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(54) **Title:** METHODS OF PURIFYING MASKED ANTIBODIES

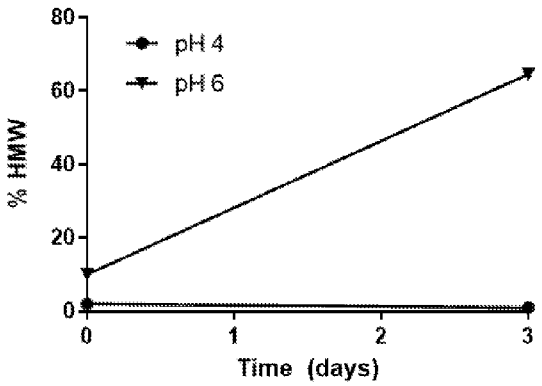


Figure 1B

(57) **Abstract:** The present invention relates to the field of antibody formulations. In particular, the present invention relates to specific methods of preparing masked antibodies with reduced aggregation. In some embodiments, the masked antibodies comprise anti-CD47 antibodies.

WO 2020/247574 A1

METHODS OF PURIFYING MASKED ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of US Provisional Application No. 62/857,367, filed June 5, 2019, which is incorporated by reference herein in its entirety for any purpose.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of purifying masked antibodies. In particular, the present invention relates to specific methods of preparing masked antibodies with reduced aggregation. In some embodiments, the masked antibodies comprise anti-CD47 antibodies.

BACKGROUND

[0003] Current antibody-based therapeutics may have less than optimal selectivity for the intended target. Although monoclonal antibodies are typically specific for binding to their intended targets, most target molecules are not specific to the disease site and may be present in cells or tissues other than the disease site.

[0004] Several approaches have been described for overcoming these off-target effects by engineering antibodies to have a cleavable linker attached to an inhibitory or masking domain that inhibits antibody binding (see, e.g., WO2003/068934, WO2004/009638, WO 2009/025846, WO2101/081173 and WO2014103973). The linker can be designed to be cleaved by enzymes that are specific to certain tissues or pathologies, thus enabling the antibody to be preferentially activated in desired locations. Masking moieties can act by binding directly to the binding site of an antibody or can act indirectly via steric hindrance. Various masking moieties, linkers, protease sites and formats of assembly have been proposed. The extent of masking may vary between different formats as may the compatibility of masking moieties with expression, purification, conjugation, or pharmacokinetics of antibodies.

[0005] The present invention relates to methods of preparing masked antibodies with reduced aggregation. In some embodiments, the masked antibodies comprise a first coiled-coil domain linked to a heavy chain variable region of the antibody and a second coiled-coil domain linked to a light chain variable region of the antibody. The presence of these potentially hydrophobic coiled-coil polypeptide sequences can lead to aggregation during purification, processing, and storage. In some embodiments, the present preparation methods may help to reduce aggregation of the masked antibodies, thus increasing yields of properly folded, unaggregated masked antibodies for use in various applications.

SUMMARY

[0006] The present disclosure addresses methods of preparing compositions comprising masked antibodies that comprise a removable masking agent (e.g., a coiled coil masking agent) that prevents binding of the antibodies to their intended targets until the masking agent is cleaved off or otherwise removed. In other words, the masking agent masks the antigen binding portion of the antibody so that it cannot interact with its targets. In certain therapeutic uses, the masking agent can be removed (e.g., cleaved) by one or more molecules (e.g., proteases) that are present in an *in vivo* environment after administration of the masked antibody to a patient. In other, for example non-therapeutic, uses, a masking agent could be removed by adding one or more proteases to the medium in which the antibody is being used. Removal of the masking agent restores the ability of the antibodies to bind to their targets, thus enabling specific targeting of the antibodies. In some embodiments herein, the antibodies are CD47 antibodies.

[0007] The presence of coiled coil masking agents, for example, could increase the chances of aggregation of the antibodies during purification processes and later during storage prior to use. Thus, the present disclosure addresses methods of preparing masked antibodies that may reduce aggregation of the masked antibodies during the process, thus potentially increasing yield. For example, in some embodiments, particular process steps are designed to reduce aggregation during those steps so as to maintain a relatively high yield during each process step.

[0008] In some embodiments, a process for purifying a masked antibody is provided, wherein the process comprises:

a) loading a starting composition comprising the masked antibody onto a protein A chromatography column under conditions suitable for binding the masked antibody to the protein A chromatography column;

b) washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH 4.5-5.5; and

c) eluting the masked antibody from the protein A column in an acidic elution buffer at pH 2.5-4 to form a protein A eluate comprising the masked antibody;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region or a light chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to the other of the heavy chain variable region or the light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second

coiled-coil domain comprises the sequence

VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

[0009] In some embodiments, the first masking domain is linked to the heavy chain variable region of the antibody and the second masking domain is linked to the light chain variable region of the antibody. In some embodiments, the starting composition is a cell lysate.

[0010] In some embodiments, the protein A chromatography is performed at room temperature. In some embodiments, the acidic wash buffer is an acetate buffer. In some embodiments, the acidic wash buffer is a glutamate buffer. In some embodiments, the acidic wash buffer comprises 10-100 mM, 10-90 mM, 10-80 mM, 10-70 mM, 10-60 mM, 10-50 mM, 10-40 mM, 15-30 mM, 20-30 mM, or 25 mM acetate, or wherein the acidic wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate. In some embodiments, the at least one acidic wash buffer of step (b) is at pH 4.7-5.4, pH 4.8, pH 4.9, pH 5, pH 5.1, or pH 5.2.

[0011] In some embodiments, the acidic elution buffer comprises 0.05-0.2M, 0.07-0.15M, 0.07-0.13M, 0.08-0.12M, 0.09M, 0.1M, or 0.11M acetic acid, or wherein the acidic elution buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamic acid. In some embodiments, the acidic elution buffer of step (c) is at pH 2.5-5, pH 3-5, pH 3-4.5, pH 3.5-4, pH 2.5-3.8, pH 2.7-3.8, or pH 2.5-3.5, pH 2.6, pH 2.7, pH 2.8, pH 2.9, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5.

[0012] In some embodiments, the process comprises washing the column at least once between (a) and (b) with a neutral wash buffer at pH 6-8, optionally wherein the neutral wash buffer is a Tris buffer, which is optionally at pH 7.5, and/or wherein the process comprises washing the column at least once between (a) and (b) with a basic wash buffer at pH 8.5-9.5, optionally wherein the basic wash buffer is an arginine buffer, which is optionally at pH 9.

[0013] In some embodiments, the process further comprises adjusting the pH of the protein A eluate to pH 3-4.2, pH 3-4, pH 3.5-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4, to form an acidified eluate. In some embodiments, the pH is adjusted using acetic acid, optionally 1M acetic acid, or using phosphoric acid, optionally 0.5 M phosphoric acid.

[0014] In some embodiments, the process comprises incubating the acidified eluate for 4-30 hours, 6-30 hours, 10-30 hours, 4-20 hours, 6-20 hours, 8-20 hours, 10-20 hours, 4-18 hours, 6-18 hours, 8-18 hours, 10-18 hours, 8-16 hours, 10-16 hours, 8-14 hours, 10-14 hours, 11-13 hours, 10 hours, 11 hours, 12 hours, 13 hours, or 14 hours after adjusting the pH.

[0015] In some embodiments, a process for purifying a masked antibody is provided, wherein the process comprises:

a) subjecting a starting composition comprising the masked antibody to one or more chromatography purification steps, to form a chromatography eluate;

b) adjusting the pH of the chromatography eluate to pH 3-4.2, pH 3-4, pH 3.5-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4, to form an acidified eluate; and

c) incubating the acidified eluate for 4-30 hours, 6-30 hours, 10-30 hours, 4-20 hours, 6-20 hours, 8-20 hours, 10-20 hours, 4-18 hours, 6-18 hours, 8-18 hours, 10-18 hours, 8-16 hours, 10-16 hours, 8-14 hours, 10-14 hours, 11-13 hours, 10 hours, 11 hours, 12 hours, 13 hours, or 14 hours;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region or a light chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to the other of the heavy chain variable region or the light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

[0016] In some embodiments, the first masking domain is linked to the heavy chain variable region of the antibody and the second masking domain is linked to the light chain variable region of the antibody. In some embodiments, the starting composition is a cell lysate.

[0017] In some embodiments, the acidified eluate is incubated at room temperature. In some embodiments, the process further comprises adjusting the pH of the acidified eluate to pH 3.5-4.5, pH 3.5-4.3, pH 3.7-4.2, pH 3.6-4, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, or pH 4.2 after incubation. In some embodiments, the pH is adjusted using tris base, optionally 1M tris base.

[0018] In some embodiments, the process comprises filtering the acidified eluate on a depth filter. In some embodiments, the process comprises chilling the acidified eluate to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C after incubation or depth filtration.

[0019] In some embodiments, the process comprises adjusting the pH of the chilled acidified eluate to pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9, to form a chilled neutral eluate. In some embodiments, the pH is adjusted using tris base, optionally 1M tris base.

[0020] In some embodiments, the process further comprises loading the chilled neutral eluate on a hydrophobic interaction chromatography (HIC) column or membrane. In some embodiments, the process comprises washing the HIC column or membrane with a HIC wash buffer that has been chilled to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C.

[0021] In some embodiments, process for purifying a masked antibody is provided, wherein the process comprises:

a) chilling a starting composition comprising the masked antibody, wherein the pH of the starting composition is adjusted to pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9 before or after chilling, to form a chilled starting composition;

b) loading the chilled starting composition on a hydrophobic interaction chromatography (HIC) column or membrane; and

c) washing the HIC column or membrane with a HIC wash buffer that has been chilled to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region or a light chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to the other of the heavy chain variable region or the light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

[0022] In some embodiments, the first masking domain is linked to the heavy chain variable region of the antibody and the second masking domain is linked to the light chain variable region of the antibody. In some embodiments, the starting composition is a cell lysate.

[0023] In some embodiments, the HIC wash buffer is a tris/sodium citrate buffer. In some embodiments, the HIC wash buffer is at pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9. In some embodiments, the HIC wash buffer comprises sodium citrate. In some embodiments, the concentration of sodium citrate in the HIC wash buffer is 200-700 mM, or 200-600 mM, or 200-500 mM, or 250-500 mM, or 300-500 mM, or 300-400 mM.

[0024] In some embodiments, the process comprises collecting a HIC effluent comprising the masked antibody. In some embodiments, the process further comprises a first diafiltration following HIC to reduce the concentration of sodium citrate to below 40 mM, or below 35 mM,

or below 30 mM, or below 25 mM, or below 20 mM, or below 15 mM, or below 10 mM, or below 5 mM, to form a diafiltered HIC effluent. In some embodiments, the first diafiltration is performed at 1-15°C, 1-10°C, or 1-9°C, or 2-8°C.

