MOLECULAR CONSTRUCTS WITH TARGETING AND EFFECTOR ELEMENTS AND THEIR APPLICATIONS

BACKGROUND OF THE INVENTION

[1] 1. Field of the Invention

[2] The present disclosure relates to the field of pharmaceuticals; more particularly, to multi-functional molecular constructs, e.g., those having targeting and effector elements for delivering the effector (e.g., therapeutic drug) to targeted sites.

[2] 2. Description of the Related Art

[4] The continual advancement of a broad array of methodologies for screening and selecting monoclonal antibodies (mAbs) for targeted antigens has helped the development of a good number of therapeutic antibodies for many diseases that were regarded as untreatable just a few years ago. According to Therapeutic Antibody Database, approximately 2,800 antibodies have been studied or are being planned for studies in human clinical trials, and approximately 80 antibodies have been approved by governmental drug regulatory agencies for clinical uses. The large amount of data on the therapeutic effects of antibodies has provided information concerning the pharmacological mechanisms how antibodies act as therapeutics.

[5] One major pharmacologic mechanism for antibodies acting as therapeutics is that, antibodies can neutralize or trap disease-causing mediators, which may be cytokines or immune components present in the blood circulation, interstitial space, or in the lymph nodes. The neutralizing activity inhibits the interaction of the disease-causing mediators with their receptors. It should be noted that fusion proteins of the soluble receptors or the extracellular portions of receptors of cytokines and the Fc portion of IgG, which act by neutralizing the cytokines or immune factors in a similar fashion as neutralizing antibodies, have also been developed as therapeutic agents.

[6] Several therapeutic antibodies that have been approved for clinical applications or subjected to clinical developments mediate their pharmacologic effects by binding to receptors, thereby blocking the interaction of the receptors with their ligands. For those

antibody drugs, Fc-mediated mechanisms, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis (CMC), are not the intended mechanisms for the antibodies.

[7] Some therapeutic antibodies bind to certain surface antigens on target cells and render Fc-mediated functions and other mechanisms on the target cells. The most important Fc-mediated mechanisms are antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis (CMC), which both will cause the lysis of the antibody-bound target cells. Some antibodies binding to certain cell surface antigens can induce apoptosis of the bound target cells.

[8] The concept and methodology for preparing antibodies with dual specificities germinated more than three decades ago. In recent year, the advancement in recombinant antibody engineering methodologies and the drive to develop improved medicine has stimulated the development bi-specific antibodies adopting a large variety of structural configurations.

[9] For example, the bi-valent or multivalent antibodies may contain two or more antigen-binding sites. A number of methods have been reported for preparing multivalent antibodies by covalently linking three or four Fab fragments via a connecting structure. For example, antibodies have been engineered to express tandem three or four Fab repeats.

[10] Several methods for producing multivalent antibodies by employing synthetic crosslinkers to associate, chemically, different antibodies or binding fragments have been disclosed. One approach involves chemically cross-linking three, four, and more separately Fab fragments using different linkers. Another method to produce a construct with multiple Fabs that are assembled to one-dimensional DNA scaffold was provided. Those various multivalent Ab constructs designed for binding to target molecules differ among one another in size, half-lives, flexibility in conformation, and ability to modulate the immune system. In view of the foregoing, several reports have been made for preparing molecular constructs with a fixed number of effector elements or with two or more different kinds of functional elements (e.g., at least one targeting element and at least one effector element). However, it is often difficult to build a molecular construct with a particular combination of the targeting and effector elements either using chemical synthesis or

recombinant technology. Accordingly, there exists a need in the related art to provide novel molecular platforms to build a more versatile molecule suitable for covering applications in a wide range of diseases.

SUMMARY

[11] The following presents a simplified summary of the disclosure in order to provide a basic understanding to the reader. This summary is not an extensive overview of the disclosure and it does not identify key/critical elements of the present invention or delineate the scope of the present invention. Its sole purpose is to present some concepts disclosed herein in a simplified form as a prelude to the more detailed description that is presented later.

[12] < I > Peptide Core-Based Multi-Arm Linkers

[13] In the first aspect, the present disclosure is directed to a linker unit that has at least two different functional elements linked thereto. For example, the linker unit may have linked thereto two different effector elements, one targeting element and one effector element, or one effector element and a polyethylene glycol (PEG) chain for prolonging the circulation time of the linker unit. The present linker unit is designed to have at least two different functional groups such that the functional elements can be linked thereto by reacting with the respective functional groups. Accordingly, the present linker unit can serve as a platform for preparing a molecular construct with two or more functional elements.

[14] According to various embodiments of the present disclosure, the linker unit comprises a center core and a plurality of linking arms. The center core is a polypeptide core comprising (1) a plurality of lysine (K) resides, in which each K residue and a next K residue are separated by a filler sequence comprising glycine (G) and serine (S) residues, and the number of K residues ranges from 2 to 15; or (2) the sequence of $(X_{aa}\text{-}K)_n$, where X_{aa} is a PEGylated amino acid having 2 to 12 repeats of ethylene glycol (EG) unit, and n is an integral from 2 to 15. Optionally, the filler sequence consists of 2 to 20 amino acid residues. In various embodiments, the filler sequence may have the sequence of GS, GGS, GSG, or SEQ ID NOs: 1-16. According to some embodiments of the present disclosure, the center core comprises 2-15 units of the sequence of $G_{1\text{-}5}SK$; preferably, the

center core comprises the sequence of $(GSK)_{2-15}$. Each of the linking arms is linked to the K residues of the center core via forming an amide linkage between the K residue and the linking arm. The linking arm linked to the center core has a maleimide, a N-hydroxysuccinimidyl (NHS) group, an azide group, an alkyne group, a tetrazine group, a cyclooctene group, or a cyclooctyne group at its free-terminus. Also, the amino acid residue at the N- or C-terminus of the center core has an azide group or an alkyne group; alternatively or additionally, the amino acid residue at the N- or C-terminus of the center core is a cysteine (C) residue, in which the thiol group of the amino acid residue is linked with a coupling arm having an azide group, an alkyne group, a tetrazine group, a cyclooctene group, or a cyclooctyne group at the free terminus of the coupling arm.

[15] According to some embodiments of the present disclosure, when the free terminus of the linking arm is the azide, the alkyne, or the cyclooctyne group, then the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the tetrazine or the cyclooctene group. According to other embodiments of the present disclosure, when the free terminus of the linking arm is the tetrazine group or cyclooctene group, then the amino acid residue at the N- or C-terminus of the center core has the azide or the alkyne group, or the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the azide, the alkyne, or the cyclooctyne group.

[16] In some embodiments, the linking arm is a PEG chain, preferably having 2 to 20 repeats of EG units. Alternatively, the linking arm is a PEG chain having 2 to 20 repeats of EG units with a disulfide linkage at the free terminus thereof (i.e., the terminus that is not linked with the K residue of the center core). In some embodiments, the coupling linking arm is a PEG chain, preferably having 2 to 12 repeats of EG units.

[17] Regarding amino acid residues having the azide group, non-limiting examples of said amino acid residues include L-azidohomoalanine (AHA), 4-azido-L-phenylalanine, 4-azido-D-phenylalanine, 3-azido-L-alanine, 3-azido-D-alanine, 4-azido-L-homoalanine, 4-azido-D-homoalanine, 5-azido-L-ornithine, 5-azido-d-ornithine, 6-azido-L-lysine, and 6-azido-D-lysine. As to the amino acid residues having the alkyne group, illustrative

examples thereof include L-homopropargylglycine (L-HPG), D-homopropargylglycine (D-HPG), and beta-homopropargylglycine (β-HPG).

[18] When the amino acid residues at the N- or C-terminus of the center core is the cysteine residue, the cyclooctene group at the free terminus of the coupling arm may be, a trans-cyclooctene (TCO) group, while the cyclooctyne group at the free terminus of the coupling arm may be a dibenzocyclooctyne (DBCO), difluorinated cyclooctyne (DIFO), bicyclononyne (BCN), or dibenzocyclooctyne (DICO) group. Alternatively, the tetrazine group at the free terminus of the coupling arm includes, but is not limited to, 1,2,3,4-tetrazine, 1,2,3,5-tetrazine, and 1,2,4,5-tetrazine, and derivatives thereof, such as, 6-methyl tetrazine.

[19] According to various embodiments of the present disclosure, the linker unit further comprises a plurality of first elements. In some embodiments, each of the first elements is linked to one of the linking arms via forming an amide bound between the linking arm and the first element. In other embodiments, each of the first elements is linked to one of the linking arms via copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction, strained-promoted azide-alkyne click chemistry (SPAAC) reaction, or inverse electron demand Diels-Alder (iEDDA) reaction occurred between the linking arm and the first element.

[20] Optionally, the present linker unit further comprises a plurality of connecting arms that are respectively linked to the plurality of linking arms via CuAAC reaction, SPAAC reaction, or iEDDA reaction. According to the embodiments of the present disclosure, each of the plurality of connecting arms has a maleimide or the NHS group at the element-linking terminus thereof (i.e., the terminus that is not linked with the linking arm). Accordingly, each of the first elements is linked to one of the connecting arms via the thiol-maleimide reaction occurred between the connecting arm and the first element; or each of the first elements is linked to one of the connecting arms via forming an amide bound between the connecting arm and the first element. In some embodiments, each of the connecting arms is a PEG chain, preferably having 2-20 repeats of EG units. In other embodiments, each of the connecting arms is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the element-linking terminus.

[21] According to various optional embodiments of the present disclosure, the first element is an effector element suitable for eliciting an intended effect (e.g., a therapeutic effect) in a subject. Alternatively, the first element may be a targeting element for directing the linker unit to the site of interest. According to the embodiments of the present disclosure, the first element is fingolimod, fingolimod phosphate, interferon-β, or a single-chain variable fragment (scFv) specific for integrin-α4, β-amyloid, a viral protein, a bacterial protein.

[22] Still optionally, the linker unit further comprises a second element that is different from the first elements. In some embodiments, the second element has an azide or alkyne group, so that it is linked to the center core or the coupling arm by coupling with the corresponding alkyne or azide group of the center core or the coupling arm via CuAAC reaction. Alternatively, in some embodiments, the second element having an azide or cyclooctyne group is linked to the center core or the coupling arm by coupling with the corresponding cyclooctyne or azide group of the center core or the coupling arm via SPAAC reaction. Still alternatively, in certain embodiments, the second element having a tetrazine or cyclooctene group is linked to the center core or the coupling arm by coupling with the corresponding cyclooctene or tetrazine group of the center core or the coupling arm via iEDDA) reaction. According to some embodiments, the linker unit comprises the connecting arm, which is linked to the linking arm via CuAAC reaction or SPAAC reaction; in these embodiments, the N- or C-terminus of the center core or the free terminus of the coupling arm has a tetrazine or cyclooctene group so that the second element having the corresponding cyclooctene or tetrazine group is linked to the center core or the coupling arm via iEDDA reaction. According to other embodiments, the linker unit comprises the connecting arm, which is linked to the linking arm via iEDDA reaction; in these conditions, the N- or C-terminus of the center core or the free terminus of the coupling arm has an azide, alkyne, or cyclooctyne group so that the second element having the corresponding chemical groups is linked to the center core or the coupling arm via CuAAC reaction or SPAAC reaction.

[23] In optional embodiments of the present disclosure, when the first element is an effector element, then the second element may be another effector element, which works additively or synergistically with or independently of the first element; alternatively, the second element may be a targeting element or an element for improving the pharmacokinetic

property of the linker unit, such as solubility, clearance, half-life, and bioavailability. In some other optional embodiments, when the first element is the targeting element, then the second element is preferably an effector element or an element for improving the pharmacokinetic property of the linker unit.

[24] In certain embodiments, the linker unit further comprises an optional third element that is different from the first and second elements. In the case where the second element is directly linked to the center core, the other terminus (i.e., the free terminus that is not linked with the second element) of the center core is optionally a cysteine residue, which can be used to introduce an optional third element. Specifically, the thiol group of the cysteine residue is reacted with a maleimide group of a PEG chain; and the thus-linked PEG chain is designated as the coupling arm, which has a tetrazine group or a cyclooctene group at its free terminus. Accordingly, the third element is then linked to the coupling arm via iEDDA reaction. In the case where the linker unit comprises both the second and third elements, it is preferable that at least one of the first and second elements is an effector as described above, while the third element may be the element for improving the pharmacokinetic property of the linker unit. One example of the element for improving the pharmacokinetic property is a long PEG chain having a molecular weight of about 20,000 to 50,000 daltons.

[25] **< II > Uses of Peptide Core-Based Multi-Arm Linkers**

[26] The linker unit according to the first aspect of the present disclosure may find its utility in clinical medicine for the treatment of various diseases. Hence, the second aspect of the present disclosure is directed to a method for treating these diseases. According to various embodiments of the present disclosure, the method for treating a particular disease includes the step of administering to the subject in need thereof a therapeutically effective amount of the linker unit according to the above-mentioned aspect and embodiments of the present disclosure. As could be appreciated, said linker unit may be administered in a pharmaceutical formulation, which comprises a pharmaceutically-acceptable excipient suitable for the intended or desired administration route, in addition to the present linker unit.

[27] Various illustrative combinations of the first and second elements of the present linker unit for treating some particular diseases are disclosed below for facilitating the understanding of some embodiments of the present disclosure.

[28] According to some embodiments of the present disclosure, the present linker unit is useful in treating a central nervous system (CNS) disease, for example, multiple sclerosis and Alzheimer's disease. For the treatment of multiple sclerosis, the first element can be fingolimod, fingolimod phosphate, interferon-β, or an scFv specific for integrin-α4. For the purpose of treating Alzheimer's disease, the element is an scFv specific for β-amyloid.

[29] According to other embodiments of the present disclosure, the linker units suitable for treating an infectious disease comprise an scFv specific for a viral protein or a bacterial protein as the first element. In one preferred embodiment, the viral protein is F protein of respiratory syncytia virus (RSV), gp120 protein of human deficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, or glycoprotein of cytomegalovirus; and the bacterial protein is endotoxin of Gram(-) bacteria, surface antigen of *Clostridium difficile*, lipoteichoic acid of *Staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, or Shiga-like toxin type I or II of *Escherichia coli*.

[30] **< III > Molecular Constructs with Targeting and Effector Moieties**

[31] In the third aspect, the present disclosure is directed to a molecular construct comprising two linker units coupling to each other either directly or indirectly, in which the core of one linker unit is configured to be linked with at least one targeting element while the core of the other linker unit is configured to be linked with at least one effector element. The present molecular construct is advantageous in that the two linker units are coupled to each other via an iEDDA reaction, a SPAAC reaction, or a CuAAC reaction. This design allows for a facile synthesis of a molecular construct with a complex structure. According to the principles and spirits of the present disclosure, the two linker units respectively carrying different numbers and/or types of functional elements can be independently prepared, and then conjugated together. In this way, it becomes feasible for a skilled artisan to construct libraries of molecular constructs respectively carrying different functional elements, and then select and combine two molecular constructs (or linker units) from the libraries to generate a desired constructs, depending on the needs and/or intended

applications. Moreover, the number of functional elements per linker unit may be controlled by adjusting the number of specific functional group(s) of the core.

[32] According to one embodiment of the present disclosure, the molecular construct comprises a first linker unit and a second linker unit. Specifically, the first linker unit comprises (1) a first center core, (2) one or more linking arms (hereinafter, the first linking arms) linked to the first center core, (3) optionally a coupling arm (hereinafter, the first coupling arms) linked to the first center core, and (4) optionally one or more connecting arms (hereinafter the first connecting arms) respectively linked to the one or more first linking arms; the second linker unit comprises (1) a second center core, (2) one or more linking arms (hereinafter, the second linking arms) linked to the second center core, (3) optionally a coupling arm (hereinafter, the second coupling arm) linked to the second center core, and (4) optionally one or more connecting arms (hereinafter the second connecting arms) respectively linked to the one or more second linking arms. The first and second linker units are coupled to each other via iEDDA, SPAAC, or CuAAC reaction occurred between any of the followings: the first and second center cores, the first coupling arm and the second center core, the first and second coupling arms, or the first center core and the second coupling arm.

[33] According to the embodiments of the present disclosure, both the first and second center cores have a plurality of amine groups. Each of the linking arms is linked to the center core via forming an amide bond therebetween, for example, between the N-hydroxysuccinimidyl (NHS) group and the amine group. After being linked to the center core, the linking arm thus has an NHS, a maleimide, an azide, an alkyne, a tetrazine, a cyclooctene, or a cyclooctyne group at the free terminus thereof.

[34] In the presence of the NHS group, a first targeting element and a first effector element are respectively linked to the first and second linking arms via forming an amide bond between the element (i.e., the first targeting element or the first effector element) and the linking arm (i.e., the first linking arm or the second linking arm). In the case where the linking arm has a maleimide, an azide, an alkyne, a tetrazine, a cyclooctene, or a cyclooctyne group at its free terminus, a first targeting element and a first effector element are respectively linked to the first and second linking arms via the thiol-maleimide, CuAAC,

iEDDA, or SPAAC reaction occurred between the element (i.e., the first targeting element or the first effector element) and the linking arm (i.e., the first linking arm or the second linking arm).

[35] Optionally, the linker unit (i.e., the first or the second linker unit) of the present molecular construct further comprises one or more connecting arms (i.e., the first or second connecting arms) that are respectively linked to the one or more linking arms via CuAAC, iEDDA, or SPAAC reaction. According to the embodiments, each of the connecting arms has an NHS or a maleimide group at it free terminus. Thus, each of the elements (i.e., the first target elements or the first effector elements) is linked to each of the connecting arms either forming an amide bond between the element and the connecting arm, or via the thiol-maleimide, CuAAC, iEDDA, or SPAAC reaction occurred between the element and the connecting arm.

[36] According to some embodiments of the present disclosure, each of the linking arms is a PEG chain having 2-20 repeats of EG units. Alternatively, each of the linking arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the free terminus thereof (i.e., the terminus that is not linked with the center core). According to some embodiments of the present disclosure, each of the coupling arms is a PEG chain having 2-12 repeats of EG units. According to some embodiments of the present disclosure, each of the connecting arms is a PEG chain having 2-20 repeats of EG units. Alternatively, each of the connecting arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the element-linking terminus (i.e., the terminus that is not linked with the linking arm).

[37] According to various embodiments of the present disclosure, each of the first and second center cores may be a compound core or a polypeptide core. In some examples, both the first and second center cores are compounds cores of the same or different compound(s). In certain preferred embodiments, both the first and second center cores are polypeptide cores having the same or different sequence(s). Alternatively, one of the two cores is a compound core, while the other is a polypeptide core.

[38] Non-limiting examples of the compound suitable for use as the present compound core include, benzene-1,3,5-triamine, 2-(aminomethyl)-2-methylpropane-1,3-diamine, tris(2-aminoethyl)-amine, benzene-1,2,4,5-tetraamine, 3,3' ,5,5'-tetraamine-1, 1'-biphenyl,

tetrakis-(2-aminoethyl)methane, tetrakis(ethylamine)-hydrazine, N,N,N',N',-tetrakis-(aminoethyl)-ethylenediamine, benzene-1,2,3,4,5,6-hexaamine, 1-N, 1-N,3-N,3-N,5-N,5-N-hexakis-(methylamine)-benzene-1, 3,5-triamine, 1-N,1-N,2-N,2-N,4-N,4-N,5-N,5-N-octakis-(methylamine)-benzene-1,2,4,5-triamine, and N,N-bis[(1-amino-3,3-diaminoethyl)-pentyl]methane-diamine.

[39] In the case where the center core is a compound core, the coupling arm is linked to one of the plurality of amine groups of the center core by forming an amide bond between the coupling arm and the center core. Meanwhile, the free terminus of the coupling arm has an azide, an alkyne, a cyclooctene, a cyclooctyne, or a tetrazine group.

[40] According to some embodiments of the present disclosure, the polypeptide suitable for use as the present polypeptide core comprises a plurality of lysine (K) residues; optionally, 2 to 15 K residues. Also, each K residue and the next K residue are separated by a filler sequence comprising glycine (G) and serine (S) residues; optionally, the filler sequence consists of 2 to 20 amino acid residues. In various embodiments, the filler sequence may have the sequence of GS, GGS, GSG, or SEQ ID NOs: 1-16. In some embodiments, the polypeptide comprises 2-15 units of the sequence of $G_{1-5}SK$, for example, $(GSK)_{2-15}$. In one embodiment, the polypeptide core has the sequence of SEQ ID NOs: 17, 18, 19, 21, 22, 23, or 24.

[41] Alternatively, the polypeptide core may comprise the sequence of $(X_{aa}\text{-}K)_n$, where X_{aa} is a PEGylated amino acid having 2 to 12 repeats of ethylene glycol (EG) unit, and n is an integral from 2 to 15. In one embodiment, the polypeptide core has the sequence of SEQ ID NO: 25 or 26.

[42] In the case where the center core is a polypeptide core, it may comprise a cysteine residue at its N- or C-terminus. In these instances, the coupling arm is linked to the cysteine residue of the center core via the thiol-maleimide reaction. The coupling arm linked to the cysteine residue has an azide, an alkyne, a cyclooctene, a cyclooctyne, or a tetrazine group at the free-terminus thereof.

[43] The first and second linker units may be coupled via various configurations, which are described in detail below, depending on the presence or absence of the first and second

coupling arms. For a linker unit having a compound core, it is preferable that it is linked with another linker unit via a coupling arm (i.e., the first or second coupling arm), while for a linker unit having a polypeptide core, the need for a coupling arm becomes optional.

[44] When the first and second linker units respectively comprise the coupling arms, then one of the coupling arms (say, for example, the first coupling arm) has a tetrazine group at the free-terminus thereof, and the other coupling arm (in this case, the second coupling arm) has a cyclooctene group at the free-terminus thereof, such that the two linker units are coupled via the iEDDA reaction occurred between the two coupling arms (i.e., the first and second coupling arms). Preferably, the tetrazine group is 1,2,3,4-tetrazine, 1,2,3,5-tetrazine, and 1,2,4,5-tetrazine, or derivatives thereof, such as, 6-methyl tetrazine; and the cyclooctene group is TCO. The same rule also applies in the case where the free termini of both coupling arms respectively have an azide group and an alkyne group; in this instance, the two linker units are coupled via the CuAAC reaction occurred between the two coupling arms (i.e., the first and second coupling arms). Alternatively, one of the coupling arms (for example, the first coupling arm) has an azide group, and the other coupling arm (in this case, the second coupling arm) has a cyclooctyne group (preferably, DBCO, DIFO, BCN, or DICO); accordingly, the two coupling arm can be coupled via the SPAAC reaction. These configurations may occur between two linker units, where both units have either compound cores or polypeptide cores, as well as in situations where one linker unit has a compound core, while the other has a polypeptide core.

[45] When only one linker unit has the coupling arm (as an example, the first linker unit with the first coupling arm), the center core of the other linker unit (for example, the second center core) is a polypeptide core. In this case, the first amino acid residue at the N- or C-terminus of one of the second center core is an amino acid residue having an azide group or an alkyne group. In some embodiments, the amino acid residue having the azide or alkyne group would undergo CuAAC reaction with the corresponding alkyne or azide group of the first coupling arm of the first linker unit, thereby coupling the first and second linker units. Alternatively, the first amino acid residue at the N- or C-terminus of one of the second center core is an amino acid residue having an azide group, which can be linked to the coupling arm of the first linker unit having a cyclooctyne group (preferably, DBCO, DIFO, BCN, or DICO) at the free-terminus via the SPAAC reaction. This configuration may occur

between two linker units, where both units have polypeptide cores, or in situations where one linker unit has a compound core, while the other has a polypeptide core.

[46] It is also possible that the first and second linker units are coupled without the presence of any coupling arms (that is, the first and second coupling arms). In other words, the first and second coupling arms are directly linked with each other. This configuration mostly occurs between two polypeptide cores. Specifically, one of the two center cores (say, for example, the first center core) has an amino acid residue having an azide group at the N- or C-terminus thereof, while the other center core (such as the second center core) has an amino acid residue having an alkyne group at the N- or C-terminus thereof. In this way, the azide group of the first center core reacts with the alkyne group of the second center core, thereby coupling the first and second linker units.

[47] Non-limiting examples of amino acid residues having the azide group include, L-azidohomoalanine (AHA), 4-azido-L-phenylalanine, 4-azido-D-phenylalanine, 3-azido-L-alanine, 3-azido-D-alanine, 4-azido-L-homoalanine, 4-azido-D-homoalanine, 5-azido-L-ornithine, 5-azido-d-ornithine, 6-azido-L-lysine, and 6-azido-D-lysine. Illustrative examples of amino acid residues having the alkyne group include, but are not limited to, L-homopropargylglycine (L-HPG), D-homopropargylglycine (D-HPG), and beta-homopropargylglycine (β-HPG).

[48] According to some embodiments of the present disclosure, one of the first and second linker units of the molecular construct further comprises an additional linking arm (hereinafter, the third linking arm) linked to the first or the second linker unit.

[49] Like the first and second linking arms, the third linking arm is configured to be linked with an element either via forming an amide bond therebetween, or via the thiol-maleimide, CuAAC, iEDDA, or SPAAC reaction. In some embodiments, the additional element is a second targeting element or a second effector element, which is used to enhance the targeting or therapeutic effect of the present molecular construct. Alternatively, a long PEG chain having a molecular weight of about 20,000 to 50,000 daltons can be used as the additional element so as to enhance the stability of the present molecular construct.

[50] In other embodiments, the present molecular construct further comprises a third linker unit. The third linker unit comprises (1) a third center core, (2) one or more linking arms (hereinafter, the third linking arms) linked to the third center core, (3) optionally a coupling arm (hereinafter, the third coupling arm) linked to the third center core, and (4) optionally one or more connecting arms (hereinafter, the third connecting arms). In this case, the third linker unit is linked to the first or the second linker unit via CuAAC reaction, iEDDA reaction, or SPAAC reaction occurred between any of the followings: the first or the second coupling arm and the third coupling arm, the first or the second center core and the third coupling arm, the first or the second center coupling arm and the third center core, or the first or the second center core and the third center core.

[51] Regarding the third linking arm of the third linker unit, it may have an NHS, a maleimide, an azide, an alkyne, a cyclooctene, a cyclooctyne, or a tetrazine group at the free terminus thereof. Accordingly, the third linking arm may be directly linked to a second effector element or targeting element either via forming an amide bond therebetween, or via the thiol-maleimide, CuAAC, iEDDA, or SPAAC reaction.. Alternatively, the third linking arm is linked to the third connecting arm either via the CuAAC, iEDDA, or SPAAC reaction; and the second effector element or targeting element is then linked to the third connecting arm via forming an amide bond therebetween, or via the thiol-maleimide reaction.

[52] As would be appreciated, the targeting/effector element (such as a drug) having an NHS group can be directly linked to the K residue of the first, second, and/or third center core via forming an amide linkage between the NHS group and the K residue without the presence of the linking arm (i.e., the first, second, or third linking arm).

[53] According to various embodiments of the present disclosure, the first, second, and optionally, the third center core may be the same or different.

[54] **< IV > Uses of Molecular Constructs with Targeting and Effector Moieties**

[55] The molecular construct according to the third aspect of the present disclosure may find its utility in clinical medicine for the treatment of various diseases. Hence, the fourth aspect of the present disclosure is directed to a method for treating these diseases. According to various embodiments of the present disclosure, the method for treating a

particular disease includes the step of administering to the subject in need thereof a molecular construct according to the third aspect of the present disclosure and embodiments thereof in a therapeutically effective amount. As could be appreciated, said molecular construct may be administered in a pharmaceutical formulation, which comprises a pharmaceutically-acceptable excipient suitable for the intended or desired administration route, in addition to the present molecular construct.

[56] Various illustrative combinations of the first and second elements of the present molecular construct for treating some particular diseases are disclosed below for facilitating the understanding of some embodiments of the present disclosure.

[57] According to some embodiments of the present disclosure, the present molecular construct is useful in treating a CNS disease, for example, multiple sclerosis and Alzheimer's disease. For treating multiple sclerosis, the first element is an scFv specific for transferrin receptor, and the second element is IFN-β, fingolimod, fingolimod phosphate, or an scFv specific for integrin α4. As to the treatment of Alzheimer's disease, an scFv specific for transferrin receptor and an scFv specific for β-amyloid are respectively employed as the first and the second elements.

[58] According to other embodiments of the present disclosure, the present molecular construct is useful in treating an infectious disease. In one embodiment, the infectious disease is caused by a virus, and the first element is an scFv specific for the viral protein, while the second element is an scFv specific for CD16b or CD32. Non-limiting examples of viral proteins include, F protein of RSV, gp120 protein of HIV-1, HA protein of influenza A virus, and glycoprotein of cytomegalovirus. In another embodiment, the infectious disease is caused by a bacterium, and the first element is an scFv specific for the bacterial protein, while the second element is an scFv specific for CD16b or CD32. The examples of bacterial protein include, but are not limited to, endotoxin of Gram(-) bacteria, surface antigen of *Clostridium difficile*, lipoteichoic acid of *Staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, or Shiga-like toxin type I or II of *Escherichia coli*.

[59] **< V > Molecular Construct for Treating Central Nervous System Diseases and Uses Thereof**

[60] In the fifth aspect, the present disclosure is directed to a fragment crystallizable (Fc)-based molecular construct that has at least one targeting element and at least one effector element linked, directly or indirectly, to a CH2-CH3 domain of an immunoglobulin. Targeting and effector elements of the present Fc-based molecular constructs are specifically selected such that these Fc-based molecular constructs are suitable for use in the treatment of central nervous system (CNS) diseases, or for use in the manufacture of a medicament for treating CNS diseases. As could be appreciated, methods for treating CNS diseases using such Fc-based molecular constructs also fall within the aspect of the present disclosure.

[61] According to certain embodiments of the present disclosure, the Fc-based molecular construct comprises a pair of CH2-CH3 segments of an IgG.Fc, a pair of effector elements, and a pair of targeting elements. The pair of effector element is interferon β1a (INF-β1a) or INF-β1b, or an antibody fragment specific for integrin α4 or β-amyloid, while the pair targeting elements is an antibody fragment specific for human transferrin receptor or human insulin receptor.

[62] In the case where the pair of effector elements is linked to the N-termini of the pair of CH2-CH3 segments, the pair of targeting elements is linked to the C-termini of the pair of CH2-CH3 segments, and vice versa. Alternatively, when the pair of effectors elements and the pair of targeting elements is both in the form of single-chain variable fragments (scFvs), then the pair of targeting elements is linked to the N-termini of the pair of effector elements in a tandem or diabody configuration, thereby forming a pair of bispecific scFvs that are linked to the N-termini of the pair of CH2-CH3 segments.

[63] In certain embodiments, the pair of CH2-CH3 segments is derived from human IgG heavy chain γ4 or human IgG heavy chain γ1.

[64] In some examples, the pair of effector elements or the pair of the targeting elements takes a Fab configuration (i.e., consisting of the V_H-CH1 domain and the V_L-C_K domain); this Fab fragment is linked to the N-termini of the first and second heavy chains, so that the Fc-based molecular construct adopts an IgG configuration. In these cases, the pair of elements that is not in the Fab configuration is linked to the C-termini of the pair of CH2-CH3 segments.

[65] According to certain optional embodiments, the effector element is INF-β1a, INF-β1b, or an scFv specific for integrin α4, while the targeting element is an scFv specific for human transferrin receptor. In particular, this molecular construct is suitable for treating multiple sclerosis.

[66] According to other optional embodiments, the effector element is an scFv specific for β-amyloid, while the targeting element is an scFv specific for human transferrin receptor. In particular, this molecular construct is suitable for treating Alzheimer's disease.

[67] Methods for treating CNS diseases in a subject in need thereof comprise the step of administering to the subject an effective amount of the molecular construct of this aspect. CNS diseases treatable by this method include multiple sclerosis and Alzheimer's disease.

[68] **< VI > Molecular Construct for Treating Infectious Diseases**

[69] In the sixth aspect, the present disclosure is directed to a fragment crystallizable (Fc)-based molecular construct that has at least one targeting element and at least one effector element linked, directly or indirectly, to a CH2-CH3 domain of an immunoglobulin. Targeting and effector elements of the present Fc-based molecular constructs are specifically selected such that these Fc-based molecular constructs are suitable for use in the treatment of diseases/conditions associated with viral or bacterial infection, or for use in the manufacture of a medicament for treating such diseases/conditions. As could be appreciated, methods for treating diseases/conditions associated with viral or bacterial infection using such Fc-based molecular constructs also fall within the aspect of the present disclosure.

[70] According to certain embodiments of the present disclosure, the Fc-based molecular construct comprises a pair of CH2-CH3 segments of an IgG.Fc, a pair of effector elements, and a pair of targeting elements. The pair of effector element is an antibody fragment specific for CD32 or CD16b, while the pair targeting elements is an antibody fragment specific for a viral protein or a bacterial protein.

[71] In the case where the pair of effector elements is linked to the N-termini of the pair of CH2-CH3 segments, the pair of targeting elements is linked to the C-termini of the pair of CH2-CH3 segments, and vice versa. Alternatively, when the pair of effectors elements

and the pair of targeting elements is both in the form of single-chain variable fragments (scFvs), then the pair of targeting elements is linked to the N-termini of the pair of effector elements in a tandem or diabody configuration, thereby forming a pair of bispecific scFvs that are linked to the N-termini of the pair of CH2-CH3 segments.

[72] In certain embodiments, the pair of CH2-CH3 segments is derived from human IgG heavy chain γ4 or human IgG heavy chain γ1.

[73] In some examples, the pair of effector elements or the pair of the targeting elements takes a Fab configuration (i.e., consisting of the V_H-CH1 domain and the V_L-C_K domain); this Fab fragment is linked to the N-termini of the first and second heavy chains, so that the Fc-based molecular construct adopts an IgG configuration. In these cases, the pair of elements that is not in the Fab configuration is linked to the C-termini of the pair of CH2-CH3 segments.

[74] According to certain optional embodiments, the effector element is an scFv specific for CD32 or CD16b, while the targeting element is an scFv specific for a viral protein. For example, the viral protein can be F protein of respiratory syncytia virus (RSV), gp120 protein of human immunodeficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, or glycoprotein of cytomegalovirus. In particular, this molecular construct is suitable for treating viral infections.

[75] According to other optional embodiments, the effector element is an scFv specific for CD32 or CD16b, while the targeting element is an scFv specific for a bacterial protein. Examples of the bacterial protein include, but are not limited to, the endotoxin of Gram(-) bacteria, the surface antigen of *Clostridium difficile*, the lipoteichoic acid of *Staphylococcus aureus*, the anthrax toxin of *Bacillus anthracis*, or the Shiga-like toxin type I or II of *Escherichia coli*. In particular, such molecular construct is suitable for treating bacterial infections.

