## Summary

**[0004]** Provided herein are methods for reducing aggregation of bispecific antibodies, in particular, bispecific T cell engager antibodies (BiTEs), that results from storage under frozen conditions. The present application is directed to the surprising findings that holding the bispecific antibodies at certain temperatures for a period of time after thaw reduces aggregates formed during storage under frozen conditions.

**[0005]** In one aspect, disclosed herein is a method for reducing aggregates of a bispecific antibody comprising holding a thawed bispecific antibody at a temperature of from about 5 ºC to about 45 ºC for at least 4 hours, wherein the bispecific antibody has been stored at a temperature of from about -20 ºC to about -40 ºC before thaw. In one example, the thawed bispecific antibody is held at the temperature for a period of from about 4 hours to about 96 hours. In one example, the thawed bispecific antibody is held at a temperature of from about 10 ºC to about 30 ºC for a period of from about 10 hours to about 48 hours. In one example, the thawed bispecific antibody is held at a temperature of from about 10 ºC to about 30 ºC for a period of from 8 hours to 48 hours.

**[0006]** In one example, the bispecific antibody is thawed at a temperature of from about 5 ºC to about 45 ºC. In one example, the bispecific antibody has been stored at a temperature of from about -20 ºC to about -35 ºC before thaw, in another example, the bispecific antibody has been stored at about -30 ºC before thaw. In one example, the aggregates comprise high molecule weight (HMW) aggregates. In one example, the HMW aggregates comprise dimers of the bispecific antibody. In one example, the bispecific antibody comprises less than about 1% of the HMW aggregates after the holding period, in another example, the bispecific antibody comprises less than about 0.5% of the HMW aggregates after the holding period.

**[0007]** In one aspect, disclosed herein is a method for preparing a composition comprising a bispecific antibody, the method comprises thawing a drug substance comprising a bispecific antibody that has been stored at a temperature of from about -20 ºC to about -40 ºC, and holding the thawed drug substance comprising the bispecific antibody at a temperature of from about 5 ºC to about 45 ºC for at least 4 hours. In one example, the drug substance is held at the temperature for a period of from about 4 hours to about 96 hours. In another example, the drug substance is held at a temperature of from about 10 °C to about 30 °C for a period of from about 10 hours to about 48 hours. In another example, the drug substance is held at a temperature of from about 10 °C to about 30 °C for a period

of from 8 hours to 48 hours. In one example, the drug substance is thawed at a temperature of from about 5 ºC to about 45 ºC. In one example, the drug substance has been stored at a temperature of from about -20 ºC to about -35 ºC, in another example, the drug substance has been stored at about -30 ºC. In one example, the method for preparing the composition further comprises filtering the drug substance, in another example, the method for preparing the composition further comprises aliquoting the composition into a drug product form. In one example, the composition is a pharmaceutical composition comprising the bispecific antibody.

**[0008]** In one aspect, disclosed herein is a method for preparing a composition comprising a bispecific antibody, the method comprises holding a thawed drug substance comprising a bispecific antibody at a temperature of from about 5 ºC to about 45 ºC for at least 4 hours, wherein the drug substance has been frozen at or above the glass transition temperature (Tg') of the drug substance before thaw. In one example, the drug substance has been frozen at a temperature of from about -10 ºC to at or above the glass transition temperature of the drug substance, in another example, the drug substance has been frozen at about -32 ºC. In one example, the drug substance is held at the temperature for a period of from about 4 hours to about 96 hours. In one example, the drug substance is thawed at a temperature of from about 5 ºC to about 45 ºC. In another example, the drug substance is held at the same temperature as the temperature at which the drug substance is thawed. In another example, the drug substance is thawed and held at the same temperature of from about 15 °C to about 30 °C for a period of from 30 hours to 50 hours. In another example, the method for preparing the composition further comprises aliquoting the composition into a drug product form. In another example, the method for preparing the composition further comprises lyophilizing the composition. In yet another example, the method for preparing the composition further comprises spray drying the composition.

**[0009]** In one example, the drug substance comprises less than about 1% of HMW aggregates after the holding period, in another example, the drug substance comprises less than about 0.5% of the HMW aggregates. In one example, the HMW aggregates comprise dimers of the bispecific antibody.

**[0010]** In one example, the drug substance comprising the bispecific antibody at a concentration of from about 0.05 mg/mL to about 20 mg/mL.

**[0011]** In one example, the bispecific antibody is a bispecific T cell engager antibody (BiTE). In one example, the bispecific antibody comprises a first binding domain that binds to a target cell surface antigen, and a second binding domain that binds to human CD3, and wherein the bispecific antibody is in (scFv)2 format. In one example, the bispecific antibody comprises a first binding domain that binds to a target cell surface antigen selected from CD19, CD33 or BCMA, and a second

binding domain that binds to human CD3, and wherein the bispecific antibody is in (scFv)2 format.

**[0012]** In one example, the bispecific antibody comprises a first binding domain and a second binding domain, wherein the first binding domain comprises a VH region and a VL region, and wherein: the VH comprises the amino acid sequence of SEQ ID NO: 77 and the VL comprises the amino acid sequence of SEQ ID NO: 78; or the VH comprises the amino acid sequence of SEQ ID NO: 28 and the VL comprises the amino acid sequence of SEQ ID NO: 32 or 33; or the VH comprises the amino acid sequence of SEQ ID NO: 132 and the VL comprises the amino acid sequence of SEQ ID NO: 133. In another example, the bispecific antibody comprises the amino acid sequence of SEQ ID NO: 17, 40 or 135.

