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(54) Title: MULTIVALENT ANTIBODIES

(57) Abstract: A multivalent antibody fusion protein comprising: a heavy chain comprising, in sequence from the N-terminal, a variable domain nominally V_H1, a C_H1 region and a further variable domain nominally V_H2, a light chain comprising, in sequence from the N-terminal, a variable domain nominally V_L1, a CL domain and a variable domain nominally V_L2, wherein said heavy and light chains are aligned to provide a first binding site formed by a first variable domain pair of V_H1 and V_L1 and a second binding site formed by a second variable domain pair of V_H2 and V_L2, and said fusion protein is conjugated to a PEG polymer.

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MULTIVALENT ANTIBODIES

The present disclosure relates to antibodies with two antigen binding sites, for example wherein the steric hindrance around each site is minimized, such that affinity to the target antigen or antigens is not detrimentally affected by the format.

Multivalent antibodies are known. However, even though the basic concept was disclosed a number of years ago, there have been practical difficulties associated with exploiting the technology and thus it has not been widely adopted for the preparation of pharmaceutical biologic products in development.

A non-natural/non-native antibody format can be difficult to express, which may significantly increase the cost of goods to an untenable level. The formats may increase the immunogenicity or reduce the *in vivo* stability in comparison to a standard antibody or fragment and/or may have undesirable pharmacokinetics.

In particular the problems associated with preparing homogenous products have been a concern for non-natural formats. If, for example, there is more than one permutation for combining the component monomers then mixtures can result. Thus elaborate purification methods may be required to isolate the desired/target entity at satisfactory purity levels.

This has been addressed in a number of ways, for example using short linkers in the production of bispecific diabodies was said to aid appropriate dimerisation. However, data has shown that the orientation of the variable domains can influence expression of the format and the formation of active binding sites.

One approach to force the assembly in the desired arrangement or orientation is referred to as the "knob-in-hole" method, in which a large "knob" is introduced in the VH domain by, for example in some antibodies exchanging valine 137 with the large residue phenyl alanine and replacing leucine 45 with tryptophan. A complementary hole can be introduced, for example in the VL domain by, in some antibodies,

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mutating phenylalanine 98 to methionine and tryptophan 87 to alanine. However, reduced antigen-binding activity was observed for several constructs.

In the present invention the provision of CL in the light chain and C_H1 in the heavy chain ensures the correct orientation of the chains.

Thus there is provided a recombinant fusion protein comprising:

a heavy chain comprising, in sequence from the N-terminal, a variable domain nominally V_H1, a C_H1 region and a further variable domain nominally V_H2,

a light chain comprising, in sequence from the N-terminal, a variable domain nominally V_L1, a CL domain and a variable domain nominally V_L2,

wherein said heavy and light chains are aligned to provide a first binding site formed by a first variable domain pair of V_H1 and V_L1 and a second binding site formed by a second variable domain pair of V_H2 and V_L2, and

said fusion protein is conjugated to a PEG polymer.

The recombinant fusion protein provided herein also advantageously has a half-life similar to that of a complete antibody and therefore is likely to be suitable for use in treatment.

Brief Description of the Figures

Figure 1	shows various fusion protein formats according to the present disclosure
Figure 2A	shows various known antibody fragments formats
Figure 2B	shows one possible arrangement for a dimer format according to the presently claimed invention
Figure 3	shows the light chain amino acid sequence for antibody 4D5
Figure 4	shows the heavy chain amino acid sequence for antibody 4D5

The heavy chain as employed herein is the chain which comprises a C_H1 domain.

Generally the heavy chain will not comprise an Fc fragment, that is to say a C_H2 and/or C_H3 fragment.

In one embodiment the heavy chain only comprises one C_H1 domain.

V_H1 and V_H2 as employed herein is intended to refer to the fact that the variable regions are located in the heavy chain of the antibody according to the present invention. It is not in itself indicative of the origin of the variable region as such.

V_L1 and V_L2 as employed herein is intended to refer to the fact that the variable regions are located in the light chain of the antibody according to the present invention. It is not in itself indicative of the origin of the variable region as such.

The light chain as employed herein comprises a CL domain. In one embodiment the light chain only comprises one CL domain.

The arrangement herein of CL as the constant region fragment in the light chain and C_H1 as the constant region fragment in the heavy chain is thought to minimize inappropriate dimerisation.

In one embodiment the fusion protein according to the disclosure comprises a hinge, for example, as a linker to a variable domain.

The hinge may be attached to the C-terminal of C_H1 in a corresponding position to that found in full length antibody and may form a link between C_H1 and V_H2. In this embodiment a linker will also be provided on the light chain so V_L2 is appropriately placed to pair with V_H2 in the heavy chain. The linker employed in the light chain may be identical, similar or completely different to the hinge linker employed in the heavy chain.

In an alternative embodiment the light chain comprises a hinge, for example attached to the C-terminal of the CL domain and linking the CL domain with V_L2. In this embodiment a linker may be required in the heavy chain to ensure the V_L2 and V_H2

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domains can pair appropriately to form an active site. The linker in the heavy chain can be identical, similar or completely different to the linker employed in the light chain.

5   Examples of suitable hinges are given below.

The variable domains are provided in each chain such that they form pre-defined pairs with suitable/adequate binding to a target antigen.

10   In one embodiment the variable domain pair has affinity for a target antigen of 100nm or less, such as 50nm or less, in particular 1 nm or less.

Suitable variable domains pairs may be identified by any means possible, for example including generation of antibodies in hosts with subsequent screening of B cells.

15   Alternatively suitable pairs may be identified by phage display.

Phage display methods known in the art and include those disclosed by Brinkman *et al.*, *J. Immunol. Methods*, 1995, 182, 41-50; Ames *et al.*, *J. Immunol. Methods*, 1995, 184, 177-186; Kettleborough *et al. Eur. J. Immunol.*, 1994, 24, 952-958; Persic *et al.*,

20   *Gene*, 1997 187, 9-18; and Burton *et al.*, *Advances in Immunology*, 1994, 57, 191-280; WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; and WO 95/20401; and US 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743; and 5,969,108.

25   Transgenic mice, or other organisms, including other mammals, may be used to generate humanized antibodies.

In one embodiment a variable domain pair (or each variable domain pair) is a cognate pair.

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Cognate pair as employed herein is intended to refer to a natural pair of variable domains, that is to say isolated from a single antibody or antibody expressing cell.

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In one example the cognate pair are a complementary VH/VL pair which bind the antigen co-operatively i.e. they are a complementary VH/VL pair.

Typically the cognate pair will be a VH/VL pair derived from the same antibody.

In one example the cognate pair are a pair of variable domains isolated as a pair from a 'library of pairs', such as a Fab phage display library.

In one example the VH/VL pair are monospecific.

First and second binding sites are relative terms (relative to each other) and are nominal labels given to the binding sites to differentiate one from the other. If one binding site is labeled "the first" then the other is labeled "the second".

First cognate pair and second pair are also relative labels to nominally differentiate the pairs. One pair labeled "first pair" herein is not definitive for the position in the molecule.

Variable domains may have been optimized and/or humanized. Optimised/humanized variable domains derived from a cognate pair will still be considered a cognate pair after optimization/humanization.

CL as employed herein refers to the constant region portion in the light chain, which may be a naturally occurring light chain constant region.

Each variable domain may directly joined or joined via a linker to the constant domain in the relevant chain.

"Directly linked to" as employed herein is intended to refer to a continuous amino acid sequence that is uninterrupted, i.e. linked directly via a peptide bond, for example directly to the sequence of the variable domain or conversely the constant region fragment and not joined via a linker. Inserting a "non-natural peptide linker" into an amino acid sequence disrupts the sequence and thus a sequence containing a "non-natural peptide linker" is not considered to be fused to the relevant portions directly, within the meaning of the present specification. The addition a natural peptide linker would also be considered interruption of the amino acid sequence, if it cannot be

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considered to form part of the sequence of one or more of the relevant components, such as a variable domain or constant region fragment (such as the CL domain or C_H1 domain).

5   V_H1 will generally be joined directly to C_H1.

V_L1 will generally be joined directly to CL.

In one embodiment V_H1 C_H1 and V_L1 CL together form a Fab or Fab'.

10

In one embodiment an amino acid in V_H2 (for example the N-terminal thereof) is directly linked to an amino acid of C_H1 by a peptide bond (for example to the C-terminal of C_H1).

15   In one embodiment an amino acid in V_H2 (for example the N-terminal thereof) is linked to C_H1 indirectly by a linker (for example to the C-terminal of C_H1).

In one embodiment an amino acid in V_L2 (for example the N-terminal thereof) is directly linked to an amino acid of CL by a peptide bond (for example to the C-

20   terminal of CL).

In one embodiment an amino acid in V_L2 (for example the N-terminal thereof) is linked to CL indirectly by a linker (for example to the C-terminal of CL).

25   In one embodiment V_H1 in the heavy chain is a variable domain from a heavy chain. That is to say is derived from the natural heavy chain of an antibody or relevant fragment thereof, or is derived from an alternative source such phage display and has the characteristics of a variable domain derived from a heavy chain.

30   In one embodiment V_H1 in the heavy chain is a variable domain from a light chain. That is to say is derived from the natural light chain of an antibody or relevant fragment thereof, or is derived from an alternative source such phage display and has the characteristics of a variable domain derived from a light chain.

In one embodiment V_H2 in the heavy chain is a variable domain from a heavy chain.

In one embodiment V_H2 in the heavy chain is a variable domain from a light chain.

In one embodiment V_L1 in the light chain is a variable domain from a heavy chain.

In one embodiment V_L1 in the light chain is a variable domain from a light chain.

In one embodiment V_L2 in the light chain is a variable domain from a heavy chain.

In one embodiment V_L2 in the light chain is a variable domain from a light chain.

In one embodiment V_H1 is a variable domain from a light chain and V_H2 is a variable domain from a light chain.

In one embodiment V_H1 is a variable domain from a heavy chain and V_H2 is a variable domain from a heavy chain.

In one embodiment V_H1 is a variable domain from a light chain and V_H2 is a variable domain from a heavy chain.

In one embodiment V_H1 is a variable domain from a heavy chain and V_H2 is a variable domain from a light chain.

In one embodiment V_L1 is a variable domain from a light chain and V_L2 is a variable domain from a light chain.

In one embodiment V_L1 is a variable domain from a heavy chain and V_L2 is a variable domain from a heavy chain.

In one embodiment V_L1 is a variable domain from a light chain and V_L2 is a variable domain from a heavy chain.

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In one embodiment V_L1 is a variable domain from a heavy chain and V_L2 is a variable domain from a light chain.

5   In one embodiment none of the variable domains of are linked by a disulfide bond.

In one embodiment the first variable domain pair bind the same epitope as the second variable domain pair.

10   In one embodiment the fusion protein of the invention avidly binds the target antigen.

In one embodiment the first variable domain pair bind the same antigen as the second variable domain pair, for example the first variable domain pair bind and the second variable domain pair bind different epitopes on the same antigen.

15

Thus in one embodiment the fusion protein according to the present disclosure is mono-specific. Monospecific as employed herein is intended to refer to the fact that all the binding sites bind the same target antigen.

20   In one aspect of this embodiment all the binding sites bind the same epitope(s) of said antigen.

In an alternative embodiment at least two binding sites bind different epitopes on the target antigen.

25

In one embodiment the first variable domain pair binds a different/distinct antigen to the second variable domain pair.

Thus in one embodiment the fusion protein according to the present disclosure is
30   bispecific, for example the two binding sites specifically bind different or distinct antigens.

Specifically binds as employed herein is intended to refer to antibodies that have high affinity for a target antigen (i.e. antigens to which they are specific) and which bind antigens to which they are not specific with a low or much lower affinity (or not at all). Methods of measuring affinity are known to those skilled in the art and include

5   such assays as BIAcore.

The amino acid pairs listed above are in the positions conducive to replacement by cysteines such that disulfide bonds can be formed. Cysteines can be engineered into these positions by known techniques.

10

In one embodiment the V_H1 is fused directly to C_H1 and V_L1 is fused directly to CL.

In one embodiment there is a disulfide bonds between the constant region fragments, such as C_H1 and CL.

15

Any of the formats provided herein may be provided with or without a disulfide bond between the constant domains.

The CL domain is derived from either Kappa or Lambda. In one embodiment CL is

20   cKappa.