[76] Methods for treating diseases/conditions associated with infections (e.g., viral or bacterial infections) in a subject in need thereof comprise the step of administering to the subject an effective amount of the molecular construct of this aspect.

BRIEF DESCRIPTION OF THE DRAWINGS

[77] The present description will be better understood from the following detailed description read in light of the accompanying drawings briefly discussed below.

[78] Figure 1A to Figure 1N are schematic diagrams illustrating linker units according to certain embodiments of the present disclosure.

[79] Figure 2 is a schematic diagram illustrating a linker unit having a compound core.

[80] Figure 3A to Figure 3D are schematic diagrams illustrating T-E molecular constructs according to some embodiments of the present disclosure.

[81] Figure 4 is a schematic diagram that illustrates libraries for constructing molecular constructs according to some embodiments of the present disclosure.

[82] Figure 5A and Figure 5B are schematic diagrams that illustrate molecular constructs according to some embodiments of the present disclosure.

[83] Figure 6 is a schematic diagram that illustrates a molecular construct according to some embodiments of the present disclosure.

[84] Figure 7A and Figure 7B are schematic diagrams illustrating molecular constructs according to various embodiments of the present disclosure.

[85] Figures 8A to 8C are schematic diagrams illustrating Fc-based molecular constructs according to various embodiments of the present disclosure.

[86] Figure 9 is a schematic diagram illustrating an Fc-based molecular construct according to various embodiments of the present disclosure.

[87] Figures 10A and 10B are schematic diagrams illustrating an Fc-based molecular constructs according to various embodiments of the present disclosure.

[88] Figure 11 shows the mass spectrometry MALDI-TOF result of a peptide core-based linker-unit carrying one linking arm with tetrazine group and three PEG linking arms with maleimide groups.

[89] Figure 12 shows the mass spectrometry MALDI-TOF result of NHS-PEG_5-conjugated fingolimod.

[90] Figure 13 shows the mass spectrometry MALDI-TOF result of a drug bundle composing of a linker unit with a free TCO functional group and a set of 5 fingolimod molecules.

[91] Figure 14 shows the mass spectrometry MALDI-TOF result of a drug bundle composing of a linker unit with a free TCO functional group and a set of 10 fingolimod molecules.

[92] Figure 15 shows the mass spectrometry MALDI-TOF result of a drug bundle composing of a linker unit with a free TCO functional group and a set of five fingolimod phosphate molecules.

[93] Figure 16 shows the SDS-PAGE analysis result of the purified ectodomain of human CD32a.

[94] Figure 17 shows the SDS-PAGE analysis result of the purified ectodomain of human TfR1.

[95] Figure 18A shows the SDS-PAGE analysis result of the purified scFv specific for Protein F of RSV; Figure 18B shows the ELISA analysis result of the purified scFv specific for Protein F of RSV; Figure 18C shows the SDS-PAGE analysis result of the purified scFv specific for endotoxin; Figure 18D shows the ELISA analysis of the purified scFv specific for endotoxin; Figure 18E shows the SDS-PAGE analysis result of the purified scFv specific for the ectodomain of CD32a; and Figure 18F shows the ELISA analysis of the purified scFv specific for the ectodomain of CD32a.

[96] Figure 19A shows the SDS-PAGE analysis result of the purified scFv specific for the ectodomain of rat TfR1; Figure 19B shows the ELISA analysis result of the purified scFv specific for the ectodomain of rat TfR1; Figure 19C shows the SDS-PAGE analysis result of the purified scFv specific for β-amyloid; and Figure 19D shows the ELISA analysis of the purified scFv specific for β-amyloid.

[97] Figure 20A shows the data of the titers of the phages bearing scFvs specific for ectodomain of human CD32a; and Figure 20B shows the single colony ELISA analysis result of phage-displayed scFvs specific for the ectodomain of human CD32a.

[98] Figure 21A shows the data of the titers of the phages bearing scFvs specific for ectodomain of human TfR1; and Figure 21B shows the single colony ELISA analysis of phage-displayed scFvs specific for the ectodomain of human TfR1.

[99] Figure 22A and Figure 22B respectively show the results of ELISA analysis and mass spectrometric analysis of TCO-conjugated scFv specific for CD32a.

[100] Figure 23A and Figure 23B respectively show the results of ELISA analysis and mass spectrometric analysis of tetrazine-conjugated scFv specific for TfR1.

[101] Figure 24A shows the FPLC elution profile of size-exclusion column S75 on the synthesized targeting linker unit composed of a linker unit with a free tetrazine functional group and a set of three scFvs specific for endotoxin as targeting elements; Figure 24B and Figure 24C respectively show the results of the SDS-PAGE analysis result and the mass spectrometric analysis result of the synthesized targeting linker unit of Figure 24A.

[102] Figure 25 shows the mass spectrometric analysis result of the synthesized targeting linker unit that was composed of a linker unit with a free tetrazine functional group and a set of three scFv specific for Protein F of RSV as targeting elements.

[103] Figure 26A shows the FPLC elution profile of cation ion exchange column on a synthesized targeting linker unit composed of a linker unit with a free TCO functional group and a set of three scFvs specific for β-amyloid as targeting elements; Figure 26B and Figure 26C respectively show the results of the SDS-PAGE analysis result and the ELISA result of the synthesized targeting linker unit of Figure 26A.

[104] Figure 27A shows the mass spectrometric analysis result of a single linker unit molecular construct with three scFvs specific for endotoxin as targeting elements and one scFv specific for ectodomain of CD32a as an effector element; Figure 27B shows the mass spectrometric analysis result of a single linker unit molecular construct with three scFvs specific for endotoxin as targeting elements and one scFv specific for ectodomain of CD32a

as an effector element; and Figure 27C shows the mass spectrometric analysis result of a single linker unit molecular construct with one scFv specific for ectodomain of TfR1 as a targeting element and three scFvs specific for β-amyloid as effector elements.

[105] Figure 28A and Figure 28B respectively show the SDS-PAGE analysis result and the mass spectrometric analysis result of the molecular construct with one scFv specific for ectodomain of TfR1 and one drug bundle bearing five fingolimod molecules.

[106] Figure 29A shows the staining analysis results of the $S1P_1$ receptor-expressing human B cells; and Figure 29B shows the transwell migration assay result of fingolimod upon the conjugation to peptide core through linking arms.

[107] Figure 30A shows the SDS-PAGE analysis result of purified recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32) fusion protein; Figure 30B and Figure 30C provide the results of ELISA analyses that respectively illustrate the binding activities of the purified recombinant fusion protein of Figure 30A to Protein F of RSV (Figure 30B) and to ectodomain of CD32a (Figure 30C).

[108] Figure 31A shows the SDS-PAGE analysis result of the purified recombinant 2-chain (scFv α endotoxin)-hIgG1.Fc-(scFv α CD32) fusion protein; and Figure 31B and Figure 31C provide the results of ELISA analyses that respectively illustrate the binding affinity of the purified recombinant fusion protein of Figure 31A to endotoxin (Figure 31B) and to ectodomain of CD32a (Figure 31C).

[109] Figure 32A and Figure 32B respectively show the SDS-PAGE analysis result and the ELISA results of the purified recombinant 2-chain (Interferon-β-1a)-hIgG4.Fc-(scFv α TfR1) fusion protein.

[110] Figure 33A and Figure 33B respectively show the SDS-PAGE analysis result and staining result of the purified recombinant 2-chain (scFv α integrin α4)-hIgG4.Fc-(scFv α TfR1) fusion protein.

[111] Figure 34 shows the ELISA analysis result of the effect of the purified recombinant 2-chain (scFv α endotoxin)-hIgG1.Fc-(scFv α CD32a) fusion protein on inhibiting TNF-α secretion.

[112] In accordance with common practice, the various described features/elements are not drawn to scale but instead are drawn to best illustrate specific features/elements relevant to the present invention. Also, like reference numerals and designations in the various drawings are used to indicate like elements/parts, where possible.

DESCRIPTION

[113] The detailed description provided below in connection with the appended drawings is intended as a description of the present examples and is not intended to represent the only forms in which the present example may be constructed or utilized. The description sets forth the functions of the example and the sequence of steps for constructing and operating the example. However, the same or equivalent functions and sequences may be accomplished by different examples.

[114] For convenience, certain terms employed in the specification, examples and appended claims are collected here. Unless otherwise defined herein, scientific and technical terminologies employed in the present disclosure shall have the meanings that are commonly understood and used by one of ordinary skill in the art.

[115] Unless otherwise required by context, it will be understood that singular terms shall include plural forms of the same and plural terms shall include the singular. Specifically, as used herein and in the claims, the singular forms "a" and "an" include the plural reference unless the context clearly indicated otherwise. Also, as used herein and in the claims, the terms "at least one" and "one or more" have the same meaning and include one, two, three, or more. Furthermore, the phrases "at least one of A, B, and C", "at least one of A, B, or C" and "at least one of A, B and/or C," as use throughout this specification and the appended claims, are intended to cover A alone, B alone, C alone, A and B together, Band C together, A and C together, as well as A, B, and C together.

[116] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in the respective testing measurements. Also, as used herein, the term "about" generally means

within 10%, 5%, 1%, or 0.5% of a given value or range. Alternatively, the term "about" means within an acceptable standard error of the mean when considered by one of ordinary skill in the art. Other than in the operating/working examples, or unless otherwise expressly specified, all of the numerical ranges, amounts, values and percentages such as those for quantities of materials, durations of times, temperatures, operating conditions, ratios of amounts, and the likes thereof disclosed herein should be understood as modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the present disclosure and attached claims are approximations that can vary as desired. At the very least, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Ranges can be expressed herein as from one endpoint to another endpoint or between two endpoints. All ranges disclosed herein are inclusive of the endpoints, unless specified otherwise.

[117] This present disclosure pertains generally to molecular constructs, in which each molecular construct comprises a targeting element (T) and an effector element (E), and these molecular constructs are sometimes referred to as "T-E molecules", "T-E pharmaceuticals" or "T-E drugs" in this document.

[118] As used herein, the term "targeting element" refers to the portion of a molecular construct that directly or indirectly binds to a target of interest (e.g., a receptor on a cell surface or a protein in a tissue) thereby facilitates the transportation of the present molecular construct into the interested target. In some example, the targeting element may direct the molecular construct to the proximity of the target cell. In other cases, the targeting element specifically binds to a molecule present on the target cell surface or to a second molecule that specifically binds a molecule present on the cell surface. In some cases, the targeting element may be internalized along with the present molecular construct once it is bound to the interested target, hence is relocated into the cytosol of the target cell. A targeting element may be an antibody or a ligand for a cell surface receptor, or it may be a molecule that binds such antibody or ligand, thereby indirectly targeting the present molecular construct to the target site (e.g., the surface of the cell of choice). The localization of the effector (therapeutic agent) in the diseased site will be enhanced or favored with the present molecular constructs as compared to the therapeutic without a

targeting function. The localization is a matter of degree or relative proportion; it is not meant for absolute or total localization of the effector to the diseased site.

[119] According to the present invention, the term "effector element" refers to the portion of a molecular construct that elicits a biological activity (e.g., inducing immune responses, exerting cytotoxic effects and the like) or other functional activity (e.g., recruiting other hapten tagged therapeutic molecules), once the molecular construct is directed to its target site. The "effect" can be therapeutic or diagnostic. The effector elements encompass those that bind to cells and/or extracellular immunoregulatory factors. The effector element comprises agents such as proteins, nucleic acids, lipids, carbohydrates, glycopeptides, drug moieties (both small molecule drug and biologies), compounds, elements, and isotopes, and fragments thereof.

[120] Although the terms, first, second, third, etc., may be used herein to describe various elements, components, regions, and/or sections, these elements (as well as components, regions, and/or sections) are not to be limited by these terms. Also, the use of such ordinal numbers does not imply a sequence or order unless clearly indicated by the context. Rather, these terms are simply used to distinguish one element from another. Thus, a first element, discussed below, could be termed a second element without departing from the teachings of the exemplary embodiments.

[121] Here, the terms "link," "couple," and "conjugates" are used interchangeably to refer to any means of connecting two components either via direct linkage or via indirect linkage between two components.

[122] The term "polypeptide" as used herein refers to a polymer having at least two amino acid residues. Typically, the polypeptide comprises amino acid residues ranging in length from 2 to about 200 residues; preferably, 2 to 50 residues. Where an amino acid sequence is provided herein, L-, D-, or beta amino acid versions of the sequence are also contemplated. Polypeptides also include amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. In addition, the term applies to amino acids joined by a peptide linkage or by other, "modified linkages," e.g.,

where the peptide bond is replaced by an α-ester, a β-ester, a thioamide, phosphoramide, carbomate, hydroxylate, and the like.

[123] In certain embodiments, conservative substitutions of the amino acids comprising any of the sequences described herein are contemplated. In various embodiments, one, two, three, four, or five different residues are substituted. The term "conservative substitution" is used to reflect amino acid substitutions that do not substantially alter the activity (e.g., biological or functional activity and/or specificity) of the molecule. Typically, conservative amino acid substitutions involve substitution one amino acid for another amino acid with similar chemical properties (e.g., charge or hydrophobicity). Certain conservative substitutions include "analog substitutions" where a standard amino acid is replaced by a non-standard (e.g., rare, synthetic, etc.) amino acid differing minimally from the parental residue. Amino acid analogs are considered to be derived synthetically from the standard amino acids without sufficient change to the structure of the parent, are isomers, or are metabolite precursors.

[124] In certain embodiments, polypeptides comprising at least 80%, preferably at least 85% or 90%, and more preferably at least 95% or 98% sequence identity with any of the sequences described herein are also contemplated.

[125] "Percentage (%) amino acid sequence identity" with respect to the polypeptide sequences identified herein is defined as the percentage of polypeptide residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percentage sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, sequence comparison between two polypeptide sequences was carried out by computer program Blastp (protein-protein BLAST) provided online by Nation Center

for Biotechnology Information (NCBI). The percentage amino acid sequence identity of a given polypeptide sequence A to a given polypeptide sequence B (which can alternatively be phrased as a given polypeptide sequence A that has a certain % amino acid sequence identity to a given polypeptide sequence B) is calculated by the formula as follows:

$$\frac{X}{Y} \times 100\ \%$$

where X is the number of amino acid residues scored as identical matches by the sequence alignment program BLAST in that program's alignment of A and B, and where Y is the total number of amino acid residues in A or B, whichever is shorter.

[126] The term "PEGylated amino acid" as used herein refers to a polyethylene glycol (PEG) chain with one amino group and one carboxyl group. Generally, the PEGylated amino acid has the formula of $NH_2(CH_2CH_2O)_n$-COOH. In the present disclosure, the value of n ranges from 1 to 20; preferably, ranging from 2 to 12.

[127] As used herein, the term "terminus" with respect to a polypeptide refers to an amino acid residue at the N- or C- end of the polypeptide. With regard to a polymer, the term "terminus" refers to a constitutional unit of the polymer (e.g., the polyethylene glycol of the present disclosure) that is positioned at the end of the polymeric backbone. In the present specification and claims, the term "free terminus" is used to mean the terminal amino acid residue or constitutional unit is not chemically bound to any other molecular.

[128] The term "antigen" or "Ag" as used herein is defined as a molecule that elicits an immune response. This immune response may involve a secretary, humoral and/or cellular antigen-specific response. In the present disclosure, the term "antigen" can be any of a protein, a polypeptide (including mutants or biologically active fragments thereof), a polysaccharide, a glycoprotein, a glycolipid, a nucleic acid, or a combination thereof.

[129] In the present specification and claims, the term "antibody" is used in the broadest sense and covers fully assembled antibodies, antibody fragments that bind with antigens, such as antigen-binding fragment (Fab/Fab'), F(ab')2 fragment (having two antigen-binding Fab portions linked together by disulfide bonds), variable fragment (Fv), single chain variable fragment (scFv), bi-specific single-chain variable fragment (bi-scFv), nanobodies, unibodies and diabodies. "Antibody fragments" comprise a portion of an intact antibody,

preferably the antigen-binding region or variable region of the intact antibody. Typically, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The well-known immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, with each pair having one "light" chain (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains, respectively. According to embodiments of the present disclosure, the antibody fragment can be produced by modifying the nature antibody or by de nova synthesis using recombinant DNA methodologies. In certain embodiments of the present disclosure, the antibody and/or antibody fragment can be bispecific, and can be in various configurations. For example, bispecific antibodies may comprise two different antigen binding sites (variable regions). In various embodiments, bispecific antibodies can be produced by hybridoma technique or recombinant DNA technique. In certain embodiments, bispecific antibodies have binding specificities for at least two different epitopes.

[130] The term "specifically binds" as used herein, refers to the ability of an antibody or an antigen-binding fragment thereof, to bind to an antigen with a dissociation constant (Kd) of no more than about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, and/or to bind to an antigen with an affinity that is at least two-folds greater than its affinity to a nonspecific antigen.

[131] The term "treatment" as used herein includes preventative (e.g., prophylactic), curative or palliative treatment; and "treating" as used herein also includes preventative (e.g., prophylactic), curative or palliative treatment. In particular, the term "treating" as used herein refers to the application or administration of the present molecular construct or a pharmaceutical composition comprising the same to a subject, who has a medical

condition a symptom associated with the medical condition, a disease or disorder secondary to the medical condition, or a predisposition toward the medical condition, with the purpose to partially or completely alleviate, ameliorate, relieve, delay onset of, inhibit progression of, reduce severity of, and/or reduce incidence of one or more symptoms or features of said particular disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition, and/or to a subject who exhibits only early signs of a disease, disorder and/or condition, for the purpose of decreasing the risk of developing pathology associated with the disease, disorder and/or condition.

[132] The term "effective amount" as used herein refers to the quantity of the present molecular construct that is sufficient to yield a desired therapeutic response. An effective amount of an agent is not required to cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered or prevented, or the disease or condition symptoms are ameliorated. The effective amount may be divided into one, two, or more doses in a suitable form to be administered at one, two or more times throughout a designated time period. The specific effective or sufficient amount will vary with such factors as particular condition being treated, the physical condition of the patient (e.g., the patient's body mass, age, or gender), the type of subject being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives. Effective amount may be expressed, for example, as the total mass of active component (e.g., in grams, milligrams or micrograms) or a ratio of mass of active component to body mass, e.g., as milligrams per kilogram (mg/kg).

[133] The terms "application" and "administration" are used interchangeably herein to mean the application of a molecular construct or a pharmaceutical composition of the present invention to a subject in need of a treatment thereof.

[134] The terms "subject" and "patient" are used interchangeably herein and are intended to mean an animal including the human species that is treatable by the molecular construct, pharmaceutical composition, and/or method of the present invention. The term "subject" or "patient" intended to refer to both the male and female gender unless on

gender is specifically indicated. Accordingly, the term "subject" or "patient" comprises any mammal, which may benefit from the treatment method of the present disclosure. Examples of a "subject" or "patient" include, but are not limited to, a human, rat, mouse, guinea pig, monkey, pig, goat, cow, horse, dog, cat, bird and fowl. In an exemplary embodiment, the patient is a human. The term "mammal" refers to all members of the class Mammalia, including humans, primates, domestic and farm animals, such as rabbit, pig, sheep, and cattle; as well as zoo, sports or pet animals; and rodents, such as mouse and rat. The term "non-human mammal" refers to all members of the class Mammalis except human.

[135] The present disclosure is based, at least on the construction of the T-E pharmaceuticals that can be delivered to target cells, target tissues or organs at increased proportions relative to the blood circulation, lymphoid system, and other cells, tissues or organs. When this is achieved, the therapeutic effect of the pharmaceuticals is increased, while the scope and severity of the side effects and toxicity is decreased. It is also possible that a therapeutic effector is administered at a lower dosage in the form of a T-E molecule, than in a form without a targeting component. Therefore, the therapeutic effector can be administered at lower dosages without losing potency, while lowering side effects and toxicity.

[136] ***Diseases that can benefit from better drug targeting***

[137] Drugs used for many diseases can be improved for better efficacy and safety, if they can be targeted to the disease sites, i.e., if they can be localized or partitioned to the disease sites more favorably than the normal tissues or organs. Certain antibody drugs, which target infectious microorganisms or their toxic products, can be improved, if they are empowered with the ability to recruit immunocytes, which phagocytose and clear the antibody-bound particles. Following are primary examples of diseases, in which drugs can be improved if they can be preferentially distributed to the disease sites or cells or if they can recruit phagocytic immunocytes.

[138] ***I Central Nervous System Diseases***

[139] For treating diseases of the central nervous system (CNS), the therapeutic agents are often required to pass through the blood-brain barrier (BBB) to get into the CNS. Some therapeutic agents do not get into the CNS; they regulate certain activities, such as immune activities, in the peripheral, which then modulates the diseased conditions in the CNS. The BBB is formed by the endothelial cells lining the capillaries of blood vessels in the CNS. Unlike the capillaries in the peripheral tissues and organs, the capillary endothelial cells in the BBB are connected by tight junctions formed by occludin, claudins, and junctional adhesion molecules.

[140] At least six antibodies, namely, aducanumab, bapinerumab, crenezumab, gantenerumab, ponezumab, and solanezumab, specific for β-amyloid, which is responsible for causing Alzheimer's disease, have been developed and placed in clinical development. These antibodies generally fall short of satisfactory therapeutic efficacy in improving Alzheimer's disease. A general belief is that if those antibodies are to achieve therapeutic efficacy, a significant portion must get across the BBB to enter the injured sites in the CNS. However, only very minute portions of those antibodies get across the BBB.

[141] Interferon-β-1a (IFN-β-1a) and interferon-β-1b (IFN-β-1b) have been used for the treatment of multiple sclerosis (MS). The pharmaceuticals, IFN-β-1a produced by mammalian cells and IFN-β-1b produced in *E. coli*, are one-chain protein of 166 amino acid residues containing one disulfide bond. It has been claimed that those therapeutic agents reduce relapse of MS in 18-38% of treated patients. The mechanisms of action of IFN-β-1a and IFN-β-1b are very complex and not completely understood, involving the increased generation of anti-inflammatory immune cells and factors and the down-regulation of pro-inflammatory cells and factors. IFN-β treatment in MS patients also reduces the trafficking of pro-inflammatory T cells across the BBB. It is yet unanswered whether IFN-β-1a and IFN-β-1b mediate their pharmacologic effects in part by getting into the injured sites in the CNS.

[142] When an antibody or protein therapeutic is administered in the body's peripheral, only a very minute amount (about 0.1%) reaches to the CNS, because only a very minute portion of the protein therapeutic gets across the BBB. However, it has also been found that in many diseases of the CNS, including Alzheimer's disease and multiple sclerosis, the

inflammation at the diseased sites renders the BBB to breakdown, leading to increased permeability. Therefore, we rationalize that if a larger proportion of an administered antibody specific for β-amyloid or IFN-β-1a and IFN-β-1b is channeled to the BBB, a higher percentage of the therapeutic agents can pass through the BBB and better therapeutic effects can be achieved.

[143] Furthermore, some therapeutic agents have been developed to inhibit the entry of inflammatory immunocytes to across the BBB. A notable example is natalizumab specific for the cell adhesion molecule integrin α4. The antibody functions by inhibiting inflammatory immune cells to attach to and pass through the epithelial layer lining the BBB. While natalizumab has been shown to be therapeutic efficacious, it has serious immunosuppressive side effect. In particular, it causes progressive multifocal leukoencephalopathy, an opportunistic infection caused by John Cunningham virus (JC virus). We therefor rationalize that if a larger proportion of an antibody specific for integrin α4 is recruited to the BBB, a smaller dose will be required, better therapeutic effects can be achieved, and fewer side effects will occur.

[144] The endothelial cells in the capillaries forming the BBB express transferrin receptors and insulin receptors, which mediate the transcytosis of transferrin and insulin molecules, respectively, to the cerebral parenchyma. For using the transferrin receptor as a ferry, only a small proportion gets through while the reaming bulk are trapped or degraded. Because the endothelial cells lining the capillaries in other parts of the vasculature do not express transferrin receptors, the transferrin receptors on the endothelial cells in the BBB can serve as site-specific antigen for recruiting administered therapeutics. Once the therapeutic is concentrated in the BBB, an increased proportion of it will pass through the capillaries.

[145] We also rationalize that when the mechanisms for channeling pharmaceuticals to the BBB is established, anti-inflammatory drugs, such as anti-TNF-α, anti-IL 12/IL-23, anti-lL 17, and anti-CD3, should be investigated for their therapeutic effects on many types of diseases of the CNS.

[146] For the antibody therapeutic specific for integrin α4, the transferrin receptor is used as a target site recruiter. For Alzheimer disease, the effector moiety can be a few copies of

scFv specific for β-amyloid; for treating multiple sclerosis, the effector moiety can be a few copies of IFN-β-1a or IFN-β-1b, or a few copies of scFv specific for integrin α4.

[147] Embodiments of the present disclosure disclose several T-E molecules respectively exist in single multi-arm linker-units or joint-linker configurations, each contains scFv specific for transferrin receptor as the targeting element and IFN-β-1a or IFN-β-1b or scFv specific for integrin α-4 as the effector element. Alternative embodiments disclose T-E molecules respectively exist in single linker-units or joint-linker configurations, each contains scFv specific for transferrin receptor as the targeting element and scFv specific for β-amyloid as the effector element.

[148] Fingolimod is an immunosuppressive drug that is derived from a natural product myriocin originally isolated from certain fungi. Fingolimod has been approved for reducing the relapse of relapsing-remitting multiple sclerosis. Fingolimod is phosphorylated in vivo to form fingolimod-phosphate, which resembles naturally occurring sphingosine-1-phosphate (S1P), an extracellular lipid mediator, and can bind to 4 of the 5 S1P receptors. The S1P receptors are expressed on lymphocytes and involved in lymphocyte migration. A generally pharmacologic mechanism of fingolimod is that it inhibits lymphocytes egress from the lymphoid tissues to the circulation and hence to the CNS. Fingolimod can cross BBB to enter CNS and many cell types in the CNS express S1P receptors, which play roles in cell proliferation, morphology, and migration. It is believed that fingolimod can have direct on the CNS. The administration of fingolimod causes common side effects of headache and fatigue, and severe side effects of skin cancer, macular edema, and fatal infections, such as hemorrhaging focal encephalitis.

[149] A fingolimod molecule has an NH2 group and thus provides a functional group to couple with a bi-functional linker with an NHS group. One preferred embodiment of the present invention is to prepare a T-E construct, which contains a targeting element for delivery to the BBB and a drug bundle of fingolimod as an effector element. For a bundle of fingolimod, 5-10 molecules are incorporated to a linker unit, using either a cleavable linker or non-cleavable linker to conjugate fingolimod molecules to the linking arms of a linker unit. Since fingolimod, after uptake in a patient, is modified to fingolimod phosphate to resemble sphingosine1-phosphate and become active, the drug bundle is alternatively

prepared with fingolimod phosphate. A linker unit with fingolimod or fingolimod phosphate bundle is conjugated with 1 or 2 scFv specific for a transferrin receptor I. Upon administration of the molecular construct, a portion of it is carried to the BBB. The fingolimod molecules released from the cleavable linkers pass through the BBB and enter the CNS. Or, a portion of the entire construct enters the CNS. Cleavable linkers can be designed by employing a number of cleaving mechanisms. An installment of S-S bond is often used, since S-S disulfide bond can be cleaved by a reduction reaction at the target tissue site. A peptide bond between amino acids, which is sensitive to proteases, such as matrix metalloproteinases in many tissues and cathepsins in endosomes in target cells, is also commonly used as a cleavable bond in many linker designs.

[150] Some illustrative T-E molecules respectively exist in single linker-units or joint-linker configurations, each contains one or two scFv specific for transferrin receptor as the targeting element and fingolimod as the effector element. In all these molecular constructs, the linkage between the linking arms and the effector elements may be non-cleavable or cleavable bonds. In applying the molecular construct platforms of this invention for the various applications in treating CNS diseases, the targeting moiety can be installed with one or two copies of scFv specific for transferrin receptor or insulin receptor. One or two scFvs specific for transferrin receptor or insulin receptor are used. If the scFv has a relatively high affinity (Kd $< 1 \times 10^{-9}$), 1 scFv is used; if the scFv has modest affinity (Kd $< 5 \times 10^{-8}$ and $> 1 \times 10^{-9}$), 2 scFv are used. It is preferred that no more than 2 copies of scFv specific for transferrin receptor or insulin receptor are used to avoid receptor cross-linking and the endocytosis of the bound drug.

[151] ***II Infectious Disease***

[152] Although large numbers of monoclonal anti bodies have been made against components of a various viruses, bacteria, and fungi, which cause serious infectious in humans and animals, few monoclonal antibodies have been developed into preventive treatments or therapeutic agents to counter infections. These shortcomings can be attributed to a few major factors. One major factor is the infectious microorganisms and their products have different serotypes and variable reactivity toward a particular antibody. Another reason is that the targeted microorganisms undergo mutations and escape the targeting of a particular antibody.

[153] The T-E molecular design of the present invention can also be applied for the prevention and treatment of infectious diseases. The plurality of the linking arms can enhance the avidity and specificity of binding to target infectious microorganisms or their products and elicit immune functions to facilitate the clearance of the microorganisms and their products. We reason that the avidity enhancement and the recruitment of immune clearance function can somehow overcome the serotypic difference and mutational problems. Such improvements should increase the efficacy of the candidate antibodies for the prevention and therapy of infectious diseases. Many antibodies, which have failed to meet expectation in clinical trials, may be configured with the present invention and re-investigated.

[154] A preferred set of embodiment of the present invention is to employ joint-linkers configuration with one linker-unit for targeting and one linker-unit for recruiting effector function. An alternative set of preferred embodiment is to employ single linker-units with multiple linking arms for targeting elements and a coupling arm for an effector element. The targeting elements may be one of the two categories: (1) scFv or sdAb specific for a surface component of a microorganism or its product, e.g., envelope protein gp120 of human immunodeficiency virus type 1 (HIV-1), F protein of respiratory syncytia virus (RSV), a surface antigen of *Clostridium difficile* or *Staphylococcus aureus*, or endotoxin of Gram-negative bacteria or Shiga-like toxin of *Escherichia coli*, or (2) the extracellular portions of cell surface receptors of viruses, such as the H IV-1 gp120-binding CD4 domain.

[155] The effector elements are 1 or 2 scFv or sdAb specific for one Fc receptor of IgG, e.g. FcγRIIA (CD32), FcγRIIIB (CD16b), or FcγRI (CD64). Those receptors are expressed on neutrophils, macrophages, and eosinophils and are the key molecules mediating phagocytosis of antibody-bound microorganisms. FcγRIIA and FcγRIIIB bind to IgG with low affinity (Kd in the range of 10^{-6} to 10^{-7} and FcγRI binds to IgG1 and IgG3 with high affinity (Kd 10^{-9}). It is advantageous to employ scFv or sdAb specific for FcγRIIA or FcγRIIIB, because they can compete favorably with IgG in binding to the receptors.

[156] The antibodies specific for carbohydrate antigens on bacterial surface are usually weak in binding affinity and are expressed in IgM rather than IgG. An IgM molecule has 10 Fv's (antigen-binding sites). However, an IgM molecule, which has a molecular weight of about 1000 kd, cannot cross capillaries and reach to extravascular space. With the configuration of the present invention, a molecular construct carrying 6 scFv or 10 sdAb will have a molecular weight of about 150 kd.

[157] In employing antibody-based therapeutics for clearing viruses, it is important that

the therapeutic does not lead to FcR-mediated enhancement of viral infection. In those cases, the bound viral particles are not phagocytosed and digested. Some viruses, such as Dengue virus can multiply in phagocytes. Thus, if the viral particles gain access to a cell and enter the bound cells without being destroyed, the virus can multiply in the infected cells. Therefore, a set of preferred embodiments of this invention is that the molecular construct contains 2 or more scFv specific for an Fcγ receptor and can bind to multiple Fcγ receptor molecules on phagocyte cell surface, so that the bound viral particles are destined to phagocytosis pathway.

[158] Among the many antibodies specific for viruses, bacteria, or their products, which have been in clinical trials, only antibodies specific for RSV have been approved for clinical uses. Even for antibodies against RSV, they are only approved for prevention, and not for treatment of on-going infection. It is desirable that an anti-RSV antibody can be developed for treating already-infected subjects. The other antibodies are still in clinical development or have failed in clinical trials. With the molecular construct platforms of this invention, all of these antibodies can be employed for improved efficacy. A partial list of those antibodies are:

(1) Palivizumab and felvizumab specific for RSV F protein

(2) Suvizumab specific for HIV-1 gp120

(3) Libivirumab, exbivirumab, tuvirumab specific for hepatitis B surface antigen (HBsAg) of HBV

(4) CR6261 mAb, diridavumab, and firivumab specific for hemagglutinin A of influenza A virus

(5) Regavirumab and sevirumab specific for glycoprotein of cytomegalovirus

(6) Rafivirumab specific for glycoprotein of rabies_virus

(7) Actoxumab and bezlotoxumab specific for surface antigen of *Clostridium difficile*

(8) Obiltoxaximab and raxibacumab specific for *Bacillus anthracis* anthrax

(9) Panobacumab (human IgM monoclonal antibody) specific for *Pseudomonas aeruginosa_serotype* IATS O11

(10) Tefibazumab and tosatoxumab specific for clumping factor A of *Staphylococcus aureus*

(11) Edobacomab specific for endotoxin of Gram-negative bacteria for treating sepsis

(12) Pagibaximab specific for lipoteichoic acid of *staphylococcus aureus* for treating staphylococcal sepsis

(13) Raxibacumab (human monoclonal antibody) specific anthrax toxin

(14) Pritoxaximab, setoxaximab, and urtoxazumab specific for Shiga-like toxin type I or II of *Escherichia coli*.

[159] According to several embodiments of the present disclosure, T-E molecules in joint-linker configurations for treating infectious diseases incorporate scFv specific for the F protein of respiratory syncytia virus (RSV) or gp120 of human immunodeficiency virus type 1 (HIV-1) as targeting/capture elements and scFv specific for FcγRIIA (CD32) or FcγRIIIB (CD16b) as effector/clearance elements. According to embodiments of the present disclosure, some T-E molecules in single linker-units or joint linkers configuration incorporate scFv specific for endotoxin of Gram(-) bacteria or lipoteichoic acid of Staphylococcus aureus as targeting/capture elements and scFv specific for CD32 or CD16b as effector/clearance elements. An accelerated removal of endotoxin during Gram(-) bacterial infection should decrease the amplitude of cytokine release, such as TNF-α, IL-1 etc., (i.e. cytokine storm) in a life-threatening septic condition. In applying the molecular construct platform of this invention for the various applications in treating infectious diseases, the effector moiety can be installed with one or two copies of scFv specific for CD32 or CD16b. One or two scFvs specific for CD32 (CD32a or CD32b) or CD16b are used. If the scFv has a relatively high affinity ($Kd < 1 \times 10^{-9}$), 1 scFv is used; if the scFv has modest affinity ($Kd < 5 \times 10^{-8}$ and $> 1 \times 10^{-9}$), 2 scFv are used. It is preferred that no more than 2 copies of scFv specific for CD32 or CD16b are used to avoid receptor cross-linking and the endocytosis of the bound drug.

