Strijbis, K. et al (Traffic 13 (2012) 780-789) report protein ligation in living cells using sortase. It has been stated by them that the Ca^{2+}-dependent S. aureus sortase A is not functional intracellularly, but that the Ca^{2+}-independent S. pyogenes sortase A is functional in the cytosol and endoplasmic reticulum (ER) lumen of both Saccharomyces cerevisiae and mammalian HEK293T cells.

Summary ~~of the Invention~~

Herein is reported a method for producing in vivo intracellularly an enzyme – catalyzed (i.e. enzymatic) conjugate of a first polypeptide domain with a second polypeptide domain by using the Ca^{2+}-dependent enzyme sortase A of Staphylococcus aureus (S. aureus), whereby one of the polypeptide domains and the soluble sortase A enzyme contain an endoplasmic reticulum retention signal sequence.

This technology is especially suited for the rapid generation e.g. of a library of combinations of a first group of polypeptide domains (e.g. a first group of binding domains such as cognate pairs of antibody variable domains) and a second group of polypeptide domains (e.g. a second group of binding domains such as cognate pairs of antibody variable domains but directed against other epitopes/antigens as those of the first group, or a group of payload molecules). This library can be easily generated e.g. by transient transfection in HEK cells and the resulting combinations can be screened thereafter e.g. for the intended biological effect or intended properties.

~~One aspect as reported herein~~ There is provided ~~is~~ a method for producing a polypeptide comprising at least two polypeptide domains comprising the step of

- cultivating a cell comprising

  a) a nucleic acid encoding a soluble sortase A with a C-terminal endoplasmic reticulum retention signal,

  b) a nucleic acid encoding a first polypeptide domain comprising at its C-terminus or in its C-terminal region a sortase motif followed by an endoplasmic reticulum retention signal, and

  c) a nucleic acid encoding a second polypeptide domain comprising at its N-terminus an oligoglycine motif of at least two glycine residues,

whereby the cell secretes the sortase A(-mediated/-catalyzed) conjugate of the first polypeptide domain and the second polypeptide domain,

thereby producing a polypeptide comprising at least two polypeptide domains.

In one aspect, there is provided a method for producing a polypeptide comprising at least two polypeptide domains comprising the step of

- cultivating a cell comprising

a) a nucleic acid encoding a soluble S. aureus sortase A with a C-terminal endoplasmic reticulum retention signal,

b) a nucleic acid encoding a first polypeptide domain comprising at its C-terminus a sortase motif followed by an endoplasmic reticulum retention signal, and

c) a nucleic acid encoding a second polypeptide domain comprising at its N-terminus at least a diglycine,

whereby the cell secretes the sortase A conjugate of the first polypeptide domain and the second polypeptide domain,

thereby producing a polypeptide comprising at least two polypeptide domains.

There is also provided ~~One aspect as reported herein is~~ a method for producing a multispecific binder comprising at least two binding entities comprising the step of

- cultivating a cell comprising

a) a nucleic acid encoding a soluble sortase A with a C-terminal endoplasmic reticulum retention signal,

b) a nucleic acid encoding a first binding entity comprising at its C-terminus or in its C-terminal region a sortase motif followed by an endoplasmic reticulum retention signal, and

c) a nucleic acid encoding a second binding entity comprising at its N-terminus at least a diglycine,

whereby the cell secretes the sortase A(-mediated/catalyzed) conjugate of the first binding entity and the second binding entity,

whereby the first binding entity specifically binds to a first antigen or target and the second binding entity specifically binds to a second antigen or target,

thereby producing a multispecific binder comprising at least two binding entities.

In another aspect, there is provided a method for producing a multispecific binder comprising at least two binding entities comprising the step of

- cultivating a cell comprising

a) a nucleic acid encoding a soluble S. aureus sortase A with a C-terminal endoplasmic reticulum retention signal,

b) a nucleic acid encoding a first binding entity comprising at its C-terminus a sortase motif followed by an endoplasmic reticulum retention signal, and

c) a nucleic acid encoding a second binding entity comprising at its N-terminus at least a diglycine,

whereby the cell secretes the sortase A conjugate of the first binding entity and the second binding entity,

whereby the first binding entity specifically binds to a first antigen or target and the second binding entity specifically binds to a second antigen or target,

thereby producing a multispecific binder comprising at least two binding entities.

~~In one embodiment of all aspects is t~~The sortase A may be the sortase A of Staphylococcus aureus (S. aureus). In one embodiment the nucleic acid encoding a (soluble) sortase A with a C-terminal endoplasmic reticulum retention signal encodes an amino acid sequence of SEQ ID NO: 51 or SEQ ID NO: 52.

Herein is reported a method for providing tailor-made, highly specific therapeutic molecules for the treatment of a disease, such as cancer or a viral infection, in a patient in need of a treatment, whereby the therapeutic molecule is adapted to the characteristics of the disease of the patient and/or to the genotype/phenotype of the patient.

Especially suited is the combination of two binding entities having a low to medium affinity for their respective targets/epitopes. Additionally, off-target binding is greatly reduced or can even be eliminated totally.

