C-terminus, but it has to be sufficiently accessible by the enzyme to efficiently partake in the sortase reaction.

[0087] Similarly, click chemistry handles can be installed N-terminally on proteins comprising a short glycine run or a protein or any compound comprising an alkylamine group (e.g., at their N-terminus for proteins), by carrying out a sortase reaction using a peptide comprising a sortase recognition motif and the desired click chemistry handle. Any protein comprising either a sortase recognition motif, or 1-10 glycine residues, or a terminal alkylamine group, can, accordingly, be derivatized with a click chemistry handle according to aspects of this invention. The installation of a click chemistry handle on a target protein confers click chemistry reactivity to the protein. For example, a protein comprising a click chemistry handle, as described herein, can react with a second molecule, for example, a second molecule, comprising a second click chemistry handle, to form a covalent bond, thus conjugating the two molecules together.

[0088] In some embodiments, proteins carrying reactive click chemistry handles are conjugated together by carrying out the respective click chemistry reaction. This results in the proteins being conjugated to each other via a covalent bond. Since the inventive strategies allow installment of a click chemistry handle on either the C- or the N-terminus of a protein, two proteins so modified can be conjugated via a covalent bond from the C-terminus of the first protein to the N-terminus of the second protein, much like a conventional protein fusion. However, installing C-terminal, reactive click chemistry handles on both target proteins allows for the generation of proteins conjugated via a covalent click chemistry bond at their C-termini (C-to-C-termini, C-C), while installing N-terminal, reactive click chemistry handles on both target proteins allows for the generation of proteins conjugated at their N-termini (N-to-N-termini, N-N). Neither covalent C-C conjugation nor covalent N-N
conjugation can be achieved by conventional protein engineering technologies, such as recombinant protein fusion technology.

**Sortase-mediated installment of click chemistry handles**

[0089] Sortases, sortase-mediated transacylation reactions, and their use in transacylation (sometimes also referred to as transpeptidation) for protein engineering are well known to those of skill in the art (see, e.g., Ploegh et al., International Patent Application PCT/US2010/000274, and Ploegh et al., International Patent Application PCT/US201 1/033303, the entire contents of each of which are incorporated herein by reference). In general, the transpeptidation reaction catalyzed by sortase results in the ligation of species containing a transamidase recognition motif with those bearing one or more N-terminal glycine residues. In some embodiments, the sortase recognition motif is a sortase recognition motif described herein. In certain embodiments, the sortase recognition motif is an LPXT motif or an LPXTG (SEQ ID NO: 2) motif. As is known in the art, the substitution of the C-terminal residue of the recognition sequence with a moiety exhibiting poor nucleophilicity once released from the sortase provides for a more efficient ligation.

[0090] The sortase transacylation reaction provides means for efficiently linking an acyl donor with a nucleophilic acyl acceptor. This principle is widely applicable to many acyl donors and a multitude of different acyl acceptors. Previously, the sortase reaction was employed for ligating proteins and/or peptides to one another, ligating synthetic peptides to recombinant proteins, linking a reporting molecule to a protein or peptide, joining a nucleic acid to a protein or peptide, conjugating a protein or peptide to a solid support or polymer, and linking a protein or peptide to a label. Such products and processes save cost and time associated with ligation product synthesis and are useful for conveniently linking an acyl donor to an acyl acceptor.

[0091] Sortase-mediated transacylation reactions are catalyzed by the transamidase activity of sortase. A transamidase is an enzyme that can form a peptide linkage (i.e., amide linkage) between an acyl donor compound and a nucleophilic acyl acceptor containing a NH$_2$-CH$_2$-moiety. In some embodiments, the sortase is sortase A (SrtA). However, it should be noted that any sortase, or transamidase, catalyzing a transacylation reaction can be used in some embodiments of this invention, as the invention is not limited to the use of sortase A. Sortases are enzymes having transamidase activity and have been isolated from Gram-positive bacteria. They have, as part of their cell wall structure, peptidoglycan as well as polysaccharides and/or teichoic acids. Gram-positive bacteria include the following genera:
Actinomyces, Bacillus, Bifidobacterium, Cellulomonas, Clostridium, Corynebacterium, Micrococcus, Mycobacterium, Nocardia, Staphylococcus, Streptococcus, and Streptomyces.

**Sortase-mediated installation of C-terminal click chemistry handles**

In certain embodiments, a sortase-mediated transacylation reaction for installing a C-terminal click chemistry handle on a protein comprises a step of contacting a protein comprising a transamidase recognition sequence of the structure:

![Chemical structure]( attachment:structure.png)

wherein

- the transamidase recognition sequence is an amino acid sequence motif recognized by a transamidase enzyme; a transamidase recognition sequence is also referred to herein as a sortase recognition sequence or a sortase recognition motif;
- \( X \) is \(-0-, -NR-, or -S-\); wherein \( R \) is hydrogen, substituted or unsubstituted aliphatic, or substituted or unsubstituted heteroaliphatic;
- \( A^1 \) is or comprises an amino acid sequence of at least 3 amino acids in length;
- \( R^1 \) is acyl, substituted or unsubstituted aliphatic, substituted or unsubstituted heteroaliphatic, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;

with a nucleophilic compound of formula:

![Chemical structure]( attachment:structure.png)

wherein

- \( B^1 \) is or comprises acyl, substituted or unsubstituted aliphatic, substituted or unsubstituted heteroaliphatic, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, an amino acid, a peptide, a protein, a polynucleotide, a carbohydrate, a tag, a metal atom, a contrast agent, a catalyst, a non-polypeptide polymer, a recognition element, a small molecule, a lipid, a linker, and/or a label;
- \( n \) is 0 or an integer from 1 to 100, inclusive;

in the presence of a transamidase enzyme, for example, a sortase, under suitable conditions to form a compound of formula:
[0093] It will be understood by those of skill in the art that the click chemistry handle may be incorporated into B\(^1\) in any manner and at any position that can be envisioned by those of skill in the art. For example, B\(^1\) may comprise an amino acid, \textit{(e.g., lysine)} and the click chemistry handle may be attached, for example, to the central carbon of the amino acid, the side chain of the amino acid, or to the carboxyl group of the amino acid, or any other position. Other ways of incorporating the click chemistry handle into B\(^1\) will be apparent to those of skill in the art, and the invention is not limited in this respect.

[0094] It will further be understood that, depending on the nature of B\(^1\), the click chemistry handle may be installed at the very C-terminus of the target protein, or, \textit{e.g.} if B\(^1\) comprises a first amino acid comprising the click chemistry handle, and a number of additional amino acids, the resulting, modified protein will comprise the click chemistry handle close to, but not directly at the C-terminus. As will be apparent to those of skill in the art, a similar situation exists for the N-terminal installation of the click chemistry handle described below.

[0095] One of ordinary skill will appreciate that, in certain embodiments, the C-terminal amino acid of the transamidase recognition sequence is omitted. That is, an acyl group \(\text{XR}^1\) replaces the C-terminal amino acid of the transamidase recognition sequence. In some embodiments, the acyl group is \(\text{OR}^1\). In some embodiments, the acyl group is \(\text{OMe}\).

[0096] In some embodiments, the sortase, or transamidase, recognition sequence is LPXT, wherein X is a standard or non-standard amino acid. In some embodiments, X is selected from D, E, A, N, Q, K, or R. In some embodiments, the recognition sequence is selected from LPXT, LPXT, SPXT, LAXT, LSXT, NPXT, VPXT, IPXT, and YPXR. In some embodiments X is selected to match a naturally occurring transamidase recognition sequence. In some embodiments, the transamidase recognition sequence is selected from: LPKT (SEQ ID NO: 48), LPIT (SEQ ID NO: 49), LPDT (SEQ ID NO: 50), SPKT (SEQ ID NO: 51), and YPXR (SEQ ID NO: 52).
NO: 51), LAET (SEQ ID NO: 52), LAAT (SEQ ID NO: 53), LAET (SEQ ID NO: 54), LAST
(SEQ ID NO: 55), LAET (SEQ ID NO: 56), LPLT (SEQ ID NO: 57), LSRT (SEQ ID NO:
58), LPET (SEQ ID NO: 59), VPDT (SEQ ID NO: 60), IPQT (SEQ ID NO: 61), YPRR
(SEQ ID NO: 62), LPMT (SEQ ID NO: 63), LPLT (SEQ ID NO: 64), LAFT (SEQ ID NO:
65), LPQT (SEQ ID NO: 66), NSKT (SEQ ID NO: 67), NPQT (SEQ ID NO: 68), NAKT
(SEQ ID NO: 69), and NPQS (SEQ ID NO: 70). In some embodiments, e.g., in certain
embodiments in which sortase A is used (see below), the transamidase recognition motif
comprises the amino acid sequence XiPX₂X₃, where X₁ is leucine, isoleucine, valine or
methionine; X₂ is any amino acid; X₃ is threonine, serine or alanine; P is proline and G is
glycine. In specific embodiments, as noted above X₁, is leucine and X₃ is threonine. In certain
embodiments, X₂ is aspartate, glutamate, alanine, glutamine, lysine or methionine. In certain
embodiments, e.g., where sortase B is utilized, the recognition sequence often comprises the
amino acid sequence NPXiTX₂, where X₁ is glutamine or lysine; X₂ is asparagine or glycine;
N is asparagine; P is proline and T is threonine. The invention encompasses the recognition
that selection of X may be based at least in part in order to confer desired properties on the
compound containing the recognition motif. In some embodiments, X is selected to modify a
property of the compound that contains the recognition motif, such as to increase or decrease
solubility in a particular solvent. In some embodiments, X is selected to be compatible with
reaction conditions to be used in synthesizing a compound comprising the recognition motif,
e.g., to be unreactive towards reactants used in the synthesis.

[0097] In some embodiments, X is -O-. In some embodiments, X is -NR-. In some
embodiments, X is -NH-. In some embodiments, X is -S-.

[0098] In certain embodiments, R¹ is substituted aliphatic. In certain embodiments, R¹ is
unsubstituted aliphatic. In some embodiments, R¹ is substituted C₁₋₁₂ aliphatic. In some
embodiments, R¹ is unsubstituted C₁₋₁₂ aliphatic. In some embodiments, R¹ is substituted C₁₋₆
aliphatic. In some embodiments, R¹ is unsubstituted C₁₋₆ aliphatic. In some embodiments,
R¹ is C₁₋₃ aliphatic. In some embodiments, R¹ is butyl. In some embodiments, R¹ is n-butyl.
In some embodiments, R¹ is isobutyl. In some embodiments, R¹ is propyl. In some
embodiments, R¹ is n-propyl. In some embodiments, R¹ is isopropyl. In some embodiments,
R¹ is ethyl. In some embodiments, R¹ is methyl.

[0099] In certain embodiments, R¹ is substituted aryl. In certain embodiments, R¹ is
unsubstituted aryl. In certain embodiments, R¹ is substituted phenyl. In certain
embodiments, R¹ is unsubstituted phenyl.
In some embodiments, A\textsuperscript{1} comprises a protein. In some embodiments, A\textsuperscript{1} comprises a peptide. In some embodiments, A\textsuperscript{1} comprises an antibody, an antibody chain, an antibody fragment, an antibody epitope, an antigen-binding antibody domain, a VHH domain, a single-domain antibody, a camelid antibody, a nanobody, an affibody, an anticalin, a DARPin, or an adnectin. In some embodiments, A\textsuperscript{1} comprises a recombinant protein, a protein comprising one or more D-amino acids, a branched peptide, a therapeutic protein, an enzyme, a polypeptide subunit of a multisubunit protein, a transmembrane protein, a cell surface protein, a methylated peptide or protein, an acylated peptide or protein, a lipidated peptide or protein, a phosphorylated peptide or protein, or a glycosylated peptide or protein. In some embodiments, A\textsuperscript{1} is an amino acid sequence comprising at least 3 amino acids. In some embodiments, A\textsuperscript{1} comprises a protein. In some embodiments, A\textsuperscript{1} comprises a peptide. In some embodiments, A\textsuperscript{1} comprises an antibody. In some embodiments, A\textsuperscript{1} comprises an antibody fragment. In some embodiments, A\textsuperscript{1} comprises an antibody epitope. In some embodiments, A\textsuperscript{1} comprises green fluorescent protein. In some embodiments, A\textsuperscript{1} comprises ubiquitin.

In some embodiments, B\textsuperscript{1} comprises a click chemistry handle. In some embodiments, B\textsuperscript{1} comprises a click chemistry handle described herein. In some embodiments, B\textsuperscript{1} comprises a click chemistry handle described in Table 1, in Table 2, or in Figure 2B. In some embodiments, B\textsuperscript{1} comprises a click chemistry handle described in Kolb, Finn and Sharpless Angewandte Chemie International Edition (2001) 40: 2004-2021; Evans, Australian Journal of Chemistry (2007) 60: 384-395; Joerg Lahann, Click Chemistry for Biotechnology and Materials Science, 2009, John Wiley & Sons Ltd, ISBN 978-0-470-69970-6; or Becer, Hoogenboom, and Schubert, click Chemistry beyond Metal-Catalyzed Cycloaddition, Angewandte Chemie International Edition (2009) 48: 4900 - 4908; the entire contents of each of which are incorporated herein by reference. For example, in certain embodiments, B\textsuperscript{1} comprises a terminal alkyne, azide, strained alkyne, diene, dieneophile, alkoxyamine, carbonyl, phosphine, hydrazide, thiol, or alkene moiety. In some embodiments, B\textsuperscript{1} comprises a click chemistry handle described in Table 1 or Table 2, or in Figure 2B.

In certain embodiments, n is an integer from 0 to 50, inclusive. In certain embodiments, n is an integer from 0 to 20, inclusive. In certain embodiments, n is 0. In certain embodiments, n is 1. In certain embodiments, n is 2. In certain embodiments, n is 3. In certain embodiments, n is 4. In certain embodiments, n is 5. In certain embodiments, n is 6.
Sortase-mediated installation of N-terminal click chemistry handles

In certain embodiments, a sortase-mediated transacylation reaction for installing an N-terminal click chemistry handle on a protein comprises a step of contacting a protein of the structure:

\[
\begin{align*}
    &\text{H}_2\text{N} & & \text{H} & & \text{O} & & \text{B}^1 & \text{n} \\
\end{align*}
\]

wherein

n is 0 or an integer between 1-100, inclusive; and

\[
\text{B}^1 \text{ is or comprises an amino acid sequence of at least three amino acid residues;}
\]

with a molecule of the structure

\[
\begin{align*}
    &\text{A}^1 & \text{Transamidase recognition sequence} & \text{O} & & \text{XR}^1 \\
\end{align*}
\]

wherein

the transamidase recognition sequence is an amino acid sequence motif recognized by a transamidase enzyme; a transamidase recognition sequence is also referred to herein as a sortase recognition sequence or a sortase recognition motif;

\[
\text{X is } -0-, -\text{NR}-, \text{ or } -\text{S}; \text{ wherein } \text{R is hydrogen, substituted or unsubstituted aliphatic, or substituted or unsubstituted heteroaliphatic;} \\
\text{A}^1 \text{ is or comprises acyl, substituted or unsubstituted aliphatic, substituted or unsubstituted heteroaliphatic, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, an amino acid, a peptide, a protein, a polynucleotide, a carbohydrate, a tag, a metal atom, a contrast agent, a catalyst, a non-polypeptide polymer, a recognition element, a small molecule, a lipid, a linker, and/or a label; wherein } \text{A}^1 \text{ comprises a click chemistry handle; and}
\]

\[
\text{R}^1 \text{ is hydrogen, acyl, substituted or unsubstituted aliphatic, substituted or unsubstituted heteroaliphatic, substituted or unsubstituted aryl, or substituted or unsubstituted heteroaryl;}
\]

in the presence of a transamidase enzyme, for example, a sortase, under suitable conditions to form a compound of formula:

\[
\begin{align*}
    &\text{A}^1 & \text{Transamidase recognition sequence} & \text{O} & & \text{N} & & \text{H} & & \text{O} & & \text{B}^1 & \text{n} \\
\end{align*}
\]
It will be understood by those of skill in the art that the click chemistry handle may be incorporated into A1 in any manner and at any position that can be envisioned by those of skill in the art. For example, A1 may comprise an amino acid, (e.g., lysine) and the click chemistry handle may be attached, for example, to the central carbon of the amino acid, the side chain of the amino acid, or to the amino group of the amino acid, or any other position. Other ways of incorporating the click chemistry handle into A1 will be apparent to those of skill in the art, and the invention is not limited in this respect.

One of ordinary skill will appreciate that, in certain embodiments, the C-terminal amino acid of the transamidase recognition sequence is omitted. That is, an acyl group

\[
\begin{align*}
\text{XR}^1 & \text{ replaces the C-terminal amino acid of the transamidase recognition sequence. In some embodiments, the acyl group is } \frac{\text{OR}^1}{\text{OMe}}. \\
\end{align*}
\]

In some embodiments, the transamidase, or sortase, recognition sequence is LPXT, wherein X is a standard or non-standard amino acid. In some embodiments, X is selected from D, E, A, N, Q, K, or R. In some embodiments, the recognition sequence is selected from LPXT, LPXT, SPXT, LAXT, LSXT, NPXT, VPXT, IPXT, and YPXR. In some embodiments X is selected to match a naturally occurring transamidase recognition sequence. In some embodiments, the transamidase recognition sequence is selected from: LPKT (SEQ ID NO: 48), LPIT (SEQ ID NO: 49), LPDT (SEQ ID NO: 50), SPKT (SEQ ID NO: 51), LAET (SEQ ID NO: 52), LAAT (SEQ ID NO: 53), LAET (SEQ ID NO: 54), LAST (SEQ ID NO: 55), LAET (SEQ ID NO: 56), LPLT (SEQ ID NO: 57), LSRT (SEQ ID NO: 58), LPET (SEQ ID NO: 59), VPDT (SEQ ID NO: 60), IPQT (SEQ ID NO: 61), YPRR (SEQ ID NO: 62), LPMT (SEQ ID NO: 63), LPLT (SEQ ID NO: 64), LAFT (SEQ ID NO: 65), LPQT (SEQ ID NO: 66), NSKT (SEQ ID NO: 67), NPQT (SEQ ID NO: 68), NAKT (SEQ ID NO: 69), and NPQS (SEQ ID NO: 70). In some embodiments, e.g., in certain embodiments in which sortase A is used (see below), the transamidase recognition motif comprises the amino acid sequence XiPX2X3, where X1 is leucine, isoleucine, valine or methionine; X2 is any amino acid; X3 is threonine, serine or alanine; P is proline and G is glycine. In specific embodiments, as noted above X1 is leucine and X3 is threonine. In certain embodiments, X2 is aspartate, glutamate, alanine, glutamine, lysine or methionine. In certain embodiments, e.g., where sortase B is utilized, the recognition sequence often comprises the
amino acid sequence NPXiTX$_2$, where X$_1$ is glutamine or lysine; X$_2$ is asparagine or glycine; N is asparagine; P is proline and T is threonine. The invention encompasses the recognition that selection of X may be based at least in part in order to confer desired properties on the compound containing the recognition motif. In some embodiments, X is selected to modify a property of the compound that contains the recognition motif, such as to increase or decrease solubility in a particular solvent. In some embodiments, X is selected to be compatible with reaction conditions to be used in synthesizing a compound comprising the recognition motif, e.g., to be unreactive towards reactants used in the synthesis.

[00106] In some embodiments, X is -0-. In some embodiments, X is -NR-. In some embodiments, X is -NH-. In some embodiments, X is -S-.

[00107] In certain embodiments, R$_1$ is substituted aliphatic. In certain embodiments, R$_1$ is unsubstituted aliphatic. In some embodiments, R$_1$ is substituted C$_{1-12}$ aliphatic. In some embodiments, R$_1$ is unsubstituted C$_{1-12}$ aliphatic. In some embodiments, R$_1$ is substituted C$_{1-6}$ aliphatic. In some embodiments, R$_1$ is unsubstituted C$_{1-6}$ aliphatic. In some embodiments, R$_1$ is Ci-3 aliphatic. In some embodiments, R$_1$ is butyl. In some embodiments, R$_1$ is w-butyl. In some embodiments, R$_1$ is isobutyl. In some embodiments, R$_1$ is propyl. In some embodiments, R$_1$ is w-propyl. In some embodiments, R$_1$ is isopropyl. In some embodiments, R$_1$ is ethyl. In some embodiments, R$_1$ is methyl.

[00108] In certain embodiments, R$_1$ is substituted ary1. In certain embodiments, R$_1$ is unsubstituted ary1. In certain embodiments, R$_1$ is substituted phenyl. In certain embodiments, R$_1$ is unsubstituted phenyl.

[00109] In some embodiments, B$_1$ comprises a protein. In some embodiments, B$_1$ comprises a peptide. In some embodiments, B$_1$ comprises an antibody, an antibody chain, an antibody fragment, an antibody epitope, an antigen-binding antibody domain, a VHH domain, a single-domain antibody, a camelid antibody, a nanobody, an affibody, an anticalin, a DARPin, or an adnectin. In some embodiments, B$_1$ comprises a recombinant protein, a protein comprising one or more D-amino acids, a branched peptide, a therapeutic protein, an enzyme, a polypeptide subunit of a multisubunit protein, a transmembrane protein, a cell surface protein, a methylated peptide or protein, an acylated peptide or protein, a lipidated peptide or protein, a phosphorylated peptide or protein, or a glycosylated peptide or protein. In some embodiments, B$_1$ is an amino acid sequence comprising at least 3 amino acids. In some embodiments, B$_1$ comprises a protein. In some embodiments, B$_1$ comprises a peptide. In some embodiments, B$_1$ comprises an antibody. In some embodiments, B$_1$ comprises an antibody fragment. In some embodiments, B$_1$ comprises an antibody epitope. In some
embodiments, B comprises green fluorescent protein. In some embodiments, B comprises ubiquitin.

[00110] In some embodiments, A comprises a click chemistry handle. In some embodiments, A comprises a click chemistry handle described herein. In some embodiments, A comprises a click chemistry handle described in Table 1, in Table 2, or in Figure 2B. In some embodiments, A comprises a click chemistry handle described in Kolb, Finn and Sharpless Angewandte Chemie International Edition (2001) 40: 2004-2021; Evans, Australian Journal of Chemistry (2007) 60: 384-395; Joerg Lahann, click Chemistry for Biotechnology and Materials Science, 2009, John Wiley & Sons Ltd, ISBN 978-0-470-69970-6; or Becer, Hoogenboom, and Schubert, click Chemistry beyond Metal-Catalyzed Cycloaddition, Angewandte Chemie International Edition (2009) 48: 4900 - 4908; the entire contents of each of which are incorporated herein by reference. For example, in certain embodiments, A comprises a terminal alkyne, azide, strained alkyne, diene, dieneophile, alkoxyamine, carbonyl, phosphine, hydrazide, thiol, or alkene moiety. In some embodiments, A comprises a click chemistry handle described in Table 1 or Table 2, or in Figure 2B.

[00111] In certain embodiments, n is an integer from 0 to 50, inclusive. In certain embodiments, n is an integer from 0 to 20, inclusive. In certain embodiments, n is 0. In certain embodiments, n is 1. In certain embodiments, n is 2. In certain embodiments, n is 3. In certain embodiments, n is 4. In certain embodiments, n is 5. In certain embodiments, n is 6.

