

## ANTIBODY-DRUG CONJUGATES AND PRODUCTION THEREOF

## FIELD OF THE INVENTION

The present invention relates to the field of antibody-drug conjugates, especially multiple-payload antibody-drug conjugates and methods of producing same.

## BACKGROUND

Antibody-drug conjugates (ADCs) refers to a group of therapeutic drugs in which a therapeutic agent, such as a cytotoxic drug, is linked to a target-specific antibody which directs the agent to the target, releases it for therapeutic effects. ADCs loaded with multiple types of drugs per antibody can circumvent ADC-associated drug resistance and provided enhanced therapeutic efficacy.

To further expand the application of the ADCs, scientists are trying to develop various new technologies of multiple-payload ADCs. The first strategy is to attach two different types of payloads into one linker, which is then conjugated to the antibody. For example, Levensgood et al. designed a linker which incorporates two thiol groups that are blocked with either S-(tert-butyl) or S-(isopropyl) disulfides <sup>[1]</sup>. Similarly, Yamazaki et al. developed a branching linker which includes azido and methyl-tetrazine functional groups <sup>[2]</sup>. Synaffix also adopted this strategy to achieve their “dual-warhead” ADCs by extending their “HydroSpace” technology to incorporate two different payloads in the same linker.

Nonetheless, two or more payloads are attached to one linker, and the linkage may introduce highly hydrophobic moieties (e.g., TCO or DBCO), the resulted linker-payloads are large in size and are more hydrophobic. This raise concerns both in process development and CMC (e.g., free drug removal) as well as in ADC immunogenicity, stability, and efficacy, especially when the desired payloads are already more hydrophobic than usual. Introducing hydrophilic groups (e.g., in the case of HydroSpace) may mitigate this problem, but a large hydrophilic masking group increases the hydrodynamic diameter of the antibody and may block the antibody binding sites.

The second strategy is to attach two different linker-payloads at different sites of an antibody. Nilchan et al. positioned a seleno-modified cysteine at heavy chain location 396 and an additional cysteine residue at heavy chain location 114 through protein engineering <sup>[3]</sup>. More recently, Boschanski et al. devised a conjugation scheme in which two types of enzymes are used to sequentially convert cysteines within either sequence “CTPSR” or “CTAGR” into formylglycine, and linker-payload conjugation is performed after each conversion <sup>[4]</sup>. Sutro Biopharma incorporated non-natural amino acid into antibody sequence, and also achieved dual-site labeling.

This strategy avoids the extra hydrophobicity problem from the first strategy. However, to produce a second type of conjugation site, antibody sequence engineering is often involved (e.g., engineered cysteine), which may affect antibody activity and/or reduce mAb production yield. Alternatively, sequence or chemical selective enzymes are employed for conjugation, but enzymes are hard to remove, which increases process development difficulties and cost.

There remains the need for a process for producing ADCs, especially multiple-payload ADCs, which allows for improved efficiency, operability, controllability, flexibility and/or scalability.

## SUMMARY OF THE INVENTION

Provided herein is a method of producing an ADC with multiple linker-payloads, preferably different linker-payloads, and more preferably linker-payloads different in the drug moiety. Also provided are ADC products of the invention obtainable with the method.

In a first aspect, provided herein is a process of producing an antibody-drug conjugate, comprising:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion; and

one or more conjugation steps, wherein the reduced antibody conjugates with a linker-payload to generate an antibody-drug conjugate;

wherein the process further comprises an optional second reduction wherein the antibody as a moiety of an antibody-drug conjugate is further reduced, and wherein the second reduction is followed by a conjugation step; and

wherein the process further comprises a deshielding treatment to remove the transitional metal incorporated in the antibody, which is concomitant with at least one of the conjugation steps or the second reduction step.

In a second aspect, provided herein is an antibody-drug conjugate product comprising an antibody-drug conjugate of interest at a purity of at least 55 mol%, wherein the antibody-drug conjugate of interest comprises four molecules of a first linker-payload linked to the Fab regions and at least two molecules of a second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different or identical.

In a third aspect, provided herein is an antibody-drug conjugate product comprising an antibody-drug conjugate of interest at a purity of at least 55 mol%, wherein the antibody-drug conjugate of interest comprises three or six molecules of a first linker-payload and optionally one or two molecules of a second linker-payload, wherein the first linker-payload and the second linker-payload are different or identical.

In a fourth aspect, provided herein is an antibody-drug conjugate product comprising an antibody-drug conjugate of interest at a purity of at least 55 mol%, wherein the antibody-drug conjugate of interest comprises two or four molecules of a first linker-payload linked to the Fab regions of the antibody and at least one molecule (e.g., one, two, three and four molecules) of a second linker-payload linked to the hinge region of the antibody, and optionally one or two molecules of a third linker-payload linked to the hinge region of the antibody; wherein the first, the second and optionally the third linker-payloads are identical or different from each other or one another.

In some embodiments, at least one of the first, the second, or the third linker-payloads is a thiol-bridge linker-payload. In some embodiments, the antibody is a bispecific antibody such as one of the WuXiBody structure or a fusion protein such as a VHH-Fc fusion protein.

In a fifth aspect, provided herein is an antibody-drug conjugate product produced by the method of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification, which are provided for illustration with no intention to limit the scope of invention in any sense.

**FIG. 1:** An overview of the strategy according to the invention. **Scheme 1-1:** an embodiment of the invention to produce an ADC of DAR6; **Scheme 1-2:** an embodiment of the invention to produce a multiple-payload ADC of 6+2 format; **Scheme 2:** an embodiment of the invention to produce a multiple-payload ADC of 4+4 format; **Scheme 3-1:** an embodiment of the invention to produce a multiple-payload ADC of 4+2 format; and **Scheme 3-1:** an embodiment of the invention to produce a multiple-payload ADC of 4+2+2 format.

**FIG. 2:** Variant embodiments of Scheme 1-1. (A): an embodiment to produce an ADC of DAR3 using a thiol-bridge linker payload; (B): an embodiment to produce an ADC of DAR4 using a VHH-Fc fusion protein; (C): an embodiment to produce an ADC of DAR4 using an antibody of the WuXiBody structure.

**FIG. 3:** LC-MS characterization of a product comprising mAb1-MMAE+MMAF of the 4+4 dual-payload format prepared in an example of the invention.

**FIG. 4:** LC-MS characterization of a product comprising mAb1-MMAE+GGFG-DXd of the 4+4 dual-payload format prepared in an example of the invention.

**FIG. 5:** LC-MS characterization of a product comprising mAb1-MMAF+MMAE of the 4+4 dual-payload format prepared in an example of the invention.

**FIG. 6:** LC-MS characterization of a product comprising mAb1-MMAF+GGFG-DXd of the

4+4 dual-payload format obtained in an example of the invention.

**FIG. 7:** LC-MS characterization of a product comprising mAb1-MMAF+CL2A-SN38 of the 4+4 dual-payload format obtained in an example of the invention.

5 **FIG. 8A-8B:** AKTA pure HIC (Hydrophobic interaction chromatography) characterization and LC-MS characterization of a product comprising mAb1-MMAE+DXd of the 4+4 dual-payload format obtained in an example of the invention.

**FIG. 9:** LC-MS characterization of a product comprising mAb2-MMAE+MMAF of the 4+4 dual-payload format obtained in an example of the invention.

10 **FIG. 10:** LC-MS characterization of a product comprising mAb2-MMAE+GGFG-DXd of the 4+4 dual-payload format obtained in an example of the invention.

**FIG. 11:** LC-MS characterization of a product comprising mAb2-MMAE+CL2A-SN38 of the 4+4 dual-payload format obtained in an example of the invention.

**FIG. 12:** LC-MS characterization of a product comprising mAb2-MMAF+MMAE of the 4+4 dual-payload format obtained in an example of the invention.

15 **FIG. 13:** LC-MS characterization of a product comprising mAb2-MMAF+CL2A-SN38 of the 4+4 dual-payload format obtained in an example of the invention.

**FIG. 14A-14E:** Purity in the crude products obtained in Process 2 in Example 1.

**FIG. 15A-15B:** Purity in the purified the products obtained in Process 3 in Example 1.

20 **FIG. 16:** LC-MS characterization of a product comprising mAb1-MMAF+MMAE of the 4+2 dual-payload format obtained in Process 1 in Example 2.

**FIG. 17:** LC-MS characterization of a product comprising mAb2-MMAF+MMAE of the 4+2 dual-payload format obtained in Process 1 in Example 2.

**FIG. 18:** Purity in crude products obtained in Process 2 in Example 2.

**FIG. 19:** Purity in purified products obtained in Process 2 in Example 2.

25 **FIG. 20:** LC-MS characterization of a product comprising mAb1-DXd+MMAE+MMAF of the 4+2+2 tri-payload format obtained in in Example 3.

**FIG. 21A-21B:** HIC characterization of the products comprising the DAR6 species obtained in Experiment 1 of Example 4.

30 **FIG. 22:** Pre-purification purity and after-purification purity of a product obtained in Experiment 1 of Example 4.

**FIG. 23:** HIC characterization of the products obtained in Experiment 2 of Example 4

**FIG. 24:** HIC characterization of the products obtained in Experiment 3 of Example 4

**FIG. 25:** HIC characterization of the products obtained in Experiment 4 of Example 4

**FIG. 26:** HIC characterization of the products obtained in Experiment 5 of Example 4

**FIG. 27:** HIC characterization of the products obtained in Experiment 6 of Example 4

**FIG. 28:** HIC characterization of the products obtained in Experiment 7 of Example 4

**FIG. 29:** LC-MS characterization of a product comprising mAb1-MMAE+Dxd of the 6+2 dual-payload format obtained in an example of the invention.

5 **FIG. 30:** LC-MS characterization of a product comprising mAb1-MMAE+MMAF of the 6+2 dual-payload format obtained in an example of the invention.

**FIG. 31:** LC-MS characterization of a product comprising mAb2-MMAE+Dxd of the 6+2 dual-payload format obtained in an example of the invention.

10 **FIG. 32:** LC-MS characterization of a product comprising mAb2-MMAE+MMAF of the 6+2 dual-payload format obtained in an example of the invention.

**FIG. 33:** PLRP result of the conjugates obtained in Process 2 of Example 5 and PLRP results of a purified conjugate obtained in Process 2 in Example 5.

**FIG. 34:** HIC characterization of the products comprising the DAR3 species obtained in Example 6

15 **FIG. 35:** HIC characterization of the products comprising the DAR4 species obtained in Example 7.

## DETAILED DESCRIPTION OF THE INVENTION

### Nomenclature

20 As used herein, singular forms preceded by “a”, “an” and “the” include plural reference, unless the context clearly dictates otherwise. As well, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein.

In the present disclosure, unless otherwise specified, a value or a value range, with or without being preceded by the term “about”, “around” or “approximately”, covers equivalents within a reasonable range of approximation as can be understood by an artisan in the related field, such as  
25 a range of  $\pm 10\%$ ,  $\pm 5\%$ ,  $\pm 3\%$ ,  $\pm 2\%$ ,  $\pm 1\%$  or  $\pm 0.5\%$  around the specified value.

In the present disclosure, one or more features in one embodiment can be combined with any one or more features in another embodiment to form an embodiment of the invention without departing from the spirit and concept of the present invention.

30 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skills in the fields to which this invention pertains. All publications and patents specifically mentioned herein are incorporated by reference for all purposes. All references cited in this specification are to be taken as indicative of the level of the skill in the art, which should not be construed as an admission that the invention is not

entitled to antedate such disclosure by virtue of prior invention.

## **Introduction**

5 Provided herein is a method of producing an ADC with multiple linker-payloads, preferably different linker-payloads, and more preferably linker-payloads different in the drug moiety. Also provided are ADC products of the invention obtainable with the method. The method allows for loading payloads, preferably different payloads onto specific cysteine sites on an antibody via conventional conjugation of cysteine chemistry in a controllable manner. The method of invention does not need any extra work such as antibody engineering, use of those branched linkers which may pose a serious hydrophilic masking group risk and extra enzyme purification. The method of invention allows for designing and producing ADC products featuring high purity of an ADC species of interest.

To some extent, the present invention partially takes advantage of the technology of transmittal ion-assisted conjugation, such as the processes described in WO2020164561. WO2020164561 described a technique of transmittal ion-assisted conjugation for producing ADC products featuring a high homogeneity of DAR4 (i.e., the species with a Drug to Antibody Ratio of 4), which is incorporated herein by reference in its entirety. Compared with the traditional practice of reduction-and-conjugation, the technology of WO2020164561 distinguishes in that the two disulfide bonds in the hinge region are shielded from reduction in the presence of a transmittal ion such as Zn(II), and thereby, the conjugation following the reduction produces predominately ADCs of DAR4 with payloads loaded onto the free thiol groups released from disulfides in the Fab regions.