[160] **PART I Multi-Arm Linkers for Treating Specific Diseases**

[161] ***I-(i) Peptide Core for Use in Multi-Arm Linker***

[162] The first aspect of the present disclosure pertains to a linker unit that comprises, (1) a center core that comprises 2-15 lysine (K) residues, and (2) 2-15 linking arms respectively linked to the K residues of the center core. The present center core is characterized in having or being linked with an azide group, an alkyne group, a tetrazine group, or a strained alkyne group at its N- or C-terminus.

[163] In the preparation of the present linker unit, a PEG chain having a N-hydroxysuccinimidyl (NHS) group at one terminus and a functional group (e.g., an NHS, a maleimide, an azide, an alkyne, a tetrazine, or a strained alkyne group) at the other terminus is linked to the K residue of the center core by forming an amide bond between the NHS group of the PEG chain and the amine group of the K residue. In the present disclosure, the PEG chain linked to the Kresidue is referred to as a linking arm, which has a functional group at the free-terminus thereof.

[164] According to the embodiments of the present disclosure, the center core is a polypeptide that has 8-120 amino acid residues in length and comprises 2 to 15 lysine (K) residues, in which each K residue and the next K residue are separated by a filler sequence.

[165] According to embodiments of the present disclosure, the filler sequence comprises glycine (G) and serine (S) residues; preferably, the filler sequence consists of 2-15 residues selected from G, S, and a combination thereof. For example, the filler sequence can be,

GS,
GGS,
GSG,
GGGS (SEQ ID NO: 1),
GSGS (SEQ ID NO: 2),
GGSG (SEQ ID NO: 3),
GSGGS (SEQ ID NO: 4),
SGGSG (SEQ ID NO: 5),
GGGGS (SEQ ID NO: 6),
GGSGGS (SEQ ID NO: 7),
GGSGGSG (SEQ ID NO: 8),
SGSGGSGS (SEQ ID NO: 9),
GSGGSGSGS (SEQ ID NO: 10),
SGGSGGSGSG (SEQ ID NO: 11),
GGSGGSGGSGS (SEQ ID NO: 12),
SGGSGGSGSGGS (SEQ ID NO: 13),
GGGGSGGSGGGGS (SEQ ID NO: 14),
GGGSGSGSGSGGGS (SEQ ID NO: 15), or

SGSGGGGGSGGGSGSG (SEQ ID NO: 16).

[166] The filler sequence placed between two lysine residues may be variations of glycine and serine residues in somewhat random sequences and/or lengths. Longer fillers may be used for a polypeptide with fewer lysine residues, and shorter fillers for a polypeptide with more lysine residues. Hydrophilic amino acid residues, such as aspartic acid and histidine, may be inserted into the filler sequences together with glycine and serine. As alternatives for filler sequences made up with glycine and serine residues, filler sequences may also be adopted from flexible, soluble loops in common human serum proteins, such as albumin and immunoglobulins.

[167] According to certain preferred embodiments of the present disclosure, the center core comprises 2-15 units of the sequence of $G_{1-5}SK$. Alternatively, the polypeptide comprises the sequence of $(GSK)_{2-15}$; that is, the polypeptide comprises at least two consecutive units of the sequence of GSK. For example, the present center core may comprises the amino acid sequence of the following,

Ac-CGGSGGSGGSKGSGSK(SEQ ID NO: 17),

Ac-CGGSGGSGGSKGSGSKGSK (SEQ ID NO: 18), or

Ac-CGSKGSKGSKGSKGSKGSKGSKGSKGSKGSK (SEQ ID NO: 19),

in which Ac represents the acetyl group.

[168] According to certain embodiments of the present disclosure, the center core is a polypeptide that comprises the sequence of $(X_{aa}\text{-}K)_n$, in which X_{aa} is a PEGylated amino acid having 2 to 12 repeats of ethylene glycol (EG) unit, and n is an integral from 2 to 15.

[169] As described above, the present center core is characterized in having or being linked with an azide group, an alkyne group, a tetrazine group, or a strained alkyne group at its N- or C-terminus. According to some embodiments of the present disclosure, the present center core comprises, at its N- or C-terminus, an amino acid residue having an azide group or an alkyne group. The amino acid residue having an azide group can be, L-azidohomoalanine (AHA), 4-azido-L-phenylalanine, 4-azido-D-phenylalanine, 3-azido-L-alanine, 3-azido-D-alanine, 4-azido-L-homoalanine, 4-azido-D-homoalanine, 5-azido-L-ornithine, 5-azido-d-ornithine, 6-azido-L-lysine, or 6-azido-D-lysine. For example, the present center core may have the sequence of,

$Ac\text{-}(GSK)_{2\text{-}7}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}A^{AH}$,

$Ac\text{-}A^{AH}\text{-}(SG_{2\text{-}4})_{1\text{-}8}\text{-}(GSK)_{2\text{-}7}$,

$Ac\text{-}A^{AH}\text{-}(SG_{2\text{-}4})_{0\text{-}7}(GSK)_{2\text{-}6}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}C$,

$Ac\text{-}C\text{-}(SG_{2\text{-}4})_{0\text{-}7}\text{-}(GSK)_{2\text{-}6}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}A^{AH}$,

$Ac\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{2\text{-}4}\text{-}Xaa_{2\text{-}12}\text{-}A^{AH}$,

$Ac\text{-}A^{AH}\text{-}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{2\text{-}4}$,

$Ac\text{-}A^{AH\text{-}}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{1\text{-}3}\text{-}Xaa_{2\text{-}12}\text{-}C$, or

$Ac\text{-}C\text{-}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{1\text{-}3}\text{-}Xaa_{2\text{-}12}A^{AH}$,

In which Xaa is a PEGylated amino acid having specified repeats of EG unit, Ac represents the acetyl group, and A^{AH} represents the AHA residue.

[170] Exemplary amino acid having an alkyne group includes, but is not limited to, L-homopropargylglycine (L-HPG), D-homopropargylglycine (D-HPG), or beta-homopropargylglycine (β-HPG). In this case, the present center core may have the sequence of,

$Ac\text{-}(GSK)_{2\text{-}7}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}G^{HP}$,

$Ac\text{-}G^{HP}\text{-}(SG_{2\text{-}4})_{1\text{-}8}\text{-}(GSK)_{2\text{-}7}$,

$Ac\text{-}G^{HP}\text{-}(SG_{2\text{-}4})_{0\text{-}7}\text{-}(GSK)_{2\text{-}6}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}C$,

$Ac\text{-}C\text{-}(SG_{2\text{-}4})_{0\text{-}7}\text{-}(GSK)_{2\text{-}6}\text{-}(G_{2\text{-}4}S)_{1\text{-}8}\text{-}G^{HP}$,

$Ac\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{2\text{-}4}\text{-}Xaa_{2\text{-}12}\text{-}G^{HP}$,

$Ac\text{-}G^{HP}\text{-}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{2\text{-}4}$,

$Ac\text{-}G^{HP}\text{-}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{1\text{-}3}\text{-}Xaa_{2\text{-}1}\text{-}C$, or

$Ac\text{-}C\text{-}Xaa_{2\text{-}12}\text{-}K\text{-}(Xaa_{2\text{-}12}\text{-}K)_{1\text{-}3}\text{-}Xaa_{2\text{-}12}\text{-}G^{HP}$,

In which Xaa is a PEGylated amino acid having specified repeats of EG unit, Ac represents the acetyl group, and G^{HP} represents the HPG residue.

[171] It is noted that many of the amino acids containing an azide or alkyne group in their side chains and PEGylated amino acids are available commercially in t-boc (tert-butyloxycarbonyl)- or Fmoc (9-fluorenylmethyloxycarbonyl)-protected forms, which are readily applicable in solid-phase peptide synthesis.

[172] According to some working examples of the present disclosure, the center core may comprise the sequence of,

$Ac\text{-}G^{HP}$GGSGGSGGSKGSGSK (SEQ ID NO: 21),

Ac-G^{HP}GGSGGSGGSKGSGSKGSK (SEQ ID NO: 22),

Ac-A^{AH}GGSGGSGGSKGSGSKGSK (SEQ ID NO: 23),

Ac-G^{HP}GGSGGSGGSKGSGSKGSGSC (SEQ ID NO: 24),

Ac-C-Xaa_2-K-Xaa_2-K-Xaa_2-K (SEQ ID NO: 25), or

Ac-C-Xaa_6-K-Xaa_6-K-Xaa_6-K-Xaa_6-K-Xaa_6-K (SEQ ID NO: 26),

in which Xaa is a PEGylated amino acid having specified repeats of EG unit, Ac represents the acetyl group, A^{AH} represents the AHA residue, and G^{HP} represents the HPG residue.

[173] Alternatively, the present center core is linked with a coupling arm, which has a functional group (e.g., an azide group, an alkyne group, a tetrazine group, or a strained alkyne group) at the free-terminus thereof (that is, the terminus that is not linked to the center core). In these cases, the present center core comprises a cysteine residue at its N- or C-terminus. To prepare a linker unit linked with a coupling arm, a PEG chain having a maleimide group at one terminus and a functional group at the other terminus is linked to the cysteine residue of the center core via thiol-maleimide reaction occurred between the maleimide group of the PEG chain and the thiol group of the cysteine residue. In the present disclosure, the PEG chain linked to the cysteine residue of the center core is referred to as the coupling arm, which has a functional group at the free-terminus thereof.

[174] Preferably, the coupling arm has a tetrazine group or a strained alkyne group (e.g., a cyclooctene or cyclooctyne group) at the free-terminus thereof. These coupling arms have 2-12 EG units. According to the embodiments of the present disclosure, the tetrazine group is 1,2,3,4-tetrazine, 1,2,3,5-tetrazine, 1,2,4,5-tetrazine, or derivatives thereof. The strained alkyne group may be a cyclooctene or a cyclooctyne group. According to the working examples of the present disclosure, the cyclooctene group is a trans-cyclooctene (TCO) group; example of cyclooctyne group includes, but is not limited to, dibenzocyclooctyne (DBCO), difluorinated cyclooctyne (DIFO), bicyclononyne (BCN), and dibenzocyclooctyne (DICO). According to some embodiments of the present disclosure, the tetrazine group is 6-methyl-tetrazine.

[175] Example of the present center core configured to be linked with the coupling arm includes, but is not limited to,

Ac-$(GSK)_{2-7}$-$(G_{2-4}S)_{1-8}$-C-Xaa_{2-12}-tetrazine,

Ac-$(GSK)_{2-7}$-$(G2\text{-}4S)_{1-8}$-C-Xaa_{2-12}-strained alkyne,

Ac-K-$(Xaa_{2-12}\text{-}K)_{2-4}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-tetrazine,

Ac-K-$(Xaa_{2-12}\text{-}K)_{2-4}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-strained alkyne,

Tetrazine-Xaa_{2-12}-C(Ac)-$(SG_{2-4})_{1-8}$-$(GSK)_{2-7}$,

Strained alkyne-Xaa_{2-12}-C(Ac)-$(SG_{2-4})_{1-8}$-$(GSK)_{2-7}$,

Tetrazine-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{2-4}$, and

Strained alkyne-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{2-4}$.

[176] Alternatively, the center core has an azide or alkyne group at one terminus and a coupling arm with tetrazine or strained alkyne group at the other terminus. Examples are the following:

Ac-A^{AH}-$(SG2\text{-}4)_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-C-Xaa_{2-12}-tetrazine,

Ac-A^{AH}-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-C-Xaa_{2-12}-strained alkyne,

Tetrazine-Xaa_{2-12}-C(Ac)-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-A^{AH},

Strained alkyne-Xaa_{2-12}-C(Ac)-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-A^{AH},

Ac-A^{AH}-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-tetrazine,

Ac-A^{AH}-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-strained alkyne,

Tetrazine-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-$A^{AH}$,

Strained alkyne-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-$A^{AH}$,

Ac-G^{HP}-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-C-Xaa_{2-12}-tetrazine,

Ac-G^{HP}-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-C-Xaa_{2-12}-strained alkyne,

Tetrazine-Xaa_{2-12}-C(Ac)-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-G^{HP},

Strained alkyne-Xaa_{2-12}-C(AC)-$(SG_{2-4})_{0-7}$-$(GSK)_{2-6}$-$(G_{2-4}S)_{1-8}$-G^{HP},

Ac-G^{HP}-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-tetrazine,

Ac-G^{HP}-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-C-$Xaa_{2-12}$-strained alkyne,

Tetrazine-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-GHP, and

Strained alkyne-Xaa_{2-12}-C(Ac)-Xaa_{2-12}-K-$(Xaa_{2-12}\text{-}K)_{1-3}$-$Xaa_{2-12}$-$G^{HP}$.

[177] The polypeptide may also be synthesized using recombinant technology by expressing designed gene segments in bacterial or mammalian host cells. It is preferable to prepare the polypeptide as recombinant proteins if the core has high numbers of lysine residues with considerable lengths. As the length of a polypeptide increases, the number of errors increases, while the purity and/or the yield of the product decrease, if solid-phase

synthesis was adopted. To produce a polypeptide in bacterial or mammalian host cells, a filler sequence ranges from a few amino acid residues to 10-20 residues may be placed between two K residues. Further, since AHA and HPG are not natural amino acids encoded by the genetic codes, the N-terminal or C-terminal residue for those recombinant polypeptides is cysteine. After the recombinant proteins are expressed and purified, the terminal cysteine residue is then reacted with short bifunctional cross-linkers, which have maleimide group at one end, which reacts with SH group of cysteine residue, and alkyne, azide, tetrazine, or strained alkyne at the other end.

[178] The synthesis of a polypeptide using PEGylated amino acids involves fewer steps than that with regular amino acids such as glycine and serine resides. In addition, PEGylated amino acids with varying lengths (i.e., numbers of repeated ethylene glycol units) may be employed, offering flexibility for solubility and spacing between adjacent amino groups of lysine residues. In addition to PEGylated amino acids, the center cores may also be constructed to comprise artificial amino acids, such as D-form amino acids, homo-amino acids, N-methyl amino acids, etc. Preferably, the PEGylated amino acids with varying lengths of polyethylene glycol (PEG) are used to construct the center core, because the PEG moieties contained in the amino acid molecules provide conformational flexibility and adequate spacing between conjugating groups, enhance aqueous solubility, and are generally weakly immunogenic. The synthesis of PEGylated amino acid-containing center core is similar to the procedures for the synthesis of regular polypeptides.

[179] Optionally, for stability purpose, the present center core has an acetyl group to block the amino group at its N-terminus.

[180] As could be appreciated, the number of the linking arms linked to the center core is mainly determined by the number of lysine resides comprised in the center core. Since there are at least two lysine residues comprised in the present center core, the present linker unit may comprise a plurality of linking arms.

[181] Reference is now made to Figure 1A. As illustrated, the linker unit 10A comprises a center core 11a comprising one HPG (G^{HP}) residue and four lysine (K) residues respectively separated by filler sequences (denoted by the dots throughout the drawings).

The filler sequences between the HPG residue and K residue or between any two K residues may comprise the same or different amino acid sequences. In this example, four linking arms 20a-20d are linked to the lysine residues by forming an amide linkage between the NHS group and the amine group of the lysine residue, respectively. As could be appreciated, certain features discussed above regarding the linker unit 10A or any other following linker units are common to other linker units disclosed herein, and hence some or all of these features are also applicable in the following examples, unless it is contradictory to the context of a specific embodiment. However, for the sake of brevity, these common features may not be explicitly repeated below.

[182] Figure 1B provides a linker unit 10B according to another embodiment of the present disclosure. The center core 11b comprises one cysteine (C) residue and six lysine (K) residues respectively separated by the filler sequences. In this example, the linker unit 10B comprises six linking arms 20a-20f that are respectively linked to the lysine residues. According to the embodiments of the present disclosure, the linking arm is a PEG chain having 2-20 repeats of EG units.

[183] Unlike the linker unit 10A of Figure 1A, the linker unit 1B further comprises a coupling arm 60. As discussed above, a PEG chain having a maleimide group at one end and a functional group at the other end is used to form the coupling arm 60. In this way, the coupling arm 60 is linked to the cysteine residue of the center core 11b via thiol-maleimide reaction. In this example, the functional group at the free terminus of the coupling arm 60 is a tetrazine group 72. According to the embodiments of the present disclosure, the coupling arm is a PEG chain having 2-12 repeats of EG units.

[184] When the release of effector elements at the targeted site is required, a cleavable bond can be installed in the linking arm. Such a bond is cleaved by acid/alkaline hydrolysis, reduction/oxidation, or enzymes. One embodiment of a class of cleavable PEG chains that can be used to form the coupling arm is NHS-PEG_{2-20}-S-S-maleimide, where S-S is a disulfide bond that can be slowly reduced, while the NHS group is used for conjugating with the amine group of the center core, thereby linking the PEG chain onto the center core. The maleimide group at the free terminus of the linking arm may be substituted by an azide, alkyne, tetrazine, or strained alkyne group. According to some embodiments of the

present disclosure, the linking arm is a PEG chain, which has 2-20 repeats of EG units with a disulfide linkage at the free terminus thereof (i.e., the terminus that is not linked with the center core). Reference is now made to FIG. 1C, in which each of the five linking arms 21a-21f respectively linked to the K resides of the center core 11b is a PEG chain with a disulfide linkage at the free terminus of the linking arm.

[185] According to the embodiments of the present disclosure, the linking arm linked to the K residue of the center core has a functional group (i.e., a maleimide, an NHS, an azide, an alkyne, a tetrazine, or a strained alkyne group) at its free terminus. Preferably, when the free terminus of the linking arm is an azide, alkyne, or cyclooctyne group, then the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is a tetrazine or cyclooctene group. Alternatively, when the free terminus of the linking arm is a tetrazine group or cyclooctene group, then the amino acid residue at the N- or C-terminus of the center core has an azide or alkyne group, or the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is an azide, the alkyne, or the cyclooctyne group.

[186] Depending on the functional group (i.e., a maleimide, an NHS, an azide, an alkyne, a tetrazine, or a strained alkyne group) present at the free terminus of the linking arm, it is feasible to design a functional element (such as, a targeting element, an effector element, or an element for improving the pharmacokinetic property) with a corresponding functional group, so that the functional element may linked to the free terminus of the linking arm via any of the following chemical reactions,

(1) forming an amide bond there between: in this case, the linking arm has an NHS group at the free terminus, and the functional element has an amine group;

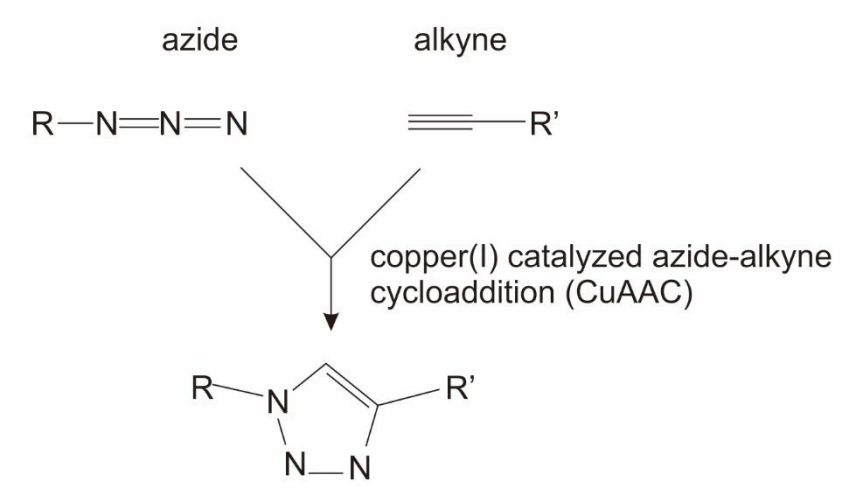
(2) the thiol-maleimide reaction: in this case, the linking arm has a maleimide group at the free terminus, and the functional element has an thiol group;

(3) the Copper(I)-catalyzed alkyne-azide cycloaddition reaction (CuAAC reaction, or the "click" reaction for short): one of the free terminus of the linking arm and the functional element has an azide group, while the other has an alkyne group; the CuAAC reaction is exemplified in Scheme 1;

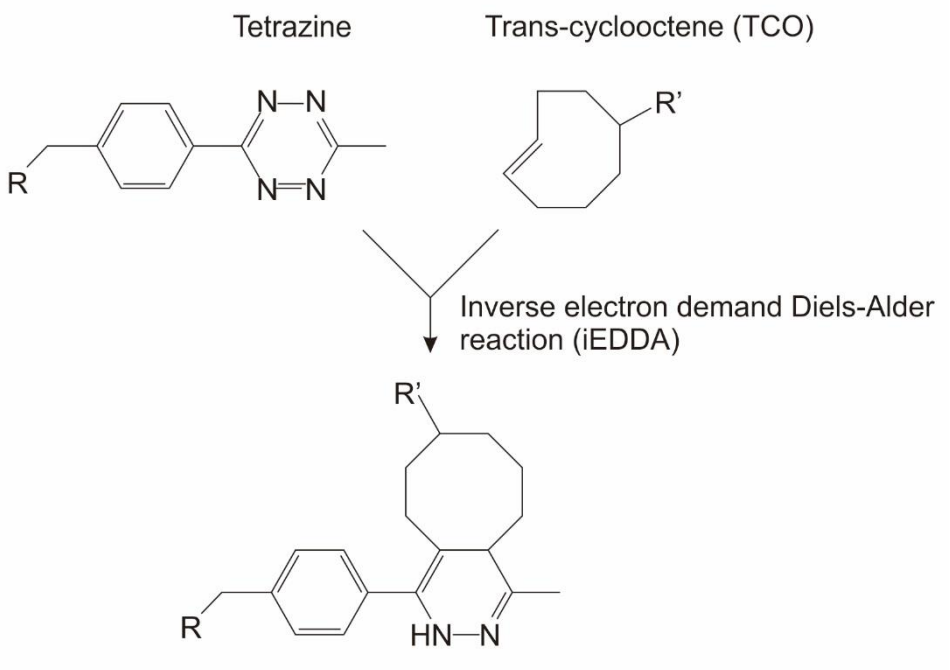
(4) the inverse electron demand Diels-Alder (iEDDA) reaction: one of the free terminus of the linking arm and the functional element has a tetrazine group, while the other has a cyclooctene group; the iEDDA reaction is exemplified in Scheme 2; or

(5) the strained-promoted azide-alkyne click chemistry (SPAAC) reaction: one of the free terminus of the linking arm and the functional element has an azide group, while the other has an cyclooctyne group; the SPAAC reaction is exemplified in Scheme 3.

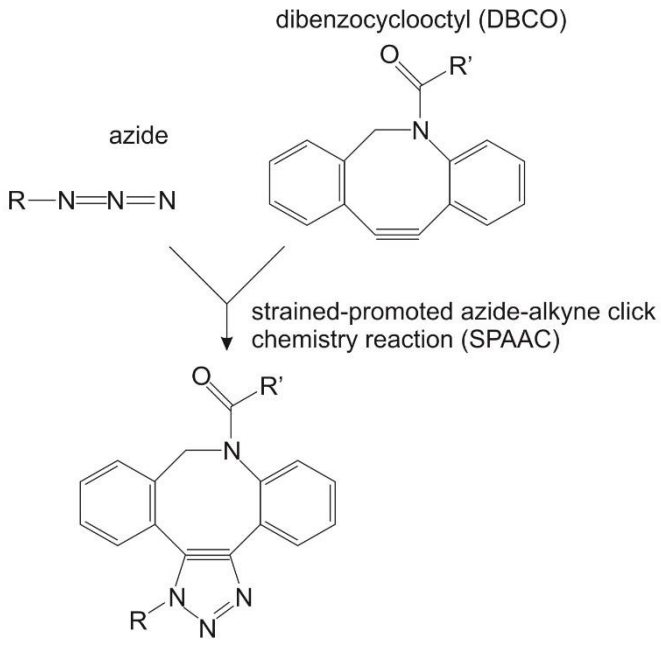
<<Scheme 1 CuAAC reaction>>



<<Scheme 2 iEDDA Reaction>>



<<Scheme 3 SPAAC reaction>>



[187] The CuAAC reaction yields 1,5 di-substituted 1,2,3-triazole. The reaction between alkyne and azide is very selective and there are no alkyne and azide groups in natural biomolecules. Furthermore, the reaction is quick and pH-insensitive. It has been suggested that instead of using copper (I), such as cuprous bromide or cuprous iodide, for catalyzing the click reaction, it is better to use a mixture of copper (11) and a reducing agent, such as sodium ascorbate to produce copper (I) in situ in the reaction mixture. Alternatively, the second element can be linked to the N- or C-terminus of the present center core via a copper-free reaction, in which pentamethylcyclopentadienyl ruthenium chloride complex is used as the catalyst to catalyze the azide-alkyne cycloaddition.

[188] For the sake of illustration, the functional elements linked to the linking arms are referred to as the first elements. As could be appreciated, the number of the first elements carried by the present linker unit depends on the number of K residues of the center core (and thus, the number of the linking arms). Accordingly, one of ordinary skill in the art may adjust the number of the first elements of the linker unit as necessary, for example, to achieve the desired targeting or therapeutic effect.

[189] An example of a linker unit 10D having the first elements is illustrated Figure 1D. Other than the features discussed hereafter, Figure 1D is quite similar to Figure 1B. First,

there are five K residues in the center core 11d, and accordingly, five linking arms 20a-20e are linked thereto, respectively. Second, the linker unit 100 has five first elements 30a-30e linked to each of the linking arms 20a-20e. As discussed below, the optional tetrazine group 72 allows for the conjugation with an additional functional element, another molecular construct (see, Part II or Part Ill below).

[190] Figure 1E provides an alternative example, in which the linker unit 10E has a similar structure with the linker unit 10, except that each of the linking arms 21a-21e has a disulfide linkage at the element-linking terminus thereof (i.e., the terminus that is linked with each of the first elements 30a-30e).

[191] Alternatively, the present linker unit further comprises a plurality of connecting arms, each of which has a functional group (i.e., a maleimide, an NHS, an azide, an alkyne, a tetrazine, or a strained alkyne group) at one terminus, and an NHS or a maleimide group at the other terminus. Using a reaction that is similar to those occurred between the first element and the linking arm, the connecting arm may be linked to the linking arm with the corresponding functional group either via forming an amide bond therebetween, or via the thiol-maleimide, CuAAC, iEOOA or SPAAC reaction. The connecting arm linked to the linking arm thus has the NHS or the maleimide group at its free terminus (or the element-linking terminus; i.e., the terminus that is not linked with the linking arm); then, the first element is linked to the element-linking terminus of the connecting arm via forming an amide bond therebetween or via the thiol-maleimide reaction.

[192] Reference is now made to Figure 1F, in which the linking arm is linked to the K residue of the center core 11d as described in Figure 1D. Compared with the linker unit 100, the linker unit 10Ffurther comprises a connecting arm 25, which is linked to the linking arms 22 via the SPAAC reaction. Then, the first element 30 is linked to the connecting arm 25 either via forming the amide bond therebetween or via the thiol-maleimide reaction. The diamond 90 as depicted in Figure 1F represents the chemical bond resulted from the SPAAC reaction occurred between the linking arm 22 and the connecting arm 25.

[193] According to some embodiments of the present disclosure, the connecting arm is a PEG chain having 2-20 repeats of EG units. Alternatively, the connecting arm is a PEG

chain having 2-20 repeats of EG units with a disulfide linkage at the element-linking terminus thereof (i.e., the free terminus that is not linked with the linking arm).

[194] In one working example, the connecting arm has three repeats of EG units, as well as a disulfide linkage at the free terminus (also referred to as the element-linking terminus) of the connecting arm. In this case, the first element linked to the element-linking terminus of the connecting arm can be efficiently released from the present linker unit by the treatment of a reductant.

[195] According to some preferred embodiments of the present disclosure, the first elements is fingolimod, fingolimod phosphate, interferon-β, or a single-chain variable fragment (scFv) specific for integrin- α4, β-amyloid, a viral protein, a bacterial protein.

[196] Non-limiting viral protein includes F protein of respiratory syncytia virus (RSV), gp120 protein of human immunodeficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, and glycoprotein of cytomegalovirus.

[197] Example of the bacterial protein includes, but is not limited to, the endotoxin of Gram(-) bacteria, the surface antigen of *Clostridium difficile*, the lipoteichoic acid of *Staphylococcus aureus*, the anthrax toxin of *Bacillus anthracis*, or the Shiga-like toxin type I or II of *Escherichia coli*.

[198] In order to increase the intended or desired effect (e.g., the therapeutic effect), the present linker unit may further comprise a second element in addition to the first element. For example, the second element can be either a targeting element or an effector element. In optional embodiments of the present disclosure, the first element is an effector element, while the second element may be another effector element, which works additively or synergistically with or independently of the first element. Still optionally, the first and second elements exhibit different properties; for example, the first element is a targeting element, and the second element is an effector element, and vice versa. Alternatively, the first element is an effector element, and the second element is an element capable of improving the pharmacokinetic property of the linker unit, such as solubility, clearance, half-life, and bioavailability. The choice of a particular first element and/or second element depends on the intended application in which the present linker unit (or multi-arm linker) is

to be used. Examples of these functional elements are discussed below in Part 1-(iii) of this specification.

[199] Structurally, the second element is linked to the azide, alkyne, tetrazine, or strained alkyne group at the N- or C-terminus of the center core. Specifically, the second element may be optionally conjugated with a short PEG chain (preferably having 2-12 repeats of EG units) and then linked to the N- or C-terminal amino acid residue having an azide group or an alkyne group (e.g., AHA residue or HPG residue). Alternatively, the second element may be optionally conjugated with the short PEG chain and then linked to the coupling arm of the center core.

[200] According to some embodiments of the present disclosure, the center core comprises an amino acid having an azide group (e.g., the AHA residue) at its N- or C-terminus; and accordingly, a second element having an alkyne group is linked to the N- or C-terminus of the center core via the CuAAC reaction. According to other embodiments of the present disclosure, the center core comprises an amino acid having an alkyne group (e.g., the HPG residue) at its N- or C-terminus; and a second element having an azide group is thus capable of being linked to the N- or C-terminus of the center core via the CuAAC reaction.

[201] Figure 1G provides an example of the present linker unit 10G carrying a plurality of first elements and one second element. In this example, the center core 11c comprises one HPG (G^{HP}) residue and five lysine (K) residues. Five linking arms 20a-20e are respectively linked to the five K residues of the center core 11c; and five first elements 30a-30e are respectively linked to said five linking arms 20a-20e via the thiol-maleimide reaction. In addition to the first elements, the linker unit 10G further comprises one second element 50 that is linked to one end of a short PEG chain 62. Before being conjugated with the center core 11c, the other end of the short PEG chain 62 has an azide group. In this way, the azide group may reacted with the HPG residue that having an alkyne group via CuAAC reaction, so that the second element 50 is linked to the center core 11c. The solid dot 40 depicted in Figure 1G represents the chemical bond resulted from the CuAAC reaction occurred between the HPG residue and the azide group.

[202] Alternatively, the second element is linked to the center core via a coupling arm. According to certain embodiments of the present disclosure, the coupling arm has a tetrazine group, which can be efficiently linked to a second element having a TCO group via the iEDDA reaction. According to other embodiments of the present disclosure, the coupling arm has a TCO group, which is capable of being linked to a second element having a tetrazine group via the iEDDA reaction. In the iEDDA reaction, the strained cyclooctenes that possess a remarkably decreased activation energy in contrast to terminal alkynes is employed, and thus eliminate the need of an exogenous catalyst.

[203] Reference is now made to Figure 1H, in which the center core 11d of the linker unit 10H comprises a terminal cysteine (C) residue and five lysine (K) residues. As depicted in Figure 1H, five linking arms 20a-20e are respectively linked to the five K residue of the center core 11d, and then five first elements 30a-30e are respectively linked to the five linking arms 20a-20e via thiol-maleimide reactions. The cysteine residue is linked to the coupling arm 60, which, before being conjugated with the second element, comprises a tetrazine group or a TCO group at its free-terminus. In this example, a second element 50 linked with a short PEG chain 62 having a corresponding TCO or tetrazine group can be linked to the coupling arm 60 via the iEDDA reaction. The ellipse 70 as depicted in Figure 1H represents the chemical bond resulted from the iEDDA reaction occurred between the coupling arm 60 and the short PEG chain 62.

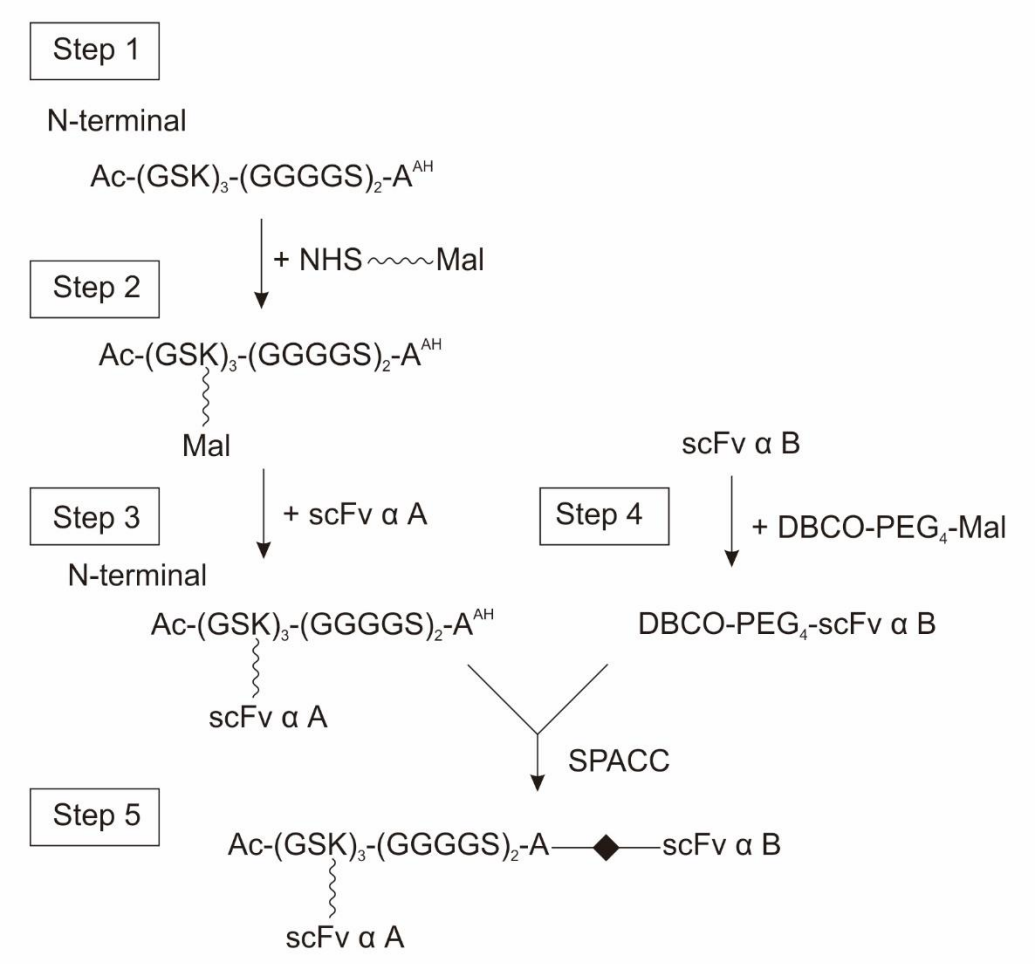
[204] According to other embodiments of the present disclosure, before the conjugation with a second element, the coupling arm has an azide group. As such, the coupling arm can be linked to the second element having a cyclooctyne group (e.g., the DBCO, DIFO, BCN, or DICO group) at the free-terminus of a short PEG chain via SPAAC reaction, and vice versa.