Suitable enzymes and recognition motifs

[00112] In certain embodiments, the transamidase is a sortase. Enzymes identified as "sortases" from Gram-positive bacteria cleave and translocate proteins to proteoglycan moieties in intact cell walls. Among the sortases that have been isolated from Staphylococcus aureus, are sortase A (Srt A) and sortase B (Srt B). Thus, in certain embodiments, a transamidase used in accordance with the present invention is a sortase A, e.g., from S. aureus. In certain embodiments, a transamidase is a sortase B, e.g., from S. aureus.

[00113] Sortases have been classified into 4 classes, designated A, B, C, and D, based on sequence alignment and phylogenetic analysis of 61 sortases from Gram positive bacterial genomes (Dramsi S, Trieu-Cuot P, Bierne H, Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res Microbiol. 156(3):289-97, 2005. These classes correspond to the following subfamilies, into which sortases have also been classified by Comfort and Clubb (Comfort D, Clubb RT. A comparative genome analysis identifies
distinct sorting pathways in gram-positive bacteria. Infect Immun., 72(5):27 10-22, 2004): Class A (Subfamily 1), Class B (Subfamily 2), Class C (Subfamily 3), Class D (Subfamilies 4 and 5). The aforementioned references disclose numerous sortases and recognition motifs. See also Pallen, M. J.; Lam, A. C ; Antonio, M.; Dunbar, K. TRENDS in Microbiology, 2001, 9(3), 97-101. Those skilled in the art will readily be able to assign a sortase to the correct class based on its sequence and/or other characteristics such as those described in Drami et al, supra. The term "sortase A" is used herein to refer to a class A sortase, usually named SrtA in any particular bacterial species, e.g., SrtA from S. aureus. Likewise "sortase B" is used herein to refer to a class B sortase, usually named SrtB in any particular bacterial species, e.g., SrtB from S. aureus. The invention encompasses embodiments relating to a sortase A from any bacterial species or strain. The invention encompasses embodiments relating to a sortase B from any bacterial species or strain. The invention encompasses embodiments relating to a class C sortase from any bacterial species or strain. The invention encompasses embodiments relating to a class D sortase from any bacterial species or strain.

[00114] Amino acid sequences of Srt A and Srt B and the nucleotide sequences that encode them are known to those of skill in the art and are disclosed in a number of references cited herein, the entire contents of all of which are incorporated herein by reference. The amino acid sequences of S. aureus SrtA and SrtB are homologous, sharing, for example, 22% sequence identity and 37% sequence similarity. The amino acid sequence of a sortase-transamidase from Staphylococcus aureus also has substantial homology with sequences of enzymes from other Gram-positive bacteria, and such transamidases can be utilized in the ligation processes described herein. For example, for SrtA there is about a 31% sequence identity (and about 44% sequence similarity) with best alignment over the entire sequenced region of the S. pyogenes open reading frame. There is about a 28% sequence identity with best alignment over the entire sequenced region of the A. naeslundii open reading frame. It will be appreciated that different bacterial strains may exhibit differences in sequence of a particular polypeptide, and the sequences herein are exemplary.

[00115] In certain embodiments a transamidase bearing 18% or more sequence identity, 20% or more sequence identity, or 30% or more sequence identity with the S. pyogenes, A. naeslundii, S. mutans, E. faecalis or B. subtilis open reading frame encoding a sortase can be screened, and enzymes having transamidase activity comparable to Srt A or Srt B from S. aureas can be utilized (e.g., comparable activity sometimes is 10% of Srt A or Srt B activity or more).
Thus in some embodiments of the invention the sortase is a sortase A (SrtA).
SrtA recognizes the motif LPXTG (SEQ ID NO: 2), with common recognition motifs being,
e.g., LPKTG (SEQ ID NO: 71), LPATG (SEQ ID NO: 96), LPNTG (SEQ ID NO: 97). In
some embodiments LPETG (SEQ ID NO: 4) is used. However, motifs falling outside this
consensus may also be recognized. For example, in some embodiments the motif comprises
an 'A' rather than a 'T' at position 4, e.g., LPXAG (SEQ ID NO: 98), e.g., LPNAG (SEQ
ID NO: 99). In some embodiments the motif comprises an 'A' rather than a 'G' at position 5,
e.g., LPXTA (SEQ ID NO: 100), e.g., LPNTA (SEQ ID NO: 101). In some embodiments
the motif comprises a 'G' rather than a 'P' at position 2, e.g., LGXTG (SEQ ID NO: 102),
e.g., LGATG (SEQ ID NO: 102). In some embodiments the motif comprises an 'L' rather
than 'L' at position 1. e.g., IPXTG (SEQ ID NO: 104), e.g., IPNTG (SEQ ID NO: 105) or
IPETG (SEQ ID NO: 106).

It will be appreciated that the terms "recognition motif" and "recognition
sequence", with respect to sequences recognized by a transamidase or sortase, are used
interchangeably. The term "transamidase recognition sequence" is sometimes abbreviated
"TRS" herein.

In some embodiments of the invention the sortase is a sortase B (SrtB), e.g., a
sortase B of S. aureus, B. anthracis, or L. monocytogenes. Motifs recognized by sortases of
the B class (SrtB) often fall within the consensus sequences NPXTX, e.g., NP[Q/K]-[T/s]-
[N/G/s] (SEQ ID NO: 107), such as NPQTN (SEQ ID NO: 108) or NPKTG (SEQ ID NO:
109). For example, sortase B of S. aureus or B. anthracis cleaves the NPQTN (SEQ ID NO:
110) or NPKTG (SEQ ID NO: 111) motif of IsdC in the respective bacteria (see, e.g.,
Other recognition motifs found in putative substrates of class B sortases are NSKTA (SEQ ID
NO: 112), NPQTG (SEQ ID NO: 113), NAKTN (SEQ ID NO: 114), and NPQSS (SEQ ID
NO: 115). For example, SrtB from L. monocytogenes recognizes certain motifs lacking P at
position 2 and/or lacking Q or K at position 3, such as NAKTN (SEQ ID NO: 116) and
NPQSS (SEQ ID NO: 117) (Mariscotti JF, Garcia-Del Portillo F, Puccionielli MG. The
listeria monocytogenes sortase-B recognizes varied amino acids at position two of the sorting

In some embodiments, the sortase is a class C sortase. Class C sortases may
utilize LPXTG (SEQ ID NO: 2) as a recognition motif.

In some embodiments, the sortase is a class D sortase. Sortases in this class are
predicted to recognize motifs with a consensus sequence NA-[E/A/S/H]-TG (SEQ ID NO:
118) (Comfort D, supra). Class D sortases have been found, e.g., in Streptomyces spp., Corynebacterium spp., Tropheryma whippelii, Thermobifida fusca, and Bifidobacterium longum. LPXTA (SEQ ID NO: 100) or LAXTG (SEQ ID NO: 120) may serve as a recognition sequence for class D sortases, e.g., of subfamilies 4 and 5, respectively. Subfamily-4 and subfamily-5 enzymes process the motifs LPXTA (SEQ ID NO: 100) and LAXTG (SEQ ID NO: 122), respectively. For example, B. anthracis Sortase C, which is a class D sortase, has been shown to specifically cleave the LPNTA (SEQ ID NO: 123) motif in B. anthracis Basl and BasH (Marrafini, supra).


[00122] The invention contemplates use of sortases found in any gram positive organism, such as those mentioned herein and/or in the references (including databases) cited herein. The invention also contemplates use of sortases found in gram negative bacteria, e.g., ColwellUap psychrerythraea, Microbulbifer degradans, Bradyrhizobium japonicum, Shewanella oneidensis, and Shewanella putrefaciens. They recognize sequence motifs LP[Q/K]T[A/S]T (SEQ ID NO: 121). In keeping with the variation tolerated at position 3 in sortases from gram positive organisms, a sequence motif LPXT[A/S] (SEQ ID NO: 119), e.g., LPXTA (SEQ ID NO: 100) or LPSTS (SEQ ID NO: 128) may be used.

[00123] The invention contemplates use of sortase recognition motifs from any of the experimentally verified or putative sortase substrates listed at http://bamics3.cmbi.kun.nl/jos/sortase_substrates/help.html, the contents of which are incorporated herein by reference, and/or in any of the above-mentioned references. In some embodiments the sortase recognition motif is selected from: LPKTG (SEQ ID NO: 71), LPITG (SEQ ID NO: 72), LPDTA (SEQ ID NO: 73), SPKTG (SEQ ID NO: 74), LAETG (SEQ ID NO: 75), LAATG (SEQ ID NO: 76), LAHTG (SEQ ID NO: 77), LASTG (SEQ ID NO: 78), LAETG (SEQ ID NO: 79), LPLTG (SEQ ID NO: 80), LSRTG (SEQ ID NO: 81), LPETG (SEQ ID NO: 4), VPDTG (SEQ ID NO: 82), IPQTG (SEQ ID NO: 83), YPRRG (SEQ ID NO: 84), LPMTG (SEQ ID NO: 85), LPLTG (SEQ ID NO: 86), LAFTG (SEQ ID NO: 87), LPQTS (SEQ ID NO: 89), it being understood that in various embodiments of the invention the 5th residue is replaced, as described elsewhere herein. For example, the sequence used may be LPXT, LAXT, LPXA, LGXT, IPXT, NPXT, NPXS, LPST (SEQ ID NO: 90), NSKT (SEQ ID NO: 91), NPQT (SEQ ID NO: 92), NAKT (SEQ ID NO: 93), LPIT
(SEQ ID NO: 94), LAET (SEQ ID NO: 95), or NPQS (SEQ ID NO: 70). The invention comprises embodiments in which 'X' in any sortase recognition motif disclosed herein or known in the art is any standard or non-standard amino acid. Each variation is disclosed. In some embodiments, X is selected from the 20 standard amino acids found most commonly in proteins found in living organisms. In some embodiments, e.g., where the recognition motif is LPXTG (SEQ ID NO: 2) or LPXT, X is D, E, A, N, Q, K, or R. In some embodiments, X in a particular recognition motif is selected from those amino acids that occur naturally at position 3 in a naturally occurring sortase substrate. For example, in some embodiments X is selected from K, E, N, Q, A in an LPXTG (SEQ ID NO: 2) or LPXT motif where the sortase is a sortase A. In some embodiments X is selected from K, S, E, L, A, N in an LPXTG (SEQ ID NO: 2) or LPXT motif and a class C sortase is used.

In some embodiments, a recognition sequence further comprises one or more additional amino acids, e.g., at the N or C terminus. For example, one or more amino acids (e.g., up to 5 amino acids) having the identity of amino acids found immediately N-terminal to, or C-terminal to, a 5 amino acid recognition sequence in a naturally occurring sortase substrate may be incorporated. Such additional amino acids may provide context that improves the recognition of the recognition motif.

The term "transamidase recognition sequence" may refer to a masked or unmasked transamidase recognition sequence. A unmasked transamidase recognition sequence can be recognized by a transamidase. An unmasked transamidase recognition sequence may have been previously masked, e.g., as described herein. In some embodiments, a "masked transamidase recognition sequence" is a sequence that is not recognized by a transamidase but that can be readily modified ("unmasked") such that the resulting sequence is recognized by a transamidase. For example, in some embodiments at least one amino acid of a masked transamidase recognition sequence has a side chain that comprises a moiety that inhibits, e.g., substantially prevents, recognition of the sequence by a transamidase of interest, wherein removal of the moiety allows the transamidase to recognize the sequence. Masking may, for example, reduce recognition by at least 80%, 90%, 95%, or more (e.g., to undetectable levels) in certain embodiments. By way of example, in certain embodiments a threonine residue in a transamidase recognition sequence such as LPXTG (SEQ ID NO: 2) is phosphorylated, thereby rendering it refractory to recognition and cleavage by SrtA. The masked recognition sequence can be unmasked by treatment with a phosphatase, thus allowing it to be used in a SrtA-catalyzed transamidation reaction.
Modified proteins comprising click chemistry handles

Some embodiments provide a modified protein (PRT) comprising a C-terminal click chemistry handle (CCH), wherein the modified protein comprises a structure according to Formula (I):

\[
PRT - \text{LPXT} - [Xaa]_y - \text{CCH} \quad \text{(I)}.
\]

Some embodiments provide a modified protein (PRT) comprising an N-terminal click chemistry handle (CCH), wherein the modified protein comprises a structure according to Formula (I) according to Formula (II):

\[
\text{CHH} - [Xaa]_y - \text{LPXT} - \text{PRT} \quad \text{(II)}.
\]

wherein, in Formulas (I) and (II):

- PRT is an amino acid sequence of at least three amino acids;
- each instance of Xaa is independently an amino acid residue;
- \(y\) is 0 or an integer between 1-100;
- LPXT is a sortase recognition motif; and
- CCH is a click chemistry handle.

In some embodiments, a modified protein is provided that consists of a structure according to Formula (I) or Formula (II).

Click Chemistry

Two proteins comprising a click chemistry handle each (e.g., a first protein comprising a click chemistry handle providing a nucleophilic (Nu) group and a second protein comprising an electrophilic (E) group that can react with the Nu group of the first click chemistry handle) can be covalently conjugated under click chemistry reaction conditions. Click chemistry is a chemical philosophy introduced by Sharpless in 2001 and describes chemistry tailored to generate substances quickly and reliably by joining small units together (see, e.g., Kolb, Finn and Sharpless *Angewandte Chemie International Edition* (2001) 40: 2004-2021; Evans, *Australian Journal of Chemistry* (2007) 60: 384-395). Additional exemplary click chemistry handles, reaction conditions, and associated methods useful according to aspects of this invention are described in Joerg Lahann, *Click Chemistry for Biotechnology and Materials Science*, 2009, John Wiley & Sons Ltd, ISBN 978-0-470-69970-6, the entire contents of which are incorporated herein by reference.

Click chemistry should be modular, wide in scope, give high chemical yields, generate inoffensive byproducts, be stereospecific, be physiologically stable, exhibit a large thermodynamic driving force (e.g., > 84 kJ/mol to favor a reaction with a single reaction
product), and/or have high atom economy. Several reactions have been identified which fit this concept:

(1) The Huisgen 1,3-dipolar cycloaddition (e.g., the Cu(I)-catalyzed stepwise variant, often referred to simply as the "click reaction"; see, e.g., Tornoe et al., *Journal of Organic Chemistry* (2002) 67: 3057-3064). Copper and ruthenium are the commonly used catalysts in the reaction. The use of copper as a catalyst results in the formation of 1,4-regioisomer whereas ruthenium results in formation of the 1,5-regioisomer;

(2) Other cycloaddition reactions, such as the Diels-Alder reaction;

(3) Nucleophilic addition to small strained rings like epoxides and aziridines;

(4) Nucleophilic addition to activated carbonyl groups; and

(4) Addition reactions to carbon-carbon double or triple bonds.

**Conjugation of proteins via click chemistry handles**

For two proteins to be conjugated via click chemistry, the click chemistry handles of the proteins have to be reactive with each other, for example, in that the reactive moiety of one of the click chemistry handles can react with the reactive moiety of the second click chemistry handle to form a covalent bond. Such reactive pairs of click chemistry handles are well known to those of skill in the art and include, but are not limited to those described in Table I:

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>1,3-dipolar cycloaddition</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁</td>
<td>R₂</td>
<td>Strain-promoted cycloaddition</td>
</tr>
<tr>
<td>R₁</td>
<td>R₂</td>
<td>Dieles-Alder reaction</td>
</tr>
<tr>
<td>R₁</td>
<td>R₂</td>
<td>Thiol-ene reaction</td>
</tr>
</tbody>
</table>

TABLE 1: Exemplary click chemistry handles and reactions, wherein each occurrence of R₁, R₂, is independently PRT-LPXT-[Xaa]ᵧ, or -[Xaa]ᵧ-LPXT-PRT, according to Formulas (I) and (II).
In some preferred embodiments, click chemistry handles are used that can react to form covalent bonds in the absence of a metal catalyst. Such click chemistry handles are well known to those of skill in the art and include the click chemistry handles described in Becer, Hoogenboom, and Schubert, *Click Chemistry beyond Metal-Catalyzed Cycloaddition*, Angewandte Chemie International Edition (2009) 48: 4900 - 4908.

<table>
<thead>
<tr>
<th>Reagent A</th>
<th>Reagent B</th>
<th>Mechanism</th>
<th>Notes on reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>Cu-catalyzed [3+2] cycloaddition</td>
<td>2 h at 60°C in H$_2$O</td>
<td>[9]</td>
</tr>
<tr>
<td>1</td>
<td>azide</td>
<td>cyclooctyne</td>
<td>strain-promoted [3+2] azide-alkyne cycloaddition</td>
<td>1 h at RT</td>
</tr>
<tr>
<td>2</td>
<td>azide</td>
<td>activated alkyne</td>
<td>[3+2] Huisgen cycloaddition</td>
<td>4 h at 50°C</td>
</tr>
<tr>
<td>3</td>
<td>azide</td>
<td>electron-deficient alkyne</td>
<td>[3+2] cycloaddition</td>
<td>12 h at RT in H$_2$O</td>
</tr>
<tr>
<td>4</td>
<td>azide</td>
<td>arylene</td>
<td>[3+2] cycloaddition</td>
<td>4 h at RT in THF with crown ether or 24 h at RT in CH$_2$CN</td>
</tr>
<tr>
<td>5</td>
<td>tetrazine</td>
<td>alkyne</td>
<td>Diels-Alder 4+2 cycloaddition</td>
<td>40 min at 25°C (100% yield)</td>
</tr>
<tr>
<td>6</td>
<td>tetrazole</td>
<td>aikene</td>
<td>1,3-dipolar cycloaddition</td>
<td>few µg UV irradiation and then overnight at 4°C</td>
</tr>
<tr>
<td>7</td>
<td>dibenzospiro[2.2]dibenzo[1,2-c:4,5-c']dicyclon</td>
<td>dibenzospiro[2.2]dibenzo[1,2-c:4,5-c']dicyclon</td>
<td>2 days at reflux in tolulene</td>
<td>[41]</td>
</tr>
<tr>
<td>8</td>
<td>anthracene</td>
<td>maleimide</td>
<td>Diels-Alder reaction</td>
<td>10 min at RT</td>
</tr>
<tr>
<td>9</td>
<td>thiou</td>
<td>enone</td>
<td>Michael addition</td>
<td>30 min UV (quantitative conv.) or 24 h at RT</td>
</tr>
<tr>
<td>10</td>
<td>thiou</td>
<td>maleimide</td>
<td>Michael addition</td>
<td>1 h at 40°C in THF or 16 h at RT in dichloromethane</td>
</tr>
<tr>
<td>11</td>
<td>thiou</td>
<td>para-fluoro</td>
<td>Nucleophilic substitution</td>
<td>overnight at RT in DMF</td>
</tr>
<tr>
<td>12</td>
<td>thiou</td>
<td>para-fluoro</td>
<td>Nucleophilic substitution</td>
<td>60 min at 40°C in DMF</td>
</tr>
</tbody>
</table>

[a] RT = room temperature, DMF = 4-N,N-dimethylaminopyridine, NMP = N,N-dimethylpropyleneurea, THF = tetrahydrofuran, CH$_2$CN = acetonitrile.


For example, in some embodiments, a first protein is provided comprising a C-terminal strained alkyne group, for example, a C-terminal cyclooctyne group as the click chemistry handle, and a second protein is provided comprising a C-terminal azide group as the click chemistry handle. The two click chemistry handles are reactive with each other, as they can carry out a strain-promoted cycloaddition, which results in the first and the second protein being conjugated via a covalent bond. In this example, the two C-termini of the proteins are conjugated together, which is also referred to as a C-C, or a C to C, conjugation.

In certain embodiments, a first molecule, for example, a first protein, comprising a nucleophilic click chemistry handle (Nu) selected from -SH, -OH, -NHR<sup>b</sup>, -NH-NHR<sup>b</sup>, or -N=NH, is conjugated to a second molecule, for example, a second protein, comprising the electrophilic partner click chemistry handle (E),

to form a chimeric protein with a conjugated group of the formula:
wherein $Z^{b9}$ is -S-, -O-, -N(R$^{b5}$)-, -NH-N(R$^{b5}$)-, or -N=N-. In some embodiments, the nucleophilic click chemistry handle Nu is -SH and $Z^{b9}$ is -S-. In certain embodiments, Nu is -OH and $Z^{b9}$ is -O-. In certain embodiments, Nu is -NHR$^{b5}$ and $Z^{b9}$ is -N(R$^{b5}$)-. In certain embodiments, Nu is -NH-NHR$^{b5}$ and $Z^{b9}$ is -NH-N(R$^{b5}$)-. In certain embodiments, Nu is -N=NH and $Z^{b9}$ is -N=N-. In certain embodiments, R$^{b5}$ is hydrogen.

In certain embodiments, Nu is -SH, -OH, -NHR$^{b5}$, -NH-NHR$^{b5}$, or -N=NH, and E is , and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:

wherein $Z^{b9}$ is -S-, -O-, -N(R$^{b5}$)-, -NH-N(R$^{b5}$)-, or -N=N-. In certain embodiments, Nu is -SH and $Z^{b9}$ is -S-. In certain embodiments, Nu is -OH and $Z^{b9}$ is -O-. In certain embodiments, Nu is -NHR$^{b5}$ and $Z^{b9}$ is -N(R$^{b5}$)-. In certain embodiments, Nu is -NH-NHR$^{b5}$ and $Z^{b9}$ is -NH-N(R$^{b5}$)-. In certain embodiments, Nu is -N=NH and $Z^{b9}$ is -N=N-. In certain embodiments, R$^{b5}$ is hydrogen.

In certain embodiments, Nu is -SH, -OH, -NHR$^{b5}$, -NH-NHR$^{b5}$, or -N=NH, and E is , and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:
wherein \( Z^{b9} \) is -S-, -O-, \( -N(R^{b5}) \), \( -\text{NH-N}(R^{b5}) \), or \( -N=N- \). In certain embodiments, \( N_u \) is -SH and \( Z^{b9} \) is -S-. In certain embodiments, \( N_u \) is -OH and \( Z^{b9} \) is -O-. In certain embodiments, \( N_u \) is -NHR and \( Z^{b9} \) is -N(R). In certain embodiments, \( N_u \) is -NH-NHR and \( Z^{b9} \) is -NH-NR. In certain embodiments, \( R^{b5} \) is hydrogen. In certain embodiments, \( R^{b6} \) is hydrogen, optionally substituted aliphatic, or optionally substituted heteroaliphatic. In certain embodiments, \( R^{b6} \) is hydrogen or \( C_{1-6} \) alkyl. In certain embodiments, \( R^{b6} \) is hydrogen or \( -\text{CH}_3 \). In certain embodiments, \( R^{b8} \) is hydrogen. In certain embodiments, \( R^{b8} \) is an amino protecting group.