In one embodiment the "natural" disulfide bond is present between C_H1 and CL. The natural position for a bond forming 'interchain' cysteine is 214 in human cKappa and cLambda (Kabat numbering 4th edition 1987). Wherever reference is made to Kabat

25   numbering the relevant reference is Kabat et al., 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA

The exact location of the bond forming cysteine or 'interchain cysteine' in C_H1 depends on the particular domain actually employed. Thus, for example in human

30   gamma-1 the natural position of the interchain cysteine forming the disulfide bond is located at position 233 (Kabat numbering 4th edition 1987). The position of the bond forming cysteine for other human isotypes such as gamma 2, 3, 4, IgM and IgD are known, for example 127.

Various interchain disulfide bonds and lack thereof are shown in Fig 2A.

In one embodiment the fusion protein according to the disclosure has a disulfide bond
in a position equivalent of corresponding to that in the naturally occurring C_H1 and
CL.

In one embodiment constant region comprising C_H1 or CL has a disulfide bond which
is in a non-naturally occurring position. This may be engineered into the molecule by
introducing cysteine(s) into the amino acid chain at the positions required. This non-
natural disulfide bond is in addition to or as an alternative to the natural disulfide bond
present between C_H1 and CL.

In one embodiment the natural disulfide bond between C_H1 and CL is absent. In one
embodiment all interchain disulfide bonds between C_H1 and CL are absent.

In one embodiment each constant region fragment is fused to at least one variable
domain.

In one embodiment each constant region fragment is also linked via a peptide, for
example an artificial/non-naturally occurring linker such as sequence in Table 1
and/or 2, to a variable domain, for example which is a non-cognate pair to the variable
domain fused thereto.

In one embodiment the constant region fragment, for example in the heavy chain,
comprises a C_H1 domain. In one embodiment the constant region fragment consists
of a C_H1 domain.

The C_H1 may be derived from be human IgA, IgD, IgE, IgG (such as IgG1, IgG2,
IgG3, IgG4) or IgM domains and isotypes thereof.

In one embodiment the light chain comprises a CL domain. In one embodiment the
constant region in the light chain consists of CL domain.

In one embodiment from the N-terminal the heavy chain is arranged as follows: a variable domain V_H1 (part of a first cognate pair) a C_H1, a variable domain V_H2 (part of a second cognate pair). In this arrangement C_H1 may, for example be fused to the variable domain V_L1 from the first cognate pair and linked via a peptide to the variable domain of the second cognate pair.

In one embodiment from the N-terminal the light chain is arranged as follows a V_L1 (part of a first cognate pair) a CL, a V_L2 (part of a second cognate pair), for example a CL may be fused to V_L1 of the first cognate pair and linked via a peptide to the V_L2 of the second cognate pair.

Examples of suitable peptide linkers are given below, for example in Table 1.

Table1. Flexible linker sequences

SEQ ID NO:	SEQUENCE
1	SGGGGSE
2	DKTHTS
3	(S)GGGGS
4	(S)GGGGSGGGGS
5	(S)GGGGSGGGGSGGGGS
6	(S)GGGGSGGGGSGGGGSGGGGS
7	(S)GGGGSGGGGSGGGGSGGGGSGGGGS
8	AAAGSG-GASAS
9	AAAGSG-XGGGS-GASAS
10	AAAGSG-XGGGSXGGGS –GASAS
11	AAAGSG- XGGGSXGGGSXGGGS –GASAS
12	AAAGSG- XGGGSXGGGSXGGGSXGGGS-GASAS
13	AAAGSG-XS-GASAS
14	PGGNRGTTTTRRPATTTGSSPGPTQSHY
15	ATTTGSSPGPT
16	ATTTGS
17	GS

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18	EPSGPISTINSPPSKESHKSP
19	GTVAAPSVFIFPPSD
20	GGGGIAPSMVGGGGS
21	GGGGKVEGAGGGGGS
22	GGGGSMKSHDGGGGS
23	GGGGNLITIVGGGGS
24	GGGGVVPSLPGGGGS
25	GGEKSIPGGGGS
26	RPLSYRPPFPFGFPSVRP
27	YPRSIYIRRRHPSPSLTT
28	TPSHLSHILPSFGLPTFN
29	RPVSPFTFPRLSNSWLPA
30	SPAAHFPRSIPRPGPIRT
31	APGPSAPSHRSLPSRAFG
32	PRNSIHFLHPLLVAPLGA
33	MPSLSGVLQVRYLSPPDL
34	SPQYPSPLTLTLPPHPSL
35	NPSLNPPSYLHRAPSRIS
36	LPWRTSLLPSLPLRRRP
37	PPLFAKGPVGLLSRSFPP
38	VPPAPVVSLRSAHARPPY
39	LRPTPPRVRSYTCCPTP-
40	PNVAHVLPLLTVPWDNLR
41	CNPLLPLCARSPAVRTFP

(S) is optional in sequences 3 to 7. The linkers employed in the present disclosure may comprise a linker shown in Table 1.

5   Examples of rigid linkers, for use in fusion proteins according to the present disclosure include, the peptide sequences GAPAPAAPAPA (SEQ ID NO:42), PPPP (SEQ ID NO:43) and PPP.

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In one embodiment the peptide link comprises an albumin binding peptide.

Examples of albumin binding peptides are provided in WO 2007/106120 and include:

Table 2

SEQ ID NO:	SEQUENCE
45	DLCLRDWGCLW
46	DICLPRWGCLW
47	MEDICLPRWGCLWGD
48	QRLMEDICLPRWGCLWEDDE
49	QGLIGDICLPRWGCLWGRSV
50	QGLIGDICLPRWGCLWGRSVK
51	EDICLPRWGCLWEDD
52	RLMEDICLPRWGCLWEDD
53	MEDICLPRWGCLWEDD
54	MEDICLPRWGCLWED
55	RLMEDICLARWGCLWEDD
56	EVRSFCTRWPAEKSCKPLRG
57	RAPESFVCYWETICFERSEQ
58	EMCYFPGICWM

In one embodiment the linker has an effector function.

In one embodiment the fusion proteins of the disclosure are dimerised, to provide a molecule referred to herein as (Fab'Fv)$_2$. Dimerisation for example may be via formation of a disulphide bond between two solvent accessible 'target' cysteines on each of two fusion protein according to the disclosure (i.e Fab-Fv), or chemical cross-linking of 'target' cysteines, lysines, sugar moieties or un-natural amino acids on each of two Fab-Fv.

Where formation of inter-Fab-Fv disulphide bond formation (intermolecular bond formation) is desired only one linker within a Fab-Fv contains cysteine or cysteines. That is to say that when the linker between CL and V$_L$2 does contain one or more cysteines, the linker between C$_H$1 and V$_H$2 will usually be devoid of cysteines.

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When the linker between C_H1 and V_H2 contains one or more cysteines, then the linker between CL and V_L2 will usually be devoid of cysteines.

5   Linkers may contain between 1 and 8 cysteines and may be composed of any peptide sequence containing 1 to 8 cysteines.

Suitable peptide sequences shown in Table 1, 2 and 3 and these may also be engineered to contain 1 to 8 cysteines, as required.

10

Hinges may be employed as linkers to promote dimerisation, since they are naturally flexible and contain sufficient secondary structure as to promote efficient and stable disulphide formation. In particular natural hinges or modified hinges of any class or isotype may be used when they contain between 1 and 8 cysteine residues.

15

When a natural or modified hinge linker sequence between CL and V_L2 does contain one or more cysteines then the linker between C_H1 and V_H2 will usually be devoid of cysteines.

20   When the hinge linker between C_H1 and V_H2 does contain one or more cysteines, then the linker between CL and V_L2 is usually devoid of cysteines.

Alternatively, inter- Fab-Fv dimerisation may be promoted by the engineering of 'target' residues on any solvent or surface accessible area of V_L1, V_H1, cKappa,

25   cLambda, C_H1, V_L2 or V_H2. Similarly, 'target' residues may be engineered to be at the C-terminus of either light chain or heavy chain polypeptides, for example after V_L2 of after V_H2. These target residues, will preferably be cysteine residues immediately after the last residues of V_L2 or V_H2 or in a linker, hinge or spacer region of polypeptide.

30

Where 'target' residues are linked by a chemical cross-linker, the length, composition, activity or hetero-functionality may be varied in order to fine tune antigen accessibility, functional avidity, serum half-life, purification properties or storage and

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formulation properties. The chemical cross-linker may be composed in part of polyethylene glycol, or may contain additional reactive groups for addition of a third specificity or active agent.

5   In one embodiment a fusion protein of the present invention is dimerised using a polymer, for example PEG, such that one chain in a first fusion protein is conjugated to a polypeptide, for example an antibody or fragment to form a heterodimer.

In one embodiment a fusion protein of the present invention is dimerised using a
10   polymer, to a second fusion protein according to the present disclosure to form a heterodimer or a homodimer.

Figure 2B shows an example of a PEG linked dimer format comprising two proteins of the present disclosure. The PEG linker is linked between C_H1 in one fusion protein
15   and C_H1 of the second fusion protein. However, a hinge region may be incorporated with suitable properties for conjugating a PEG linker molecule thereto. Alternatively a CL domain may be employed to conjugate the PEG thereto.

The position of the disulfide bond between one or more variable domain pairs can be
20   in any of the positions, described herein.

The format may also be provided with or without a disulfide bond between the constant regions, examples of which are discussed above in more detail.

25   Hinges may be natural hinges or modified hinges. Modified hinges may be employed as per Table 3.

A number of modified hinge regions have already been described for example, in US5,677,425, US6642356, WO9915549, WO2005003170, WO2005003169,
30   WO2005003170, WO9825971 and WO2005003171 and these are incorporated herein by reference. Particular examples of hinges include those shown in Table 3.

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Table 3. Hinge linker sequences

SEQ ID NO:	SEQUENCE
59	DKTHTCXX
60	DKTHTCPPCPA
61	DKTHTCPPCPATCPPCPA
62	DKTHTCPPCPATCPPCPATCPPCPA
63	DKTHTCPPCPAGKPTLYNSLVMSDTAGTCY
64	DKTHTCPPCPAGKPTHVNVSVVMAEVDGTCY
65	DKTHTCCVECPPCPA
66	DKTHTCPRCPEPKSCDTPPPCPRCPA
67	DKTHTCPSCPA
68	DKTHTSXX
69	DKTHTCPPSPA
70	DKTHTSPPCPA
71	DKTHTSPPSPA
72	DKTHTCPPCPATCPPSPA
73	DKTHTCPPCPATSPPCPA
74	DKTHTCPPSPATCPPCPA
75	DKTHTSPPCPATCPPCPA
76	DKTHTCPPCPATSPPSPA
77	DKTHTCPPSPATCPPSPA
78	DKTHTSPPCPATCPPSPA
79	DKTHTSPPSPATCPPCPA
80	DKTHTSPPCPATSPPCPA
81	DKTHTCPPSPATSPPCPA
82	DKTHTSPPSPATSPPSPA
83	DKTHTCPPSPATSPPSPA
84	DKTHTSPPCPATSPPSPA
85	DKTHTSPPSPATCPPSPA
86	DKTHTSPPSPATSPPCPA
87	DKTHTCPPCPATCPPCPATCPPSPA
88	DKTHTCPPCPATCPPCPATSPPCPA

