Glycosylated repeat-motif-molecule conjugates

Herein are reported GEMOCs (glycosylated repeat-motif-molecule conjugates), which are produced recombinantly and comprise an in vivo glycosylation, i.e. from the cell type expressing the EMOC (repeat-motif-molecule conjugate), as well as a method for producing these conjugates and the use of these conjugates.

Background of the Invention

Different repeat-motif-molecules have been developed to provide an alternative to classical antigen binding molecules, such as immunoglobulins.

Exemplary repeat-motif-molecules are designed ankyrin repeat proteins (DARPins). DARPins can be expressed in functional form in the cytoplasm of E.coli strains. The repeat motif of DARPins comprises a 33-residue amino acid sequence. The repeats comprise a β-turn motif followed by a pair of antiparallel α -helices and a loop leading to the turn of the next repeat. Generally between 1 to more than 30 repeat motifs are present in ankyrin repeat-motif-molecules, whereby 4 to 6 are most frequent.

Kohl et al. (Kohl, A., et al., Proc. Natl. Acad. Sci. USA 100 (2003) 1700 -1705) developed an artificial ankyrin repeat module based on the alignment of about 2000 naturally occurring ankyrin repeat sequences. This artificial consensus ankyrin repeat sequence comprises 27 fixed amino acid residues and 6 variable amino acid residues forming a (part of a) binding site (Forrer, P., et al., ChemBioChem 5 (2004) 183 -189).

In order to obtain DARPins binding to a predetermined target molecule the variable amino acid residues of the artificial consensus ankyrin repeat sequence are randomized and a specific binder is identified via ribosome display (see e.g. WO 02/020565; Hanes, Proc. Natl. Acad. Sci. USA 94 (1997) 4937-4942).

In US 2009/0148905 antigen binding constructs are reported. Complement factor H-derived short consensus repeat-antibody constructs are reported in WO 2008/135237. Antibody-RNAse-conjugates are reported in WO 2007/122511. A Newcastle Disease Virus comprising a recombinant nucleic acid, wherein the nucleic acid codes for a binding protein that has a therapeutic activity when expressed by the virus-infected tumor cell is reported in US 2008/0206201. In US 2008/0248026 PTEN/AKT methods and compositions relating to BMP are

reported. Recombinant expression of proteins in a disulfide-bridged, two-chain form is reported in WO2005/076902 and US 2008/0103098. In WO 2009/068649 antigen-binding constructs are reported.

Summary

Herein are reported repeat-motif-molecule conjugates in glycosylated form, i.e. expressed in mammalian cells.

The disclosure relates to a glycosylated repeat-motif-molecule conjugate of the following formula

(repeat-motif-molecule – $linker_n$)$_m$ – (conjugation partner)$_q$ – ($linker_o$ – repeat-motif-molecule)$_p$

wherein n and o are independently of each other and independently for each value of m and p and q integer values of 0 or 1, and m and p are independently of each other integer values of 0 or 1 or 2 or 3 or 4 or 5 or 6 or 7, and q is independently of the values of n, m, o, and p an integer value of 0 or 1,

and wherein the repeat-motif-molecule conjugate comprises at least one oligosaccharide attached to a glycosylation site,

and wherein at least m=q=1 or p=q=1.

In a first aspect, there is provided a glycosylated repeat-motif-molecule conjugate of the following formula

(repeat-motif-molecule – $linker_n$)$_m$ – (conjugation partner)$_q$ – ($linker_o$ – repeat-motif-molecule)$_p$

wherein n=1, m=1, q=1, p=0,

wherein the repeat-motif-molecule conjugate comprises at least one oligosaccharide attached to a glycosylation site, and

wherein the conjugation partner comprises an engineered antibody heavy chain hinge region with three cysteine residues, and

wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

In a second aspect, there is provided a glycosylated repeat-motif-molecule conjugate of the following formula

(repeat-motif-molecule – $linker_n)_m$ – $(conjugation\ partner)_q$ – $(linker_o$ – repeat-motif-molecule$)_p$

wherein n=0, m=1, q=1, p=0,

wherein the repeat-motif-molecule conjugate comprises at least one oligosaccharide attached to a glycosylation site, and

wherein the conjugation partner comprises an engineered antibody heavy chain hinge region with three cysteine residues, and

wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

In one embodiment the repeat-motif-molecule is an ankyrin-repeat-motif-molecule or a leucine-rich-repeat-motif-molecule. In a further embodiment the ankyrin-repeat-motif-molecule has an amino acid sequence of SEQ ID NO: 4 or a variant thereof. In another embodiment the linker is a peptidic linker selected from SEQ ID NO: 14 and SEQ ID NO: 25 to SEQ ID NO: 33. In one embodiment the conjugation partner is a multimerizing conjugation partner. In still a further embodiment the multimerizing conjugation partner is selected from natural or engineered pairs of heavy chain CH2 and CH3 domains, from natural or engineered pairs of heavy chain CH1 domain and light chain constant domain, from natural or engineered heavy chain hinge region, from natural or engineered sequences of heavy chain hinge region and CH2 and CH3 domains, from leucine zipper domain, from isoleucine zipper domain, from 4-helix-bundles, and from p53 tetramerization domain, or from combinations thereof.

In one embodiment the conjugate according to the disclosure is characterized in that it has the formula of

$((\text{repeat-motif-molecule} - \text{linker}_n)_m - (\text{conjugation partner})_q - (\text{linker}_o - \text{repeat-motif-molecule})_p)_r$

with

i) n=1, m=1, q=1, p=0, r=1, or
ii) n=1, m=1, q=1, p=0, r=2, or
iii) n=0, m=1, q=1, p=0, r=1, or
iv) n=0, m=1, q=1, p=0, r=2, or
v) m=0, o=1, q=1, p=1, r=1, or
vi) m=0, o=1, q=1, p=1, r=2, or
vii) m=0, o=0, q=1, p=1, r=1, or
viii) m=0, o=0, q=1, p=1, r=2, or
ix) m=1, n=0, o=1, q=1, p=1, r=1, or
x) m=1, n=0, o=1, q=1, p=1, r=2, or
xi) m=1, n=1, o=1, q=1, p=1, r=1, or
xii) m=1, n=1, o=1, q=1, p=1, r=1,

wherein the conjugation partner is selected from natural or engineered pairs of heavy chain CH2 and CH3 domains, natural or engineered pairs of heavy chain CH1 domain and light chain constant domain, natural or engineered heavy chain hinge regions, natural or engineered sequences of heavy chain hinge region and CH2 and CH3 domains,

and wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

In a further embodiment the conjugate is characterized in that the at least one oligosaccharide is a mixture of oligosaccharides obtained by the expression of the conjugate in CHO cells or HEK293 cells.

In one embodiment the conjugate is characterized in that

m=0, q=1, p=1, and o=0 or o=1, and r=1 or 2,

wherein the conjugation partner is selected from a conjugate of N-terminal SEQ ID NO: 50 or 51 or 52 and C-terminal of SEQ ID NO: 43 or 44, or of N-terminal of SEQ ID NO: 53 or 54 or 55, and C-terminal of SEQ ID NO: 45 or 46, and

wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

Another aspect as reported herein is a glycosylated repeat-motif-molecule conjugate, characterized in comprising two glycosylated repeat-motif-molecule conjugates as reported herein.

In another embodiment the glycosylated repeat-motif-molecule conjugate comprises two glycosylated repeat-motif-molecule conjugates according to one of the previous embodiments.

In a further embodiment the glycosylated repeat-motif-molecule conjugate further comprises

a) in case the conjugation partner of the repeat-motif-molecule is selected from SEQ ID NO: 50, 51 and 52 two conjugates of N-terminal of SEQ ID NO: 53 or 54 or 55 and C-terminal of SEQ ID NO: 45 or 46, or

b) in case the conjugation partner of the repeat-motif-molecule is selected from SEQ ID NO: 53, 54 and 55 two conjugates of N-terminal of SEQ ID NO: 50 or 51 or 52 and C-terminal of SEQ ID NO: 43 or 44.

A further aspect as reported herein is a nucleic acid comprising the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of an antibody germline gene,
- an immunoglobulin heavy chain signal sequence,
- a repeat-motif-molecule conjugate's encoding sequence,
- a polyadenylation ("poly A") signal sequence.

Another aspect as reported herein is a method for producing a glycosylated repeat-motif-molecule conjugate as reported herein comprising

- cultivating a mammalian cell comprising a nucleic acid as reported herein under conditions suitable for the expression of the repeat-motif-molecule,
- recovering the glycosylated repeat-motif-molecule from the cell or the cultivation medium and thereby producing the glycosylated repeat-motif-molecule,
- optionally purifying the recovered glycosylated repeat-motif-molecule.

In one embodiment the mammalian cell is selected from CHO cells and HEK293 cells.

In one embodiment the multimerizing conjugation partner is an engineered heavy chain hinge region with three cysteine residues. In another embodiment the conjugation partner comprises the engineered antibody heavy chain hinge region, a CH2 domain and a CH3 domain. In a further embodiment the repeat-motif-molecule is an anticalin. In still another embodiment the repeat-motif-molecule is conjugated to the N-terminus of the conjugation partner.