In certain embodiments, \( N_u \) is -SH, -OH, -NHR, -NH-NHR, or -N=NH, and \( E \) is , and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein \( N_u \) and \( E \) are joined to form a conjugated group of the formula:

![Diagram](image)

wherein \( Z^{b9} \) is -S-, -O-, \( -N(R^{b5}) \), \( -\text{NH-N}(R^{b5}) \), or \( -N=N- \). In certain embodiments, \( N_u \) is -SH and \( Z^{b9} \) is -S-. In certain embodiments, \( N_u \) is -OH and \( Z^{b9} \) is -O-. In certain embodiments, \( N_u \) is -NHR and \( Z^{b9} \) is -N(R). In certain embodiments, \( N_u \) is -NH-NHR and \( Z^{b9} \) is -NH-NR. In certain embodiments, \( R^{b5} \) is hydrogen. In certain embodiments, \( R^{b6} \) is hydrogen, optionally substituted aliphatic, or optionally substituted heteroaliphatic. In certain embodiments, \( R^{b6} \) is hydrogen or \( C_{1-6} \) alkyl. In certain embodiments, \( R^{b6} \) is hydrogen or \( -\text{CH}_3 \). In certain embodiments, \( R^{b11} \) is hydrogen. In certain embodiments, \( R^{b11} \) is an oxygen protecting group.

In certain embodiments, \( N_u \) is -SH, -OH, -NHR, -NH-NHR, or -N=NH, and \( E \) is \(-C0_2\text{R}^{b6}, -\text{COX}^{b7}\), and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein \( N_u \) and \( E \) are joined to form a conjugated group of the formula:

![Diagram](image)

wherein \( Z^{b9} \) is -S-, -O-, \( -N(R^{b5}) \), \( -\text{NH-N}(R^{b5}) \), or \( -N=N- \). In certain embodiments, \( N_u \) is -SH and \( Z^{b9} \) is -S-. In certain embodiments, \( N_u \) is -OH and \( Z^{b9} \) is -O-. In certain embodiments, \( N_u \) is -NHR and \( Z^{b9} \) is -N(R). In certain embodiments, \( N_u \) is -NH-NHR and \( Z^{b9} \) is -NH-NR. In certain embodiments, \( N_u \) is -NH-NHR. In certain embodiments, \( N_u \) is -NH-NHR.
and Z\textsuperscript{b9} is -NH-N(R\textsuperscript{b5})-. In certain embodiments, Nu is -N=NH and Z\textsuperscript{b9} is -N=N-. In certain embodiments, R\textsuperscript{b5} is hydrogen.

[00139] In certain embodiments, Nu is -SH, -OH, -NHR\textsuperscript{b5}, -NH-NHR\textsuperscript{b5}, or -N=NH, and E is , and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula: 

![Diagram](image)

wherein Z\textsuperscript{b9} is -S-, -O-, -N(R\textsuperscript{b5})-, -NH-N(R\textsuperscript{b5})-, or -N=NH-. In certain embodiments, Nu is -SH and Z\textsuperscript{b9} is -S-. In certain embodiments, Nu is -OH and Z\textsuperscript{b9} is -O-. In certain embodiments, Nu is -NHR\textsuperscript{b5} and Z\textsuperscript{b9} is -N(R\textsuperscript{b5})-. In certain embodiments, Nu is -NH-NHR\textsuperscript{b5} and Z\textsuperscript{b9} is -NH-N(R\textsuperscript{b5})-. In certain embodiments, Nu is -N=NH and Z\textsuperscript{b9} is -N=N-. In certain embodiments, R\textsuperscript{b5} is hydrogen. In certain embodiments, R\textsuperscript{b6} is hydrogen, optionally substituted aliphatic, or optionally substituted heteroaliphatic. In certain embodiments, R\textsuperscript{b6} is hydrogen or C\textsubscript{1-4}alkyl. In certain embodiments, R\textsuperscript{b6} is hydrogen or -CH\textsubscript{3}.

[00140] In certain embodiments, Nu is -SH, -OH, -NHR\textsuperscript{b5}, -NH-NHR\textsuperscript{b5}, or -N=NH, and E is , and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula: 

![Diagram](image)

wherein Z\textsuperscript{b9} is -S-, -O-, -N(R\textsuperscript{b5})-, -NH-N(R\textsuperscript{b5})-, or -N=NH-. In certain embodiments, Nu is -SH and Z\textsuperscript{b9} is -S- (a thiol-yne reaction). In certain embodiments, Nu is -OH and Z\textsuperscript{b9} is -O-. In certain embodiments, Nu is -NHR\textsuperscript{b5} and Z\textsuperscript{b9} is -N(R\textsuperscript{b5})-. In certain embodiments, Nu is -NH-NHR\textsuperscript{b5} and Z\textsuperscript{b9} is -NH-N(R\textsuperscript{b5})-. In certain embodiments, Nu is -N=NH and Z\textsuperscript{b9} is -N=N-. In certain embodiments, R\textsuperscript{b5} is hydrogen. In certain embodiments, R\textsuperscript{b6} is hydrogen, optionally substituted aliphatic, or optionally substituted heteroaliphatic. In certain embodiments, R\textsuperscript{b6} is hydrogen or C\textsubscript{1-4}alkyl. In certain embodiments, R\textsuperscript{b6} is hydrogen or -CH\textsubscript{3}.
In certain embodiments, Nu is -SH, -OH, -NHR<sub>b</sub>, -NH-NHR<sub>b</sub>, or -N=NH, and

![Diagram](image)

E is X<sub>b</sub><sup>9</sup>, and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:

![Diagram](image)

wherein Z<sub>b</sub><sup>9</sup> is -S-, -O-, -N(R<sup>b</sup><sub>5</sub>)-, -NH-N(R<sup>b</sup><sub>5</sub>)-, or -N=N-. In certain embodiments, Nu is -SH and Z<sub>b</sub><sup>9</sup> is -S- (a thiol-yne reaction). In certain embodiments, Nu is -OH and Z<sub>b</sub><sup>9</sup> is -0-. In certain embodiments, Nu is -NHR<sub>b</sub> and Z<sub>b</sub><sup>9</sup> is -N(R<sup>b</sup><sub>5</sub>)-. In certain embodiments, Nu is -NH-NHR<sub>b</sub> and Z<sub>b</sub><sup>9</sup> is -NH-N(R<sup>b</sup><sub>5</sub>)-. In certain embodiments, Nu is -N=N=NH and Z<sub>b</sub><sup>9</sup> is -N=N-. [00142]

In certain embodiments, Nu is -SH, -OH, -NHR<sub>b</sub>, -NH-NHR<sub>b</sub>, or -N=NH, and

![Diagram](image)

E is X<sub>b</sub><sup>9</sup>, and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:

![Diagram](image)

wherein Z<sub>b</sub><sup>9</sup> is -S-, -O-, -N(R<sup>b</sup><sub>5</sub>)-, -NH-N(R<sup>b</sup><sub>5</sub>)-, or -N=N-. In certain embodiments, Nu is -SH and Z<sub>b</sub><sup>9</sup> is -S- (a thiol-yne reaction). In certain embodiments, Nu is -OH and Z<sub>b</sub><sup>9</sup> is -0-. In certain embodiments, Nu is -NHR<sub>b</sub> and Z<sub>b</sub><sup>9</sup> is -N(R<sup>b</sup><sub>5</sub>)-. In certain embodiments, Nu is -NH-NHR<sub>b</sub> and Z<sub>b</sub><sup>9</sup> is -NH-N(R<sup>b</sup><sub>5</sub>)-. In certain embodiments, Nu is -N=N=NH and Z<sub>b</sub><sup>9</sup> is -N=N-. [00143]

In certain embodiments, Nu is -SH, -OH, -NHR<sub>b</sub>, -NH-NHR<sub>b</sub>, or -N=NH, and

![Diagram](image)

E is X<sub>b</sub><sup>9</sup>, and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:
wherein \( Z_{b9} \) is \(-S-, -O-, -N(R^{b5})-\), \(-\text{NH-N}(R^{b5})-\), or \(-N=N-\). In certain embodiments, \( N u \) is \(-\text{SH}\) and \( Z_{b9} \) is \(-S-\) (a thiol-yne reaction). In certain embodiments, \( N u \) is \(-\text{OH}\) and \( Z_{b9} \) is \(-O-\). In certain embodiments, \( N u \) is \(-\text{NHR}^{b5}\) and \( Z_{b9} \) is \(-\text{N}(R^{b5})-\). In certain embodiments, \( N u \) is \(-\text{NH-NHR}^{b5}\) and \( Z_{b9} \) is \(-\text{NH-N}(R^{b5})-\). In certain embodiments, \( N u \) is \(-N=\text{NH}\) and \( Z_{b9} \) is \(-N=N-\).

[00144] In certain embodiments, \( N u \) is \(-\text{SH}, -\text{OH}, -\text{NHR}^{b5}, -\text{NH-NHR}^{b5}\), or \(-N=\text{NH}\), and \( E \) is \(-\text{CHO}\), and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein \( N u \) and \( E \) are joined to form a conjugated group of the formula:

\[
\begin{array}{c}
\overset{Y_2}{Z_{b9}} \overset{Y_3}{N} \overset{Y_4}{Z_{b9}} \\
\end{array}
\]

wherein \( Z_{b9} \) is \(-S-, -O-, -N(R^{b5})-\), \(-\text{NH-N}(R^{b5})-\), or \(-N=N-\). In certain embodiments, \( N u \) is \(-\text{SH}\) and \( Z_{b9} \) is \(-S-\) (a thiol-yne reaction). In certain embodiments, \( N u \) is \(-\text{OH}\) and \( Z_{b9} \) is \(-O-\). In certain embodiments, \( N u \) is \(-\text{NHR}^{b5}\) and \( Z_{b9} \) is \(-\text{N}(R^{b5})-\). In certain embodiments, \( N u \) is \(-\text{NH-NHR}^{b5}\) and \( Z_{b9} \) is \(-\text{NH-N}(R^{b5})-\). In certain embodiments, \( N u \) is \(-N=\text{NH}\) and \( Z_{b9} \) is \(-N=N-\).

[00145] In certain embodiments, \( N u \) is \(-N=\text{NH}\) and \( E \) is \(-\text{CHO}\), are conjugated to form a homodimer or a heterodimer polypeptide of Formula (III) wherein \( N u \) and \( E \) are joined to form a conjugated group of the formula:

\[
\begin{array}{c}
\overset{N}{\text{N}} \\
\end{array}
\]

[00146] In certain embodiments, \( N u \) is \(-\text{NHR}^{b5}\), \( R^{b5} \) is hydrogen, and \( E \) is \(-\text{CHO}\), and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein \( N u \) and \( E \) are joined to form a conjugated group of the formula:

\[
\begin{array}{c}
\overset{N}{\text{N}} \\
\end{array}
\]

[00147] In certain embodiments, \( N u \) is \(-\text{NH-N}(R^{b5})-\), \( R^{b5} \) is hydrogen, and \( E \) is \(-\text{CHO}\), and the two molecules, for example, two proteins, are conjugated to form a chimeric molecule, for example, a chimeric protein wherein \( N u \) and \( E \) are joined to form a conjugated group of the formula:

\[
\begin{array}{c}
\overset{N}{\text{N}} \\
\end{array}
\]
In certain embodiments, Nu is \( \text{R}^{b10} \), and E is \( \text{R}^{b6} \), and the two molecules, for example, two proteins, are conjugated via a Diels-Alder reaction to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:

\[
\begin{align*}
\text{Nu} & \quad \text{E} \\
\text{R}^{b10} & \quad \text{R}^{b6}
\end{align*}
\]

In certain embodiments, \( \text{R}^{b10} \) is hydrogen. In certain embodiments, \( \text{R}^{b6} \) is hydrogen or optionally substituted aliphatic, \( \text{e.g.} \), acyl.

In certain embodiments, Nu is \( \text{N}_{3} \), and the two molecules, for example, two proteins, are conjugated via a Huisgen 1,3-dipolar cycloaddition reaction to form a chimeric molecule, for example, a chimeric protein wherein Nu and E are joined to form a conjugated group of the formula:

\[
\begin{align*}
\text{Nu} & \quad \text{E} \\
\text{R}^{b6}
\end{align*}
\]

(1,4 regioisomer) or

(1,5 regioisomer).

In certain embodiments, \( \text{R}^{b6} \) is hydrogen, optionally substituted aliphatic, or optionally substituted heteroaliphatic. In certain embodiments, \( \text{R}^{b6} \) is hydrogen or \( \text{C}_{1-6} \text{alkyl} \). In certain embodiments, \( \text{R}^{b6} \) is hydrogen or \(-\text{CH}_{3}\). In certain embodiments, \( \text{R}^{b6} \) is hydrogen.

In certain embodiments, two proteins, each comprising a click chemistry handle Nu, wherein each Nu is independently \(-\text{SH}, -\text{OH}, -\text{NHR}^{b5}, -\text{NH-NHR}^{b5}, \) or \(-\text{N}=\text{NH}\), are conjugated by reacting the two polypeptides with a bis-electrophile of formula

\[
\text{X}^{b7} - \text{W}^{3} - \text{X}^{b7}
\]

wherein \( \text{X}^{b7} \) is a leaving group, and \( \text{W}^{3} \) is selected from the group consisting of optionally substituted alkyne; optionally substituted alkenylene; optionally substituted alkynylene; optionally substituted heteroalkylene; optionally substituted heteroalkenylene; optionally
substituted heteroalkynylene; optionally substituted arylene; or optionally substituted heteroarylene, to provide a conjugated group of formula:

\[
\begin{array}{c}
\text{Z}^{b7} - W_3 - Z^{b9} - \\
\end{array}
\]

wherein \( Z^{b7} \) is -0-, -S-, -N(R^5)-, -NH-N(R^5)-, or -N=N-. In certain embodiments, each Nu is -SH and each \( Z^{b9} \) is -S-. In certain embodiments, each Nu is -OH and each \( Z^{b9} \) is -0-. In certain embodiments, each Nu is -NHR^5 and each \( Z^{b9} \) is -N(R^5)-. In certain embodiments, each Nu is -NH-NHR^5 and each \( Z^{b9} \) is -NH-N(R^5)-. In certain embodiments, each Nu is -N=NH and each \( Z^{b9} \) is -N=N-. In certain embodiments, \( W_3 \) is optionally substituted alkylene. In certain embodiments, \( W_3 \) is optionally substituted arylene. In certain embodiments, \( W_3 \) is optionally substituted heteroarylene. Various combinations of the two Nu groups and two \( X^{b7} \) groups are contemplated. In certain embodiments, the two Nu groups, and thus the two \( Z^{b9} \) groups, are the same. In certain embodiments, the two Nu groups, and thus the two \( Z^{b9} \) groups, are different. In certain embodiments, the two \( X^{b7} \) groups are the same. In certain embodiments, the two \( X^{b7} \) groups are different.

[00151] In certain embodiments, wherein \( W_3 \) is optionally substituted alkylene, the bis-electrophile is of the formula:

\[
\begin{array}{c}
\text{X}^{b7} \\
\end{array}
\]

wherein \( X^{b7} \) is -Br, -Cl, or -I.

[00152] For example, when the bis-electrophile is of the formula:

\[
\begin{array}{c}
\text{X}^{b7} \\
\end{array}
\]

, the resulting conjugated group is of the formula:

\[
\begin{array}{c}
\text{Z}^{b9} - W_3 - Z^{b9} - \\
\end{array}
\]

[00153] In certain embodiments, wherein \( W_3 \) is optionally substituted heteroarylene, the bis-electrophile is of the formula:

\[
\begin{array}{c}
\text{X}^{b7} \\
\end{array}
\]

wherein \( X^{b7} \) is -Br, -Cl, or -I.
For example, when the bis-electrophile is of the formula:

\[ X^b7 \begin{array}{c} Y_2 \ \ Y_3 \end{array} \begin{array}{c} X^b7 \end{array}, \]

the resulting conjugated group is of the Formula:

\[ \begin{array}{c} Y_2 \ \ Y_3 \end{array} \begin{array}{c} Z^b9 \ \ Z^b9 \end{array}. \]

In certain embodiments, two proteins, each comprising a click chemistry handle E, wherein each E is independently selected from a leaving group, \(-\text{CHO}, -\text{CO}_2\text{R}^{b6}, -\text{COX}^{b7}, \)

\[ R^{b6}, \quad \begin{array}{c} O \end{array} R^{b6}, \quad \begin{array}{c} \equiv \end{array} R^{b6}, \quad Y_2 Y_3, \quad X^b7, \quad \begin{array}{c} \equiv \end{array} R^{b6} \]

are conjugated by reacting the two polypeptides with a bis-nucleophile Nu—\( W_4 \)—Nu wherein each Nu is -\text{SH}, -\text{OH}, -\text{NHR}^{b5}, -\text{NH-NHR}^{b5}, -\text{N=N=NH}, -\text{N=C}, -\text{N}_3, \) or \(-\equiv R^{b10}, \) and \( W_4 \) is independently represents optionally substituted alkylene; optionally substituted alkenylene; optionally substituted alkynylene; optionally substituted heteroalkylene; optionally substituted heteroalkenylene; optionally substituted heteroalkynylene; optionally substituted arylene; optionally substituted heteroarylene; or a combination thereof; to provide a conjugated polypeptide. The two E groups conjugated to \( W_4 \) independently correspond to any of the above described conjugated groups, also listed below:

\[ R^{b6}, \quad \begin{array}{c} N \end{array} R^{b6}, \quad \begin{array}{c} \equiv \end{array} R^{b6}, \quad \begin{array}{c} \equiv \end{array} R^{b6}, \quad \begin{array}{c} \equiv \end{array} R^{b6} \]
Various combinations of the two E groups are contemplated. In certain embodiments, the two E groups are the same. In certain embodiments, the two E groups are different. In certain embodiments, the two Nu groups, and thus the two Z₉ groups, are different. In certain embodiments, the two X₇ groups are the same. In certain embodiments, the two X₇ groups are different.

**Chimeric proteins and uses thereof**

[00156] Some embodiments of this invention provide chimeric proteins, for example, proteins comprising a sortase recognition motif and conjugated to a second molecule via click chemistry. In some embodiments, the chimeric protein comprises an antibody or antibody fragment, for example, a nanobody. In some embodiments, the antibody, or antibody fragment, is a therapeutic antibody or antibody fragment, for example, an antibody or antibody fragment that binds to a therapeutic target antigen. Some embodiments embrace any therapeutic antibody known to those of skill in the art, since the invention is not limited in this respect. Further, any antibody or antibody fragment binding to a therapeutic antigen, for example, to the same or a different epitope of the therapeutic antigen as a known therapeutic antibody, can be employed in some embodiments of this invention, for example, for the generation of chimeric antibodies as described herein. Some embodiments provide chimeric antibodies that are generated as the result of derivatizing such therapeutic antibodies, or antibodies binding therapeutic antigens, according to methods described herein.

[00157] In some embodiments, a chimeric protein targets a specific antigen, cell type, or site in a cell population, tissue, organism, or subject. For example, in some embodiments, a chimeric, bi-specific antibody is provided that comprises a first antigen binding domain that
targets the antibody to a target site (e.g., an organ, a cell or cell type (e.g., a diseased cell, such as a tumor cell), a tissue, or a site of disease) and a second antigen binding domain that provides a function, e.g., a therapeutic function. Such therapeutic function may be provided by a toxin, or by a molecule attracting a specific cell or cell type to the target site. In some embodiments, a chimeric protein is provided that comprises an antibody targeting a specific cell, cell type, tissue, or site, for example, in a subject, wherein the antibody is conjugated via click chemistry to a therapeutic agent, for example, a small molecule, or a therapeutic polypeptide. In some embodiments, a therapeutic protein as provided herein binds to a tumor antigen as target antigens. In some embodiments, a therapeutic protein as provided herein binds to antigens of a known or potential pathogen (e.g., a virus, a bacterium, a fungus, or a parasite).

[00158] Those of skill in the art will understand that chimeric polypeptides and proteins as provided herein may comprise any therapeutic agent that either comprises or can be linked to a click chemistry handle.

[00159] In some embodiments, the methods and reagents described herein are used to attach a target protein to a solid or semi-solid support or a surface, e.g., a particle (optionally magnetic), a microparticle, a nanoparticle, a bead, a slide, a filter, or a well (e.g., of multiwell/microtiter plate).

[00160] In some embodiments, the methods and reagents described herein, and the modified proteins, for example, the chimeric proteins, or the chimeric antibodies described herein, are used in vitro, in vivo, in research, for detection, for screening, in diagnostic assays, or in therapeutic applications. Exemplary, non-limiting therapeutic applications include treatment of infectious diseases, treatment of cancer, and treatment of metabolic disease. Other therapeutic uses will be evident to those of skill in the art, since the invention is not limited in this respect.

Selected target proteins

[00161] Without limiting the invention in any way, this section discusses certain target proteins. In general, any protein or polypeptide can be modified to carry a click chemistry handle and/or conjugated to another molecule via click chemistry according to methods provided herein. In some embodiments the target protein comprises or consists of a polypeptide that is at least 80%, or at least 90%, e.g., at least 95%, 86%, 97%, 98%, 99%, 99.5%, or 100% identical to a naturally occurring protein or polypeptide. In some embodiments, the target protein has no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid
differences relative to a naturally occurring sequence. In some embodiments the naturally occurring protein is a mammalian protein, e.g., of human origin. In some embodiments, the protein is an antibody, an antibody fragment, or protein comprising an antigen-binding domain. In some embodiments the naturally occurring protein is a cytokine, e.g., a type I cytokine. In some embodiments of particular interest, the target protein is a four-helix bundle protein, e.g., a four-helix bundle cytokine. Exemplary four-helix bundle cytokines include, e.g., certain interferons (e.g., a type I interferon, e.g., IFN-Cc), interleukins (e.g., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-12), and colony stimulating factors (e.g., G-CSF, GM-CSF, M-CSF). The IFN can be, e.g., interferon alpha 2a or interferon alpha 2b. See, e.g., Mott HR and Campbell ID. "Four-helix bundle growth factors and their receptors: protein-protein interactions." Curr Opin Struct Biol. 1995 Feb;5(1):114-21; Chaiken IM, Williams WV. "Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design." Trends Biotechnol. 1996 Oct;14(10):369-75; Klaus W, et al, "The three-dimensional high resolution structure of human interferon alpha-2a determined by heteronuclear NMR spectroscopy in solution". J Mol Biol., 274(4):661-75, 1997, for further discussion of certain of these cytokines.

[00162] In some embodiments, the cytokine has a similar structure to one or more of the afore-mentioned cytokines. For example, the cytokine can be an IL-6 class cytokine such as leukemia inhibitory factor (LIF) or oncostatin M. In some embodiments, the cytokine is one that in nature binds to a receptor that comprises a GP130 signal transducing subunit. Other four-helix bundle proteins of interest include growth hormone (GH), prolactin (PRL), and placental lactogen. In some embodiments, the target protein is an erythropoiesis stimulating agent, e.g., erythropoietin (EPO), which is also a four-helix bundle cytokine. In some embodiments, an erythropoiesis stimulating agent is an EPO variant, e.g., darbepoetin alfa, also termed novel erythropoiesis stimulating protein (NESP), which is engineered to contain five N-linked carbohydrate chains (two more than recombinant HuEPO). In some embodiments, the protein comprises five helices. For example, the protein can be an interferon beta, e.g., interferon beta-la or interferon beta-lb, which (as will be appreciated) is often classified as a four-helix bundle cytokine. In some embodiments, a target protein is IL-9, IL-10, IL-11, IL-13, or IL-15. See, e.g., Hunter, CA, Nature Reviews Immunology 5, 521-531, 2005, for discussion of certain cytokines. See also Paul, WE (ed.), Fundamental Immunology, Lippincott Williams & Wilkins; 6th ed., 2008. Any protein described in the
references cited herein, all of which are incorporated herein by reference, can be used as a target protein.