89	DKTHTCPPCPATCPPSPATCPPCPA
90	DKTHTCPPCPATSPPCPATCPPCPA
91	DKTHTCPPSPATCPPCPATCPPCPA
92	DKTHTSPPCPATCPPCPATCPPCPA
93	DKTHTCPPCPATCPPCPATSPPSPA
94	DKTHTCPPCPATCPPSPATCPPSPA
95	DKTHTCPPCPATSPPCPATCPPSPA
96	DKTHTCPPSPATCPPCPATCPPSPA
97	DKTHTSPPCPATCPPCPATCPPSPA
98	DKTHTCPPCPATCPPSPATSPPCPA
99	DKTHTCPPCPATSPPCPATSPPCPA
100	DKTHTCPPSPATCPPCPATSPPCPA
101	DKTHTSPPCPATCPPCPATSPPCPA
102	DKTHTCPPCPATSPPSPATCPPCPA
103	DKTHTCPPSPATCPPSPATCPPCPA
104	DKTHTSPPCPATCPPSPATCPPCPA
105	DKTHTCPPSPATSPPCPATCPPCPA
106	DKTHTSPPCPATSPPCPATCPPCPA
107	DKTHTSPPSPATCPPCPATCPPCPA
108	DKTHTCPPCPATCPPSPATSPPSPA
109	DKTHTCPPCPATSPPCPATSPPSPA
110	DKTHTCPPSPATCPPCPATSPPSPA
111	DKTHTSPPCPATCPPCPATSPPSPA
112	DKTHTCPPCPATSPPSPATCPPSPA
113	DKTHTCPPSPATCPPSPATCPPSPA
114	DKTHTSPPCPATCPPSPATCPPSPA
115	DKTHTCPPSPATSPPCPATCPPSPA
116	DKTHTSPPCPATSPPCPATCPPSPA
117	DKTHTSPPSPATSPPCPATCPPCPA
118	DKTHTSPPSPATCPPSPATCPPCPA
119	DKTHTSPPSPATCPPCPATSPPCPA
120	DKTHTSPPSPATCPPCPATCPPSPA

121	DKTHTCPPSPATSPPSPATCPPCPA
122	DKTHTCPPSPATSPPCPATSPPCPA
123	DKTHTCPPSPATCPPSPATSPPSPA
124	DKTHTCPPSPATSPPSPATCPPSPA
125	DKTHTSPPSPATSPPCPATCPPSPA
126	DKTHTSPPSPATSPPSPATCPPCPA
127	DKTHTSPPSPATSPPCPATSPPCPA
128	DKTHTSPPSPATSPPCPATCPPSPA
129	DKTHTCPPSPATSPPSPATSPPCPA
130	DKTHTCPPSPATSPPSPATSPPSPA
131	DKTHTSPPSPATSPPSPATSPPCPA
132	DKTHTSPPCPATSPPSPATSPPSPA
133	DKTHTSPPSPATCPPSPATSPPSPA
134	DKTHTSPPSPATSPPCPATSPPSPA
135	DKTHTSPPSPATSPPSPATCPPSPA
136	DKTHTSPPSPATSPPSPATSPPSPA
137	DKTHTCPPCPAGKPTLYNSLVMSDTAGTSY
138	DKTHTCPPSPAGKPTLYNSLVMSDTAGTCY
139	DKTHTSPPCPAGKPTLYNSLVMSDTAGTCY
140	DKTHTCPPSPAGKPTLYNSLVMSDTAGTSY
141	DKTHTSPPSPAGKPTLYNSLVMSDTAGTCY
142	DKTHTSPPCPAGKPTLYNSLVMSDTAGTSY
143	DKTHTSPPSPAGKPTLYNSLVMSDTAGTSY
1441	DKTHTCPPCPAGKPTHVNVSVVMAEVDGTSY
145	DKTHTCPPSPAGKPTHVNVSVVMAEVDGTCY
146	DKTHTSPPCPAGKPTHVNVSVVMAEVDGTCY
147	DKTHTCPPSPAGKPTHVNVSVVMAEVDGTSY
148	DKTHTSPPCPAGKPTHVNVSVVMAEVDGTSY
149	DKTHTSPPSPAGKPTHVNVSVVMAEVDGTCY
150	DKTHTSPPSPAGKPTHVNVSVVMAEVDGTSY
151	DKTHTCCVECPPSPA
152	DKTHTCCVESPPCPA

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153	DKTHTCSVECPPCPA
154	DKTHTSCVECPPCPA
155	DKTHTCCVESPPSPA
156	DKTHTCSVECPPSPA
157	DKTHTSCVECPPSPA
158	DKTHTCSVESPPCPA
159	DKTHTSSVECPPCPA
160	DKTHTCSVESPPSPA
161	DKTHTSSVECPPSPA
162	DKTHTSSVESPPCPA
163	DKTHTSSVESPPSPA
164	DKTHTCPRCPEPKSCDTPPPCPRSPA
165	DKTHTCPRCPEPKSCDTPPPSPRCPA
166	DKTHTCPRCPEPKSSDTPPPCPRCPA
167	DKTHTCPRSPEPKSCDTPPPCPRCPA
168	DKTHTSPRCPEPKSCDTPPPCPRCPA
169	DKTHTCPRCPEPKSCDTPPPSPRSPA
170	DKTHTCPRCPEPKSSDTPPPCPRSPA
171	DKTHTCPRSPEPKSCDTPPPCPRSPA
172	DKTHTSPRCPEPKSCDTPPPCPRSPA
173	DKTHTCPRCPEPKSSDTPPPSPRSPA
174	DKTHTCPRSPEPKSCDTPPPSPRSPA
175	DKTHTSPRCPEPKSCDTPPPSPRSPA
176	DKTHTCPRSPEPKSSDTPPPCPRSPA
177	DKTHTSPRCPEPKSSDTPPPCPRSPA
178	DKTHTSPRSPEPKSCDTPPPCPRSPA
179	DKTHTCPRSPEPKSSDTPPPSPRCPA
180	DKTHTSPRCPEPKSSDTPPPSPRCPA
181	DKTHTSPRSPEPKSSDTPPPCPRCPA
182	DKTHTCPRSPEPKSSDTPPPSPRSPA
183	DKTHTSPRSPEPKSSDTPPPCPRSPA
184	DKTHTSPRSPEPKSCDTPPPSPRSPA

20

185	DKTHTSPRCPEPKSSDTPPPSPRSPA
186	DKTHTSPRSPEPKSSDTPPPSPRSPA
187	DKTHTCPSSPA
188	DKTHTSPSCPA
189	DKTHTSPSSPA

wherein in X represents any amino acid, for example XX may represent AA.

In one embodiment the fusion protein according to the present disclosure does not comprise a hinge.

The inventors believe that by providing variable domains as cognate pairs in the final construct optimises and maintains the antigen bind properties of the binding site formed by the relevant pair.

It will be appreciated that one or more amino acid substitutions, additions and/or deletions may be made to the antibody variable domains, provided by the present invention, without significantly altering the ability of the antibody to bind to target antigen and to neutralise activity thereof. The effect of any amino acid substitutions, additions and/or deletions can be readily tested by one skilled in the art, for example by using the *in vitro* assays, for example a BIAcore assay.

In one embodiment the interchain disulfide bond between the light chain and heavy chain is not present and one or two of the cysteines that would not normally form the bond (interchain cysteines) are conjugated to a polymer. One interchain cysteine can be selectively conjugated employing genetic engineering techniques to replace the corresponding interchain cysteine with an alternative amino acid such as serine.

Both interchain cysteines may be conjugated to a polymer by employing suitably strong reducing agents. Various arrangements of interchain cysteines or lack of them are represented in Figure 2A.

21

The kappa or lambda domain may be modified by chemical conjugation with a suitable polymer.

In one embodiment a human cKappa in a fusion protein of the present disclosure comprises at the C-terminal a sequence selected from:

SFNRGEC (SEQ ID NO:190);

SFNRGCS (SEQ ID NO:191);

SFNRCES (SEQ ID NO:192);

SFNCGES (SEQ ID NO:193);

SFCRGES (SEQ ID NO:194); and

SCNRGES (SEQ ID NO:195).

One or more cysteines in the C-terminal of cKappa, for example as described directly above, may be used to conjugate a PEG polymer thereto.

Suitable positions in cKappa for conjugation include:

1. **Upper cKappa**, which is approximately equidistant from V_L1 and V_L2 in a fusion protein according to the present disclosure, for example Glu143, Gln199 and/or Val110 (linear and Kabat numbering),,

2. **Middle cKappa** which is distant from all mobile and flexible motifs which might be required for effective antigen binding, for example Lys145 and/or Gln147, (linear and Kabat numbering),and/or

3. **Lower cKappa,** for example Lys190, Asn210, Arg211, (linear and Kabat numbering),and/or Glu213.

These amino acids may be replaced, for example with cysteine, as required, employing known techniques.

In one embodiment the fusion protein comprises the sequence:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS^{156}GNSQES VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS^{202}S^{203}PVTKSFNRGEC (SEQ ID NO:196)

22

for example in cKappa, in particular in the position 108-214 (Kabat numbering) in the amino acid sequence.

5   In one embodiment a human C_H1 in a fusion protein of the present disclosure comprises at the C-terminal a sequence selected from:

        KSC,

        KSS, and

        KCS.

10

In one embodiment the fusion protein comprises the sequence:

S^{120}ASTKGPSVFPLAPSSKSTS^{139}GGTAALGCLVKDYFPEPVTVSWNS^{163}GALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSC^{222}

15   (SEQ ID NO:197)

Amino acid S^{120} shown in bold is not part of the genetic / exon definition of C_H1, but is considered to be part of the strucural 'elbow'

Underlined sequence shown is not part of the genetic / exon definition of C_H1, but is

20   part of upper hinge

for example in C_H1, in particular in the position 121-218 (Kabat numbering) in the amino acid sequence.

25   Suitable positions in C_H1 for conjugation include:

        1. **Upper C_H1** which is approximately equidistant from V_H1 and V_H2 in fusion proteins according to the present invention, for example Asn211 (216) and/or Thr123 (Asn 216 and/or Thr 116 by Kabat numbering),,

30   2. **Middle C_H1** which is distant from all mobile and flexible motifs which might be required for effective antigen binding, for example Ser163 and/or Ser127 (Ser163 and/or Ser120 by Kabat numbering), and/or

        3. **Lower C_H1** for example Lys136, Ser137, and/or Ser222 (Lys129, Ser130, Ser232 by Kabat numbering).

23

These amino acids may be replaced, for example with cysteine, as required employing known techniques.

In one embodiment therefore an engineered cysteine according to the present
5   invention refers to where the naturally occurring residue at a given amino acid position has been replaced with a cysteine residue.

Introduction of engineered cysteines can be performed using any method known in the art. These methods include, but are not limited to, PCR extension overlap
10   mutagenesis, site-directed mutagenesis or cassette mutagenesis (see, generally, Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, 1989; Ausbel et al., Current Protocols in Molecular Biology, Greene Publishing & Wiley-Interscience, NY, 1993). Site-directed mutagenesis kits are commercially available, e.g. QuikChange® Site-
15   Directed Mutagenesis kit (Stratagen, La Jolla, CA). Cassette mutagenesis can be performed based on Wells et al., 1985, Gene, 34:315-323. Alternatively, mutants can be made by total gene synthesis by annealing, ligation and PCR amplification and cloning of overlapping oligonucleotides.

20   In one embodiment the fusion proteins has no cysteines in the C_H1 and/or CL regions for conjugation to a polymer.

In one embodiment V_H1 is fused to C_H1 and the amino acid sequence connecting the same is referred to herein as the elbow. In one embodiment said elbow has one or
25   more, such as one, PEG polymers conjugated thereto, for example conjugated at a position equidistant between V_H1 and V_H2 such as conjugated to Thr123 (Thr 116 by Kabat numbering) and/or S119 (Ser 112 by Kabat numbering) and/or S122 (Ser115 by Kabat numbering) residues. The disulfide bond is provided, for example by engineering a cysteine or cysteines into suitable positions in the molecule to provide a
30   substrate for conjugating a PEG polymer thereto.

24

In one embodiment V_L1 is fused to CL and the amino acid sequence connecting the same is referred to herein as the elbow. In one embodiment said elbow has one or more, such as one, PEG polymer conjugated thereto for example conjugated at a position equidistant between V_L1 and V_L2 such as conjugated to Lys107, Arg108

5   and/or Thr109 residues (linear and Kabat numbering). As discussed above, cysteines can be provided as required for conjugation.

In one embodiment the PEG is conjugated to a residue that is spatially close to areas of aggregation propensity. Advantageously, PEGylation close to such areas may

10   substantially mask the region of interest from inter-molecular interactions at concentrations in solution suitable for therapeutic doses. In the light chain the conjugation may for example be at or about Thr109 or Gln199 (linear and Kabat numbering), for example to negate the ValAla-AlaLys spot, (linear and Kabat numbering) and/or Lys149 or Asn152 (linear and Kabat numbering), for example to

15   negate the single Leu spot. In the heavy chain the conjugation may, for example be at or about Gln17 Gln178 (Gln 179 by Kabat numbering) or Ser180 (Ser 182 by Kabat numbering) or Gly181 (Gly 183 by Kabat numbering) to deal with the ValLys-Tyr spot.