The present invention is based on the inventors' finding that the disulfide bonds in the hinge region which are shielded from reduction in the transition ion-assisted conjugation can be selectively reduced under control to provide free thiol groups for further conjugation, which thereby allows for producing multiple-payload ADCs with designed formats of drug loading in a controllable manner.

## **Exemplary Embodiments of The Invention**

### **1. Method Of ADC Production**

30 Generally, in a first aspect, provided is a process of producing an antibody-drug conjugate, comprising: a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion; and one or more conjugation steps, wherein the reduced antibody conjugates with a linker-payload to generate an antibody-drug conjugate; wherein the process further comprises an optional second reduction wherein the antibody as a moiety of an antibody-drug conjugate is

further reduced, and wherein the second reduction is followed by a conjugation step; wherein the process further comprises a deshielding treatment to remove the transitional metal incorporated in the antibody, which is concomitant with at least one of the conjugation steps or the second reduction step. The product of the last conjugation step is the final antibody-drug conjugate product and the process may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product.

There is no limit on the antibodies that can be use in the present invention, and every antibody contemplated for use in ADCs can be used. In some embodiments, antibodies comprising the inter-chain disulfide bonds in the Fab regions and the inter-chain disulfide bonds in the hinge region as in any one of the IgG classes are suitable for the present invention, especially those having the inter-chain disulfide bonds as in the case of IgG1 and IgG4. In some embodiments, the antibody is of the IgG1 or the IgG4 class. In some embodiments, the antibody is a monoclonal antibody (mAb).

The term “antibody”, in a broad sense, refers to a specific binding protein that can be used as the antibody moiety in an ADC, especially those having at least the hinge region comprising the inter-chain disulfide bonds for conjugation as in an IgG molecule said above. For example, in the context of the present invention, in addition to the IgG molecules said above, useful antibody molecules may further include engineered antibodies such as bispecific or multispecific antibodies like those of a WuXiBody structure (i.e., a multispecific antibody comprising an IgG structure wherein the LC+CH1 fragment in one of the light chain-heavy chain (LC-HC) pairs is replaced by a TCR constant region (e.g., C $\alpha$  and C $\beta$ )) and Fc fusion proteins such as VHH-Fc fusions. Specific examples of antibody useful in the invention include but are not limited to Trastuzumab, Rituximab, Cetuximab, Adalimumab, Alemtuzumab, Atezolizumab, Basiliximab, Certolizumab, Daclizumab, Daratumumab, Golimumab, Ipilimumab, Mepolizumab, Necitumumab, Obinutuzumab, Ofatumumab, etc..

In the present disclosure, the ordinal numerals “first”, “second” and “third” before linker-payloads do not necessarily mean that the linker-payloads are each distinct. In some embodiments, they refer to the linker-payloads that are introduced in the first, the second and the third conjugation reaction respectively. And, in some descriptions herein, they may refer to the three types of conjugation site as categorized herein below. When linker-payloads labeled with different numerals (e.g., the second and the third linker-payloads) are identical in identity, they can be summed to under the smaller one (e.g., the second linker payload).

In the present disclosure, the term “multiple-payload”, when used as an attributive, encompasses linker-payloads that are different in the linker structure, the drug moiety or both, and

preferably in the drug moiety. In the present disclosure, the term “linker-payload” (LP) encompasses the payload molecules *per se* that can conjugate with an antibody without the linker. In the present disclosure, as can be understood from the context, the term “linker-payload” may refer to a linker-payload moiety as part of the ADC, especially when being followed by “loaded/linked on/onto” an antibody, or the corresponding intact linker-payload molecule from which the linker-payload moiety derives via conjugation between the linker-payload molecule and the antibody. In the present disclosure, the term “payload” and the term “drug” are used exchangeably as referring to a therapeutically or diagnostically active agent or the moiety derived from the agent. Accordingly, as can be understood from the context in the present disclosure, the “multiple-payload” ADCs encompasses the scenario of ADCs comprising different linker-payloads (i.e., in a broad sense) and the scenario of ADCs comprising different payloads (i.e., in a narrow sense). Examples of useful linker-payload molecule include but are not limited to MC-vc-MMAE, MC-MMAF, MC-GGFG-DXd, MC-CL2A-SN38, mc-Extecan, DBM-MMAF, DM21-Glucose-DXd, YLink-Glucose-DXd and MC-Glucose-DXd. In some embodiments, thiol-bridge linker-payloads can be used, such as those featuring a dibromomaleimide linker, a 4,5-dibromo-1,2-dihydropyridazine-3,6-dione linker or a bismaleimide linker.

The process of the invention allows for producing multiple-payload ADCs with designed formats of drug loading in a controllable manner and ADC products having a high purity of the designed species of interest (also referred to as “target species” herein below). For example, the designed formats of drug loading may include the “4+4 format”, the “4+2 format”, the “DAR4 format”, the “DAR6 format”, the “6+2 format” and the “4+2+2 format”, wherein each numeral refers to the DAR value for the corresponding linker-payload. In the present disclosure, a DAR value refers to the theoretical value as indicated or testified by the measurement, as can be understood from the context.

In the present disclosure, the “4+4 format” refers to a species of ADC comprising four molecules of a first linker-payload linked to the Fab regions and four molecules of a second linker-payload linked to the hinge region; the “4+2 format” refers to the species comprising four molecules of a first linker-payload linked to the Fab regions and two molecules of a second linker-payload linked to the hinge region; the “DAR4 format” refers to the species comprising two molecules of linker-payload linked to the Fab regions and two molecules of the linker-payload linked to the hinge region; the “DAR6 format” refers to the species comprising four molecules of linker-payload linked to the Fab regions and two molecules of the linker-payload linked to the hinge region; the “6+2 format” refers to the species comprising four molecules of a first linker-payload linked to the Fab regions, two molecules of the first linker-payload linked to a first site in

the hinge region and two molecules of a second linker-payload linked to a second site in the hinge region; and the “4+2+2 format” refers to the species comprising four molecules of a first linker-payload linked to the Fab regions, two molecules of a second linker-payload linked to a first site in the hinge region and two molecules of a third linker-payload linked to a second site in the hinge region. Preferably, the first, the second and optionally the third linker-payloads are different from each other or one another and more preferably different from each other or one another in the drug moiety. Even more preferably, each of the first, the second and the third linker-payloads is unique and most preferably unique in the drug moiety.

Specifically, in a first embodiment, the process of invention comprises:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion;

a first conjugation step, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate;

a second reduction step coupled with a concomitant deshielding treatment, wherein the antibody moiety of the first antibody-drug conjugate is further reduced; and

a second conjugation step, wherein the further reduced antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.

In some instances, this process can produce a multiple-payload ADC of the 4+4 format, wherein four molecules of the first linker-payload are loaded onto the Fab regions in the first conjugation step and four molecules of the second linker-payload are loaded onto the hinge region in the second conjugation step. An example of this process is schematically depicted as Scheme 2 in FIG. 1.

#### First Reduction in Presence of a Transition metal Ion

In this and all the following embodiments of the method of invention, in the first reduction, the antibody is partially reduced by a reductant in the presence of a transition metal ion to open the two interchain disulfide bonds (S-S) in the Fab regions, whereby the partially reduced antibody comprises four free thiol groups released from the interchain disulfide bonding at the cysteine sites for conjugation with linker-payloads. This partial reduction in presence of a transition metal can be conducted according to WO2020164561, which is incorporated herein by reference in its entirety.

In particular, any reductant useful in production of ADCs can be used in the present invention,

such as Tris (2-carboxyethyl) phosphine (TCEP), Diphenylarsinic acid (DPAA), diPPBs (2-diphenylphosphanylbenzenesulfonic acid), 2-diphenylphosphanylbenzenesulfonic acid (diPPBs), Dithiothreitol (DTT), Dithioerythritol (DTE),  $\beta$ -Mercaptoethanol,  $\text{LiAlH}_4$ ,  $\text{Na}_2\text{S}_2\text{O}_3$ ,  $\text{KBH}_4$ , and Hydrazine. The reductant may be used at a reductant/mAb ratio ranging from 1 to 20, such as 2 to 16, 3 to 10, 4, 6, 8, 10 or 14. Transition metal ions useful in the present invention include, but are not limited to,  $\text{Zn}^{2+}$  (Zn(II)),  $\text{Mn}^{2+}$  (Mn(II)),  $\text{Ni}^{2+}$  (Ni (II)),  $\text{Fe}^{2+}$  (Fe (II)),  $\text{Fe}^{3+}$  (Fe (III)),  $\text{Ca}^{2+}$  (Ca (II)) and  $\text{Cu}^{2+}$  (Cu (II)). Mixture of transition metal ions can be used. The transition metal ion(s) may be added in the form of a soluble salt, such as halides (e.g.,  $\text{ZnCl}_2$ ,  $\text{MnI}_2$ ,  $\text{CuBr}_2$ ), sulfates (e.g.,  $\text{ZnSO}_4$ ), nitrates (e.g.,  $\text{Zn}(\text{NO}_3)_2$ ), acetates (e.g.,  $\text{Zn}(\text{CH}_3\text{COO})_2$ ), formates and tetrafluoroborate. The ions may be present at a ratio to antibody ranging from 0.5 to 50, such as 1 to 16, 2 to 8, or such as a ratio of about 4, 5 or 6. The reaction can be conducted at temperature in the range of about  $0^\circ\text{C}$  -  $37^\circ\text{C}$  (such as  $2^\circ\text{C}$  -  $25^\circ\text{C}$ ,  $4^\circ\text{C}$ ,  $12^\circ\text{C}$ ,  $16^\circ\text{C}$  or  $22^\circ\text{C}$ ) for about 1 – 24 hours (such as around 12, 16 or 20 hours) in an appropriate buffer, such as a buffer of about pH 6-8 such as about pH 6.5, pH 7.0 or pH 7.5 and salt concentration ranging from about 20 - 80mM, such as about 40 mM. Examples of reduction buffer include but are not limited to PB, HEPES, MES, Tris and MOPSO. Optionally, the reduction buffer may comprise an additive as desired, such as Tween 20, Benzyl Alcohol and Trehalose. The resultant reduction solution, which comprises the partially reduced antibody with four free thiol groups for conjugation at the cysteine sites in the Fab regions, can be directly used in the next step of conjugation without removal of the reductant, while a step of removing the reductant can be included if desired.

In some examples, for the first reduction, the buffer can be for example a PB buffer (40mM, pH 6.90) or a HEPES buffer (40mM, pH 7.00); the reductant/mAb ratio can be about 3 - 10 such as about 3.25 and about 10, wherein the reductant can be for example TCEP; and/or the reaction can be conducted at temperature in the range of about  $4^\circ\text{C}$  -  $12^\circ\text{C}$  for about 16-20 hours, such as at about  $4^\circ\text{C}$  for about 16 hours or at about  $12^\circ\text{C}$  for about 20 hours.

#### First Conjugation

The first conjugation can be conducted according to common practices in ADC production. One of ordinary skills in the art knows how to formulate the conjugation according to the given antibody, the payload or the linker-payload. For example, the conjugation may be conducted a linker-payload/mAb ratio ranging from 6 to 20 (such as about 8, 10, 12, 14 or 16) in a buffer of about pH 4.0 to 8.0 at a temperature in the range of about  $0^\circ\text{C}$  -  $37^\circ\text{C}$  (such as  $2^\circ\text{C}$  -  $25^\circ\text{C}$ ,  $4^\circ\text{C}$ ,  $12^\circ\text{C}$

°C, 16°C or 22°C) for 1 – 4 hours (such as 1, 2 or 3 hours). In some examples, the first conjugation can be kept at about 4-12°C for about 1-2 hours, such as at about 4°C for about 2 hours or at about 12°C for about 1 hour. The first conjugation subsequent to the first reduction produces a first intermediate ADC of DAR4 for the first linker-payload.

5        Optionally, the resultant first conjugation solution may be subjected to a treatment to deplete the residual un-conjugated linker-payloads, if any. The step of depletion may be preferred when there is a next conjugation with a different linker-payload. In some cases, the depletion may be conducted via incubation with a quenching reagent such as a cysteine agent, including cysteine and derivatives thereof like acetylcysteine (NAC). The incubation may be conducted at the same  
10        environment as the conjugation for a period such as around 30 to 60 minutes (i.e., around 0.5 - 1 hour). In some examples, the incubation may be conducted at around 4-12°C for about 0.5 hours. The quenching reagent may be added at a ratio of 6-40 such as 10, 12, 14, 16, 21 relative to the antibody.

15        Optionally, the first intermediate ADC may be purified. The purification may be preferred for removing the existing reagents and for enriching the ADC. In some cases, the intermediate ADC is purified or buffer exchanged using a desalting column. In some cases, the intermediate ADC is first subjected to the treatment of depletion and then the purification.

Second reduction coupled with concomitant deshielding treatment

20        This step includes (i) a second reduction and (ii) a concomitant deshielding treatment. The term “second reduction” by itself refers to the reaction of reduction *per se*, not in combination with a concomitant deshielding treatment.