[00163] In some embodiments, a target protein is a protein that is approved by the US Food & Drug Administration (or an equivalent regulatory authority such as the European Medicines Evaluation Agency) for use in treating a disease or disorder in humans. Such proteins may or may not be one for which a PEGylated version has been tested in clinical trials and/or has been approved for marketing.

[00164] In some embodiments, a target protein is a neurotrophic factor, i.e., a factor that promotes survival, development and/or function of neural lineage cells (which term as used herein includes neural progenitor cells, neurons, and glial cells, e.g., astrocytes, oligodendrocytes, microglia). For example, in some embodiments, the target protein is a factor that promotes neurite outgrowth. In some embodiments, the protein is ciliary neurotrophic factor (CNTF; a four-helix bundle protein) or an analog thereof such as Axokine, which is a modified version of human Ciliary neurotrophic factor with a 15 amino acid truncation of the C terminus and two amino acid substitutions, which is three to five times more potent than CNTF in in vitro and in vivo assays and has improved stability properties.

[00165] In some embodiments, the target protein is one that forms homodimers or heterodimers, (or homo- or heterooligomers comprising more than two subunits, such as tetramers). In certain embodiments the homodimer, heterodimer, or oligomer structure is such that a terminus of a first subunit is in close proximity to a terminus of a second subunit. For example, an N-terminus of a first subunit is in close proximity to a C-terminus of a second subunit. In certain embodiments the homodimer, heterodimer, or oligomer structure is such that a terminus of a first subunit and a terminus of a second subunit are not involved in interaction with a receptor, so that the termini can be joined via a non-genetically encoded peptide element without significantly affecting biological activity. In some embodiments, termini of two subunits of a homodimer, heterodimer, or oligomer are conjugated via click chemistry using a method described herein, thereby producing a dimer (or oligomer) in which at least two subunits are covalently joined. For example, the neurotrophins nerve growth factor (NGF); brain-derived neurotrophic factor (BDNF); neurotrophin 3 (NT3); and neurotrophin 4 (NT4) are dimeric molecules which share approximately 50% sequence identity and exist in dimeric forms. See, e.g., Robinson RC, et al, "Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer.", Biochemistry. 34(13):4139-46, 1995; Robinson RC, et al, "The structures of the neurotrophin 4 homodimer and the brain-
derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site.” Protein Sci. 8(12):2589-97, 1999, and references therein. In some embodiments, the dimeric protein is a cytokine, e.g., an interleukin.

[00166] In some embodiments, the target protein is an enzyme, e.g., an enzymes that is important in metabolism or other physiological processes. As is known in the art, deficiencies of enzymes or other proteins can lead to a variety of disease. Such diseases include diseases associated with defects in carbohydrate metabolism, amino acid metabolism, organic acid metabolism, porphyrin metabolism, purine or pyrimidine metabolism, lysosomal storage disorders, blood clotting, etc. Examples include Fabry disease, Gaucher disease, Pompe disease, adenosine deaminase deficiency, asparaginase deficiency, porphyria, hemophilia, and hereditary angioedema. In some embodiments, a protein is a clotting or coagulation factor, (e.g., factor VII, Vila, VIII or IX). In other embodiments a protein is an enzyme that plays a role in carbohydrate metabolism, amino acid metabolism, organic acid metabolism, porphyrin metabolism, purine or pyrimidine metabolism, and/or lysosomal storage, wherein exogenous administration of the enzyme at least in part alleviates the disease.

[00167] In some embodiments, a target protein comprises a receptor or receptor fragment (e.g., extracellular domain). In some embodiments the receptor is a TNFcc receptor. In certain embodiments, the target protein comprises urate oxidase.

[00168] One of skill in the art will be aware of the sequences of proteins described herein. Without limitation, sequences of certain target protein are found in, e.g., USSN 10/773,530; 11/531,531; USSN 11/707,014; 11/429,276; 11/365,008. In some embodiments, a target protein is listed in Table 3. The invention encompasses application of the inventive methods to any of the proteins described herein and any proteins known to those of skill in the art.

[00169] In some embodiments, the invention provides modified versions of any target protein, wherein the modified version comprises (i) one or more nucleophilic residues such as glycine at the N-terminus (e.g., between 1 and 10 residues) and, optionally, a cleavage recognition sequence, e.g., a protease cleavage recognition sequence that masks the nucleophilic residue(s); or (ii) a sortase recognition motif at or near the C-terminus. In some embodiments, the target protein comprises both (i) and (ii). Such modified proteins can be used in the methods of protein conjugation as described herein.

[00170] One of skill in the art will be aware that certain proteins, e.g., secreted eukaryotic (e.g., mammalian) proteins, often undergo intracellular processing (e.g., cleavage of a
secretion signal prior to secretion and/or removal of other portion(s) that are not required for biological activity), to generate a mature form. Such mature, biologically active versions of target proteins are used in certain embodiments of the invention.

Table 3: selected target protein sequences

<table>
<thead>
<tr>
<th>Chain A: TTCCGLRQY (SEQ ID NO: 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain B: IKGGLFADIAHSPWQAAIFAKHHRGGERF/LCGGILSSCWLSA</td>
</tr>
<tr>
<td>HCFQQCQEQEEEEERFFFFPPPHHHHVTILGTRYVVPGE</td>
</tr>
<tr>
<td>EEQQFEEVYKIYVHKEFDFTYNDIAL.LQKSSSSSDDDSSSSSS</td>
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<tr>
<td>SSSSSSSRRCQAQESSVVRTVCPLPADLQLPDWTCELSGYGKHE</td>
</tr>
<tr>
<td>ALSPFYSERLKEAHVRLYPSSRCTTSSSSQQQHLNRTVTDNLMC</td>
</tr>
<tr>
<td>GLGCGRGQKDPVGVYKTNVYLDIWMRNRP (SEQ ID NO: 47)</td>
</tr>
</tbody>
</table>

| Chain A: VVGGEDAKPGQFQWPQVLNKGVAFCGGSIVNEKWIVTA_AHCV |
| Chain B: EETTTYKTVVAGHEINNIEETETQKRNVIRIPHHNYNNAA |
| AANKYNYHDIAL.LEDEPVLSNYYTPICAKDEYKTNINNNFL |
| FOSGYGSQGVORFVKGRSALQTYLVRVLVDVATLCLSTKFTTSQL |
| NNMFCAGGFTHFGGGRRDSQDGSGPHVTVEGTST.E.TGHISW |
| GEECAAMMKGYKTYKVSYNYNWEKTLT (SEQ ID NO: 6) |

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<thead>
<tr>
<th>Factor IX</th>
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<tr>
<td>EFARPCIPSFGYSSVVCNATYCDSDPADLPGTFYRESTRGSGR</td>
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<td>RMELSMGPIQANHTGTLLLT.LQPEQPKQKVKGFGAMTDAA</td>
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<td>DAVMLMNPDGSAVVVLNRSSKDPVLTIDPAPVGFLEITPQYSI</td>
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<table>
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<tr>
<td>LDNGLARTPTMGWLHWERFMCNLCDQEEDPCSEKLIFMEMAE</td>
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<td>RQLANYVHSIGKLGKYIADVCAFLGIGSFYYIDQTAQD</td>
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<tr>
<td>WGVDLKLDFGCYDCCLENADYKHMSLMLNRGTRISVYCEW</td>
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<tr>
<td>PLYMWPIQFQPNYETIRQCNHWRNFADDYDSKISKILDWTSF</td>
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<tr>
<td>NQRVDVAGPGWNPDMILVINGFLSWQOVQTMALWAIM</td>
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<tr>
<td>AAPLFMSNDLRHISPDKALKVLDQDKVIAINQDPGLQKGQLYRQG</td>
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<tr>
<td>DNFEVWERPLGLAAYADNIQGEPGPRSYITASIWSLKGKAV</td>
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<td>NPCFCTTIQLLPVKRKLGFYEWTSRSLRSHNPITGVQLLQENTM (SEQ ID NO: 9)</td>
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<table>
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<tr>
<th>alpha galactosidase A</th>
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<tr>
<td>RPPNIVLIFYADDLGCGDLCGHPSSTTPNLDQLAAAGGLRFTDFY</td>
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<td>VPVSLPSRAALLTGRLPVRMGMYPGVLYPSSRGGPLPLEEVTVAE</td>
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<tr>
<td>VLAARGLYIAGMKWHLGVGPEGALPFPQFHRFLGIPYSHD</td>
</tr>
<tr>
<td>QGFCQNHLCFPPATPCDGCDQQLVPIPLANL.SVEAQPPWPLGL</td>
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<tr>
<td>EARYMYAFHDMLMADAQQRDRPFLFLYASLHITHYPFQSGSFAE</td>
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<tr>
<td>RSRGPRPGDSMLMELDAAVGTLMTAIGDLGLEEITAVIFDANGPE</td>
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<tr>
<td>TMRRSRGCGCSGLRRCRGKTTYEEGVRPEALFWPHIGAIVTGHE</td>
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<td>LASSLDDLPTALAAAGAPLVPNTRDFDLPLLLGTTGKSPRQSLLF (SEQ ID NO: 10)</td>
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<table>
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<th>arylsulfatase-A (iduronidase, α-L-)</th>
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<td>RPPNIVLIFYADDLGCGDLCGHPSSTTPNLDQLAAAGGLRFTDFY</td>
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<td>VPVSLPSRAALLTGRLPVRMGMYPGVLYPSSRGGPLPLEEVTVAE</td>
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<td>TMRRSRGCGCSGLRRCRGKTTYEEGVRPEALFWPHIGAIVTGHE</td>
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<td>LASSLDDLPTALAAAGAPLVPNTRDFDLPLLLGTTGKSPRQSLLF (SEQ ID NO: 10)</td>
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</table>
YPSYPDEVRGFVAFAVRTGKYKAHFFTQGSAHSDTTADPACHASSS LTAHEPPLL YLDSKGDPGYLNLLGATPVQALKQLQLKAQLD AAVTFGPQVARDEPDALQICCHPGCTPRPACCHCP (SEQ ID NO: 10)

aryl sulfatase B (N-acetylgalactosamine-4-sulfatase) (lfsu)

SRPPLHLADDLLGWNVDVGFGSRTPLHLDAAGGVLLENY YTQLTPRSSQRLLLGYRQITQLQHQLQPSCPSCPVLDEKLLQQ LKEAGYTHVMGKWHLMYKRECLPRTGRDFTYFLYGLLEDY YSHERCITLALDNRACLDREDGEEVATGYNMSTNFTKRAI ALTHNIEPEKPLFLALQSVEHLPQPEEYLYPQDIFQKDNHHR YAGYMVLSDMDEAVGNTAALKSSLGWNTVFIPTDNGGQITLAG GNNWPLRGRKWLSWEGVVGRQVAPLKKQGKVRKNRELHIIS DWLPQKLARGHNTGTKPLDFDVKWKTISEGSPRIELHNID PNFPVSSCASIANTSVHAIRHGNWKLTTGYPGCYWFPSPSQY NVSEIPEPSDPFPTKTLWLDIDDRDPEERHDLRSREYPHIVTKLSSLQF YHKKHSSVPYFFPAQDPRCDPKATGVWGWM (SEQ ID NO: 11)

beta-hexosaminidase A (2gjx)

LWPWPQFNFQITSDQRYLVPNNFQFPQYDVSAAQPGCSVLDEAF QRYRDLLFTGLTEKNLVVSVTVPGCNQLPTLESVENYLTINQDO CLLSETWVLARGLETFSQVLWKSASEGTTFKINETEIDRFPFRPH GLLDDTSRYHLPLSSLDLTDLVMAYNKLNVHHLVWDDSFPYSES FFTPELMKRGSYNVPVTTHYTAQDVKEVIEYARLGRIRVLAEDTFTPH GHTLSWGPQGPLLtTTPCSIESPSTFTPVPVNSLPNLTYEMSTFLL EVSSVFPFDYLHHLGDEVDFTCWSNPEIQDMRKKGGEDFKQL LFSYIQITDLLDIVSSYKGYVQWQVFDPNKKIQIDTIQVWREDI PNYMKELELVTAKAFRALLSAPWYLNRSYGPDWFDVFYVEPL AFEGTPQKALVIGGEACMWGEYVDTNVLVPLRPWPRAGAVAE RLWSNKLTSDLTFAYERLHFSRCELLLRGQVAQPLNVGFCEQEFQ (SEQ ID NO: 12)

Hexosaminidase A and B (2gjx)

CHAIN A:

LWPWPQFNFQTSQRYLVPNNFQFPQYDVSAAQPGCSVLDEAF QRYRDLLFTGLTEKNLVVSVTVPGCNQLPTLESVENYLTINQDO CLLSETWVLARGLETFSQVLWKSASEGTTFKINETEIDRFPFRPH GLLDDTSRYHLPLSSLDLTDLVMAYNKLNVHHLVWDDSFPYSES FFTPELMKRGSYNVPVTTHYTAQDVKEVIEYARLGRIRVLAEDTFTPH GHTLSWGPQGPLLtTTPCSIESPSTFTPVPVNSLPNLTYEMSTFLL EVSSVFPFDYLHHLGDEVDFTCWSNPEIQDMRKKGGEDFKQL LFSYIQITDLLDIVSSYKGYVQWQVFDPNKKIQIDTIQVWREDI PNYMKELELVTAKAFRALLSAPWYLNRSYGPDWFDVFYVEPL AFEGTPQKALVIGGEACMWGEYVDTNVLVPLRPWPRAGAVAE RLWSNKLTSDLTFAYERLHFSRCELLLRGQVAQPLNVGFCEQEFQ (SEQ ID NO: 13)
DIIATINKGSIVWQEVFDDKAKLAPGTIVEVWKDSAYPEELSRVT
ASGFPVLSAPWYLDSLISYGQDWRKYYKVEPDLFGGTQKQQLFI
GGEACLWGEYVDATNLTPRLWRPRASVLAERLWSSKDVRRMD
A YDLTRLHRCRMVERGIAAQQPLGYAGYN (SEQ ID NO: 15)

Chain D:
LWPWPQNFQTDSRQNLVPNNFQQDVPNSSAAQPGCSVLD
EFQRYYLDLISYGQDWRKYYKVEPLDFGGTQKQKQLFI
GGEACLWGEYVDATNLTPRLWPRASAVGERLWSSKDVRD
AYDRLTRHRCRMVERGIAAQPLYAGYCN (SEQ ID NO: 16)

phenylalanine hydroxylase (PAH) (lj8u)

Cathepsin A

G-CSF

GM-CSF

Interferon alfa-2

Interferon beta-1

Interferon gamma-1b

IL-2 (1M47)

IL-1 (2nvh)
<table>
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<tr>
<th>Protein</th>
<th>Sequence</th>
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<td>TNF-alpha (4tsv)</td>
<td>DPKVHV/VANPQAEQLQWNSRANALLANGVELRDQNQLVVP1 EGLFLYISQVLFKQGQCPSTHVLLHTTISRIASYQTVKVNLLSAIKS PCQRETPEGEAKPWYEPYLGQVFQLEKEGDRSLEAINEPDLYDFF AESQVYYFGIAL</td>
<td>(SEQ ID NO: 26)</td>
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<td>TNF-beta (lymphotoxin) (ltnr)</td>
<td>KPAAAHLGDPSKQNSSLWRANTDRAFLQDGFSLSNNSLVPTSG1 YFVYQVYVFSGKAYSPKATSSPLYLAHEVQLFSQYPFHVLPILS QKMYVGPQLEPWLHSMYHGAAFQLTQDQLSTHTDGPILVSLP STVFVFGAFAL</td>
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<tr>
<td>Erythropoietin</td>
<td>APPRLCDSRVLERLYLAEKAEEKTGGCAEHSNLEKIVTPDFTKV NPYAWKRMEVQGQAVEQWQLALLSEAVLRGQALLVKSSQPWP EPLQLHVDKAVSGLRLTTLRLALGAQKEAISNDSAASAPLRTI TADTFRKLFRVYSNFLRGLKLYTGEACRTGD</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>Chain A: GIVEQCCTSICSLYQLENYCN (SEQ ID NO: 29)</td>
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<tr>
<td></td>
<td>Chain B: FNQHLCGSHLVEALYLVCGERGFFYTPK</td>
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<tr>
<td>Growth hormone (GH) (Somatomedin) (Ihwu)</td>
<td>FPTIPLSRLADNWLARADLNQLAFLDPTYQEEAEAYIPKEQISHFW WNPQTSLCPSEIPSTPSNEKTQKSNLLELRLSISLQSWLPEVFQ PVR SVFANSLVYGASDNSVYLLDLKDEEQITLMGLREALLKNYG LLLCYCFNKMSKVSTYLTQVCRSVECGF (SEQ ID NO: 31)</td>
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<td>Follicle-stimulating hormone (FSH)</td>
<td>CHHRICSNRFVLQCQSKVEITPSDLPRNAELRFVLTKLVRQQQ GAFSFGDLEKIESNDQVLEIDVFSNLPKHEIREKANNS NLPEAFQNLPLQYLISNTGKHLPDVHKIIHRQKVLLDQDNINI HTIERNFSVGFLSESFLVNLKNGQPJEHNACNTQDLNELSDN NNLEELPNDVFHAGSVPVILDISRTHLPSYQLENKLKARRST YNLKKPLITE (SEQ ID NO: 32)</td>
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<td>Leptin (lax8)</td>
<td>IQKQVDDTDLTIKLTVRTRINDLDIFPGLPILILTSMPSRNQISNDLNLDDLHFSAKSCHELPEASGLDELSDL GGIVLEASGYSTEVVALSRQGLSVDQMQLQDLSPPGC (SEQ ID NO: 33)</td>
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</tr>
<tr>
<td>Insulin-like growth factor (or somatomedin) (Iwq)</td>
<td>PETLCGAELDALAQFCGDRGFGFYNKPFGYSSSRAPQGTIQVE CCFRSCDLRRLLEMYCAP (SEQ ID NO: 34)</td>
<td></td>
</tr>
<tr>
<td>Adiponectin (lac28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chain A:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MYRSAFSVGLETRETVTPVPVIPRTFKIFYNQNHYDGSTGKFGYCN1 PGLYYFYSHITTVMKDVKYSFKKDKAFLTVFYDQYQVENQGAS GSVLHLHVEGDQVWQLQVYADVNVNDSTFTGFLLHYDT (SEQ ID NO: 35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chain B:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MYRSAFSVGGLPVPVIIRFTKIFYNQNHYGDSGKFGYCNIPGLFYSFYHVYHMYKDVKYSFKKDKAFLTVFYDQYQVENQGAS GSVLHLHVEGDQVWQLQVYADVNVNDSTFTGFLLHYDT (SEQ ID NO: 36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chain C:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MYRSAFSVGLETRETVTPVPVIPRTFKIFYNQNHYGDSGKFGYCNIPGLFYSFYHVYHMYKDVKYSFKKDKAFLTVFYDQYQVENQGAS GSVLHLHVEGDQVWQLQVYADVNVNDSTFTGFLLHYDT (SEQ ID NO: 37)</td>
<td></td>
</tr>
<tr>
<td>Factor VIII (aka antihemophilic factor) (2r7e)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Human serum albumin (lao6) (SEQ ID NO: 39)  

Chain A: 
SEVAHRFKDLGEENFKALVLLIAFAQYLQQCPFEDHVKLVNEVT 
AKTVCVADESAECDKSLHTLFQDGLCTVATLRETYGEMADCCA 
KQEPRNECFLQFHDDNPNLPRVRPVEVDVMCTAFHDEETFLK 
KLYSEEARRHPYYAPELLFAKRYAANFEQCAADAKAACLLP 
KLDELREDGKASSAKQRLCKASLQKFGERAFAKAVARLSQRF 
KAESFAESKLYTDLTKVHTECCGDLEACDDARLAKYICENQ 
DSISSKLKECEKPKLEHKCEIAEVENMDADLPFLAADFVEKSD 
VCKNYYAEADVFLGMFLYEEARHPDYSVLLRRLAKTYETTLE 
KCCAAADPHECYAVKFDEFKPVEEPQNIKONCELEFLQGEYKF 
QNALLVRYTKKVQPVSTPTVEVSRLGKVGSKCCKKHEAKRMP 
CAEDYLSVLQLCVLHEKTPVSDRTVKCETESLVNRPCFSALE 
VDETYVPKEFNAETFTHADICTLSEKERQIKKQTALVEVHVHP 
KATKEQLAKVMDDFFAAVEKCKCDKETCFAEEGKKLVAASQ 
AA (SEQ ID NO: 40)  

Chain B: 
SEVAHRFKDLGEENFKALVLLIAFAQYLQQCPFEDHVKLVNEVT 
AKTVCVADESAECDKSLHTLFQDGLCTVATLRETYGEMADCCA 
KQEPRNECFLQFHDDNPNLPRVRPVEVDVMCTAFHDEETFLK 
KLYSEEARRHPYYAPELLFAKRYAANFEQCAADAKAACLLP 
KLDELREDGKASSAKQRLCKASLQKFGERAFAKAVARLSQRF 
KAESFAESKLYTDLTKVHTECCGDLEACDDARLAKYICENQ 
DSISSKLKECEKPKLEHKCEIAEVENMDADLPFLAADFVEKSD 
VCKNYYAEADVFLGMFLYEEARHPDYSVLLRRLAKTYETTLE 
KCCAAADPHECYAVKFDEFKPVEEPQNIKONCELEFLQGEYKF 
QNALLVRYTKKVQPVSTPTVEVSRLGKVGSKCCKKHEAKRMP 
CAEDYLSVLQLCVLHEKTPVSDRTVKCETESLVNRPCFSALE 
VDETYVPKEFNAETFTHADICTLSEKERQIKKQTALVEVHVHP 
KATKEQLAKVMDDFFAAVEKCKCDKETCFAEEGKKLVAASQ 
AA (SEQ ID NO: 40)  

Hemoglobin (lbzO) (SEQ ID NO: 44)  

Chain A: 
VLSAPDKTNVKAAGWKVGAHAVEGYGAEALERMFLSFTPTKTYF 
PHFDLSHGSQVKGKHKKVADALTNAVAHVDPPMNALSDFDL 
HAIKLRLDPVNVKLLSHCLLLVLAHLPAEFTPAVHASLDF 
SVSTVLTSDKYR (SEQ ID NO: 43)  

Chain B: 
VHHTPEEASKAVTLWKGKVNVDEVGEALGRLLVYVPWTQRFF 
SFGDLSTPDAVMGNPKVKAHGGKVLGA5DGLAHLNLKGTFA 
TSLLEJHCDDHLHVDPENRLLGNVLVCYAHFHGFKEETPVPQAAY 
QKVVAGVANALARHKY (SEQ ID NO: 44)
[00171] It will be appreciated that considerable structure/function information is available regarding many of the afore-mentioned proteins, as well as sequences from different mammalian species, that can be used to design variants of the naturally occurring sequence that retain significant biological activity (e.g., at least 25%, 75%, 90% or more of the activity of the naturally occurring protein). For example, crystal structures or NMR structures of a number of proteins, in some instances in a complex with the corresponding receptor, are available. In addition, it will be understood that, if the naturally occurring N- and C-termini are not located in close proximity to each other in the native structure, a naturally occurring sequence can be extended at the N- and/or C-termini, e.g., with a flexible peptide spacer so that the termini can come into close proximity.