**[0013]** In one example, the bispecific antibody is a BiTE, wherein the BiTE further comprises a third domain comprising two polypeptide monomers, each comprises a hinge, a CH2 and a CH3 domain, wherein the two polypeptide monomers are linked to each other via a peptide linker. In another example, the third domain comprises in an amino to carboxyl order hinge-CH2-CH3-linker-hinge-CH2-CH3. In one example, the third domain is a half-life extended (HLE) domain.

**[0014]** In one example, the bispecific antibody comprises a first binding domain, a second binding domain, and a third domain, wherein the first binding domain binds to at least one target cell surface antigen selected from CD19, CD33, EGFRvIII, MSLN, CDH19, FLT3, DLL3, CDH3, CD70, BCMA or PSMA, the second binding domain binds to human CD3, and the third domain comprises two polypeptide monomers, each comprises a hinge, a CH2 and a CH3 domain, wherein the two polypeptide monomers are linked to each other via a peptide linker.

**[0015]** In one example, the bispecific antibody comprises a first binding domain, a second binding domain, and a third domain, the first binding domain comprises a VH region and a VL region and binds to at least one target cell surface antigen selected from CD19, CD33, EGFRvIII, MSLN, CDH19, FLT3, DLL3, CDH3, CD70, BCMA or PSMA, the second binding domain binds to human CD3, and the third domain comprises two polypeptide monomers, each comprises a hinge, a CH2 and a CH3 domain, wherein the two polypeptide monomers are linked to each other via a peptide linker, and wherein: (a) the VH comprises the amino acid sequence of SEQ ID NO: 108 and the VL comprises the amino acid sequence of SEQ ID NO: 109; or (b) the VH comprises the amino acid sequence of SEQ ID NO: 27 and the VL comprises the amino acid sequence of SEQ ID NO: 32; or (c) the VH comprises the amino acid sequence of SEQ ID NO: 48 and the VL comprises the amino acid sequence of SEQ ID NO: 49; or (d) the VH comprises the amino acid sequence of SEQ ID NO: 59 and the VL comprises the amino acid sequence of SEQ ID NO: 60; or (e) the VH comprises the amino acid sequence of SEQ ID NO: 77 and the VL comprises the amino acid sequence of SEQ ID

NO: 78; or (f) the VH comprises the amino acid sequence of SEQ ID NO: 108 and the VL comprises the amino acid sequence of SEQ ID NO: 112; or (g) the VH comprises the amino acid sequence of SEQ ID NO: 89 and the VL comprises the amino acid sequence of SEQ ID NO: 90; or (h) the VH comprises the amino acid sequence of SEQ ID NO: 100 and the VL comprises the amino acid sequence of SEQ ID NO: 101; or (i) the VH comprises the amino acid sequence of SEQ ID NO: 121 and the VL comprises the amino acid sequence of SEQ ID NO: 122; or (j) the VH comprises the amino acid sequence of SEQ ID NO: 188 and the VL comprises the amino acid sequence of SEQ ID NO: 189; or (k) the VH comprises the amino acid sequence of SEQ ID NO: 132 and the VL comprises the amino acid sequence of SEQ ID NO: 133; or (l) the VH comprises the amino acid sequence of SEQ ID NO: 173 and the VL comprises the amino acid sequence of SEQ ID NO: 174.

**[0016]** In one example, the bispecific antibody comprises a first binding domain, a second binding domain, and a third domain, the first binding domain binds to at least one target cell surface antigen selected from CD19, CD33, EGFRvIII, MSLN, CDH19, FLT3, DLL3, CDH3, CD70, BCMA or PSMA, the second binding domain binds to human CD3, and the third domain comprises two polypeptide monomers, each comprises a hinge, a CH2 and a CH3 domain, wherein the two polypeptide monomers are linked to each other via a peptide linker, and wherein the bispecific antibody comprising or consisting of the amino acid sequence selected from SEQ ID NO: 63, 114, 41, 82, 136, 104, 93, 177, 125, 190 or 52.

**[0017]** In one example, the bispecific antibody is a bispecific masked antigen binding protein**.** In one example, the bispecific masked antigen binding protein comprises (a) a first antibody or antigen binding fragment thereof (AB1) that binds to a first antigen and a masking domain (MD1) coupled to AB1, wherein the MD1 comprises (1) a first masking peptide (MP1) that inhibits or reduces the binding of AB1 to its antigen, and (2) a protein recognition site (PR1), wherein binding to or cleavage of the PR1 by a protein or a protease increases AB1 binding to its antigen; (b) a second antibody or antigen binding fragment there of (AB2) that binds to a second antigen, and a second masking domain (MD2) coupled to AB2, wherein the MD2 comprises: (1) a second masking peptide (MPs) that inhibits or reduces the binding of AB2 to its antigen, and (2) a second protein recognition site (PR2), wherein binding to or cleavage of the PR2 by a protein or a protease increases AB2 binding to its antigen. In one example, the PR1 and PR2 comprise the same protein recognition sequence. In another example, the AB1 binds to human CD3 and the AB2 binds to human EGFR.