(i) Second reduction

25        The second reduction is formulated to open one or more of the remaining interchain disulfide bond(s) other than those in the Fab regions and the shielded one(s) as possible and as needed. In some embodiments, the second reduction is conducted in absence of the transition metal ion that is used or contemplated as can be used for the first reduction above. It may optionally have some adaptive modifications. For example, the second reduction may be conducted at a reduced reductant/mAb ratio, such as a reduction of 2, 4, 6 or 8 relative to the ratio in the first reduction, and such as a ratio of about 6 or 8. This can be empirically determined by a skilled according to  
30        specific conditions and actual requirements. For example, when diPPBS is used, the reductant/mAb ratio can be increased to 35. The second reduction may be conducted at an elevated temperature, such as a temperature in the range of around 4-37°C or the range of room temperature (eg., around 20-22°C) to around 37°C, such as at a temperature of about 4°C, 12°C, 16°C, 22°C or 37°C.

In some examples, for the second reduction, the reductant can be TCEP or DPAA; the reductant/mAb ratio can be around 6 - 8; the reaction can be conducted at about 20 – 37°C (e.g., 22 °C or 37 °C) for about 4 - 16 hours, such as at about 37 °C for about 16 hours or at about 22 °C for about 4 hours.

5           (ii) Concomitant deshielding treatment

In the present disclosure and in the context the deshielding treatment, the term “coupled” and the term “concomitant” are both used to mean that an operation or a reaction proceeds immediately after or simultaneously with or in overlapping with the treatment and relies on or takes advantage of the result of the deshielding. Accordingly, in this step, the second reduction may proceed immediately after or simultaneously with or in overlapping with the treatment. The second reduction takes advantage of the result of deshielding to produce a further reduced antibody with additional free cysteine sites for conjugation.

The deshielding treatment according to the present invention comes from the inventors' finding that part of the disulfide bonds in the hinge region (e.g., one of the S-S bonds in the hinge of IgG1 and IgG4) that is(are) shielded from reduction in the transition metal ion-assisted process can be selectively opened by removing the transition metal ion complexed with the thiol groups and the free thiols released thereby can be utilized for additional conjugation in a controlled manner. This advantageously allows for alternating conjugations to facilitate design and production of multiple-payload ADCs.

The deshielding treatment can be a decomplexing treatment. In a case of the invention, the deshielding treatment is conducted using a chelating agent. In some cases, the second reduction may proceed in the presence of the chelating agent. Examples of chelating agent include but are not limited to polyphosphates, amino carboxylic acids, hydroxycarboxylic acids, polyamines, fatty amines, phenanthrolines, etc., such as Ethylenediamine, 2,2'-Bipyridine, 1,10-phenanthroline, EDTA, EGTA, DTPA, etc. The chelating agent may be added in excess, such as a final concentration of 0.05 – 10 mM, such as 1 – 5mM, 0.1mM, 1mM, 2mM, 5mM and 8mM.

In particular, the second reduction coupled with the concomitant deshielding treatment can provide a further reduced antibody (i.e., the antibody moiety of the first ADC) featuring the free thiols at the cysteine residues once shielded in the presence of the transition metal ion and additional free thiols at one or more other sites of interchain S-S bonds. For example, in the case of an IgG1 or IgG4 antibody, the second reduction coupled with concomitant deshielding treatment open both S-S bonds in the hinge region and thus four additional sites available for conjugation.

30           Second Conjugation

The second reduction coupled with concomitant deshielding treatment is followed by a

second conjugation, wherein a second linker-payload conjugates with the further reduced antibody at the additional sites of conjugation to thereby provide a second ADC. For example, the second ADC may be a multiple-payload ADC of the 4+4 format, wherein the first numeral “4” refers to the DAR of the first linker-payload molecules linked to the Fab regions and the second numeral “4” refers to the DAR of the second linker-payload molecules linked to the hinge region, especially in the case of an IgG1 antibody and an IgG4 antibody. In a preferred case, obtained is a dual-payload ADC of the 4+4 format wherein the first and the second payloads are different.

The second conjugation may be the same as the first conjugation except for some optional modifications. For example, the second conjugation may be conducted using a reduced linker-payload/antibody ratio according to and optionally in excess to the stoichiometric ratio between the linker-payload and antibody for this second conjugation, such as 6 to 10. In some examples, the second conjugation can be was kept at around 4-22 °C such as around 4-12 °C for around 1-2 hours, such as at 4 °C for 2 hours, at 12 °C for 1 hour or at 22 °C for 1 hour.

#### Recovery

The embodiment may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product. The second ADC, i.e., the multiple-payload ADC of the 4+4 format, may be recovered as the final ADC product. The final ADC may be purified, whereby the species of interest can be enriched. Various techniques for ADC purification can be used in the present invention. Useful techniques of purification include but are not limited to filtration, ultrafiltration, dialysis, UF-DF, desalting, buffer exchange, chromatography (e.g., HIC) or a combination thereof. In some cases, the final product is buffer exchanged using a desalting column. In some cases, the final product is further purified by chromatography such as HIC-HPLC. The resultant product will be an ADC product having a high purity of the multiple-payload ADC of the 4+4 format.

In the above, the embodiments and especially the DAR formats are largely described with reference to linker-payload molecules that react with the free thiol groups for conjugation on the antibody molecule at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the first and/or the second linker-payload molecules are thiol-bridge linker-payloads that can react with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:2.

Accordingly, when the first linker-payload molecule in the first conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said first antibody-drug conjugate) with two (2) molecules of the first linker-payload loaded onto the Fab regions per antibody; and/or when the second linker-payload molecule in the second conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said second ADC)

with two (2) molecules of the second linker-payload loaded onto the hinge region per antibody. Accordingly, the process can produce a multiple-payload ADC of a  $2^s+4$ ,  $4+2^s$  or  $2^s+2^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, when a thiol-bridge linker-payload molecule is used in one or both of the first and the second conjugations. The superscript "s" indicates thiol-bridge linker-payloads.

In a second embodiment, the process of invention comprises:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion;

a first conjugation step, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate; and

a second conjugation step coupled with a concomitant deshielding treatment, wherein the antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.

This process can produce a multiple-payload ADC of the 4+2 format, wherein four molecules of the first linker-payload are loaded onto the Fab regions in the first conjugation step and two molecules of the second linker-payload are loaded onto the hinge region in the second conjugation step. An example of this process is schematically depicted as Scheme 3-1 in FIG. 1.

The first reduction followed by the first conjugation produces a first intermediate ADC of DAR4 featuring the first linker-payload, which may be optionally subjected to the treatment of depletion, the purification or both as specified in the first embodiment.

Second conjugation coupled with concomitant deshielding treatment

This step includes a second conjugation and a concomitant deshielding treatment. The term "second conjugation" by itself refers to the reaction of conjugation *per se*, not in combination with a concomitant deshielding treatment.

The second embodiment does not include a second reduction before the second conjugation, and the first conjugation is followed by a second conjugation coupled with the concomitant deshielding treatment, wherein the antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.

Both the concomitant deshielding treatment and the second conjugation are as specified in the first embodiment. In some examples of the second embodiment, the second conjugation can be was kept at around 4-12 °C for around 1-2 hours, such as at 4 °C for 2 hours or at 12 °C for 1 hour.

The second conjugation may proceed immediately after or simultaneously or in overlapping with the deshielding treatment. In some cases, the second conjugation may proceed in the presence of the chelating agent. The second conjugation relies on the result of deshielding that provides additional free cysteine sites for conjugation. Thereby, resultant second antibody-drug conjugate may be a multiple-payload ADC of the 4+2 format, wherein four molecules of the first linker-payload are linked to the Fab regions and two molecules of the second linker-payload are loaded at the cysteine sites in the hinge region that is made available by the deshielding treatment. In a preferred case, obtained is a dual-payload ADC of the 4+2 format wherein the first and the second payloads are different.

The embodiment may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product. The second ADC, i.e., the multiple-payload ADC of the 4+2 format may be recovered as the final ADC product and may optionally be purified as specified in the first embodiment. The resultant product may be an ADC product having a high purity of the multiple-payload ADC of the 4+2 format.

In the above, the embodiments and especially the DAR formats are largely described with reference to linker-payload molecules that react with the free thiol groups for conjugation on the antibody molecule at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the first and/or the second linker-payload molecules are thiol-bridge linker-payloads that can react with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:2. Accordingly, when the first linker-payload molecule in the first conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said first antibody-drug conjugate) with two (2) molecules of the first linker-payload loaded onto the Fab regions per antibody; and/or when the second linker-payload molecule in the second conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said second ADC) with one (1) molecule of the second linker-payload loaded onto the hinge region per antibody. Accordingly, the process can produce a multiple-payload ADC of a  $2^s+2$ ,  $4+1^s$  or  $2^s+1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, when a thiol-bridge linker-payload molecule is used in one or both of the first and the second conjugations.

In a third embodiment, the process of invention comprises:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion;

a first conjugation step, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate;

a second conjugation step coupled with a concomitant deshielding treatment, wherein the antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate;

a second reduction step, wherein the antibody moiety of the second antibody-drug conjugate is further reduced; and

a third conjugation step, wherein the antibody moiety of the second antibody-drug conjugate conjugates with a third linker-payload to generate a third antibody-drug conjugate.

This process, compared with the second embodiment, further comprises a second reduction followed by a third conjugation after the second conjugation coupled with the concomitant deshielding treatment. An example of this process is schematically depicted as Scheme 3-2 in FIG. 1. The second intermediate ADC is thus preferably subjected to the treatment of depletion to remove the residual un-conjugated second linker-payloads. Optionally and preferably, the second intermediate ADC of the 4+2 format may be purified as specified in the first embodiment.

The second reduction and the third conjugation may be conducted according to the second reduction and the second conjugation in the first embodiment. for the second reduction, the reductant can be TCEP or DPAA; the reductant/mAb ratio can be around 6 - 10; the reaction can be conducted at about 22°C – 37°C for about 4 - 16 hours. In a preferred embodiment, the second reduction may be performed in the presence of a chelating agent such as EDTA to eliminate the risk of metal ion-mediated oxidation caused by metal ions other than those useful in the first reduction. Thereby, the third conjugation loads additional linker-payloads at the cysteine sites released from the remaining S-S bonds by the second reduction in the hinge region. For example, the resultant third ADC may be a triple-payload ADC of the 4+2+2 format wherein four molecules of the first linker-payload are linked to the Fab region, two molecules of a second linker-payload are linked to the hinge region at the deshielded cysteine sites and two molecules of a third linker-payloads are linked to the hinge region at the other cysteine residues. The embodiment may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product. The third ADC of 4+2+2 format may be recovered as the final ADC product and may optionally be purified as specified in the first embodiment. The resultant product may be an ADC product having a high purity of the multiple-payload ADC of the 4+2+2 format.

In the above, the embodiments and especially the DAR formats are largely described with reference to linker-payload molecules that react with the free thiol groups for conjugation on the antibody molecule at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the first

and/or the second linker-payload molecules are thiol-bridge linker-payloads that can react with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:2. Accordingly, when the first linker-payload molecule in the first conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said first antibody-drug conjugate) with two (2) molecules of the first linker-payload loaded onto the Fab regions per antibody; and/or when the second linker-payload molecule in the second conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said second ADC) with one (1) molecule of the second linker-payload loaded onto the hinge region of the antibody; and/or when the third linker-payload in the third conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said third ADC) with one (1) molecule of the third linker-payload loaded onto the hinge region of the antibody. Accordingly, the process can produce a multiple-payload ADC of a  $2^s + 2 + 2$ ,  $2^s + 2 + 1^s$ ,  $2^s + 1^s + 2$ ,  $2^s + 1^s + 1^s$ ,  $4 + 2 + 1^s$ ,  $4 + 1^s + 2$  or  $4 + 1^s + 1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody, the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and the third numeral refers to the count of the third linker-payload moiety loaded on the hinge region of the antibody, when a thiol-bridge linker-payload molecule is used in one or more of the first, the second and the third conjugations.

In a fourth embodiment, the process of invention comprises:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion; and

a first conjugation step coupled with a concomitant deshielding treatment, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate.

In some examples of the fourth embodiment, for the first reduction, the buffer can be for example PB (40mM, pH 6.90), HEPES (20 – 80mM, pH 6.8 – 8.0), Tris (40mM, pH 7.0), MES (40mM, pH 6.5) or MOPSO (40mM, pH 7.0); the reductant/mAb ratio can be 2 to 14 such as 2.75, 3.00, 3.25, 3.75, 4, 8 or 14, wherein the reductant can be for example TCEP; and/or the reaction can be conducted at temperature in the range of about 4 -22 °C for about 16-20 hours, such as at 4 °C for 16 hours, at 12 °C for 20 hours, at 16°C for 16 hours or at 22 °C for 16 hours.

This process is characterized in the first conjugation step coupled with the concomitant deshielding treatment. This step includes a first conjugation and a concomitant deshielding treatment. The term “first conjugation” by itself refers to the reaction of conjugation *per se*, not in combination with a concomitant deshielding treatment.