[0025] In some embodiments, the process further comprises a second diafiltration in an acetate buffer, wherein the second diafiltration is performed at room temperature, optionally 15-28°C, or 18-25°C. In some embodiments, the acetate buffer comprises 20-100 mM, 20-90 mM, 20-80 mM, 20-70 mM, 30-50 mM, 35 mM, 40 mM, or 45 mM acetate. In some embodiments, prior to the second diafiltration, the pH of the diafiltered HIC effluent is adjusted to pH 3.5-4.5, pH 3.7-4.5, pH 3.7-4.3, pH 3.8, pH 3.9, pH 4, pH 4.1, or pH 4.2. In some embodiments, the pH is adjusted using 25% v/v glacial acetic acid, to form an acidified diafiltered HIC effluent. In some embodiments, the acidified diafiltered HIC effluent is subjected to ultrafiltration to form a concentrated masked antibody composition. In some embodiments, the concentration of the masked antibody in the concentrated masked antibody composition is 15-35 mg/mL, or 20-35 mg/mL, or 25-35 mg/mL.

[0026] In some embodiments, the process further comprises performing virus removal. In some embodiments, virus removal is performed by nanofiltration. In some embodiments, the nanofiltration is performed at acidic pH. In some embodiments, the acidic pH is pH 3-4.4, pH 3.5-4.4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4. In some embodiments, the virus removal is performed at room temperature. In some embodiments, virus removal follows the ultrafiltration.

[0027] In some embodiments, the process optionally comprises adjusting the pH of the acidified eluate to pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5. In some embodiments, the pH is adjusted using tris base, optionally 1M tris base.

[0028] In some embodiments, the process further comprises loading the optionally pH adjusted acidified eluate on a hydrophobic interaction chromatography (HIC) column or membrane. In some embodiments, HIC is performed at room temperature.

[0029] In some embodiments, a process for purifying a masked antibody is provided, wherein the process comprises:

a) obtaining a starting composition comprising the masked antibody, wherein the pH of the starting composition is adjusted to pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5;

b) loading the starting composition on a hydrophobic interaction chromatography (HIC) column or membrane; and

c) washing the HIC column or membrane with a HIC wash buffer at pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5; wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

[0030] In some embodiments, the starting composition is a cell lysate. In some embodiments, the HIC wash buffer is a glutamate buffer. In some embodiments, the HIC wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate. In some embodiments, the HIC wash buffer is at pH 3.6 to 4, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4. In some embodiments, the HIC step is conducted at room temperature.

[0031] In some embodiments, the process comprises collecting a HIC effluent comprising the masked antibody. In some embodiments, the process further comprises exchanging the buffer of the HIC effluent to a glutamate buffer at pH pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5, wherein exchanging the buffer is through diafiltration or tangential flow filtration. In some embodiments, the glutamate buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate.

[0032] In some embodiments, the process further comprises ultrafiltration prior to diafiltration or tangential flow filtration to concentrate the masked antibody to 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL. In some embodiments, the process further comprises performing virus removal. In some embodiments, virus removal is performed by nanofiltration. In some embodiments, the nanofiltration is performed at acidic pH. In some embodiments, the acidic pH is pH 3-4.4, pH 3.5-4.4, pH 3.6-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4. In some embodiments, the virus removal is performed at room temperature. In some embodiments, virus removal precedes the diafiltration and ultrafiltration.

[0033] In some embodiments, each pH >5 step of the process is performed at a temperature of 1°C to 15°C, and/or wherein each room temperature step of the process is performed at pH <4.5.

[0034] In some embodiments, each masking domain comprises a protease-cleavable linker and is linked to the heavy chain or light chain via the protease-cleavable linker. In some embodiments, the protease-cleavable linker comprises a matrix metalloprotease (MMP) cleavage site, a urokinase plasminogen activator cleavage site, a matriptase cleavage site, a legumain cleavage site, a Disintegrin and Metalloprotease (ADAM) cleavage site, or a caspase cleavage site. In some embodiments, the protease-cleavable linker comprises a matrix metalloprotease (MMP) cleavage site. In some embodiments, the MMP cleavage site is selected from an MMP2 cleavage site, an MMP7 cleavage site, an MMP9 cleavage site and an MMP13 cleavage site. In some embodiments, the MMP cleavage site comprises the sequence IPVSLRSG (SEQ ID NO: 19) or GPLGVR (SEQ ID NO: 21).

[0035] In some embodiments, the first masking domain comprises the sequence GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG (SEQ ID NO: 4). In some embodiments, the second masking domain comprises the sequence GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG (SEQ ID NO: 3). In some embodiments, the first masking domain comprises the sequence GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG (SEQ ID NO: 4), and the second masking domain comprises the sequence GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG (SEQ ID NO: 3).

[0036] In some embodiments, the first masking domain is linked to the amino-terminus of the heavy chain and the second masking domain is linked to the amino-terminus of the light chain. In some embodiments, the first masking domain is linked to the amino-terminus of the light chain and the second masking domain is linked to the amino-terminus of the heavy chain.

[0037] In some embodiments, the antibody binds an antigen selected from CD47, CD3, CD19, CD20, CD22, CD30, CD33, CD34, CD40, CD44, CD52, CD70, CD79a, CD123, Her-2, EphA2, lymphocyte associated antigen 1, VEGF or VEGFR, CTLA-4, LIV-1, nectin-4, CD74, SLTRK-6, EGFR, CD73, PD-L1, CD163, CCR4, CD147, EpCam, Trop-2, CD25, C5aR, Ly6D, alpha v integrin, B7H3, B7H4, Her-3, folate receptor alpha, GD-2, CEACAM5, CEACAM6, c-MET, CD266, MUC1, CD10, MSLN, sialyl Tn, Lewis Y, CD63, CD81, CD98, CD166, tissue factor (CD142), CD55, CD59, CD46, CD164, TGF beta receptor 1 (TGFβR1), TGFβR2, TGFβR3, FasL, MerTk, Axl, Clec12A, CD352, FAP, CXCR3, and CD5.

[0038] In some embodiments, the antibody binds CD47. In some embodiments, the antibody comprises a light chain variable region and a heavy chain variable region, wherein the heavy chain variable region comprises HCDR1 comprising SEQ ID NO: 25; HCDR2 comprising SEQ ID NO: 26; and HCDR3 comprising SEQ ID NO: 27; wherein the light chain variable region comprises LCDR1 comprising SEQ ID NO: 31; LCDR2 comprising SEQ ID NO: 32; and

LCDR3 comprising SEQ ID NO: 33 or 34. In some embodiments, the heavy chain variable region comprises an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence selected from SEQ ID NO: 22. In some embodiments, the light chain variable region comprises an amino acid sequence with at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 23 or 24. In some embodiments, the antibody comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising SEQ ID NOs: 25, 26, 27, 31, 32, and 33.

[0039] In some embodiments, the antibody that binds CD47 comprises a light chain variable region and a heavy chain variable region, wherein the heavy chain variable region comprises HCDR1 comprising SEQ ID NO: 28; HCDR2 comprising SEQ ID NO: 29; and HCDR3 comprising SEQ ID NO: 30; and wherein the light chain variable region comprises LCDR1 comprising SEQ ID NO: 35; LCDR2 comprising SEQ ID NO: 36; and LCDR3 comprising SEQ ID NO: 37 or 38. In some embodiments, the heavy chain variable region comprises an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 22. In some embodiments, the light chain variable region comprises an amino acid sequence with at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 23 or 24. In some embodiments, the antibody comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising SEQ ID NOs: 28, 29, 30, 35, 36, and 37.

[0040] In some embodiments, the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 22. In some embodiments, the light chain variable region comprises the amino acid sequence of SEQ ID NO: 23 or 24. In some embodiments, the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 22 and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 23.

[0041] In some embodiments, the masked antibody comprises a first masking domain linked to a heavy chain and a second masking domain linked to a light chain, wherein the first masking domain and the heavy chain comprises or consists of the sequence of SEQ ID NO: 39 or SEQ ID NO: 40, and the second masking domain and the light chain comprises or consists of the sequence of SEQ ID NO: 42.

[0042] In some embodiments, the antibody that binds CD47 blocks an interaction between CD47 and SIRPα.

[0043] In some embodiments, the antibody has reduced core fucosylation. In some embodiments, the antibody is afucosylated.

[0044] In some embodiments, the masked antibody is conjugated to a cytotoxic agent. In some embodiments, the cytotoxic agent is an antitubulin agent, a DNA minor groove binding agent, a DNA replication inhibitor, a DNA alkylator, a topoisomerase inhibitor, a NAMPT inhibitor, or a chemotherapy sensitizer. In some embodiments, the cytotoxic agent is an anthracycline, an auristatin, a camptothecin, a duocarmycin, an etoposide, an enediyine antibiotic, a lexitropsin, a taxane, a maytansinoid, a pyrrolobenzodiazepine, a combretastatin, a cryptophysin, or a vinca alkaloid. In some embodiments, the cytotoxic agent is auristatin E, AFP, AEB, AEVB, MMAF, MMAE, paclitaxel, docetaxel, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, melphalan, methotrexate, mitomycin C, a CC-1065 analogue, CBI, calicheamicin, maytansine, an analog of dolastatin 10, rhizoxin, or palytoxin, epothilone A, epothilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, eleutherobin, a tubulysin, a plocabulin, or maytansine. In some embodiments, the cytotoxic agent is an auristatin. In some embodiments, the cytotoxic agent is MMAE or MMAF.

[0045] In some embodiments, following purification of the masked antibody, the masked antibody is conjugated to a cytotoxic agent. In some embodiments, the cytotoxic agent is an antitubulin agent, a DNA minor groove binding agent, a DNA replication inhibitor, a DNA alkylator, a topoisomerase inhibitor, a NAMPT inhibitor, or a chemotherapy sensitizer. In some embodiments, the cytotoxic agent is an anthracycline, an auristatin, a camptothecin, a duocarmycin, an etoposide, an enediyine antibiotic, a lexitropsin, a taxane, a maytansinoid, a pyrrolobenzodiazepine, a combretastatin, a cryptophysin, or a vinca alkaloid.

[0046] In some embodiments, the cytotoxic agent is auristatin E, AFP, AEB, AEVB, MMAF, MMAE, paclitaxel, docetaxel, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, melphalan, methotrexate, mitomycin C, a CC-1065 analogue, CBI, calicheamicin, maytansine, an analog of dolastatin 10, rhizoxin, or palytoxin, epothilone A, epothilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, eleutherobin, a tubulysin, a plocabulin, or maytansine. In some embodiments, the cytotoxic agent is an auristatin. In some embodiments, the cytotoxic agent is MMAE or MMAF.

[0047] In some embodiments, a masked antibody is provided that has been purified by a process disclosed herein.