[00172] In various embodiments, an antibody binds to an antigen of interest. An antigen of interest may be or may comprise, for example, a polypeptide, a polysaccharide, a carbohydrate, a lipid, a nucleic acid, or combination thereof. An antigen may be naturally occurring or synthetic in various embodiments. In some embodiments, an antigen is naturally produced by and/or comprises a polypeptide or peptide that is genetically encoded by a pathogen, an infected cell, or a neoplastic cell (e.g., a cancer cell). In some embodiments, an antigen is an autoantigen ("self antigen"), or an agent that has the capacity to initiate or enhance an autoimmune response. In some embodiments, an antigen is produced or genetically encoded by a virus, bacteria, fungus, or parasite which, in some embodiments, is a pathogenic agent. In some embodiments, an agent (e.g., virus, bacterium, fungus, parasite) infects and, in some embodiments, causes disease in, at least one mammalian or avian species, e.g., human, non-human primate, bovine, ovine, equine, caprine, and/or porcine species. In some embodiments, a pathogen is intracellular during at least part of its life cycle. In some embodiments, a pathogen is extracellular. It will be appreciated that an antigen that originates from a particular source may, in various embodiments, be isolated from such source, or produced using any appropriate means (e.g., recombinantly, synthetically, etc.), e.g., for purposes of using the antigen, e.g., to identify, generate, test, or use an antibody thereto). An antigen may be modified, e.g., by conjugation to another molecule or entity (e.g., an adjuvant), chemical or physical denaturation, etc. In some embodiments, an antigen is an envelope protein, capsid protein, secreted protein, structural protein, cell wall protein or polysaccharide, capsule protein or polysaccharide, or enzyme. In some embodiments an antigen is a toxin, e.g., a bacterial toxin.
Exemplary viruses include, e.g., Retroviridae (e.g., lentiviruses such as human immunodeficiency viruses, such as HIV-I); Caliciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses, hepatitis C virus); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. Ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bunyaviridae (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (paroviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae; Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), EBV, KSV); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Picornaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses).

Exemplary bacteria include, e.g., Helicobacter pylori, Borrelia burgdorferi, Legionella pneumophila, Mycobacteria (e.g., M. tuberculosis, M. avium, M. intracellular, M. kansasii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus viridans group, Streptococcus faecalis, Streptococcus bovis, Streptococcus anaerobic spp., Streptococcus pneumoniae, Campylobacter sp., Enterococcus sp., Chlamydia sp., Haemophilus influenzae, Bacillus anthracis, Corynebacterium diphtheriae, Erysipelothrix rhusiopathiae, Clostridium perfringens, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasteurella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidum, Treponema pertenue, Leptospira, Actinomyces israelii and Francisella tularensis.

Exemplary fungi include, e.g., Aspergillus, such as Aspergillus flavus, Aspergillus jumigatus, Aspergillus niger, Blastomyces, such as Blastomyces dermatitidis, Candida, such as Candida albicans, Candida glabrata, Candida guilliermondii, Candida krusei, Candida parapsilosis, Candida tropicalis, Coccidioides, such as Coccidioides immitis, Cryptococcus, such as Cryptococcus neoformans, Epidermophyton, Fusarium, Histoplasma, such as Histoplasma capsulatum, Malassezia, such as Malassezia furfur, Microsporum, Mucor, Paracoccidioides, such as Paracoccidioides brasiliensis, Penicillium, such as Penicillium
marneffei, Pichia, such as Pichia anomala, Pichia guilliermondii, Pneumocystis, such as Pneumocystis carinii, Pseudallescheria, such as Pseudallescheria boydii, Rhizopus, such as Rhizopus oryzae, Rhodotorula, such as Rhodotorula rubra, Scedosporium, such as Scedosporium apiospermum, Schizophyllum, such as Schizophyllum commune, Sporothrix, such as Sporothrix schenckii, Trichophyton, such as Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton verrucosum, Trichophyton violaceum, Trichosporon, such as Trichosporon asahii, Trichosporon cutaneum, Trichosporon inkin, and Trichosporon mucoides.

Exemplary parasites include, e.g., Plasmodium spp. (e.g., P. falciparum, P. malariae, P. yoelii, P. berghei), Entamoeba spp. (e.g., Entamoeba histolytica), Giardia spp. (e.g., G. intestinalis, G. duodenalis, G. lamblia, G. muris, G. agilis, G. ardae, and G. psittaci), Toxoplasma spp. (e.g., T. gondii), Cryptosporidium spp. (e.g., C. parvum, C. muris, C. felis, C. wrairi, C. baileyi, C. meleagris, C. serpentis, and C. nasorum), Cyclospora spp. (e.g., C. cayetanensis), Naegleria spp. (e.g., Naegleria fowleri), Acanthamoeba spp., Leishmania spp. (e.g., L. major, L. tropica, L. aethiopica, L. mexicana, L. braziliensis, L. donovani, L. infantum, L. chagasi), Schistosoma spp. (e.g., S. mansoni), and Trypanosoma spp. (e.g., T.ambiostoma, T. avium, T. brucei, T. cruzi, T. congolense, T. equinum, T. lewisi, T. theileri, and T. vivax).

In some embodiments, an antigen is a tumor antigen (TA). In general, a tumor antigen can be any antigenic substance produced by tumor cells (e.g., tumorigenic cells or in some embodiments tumor stromal cells, e.g., tumor-associated cells such as cancer-associated fibroblasts). In many embodiments, a tumor antigen is a molecule (or portion thereof) that is differentially expressed by tumor cells as compared with non-tumor cells. Tumor antigens may include, e.g., proteins that are normally produced in very small quantities and are expressed in larger quantities by tumor cells, proteins that are normally produced only in certain stages of development, proteins whose structure (e.g., sequence or post-translational modification(s)) is modified due to mutation in tumor cells, or normal proteins that are (under normal conditions) sequestered from the immune system. Tumor antigens may be useful in, e.g., identifying or detecting tumor cells (e.g., for purposes of diagnosis and/or for purposes of monitoring subjects who have received treatment for a tumor, e.g., to test for recurrence) and/or for purposes of targeting various agents (e.g., therapeutic agents) to tumor cells. For example, in some embodiments, a chimeric antibody is provided, comprising an antibody of antibody fragment that binds a tumor antigen, and conjugated via click chemistry to a therapeutic agent, for example, a cytotoxic agent. In some embodiments, a TA is an
expression product of a mutated gene, e.g., an oncogene or mutated tumor suppressor gene, an overexpressed or aberrantly expressed cellular protein, an antigen encoded by an oncogenic virus (e.g., HBV; HCV; herpesvirus family members such as EBV, KSV; papilloma virus, etc.), or an oncofetal antigen. Oncofetal antigens are normally produced in the early stages of embryonic development and largely or completely disappear by the time the immune system is fully developed. Examples are alphafetoprotein (AFP, found, e.g., in germ cell tumors and hepatocellular carcinoma) and carcinoembryonic antigen (CEA, found, e.g., in bowel cancers and occasionally lung or breast cancer). Tyrosinase is an example of a protein normally produced in very low quantities but whose production is greatly increased in certain tumor cells (e.g., melanoma cells). Other exemplary TAs include, e.g., CA-125 (found, e.g., in ovarian cancer); MUC-1 (found, e.g., in breast cancer); epithelial tumor antigen (found, e.g., in breast cancer); melanoma-associated antigen (MAGE; found, e.g., in malignant melanoma); prostatic acid phosphatase (PAP, found in prostate cancer). In some embodiments, a TA is at least in part exposed at the cell surface of tumor cells. In some embodiments, a tumor antigen comprises an abnormally modified polypeptide or lipid, e.g., an aberrantly modified cell surface glycolipid or glycoprotein. It will be appreciated that a TA may be expressed by a subset of tumors of a particular type and/or by a subset of cells in a tumor.

Exemplary therapeutic antibodies that are useful in the production of chimeric antibodies or proteins according to methods provided herein include, but are not limited to, the following antibodies (target of the antibody is listed in parentheses together with exemplary non-limiting therapeutic indications):

Abciximab (glycoprotein IIb/IIIa; cardiovascular disease), Adalimumab (TNF-a, various auto-immune disorders, e.g., rheumatoid arthritis), Alemtuzumab (CD52; chronic lymphocytic leukemia), Basiliximab (IL-2Ra receptor (CD25); transplant rejection), Bevacizumab (vascular endothelial growth factor A; various cancers, e.g., colorectal cancer, non-small cell lung cancer, glioblastoma, kidney cancer; wet age-related macular degeneration), Catumaxomab, Cetuximab (EGF receptor, various cancers, e.g., colorectal cancer, head and neck cancer), Certolizumab (e.g., Certolizumab pegol) (TNF alpha; Crohn's disease, rheumatoid arthritis), Daclizumab (IL-2Ra receptor (CD25); transplant rejection), Eculizumab (complement protein C5; paroxysmal nocturnal hemoglobinuria), Efalizumab (CDlla; psoriasis), Gemtuzumab (CD33; acute myelogenous leukemia (e.g., with calicheamicin)), Ibritumomab tiuxetan (CD20; Non-Hodgkin lymphoma (e.g., with yttrium-90 or indium-111)), Infliximab (TNF alpha; various autoimmune disorders, e.g., rheumatoid
arthriti
Muromonab-CD3 (T Cell CD3 receptor; transplant rejection), Natalizumab (alpha-4
(a4) integrin; multiple sclerosis, Crohn's disease), Omalizumab (IgE; allergy-related asthma),
Palivizumab (epitope of RSV F protein; Respiratory Syncytial Virus infection), Panitumumab
(EGF receptor; cancer, e.g., colorectal cancer), Ranibizumab (vascular endothelial growth
factor A; wet age-related macular degeneration) Rituximab (CD20; Non-Hodgkin
lymphoma), Tositumomab (CD20; Non-Hodgkin lymphoma), Trastuzumab (ErbB2; breast
cancer), and any antigen-binding fragment thereof.

In some embodiments, a therapeutic monoclonal antibody and a second agent
useful for treating the same disease are conjugated using an inventive approach described
herein. In some embodiments, the second agent comprises a polypeptide, peptide, small
molecule, or second antibody.

In some embodiments, a monoclonal antibody and a cytokine, e.g., an interferon,
e.g., interferon alpha, are conjugated using an inventive approach described herein.
Optionally, the monoclonal antibody and cytokine are both useful for treating the same
disease.

In some embodiments, an inventive approach described herein is used to
conjugate two (or more) subunits (e.g., separate polypeptide chains) of a multi-subunit
protein. In some embodiments, a multi-subunit protein is a receptor (e.g., a cell surface
receptor). In some embodiments, a multi-subunit protein is an enzyme. In some
embodiments, a multi-subunit protein is a cytokine. In some embodiments, a multi-subunit
protein is a channel or transporter. In some embodiments, such linkage facilitates proper
folding of the multi-subunit protein (e.g., accelerates folding or increases proportion of
correctly folded functional proteins).

In some embodiments, a target protein or a polypeptide comprises a protein
transduction domain. For example, an inventive approach may be used to link a protein
transduction domain to a polypeptide of interest.

In some embodiments, an inventive approach described herein is used to produce
a vaccine, e.g., a monovalent or polyvalent vaccine. For example, two or more antigens (e.g.,
of one or more pathogenic agents such as those mentioned above or tumor antigen) may be
joined using an inventive approach. In some embodiments, the resulting agent may be
administered to a subject, e.g., in an appropriate composition, optionally comprising suitable
carrier(s) or excipient(s). In some embodiments, the resulting agent is used ex vivo, e.g.,
stimulate or be taken up by immune system cells, e.g., T cells, antigen-presenting cells (e.g.,
dendritic cells), which may have been previously obtained from a donor. In some
embodiments, a donor is a subject to whom the cells are subsequently to be administered. In some embodiments, a vaccine is of use to immunize a mammalian or avian subject against a pathogen or tumor, *e.g.*, to induce or augment an immune response directed to the pathogen (or cells infected by the pathogen) or tumor.

[00185] In some embodiments, an antigen and a cytokine are conjugated using the inventive approach described herein, wherein the cytokine optionally modulates, *e.g.*, stimulates, proliferation, differentiation, and/or at least one activity of immune system cells, *e.g.*, T cells (*e.g.*, T cells belonging to a subset such as cytotoxic, helper, regulatory, or natural killer cells), B cells, macrophages, etc.

[00186] It will be understood that in some aspects, the invention encompasses agents produced according to methods described herein, and compositions comprising such agents. It will be understood that, in some aspects, the invention encompasses methods of using such agents, *e.g.*, for one or more purposes described herein, or other purposes.

**Pharmaceutical Compositions**

[00187] In some embodiments, the invention provides pharmaceutical compositions comprising any of the modified proteins described herein, for example, a protein that has been modified to carry a click chemistry handle, or a chimeric protein conjugated to a second molecule, for example, another protein, via click chemistry. In some embodiments the protein is conjugated to a polymer, *e.g.*, PEG, via click chemistry.

[00188] A pharmaceutical composition may comprise a variety of pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water, 5% dextrose, or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil, or injectable organic esters that are suitable for administration to a human or non-human subject. See, *e.g.*, Remington: *The Science and Practice of Pharmacy*, 21st edition; Lippincott Williams & Wilkins, 2005. In some embodiments, a pharmaceutically acceptable carrier or composition is sterile. A pharmaceutical composition can comprises, in addition to the active agent, physiologically acceptable compounds that act, for example, as bulking agents, fillers, solubilizers, stabilizers, osmotic agents, uptake enhancers, etc. Physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose, lactose; dextrins; polyols such as mannitol; antioxidants, such as ascorbic acid or glutathione; preservatives; chelating agents; buffers; or other stabilizers or excipients. The choice of a pharmaceutically acceptable carrier(s) and/or physiologically acceptable compound(s) can depend for example,
on the nature of the active agent, e.g., solubility, compatibility (meaning that the substances can be present together in the composition without interacting in a manner that would substantially reduce the pharmaceutical efficacy of the pharmaceutical composition under ordinary use situations) and/or route of administration of the composition. The pharmaceutical composition could be in the form of a liquid, gel, lotion, tablet, capsule, ointment, cream, transdermal patch, etc. A pharmaceutical composition can be administered to a subject by various routes including, for example, parenteral administration. Exemplary routes of administration include intravenous administration; respiratory administration (e.g., by inhalation), intramuscular administration, nasal administration, intraperitoneal administration, oral administration, subcutaneous administration and topical administration. For oral administration, the compounds can be formulated with pharmaceutically acceptable carriers as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc. In some embodiments a compound may be administered directly to a target tissue. Direct administration could be accomplished, e.g., by injection or by implanting a sustained release implant within the tissue. Of course a sustained release implant could be implanted at any suitable site. In some embodiments, a sustained release implant may be particularly suitable for prophylactic treatment of subjects at risk of developing a recurrent cancer. In some embodiments, a sustained release implant delivers therapeutic levels of the active agent for at least 30 days, e.g., at least 60 days, e.g., up to 3 months, 6 months, or more. One skilled in the art would select an effective dose and administration regimen taking into consideration factors such as the patient's weight and general health, the particular condition being treated, etc. Exemplary doses may be selected using in vitro studies, tested in animal models, and/or in human clinical trials as standard in the art.

[00189] A pharmaceutical composition comprising a modified protein according to aspects of this invention may be delivered in an effective amount, by which is meant an amount sufficient to achieve a biological response of interest, e.g., reducing one or more symptoms or manifestations of a disease or condition. The exact amount required will vary from subject to subject, depending on factors such as the species, age, weight, sex, and general condition of the subject, the severity of the disease or disorder, the particular compound and its activity, its mode of administration, concurrent therapies, and the like. In some embodiments, a compound, e.g., a protein, is formulated in unit dosage unit form for ease of administration and uniformity of dosage, which term as used herein refers to a physically discrete unit of agent appropriate for the patient to be treated. It will be understood, however, that the total daily dosage will be decided by the attending physician within the scope of sound medical
judgment. In some embodiments, e.g., when administering a PEG-conjugated protein, information available regarding a suitable dose of the unPEGylated version, optionally in conjunction with in vitro activity data, can be used as a guideline in selecting an appropriate dose for preclinical testing and/or for clinical use.

[00190] The pharmaceutical compositions can be used to treat a wide variety of different diseases and disorders. In some embodiments, a pharmaceutical composition is used, e.g., to treat any disease or condition for which the unmodified protein is of use. Thus the invention provides methods of treatment comprising administering an inventive protein to a subject in need thereof. The subject is typically a mammalian subject, e.g., a human. In some embodiments the subject is a non-human animal that serves as a model for a disease or disorder that affects humans. The animal model may be used, e.g., in preclinical studies, e.g., to assess efficacy and/or determine a suitable dose.

[00191] In some embodiments, an inventive protein is administered prophylactically, e.g., to a subject who does not exhibit signs or symptoms of the disease or disorder (but may be at increased risk of developing the disorder or is expected to develop the disease or disorder). In some embodiments an inventive protein is administered to a subject who has developed one or more signs or symptoms of the disease or disorder, e.g., the subject has been diagnose as having the disease or disorder. Optionally, the method comprises diagnosing the subject as having a disease or disorder for which the protein is an appropriate treatment. For example, interferons have a variety of uses, e.g., in the treatment of autoimmune diseases (e.g., multiple sclerosis) and infectious diseases (e.g., viral infections such as those caused by viruses belonging to the Flaviviridae family, e.g., HBV, HCV; bacterial infections, fungal infections, parasites). Exemplary viruses include, but are not limited to, viruses of the Flaviviridae family, such as, for example, Hepatitis C Virus, Yellow Fever Virus, West Nile Virus, Japanese Encephalitis Virus, Dengue Virus, and Bovine Viral Diarrhea Virus; viruses of the Hepadnaviridae family, such as, for example, Hepatitis B Virus; viruses of the Picornaviridae family, such as, for example, Encephalomyocarditis Virus, Human Rhinovirus, and Hepatitis A Virus; viruses of the Retroviridae family, such as, for example, Human Immunodeficiency Virus, Simian Immunodeficiency Virus, Human T-Lymphotrophic Virus, and Rous Sarcoma Virus; viruses of the Coronaviridae family, such as, for example, SARS coronavirus; viruses of the Rhabdoviridae family, such as, for example, Rabies Virus and Vesicular Stomatitis Virus, viruses of the Paramyxoviridae family, such as, for example, Respiratory Syncytial Virus and Parainfluenza Virus, viruses of the Papillomaviridae family,
such as, for example, Human Papillomavirus, and viruses of the Herpesviridae family, such as, for example, Herpes Simplex Virus.

[00192] Interferon therapy is used (often in combination with chemotherapy and radiation) as a treatment for many cancers, which term is used herein to encompass solid tumors (carcinomas, sarcomas), and leukemias. In some embodiments the tumor is an adenocarcinoma. In some embodiments the tumor is a sarcoma. In some embodiments the tumor affects an organ or organ system selected from breast, lymph node, prostate, kidney, bladder, lung, liver, gastrointestinal tract, colon, testis, stomach, pancreas, thyroid, skin, ovary, uterus, cervix, skin, nerve, bone, and nervous system (e.g., brain). In some embodiments, an interferon is used for treating a hematological malignancy, e.g., a leukemia or a lymphoma, e.g., hairy cell leukemia, chronic myeloid leukemia, nodular lymphoma, cutaneous T-cell lymphoma. In some embodiments an IFN, e.g., IFN-a2b, is used to treat a melanoma.

[00193] Erythropoiesis stimulating agents such as EPO are of use to treat anemia, which may result from a variety of causes. For example, the anemia may be an anemia of chronic disease, anemia associated with medications (e.g., cancer chemotherapy), radiation, renal disease (e.g., diabetes), infectious diseases, or blood loss. Colony stimulating factors such as G-CSF, GM-CSF, and/or M-CSF may be used to treat leukopenia, e.g., neutropenia and/or lymphopenia, which may result, e.g., from medications (e.g., cancer chemotherapy), radiation, infectious disease, or blood loss.

[00194] Neurotrophic factor proteins may be used, e.g., to treat neurodegenerative diseases (e.g., amyotrophic lateral sclerosis, Huntington disease, Alzheimer disease, Parkinson disease), central or peripheral nervous system injury.

[00195] Growth hormone may be used, e.g., to treat children's growth disorders and adult growth hormone deficiency.

[00196] Interleukins are of use to modulate the immune response for a wide variety of purposes, e.g., to stimulate an immune response against an infectious agent or cancer. In some embodiments, an interleukin stimulates immune system cells and/or increases the intensity and/or duration of innate and/or adaptive immune responses. As known in the art, certain interleukins help to limit the intensity and/or duration of innate and/or adaptive immune responses. Administration of such interleukins may be of use in treatment of autoimmune diseases, sepsis, or other conditions in which an aberrant or overactivated immune response can be deleterious.
Autoimmune disorders include type I diabetes (e.g., juvenile onset diabetes), multiple sclerosis, scleroderma, ankylosing spondylitis, sarcoid, pemphigus vulgaris, myasthenia gravis, systemic lupus erythematosus, rheumatoid arthritis, juvenile arthritis, Behcet's syndrome, Reiter's disease, Berger's disease, dermatomyositis, Wegener's granulomatosis, autoimmune myocarditis, anti-glomerular basement membrane disease (including Goodpasture's syndrome), dilated cardiomyopathy, thyroiditis (e.g., Hashimoto's thyroiditis, Graves' disease), and Guillane-Barre syndrome.

Diseases caused by gram-positive or gram-negative bacteria, mycobacteria, fungi such as Candida or Aspergillus, helminths, etc., are of interest in certain embodiments. Exemplary bacteria and fungi include those falling within the following groups:

- Actinomycetales (e.g., Corynebacterium, Mycobacterium, Norcardia), Aspergillosis,
- Bacillaceae (e.g., Anthrax, Clostridium), Bacteroidaceae, Blastomycosis, Bordetella,
- Borrelia, Brucellosis, Candidiasis, Campylobacter, Coccidioidomycosis, Cryptococciosis,
- Dermatocycoses, Enterobacteriaceae (Klebsiella, Salmonella, Serratia, Yersinia),
- Erysipelothrix, Helicobacter, Legionella, Leptospires Listeria, Mycoplasmatales,
- Neisseriaceae (e.g., Acinetobacter, Menigococci), Pasteurellacea (e.g., Actinobacillus,
- Heamophilus, Pasteurella), Pseudomonas, Rickettsiaceae, Chlamydiaceae, Treponema, and Staphylococci.

In some embodiments a modified, e.g., PEGylated protein exhibits increase efficacy relative to an unmodified form and/or requires a lower dose or less frequent administration (greater dosing interval) to achieve equivalent efficacy and/or exhibits reduced toxicity (reduced side effects, greater tolerability, greater safety) and/or can be administered by a more convenient or preferable route of administration.