[205] Reference is now made to Figure 1I, in which the linker unit 10I has a structure similar to the linker unit 10H of Figure 1H, except that the coupling arm 60 comprises an azide or a cyclooctyne group (e.g., the DBCO, DIFO, BCN, or DICO group), instead of the tetrazine or TCO group. Accordingly, the second element 50 linked with a short PEG chain 62 may have a corresponding cyclooctyne (e.g., DBCO, DIFO, BCN, or DICO) or azide group, so that it can be linked to the coupling arm 60 via the SPAAC reaction. The

diamond 90 as depicted in Figure 1I represents the chemical bond resulted from the SPAAC reaction occurred between the coupling arm 60 and the short PEG chain 62.

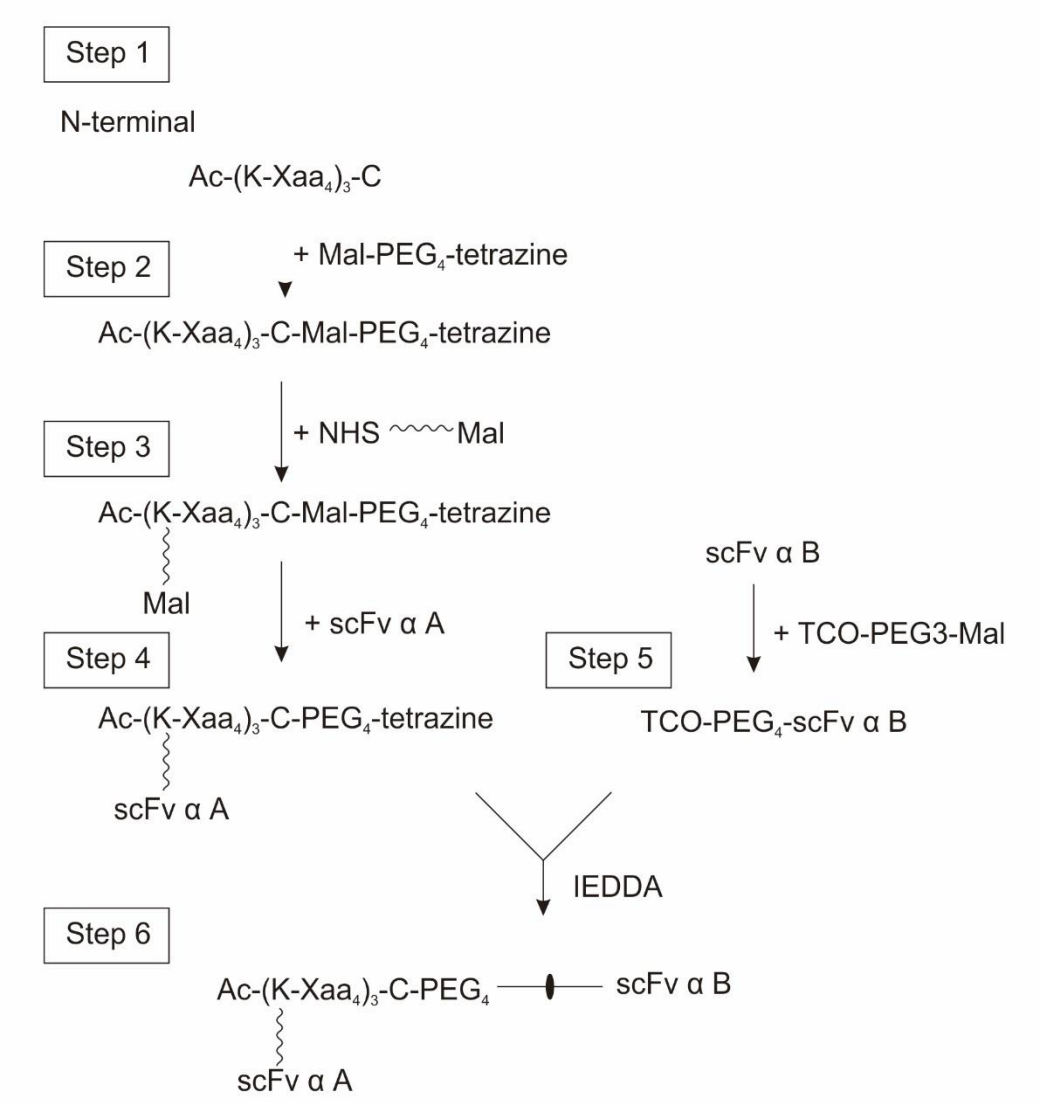
[206] Scheme 4 is an exemplary illustration of the process of preparing the present linker unit. In step 1, the center core comprising the amino acid sequence of (GSK)3 and a L-azidohomoalanine (AHA) residue at the C-terminus thereof is prepared. In step 2, three linking arms are respectively linked to the lysine (K) residues of the center core via forming an amide bond between the NHS group and the amine group; the linking arm linked to the center core has a maleimide (Mal) group at the free-terminus thereof. In step 3, three anti-A antigen scFvs (scFv α A) as the first element are respectively linked to the linking arms via the thiol-maleimide reaction. Meanwhile, in step 4, one anti-B antigen scFv (scFv α B) as the second element is linked with a short PEG chain that has 4 repeats of EG units and a DBCO group at the free terminus. Finally, in step 5, the second element is linked to the AHA residue of the center core via the SPAAC reaction.

<<Scheme 4 Preparation of linker unit linked with two different scFvs via linking arm and C-terminal amino acid residue>>



[207] Scheme 5 illustrates another example of the process for preparing the present linker unit. In step 1, the center core comprising the amino acid sequence of $(K\text{-}Xaa)_3$ and a cysteine residue at the C-terminus thereof is prepared. In step 2, a PEG chain (as the coupling arm) that has the maleimide (Mal) group at one terminus and a tetrazine group at the other terminus is linked to the cysteine residue via the thiol-maleimide reaction. Then, in step 3, three linking arm are respectively linked to the lysine (K) residues of the center core. Next, three anti-A antigen scFvs (scFv α A) as the first elements are respectively linked to the linking arms via the thiol-maleimide reaction as described in step 4. Meanwhile, in step 5, one anti-B antigen scFv (scFv α B) as the second element is linked with a short PEG chain that has 3 repeats of EG units and a TCO group at the free terminus. Finally, in step 6, the second element is linked to the coupling arm via the iEDDA reaction.

<<Scheme 5 Preparation of linker unit linked with two different scFvs via linking arm and coupling arm>>



[208] PEGylation is a process, in which a PEG chain is attached or linked to a molecule (e.g., a drug or a protein). It is known that PEGylation imparts several significant pharmacological advantages over the unmodified form, such as improved solubility, increased stability, extended circulating life, and decreased proteolytic degradation. According to one embodiment of the present disclosure, the second element is a PEG chain, which has a molecular weight of about 20,000 to 50,000 daltons.

[209] Figure 1J provides an alternative example of the present linker unit (linker unit 10J), in which five first elements 30 are respectively linked to the lysine residues via the linking arms 20, and the HPG (G^{HP}) residue of the center core 11e is linked with a PEG chain 80 via the CuAAC reaction. The solid dot 40 depicted in Figure 1J represents the chemical bond resulted from the CuAAC reaction occurred between the HPG residue and the PEG chain 80.

[210] Figure 1K provides another example of the present disclosure, in which the N-terminus of the center core 11 d is a cysteine residue that is linked to a coupling arm 60. A PEG chain 80 can be efficiently linked to the coupling arm 60 via the iEDDA reaction. The ellipse 70 of the linker unit 10K represents the chemical bond resulted from the iEDDA reaction occurred between the coupling arm 60 and the PEG chain 80.

[211] Figure 1L provides an alternative example of the present linker unit, in which the linker unit 10L has a structure similar to the linker unit 10J of Figure 1J, except that the PEG chain 80 is linked to the coupling arm 60 via the SPAAC reaction. The diamond 90 depicted in Figure 1L represents the chemical bond resulted from the SPAAC reaction occurred between the coupling arm 60 and the PEG chain 80.

[212] According to some embodiments of the present disclosure, in addition to the first and second elements, the present linker unit further comprises a third element. In this case, one of the N- and C-terminus of the center core is an amino acid having an azide group or an alkyne group, while the other of the N- and C-terminus of the center core is a cysteine residue. The lysine residues of the center core are respectively linked with the linking arms, each of which has a maleimide group at its free terminus; whereas the cysteine residue of the center core is linked with the coupling arm, which has a tetrazine group or a strained alkyne group at its free terminus. As described above, the first element is therefore linked to the linking arm via the thiol-maleimide reaction, and the second element is linked to the coupling arm via the iEDDA reaction. Further, a third element is linked to the terminal amino acid having an azide group or an alkyne group via the CuAAC reaction or SPAAC reaction.

[213] Reference is now made to the linker unit 10M of Figure 1M, in which the center core 11f has an HPG (G^{HP}) residue at the N-terminus thereof and a cysteine residue at the C-terminus thereof. The linking arms 20 and the coupling arm 60 are respectively linked to the lysine (K) residues and the cysteine (C) residue of the center core 11f. Further, five first elements 30 are respectively linked to the five linking arms 20, the second element (i.e., the PEG chain) 80 is linked to the coupling arm 60, and the third element 50 is linked to the HPG residue via the short PEG chain 62. The solid dot 40 indicated the chemical bond resulted from the CuAAC reaction occurred between the HPG residue and the short PEG

chain 62; while the ellipse 70 represents the chemical bond resulted from the iEDDA reaction occurred between the coupling arm 60 and the PEG chain 80.

[214] Figure 1N provides another embodiment of the present disclosure, in which the linker unit 10N has the similar structure with the linker unit 10M of Figure 1M, except that the short PEG chain 62 is linked with the HPG residue via the SPAAC reaction, instead of the iEDDA reaction. The diamond 90 in Figure 1N represents the chemical bond resulted from the SPAAC reaction occurred between the short PEG chain 62 and the HPG residue.

[215] In the preferred embodiments of this disclosure, the linking arms have a maleimide group in the free terminus for conjugating with first elements having the sulfhydryl group via the thiol-maleimide reaction. Also, there is one cysteine residue or an amino acid residue with an azide or alkyne group at a terminus of the peptide core for attaching a coupling arm for linking a second element.

[216] It is conceivable for those skilled in the arts that variations may be made. A conjugating group, other than maleimide, such as azide, alkyne, tetrazine, or strained alkyne may be used for the free terminus of the linking arms, for linking with first elements with a CuAAC, iEDDA, or SPAAC reaction. Also the cysteine residue (or an amino acid residue with an azide or alkyne group) of the peptide core needs not to be at the N- or C-terminus. Furthermore, two or more of such residues may be incorporated in the peptide core to attach multiple coupling arms for linking a plural of second elements.

[217] ***I-(ii) Compound Core for Use in Multi-arm Linker***

[218] In addition to the linker unit described in part *I-(i)* of the present disclosure, also disclosed herein is another linker unit that employs a compound, instead of the polypeptide, as the center core. Specifically, the compound is benzene-1,3,5-triamine, 2-(aminomethyl)-2-methylpropane-1,3-diamine, tris(2-aminoethyl)amine, benzene-1 ,2,4,5-tetraamine, 3,3' ,5,5'-tetraamine-1, 1'-biphenyl, tetrakis(2-aminoethyl)methane, tetrakis-(ethylamine)hydrazine, N,N ,N', N' ,-tetrakis(aminoethyl)ethylenediamine, benzene-1,2,3,4,5,6-hexaamine, 1-N, 1-N,3-N,3-N,5-N,5-N-hexakis(methylamine)-benzene-1,3,5-triamine, 1-N, 1-N ,2-N,2-N,4-N ,4-N ,5-N,5-N,-octakis(methylamine)-benzene-1 ,2,4,5-triamine, benzene-1,2,3,4,5,6-hexaamine, or N,N-bis[(1-amino-3,3-diaminoethyl)pentyl]-

methanediamine. Each of these compounds has 3 or more amine groups in identical or symmetrical configuration. Therefore, when one of the amine groups of a compound is conjugated with a coupling arm, all of the molecules of the compound have the same configuration.

[219] Similar to the mechanism of linkage described in Part 1-(i) of the present disclosure, each compound listed above comprises a plurality of amine groups, and thus, a plurality of PEG chains having NHS groups can be linked to the compound via forming an amide linkage between the amine group and the NHS group; the thus-linked PEG chain is designated as linking arm, which has a functional group (e.g., an NHS, a maleimide, an azide, an alkyne, a tetrazine, a cyclooctene, or a cyclooctynep group) at the free-terminus thereof. Meanwhile, at least one of the amine groups of the compound core is linked to another PEG chain, which has an NHS group at one end, and a functional group (e.g., an azide, alkyne, tetrazine, a cyclooctene, or a cyclooctynep group) at the other end; the thus-linked PEG chain is designated as coupling arm, which has a functional group at the free-terminus thereof.

[220] Accordingly, a first element can be linked to the linking arm via (1) forming an amide bond therebetween, (2) the thiol-maleimide reaction, (3) the CuAAC reaction, (4) the iEDDA reaction, or (5) SPAAC reaction. Meanwhile, the second element can be linked to the coupling arm via the CuAAC, iEDDA or SPAAC reaction.

[221] According to some embodiments of the present disclosure, the linking arm is a PEG chain having 2-20 repeats of EG units; preferably, the linking arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the free terminus thereof (i.e., the terminus that is not with the center core). The coupling arm is a PEG chain having 2-12 repeats of EG unit. In one embodiment, both the linking and coupling arms have 12 repeats of EG unit, in which one terminus of the coupling arm is an NHS group, and the other terminus of the coupling arm is an alkyne group.

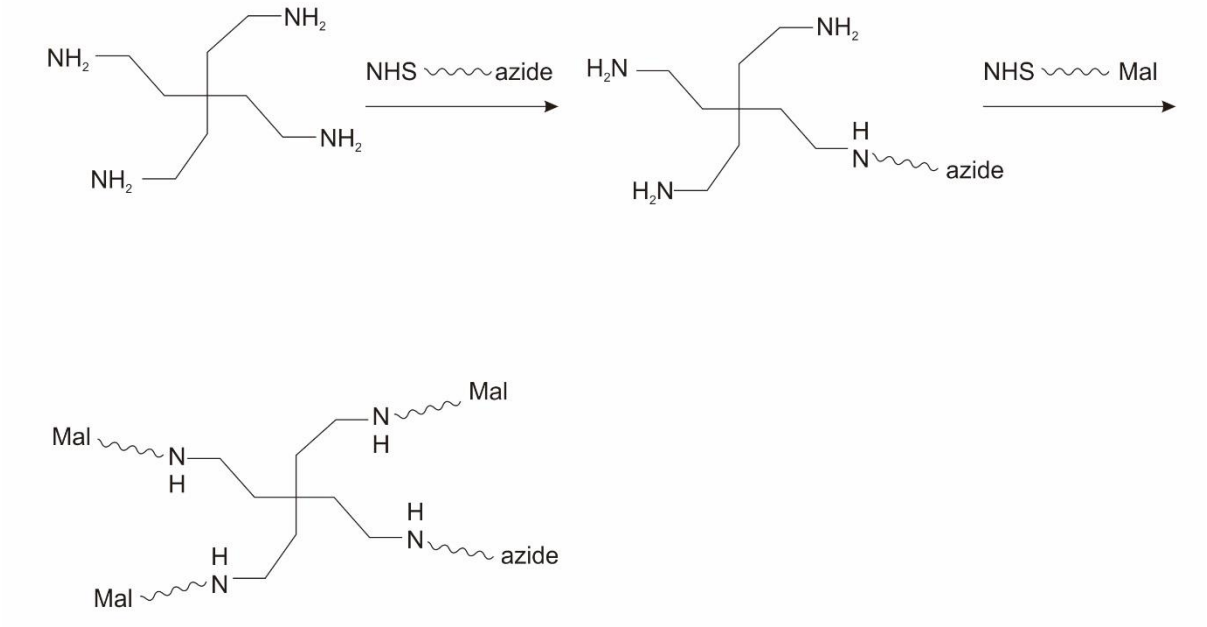
[222] According to an alternative embodiment of the present disclosure, the linker unit further comprises a plurality of connecting arms, each of which is linked to each of the linking arm. Then, a plurality of the first elements are respectively linked to the plurality of connecting arms. In one embodiment, the connecting arm is a PEG chain having 2-20

repeats of EG units. In another embodiment, the connecting arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the element-linking terminus that is not linked with the linking arm.

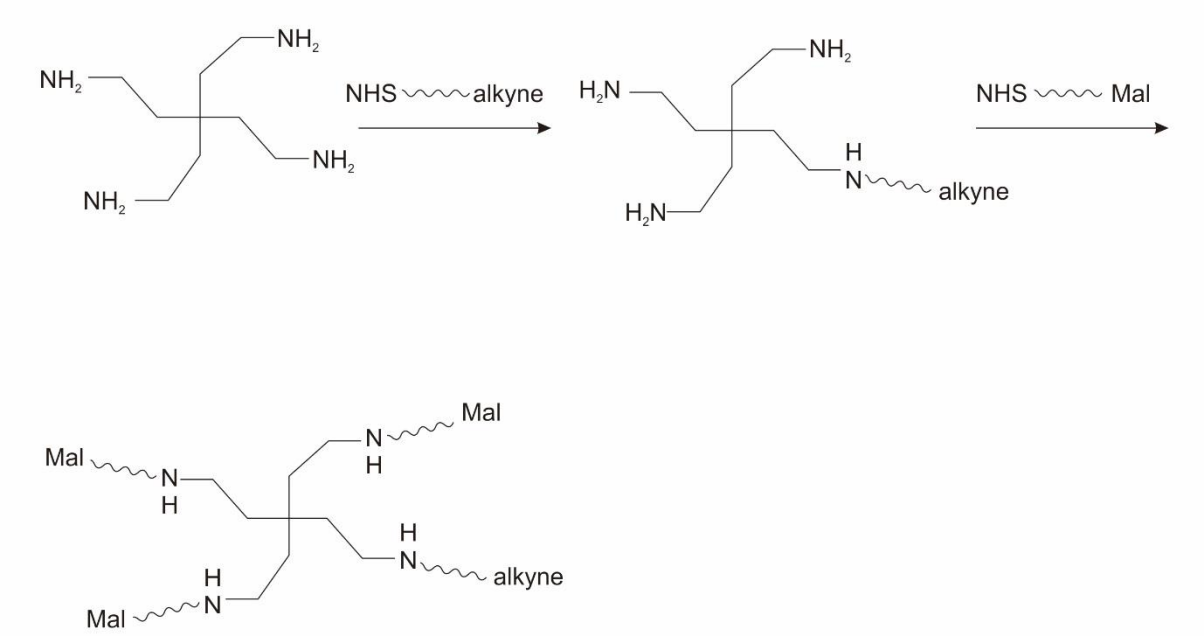
[223] Schemes 6 and 7 respectively depict the linkages between the center compound core and the linking arm, as well as the coupling arm. In schemes 6 and 7, "NHS" represents the NHS ester, "Mal" represents the maleimide group, "azide" represents the azide group, and "alkyne" represents the alkyne group.

[224] The requirement of having multiple NH_2 groups exist in a symmetrical and identical orientation in the compound serving as the center core is for the following reason: when one of the NH_2 group is used for connecting a bifunctional linker arm with N-hydroxysuccinimidyl (NHS) ester group and alkyne, azide, tetrazine, or strained alkyne group, the product, namely, a core with a coupling arm having alkyne, azide, tetrazine or strained alkyne, is homogeneous and may be purified. Such a product can then be used to produce multi-arm linker units with all other NH_2 groups connected to linking arms with maleimide or other coupling groups at the other ends. If a compound with multiple NH2 groups in non-symmetrical orientations, the product with one bifunctional linking arm/coupling arms is not homogeneous.

<<Scheme 6 Linkage of linking and coupling arms respectively having maleimide group and azide group to center core>>



<<Scheme 7 Linkage of linking and coupling arms respectively having maleimide group and alkyne group to center core>>



[225] Some of those symmetrical compounds can further be modified to provide center cores with more linking arms/coupling arms. For example, tetrakis(2-aminoethyl)methane, which can be synthesized from common compounds or obtained commercially, may be used as a core for constructing linker units with four linking arms/coupling arms. Tetrakis(2-aminoethyl)methane can react with bis(sulfosuccinimidyl)suberate to yield a condensed product of two tetrakis(2-aminoethyl)methane molecules, which can be used as a core for constructing linker units having six linking arms/coupling arms. The linker units, respectively having 3 linking arms/coupling arms, 4 linking arms/coupling arms and 6 linking arms/coupling arms, can fulfil I most of the need for constructing targeting/effector molecules with joint-linker configuration.

[226] As would be appreciated, the numbers of the linking arm and/or the coupling arm and the element linked thereto may vary with the number of amine groups comprised in the center core. In some preferred embodiments, the numbers of the linking arm/coupling arm and the corresponding linking element linked thereto ranges from about 1-7.

[227] Reference is now made to Figure 2, in which benzene-1,2,4,5-tetraamine having 4 amine groups is depicted. Three of the amine groups are respectively linked to the linking arms 20, and one of the amine group is linked to the coupling arm 60, which has an azide group at its free-terminus. Three first elements 30 are then respectively linked to the three linking arms 20 via the thiol-maleimide reactions, and one second element 50 is linked to

the coupling arm 60 via the CuAAC reaction. The solid dot 40 as depicted in Figure 2 represents the chemical bond resulted from the CuAAC reaction occurred between the coupling arm 60 and the second element 50.

[228] ***I-(iii) Functional Elements Suitable for Use in Multi-arm Linker***

[229] In the case where the linker unit (or multi-arm linker) comprises only the first element but not the second and/or third element(s), the first element is an effector element that may elicit a therapeutic effect in a subject. On the other hand, when the present linker unit comprises elements in addition to first element(s), then at least one of the elements is an effector element, while the other may be another effector element, a targeting element, or an element capable of enhancing one or more pharmacokinetic properties of the linker unit (e.g., solubility, clearance, half-life, and bioavailability). For example, the linker unit may have two different kinds of effector element, one effector element and one targeting element or one pharmacokinetic property-enhancing element, two different kinds of targeting elements and one kind of effector element, two different kinds of effector elements and one kind of targeting element, or one kind of targeting element, one kind of effector element and one element capable of improving the pharmacokinetic property of the linker unit.

[230] According to certain embodiments of the present disclosure, the targeting element or the effector element is fingolimod, fingolimod phosphate, interferon-β, or a single-chain variable fragment (scFv) specific for integrin-α4, β-amyloid, a viral protein, or a bacterial protein.

[231] Examples of viral proteins include, but are not limited to, F protein of respiratory syncytia virus (RSV), gp120 protein of human immunodeficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, and glycoprotein of cytomegalovirus.

[232] Illustrative examples of bacterial protein include endotoxin of Gram(-) bacteria, surface antigen of Clostridium difficile, lipoteichoic acid of *Staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, and Shiga-like toxin type I or II of *Escherichia coli*.

[233] Elements that enhance one or more pharmacokinetic properties of the linker unit can be a long PEG chain having a molecular weight of about 20,000 to 50,000 daltons.

[234] Specific examples of the functional elements incorporated in the present multi-arm linker for treating a particular disease are discussed below.

[235] To treat a CNS disease, such as multiple sclerosis, one exemplary linker unit may use fingolimod, fingolimod phosphate, interferon-β, or an scFv specific for integrin-a4 as the first element (effector element). To treat Alzheimer's disease, the present linker unit may use an scFv specific for β-amyloid as the first element (effector element). Optionally, the linker unit for treating multiple sclerosis, Alzheimer's disease, or other CNS diseases may further comprise a second element of an scFv specific for transferrin receptor as the targeting element.

[236] Similarly, an scFv specific for a viral or bacterial protein can be used as the targeting element (first element) in order to treat the infection caused by the virus or bacterium. In these cases, the linker unit may comprise an optional second element, such as an scFv specific for CD32 or CD16b, as the effector element.

[237] ***I-(iv) Use of Multi-arm Linker***

[238] The present disclosure also pertains to method for treating various diseases using the suitable linker unit. Generally, the method comprises the step of administering to a subject in need of such treatment an effective amount of the linker unit according to embodiments of the present disclosure.

[239] Compared with previously known therapeutic constructs, the present linker unit discussed in Part I is advantageous in two points:

(1) The number of the functional elements may be adjusted in accordance with the needs and/or applications. The present linker unit may comprise two elements (i.e., the first and second elements) or three elements (i.e., the first, second, and third elements) in accordance with the requirements of the application (e.g., the disease being treated, the route of administration of the present linker unit, and the binding avidity and/or affinity of the antibody carried by the present linker unit). For example, when the present linker unit is directly delivered into the tissue/organ (e.g., the treatment of eye), one element acting as the effector element may be enough, thus would eliminate the need of a second element acting as the targeting element. However, when the present linker unit is delivered

peripherally (e.g., oral, enteral, nasal, topical, transmucosal, intramuscular, intravenous, or intraperitoneal injection), it may be necessary for the present linker unit to simultaneously comprise a targeting element that specifically targets the present linker unit to the lesion site; and an effector element that exhibits a therapeutic effect on the lesion site. For the purpose of increasing the targeting or treatment efficacy or increasing the stability of the present linker unit, a third element (e.g., a second targeting element, a second effector element, or a PEG chain) may be further included in the present linker unit.

(2) The first element is provided in the form of a bundle. As described above, the number of the first element may vary with the number of lysine residue comprised in the center core. If the number of lysine residue in the center core ranges from 2 to 15, then at least two first elements may be comprised in each linker unit. Thus, instead of providing one single molecule (e.g., cytotoxic drug and antibody) as traditional therapeutic construct or method may render, the present linker unit is capable of providing more functional elements (either as targeting elements or as effector elements) at one time, thereby greatly improves the therapeutic effect.

[240] In certain therapeutic applications, it is desirable to have a single copy of a targeting or effector element. For example, a single copy of a targeting element can be used to avoid unwanted effects due to overly tight binding. This consideration is relevant, when the scFv has a relatively high affinity for the targeted antigen and when the targeted antigen is a cell surface antigen on normal cells, which are not targeted diseased cells. As an example, in using scFv specific for CD3 or CD16a to recruit T cells or NK cells to kill targeted cells, such as thyroid gland cells in patients with Graves' disease, a single copy of the scFv specific for CD3 or CD16a is desirable, so that unwanted effects due to cross-linking of the CD3 or CD16a may be avoided. Similarly, in using scFv specific for CD32 or CD16b to recruit phagocytic neutrophils and macrophages to clear antibody-bound viral or bacterial particles or their products, a single copy of scFv may be desirable. Also, in using scFv specific for transferrin receptor to carry effector drug molecules to the BBB for treating CNS diseases, a single copy of scFv specific for transferrin receptor is desirable. In still another example, it is desirable to have only one copy of long-chain PEG for enhancing pharmacokinetic properties. Two or more long PEG chains may cause tangling and affect the binding properties of the targeting or effector elements.

[241] **PART II Joint-linker Molecular Constructs for Treating Specific Diseases**

[242] Another aspect of the present disclosure pertains to a molecular construct comprising at least two linker units, in which one linker unit carries one or more targeting element, whereas another other linker unit carries one or more effector elements or pharmacokinetic property-enhancing elements. In the present disclosure, molecular constructs with both the targeting and effector moieties (whether a therapeutic or pharmacokinetic one) are referred to as joint-linker molecular constructs. According to various embodiments of the present disclosure, each of the linker unit comprised in such joint-linker molecular constructs may be either a peptide core-based or a compound core-based multi-arm linkers discussed above in Part I of the present disclosure. According to certain embodiments of the present disclosure, at least one of the linker units of the present molecular construct comprises the polypeptide core. Preferably, at least two linker units of the present molecular construct comprise the polypeptide cores. More preferably, all the linker units of present molecular construct respectively comprise the polypeptide cores.

[243] ***II-(i) Structure of Joint-linker Molecular Construct***

[244] According to some embodiments of the present disclosure, the molecular construct comprises two linker units, and the linker units are coupled to each other via either the CuAAC reaction (using copper or pentamethylcyclopentadienyl ruthenium chloride complex as catalyst), the SPAAC reaction, or the iEDDA reaction. In the embodiments, one of the linker units is linked with a plurality of first elements, which act as the targeting elements, and the other of the linker units is linked with a plurality of second elements, which act as the effector elements.

[245] According to other embodiments of the present disclosure, the molecular construct comprises three linker units, in which the first and second linker units are coupled to each other via the iEDDA reaction, and then, the third linker unit is coupled to the first or second linker unit via the CuAAC reaction. Alternatively, the first and second linker units are coupled to each other via the iEDDA reaction, and the third linker unit is coupled to the first or second linker unit via the SPAAC reaction. In the embodiments, the first, second, and third linker units respectively carry a plurality of first, second, and third elements, in which

the first, second, and third elements are different. According to one embodiment, two of the three elements (i.e., the first, second, and third elements) are targeting elements, and one of the three elements is an effector element. According to another embodiment, two of the three elements are effector elements, and one of the three elements is a targeting element. According to still another embodiment, one of the three elements is a targeting element, another of the three elements is an effector element, and the other of the three elements is an element capable of improving the pharmacokinetic property of the molecular construct, such as solubility, clearance, half-life, and bioavailability.

[246] Reference is first made to Figures 3A-3D, which respectively depict the linkage between the two linker units. Figure 3A depicts a molecular construct comprising two linker units (100A, 200A), which are coupled to each other via the iEDDA reaction. The first linker unit 100A comprises a first center core 110a, a linking arm 120 (as the first linking arm), and a coupling arm 130a (as the first coupling arm), in which the linking and coupling arms are respectively linked to the first center core 110a at one ends. Similarly, the second linker unit 200A comprises a second center core 21Oa, a linking arm 220 (as the second linking arm), and a coupling arm 230a (as the second coupling arm), in which the linking and coupling arms are respectively linked to the second center core 210a at one ends. One of the coupling arms 130a, 230a has a tetrazine group at its free terminus, while the other of the coupling arms 130a, 230a has a TCO group. Specifically, if the coupling arm 130a has a tetrazine group 152 at its free terminus (i.e., the terminus not connected to the first center core 110a), then the coupling arm 230a would have a TCO group 154 at its free terminus (i.e., the terminus not connected to the second center core 210a), and vice versa. Accordingly, the two linker units (100A, 200A) are coupled to each other via the iEDDA reaction occurred between the respective free ends of the coupling arms 130a, 230a. The ellipse 156 as depicted in Figure 3A represents the chemical bond resulted from the iEDDA reaction occurred between the coupling arms 130a, 230a.

[247] In the depicted embodiment, each of the linking arms 120, 220 has a maleimide group at its free terminus. Accordingly, a first targeting element 140 and a first effector element 240, each has a thiol group are respectively linked to the linking arms 120, 220 via the thiol-maleimide reaction.

[248] According to one embodiment, both the first and second center cores 110a, 210a depicted in Figure 3A are polypeptide cores. According to another embodiment, both the first and second center cores 110a, 210a depicted in Figure 3A are compound cores. According to still another embodiment, one of the first and second center cores 110a, 210a depicted in Figure 3A is a polypeptide core, while the other of the first and second center cores 110a, 210a depicted in Figure 3A is a compound core.

[249] Figure 3B provides an alternative embodiment of the present disclosure, in which both the first and second center cores 110b, 210b are polypeptide cores, and are respectively linked to a first targeting element 140 and a first effector element 240 via the linking arms 120, 220. The unique feature in this embodiment is that, one of the center cores 110b, 210b comprises an amino acid residue having an azide group (e.g., the AHA residue) at it N- or C-terminus, while the other of the center cores 110b, 210b comprises an amino acid residue having an alkyne group (e.g., the HPG residue) at it N- or C-terminus, such configuration allows the center cores 110a, 210a to be directly linked to each other, that is, without connecting through any coupling arms as that depicted in Figure 3A. Specifically, if the center core 110b comprises the amino acid residue having the azide group 162 at its N- or C-terminus, then the center core 210b would comprises the amino acid residue having the alkyne group 164 at its N- or C-terminus, and vice versa. Accordingly, the linker units 100B, 200B can couple together directly via the CuAAC reaction occurred between the N- or C-terminal amino acid residues of the center cores 110b, 210b. The solid dot 166 as depicted in Figure 3B represents the chemical bond formed between the N- or C-terminal amino acid residues.

[250] Figure 3C is another embodiment of the present disclosure. The linker units 100C, 200C have the similar structures as the linker units 100A, 200A, except that the coupling arms 130b, 230b respectively have an azide group 162 and a DBCO group 172, instead of the azide group 152 and the alkyne group 154 as depicted in the linker units 100A, 200A of Figure 3A. Specifically, the center core 110a is linked with a coupling arm 130b (as the first coupling arm) having an azide group 162 at its free-terminus; and the center core 210a is linked with a coupling arm 230b (as the second coupling arm) having a DBCO group 172 at its free-terminus. The linker units 100C, 200C are then coupled via the

SPAAC reaction occurred between the coupling arms 130b, 230b; and forming the chemical bond 182, depicted as a diamond.

[251] In one embodiment, both the first and second center cores 110a, 210a depicted in Figure 3C are polypeptide cores. In another embodiment, both the first and second center cores 110a, 210a depicted in Figure 3C are compound cores. In still another embodiment, one of the first and second center cores 110a, 210a depicted in Figure 3C is a polypeptide core, while the other of the first and second center cores 110a, 210a depicted in Figure 3C is a compound core.