20   In one embodiment the PEG molecule is attached to a solvent accessible, reactive and/or surface exposed/accessible amino acid such as cysteine.

A surface exposed cysteine (free cysteine) as employed herein is intended to refer to cysteine that when the fusion protein in a "natual" folded conformation is accessible

25   for conjugating an effector molecule, such as a PEG molecule, thereto. A surface cysteine is one found in a hydrophilic part of the antibody or fragment. Examples of how to engineer free cysteines of this type are also provided in US7,521,541.

Suitable amino acids in the light chain of 4D5 (sequence shown in Fig 3), which may

30   be replaced by cysteine include:
Serine (S): 7,9, 10, 12, 14, 26, 56, 60, 63, 67, 76, 77, 114, 121, 127, 156, 159, 168, 171, 202, 203,
Threonine (T): 5,20, 22, 31, 69, 72, 74, 129, 197, 206,

25

Glycine (G): 16, 41, 57, 68, 128, 143, 157, 200, 212

Aspartate (D): 17, 28, 70, 122, 151, 167, 170

Arginine (R): 18, 24, 61, 211,

Glutamine (Q): 27, 79, 147, 160, 195, 199

5   Asparagine (N): 30, 152, 158, 210,

Alanine (A): 34, 153,

Lysine (K): 39, 42, 126, 145, 149, 169,

Glutamate (E): 55, 81, 123, 161, 213.

The numbers employed above are the antibody primary sequence numbering as shown

10   in PDB crystal structure sequence file, 1FVE. These numbers also correspond to

Kabat numbering. The disclosure also extends to replacement of corresponding amino

acids (employing Kabat numbering) in other antibodies or fragments comprised in

fusion proteins of the invention.

15   Accordingly in one embodiment the antibody fusion protein light chain comprises an

engineered cysteine wherein the position of said engineered cysteine is selected from

the group consisting of S7, S9, S10, S12, S14, S26, S56, S60, S63, S67, S76, S77,

S114, S121, S127, S156, S159, S168, S171, S202, S203, T5, T20, T22, T31, T69,

T72, T74, T129, T197, T206, G16, G41, G57, G68, G128, G143, G157, G200, G212,

20   D17, D28, D70, D122, D151, D167, D170, R18, R24, R61, R211, Q27, Q79, Q147,

Q160, Q195, Q199, N30, N152, N158, N210, A34, A153, K39, K42, K126, K145,

K149, K169, E55, E81, E123, E161 and E213.

In one embodiment the antibody fusion protein light chain comprises an engineered

25   cysteine wherein the position of said engineered cysteine is selected from the group

consisting of S7, S9, S10, S12, S14, S26, S56, S60, S63, S67, S76, S77, S114, S121,

S127, S156, S159, S168, S171, S202, S203, T5, T20, T22, T31, T69, T72, T74, T109,

T129, T197, T206, G16, G41, G57, G68, G128, G143, G157, G200, G212, D17, D28,

D70, D122, D151, D167, D170, R18, R24, R61, R108, R211, Q27, Q79, Q147, Q160,

30   Q195, Q199, N30, N152, N158, N210, A34, A153, K39, K42, K107, K126, K145,

K149, K169, K190, E55, E81, E123, E143, E161 and E213.

26

Other suitable residues have been described in the literature including WO2006/034488 and WO2008/038024. Accordingly, in one embodiment a cysteine is engineered into the fusion protein light chain, for example:

V15 is replaced by C,

5   A43 is replaced by C,

V110 is replaced by C,

S114 is replaced by C,

S121 is replaced by C,

S127 is replaced by C,

10   A144 is replaced by C,

A153 is replaced by C,

N158 is replaced by C,

S168 is replaced by C,

V205 is replaced by C,

15   S171 is replaced by C,

S156 is replaced by C,

S202 is replaced by C, and/or

S203 is replaced by C.

The numbering above is by reference to the light chain sequence shown in Figure 3,

20   but the disclosure herein also extends to corresponding Kabat positions in other light chains.

In one embodiment a cysteine is engineered into the fusion protein light chain, for example:

25   S171 is replaced by C,

S156 is replaced by C,

S202 is replaced by C, and/or

S203 is replaced by C.

The numbering above is by reference to the light chain sequence shown in Figure 3,

30   but the disclosure herein also extends to corresponding Kabat positions in other light chains.

27

In one embodiment the antibody fusion protein light chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S7, S9, S10, S12, S14, S26, S56, S60, S63, S67, S76, S77, S114, S121, S127, S156, S159, S168, S171, S202, S203, T5, T20, T22, T31, T69, T72, T74, T109,

5   T129, T197, T206, V15, V110, V205, G16, G41, G57, G68, G128, G143, G157, G200, G212, D17, D28, D70, D122, D151, D167, D170, R18, R24, R61, R108, R211, Q27, Q79, Q147, Q160, Q195, Q199, N30, N152, N158, N210, A34, A43, A144, A153, K39, K42, K107, K126, K145, K149, K169, K190, E55, E81, E123, E143, E161 and E213.

10

In one embodiment the antibody fusion protein light chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S7, S9, S10, S12, S14, S26, S56, S60, S63, S67, S76, S77, S159, T5, T20, T22, T31, T69, T72, T74, T109, T129, T197, T206, G16, G41, G57, G68, G128,

15   G143, G157, G200, G212, D17, D28, D70, D122, D151, D167, D170, R18, R24, R61, R108, R211, Q27, Q79, Q147, Q160, Q195, Q199, N30, N152, N210, A34, K39, K42, K107, K126, K145, K149, K169, K190, E55, E81, E123, E143, E161 and E213.

20   In one embodiment a cysteine is engineered into the fusion protein light chain constant region, CL, for example:

        T109 is replaced by C,
        E143 is replaced by C,
25   K145 is replaced by C,
        K149 is replaced by C and/or
        N210 is replaced by C.

    In one embodiment a cysteine is engineered into the fusion protein light chain variable
30   region, for example:
        S77 is replaced by C and/or
        K107 is replaced by C.

28

In one embodiment the antibody fusion protein light chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S77, K107, T109, E143, K145, K149 and N210.

5   All the residues specified herein above are derived from IgG1 but the disclosure herein also extends to corresponding Kabat positions in other light chains from other classes and isotypes of antibodies.

Accordingly, in one embodiment the antibody fusion protein light chain comprises an
10   engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of 5, 7, 9, 10, 12, 14, 15, 16, 17, 18, 20, 22, 24, 26, 27, 28, 30, 31,34, 39, 41, 42, 43, 55, 56, 57, 60, 61, 63, 67, 68, 69, 70, 72, 74, 76, 77, 79, 81, 107, 108, 109, 110, 114, 121, 122, 123, 126, 127, 128, 129, 143, 144, 145, 147, 149, 151, 152, 153, 156, 157, 158, 159, 160, 161, 167, 168, 169, 170, 171, 190, 195, 197, 199,
15   200, 202, 203, 205, 206, 210, 211, 212 and 213 numbered according to the Kabat numbering system.

In one embodiment the antibody fusion protein light chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group
20   consisting of 5, 7, 9, 10, 12, 14, 16, 17, 18, 20, 22, 24, 26, 27, 28, 30, 31,34, 39, 41, 42, 55, 56, 57, 60, 61, 63, 67, 68, 69, 70, 72, 74, 76, 77, 79, 81, 107, 108, 109, 122, 123, 126, 128, 129, 143, 145, 147, 149, 151, 152, 157, 159, 160, 161, 167, 169, 170, 190, 195, 197, 199, 200, 206, 210, 211, 212 and 213 numbered according to the Kabat numbering system.

25

Accordingly, in one embodiment the antibody fusion protein light chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of position 77, 107, 109, 143, 145, 149 and 210 numbered according to the Kabat numbering system.

30

In fusion proteins comprising the one or more of the following amino acids:
Suitable amino acids in the heavy chain of 4D5 (sequence shown in Fig 4), which may be replaced by cysteine include:

29

Serine (S): 7, 17, 21, 63, 71, 86, 119, 120, 122, 127, 134, 135, 137, 139, 160, 163, 168, 179, 180, 193, 194, 195, 210, 222

Threonine (T): 58, 69, 123, 138, 167, 198, 200,

Glycine (G): 8, 9, 10, 15, 16, 26, 66, 100, 101, 103, 140, 141, 164, 169, 181, 197,

5   Aspartate (D): 62, 73, 102, 215,

Arginine (R): 59, 67, 87,

Glutamine (Q): 3, 13, 112, 155, 199, 219,

Asparagine (N): 84, 211,

Alanine (A): 88, 121, 165

10   Lysine (K): 43, 65, 124, 136, 208, 213, 217,

Glutamate (E): 89

The numbers employed above are the antibody primary sequence numbering as shown in PDB crystal structure sequence file, 1FVE. The disclosure also extends to replacement of corresponding amino acids (employing Kabat numbering) in other

15   antibodies or fragments comprised in fusion proteins of the invention.

The equivalent Kabat sequence numbering for the positions in the heavy chain is:

Serine (S): 7, 17, 21, 62, 70, 82b, 112, 113, 115, 120, 127, 128, 130, 134, 156, 163,168, 180, 182, 195, 196, 197, 215, 232

20   Threonine (T): 57, 68, 116, 133, 167, 200, 205,

Glycine (G): 8, 9, 10, 15, 16, 26, 65, 96, 97, 99, 135, 136, 164, 169, 183, 199,

Aspartate (D): 61, 72, 98, 220,

Arginine (R): 58, 66, 86,

Glutamine (Q): 3, 13, 105, 203

25   Asparagine (N): 82a, 216,

Alanine (A): 84, 114, 165

Lysine (K): 43, 64, 117, 129, 213, 218, 222,

Glutamate (E): 85

30   Accordingly in one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S7, S17, S21, S62, S70, S82b, S112, S113, S115, S120, S127, S128, S130, S134, S156, S163, S168, S180, S182, S195, S196, S197, S215, S232,

30

T57, T68, T116, T133, T167, T200, T205, G8, G9, G10, G15, G16, G26, G65, G96,

G97, G99, G135, G136, G164, G169, G183, G199, D61, D72, D98, D220, R58, R66,

R86, Q3, Q13, Q105, Q203, N82a, N216, A84, A114, A165, K43, K64, K117, K129,

K213, K218, K222 and E85.

5

In one embodiment the antibody fusion protein heavy chain comprises an engineered

cysteine wherein the position of said engineered cysteine is selected from the group

consisting of S7, S17, S21, S62, S70, S82b, S112, S113, S115, S120, S127, S128,

S130, S134, S156, S163, S168, S180, S182, S195, S196, S197, S215, S232, T57,

10   T68, T116, T133, T167, T200, T205, G8, G9, G10, G15, G16, G26, G65, G96, G97,

G99, G135, G136, G164, G169, G183, G199, D61, D72, D98, D220, R58, R66, R86,

Q3, Q13, Q105, Q179, Q203, N82a, N216, A84, A114, A165, K43, K64, K117,

K129, K213, K218, K222 and E85.

15   Other suitable residues have been described in the literature including

WO2006/034488 and WO2008/038024. Accordingly, in one embodiment a cysteine

is engineered into the fusion protein heavy chain, for example:

            A40 is replaced by C,

            L86 is replaced by C,

20   A88 is replaced by C,

            A121 is replaced by C,

            S119 is replaced by C,

            S120 is replaced by C,

            A121 is replaced by C,

25   S122 is replaced by C,

            A175 is replaced by C,

            V176 is replaced by C, and/or

            S179 is replaced by C.

The numbering above is by reference to the heavy chain sequence shown in Figure 4,

30   but the disclosure herein also extends to corresponding Kabat positions in other heavy

chains.

31

The numbering above is by reference to the heavy chain sequence shown in Figure 4, but the disclosure herein also extends to corresponding Kabat positions in other heavy chains.