Detailed Description of the Invention

Herein are reported glycosylated repeat-motif-molecule conjugates (GEMOCs) of the general formula:

(repeat-motif-molecule – $linker_n)_m$ – $(conjugation\ partner)_q$ – $(linker_o$ – repeat-motif-$molecule)_p$

wherein n and o are independently of each other and independently for each value of m and p integer values of 0 or 1, and m and p are independently of each other integer values of 0 or 1 or 2 or 3 or 4 or 5 or 6 or 7, q is independently 0 or 1,

wherein the conjugation partner comprises at least one glycosylation site and the GEMOC is expressed in a mammalian cell.

The term "glycosylated" or grammatical equivalents thereof denotes that the respective repeat-motif-molecule comprise a saccharide residue covalently linked to an amino acid of the amino acid backbone of the repeat-motif-molecule. In one embodiment the repeat-motif-molecule comprises at least one N- or O-glycosylation site motif, either a natural occurring or engineered motif (see SEQ ID NO: 56 and 58). In one embodiment the N-glycosylation site motif is selected from asp-X-thr, asp-X-ser, or asp-X-cys, wherein X can be any amino acid residues but

not proline (pro, P). In another embodiment a glycosylation tag is added to the repeat-motif-molecule (see e.g. SEQ ID NO: 60, 61, 62, and 63; see also Meder, D., et al., J. Cell Biol. 168 (2005) 303-313; Bulbarelli, A., et al., J. Cell Sci., 115 (2002) 1689-1702). Thus, in one embodiment the conjugate comprises as repeat-motif-molecule a molecule of SEQ ID NO: 62 or 63. With these glycosylation tag comprising repeat-motif-molecules a good glycosylation can be achieved.

The term "mammalian cell" denotes a cell selected from CHO cells, BHK cells, HEK cells, COS cells, Per.C6® cells, or hybridoma cells.

The term "amino acid" as used within this application denotes the group of carboxy α-amino acids, which directly or in form of a precursor can be encoded by a nucleic acid. The individual amino acids are encoded by nucleic acids consisting of three nucleotides, so called codons or base-triplets. Each amino acid is encoded by at least one codon. This is known as "degeneration of the genetic code". The term "amino acid" as used within this application denotes the naturally occurring carboxy α-amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

The term "antibody" refers to a molecule consisting of one or more polypeptide(s) substantially encoded by antibody genes. An antibody in general comprises two so called light chain polypeptides (light chain) and two so called heavy chain polypeptides (heavy chain). Each of the heavy and light chain polypeptides contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chain polypeptides comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (C1q). The variable domain of an antibody's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

The term "hinge region" denotes the fragment of a, in one embodiment human, full length antibody heavy chain of from residue 216, which is normally a glutamic acid residue, to residue 226, which is normally a cysteine residue, or to residue 230, which is normally a proline residue. The hinge region comprises cysteine residues which can form disulfide bonds with the corresponding cysteine residues of a second hinge region, e.g. of a second antibody heavy chain. In one embodiment the hinge region comprises two cysteine residues.

The terms "second heavy chain constant domain" and "CH2 domain" and "CH2" which can be used interchangeably denote the fragment of a, in one embodiment human, full length antibody heavy chain of from residue 231 to residue 340. The CH2 domain comprises residue 297, which is normally the amino acid asparagine, at which a saccharide is covalently attached to the amino acid backbone.

The terms "third heavy chain constant domain" and "CH3 domain" and "CH3" which can be used interchangeably denote the fragment of a, in one embodiment human, full length antibody heavy chain of from residue 341 to residue 447, i.e. C-terminal to the CH2 domain.

The terms "antibody-dependent cell-mediated cytotoxicity" and "ADCC" which can be used interchangeably within this application denote a mechanism of cell lysis effected by nonspecific cytotoxic cells having an Fc-Receptor (FcR) on its cell surface, such as natural killer cells (NK cells), neutrophils and macrophages. These cells recognize and lyse cells with surface bound antibody having an Fc-Receptor binding portion, such as an Fc-part.

The terms "complement dependent cytotoxicity" and "CDC" which can be used interchangeably within this application denote a mechanism of cell lysis effected by complement, which is initiated by binding of C1q to e.g. an antibody bound to its antigen.

The term "binding to a target molecule" denotes a specific interaction between a binding, i.e. complementarity providing, molecule and its binding partner, i.e. a specific target molecule. The strength of this specific interaction denoted as binding affinity is given as K_D-value. The term "not specifically binding to a target molecule" denotes a non-specific interaction between a binding molecule and its target molecules with a K_D-value of 10^{-5} mol/l or higher (e.g. 10^{-3} mol/l), in one embodiment with a K_D-value of 10^{-6} mol/l or higher. The binding affinity is

determined with a standard binding assay, such as surface plasmon resonance technique (BIAcore®). This binding affinity value has not to be treated as an exact value, it is merely a point of reference. The term "specifically binding to a target molecule" denotes a specific interaction between a binding molecule and its target molecule with a K_D-value of 10^{-7} mol/l or lower (e.g. 10^{-10} mol/l), in one embodiment with a K_D-value of 10^{-8} mol/l or lower.

In one embodiment the repeats of the repeat-motif-molecule are of from 20 to 40 amino acid residues in length. The repeats are arranged in individual structural units forming together the repeat-motif-molecule. In one embodiment the repeat-motif-molecule is selected from ankyrin-repeat-motif-molecule and leucine-rich-repeat-motif-molecule (LRRPs).

In one embodiment the conjugation partner comprises an immunoglobulin membrane anchor domain or a GPI-anchor domain.

The term "repeat-motif-molecule" denotes a natural occurring or artificial polypeptide that can be expressed as single molecule, i.e. not conjugated to a second molecule or to a second copy of itself, in soluble form in the cytoplasm or periplasm of E. coli. In one embodiment the repeat-motif-molecule comprises at least two structurally identical units (repeats). These units do not need to be identical in their amino acid sequence. These units can but do not need to be independently folding domains. The repeat-motif-molecule can but does not have to comprise other sequence stretches in addition to the repetitive units. In one embodiment the repeat-motif-molecule is an ankyrin-repeat-motif-molecule. In another embodiment the repeat-motif-molecule is a leucine-rich-repeat-motif-molecule.

The term "ankyrin-repeat-motif-molecule" denotes an artificial polypeptide comprising of from 1 to 30 consensus 33-residue amino acid sequences. Each of the repeats comprises a β-turn motif followed by a pair of antiparallel α-helices and a loop leading to the turn of the next repeat. In one embodiment the ankyrin-repeat-motif-molecule comprises of from 1 to 30 repeats, in another embodiment of from 2 to 10 repeat, and in a further embodiment of from 3 to 6 repeats.

In one embodiment the ankyrin-repeat-motif-molecule is derived from one of the following consensus sequences by random mutagenesis and selection for target molecule binding, e.g. by ribosome display, yeast display or phage display:

DGNT(P,A)LHLA(A,V) ENG(H,N)LE(I,V)VKL L(L,I)EAGA(D,N)INA (SEQ ID NO: 01),

DSDGNTPLHL AAENGQLEVV KLLLEAGADV NAR (SEQ ID NO: 02),

(D,T)KNGLTPLH(L,I) AAQEGHLEVV KLLLENGA(D,N)(V,I) NAK (SEQ ID NO: 03),

DxxGxTPLHL AAxxGHLEIV EVLLK(H,N,Y)GADV NAx (SEQ ID NO: 04),

ADVNAKDKDG YTPLHLAARE GHLEIVEVLL KAG (SEQ ID NO: 05),

DxxGxTPLHLAaxxGpxpaVpxLLpxGADVNAx (SEQ ID NO: 06),

DxxGxTPLHLAxxxGxxxVVxLLLxxGADVNAx (SEQ ID NO: 07),

DxxGxTPLHLAxxxGxxxIVxVLLxxGADVNAx (SEQ ID NO: 08),

D11G1TPLHLAA11GHLEIVEVLLK2GADVNA1 (SEQ ID NO: 09),

with "x" denoting a fully variable position,

with the amino acids given in brackets denoting the specific variants possible at this position,

with "a" denoting an amino acid with an apolar side chain,

with "p" denoting an amino acid residue with a polar side chain,

with "1" denoting an amino acid residue selected from the group consisting of (in one letter code) amino acid residues A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y, and

with "2" denoting an amino acid residue selected from the group consisting of the amino acid residues H, N and Y.

GEMOCs comprising ankyrin-repeat-motif-molecules binding to any predetermined target molecule can be obtained by screening libraries comprising randomized GEMOCs or randomized ankyrin-repeat-motif-molecules e.g. by ribosome display (see e.g. WO 02/120565; Hanes, J. and Plückthun, A., Proc. Natl. Acad. Sci. USA 94 (1997) 4937-4942).