[0048] The summary of the disclosure described above is non-limiting, and other features and advantages of the disclosed antibodies and methods of making and using them will be apparent from the following drawings, the detailed description, the examples and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] Figures 1A-1B show that stability of an anti-CD47 masked antibody (Vel-IPV-hB6H12.3; also called CD47M) is sensitive to pH. (A) Stability of Vel-IPV-hB6H12.3 (measured as percentage high molecular weight [HMW]) after 3 days at 25°C in formulations of different pH with 150 mM NaCl (salt) or without added salt (no salt). (B) Stability of Vel-IPV-hB6H12.3 over 3 days at 25°C in formulations at pH 4 and pH 6.

[0050] Figures 2A-2B show data on demasking of Vel-IPV-hB6H12.3. (A) Levels of demasked Vel-IPV-hB6H12.3 increased over a 2-hour demasking reaction with MMP2. (B) The percentage of HMW Vel-IPV-hB6H12.3 over time during the demasking reaction.

[0051] Figures 3A-3B depict representative cytokine production induced by incubation of cancer patient whole blood samples incubated with hB6H12.3 or Vel-IPV-hB6H12.3 (CD47M) for 20 hours at 37°C. Figure 3A shows production of IP-10 and Figure 3B shows production of IL-1RA.

[0052] Figure 4 shows annexin V staining on HT1080 tumor cells from HT1080 xenograft model mice administered hB6H12.3, Vel-IPV-hB6H12.3 (CD47M), or hIgG1 isotype control ("h00 isotype").

DETAILED DESCRIPTION

[0053] This disclosure includes processes of preparing compositions comprising masked antibodies. Masked antibodies may comprise antibodies in which variable regions are masked by linkage of the amino-termini of variable regions chains to coiled-coil forming peptides. The coiled-coil forming peptides associate with one another to form coiled coils (i.e., the respective peptides each form coils and these coils are coiled around each other) and, in some embodiments, sterically inhibit binding of the antibody binding site to its target. Masking of antibodies by this format can reduce binding affinities (and cytotoxic activities in the case of ADC's) by over a hundred-fold, and in some embodiments, can reduce off-target effects. In some instances, however, masked antibodies may aggregate in solution, which may be undesirable. In some embodiments, the present processes reduce the aggregation of masked antibodies during and/or after their purification and concentration into a formulation for storage and use. In some embodiments, the process involves performing chromatography or other purification steps under conditions that may reduce aggregation.

[0054] Because this coiled-coil masking can be applied to any antibody, as it is independent of the specific CDR and variable region sequences of the antibody and independent of the target or epitope that an antibody binds, the processes herein are applicable to a wide variety of masked antibodies comprising coiled-coil masking polypeptides.

[0055] In some embodiments, the antibody is an anti-CD47 antibodies. It may be useful to administer anti-CD47 antibodies to patients in a masked form. For example, anti-CD47 IgG3 antibodies have been known to exhibit toxicities such as peripheral red blood cell depletion and platelet depletion, which decrease their usefulness as effective therapeutics against CD47-associated disorders such as, e.g., CD47 expressing cancers. Masked anti-CD47 antibodies may therefore be less toxic, for example, in that they can be activated by unmasking in the context of a tumor microenvironment, to effectively target the antibodies of the present invention specifically to CD47-expressing solid tumors. Accordingly, the processes herein are compatible with a variety of anti-CD47 antibodies, such as those specifically disclosed herein.

[0056] In certain exemplary embodiments, antibodies are provided that comprise a removable mask (e.g., a mask comprising a coiled coil domain) that blocks binding of the antibody to its antigenic target. In certain embodiments, a coiled coil domain is attached to the amino-terminus of one or more of the heavy and/or light chains of the antibody via a matrix metalloproteinase (MMP)-cleavable linker sequence. In a tumor microenvironment, for example, altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis (Kessenbrock (2011) Cell 141:52). MMPs represent the most prominent family of proteinases associated with tumorigenesis, and MMPs mediate many of the changes in the microenvironment during tumor progression. *Id.* Upon exposure of the antibody of the present invention to an MMP, the MMP linker sequence is cleaved, thus allowing removal of the coiled coil mask and enabling the antibody to bind its target antigen in a tumor microenvironment-specific manner.

[0057] In other embodiments, such as for use *in vitro*, such as in medical diagnostics, chemical processing, or industrial uses, masked antibodies may be useful so that antibody activity can be controlled by addition of an exogenous protease to the solution at an appropriate point to cleave off the coiled-coils of the mask and allow the antibodies to bind to their targets. Regardless of the application, however, addition of coiled-coil masks to antibodies could increase the risk of aggregation when the antibodies are purified or when they are stored in concentrated form. The preparation processes described herein may address this concern by reducing aggregation of masked antibodies as the process proceeds, allowing in some embodiments for higher masked antibody yield.

Definitions

[0058] So that the invention may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

[0059] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0060] Compositions or methods "comprising" one or more recited elements or steps may include other elements or steps not specifically recited. For example, a composition that comprises antibody may contain the antibody alone or in combination with other ingredients.

[0061] Compositions or methods "consisting essentially of" one or more steps may include elements or steps not specifically recited so long as any additional element or step does not materially alter the essential nature of the composition or method as recited in the claim. For example, other steps may be included so long as they do not materially alter the overall preparation process, such as wash steps or buffer changes.

[0062] Unless otherwise apparent from the context, when a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

[0063] Solvates in the context of the invention are those forms of the compounds of the invention that form a complex in the solid or liquid state through coordination with solvent molecules. Hydrates are one specific form of solvates, in which the coordination takes place with water. In certain exemplary embodiments, solvates in the context of the present invention are hydrates.

[0064] In processes herein, certain steps may be carried out at "neutral pH" or at "acidic pH." "Neutral pH" is broadly defined herein to encompass a pH roughly between 6.5 and 8.5, while "acidic pH" refers to a pH generally of 6.0 or less. Steps performed at acidic pH are often performed at pH 5 or less or pH 4.5 or less, however, as described further below.

[0065] In processes herein, "room temperature" means either that the process is performed without temperature control, such as without temperature control other than that imposed on the overall facility or building in which the process is performed, or that is otherwise conducted at a temperature of about 15 °C to 28 °C, such as 18 °C to 25 °C, or 20 °C to 25 °C.

[0066] The term "virus removal" refers to a process of treating a composition to remove viruses or potential viruses or virus particles from a composition. Virus removal, for example, can be performed by filtering the composition with a filter that is large enough for the desired protein materials to pass through but small enough for virus particles to be trapped in the filter. Virus removal can also be performed by irradiation or other chemical procedures so long as the process does not damage the protein in the composition.

[0067] The term "nanofiltration" refers to a process of virus removal using a filter with, for example, 1-30 nm, 10-30 nm, or 20 nm pores.

[0068] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0069] The term "antibody" denotes immunoglobulin proteins produced by the body in response to the presence of an antigen and that bind to the antigen, as well as antigen-binding fragments and engineered variants thereof. Hence, the term "antibody" includes, for example, intact monoclonal antibodies (e.g., antibodies produced using hybridoma technology) and it also encompasses antigen-binding antibody fragments, such as a $F(ab')_2$, a Fv fragment, a diabody, a single-chain antibody, an scFv fragment, or an scFv-Fc. Genetically engineered intact antibodies and fragments such as chimeric antibodies, humanized antibodies, single-chain Fv fragments, single-chain antibodies, diabodies, minibodies, linear antibodies, bispecific or bivalent, multivalent or multi-specific (e.g., bispecific) hybrid antibodies, and the like. Thus, the term "antibody" is used expansively to include any protein that comprises an antigen-binding site of an antibody and is capable of specifically binding to its antigen.

[0070] The term "antibody" includes a "naked" antibody that is not bound (i.e., covalently or non-covalently bound) to a masking compound of the invention. The term antibody also embraces a "masked" antibody, which comprises an antibody that is covalently or non-covalently bound to one or more masking compounds such as, e.g., coiled coil peptides, as described further herein. The term antibody includes a "conjugated" antibody or an "antibody-drug conjugate (ADC)" in which an antibody is covalently or non-covalently bound to a pharmaceutical agent, e.g., to a cytostatic or cytotoxic drug. In certain embodiments, an antibody is a naked antibody or antigen-binding fragment that optionally is conjugated to a pharmaceutical agent, e.g., to a cytostatic or cytotoxic drug. In other embodiments, an antibody is a masked antibody or antigen-binding fragment that optionally is conjugated to a pharmaceutical agent, e.g., to a cytostatic or cytotoxic drug.

[0071] Antibodies typically comprise a heavy chain variable region and a light chain variable region, each comprising three complementary determining regions (CDRs) with surrounding framework (FR) regions, for a total of six CDRs. An antibody light or heavy chain variable region (also referred to herein as a "light chain variable domain" ("VL domain") or "heavy chain variable domain" ("VH domain"), respectively) comprises "framework" regions interrupted by three "complementarity determining regions" or "CDRs." The framework regions serve to align the CDRs for specific binding to an epitope of an antigen. Thus, the term "CDR" refers to the amino acid residues of an antibody that are primarily responsible for antigen binding. From amino-terminus to carboxyl-terminus, both VL and VH domains comprise the following framework (FR) and CDR regions: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[0072] Naturally occurring antibodies are usually tetrameric and consist of two identical pairs of heavy and light chains. In each pair, the light and heavy chain variable regions (VL and VH) are together primarily responsible for binding to an antigen, and the constant regions are primarily responsible for the antibody effector functions. Five classes of antibodies (IgG, IgA, IgM, IgD, and IgE) have been identified in higher vertebrates. IgG comprises the major class, and it normally exists as the second most abundant protein found in plasma. In humans, IgG consists of four subclasses, designated IgG1, IgG2, IgG3, and IgG4. Each immunoglobulin heavy chain possesses a constant region that comprises constant region protein domains (CH1, hinge, CH2, and CH3; IgG3 also contains a CH4 domain) that are substantially invariant for a given subclass in a species. Antibodies as defined herein, may include these natural forms as well as various antigen-binding fragments, as described above, antibodies with modified heavy chain constant regions, bispecific and multispecific antibodies, and masked antibodies.

[0073] The assignment of amino acids to each variable region domain is in accordance with the definitions of Kabat, Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD, 1987 and 1991). Kabat also provides a widely used numbering convention (Kabat numbering) in which corresponding residues between different heavy chain variable regions or between different light chain variable regions are assigned the same number. CDRs 1, 2 and 3 of a VL domain are also referred to herein, respectively, as CDR-L1, CDR-L2 and CDR-L3. CDRs 1, 2 and 3 of a VH domain are also referred to herein, respectively, as CDR-H1, CDR- H2 and CDR-H3. If so noted, the assignment of CDRs can be in accordance with IMGT® (Lefranc et al., Developmental & Comparative Immunology 27:55-77; 2003) in lieu of Kabat.