5   The equivalent positions by Kabat numbering are as follows:

A40 is replaced by C,

L82c is replaced by C,

A84 is replaced by C,

S112 is replaced by C,

10   S113 is replaced by C,

A114 is replaced by C,

S115 is replaced by C,

A176 is replaced by C,

V177 is replaced by C, and/or

15   S180 is replaced by C.

Accordingly in one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S7, S17, S21, S62, S70, S82b, S112, S113, S115, S120, S127,

20   S128, S130, S134, S156, S163, S168, S180, S182, S195, S196, S197, S215, S232, T57, T68, T116, T133, T167, T200, T205, G8, G9, G10, G15, G16, G26, G65, G96, G97, G99, G135, G136, G164, G169, G183, G199, D61, D72, D98, D220, R58, R66, R86, Q3, Q13, Q105, Q179, Q203, N82a, N216, A40, A84, A114, A165, A176, V177, L82c, K43, K64, K117, K129, K213, K218, K222 and E85.

25

In one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of S7, S17, S21, S62, S70, S82b, S120, S127, S128, S130, S134, S156, S163, S168, S182, S195, S196, S197, S215, S232, T57, T68, T116, T133, T167,

30   T200, T205, G8, G9, G10, G15, G16, G26, G65, G96, G97, G99, G135, G136, G164, G169, G183, G199, D61, D72, D98, D220, R58, R66, R86, Q3, Q13, Q105, Q179, Q203, N82a, N216, A165, K43, K64, K117, K129, K213, K218, K222 and E85.

32

In one embodiment a cysteine is engineered into the fusion protein heavy chain constant region CH1, for example:

        T116 is replaced by C,

5   S163 is replaced by C,

        S182 is replaced by C, and/or

        N216 is replaced by C.

In one embodiment a cysteine is engineered into the fusion protein heavy chain

10   variable domain, VH, for example:

        S82b is replaced by C.

In one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group

15   consisting of S82b, T116, S163, S182 and N216.

All the residues specified herein above are derived from IgG1 but the disclosure herein also extends to corresponding Kabat positions in other heavy chains from other classes and isotypes of antibodies.

20

Accordingly, in one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of position 3, 7, 8, 9, 10, 13, 15, 16, 17, 21, 26, 40, 43, 57, 58, 61, 62, 64, 65, 66, 68, 70, 72, 82a, 82b, 82c, 84, 85, 86, 96, 97, 98, 99, 105, 112,

25   113, 114, 115, 116, 117, 120, 127, 128, 129, 130, 133, 134, 135, 136, 156, 163, 164, 165, 167, 168, 169, 176, 177, 179, 180, 182, 183, 195, 196, 197, 199, 200, 203, 205, 213, 215, 216, 218, 220, 222 and 232 numbered according to the Kabat numbering system.

30   In one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of position 3, 7, 8, 9, 10, 13, 15, 16, 17, 21, 26, 43, 57, 58, 61, 62, 64, 65, 66, 68, 70, 72, 82a, 82b, 85, 86, 96, 97, 98, 99, 105, 116, 117, 120, 127, 128, 129,

130, 133, 134, 135, 136, 156, 163, 164, 165, 167, 168, 169, 179, 182, 183, 195, 196, 197, 199, 200, 203, 205, 213, 215, 216, 218, 220, 222 and 232 numbered according to the Kabat numbering system.

5   In one embodiment the antibody fusion protein heavy chain comprises an engineered cysteine wherein the position of said engineered cysteine is selected from the group consisting of position 82b, 116, 163, 182 and 216 numbered according to the Kabat numbering system.

10   In one embodiment the fusion protein comprises a free cysteine to which a PEG molecule is attached or to which a PEG molecule can be attached. In one embodiment the fusion protein or part thereof comprises the one or more sequences selected from:

LVTVCSASTKGPS   (SEQ ID NO:198)

15   LVTVSCASTKGPS   (SEQ ID NO:199)

LVTVSSCSTKGPS, (SEQ ID NO:200)and/or

HTFPCVLQSSGLYS. (SEQ ID NO:201)

In one embodiment provides an cysteine engineered fusion protein comprises a free

20   cysteine and optionally comprises a sequence selected from one or more of the following:

34

SLSASCGDRVT (SEQ ID NO:202)

QKPGKCPKLLI (SEQ ID NO:203)

EIKRTCAAPSV (SEQ ID NO:204)

TCAAPCVFIFPP (SEQ ID NO:205)

FIFPPCDEQLK (SEQ ID NO:206)

DEQLKCGTASV (SEQ ID NO:207)

FYPRECKVQWK (SEQ ID NO:208)

WKVDNCLQSGN (SEQ ID NO:209)

ALQSGCSQESV (SEQ ID NO:210)

VTEQDCKDSTY (SEQ ID NO:211)

GLSSPCTKSFN (SEQ ID NO:212)

NWIRQCPGNK (SEQ ID NO:213)

LNSCTTEDTAT (SEQ ID NO:214)

GQGTLVTVSACSTKGPSVFPL (SEQ ID NO:215)

HTFPCVLQSSGLYS (SEQ ID NO:216)

HTFPACLQSSGLYS (SEQ ID NO:217)

FLSVSCGGRVT (SEQ ID NO:218)

QKPGNCPRLLI (SEQ ID NO:219)

EIKRTCAAPSV (SEQ ID NO:220)

FYPRECKVQWK (SEQ ID NO:221)

VTEQDCKDSTY (SEQ ID NO:222)

In one embodiment there is provided a light chain comprising the sequence:

DIQMTQSPSSLSASVGDRVT²⁰ITCRASQDVNTAVAWYQQKP⁴⁰GKAPKLLIYSASFLE
SGVPS⁶⁰RFSGSRSGTDFTLTISSLQP⁸⁰EDFATYYCQQHYTTPPTFGQ¹⁰⁰GTKVEIKR
TVAPSVFIFPP¹²⁰SDEQLKSGTASVVCLLNNFY¹⁴⁰PREAKVQWKVDNALQSGNSQ¹⁶⁰

35

⁰ESVTEQDSKDSTYSLSSTLT¹⁸⁰LSKADYEKHKVYACEVTHQG²⁰⁰LSSPVTKSFNRGE
C²¹⁴

(SEQ ID NO:223)

Key

underlined	surface accessible side chain
double-underlined	residues suitable for replacing by cysteine
bold	areas of particular practical interest for conjugation (distant from CDR's & outer/flexible loops)
italic	areas of improbable practical interest (near to or in CDR residues)..
light shading	buried/inaccessible regions
dark shading	CH1 residues with 'aggregation propensity"

In one embodiment there is provided a heavy chain comprising the sequence:

EVQLVESGGGLVQPGGSLRL²⁰SCAASG*FNIKDTYIH*WVRQA⁴⁰PGKGLEWVARI *YPTN
GYTRY*⁶⁰ADSVKGRFTISADT*SKNTAY*⁸⁰LQMNSLRAEDTAVYYCSRWG*¹⁰⁰GDGFYAMD
YWGQGTLVTVSS*¹²⁰ASTKGPSVFPLAPSSKSTSG¹⁴⁰GTAALGCLVKDYFPEPVTVS¹⁶
⁰WNSGALTSGVHTFPAVLQSS¹⁸⁰GLYSLSSVVTVPSSSLGTQT²⁰⁰YICNVNHKPSNTK
VDKKVEP²²⁰KSC

(SEQ ID NO:44)

In one embodiment the PEG polymer is conjugated to a position in a lower light chain
variable domain, for example V$_L$1 and/or V$_L$2 suitably distant from CDR's, and
near/adjacent to the elbow region but not encoded therewithin, such as Ser12, Ser14,
Gln79, or Glu81, such that the binding of the of the entity is not in any way
diminished or adversely affected. The numbering of the these light chain residues is
the same using Kabat numbering.

In one embodiment the PEG polymer is conjugated to a position in a lower heavy
chain variable domain, for example V$_H$1 and/or V$_H$2, suitably distant from CDR's,
and near/adjacent to the elbow region but not encoded therewithin, such as Gln13
(Gln 13 by Kabat numbering) or Glu89 (Glu85 by Kabat numbering), such that the
binding of the entity is not in any way diminished or adversely affected.

All embodiments herein are such that the conjugation of the PEG polymer to the
entity does not diminish or adversely affect the binding/ affinity of the fusion protein
of the disclosure.

Suitable polymers include a polyalkylene polymer, such as a poly(ethyleneglycol) or, especially, a methoxypoly(ethyleneglycol) or a derivative thereof, and especially with a molecular weight in the range from about 15000Da to about 40000Da. Other suitable polymers such as starches, complex glycoforms such as N-GlucNac and

5   others eg Polymers investigated include those based on amino acids such as poly-GGGGS, polyglutamate and polyaspartate (Schlapschy et al., 2007; Jultani et al., 1997; Zunino et al., 1982); those based on carbohydrates such as oxidized dextran, carboxymethyl dextran, starch and polysialic acid (Fagnani et al., 1990; Baudys et al., 1998; Gregoriadis et al., 2000); and completely synthetic polymers such as poly(N-

10   vinylpyrrolidone), poly(N-acryloilmorpholine), polyoxyethylated glycerol, hydroxypropyl methacrylamide, polymethacrylate, bow tie dendrimers and PEG (Kaneda et al., 2004; Caliceti et al., 1999; Soucek et al., 2002).

      The size of the polymer may be varied as desired, but will generally be in an average

15   molecular weight range from 500Da to 80000Da, for example from 5000 to 50000Da such as from 20000 to 40000Da. The polymer size may in particular be selected on the basis of the intended use of the product for example ability to localize to certain tissues such as tumors or extend circulating half-life (for review see Chapman, 2002, Advanced Drug Delivery Reviews, 54, 531-545). Thus, for example, where the

20   product is intended to leave the circulation and penetrate tissue, for example for use in the treatment of a tumour, it may be advantageous to use a small molecular weight polymer, for example with a molecular weight of around 5000Da. For applications where the product remains in the circulation, it may be advantageous to use a higher molecular weight polymer, for example having a molecular weight in the range from

25   20000Da to 40000Da.

      PEG molecules may be attached through any available amino acid side-chain or terminal amino acid functional group located in the antibody fragment, for example any free amino, imino, thiol, hydroxyl or carboxyl group. Such amino acids may

30   occur naturally in the antibody fragment or may be engineered into the fragment using recombinant DNA methods (see for example US 5,219,996; US 5,667,425; WO98/25971). In one example the antibody molecule of the present invention is a modified Fab fragment wherein the modification is the addition to the C-terminal end

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of its heavy chain one or more amino acids to allow the attachment of an effector

molecule. Suitably, the additional amino acids form a modified hinge region

containing one or more cysteine residues to which the effector molecule may be

attached. Multiple sites can be used to attach two or more PEG molecules.

5

In one embodiment the Fab or Fab' is PEGylated with one or two PEG molecules.

In one embodiment a PEG molecule is linked to a cysteine 171 in the light chain, for

example see WO2008/038024 incorporated herein by reference.

10

In one the Fab or Fab' is PEGylated through a solvent or surface accessible cysteine.

Suitably PEG molecules are covalently linked through a thiol group of at least one

cysteine residue located in the fusion protein. Each polymer molecule attached to the

15   fusion protein may be covalently linked to the sulphur atom of a cysteine residue

located in the protein. The covalent linkage will generally be a disulphide bond or, in

particular, a sulphur-carbon bond. Where a thiol group is used as the point of

attachment appropriately activated PEG molecules, for example thiol selective

derivatives such as maleimides and cysteine derivatives may be used. An activated

20   PEG molecule may be used as the starting material in the preparation of polymer-

fusion protein containing molecules as described above. The activated PEG molecule

may be any polymer containing a thiol reactive group such as an α-halocarboxylic

acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone or a

disulphide. Such starting materials may be obtained commercially (for example from

25   Nektar, USA; Nippon Oils and Fats (NOF), Japan; Dr Reddy, UK; JenKem, China;

Pan Asia Bio, China; SunBio, South Korea; Biovectra, USA) or may be prepared

from commercially available starting materials using conventional chemical

procedures. Particular PEG molecules include 20K methoxy-PEG-amine and 20K

methoxy-PEG-N-hydroxy succinimde ester (for example from Nippon Oils and Fats

30   (NOF), Japan; Dr Reddy, UK; JenKem, China; Pan Asia Bio, China; SunBio, South

Korea; Rapp Polymere, Germany).

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Effector molecules such as PEG molecules may be attached to fusion proteins by a number of different methods, including through aldehyde sugars or more commonly through any available amino acid side-chain or terminal amino acid functional group located in the antibody fragment, for example any free amino, imino, thiol, hydroxyl

5   or carboxyl group. The site of attachment of effector molecules can be either random or site specific.