In one embodiment the GEMOC comprises an N-terminal capping repeat of the amino acid sequence of DLGKKLLE AARAGQDDEVRILMANGADV (SEQ ID NO: 47).

In one embodiment the GEMOC comprises a C-terminal capping repeat of the amino acid sequence of VNAQDKFGKT AFDISIDNGNEDLAEILQ (SEQ ID NO: 48).

In another embodiment the repeat-motif-molecule is a leucine-rich-repeat-motif-molecule (LRR). Methods for making leucine-rich-repeat-motif-molecules are reported in WO 2006/083275.

In general antibodies have activities selected from a neutralizing activity, a targeting activity for a toxic moiety, or an effector function.

In Figure 1 the domain architecture of an antibody is shown. A full length antibody comprises two so called light chains and two so called heavy chains. Each of the light chains in turn comprises a light chain variable domain and a light chain constant domain. The light chain constant domain is selected from a kappa type domain or a lambda type domain. Each of the heavy chains in turn comprises a heavy chain variable domain, a first heavy chain constant domain (CH1), a hinge region, a second heavy chain constant domain (CH2), a third heavy chain constant domain (CH3), and optionally a fourth heavy chain constant domain (CH4). In addition comprises a full length antibody disulfide bonds between the light chain constant domain and the first heavy chain constant domain as well as two disulfide bonds between the second heavy chain constant domains of two heavy chains.

In one embodiment the conjugation partner in the GEMOC is a full length antibody heavy chain or a full length antibody light chain but each without a variable domain. In one embodiment the conjugation partner in the GEMOC is an antibody heavy chain or an antibody light chain each with a variable domain. In another embodiment the repeat-motif-molecule is conjugated to the C-terminus of the respective antibody chain either directly of via a peptidic linker. In one embodiment the GEMOC comprises two GEMOCs of the previous embodiment.

In one embodiment the conjugation partner is an antibody light or heavy chain of an anti-Aβ antibody. Such an anti-Aβ antibody and the corresponding nucleic acid

sequences are, for example, reported in WO 2003/070760 or US 2005/0169925 or in SEQ ID NO: 50 to 55 or a variant thereof.

The conjugates as reported herein comprise various variants containing one or more repeat-motif-molecule, each of them binding to the same target molecule or to different target molecules. In the conjugates as reported herein to one terminus of the non-repeat-motif-molecule, i.e. the conjugation partner, irrespective of being the C-terminus or the N-terminus, only one, i.e. a single repeat-motif-molecule is covalently linked.

A first variant is a conjugate comprising two or more repeat-motif-molecules, each binding to the same or different target molecules, wherein each pair of repeat-motif-molecules is joined by a peptidic linker. In one embodiment the number of repeat-motif-molecules is selected from two, three, four, five, six, seven, eight, nine, or ten. In Figure 2 exemplary conjugates comprising two or more repeat-motif-molecules joint by peptidic linkers are shown.

A second variant is a conjugate comprising one or more repeat-motif-molecules and one or more non-repeat-motif-molecule. Each of the repeat-motif-molecules binds to the same or a different target molecule. The non-repeat-motif-molecule provides the scaffold to which the one or more repeat-motif-molecules are covalently linked, optionally and independently of each other via a peptidic linker. In one embodiment the non-repeat-motif-molecule is selected from antibody CL domain, antibody CH1 domain, antibody hinge region, antibody CH2 domain, antibody CH3 domain, antibody CH4 domain, membrane anchor motifs, transmembrane domains, glycosylation tag sequences, purification or detection tag sequences, or a combination of one or more thereof, either natural occurring or engineered variants thereof. In one embodiment the non-repeat-motif-molecule comprises two or more polypeptides covalently linked together by one or more disulfide bond(s). In one embodiment the non-repeat-motif-molecule is an antibody Fc-part. In one embodiment the non-repeat-motif-molecule is a dimer consisting of two monomers each comprising in N- to C-terminal direction a heavy chain hinge region, a CH2 domain and a CH3 domain, or a dimer consisting of two monomers each comprising in N- to C-terminal direction a CH1 domain, a heavy chain hinge region, a CH2 domain and a CH3 domain, or a tetramer comprising two monomers each comprising in N- to C-terminal direction a CH1 domain, a heavy chain hinge region, a CH2 domain and a CH3 domain and two monomers comprising a light

chain constant domain. In another embodiment each of the monomers independently of each other further comprises a variable domain.

The term "peptidic linker" denotes linkers of natural and/or synthetic origin comprising amino acid residues connected to each other via peptide bonds. They consist of a linear amino acid chain wherein the 20 naturally occurring amino acids are the monomeric building blocks. The chain has a length of from 1 to 50 amino acid residues, in one embodiment between 3 and 28 amino acid residues, in a further embodiment between 4 and 20 amino acid residues. The linker may contain repetitive amino acid sequences or sequences of naturally occurring polypeptides. The linker has the function to ensure that the two components connected through the linker can fold correctly and be presented properly due to steric and rotational freedom. In one embodiment the linker is a "synthetic peptidic linker" that is designated to be rich in glycine, glutamine, and/or serine residues. These residues are arranged e.g. in small repetitive units of up to five amino acids, such as (G)GGGS, (Q)QQQG, or (S)SSSG (SEQ ID NO: 14, 15, and 16). This small repetitive unit may be repeated for two to five times to form a multimeric unit. Other synthetic peptidic linkers are composed of a single amino acid, that is repeated between 10 to 20 times, such as e.g. serine in the linker GSSSSSSSSSSSSSSG (SEQ ID NO: 17). In one embodiment the linker is selected from [GQ4]3GNN (SEQ ID NO: 18), LSLSPGK (SEQ ID NO: 19), LSPNRGEC (SEQ ID NO: 20), LSLSGG (SEQ ID NO: 21), LSLSPGG (SEQ ID NO: 22), G3[SG4]2SG (SEQ ID NO: 23), or G3[SG4]2SG2 (SEQ ID NO: 24).

In one embodiment the linker has from 4 to 20 amino acid residues. In one embodiment the linker is the same between the repeat-motif-molecules, in another embodiment the conjugate contains linker with two or more different amino acid sequences. In a further embodiment the linker is selected from (G3S), (G3S)2, (G3S)3, (G3S)4, (G3S)5, (G4S), (G4S)2, (G4S)3, (G4S)4, (G4S)5 (SEQ ID NO: 14 and 25 to 33) , preferably from (G4S)3 and (G4S)4 (SEQ ID NO: 31 and SEQ ID NO: 32).

In one embodiment the conjugate as reported herein comprises one repeat-motif-molecule and one non-repeat-motif-molecule, and optionally a peptidic linker in between these molecules. The repeat-motif-molecule is covalently linked either to the C-terminus or to the N-terminus of the non-repeat-motif-molecule or to one of

the C-termini or to one of the N-termini of the polypeptides forming the non-repeat-motif-molecule.

In Figures 3 to 5 exemplary conjugates of the embodiments of the second variant are shown. In Figure 3 conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of the different individual antibody domains are shown. In Figure 4 conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of combinations of two, three or four antibody domains are shown. In Figure 5 conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of disulfide bond linked antibody domains (CL-CH1 or hinge-hinge) are shown, optionally these conjugates may comprise in addition further antibody domains except variable domains.

In another embodiment the conjugate as reported herein comprises two repeat-motif-molecule and one non-repeat-motif-molecule, and optionally up to two peptidic linker(s). One repeat-motif-molecule is covalently linked to the C-terminus and one to the N-terminus of the non-repeat-motif-molecule or both are covalently linked to the C-termini or to the N-termini of the polypeptides forming the non-repeat-motif-molecule or one of the repeat-motif-molecules is covalently linked to a C-terminus and one is covalently linked to an N-terminus of the polypeptides forming the non-repeat-motif-molecule. Therein the C-terminus and the N-terminus are the termini of the same polypeptide or of different polypeptides. In Figures 6 to 8 exemplary conjugates of this embodiment are shown. In Figure 6 the two repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising only one C-terminus and one N-terminus, wherein the non-repeat-motif-molecule may comprise one or more antibody domains. In Figures 7 and 8 the two repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains, optionally these conjugates may comprise in addition further antibody domains except variable domains.

In a further embodiment the conjugate comprises four repeat-motif-molecule and one non-repeat-motif-molecule, and optionally up to four peptidic linker(s). In this embodiment the non-repeat-motif-molecule comprises at least two polypeptides covalently associated together, in one embodiment via one or more disulfide bonds. In one embodiment the repeat-motif-molecules are covalently linked to the respective number of C-termini and N-termini of the non-repeat-motif-molecule. In another embodiment two of the repeat-motif-molecules are conjugated to each

other forming dimeric repeat-motif-molecules and the dimeric repeat-motif-molecule is in turn conjugated to the non-repeat-motif-molecule. In Figures 9 to 11 exemplary conjugates of this embodiment are shown. In Figure 9 the four repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising two C-termini and two N-termini, wherein the non-repeat-motif-molecule may comprise one or more antibody domains. In Figures 10 and 11 the four repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains, optionally these conjugates may comprise in addition further antibody domains except variable domains.