[0074] An "antigen-binding site" of an antibody is that portion of an antibody that is sufficient to bind to its antigen. The minimum such region is typically a fragment of a variable domain comprising six CDRs (or three CDRs in the case of a single-domain antibody). In some

embodiments, an antigen-binding site of an antibody comprises both a heavy chain variable (VH) domain and a light chain variable (VL) domain that bind to a common epitope. Within the context of the present invention, an antibody may include one or more components in addition to an antigen-binding site, such as, for example, a second antigen-binding site of an antibody (which may bind to the same or a different epitope or to the same or a different antigen), a peptide linker, an immunoglobulin constant region, an immunoglobulin hinge, an amphipathic helix (see Pack and Pluckthun, Biochem. 31: 1579- 1584, 1992), a non-peptide linker, an oligonucleotide (see Chaudri et al, FEBS Letters 450:23-26, 1999), a cytostatic or cytotoxic drug, and the like, and may be a monomeric or multimeric protein. Examples of molecules comprising an antigen-binding site of an antibody are known in the art and include, for example, Fv, single-chain Fv (scFv), Fab, Fab', F(ab')2, F(ab)c, diabodies, minibodies, nanobodies, Fab-scFv fusions, bispecific (scFv)4-IgG, and bispecific (scFv)2-Fab. (See, *e.g.*, Hu et al , Cancer Res. 56:3055-3061, 1996; Atwell et al., Molecular Immunology 33: 1301-1312, 1996; Carter and Merchant, Curr. Op. Biotechnol. 8:449-454, 1997; Zuo et al., Protein Engineering 13:361-367, 2000; and Lu et al., J. Immunol. Methods 267:213-226, 2002.)

[0075] Numbering of the heavy chain constant region is via the EU index as set forth in Kabat (Kabat, Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD, 1987 and 1991).

[0076] Unless the context dictates otherwise, the term "monoclonal antibody" is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" can include an antibody that is derived from a single clone, including any eukaryotic, prokaryotic or phage clone. In particular embodiments, the antibodies described herein are monoclonal antibodies.

[0077] The term "chimeric antibody" refers to an antibody in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in an antibody derived from a particular species (e.g., human) or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in an antibody derived from another species (e.g., mouse) or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity.

[0078] The term "humanized VH domain" or "humanized VL domain" refers to an immunoglobulin VH or VL domain comprising some or all CDRs entirely or substantially from a non-human donor immunoglobulin (e.g., a mouse or rat) and variable domain framework sequences entirely or substantially from human immunoglobulin sequences. The non-human immunoglobulin providing the CDRs is called the "donor" and the human immunoglobulin

providing the framework is called the "acceptor." In some instances, humanized antibodies will retain some non-human residues within the human variable domain framework regions to enhance proper binding characteristics (e.g., mutations in the frameworks may be required to preserve binding affinity when an antibody is humanized).

[0079] A "humanized antibody" is an antibody comprising one or both of a humanized VH domain and a humanized VL domain. Immunoglobulin constant region(s) need not be present, but if they are, they are entirely or substantially from human immunoglobulin constant regions.

[0080] Although humanized antibodies often incorporate all six CDRs (preferably as defined by Kabat or IMGT®) from a mouse antibody, they can also be made with fewer than all six CDRs (e.g., at least 3, 4, or 5) from a mouse antibody (e.g., Pascalis et al., J. Immunol. 169:3076, 2002; Vajdos et al., Journal of Molecular Biology, 320: 415-428, 2002; Iwahashi et al., Mol. Immunol. 36:1079-1091, 1999; Tamura et al, Journal of Immunology, 164: 1432- 1441, 2000).

[0081] A CDR in a humanized antibody is "substantially from" a corresponding CDR in a non-human antibody when at least 60%, at least 85%, at least 90%, at least 95% or 100% of corresponding residues (as defined by Kabat (or IMGT)) are identical between the respective CDRs. In particular variations of a humanized VH or VL domain in which CDRs are substantially from a non-human immunoglobulin, the CDRs of the humanized VH or VL domain have no more than six (*e.g.*, no more than five, no more than four, no more than three, no more than two, or nor more than one) amino acid substitutions (preferably conservative substitutions) across all three CDRs relative to the corresponding non-human VH or VL CDRs. The variable region framework sequences of an antibody VH or VL domain or, if present, a sequence of an immunoglobulin constant region, are "substantially from" a human VH or VL framework sequence or human constant region, respectively, when at least about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% of corresponding residues (as defined by Kabat numbering for the variable region and EU numbering for the constant region), or about 100% of corresponding residues (as defined by Kabat numbering for the variable region and EU numbering for the constant region) are identical. Hence, all parts of a humanized antibody, except the CDRs, are typically entirely or substantially from corresponding parts of natural human immunoglobulin sequences.

[0082] Two amino acid sequences have "100% amino acid sequence identity" if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced

by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art. (See, *e.g.*, Peruski and Peruski, The Internet and the New Biology: Tools for Genomic and Molecular Research (ASM Press, Inc. 1997); Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in Methods in Gene Biotechnology 123-151 (CRC Press, Inc. 1997); Bishop (ed.), Guide to Human Genome Computing (2nd ed., Academic Press, Inc. 1998).) Two amino acid sequences are considered to have "substantial sequence identity" if the two sequences have at least about 80%, at least about 85%, at about least 90%, or at least about 95% sequence identity relative to each other.

[0083] Percentage sequence identities are determined with antibody sequences maximally aligned by the Kabat numbering convention. After alignment, if a subject antibody region (*e.g.*, the entire variable domain of a heavy or light chain) is being compared with the same region of a reference antibody, the percentage sequence identity between the subject and reference antibody regions is the number of positions occupied by the same amino acid in both the subject and reference antibody region divided by the total number of aligned positions of the two regions, with gaps not counted, multiplied by 100 to convert to percentage.

[0084] Specific binding of an antibody to its target antigen typically refers an affinity of at least about 10^6, about 10^7, about 10^8, about 10^9, or about 10^{10} M^{-1}. Specific binding is detectably higher in magnitude and distinguishable from non-specific binding occurring to at least one non-specific target. Specific binding can be the result of formation of bonds between particular functional groups or particular spatial fit (e.g., lock and key type), whereas nonspecific binding is typically the result of van der Waals forces.

[0085] The term "epitope" refers to a site of an antigen to which an antibody binds. An epitope can be formed from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of one or more proteins. Epitopes formed from contiguous amino acids are typically retained upon exposure to denaturing agents, e.g., solvents, whereas epitopes formed by tertiary folding are typically lost upon treatment with denaturing agents, e.g., solvents. An epitope typically includes at least about 3, and more usually, at least about 5, at least about 6, at least about 7, or about 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and two-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols, in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed. (1996).

[0086] Antibodies that recognize the same or overlapping epitopes can be identified in a simple immunoassay showing the ability of one antibody to compete with the binding of another

antibody to a target antigen. The epitope of an antibody can also be defined by X-ray crystallography of the antibody bound to its antigen to identify contact residues.

[0087] Alternatively, two antibodies have the same epitope if all amino acid mutations in the antigen that reduce or eliminate binding of one antibody reduce or eliminate binding of the other (provided that such mutations do not produce a global alteration in antigen structure). Two antibodies have overlapping epitopes if some amino acid mutations that reduce or eliminate binding of one antibody reduce or eliminate binding of the other antibody.

[0088] Competition between antibodies can be determined by an assay in which a test antibody inhibits specific binding of a reference antibody to a common antigen (see, e.g., Junghans et al., Cancer Res. 50: 1495, 1990). A test antibody competes with a reference antibody if an excess of a test antibody inhibits binding of the reference antibody.

[0089] Antibodies identified by competition assay (competing antibodies) include antibodies that bind to the same epitope as the reference antibody and antibodies that bind to an adjacent epitope sufficiently proximal to the epitope bound by the reference antibody for steric hindrance to occur. Antibodies identified by a competition assay also include those that indirectly compete with a reference antibody by causing a conformational change in the target protein thereby preventing binding of the reference antibody to a different epitope than that bound by the test antibody.

[0090] An antibody effector function refers to a function contributed by an Fc region of an Ig. Such functions can be, for example, antibody-dependent cellular cytotoxicity (ADCC), antibody- dependent cellular phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC). Such function can be affected by, for example, binding of an Fc region to an Fc receptor on an immune cell with phagocytic or lytic activity or by binding of an Fc region to components of the complement system. Typically, the effect(s) mediated by the Fc -binding cells or complement components result in inhibition and/or depletion of the targeted cell. Fc regions of antibodies can recruit Fc receptor (FcR)-expressing cells and juxtapose them with antibody-coated target cells. Cells expressing surface FcR for IgGs including FcγRIII (CD16), FcγRII (CD32) and FcγRIII (CD64) can act as effector cells for the destruction of IgG-coated cells. Such effector cells include monocytes, macrophages, natural killer (NK) cells, neutrophils and eosinophils. Engagement of FcγR by IgG activates ADCC or ADCP. ADCC is mediated by CD16+ effector cells through the secretion of membrane pore-forming proteins and proteases, while phagocytosis is mediated by CD32+ and CD64+ effector cells (see Fundamental Immunology, 4th ed., Paul ed., Lippincott-Raven, N.Y., 1997, Chapters 3, 17 and 30; Uchida et al., J. Exp. Med. 199:1659-69, 2004; Akewanlop et al., Cancer Res. 61:4061-65, 2001; Watanabe et al., Breast Cancer Res. Treat. 53: 199-207, 1999).

[0091] In addition to ADCC and ADCP, Fc regions of cell-bound antibodies can also activate the complement classical pathway to elicit CDC. C1q of the complement system binds to the Fc regions of antibodies when they are complexed with antigens. Binding of C1q to cell-bound antibodies can initiate a cascade of events involving the proteolytic activation of C4 and C2 to generate the C3 convertase. Cleavage of C3 to C3b by C3 convertase enables the activation of terminal complement components including C5b, C6, C7, C8 and C9. Collectively, these proteins form membrane-attack complex pores on the antibody-coated cells. These pores disrupt the cell membrane integrity, killing the target cell (see Immunobiology, 6th ed., Janeway et al, Garland Science, N. Y., 2005, Chapter 2).

[0092] The term "antibody-dependent cellular cytotoxicity" or "ADCC" refers to a mechanism for inducing cell death that depends on the interaction of antibody-coated target cells with immune cells possessing lytic activity (also referred to as effector cells). Such effector cells include natural killer cells, monocytes/macrophages and neutrophils. The effector cells attach to an Fc region of Ig bound to target cells via their antigen-combining sites. Death of the antibody-coated target cell occurs as a result of effector cell activity.

[0093] The term "antibody-dependent cellular phagocytosis" or "ADCP" refers to the process by which antibody-coated cells are internalized, either in whole or in part, by phagocytic immune cells (e.g., by macrophages, neutrophils and/or dendritic cells) that bind to an Fc region of Ig.