[252] As would be appreciated, two linker units can be coupled to each other via the CuAAC reaction occurred between the center core and the coupling arm. Reference is now made to Figure 30, in which the center core 110b comprises a N- or C-terminal amino acid residue that has an azide group 162 (e.g., the AHA residue), and the center core 210a is linked with a coupling arm 230b having a TCO group 172 at its free-terminus. Accordingly, the linker units 100B and 200C can be coupled via the SPAAC reaction occurred between the center core 110b and the coupling arm 230b; and forming the chemical bond 182.

[253] According to one embodiment, the linker units 100B, 200C depicted in Figure 30 respectively comprise polypeptide cores. According to another embodiment, the center core 100B depicted in Figure 30 is a polypeptide core, while the center core 200C depicted in Figure 30 is a compound core.

[254] Alternatively, the linker unit that comprises a N- or C-terminal amino acid residue having an alkyne group (e.g., the HPG residue), and the linker unit comprising the coupling arm with an azide group at its free-terminus can be coupled together via the azide-alkyne cycloaddition occurred between the center core and the coupling arm.

[255] As would be appreciated, at least one of the linker units of the present molecular construct may further comprise a connecting arm, in which one terminus of the connecting arm is linked with the linking arm, while the other terminus is linked with the functional element (either the targeting element or the effector element) as depicted in *Part I*. For

example, the present molecular construct may comprise two linker units, in which the first element is directly linked to the first linking arm, while the second element is linked to the second linking arm via the linkage of the connecting arm. Alternatively, the present molecular construct may comprise two linker units, in which the first and second element are respectively linked to the first and second linking arms through the linkages of the first and second connecting arms.

[256] Preferably, when at least one of the first and second linking arms is linked to the connecting arm/functional element via the CuAAC or SPAAC reaction, then the first and second linker units are coupled to each other via the iEDDA reaction. Alternatively, when at least one of the first and second linking arms is linked to the connecting arm/functional element via the iEDDA reaction, then the first and second linker units are coupled to each other via the CuAAC or SPAAC reaction.

[257] According to some embodiments, the connecting arm is a PEG chain having 2-20 repeats of EG units. According to other embodiments, the connecting arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the element-linking terminus that is not linked with the linking arm.

[258] According to one embodiment of the present disclosure, the first element is an scFv specific for transferrin receptor, and the second element is interferon-β (IFN-β), fingolimod, fingolimod phosphate, or an scFv specific for integrin α4 or β-amyloid. According to another embodiment of the present disclosure, the first element is an scFv specific for a viral protein or a bacterial protein, and the second element is an scFv specific for CD16b or CD32.

[259] Compared with other therapeutic construct, the present molecular construct is advantageous in at least the three following aspects:

(1) the linker unit comprising a specified number and/or type of targeting/effector element can be prepared independently, then proceed to be coupled together via the CuAAC reaction, the iEDDA reaction, or the SPAAC reaction;

(2) the number and kind of the targeting and/or effector elements may vary in accordance with the requirements of application (e.g., the disease being treating, and the binding avidity and/or affinity of the targeting and/or effector element). The combination of

the targeting and effector elements may be adjusted according to specific needs and/or applications. Each of the present targeting and effector elements may vary with such factors like particular condition being treated, the physical condition of the patient, and/or the type of disease being treated. The clinical practitioner may combine the most suitable targeting element and the most suitable effector element so as to achieve the best therapeutic effect. According to embodiments of the present disclosure, the targeting element may be a growth factor, a peptide hormone, a cytokine, or an antibody fragment; and the effector element may be an immunomodulant, a chelator complexed with a radioactive nuclide, a cytotoxic drug, a cytokine, a soluble receptor, or an antibody; and

(3) compared with other coupling reactions, the CuAAC reaction, the iEDDA reaction, or the SPAAC reaction is more efficient in terms of coupling any two linker units.

[260] Reference is now made to Figure 4, in which six libraries are illustrated, and are prepared independently. In this embodiment, Libraries 1-6 respectively comprise a plurality of linker units 300A, 300B, 300C, 400A, 400B, and 400C that are linked with functional elements. Each linker units 300A, 300B, and 300C are similar in structures; in which each of the linker units 300A, 300B, and 300C comprises one center core 310, one coupling arm 330 linked thereto and has a tetrazine group 350 at its free terminus, and a specified number of the linking arm 320. For instance, Linker unit 300A comprises four linking arms 320, and accordingly, four targeting elements 340a can be respectively linked to the four linking arms 320. Similarly, two targeting elements 340b and five targeting elements 340c can be respectively linked to the linker units 300B and 300C. The targeting elements 340a, 340b, and 340c can be the same or different. As to the linker units 400A, 400B and 400C, each of these linker units comprises one center core 410, one coupling arm 430 linked thereto and has a strained alkyne group 450 at its free terminus, and a specified number of the linking arm 420. As depicted, three effector elements 440a, five effector elements 440b, and eight effector elements 440c can be respectively linked to the linker units 400A, 400B and 400C. The effector elements 440a, 440b, and 440c can be the same or different. The Libraries 1-6 may be prepared independently. One skilled artisan may select the first linker unit from Libraries 1, 2 and 3, and the second linker unit from Libraries 4, 5, and 6, then proceed to couple the first and second linker units via the iEDDA reaction occurred between the tetrazine group 350 and the strained alkyne group 450 so as

to produce the molecular construct with the specified number of targeting and effector elements.

[261] Based on the library concept, the present molecular construct can be produced with different configurations depending on the libraries selected. Figure 5A provides an example of the present molecular construct, in which each of the first and second center cores (310, 410) is linked with three linking arms (320, 420) and one coupling arm (330, 430). Three of the first targeting elements 340 are respectively linked to the linking arms 320; and three of the first effector elements 440 are respectively linked to the linking arms 420. The two linker units are coupled to each other via the iEDDA reaction occurred between two coupling arms 330, 430, and forming the chemical bond 356. By this configuration, equal numbers of multiple targeting and/or effector elements may be carried in one molecular construct.

[262] Figure 5B provides another example of the present molecular construct, in which the first and second center cores respectively contain different numbers of amine groups (e.g., lysine residues), and accordingly, the molecular construct contains non-equal numbers of targeting and effector elements. In the depicted example, the first center core 310 is linked to one coupling arm 330, and two linking arms 320. The second center core 410 is linked to one coupling arm 430, and five linking arms 420. Accordingly, two targeting elements 340 are respectively linked to the linking arms 320; and five effector elements 440 are respectively linked to the linking arms 420. The ellipse 356 in Figure 5B represents the linkage between two coupling arms 330, 430.

[263] In optional embodiments, the present molecular construct may further comprise a relatively long PEG chain connected to either the first or second center core, so that the present molecular construct may be segregated further away from the reticuloendothelial system and attains a longer half-life after being administered to a subject. In the case where a protein is modified by a PEG chain so as to improve its pharmacokinetic properties and/or to decrease immunogenicity, PEG up to 20,000-50,000 daltons in length, is preferred. Accordingly, in one preferred embodiment of the present invention, linking arms of relatively shorter lengths are used to connect the targeting and effector elements, while a PEG chain

of 20,000 to 50,000 daltons is connected to any of the linker units with the purpose of increasing *in vivo* half-life of the present molecular construct.

[264] In some embodiments, multiple scFv fragments are used as the targeting and/or effector elements to construct the present molecular construct. The targeting element/effector element pharmaceuticals based on molecular constructs comprising scFv fragments should have longer *in vivo* half-lives than individual antibody fragments. For some clinical applications, much extended half-lives of the pharmaceuticals are desired, so as to eliminate the need of frequent administration of the drugs; in these cases, PEG chains that are 20,000 to 50,000 daltons by weight, may be used as the linking arms to link the scFv fragments that serve as targeting or effector elements. PEGs of these lengths have been used to modify a large number of therapeutic proteins to increase their half-lives.

[265] According to some embodiments of the present disclosure, the linker unit may comprise two linking arms respectively linked to the different functional elements. Reference is now made to Figure 6, in which the molecular construct comprises two linker units 100A and 200D. The first and second functional elements 140, 240 (one serves as the targeting element, and the other serves as the effector element) are respectively linked to the first center core 110a and the second center core 210c via the linking arms 120, 220; and the two center cores 110a, 210c are coupled to each other via the iEDDA reaction occurred between the coupling arms 130a, 230a, in which the ellipse 156 represents the chemical bond forming therebetween. In addition to the functional element 240, the second center core 210c is further linked to a PEG chain 260. Specifically, the second center core 210c comprises an AHA residue, which can be reacted with and linked to the PEG chain 260 having a stained alkyne group via the SPAAC reaction, in which the diamond 182 represents the chemical bond forming from the SPAAC reaction. Depending on the intended and desired use, the third element can be a second targeting element, a second effector element, or an element capable of improving the pharmaceutical property of the molecular construct. According to one embodiment of the present disclosure, the PEG chain 260 has a molecular weight about 20,000 to 50,000 daltons.

[266] Based on the concept, a linker unit may comprise a plurality of linking arms, which can be linked to a plurality of functional elements. For example, a linker unit may

comprises 5-12 linking arms, which can be linked to 5-12 functional elements. This is especially useful when the functional elements are small molecules, such as therapeutic drugs or toll-like receptor agonists. The linker unit carrying multiple molecules of a therapeutic drug is herein referred to as a drug bundle.

[267] Further, the polypeptide cores can be employed to prepare the molecular construct comprising three linker units. Accordingly, another aspect of the present disclosure is directed to a molecular construct comprising three linker units. Among the three linker units, two of them may be connected to each other via the iEDDA reaction, while the third linker unit is connected to any of the two linker units by the SPAAC reaction or CuAAC reaction. The rationale for constructing a multi-linker unit (e.g., three linker units) is that two different sets of targeting elements or two different sets of effector elements can be incorporated therein.

[268] Reference is now made to Figure 7, in which the molecular construct comprises three linker units (500, 600, 700A). The linker units 500, 600, 700A respectively comprise a center core (510, 610, 710), and an linking arm (520, 620, 720) with a functional element (540, 640, 740) linked thereto. The linker unit 600 is characterized in comprising a cysteine residue at one of its N- or C- terminus that is linked with a coupling arm 630; and an amino acid residue having an azide or alkyne group at the other of its N- or C- terminus. One of the coupling arms 530, 630 has a tetrazine group at its free terminus, and the other of the coupling arms 530, 630 has a strained alkyne group at its free terminus. Accordingly, the linker units 500, 600 can be coupled to each other via the iEDDA reaction occurred between the coupling arms 530, 630 as the linkage manner described in Figure 3A. As to the linkage of the linker unit 700, when the N- or C-terminal amino acid residue of the center core 610 has an azide group (e.g., the AHA residue), the center core 710 comprises an amino acid having an alkyne group (e.g., the HPG residue) at its N- or C- terminus; or, when the N- or C-terminal amino acid residue of the center core 610 has an alkyne group (e.g., the HPG residue), then the center core 710 comprises an amino acid having an azide group (e.g., the AHA residue) at its N- or C- terminus. Thus, as the linkage manner described in Figure 3B, the linker units 600, 700A can be directly coupled to each other via the CuAAC reaction occurred between the N- or C-terminal amino acid residues of the center cores 610, 710 without the presence of the coupling arms. The ellipse 560 and the solid dot 670 in

Figure 7 respectively represent the chemical bonds resulted from the iEDDA reaction and the CuAAC reaction.

[269] Alternatively, two of the three linker units may be connected to each other via the iEDDA reaction, while the third linker unit is connected to any of the two linker units by the SPAAC reaction. Reference is now made to Figure 7B, in which the linker units 500, 600 are coupled together via the iEDDA reaction as described in Figure 7A, whereas the linker unit 700B is linked to the linker unit 600 via the SPAAC reaction occurred between the center core 610 and the coupling arm 730. The diamond 672 in Figure 7B represents the chemical bond resulted from the SPAAC reaction.

[270] As would be appreciated, each number of the functional elements 540, 640, 740 respectively linked to the linker units 500, 600, 700A or 700B are different depending on the intended use. With the library concept depicted in Figure 4, the linker units respectively carrying different numbers and/or types of functional elements can be prepared separately as different libraries, and one skilled artisan may select and combine the desired linker units from the libraries in accordance with the various applications.

[271] Basically, the coupling arm of the present molecular construct described in above aspects and/or embodiments of the present disclosure that has an azide, alkyne, tetrazine, or strained alkyne group at the terminus is designed as a PEG chain having 2-12 repeats of EG units. The linking arm is designed as a PEG chain having 2-20 repeats of EG units; preferably, the linking arm is a PEG chain having 2-20 repeats of EG units with a disulfide linkage at the free terminus that is not linked with the center core.

[272] Adopting a polypeptide as the center core provides versatility in the present molecular construct, in which multiple copies or types of targeting/effector elements may be present in one construct, accordingly, enhanced specificity of drug delivery and potency in the intended target sites are achieved. A large number of configurations can be adopted by employing the molecular construct comprising multiple linker units. A few examples are: a first linker unit carrying three scFvs targeting elements, and a second linker unit carrying 5 therapeutic drugs; a first linker unit carrying three scFvs targeting elements, and a second linker unit carrying three scFvs effector elements; a first linker unit carrying two scFvs of the first set targeting elements, a second linker unit carrying two scFvs of the second set

targeting elements, and a third linker unit carrying 5 therapeutic drugs; a first linker unit carrying 2 bi-scFv targeting elements, and a second linker unit carrying two scFvs effector elements; or a first linker unit carrying three scFvs targeting elements, a second linker unit carrying two scFvs effector elements plus a linking arm attached with a long PEG of 20,000-50,000 daltons for the purpose of increasing pharmacokinetic properties.

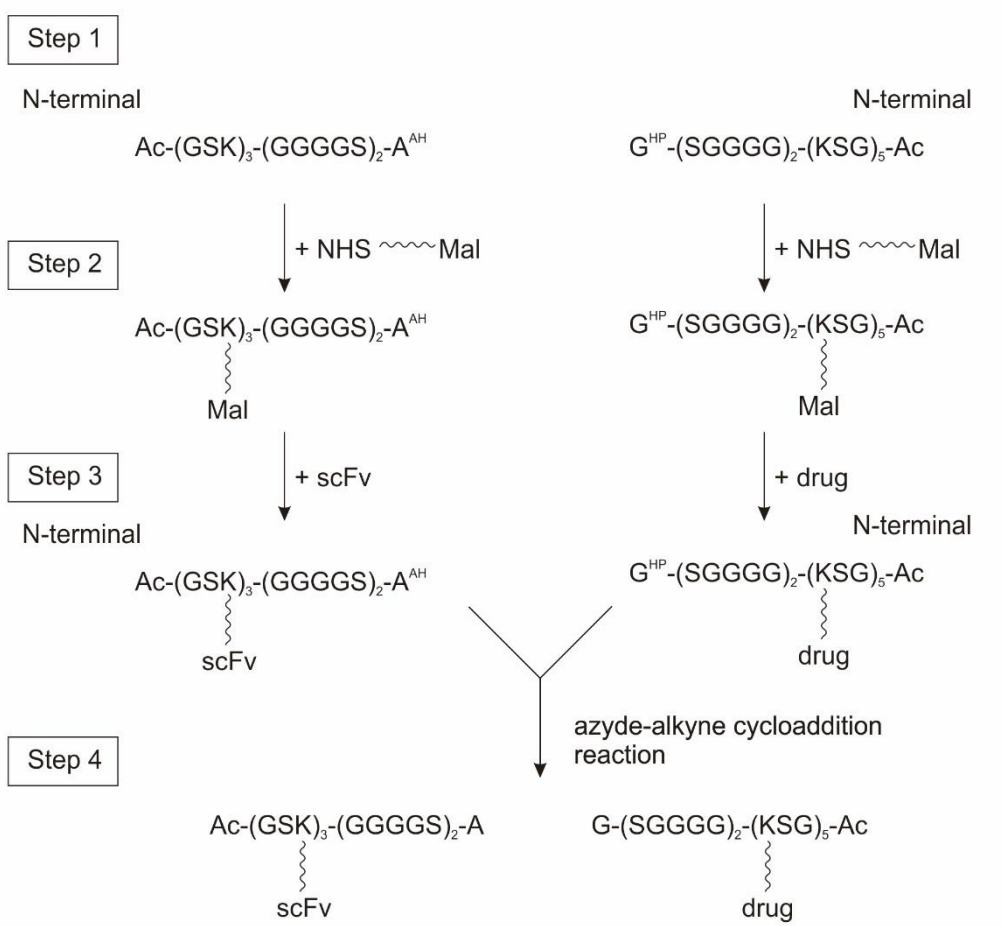
[273] In some embodiments of this invention, a bi-functional PEG acting as a linking arm is used to link the antigen-binding fragments of antibodies, which serve as targeting or effector elements, to the amine groups located in the polypeptide core. Each PEG may have NHS group at one end and maleimide group at the other end. The NHS group may couple with amine group in the polypeptide core, while the maleimide group may couple with sulfhydryl group of a cysteine residue of an scFv, bi-scFv, or Fab fragment of an antibody. The scFv and bi-scFv are engineered to have a polypeptide linker with terminal cysteine residue at the C-terminal. Fab may be derived from a whole IIG by pepsin cleavage, and the free sulfhydryl groups are derived from the inter-chain disulfide bond by a mild reduction reaction.

[274] Schemes 8-12 provide several working example respectively depicting the coupling and preparation of specified linker units.

[275] Scheme 8 is a schematic diagram depicting the preparation of the present molecular construct in accordance with one embodiment of the present disclosure, in which NHS represents NHS ester, Mal represents maleimide group, A^{AH} represents L-azidohomoalanine (AHA) residue, G^{HP} represents homopropargylglycine (HPG) residue, Ac represents acetyl group, and scFv represent single-chain variable fragment. In step 1, the first center core comprising the amino acid sequence of $(GSK)_5$ and a L-azidohomoalanine (AHA) residue at the C-terminus thereof; and the second center core comprising the amino acid sequence of $(GSK)_5$ and a homopropargylglycine (HPG) residue at the C-terminus thereof, are respectively prepared. For the purpose of stabilizing the polypeptide, the N-terminuses of the first and second center cores are respectively modified with an acetyl group. In step 2, the linking arms are respectively linked to the lysine residues in the first and second center cores via forming an amide linkage there between; the linked arm linked to the center core has a maleimide group at the free-terminus. In

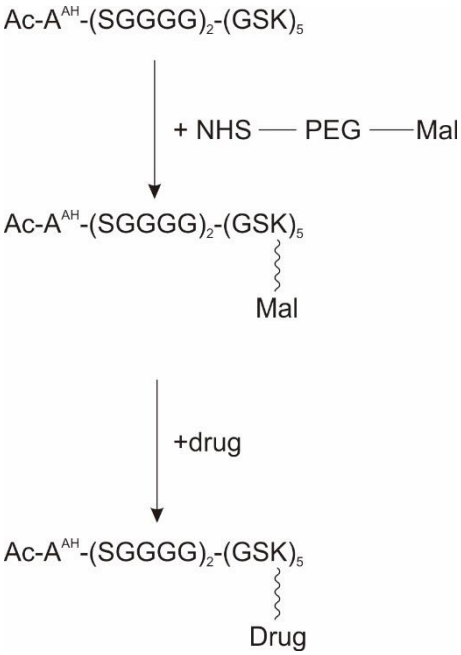
step 3, the first targeting element (i.e., the antibody) having a thiol group (e.g., a cysteine residue) is linked to the linking arm linked with the first center core via the thiol-maleimide reaction; similarly, the effector element (i.e., the drug) having a thiol group is linked to the linking arm linked with the second center core via the thiol-maleimide reaction. In step 4, the two linker units are coupled via a CuAAC reaction occurred between the AHA and HPG residues.

<<Scheme 8 Coupling linker units via c-terminal amino acid residues>>



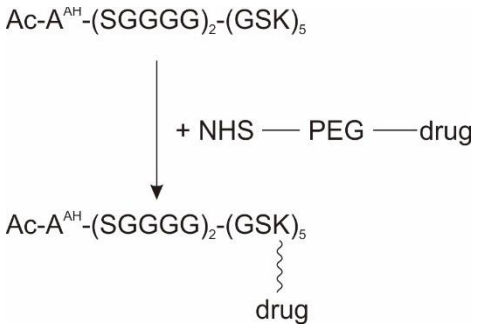
[276] Optionally, the targeting/effector element can be linked to the center core in an alternative method. Scheme 9 is a scheme illustrating the coupling of the effector element with the polypeptide core, in which the linking arm is first linked to the center core, and then the effector element (i.e., the drug) is linked to the linking arm via the thiol-maleimide reaction.

<<Scheme 9 Method of coupling of effector element with polypeptide core through linking to linking arms>>



[277] In the alternative method of scheme 10, the effector element (i.e., the drug) is coupled to the linking arm so as to produce a linking arm-effector conjugate (i.e., PEG-drug); next, the linking arm-effector conjugate is linked to the center core via forming an amide linkage between the lysine residues and the NHS esters.

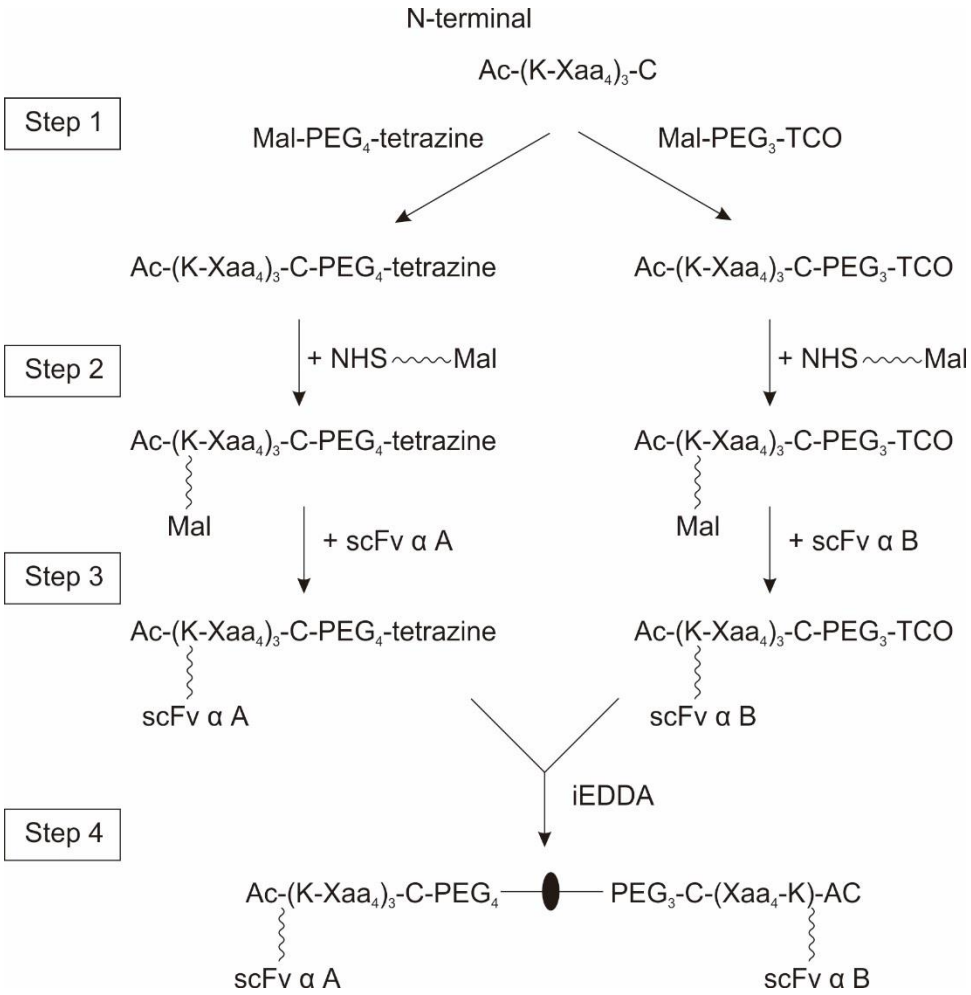
<<Scheme 10 Alternative method of coupling of effector element with polypeptide core by first conjugating with PEG chain and then linking to amino groups of lysine residues>>



[278] Alternatively, the linking arms for the joint-linker configuration may also be used to link bispecific scFv, which act as targeting elements or effector elements. These configurations will increase the specificity of targeting and/or the potency of the effector mechanisms.

[279] Scheme 11 provides an example of preparing the present molecular construct, which comprises two linker units; both linker units comprises the amino acid sequence of (K-Xaa4)3 and a cysteine (C) residue at the C-terminus thereof.

<<Scheme 11 Preparation of molecular construct via iEDDA reaction occurred between coupling arms>>

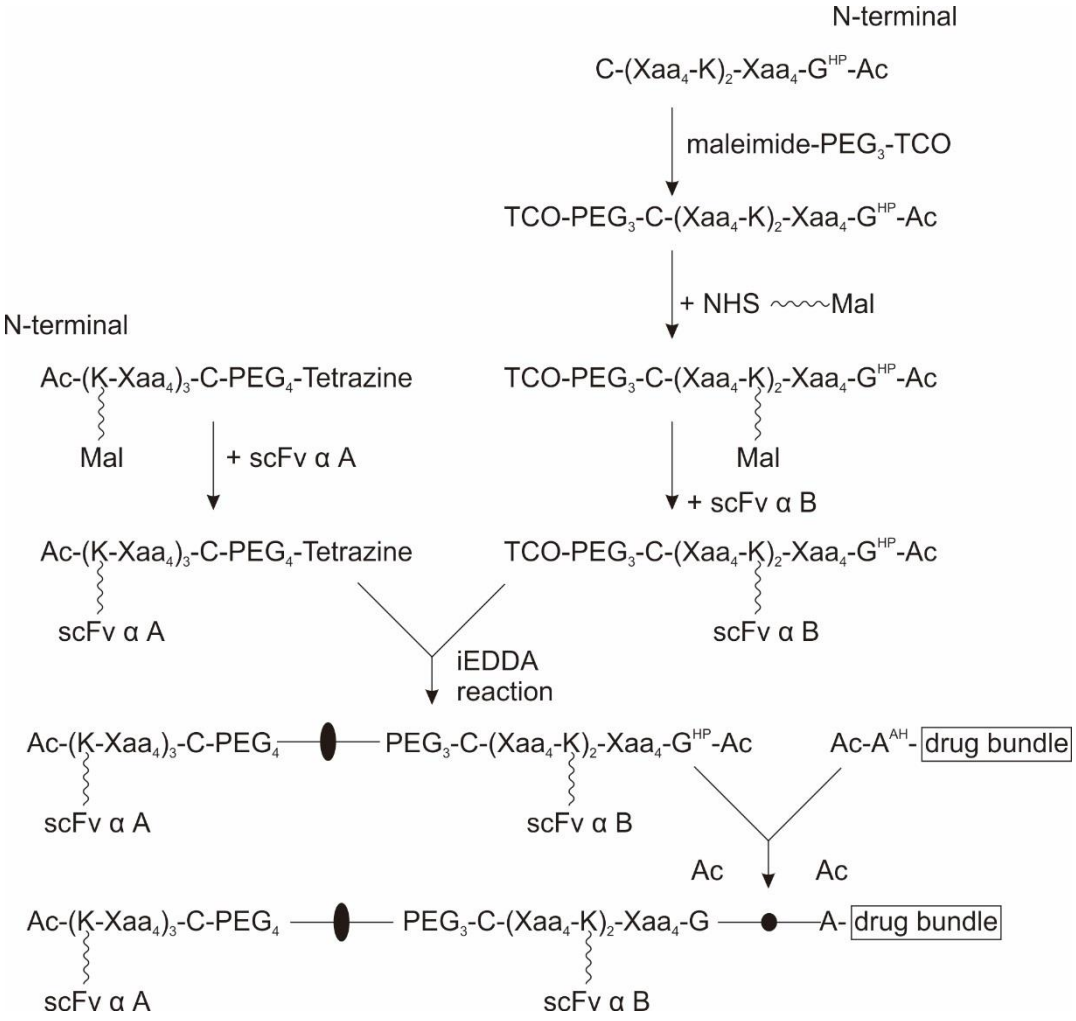


[280] In step 1, two coupling arms are respectively linked to the C residues of the linker units, in which one of the coupling arms has a maleimide (Mai) group at one terminus and a tetrazine group at the other terminus, while the other coupling arm has a Mai group at one terminus and a TCO group at the other terminus. In step 2, the linking arms are respectively linked to the lysine (K) residues via forming the amide bond between the linking arm and the K residue. Then, in step 3, three anti-A antigen scFvs (scFv α A) and three anti-B antigen scFvs (scFv α B) are respectively linked to the linking arms of the linker units

via the thiol-maleimide reaction. Finally, in step 4, the two linker unit are coupled to each other via the iEDDA reaction occurred between the tetrazine and TCO group.

[281] Scheme 12 provides an example of preparing a molecular construct comprising three linker units, in which two linker units respectively linked with the scFv α A and scFv α B are coupled to each other via the iEDDA reaction as described in Scheme 11 and a third linker unit couples with the second linker unit via a CuAAC reaction.

<<Scheme 12 Preparation of molecular construct having three linker units with three functional elements>>



[282] In this example, the third linker unit is a drug bundle. However, this reaction scheme can be applied to a third linker unit with other elements, such as scFv. In the present example, the center linker unit (that is, the second linker unit) comprises an HPG

(G^{HP}) residue at its N-terminus, and accordingly, a drug bundle conjugated with an AHA (A^{AH}) residue can be linked to the second linker unit via the CuAAC reaction occurred between the HPG and AHA residues. Alternatively, the center linker unit may comprise an AHA residue at its Nor C-terminus, and can couple with a third linker unit carrying a coupling arm with a DBCO or another strained alkyne group via a SPAAC reaction. The thus-formed molecular construct in scheme 12 has three functional elements: scFv α A, scFv α B, and drug molecule. The molecular constructs with three linker units can carry three sets of scFv, of which two sets as targeting elements and one set as effector elements, or one set as targeting elements and two sets as effector elements.

[283] When the targeting and effector elements are all scFv, and linking arms of 600 daltons (12 EG units) are used, a molecular construct with a total of six scFvs has a molecular weight of about 170,000 daltons. A molecular construct with seven scFvs has a molecular weight of about 200,000 daltons, and a molecular construct with eight scFvs has a molecular weight of about 230,000 daltons. Most of the molecular constructs of this invention have molecular weights smaller than 200,000 daltons, and a few molecular constructs have molecular weights in 200,000-250,000 daltons.

[284] When four different sets of scFv are to be carried in one molecular construct, it is preferable to have one linker unit carrying a joined single-chain, bi-specific scFv (bi-scFv), such as scFv1-scFv2 (e.g., specific for HER2 and HER3), and the other two linker units each carrying one scFv (i.e., scFv3 and scFv4 respectively). There are two ways to construct bi-specific scFv1-scFv2. In the "tandem" configuration, V_L1-V_H1-V_L2-V_H2 or V_H1-V_L1-V_H2-V_L2 is arranged; in the "diabody" configuration, V_L2-V_L1-V_H1-V_H2 or V_H2-V_H1-V_L1-V_L2 is arranged. Proper linkers with GGGGS (SEQ ID NO: 6) repeats or other sequences are placed between the immunoglobulin domains.

[285] In our experience, a peptide or a PEG linker, which contain maleimide and azide groups may become polymerized upon long-term storage, due to the automatic coupling reaction between the maleimide and azide groups. Therefore, it is preferable that each linker unit is prepared freshly and independently, and processed to connecting the targeting or effector elements onto the linker units, and the coupling of the linker units through click reaction without delay. An alternative preferred embodiment is that the targeting elements

group in one of the linker units is then converted to azide with a short homo-bifunctional linker with azide at both ends. The linker units, one with alkyne and the other with azide, are then coupled via a click reaction.

[286] The preferred linking arms for this invention are PEG. The length of the linking arms is important for several considerations. It should be long enough to allow flexibility of the linked scFv or other types of functional elements to reach targeted antigenic sites on targeted cell surface without steric constraints; yet not long enough to cause intra-molecular and inter-molecular tangling of the linking arms and their linked scFv fragments or functional elements, or to unnecessarily increase the size of the whole molecular construct for hindering tissue penetration. Linking arms that are too long may also fail to pull antigen molecules to form compacted clusters, if such clusters are required to initiate signal-transducing process for apoptosis or other cellular effects. The optimal length of linking arms for different types of combinations of targeted antigens and their binding agents may be determined by any skilled artisan in the related field without undue experimentation. A linking arm of NHS-$(PEG)_{12}$-Maleimide (approximately 500 daltons) is preferred in a number of molecular construct of this invention. A fully stretched $(PEG)_{12}$ has a length of 40-50 Å.

[287] Applicable linking arms and coupling arms are not limited by PEG chains. Peptides comprising glycine, serine and other amino acid hydrophilic residues, and polysaccharides, and other biocompatible linear polymers, which are modified to contain NHS and maleimide groups, can be used.

[288] For certain therapeutic applications, it is desirable that the effector elements in the molecular constructs of this disclosure be released from the linking arms, so that they can get into cells in the targeted site, including cells bound by the targeting elements or surrounding cells, to cause pharmacological effects. In those cases, a cleavable bond is engineered in the linking arm. Cleavable bonds, which are susceptible for cleavage by hydrolysis, acid exposure, reduction, and enzymes, have been developed. For example, peptide segments susceptible to matrix metalloproteinases, which are present in inflammatory tissues, have been used in constructing therapeutic constructs. One

embodiment of the present invention is to use PEG linkers with S-S bond adjacent to the maleimide group NHS-PEG_{2-12}-S-S-maleimide, wherein S-S is a disulfide bond, which can be slowly reduced.

[289] According to some embodiments of the present disclosure, the targeting element described in above-mentioned embodiments is selected from the group consisting of a growth factor, a peptide hormone, a cytokine, and an antibody fragment; and the effector element is an immunomodulant, a chelator complexed with a radioactive nuclide, a therapeutic drug, a cytokine, a soluble receptor, or an antibody.

[290] In the embodiments, the antibody is in the form of an antigen-binding fragment (Fab), a variable fragment (Fv), a single-chain variable fragment (scFv), a single domain antibody (sdAb), or a bi-specific single-chain variable fragment (bi-scFv). According to one embodiment, the bi-scFv is a bi-specific tandem scFv or a bi-specific diabody scFv.