In still another embodiment the conjugate comprises six repeat-motif-molecule and one non-repeat-motif-molecule, and optionally up to six peptidic linker(s). In this embodiment the non-repeat-motif-molecule comprises at least two polypeptides covalently associated together, in one embodiment via one or more disulfide bonds. In one embodiment the repeat-motif-molecules are covalently linked to the respective number of C-termini and N-termini of the non-repeat-motif-molecule. In another embodiment four of the repeat-motif-molecules are conjugated to each other forming dimeric repeat-motif-molecules and the dimeric repeat-motif-molecule is in turn conjugated to the non-repeat-motif-molecule and two repeat-motif-molecules are not conjugated to each other but are each conjugated to a single terminus of the non-repeat-motif-molecule. In Figure 12 an exemplary conjugate of this embodiment is shown.

In a further embodiment the conjugate comprises eight repeat-motif-molecule and one non-repeat-motif-molecule, and optionally up to eight peptidic linker(s). In this embodiment the non-repeat-motif-molecule comprises at least two polypeptides covalently associated together, in one embodiment via one or more disulfide bonds. In one embodiment the repeat-motif-molecules are covalently linked to the respective number of C-termini and N-termini of the non-repeat-motif-molecule. In another embodiment of from two to eight of the repeat-motif-molecules are conjugated to each other forming dimeric repeat-motif-molecules and the dimeric repeat-motif-molecule is in turn conjugated to the non-repeat-motif-molecule and the remaining number of repeat-motif-molecules are not conjugated to each other but are each conjugated to a single terminus of the non-repeat-motif-molecule.

The repeat-motif-molecule may be a derivative of a scaffold selected from the group consisting of CTLA-4 (Evibody), lipocalin, protein A derived molecules

such as the Z-domain of protein A (Affibody, SpA), A-domain (Avimer, Maxibody), heat shock proteins such as GroEl and GroES, transferrin (transbody), peptide aptamer, C-type lectin domain (Tetranectin), human y-crystallin and human ubiquitin (affilins), PDZ domains, scorpion toxin, kunitz type domains of human protease inhibitors, and fibronectin (adnectin), repeat-motif-molecules derived from the Src homology domains (e.g. SH2 or SH3 domains), repeat-motif-molecules derived from PDZ domains, repeat-motif-molecules derived from the beta-lactamase, repeat-motif-molecules derived from high affinity protease inhibitors, repeat-motif-molecules derived from small disulfide binding protein scaffolds such as scorpion toxins, repeat-motif-molecules comprising repeat binding domains such as the EGF-like domain, the Kringle-domain, the PAN domain, the Gla domain, the SRCR domain, the Kunitz domain, the Bovine pancreatic trypsin inhibitor domain, the Kazal-type serine protease inhibitor domain, the Trefoil (P-type) domain, the von Willebrand factor type C domain, the anhaphylatoxin-like domain, the CUB domain, the thyroglobulin type I repeat, the LDL-receptor class A domain, the Sushi domain, the Link domain, the Thrombospondin type I domain, the immunoglobulin-like domains, the C-type lectin domain, the MAM domain, the von Willebrand factor type A domain, the Somatomedin B domain, the WAP-type four disulfide core domain, the F518 type C domain, the Hemopexin domain, the Laminin-type EGF-like domain, the C2 domain, repeat-motif-molecules derived from Avimers, repeat-motif-molecules derived from Telobodies, repeat-motif-molecules derived from Evibodies, repeat-motif-molecules derived from Microbodies (see e.g. Jeong, K.J. and Silverman, J., Nat. Biotechnol. 23 (2005) 1493-1494; Panni, S., et al., J. Biol. Chem. 277 (2002) 21666-21674; Schneider, S., et al., Nat. Biotechnol. 17 (1999) 170-175; Legendre, D., et al., Protein Sci. 11 (2002) 1506-1518; Stoop, A.A., et al., Nat. Biotechnol. 21 (2003) 1063-1068; Vita, C., et al., Proc. Natl. Acad. Sci. USA 92 (1995) 6404-6408; WO 2006/055689; US 2006/0234299).

In one embodiment the GEMOC comprises a conjugation partner selected from an antibody substructure, minibody, adnectin, anticalin, affibody, affilin, knottin, glybody, C-type lectin-like domain protein, designed ankyrin-repeat proteins (DARPin), Tetranectin, kunitz domain protein, Thioredoxin, cytochrome b562, zinc finger scaffold, Staphylococcal nuclease scaffold, fibronectin or fibronectin dimer, tenascin, N-cadherin, E-cadherin, ICAM, titin, GCSF-receptor, cytokine receptor, glycosidase inhibitor, antibiotic chromoprotein, myelin membrane adhesion molecule PO, CD8, CD4, CD2, class I MHC, T-cell antigen receptor, CD

1, C2 and I-set domains of VCAM-1, 1-set immunoglobulin domain of myosin-binding protein C, 1-set immunoglobulin domain of myosin-binding protein H, I-set immunoglobulin domain of telokin, NCAM, twitchin, neuroglian, growth hormone receptor, erythropoietin receptor, prolactin receptor, interferon-gamma receptor, ß-galactosidase/glucuronidase, ß-glucuronidase, transglutaminase, T-cell antigen receptor, superoxide dismutase, tissue factor domain, cytochrome F, green fluorescent protein, GroEL, and thaumatin.

In one embodiment the conjugation partner is an anticalin. In a further embodiment the anticalin is anticalin A-44. In one embodiment the conjugate comprises the amino acid sequence of SEQ ID NO: 64, and/or SEQ ID NO: 65, and/or SEQ ID NO: 66. It has been found that GEMOCS comprising an anticalin can be expressed at higher yields compared to GEMOCS comprising DARPins. In one embodiment the anticalin is fused to an antibody Fc-part. In another embodiment the Fc-part is the Fc-part of human IgG1 or human IgG4 with or without mutations. Exemplary mutations are the amino acid exchanges L234A and L235A for IgG1, and S228P and L235E for IgG4.

It has been found that it is advantageous for the production of GEMOCS comprising repeat-motif-molecules fused to the N-termini of an antibody Fc-part, such as a human IgG1 Fc-part or a human IgG4 Fc-part with or without the above identified amino acid exchanges, that the hinge region comprises three interchain disulfide bonds. Compared to conjugates containing two interchain disulfide bridges in the hinge region conjugates with three interchain disulfide bridges in the hinge region can be produced with reduced by product content, especially with respect to fragmentation.

The herein before reported GEMOCs comprising two or more repeat-motif-molecules provide a possibility for bispecific, trispecific or tetraspecific target molecule binding constructs. The term "bispecific" denotes that the GEMOC comprises at least two repeat-motif-molecules binding to two different target molecules. The term "trispecific" denotes that the GEMOC comprises at least three repeat-motif-molecules binding to three different target molecules. The term "tetraspecific" denotes that the GEMOC comprises at least four repeat-motif-molecules binding to four different target molecules.

In case of a GEMOC comprising antibody domains the production of mispaired byproducts as well as fragments has to be circumvented or at least minimized in order to achieve acceptable production yields, and to avoid the requirement for sophisticated purification procedures. An approach to circumvent the problem of mispaired byproducts, which is known as 'knobs-into-holes', aims at forcing the pairing of two different conjugates each comprising an antibody heavy chains CH3 domain by introducing mutations into the CH3 domains to modify the contact interface. On one chain bulky amino acids are replaced by amino acids with short side chains to create a 'hole'. Concomitantly, amino acids with large side chains are introduced into the other CH3 domain, to create a 'knob'. By coexpressing these two modified conjugates high yields of heterodimer formation ('knob-hole') versus homodimer formation ('hole-hole' or 'knob-knob') was observed (see e.g. Ridgway, J.B., Protein Eng. 9 (1996) 617-621; WO 96/027011). A further approach to clearly reduce the formation of mispaired byproducts is the use of a technology called "domain-exchange" or "cross-over". Therein the specific interaction between antibody CL and CH1 domains is exploited. In one embodiment the conjugation partner in the GEMOCs as reported herein comprise two polypeptides whereof one comprises an antibody light chain constant domain (CL) and the other comprises an antibody heavy chain first constant domain (CH1). Exemplary bispecific GEMOCs are shown in Figure 13.

The term "target molecule" denotes a molecule that is complementary to and provides for interaction sites for interacting with a repeat-motif-molecule. In one embodiment the target molecule is selected from cell surface molecules, soluble cell surface molecules, cytokines, hormones, enzymes, immunoglobulins, toxins, virus particle proteins, plaque proteins and plaque precursor proteins, or nanoparticles.

The GEMOCs as reported herein comprise a conjugation partner providing a scaffold for presenting the repeat-motif-molecule(s). This conjugation partner can provide in addition further functionality, such as e.g. a receptor binding functionality. The conjugation partner is in one embodiment selected from antibody domains, in one embodiment it comprises the CH2 domain of an antibody. The antibody CH2 domain provides for the Fc-Receptor binding functionality which is required for ADCC or CDC. In another embodiment the conjugation partner is a molecule that can multimerize or associate with other GEMOCs comprising the same conjugation partner or the respective complementary association partner.