It has been found that with the method as reported herein it is possible to tailor-make e.g. bispecific binders such as e.g. bispecific antibodies specifically directed to two surface markers found on the surface of a cell, such as a cancer cell. As the binding specificities are individually provided by the starting components it is possible to tailor-make a multispecific targeting and binding molecule simply by determining the surface markers present on a cell, e.g. on a cancer cell, and conjugating the respective antibody fragments that specifically bind to these surface markers or their respective ligands by an enzymatic procedure. As the enzymatic conjugation is performed by the enzyme sortase A, in one embodiment by the sortase A of S. aureus, the resulting bispecific binder (bispecific antibody) is characterized by the presence of the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue).

There is also provided ~~One aspect as reported herein is~~ a method for selecting a multispecific binder that specifically binds to two different epitopes or antigens comprising the step of

- selecting from a multitude of multispecific binders comprising different combinations of a first binding entity and a second binding entity a multispecific binder that specifically binds to two different epitopes or antigens.

In another aspect, there is provided a method for selecting a multispecific binder that specifically binds to two different epitopes or antigens comprising the step of

- selecting from a multitude of multispecific binders comprising different combinations of a first binding entity and a second binding entity a multispecific binder that specifically binds to two different epitopes or antigens,

wherein the members of the multitude of multispecific binders are each obtained by a method as defined herein.

There is also provided ~~One aspect as reported herein is~~ a method for selecting a bispecific antibody comprising the following steps

(i) determining the cell surface makers present in a cell containing sample and selecting thereof at least a first surface marker and a second surface marker,

(ii) transfecting a cell with (a) a nucleic acid encoding an antibody Fab, or scFab fragment, or an scFv antibody comprising within the 20 (twenty) C-terminal amino acid residues the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) followed by an endoplasmic reticulum retention signal KDEL (SEQ ID NO: 02), whereby the Fab, or scFab fragment, or scFv antibody specifically binds to the first surface marker or its ligand, (b) a nucleic acid encoding a one-

armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second surface marker or its ligand, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, and (c) a nucleic acid encoding a soluble sortase A with a C-terminal endoplasmic reticulum retention signal,

and thereby producing the bispecific antibody.

In another aspect, there is provided a method for selecting a bispecific antibody comprising the following steps

(i) determining the cell surface makers present in a cell containing sample and selecting thereof at least a first surface marker and a second surface marker,

(ii) transfecting a cell with (a) a nucleic acid encoding an antibody Fab fragment, or an antibody scFab, or a scFv antibody comprising within the 20 C-terminal amino acid residues the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) followed by an endoplasmic reticulum retention signal KDEL (SEQ ID NO: 02), whereby the Fab fragment or scFv antibody specifically binds to the first surface marker or its ligand, (b) a nucleic acid encoding an one-armed antibody fragment comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to the second surface marker or its ligand, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge

region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, and (c) a nucleic acid encoding a soluble S. aureus sortase A with a C-terminal endoplasmic reticulum retention signal,

and thereby producing the bispecific antibody.

There is also provided ~~One aspect as reported herein is~~ a method for determining a combination of antigen binding sites comprising the following steps:

(i) determining the binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life of a multitude of bispecific antibodies prepared by combining (a) each member of a first multitude of antibody Fab, or scFab fragments, or scFv antibody fragments whereby each member comprises within the 20 C-terminal amino acid residues the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) followed by an endoplasmic reticulum retention signal KDEL (SEQ ID NO: 02), whereby the Fab, or scFab fragment, or scFv antibody specifically binds to a first epitope or antigen, with (b) each member of a multitude of one-armed antibody fragments comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to a second epitope or antigen, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby

the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, covalently by a sortase A catalyzed enzymatic reaction,

and

(ii) choosing the bispecific antibody with suitable binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

In another aspect, there is provided a method for determining a combination of antigen binding sites comprising the following steps

(i) determining the binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life of a multitude of bispecific antibodies prepared by combining (a) each member of a first multitude of antibody Fab fragments or scFv antibody fragments whereby each member comprises within the 20 C-terminal amino acid residues the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) followed by an endoplasmic reticulum retention signal KDEL (SEQ ID NO: 02), whereby the Fab fragment or scFv antibody specifically binds to a first epitope or antigen, with (b) each member of a multitude of one-armed antibody fragments comprising a full length antibody heavy chain, a full length antibody light chain, and an antibody heavy chain Fc-region polypeptide, whereby the full length antibody heavy chain and the full length antibody light chain are cognate antibody chains complementary to each other and the pair of variable domains (VH and VL) thereof forms an antigen binding site that specifically binds to a second epitope or antigen, whereby the full length antibody heavy chain and the antibody heavy chain Fc-region polypeptide are covalently linked to each other via one or more disulfide bonds forming an antibody hinge region, and whereby the antibody heavy chain Fc-region polypeptide has an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus, via a S.aureus sortase A-mediated intracellular enzymatic coupling reaction,

and

(ii) choosing the bispecific antibody with suitable binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life and thereby determining a combination of antigen binding sites.