Random attachment is often achieved through amino acids such as lysine and this results in effector molecules, such as PEG molecules, being attached at a number of

10   sites throughout the antibody fragment depending on the position of the lysines. While this has been successful in some cases the exact location and number of effector molecules, such as PEG molecules, attached cannot be controlled and this can lead to loss of activity for example if too few are attached and/or loss of affinity if for example they interfere with the antigen binding site (Chapman 2002 Advanced Drug

15   Delivery Reviews, 54, 531-545). As a result, controlled site specific attachment of effector molecules, such as PEG molecules, is usually the method of choice.

Site specific attachment of effector molecules, such as PEG molecules, is most commonly achieved by attachment to cysteine residues since such residues are

20   relatively uncommon in antibody fragments. Antibody hinges are popular regions for site specific attachment since these contain cysteine residues and are remote from other regions of the fusion protein likely to be involved in antigen binding. Suitable hinges either occur naturally in the fragment or may be created using recombinant DNA techniques (See for example US 5,677,425; WO98/25971; Leong et al., 2001

25   Cytokine, 16, 106-119; Chapman et al., 1999 Nature Biotechnology, 17, 780-783). Alternatively, or in addition, site-specific cysteines may also be engineered into the antibody fragment for example to create surface exposed cysteine(s) for effector molecule attachment (US 5,219,996).

30   In one embodiment the fusion protein according to the invention comprises a hinge as a linker between CL and VL2 spacer of approximately corresponding length to the hinge.

39

Techniques have been described in which native and engineered cysteines are used for
the site-specific attachment of effector molecules, such as PEG molecules, (See
WO2005003169, WO2005003170 and WO2005003171). In all of these fragments
the native interchain disulphide bond between the heavy and light chain constant

5   regions (C_H1 and CL) is absent either because the interchain cysteines have been used
as a site of attachment for effector molecules or because the interchain cysteines have
been replaced by another amino acid to avoid effector molecule attachment to those
cysteines. These fragments may also comprise engineered cysteines for use as sites of
effector molecule attachment. In one example these engineered cysteines are a pair of

10   engineered cysteines which form a disulphide link between the heavy and light chain
constant regions of the antibody fragment starting material; said disulphide linkage is
lost however once effector molecules are attached to those cysteines.

In one embodiment the fusion protein PEG molecule conjugates are provided in

15   which the heavy and light chains of the antibody fragments are linked by an
engineered interchain disulphide bond which is not the native interchain disulphide
bond. This engineered interchain disulphide bond is retained during effector molecule
attachment even when strong reducing agents are used. There are also provided sites
in the light chain:heavy chain interface where pairs of cysteines can be successfully

20   engineered to introduce a disulphide bond that is sufficiently buried that it is largely
inaccessible to reducing agents and effector molecules, examples of 'buried
disulphides' are shown in WO2007010231.

A particular advantage of these fragments lies in that the disulphide bond between the

25   engineered interchain cysteines remains intact during effector molecule attachment.

Thus in one embodiment according to the present invention there is provided a fusion
protein to which one or more effector molecules is/are attached characterized in that
the native interchain disulphide bond between the heavy (C_H1) and light (CL) chain

30   constant regions is absent and the heavy chain (C_H1) and light chain (CL) constant
regions are linked by an interchain disulphide bond between a pair of engineered
cysteines, one in the light chain (CL) constant region and the other in the heavy chain
constant (C_H1) region.

40

In one embodiment the PEG is conjugated to a surface accessible cysteine.

The term 'native interchain disulphide bond' as used herein refers to the interchain
5   disulphide bond that exists between the cysteine in the heavy and light chain constant
regions encoded in a naturally occurring germline antibody gene. In particular the
native interchain cysteines are a cysteine in the constant region of the light chain (CL)
and a cysteine in the first constant region of the heavy chain (C_H1) that are disulphide
linked to each other in naturally occurring antibodies. Examples of such cysteines
10   may typically be found at position 214 of the light chain and 233 of the heavy chain of
human IgG1, 127 of the heavy chain of human IgM, IgE, IgG2, IgG3, IgG4 and 128
of the heavy chain of human IgD and IgA2B, as defined by Kabat et al., 1987, in
Sequences of Proteins of Immunological Interest, US Department of Health and
Human Services, NIH, USA. It will be appreciated that the exact positions of these
15   cysteines may vary from that of naturally occurring antibodies if any modifications,
such as deletions, insertions and/or substitutions have been made to the antibody
fragment.

Thus in one or more embodiment the native interchain disulphide bond is absent. The
20   native interchain disulphide bond may be absent because one or more effector
molecules are attached thereto.

In another embodiment the native interchain disulphide bond is absent in the antibody
fragments of the present invention because the interchain cysteines have been
25   replaced with another amino acid, such as serine.

In the antibody fragments of the present invention the heavy and light chain constant
regions are linked by an interchain disulphide bond between an engineered cysteine in
the light and/or heavy chain.
30

The fusion proteins of the present invention suitably have a high binding affinity, in
particular picomolar. Affinity may be measured using any suitable method known in
the art, including BIAcore. In one embodiment the antibody molecule of the present

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invention has a binding affinity of about 100 pM or better. In one embodiment the

fusion protein of the present invention has a binding affinity of about 50pM or better.

In one embodiment the fusion protein of the present invention has a binding affinity

of about 40pM or better. In one embodiment the fusion protein of the present

5   invention has a binding affinity of about 30pM or better. In one embodiment the

fusion protein of the present invention is fully human or humanised and has a binding

affinity of about 100pM or better.

If desired a fusion protein of the present invention may be conjugated to one or more

10   further effector molecule(s). It will be appreciated that the effector molecule may

comprise a single effector molecule or two or more such molecules so linked as to

form a single moiety that can be attached to the antibodies of the present invention.

Where it is desired to obtain an antibody fragment linked to an effector molecule, this

may be prepared by standard chemical or recombinant DNA procedures in which the

15   antibody fragment is linked either directly or via a coupling agent to the effector

molecule. Techniques for conjugating such effector molecules to antibodies are well

known in the art (see, Hellstrom et al., Controlled Drug Delivery, 2nd Ed., Robinson

et al., eds., 1987, pp. 623-53; Thorpe et al., 1982 , Immunol. Rev., 62:119-58 and

Dubowchik et al., 1999, Pharmacology and Therapeutics, 83, 67-123). Particular

20   chemical procedures include, for example, those described in WO 93/06231, WO

92/22583, WO 89/00195, WO 89/01476 and WO03031581. Alternatively, where the

effector molecule is a protein or polypeptide the linkage may be achieved using

recombinant DNA procedures, for example as described in WO 86/01533 and

EP0392745.

25

The term effector molecule as used herein includes, for example, antineoplastic

agents, drugs, toxins, biologically active proteins, for example enzymes, other

antibody or antibody fragments, synthetic or naturally occurring polymers, nucleic

acids and fragments thereof e.g. DNA, RNA and fragments thereof, radionuclides,

30   particularly radioiodide, radioisotopes, chelated metals, nanoparticles and reporter

groups such as fluorescent compounds or compounds which may be detected by NMR

or ESR spectroscopy.

42

Examples of effector molecules may include cytotoxins or cytotoxic agents including any agent that is detrimental to (*e.g.* kills) cells. Examples include combrestatins, dolastatins, epothilones, staurosporin, maytansinoids, spongistatins, rhizoxin, halichondrins, roridins, hemiasterlins, taxol, cytochalasin B, gramicidin D, ethidium

5   bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof.

10   Effector molecules also include, but are not limited to, antimetabolites (*e.g.* methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.* mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II)

15   (DDP) cisplatin), anthracyclines (*e.g.* daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.* dactinomycin (formerly actinomycin), bleomycin, mithramycin, anthramycin (AMC), calicheamicins or duocarmycins), and anti-mitotic agents (*e.g.* vincristine and vinblastine).

20   Other effector molecules may include chelated radionuclides such as ^{111}In and ^{90}Y, Lu^{177}, Bismuth^{213}, Californium^{252}, Iridium^{192} and Tungsten^{188}/Rhenium^{188}; or drugs such as but not limited to, alkylphosphocholines, topoisomerase I inhibitors, taxoids and suramin.

25   Other effector molecules include proteins, peptides and enzymes. Enzymes of interest include, but are not limited to, proteolytic enzymes, hydrolases, lyases, isomerases, transferases. Proteins, polypeptides and peptides of interest include, but are not limited to, immunoglobulins, toxins such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin, a protein such as insulin, tumour necrosis factor, α-interferon, β-

30   interferon, nerve growth factor, platelet derived growth factor or tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.* angiostatin or endostatin, or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), granulocyte macrophage colony stimulating factor (GM-

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CSF), granulocyte colony stimulating factor (G-CSF), nerve growth factor (NGF) or other growth factor and immunoglobulins.

Other effector molecules may include detectable substances useful for example in
5   diagnosis. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as
10   diagnostics. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include
15   luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include ^{125}I, ^{131}I, ^{111}In and ^{99}Tc.

In another example the effector molecule may increase the half-life of the antibody *in vivo*, and/or reduce immunogenicity of the antibody and/or enhance the delivery of an
20   antibody across an epithelial barrier to the immune system. Examples of suitable effector molecules of this type include polymers, albumin, albumin binding proteins or albumin binding compounds such as those described in WO05/117984.

Where the effector molecule is a polymer it may, in general, be a synthetic or a
25   naturally occurring polymer, for example an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide, e.g. a homo- or hetero- polysaccharide.

Specific optional substituents which may be present on the above-mentioned synthetic
30   polymers include one or more hydroxy, methyl or methoxy groups.

Specific examples of synthetic polymers include optionally substituted straight or branched chain poly(propyleneglycol) poly(vinylalcohol) or derivatives thereof,

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especially optionally substituted poly(ethyleneglycol) such as

methoxypoly(ethyleneglycol) or derivatives thereof.

Specific naturally occurring polymers include lactose, amylose, dextran, glycogen or

5   derivatives thereof.

"Derivatives" as used herein is intended to include reactive derivatives, for example

thiol-selective reactive groups such as maleimides and the like. The reactive group

may be linked directly or through a linker segment to the polymer. It will be

10   appreciated that the residue of such a group will in some instances form part of the

product as the linking group between the fusion protein and the polymer.

The present invention also provides isolated DNA encoding an antibody described

herein or a fragment thereof of a heavy or light chain thereof.

15

In a further aspect there is provided a vector comprising said DNA.

General methods by which the vectors may be constructed, transfection methods and

culture methods are well known to those skilled in the art. In this respect, reference is

20   made to "Current Protocols in Molecular Biology", 1999, F. M. Ausubel (ed), Wiley

Interscience, New York and the Maniatis Manual produced by Cold Spring Harbor

Publishing.

In a further aspect there is provided a host cell comprising said vector and/or DNA.

25

Any suitable host cell/vector system may be used for expression of the DNA

sequences encoding the fusion protein molecule of the present invention. Bacterial,

for example *E. coli*, and other microbial systems may be used or eukaryotic, for

example mammalian, host cell expression systems may also be used. Suitable

30   mammalian host cells include CHO, myeloma or hybridoma cells.

The present invention also provides a process for the production of an fusion protein

molecule according to the present invention comprising culturing a host cell

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containing a vector (and/or DNA) of the present invention under conditions suitable
for leading to expression of protein from DNA encoding the antibody molecule of the
present invention, and isolating the antibody molecule.

5   For production of products comprising both heavy and light chains, the cell line may
be transfected with two vectors, a first vector encoding a light chain polypeptide and a
second vector encoding a heavy chain polypeptide. Alternatively, a single vector may
be used, the vector including sequences encoding light chain and heavy chain
polypeptides.

10

The fusion protein molecules according to the present disclosure are expressed at
good levels from host cells. Thus the properties of the fusion protein molecule are
optimised and conducive to commercial processing.

15   The fusion protein molecules of the present invention are useful in the treatment
and/or prophylaxis of a pathological condition.

Thus there is provided a fusion protein molecule for use in treatment, for by
administering a therapeutically effective amount thereof. In one embodiment the
20   fusion protein molecules is administered in as a pharmaceutical formulation,
comprising a pharmaceutically acceptable excipient.