It should be noted that the invention is not limited to the foregoing, exemplary click chemistry handles, and additional click chemistry handles, reactive click chemistry handle pairs, and reaction conditions for such click chemistry handle pairs will be apparent to those of skill in the art.

The following working examples are intended to describe exemplary reductions to practice of the methods, reagents, and compositions provided herein and do not limit the scope of the invention.
EXAMPLES

Example 1: Production of N-to-N and C-to-C protein fusions created by combining click chemistry with a sortase-catalyzed transacylation.

[00202] Protein fusions are useful tools in biochemistry. Using genetic constructs, a large variety of proteins fused to GFP have been expressed. One major disadvantage of protein fusion technology is, however, that only C-to-N linked protein fusions can be achieved, in which the C-terminus of one protein is fused to the N-terminus of another protein. This limits the scope of such protein fusions to those that do not require an unoccupied, or unfused N- or C-terminus. For example, the N-terminus of antibodies is required for antigen recognition and therefore bispecific antibodies cannot be produced using conventional recombinant technologies, including protein fusion techniques. Other proteins, such as ubiquitin, require an unmodified C-terminus for normal activity.

[00203] Some aspects of this invention provide methods and reagents for the preparation of N-to-N and C-to-C protein fusions using a combination of the sortase reaction and click chemistry. The sortase-catalyzed transacylation allows the facile installation of all manner of substituents at the C-terminus of a suitably modified protein. The sole requirement for a successful transacylation reaction is the presence of a suitably exposed LPXTG (SEQ ID NO: 2) motif in the target protein. The design of nucleophiles that can be used in a sortase catalyzed reaction is likewise straight-forward: a short run of glycine residues, or even an alkylamine suffices to allow the reaction to proceed. For an exemplary scheme for the generation of C-C and N-N conjugated proteins via sortase-mediated installation of click chemistry handles and subsequent click chemistry reaction, see Figure 1. The click handles azide and cyclooctyne are represented by N3 and an octagon, respectively.

[00204] The key advantages of the installation of click chemistry handles on proteins via a sortase reaction are ease of synthesis of the required nucleophile for the sortase reaction, and execution of the reaction on native proteins under physiological conditions (Figure 2A). The nucleophiles that have previously been used in the sortase reaction contained any of the following modifications: biotin, fluorophores, fatty acids, nucleic acids, lipids, radioisotopes, carbohydrates or even proteins with a suitably exposed N-terminal stretch of glycine residues (e.g., 1-10 G residues).

[00205] Some aspects of this invention provide an extended range of protein modifications through the synthesis of nucleophiles that provide the handles for click-reaction. This allows for the creation of proteins fused at their C-termini. Any type of bioorthogonal click-reaction can be used for this purpose and some examples that can be applied, but not limited to, are
the copper-catalyzed click reaction, the (traceless) Staudinger ligation, the strain-promoted click reaction, thio-ene reaction, (inverse-electron demand) Diels-Alder reaction, oxime ligation and the native chemical ligation (see Table I and Figure 2B). In some embodiments, these functionalities are introduced on the side-chain of natural amino acids or by incorporation of non-natural amino acids.

[00206] Some aspects of this invention provide methods and reagents for the generation of bi-specific, chimeric antibodies. In some embodiments, two antibodies are conjugated via click chemistry at their C termini to form a chimeric antibody. C-C terminal conjugation allows the antigen-binding N-termini of the conjugated antibodies to retain their antigen-binding properties. If two antibodies so conjugated bind different antigens, the resulting chimeric antibody is bi-specific.

[00207] Some aspects of this invention provide a strategy for the preparation of bispecific antibodies according to some embodiments of this invention. In some embodiments, antibodies are provided that contain a C-terminal sortase recognition sequence, for example, a C-terminal LPXTGG (SEQ ID NO: 3) sequence. In some embodiments, the antibodies further comprise a C-terminal tag, for example, a hexahistidine (His6) tag. Such antibodies can be obtained via recombinant methods and using reagents that are well known to those of skill in the art.

[00208] In some embodiments, the nucleophile for the sortase reaction, for example, a GGG-peptide, comprising a click chemistry handle, is synthesized employing standard solid phase peptide synthesis.

[00209] In some embodiments, a first antibody comprising a C-terminal sortase recognition motif is modified by a sortase catalyzed reaction in the presence of a nucleophile comprising a first click chemistry handle (e.g., handle A, see Figure 2B). A second antibody comprising a sortase recognition motif, for example, an antibody binding a different antigen than the first antibody, is modified by a sortase catalyzed reaction in the presence of a nucleophile comprising a second click chemistry handle (e.g., handle B, see Figure 2B). The two click chemistry handles (e.g., handle A and B) are typically click "partners," meaning that they can react in a click chemistry reaction to form a covalent bond. Some exemplary click reactions and partner click handles are described in Table 1 and Figure 2B. As result of the sortase reaction, antibodies on which a C-terminal click chemistry handle is installed, are obtained (Figure 2C).

[00210] In some embodiments, the sortase-modified antibodies are isolated or purified, for example, using His-tag purification, size exclusion chromatography and/or ion exchange
chromatography. In some embodiments, the first and the second sortase-modified antibody are mixed under physiological conditions suitable for the respective click reaction to take place. For example, if the click reaction requires a catalyst, such as copper, to take place under physiological conditions, conditions suitable for the reaction to take place would include the provision of a copper catalyst in an amount effective to catalyze the click reaction. In some embodiments, the click reaction is followed using LC/MS and gel chromatography, for example, to determine completion of the reaction. In some embodiments, when the reaction is complete, the C-to-C-fused proteins are isolated or purified, for example, with the above-mentioned methods (Figure 2D).

Example 2: Installation of non-click functionalities via sortase reaction

The functionalities that can be incorporated in the nucleophiles for the sortase reaction are not limited to click chemistry handles. Sortase nucleophiles may be equipped with any of the functionalities that previously have been used in the sortase reaction (Figure 3A). For example, in some embodiments, biotin is incorporated, which allows for visualization, purification and tetramerization of the modified protein, e.g., the sortase-modified antibody, using streptavidin. In some embodiments, a fluorophore is incorporated, for example, a fluorescent protein, or a fluorescent moiety, which allows for visualization of protein dimers. Especially for bispecific antibodies, this is a useful feature allowing them to be used in FACS and microscopy experiments. Moreover, combinations of compatible click handles may be used for the synthesis of even more complex structures, such as protein trimers, and PEGylated protein dimers (Figure 3B).

Taking into account the flexibility afforded by solid phase synthesis, the inclusion of yet other functionalities at the site of suture can be used to further expand the range of properties imparted on such chimeric protein. For example, sortase-mediated installation of a synthetic polymer, for example, a PEG moiety, can extend the half-life of peptides and proteins, for example, such a modification extends the circulatory half-life of cytokines. Incorporation of detectable labels, such as fluorophores, fluorescent proteins, dyes, bioluminescent enzymes and probes, or radioisotopes enables access to all commonly used imaging modalities.

Example 3: generation of bi-specific, chimeric antibodies

An exemplary strategy of sortase-mediated installation of click chemistry handles was applied to generate bispecific antibody fragments based on the use of the VHH domains.
typical of camelid antibodies. Unlike other mammalian species, camelids possess an additional class of antibodies whose binding site is constructed from a VH domain only. These domains can be expressed in bacteria as so-called nanobodies. Their small size and ease of manipulation make them attractive targets for the construction of therapeutics. Especially the ability to combine two distinct recognition specificities in a single reagent holds promise for the construction of so called bi-specific antibodies.

[00214] VHH fragments were expressed in E. coli as nanobodies. The VHH fragments were based on an antibody raised in vicuna against GFP and an antibody raised in llama against 2-microglobulin. Both nanobodies were equipped with an LPXTG (SEQ ID NO: 2) motif to prepare them for a sortagging reaction. The design of the nucleophiles involved the installation of a strained cyclooctyne on one nanobody, and of an azide on the other nanobody, respectively, to allow a copper-free click reaction to proceed.

[00215] Optimal conditions for the click reaction were established using an N-terminal labeling reaction executed on suitably modified ubiquitin (Ub, Figure 4, scheme), ubiquitin vinyl methyl ester (UbVME), an electrophilic Ub derivative that covalently modifies ubiquitin-specific proteases. For this reaction a (Gly)₃ extended version of UbVME was chosen. Execution of the click reaction yielded a UbVME dimer, the functionality of which was assessed by modification of the ubiquitin C-terminal hydrolase, UCHL3 (Figure 4, gel image). An important aspect of the chemistry employed is the avoidance of harsh conditions that might inflict damage on the proteins that are the substrates in this reaction. All transformations are performed in an aqueous environment at neutral pH.

[00216] It was observed that the N- and C-terminal sortagging reactions proceed with comparable efficiency (Figure 5), and so the scheme employed here not only allows C-to-C but also N-to-N fusions, both of which are impossible to accomplish by conventional recombinant technologies. In some embodiments, where the reactants of the sortase reaction (e.g., input nanobodies) as well as the sortase used in the reaction are equipped with a tag, for example, a His6 tag, adsorption onto an appropriate binding agent, e.g., NiNTA agarose, effectively depletes these reactants, allowing for a one-step purification of the desired, "sortagged" product.

[00217] The kinetics of the dimerization reaction of azide-modified Ub and cyclooctyne-modified Ub was investigated (Figure 6). Dimerization was not observed in samples comprising only either N3-Ub or cyclooctyne-Ub. When incubated together, however, dimerization was detectable after 30 minutes of incubation, and reached a plateau at 1 hr of
incubation time. The reaction was efficient at different mixing ratios of N3- and
cyclooctyne-Ub.

[00218] The two nanobodies were subjected to a sortase-mediated installation of a click
chemistry handle, an azide, and a cyclooctyne, respectively under the optimized reaction
conditions determined for Ub (see Example 4 for reaction conditions, Figure 7). The
resulting nanobodies comprising a suitable click handle each, were purified by size exclusion
chromatography to remove any unincorporated sortase reaction nucleophile (Figure 8). The
purified nanobodies can be conjugated via a click chemistry reaction analogous to the
dimerization of ubiquitin. The conjugation products can be purified by size exclusion
chromatography on an S75 column, and the desired product characterized by SDS-PAGE and
MS/MS to confirm the identity of the C-to-C nanobody fusion product.

[00219] A crude reaction mixture can be prepared and incubated with saturating amounts
of the target antigens, beta-2-microglobulin and eGFP, both expressed in E. coli. Size
exclusion chromatography followed by SDS-PAGE and silver staining of individual fractions
allows for the identification of unbound antigen at their expected Stokes' radii, as well as that
of the separate nanobodies, each complexed with their cognate antigen. The examples of N-
to-N and of C-to-C protein conjugation demonstrate that chimeric proteins, inaccessible by
standard genetic methods, may be obtained in good yields using the methods and reagents
provided herein.

[00220] Figure 9 shows sortagging of an anti-GFP nanobody. Figure 10 shows sortagging
of interferon alpha (INFA) and anti-GFP (anti-eGFP) nanobody. 37: C-terminal azide; 57: C-
terminal cyclooctyne; 40: N-terminal cyclooctyne; 41: N-terminal azide. Figure 11 shows
sortagging of INFA and anti-GFP.

Example 4: Materials and Methods

Solid phase peptide synthesis of the sortase reaction peptides

[00221] Rink-amide resin was solvated in NMP and after removal of the Fmoc-group by
treating the resin with 20% piperidine in NMP, the resin was loaded and elongated using the
consecutive steps. (I) The resin was washed with NMP (3x), CH₂Cl₂ (3x) and NMP. (II)
Fmoc-protected amino acid (either commercially available or home-made) were condensed
under the agency of HOBt (3 equiv.), PyBOP (3 equiv.) and DiPEA (6 equiv.). (III) The resin
was washed again using the same conditions as in step (I). (IV) The coupling was monitored
using Kaiser test and if complete, (V) the Fmoc-protective group was removed using 20%
piperidine in NMP.
Finally, the peptides were cleaved off resin by agitating the resin in the presence of 95% TFA, 2.5% TIS, 2.5% H₂O for 3h. Ice-cold Et₂O was added to the cleavage solution and the formed precipitate was pelleted by centrifugation of the solution for 30 min at 4°C. The crude peptides were purified by reverse phase HPLC purification (buffers used: A: H₂O, B: ACN, C: 10% TFA in H₂O).

C-terminal peptides

H₂N-GGGK(Azidohexanoic acid)-CONH₂

Rink amide resin (100mg, 50 μmol) was loaded with Fmoc-Lys(Mtt)-OH and elongated with Fmoc-GGG-OH as described in the general method. After washing the resin with CH₂Cl₂, the Mtt protective group was removed by treating the resin twice with 1% TFA, 1% TIS in CH₂Cl₂ for 30 min (or until the yellow color completely disappeared). The resin was washed with CH₂Cl₂ (5x), NMP (5x) and NMP containing 5 equivalents of DiPEA. Azidohexanoic acid (31 mg, 200 μmol) was condensed using PyBOP (104 mg, 200 μmol) and DiPEA (70 μL, 400 μmol). After 2 hours shaking, the Kaiser test showed complete conversion. The N-terminal Fmoc group was removed and the peptide was cleaved off resin as described in the general method. Reverse phase HPLC purification (15-24% B in 12 min (3 CV), Rt= 8 min) gave the title compound (15.4 mg, 33 μmol, 67%) as an off-white solid.

H₂N-GGGC(DBCO)-CONH₂

Rink amide resin (167 mg, 100 μmol) was loaded with Fmoc-Cys(Trt)-OH and elongated with Fmoc-GGG-OH, and cleaved off the resin as described in the general method affording crude H₂N-GGGC-CONH₂ (SEQ ID NO: 129) in quantitative yield. This peptide (38 mg, 83 μmol) was dissolved in PBS (0.25 mL) and to this was added DBCO-maleimide (17 mg, 40 μmol) in DMF (0.25 mL). The reaction was stirred overnight, acidified with TFA and purified by RP-HPLC (20-35% B in 20 min (5 CV)) gave the title compound (15.3 mg, 22 μmol, 27%) as a white solid.

N-terminal peptides

Azidohexanoic acid-LPETGG-CONH₂

Rink amide resin (60 μmol) was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids and cleaved off the resin as described in the general
method. For the final coupling azidohexanoic acid was used. RP-HPLC (26-35% B in 12 min (3 CV)) gave the title compound (9.5 mg, 13 µmol, 13%) as a white solid.

**DBCO-LPETGG-CONH₂**

[00226] Rink amide resin was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids and cleaved off the resin as described in the general method. Precipitation from Et₂O afforded crude H₂N-LPETGG-CONH₂ (SEQ ID NO: 132) (17.9 mg, 31.3 µmol), which was dissolved in DMF (0.5 mL). DBCO-OSu (14 mg, 20 µmol) was added and the reaction was stirred overnight. The solution was diluted before being purified by RP-HPLC (25-34% B in 12 min (3 CV)) gave the title as an off-white solid.

**Sortagging of Ubiquitin**

[00227] Sortase (7.2 µL, 700 µM) and probe (10 µL, 5 mM) were added to ubiquitin (58 µM) in 100 µL sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl₂). The resulting mixture was incubated at 37°C for 2h. Next, the solution was acidified and purified by reverse phase HPLC. The resulting purified protein was concentrated in vacuo, redissolved in H₂O and quantified by gel-electrophoresis.

**Sortagging of nanobodies**

[00228] Sortase (7.2 µL, 700 µM) and probe (10 µL, 5 mM) were added to the nanobody (15 µM) in 100 µL sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl₂). The resulting mixture was incubated at 37°C overnight. Next, the solution was diluted with Et₃N HOAc (pH 5) and purified by size exclusion HPLC. The resulting purified protein was concentrated in vacuo, redissolved in H₂O and quantified by gel-electrophoresis.

**Dimerization of Ubiquitin**

[00229] Azido-modified ubiquitin and DBCO-modified ubiquitin were mixed in a one to one ratio and incubated for 0.5-7h at 37°C. The conversion to the dimerized product was analyzed using gel electrophoresis.

**Activity-assay**

[00230] Azido-modified UbVME and DBCO-modified UbVME were mixed in a one to one ratio and were incubated overnight at 37°C. After dimerization, the samples were diluted
with Tris buffer (7 µl) and UCHL3 (2 µl, 5 fold excess to UbVME) was added. The resulting mixture was incubated for 2 h, denatured with sample buffer (4x) and loaded on 15% gel. The proteins were transferred to a PVDF-membrane. The membrane was blocked with 4% milk in PBS/Tween (0.1%). Rabbit polyclonal anti-ubiquitin (1:100) was added and the membrane was agitated for 30 min at room temperature. The membrane was four times washed with 0.1% Tween in PBS before the secondary antibody (HRP-goat anti rabbit, 1:25000) was added. After 30 min shaking at room temperature, the membrane was washed with 0.1% Tween in PBS (4x) and the proteins were visualized using ECL plus.

Example 5: The preparation of unnatural N-N and C-C protein fusions
[00231] The strategies described herein were employed to produce N-to-N and C-to-C protein fusions with full retention of the biological activity of the fusion partners and without inflicting chemical damage on the joined proteins. Sortase A was used to install on the N- or C-terminus of proteins of interest the requisite modifications to execute a strain-promoted copper-free Huisgen cycloaddition. Applied here to protein-protein fusions, the methods described can be used to conjugate any protein with any entity of interest.

Materials and Methods
[00232] General experimental. All chemicals were of commercial sources and were used as received. Fmoc-Lys(Mtt)-OH, Fmoc-Gly-OH, Fmoc-Thr-OH, Fmoc-Pro-OH, Fmoc-Glu-OH, Fmoc-Leu-OH, 0-benzotriazole-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU), benzotriazol-l-yl oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from EMD Biosciences/Novabiochem. Rink amide resin was purchased from Advanced Chemtech. Cyclooctyne reagents were purchased from Click Chemistry Tools. Water used in biological procedures or as a reaction solvent was purified using a MilliQ purification system (Millipore). DriSolv® anhydrous CH2Cl2, DriSolv® anhydrous MeOH, DriSolv® anhydrous DMF were purchased from EMD Chemicals. Redistilled, anhydrous N,N-diisopropylethylamine (DiPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS) N-methylpyrrolidone (NMP) was obtained from Sigma-Aldrich.

[00233] Mass Spectrometry. LC-ESTMS analysis was performed using a Micromass LCT mass spectrometer (Micromass® MS Technologies, USA) and a Paradigm MG4 HPLC system equipped with a HTC PAL autosampler (Michrom BioResources, USA) and a Waters Symmetry 5 µιη C8 column (2.1 x 50 mm, MeCN:H2O (0.1% formic acid) gradient mobile
phase, 150 µL/µη).

**HPLC/FPLC.** HPLC purifications were achieved using an Agilent 1100 Series HPLC system equipped with a Waters Delta Pak 15 µη, 100 Å C18 column (7.8 x 300 mm, MeCN:H2O gradient mobile phase, 3 mL/min) as indicated below. Size exclusion and cation exchange chromatography were performed on a Pharmacia AKTA Purifier system equipped with a HiLoad 16/60 Superdex 75 column (Amersham) or a Mono S 5/50 GL column (Amersham), respectively.

**UV-vis Spectroscopy.** UV-vis spectroscopy was performed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

**In-gel Fluorescence.** Fluorescent gel images were obtained using a Typhoon 9200 Variable Mode Imager (GE Healthcare).

**General procedure for the solid phase peptide synthesis of the probes.** Rink-amide resin was solvated in NMP and after removal of the Fmoc-group by treating the resin with 20% piperidine in NMP, the resin was loaded and elongated using the consecutive steps. (I) The resin was washed with NMP (3x), CH₂Cl₂ (3x) and NMP. (II) Fmoc-protected amino acid were condensed under the agency of HOBT (3 equiv.), PyBOP (3 equiv.) and DiPEA (6 equiv.). (III) The resin was washed again using the same conditions as in step (I). (IV) The coupling was monitored using Kaiser test and if complete, (V) the Fmoc-protective group was removed using 20% piperidine in NMP. In the final step, the peptides were cleaved off resin by agitating the resin in the presence 95%TFA, 2.5% TIS, 2.5% H₂O for 3h. Ice-cold Et₂O was added to the cleavage solution and the formed precipitate was collected by centrifugation of the solution for 30 min at 4°C. The crude pellet was purified by reverse phase HPLC purification (buffers used: A : H₂O , B : ACN, C : 10% TFA in H₂O).

**N-terminal probes**

**Azidohexanoic acid-LPETGG-CONH₂ (I).** Rink amide resin (60 µηο) was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids and cleaved off the resin as described in the general method. For the final coupling azidohexanoic acid was used. RP-HPLC (26-35% B in 12 min (3 CV)) gave the title compound (9.5 mg, 13 µηο, 13%) as a white solid. LC/MS: Rₜ 6.34 min; linear gradient 5→45% B in 10 min; ESI/MS: m/z = 711.1 [M+H]+. NMR (400 MHz, CDC₁₃) δ ppm 4.65 (dd, J = 10.0, 4.4 Hz, 1H), 4.42 (dd, J = 8.4, 6.0 Hz, 1H), 4.35 (dd, J = 9.2, 5.2 Hz, 1H), 4.30-4.24 (m, 2H), 4.00 (s, 2H), 3.96 (s, 2H), 3.91-3.84 (m, 4H), 3.70-3.64 (m, 1H), 2.48 (t, J = 7.2 Hz, 2H), 2.26 (t, J =
7.6), 2.24-1.96 (m, 6H), 1.78-1.70 (m, 1H), 1.69-1.56 (m, 7H), 1.44-1.38 (m, 3H), 1.21 (d, J 6.4 Hz, 3H), 0.97 (t, 6.4 Hz, 6H).

[00239] DIBAC-LPETGG-CONH$_2$(2). Rink amide resin was loaded with Fmoc-Glyc-OH, elongated with the appropriately protected amino acids and cleaved off the resin as described in the general method. Precipitation from Et$_2$O afforded crude H$_2$N-LPETGG-CONH$_2$ (SEQ ID NO: 132) (17.9 mg, 31.3 µmol), which was dissolved in DMF (0.5 mL). DIBAC-OSu (14 mg, 20 µmol) was added and the reaction was stirred overnight. The solution was diluted before being purified by RP-HPLC (25-34% B in 12 min (3 CV)) gave the title compound (13.1 mg, 12.3 µmol, 39%) as an off-white solid. LC/MS: R$_f$ 9.42 min; linear gradient 5→45% B in 10 min; ESI/MS: $m/z$ =1066.14 [M+H]$^+$. H NMR (400 MHz, CDCl$_3$) δ ppm 7.65 (dd, J =13.2, 7.2 Hz, 1H), 7.46-7.28 (m, 7H), 5.05 (d, J = 14.4 Hz, 1H), 4.72-4.65 (m, 1H), 4.60-4.50 (m, 1H), 4.48-4.38 (m, 2H), 4.36 (d, J = 4 Hz, 1H), 4.26-4.23 (m, 1H), 4.04-3.87 (m, 5H), 3.73-3.62 (m, 2H), 3.52-3.38 (m, 1H), 3.10-2.92 (m, 1H), 2.82-2.67 (m, 1H), 2.56-2.39 (m, 5H), 2.34-2.09 (m, 6H), 2.07-1.98 (m, 4H), 1.94-1.85 (m, 2H), 1.72-1.52 (m, 6H), 1.50-1.40 (m, 1H), 1.20 (d, J = 6.0 Hz, 3H), 0.98-0.90 (m, 6H).