An example of this process is schematically depicted as Scheme 1-1 in FIG. 1. In this process,

the reduction solution resultant from the first reduction can be directly used in the first conjugation coupled with the concomitant deshielding treatment directly without removal of the reductant. The first conjugation and the concomitant deshielding treatment are each as specified in the first embodiment. In some cases, the first conjugation may proceed in the presence of the chelating agent. Taking advantage of the result of deshielding, the first conjugation produces a first ADC of the DAR6 format, wherein four molecules of the first linker-payload are linked to the Fab regions and two molecules of the first linker-payload are loaded at the cysteine sites in the hinge region that are made available via deshielding.

In some examples of the fourth embodiment, for the deshielding treatment, the chelating agent may be added in excess, such as a final concentration of 2 - 5mM, wherein the chelating agent can be for example EDTA; and/or the first conjugation reaction can be kept at about 4-22°C for about 1-2 hours, such as at about 4 °C for about 2 hours, at about 12 °C for 1 hour, at about 16 °C for about 1 hour or at 22 °C for about 1 hour. Optionally, the resultant first conjugation solution may be subjected to the treatment to deplete the residual un-conjugated linker-payloads, if any. In some examples of the fourth embodiment, the quenching reagent such as NAC may be added at a ratio of 10-16 relative to the antibody, and/or the incubation may be conducted at around 4-22 °C such as about 4 °C, 12 °C, 16 °C or 22 °C for 0.5 hours.

The embodiment may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product. The first ADC of the DAR6 format may be recovered as the final ADC product and may optionally be purified as specified in the first embodiment. This process advantageously allows for production of an ADC product having a high purity of ADC of the DAR6 format.

In the above, the embodiments and especially the DAR formats are largely described with reference to linker-payload molecules that react with the free thiol groups for conjugation on the antibody molecule at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the linker-payload molecule is a thiol-bridge linker-payload molecule that can react with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:2. Accordingly, as depicted in panel (A) of FIG.2, the process can produce an ADC of a DAR3 format with two molecules of the linker-payload loaded onto the Fab regions of an antibody and one molecule of the linker-payload loaded onto the hinge region of the antibody, when a thiol-bridge linker-payload molecule is used as the linker-payload in the conjugation.

In the above, the embodiments and especially the DAR formats are largely described with reference to an IgG antibody that has two inter-chain disulfide bonds in the two Fab regions in addition to the two inter-chain disulfide bonds in the hinge region. In some embodiments, the

antibody may be an engineered antibody that has more or less than two disulfide bonds as in a typical Fab region, whereby more formats of DAR can be obtained.

For example, in some embodiments as depicted in panel (B) of FIG. 2, the antibody is a VHH-Fc protein and the linker-payload molecule reacts with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:1, thereby the target product of this embodiment is a DAR4 species which comprise two molecules of linker-payload loaded onto the Fab regions and two molecules of the linker-payload loaded onto the hinge region. Accordingly, the process can produce a multiple-payload ADC of a 1s+2 or 2+1s or 1s +1s format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab region and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region, when a thiol-bridge linker-payload molecule is used in one or both of the first and the second conjugations.

In some other embodiments as depicted in panel (C) of FIG. 2, the antibody is a bispecific antibody of the WuXiBody structure, wherein the LC+CH1 fragment in one of the light chain-heavy chain (LC-HC) pairs is replaced by a TCR constant region (e.g., C $\alpha$  and C $\beta$ ), and thereby the target product of this embodiment is a DAR4 species which comprises two molecules of linker-payload moiety loaded onto the Fab region in the other LC-HC pair and two molecules of the linker-payload loaded onto the hinge region. Accordingly, the process can produce a multiple-payload ADC of a 1s+2 or 2+1s or 1s +1s format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab region and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region, when a thiol-bridge linker-payload molecule is used in one or both of the first and the second conjugations.

In a fifth embodiment, the process of invention comprises:

a first reduction step, wherein an antibody is reduced in the presence of a transition metal ion;

a first conjugation step coupled with a concomitant deshielding treatment, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate;

a second reduction step, wherein the antibody moiety of the first antibody-drug conjugate is further reduced; and

a second conjugation step, wherein the antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.

This process, compared with the fourth embodiment, further comprises a second reduction followed by a second conjugation after the first conjugation step coupled with the concomitant

deshielding treatment. An example of this process is schematically depicted as Scheme 1-2 in FIG. 1. The first conjugation solution is thus preferably subjected to the treatment of depletion to remove the residual un-conjugated first linker-payloads as specified in the first embodiment. Optionally and preferably, the first intermediate ADC of the DAR6 format may be purified as specified in the first embodiment.

The second reduction and the second conjugation are each as described in the first embodiment. In some examples of the fifth embodiment, for the second reduction, the reductant can be TCEP or DPAA; the reductant/mAb ratio can be around 6 - 10; the reaction can be conducted at about 22°C – 37°C for about 4 - 16 hours.

In a preferred embodiment, the second reduction may be performed in the presence of a chelating agent such as EDTA to eliminate the risk of metal ion-mediated oxidation caused by metal ions other than those useful in the first reduction. Thereby, the second conjugation loads additional linker-payloads at the cysteine sites released from the remaining S-S bonds by the second reduction in the hinge region. For example, the resultant second ADC may be a dual-payload ADC of the 6+2 format wherein four molecules of the first linker-payload are linked to the Fab region, two molecules of the first linker-payload are linked to the hinge region at the deshiielded cysteine residues and two molecules of the second linker-payloads are linked to the hinge region at another cysteine site. The embodiment may optionally include a step to recover the product of the last conjugation step as the final antibody-drug conjugate product. The second ADC of the 6+2 format may be recovered as the final ADC product and may optionally be purified as specified in the first embodiment. The resultant product may be an ADC product having a high purity of the multiple-payload ADC of the 6+2 format.

In the above, the embodiments and especially the DAR formats are largely described with reference to linker-payload molecules that react with the free thiol groups for conjugation on the antibody molecule at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the first and/or the second linker-payload molecules are thiol-bridge linker-payloads that can react with the free thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:2. Accordingly, when the first linker-payload molecule in the first conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said first antibody-drug conjugate) with two molecules of the first linker-payload loaded onto the Fab regions and one molecule of the first linker-payload loaded onto the hinge region per antibody; and/or when the second linker-payload molecule in the second conjugation is a thiol-bridge linker-payload molecule, it results in an intermediate (i.e., the above-said second ADC) with one (1) molecule of the second linker-payload loaded onto the hinge region per antibody. Accordingly, the process can produce a multiple-

payload ADC of a  $3^s+2$ ,  $6+1^s$  or  $3^s+1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab region and the hinge of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, when a thiol-bridge linker-payload molecule is used in one or both of the first and the second conjugations.

## **2. Antibody-Drug Conjugate**

In a second aspect of the disclosure, provided is an antibody-drug conjugate (ADC) product comprising a species of interest at a high purity of at least 55 mol%, at least 60 mol%, at least 65 mol%, at least 68 mol%, at least 70 mol%, at least 75 mol%, at least 80 mol%, at least 85 mol%, at least 87 mol%, at least 88 mol%, at least 90 mol%, at least 93 mol%, at least 95 mol%, at least 97 mol%, at least 98 mol% or at least 99 mol%.

In one embodiment, the species of interest, i.e., the target ADC, comprises four molecules of a first linker-payload linked to the Fab regions and at least two molecules of a second linker-payload linked to the hinge region. In particular, the species of interest may be an ADC of the DAR6 format, a dual-payload ADC of the 4+4 format, a dual-payload ADC of the 4+2 format, a dual-payload ADC of the 6+2 format or a triple-payload ADC of the 4+2+2 format.

In another embodiment, the species of interest, i.e., the target ADC, comprises three or six molecules of a first linker-payload and optionally one or two molecules of a second linker-payload, wherein the first linker-payload and the second linker-payload are different or identical.

In a further embodiment, the species of interest, i.e., the target ADC, comprises two or four molecules of a first linker-payload linked to the Fab regions of the antibody and at least one molecule (e.g., one, two, three and four molecules) of a second linker-payload linked to the hinge region of the antibody, and optionally one or two molecules of a third linker-payload linked to the hinge region of the antibody; wherein the first, the second and optionally the third linker-payloads are identical or different from each other or one another.

In one embodiment, the ADC products are produced by the method of the present invention as specified above.

In the present disclosure, the term “antibody-drug conjugate product” refers to a population of ADC molecules that are heterogenous or homogenous in drug loading format. In the present disclosure and in the context of the ADC product, the term “purity” refers to the percentage of the species of interest in the ADC population. The purity may be determined by HIC-HPLC, PLRP-HPLC, LC-MS, among various methods known to a person in the art.

In some embodiments, the ADC of interest is a dual-payload ADC of the 4+4 format which

comprises four molecules of the first linker-payload linked to the Fab regions and four molecules of the second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different. In one embodiment, the ADC product of the invention has a purity of the dual-payload ADC of the 4+4 format being at least 68 mol%, at least 72 mol%, at least 73 mol%, at least 74 mol%, at least 76 mol%, at least 77 mol%, at least 88 mol%, at least 93 mol%, at least 95 mol%, at least 97 mol%, at least 98 mol% or at least 99 mol%.

In some other embodiments, the ADC of interest is a multiple-payload ADC of a  $2^s+4$ ,  $4+2^s$  or  $2^s+2^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and wherein one or both of the first and the second linker-payload moieties derive from a thiol-bridge linker-payload molecule. In the context of ADCs and also the context of the present disclosure, a linker-payload moiety derived from a linker-payload molecule refers the part of the molecule that is incorporated into the ADC molecule as an integral part thereof. The superscript "s" indicates thiol-bridge linker-payloads.

In some embodiments, the ADC of interest is a dual-payload ADC of the 4+2 format which comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different. In one embodiment, the ADC product of the invention has a purity of the dual-payload ADC of the 4+2 format being at least 61 mol%, at least 62 mol%, at least 64 mol%, at least 69 mol%, at least 76 mol%, at least 84 mol%, at least 87 mol% or at least 88 mol%.

In some other embodiments, the ADC of interest is a multiple-payload ADC of a  $2^s+2$ ,  $4+1^s$  or  $2^s+1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and wherein one or both of the first and the second linker-payload moieties each derive from a thiol-bridge linker-payload molecule.

In some embodiments, the ADC of interest is a triple-payload ADC of the 4+2+2 format which comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the second linker-payload linked to the hinge region as well as two molecules of the third linker-payload linked to the hinge region, wherein the first, the second and the third linker-payloads are different to one another. In one embodiment, the ADC product of the invention has a purity of the triple-payload ADC of the 4+2+2 format being at least 60 mol%, at least 70 mol%

and at least 90 mol%.

In some other embodiments, the ADC of interest is a multiple-payload ADC of a  $2^s + 2 + 2$ ,  $2^s + 2 + 1^s$ ,  $2^s + 1^s + 2$ ,  $2^s + 1^s + 1^s$ ,  $4 + 2 + 1^s$ ,  $4 + 1^s + 2$  or  $4 + 1^s + 1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody, the second  
5 numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and the third numeral refers to the count of the third linker-payload moiety loaded on the hinge region of the antibody, and wherein one, two or three of the first, the second and the third linker-payload moieties each derive from a thiol-bridge linker-payload molecule.

In some embodiments, the ADC of interest is an ADC of the DAR6 format which comprises  
10 four molecules of the linker-payload linked to the Fab regions and two molecules of the linker-payload linked to the hinge region. In one embodiment, the ADC product of the invention has a purity of the ADC of the DAR6 format being at least 74 mol%, at least 75 mol%, at least 76 mol%, at least 77 mol%, at least 78 mol%, at least 79 mol%, at least 81 mol% and at least 90 mol%.

In some other embodiments, the ADC of interest is an ADC of a DAR3 format with two  
15 molecules of a linker-payload moiety loaded onto the Fab regions of an antibody and one molecule of the linker-payload moiety loaded onto the hinge region of the antibody, wherein the linker-payload moiety derives from a thiol-bridge linker-payload molecule. In some embodiments, the ADC product of the invention has a purity for the DAR3 species being at least 60 mol% or at least  
20 70 mol%.

In some other embodiments, the ADC of interest is a DAR4 species which comprises a VHH-Fc protein with two molecules of linker-payload moiety loaded onto the Fab regions of the protein and two molecules of the linker-payload loaded onto the hinge region of the protein, wherein the linker-payload moiety may derive from a linker-payload molecule reacting with free thiol groups  
25 for conjugation at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the ADC product of the invention has a purity for the DAR4 species being at least 60 mol%, at least 65 mol%, at least 70 mol% or at least 75 mol%.