Thus the present invention also provides a pharmaceutical or diagnostic composition
comprising a fusion protein molecule of the present invention in combination with
25   one or more of a pharmaceutically acceptable excipient, diluent or carrier.
Accordingly, provided is the use of a fusion protein molecules of the invention for the
manufacture of a medicament. The composition will usually be supplied as part of a
sterile, pharmaceutical composition that will normally include a pharmaceutically
acceptable carrier. A pharmaceutical composition of the present invention may
30   additionally comprise a pharmaceutically-acceptable adjuvant.

The present invention also provides a process for preparation of a pharmaceutical or
diagnostic composition comprising adding and mixing the fusion protein molecule of

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the present invention together with one or more of a pharmaceutically acceptable excipient, diluent or carrier.

The fusion protein molecule may be the sole active ingredient in the pharmaceutical

5   or diagnostic composition or may be accompanied by other active ingredients including other antibody ingredients, for example anti-TNF, anti- IL-1β, anti-T cell, anti-IFNγ or anti-LPS antibodies, or non-antibody ingredients such as xanthines. Other suitable active ingredients include antibodies capable of inducing tolerance, for example, anti-CD3 or anti-CD4 antibodies.

10

In a further embodiment the fusion protein molecule or composition according to the disclosure is employed in combination with a further pharmaceutically active agent, for example a corticosteroid (such as fluticasonoe propionate) and/or a beta-2-agonist (such as salbutamol, salmeterol or formoterol) or inhibitors of cell growth and

15   proliferation (such as rapamycin, cyclophosphmide, methotrexate) or alternative a CD28 and /or CD40 inhibitor. In one embodiment the inhitor is a small molecule. In another embodiment the inhibitor is an antibody specific to the target.

The pharmaceutical compositions suitably comprise a therapeutically effective

20   amount of the antibody of the invention. The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent needed to treat, ameliorate or prevent a targeted disease or condition, or to exhibit a detectable therapeutic or preventative effect. For any antibody, the therapeutically effective amount can be estimated initially either in cell culture assays or in animal models, usually in rodents,

25   rabbits, dogs, pigs or primates. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

The precise therapeutically effective amount for a human subject will depend upon

30   the severity of the disease state, the general health of the subject, the age, weight and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities and tolerance/response to therapy. This amount can be determined by routine experimentation and is within the judgement of the clinician.

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Generally, a therapeutically effective amount will be from 0.01 mg/kg to 50 mg/kg, for example 0.1 mg/kg to 20 mg/kg. Pharmaceutical compositions may be conveniently presented in unit dose forms containing a predetermined amount of an active agent of the invention per dose.

5

Compositions may be administered individually to a patient or may be administered in combination (*e.g.* simultaneously, sequentially or separately) with other agents, drugs or hormones.

10   The dose at which the fusion protein molecule of the present invention is administered depends on the nature of the condition to be treated, the extent of the inflammation present and on whether the antibody molecule is being used prophylactically or to treat an existing condition.

15   The frequency of dose will depend on the half-life of the antibody molecule and the duration of its effect. If the antibody molecule has a short half-life (e.g. 2 to 10 hours) it may be necessary to give one or more doses per day. Alternatively, if the antibody molecule has a long half life (e.g. 2 to 15 days) it may only be necessary to give a dosage once per day, once per week or even once every 1 or 2 months.

20

The pharmaceutically acceptable carrier should not itself induce the production of antibodies harmful to the individual receiving the composition and should not be toxic. Suitable carriers may be large, slowly metabolised macromolecules such as proteins, polypeptides, liposomes, polysaccharides, polylactic acids, polyglycolic

25   acids, polymeric amino acids, amino acid copolymers and inactive virus particles.

Pharmaceutically acceptable salts can be used, for example mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulphates, or salts of organic acids, such as acetates, propionates, malonates and benzoates.

30

Pharmaceutically acceptable carriers in therapeutic compositions may additionally contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents or pH buffering substances, may be

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present in such compositions. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

5   Suitable forms for administration include forms suitable for parenteral administration, e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the product is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents, such as suspending, preservative, stabilising and/or dispersing agents.

10   Alternatively, the antibody molecule may be in dry form, for reconstitution before use with an appropriate sterile liquid.

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals. However, in one or more

15   embodiments the compositions are adapted for administration to human subjects.

Suitably in formulations according to the present disclosure, the pH of the final formulation is not similar to the value of the isoelectric point of the antibody or fragment, for example if the pH of the formulation is 7 then a pI of from 8-9 or above

20   may be appropriate. Whilst not wishing to be bound by theory it is thought that this may ultimately provide a final formulation with improved stability, for example the antibody or fragment remains in solution.

The pharmaceutical compositions of this invention may be administered by any

25   number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, transcutaneous (for example, see WO98/20734), subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, intravaginal or rectal routes. Hyposprays may also be used to administer the pharmaceutical compositions of the invention. Typically, the therapeutic

30   compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

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Direct delivery of the compositions will generally be accomplished by injection, subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Dosage treatment may be a single dose schedule or a multiple dose schedule.

5

It will be appreciated that the active ingredient in the composition will be a fusion protein molecule. As such, it will be susceptible to degradation in the gastrointestinal tract. Thus, if the composition is to be administered by a route using the gastrointestinal tract, the composition will need to contain agents which protect the

10   antibody from degradation but which release the antibody once it has been absorbed from the gastrointestinal tract.

A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

15

In one embodiment the formulation is provided as a formulation for topical administrations including inhalation.

Suitable inhalable preparations include inhalable powders, metering aerosols

20   containing propellant gases or inhalable solutions free from propellant gases. Inhalable powders according to the disclosure containing the active substance may consist solely of the abovementioned active substances or of a mixture of the abovementioned active substances with physiologically acceptable excipient.

25   These inhalable powders may include monosaccharides (e.g. glucose or arabinose), disaccharides (e.g. lactose, saccharose, maltose), oligo- and polysaccharides (e.g. dextranes), polyalcohols (e.g. sorbitol, mannitol, xylitol), salts (e.g. sodium chloride, calcium carbonate) or mixtures of these with one another. Mono- or disaccharides are suitably used, the use of lactose or glucose, particularly but not exclusively in the

30   form of their hydrates.

Particles for deposition in the lung require a particle size less than 10 microns, such as 1-9 microns for example from 0.1 to 5 μm, in particular from 1 to 5 μm. The particle

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size of the active ingredient (such as the antibody or fragment) is of primary importance.

The propellent gases which can be used to prepare the inhalable aerosols are known in
the art. Suitable propellent gases are selected from among hydrocarbons such as n-
propane, n-butane or isobutane and halohydrocarbons such as chlorinated and/or
fluorinated derivatives of methane, ethane, propane, butane, cyclopropane or
cyclobutane. The abovementioned propellent gases may be used on their own or in
mixtures thereof.

Particularly suitable propellent gases are halogenated alkane derivatives selected from
among TG 11, TG 12, TG 134a and TG227. Of the abovementioned halogenated
hydrocarbons, TG134a (1,1,1,2-tetrafluoroethane) and TG227 (1,1,1,2,3,3,3-
heptafluoropropane) and mixtures thereof are particularly suitable.

The propellent-gas-containing inhalable aerosols may also contain other ingredients
such as cosolvents, stabilisers, surface-active agents (surfactants), antioxidants,
lubricants and means for adjusting the pH. All these ingredients are known in the art.

The propellant-gas-containing inhalable aerosols according to the invention may
contain up to 5 % by weight of active substance. Aerosols according to the invention
contain, for example, 0.002 to 5 % by weight, 0.01 to 3 % by weight, 0.015 to 2 % by
weight, 0.1 to 2 % by weight, 0.5 to 2 % by weight or 0.5 to 1 % by weight of active
ingredient.

Alternatively topical administrations to the lung may also be by administration of a
liquid solution or suspension formulation, for example employing a device such as a
nebulizer, for example, a nebulizer connected to a compressor (e.g., the Pari LC-Jet
Plus(R) nebulizer connected to a Pari Master(R) compressor manufactured by Pari
Respiratory Equipment, Inc., Richmond, Va.).

The fusion protein molecule of the invention can be delivered dispersed in a solvent,
e.g., in the form of a solution or a suspension. It can be suspended in an appropriate

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physiological solution, e.g., saline or other pharmacologically acceptable solvent or a buffered solution. Buffered solutions known in the art may contain 0.05 mg to 0.15 mg disodium edetate, 8.0 mg to 9.0 mg NaCl, 0.15 mg to 0.25 mg polysorbate, 0.25 mg to 0.30 mg anhydrous citric acid, and 0.45 mg to 0.55 mg sodium citrate per 1 ml

5   of water so as to achieve a pH of about 4.0 to 5.0. A suspension can employ, for example, lyophilised antibody.

The therapeutic suspensions or solution formulations can also contain one or more excipients. Excipients are well known in the art and include buffers (e.g., citrate

10   buffer, phosphate buffer, acetate buffer and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (e.g., serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. Solutions or suspensions can be encapsulated in liposomes or biodegradable microspheres. The formulation will generally be provided in a substantially sterile form employing sterile manufacture

15   processes.

This may include production and sterilization by filtration of the buffered solvent/solution used for the for the formulation, aseptic suspension of the antibody in the sterile buffered solvent solution, and dispensing of the formulation into sterile

20   receptacles by methods familiar to those of ordinary skill in the art.

Nebulizable formulation according to the present disclosure may be provided, for example, as single dose units (e.g., sealed plastic containers or vials) packed in foil envelopes. Each vial contains a unit dose in a volume, e.g., 2 ml, of solvent/solution

25   buffer.

The fusion protein molecule of the present disclosure are thought to be suitable for delivery via nebulisation.

30   Comprising in the context of the present specification is intended to meaning including.

Where technically appropriate embodiments of the invention may be combined.

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Embodiments are described herein as comprising certain features/elements. The disclosure also extends to separate embodiments consisting or consisting essentially of said features/elements.
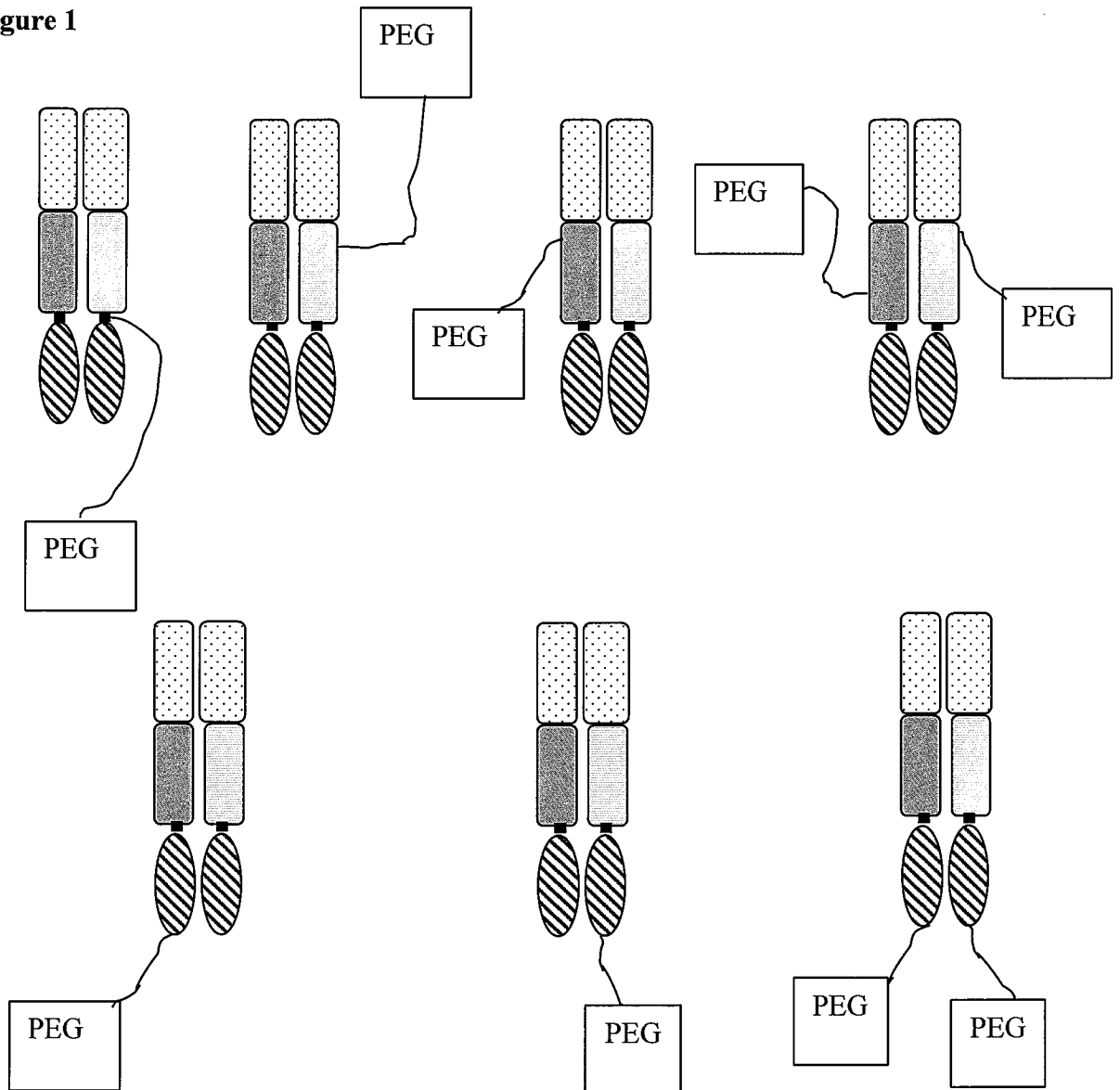
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CLAIMS

1. A recombinant fusion protein comprising:

    a heavy chain comprising, in sequence from the N-terminal, a variable domain nominally V_H1, a C_H1 region and a further variable domain nominally V_H2,

    a light chain comprising, in sequence from the N-terminal, a variable domain nominally V_L1, a CL domain and a variable domain nominally V_L2,

wherein said heavy and light chains are aligned to provide a first binding site formed by a first variable domain pair of V_H1 and V_L1 and a second binding site formed by a second variable domain pair of V_H2 and V_L2, and

said fusion protein is conjugated to a PEG polymer.