Such a multimerizing conjugation partner or pair of conjugation partner and complementary association partner is in one embodiment selected from leucine zippers, the pair comprising the regulatory subunit of cAMP-dependent protein kinase A (PKA) and the anchoring domain of a kinase anchoring proteins (AKA).

The employed conjugation partner in the GEMOCs should be of human origin, highly stable with regard to fold and degradation, „inert", i.e. no cytokine or signaling function, easy and economic in production, allow for further processing (e.g. proteolytic cleavage, chemical modification of peptide, post translational modification), of appropriate size (no renal filtration) or other systemic elimination, and not immunogenic.

The term "multimerizing conjugation partner" denotes molecules capable of covalently or non-covalently associating two or more individual GEMOCs. Multimerizing conjugation partners are in one embodiment selected from the leucine zipper domain (Kostelny, S.A., et al., J. Immunol. 148 (1992) 1547-1553; de Kruif, J., et al., J. Biol. Chem. 271 (1996) 7630-7634), a yeast GCN4 leucine zipper, an isoleucine zipper domain, the helix-turn-helix motif (Pack, P., et al., Biotechnol. 11 (1993) 1271-1277), the max-interacting proteins and related molecules (US 5,512,473), the polyglutamic acid-polylysine domains (US 5,582,996), the natural or engineered pair of heavy chain CH2-CH3 domain, the natural or engineered pair of heavy chain CH1 domain-light chain constant domain (Mueller, K.M., et al., FEBS Lett. 422 (1998) 259-264), the 180 amino acid carboxyl terminal domain of the TATA binding protein (Colemen, R.A., et al., J. Biol. Chem. 270 (1995) 13842-13849), VCAM and VLA-4, integrins and extracellular matrix proteins, integrins and cell surface molecules (e.g. CD54 or CD102), ALCAMs, glutathione transfereases, SRCR domains, a receptor dimer pair (e.g., interleukin-8 receptor (IL-8R), integrin heterodimers (such as LFA-1 and GPIIIb/IIIa), or only the dimerization region(s) thereof, dimeric ligand polypeptides (e.g. nerve growth factor (NGF), neurotrophin-3 (NT-3), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF) (see e.g. Arakawa, T., et al., J. Biol. Chem. 269 (1994) 27833-27839; Radziejewski, C., et al., Biochem. 32 (1993) 13350-6)), or only the dimerization region(s) thereof, generally a pair of cysteine residues able to form a disulfide bond, a pair of peptides or polypeptides, each comprising at least one cysteine residue (e.g. from about one, two or three to about ten cysteine residues) such that disulfide bond(s) can form between the peptides or polypeptides, generally coiled-

coil domains (see e.g. Lupas, A., et al., Science 252 (1991) 1162-1164), the tetramerization domain of p53, the N-terminal residues (amino acids 20-80) of cartilage oligomeric matrix protein (COMP), alpha-helical sequences (see e.g. Eisenberg, D., et al., Protein 1 (1986) 16-22; Ho, S.P. and DeGrado, W.F., J. Am. Chem. Soc. 109 (1987) 6751-6758; Regan, L. and DeGrado, W.F., Science 241 (1988) 976-978; Hill, C.P., et al., Science 249 (1990) 543-546; Plückthun, A. and Pack, P., Immunotechnol. 3 (1997) 83-105). In one embodiment the multimerizing conjugation partner is selected from natural or engineered pairs of heavy chain CH2 and CH3 domains, natural or engineered pairs of heavy chain CH1 domain and light chain constant domain, natural or engineered heavy chain hinge region. In a further embodiment the multimerizing conjugation partner is selected from natural or engineered pairs of heavy chain CH2 and CH3 domains, natural or engineered pairs of heavy chain CH1 domain and light chain constant domain, from natural or engineered sequences of heavy chain hinge region and CH2 and CH3 domains. In another embodiment the multimerizing conjugation partner is selected from natural or engineered pairs of heavy chain CH2 and CH3 domains, natural or engineered pairs of heavy chain CH1 domain and light chain constant domain.

Antibodies can proteolytically be cleaved by the enzyme papain close to the N-terminal side of each hinge region resulting in two antigen binding fragments (Fab) and one constant region fragment (Fc). The Fab fragment consists of the entire light chain and the N-terminal a fragment of the heavy chain comprising the VH and CH1 domain. The Fc-part in turn comprises the remaining domains of the two heavy chains linked by the disulfide bonds in the hinge region.

The Fc-part of an antibody interacts with components of the immune system, e.g. the complement cascade (complement dependent cytotoxicity (CDC)) or by binding to the Fc-gamma receptors (antibody dependent cell cytotoxicity (ADCC)) or cytotoxic effector cells. To alter the effector function of the Fc-part substitutions of amino acid residues can be made (US 5,648,260; US 5,624,821). The Fc-part is responsible for mediating effector functions such as cytokine induction, ADCC, phagocytosis, complement dependent cytotoxicity (CDC) and half-life/clearance rate of antibody and antigen-antibody complexes. The Fc-part generally starts at position 226 (Cys) or 230 (Pro) and extends to the C-terminus of the antibody heavy chains.

The GEMOCs as reported herein are glycosylated proteins comprising one or more carbohydrate residues. In vivo protein production comprises post-translational

modification steps, especially glycosylation. The sugar (glycosyl) residues are added enzymatically to the protein amino acid sequence at N- or O-glycosylation motifs. Antibodies for example are glycosylated proteins with one or more carbohydrate moieties in the Fc-part and also in the variable domains. The carbohydrate moieties of the Fc-part are at least partly responsible for the effector function and only less important for antigen binding or half-life of the antibody (Jefferis, R., Biotechnol. Prog. 21 (2005) 11-16).

Depending on the amino acid sequence of the constant region of the heavy chains, antibodies (immunoglobulins) are divided in the classes: IgA, IgD, IgE, IgG, and IgM. Some of these classes are further divided into subclasses (isotypes), i.e. IgG in IgG1, IgG2, IgG3, and IgG4, or IgA in IgA1 and IgA2. According to the immunoglobulin class to which an antibody belongs are the heavy chain constant regions of immunoglobulins are called α (IgA), δ (IgD), ε (IgE), γ (IgG), and μ (IgM), respectively. Depending on the amino acid sequence of the constant domain the antibody light chains are denoted as kappa (κ) or lambda (λ) light chain.

The term "glycosylated" denotes that oligosaccharides are attached to at least one amino acid residue. Due to the involvement of a multitude of enzymes in the post-translational glycosylation in cells, i.e. in vivo, a recombinantly produced polypeptide is obtained having a glycosylation patter, i.e. a glycosylation heterogeneity, due to the cell expressing the polypeptide. Thus, therefore a recombinantly produced polypeptide comprises not only a single, defined N- or O-linked oligosaccharide at a specified amino acid residue, but is a mixture of polypeptides each having the same amino acid sequence but comprising different oligosaccharides at this specified amino acid position, i.e. has a glycosylation pattern. Therefore, a glycosylated EMOC comprises a group of conjugates each with the same amino acid sequence but with different oligosaccharides attached to a specified amino acid position. The term "oligosaccharide" as used within this application denotes a polymeric saccharide comprising two or more covalently linked monosaccharide units. In one embodiment the oligosaccharide attached to a glycosylation site is a mixture of oligosaccharides obtained by the expression of the conjugate in a CHO cell or HEK293 cell, i.e. the oligosaccharide is a mixture of oligosaccharide obtained by post-translational modification of the cell expressing the conjugate, i.e. it is characteristic for the cell expressing the conjugate. The glycosylation and glycosylation pattern obtained by a cell expressing a polypeptide,

i.e. in vivo, is completely different from the glycosylation or glycosylation pattern obtained in vitro.

Another aspect of the current invention is a method for producing the GEMOCs as reported herein by expressing the respective encoding nucleic acid in a mammalian cell.

In detail, herein is reported a method for producing a glycosylated repeat-motif-molecule conjugate in a mammalian cell comprising

- providing a mammalian cell comprising a nucleic acid encoding the repeat-motif-molecule conjugate,
- cultivating the cell under conditions suitable for the expression of the repeat-motif-molecule,
- recovering the glycosylated repeat-motif-molecule from the cell or the cultivation medium and thereby producing the glycosylated repeat-motif-molecule,
- optionally purifying the recovered glycosylated repeat-motif-molecule.

GEMOCs as reported herein can be produced in mammalian cells, i.e. as in vivo glycosylated repeat-motif-molecule conjugates. It is herein reported a method for the production of repeat-motif-molecule conjugates in a glycosylated form, in one embodiment in an in vivo glycosylated form. Such an in vivo glycosylation, i.e. a glycosylation in the same cell also expressing the repeat-motif-molecule conjugate, is a cell specific post-translational modification. Recombinantly produced polypeptides can be glycosylated in vitro, i.e. after isolation from the producing cell (see e.g. Leung, S., et al., J. Immunol. 154 (1995) 5919-5926; US 5,443,953 for antibodies).