[291] In order to retain diffusing ability of the molecular constructs, a molecular size smaller than 250,000 daltons is preferred. Thus, scFv fragments are preferred for most of the embodiments. At the DNA level, genes are constructed so that the V_L and V_H are linked as a single polypeptide in either order (V_L-V_H or V_H-V_L) by a peptide linker of 10-25 amino acid residues with glycine and serine being the major residues. At the C-terminal, a short stretch with glycine and serine and a terminal residue cysteine is engineered. Recombinant scFv and bi-scFv can be produced in bacteria, such as *E. coli* and *Pseudomonas putida*, in yeast, such as *Pichia pastoris*, or in mammalian cells, such as CHO and HEK293 cell lines.

[292] The inventors' laboratory have produced a large number of IgG antibodies, Fab, scFv and various antibody fragments, Fc-based proteins, and other recombinant antibodies in HEK293 and CHO cell lines for experimentation in *in vitro* systems and in animal models. Our laboratory has also developed cell lines for producing antibodies for human clinical trials. The HEK293 transient expression system can be conveniently employed to produce up to 1 g of IgG or antibody fragments using a few flasks of 1-2 liters in the research laboratory. The scFv fragments to be used in the molecular constructs of this invention generally do not have a carbohydrate modification, and carbohydrate modification is not required for the binding activity of the scFv to their antigenic targets. Furthermore, only

one disulfide bond and one terminal cysteine are present in the scFv fragment. Therefore, small-scale bacterial expression systems have been developed as a manufacturing alternative for producing scFv. With *E. coli*, expression systems for recovering scFv in intracellular inclusion bodies, in periplasm, and in secreted form have been employed. The scFv can be purified in most cases with an affinity column with Protein L, which interacts with V_H of most K light chain, or in other cases with ion-exchange columns.

[293] The examples of this invention based on the joint-linker platform employ mainly scFv and Fab as the targeting and/or effector elements. However, specific binding molecules may also be screened from large libraries of binding molecules based on sdAb or other antibody fragments. Libraries of binding molecules, which are not based on immunoglobulin domains but resemble antibodies in having specific binding affinities to selected target molecules, include (1) aptamers, which are oligonucleotides or short peptides selected for binding to target molecules, (2) fynomers, which are small binding proteins derived from the human Fyn SH3 domain, (3) affimers, which are binding proteins derived from the cysteine protein inhibitor family of cystatins, and (4) DARPins (designed ankyrin repeat proteins), which are genetically engineered proteins with structures derived from the natural ankyrin proteins and consist of 3, 4, or 5 repeat motifs of these proteins. These antibody-mimetics have molecular weights of about 10K to 20K daltons.

[294] ***II-(ii) Functional Elements Suitable for Use with Joint-linker Molecular Construct***

[295] As discussed above, the present joint-linker comprises at least two linker units, in which the first linker unit carries one or more targeting elements, and the second linker unit carries one or more effector elements or pharmacokinetic property-enhancing elements, and vice versa. Specific examples of the functional elements incorporated in the present joint-linker molecular construct for treating a particular disease are discussed below.

[296] To treat central nervous system (CNS) diseases, an scFv specific for transferrin receptor can be used as the targeting element of the present joint-linker molecular constructs, in connection with effector elements suitable for the particular CNS disease. For example, joint-linker molecular constructs for the treatment of multiple sclerosis may use fingolimod, fingolimod phosphate, interferon-β, or an scFv specific for integrin-α4 as the

effector element. In the case of Alzheimer's disease, illustrative joint-linker molecular constructs can use an scFv specific for β-amyloid as the effector element.

[297] For diseases or conditions associated with infection, an scFv specific for CD16b or CD32 can be used as the effector element, while the targeting element depends on the cause of the infection. Specifically, when the infection is caused by a virus, an scFv specific for a viral protein is used as the targeting element, whereas when the infection is caused by a bacterium, an scFv specific for a bacterial protein is used as the targeting element. Illustrative examples of viral proteins include, but are not limited to, F protein of respiratory syncytia virus (RSV), gp120 protein of human immunodeficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, and glycoprotein of cytomegalovirus. Non-limiting examples of bacterial protein include endotoxin of Gram(-) bacteria, surface antigen of Clostridium difficile, lipoteichoic acid of *Staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, and Shiga-like toxin type I or II of *Escherichia coli*.

[298] ***II-(iii) Use of Joint-linker Molecular Construct***

[299] The present disclosure also pertains to method for treating various diseases using the suitable joint-linker molecular construct. Generally, the method comprises the step of administering to a subject in need of such treatment an effective amount of the joint-linker molecular construct according to embodiments of the present disclosure.

[300] **PART III Fc-based Molecular Constructs for Treating Central Nervous System Diseases or Infectious Diseases**

[301] ***III-(1) Structure of Fc-based Molecular Construct***

[302] In the broad sense of the Fc-based configuration, immunoglobulin antibody can serve as the base of a targeting or effector element, and its corresponding effector or targeting element can be incorporated at the C-terminal of its two heavy γ chains in the form of scFv domains. For a typical "Fc-based" configuration, two-chain IgG.Fc is used as the base of the molecular platform. Each of the polypeptide chain is fused with one or two targeting and one or two effector elements, for a total of two to three elements on each chain. The T-E molecule with an Fc-based configuration will have a total of four to six elements (e.g., scFv or any other antibody fragments). Optionally, the Fc portion of the molecular constructs also carries Fc-mediated effector functions, ADCC, and/or

complement-mediated activation. While in certain other applications, such Fc-mediated effector functions are avoided.

[303] In designing the Fc-based molecular constructs, targeting elements are positioned at the N- or C-terminus. If the effector elements function by binding to a cell surface component, they should also be positioned at the terminus. If the effector elements function by binding to and neutralizing soluble factors, they can be positioned between a terminal targeting or effector element and CH2-CH3.

[304] By selecting the T-E elements of the present Fc-based molecular construct, the molecular construct can be used to treat central nervous system (CNS) diseases or infectious diseases. The present disclosure is also advantageous in that, in some embodiments, it utilizes the linker unit according to the first aspect of the present disclosure, which provides a facile means for controlling the number of the targeting and effector elements of the present Fc-based molecular constructs. Depending on the targeting and/or effector elements selected, the present Fc-based molecular construct may take different configurations, which are discussed below, respectively.

[305] In the present Fc-based molecular constructs, both the targeting element and effector element are antibodies or fragments thereof.

[306] Referring to Figure 8A, which is a schematic diagram illustrating an Fc-based molecular construct 800A according to certain embodiments of the present disclosure. As illustrated, the Fc-based molecular construct 800A comprises two identical CH2-CH3 chains 810, a pair of effector elements E1 linked to the N-termini of the CH2-CH3 chains 810, and a pair of targeting elements T1 linked to the C-termini of the CH2-CH3 chains 810. In this illustrative configuration, both the targeting element T1 and effector element E1 are scFvs.
[3067] The Fc-based molecular construct 800B illustrated in Figure 8B is quite similar to the Fc-based molecular construct 800A of Figure 8A in structure, except that the two effector elements E1 are respectively linked to the C-termini of the CH2-CH3 chains 810, while the two targeting effectors are respectively linked to the C-termini of the CH2-CH3 chains 810.

[308] According to certain embodiments, both the effector elements and targeting elements are linked to the N-termini of the CH2-CH3 chains. For example, when both the effector element and the targeting element are in the form of single-chain variable fragments (scFvs), the effector element and the targeting element may be linked in a tandem or diabody coriiguration, thereby forming a bispecific scFv that is linked to the N-terminus of the CH2-CH3 chain.

[309] The Fc-based molecular construct 800C (Figure SC) comprises an Fe portion, and accordingly, each CH2-CH3 chain 810 has a T1-E1 bispecific scFv linked to the N-terminus thereof.

[310] In some examples, the pair of effector elements or the pair of the targeting elements takes a Fab configuration (i.e., consisting of the V_H-CH1 domain and the V_L-C_K domain); this Fab fragment is linked to the N-termini of the CH2-CH3 chains, so that the Fc-based molecular construct adopts an IgG configuration. In these cases, the pair of elements that is not in the Fab configuration may be linked to the C-termini of the pair of CH2-CH3 segments.

[311] For example, in the Fc-based molecular construct 900 of Figure 9, each of the two targeting elements T1 comprises the V_H-CH1 domain 820 and the V_L-C_K domain 825, thereby forming a Fab configuration 830 that is linked to the N-termini of the CH2-CH3 chains 810, so that the Fc-based molecular construct 900 adopts the IgG configuration. In this case, the pair of effector elements E1 is linked to the C-termini of the pair of CH2-CH3 chains 810.

[312] According to some embodiments, the present Fc-based molecular construct has an effector element which is a peptide.

[313] For example, according to certain embodiments of the present disclosure, the effector element can be a peptide with certain therapeutic effect, while the targeting element is an antibody or a fragment thereof (see, Figures 10A and 10B). As illustrated, the Fc-based molecular construct 1000A of Figure 10A comprises a pair of targeting elements T1 (as scFvs) linked to the N-termini of the pair of CH2-CH3 segments 1210, and a pair of

effector elements E1 (in the form of therapeutic peptides) linked to the C-termini of the pair of CH2-CH3 segments 1210.

[314] Similarly, in the Fc-based molecular construct 1000B of Figure 10B, the pair of targeting elements T1 (as scFvs) is linked to the C-termini of the pair of CH2-CH3 segments 1210, whereas the pair of effector elements E1 (in the form of therapeutic peptides) is linked to the N-termini of the pair of CH2-CH3 segments 1210.

[315] As could be appreciated, for Fc-based molecular constructs that use a peptide as the effector element, the targeting element can be constructed into a Fab fragment, so that the molecular constructs take the IgG configuration.

[316] In the configuration illustrated in Figures 8A to 10B, the CH2-CH3 chains are adopted from human immunoglobulins γ1 or γ4. In general, γ1 is chosen, when Fc-mediated functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated activity (inflammatory activation or target cell lysis), are desired. In the case where Fc-mediated functions are avoided, γ4 is chosen for constructing the present Fc-based molecular constructs.

[317] ***III-(ii) Functional Elements Suitable for Use with Fc-based Molecular Construct***

[318] Now that the basic structural arrangements of the Fc-based molecular constructs have been discussed above, certain combinations of particular effector element(s) and targeting element(s) are provided below for the illustration purpose.

[319] To treat central nervous system (CNS) diseases, an antibody (or a fragment thereof) specific for transferrin receptor can be used as the targeting element, in connection with effector elements suitable for the particular CNS disease. For example, Fc-based molecular constructs for the treatment of multiple sclerosis may use an scFv specific for integrin-α4 as the effector element. In the case of Alzheimer's disease, illustrative Fc-based molecular constructs can use an scFv specific for β-amyloid as the effector element. The above-mentioned Fc-based molecular constructs for treating CNS diseases may take the configuration described in connection with any of Figures 8A to 8C, and Figure 9.

[320] Fc-based molecular constructs for treating multiple sclerosis may also use INF-β1a or INF-β1b as the effector elements. In this case, the Fc-based molecular constructs may take the configuration described in connection with Figures 10A or 10B.

[321] In constructing Fc-based molecular constructs for treating diseases/conditions associated with infection (such as viral infections or bacterial infections), one may use an antibody (or a fragment thereof) specific for a viral protein or bacterial protein as the targeting element. As to the effector elements for treating infections, an antibody (or a fragment thereof) specific for CD32 or CD16b can be used. These Fc-based molecular constructs may take the configuration described in connection with any of Figures 8A to 8C, and Figure 9.

[322] The essence of this invention is the rationalization and conception of the specific combination or pairing of the targeting and effector elements. The adoption of Fc-fusion configuration in the molecular constructs is a preferred embodiment. It is conceivable for those skilled in the arts to link the pairs of targeting and effector elements of this invention employing other molecular platforms, such as peptides, proteins (e.g., albumin), polysaccharides, polyethylene glycol, and other types of polymers, which serve as a structural base for attaching multiple molecular elements.

[323] ***III-(iii) Use of Fc-based Molecular Construct***

[324] The present disclosure also pertains to method for treating CNS diseases using the suitable Fc-based molecular construct. Generally, the method comprises the step of administering to a subject in need of such treatment an effective amount of the Fc-based molecular construct according to embodiments of the present disclosure.

[325] The present disclosure further pertains to method for treating infections using the suitable Fc-based molecular construct. Generally, the method comprises the step of administering to a subject in need of such treatment an effective amount of the Fc-based molecular construct according to embodiments of the present disclosure.

[326] **EXPERIMENTAL EXAMPLES**

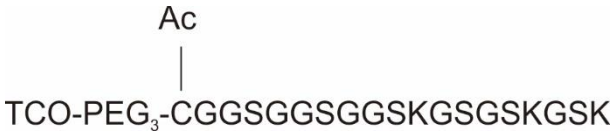
[327] **Example 1: Synthesis of peptide 1 (SEQ ID NO: 18), peptide 2 (SEQ ID NO: 27)**

and peptide 3 (SEQ ID NO: 19) as peptide cores, and conjugation of the SH group of their cysteine residue with maleimide-PEG_3-transcyclooctene (TCO) as a coupling arm

[328] The synthesized peptides 1, 2 and 3 (Chinapeptide Inc., Shanghai, China) were processed similarly. Each peptide was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl and 5 mM EDTA at a final concentration of 2 mM. The dissolved peptide was reduced by 1 mM tris(2-carboxyethyl)phosphine (TCEP) at 25°C for 2 hours. For conjugating the SH group of the cysteine residue with maleimide-PEG_3-TCO (Conju-probe Inc.) to create a functional linking group TCO, the peptide and maleimide-PEG_3-TCO were mixed at a 1/7.5 molar ratio and incubated at pH 7 and 25°C for 18 hours. The TCO-conjugated peptide was purified by reverse phase HPLC on a Supelco C18 column (250 mm × 10 mm; 5 μm), using a mobile phase of acetonitrile and 0.1% trifluoroacetic acid, a linear gradient of 0% to 100% acetonitrile over 30 minutes, at a flow rate of 1.0 ml/min and a column temperature of 25°C.

[329] The identification of the synthesized TCO-peptides (illustrated below) was carried out by MALDI-TOF mass spectrometry. Mass spectrometry analyses were performed by the Mass Core Facility at the Institute of Molecular Biology (IMB), Academia Sinica, Taipei, Taiwan. Measurements were performed on a Bruker Autoflex Ill MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany).

[330] The synthesized TCO-peptide 1, as illustrated below, had a molecular weight (m.w.) of 2,078.9 daltons.



[331] The synthesized TCO-peptide 2, as illustrated below, had a m.w. of 2,020.09 daltons.

[332] The TCO-peptide 3, as illustrated below, had a m.w. of 3,381.85 daltons.

[333] **Example 2: Synthesis of peptide 1 as a peptide core, and conjugation of the SH group of its cysteine residue with maleimide-PEG$_4$-tetrazine as a coupling arm**

[334] The synthesized peptide 1 (Chinapeptide Inc., Shanghai, China) was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl and 5 mM EDTA at a final concentration of 2 mM. The dissolved peptide was reduced by 1 mM tris(2-carboxyethyl)phosphine (TCEP) at 25°C for 2 hours. For conjugating the SH group of the cysteine residue with maleimide-PEG$_4$-tetrazine (Conju-probe Inc., San Diego, USA) to create a functional linking group tetrazine, the peptide and maleimide-PEG$_4$-tetrazine were mixed at a 1/5 ratio and incubated at pH 7 and 4°C for 18 hours. The tetrazine-conjugated peptide was purified by reverse phase HPLC on a Supelco C 18 column (250 mm × 10 mm; 5 µm), using a mobile phase of acetonitrile and 0.1 % trifluoroacetic acid, a linear gradient of 0% to 100% acetonitrile over 30 minutes, at a flow rate of 1.0 ml/min and a column temperature of 25°C.

[335] The synthesized tetrazine-peptide 1, as illustrated below, had a m.w. of 2,185.2 daltons.

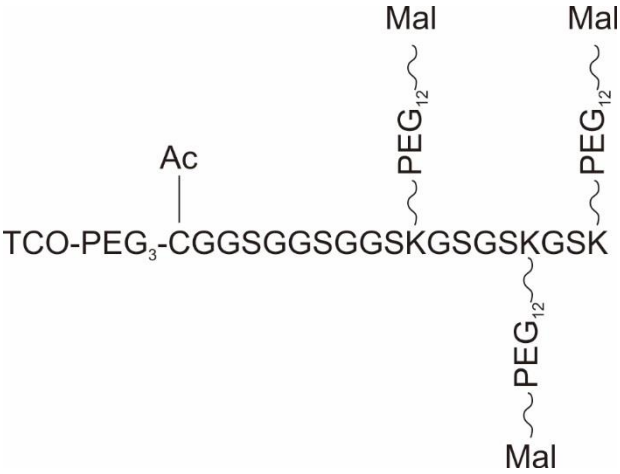
[336] **Example 3: Synthesis of a linker unit by conjugating NHS-PEG$_{12}$-Mal to NH$_2$ groups of TCO-peptide 1 as linking arms**

[337] Three linking arms of PEG$_{12}$-maleimide were attached to the peptide core, TCO-peptide 1. The crosslinker, NHS-PEG$_{12}$-maleimide (succinimidyl-[(N-maleimido-propionamido)-dodecaethyleneglycol] ester, was purchased from Thermo Fisher Scientific Inc. (Waltham, USA). The conjugation procedure was performed per the manufacturer's instruction. Briefly, the peptide with lysine residues was dissolved in the conjugation buffer, phosphate buffered saline (pH 7.5) at 100 mM. NHS-PEG$_{12}$-maleimide crosslinker was then added to the dissolved peptide at a 1 mM final concentration (10-fold molar excess over 0.1 mM peptide solution). The reaction mixtures were incubated for 18 hours at room temperature. The maleimide-PEG$_{12}$-conjugated TCO-peptide 1 was purified by reverse phase HPLC on a Supelco C18 column (250 mm X 4.6 mm; 5 µm), using a mobile phase of

acetonitrile and 0.1% trifluoroacetic acid, a linear gradient of 0% to 100% acetonitrile over 30 minutes, at a flow rate of 1.0 ml/min and a column temperature of 25°C.

[338] The identification of the maleimide-PEG_{12}-conjugated TCO-peptide 1 was carried out by mass spectrometry MALDI-TOF.

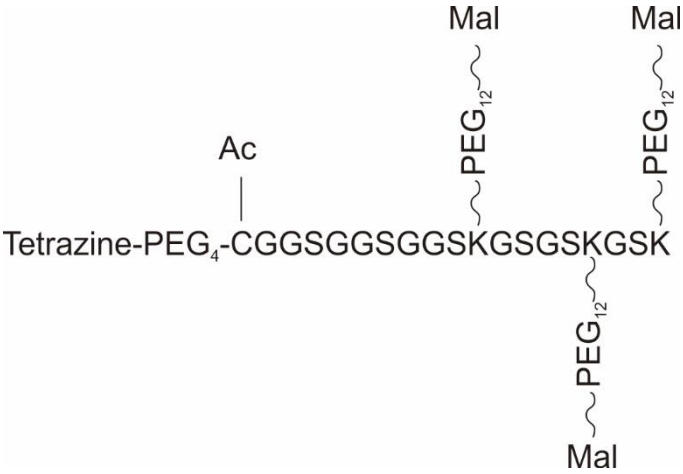
[339] The synthesized maleimide-PEG_{12}-conjugated TCO-peptide1 had a m.w. of 4,332 daltons. As illustrated below, the maleimide-PEG_{12}-conjugated TCO-peptide1 is a peptide-core based linker unit carrying one TCO group and three PEG linking arms with maleimide groups.



[340] **Example 4: Synthesis of a linker unit by conjugating NHS-PEG_{12}-Mal to NH_2 groups of tetrazine-peptide 1 as linking arms**

[341] Three linking arms of PEG_{12}-maleimide were attached to the peptide core, tetrazine-peptide 1. The crosslinker, NHS-PEG_{12}-maleimide (succinimidyl-[(N-maleimido-propionamido)-dodecaethyleneglycol] ester, was purchased from Thermo Fisher Scientific Inc. (Waltham, USA). The conjugation procedure was performed per the manufacturer's instruction. Briefly, the peptide with lysine residues was dissolved in the conjugation buffer, phosphate buffered saline (pH 7.5) at 100 mM. NHS-PEG_{12}-maleimide crosslinker was then added to the dissolved peptide at a 1 mM final concentration (10-fold molar excess over 0.1 mM peptide solution). The reaction mixtures were incubated for 18 hours at room temperature. The maleimide-PEG_{12}-conjugated tetrazine-peptide 1 was purified by reverse phase HPLC on a Supelco C18 column (250 mm X 4.6 mm; 5 µm), using a mobile phase of acetonitrile and 0.1 % trifluoroacetic acid, a linear gradient of 0% to 100% acetonitrile over 30 minutes, at a flow rate of 1.0 ml/min and a column temperature of 25°C.

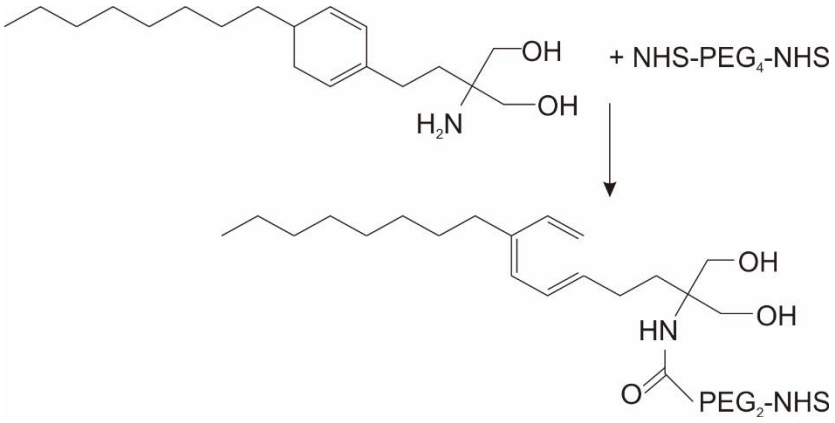
[342] The synthesized maleimide-PEG12-conjugated tetrazine-peptide1, as illustrated below, was a peptide-core based linker unit carrying one tetrazine group and three PEG linking arms with maleimide groups. Figure 11 shows the MALDI-TOF result, indicating that the construct had a m.w. of 4,461 daltons.



[343] **Example 5: Conjugation of fingolimod and fingolimod phosphate molecule with an NHS-PEG$_5$-NHS cross-linker**

[344] Fingolimod was purchased from Biotang Inc. (Lexington, USA) and fingolimod phosphate from KM3 Scientific Corporation (New Taipei City, Taiwan). The NH2 group of fingolimod molecule was reacted with a homo-bifunctional crosslinker, NHS-PEG$_5$-NHS, as shown in scheme 13. Fingolimod was dissolved in 100% DMSO at a final concentration of 10 mM; NHS-PEG$_5$-NHS was dissolved in 100% DMSO at a 250 mM final concentration. To activate the NH2 group of fingolimod, 6% (v/v) of basic sodium phosphate buffer (pH 12.7) was added to the fingolimod solution and then incubated for 10 minutes. NHS-PEG$_5$-NHS crosslinker was added to the dissolved fingolimod solution at a final concentration of 30 mM (3-fold molar excess over 10 mM fingolimod solution). The reaction mixture was incubated for 3 hours at room temperature.

<<Scheme 13 Conjugation of fingliomod molecule with an NHS-PEG$_5$-NHS cross-linker>>

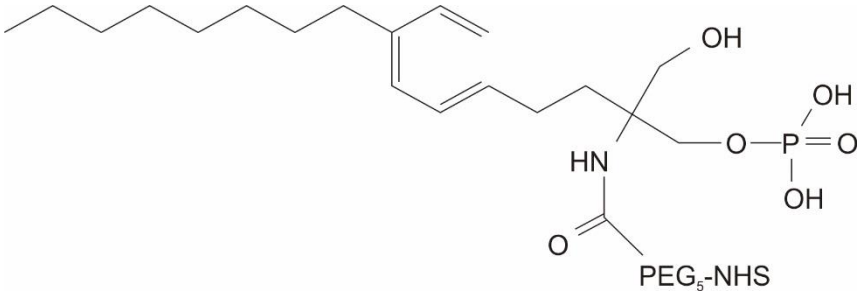


[345] Fingolimod phosphate was dissolved in 100% DMSO at a final concentration of 5 mM, and NHS-PEG_5-NHS crosslinker was dissolved in 100% DMSO at a final concentration of 250 mM. NHS-PEG_5-NHS crosslinker was added to the dissolved fingolimod phosphate solution at a 15 mM final concentration (3-fold molar excess over 5 mM fingolimod phosphate solution). The reaction mixture was incubated for 3 hours at room temperature, then 18% (v/v) acid sodium phosphate buffer (pH=0.88) was added to quench the reaction. The solvent was evaporated under vacuum.

[346] NHS-PEG_5-conjugated fingolimod and NHS-PEG_5-conjugated fingolimod phosphate were dissolved in 30% acetonitrile, and then purified using reverse phase HPLC on a Supelco C18 column (250 mm X 4.6 mm; 5 µm), using a mobile phase of acetonitrile and 0.1 % trifluoroacetic acid, a linear gradient of 30% to 100% acetonitrile over 30 minutes, at a flow rate of 1.0 ml/min and a column temperature of 25°C.

[347] Figure 12 shows that the synthesized NHS-PEG_5-conjugated fingolimod, as illustrated in scheme 13, had a m.w. of 725.41 daltons.

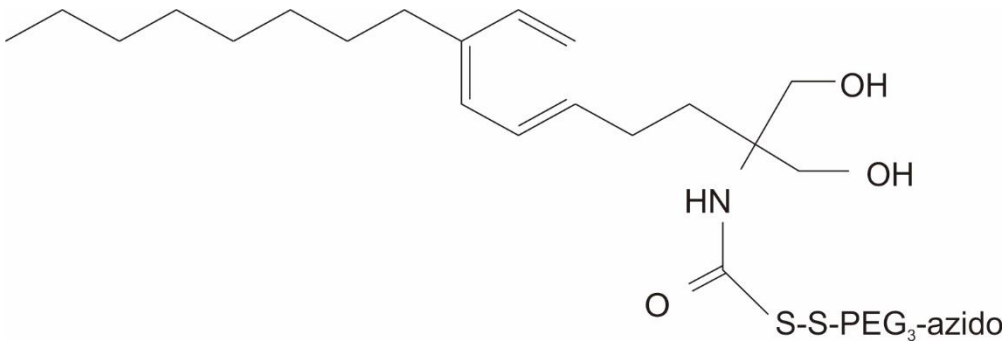
[348] The synthesized NHS-PEG_5-conjugated fingolimod phosphate, as illustrated below, had a m.w. of 803.3 daltons.



[349] **Example 6: Conjugation of fingolimod molecule with an NHS-S-S-PEG_3-azido linking arm**

[350] The NH_2 group of fingolimod molecule was reacted with a hetero-bifunctional cleavable linker, NHS-S-S-PEG_3-azido (Conju-probe Inc.), at a 1:3 molar ratio. The product, azido-PEG_3-S-S-fingolimod was purified by HPLC to remove the excess, unreacted fingolimod molecules. The procedures for conjugation and purification were similar to those described in the preceding example.

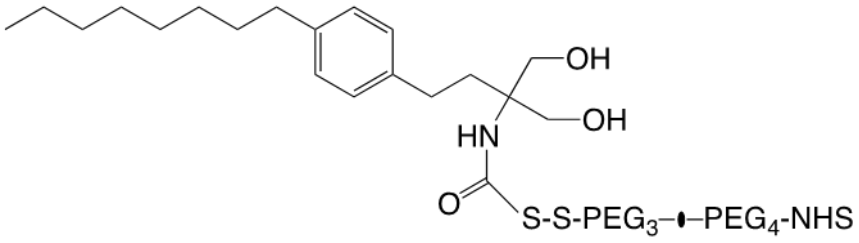
[351] The synthesized azido-PEG_3-S-S-conjugated fingolimod, as illustrated below, had a m.w. of 629.33 daltons.



[352] **Example 7: Conjugation of azido-PEG_3-S-S-conjugated fingolimod molecule with a NHS-PEG_4-dibenzylcyclooctyne (DBCO) crosslinker**

[353] Azido-PEG_3-S-S-conjugated fingolimod molecule was dissolved in 100% DMSO at a final concentration of 10 mM, and NHS-PEG_4-0BCO crosslinker was dissolved in 100% DMSO at a final concentration of 250 mM. 5 µl of NHS-PEG_4-DBCO crosslinker was added to 400 µl of the dissolved azido-PEG_3-S-S-conjugated fingolimod solution to a final molar ratio of 1/3.2 (NHS-PEG_4-0BCO: azido-PEG_3-S-S-conjugated fingolimod) in 100 mM sodium phosphate buffer at pH 7.5. The reaction mixture was incubated for 3 hours at room temperature.

[354] The synthesized NHS-PEG_4-PEG_3-S-S-conjugated fingolimod, as illustrated below, had a m.w. of 1,278.61 daltons. The two isotopic peaks were also visible in the MS spectrum at 1,279.64 and 1,280.635, corresponding to $[M+H+1]^+$ and $[M+H+2]^+$.

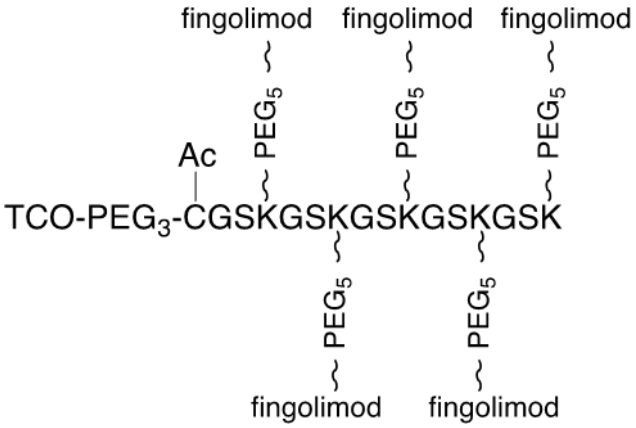


[355] **Example 8: Conjugation of NHS-PEG_5-conjugated fingolimod molecules to TCO-peptide 2 and 3**

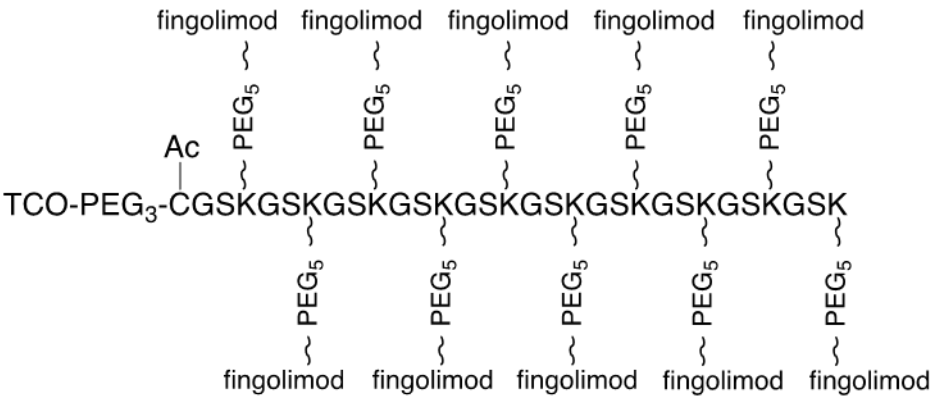
[356] TCO-peptide 2 was dissolved in 100 mM sodium phosphate buffer at pH 7.5 to a concentration of 20 mM, and NHS-PEG_5-conjugated fingolimod was dissolved in 100% DMSO to a concentration of 50 mM. TCO-peptide 2 and NHS-PEG_5-conjugated fingolimod were mixed at 1/42 molar ratio in 100% DMSO and incubated for 3 hours at room temperature. Subsequently, additional TCO-peptide 2 was added to the reaction solution to a final molar ratio of 1/13.5 (TCO-peptide 2: NHS PEG_5-conjugated fingolimod) in 100% DMSO. The mixture was further incubated for 3 hours at room temperature. Figure 13 shows that the drug bundle of TCO-peptide 2 with fingolimod had a m.w. of 5,069 daltons.

[357] TCO-peptide 3•was dissolved in 100 mM sodium phosphate buffer at pH 7.5 to a concentration of 10 mM, and NHS-PEG_5-conjugated fingolimod was dissolved in 100% DMSO to a concentration of 50 mM. TCO-peptide 3 and PEG_5-NHS-conjugated fingolimod were mixed at 1/42 molar ratio at room temperature for overnight. Figure 14 shows that the drug bundle of TCO-peptide 3 with fingolimod had a m.w. of 9,479 daltons, indicating that ten fingolimod molecules were conjugated to the TCO-peptide 3 linker unit.

[358] The synthesized drug bundle, as illustrated below, was composed of a linker unit with a free TCO functional group and a set of five fingolimod molecules.



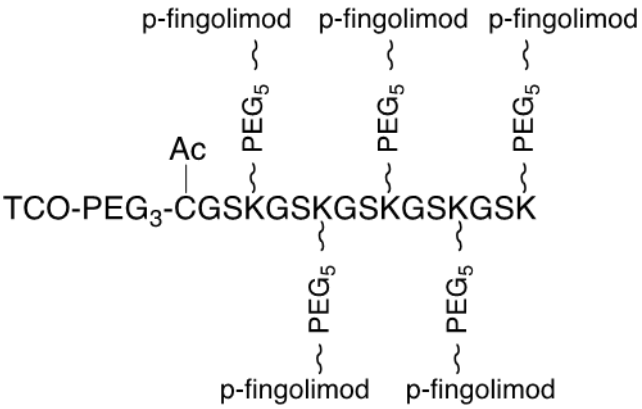
[359] The second synthesized drug bundle, as illustrated below, was composed of a linker unit with a free TCO functional group and a set of ten fingolimod molecules.



[360] **Example 9: Conjugation of NHS-PEG$_5$-conjugated fingolimod phosphate molecules to TCO-peptide 2**

[361] TCO-peptide 2 and NHS-PEG$_5$-conjugated fingolimod phosphate were mixed at 1/42 molar ratio in 100 mM sodium phosphate buffer at pH 7.5 at room temperature for 3 hours. Mass spectrometric analysis shows that the drug bundle of TCO-peptide 2 with fingolimod phosphate had a m.w. of 5,379.16 daltons (Figure 15).

[362] The synthesized drug bundle, as illustrated below, was composed of a linker unit with a free TCO functional group and a set of five fingolimod phosphate molecules as effector elements.