[0094] The term "complement-dependent cytotoxicity" or "CDC" refers to a mechanism for inducing cell death in which an Fc region of a target-bound antibody activates a series of enzymatic reactions culminating in the formation of holes in the target cell membrane.

[0095] Typically, antigen-antibody complexes such as those on antibody-coated target cells bind and activate complement component C1q, which in turn activates the complement cascade leading to target cell death. Activation of complement may also result in deposition of complement components on the target cell surface that facilitate ADCC by binding complement receptors (e.g., CR3) on leukocytes.

[0096] An "antibody-drug conjugate" refers to an antibody conjugated to a cytotoxic agent or cytostatic agent. Typically, antibody-drug conjugates bind to a target antigen on a cell surface, followed by internalization of the antibody-drug conjugate into the cell and subsequent release of the drug into the cell.

[0097] Typically, antigen-antibody complexes such as those on antibody-coated target cells bind and activate complement component C1q, which in turn activates the complement cascade leading to target cell death. Activation of complement may also result in deposition of

complement components on the target cell surface that facilitate ADCC by binding complement receptors (e.g., CR3) on leukocytes.

[0098] A "cytotoxic effect" refers to the depletion, elimination and/or killing of a target cell. A "cytotoxic agent" refers to a compound that has a cytotoxic effect on a cell, thereby mediating depletion, elimination and/or killing of a target cell. In certain embodiments, a cytotoxic agent is conjugated to an antibody or administered in combination with an antibody. Suitable cytotoxic agents are described further herein.

[0099] A "cytostatic effect" refers to the inhibition of cell proliferation. A "cytostatic agent" refers to a compound that has a cytostatic effect on a cell, thereby mediating inhibition of growth and/or expansion of a specific cell type and/or subset of cells. Suitable cytostatic agents are described further herein.

[0100] The terms "patient" and "subject" refer to organisms to be treated by the methods described herein and includes human and other mammalian subjects such as non-human primates, mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), rabbits, rats, mice, and the like and transgenic species thereof, that receive either prophylactic or therapeutic treatment. In certain exemplary embodiments, a subject is a human patient suffering from or at risk of developing cancer, e.g., a solid tumor, that optionally secretes one or more proteases capable of cleaving a masking domain (e.g., a coiled coil masking domain) of an antibody described herein.

[0101] As used herein, the terms, "treat," "treatment" and "treating" includes any effect, e.g., lessening, reducing, modulating, ameliorating or eliminating, that results in the improvement of the condition, disease, disorder, and the like, or ameliorating a symptom thereof, such as for example, reduced number of cancer cells, reduced tumor size, reduced rate of cancer cell infiltration into peripheral organs, or reduced rate of tumor metastasis or tumor growth.

[0102] As used herein, the term "effective amount" refers to the amount of a compound (e.g., an anti-CD47 antibody or masked antibody) sufficient to effect beneficial or desired results. The term "effective amount," in the context of treatment of a CD47-expressing disorder by administration of an anti-CD47 antibody as described herein, refers to an amount of such antibody that is sufficient to inhibit the occurrence or ameliorate one or more symptoms of a CD47-related disorder (e.g., a CD47-expressing cancer). An effective amount of an antibody is administered in an "effective regimen." The term "effective regimen" refers to a combination of amount of the antibody being administered and dosage frequency adequate to accomplish prophylactic or therapeutic treatment of the disorder (e.g., prophylactic or therapeutic treatment of a CD47-expressing cancer).

[0103] The term "pharmaceutically acceptable" means approved or approvable by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "pharmaceutically compatible ingredient" refers to a pharmaceutically acceptable diluent, adjuvant, excipient, or vehicle with which an antibody is formulated.

[0104] The phrase "pharmaceutically acceptable salt," refers to pharmaceutically acceptable organic or inorganic salts. Exemplary salts include sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p toluenesulfonate, and pamoate (i.e., 1,1'-methylene bis-(2 hydroxy-3-naphthoate) salts. A pharmaceutically acceptable salt may further comprise an additional molecule such as, e.g., an acetate ion, a succinate ion or other counterion. A counterion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

I. Masking Domains Comprising Coiled-Coils

[0105] In certain embodiments, an antibody is associated with a masking domain comprising coiled coil domains (also referred to as a "coiled coil masking domain") that blocks binding of the antibody to its antigen target. In various embodiments, an antibody associated with a masking domain is referred to as a "masked antibody."

[0106] A coiled coil is a structural motif in proteins and peptides in which two or more alpha-helices wind around each other to form a supercoil. There can be two, three or four helices in a coiled coil bundle and the helices can either run in the same (parallel) or in the opposite (antiparallel) directions.

[0107] Coiled coils typically comprise sequence elements of three and four residues whose hydrophobicity pattern and residue composition are compatible with the structure of amphipathic alpha-helices. The alternating three and four residue sequence elements constitute heptad repeats in which the amino acids are designated 'a,' 'b,' 'c,' 'd,' 'e,' 'f' and 'g.' Residues in positions 'a' and 'd' are generally hydrophobic and form a zig-zag pattern of knobs and holes that interlock with a similar pattern on another strand to form a tight-fitting hydrophobic core. Of the remaining residues, 'b,' 'c' and 'f' tend to be charged. Therefore, the formation of a heptad repeat depends on the physical properties of hydrophobicity and charge

that are required at a particular position, not on a specific amino acid. In certain exemplary embodiments, coiled coils of the present invention are formed from two coiled coil-forming peptides.

[0108] Examples of consensus formulae for heptad repeats in coiled coil-forming peptides are provided by W02011034605, incorporated herein by reference in its entirety for all purposes.

[0109] Exemplary consensus formulae according to certain embodiments are set forth below:

Formula 1: (X1, X2, X3, X4, X5, X6, X7)n, wherein:

X1 is a hydrophobic amino acid or asparagine;

X2, X3 and X6 are any amino acid;

X4 is a hydrophobic amino acid;

X5 and X7 are each a charged amino acid residue; and

n is a positive integer.

Formula 2: (X1', X2', X3', X4', X5, X6, X7)n, wherein:

X1' is a hydrophobic amino acid or asparagine;

X2', X'3 and X'6 are each any amino acid residue;

X4' is hydrophobic amino acid;

X5' and X7' are each a charged amino acid residue;

wherein n in formula 1 and 2 is greater or equal to 2; and

n is a positive integer.

[0110] In certain embodiments in which peptides of Formula 1 and Formula 2 form a coiled coil, X5 of Formula 1 is opposite in charge to X'7 of Formula 2, and X7 or Formula 1 is opposite in charge to X'5 of Formula 2. Heptad repeats within a coiled coil forming peptide can be the same or different from each other while conforming to Formula 1 and/or 2.

[0111] Coiled coils can be homodimeric or heterodimeric. Examples of peptides that can form coiled coil according to certain exemplary embodiments are shown in Table 1 below (SEQ ID NOs: 1-4). The peptide sequences can be used as is, or their components can be used in other combinations. For example, the Vel coiled coil-forming peptide can be used with other linker sequences. Sequences shown for light chains can also be used with heavy chains and vice versa.

[0112] In certain exemplary embodiments, a bivalent antibody comprising two light and heavy chain pairs is provided, wherein the amino-termini of one or more of the light chains and/or the heavy chains are linked via linkers comprising a protease cleavage site to coiled coil-forming peptides that associate to form a coiled coil, reducing binding affinity of the light and heavy chain pair to a target. Optionally, the peptides associate without forming a disulfide bridge.

[0113] Optionally, the two light and heavy chain pairs are the same. Optionally, the two light and heavy chain pairs are different. Optionally, the light chains include a light chain variable region and light chain constant region and the heavy chains include a heavy chain variable region and heavy chain constant region. Optionally, the heavy chain region includes CH1, hinge, CH2 and CH3 regions. Optionally, the two light chain are linked to a first heterologous peptide and the two heavy chains to a second heterologous peptide.

[0114] Optionally, the protease cleavage site is an MMP1, MMP2, and/or MMP12 cleavage site.

[0115] In some cases, antigen binding is reduced at least 100-fold by the presence of a masking domain (e.g., a coiled coil masking domain). In some embodiments, antigen binding is reduced 200-1500-fold by the presence of a masking domain (e.g., a coiled coil masking domain). In some embodiments, cytotoxicity of the conjugate is reduced at least 100-fold by the presence of a masking domain (e.g., a coiled coil masking domain). In some embodiments, cytotoxicity of the conjugate is reduced at least 200-1500-fold by the presence of a masking domain (e.g., a coiled coil masking domain).

[0116] Optionally, the coiled coil forming peptides are linked to the amino-termini of the heavy and light chains in the same orientation. Optionally, the coiled coil-forming peptides are linked to the amino-termini of the heavy and light chains in opposing orientations. Optionally, multiple copies of the coiled coil forming peptide are linked in tandem to the amino-termini of the heavy and light chains.

[0117] In some embodiments, a masking domain comprises a VelA coiled-coil domain (SEQ ID NO: 1). In some embodiments, a masking domain comprises a VelB coiled-coil domain (SEQ ID NO: 2). In some embodiments, a masked antibody comprises a first masking domain comprising a VelA coiled-coil domain and a second masking domain comprising a VelB coiled-coil domain, wherein the first masking domain is linked to the light chain and the second masking domain is linked to the heavy chain, or vice versa. In some embodiments, each masking domain is linked to the amino-terminus of the heavy chain or light chain.

Table 1: Nonlimiting exemplary coiled-coil masking domains

Description	Sequence	SEQ ID NO
VelA coiled-coil	VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL	1
VelB coiled-coil	VDELQAEVDQLEDENYALKTKVAQLRKKVEKL	2
VelA-IPV	GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG	3
VelB-IPV	GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG	4

[0118] In certain exemplary embodiments, amino acid substitutions in a variant peptide that forms a coiled coil are conservative substitutions. For purposes of classifying amino acids

substitutions as conservative or nonconservative, the following amino acid substitutions are considered conservative substitutions: serine substituted by threonine, alanine, or asparagine; threonine substituted by proline or serine; asparagine substituted by aspartic acid, histidine, or serine; aspartic acid substituted by glutamic acid or asparagine; glutamic acid substituted by glutamine, lysine, or aspartic acid; glutamine substituted by arginine, lysine, or glutamic acid; histidine substituted by tyrosine or asparagine; arginine substituted by lysine or glutamine; methionine substituted by isoleucine, leucine or valine; isoleucine substituted by leucine, valine, or methionine; leucine substituted by valine, isoleucine, or methionine; phenylalanine substituted by tyrosine or tryptophan; tyrosine substituted by tryptophan, histidine, or phenylalanine; proline substituted by threonine; alanine substituted by serine; lysine substituted by glutamic acid, glutamine, or arginine; valine substituted by methionine, isoleucine, or leucine; and tryptophan substituted by phenylalanine or tyrosine. Conservative substitutions can also mean substitutions between amino acids in the same class. Classes are as follows: Group I (hydrophobic side chains): met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gin, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe.