Methods for purifying polypeptides and immunoglobulins are well established and widespread used and are employed either alone or in combination. Such methods are, for example and in certain embodiments, affinity chromatography using microbial-derived proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange chromatography), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands),

hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and preparative electrophoretic methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102). In one embodiment the chromatography column packing is a chromatography material selected from an affinity chromatography material, or an ion exchange chromatography material, or a thiophilic adsorption chromatography material, or a hydrophobic interaction chromatography material, or an aromatic adsorption chromatography material, or a metal chelate affinity chromatography material, or a size exclusion chromatography material.

To produce a secreted polypeptide, the structural gene of interest also may comprise a DNA segment that encodes a signal sequence/leader peptide. The signal sequence directs the newly synthesized polypeptide to and through the membrane of the Endoplasmic Reticulum (ER) where the polypeptide can be routed for secretion. The signal sequence is cleaved off by a signal peptidases during the protein crosses the ER membrane. As for the function of the signal sequence the recognition by the host cell's secretion machinery is essential. Therefore the used signal sequence has to be recognized by the host cell's proteins and enzymes of the secretion machinery.

Translational regulatory elements include a translational initiation (AUG) and stop codon (TAA, TAG or TGA). An internal ribosome entry site (IRES) can be included in some constructs.

Thus, another aspect as reported herein is a nucleic acid for expression of a repeat-motif-molecule conjugate in a mammalian cell comprises the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of an antibody germline gene,
- an immunoglobulin heavy chain signal sequence,
- the repeat-motif-molecule conjugate's encoding sequence,
- a polyadenylation ("poly A") signal sequence.

In one embodiment the 5'-untranslated region of an antibody germline gene is of a human antibody germline gene. In another embodiment the immunoglobulin heavy chain signal sequence is a murine or human immunoglobulin heavy chain signal sequence. In a further embodiment the immunoglobulin heavy chain signal sequence includes a signal sequence intron (signal sequence 1, intron, signal sequence 2 [L1-intron-L2]).

The subject matter presented herein will be exemplified in the following with two model repeat-motif-molecules binding to HER2 (DARPin H10-2-G3, scaffold N2C (2 repeats), Zahnd, C., et al., J. Mol. Biol. 369 (2007) 1015-1028; DARPin 9-26, scaffold N3C (3 repeats), Steiner, D., et al., J. Mol. Biol. 382 (2008) 1211-1227). Four expression constructs for the expression of these GEMOCs in HEK293 cells were tested (in N- to C-terminal direction):

1) leader-peptide – repeat-motif-molecule - hexa-histidine-tag,

2) leader-peptide – repeat-motif-molecule - immunoglobulin Fc fragment,

3) leader-peptide - immunoglobulin Fc fragment - repeat-motif-molecule,

4) leader-peptide - immunoglobulin Fab-fragment - repeat-motif-molecule.

Thus, further aspects as reported herein are the GEMOCs of SEQ ID NO: 10, 11, 12, 13, 34, 35, 36, 37, 38, 39, 40, 41, 42, the GEMOCs comprising SEQ ID NO: 11 and 11, SEQ ID NO: 13 and 13, SEQ ID NO: 11 and 13, SEQ ID NO: 34 and 34, SEQ ID NO: 35 and 35, SEQ ID NO: 34 and 35, SEQ ID NO: 36 and 37, SEQ ID NO: 39 and 39, as well as the respective nucleic acids encoding them, as well as the respective nucleic acids with these encoding nucleic acid sequences and sequence combinations, as well as the methods for producing these GEMOCs.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Sequence Listing

SEQ ID NO: 01 Ankyrin-repeat-motif-molecule amino acid consensus sequence 1.

SEQ ID NO: 02 Ankyrin-repeat-motif-molecule amino acid consensus sequence 2.

SEQ ID NO: 03	Ankyrin-repeat-motif-molecule amino acid consensus sequence 3.
SEQ ID NO: 04	Ankyrin-repeat-motif-molecule amino acid consensus sequence 4.
SEQ ID NO: 05	Ankyrin-repeat-motif-molecule amino acid consensus sequence 5.
SEQ ID NO: 06	Ankyrin-repeat-motif-molecule amino acid consensus sequence 6.
SEQ ID NO: 07	Ankyrin-repeat-motif-molecule amino acid consensus sequence 7.
SEQ ID NO: 08	Ankyrin-repeat-motif-molecule amino acid consensus sequence 8.
SEQ ID NO: 09	Ankyrin-repeat-motif-molecule amino acid consensus sequence 9.
SEQ ID NO: 10	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated hexa-histidine-tag amino acid sequence.
SEQ ID NO: 11	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated IgG1 Fc-part.
SEQ ID NO: 12	Model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag amino acid sequence.
SEQ ID NO: 13	Model repeat-motif-molecule 9-26 with C-terminally conjugated IgG1 Fc-part.
SEQ ID NO: 14 to 33	Peptidic linker sequences.
SEQ ID NO: 34	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated IgG1 Fc-part with two disulfide bonds in the hinge region.
SEQ ID NO: 35	Model repeat-motif-molecule 9-26 with C-terminally conjugated IgG1 Fc-part with two disulfide bonds in the hinge region.
SEQ ID NO: 36	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated IgG1 Fc-part with CH3-knob-domain.
SEQ ID NO: 37	Model repeat-motif-molecule 9-26 with C-terminally conjugated IgG1 Fc-part with CH3-hole-domain.
SEQ ID NO: 38	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated model repeat-motif-molecule 9-26.
SEQ ID NO: 39	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated IgG1 Fc-part with two disulfide bonds in the

hinge region with C-terminally conjugated model repeat-motif-molecule 9-26.

SEQ ID NO: 40 Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag.

SEQ ID NO: 41 Model repeat-motif-molecule 9-26 with C-terminally conjugated model repeat-motif-molecule H10-2-G3 with C-terminally conjugated hexa-histidine-tag.

SEQ ID NO: 42 Dimeric model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag.

SEQ ID NO: 43 Human IgG1 constant region.

SEQ ID NO: 44 Human IgG4 constant region.

SEQ ID NO: 45 Human kappa constant region.

SEQ ID NO: 46 Human lambda constant region.

SEQ ID NO: 47 N-terminal capping repeat.

SEQ ID NO: 48 C-terminal capping repeat.

SEQ ID NO: 49 CMV promoter sequence.

SEQ ID NO: 50 Anti-Aβ antibody variable heavy chain domain amino acid sequence.

SEQ ID NO: 51 Anti-Aβ antibody variable heavy chain domain amino acid sequence.

SEQ ID NO: 52 Anti-Aβ antibody variable heavy chain domain amino acid sequence.

SEQ ID NO: 53 Anti-Aβ antibody variable light chain domain amino acid sequence.

SEQ ID NO: 54 Anti-Aβ antibody variable light chain domain amino acid sequence.

SEQ ID NO: 55 Anti-Aβ antibody variable light chain domain amino acid sequence.

SEQ ID NO: 56 Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated hexa-histidine-tag amino acid sequence and introduced engineered glycosylation site at the beginning of the C-capping repeat.

SEQ ID NO: 57 Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated IgG1 Fc-part and thereto C-terminally conjugated membrane anchor domain.

SEQ ID NO: 58	Model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag amino acid sequence and introduced engineered glycosylation site at the beginning of the C-capping repeat.
SEQ ID NO: 59	Model repeat-motif-molecule 9-26 with C-terminally conjugated IgG1 Fc-part and thereto C-terminally conjugated membrane anchor domain.
SEQ ID NO: 60	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated hexa-histidine-tag amino acid sequence and glycosylation tag in the C-terminal region.
SEQ ID NO: 61	Model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag amino acid sequence and glycosylation tag in the C-terminal region.
SEQ ID NO: 62	Model repeat-motif-molecule H10-2-G3 with C-terminally conjugated hexa-histidine-tag amino acid sequence and glycosylation tag.
SEQ ID NO: 63	Model repeat-motif-molecule 9-26 with C-terminally conjugated hexa-histidine-tag amino acid sequence and glycosylation tag.
SEQ ID NO: 64	Model conjugate comprising anticalin A-44 with hexa-histidine-tag.
SEQ ID NO: 65	Model anticalin A-44 with Fc-tag and three disulfide bridges in the hinge region.
SEQ ID NO: 66	Model anticalin A-44 with Fc-tag and two disulfide bridges in the hinge region.

Description of the Figures

Figure 1	Schematic presentation of the domain architecture of an antibody and symbol legend for further figures.
Figure 2	Exemplary conjugates comprising two or more repeat-motif-molecules.
Figure 3	Exemplary conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of different individual antibody domains.

Figure 4 Exemplary conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of combinations of two, three or four antibody domains.

Figure 5 Exemplary conjugates of one repeat-motif-molecule to the N-terminus and C-terminus of disulfide bond linked antibody domains.