C-terminal probes

[00240] H$_2$N-GGGK(N$_3$K(TAMRA))-CONH$_2$(3). Rink amide resin (60 µmol) was loaded with Fmoc-Lys(Mtt)-OH and elongated with Fmoc-Azidolysine-OH and Fmoc-GGG-OH as described in the general method. After washing the resin with CH$_2$C$_2$·6H$_2$O, the Mtt protective group was removed by treating the resin twice with 1% TFA, 1% TIS in CH$_2$C$_2$·6H$_2$O for 30 min (or until the yellow color completely disappeared). The resin was washed with CH$_2$C$_2$·6H$_2$O (5x), NMP (5x) and NMP containing DiPEA (43.5 µL, 250 µmol, 5 equiv). 5(6)-Carboxytetramethylrhodamine (77 mg, 180 µmol, 3 equiv.) was condensed using PyBOP (94 mg, 180 µmol, 3 equiv.) and DiPEA (65 µL, 370 µmol, 6 equiv.). After 16 hours shaking, the Kaiser test showed complete conversion. The N-terminal Fmoc group was removed and the peptide was cleaved off resin as described in the general method. Reverse phase HPLC purification (25-34% B in 12 min (3 CV)) gave the title compound (41.4 mg, 50.5 µmol, 81%) as a purple solid. LC/MS: R$_f$ 5.50 and 6.10 min; linear gradient 5→45% B in 10 min; ESI/MS: $m/z$ = 883.3 [M+H]$^+$. H NMR (400 MHz, CDCl$_3$) δ ppm 8.78 (d, J = 1.6 Hz, 1H), 8.28 (dd, J = 7.6, 1.6 Hz, 1H), 7.53 (d, J = 8.0 Hz), 7.14 (d, J = 9.6 Hz, 2H), 7.06 (dd, J = 9.6, 2.4 Hz, 2H), 6.98 (d, J = 2.4 Hz, 2H), 4.34 (dd, J = 9.2, 5.2 Hz, 2H), 3.98 (d, 14.8 Hz, 1H),
3.96 (s, 2H), 3.82 (d, 18.4 Hz, 1H), 3.80 (s, 2H), 3.54-3.46 (m, 2H), 3.32-3.28 (m, 14H), 1.94-1.45 (m, 12H).

**H$_2$N-GGGC(DIBAC)-CONH$_2$ (4).** Rink amide resin (167 mg, 100 μmol) was loaded with Fmoc-Cys(Trt)-OH, elongated with Fmoc-GGG-OH, and cleaved off the resin as described in the general method affording crude tetrapeptide, H$_2$N-GGGC-CONH$_2$ (SEQ ID NO: 129), in quantitative yield. This peptide (38 mg, 83 μmol, 2 equiv.) was dissolved in PBS (0.25 mL) and to this was added DIBAC-maleimide (17 mg, 40 μmol, 1 equiv.) in DMF (0.25 mL). The reaction was stirred overnight, acidified with TFA and purified by RP-HPLC (20-35% B in 20 min (5 CV)) giving the title compound (15.3 mg, 22 μmol, 27%) as a white solid. LC/MS: $R_t$ 6.90 min; linear gradient 5→45% B in 10 min; ESI/MS: $m/z$ = 719.3 [M+H]$^+$. NMR (400 MHz, M) δ ppm 7.66 (d, $J$ = 7.2 Hz, 1H), 7.55-7.51 (m, 1H), 7.48-7.45 (m, 3H), 7.38 (dt, $J$ =7.6, 1.4 Hz, 1H), 7.37-7.33 (m, 1H), 7.28 (d, $J$ = 7.2 Hz, 1H), 5.14 (d, $J$ =14 Hz, 1H), 4.69-4.64 (m, 1H), 4.01-3.85 (m, 6H), 3.77 (d, $J$ = 4.8 Hz, 1H) 3.73 (s, 1H), 3.70 (s, 1H), 3.67-3.63 (m, 2H), 3.39 (dd, $J$ = 14.0, 5.2, 2.8 Hz, 1H), 3.27-3.05 (m, 5H), 2.97 (ddd, $J$ =14, 8.4, 5.2 Hz, 1H), 2.48-2.41 (m, 3H), 2.33-2.87 (m, 2H) 2.08-1.99 (m, 1H).

**H$_2$N-GGGK(Azidohexanoic acid)-CONH$_2$ (5).** Rink amide resin (100 mg, 50 μmol) was loaded with Fmoc-Lys(Mtt)-OH and elongated with Fmoc-GGG-OH as described in the general method. After washing the resin with CH$_2$Cl$_2$, the Mtt protective group was removed by treating the resin twice with 1% TFA, 1% TIS in CH$_2$Cl$_2$ for 30 min (or until the yellow color completely disappeared). The resin was washed with CH$_2$Cl$_2$ (5x), NMP (5x) and NMP containing DiPEA (43.5 μL, 250 μmol, 5 equiv). Azidohexanoic acid (31 mg, 200 μmol, 4 equiv.) was condensed using PyBOP (104 mg, 200 μmol, 4 equiv.) and DiPEA (70 μL, 400 μmol, 8 equiv.). After 2 hours shaking, the Kaiser test showed complete conversion. The N-terminal Fmoc group was removed and the peptide was cleaved off resin as described in the general method. Reverse phase HPLC purification (15-24% B in 12 min (3 CV)) gave the title compound (15.4 mg, 33 μmol, 67%) as an off-white solid. LC/MS: $R_t$ 2.77 min; linear gradient 5→45% B in 10 min; ESI/MS: $m/z$ = 456.3 [M+H]$^+$. NMR (400 MHz, CDCl$_3$) δ ppm 4.35 (dd, $J$ = 9.2, 4.8 Hz, 1H), 3.98 (d, $J$ = 16.8 Hz, 1H), 3.97 (s, 2H), 3.86 (d, $J$ = 16.8 Hz, 1H), 3.78 (s, 2H), 3.29 (t, $J$ = 6.8 Hz, 2H), 3.17 (dt, $J$ =6.8, 2.0 Hz, 2H), 2.20 (t, $J$ = 12 Hz, 2H), 1.86-1.81 (m, 1H), 1.73 (ddd, $J$ = 18.4, 9.4, 5.0 Hz, 1H), 1.67-1.57 (m, 4H), 1.55-1.47 (m, 2H), 1.43-1.38 (m, 4H).
Cloning and Expression of proteins. Ubiquitin N-terminally fused to N-terminal his tag followed by a thrombin cleavage site (MGSSHHHHHHSSGLVPRGGGSH, SEQ ID NO: 130) was cloned into a pET28 vector. The vector was transformed into BL21(DE3)pLysS. A starter culture was grown in LB. The expression culture was started at OD₆₀₀ of 0.2. When the culture reached an OD₆₀₀ of 0.6-0.8, the bacteria were induced with 1 mM IPTG and cultured for 6 at 37°C. The bacteria were collected by centrifugation at 6000 xg for 15 min and the pellet was resuspended in lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, 50 μg/mL DNAsel (Roche) and 1 tablet/25 mL complete protease inhibitor (Roche)) and sonificated. The lysate was clarified by centrifugation. Soluble protein was purified by Ni-NTA (Qiagen). The thrombin sequence was removed using a Thrombin CleanCleave kit (sigma Aldrich).

Ubiquitin (1-75) N-terminally fused to thrombin cleavage site followed by GGG (MGSSHHHHHHSSGLVPRGGGSH, SEQ ID NO: 131) and C-terminally fused to intein was cloned into a pTYB2. The vector was transformed into BL21(DE3)pLysS. The ubiquitin-intein constructed was expressed, purified and converted into the UbVME adduct as previously described for HA-tagged UbVME. Thrombin CleanCleave kit was used to expose the N-terminal glycine residues.

Synthetic version of anti GFP containing a C-terminal LPETGG (SEQ ID NO: 1) was sub-cloned into a pET28A+ vector. The vector was transformed into E. coli BL21(DE3)pLysS. A starter culture (250 mL, LB medium) was grown to saturation overnight at 37°C. An expression culture, started at OD₆₀₀ of 0.2, (2L, Yeast/Tryptone (2YT) medium) was grown at 37°C until the OD₆₀₀ =0.6. The bacteria were induced with IPTG (1 mM) and grown for 16 h at 25°C. Bacteria were collected by centrifugation at 6000 xg for 15 min and they were lysed by sonification in lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazole, 50 μg/mL DNAsel (Roche) and 1 tablet/25 mL complete protease inhibitor (Roche)). The lysate was clarified by centrifugation. Soluble protein was purified by Ni-NTA (Qiagen) followed by size-exclusion chromatography on a Superdex™ 75.

VHH7 containing a C-terminal LPETGGHHHHHHH (SEQ ID NO: 45), was cloned into a pHEN vector N-terminally preceded by the pelB leader sequence. The vector was transformed into E. coli WK6. A started culture (250 mL) was grown in 2YT to saturation overnight at 37°C. The expression culture was started at OD₆₀₀ of 0.2. When the culture reached an OD₆₀₀ of 0.7, the expression of protein was induced by the addition of 1 mM IPTG. The bacteria were cultured overnight at 37°C. The periplasmic fraction was
isolated by incubating the bacterial pellet in 1 volume of 1x TES buffer (Tris 0.2M, EDTA 0.65 mM, Sucrose 0.5M) for 1h at 4°C and subsequently 2 volumes of 0.25x TES buffer were added. The resulting suspension was stirred overnight at 4°C. The solution was clarified by centrifugation, was concentrated using amicon ultra 3K spin concentrators and the proteins were subjected Ni-NTA. The proteins were further purified by size-exclusion chromatography.

[00247] Human interleukin-2 lacking the leader sequence and fused at the C terminus to the sequence GGLPETGHHHHHH (SEQ ID NO: 46) was cloned into the pET28a+ vector (Novagen). The vector was transformed into E. coli BL21(DE3)pLysS and a starter culture was grown overnight at 37°C. The starter culture was added to the expression culture (3 L, 2YT) and grown until the OD600 reached 0.6. To induce expression, 1 mM IPTG (final concentration) was added and the bacteria were grown at 37°C for 4h. The bacteria were collected by centrifugation at 6000 xg for 15 min at 4°C. The bacteria were lysed in by sonification in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 μg/mL DNaseI (Roche) and 1 tablet/25 mL complete protease inhibitor (Roche)). The inclusion bodies were collected by centrifugation (12000 xg for 15 min at 4°C). Before being dissolved in 50 mM Tris, pH 7.4, 150 mM NaCl, 6M guanidinium, the inclusions were first washed by resuspending the pellet lysis buffer (1x), n-butanol (1x), and 50 mM Tris pH7.4, 150 mM NaCl, 1M guanidinium HC1 (2x) and subsequent centrifugation.

[00248] The unfolded protein (6 mg/mL, 0.7 mL) was pretreated with TCEP (1 mM) and subsequently added (0.1 mL/h) to refolding buffer (200 mL, 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM glutathione, 0.5 mM oxidized glutathione) at 25°C. The reaction was stirred for 2 days, concentrated on a Ni-NTA column and subsequently purified by size exclusion chromatography.


[00250] Modification of ubiquitin with N3-LPETGG (1) and DIBAC-LPETGG (2). Ubiquitin was modified with 1 and 2 as described for UbVME. N3-Ub: Rf 7.17 min; linear
gradient 5→45% B in 10 min; ESI/MS: m/z = 9542 (M+H)+. DIBAC-Ub: R, 7.37 min; linear gradient 5→45% B in 10 min; ESI/MS: m/z = 9898 (M+H)+.

**Dimerization of Ubiquitin.** Azido-modified ubiquitin (5 μL, 4 μg/μL) and DIBAC-modified ubiquitin (8 μL, 2.5 μg/μL) were mixed (final concentration of the proteins 170 μM) and incubated for 0.5-7h at 37°C. The conversion to the dimerized product was analyzed using gel electrophoresis.

**N-terminal sortagging.** Sortase A of *S. aureus* (150 μM final concentration, 4.5x stock in 50 mM Tris, pH 7.4, 150 mM NaCl) and probe 1 or 2 (0.5 mM final concentration, IOx stock) were added to UbVME (58 μM final concentration) in sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl\(_2\)). The resulting mixture was incubated at 37°C for 3h. Next, the solution was acidified with 1% TFA in H\(_2\)O and purified by reverse phase HPLC (30→45% B in 20 min, 3 mL/min). The resulting purified protein was neutralized with sat. aq. NaHCO\(_3\) concentrated in vacuo, redissolved in H\(_2\)O and quantified by gel-electrophoresis. The protein was analyzed by LC/MS. N\(_3\)-UbVME: R, 7.70 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 9714 (M+H)+. DIBAC-UbVME: R, 7.54 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 9360 (M+H)+.

**C-terminal sortagging.** Sortase A of *S. aureus* (150 μM final concentration, 4.5x stock in 50 mM Tris, pH 7.4, 150 mM NaCl) and probe (0.5 mM final concentration, IOx stock) were added to the VHH (15 μM final concentration) in sortase buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl\(_2\)). The resulting mixture was incubated at 25°C overnight. The protein was purified by size exclusion on a Superdex™ 75. The resulting purified protein was concentrated in centrifugal filter units and analyzed by gel-electrophoresis and LC/MS. Anti GFP-3: R, 6.02 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 14330 (M+H)+. Anti GFP-4: R, 7.90 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 14170 (M+H)+. **VHH7-3**: R, 7.20 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 15549 (M+H)+. **VHH7-5**: R, 7.00 min; linear gradient 5→45% B in 20 min; ESI/MS: m/z = 15139 (M+H)+.

**Synthesis of dimeric UbVME constructs.** A mixture of azido modified UbVME (42.5 μM, 80 μM) and cyclooctyne modified UbVME (42.5 μM, 70 μM) was incubated overnight and subsequently purified by reverse phase HPLC (30→45% B in 20 min, 3 mL/min). After purification, the solution was neutralized with sat. aq. NaHCO\(_3\) and concentrated in vacuo. Dimeric ubiquitin constructs containing only one reactive vinylmethyl
ester were obtained by either incubating azido modified ubiquitin (42.5 µL, 80 µM) with cyclooctyne modified UbVME (42.5 µL, 70 µM) or azido modified UbVMe (42.5 µL, 80 µM) with cyclooctyne modified ubiquitin (42.5 µL, 70 µM). After dimerization, the proteins were purified and handled as described above.

**[00255] Labeling of UCHL3 with dimeric UbVME constructs.** Purified dimeric constructs (0.5 µg, 24.5 pmol) were diluted in 20 µL Tris buffer (20 mM, pH 8, 100 mM NaCl, 0.1 mM TCEP) in the presence or absence of UCHL3 (94 pmol). The resulting mixture was incubated for 2 h, denatured with Laemmli sample buffer (4x) and loaded on a TRIS-tricine gel. The proteins were either directly analyzed by Coomassie brilliant blue staining or they were transferred to a PVDF-membrane. The membrane was blocked with 4% BSA in PBS/Tween (0.1% v/v). Penta-His HRP (1:12500) was added and the membrane was agitated for 30 min at room temperature. The membrane was four times washed with 0.1% v/v Tween in PBS before the proteins were visualized using ECL plus.

**[00256] Dimerization of nanobodies.** Homodimeric anti GFP nanobody was prepared by incubating anti GFP-3 (100 µL, 80 µM) and anti GFP-4 (100 µL, 85 µM) overnight at room temperature. Heterodimeric VHH7-3-anti GFP-4 and VHH7-5-anti GFP-4 were obtained by reacting either VHH7-3 (200 µL of a 20 µM solution) or VHH7-5 (200 µL of a 60 µM solution) with anti GFP-4 (100 µL of a 120 µM solution) overnight at 25°C. The dimeric nanobodies were purified by size exclusion on a Superdex™ 75. Fractions were collected and concentrated in centrifugal filter units. The purified dimers were analyzed on a 15% SDS-PAGE. (Anti GFP)₂: \( R_t \) 10.07 min; linear gradient 5→45% B in 20 min; ESI7MS: \( m/z = 28526 \) (M+H₂O+H)⁺. VHH7-3-anti GFP-4: \( R_t \) 10.91 min; linear gradient 5→45% B in 20 min; ESI7MS: \( m/z = 29755 \) (M+H₂O+H)⁺. VHH7-5-anti GFP-4: \( R_t \) 10.87 min; linear gradient 5→45% B in 20 min; ESI7MS: \( m/z = 29329 \) (M+H₂O+H)⁺.

**[00257] Functionality assay of homodimeric nanobodies.** Homodimeric anti GFP nanobody (20 µL, 25 µM) was incubated with GFP (2.5 µL, 10 µL, and 30 µL of a 80 µM solution). The formed nanobody-GFP complex was subjected to size exclusion on a Superdex™ 200.

**[00258] Functionality assay of heterodimeric nanobodies.** Lymph node cells were harvested from C57BL/6 (Jackson labs) or MHCII-deficient mice (Jackson labs), washed and incubated for 10 minutes with VHH7-anti GFP, GFP and VHH7-anti GFP+GFP at 4°C. The cells were collected by centrifugation, washed with PBS and analyzed by flow cytometry.
In vivo delivery assay. For the delivery assays, BALB/c mice (Jackson labs) were injected in the tail vein with either the bispecific antibody or GFP (50 µg per mouse). The mice receiving the bispecific antibody either directly received GFP (50 µg) intraperitoneally or received GFP (50 µg) intravenously after 1h. After 5.5h, blood was harvested, the mice were sacrificed and cells were isolated from lymph nodes, thymus, and spleen. Cells were washed with PBS and incubated with anti CD19-APC (BD Pharmingen), and 7-AAD (Viaprobe, BD) for 10 min at 4°C. The cells were washed with PBS and analyzed by flow cytometry.

Results
To construct N-to-N protein dimers, LPXTGG (SEQ ID NO: 3) peptides 1 and 2 were synthesized, N-terminally equipped with an azidohexanoic acid or a dibenzoazacyclooctyne (DIBAC) (25), (Figure 12A). Using sortase A from S. aureus, these peptides were ligated to the N-terminus of a substrate, G3-ubiquitin (G3Ub), with a suitably exposed short run of Gly residues to serve as the incoming nucleophile. Peptides 1 and 2 were transacylated efficiently onto G3-ubiquitin (Figure 13). With the modified proteins in hand, the requirements for dimerization were established. Azido-modified ubiquitin (80 µM) was mixed and incubated at 37°C with a stoichiometric amount of ubiquitin equipped with a cyclooctyne. After 30 minutes, a 18 kDa polypeptide corresponding to the ubiquitin dimer was observed as revealed by Coomassie brilliant blue-staining and in an anti-ubiquitin immunoblot (Figure 13). Extending the incubation time to 7h resulted in -70% conversion to dimeric ubiquitin as quantified by SDS-PAGE using ImageJ. At lower concentrations (15 µM), the reaction still proceeded, albeit at a somewhat slower rate (-70% conversion after 16h).

To evaluate whether the proteins joined retained their biological activity, a bivalent version (N-to-N fusion) of ubiquitin vinylmethyl ester (UbVME) was constructed. UbVME is an active site-directed probe that covalently modifies a large number of ubiquitin-specific proteases (USP) (26). The formation of these adducts is readily visualized by a shift in mobility upon analysis by SDS-PAGE. Modification of a USP with the bivalent version of UbVME should yield a complex that contains two UbVME units and two copies of the USP, with a corresponding increase in molecular weight of the adduct formed. The synthesis of the dimeric UbVME construct thus exploits the combined action of two bio-orthogonal reactions, an intein-based native ligation to obtain the C-terminally modified version of ubiquitin.
bearing the vinylmethylester moiety (26), and the N-terminal sortagging reaction (27).
Starting with G3-UbVME, prepared as described, the azido- and strained cyclooctyne-
modified versions were obtained. By reacting equimolar amounts of azido- and cyclooctyne-
modified UbVME and subsequent purification by reverse phase HPLC to remove any
unreacted UbVME monomers, the bivalent adduct was obtained. The reactivity of this
bivalent adduct was evaluated using ubiquitin carboxy-terminal hydrolase isozyme L3
(UCHL3), for which the crystal structure in complex with UbVME is known (28). As
controls, a dimeric construct in which one of the C-termini is equipped with a reactive
vinylmethyl ester and the other with a non-reactive carboxylic acid was produced. The
resulting UbVME-ubiquitin is therefore capable of binding a single UCHL3 molecule.
Incubation of bivalent UbVME with an excess of N-terminally His-tagged UCHL3 (2
equivalents per vinylmethyl ester) (Figure 14B) yielded the bivalent adduct bound to two
UCHL3 molecules (-67 kDa). When UCHL3 was incubated with either the control UbVME-
ubiquitin constructs or with UbVME, the expected molecular weights shifts were observed,
i.e. UCHL3 modified with an UbVME-ubiquitin dimer (-47 kDa) and UCHL3 modified with
an UbVME monomer (-37 kDa, see Figure 13), respectively. Immunoblotting for His6
(Figure 14C) confirmed that the newly formed adduct indeed contains the His6 tag embodied
in the UCHL3 input material. Both UbVME units in the bivalent adduct produced by the
click reaction thus retain full activity, as evident form their ability to covalently modify the
intended target.

[00262] A second example was explored. Camelids produce unusual antibodies composed
of heavy chains only (29). Their variable regions, when expressed recombinantly as single
domain constructs, also known as VHH, retain full antigen binding capability (30). Bivalent
single domain VHH proteins were synthesized by conjugating them via their C-termini using
the combined sortagging-click strategy. Triglycine peptides containing an azide 3 or a
cyclooctyne 4 were synthesized (Figure 15A) and a synthetic version of a camelid VHH
specific for green fluorescent protein (GFP) was produced recombinantly (31). This VHH
was modified to contain a sortase substrate motif followed by a (His)6 tag to facilitate
purification. Excellent conversion to anti GFP VHH labeled with the click handles was
achieved after incubating at 25°C overnight as judged by SDS-PAGE and LC/MS. Excess
triglycine nucleophile was removed by size exclusion chromatography to avoid interference
with the subsequent dimerization reaction (Figure 16). Using these modified VHHs, the
corresponding C-to-C fused homodimer was generated (Figure 15B), which was purified to homogeneity by size exclusion chromatography (Figure 16).

[00263] The VHH monomers and dimers were incubated with their target antigen, GFP, to assess complex formation. The modified VHH monomers, when incubated with GFP, showed the expected increase in Stokes' radius in a size exclusion chromatography experiment (Figure 17). The C-to-C VHH dimer was then incubated with increasing concentrations of GFP, and the complexes formed between the dimer and GFP were analyzed by size exclusion chromatography (Figure 15C). The free VHH dimer was readily resolved from the dimer occupied by a single GFP at low concentrations of added GFP, which in turn was readily resolved from the dimer occupied by two GFP moieties at the higher GFP concentration (Figure 18).