Linkers and Cleavage Sites

[0119] In certain embodiments of the invention, a masking domain comprises a linker, which is located between the coiled-coil domain and the antibody chain to which the coiled-coil domain is attached. The linkers can be any segments of amino acids conventionally used as linker for joining peptide domains. Suitable linkers can vary in length, such as from 1-20, 2-15, 3-12, 4-10, 5, 6, 7, 8, 9 or 10. Some such linkers include a segment of polyglycine. Some such linkers include one or more serine residues, often at positions flanking the glycine residues. Other linkers include one or more alanine residues. Glycine and glycine-serine polymers are relatively unstructured, and therefore may be able to serve as a neutral tether between components. Glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (see Scheraga, Rev. Computational Chem. 11173-142 (1992)). Some exemplary linkers are in the form S(G)nS, wherein n is from 5-20. Other exemplary linkers are (G)n, glycine-serine polymers (including, for example, (GS)n, (GSGGS)n [(GSGGS) is SEQ ID NO: 5) and (GGGS)n, [(GGGS) is SEQ ID NO: 6) where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers known in the art. Some examples of linkers are Ser-(Gly)10-Ser (SEQ ID NO: 7), Gly-Gly- Ala-Ala (SEQ ID NO: 8), Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 9), Leu-Ala-Ala-Ala-Ala (SEQ ID NO: 10), Gly-Gly-Ser-Gly (SEQ ID NO: 11), Gly-Gly-Ser-Gly-Gly (SEQ ID NO: 12), Gly-Ser-

Gly-Ser-Gly (SEQ ID NO: 13), Gly-Ser-Gly-Gly-Gly (SEQ ID NO: 14), Gly-Gly-Gly-Ser-Gly (SEQ ID NO: 15), Gly-Ser-Ser-Ser-Gly (SEQ ID NO: 16), and the like.

[0120] The protease site is preferably recognized and cleaved by a protease expressed extracellularly so it contacts a masked antibody, releasing the masked antibody and allowing it to contact its target, such as a receptor extracellular domain or soluble ligand. Several matrix metalloproteinase sites (MMP1-28) are suitable. MMPs play a role in tissue remodeling and are implicated in neoplastic processes such as morphogenesis, angiogenesis and metastasis. Some exemplary protease sites are PLG-XXX (SEQ ID NO: 17), a well-known endogenous sequence for MMPs, PLG-VR (SEQ ID NO: 18) (W02014193973) and IPVSLRSG (SEQ ID NO: 19) (Turk et al., Nat. Biotechnol., 2001, 19, 661-667), LSGRSDNY (SEQ ID NO: 20) (Cytomyx) and GPLGVR (SEQ ID NO: 21) (Chang et al., Clin. Cancer Res. 2012 Jan 1; 18(1):238-47). Additional examples of MMPs are provided in US 2013/0309230, WO 2009/025846, WO 2010/081173, WO 2014/107599, WO 2015/048329, US 20160160263, and Ratnikov et al., Proc. Natl. Acad. Sci. USA, 111: E4148-E4155 (2014).

Table 2. Protease cleavage sequences. The MMP-cleavage site is indicated by * while the uPA/matriptase/legumain cleavage sites are indicated by **.

Cleavage Site Name	Sequence
M2	GPLG*VR** (SEQ ID NO: 21)
IPV	IPVS*LR**SG (SEQ ID NO: 19)

[0121] In some embodiments, a masking domain comprises a coiled-coil domain, a linker, and a protease cleavage sequence. In some such embodiments, a masking domain is VelA-IPV (SEQ ID NO: 3), wherein the coiled-coil domain is VelA (SEQ ID NO: 1), the linker is GS, and the protease cleavage sequence is IPVSLRSG (SEQ ID NO: 19). In some embodiments, a masking domain comprises a coiled-coil domain, a linker, and a protease cleavage sequence. In some such embodiments, a masking domain is VelB-IPV (SEQ ID NO: 4), wherein the coiled-coil domain is VelB (SEQ ID NO: 2), the linker is GS, and the protease cleavage sequence is IPVSLRSG (SEQ ID NO: 19).

[0122] In some embodiments, a first masking domain is a VelA-IPV masking domain (SEQ ID No: 3), which includes an MMP protease site, and a second masking domain is a VelB-IPV masking domain (SEQ ID NO: 4), which also includes an MMP protease site. In some embodiments, the first masking domain is linked to the light chain and the second masking domain is linked to a heavy chain, or vice versa. In some embodiments, each masking domain is linked to the amino-terminus of the heavy chain or light chain.

II. Linking Coiled Coil Masking Agents to Antibodies

[0123] Coiled coil forming peptides are linked to the amino-termini of antibody variable regions via a linker including a protease site. A typical antibody includes a heavy and light chain variable region, in which case a coiled coil forming peptide is linked to the amino-termini of each. A bivalent antibody has two binding sites, which may or may not be the same. In a normal monospecific antibody, the binding sites are the same and the antibody has two identical light and heavy chain pairs. In this case, each heavy chain is linked to the same coiled coil forming peptide and each light chain to the same coiled coil forming peptide (which may or may not be the same as the peptide linked to the heavy chain). In a bispecific antibody, the binding sites are different and formed from two different heavy and light chain pairs. In such a case, the heavy and light chain variable region of one binding site are respectively linked to coiled coil forming peptides as are the heavy and light chain variable regions of the other binding site. Typically both heavy chain variable regions are linked to the same type of coiled coil forming peptide as are both light chain variable regions.

[0124] A coiled coil-forming peptide can be linked to an antibody variable region via a linker including a protease site. Typically, the same linker with the same protease cleavage site is used for linking each heavy or light chain variable region of an antibody to a coiled coil peptide. The protease cleavage site should be one amenable to cleavage by a protease present extracellularly in the intended target tissue or pathology, such as a cancer, such that cleavage of the linker releases the antibody from the coiled coil masking its activity allowing the antibody to bind to its intended target, such as a cell-surface antigen or soluble ligand.

[0125] As well as the variable regions, a masked antibody typically includes all or part of a constant region, which can include any or all of a light chain constant region, CH1, hinge, CH2 and CH3 regions. As with other antibodies one or more carboxy-terminal residues can be proteolytically processed or derivatized.

[0126] Coiled coils can be formed from the same peptide forming a homodimer or two different peptides forming a heterodimer. For formation of a homodimer, light and heavy antibody chains are linked to the same coiled coil forming peptide. For formation of a heterodimer, light and heavy antibody chains are linked to different coiled coils peptides. For some pairs of coiled coil forming peptides, it is preferred that one of the pair be linked to the heavy chain and the other to the light chain of an antibody although the reverse orientation is also possible.

[0127] Each antibody chain can be linked to a single coiled coil forming peptide or multiple such peptides in tandem (*e.g.*, two, three, four or five copies of a peptide). If the latter, the peptides in tandem linkage are usually the same. Also if tandem linkage is employed, light and heavy chains are usually linked to the same number of peptides.

[0128] Linkage of antibody chains to coiled coil forming peptides can reduce the binding affinity of an antibody by at least about 10-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold, at least about 1000-fold or at least about 1500-fold relative to the same antibody without such linkage or after cleavage of such linkage. In some such antibodies, binding affinity is reduced between about 50-5000-fold, between about 50-1500-fold, between about 100-1500-fold, between about 200-1500-fold, between about 500-1500-fold, between about 500-5000-fold, between about 50-1000-fold, between about 100-1000-fold, between about 200-1000-fold, between about 500-1000-fold, between about 50-500-fold, or between about 100-500-fold. Effector functions of the antibody, such as ADCC, phagocytosis, and CDC or cytotoxicity as a result of linkage to a drug in an antibody drug conjugate can be reduced by the same factors or ranges. Upon proteolytic cleavage that serves to unmask an antibody or otherwise remove the mask from the antibody, the restored antibody typically has an affinity or effect function that is within a factor of 2, 1.5 or preferably unchanged within experimental error compared with an otherwise identical control antibody, which has never been masked.

III. Processes for Preparing Masked Antibody Compositions

[0129] Masked antibody compositions may be made according to the following processes. A starting composition for the preparation processes, which contains the polypeptides intended to make up the composition, may be obtained from various sources, for example, from a cell lysate or a cell lysate after certain initial purification steps have been performed. In some embodiments, for example, cells expressing the polypeptides may be filtered to remove cellular components and to place the starting composition into an appropriate buffer for subsequent steps. In some embodiments, the starting composition is treated with a detergent, for example, to remove retroviruses, cellular components, or phospholipid membrane-comprising components.

[0130] In some embodiments, the starting composition is subjected to protein A chromatography. In some embodiments, the starting composition has not previously been subjected to any chromatography procedures prior to the protein A chromatography. In some embodiments, the starting composition comprising the masked antibody is loaded onto a protein A chromatography column under conditions suitable for binding the masked antibody to the protein A chromatography column, the column is then washed, and following at least one wash, the desired polypeptide material is eluted from the column. In some embodiments, the protein A chromatography is performed at room temperature. In some embodiments, the protein A chromatography is performed at neutral pH, meaning that the column equilibration buffer and the starting composition in the loading buffer are at neutral pH. For example, the

chromatography may be conducted at pH 7.0-8.0, such as at pH 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, or 8.0. Thus, for example, the equilibration buffer for the column may be at neutral pH and/or the starting composition loaded onto the column may be at neutral pH.

[0131] As noted above, the chromatography process may include one or more wash steps following column loading and preceding elution. The wash steps may comprise washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH of less than 5.5 or at pH 4.5-5.5. In some embodiments, the at least one acidic wash buffer is at pH 4.7-5.4, pH 4.8, pH 4.9, pH 5, pH 5.1, or pH 5.2. In some embodiments, the at least one acidic wash buffer is at pH 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, or 5.5.

[0132] In some embodiments, washes may be conducted in buffers of increasing acidity from initial to final wash. In some embodiments, one or more wash steps may be performed in the column equilibration buffer, and then may include at least one final wash step prior to elution conducted at acidic pH in an acidic wash buffer, such as the acetate wash buffer described above.

[0133] Following washing, the process may comprise eluting the masked antibody from the protein A column in an acidic elution buffer to form a protein A eluate comprising the masked antibody. In some embodiments, the elution buffer may be more acidic than the preceding wash buffer(s). The masked antibody may be eluted from the column at acidic pH, such as at pH less than 4 or a pH of less than 4.5, or pH 2.5-4.5, pH 2.5-4, pH 3-4.5, pH 3-4, pH 2.5-3.8, pH 2.7-3.8, or pH 2.5-3.5, pH 2.6, pH 2.7, pH 2.8, pH 2.9, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4.0, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5.