Figure 6 Exemplary conjugates of two repeat-motif-molecules conjugated to a non-repeat-motif-molecule comprising only one C-terminus and one N-terminus.

Figure 7 Exemplary conjugates of two repeat-motif-molecules conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains (part 1).

Figure 8 Exemplary conjugates of two repeat-motif-molecules conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains (part 2).

Figure 9 Exemplary conjugates of four repeat-motif-molecules conjugated to a non-repeat-motif-molecule comprising two C-termini and two N-termini.

Figure 10 Exemplary conjugates of four repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains (part 1).

Figure 11 Exemplary conjugates of four repeat-motif-molecules are conjugated to a non-repeat-motif-molecule comprising disulfide bond linked antibody domains (part 2).

Figure 12 Exemplary conjugate of six repeat-motif-molecules and one non-repeat-motif-molecule.

Figure 13 Exemplary domain exchanged GEMOCs.

Figure 14 Plasmid map of expression plasmids 9800.

Figure 15 Plasmid map of the expression plasmid 9801.

Figure 16 Plasmid map of the heavy chain-polypeptide-conjugate expression plasmid 9807.

Figure 17 Plasmid map of the antibody light chain expression vector 5170.

Figure 18 SDS-PAGE gels of expressed ankyrin-repeat-motif-molecule conjugates in the supernatant of transiently transfected HEK293 cells on day 7; Construct 1 = protein of SEQ ID NO: 10, construct 2 = protein of SEQ ID NO: 12, construct 3 = protein of SEQ ID NO: 11, construct 4 = protein of SEQ ID NO: 13.

Figure 19 BIAcore analysis of the binding of GEMOCs according to the invention to soluble HER2.

Example 1

Plasmid construction

Standard methods were used to manipulate DNA as described in Sambrook, J. et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions. Desired gene segments were prepared by gene synthesis. The synthesized gene fragments were cloned into a specified expression vector. The DNA sequence of the subcloned gene fragments were confirmed by DNA sequencing.

Expression plasmids 9800 and 9803

A gene segment encoding the conjugates of SEQ ID NO: 10 or SEQ ID NO: 12 was cloned into a specified expression plasmid via the unique restriction sites BsmI and Bpu10I. The expression plasmid was designed to combine the conjugate's encoding sequence with a C-terminal hexa-histidine-tag, e.g. for expression in HEK293 cells and subsequent purification. Beside the expression cassette for the conjugate the plasmid comprises:

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the conjugate's encoding sequence comprises the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of a human antibody germline gene,
- a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (signal sequence 1, intron, signal sequence 2 [L1-intron-L2]) and the unique restriction site BsmI at the 3' end of L2,
- the conjugate's encoding sequence,
- a GSG linker encoding a unique Bpu10I at the 3' end of the conjugate's encoding sequence and in frame with the conjugate's encoding sequence,
- a hexa-histidine-tag at the 3' end of the GSG linker, in frame with the

conjugate's encoding sequence and the GSG linker,
- a polyadenylation ("poly A") signal sequence, and
- the unique restriction sites NheI and EagI at the 3'-end of the expressed sequence.

The plasmid map of expression plasmids 9800 and 9803 accordingly, is shown in Figure 14. The amino acid sequences of the mature (without signal sequence) conjugates are denoted in SEQ ID NO: 10 and 12.

Expression plasmids 9801 and 9804

A gene segment encoding conjugate of SEQ ID NO: 11 or SEQ ID NO: 13 was cloned into a specified expression plasmid via the unique restriction sites HindIII and PmlI. The expression plasmid was designed to conjugate the conjugate to the N-terminus of the hinge region of an immunoglobulin of class IgG1, thereby replacing the naturally present VH and CH1 domains. The resulting expression plasmid was useful for the expression of the conjugates of SEQ ID NO: 11 and 13, respectively, e.g. in HEK293 cells. Beside the expression cassette for the repeat-motif-molecule conjugated to the IgG1-hinge, -CH2 and -CH3 sequences the plasmid comprises:

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the repeat-motif-molecule-hinge-CH2-CH3 conjugate is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- the Intron A sequence of the human cytomegalovirus,
- a 5'-untranslated region of a human antibody germline gene,
- a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (signal sequence 1, intron, signal sequence 2 [L1-intron-L2]) and the unique restriction site BsmI at the 3' end of L2,
- the conjugate's encoding sequence,
- sequences encoding the hinge region, the CH2- and CH3-domain of human IgG1, with a unique PmlI restriction site in the hinge region to allow cloning of the conjugate's encoding sequence, and

- a polyadenylation ("poly A") signal sequence.

The plasmid map of the expression plasmid 9801, and 9804 accordingly, is shown in Figure 15. The amino acid sequences of the mature (without signal sequence) conjugates are shown in SEQ ID NO: 11 and 13.

Expression plasmids 9807 and 9808

An example antibody with which the repeat-motif-molecules of SEQ ID NO: 10 and 12 without hexa-histidine-tag can be conjugated is an antibody against the amyloid β-A4 peptide (anti-Aβ antibody). Such an antibody and the corresponding nucleic acid sequences are, for example, reported in WO 2003/070760 or US 2005/0169925. A gene segment encoding the conjugate was cloned into a specified expression plasmid via the unique restriction sites MroI and NheI. The expression plasmid was designed to conjugate the polypeptide to the C-terminus of a human immunoglobulin heavy chain of the subclass IgG1 (genomically organized expression cassette; exon-intron organization). The resulting expression plasmid was useful for the expression of the conjugates e.g. in HEK293 cells. Beside the expression cassette of the heavy chain of a monoclonal antibody-repeat-motif-molecule conjugate the plasmid comprises:

- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the heavy chain-repeat-motif-molecule conjugate is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of a human antibody germline gene,
- a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (signal sequence 1, intron, signal sequence 2 [L1-intron-L2]),
- an anti-Ab antibody variable heavy chain domain encoding segment arranged with a splice donor site and a unique BamHI restriction site at the 3'-end,
- a nucleic acid encoding the human γ1 constant domains CH1, hinge, CH2 and CH3 separated by introns,
- a (G4S)3 linker fused in frame to the 3' end of the CH3 domain,
- the unique restriction sites MroI and NheI at the linker's 3' end to allow the

cloning of the polypeptide in frame to the C-terminus of the linker, and
- a polyadenylation ("poly A") signal sequence.

The plasmid map of the heavy chain-repeat-motif-molecule conjugate expression plasmid 9807, and 9808 accordingly, is shown in Figure 15.

Expression plasmid 5170

For the expression of the light chain of the antibody the plasmid 5170 was used. Plasmid 5170 is an expression plasmid e.g. for transient expression of an antibody light chain (genomically organized expression cassette; exon-intron organization) in HEK293 cells.

Beside the antibody κ-light chain expression cassette this plasmid comprises:

- a neomycine resistance gene as a selectable marker,
- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli, and
- a ß-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the antibody κ-light chain gene is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of a human antibody germline gene,
- a murine immunoglobulin heavy chain signal sequence including a signal sequence intron (signal sequence 1, intron, signal sequence 2 [L1-intron-L2]),
- an anti-Aβ antibody variable light chain domain encoding segment arranged with a splice donor site and a unique BamHI restriction site at the 3'-end, a truncated human κ-light chain intron 2,
- the human κ-light gene constant domain,
- a polyadenylation ("poly A") signal sequence, and
- the unique restriction sites AscI and SgrAI at the 3'-end.

The plasmid map of the antibody κ-light chain expression vector 5170 is shown in Figure 17.

Example 2

Transient transfection and expression

Recombinant conjugates according to the invention as exemplified in Example 1 were obtained by transient transfection of HEK293-Freestyle cells (human embryonic kidney cell line 293, Invitrogen) growing in suspension. The transfected cells were cultivated in F17 medium (Gibco) or Freestyle 293 medium (Invitrogen), supplemented with 6 mM Glutamine, either Ultra-Glutamine (Biowhittake/Lonza) or L-Glutamine (Sigma), with 8 % CO_2 at 37 °C in shake flasks in the scale of 30 ml to 250 ml medium. For transfection Fectin (Invitrogen) was used in a ratio of reagent (µl) to DNA (µg) of 4:3. In the case of conjugates to the C-terminus of an antibody heavy chain, light and heavy chains were expressed from two different plasmids using a molar ratio of light chain to heavy chain encoding plasmid ranging from 1:2 to 2:1, respectively. The conjugate containing cell culture supernatants were harvested at day 6 to 8 after transfection. General information regarding the recombinant expression of human immunoglobulins in e.g. HEK293 cells is given in: Meissner, P. et al., Biotechnol. Bioeng. 75 (2001) 197-203.