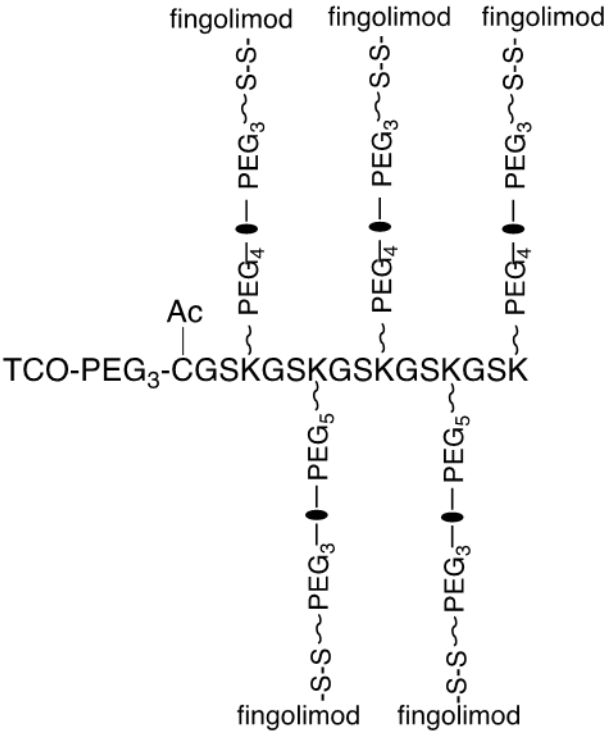


[363] **Example 10: Conjugation of NHS-PEG$_4$-PEG$_3$-S-S-conjugated fingolimod molecules to TCO-peptide2**

[364] Five NHS-PEG$_4$-PEG$_3$-S-S-conjugated fingolimod molecules were attached to TCO-peptide 2. The conjugation of NHS-PEG$_4$-PEG$_3$-S-S-conjugated fingolimod molecules to the NH_2 groups of lysine residues of the TCO-peptide 2 was performed

similarly as in the preceding example. The identification was carried out by mass spectrometry MALDI-TOF.

[365] The synthesized drug bundle, as illustrated below, had a m.w. of 7,815 daltons; it was composed of a linker unit with a free TCO functional group and a set of five fingolimod molecules



[366] **Example 11: Production of recombinant ectodomain of human CD32a by HEK293F overexpression system**

[367] The gene-encoding sequence was placed in pG1 K expression cassette. The amino acid sequence of the extracellular portion of human CD32a, which was expressed as a recombinant protein with a histidine-tag, is set forth in SEQ ID NO: 28. Recombinant ectodomain of human CD32a was expressed in Freestyle 293F suspension culture cell expression system and medium (Invitrogen, Carlsbad, USA). Freestyle 293F cells were seeded at a cell density of 1.0×10^6 viable cells/ml in 600-ml culture and maintained for 18 to 24 hours prior to transfection to ensure that the cells were actively dividing at the time of transfection. At the time of transfection, 1.0×10^7 cells in a 96-ml medium in a 2-liter Erlenmeyer shaker flask were transfected by using linear polyethylenimine with an average

molecular weight of 25 kDa (Polysciences, Warrington, USA) as a transfection reagent. The transfected cells were incubated at 37°C for 4 hours post-transfection in an orbital shaker (125 rpm), and their cell density was then adjusted to 2.5×10^6 cells/ml with a fresh medium and incubated for 4 to 5 days. Culture supernatants were harvested and protein in the media was purified using nickel affinity chromatography. Figure 16 shows SDS-PAGE analysis of purified protein of ectodomain of human CD32a.

[368] **Example 12: Production of recombinant ectodomain of human transferrin-1 receptor (TfR) by HEK293F overexpression system**

[369] The gene-encoding sequence was placed in pG1K expression cassette. The amino acid sequence of the ectodomain of human TfR1, which was expressed as a recombinant protein with a histidine-tag, is set forth in SEQ ID NO: 29. Recombinant ectodomain of human TfR1 was expressed in Freestyle 293F suspension culture cell expression system and medium (Invitrogen, Carlsbad, USA). Freestyle 293F cells were seeded at a cell density of 1.0×10^6 viable cells/ml in 600-ml culture and maintained for 18 to 24 hours prior to transfection to ensure that the cells were actively dividing at the time of transfection. At the time of transfection, 1.0×10^7 cells in 96-ml medium in a 2-liter Erlenmeyer shaker flask were transfected by using linear polyethylenimine with an average molecular weight of 25 kDa (Polysciences, Warrington, USA) as a transfection reagent. The transfected cells were incubated at 37°C for 4 hours post-transfection in an orbital shaker (125 rpm), and their cell density was then adjusted to 2.5×10^6 cells/ml with a fresh medium and incubated for 4 to 5 days. Culture supernatants were harvested and protein in the media was purified using nickel affinity chromatography. Figure 17 shows SDS-PAGE analysis of the purified protein of ectodomain of human TfR1.

[370] **Example 13: Production of scFv of mAb specific for Protein F of RSV, mAb specific for endotoxin, and mAb specific for ectodomain of CD32a by Expi293F overexpression system**

[371] The V_L and V_H of the scFv specific for Protein F of RSV were from monoclonal antibody palivizumab; the V_L and V_H of the scFv specific for endotoxin were from monoclonal antibody WN1 222-5 (Patent US 5858728); V_L and V_H of the scFv specific for ectodomain of CD32a were from MDE-8 (US Patent Application publication

US2007/0253958). The scFv derived from those antibodies were designed to contain a flexible linker of GGGGSGGGGS and a terminal cysteine residue at the C-terminus. The cysteine residue provides a sulfhydryl group for conjugation with maleimide group present at the free ends of linking arms in various linker units. To produce the scFv of mAb specific for Protein F of RSV, mAb specific for endotoxin, and mAb specific for extracellular component of CD32a, the V_L and V_H DNA sequences of the three antibodies with further codon optimization were used. DNA sequences encoding V_L-GSTSGSGKPGSGEGSTKG-V_H-$(GGGGS)_2$-C were synthesized. The amino acid sequences of the scFv of mAb specific for Protein F of RSV, mAb specific for endotoxin, and mAb specific for ectodomain of CD32a prepared for the experiments of the invention are set forth in SEQ ID NO: 30 to 32, respectively.

[372] For preparing scFv proteins using a mammalian expression system, an overexpression system based on Expi293F™ cell line were used for experimentation. The system employed ExpiFectamine™ 293 transfection kit (Life Technologies, Carlsbad, USA) consisting of the Expi293F™ cell line, the cationic lipid-based ExpiFectamine™ 293 Reagent and ExpiFectamine™ 293 transfection Enhancers 1 and 2, and the medium, which was part of the expression system (Gibco, New York, USA).

[373] The scFv-encoding sequence was placed in pG1 K expression cassette. Expi293F cells were seeded at a density of 2.0×10^6 viable cells/ml in Expi293F expression medium and maintained for 18 to 24 hours prior to transfection to ensure that the cells were actively dividing at the time of transfection. At the time of transfection, 7.5×10^8 cells in 255ml medium in a 2-liter Erlenmeyer shaker flask were transfected by ExpiFectamine™ 293 transfection reagent. The transfected cells were incubated at 37°C for 16 to 18 hours post-transfection in an orbital shaker (125 rpm) and the cells were added ExpiFectamine™ 293 transfection enhancer 1 and enhancer 2 to the shaker flask, and incubated for 5 to 6 days. Culture supernatants were harvested and scFv proteins in the media were purified using Protein L affinity chromatography.

[374] Figures 18A and 18B show SDS-PAGE and ELISA analyses of purified scFv of mAb specific for Protein F of RSV; Figures 18C and 18D show SDS-PAGE and ELISA analyses of purified scFv of mAb specific for endotoxin; Figures 18E and 18F show

SDS-PAGE and ELISA analyses of purified scFv of mAb specific for ectodomain of CD32a. The 96-well ELISA plates (Greiner Bio-one) were coated with 5 μg/ml of Protein F of RSV, μg/ml of endotoxin, and 5 μg/ml of ectodomain of CD32a, respectively. Purified scFvs were detected by HRP-conjugated protein Lat a ratio of 1:5000.

[375] The ELISA results show that each purified scFv protein bound specifically to its antigen (Protein F of RSV, endotoxin, or ectodomain of CD32a), using adalimumab scFv (anti-TNF-α scFv) as a negative control.

[376] **Example 14: Production of scFv of mAb specific for ectodomain of TfR1 and mAb specific for β-amyloid by Expi293F overexpression system**

[377] The V_L and V_H of the scFv specific for ectodomain of TfR1 were from monoclonal antibody OX26; the V_L and V_H of the scFv specific for β-amyloid were from monoclonal antibody bapineuzumab. The scFv derived from those antibodies were designed to contain a flexible linker of GGGGSGGGGS and a terminal cysteine residue at the C-terminus. The cysteine residue provides a sulfhydryl group for conjugation with maleimide group present at the free ends of linking arms in various linker units. To produce the scFv of mAb specific for ectodomain of TfR1 and mAb specific for -β-amyloid, the V_L and V_H DNA sequences of the two antibodies with further codon optimization were used. DNA sequences encoding V_L-GSTSGSGKPGSGEGSTKG-V_H-$(GGGGS)_2$-C were synthesized. The amino acid sequences of the scFv of mAb specific for ectodomain of TfR1 and mAb specific for β-amyloid prepared for the experiments of the invention are set forth in SEQ ID NO: 33 and 34, respectively.

[378] For preparing scFv proteins using mammalian expression systems, the overexpression system based on Expi293F™ cell line were used. The system employed ExpiFectamine™ 293 transfection kit (Life Technologies, Carlsbad, USA) consisting of the Expi293F™ cell line, the cationic lipid-based ExpiFectamine™ 293 Reagent and ExpiFectamine™ 293 transfection Enhancers 1 and 2, and the medium (Gibco, New York, USA).

[379] The scFv-encoding sequence was placed in pG1K expression cassette. Expi293F cells were seeded at a density of 2.0×10^6 viable cells/ml in Expi293F expression

medium and maintained for 18 to 24 hours prior to transfection to ensure that the cells were actively dividing at the time of transfection. At the time of transfection, 7.5×10^8 cells in 255ml medium in a 2-liter Erlenmeyer shaker flask were transfected by ExpiFectamine™ 293 transfection reagent. The transfected cells were incubated at 37°C for 16 to 18 hours 5 post-transfection in an orbital shaker (125 rpm) and the cells were added ExpiFectamine™ 293 transfection enhancer 1 and enhancer 2 to the shaker flask, and incubated for 5 to 6 days. Culture supernatants were harvested and scFv proteins in the media were purified using Protein L affinity chromatography. Figures 19A and 19B respectively show SDS-PAGE and ELISA analyses of purified scFv of mAb specific for ectodomain of TfR1. Figures 19C and 19D respectively show SDS-PAGE and ELISA analyses of purified scFv of mAb specific for β-amyloid. The ELISA plates were coated with 5 μg/ml of ectodomain of TfR1 and 5 μg/ml of β-amyloid, respectively. Purified scFvs were detected by HRP-conjugated protein Lat a ratio of 1:5000.

[380] The ELISA results show that each purified scFv protein bound specifically to its antigen (ectodomain of TfR1 or β-amyloid), using HRP-conjugated protein L alone as a negative control.

[381] **Example 15: Construction and selection of phage-displayed scFvs specific for ectodomain of human CD32a**

[382] The phage clones carrying the scFv specific for the ectodomain of human CD32a were obtained through a contractual arrangement with Dr. An-Suei Yang's laboratory at the Genomics Research Center, Academia Sinica, Taipei, Taiwan. The framework sequence of the GH2 scFv library was derived from G6 anti-VEGF Fab (Protein Bank Code 2FJG) and cloned into restriction sites *SfiI* and *NotI* of phagemid vector pCANTAB5E (GE Healthcare), carrying an ampicillin resistance, a lacZ promotor, a pelB leader sequence for secretion of scFv fragments into culture supernatants, a E-tag applicable for detection. The V_H and V_L domains of the scFv template were diversified separately based on the oligonucleotide-directed mutagenesis procedure; the three CDRs in each of the variable domains were diversified simultaneously. The scFv library of over 109 clones was used for selections on ectodomain of CD32a.

[383] Maxisorp 96-well plates (Nunc) coated with recombinant CD32a proteins (1

μg/100 μL PBS per well) were used for panning anti-CD32a antibodies. In brief, the wells were coated with human CD32a by shaking the coating solution in the wells for 2 hours at room temperature. The CD32a-coated wells were then treated with blocking buffer (5% skim milk in PBST (phosphate buffered saline with 0.1% tween-20)) for 1 hour at room temperature. Recombinant phages in the blocking buffer diluted to 8×10^{11} CFU/ml was added to the CD32a-coated wells for 1 hour with gentle shaking; CFU stands for colony-forming unit. The wells were then washed vigorously 10 times with PBST, followed by 6 times with PBS to remove nonspecific binding phages. The bound phages were eluted using 0.1 M HCl/glycine buffer at pH 2.2, and the elution solution was neutralized immediately by 2 M Tris-base buffer at pH 9.0. *E. coli* strain ER2738 (OD600 = ~0.6) was used for phage infection at 37 °C for 30 minutes; non-infected *E. coli* was eliminated by treating with ampicillin for 30 minutes. After ampicillin treatment, helper phage M13KO7 carrying kanamycin resistance was added for another one-hour incubation. The selected phages rescued by helper phage in the *E. coli* culture were amplified with vigorously shaking overnight at 37°C in the presence of kanamycin. The amplified phages were precipitated in PEG/NaCl, and then resuspended in PBS for the next selection-amplification cycles. A total of three consecutive panning rounds was performed on ectodomain of CD32a by repeating this selection-amplification procedure.

[384] Phage-infected ER2738 colonies of plates with dilution series were counted and phage titers were calculated, yielding the output titer/ml (CFU/ml) per panning round. A 1000-fold increase in phage output title from 1.6E+04 CFU/well to 2.2E+07 CFU/well was obtained after three rounds of panning. The phage output/input titer ratios from each round are shown in Figure 20A. For each panning round, the phage output/input titer ratios are given on the y-axis. There was clear enrichment of the positive clones over the three rounds of panning. The third panning round resulted in a 100-fold on the ratios of phage output/input titer over the first round, as the binding clones became the dominant population in the library.

[385] In a typical selection procedure, after three rounds of antigen-panning on human CD32a-coated wells in ELISA plates, approximately 80% of the bound phage particles bound to CD32a specifically in ELISA with coated CD32a.

[386] **Example 16: Single colony ELISA analysis of phage-displayed scFvs specific for ectodomain of human CD32a**

[387] *E. coli* strain ER2738 infected with single-clonal phages each harboring a selected scFv gene in its phagemid was grown in the mid-log phase in 2YT broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) with 100 μg/ml ampicillin in deep well at 37°C with shaking. After broth reaching an OD600 of 1.0, IPTG was added to a final concentration of 1 μg/ml. The plates were incubated at 37°C overnight with rigorously shaking; thereafter, the plates were centrifuged at 4000 g for 15 minutes at 4°C.

[388] For soluble scFv binding test, ELISA was carried out. In brief, Maxisorp 96-well plate (Nunc) was coated with ectodomain of CD32a (0.5 μg/100 μl PBS per well) or a negative control antigen human transferrin-1 receptor, for 18 hours with shaking at 4°C. After treated with 300 μl of blocking buffer for 1 hour, 100 μl of secreted scFv in the supernatant was mixed with 100 μl of blocking buffer and then added to the coated plate for another 1 hour. Goat anti-E-tag antibody (conjugated with H RP, 1:4000, Cat. No. AB19400, Abcam) was added to the plate for 1 hour. TM B substrate (50 μl per well) was added to the wells and the absorbance at 450 nm was measured after reactions were stopped by adding 1N HCl (50 μl per well).

[389] A total of 192 phage clones after the third round of panning were subjected to the present analysis. Among them, 12 scFv clones that bound to CD32a with a differential of OD450 greater than 10 were further characterized by sequencing genes encoding these scFvs. Six different DNA sequences were identified. Figure 20B shows the ELISA result of an scFv clone 22D1. The amino acid sequence of an scFV clone 22D1, which binds to human CD32a with an OD450 of 0.8, is shown in SEQ ID NO: 35.

[390] **Example 17: Construction and selection of phage-displayed scFvs specific for ectodomain of human TfR1**

[391] The phage clones carrying the scFv specific for the ectodomain of human TfR1 were obtained through a contractual arrangement with Dr. An-Suei Yang's laboratory at the Genomics Research Center, Academia Sinica, Taipei, Taiwan. The framework sequence of the GH2 scFv library was derived from G6 anti-VEGF Fab (Protein Bank Code 2FJG) and

cloned into restriction sites *SfiI* and *NotI* of phagemid vector pCANTAB5E (GE Healthcare), carrying an ampicillin resistance, a lacZ promotor, a pelB leader sequence for secretion of scFv fragments into culture supernatants, an E-tag applicable for detection. The V_H and V_L domains of the scFv template were diversified separately based on the oligonucleotide-directed mutagenesis procedure; the three CDRs in each of the variable domains were diversified simultaneously. The scFv library of over 109 clones was used for selections on ectodomain of CD32a.

[392] Maxisorp 96-well plates (Nunc) coated with recombinant ectodomain of TfR1 proteins (1 µg/100 µL PBS per well) were used for panning anti-TfR1 antibodies. In brief, the wells were coated with human TfR1 by shaking the coating solution in the wells for 2 hours at room temperature. The TfR1-coated wells were then treated with blocking buffer (5% skim milk in PBST (phosphate buffered saline with 0.1 % tween-20)) for 1 hour at room temperature. Recombinant phages in the blocking buffer diluted to 8×10^{11} CFU/ml was added to the TfR1-coated wells for 1 hour with gentle shaking; CFU stands for colony-forming unit. The wells were then washed vigorously 10 times with PBST, followed by 6 times with PBS to remove nonspecific binding phages. The bound phages were eluted using 0.1 M HCl/glycine buffer at pH 2.2, and the elution solution was neutralized immediately by 2 M Tris-base buffer at pH 9.0. *E. coli* strain ER2738 (OD600 = ~0.6) was used for phage infection at 37 °C for 30 minutes; non-infected *E. coli* was eliminated by treating with ampicillin for 30 minutes. After ampicillin treatment, helper phage M13K07 carrying kanamycin resistance was added for another one-hour incubation. The selected phages rescued by helper phage in the *E. coli* culture were amplified with vigorously shaking overnight at 37 °C in the presence of kanamycin. The amplified phages were precipitated in PEG/NaCl, and then resuspended in PBS for the next selection-amplification cycles. A total of three consecutive panning rounds was performed on ectodomain of TfR1 by repeating this selection-amplification procedure.

[393] Phage-infected ER2738 colonies of plates with serial dilutions were counted and phage titers were calculated, yielding the output titer/ml (CFU/ml) per panning round. A 10^4-fold increase in phage output title from 3.74E+03 CFU/well to 1.5E+08 CFU/well was obtained after three rounds of panning. The phage output/input titer ratios from each round are shown in Figure 21A For each panning round, the phage output/input titer

ratios are given on the y-axis. There was clear enrichment of the positive clones over the three rounds of panning. The third panning round resulted in a 104-fold on the ratios of phage output/input titer over the first round, as the binding clones became the dominant population in the library.

[394] In a typical selection procedure, after three rounds of antigen-panning on human TfR1-coated wells in ELISA plates, approximately 80% of the bound phage particles bound to TfR1 specifically in ELISA with coated TfR1.

[395] **Example 18: Single colony ELISA analysis of phage-displayed scFvs specific for ectodomain of human TfR1**

[396] *E.coli* strain ER2738 infected with single-clonal phages each harboring a selected scFv gene in its phagemid was grown in the mid-log phase in 2YT broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) with 100 μg/ml ampicillin in deep well at 37°C with shaking. After broth reaching an 00600 of 1.0, IPTG was added to a final concentration of 1 μg/ml. The plates were incubated at 37°C overnight with rigorously shaking; thereafter, the plates were centrifuged at 4000 g for 15 minutes at 4°C.

[397] For soluble scFv binding test, ELISA was carried out. In brief, Maxisorp 96-well plate (Nunc) was coated with ectodomain of TfR1 (0.5 μg/100 μL PBS per well) or a negative control antigen CD16b, for 18 hours with shaking at 4°C. After treated with 300 μL of blocking buffer for 1 hour, 100 μL of secreted scFv in the supernatant was mixed with 100 μl of blocking buffer and then added to the coated plate for another one-hour. Goat anti-E-tag antibody (conjugated with HRP, 1:4000, Cat. No. AB19400, Abcam) was added to the plate for 1 hour. TMB substrate (50 μl per well) was added to the wells and the absorbance at 450 nm was measured after reactions were stopped by adding 1 NHCl (50 μl per well).

[398] A total of 192 phage clones after the third round of panning were subjected to the present analysis. Among them, 23 scFv clones that bound to TfR1 with a differential of OD450 greater than 10 were further characterized by sequencing the genes encoding these scFvs. Sixteen different DNA sequences were identified. Figure 21B shows the ELISA result of an scFv clone 12A1. The amino acid sequence of the scFV clone 12A1, which binds to human TfR1 with an OD450 of 1.7, is shown in SEQ ID NO: 36.

[399] **Example 19: Preparation of TCO-scFv specific for the ectodomain of CD32a**

[400] The DNA sequence encoding SEQ ID NO: 32 was synthesized and expressed as in the above Examples. For the conjugation with Mal-PEG_3-TCO (Conju-probe, Inc.), the cysteine residue at the C-terminal end of the purified scFv of anti-CD32a mAb was reduced by incubating with 5 mM dithiothreitol (OTT) at room temperature for 4 hours with gentle shaking. The buffer of reduced scFv proteins were exchanged to sodium phosphate buffer (100 mM sodium phosphate, pH7.0, 50 mM NaCl, and 5 mM EDTA) by using NAP-10 Sephadex G-25 column. After the reduction reaction and buffer exchange, conjugation was conducted overnight at room temperature in a reaction molar ratio of 10:1 ([Mal-PEG_3-TCO:[scFv]]. The excess crosslinker was removed by a desalting column and the TCO-conjugated scFv product was analyzed.k

[401] The results of mass spectroscopy MALDI-TOF analysis indicated that the sample of TCO-conjugated scFv specific for CD32a had a m.w. of 27,337 daltons. The purity of TCO-conjugated scFvs specific for CD32a was identified through Coomassie blue staining of 12% SDS-PAGE. Figure 22A and Figure 22B show, respectively, the ELISA and Mass spectrometric analysis of TCO-conjugated scFv specific for CD32a, in which unmodified scFv specific for CD32a was used as a positive control. According to the ELISA results, TCO-conjugated scFv specific for CD32a bound to recombinant ectodomain of human CD32a.

[402] **Example 20: Preparation of tetrazine-scFv specific for the ectodomain of TfR1**

[403] The DNA sequence encoding SEQ ID NO: 33 was synthesized and expressed as in the above Examples. For the conjugation with Mal-PEG_4-tetrazine (Conju-probe, Inc.), the cysteine residue at the C-terminal end of the purified scFv of mAb specific for TfR1 was reduced by incubating with 5 mM OTT at room temperature for 4 hours with gentle shaking. The buffer of reduced scFv proteins were exchanged to sodium phosphate buffer (100 mM sodium phosphate, pH 7.0, 50 mM NaCl, and 5 mM EDTA) by using NAP-10 Sephadex G-25 column. After the reduction reaction and buffer exchange, conjugation was conducted overnight at 4°C in a reaction molar ratio of 10:1 ([Mal-PEG_4-tetrazine:[scFv]]. The excess crosslinker was removed by a desalting column and the tetrazine-conjugated

scFv product was analyzed.

[404] The results of mass spectroscopy MALDI-TOF analysis indicated that the sample of tetrazine-conjugated scFv specific for TfR1 had a m.w. of 27,086 daltons. The purity of tetrazine-conjugated scFv specific for TfR1 was identified through Coomassie blue staining of 12% SDS-PAGE. Figure 23A and Figure 238 show, respectively, the ELISA and Mass spectrometric analysis of tetrazine-conjugated scFv specific for TfR1, in which unmodified scFv specific for TfR1 was used as a positive control. According to the ELISA results, tetrazine-conjugated scFv specific for TfR1 bound to recombinant ectodomain of TfR1.

[405] **Example 21: Conjugation of three scFvs specific for endotoxin to the three maleimide-PEG_{12} linking arms based on tetrazine-peptide 1**

[406] This example demonstrates that three scFvs can be conjugated to the three PEG_{12}-maleimide linking arms based on tetrazine-peptide 1. Prior to conjugation with the tetrazine-peptide 1 that had three PEG_{12}-maleimide linking arms, scFv specific for endotoxin was incubated with DTT at a molar ratio of 2: 1 ([DTT]:[scFv]) at 25°C for 4 hours with gentle shaking to keep its C-terminal cysteine in a reduced form. Subsequently, the buffer of reduced scFv specific endotoxin was exchanged to maleimide-SH coupling reaction buffer (100 mM sodium phosphate, pH 7.0, 50 mM NaCl and 5 mM EOTA) by using an NAP-10 Sephadex G-25 column (GE Healthcare). After the reduction and buffer exchange, the conjugation to the tetrazine-peptide 1 having three maleimide-PEG_{12} linking arms was conducted overnight at 4°C at a molar ratio of 1:4 ([linker]:[Protein]).

[407] The PEG_{12}-maleimide-conjugated tetrazine-peptide 1 conjugated with three scFvs specific for endotoxin was separated from the free scFv, free PEG_{12}-maleimide-conjugated tetrazine-peptide 1 and the PEG_{12}-maleimide-conjugated tetrazine-peptide 1 conjugated with one and two scFvs specific for endotoxin by size exclusion chromatography column S75.

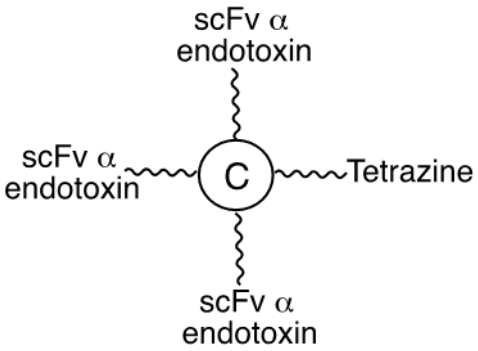
[408] Figure 24A is the FPLC elution profile on a synthesized targeting linker unit composed of a linker unit with a free tetrazine functional group and a set of three scFvs specific for endotoxin as targeting elements with retention volume of 9.5 ml. The product (i.e., the PEG_{12}-maleimide-conjugated tetrazine-peptide 1 having a free tetrazine functional

group and being conjugated with a set of three scFvs specific for endotoxin) was purified in the elution fraction and shown in lane 4 (indicated by arrow) of the 10% SDS-PAGE analysis shown in Figure 24B.

[409] **Example 22: Analysis of a targeting linker unit containing three scFvs specific for endotoxin linked to the three maleimide-PEG_{12} linking arms based on tetrazine-peptide 1 by MALDI-TOF**

[410] The sample of the targeting linker unit with three scFvs specific for endotoxin linked to the three maleimide-PEG_{12} linking arms based on tetrazine-peptide 1 was analyzed by MALDI-TOF. The median of the experimental molecular weight was consistent with the median of theoretical molecular weight of three scFvs specific for endotoxin conjugated to tetrazine-peptide 1 with three maleimide-PEG_{12} linking arms. According to the mass spectrometric profile in Figure 24C, the present targeting linker unit had a median molecular weight of 81,727 daltons.

[411] Illustrated below is the synthesized targeting linker unit that was composed of a linker unit with a free tetrazine functional group and a set of three scFvs specific for endotoxin as targeting elements.

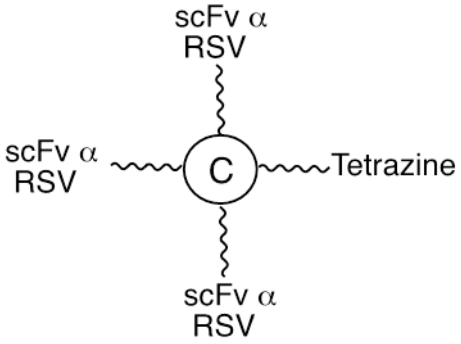


[412] **Example 23: Preparation of a targeting linker unit based on tetrazine-peptide with three scFvs specific for Protein F of RSV**

[413] The conjugation of scFv to the linker unit and the purification and analysis of the product were the same as in the preceding Examples.

[414] Shown in Figure 25 is the mass spectrometric analysis of the synthesized targeting linker unit that was composed of a linker unit with a free tetrazine functional group and a set of-three scFv specific for Protein F of RSV as targeting elements (illustrated below). As

indicated in Figure 25, this targeting linker unit had a molecular weight of 81,978 daltons.



[415] **Example 24: Conjugation of three scFvs specific for β-amyloid to three maleimide-PEG_{12} linking arms based on TCO-peptide 1**

[416] This example was performed to demonstrate that three scFvs could be conjugated to the three maleimide-PEG_{12} linking arms based on TCO-peptide 1. Prior to conjugation with the TCO-peptide 1 that had three maleimide-PEG_{12} linking arms, scFv specific for β-amyloid was incubated with OTT at a molar ratio of 2:1 ([DTT]:[scFv]) at room temperature for 4 hours with gentle shaking to keep its C-terminal cysteine in a reduced form. Subsequently, the buffer of reduced scFv specific for β-amyloid was exchanged to maleimide-SH coupling reaction buffer (100 mM sodium phosphate, pH 7.0, 50 mM NaCl and 5 mM EDTA) by using an NAP-10 Sephadex G-25 column (GE Healthcare). After the reduction and buffer exchange, the conjugation to the TCO-peptide 1 having three maleimide-PEG_{12} linking arms was conducted overnight at room temperature at a molar ratio of 1:4 ([linker]:[Protein]).

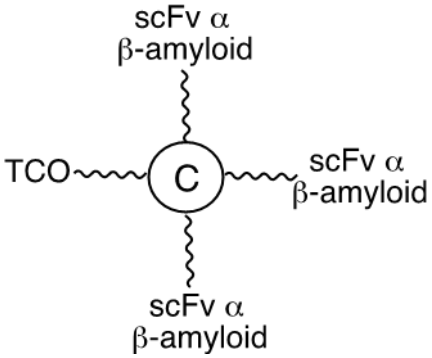
[417] The reaction mixture of the preceding examples was adjusted to pH 5.0 and then applied to pre-equilibrated (5 mM EDTA, and 50 mM sodium acetate at pH 5.0) cation exchange column SP Sepharose FF (GE Healthcare). The maleimide-PEG_{12}-conjugated TCO-peptide 1 conjugated with three scFvs specific for β-amyloid was eluted using a linear gradient of 0-500 mM sodium chloride in a flow rate of 0.5 ml/min for 100 minutes. The maleimide-PEG_{12}-conjugated TCO-peptide 1 conjugated with three scFvs specific for β-amyloid was separated from the free scFv, free maleimide-PEG_{12}-conjugated TCO-peptide 1 and the maleimide-PEG_{12}-conjugated TCO-peptide 1 conjugated with one and two scFvs specific for β-amyloid by cation exchange column SP Sepharose FF. The purified product, maleimide-PEG_{12}-conjugated TCO-peptide 1 conjugated with three scFvs

specific for β-amyloid, was concentrated and buffer-exchange into click reaction buffer, 100 mM potassium phosphate at pH 7.0.

[418] Figure 26A is the FPLC elution profile of cation exchange column SP Sepharose FF on a synthesized effector linker unit composed of a linker unit with a free TCO functional group and a set of three scFvs specific for β-amyloid as effector elements. Symbol #1 and #2 respectively represented the eluted peaks of maleimide-PEG_{12}-conjugated TCO-peptide 1 conjugated with two scFvs and three scFvs specific for β-amyloid. The product, the maleimide- PEG_{12}-conjugated TCO-peptide 1 bearing a free TCO functional group and three scFvs specific for β-amyloid was purified and revealed in lane 2 of the 8% SDS-PAGE analysis shown in Figure 26B.

[419] **Example 25: Analysis of an effector linker unit containing three scFvs specific for β-amyloid linked to the three maleimide-PEG_{12} linking arms based on TCO-peptide 1 by MALDI-TOF**

[420] The sample of the effector linker unit of three scFvs specific for β-amyloid linked to the three maleimide-PEG_{12} linking arms based on TCO-peptide 1 was analyzed by MALDI-TOF. The median of the experimental molecular weight was consistent with the median of theoretical molecular weight of three scFvs specific for β-amyloid conjugated to TCO-peptide 1 with three maleimide-PEG_{12} linking arms. According to the mass spectrometric profile in Figure 26C, the synthesized effector linker unit had the median molecular weight of 87,160 daltons.



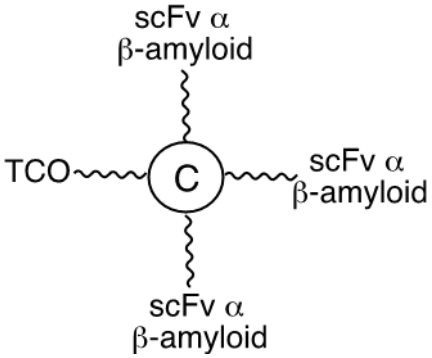
[421] Illustrated herein is the synthesized targeting linker unit that was composed of a linker unit with a free TCO functional group and a set of three scFvs specific for β-amyloid as targeting elements.

[422] **Example 26: Preparation of molecular construct with three scFvs specific for Protein F of RSV as targeting elements and one scFv specific for ectodomain of CD32a as an effector element**

[423] In this example, the targeting linker unit of the preceding examples and a TCO-scFv specific for extodomain of CD32a were coupled via a tetrazine-TCO iEDDA reaction. Specifically, the targeting linker unit had three scFv specific for Protein F of RSV and one free tetrazine group.

[424] the procedure for tetrazine-TCO ligation was performed per the manufacturer's instructions (Jena Bioscience GmbH, Jena, Germany). Briefly, 100 µl of the targeting linker unit (0.3 mg/ml) was added to the solution containing the effector element at a molar ratio of 1:1.2 ([tetrazine]:[TCO]). The reaction mixture was incubated for 1 hour at room temperature. The product was subjected to mass spectrometric analysis, and the result indicated a molecular weight of 113,036 daltons (Figure 27A).

[425] The product, a single linker unit molecular construct with three scFvs specific for Protein F of RSV as targeting elements and one scFv specific for ectodomain of CD32a as an effector element, is illustrated below.

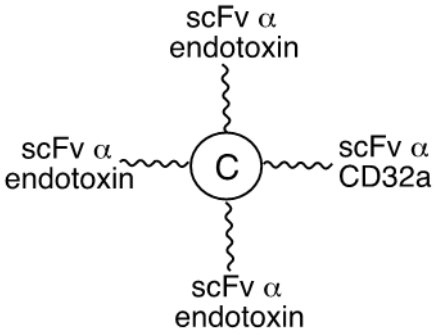


[426] **Example 27: Preparation of molecular construct with three sc Fvs specific for endotoxin as targeting elements and one scFv specific for ectodomain of CD32a as an effector element**

[427] The targeting linker unit prepared in an earlier Example and the TCO-scFv specific for ectodomain of CD32a were coupled via a tetrazine-TCO iEDDA reaction.