[0134] In some embodiments, the at least one acidic wash buffer comprises acetate. In some embodiments, the at least one acidic wash buffer comprises 10-100 mM, 10-90 mM, 10-80 mM, 10-70 mM, 10-60 mM, 10-50 mM, 10-40 mM, 15-30 mM, 20-30 mM, or 25 mM acetate. In some embodiments, particularly where the wash buffer comprises acetate, the elution buffer comprises acetic acid. In some embodiments, the elution buffer may comprise 0.05-0.2M, 0.07-0.15M, 0.07-0.13M, 0.08-0.12M, 0.09M, 0.1M, or 0.11M acetate (such as acetic acid).

[0135] In some embodiments, the at least one acidic wash buffer comprises glutamate. In some embodiments, the at least one acidic wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate. In some such embodiments, the elution buffer comprises glutamic acid. In some embodiments, the elution buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamic acid.

[0136] As a nonlimiting example, in some embodiments, the purification process comprises (a) loading a starting composition comprising the masked antibody onto a protein A chromatography column under conditions suitable for binding the masked antibody to the protein A chromatography column; (b) washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH 4.5-5.5; and (c) eluting the masked antibody from the protein A column in an acidic elution buffer at pH 2.5-4 to form an eluate comprising the masked antibody. In some embodiments, the process comprises washing the column at least once between (a) and (b) with a neutral wash buffer at pH 6-8, such as a Tris buffer at pH 7-8, such as pH 7.5, and/or with a basic wash buffer at pH 8.5-9.5, such as an arginine buffer at pH 9.

[0137] In some embodiments, the composition following protein A chromatography is adjusted to acidic pH and incubated prior to subsequent steps. For example, in some embodiments, the process further comprises adjusting the pH of the eluate comprising the masked antibody to pH 3-4.2, pH 3-4, pH 3.5-4, pH 3.6-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4, to form an acidified eluate. The pH, for example, may be adjusted using acetic acid, optionally 1M acetic acid, or phosphoric acid, optionally 0.5 M phosphoric acid.

[0138] In some embodiments, the protein A chromatography process comprises equilibrating the protein A column and loading the sample comprising masked antibody at neutral pH, such as at pH 6-8 or pH 7-8, or pH 7.5 (step (a) above), and optionally washing the column after step (a) at least once with a neutral wash buffer at pH 6-8, such as a Tris buffer at pH 7-8, such as pH 7.5, followed by (b) washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH 4.5-5.5; and (c) eluting the masked antibody from the protein A column in an acidic elution buffer at pH 2.5-4 to form an eluate comprising the masked antibody, wherein the acidic wash buffer and acidic elution buffer comprise acetate/acetic acid as described above.

[0139] In some embodiments, the protein A chromatography process comprises equilibrating the protein A column and loading the sample comprising masked antibody at neutral pH, such as at pH 6-8 or pH 7-8, or pH 7.5 (step (a) above), and optionally washing the column after step (a) at least once with a neutral wash buffer at pH 6-8, such as a Tris buffer at pH 7-8, such as pH 7.5, and washing the column after step (a) at least once with a basic wash buffer at pH 8.5-9.5, such as an arginine buffer at pH 9, followed by (b) washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH 4.5-5.5; and (c) eluting the masked antibody from the protein A column in an acidic elution buffer at pH 2.5-4 to form an eluate comprising the masked antibody, wherein the acidic wash buffer and acidic elution buffer comprise glutamate/glutamic acid as described above.

[0140] In some embodiments, the protein A eluate collected is then incubated at acidic pH for a time. In some embodiments, the process comprises incubating the protein A chromatography eluate for 4-30 hours, 6-30 hours, 10-30 hours, 4-20 hours, 6-20 hours, 8-20 hours, 10-20 hours, 4-18 hours, 6-18 hours, 8-18 hours, 10-18 hours, 8-16 hours, 10-16 hours, 8-14 hours, 10-14 hours, 11-13 hours, 10 hours, 11 hours, 12 hours, 13 hours, or 14 hours after adjusting the pH. In some embodiments, the eluate is incubated at room temperature. In some embodiments, the composition is incubated at room temperature at pH 3-4.2, pH 3-4, pH 3.5-4, pH 3.6-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4.

[0141] In some embodiments, following incubation, the pH of the eluate is adjusted to pH 3.5-4.5, pH 3.5-4.3, pH 3.7-4.2, pH 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5. In some embodiments, when the pH is adjusted to be more basic, the pH is adjusted using Tris base, optionally 1M Tris base.

[0142] In some embodiments, a filtration step may be conducted at acidic pH prior to or during the incubation, for example, to remove additional particulates or non-protein materials. In some embodiments, the eluate is filtered on a depth filter. For example, this may be done before or after incubation.

[0143] In some embodiments, the incubation at acidic pH following protein A chromatography reduces aggregation in the final masked antibody composition compared to a masked antibody composition prepared using the same process but without the several hours incubation at acidic pH following protein A chromatography.

[0144] In some embodiments, a hydrophobic interaction chromatography (HIC) process is conducted following protein A chromatography, or following protein A chromatography and the low pH incubation, or following protein A chromatography, low pH incubation, and filtration. As described herein, the HIC process may be conducted at neutral pH at reduced temperature, or may be conducted at acidic pH. Where the HIC process is conducted at acidic pH, in some embodiments the process from HIC through to diafiltration/tangential flow filtration and viral filtration, for example, is conducted at acidic pH, such as at pH 3-4.5, pH 3.5-4.5, pH 3.5-4, pH 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5. In some embodiments, the process herein beginning with HIC is conducted at pH 3.6-4, such as pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4. In some cases, it is conducted at pH 4.

[0145] In some embodiments, HIC is conducted at neutral pH, i.e., the column equilibration buffer and the composition in the loading buffer are at neutral pH. For example, the chromatography may be conducted at pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9. In some embodiments, HIC is conducted at neutral pH, and the eluate is chilled to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C after low

pH incubation and/or depth filtration. In some embodiments, the pH of the chilled eluate is adjusted to pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9. In some embodiments, the pH is adjusted using Tris base, optionally 1M Tris base. In some embodiments, the HIC membrane comprises Sartobind® phenyl, which comprises phenyl on a cellulose membrane backbone. In some such embodiments, HIC is conducted at reduced temperature, for example, ≤ 15 °C or at a range of 1 °C to 15 °C, or at 2 °C to 10 °C, or 4 °C to 15 °C, or 4 °C to 12 °C, 4 °C to 8 °C, or 6 °C to 12 °C, or 6 °C to 10 °C. In some embodiments, the HIC is performed at a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C, and at neutral pH. Thus, in some embodiments, the equilibration, loading, and wash buffers are chilled to, for example, ≤ 15 °C or at a range of 1 °C to 15 °C, or at 1 °C to 10 °C, 1 °C to 9°C, or 2 °C to 8°C, or 4 °C to 15 °C, or 4 °C to 12 °C, 4 °C to 8 °C, or 6 °C to 12 °C, or 6 °C to 10 °C, in order to maintain the procedure at low temperature. Thus, in some embodiments, the HIC column is loaded with a chilled, neutral protein A eluate solution. In some embodiments, the HIC column may also be pre-chilled. In some embodiments, the wash buffers are also kept chilled. In some embodiments, the reduced temperature mitigates aggregation in the solution as the pH is raised to neutral. In some embodiments, the HIC column equilibration, loading composition, wash buffer are all at neutral pH, for example a pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9.

[0146] In some such embodiments, the HIC wash buffer is a sodium citrate buffer. In some embodiments, the HIC wash buffer comprises 200-700 mM, or 200-600 mM, or 200-500 mM, or 250-500 mM, or 300-500 mM, or 300-400 mM sodium citrate. In various embodiments, the masked antibody is collected in a HIC effluent.

[0147] In other embodiments, the HIC is performed at acidic pH. In some such cases, HIC can be performed at room temperature (i.e., 15-28°C or 18-25°C) rather than at reduced temperature. In such cases, the pH of the composition following protein A chromatography, or following protein A chromatography and the low pH incubation, or following protein A chromatography, low pH incubation, and filtration may be adjusted to pH 3-4.5, pH 3.5-4.5, pH 3.5-4, pH 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5. In some embodiments, pH may be adjusted using is adjusted using Tris base, optionally 1M Tris base. In some embodiments, the HIC resin comprises butyl Sepharose® HP, which comprises aliphatic butyl groups on an agarose backbone. In some embodiments, the HIC resin is Capto Butyl ImpRes resin (Capto base matrix with butyl ligands), Capto Phenyl ImpRes (Capto base matrix with phenyl ligands), Zetarous Dodecyl (C12) FF6, Low substitute, Zetarous Dodecyl (C12) FF6, High substitute, Zetarous Hexadecyl (C16) FF6, Low substitute, or Zetarous Hexadecyl (C16) FF6, High substitute.

[0148] In some such embodiments, the HIC wash buffer is a glutamate buffer. In some embodiments, the HIC wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate. In some embodiments, the HIC is conducted at a pH of 3.6 to 4, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4.

[0149] In some embodiments, protein A and HIC are the only chromatography procedures conducted to prepare the masked antibody composition. In other embodiments, further chromatography is conducted, such as before or after or in between the protein A and HIC steps.

[0150] In some embodiments, following chromatography, the masked antibody in the composition are further concentrated and/or the pH is adjusted from neutral to acidic and/or the temperature is raised to room temperature.

[0151] In some embodiments, at least one diafiltration step is conducted following HIC. In some embodiments, the diafiltration is performed by tangential flow filtration (TFF). For example, the diafiltration may be used to reduce salt, such as sodium citrate, concentrations, re-equilibrate the composition to an acidic pH buffer, remove additional impurities, as well as to further concentrate the protein.

[0152] In some embodiments, the process comprises a first diafiltration to reduce the concentration of salt, such as sodium citrate, to below 40 mM, or below 35 mM, or below 30 mM, or below 25 mM, or below 20 mM, or below 15 mM, or below 10 mM, or below 5 mM, and/or to exchange the buffer, for example to a specific salt and concentration such as 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate or 10-100 mM, 10-90 mM, 20-80 mM, 20-70 mM, 30-50 mM, 35 mM, 40 mM, or 45 mM acetate, therefore forming a diafiltered HIC effluent. In some embodiments, the first diafiltration is performed at 1-15°C, 1-10°C, or 1-9°C, or 2-8°C. In some embodiments, the process further comprises a second diafiltration in an acetate buffer or glutamate buffer. In some embodiments, the second diafiltration is performed at room temperature, optionally 15-28°C, or 18-25°C. In some embodiments, the acetate buffer for the second diafiltration comprises 10-100 mM, 10-90 mM, 20-80 mM, 20-70 mM, 30-50 mM, 35 mM, 40 mM, or 45 mM acetate. In some embodiments, the glutamate buffer for the second diafiltration comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate. In some embodiments, prior to the second diafiltration, the pH of the diafiltered HIC eluate is adjusted to acidic pH, such as to pH 3.5-4.5, pH 3.7-4.5, pH 3.7-4.3, pH 3.8, pH 3.9, pH 4, pH 4.1, or pH 4.2. In some embodiments, the pH is adjusted to pH 3.5 to 4.5, or pH 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, or 4.5. In some embodiments, where the composition is exchanged into an

acetate buffer, the pH is adjusted using 25% v/v glacial acetic acid, to form an acidified diafiltered HIC effluent.