~~In one embodiment of all aspects is t~~The sortase A may be the sortase A of Staphylococcus aureus (S. aureus). In one embodiment the nucleic acid encoding a (soluble) sortase A with a C-terminal endoplasmic reticulum retention signal encodes an amino acid sequence of SEQ ID NO: 51 or SEQ ID NO: 52.

In another ~~One~~ aspect, there is provided ~~as reported herein is~~ a bispecific antibody obtained by a method as reported herein.

In another ~~One~~ aspect, there is provided ~~as reported herein is~~ a bispecific antibody comprising the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) in one of its heavy chains.

~~In t~~The following embodiments ~~of all aspects~~ as reported herein are given.

In one embodiment the members of the multitude of multispecific binders are each obtained by a method as reported herein.

In one embodiment a multispecific binder is selected based on its binding specificity and/or selectivity and/or affinity and/or effector function and/or in vivo half-life.

In one embodiment the binding entity is a cognate pair of an antibody heavy chain variable domain and an antibody light chain variable domain.

In one embodiment the multispecific binder is a bispecific antibody comprising two or four binding entities.

In one embodiment the first polypeptide domain and the second polypeptide domain are selected independently of each other from full length antibody, scFv, scFab, antibody heavy chain, antibody light chain, antibody heavy chain Fc-region fragment, pair of antibody light chain variable domain and antibody heavy chain

In one embodiment the first polypeptide domain comprises i) the amino acid sequence LPXTG (SEQ ID NO: 01, wherein X can be any amino acid residue) in its C-terminal amino acid sequence region (i.e. within the twenty C-terminal amino acid residues) and ii) the endoplasmic reticulum retention signal KDEL (SEQ ID NO: 02) at its C-terminus, and the second polypeptide domain comprises an oligoglycine G_m (m = 2, or 3, or 4, or 5) at its N-terminus.

In one embodiment the second polypeptide domain or the second binding entity comprises an oligoglycine G_m (m = 2, or 3, or 4, or 5) amino acid sequence at its N-terminus.

The present disclosure may relate to ~~One aspect as reported herein is~~ a pharmaceutical formulation comprising a multispecific binder as reported herein.

The present disclosure may relate to ~~One aspect as reported herein is the~~ use of a multispecific binder as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of cancer.

The present disclosure may relate to ~~One aspect as reported herein is~~ a method of treating an individual having cancer comprising administering to the individual an effective amount of a multispecific binder as reported herein.

The present disclosure may relate to ~~One aspect as reported herein is~~ a method for destroying cancer cells in an individual comprising administering to the individual an effective amount of a multispecific binder as reported herein.

In another aspect, there is provided ~~One aspect as reported herein is~~ a pharmaceutical formulation comprising a bispecific antibody as reported herein.

In another aspect, there is provided ~~One aspect as reported herein is the~~ use of a bispecific antibody as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of cancer.

The present disclosure may relate to ~~One aspect as reported herein is~~ a method of treating an individual having cancer comprising administering to the individual an effective amount of a bispecific antibody as reported herein.

The present disclosure may relate to ~~One aspect as reported herein is~~ a method for destroying cancer cells in an individual comprising administering to the individual an effective amount of a

bispecific antibody as reported herein. In one embodiment, ~~of all aspects as reported herein~~ the Fc-region is a human Fc-region or a variant thereof.

In one embodiment the human antibody Fc-region is of human IgG1 subclass, or of human IgG2 subclass, or of human IgG3 subclass, or of human IgG4 subclass.

In one embodiment the antibody Fc-region is a human antibody Fc-region of the human IgG1 subclass, or of the human IgG4 subclass.

In one embodiment the human antibody Fc-region comprises a mutation of the naturally occurring amino acid residue at least at one of the following amino acid positions 228, 233, 234, 235, 236, 237, 297, 318, 320, 322, 329, and/or 331 to a different residue, wherein the residues in the antibody Fc-region are numbered according to the EU index of Kabat.

In one embodiment the human antibody Fc-region comprises a mutation of the naturally occurring amino acid residue at position 329 and at least one further mutation of at least one amino acid residue selected from the group comprising amino acid residues at position 228, 233, 234, 235, 236, 237, 297, 318, 320, 322 and 331 to a different residue, wherein the residues in the Fc-region are numbered according to the EU index of Kabat. The change of these specific amino acid residues results in an altering of the effector function of the Fc-region compared to the non-modified (wild-type) Fc-region.

In one embodiment the human antibody Fc-region has a reduced affinity to the human FcγRIIIA, and/or FcγRIIA, and/or FcγRI compared to a conjugate comprising the corresponding wild-type IgG Fc-region.

In one embodiment the amino acid residue at position 329 in the human antibody Fc-region is substituted with glycine, or arginine, or an amino acid residue large enough to destroy the proline sandwich within the Fc-region.

In one embodiment the mutation in the human antibody Fc-region of the naturally occurring amino acid residue is at least one of S228P, E233P, L234A, L235A, L235E, N297A, N297D, P329G, and/or P331S.

In one embodiment the mutation is L234A and L235A if the antibody Fc-region is of human IgG1 subclass, or S228P and L235E if the antibody Fc-region is of human IgG4 subclass.