[428] The procedure for tetrazine-TCO ligation was performed as described in the previous Example. The product, as illustrated below, was a single linker unit molecular

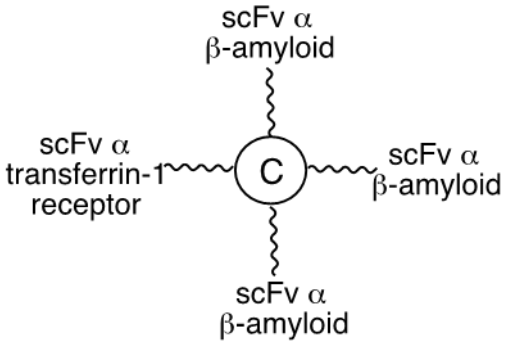
construct with three scFvs specific for endotoxin as targeting elements and one scFv specific for ectodomain of CD32a as an effector element. The mass spectrometric analysis shown in Figure 27B indicated that this molecular construct had a molecular weight of 113,761 daltons.



[429] **Example 28: Preparation of molecular construct with one scFv specific for ectodomain of TfR1 as a targeting element and three scFvs specific for β-amyloid as effector elements**

[430] The targeting linker unit prepared in an earlier Example and the tetrazine-scFv specific for ectodomain of TfR1 were coupled via a tetrazine-TCO iEDDA reaction.

[431] The procedure for tetrazine-TCO ligation was performed per the manufacturer's instructions (Jena Bioscience GmbH, Jena, Germany). Briefly, 12.6 µl of the targeting element (5.65 mg/ml) was added to the solution containing the linker unit with effector elements at a molar ratio of 10:1 ([tetrazine]:[TCO]). The reaction mixture was incubated for 3 hours at room temperature. The product was subjected to mass spectrometric analysis, and the result indicated a molecular weight of 114,248 daltons (Figure 27C).



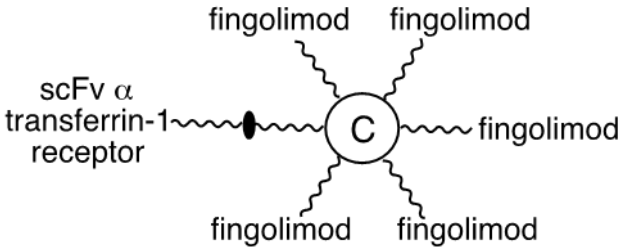
[432] The product, as illustrated herein, was a single linker unit molecular construct with one scFv specific for ectodomain of TfR1 as a targeting element and three scFvs specific for β-amyloid as effector elements.

[433] **Example 29: Preparation of molecular construct with one scFv specific for ectodomain of TfR1 as a targeting element and five fingolimod molecules as effector elements**

[434] In this example, the molecular construct with one scFv specific for ectodomain of TfR1 and a drug bundle of five fingolimod molecules was constructed. The molecular construct was made by a TCO-tetrazine iEDDA reaction. The procedure for tetrazine-TCO ligation was performed per the manufacturer's instructions (Jena Bioscience GmbH, Jena, Germany). Briefly, 277 µl of the effector linker unit (0.126 µmole) was added to the solution containing the targeting element of one scFv specific for ectodomain of TfR1 at a molar ratio of 5:1 ([TCO]:[tetrazine]). The reaction mixture was incubated for 3 hours at room temperature.

[435] The product, as illustrated below, was the molecular construct with one scFv specific for ectodomain of TfR1 and one drug bundle bearing five fingolimod molecules. Figure 28A and Figure 28B respectively show the SDS-PAGE and mass spectrometric analyses of the present the molecular construct. A major band, arrow #1, is the molecular construct with one scFv specific for ectodomain of TfR1 and a drug bundle with five fingolimod molecules, and arrow #2 is unconjugated scFv specific for ectodomain of TfR1.

[436] The mass spectrometric analysis shows that the molecular construct with one scFv specific for ectodomain of TfR1 and a drug bundle of five fingolimod molecules has a molecular weight of 32,105 daltons.



[437] **Example 30: Assay of biological activity of fingolimod upon the conjugation to peptide core through linking arms**

[438] Modified fingolimod molecules (NHS-PEG_5-conjugated fingolimod and the drug bundle with a free TCO functional group and with five fingolimod molecules) were synthesized as described in the preceding examples. To examine the biological activities

of the three compounds, S1P-driven Transwell migration assay was performed with human primary B cells isolated from human PBMC (peripheral blood mononuclear cells).

[439] In the preparation of human primary B cells, human B cells were isolated from human PBMC (peripheral blood mononuclear cells) by B cell isolation kit (Myltenyi Biotech). Then, the isolated B cells were seeded and maintained in a 15-cm dish in IMDM medium supplemented with 10% feta l bovine serum (Gibco) and 20 ng/ml IL2 (Peprotech Inc.).

[440] Figure 29A shows that staining analysis of the isolated S1P1-expressing human B cells, 2×10^5 B cells were incubated with 10 μg/ml of anti-S1P1 receptor antibody (AbD Serotec) in PBS containg 1% BSA on ice for 30 mininutes. Cells were washed and incubated with FITC-conjugated goat anti-mouse IgG, diluted 1:200 in PBS/BSA, on ice for 30 minutes in the dark. The cells were then analyzed by FACS (FACSCanto II, BD Biosciences).

[441] For chemotaxis assays, 100 μl of the maintained human B cells (4×10^5 cells) were transferred into 1.5-ml eppendorf tube and added with fingolimod, fingolimod phosphate, NHS-PEG_5-conjugated fingolimod, and the drug bundle with a free TCO functional group and with five fingolimod molecules, respectively, at a final concentration of 1 and 10 μM at 37°C for 4 hours. Subsequently, 100 μl of treated B cells were added to the upper chamber of a 6.5-mm Trans-well with a 5 μm pore polyester membrane insert (Corning), and the lower chamber of the Trans-well had contained 500 μl of IMDM medium with sphingosine-1-Phosphate molecule at a final concentration of 10 nM. After 3 hours, the migrated cells in the lower chambers were collected and further stained with trypan blue and counted by hemocytometer. For each measurement, the specific migration was calculated as follows: [(Number of cells in lower chamber)/ (Number of cells in lower + upper chamber) ×100]- (cell migration percentage at 0 nM attractant)]. The result of the percentage of specific migrated cells is shown in Figure 29B.

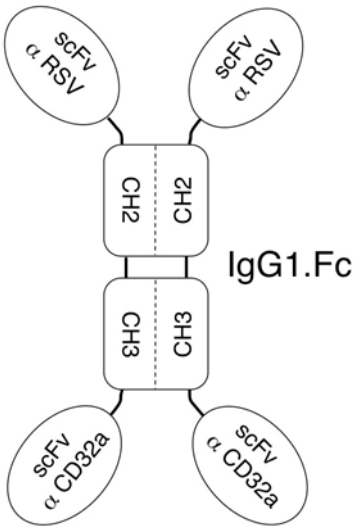
[442] Figure 29B shows the assay results of the biological activity of NHS-PEG_5-conjugated fingolimod and the drug bundle with a free TCO functional group and with five fingolimod molecules. The result indicates that the fingolimod molecule conjugated with a linking arm had similar biological activity to block B-cell migration as the unmodified fingolimod.

[443] **Example 31: Construction of a gene segment encoding 2-chain IgG1.Fc fusion protein containing scFv specific for Protein F of RSV and scFv specific for ectodomain of CD32a**

[444] The scFv1-CH2-CH3-scFv2 (human γ1) recombinant chain was configured by fusing two scFvs, in which the first one specific for Protein F of RSV fused to the N-terminal of CH2 domain of IgG1.Fc through a flexible hinge region, while the second one specific for ectodomain of CD32a was fused to the C-terminal of CH3 domain through a flexible linker, $(GGGGS)_3$.

[445] Both of the scFvs had an orientation of V_L-linker-V_H. The V_L and V_H in each of the two scFv were connected by a hydrophilic linker, GSTSGSGKPGSGEGSTKG. The sequence of the recombinant chain in the IgG1.Fc fusion protein molecular construct is shown as SEQ ID NO: 37.

[446] Illustrated below is the configuration of the prepared 2-chain (scFv α RSV)-(scFv α CD32a)-hIgG1.Fc molecular construct.



[447] **Example 32: Expression and purification of recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32) fusion protein**

[448] In this Example, the gene-encoding sequence was placed in pcDNA3 expression cassette. Expi293F cells were seeded at a density of 2.0×10^6 viable cells/ml in Expi293F expression medium and maintained for 18 to 24 hours prior to transfection to ensure that

the cells were actively dividing at the time of transfection. At the time of transfection, 7.5×10^8 cells in 255-ml medium in a 2-liter Erlenmeyer shaker flask were transfected by ExpiFectamine™ 293 transfection reagent. The transfected cells were incubated at 37°C for 16 to 18 hours post-transfection in an orbital shaker (125 rpm) and the cells were added ExpiFectamine™ 293 transfection enhancer 1 and enhancer 2 to the shaker flask, and incubated for 7 days. Culture supernatants were harvested and recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) fusion protein in the media was purified using Protein A chromatography. Following buffer exchange to PBS, the concentration of (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) protein was determined and analyzed by 12% SdS-PAGE shown in Figure 30A. The Fc-fusion molecular construct was revealed as the major band at about 85 kDa, consistent with the expected size.

[449] **Example 33: ELISA analysis of the binding of recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) fusion protein**

[450] To examine the binding ability of recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) fusion protein to both Protein F of RSV and ectodomain of CD32a, ELISA assay was performed. ELISA plates were coated with 2 μg/ml of Protein F of RSV (Sino biological Inc.). Recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) fusion protein and (scFv α RSV)-hIgG1.Fc were detected by HRP-conjugated goat anti-human IgG1.Fc. The ELISA results in Figure 308 show that the recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) fusion protein bind to Protein F of RSV, using adalimumab scFv as a control scFv.

[451] Figure 30C shows binding activity of the recombinant Fc-fusion protein to ectodomain of CD32a. ELISA plates were coated with 5 μg/ml of recombinant ectodomain of CD32a. Recombinant 2-chain (scFv α RSV)-hIgG 1.Fc-(scFv α CD32a) fusion protein was detected by HRP-conjugated goat anti-human IgG1.Fc. Figure 30C shows that the recombinant 2-chain (scFv α RSV)-hIgG1.Fc-(scFv α CD32a) Fc-fusion protein has binding activity to recombinant ectodomain of CD32a. Recombinant 2-chain (scFv α endotoxin)-IgG1.Fc protein was used as a control antibody.

[452] **Example 34: Preparation of 2-chain IgG1.Fc fusion protein containing scFv specific for endotoxin and scFv specific for ectodomain of CD32a**

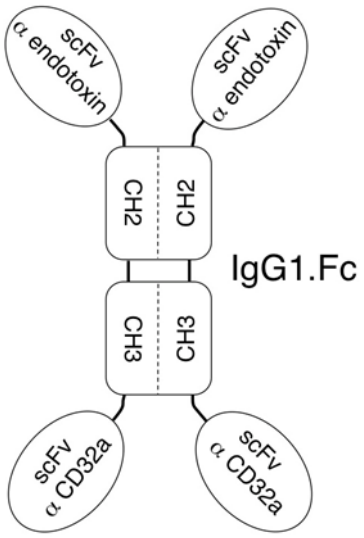
[453] The scFv1-CH2-CH3-scFv2 (human γ1) recombinant chain was configured by fusing two scFvs, in which the first one specific for endotoxin was fused to the N-terminal of CH2 domain of IgG1.Fc through a flexible hinge region, and the second one specific for ectodomain was fused to the C-terminal of CH3 domain through a flexible linker, $(GGGGS)_3$.

[454] Both of the scFvs had an orientation of V_L-linker-V_H. The V_L and V_H in each of the two scFv were connected by a hydrophilic linker, GSTSGSGKPGSGEGSTKG.

[455] The sequence of the recombinant chain in the IgG1.Fc fusion protein molecular construct is shown as SEQ ID NO: 38. The expression of the constructed gene in Expi293F cells and the purification of the expressed fusion protein were performed as in preceding Examples. Characterization of the new construct was performed with SDS-PAGE and ELISA The SDA-PAGE results in Figure 31A shows that the recombinant chain of the new construct has a size of about 85 kDa, consistent with the expected size.

[456] Figure 31B shows ELISA results of the recombinant 2-chain (scFv α endotoxin)-(scFv α CD32a)-hIgG1.Fc binding to E.coli LPS 0111:B4 (Sigma Aldrich). ELISA plates were coated with 50 g/ml poly-L-lysine. Subsequently, the poly-L-lysine-coated plates were further coated with 10 μg/ml E.coli LPS 0111:B4. The recombinant fusion protein was detected by HRP-conjugated goat anti-human IgG.Fc. The ELISA results show that the present recombinant Fc-fusion protein has binding activity to E.coli LPS 0111:B4 (Sigma Aldrich); Figure 31C shows that the recombinant Fc-fusion protein has binding activity to ectodomain of CD32a.

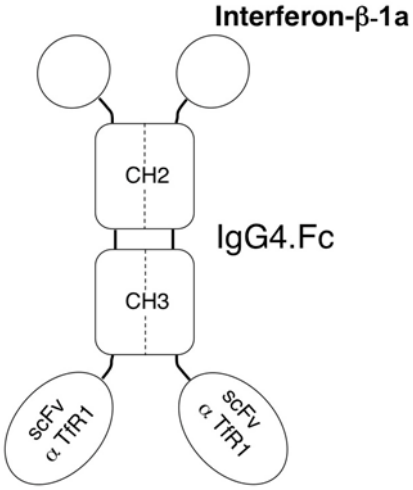
[457] Illustrated below is the configuration of the thus-prepared 2-chain (scFv α endotoxin)-(scFv α CD32)-hIgG1.Fc molecular construct.



[458] **Example 35: Construction of a gene segment encoding 2-chain IgG4.Fc fusion protein containing interferon-β-1a and scFv specific for ectodomain of TfR1**

[459] The 2-chain IgG.Fc fusion protein was prepared by configuring (interferon-β-1a)-CH2-CH3-(scFv α TfR1) (human γ4) in a recombinant chain. The C-terminal of the interferon-β-1a was fused to the N-terminal of CH2 via a linker, GGGGSGGGASGGS. The scFv specific for ectodomain of TfR1 was fused to the C-terminal of CH3 domain through a flexible linker, $(GGGGS)_3$.

[460] The scFv (specific for ectodomain of TfR1) had an orientation of VL-linker-VH. The V_L and V_H in the scFv were connected by a hydrophilic linker, GSTSGSGKPGSGEGSTKG. The sequence of the recombinant chain in the IgG4.Fc fusion protein molecular construct is shown as SEQ ID NO: 39.



[461] Illustrated herein is the configuration of the prepared 2-chain (interferon-β-1a)-IgG4.Fc-(scFv α TfR1) molecular construct.

[462] **Example 36: Expression and purification of recombinant 2-chain (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) fusion protein**

[463] In this Example, the gene-encoding sequence was placed in pcDNA3 expression cassette. Expi293F cells were seeded at a density of 2.0×10^6 viable cells/ml in Expi293F expression medium and maintained for 18 to 24 hours prior to transfection to ensure that the cells were actively dividing at the time of transfection. At the time of transfection, 7.5×10^8 cells in 255-ml medium in a 2-liter Erlenmeyer shaker flask were transfected by ExpiFectamine™ 293 transfection reagent. The transfected cells were incubated at 37°C for 16 to 18 hours post-transfection in an orbital shaker (125 rpm) and the cells were added ExpiFectamine™ 293 transfection enhancer 1 and enhancer 2 to the shaker flask, and incubated for 7 days. Culture supernatants were harvested and recombinant 2-chain (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) fusion protein in the media was purified using Protein A chromatography. Following buffer exchange to PBS, the concentration of (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) protein was determined and analyzed by 8% SDS-PAGE shown in Figure 32A. The Fe-fusion molecular construct was revealed as the major band at about 80 kDa, consistent with the expected size.

[464] **Example 37: Binding analysis of recombinant 2-chain (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) fusion protein using ELISA and flow cytometry**

[465] Binding activity of recombinant (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) was assayed by ELISA using a 96-well plate coated with recombinant assayed by ELISA using a 96-well plate coated with recombinant (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) protein in 5 g/ml concentration, 100 µl per well. The scFv specific for ectodomain of TfR1 is as a negative control. Recombinant 2-chain (Interferon-β-1a)-hIgG.Fc-(scFv α TfR1) was detected by HRP-conjugated rabbit anti-human interferon-β polyclonal antibody (Santa Cruz Biotechnology, Dallas, USA). Next, 50 µl of TMB substrate was added for color development. The reaction was stopped by 50 µl of 1 M HCl. Absorbance at 450 nm was measured with a plate reader. Each bar

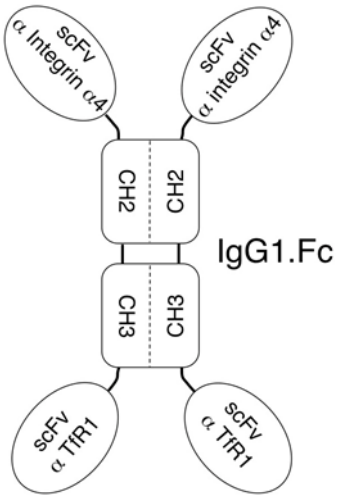
represents the mean OD450 value of duplicate samples.

[466] Figure 32B shows ELISA analysis of the present the molecular construct. The ELISA results show that (Interferon-β-1a)-hIgG1.Fc-(scFv α TfR1) fusion protein bound specifically to recombinant ectodomain of TfR1 protein.

[467] **Example 38: Preparation of 2-chain IgG4.Fc fusion protein containing scFv specific for integrin a4 and scFv specific for ectodomain of TfR1**

[468] The VL and VH of the scFv specific for integrin α4 were from monoclonal antibody natalizumab. The 2-chain IgG.Fc fusion protein was prepared by configuring (scFv α integrin α4)-CH2-CH3-(scFv α TfR1) (human γ4) in a recombinant chain. The C-terminal of the scFv specific for integrin a4 was fused to the N-terminal of CH2 via a linker, GGGGSGGGASGGS. The scFv specific for ectodomain of TfR1 was fused to the C-terminal of CH3 domain through a flexible linker, (GGGGS)3. The result of 8% SDA-PAGE in Figure 33A shows that the recombinant chain of the new construct has a size of about 85 kDa (indicated by arrow), consistent with the expected size.

[469] The two scFv had the orientation of VL-linker-VH. The VL and VH in each of the two scFv were connected by a hydrophilic linker, GSTSGSGKPGSGEGSTKG. The sequence of the recombinant chain in the IgG4.Fc fusion protein molecular construct is shown as SEQ ID NO: 40.



[470] Illustrated herein is the configuration of the prepared 2-chain (scFv α integrin

a4)-IgG4.Fc-(scFv α TfR1) molecular construct.

[471] To examine the binding ability of recombinant 2-chain (scFv α integrin α4)-IgG4.Fc-(scFv α TfR1) protein to integrin a4-expressing Jurkat T cells, cell-binding assay was performed by flow cytometry.

[472] 1×10^6 Jurkat T cells was maintained in the RPMI1640 medium supplemented with 10% FBS at a density of 1×10^5. The cells were kept in 37°C with 5% CO_2 in a humidified chamber. 1×10^6 Jurkat T cells were washed with the binding buffer (phosphate-buffered saline with 0.1% FBS, 2mM EOTA and 20ng/ml NaN3) twice. 10 μg/ml of Human BO Fc block™ (BO Biosciences, San Jose, US) was added to the washed JurkatT cells to block Fc receptor mediated. Cells were washed and incubated with 10 μg/ml of recombinant (scFv α integrin α 4)-IgG4.Fc-(scFv α TfR1) protein on ice for 15 minutes, using recombinant 2-chain (interferon-β-1a)-gG4.Fc-(scFv α TfR1) as a negative control. Cells were washed again and incubated with FITC-conjugated goat anti-human IgG.Fc (Caltag, Buckingham, UK), diluted 1:200 in blocking buffer, at on ice for 15 min in the dark. The stained cells were analyzed on a FACSCanto II flow cytometer (BO Biosciences).

[473] Figure 33B shows results of the cell staining analysis of recombinant 2-chain (scFv α integrin α4)-IgG4.Fc-(scFv α TfR1) protein on integrin a4-expressing Jurkat T cells. The construct bound to Jurkat T cells substantially positively.

[474] **Example 39: Assay of biological activity of 2-chain IgG1.Fc containing scFv specific for endotoxin and scFv specific for ectodomain of CD32a on macrophage-like U937 cells**

[475] To test the effects of recombinant 2-chain (scFv α endotoxin)-hIgG1.Fc-(scFv α C032a) fusion protein on inhibiting TNF-α secretion, ELISA was to determine the amount of secreted TNF-α in the supernatant by macrophage-like U937 cells.

[476] U937 cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin-streptomycin (Gibco), at the density between 3×10^5 and 2×10^6 cells/ml. The cells were kept in 37°C with 5% CO_2 in a humidified chamber. To differentiate U937 into macrophage-like cells, 1×10^6 cells/ml of U937 were incubated with 10

ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich). After 48 hours, non-adherent cells were removed, and adherent cells were washed and seeded into 96-well plates.

[477] 5×10^4 cells/well of differentiated U937 were seeded into 96-well plates the day before assay. Cells were stimulated with 1 μg/ml E. coli LPS 0111: B4 (Sigma Aldrich) alone, or premixes of LPS and 10 μg/ml of (scFv α endotoxin)-hIgG1Fc, 15 μg/ml of (scFv α endotoxin)-hIgG1Fc-(scFv α CD32a) or 2.5 μg/ml of anti-CD32a scFv. The stimulation proceeded for 2 hours before the supernatant was collected. TNF-a production was measured by commercially available ELISA kit (Biolegend).

[478] TNF-α levels in U937 supernatant were measured using an ELISA kit from R&D Systems. The wells of ELISA plates (Greiner Bio-One) were coated with 4 μg/ml of capture antibody in PBS at 4°C overnight. Wells were subsequently blocked by 0.5% in PBS for 1 hour and incubated with diluted culture supernatant for 2 hours. 400 ng/ml of biotin-labeled detection antibody was used followed by Streptavidin-HRP to detect bound TNF-a. Chromogenic reaction was carried out using TMB substrate (Clinical Science Products), and stopped by adding 1 N HCl. Plates were read at 450 nm absorbance. Concentrations of TNF-α were determined by extrapolation from four-parameter logistic fit standard curves generated from dilutions of standard protein supplied by the manufacturer.

[479] Figure 34 shows that recombinant 2-chain (scFv α endotoxin)-hIgG1Fc-(scFv α CD32a) significantly reduced TNF-cx secretion stimulated by E.coli LPS 0111:B4, compared to control antibodies 2-chain (scFv α endotoxin)-hIgG1Fc protein and anti-CD32a scFv or medium alone.

[480] It will be understood that the above description of embodiments is given by way of example only and that various modifications may be made by those with ordinary skill in the art. The above specification, examples and data provide a complete description of the structure and use of exemplary embodiments of the invention. Although various embodiments of the invention have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those with ordinary skill in the art could make numerous alterations to the disclosed embodiments without departing from the spirit or scope of this invention.

WHAT IS CLAIMED IS:

1. A linker unit comprising, a center core, a plurality of linking arms, and optionally a coupling arm, wherein,

the center core comprises, (1) a first polypeptide comprising a plurality of lysine (K) residues, wherein each K residue and its next K residue are separated by a filler sequence comprising glycine (G) and serine (S) residues, and the number of K residues ranges from 2 to 15; or (2) a second polypeptide comprising the sequence of $(X_{aa}\text{-}K)_n$, where each X_{aa} is a PEGylated amino acid having 2 to 12 repeats of ethylene glycol (EG) unit, and n is an integral from 2 to 15;

the plurality of linking arms are respectively linked to the K residues of the center core;

each of the plurality of linking arms has a N-hydroxysuccinimidyl (NHS), an azide, an alkyne, a tetrazine, a cyclooctene, or a cyclooctyne group at its free terminus; and

the amino acid residue at the N- or C-terminus of the center core has the azide or the alkyne group; or the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the thiol group of the cysteine residue is linked with the coupling arm having the azide, the alkyne, the tetrazine, the cyclooctene, or the cyclooctyne group at the free terminus of the coupling arm, wherein,

when the free terminus of the linking arm is the azide, the alkyne, or the cyclooctyne group, then the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the tetrazine or the cyclooctene group; or

when the free terminus of the linking arm is the tetrazine group or cyclooctene group, then the amino acid residue at the N- or C-terminus of the center core has the azide or the alkyne group, or the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the azide, the alkyne, or the cyclooctyne group.

2. The linker unit of claim 1, wherein the filler sequence has the sequence of GS, GGS, GSG, or SEQ ID NOs: 1-16.

3. The linker unit of claim 1, wherein each of the linking arms is a PEG chain having 2-20 repeats of EG units, and coupling arm is a PEG chain having 2-12 repeats of EG units, wherein the EG unit of the PEG chain of t linking arm has, optionally, a disulfide linkage at the free terminus thereof.

8. The linker unit of claim 1, wherein,

the amino acid residue having the azide group is L-azidohomoalanine (AHA), 4-azido-L-phenylalanine, 4-azido-D-phenylalanine, 3-azido-L-alanine, 3-azido-D-alanine, 4-azido-L-homoalanine, 4-azido-D-homoalanine, 5-azido-L-ornithine, 5-azido-d-ornithine, 6-azido-L-lysine, or 6-azido-D-lysine;

the amino acid residue having the alkyne group is L-homopropargylglycine (L-HPG), D-homopropargylglycine (D-HPG), or beta-homopropargylglycine (β-HPG);

the cyclooctene group is trans-cyclooctene (TCO); and the cyclooctyne group is dibenzocyclooctyne (DBCO), difluorinated cyclooctyne(DIFO), bicyclononyne (BCN), or dibenzocyclooctyne (DICO); or

the tetrazine group is 1,2,3,4-tetrazine, 1,2,3,5-tetrazine or 1,2,4,5-tetrazine, or derivatives thereof.

5. The linker unit of claim 1, further comprising

a plurality of first elements that are respectively linked to the plurality of linking arms via forming an amide bound therebetween, or via copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction, strained-promoted azide-alkyne click chemistry (SPAAC) reaction, or inverse electron demand Diels-Alder (iEDDA) reaction; and

optionally, a second element that is linked to the center core via any of the following reactions,

CuAAC reaction occurred between the azide or the alkyne group and the second element;

SPAAC reaction occurred between the azide or cyclooctyne group and the second element; and

iEDDA reaction occurred between the cyclooctene group or tetrazine group and the second element.

6. The linker unit of claim 5, wherein,

the plurality of first elements are respectively linked to the plurality of linking arms via forming an amide bound therebetween,

the second element is linked to the azide or alkyne group at the N- or C- terminus of the center core via CuAAC reaction, and the linker unit further comprising, optionally, a third element that is linked to the coupling arm via iEDDA reaction; or

the plurality of first elements are respectively linked to the plurality of linking arms via forming an amide bound therebetween, the second element is linked to the azide group at the N- or C- terminus of the center core via SPAAC reaction, and the linker unit further comprising, optionally, a third element that is linked to the coupling arm via iEDDA reaction.

7. The linker unit of claim 1 or 3, further comprising a plurality of connecting arms that are respectively linked to the plurality of linking arms via CuAAC reaction, SPAAC reaction, or iEDDA reaction, wherein each of the plurality of connecting arms has a maleimide or the NHS group at its free terminus.

8. The linker unit of claim 7, further comprising a plurality of first elements that are respectively linked to the plurality of linking arms via thiol-maleimide reaction or forming an amide bound therebetween; and

optionally, a second element that is linked to the center core via any of the following reactions:

CuAAC reaction occurred between the azide or the alkyne group and the second element;

SPAAC reaction occurred between the azide or cyclooctyne group and the second element; and

iEDDA reaction occurred between the cyclooctene group or tetrazine group and the second element.

9. The linker unit of claim 5 or 8, wherein when the first element is the fingolimod, fingolimod phosphate, interferon-β, or the scFv specific for integrin-α4 or β-amyloid, the second
element is an scFv specific for transferrin receptor.

10. The linker unit of claim 5 or 8, wherein when the first element is the scFv specific for the viral protein or the bacterial protein, the second element is an scFv specific for CD32 or CD16b;

the viral protein is F protein of respiratory syncytia virus (RSV), gp120 protein of human deficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, or glycoprotein of cytomegalovirus; and

the bacterial protein is endotoxin of Gram(-) bacteria, surface antigen of *Clostridium difficile*, lipoteichoic acid of *staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, or Shiga-like toxin type I or II of *Escherichia coli*.

11. A linker unit comprising,

a center core that comprises, (1) a first polypeptide comprising a plurality of lysine (K) residues, wherein each K residue and its next K residue are separated by a filler sequence comprising glycine (G) and serine (S) residues, and the number of K residues ranges from 2 to 15; or (2) a second polypeptide comprising the sequence of $(X_{aa}\text{-}K)_n$, where each X_{aa} is a PEGylated amino acid having 2 to 12 repeats of ethylene glycol (EG) unit, and n is an integral from 2 to 15; wherein at least one of the N- and C-terminal amino acid residues of the center core is an amino acid having an azide or an alkyne group or is a cysteine residue, wherein when one of the N- and C-terminal amino acid residues is the cysteine residue, the linker unit further comprises, optionally, a coupling arm that is linked to the cysteine residue via the thiol group of the cysteine residue and has the azide, the alkyne, a tetrazine, a cyclooctene, or a cyclooctyne group at the free terminus thereof,

a plurality of linking arms respectively linked to the K residues of the center core;

optionally, a plurality of connecting arms respectively linked to the plurality of the linking arms via CuAAC reaction, SPAAC reaction, or iEDDA reaction;

a plurality of first elements that are respectively linked to the plurality of linking arms via forming an amide bound therebetween, or via thiol-maleimide reaction, CuAAC reaction, SPAAC reaction, or iEDDA reaction; or respectively linked to the plurality of connecting arms via forming an amide bound therebetween, or via thiol-maleimide reaction, wherein,

each of the first elements is fingolimod, fingolimod phosphate, interferon-β, or a single-chain variable fragment (scFv) specific for integrin-α4, β-amyloid, a viral protein, a bacterial protein; and

when the plurality of linking arms are linked to the plurality of connecting arms or the plurality of first elements via CuAAC reaction or SPAAC reaction, then the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the tetrazine or the cyclooctene group; or

when the plurality of linking arms are linked to the plurality of connecting arms or the plurality of first elements via iEDDA reaction, then the amino acid residue at the N- or C-terminus of the center core has the azide or the alkyne group, or the amino acid residue at the N- or C-terminus of the center core is a cysteine residue, and the free terminus of the coupling arm is the azide, the alkyne, or the cyclooctyne group; and

optionally, a second element that is linked to the center core via any of the following reactions: CuAAC reaction occurred between the azide or alkyne group and the second element; SPAAC reaction occurred between the azide or cyclooctyne group and the second

element; and iEDDA reaction occurred between the cyclooctene or tetrazine group and the second element.

12. The linker unit of claim 11, wherein the filler sequence has the sequence of GS, GGS, GSG, or SEQ ID NOs: 1-16.

13. The linker unit of claim, wherein each of the linking arms is a PEG chain having 2-20 repeats of EG units, and coupling arm is a PEG chain having 2-12 repeats of EG units, wherein the EG unit of the PEG chain of t linking arm has, optionally, a disulfide linkage at the free terminus thereof.

14. The linker unit of claim 11, wherein

the amino acid residue having the azide group is L-azidohomoalanine (AHA), 4-azido-L-phenylalanine, 4-azido-D-phenylalanine, 3-azido-L-alanine, 3-azido-D-alanine, 4-azido-L-homoalanine, 4-azido-D-homoalanine, 5-azido-L-ornithine, 5-azido-d-ornithine, 6-azido-L-lysine, or 6-azido-D-lysine;

the amino acid residue having the alkyne group is L-homopropargylglycine (L-HPG), D-homopropargylglycine (D-HPG), or beta-homopropargylglycine (β-HPG);

the cyclooctene group is trans-cyclooctene (TCO); and the cyclooctyne group is dibenzocyclooctyne (DBCO), difluorinated cyclooctyne(DIFO), bicyclononyne (BCN), or dibenzocyclooctyne (DICO);

the tetrazine group is 1,2,3,4-tetrazine, 1,2,3,5-tetrazine or 1,2,4,5-tetrazine, or derivatives thereof.

15. The linker unit of claim 11, wherein

when the first element is the scFv specific for the viral protein or the bacterial protein, the second element is an scFv specific for CD32 or CD16b;

the viral protein is F protein of respiratory syncytia virus (RSV), gp120 protein of human immunodeficiency virus type 1 (HIV-1), hemagglutinin A (HA) protein of influenza A virus, or glycoprotein of cytomegalovirus; and

the bacterial protein is endotoxin of Gram(-) bacteria, surface antigen of *Clostridium difficile*, lipoteichoic acid of *Staphylococcus aureus*, anthrax toxin of *Bacillus anthracis*, or Shiga-like toxin type I or II of *Escherichia coli*.

16. The linker unit of claim 11, wherein,

when the first element is the fingolimod, fingolimod phosphate, interferon-β, or the scFv specific for integrin-aα or β-amyloid, the second element is an scFv specific for transferrin receptor.

17. The linker unit of claim 11, wherein,

when the plurality of first elements that are respectively linked to the plurality of linking arms via forming the amide bound therebetween or via thiol-maleimide reaction, and

the second element is linked to the azide or alkyne group of the N- or C-terminal amino acid residues of the center core via CuAAC reaction;

the linker unit further comprises optionally, a third element that is linked to the coupling arm via iEDDA reaction; or

when the plurality of first elements that are respectively linked to the plurality of linking arms via forming the amide bound therebetween or via thiol-maleimide reaction, and

the second element is linked to the azide group of the N- or C-terminal amino acid residues of the center core via SPAAC reaction.

the linker unit further comprises, optionally, a third element that is linked to the coupling arm via iEDDA reaction.

18. A pharmaceutical for the treatment of a central nervous system (CNS) disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the linker unit of claim 9 or 16.

19. The pharmaceutical of claim 18, wherein,

the CNS disease is multiple sclerosis, and the first element is the fingolimod, fingolimod phosphate, interferon-β, or the scFv specific for integrin-α4; or

the CNS disease is Alzheimer's disease, and the first element is the scFv specific for β-amyloid.

20. A pharmaceutical for the treatment of an infectious disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the linker unit of claim 10 or 15.