Example 3

Expression analysis using SDS-PAGE

LDS sample buffer, fourfold concentrate (4xLDS): 4 g glycerol, 0.682 g TRIS (tris-(hydroxymethyl)-aminomethane), 0.666 g TRIS-HCl (tris-(hydroxymethyl)-aminomethane-hydrochloride), 0.8 g LDS (lithium dodecyl sulfate), 0.006 g EDTA (ethylene diamin tetra acid), 0.75 ml of a 1 % by weight (w/w) solution of Serva Blue G250 in water, 0.75 ml of a 1 % by weight (w/w) solution of phenol red, add water to make a total volume of 10 ml.

The culture broth containing the secreted conjugate was centrifuged to remove cells and cell debris. An aliquot of the clarified supernatant was admixed with 1/4 volumes (v/v) of 4xLDS sample buffer and 1/10 volume (v/v) of 0.5 M 1,4-dithiotreitol (DTT). Then the samples were incubated for 10 min. at 75 °C and protein separated by SDS-PAGE. The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10 % NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MES running buffer was used. The polypeptides and polypeptide-conjugates could be clearly detected (see Figure 18).

Example 4

Protein purification by affinity chromatography and gel filtration chromatography

Fc- and antibody-conjugates

The expressed and secreted Fc- and antibody-conjugates were purified by affinity chromatography using the protein A affinity material MabSelectSure (GE Healthcare). Briefly, after centrifugation (10,000 g for 10 minutes) and filtration through a 0.45 µm filter the conjugate containing clarified culture supernatants was applied on a MabSelectSure column equilibrated with PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Unbound proteins were removed by washing with equilibration buffer. The conjugate was eluted with 0.1 M citrate buffer, pH 3.3, and the product containing fractions were neutralized with 1 M TRIS pH 9.0. Afterwards, the solution was dialyzed against PBS buffer at 4 °C, concentrated with an Amicon Centricon concentration device, and stored in an ice-water bath at 0 °C. Aggregation of the Fc- and antibody-conjugates was analyzed by analytical size exclusion chromatography. Fractions showing high molecular weight aggregates were subjected to preparative size exclusion chromatography. Briefly, the buffer of the conjugate was exchanged to histidine buffer (20 mM histidine, 140 mM NaCl, pH 6.0) by repeated concentration and dilution in a Centricon device (Amicon). After concentration to 1-4 ml, the conjugate containing solution was applied to a Superdex200 High Load column (GE HealthCare) equilibrated with the same histidine buffer. Fractions of 1 ml were collected. All fractions were analyzed by analytical SEC (Superdex200, GE HealthCare) and fractions with purely monomeric conjugate were pooled. The integrity of the conjugates were analyzed by SDS-PAGE in the presence and absence of a reducing agent and staining with Coomassie brilliant blue as described in the previous paragraph.

Conjugates of SEQ ID NO: 10 and 12

The expressed and secreted conjugates comprising an hexa-histidine-tag (SEQ ID NO: 10 and 12) were purified by affinity chromatography using Nickel chelating affinity material Ni-Sepharose HP HighTrap (GE Healthcare) according to known methods. Briefly, after centrifugation (10,000 g for 10 minutes) and filtration through a 0.45 µm filter the conjugate containing clarified culture supernatant was applied on a Ni-Sepharose column equilibrated with phosphate buffer (50 mM

Na_3PO_4, 300 mM NaCl, pH 8.0). Unbound proteins were removed by washing with phosphate equilibration buffer containing 20 mM imidazole. The conjugate was eluted with a conductivity gradient. Afterwards, the conjugate containing solution was extensively dialyzed against histidine buffer (20 mM histidine, 140 mM NaCl, pH 6.0) at 4 °C, concentrated with an Amicon Centricon concentration device, and stored in an ice-water bath at 0 °C. Aggregation of the Fc- and antibody-conjugates was analyzed by analytical size exclusion chromatography. Fractions showing high molecular weight aggregates were subjected to preparative size exclusion chromatography according to known methods. Briefly, the concentrated conjugate containing solution was applied to a Superdex200 High Load column (GE HealthCare) equilibrated with the same histidine buffer as reported above. Fractions of 1 ml were collected. All fractions were analyzed by analytical SEC (Superdex200, GE HealthCare) and fractions with purely monomeric conjugate were pooled. The integrity of the conjugate were analyzed by SDS-PAGE in the presence and absence of a reducing agent and staining with Coomassie brilliant blue as described in the previous paragraph.

Example 5

Binding assay by BIAcore

All surface plasmon resonance measurements were performed on a BIAcore 3000 instrument (GE Healthcare Biosciences AB, Sweden) at 25 °C. Running and dilution buffer was PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 105 mM NaCl, 2.7 mM KCl), pH 6.0, 0.005 % (v/v) Tween 20. Soluble protein A was diluted in 10 mM sodium acetate buffer, pH 5.0, and immobilized on a CM5 biosensor chip using the standard amine coupling kit (GE Healthcare Biosciences AB, Sweden) to obtain protein A surface densities of approximately 1000 RU. HBS-P (10 mM HEPES, pH 7.4, 118 mM NaCl, 0.005 % surfactant P20; GE Healthcare Biosciences AB, Sweden) was used as running buffer during immobilization. The Fc- and antibody-conjugates in question were diluted with PBS, 0.005 % (v/v) Tween 20, pH 6.0 to a concentration of 450 nM and injected over 3 minutes at a flow rate of 30 µl/minute. Then the soluble ligand was diluted into the same buffer to different concentrations between 70 and 680 nM and injected over 3 minutes at a flow rate of 30 µl/minute. Afterwards the sensor chip was regenerated for 1 minute with PBS, pH 8.0, 0.005 % (v/v) Tween 20. Data analysis was performed with the BIAevaluation software (BIAcore, Sweden) (see Figure 19).

Example 6

Binding assay by ELISA

The binding of the Fc- and antibody-conjugates were determined by means of an ELISA test (enzyme-linked immunosorbent assay). Herein a sandwich assay based on the streptavidin/biotin technology was used. A streptavidin-coated microtiter plate was used for the assay.

Example 7

Binding assay by FACS

The HER2 overexpressing stable cell line SK-BR-3 (ATCC# HTB-30) was cultivated in McCoy's 5a medium (PAN) supplemented with 10 % fetal calf serum (FCS) and 2 mM L-glutamine. For detection of HER2 on the cell surface by the conjugate according to the invention, the cells were washed with PBS supplemented with 1 % FCS as blocking reagent. The conjugate was incubated in PBS/1 % FCS with the cells at 4 °C for 15 min., washed and incubated with an appropriate fluorescently labeled secondary antibody detecting the conjugate. The labeled cells were sorted by fluorescence assisted cell sorting (FACS) for the binding of the conjugate onto the cells.

Patent Claims

1. Glycosylated repeat-motif-molecule conjugate of the following formula

(repeat-motif-molecule – $linker_n)_m$ – $(conjugation\ partner)_q$ – $(linker_o$ – repeat-motif-molecule$)_p$

wherein n=1, m=1, q=1, p=0,

wherein the repeat-motif-molecule conjugate comprises at least one oligosaccharide attached to a glycosylation site, and

wherein the conjugation partner comprises an engineered antibody heavy chain hinge region with three cysteine residues, and

wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

2. Glycosylated repeat-motif-molecule conjugate of the following formula

(repeat-motif-molecule – $linker_n)_m$ – $(conjugation\ partner)_q$ – $(linker_o$ – repeat-motif-molecule$)_p$

wherein n=0, m=1, q=1, p=0,

wherein the repeat-motif-molecule conjugate comprises at least one oligosaccharide attached to a glycosylation site, and

wherein the conjugation partner comprises an engineered antibody heavy chain hinge region with three cysteine residues, and

wherein the repeat-motif-molecule is an ankyrin-repeat-motif-molecule.

3. Conjugate according to any one of claims 1 to 2, characterized in that said ankyrin-repeat-motif-molecule has an amino acid sequence of SEQ ID NO: 4 or a variant thereof.

4. Conjugate according to any one of the preceding claims, characterized in that said linker is a peptidic linker selected from SEQ ID NO: 14 and SEQ ID NO: 25 to SEQ ID NO: 33.

5. Glycosylated repeat-motif-molecule conjugate, characterized in comprising two glycosylated repeat-motif-molecule conjugates according to any one of claims 1 to 4.

6. Nucleic acid comprising the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region of an antibody germline gene,
- an immunoglobulin heavy chain signal sequence,
- a glycosylated repeat-motif-molecule conjugate's according to any one of claims 1 to 5 encoding sequence,
- a polyadenylation ("poly A") signal sequence.

7. Method for producing a glycosylated repeat-motif-molecule conjugate according to any one of claims 1 to 5 comprising

- providing a mammalian cell comprising a nucleic acid according to claim 6,
- cultivating the cell under conditions suitable for the expression of the repeat-motif-molecule conjugate,
- recovering the glycosylated repeat-motif-molecule conjugate from the cell or the cultivation medium and thereby producing the glycosylated repeat-motif-molecule conjugate,
- optionally purifying the recovered glycosylated repeat-motif-molecule conjugate.

8. Method according to claim 7, characterized in that said mammalian cell is selected from CHO cells, NS0 cells, Sp2/0 cells, Per.C6 cells, and HEK293 cells.