In some other embodiments, the ADC of interest is a DAR4 species which comprises a bispecific antibody of the WuXiBody structure with two molecules of linker-payload moiety  
30 loaded onto the Fab region in one of the LC-HC pairs and two molecules of the linker-payload moiety loaded onto the hinge region, wherein the WuXiBody structure is characterized in that the LC+CH1 fragment in one of the light chain-heavy chain (LC-HC) pairs is replaced by a TCR constant region (e.g., C $\alpha$  and C $\beta$ ) and the Fab region in the other LC-HC pair is retained and wherein the linker-payload moiety may derive from a linker-payload molecule reacting with free

thiol groups for conjugation at a linker-payload/free thiol group ratio of 1:1. In some embodiments, the ADC product of the invention has a purity for the DAR4 species being at least 60 mol%, at least 65 mol%, at least 70 mol% or at least 75 mol%.

In some other embodiments, the ADC of interest is a multiple-payload ADC of a  $1^s+2$ ,  $2+1^s$  or  $1^s+1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab regions of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and wherein one or both of the first and the second linker-payload moieties each derive from a thiol-bridge linker-payload molecule.

In some embodiments, the ADC of interest is a dual-payload ADC of the 6+2 format which comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the first linker-payload linked to the hinge region as well as two molecules of the second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different. In one embodiment, the ADC product of the invention has a purity of the dual-payload ADC of the 6+2 format being at least 60 mol%, at least 64 mol%, at least 65 mol%, at least 66 mol%, at least 67 mol% or at least 74 mol%.

In some other embodiments, the ADC of interest is a multiple-payload ADC of a  $3^s+2$ ,  $6+1^s$  or  $3^s+1^s$  format, wherein the first numeral refers to the count of the first linker-payload moiety loaded on the Fab region and the hinge of an antibody and the second numeral refers to the count of the second linker-payload moiety loaded on the hinge region of the antibody, and wherein one or both of the first and the second linker-payload moieties each derive from a thiol-bridge linker-payload molecule.

In another aspect, provided herein is an ADC product produced by the method of the present invention, wherein the product comprises a species of interest featuring the DAR format and optionally the purity as described above.

## Examples

The following Examples are provided merely for illustration, with no intention to limit scope of the invention.

In the examples, the following antibodies were used:

mAb 1 = Trastuzumab, mAb 2 = Rituximab, mAb 3 = Cetuximab, mAb 4 = Adalimumab, mAb 5 = Alemtuzumab, mAb 6 = Atezolizumab, mAb 7 = Basiliximab, mAb 8 = Certolizumab, mAb 9 = Daclizumab, mAb 10 = Daratumumab, mAb 11 = Golimumab, mAb 12 = Ipilimumab, mAb 13 = Mepolizumab, mAb 14 = Necitumumab, mAb 15 = Obinutuzumab, mAb 16 =

Ofatumumab, mAb 17 = anti claudin 18.2 Fc fusion protein (VHH-FC) (cAb17), mAb 18 = W329001-U5T5.E17R-57.uIgG1) cAb 18 (WuXiBody).

### Example 1: Production of dual-payload ADCs of the 4+4 species

In this example, described are exemplary processes according to Scheme 2 in FIG. 1.

#### Process 1

5mg/mL mAb1 (Trastuzumab, characterized in the light chain of SEQ ID NO:1 and the heavy chain of SEQ ID NO:2, WuXi Biologics) in PB buffer (40mM, pH 6.90) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio of 10, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at 4 °C for 16 hours (h). The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, a linker-payload/mAb molar ratio (LP/mAb) of 12.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain an intermediate ADC of mAb1 conjugated with 4 linker-payload 1.

Acetylcysteine (NAC) (A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the intermediate ADC, to react at 4 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90).

Concentrated EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH<sub>2</sub>O) was added to the purified intermediate in PB buffer (40 mM, pH 6.90) to a final concentration of 5 mM, and TCEP (TCEP/mAb molar ratio of 6) was added into the intermediate exposed to the EDTA treatment to form a second reduction reaction, which was kept at 37 °C for 16 hours. The resultant second reduction solution, which comprised the further reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 2 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), followed by incubation at 4 °C for 2 hours for conjugation.

The product of conjugation was buffer exchanged into the storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 1-1 and FIGs. 3 to 7.

Table 1-1: Purity of 4+4 dual-payload species determined by PLRP HPLC in crude product

| ADC Name            | Linker-payload 1 | Linker-payload 2 | Purity (mol%) |
|---------------------|------------------|------------------|---------------|
| mAb1-MMAE+MMAF      | mc-vc-PAB-MMAE   | mc-MMAF          | 76.82%        |
| mAb1-MMAE+GGFG-DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 73.67%        |
| mAb1-MMAF+MMAE      | mc-MMAF          | mc-vc-PAB-MMAE   | 73.96%        |
| mAb1-MMAF+GGFG-DXd  | mc-MMAF          | GGFG-DXd         | 72.75%        |
| mAb1-MMAF+CL2A-SN38 | mc-MMAF          | CL2A-SN38        | 74.76%        |

The crude product was further subjected to preparative HIC purification on an AKTA pure 25M system. All chromatographic steps were performed at room temperature. A ProPac™ HIC-10, 7.8\*75 mm column (Thermo Scientific) was equilibrated with over 5 column volumes of buffer A (0.7M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 25mM PB, pH 6.5). To prepare the sample for loading onto the column, 1 mL of the crude product comprising the 4+4 dual payload ADC mab1-4MMAE-4GGFG-DXd (3.5 mg/mL) obtained above were mixed with an equivalent volume of buffer A. After loading, the column was washed with buffer A until an A280 nm baseline was achieved. The column was eluted with a 0-100% gradient of buffer B (25mM PB, 25%IPA, pH 6.5) in buffer A for 50 column volumes followed by 100%B for 3 CVs, and fractions were collected at 280nm, 248nm and 370nm wave length. The fractions of interest thus collected were buffer exchanged into PB (PB 40mM, pH 6.90; Na<sub>2</sub>HPO<sub>4</sub> 24.4mM, NaH<sub>2</sub>PO<sub>4</sub> 15.6mM) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

Purity of the HIC-purified product was determined by HIC-HPLC and LC-MS, and the results are shown in Table 1-2 and FIG. 8A-8B. The purified ADC was characterized by LC-MS (Agilent 1290 Infinity II; QTOF: Agilent 6530 LC/Q-TOF).

Table 1-2: Purity of 4+4 dual payload species in HIC purified product

| ADC Name      | Linker-payload 1 | Linker-payload 2 | PLRP Purity <sup>1</sup><br>(mol%) | PLRP Purity <sup>2</sup><br>(mol%) | HIC-HPLC Purity <sup>3</sup><br>(mol%) |
|---------------|------------------|------------------|------------------------------------|------------------------------------|----------------------------------------|
| mAb1-MMAE+DXd | mc-vc-PAB-MMAE   | GGFG-DXd         | 73.67%                             | 97.02%                             | 88.17%                                 |

Notes:

- 1: Pre-purification purity
- 2: After-purification purity
- 3: After-purification purity

The processes were repeated as described above except that mAb2 (Rituximab, characterized in the light chain of SEQ ID NO:3 and the heavy chain of SEQ ID NO:4, WuXi Biologics) was used instead of mAb1. Characterization of the obtained products were shown in Table 1-3 and

FIGs. 9 to 13.

Table 1-3: Purity of 4+4 dual payload species determined by PLRP HPLC in crude product

| ADC Name            | Linker-payload 1 | Linker-payload 2 | Purity (mol%) |
|---------------------|------------------|------------------|---------------|
| mAb2-MMAE+MMAF      | mc-vc-PAB-MMAE   | mc-MMAF          | 74.38%        |
| mAb2-MMAE+GGFG-DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 77.33%        |
| mAb2-MMAE+CL2A-SN38 | mc-vc-PAB-MMAE   | CL2A-SN38        | 72.58%        |
| mAb2-MMAF+MMAE      | mc-MMAF          | mc-vc-PAB-MMAE   | 71.20%        |
| mAb2-MMAF+CL2A-SN38 | mc-MMAF          | CL2A-SN38        | 68.24%        |

The crude product was subjected to preparative HIC purification as described above.

## Process 2

5mg/mL mAb1 /mAb2/mAb3in HEPES buffer (40mM, pH 6.90) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at 12 °C for 22 hours (h). The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, a linker-payload/mAb molar ratio of 12.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate ADC of mAb1 conjugated with 4 linker-payload 1.

Acetylcysteine (NAC) (A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the intermediate ADC, to react at 12 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90).

Concentrated EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH<sub>2</sub>O) was added to the purified intermediate in HEPES buffer (40 mM, pH 6.90) to a final concentration of 5 mM, and TCEP (TCEP/mAb molar ratio of 6) was added into the intermediate exposed to the EDTA treatment to form a second reduction reaction, which was kept at 22 °C for 16 hours. The resultant second reduction solution, which comprised the further reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 2 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), followed by incubation at 12 °C for 1 hours for conjugation.

The product of conjugation was buffer exchanged into the storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 1-4 and FIGs. 14A – 14E.

5 Table 1-4: Purity of 4+4 dual payload species determined by PLRP HPLC in crude product

| ADC Name       | Linker-payload 1 | Linker-payload 2 | Purity (mol%) |
|----------------|------------------|------------------|---------------|
| mAb1-MMAE+DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 70.60%        |
| mAb2- MMAE+DXd | mc-vc-PAB-MMAE   | GGFG-DXd         | 79.93%        |
| mAb3- MMAE+DXd | mc-vc-PAB-MMAE   | GGFG-DXd         | 74.27%        |
| mAb1-MMAE+MMAF | mc-vc-PAB-MMAE   | mc-MMAF          | 80.78%        |
| mAb2-MMAE+MMAF | mc-vc-PAB-MMAE   | mc-MMAF          | 89.07%        |

### Process 3

5mg/mL mAb1 in PB buffer (40mM,pH 6.90) was reduced by TCEP at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub> at a Zn(II)/mAb molar ratio of 4, at 12 °C for 20 hours  
 10 (h). The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, a linker-payload/mAb molar ratio of 12.0) in DMA was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hours to obtain an intermediate ADC of mAb1  
 15 conjugated with 4 linker-payload 1.

NAC was added into the first conjugation solution, which comprised the intermediate ADC, to deplete excessive linker-payload as described above in Process 1.

The crude intermediate was further subjected to preparative HIC purification on an AKTA pure 25M system. All chromatographic steps were performed at room temperature. A HiTrap Butyl  
 20 HP 1mL column (Cytiva) (Thermo Scientific) was equilibrated with over 5 column volumes of buffer A (0.7M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 25mM PB, pH 6.5). To prepare the sample for loading onto the column, 1 mL of the crude product comprising the 4 payload ADC mab1-4MMAE (3.5 mg/mL) obtained above were mixed with an equivalent volume of buffer A. After loading, the column was washed with buffer A until an A280 nm baseline was achieved. The column was eluted with a 0-  
 25 100% gradient of buffer B (25mM PB, 25%IPA, pH 6.5) in buffer A for 50 column volumes followed by 100%B for 3 CVs, and fractions were collected at 280nm, 248nm and 370nm wave length. The fractions of interest thus collected were buffer exchanged into PB (PB 40mM, pH 6.90; Na<sub>2</sub>HPO<sub>4</sub> 24.4mM, NaH<sub>2</sub>PO<sub>4</sub> 15.6mM) using spin desalting column (40 kDa, 0.5 mL, REF: 87766,

Lot #SJ251704, Manufacturer: Thermo fisher.

Concentrated EDTA (50mM stock in ddH<sub>2</sub>O) was added to the purified intermediate in PB buffer (40 mM, pH 6.90) to a final concentration of 5 mM, and TCEP (TCEP/mAb molar ratio of 6) was added into the intermediate exposed to the EDTA treatment to form a second reduction reaction, which was kept at 22 °C for 16 hours. The resultant second reduction solution, which comprised the further reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 2 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), followed by incubation at 22 °C for 1 hour for conjugation.

The product of conjugation was buffer exchanged into the storage buffer as as described above in Process 1. The resultant crude product was further subjected to HIC purification as described above in Process 1. Purity of the HIC-purified product was determined by PLRP HPLC, and the results are shown in Table 1-5 and FIGs. 15A and 15B.

Table 1-5: Purity of 4+4 dual payload species in purified product

| ADC Name           | Linker-payload 1 | Linker-payload 2 | PLRP Purity<br>(mol%) |
|--------------------|------------------|------------------|-----------------------|
| mAb1-MMAE+MMAF     | mc-vc-PAB-MMAE   | mc-MMAF          | 97.00%                |
| mAb1-MMAE+GGFG-DXd | mc-vc-PAB-MMAE   | GGFG-DXd         | 98.98%                |

## Example 2: Production of dual-payload ADCs of the 4+2 species

In this example, described are exemplary processes according to Scheme 3-1 in FIG. 1.