2. A recombinant fusion protein according to claim 1, wherein an amino acid of V_H2 is directly linked to an amino acid of C_H1 by a peptide bond.

3. A recombinant fusion protein according to claim 1, wherein V_H2 is linked to C_H1 indirectly by a linker.

4. A recombinant fusion protein according to any one of claims 1 to 3, wherein V_L2 is directly linked to an amino acid of CL by a peptide bond.

5. A recombinant fusion protein according to any one of claims 1 to 3 wherein V_L2 is linked to CL indirectly by a linker.

6. A recombinant fusion protein according to claim 3 or claim 5 wherein the linker has the sequence given in SEQ ID NO:226.

7. A recombinant fusion protein according to any one of claims 1 to 6, wherein the recombinant fusion protein is PEGylated through a solvent accessible cysteine.

8. A recombinant fusion protein according to any one of claims 1 to 7 wherein the recombinant fusion protein is PEGylated through the interchain cysteine of CH1 and/or CL.

9. A recombinant fusion protein according to any one of claims 1 to 8 wherein the recombinant fusion protein is PEGylated through an engineered cysteine in the light chain wherein the position of said engineered cysteine is selected from the group consisting of position 5, 7, 9, 10, 12, 14, 15, 16, 17, 18, 20, 22, 24, 26, 27, 28, 30, 31,34, 39, 41, 42, 43, 55, 56, 57, 60, 61, 63, 67, 68, 69, 70, 72, 74, 76, 77, 79, 81, 107, 108, 109, 110, 114, 121, 122, 123, 126, 127, 128, 129, 143, 144, 145, 147, 149, 151, 152, 153, 156, 157, 158, 159, 160, 161, 167, 168, 169, 170, 171,

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190, 195, 197, 199, 200, 202, 203, 205, 206, 210, 211, 212 and 213, numbered according to the Kabat numbering system.

10. A recombinant fusion protein according to any one of claims 1 to 9 wherein the recombinant fusion protein is PEGylated through an engineered cysteine in the light chain wherein the position of said engineered cysteine is selected from the group consisting of position 77, 107, 109, 143, 145, 149 and 210, numbered according to the Kabat numbering system.

11. A recombinant fusion protein according to any one of claims 1 to 10 wherein the recombinant fusion protein is PEGylated through an engineered cysteine in the heavy chain wherein the position of said engineered cysteine is selected from the group consisting of position 3, 7, 8, 9, 10, 13, 15, 16, 17, 21, 26, 40, 43, 57, 58, 61, 62, 64, 65, 66, 68, 70, 72, 82a, 82b, 82c, 84, 85, 86, 96, 97, 98, 99, 105, 112, 113, 114, 115, 116, 117, 120, 127, 128, 129, 130, 133, 134, 135, 136, 156, 163, 164, 165, 167, 168, 169, 176, 177, 179, 180, 182, 183, 195, 196, 197, 199, 200, 203, 205, 213, 215, 216, 218, 220, 222 and 232, numbered according to the Kabat numbering system.

12. A recombinant fusion protein according to any one of claims 1 to 11 wherein the recombinant fusion protein is PEGylated through an engineered cysteine in the heavy chain wherein the position of said engineered cysteine is selected from the group consisting of position 82b, 116, 163, 182 and 216, numbered according to the Kabat numbering system.

13. A recombinant fusion protein according to any one of claims 1 to 12, wherein the recombinant fusion protein is PEGylated with one or two PEG molecules.

14. A recombinant fusion protein according to claim 13, wherein the PEG molecules are in the range 5000 to 80000Da.

15. A recombinant fusion protein according to any one of claims 1 to 14, wherein V_H1 in the heavy chain is a variable domain from a heavy chain or a variable domain from a light chain.

16. A recombinant fusion protein according to any one of claims 1 to 15, wherein V_H2 in the heavy chain is a variable domain from a heavy chain or a variable domain from a light chain.

17. A recombinant fusion protein according to any one of claims 1 to 16, wherein V_L1 in the light chain is a variable domain from a light chain or a heavy chain.

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18. A recombinant fusion protein according to any one of claims 1 to 17, wherein V_L2 in the light chain is a variable domain from a light chain or a heavy chain.

19. A recombinant fusion protein according to any one of claims 1 to 18 wherein the two variable domain pairs are each a cognate pair.

20. A recombinant fusion protein according to any one of claims 1 to 19 wherein the two variable domain pairs are each a complementary VH/VL pair which bind antigen co-operatively.

21. A recombinant fusion protein according to any one of claims 1 to 20 wherein the CH1 and CL domains are derived from IgG1.

22. A recombinant fusion protein according to any one of claims 1 to 21 which is dimerised.

23. A recombinant fusion protein according to any one of claim 22 which is dimerised via solvent accessible cysteines.

24. A recombinant fusion protein according to claim 22 or 23 which is dimerised directly via a disulphide bond.

25. A recombinant fusion protein according to claim 24 which is dimerised via a chemical linker.

26. A recombinant fusion protein according to claim 25 which is dimerised via a PEG linker.

27. A recombinant fusion protein according to claim 22 which is dimerised via a peptide linker.

28. A recombinant fusion protein according to any one of claims 1 to 27 which is bispecific or monospecific.

Figure 1

Each of the above representation may also have a disulfide bond between CH and CL.
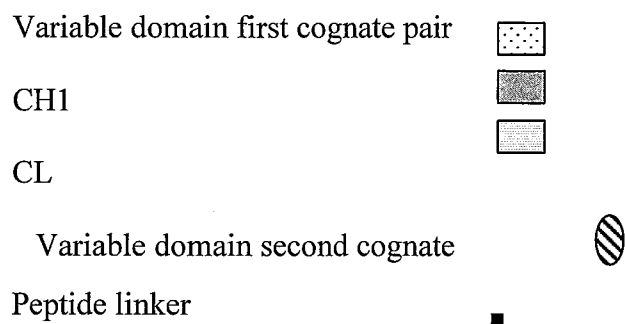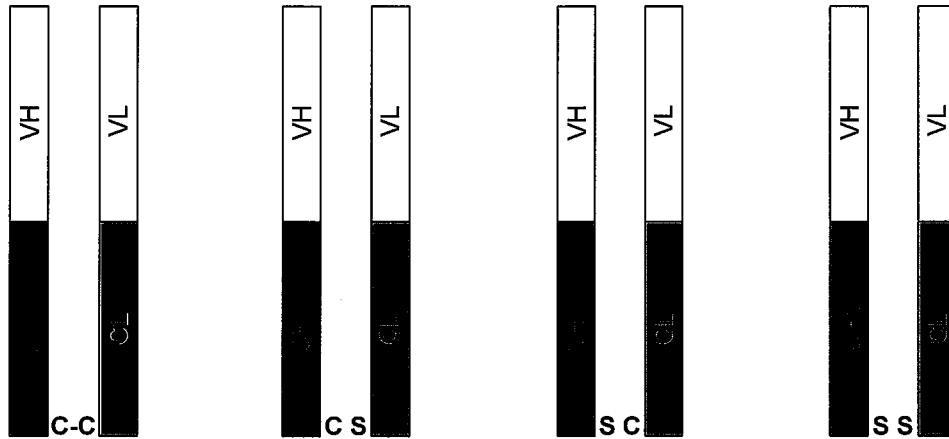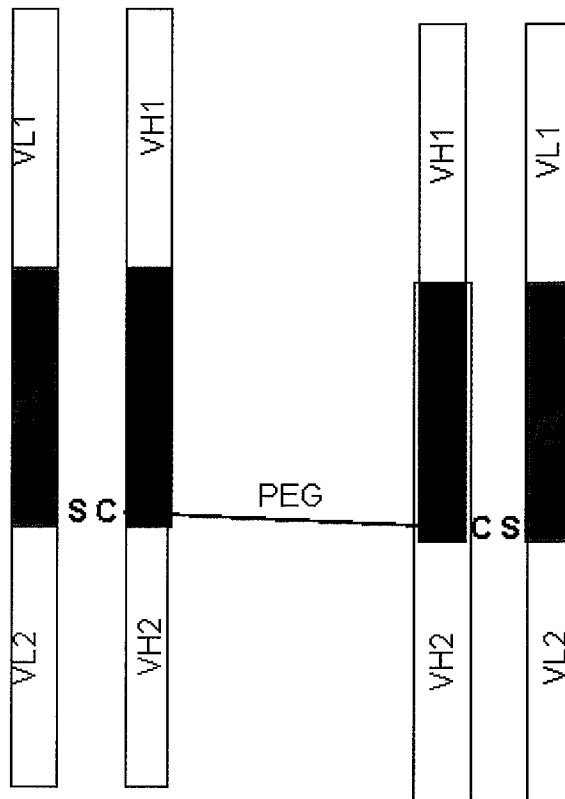
Variable domain first cognate pair

CH1

CL

Variable domain second cognate

Peptide linker

Figure 2A

Various interchain "cysteine" arrangements.

C = cysteine,   S = serine

Figure 2B

Figure 3

Light Chain Sequence 4D5

DIQMTQ**SPSSLSASVGDRVT**[20]**ITC**_RASQDVNTA_VAWYQQKP[40]GK_A_PKLLIYSASFLE
SGVP**S**[60]**R**FSGSR_SGTD_FTLTI**SS**LQP[80]EDFATYYCQQHY_TT_PPTFGQ[100]GTKVEI**KR**
TVAAP**S**VFIFPP[120]**SDE**QL**KSGT**ASVVCLLNNFY[140]PRE**A**KVQWKVDN**A**LQ**SGNSQ**[16]
[0]**E**SVTEQD**SKD**STYSLSSTLT[180]L**SKADYEKHK**VYACEVTHQG[200]**LSS**PVTKSFNRGE
C[214]
SEQ ID NO:223

Figure 4

Heavy Chain Sequence 4D5

EVQLVESGGGLVQPGGSLRL[20]SCAASG_FNIKDTY_IHWVRQA[40]PGKGLEWVARI_YPTN_
GYTRY[60]ADSVKGRFTISAD_TSKNTAY_[80]LQMNSLRAEDTAVYYCSRWG[100]_GDGFYAMD_
YWGQGTLVTVSS[120]ASTKGPSVFPLAPSSKSTSG[140]GTAALGCLVKDYFPEPVTVS[16]
[0]WNSGALTSGVHTFPAVLQSS[180]GLYSLSSVVTVPSSSLGTQT[200]YICNVNHKPSNTK
VDKKVEP[220]KSCDKTHTC[223]

SEQ ID NO:44