[00264] This data shows that C-to-C fusion of an antibody fragment, in this case a single VHH domain, is readily achieved using sortase in combination with click chemistry according to aspects of this invention. Not only is the conversion excellent (-90%), but the resulting products retain their full function. Because most of the nucleophiles used in the sortase reaction are water-soluble, and all necessary functional groups that require harsh and/or non-selective reaction conditions are introduced during the synthesis of the nucleophile, this approach minimizes unwanted side reactions (such as acylation of available amino groups (18), denaturation of proteins) that might affect biological activity.

[00265] The above experiment was extended to generate a VHH that is specific for mouse Class II MHC products (VHH7), an alpaca-derived VHH, linked to the anti GFP VHH via their C-termini to create a heterobispecific product. Two adducts were prepared as described above, one containing a tetramethylrhodamine (TAMRA) fluorophore at the junction (using peptide 3) and a non-fluorescent conjugate (using peptide 5). The two adducts were purified to obtain the fluorescent and non-fluorescent bispecific VHH preparations (Figure 19) and added to mouse lymph node cells, the B cells amongst which are uniformly positive for Class II MHC products. When cells were exposed to the bispecific fluorescent VHH, specific staining of B cells in the TAMRA channel was observed (Figure 20A). No staining was detected for the non-fluorescent bispecific antibody (Figure 20). GFP was then added to cells exposed to bispecific VHHs. This resulted in staining in the GFP channel for both bispecifics. This result shows that in this case, too, each of the fusion partners retains its activity and specificity. Lymph node cells of a MHC class II knockout mouse failed to stain with the bispecific VHHs, demonstrating specificity.
To demonstrate the use of such bispecific antibody derivatives for the creation of a deep tissue reservoir (32), the anti GFP-VHH7 bispecific construct was injected intravenously with the goal to first target a relevant cell population (B cells) with this reagent. A single bolus of recombinant GFP (50 μg) was either directly administered intraperitoneally, or one hour later intravenously and the animals were sacrificed 5.5 hrs later. Splenocytes were harvested and analyzed by flow cytometry (Figure 20B). Most CD19+ cells (B cells) were GFP+, indicating successful capture of GFP in vivo. Administration of GFP into control animals that had not received the bispecific construct showed no GFP staining on CD19+ or CD19- cells. This experiment thus shows that a bispecific construct can be used to first target a cell population of interest, which can then be addressed with a ligand for the remaining free second binding site. Construction of bispecific reagents of this type allows for the targeted delivery of biologicals in a manner that might avoid acute toxicity, as is observed, e.g., for systemic interleukin-2 (IL2) administration.

With the methods and reagents provided herein it is now possible to connect any entity proven to be a substrate in a sortase reaction and in all possible topologies. For example, C-terminally conjugated human IL2 and interferon-cc was successfully conjugated to anti GFP and VHH-7 using this approach (Figure 22), thus showing the general applicability of these tools.

Discussion

The ability to fuse proteins via their N- or C-termini creates immediate opportunities for the production of molecules not accessible by standard genetic means. Proteins connected in this manner retain functionality for N-to-N and for C-to-C fusions. As an instructive example, the ability to create C-terminally fused bispecific camelid-derived VHH constructs with full retention of the binding capacity of both fusion partners has been demonstrated. Possible applications extend to other fusions as well. For those situations where the desired combination demands that both C- or both N-termini remain available for proper function, standard genetic approaches fall short. Click chemistry has developed to the point where off-the-shelf reagents suitable for solid phase peptide synthesis allow ready access to the peptides that enable these types of fusion. Although demonstrated here for protein-protein fusions, further modifications of the click handles used to connect the two proteins allow installation of yet other functionalities, such as fluorophores, or pharmacologically active small molecules. Ease of modification of proteins of interest, ready
access to recombinant sortases of different origin, and the flexibility afforded in nucleophile
design through use of standard peptide synthetic methodology add to the versatility of
sortase-mediated transacylation.

[00269] Protein fusions not easily accessed by other means are within easy reach using the
technology described herein. Of note, Hudak et al. described the use of aldehyde tag in
combination with strain-promoted click chemistry to achieve similar goals and produced
hlgG fused to human growth hormone and maltose binding protein(33). This approach is
orthogonal to our sortagging strategy and immediately suggests the possibility of combining
methods such as the aldehyde tag-click chemistry method developed by Hudak et al. with the
chemo-enzymatic method developed here to access even more challenging protein-protein
fusions.

References
communication between protomers of dopamine class A GPCR dimers modulates activation.
NatMeth 5:688-695.
4. Leong SR et al. (1997) IL-8 single-chain homodimers and heterodimers: interactions
with chemokine receptors CXCR1, CXCR2, and DARC. Protein Sci 6:609-617.
6. Drury LJ et al. (2011) Monomeric and dimeric CXCL12 inhibit metastasis through
Monoclonal Antibody- Avidin Fusion Protein for Receptor-Mediated Delivery of
Erythropoietin as an IgG Fusion Protein That Penetrates the Blood-Brain Barrier in the
Mouse. Mol Pharmaceutics 7:2148-2155.


[00270] The entire contents of all references listed in the Summary, Detailed Description, and Examples sections are incorporated herein by reference, as if each reference was individually incorporated by reference. In case of a conflict between an incorporated reference and the instant specification, the instant specification shall control.

[00271] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.
EQUIVALENTS AND SCOPE

[00272] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

[00273] In the claims articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" or "and/or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are comprised in, present in, employed in, or otherwise relevant to a given product, formula, or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[00274] Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[00275] Where elements are presented as lists, e.g., in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It is also noted that the term "comprising" is intended to be open and permits the inclusion of additional elements or steps. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, steps, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, steps, etc. For purposes of simplicity those embodiments have not been specifically set forth in haec verba.
herein. Thus for each embodiment of the invention that comprises one or more elements, features, steps, *etc.*, the invention also provides embodiments that consist or consist essentially of those elements, features, steps, *etc.*

[00276] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, where ranges are provided, all specific values within the range are provided as well in some embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

[00277] In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value or group of values within the range, may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.
CLAIMS

What is claimed is:

1. A method of installing a click chemistry handle to the C-terminus of a target protein, the method comprising the steps of:
   (a) providing the target protein with a C-terminal sortase recognition sequence;
   (b) contacting the target protein with a peptide or agent comprising 1-10 N-terminal glycine residues or an N-terminal alkylamine group and the click chemistry handle; in the presence of a sortase enzyme under conditions suitable for the sortase to transamidate the target protein and the peptide comprising the click chemistry handle, thus conjugating the target protein to the click-chemistry handle.

2. The method of claim 1, wherein the target protein is fused to the sortase recognition sequence at the protein's C-terminus.

3. A method of installing a click chemistry handle to the N-terminus of a target protein, the method comprising
   (a) providing the target protein with 1-10 N-terminal glycine residues or an N-terminal alkylamine group;
   (b) contacting the target protein with a peptide comprising a sortase recognition motif, and the click chemistry handle; in the presence of a sortase enzyme under conditions suitable for the sortase to transamidate the target protein and the peptide, thus conjugating the target protein to the click-chemistry handle.

4. The method of claim 3, wherein the target protein is fused to the 1-10 N-terminal glycine residues or an N-terminal alkylamine group at the protein's N-terminus.

5. The method of any of claims 1-4, wherein the sortase recognition motif is a sortase A recognition motif.

6. The method of any of claims 1-5, wherein the sortase recognition motif comprises the sequence LPXT.
7. The method of any of claims 1-6, wherein the 1-10 N-terminal G glycine residues are three N-terminal glycine residues.

8. The method of any of claims 1-7, wherein the peptide comprises a linker between the click chemistry group and the 1-10 glycine residues or the N-terminal alkylamine group, or between the click chemistry group and the sortase recognition sequence.

9. The method of claim 8, wherein the linker comprises an amino acid sequence of 1-100 amino acid residues.

10. The method of any of claims 1-9, wherein the click chemistry handle is selected from the group consisting of terminal alkyne, azide, strained alkyne, diene, dieneophile, alkoxyamine, carbonyl, phosphine, hydrazide, thiol, tetrazine, and alkene.

11. The method of claim 10, wherein the click chemistry handle is selected from the group consisting of cyclooctyne and azide.

12. The method of any of claims 1-11, wherein the sortase recognition sequence is LPETG (SEQ ID NO: 4).

13. A method of post-translationally conjugating two proteins to form a chimeric protein, the method comprising:

   contacting a first protein conjugated to a first click-chemistry handle with a second protein conjugated to a second click chemistry handle under conditions suitable for the first click chemistry handle to react with the second click chemistry handle, thus generating a chimeric protein comprising the two proteins linked via a covalent bond.

14. The method of claim 13, wherein the first click chemistry handle is conjugated to the N-terminus of the first protein, and the second click chemistry handle is conjugated to the N-terminus of the second protein, and the chimeric protein is an N-terminus-to-N-terminus conjugation of the first and the second protein.
15. The method of claim 13, wherein the first click chemistry handle is conjugated to the C-terminus of the first protein, and the second click chemistry handle is conjugated to the C-terminus of the second protein, and the chimeric protein includes a C-terminus-to-C-terminus conjugation of the two proteins.

16. The method of any of claims 13-15, wherein the click chemistry handle of the first protein is selected from the group consisting of terminal alkyne, strained alkyne, diene, alkoxyamine, phosphine, hydrazide, tetraine, and thiol.

17. The method of any of claims 13-16, wherein the click chemistry handle of the second protein is selected from the group consisting of azide, dieneophile, carbonyl, and alkene.

18. The method of any of claims 13-17, wherein
   (i) the click chemistry handle of the first protein is a terminal alkyne, and the click chemistry handle of the second protein is an azide;
   (ii) the click chemistry handle of the first protein is a strained alkyne, and the click chemistry handle of the second protein is an azide;
   (iii) the click chemistry handle of the first protein is a diene, and the click chemistry handle of the second protein is a dieneophile;
   (iv) the click chemistry handle of the first protein is an alkoxyamine, and the click chemistry handle of the second protein is a carbonyl;
   (v) the click chemistry handle of the first protein is a phosphine, and the click chemistry handle of the second protein is an azide;
   (vi) the click chemistry handle of the first protein is a hydrazide, and the click chemistry handle of the second protein is a carbonyl; or
   (vii) the click chemistry handle of the first protein is a thiol, and the click chemistry handle of the second protein is an alkene.(viii) the click chemistry handle of the first protein is a cyclooctyne, and the click chemistry handle of the second protein is an azide.

19. A bispecific, chimeric antibody comprising
   a first antibody or antigen-binding antibody fragment, comprising a sortase recognition sequence; and
a second antibody or antigen-binding antibody fragment, comprising a sortase recognition sequence; wherein the first and the second antibody or antibody fragment are conjugated together via click chemistry.

20. The chimeric antibody of claim 19, wherein the first and the second antibody or antibody fragment are conjugated together via a covalent bond at their C-termini (C-C) or at their N-termini (N-N).

21. The chimeric antibody of claim 19 or 20, wherein the first and/or the second antibody comprises a single-domain antibody or an antigen-binding fragment thereof.

22. The chimeric antibody of any of claims 19-21, wherein the first and/or the second antibody comprises a camelid antibody or an antigen-binding fragment thereof.

23. The chimeric antibody of any of claims 19-22, wherein the first and/or the second antibody comprises a VHH domain or an antigen-binding fragment thereof.

24. The chimeric antibody of any of claims 19-23, wherein the first and/or the second antibody comprises an scFv or an antigen-binding fragment thereof.

25. The chimeric antibody of any of claims 19-24, wherein the first and/or the second antibody comprises a nanobody or an antigen-binding fragment thereof.

26. The chimeric antibody of any of claims 19-25, wherein the first and the second antibody, or antigen-binding fragment thereof, bind different antigens.

27. The chimeric antibody of any of claims 19-25, wherein the first and the second antibody, or antigen-binding fragment thereof, bind the same antigen.

28. The chimeric antibody of claim 27, wherein the first and the second antibody, or antigen-binding fragment thereof, bind different epitopes of the same antigen.

29. A protein, comprising a target protein with a sortase recognition motif, and a second molecule conjugated to the protein via click chemistry.
30. The protein of claim 29, wherein the sortase recognition motif comprises the sequence LPXT.

31. The protein of claim 29 or 30, wherein the protein is generated by post-translationally installing a click chemistry handle on the target protein and contacting the target protein with the second molecule, wherein the second molecule comprises a second click chemistry handle that can react with the click chemistry handle of the target protein to form a covalent bond under suitable conditions.

32. The protein of any of claims 27-31, wherein the second molecule is a second protein, a small chemical compound, a nucleic acid, or a lipid.

33. The protein of claim 32, wherein the second protein comprises a sortase recognition motif.

34. The protein of claim 32 or 33, wherein the target protein and the second protein are posttranslationally conjugated via click chemistry at their N-termini (N-N), or at their C-termini (C-C).

35. The protein of any of claims 29-34, wherein the target protein comprises an antigen-binding domain.

36. The protein of any of claims 29-35, wherein the second molecule comprises an antigen-binding domain.

37. The protein of any of claims 32-36, wherein the target protein and the second molecule comprise an antigen-binding domain each, and wherein the antigen-binding domains of the target protein and of the second molecule are different.

38. The protein of claim 37, wherein the antigen-binding domains of the protein and the second molecule bind different antigens.
39. The protein of claim 37 or 38, wherein the antigen-binding domain of the protein and/or of the second molecule comprises an antibody, an antigen-binding antibody fragment, an adnectin, an affibody, an anticalin, a DARPin, or an aptamer.

40. The protein of any of claims 37-39, wherein the antigen-binding domain of the protein and/or of the second molecule comprise a camelid antibody, a VHH domain, a single-domain antibody, an scFv, a nanobody, or an antigen-binding fragment thereof.

41. The protein of claim 29, wherein the second molecule comprises a synthetic polymer.

42. The protein of claim 41, wherein the synthetic polymer comprises a PEG moiety.

43. The protein of claim 29, wherein the additional molecule comprises a detectable label.

44. The protein of claim 43, wherein the detectable label comprises a fluorophore, an enzyme, or a radioisotope.

45. The protein of claim 44, wherein the detectable label is selected from the group consisting of a fluorescent protein, a fluorescent dye, a luciferase, and a peroxidase.

46. A protein comprising a sortase recognition motif; and a click chemistry handle conjugated to the sortase recognition motif.

47. The protein of claim 46, wherein the sortase recognition motif comprises the sequence LPXT.

48. The protein of claim 46 or 47, wherein the protein comprises an antigen-binding domain.

49. The protein of any of claims 46-48, wherein the protein comprises an antibody, or an antigen-binding antibody fragment.
50. The protein of claim 49, wherein the protein comprises a camelid antibody or antigen-binding fragment thereof, a VHH domain, a single-domain antibody, a nanobody, an scFv, an affibody, an anticalin, a DARPin, or an adnectin.

51. The protein of any of claims 29-50, wherein the protein comprises a linker between the click chemistry group and sortase recognition sequence.

52. The protein of claim 51, wherein the linker comprises an amino acid sequence of 1-100 amino acid residues.

53. The protein of any of claims 46-50, wherein the click chemistry handle is at the C-terminus of the protein.

54. The protein of any of claims 46-50, wherein the click chemistry handle is at the N-terminus of the protein.

55. The protein of any of claims 46-50, wherein the click chemistry handle is selected from the group consisting of terminal alkyne, azide, strained alkyne, diene, dieneophile, alkoxyamine, carbonyl, phosphine, hydrazide, thiol, and alkene.

56. A kit comprising
   (a) a first peptide comprising 1-10 glycine residues or a terminal alkyamine conjugated to a first click chemistry handle; and
   (b) a second peptide comprising a sortase recognition motif conjugated to a second click chemistry handle; wherein the click chemistry handle of the first and the second peptide can react under suitable conditions; and, optionally,
   (c) a sortase enzyme.

57. A kit comprising
   (a) a first peptide comprising 1-10 glycine residues or a terminal alkyamine conjugated to a first click chemistry handle; and
   (b) a second peptide comprising a 1-10 glycine residues or a terminal alkyamine conjugated to a second click chemistry handle; wherein the click chemistry handle of the first and the second peptide can react under suitable conditions; and, optionally,
(c) a sortase enzyme.

58. A kit comprising
   (a) a first peptide comprising a sortase A recognition motif conjugated to a first click chemistry handle; and
   (b) a second peptide comprising a sortase recognition motif conjugated to a second click chemistry handle; wherein the click chemistry handle of the first and the second peptide can react under suitable conditions; and, optionally,
   (c) a sortase enzyme.

59. The kit of any of claims 56-58, wherein the first click chemistry handle is selected from the group consisting of terminal alkynes, strained alkynes, dienes, alkoxyamines, phosphines, hydrazides, and thiols.

60. The kit of any of claims 56-59, wherein the second click chemistry handle is selected from the group consisting of azides, dieneophiles, carboxyls, and alkenes.

61. The kit of any of claims 56-60, wherein
   (i) the first click chemistry handle is a terminal alkyne, and the second click chemistry handle is an azide;
   (ii) the first click chemistry handle is a strained alkyne, and the second click chemistry handle is an azide;
   (iii) the first click chemistry handle is a diene, and the second click chemistry handle is a dieneophile;
   (iv) the first click chemistry handle is an alkoxyamine, and the second click chemistry handle is a carbonyl;
   (v) the first click chemistry handle is a phosphine, and the second click chemistry handle is an azide;
   (vi) the first click chemistry handle is a hydrazide, and the second click chemistry handle is a carbonyl; or
   (vii) the first click chemistry handle is a thiol, and the second click chemistry handle is an alkene.
   (viii) the first click chemistry handle is a cyclooctyne, and the second click chemistry handle is an azide.
62. The kit of any of claims 56-61, wherein the kit further comprises, a catalyst, a reaction buffer, and/or instructions for use of the kit.
C-C Dimers

"Click" chemistry

\[ \text{LEPTGGGK-CONH}_2 \rightarrow \text{-LEPTGG-His}_6 \]

\[ + \text{N}_3 \]

\[ \text{H}_2\text{N-GGGK-CONH}_2 \]

N-N Dimers

"Click" chemistry

\[ \text{LPETGGG} \rightarrow \text{-LPETGG-CONH}_2 \]

\[ + \text{N}_3\text{LPETGG-CONH}_2 \]

\[ \text{H}_2\text{N-GGG-CONH}_2 \]

**Fig. 1**

SUBSTITUTE SHEET (RULE 26)
Fig. 2A
Fig. 2C

Fig. 2D
Fig. 3A
Fig. 3B-2

SUBSTITUTE SHEET (RULE 26)
N-N Dimers: monomer synthesis

\[
\begin{align*}
\text{H}_2\text{N-GGG} & \quad \text{Ub} \quad \text{GGG-Ub-VME} \\
\text{H}_2\text{N-GGG} & \quad \text{Ub} \quad \text{GGG-Ub-VME} \\
\text{N}_3\text{-LPETGG-CONH}_2 & \quad \text{Ub} \\
\text{SrtA} \\
\text{N}_3\text{-LPETGGG} & \quad \text{Ub} \\
\end{align*}
\]

N-N Dimerization

\[
\begin{align*}
\text{N}_3\text{-LPETGGG} & \quad \text{Ub} \\
+ & \quad \text{LPETGGG} \quad \text{Ub} \\
\text{LPETGGG} & \quad \text{Ub} \\
\end{align*}
\]

N3UbVME  +  +  +  +  -  -  +  +  -  -  -

DBCO-UbVME -  +  +  -  +  +  +  +  +  +  +

N3UbOH -  -  -  -  -  -  -  +  +  +  +

UCHL3 -  -  -  +  +  +  +  -  +  +  +

Fig. 4
$ub + \text{N3-LPETGG}$

110308_01_MDW_N3_ub

TOF MS ES+
TIC
$1.33e5$

$G_3Ub \xrightarrow{\text{SrtA-H}_6} N_3\text{LPETG}_3Ub$

Fig. 5-2
ub + cyclooctyne-LPETGG
110308_03_MDW_cyclooctyne_ub 221 (7.370) M1 [Ev-214219,lt5]
(Gs, 1.000,400: 1800,2.00,133,R33): Cm (214:252)

TOF MS ES+
4.07e4

ub + cyclooctyne-LPETGG
110308_03_MDW_cyclooctyne_ub 221 (7.370) Cm (214:252)

TOF MS ES+
4.07e4

Fig. 5-3
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- + + + + + + + +

+ - + + + + + +

7 7 0.5 1 2 4 7

---

Fig. 6

---
C-C Dimers: monomer synthesis

LEPTGGG-H₆
anti β2M

LEPTGGG-H₆
anti GFP

N₃
H₂N-GGGK-CONH₂
SrtA

LEPTGGGK-CONH₂
anti β2M

H₂N-GGGK-CONH₂
SrtA

LEPTGGGK-CONH₂
anti GFP

C-C Dimerization

N₃
LEPTGGGK-CONH₂

LEPTGGGK-CONH₂

LEPTGGGK-CONH₂
anti β2M anti GFP

Fig. 7
DAD1 C, Sig=210.8 Ref=360.100 (MDWA_B2M_N3_SE0004.D)

mAU
2250
2000
1750
1500
1250
1000
750
500
250

min
0
5
10
15
20
25

anti b2m pure check
110404_03_MDWa_b2m

TOF MS ES+
TIC
3.75e5

Fig. 8-1

SUBSTITUTE SHEET (RULE 26)
anti b2m pure check
110404_03_MDWa_b2m 348 (11.606) Cm (167:368)

Fig. 8-2
SUBSTITUTE SHEET (RULE 26)
anti gfp enh. sortagged with mdw57
110404_01_MDWa_gfp_sort 199 (6.636) M1 [Ev-464492,lt5]
(Gs, 1.000,400: 1800, 1.00,L33,R33); Cm (198:365)

TOF MS ES+ 2.99e4

mass

Fig. 9-2
Coomassie gel after sortagging and size exclusion

Fr 1  Fr 2  Fr 1  Fr 2

Coomassie gel after dimerizing and size exclusion

Fig. 10
Fig. 12
Fig. 13A

Fig. 13B

TOF MS ES+ 4.07e4

TOF MS ES+ 3.11e4

SUBSTITUTE SHEET (RULE 26)
Fig. 13C

Fig. 13D

Fig. 13E

Fig. 13F
Fig. 15A
Fig. 15B
Fig. 16D
Fig. 16E

Fig. 16F
Fig. 17
Fig. 20A

Fig. 20B
Fig. 21

Fig. 22
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

C07K 1/06 (2006.01)
C12P 21/04 (2006.01)
C07K 16/46 (2006.01)
C07K 19/00 (2006.01)
C12N 9/00 (2006.01)
C12N 9/10 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 1/06, C12P 21/04, C07K 16/46, 19/00, C12N 9/00.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PAJ, Esp@cenet, PCT Online, USPTO DB, WIPO, RUPTO, EAPATIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>DAVID A. LEVARY et al. &quot;Protein-Protein Fusion Catalyzed by Sortase A&quot;. Plos One, April 6, 2011, vol. 6, Issue 4, e18342, pp. 1-6, fig. 1, 2</td>
<td>19,20,21-25, 26, 27-28, 35-40, 46-50</td>
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Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search

29 October 2012 (29.10.2012)

Date of mailing of the international search report

15 November 2012 (15.1.2012)

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