### Process 1

5mg/mL antibody in PB buffer (40mM,pH 6.90) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at aTCEP/mAb molar ratio of 10, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at 4 °C for 16h. The reduction solution comprising the reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, a linker-payload/mAb molar ratio of 12.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain an intermediate mAb1 conjugated with 4 linker-payload 1.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution comprising the intermediate ADC to react at 4 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate was purified using a spin desalting column (40 kDa, 0.5 mL, REF:

87766, Lot #SJ251704, Manufacturer: Thermo fisher) by PB buffer (40mM, pH 6.90).

Concentrated EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH<sub>2</sub>O) was added to the eluate to a final concentration of 5 mM. Solution of linker-payload 2 in DMA (overall DMA 10% v/v) was added to the EDTA treated eluate at a linker-payload/mAb molar ratio of 10, and incubated at 4 °C for 2 hours for conjugation

The product of conjugation was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 2-1 and FIGs. 16 and 17.

Table 2-1: Purity of 4+2 dual payload species in crude product

| ADC Name       | Antibody | Linker-payload 1 | Linker-payload 2 | LRP-HPLC Purity (mol%) |
|----------------|----------|------------------|------------------|------------------------|
| mAb1-MMAF+MMAE | mAb1     | mc-MMAF          | mc-vc-PAB-MMAE   | 61.71%                 |
| mAb2-MMAF+MMAE | mAb2     | mc-MMAF          | mc-vc-PAB-MMAE   | 64.84%                 |

The crude product was subjected to preparative HIC purification as described above.

## Process 2

5mg/mL mAb1 in HEPES buffer (40mM, pH7) was reduced by TCEP at aTCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub>, at a Zn(II)/mAb molar ratio of 4, at 12 °C for 20 h. The reduction solution comprising the reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, a linker-payload/mAb molar ratio of 12.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hour to obtain an intermediate mAb1 conjugated with 4 linker-payload 1.

NAC (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution comprising the intermediate ADC to react at 12 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by HEPES (40mM, pH7).

Concentrated EDTA was added to the eluate to a final concentration of 2 mM. Solution of linker-payload 2 in DMA (overall DMA 10% v/v) was added to the EDTA treated eluate at a linker-payload/mAb molar ratio of 10, and incubated at 12 °C for 1 hour for conjugation

The product of conjugation was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704,

Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 2-2 and FIG. 18. The crude product was further subjected to preparative HIC purification as specified in example 1. The purified ADC was characterized by LC-MS (Agilent 1290 Infinity II; QTOF: Agilent 6530 LC\Q-TOF). The results are shown in Table 2-2 and FIG. 19.

Table 2-2: Purity of 4+2 dual payload target species

| ADC Name       | Linker-payload 1 | Linker-payload 2 | Purity <sup>1</sup> | Purity <sup>2</sup> |
|----------------|------------------|------------------|---------------------|---------------------|
| mAb1-MMAE+MMAF | mc-vc-PAB-MMAE   | mc-MMAF          | 69.4% [HIC-HPLC]    | 87.8% [HIC-HPLC]    |
| mAb1-MMAE+DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 70.1% [PLRP-HPLC]   | NA                  |
| mAb1-DXd+MMAE  | GGFG-DXd         | mc-vc-PAB-MMAE   | 67.7% [PLRP-HPLC]   | 88.1% [PLRP-HPLC]   |

Notes:

- 1: Pre-purification purity determined as specified in the brackets  
 2: After-purification purity determined as specified in the brackets

### Example 3: Production of triple-payload ADCs of the 4+2+2 species

In this example, described is an exemplary process according to Scheme 3-2 in FIG. 1.

5mg/mL mAb1 (Trastuzumab, *supra*) in HEPES buffer (40mM, pH 6.90) were reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at 12 °C for 20h. The reduction reaction comprising the reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA (Dimethylacetamide, ARK2190-1L, SAFC) was added into and mixed with the reduction solution to thereby form a first conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hour to obtain the first intermediate, i.e., mAb conjugated with 4 linker-payload 1.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution comprising the intermediate ADC to react at 12 °C for 1 hours to deplete excessive linker-payload.

Then, intermediate DAR4 ADC product was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by HEPES buffer (40mM, pH 6.90).

Concentrated EDTA (EDTA·2Na, 10009719, SCR) (50mM stock in ddH<sub>2</sub>O) was added to the eluate to a final concentration of 5 mM. Solution of linker-payload 2 in DMA was added to the EDTA treated eluate (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), and incubated at 12 °C for 1 hours for conjugation.

Acetylcysteine (NAC) (10mM stock in ddH<sub>2</sub>O, NAC/mAb molar ratio of 16) was added into the second conjugation solution to react at 12 °C for 0.5 hours to deplete excessive linker-payload.

The second intermediate, i.e., the 4+2 dual-payload ADC product, was purified using a spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher) by HEPES buffer (40mM, pH 6.90).

The second intermediate ADC product in HEPES buffer (40 mM, pH 7.0) was reduced by DPAA at a DPAA/mAb molar ratio of 6, and the second reduction was kept at 22 °C for 16 hours. The second reduction solution, which comprised the further reduced antibody, was directly used in the next step of conjugation without DPAA removal.

Solution of linker-payload 3 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10) to obtain a third conjugation solution, and the third conjugation solution was incubated at 22 °C for 2 hours.

The product of third conjugation was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized as specified in example 1, and the results are shown in Table 3-1 and FIG. 20.

Table 3-1: Purity of triple-payload 4+2+2 ADC

| Lot.               | Linker-payload 1 | Linker-payload 2 | Linker-payload 3 | purity (mol%) |
|--------------------|------------------|------------------|------------------|---------------|
| mAb1-DXd+MMAE+MMAF | mc-GGFG-DXd      | mc-vc-PAB-MMAE   | mc-MMAF          | 88.3          |

Example 4: Production of ADCs of DAR6

In this example, described are exemplary processes according to Scheme 1-1 in FIG. 1.

Experiment 1

5mg/mL antibody (as indicated by the ADC names) in PB buffer (40mM, pH 6.90) or HEPES (40mM, pH7) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio as specified below, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at a temperature for a time period as specified below. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA at a final concentration as specified were added into and mixed with the reduction

solution to thereby form the conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratios as specified below) was added into the first conjugation solution, which comprised the ADC product to react at a temperature for a time period as specified below to deplete excessive linker-payload.

Then, the product was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 4-1 and FIGs. 21A and 21B.

Table 4-1: Production and characterization of DAR6 ADC products

| ADC name   | Linker-payload | Reduction Buffer, TCEP/mAb, T (°C) & T (hr) | EDTA (mM) | NAC/mAb ratio, T (°C) & T (hr) | Purity* (mol %) | Average DAR |
|------------|----------------|---------------------------------------------|-----------|--------------------------------|-----------------|-------------|
| mAb1-MMAE  | mc-vc-PAB-MMAE | PB, 8, 4°C, 20 hr                           | 5         | 16, 4°C, 0.5 hr                | 75.5            | 6.15        |
| mAb2-MMAE  | mc-vc-PAB-MMAE | PB, 8, 4°C, 20 hr                           | 5         | 16, 4°C, 0.5 hr                | 74.4            | 6.06        |
| mAb3-MMAE  | mc-vc-PAB-MMAE | PB, 8, 4°C, 20 hr                           | 5         | 16, 4°C, 0.5 hr                | 77.1            | 6.08        |
| mAb4-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 68.97           | 5.92        |
| mAb5-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 73.44           | 5.99        |
| mAb6-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 73.36           | 5.89        |
| mAb7-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 69.67 *         | 5.75        |
| mAb8-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 75.48           | 6.03        |
| mAb9-MMAE  | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 73.39           | 5.87        |
| mAb10-MMAE | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 76.7            | 6.01        |
| mAb11-MMAE | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 76.26           | 6.00        |
| mAb12-MMAE | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 76.32           | 5.99        |
| mAb13-MMAE | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 76.83           | 6.05        |
| mAb14-MMAE | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr                    | 2         | 16, 12°C, 0.5 hr               | 77.45           | 6.11        |

|              |                |                          |   |                  |       |      |
|--------------|----------------|--------------------------|---|------------------|-------|------|
| mAb15-MMAE   | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 75.48 | 5.92 |
| mAb16-MMAE   | mc-vc-PAB-MMAE | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 75.97 | 5.93 |
| mAb1-MMAF    | mc-MMAF        | PB, 8, 4°C, 20 hr        | 5 | 16, 4°C, 0.5 hr  | 78.9  | 6.06 |
| mAb2-MMAF    | mc-MMAF        | PB, 8, 4°C, 20 hr        | 5 | 16, 4°C, 0.5 hr  | 81.6  | 5.99 |
| mAb3-MMAF    | mc-MMAF        | PB, 8, 4°C, 20 hr        | 5 | 16, 4°C, 0.5 hr  | 79.0  | 6.03 |
| mAb1-DXd     | mc-GGFG-DXd    | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 69.4  | 6.05 |
| mAb2-DXd     | mc-GGFG-DXd    | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 70.5  | 6.1  |
| mAb3-DXd     | mc-GGFG-DXd    | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 70.5  | 5.94 |
| mAb1-Extecan | mc-Extecan     | HEPES, 3.25, 12°C, 20 hr | 2 | 16, 12°C, 0.5 hr | 65.2* | 6.5  |

**Note:** The purity was determined via HIC-HPLC, except for those labeled with “\*” which refers to a purity determined via PLRP-HPLC.

Purification can improve target purity further, as demonstrated in FIG. 22. Therein, the mAb1-MMAE was produced in the same way as for mAb4-MMAE above, and crude product obtained thereby was subjected to preparative HIC purification as specified in example 1.

## Experiment 2

In this experiment, various buffers were used in the reduction step. Specifically, 5mg/mL mAb1 in a PB buffer (40mM, pH 6.90) or some other buffers according to the Table below was reduced by TCEP at a TCEP/mAb molar ratio of 8 (for PB only) or 3.25, with the presence of ZnCl<sub>2</sub>, at a Zn(II)/mAb molar ratio of 4, at 4 °C for 16h (for PB only) or at 12°C for 20 h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 5mM or PB and 2mM for the others) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the ADC product to react at 4 °C (for PB) or 12°C (for the others) for 0.5 hours to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized, and the results are shown in Table 4-2 and FIG. 23.

Table 4-2: Tests on different reduction buffers

| ADC name | Reduction buffer | HIC-HPLC Purity (mol%) | Average DAR |
|----------|------------------|------------------------|-------------|
|----------|------------------|------------------------|-------------|

|                 |                    |      |     |
|-----------------|--------------------|------|-----|
| mAb1-DAR6-PB    | PB (40mM, pH 6.9)  | 75.5 | 6.1 |
| mAb1-DAR6-HEPES | HEPES (40mM, pH 7) | 75.1 | 5.9 |
| mAb1-DAR6-MES   | MES (40mM, pH 6.5) | 70   | 5.7 |
| mAb1-DAR6-Tris  | Tris (40mM, pH 7)  | 75.5 | 6.2 |
| mAb1-DAR6-MOPSO | MOPSO (40mM, pH 7) | 76.5 | 6.0 |

Experiment 3

In this experiment, various salt concentrations in reduction buffer were tested. Specifically, 5mg/mL mAb1 in HEPES buffer at pH 7 and a varying salt concentration was reduced by TCEP at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl2, at a Zn(II)/mAb molar ratio of 4, at 12°C for 20 h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (mc-vc-PAB-MMAE, 10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 2mM) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hour to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (10mM stock in ddH2O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the ADC product to react at 12°C for 0.5 hours to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized, and the results are shown in Table 4-3 and FIG. 24.

Table 4-3: Tests on salt concentration in reduction buffers

| ADC name             | Buffer Concentration (mM) | HIC-HPLC Purity (mol%) | Average DAR |
|----------------------|---------------------------|------------------------|-------------|
| mAb1-DAR6-HEPES-20mM | 20                        | 74.7                   | 5.9         |
| mAb1-DAR6-HEPES-40mM | 40                        | 76.7                   | 6.1         |
| mAb1-DAR6-HEPES-60mM | 60                        | 76.1                   | 6.1         |
| mAb1-DAR6-HEPES-80mM | 80                        | 76.0                   | 6.2         |

Experiment 4

In this experiment, various mAb concentrations in reduction were tested. Specifically, mAb1 at the specified concentration in HEPES (40 mM, pH 7) was reduced by TCEP at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl2, at a Zn(II)/mAb molar ratio of 4, at 12°C for 20 h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (mc-vc-PAB-MMAE, 10 mg/mL, i.e., a linker-payload/mAb molar

ratio of 12.0) in DMA and EDTA (final 2mM) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hour to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the ADC product to react at 12°C for 0.5 hours to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized, and the results are shown in Table 4-4 and FIG. 25.

Table 4-4: Tests on different mAb concentrations

| ADC name                         | mAb Concentration (mg/mL) | HIC-HPLC Purity (mol%) | Average DAR |
|----------------------------------|---------------------------|------------------------|-------------|
| mAb1-DAR6-1mg·mL <sup>-1</sup>   | 1                         | 65.4                   | 5.4         |
| mAb1-DAR6-2.5mg·mL <sup>-1</sup> | 2.5                       | 72.9                   | 5.9         |
| mAb1-DAR6-5mg·mL <sup>-1</sup>   | 5                         | 73.4                   | 6.1         |
| mAb1-DAR6-10mg·mL <sup>-1</sup>  | 10                        | 72.6                   | 6.1         |
| mAb1-DAR6-15mg·mL <sup>-1</sup>  | 15                        | 70.5                   | 6.2         |
| mAb1-DAR6-20mg·mL <sup>-1</sup>  | 20                        | 72.1                   | 6.2         |

### Experiment 5

In this experiment, various temperatures and TCEP equivalents (i.e., TCEP/mAb ratio) were tested. Specifically, 5mg/mL mAb1 in PB buffer (40 mM, pH 6.9) or HEPES buffer (40 mM, pH 7) was reduced by TCEP at the TCEP/mAb molar ratio as indicated by the ADC names, with the presence of ZnCl<sub>2</sub> at a Zn(II)/mAb molar ratio of 4, at the temperature for the time as specified below. The reduction solution, which comprised the reduced antibody in HEPES, was directly used in the next step of conjugation without TCEP removal. The reduction solution in PB buffer, which comprised the reduced antibody, was subjected to a treatment for removing TCEP. Specifically, the reduction solution was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

Solution of linker-payload (mc-vc-PAB-MMAE, 10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA were added into and mixed with the reduction solution to thereby form the conjugation reaction, wherein the final concentration of EDTA was as shown below. The conjugation reaction was kept at the temperature for the time as specified below to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added

into the first conjugation solution, which comprised the ADC product to react at the temperature as specified below for 0.5h to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized as shown in, and the results are shown in Table 4-5 and FIG. 26.

Table 4-5: Tests on reduction at various temperatures and TCEP equivalents

| ADC name                   | Reduction buffer | T(°C) & T (hr) <sup>1</sup> | EDTA (mM) | T(°C) & T (hr) <sup>2</sup> | T(°C) <sup>3</sup> | Target species ratio <sup>4</sup> (mol%) | Average. DAR |
|----------------------------|------------------|-----------------------------|-----------|-----------------------------|--------------------|------------------------------------------|--------------|
| mAb1-DAR6-4°C / 10.00 TCEP | PB               | 4°C, 20hr                   | 5         | 4°C, 2hr                    | 4°C                | 70.5                                     | 6.2          |
| mAb1-DAR6-4°C / 12.00 TCEP | PB               | 4°C, 20hr                   | 5         | 4°C, 2hr                    | 4°C                | 69.6                                     | 6.3          |
| mAb1-DAR6-4°C / 14.00 TCEP | PB               | 4°C, 20hr                   | 5         | 4°C, 2hr                    | 4°C                | 70.8                                     | 6.3          |
| mAb1-DAR6-12°C / 3.25 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 72.4                                     | 5.8          |
| mAb1-DAR6-12°C / 3.75 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 75.7                                     | 6.3          |
| mAb1-DAR6-16°C / 3.00 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 74.7                                     | 6            |
| mAb1-DAR6-16°C / 3.25 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 75.7                                     | 6.1          |
| mAb1-DAR6-22°C / 2.85 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 67.5                                     | 5.5          |
| mAb1-DAR6-22°C / 3.00 TCEP | HEPES            | 12°C, 20hr                  | 2         | 12°C, 1hr                   | 12°C               | 73.4                                     | 5.8          |

**Note:**

1. The temperature and time in the step of reduction
2. The temperature and time in the step of conjugation
3. The temperature of NAC treatment
4. Target species ratio determined via PLRP-HPLC

### Experiment 6

In this experiment, reduction buffers at various pH values were tested. Specifically, 5mg/mL mAb1 in HEPES buffer (40 mM) at the pH as specified below was reduced by TCEP at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub>, at a Zn(II)/mAb molar ratio of 4, at 12°C for 20 h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (mc-vc-PAB-MMAE, 10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 2mM) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hours to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the ADC product to react at 12°C for 0.5 hours to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized, and the results are shown in Table 4-6 and

FIG. 27.

Table 4-6: Tests on reduction buffers at different pH values

| ADC name         | Buffer pH | HIC-HPLC Purity (mol%) | Average. DAR |
|------------------|-----------|------------------------|--------------|
| mAb1-DAR6-pH 6.8 | 6.8       | 74.9                   | 5.9          |
| mAb1-DAR6-pH 7.0 | 7         | 72.4                   | 5.8          |
| mAb1-DAR6-pH 7.4 | 7.4       | 73.8                   | 5.9          |
| mAb1-DAR6-pH 8.0 | 8         | 66.2                   | 5.8          |

### Experiment 7

In this experiment, reduction buffer comprising different additives were tested. Specifically, 5mg/mL mAb1 in HEPES buffer (40 mM, pH 7) was reduced by TCEP at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub>, at a Zn(II)/mAb molar ratio of 4, at 12°C for 20 h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (mc-vc-PAB-MMAE, 10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 2mM) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hours to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the ADC product to react at 12°C for 0.5 hours to deplete excessive.

Then, the product was buffer exchanged into its storage buffer as described in Experiment 1. The crude product obtained thereby was characterized, and the results are shown in Table 4-7 and FIG. 28.

Table 4-7: Tests on additives in reduction buffer

| ADC name            | Additives                         | HIC-HPLC Purity (mol%) | Average. DAR |
|---------------------|-----------------------------------|------------------------|--------------|
| mAb1-Tween 20       | 0.02 mg·mL <sup>-1</sup> Tween 20 | 70.2                   | 5.6          |
| mAb1-Benzyl Alcohol | 0.3% (v/v) Benzyl Alcohol         | 65.9                   | 5.5          |
| mAb1-Trehalose      | 5 mg·mL <sup>-1</sup> Trehalose   | 69.5                   | 5.6          |

### Example 5: Production of dual-payload ADCs of the 6+2 species

In this example, described are exemplary processes according to Scheme 1-2 in FIG. 1.

#### Process 1

5mg/mL antibody in PB buffer (40mM, pH 6.90) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio of 8 with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at 4 °C for 20h. The reduction solution comprising the reduced

antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 5mM) were added into and mixed with the reduction solution to thereby form the first conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain the DAR6 ADC product as an intermediate.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the intermediate ADC, to react at 4 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate DAR6 ADC was buffer exchanged into PB buffer (40mM, pH 6.9) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher).

Concentrated EDTA was added to a final concentration of 5 mM, and the intermediate DAR6 ADC was reduced by TCEP (TCEP/mAb molar ratio of 6) in the presence of EDTA at 37 °C for 16 hours. The second reduction reaction comprising the further reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 2 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), followed by incubation at 4 °C for 2 hours for conjugation.

The product of conjugation was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 5-1 and FIGs.29-32.

Table 5-1: Purity of 6+2 dual-payload species in crude product

| ADC Name       | Linker-payload 1 | Linker-payload 2 | PLRP-HPLC Purity (mol%) |
|----------------|------------------|------------------|-------------------------|
| mAb1-MMAE+DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 64.0%                   |
| mAb1-MMAE+MMAF | mc-vc-PAB-MMAE   | mc-MMAF          | 60.0%                   |
| mAb2-MMAE+DXd  | mc-vc-PAB-MMAE   | GGFG-DXd         | 67.0%                   |
| mAb2-MMAE+MMAF | mc-vc-PAB-MMAE   | mc-MMAF          | 66.0%                   |

The crude product was submitted to do the preparative hydrophobic interaction chromatography purification as specified in example 1.

## Process 2

5mg/mL antibody in HEPES buffer (40mM, pH 7) was reduced by TCEP at a TCEP/mAb molar ratio of 3.25 with the presence of ZnCl<sub>2</sub> at a Zn(II)/mAb molar ratio of 4, at 12 °C for 20h.

The reduction solution comprising the reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 1 (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA (final 2 mM) were added into and mixed with the reduction solution to thereby form the first conjugation reaction. The conjugation reaction was kept at 12 °C for 1 hour to obtain the DAR6 ADC product as an intermediate.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio of 16) was added into the first conjugation solution, which comprised the intermediate ADC, to react at 12 °C for 0.5 hours to deplete excessive linker-payload.

Then, the intermediate DAR6 ADC was buffer exchanged into HEPES buffer (40mM, pH 7) using spin desalting column (*Supra*).

Concentrated EDTA was added to a final concentration of 2 mM, and the intermediate DAR6 ADC was reduced by DPAA (DPAA /mAb molar ratio of 6) in the presence of EDTA at 22 °C for 16 hours. The second reduction reaction comprising the further reduced antibody was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload 2 in DMA was added to the second reduction solution (overall DMA 10% v/v, at a linker-payload/mAb molar ratio of 10), followed by incubation at 22 °C for 1 hour for conjugation.

The product of conjugation was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 5-2 and FIG. 33. The crude product was submitted to do the preparative hydrophobic interaction chromatography purification and characterized as specified in example 1. The results are shown in Table 5-2 and FIG. 33.

Table 5-2: Purity of 6+2 dual-payload species

| ADC Name          | Linker-payload 1 | Linker-payload 2 | PLRP-HPLC Purity (mol%) |                    |
|-------------------|------------------|------------------|-------------------------|--------------------|
|                   |                  |                  | Pre-purification        | After purification |
| mAb1-DXd+MMAE     | GGFG-DXd         | mc-vc-PAB-MMAE   | 74.9                    | 84.9               |
| mAb1-Extecan+MMAE | mc-Extecan       | mc-vc-PAB-MMAE   | 66.4                    | <i>NA</i>          |

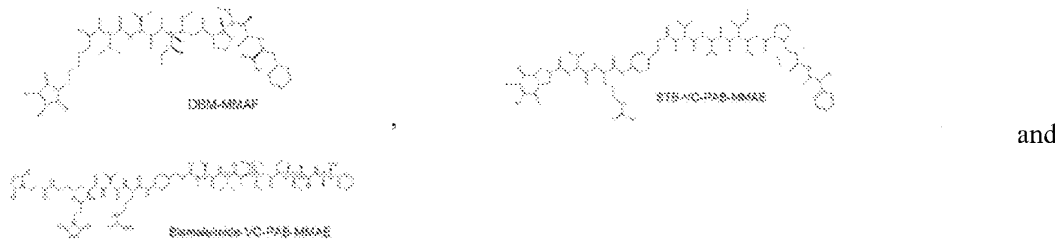
### Example 6: Production of ADCs of DAR3

In this example, described is an exemplary process according to the scheme (A) in FIG. 2.

5mg/mL mAb1 (Trastuzumab, *supra*) in HEPES buffer (40mM, pH 7.0) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio of 3.25, with the presence of ZnCl<sub>2</sub>

(14422-500G, Sigma-Aldrich, at a Zn(II)/mAb molar ratio of 4, at 12 °C for 20h. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of the linker-payload at the concentration (Conc.) and LP-mAb molar ratio as specified below in DMA and EDTA (final 2mM) were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at the temperature and time as specified below to obtain the ADC product of a DAR3 species. The linker-payload molecules are:



Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio 8) was added into the first conjugation solution, which comprised the ADC product to react at 12 °C for 0.5 hours to deplete excessive linker-payload.

Then, the product was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). and the results are shown in Table 6-1 and FIG. 34

Table 6-1 : Production and characterization of DAR3 ADC products

| ADC Name              | LP Conc.,<br>LP/mAb | Temp.<br>&Time <sup>1</sup> | Target Species<br>Purity (mol%) <sup>2</sup> | Average DAR |
|-----------------------|---------------------|-----------------------------|----------------------------------------------|-------------|
| mAb1-DBM-MMAF         | 4                   | 12C 3h                      | 70.5                                         | 3.15        |
| mAb1-STB-MMAE         | 8                   | 22C 16h                     | 61.2                                         | 2.97        |
| mAb1-Bimaleimide-MMAE | 3.5                 | 4C 16h                      | 61.6                                         | 3.20        |

Note:

1. The temperature and time in the step of conjugation
2. Purity determined via PLRP-HPLC

Example 7: Production of DAR4 ADCs comprising a bispecific antibody or a Fc fusion

In this example, described are exemplary processes according to the scheme (B) and (C) in FIG. 2.

5mg/mL antibody (as indicated by the ADC names) in PB buffer (40mM, pH 6.90) or HEPES (40mM, pH7) was reduced by TCEP (C4706-2G, Sigma-Aldrich) at a TCEP/mAb molar ratio as

specified below, with the presence of ZnCl<sub>2</sub> (14422-500G, Sigma-Aldrich) at a Zn(II)/mAb molar ratio of 4, at a temperature for a time period as specified below. The reduction solution, which comprised the reduced antibody, was directly used in the next step of conjugation without TCEP removal.

Solution of linker-payload (10 mg/mL, i.e., a linker-payload/mAb molar ratio of 12.0) in DMA and EDTA at a final concentration as specified were added into and mixed with the reduction solution to thereby form the conjugation reaction. The conjugation reaction was kept at 4 °C for 2 hours to obtain the DAR6 ADC product.

Acetylcysteine (NAC) (Acetylcysteine, A9165-100G, Sigma-Aldrich) (10mM stock in ddH<sub>2</sub>O, a NAC/mAb molar ratio as specified below) was added into the first conjugation solution, which comprised the ADC product to react at a temperature for a time period as specified below to deplete excessive linker-payload.

Then, the product was buffer exchanged into its storage buffer (20mM histidine-ace buffer, pH 5.5) using spin desalting column (40 kDa, 0.5 mL, REF: 87766, Lot #SJ251704, Manufacturer: Thermo fisher). The crude product obtained thereby was characterized, and the results are shown in Table 7 and FIG. 35.

Table 7-1 : Production and characterization of DAR4 ADC products

| ADC name   | Linker-payload | Reduction Buffer, TCEP/mAb, T (°C) & T (hr) | EDTA (mM) | NAC/mAb ratio, T (°C) & T (hr) | Purity* (mol %) | Average DAR |
|------------|----------------|---------------------------------------------|-----------|--------------------------------|-----------------|-------------|
| mAb17-MMAE | mc-vc-PAB-MMAE | HEPES, 6, 12°C, 20 hr                       | 2         | 21, 12°C, 1 hr                 | 70.3            | 3.87        |
| mAb17-MMAF | mc-MMAF        | HEPES, 7, 12°C, 20 hr                       | 0.27      | 21, 12°C, 1 hr                 | 66.8 *          | 3.94        |
| mAb17-DXd  | GGFG-DXd       | HEPES, 7, 12°C, 20 hr                       | 0.27      | 21, 12°C, 1 hr                 | 70.6 *          | 3.87        |
| mAb17-DM21 | mc-DM21        | HEPES, 7.5, 12°C, 20 hr                     | 0.27      | 21, 12°C, 1 hr                 | 71.5 *          | 3.63        |
| mAb18-MMAE | mc-vc-PAB-MMAE | HEPES, 10, 12°C, 20 hr                      | 5         | 40, 4°C, 1 hr                  | 74.8            | 4.08        |

**Note:** The purity was determined via HIC-HPLC, except for those labeled with “\*” which refers to a purity determined via PLRP-HPLC.

#### Sequence List:

| SEQ ID NO: | Description                | SEQ ID NO: | Description             |
|------------|----------------------------|------------|-------------------------|
| 1          | Light chain of Trastuzumab | 19         | Daratumumab heavy chain |
| 2          | Heavy chain of Trastuzumab | 20         | Daratumumab light chain |
| 3          | Light chain of Rituximab   | 21         | SUBUNIT_1 of Golimumab  |

|    |                          |    |                                                              |
|----|--------------------------|----|--------------------------------------------------------------|
| 4  | Heavy chain of Rituximab | 22 | SUBUNIT_2 of Golimumab                                       |
| 5  | Light chain of Cetuximab | 23 | Ipilimumab heavy chain                                       |
| 6  | Heavy chain of Cetuximab | 24 | Ipilimumab light chain                                       |
| 7  | Adalimumab Light chain   | 25 | SUBUNIT_1 of Mepolizumab                                     |
| 8  | Adalimumab Heavy chain   | 26 | SUBUNIT_2 of Mepolizumab                                     |
| 9  | Alemtuzumab Heavy Chain  | 27 | SUBUNIT_1 of Necitumumab                                     |
| 10 | Alemtuzumab Light Chain  | 28 | SUBUNIT_2 of Necitumumab                                     |
| 11 | Atezolizumab Heavy Chain | 29 | SUBUNIT_1 of Obinutuzumab                                    |
| 12 | Atezolizumab Light Chain | 30 | SUBUNIT_2 of Obinutuzumab                                    |
| 13 | Basiliximab Heavy chain  | 31 | SUBUNIT_1 of Ofatumumab                                      |
| 14 | Basiliximab Light chain  | 32 | SUBUNIT_2 of Ofatumumab                                      |
| 15 | Certolizumab Light chain | 33 | anti claudin 18.2 Fc fusion protein (VHH-FC) (cAb17)         |
| 16 | Certolizumab Heavy chain | 34 | W329001-U5T5.E17R-57.uIgG1) cAb 18 (WuXiBody) -Heavy chain 1 |
| 17 | Daclizumab Heavy chain   | 35 | W329001-U5T5.E17R-57.uIgG1) cAb 18 (WuXiBody) -Light chain 1 |
| 18 | Daclizumab Light chain   | 36 | W329001-U5T5.E17R-57.uIgG1) cAb 18 (WuXiBody) -Heavy chain 2 |
|    |                          | 37 | W329001-U5T5.E17R-57.uIgG1) cAb 18 (WuXiBody) -Heavy chain 2 |

\*\*\*\*\*

#### References:

- [1]. Levensgood, M. R. et al. Orthogonal Cysteine Protection Enables Homogeneous Multi-Drug Antibody–Drug Conjugates. *Angew. Chem. Int. Ed.* 56, 733–737 (2017).
- [2]. Yamazaki, C. M. et al. Antibody-drug conjugates with dual payloads for combating breast tumor heterogeneity and drug resistance. *Nat. Commun.* 12, 3528 (2021).
- [3]. Nilchan, N. et al. Dual-mechanistic antibody-drug conjugate via site-specific selenocysteine/cysteine conjugation. *Antib. Ther.* 2, 71–78 (2019).
- [4]. Boschanski, M. et al. Site-Specific Conjugation Strategy for Dual Antibody–Drug Conjugates Using Aerobic Formylglycine-Generating Enzymes. *Bioconjug. Chem.* 32, 1167–1174 (2021).

## CLAIMS

## WHAT IS CLAIMED IS

1. A process of producing an antibody-drug conjugate, comprising:  
a first reduction step, wherein an antibody is reduced in the presence of a transition metal  
5 ion; and  
one or more conjugation steps, wherein the reduced antibody conjugates with a linker-payload to generate an antibody-drug conjugate;  
  
wherein the process further comprises an optional second reduction wherein the antibody  
10 as a moiety of an antibody-drug conjugate is further reduced, and wherein the second reduction is followed by a conjugation step; and  
wherein the process further comprises a deshielding treatment to remove the transitional metal incorporated in the antibody, which is concomitant with at least one of the conjugation steps or the second reduction step.  
15
2. The process of claim 1, wherein the deshielding treatment comprises treatment with a chelating agent.
3. The process of claim 1, wherein the process comprises two or three conjugation steps and  
20 the linker payloads are different between at least two of the conjugation steps.
4. The process of claim 1, comprising, after the first reduction step,  
a first conjugation step, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate;  
25 a second reduction step coupled with the concomitant deshielding treatment, wherein the antibody moiety of the first antibody-drug conjugate is further reduced; and  
a second conjugation step, wherein the further reduced antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.  
30
5. The process of claim 1, comprising, after the first reduction step,  
a first conjugation step, wherein the antibody conjugates with a first linker-payload to generate a first antibody-drug conjugate; and  
a second conjugation step coupled with the concomitant deshielding treatment, wherein

the antibody moiety of the first antibody-drug conjugate conjugates with a second linker-payload to generate a second antibody-drug conjugate.

- 5           6. The process of claim 1, comprising, after the first reduction step,  
a first conjugation step coupled with the concomitant deshielding treatment, wherein the  
antibody conjugates with a first linker-payload to generate a first antibody-drug  
conjugate.
- 10           7. The process of claim 1, comprising, after the first reduction step,  
a first conjugation step coupled with the concomitant deshielding treatment, wherein the  
antibody conjugates with a first linker-payload to generate a first antibody-drug  
conjugate;  
a second reduction step, wherein the antibody moiety of the first antibody-drug conjugate  
is further reduced; and  
15           a second conjugation step, wherein the antibody moiety of the first antibody-drug  
conjugate conjugates with a second linker-payload to generate a second antibody-drug  
conjugate.
- 20           8. The process of claim 1, comprising, after the first reduction step,  
a first conjugation step, wherein the antibody conjugates with a first linker-payload to  
generate a first antibody-drug conjugate;  
a second conjugation step coupled with the concomitant deshielding treatment, wherein  
the antibody moiety of the first antibody-drug conjugate conjugates with a second  
linker-payload to generate a second antibody-drug conjugate;  
25           a second reduction step, wherein the antibody moiety of the second antibody-drug  
conjugate is further reduced; and  
a third conjugation step, wherein the antibody moiety of the second antibody-drug  
conjugate conjugates with a third linker-payload to generate a third antibody-drug  
conjugate.
- 30           9. An antibody-drug conjugate product comprising an antibody-drug conjugate of interest  
at a purity of at least 55 mol%, wherein,  
(i) the antibody-drug conjugate of interest comprises two or four molecules of a first linker-  
payload linked to the Fab regions and at least two molecules of a second linker-payload

linked to the hinge region, wherein the first linker-payload and the second linker-payload are different or identical;

- (ii) the antibody-drug conjugate of interest comprises three or six molecules of a first linker-payload and optionally one or two molecules of a second linker-payload, wherein the first linker-payload and the second linker-payload are different or identical; or
- (iii) the antibody-drug conjugate of interest comprises two or four molecules of a first linker-payload linked to the Fab regions of the antibody and at least one molecule (e.g., one, two, three and four molecules) of a second linker-payload linked to the hinge region of the antibody, and optionally one or two molecules of a third linker-payload linked to the hinge region of the antibody; wherein the first, the second and optionally the third linker-payloads are identical or different from each other or one another.

10. The antibody-drug conjugate product of claim 9, wherein

- (i) the antibody-drug conjugate of interest comprises four molecules of the first linker-payload linked to the Fab regions and four molecules of the second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different;
- (ii) the antibody-drug conjugate of interest comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different;
- (iii) the antibody-drug conjugate of interest comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the first linker-payload linked to the hinge region, and wherein the purity is at least 65 mol%;
- (iv) the antibody-drug conjugate of interest comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the first linker-payload linked to the hinge region as well as two molecules of a second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different;
- (v) the antibody-drug conjugate of interest comprises four molecules of the first linker-payload linked to the Fab regions and two molecules of the second linker-payload linked to the hinge region as well as two molecules of the third linker-payload linked to the hinge region, wherein the first, the second and the third linker-payloads are different to one another; or
- (vi) the antibody-drug conjugate of interest comprises two molecules of a linker-payload

moiety loaded onto the Fab regions and one molecule of the linker-payload moiety loaded onto the hinge region, wherein the linker-payload moiety derives from a thiol-bridge linker-payload molecule; or

5 (vii) the antibody-drug conjugate of interest comprises two molecules of a linker-payload moiety loaded onto the Fab regions of the antibody and two molecules of the linker-payload loaded onto the hinge region of the antibody, wherein the antibody is a VHH-Fc protein; or

10 (viii) the antibody-drug conjugate of interest comprises two molecules of a linker-payload moiety loaded onto the Fab region in one of the LC-HC pairs in the antibody and two molecules of the linker-payload moiety loaded onto the hinge region, wherein the antibody is a bispecific antibody of the WuXiBody structure wherein the LC+CH1 fragment in one of the light chain-heavy chain (LC-HC) pairs is replaced by a TCR constant region and the Fab region in the other LC-HC pair is retained.

15 11. The antibody-drug conjugate product of claim 10, wherein the antibody-drug conjugate of interest (i) is present at a purity of at least 68 mol%; the antibody-drug conjugate of interest (ii) is present at a purity of at least 61 mol%; the antibody-drug conjugate of interest (iv) is present at a purity of at least 60 mol%; the antibody-drug conjugate of interest (v) is present at a purity of at least 56 mol%; the antibody-drug conjugate of interest (vi) is present at a purity of at least 60  
20 mol%; the antibody-drug conjugate of interest (vii) is present at a purity of at least 60 mol%; or the antibody-drug conjugate of interest (viii) is present at a purity of at least 60 mol%.

12. The antibody-linker-payload conjugate according to claim 9, which is produced by the method of claim 1.

25

13. An antibody-drug conjugate product produced by the method of claim 1, wherein,

30 (i) the product comprises an antibody-drug conjugate of interest comprising two or four molecules of a first linker-payload linked to the Fab regions and at least two molecules of a second linker-payload linked to the hinge region, wherein the first linker-payload and the second linker-payload are different or identical;

(ii) the antibody-drug conjugate of interest comprises three or six molecules of a first linker-payload and optionally one or two molecules of a second linker-payload, wherein the first linker-payload and the second linker-payload are different or identical; or

(iii) the antibody-drug conjugate of interest comprises two or four molecules of a first linker-

payload linked to the Fab regions of the antibody and at least one molecule (e.g., one, two, three and four molecules) of a second linker-payload linked to the hinge region of the antibody, and optionally one or two molecules of a third linker-payload linked to the hinge region of the antibody; wherein the first, the second and optionally the third linker-payloads are identical or different from each other or one another.

5