[0153] In some embodiments, for example, when HIC is performed at neutral or nonacidic pH, the acidified diafiltered HIC effluent is subjected to ultrafiltration (UF) to concentrate the masked antibody, for instance, to form a concentrated masked antibody composition. In some embodiments, the ultrafiltration processes allow for further concentration of the masked antibody while avoiding aggregation. In some embodiments, following ultrafiltration, the concentration of masked antibody polypeptides in the composition is 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL.

[0154] Alternatively, for example, when HIC is performed at acidic pH, an ultrafiltration step may precede diafiltration. In some such embodiments, before ultrafiltration, pH is adjusted to < pH 3.6, pH 3.6, 3.7, pH 3.8, pH 3.9, or pH 3.6-4. In some embodiments, following ultrafiltration, the concentration of masked antibody polypeptides in the composition is 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL.In these embodiments, diafiltration may be performed using the ultrafiltration eluate.

[0155] In some embodiments, following chromatography and additional protein concentration, the composition is subjected to virus removal or inactivation, for example, by further filtration such as nanofiltration. For example, nanofiltration may follow ultrafiltration, for example, in embodiments where HIC is conducted at reduced temperature and neutral pH. In other embodiments, nanofiltration may precede diafiltration and/or ultrafiltration, such as in embodiments where HIC is conducted at acidic pH and room temperature.

[0156] In some embodiments, the nanofiltration is performed at room temperature. In some embodiments, the nanofiltration is performed at acidic pH. In some embodiments, the acidic pH is pH 3-4.4, pH 3-4, pH 3.5-4, pH 3.6-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, or pH 4.4. In some embodiments where HIC is performed at neutral pH and reduced temperature, the composition is re-equilibrated to acidic pH following HIC, and the virus removal, such as by nanofiltration, is performed at acidic pH. In some such embodiments, the temperature of the composition is increased to room temperature before, at approximately the same time, or after, the pH is lowered.

[0157] In some embodiments, considering the process as a whole from the starting composition to virus removal, one or more, or each, neutral pH step of the process is performed at a temperature of 1°C to 15°C, and/or one or more, or each, room temperature step of the process is performed at acidic pH. In other embodiments, the entire process is performed at acidic pH starting with the acidic washing and elution of the protein A column.

[0158] In some embodiments, following virus removal or otherwise at the end of the above process, the composition may be further treated, such as to exchange the buffer, chill it to a low temperature, or further concentrate the masked antibodies, or for some or all of these purposes.

[0159] In some embodiments, the final concentration of masked antibody in the purified composition is 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL. For example, in some embodiments, after the above process steps (i.e. after virus removal), the composition is formulated into an aqueous formulation, either for storage or for lyophilization or for immediate use. Formulating the composition into the aqueous formulation may be through a buffer exchange process and/or via the wash solution that is used during nanofiltration and/or by adding concentrated formulation components to reach the desired final concentration. The formulation step may also include further concentrating or diluting the masked antibody.

[0160] In some embodiments, the pH of the resulting aqueous formulation, comprising at least the masked antibody and one or more buffer ingredients, is from pH 3.5 to 4.3, such as from pH 3.6 to 4.0, or pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4.0, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5.

[0161] In some embodiments, the process includes a further step of lyophilizing the formulation. Such a process may entail freeze drying the aqueous formulation, optionally in the presence of a lyoprotectant substance. Where the formulation is lyophilized, it may later be reconstituted into another aqueous formulation, for example just prior to use.

IV. Exemplary Antibodies

[0162] Antibodies include non-human, humanized, human, chimeric, and veneered antibodies, nanobodies, dAbs, scFV's, Fabs, and the like. Some such antibodies are immunospecific for a cancer cell antigen, preferably one on the cell surface internalizable within a cell on antibody binding. In some embodiments, the antibody portion of a masked antibody binds a therapeutic antigen. Such therapeutic antigens include antigens that may be targeted for treatment of any disease or disorder, including, but not limited to, cancer, autoimmune disorders, and infections.

[0163] Targets to which antibodies can be directed include receptors on cancer cells and their ligands or counter-receptors (i.e., tumor-associated antigens). Such targets include, but are not limited to, CD3, CD19, CD20, CD22, CD30, CD33, CD34, CD40, CD44, CD47, CD52, CD70, CD79a, CD123, Her-2, EphA2, lymphocyte associated antigen 1, VEGF or VEGFR, CTLA-4, LIV-1, nectin-4, CD74, SLTRK-6, EGFR, CD73, PD-L1, CD163, CCR4, CD147, EpCam, Trop-2, CD25, C5aR, Ly6D, alpha v integrin, B7H3, B7H4, Her-3, folate receptor alpha, GD-2, CEACAM5, CEACAM6, c-MET, CD266, MUC1, CD10, MSLN, sialyl Tn, Lewis Y, CD63, CD81, CD98, CD166, tissue factor (CD142), CD55, CD59, CD46, CD164, TGF beta receptor 1 (TGFβR1), TGFβR2, TGFβR3, FasL, MerTk, Axl, Clec12A, CD352, FAP, CXCR3, and CD5.

[0164] In some embodiments, a masked antibody provided herein may be useful for treating an autoimmune disease. Nonlimiting antigens that may be bound by an antibody useful for treating an autoimmune disease include TNF-α, IL-1, IL-2R, IL-6, IL-12, IL-23, IL-17, IL-17R, BLyS, CD20, CD52, α4β7 integrin, and α4-integrin.

[0165] Some examples of commercial antibodies and their targets suitable for use in the masked antibodies described herein include, but are not limited to, brentuximab or brentuximab vedotin, CD30, alemtuzumab, CD52, rituximab, CD20, trastuzumab Her/neu, nimotuzumab, cetuximab, EGFR, bevacizumab, VEGF, palivizumab, RSV, abciximab, GpIIb/IIIa, infliximab, adalimumab, certolizumab, golimumab TNF-alpha, baciliximab, daclizumab, IL-2R, omalizumab, IgE, gemtuzumab or vadastuximab, CD33, natalizumab, VLA-4, vedolizumab alpha4beta7, belimumab, BAFF, otelixizumab, teplizumab CD3, ofatumumab, ocrelizumab CD20, epratuzumab CD22, alemtuzumumab CD52, eculizumab C5, canakimumab IL-1beta, mepolizumab IL-5, reslizumab, tocilizumab IL-6R, ustekinumab, briakinumab IL-12, 23, hBU12 (CD19) (US20120294853), humanized 1F6 or 2F12 (CD70) (US20120294863), BR2-14a and BR2-22a (LIV-1) (WO2012078688).

Exemplary Anti-CD47 Antibodies

[0166] The present formulations may comprise masked versions of isolated, recombinant and/or synthetic anti-CD47 human, primate, rodent, mammalian, chimeric, humanized and/or CDR-grafted antibodies. In certain exemplary embodiments, the formulations herein comprise masked humanized anti-CD47 IgG1 antibodies.

[0167] In particular embodiments of the invention, the humanized anti-CD47 antibodies have one or more of the following activities: 1) enhanced antigen binding relative to a reference antibody (e.g., a murine parental antibody); 2) enhanced Antibody Dependent Cellular Cytotoxicity (ADCC) relative to a reference antibody (e.g., a murine parental antibody); 3) enhanced phagocytosis (e.g., Antibody Dependent Cellular Phagocytosis (ADCP)) relative to a reference antibody (e.g., a murine parental antibody); 4) reduced red blood cell hemagglutination (HA), relative to a reference antibody (e.g., a murine parental antibody); 5) binding to a three-dimensional (i.e., non-linear) CD47 epitope. Antibodies hB6H12.3 and hB6H12.3 (deamidation mutant) have one or more, or all, of the foregoing properties, wherein the reference antibody is mB6H12. In some embodiments, antibody hB6H12.3 has at least the property of resulting in reduced red blood cell HA relative to murine B6H12 antibody.

[0168] Exemplary anti-CD47 antibodies that may be included in the masked antibodies herein include the CD47 antibody heavy chain / light chain pair of hB6H12.3 (hvH1 / hvK3) or hB6H12.3 (deamidation mutant) (hvH1 / hvK3 G91A). Exemplary anti-CD47 antibody heavy

chain variable region sequences, light chain variable regions, heavy chain CDRs and light chain CDRs can be found at Table 3 – Table 8. The amino acid sequences for the heavy chain and light chain of an exemplary humanized anti-CD47 antibody can be found at Table 9.

Table 3. Heavy chain variable sequence of hB6H12.3 and hB6H12.3 (deamidation mutant). Kabat CDRs are underlined, and IMGT CDRs are bolded.

Heavy Chain	Sequence
hvH1	EVQLLESGGGLVQPGGSLRLSCAAS**GFTFSGYGMS**WVRQAPGKRLEW VAT**ITSGGTYT**YYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYFC **ARSLAGNAMDY**WGQGTLVTVSS **(SEQ ID NO: 22)**

Table 4. Light chain variable sequence of hB6H12.3 and hB6H12.3 (deamidation mutant). Kabat CDRs are underlined, and IMGT CDRs are bolded.

Light Chain	Sequence
hvK3	EIVMTQSPDFQSVTPKEKVTLTCRAS**QTISDY**LHWYQQKPDQSPKLLIK **FASQSIS**GVPSRFSGSGSGSDFTLTINSLEAEDAATYYC**QNGHGFPRT**FG QGTKLEIK(R) **(SEQ ID NO: 23)**
hvK3 (G91A)	EIVMTQSPDFQSVTPKEKVTLTCRAS**QTISDY**LHWYQQKPDQSPKLLIK **FASQSIS**GVPSRFSGSGSGSDFTLTINSLEAEDAATYYC**QNAHGFPRT**FG QGTKLEIKR **(SEQ ID NO: 24)**

Table 5. Heavy chain CDR sequences of hB6H12.3 and hB6H12.3 (deamidation mutant) (Kabat).

CDR	Sequence
hvH1 HCDR1 (Kabat)	GYGMS (SEQ ID NO: 25)
hvH1 HCDR2 (Kabat)	TITSGGTYTYYPDSVKG (SEQ ID NO: 26)
hvH1 HCDR3 (Kabat)	SLAGNAMDY (SEQ ID NO: 27)

Table 6. Heavy chain CDR sequences of hB6H12.3 and hB6H12.3 (deamidation mutant)(IMGT).

CDR	Sequence
hvH1 HCDR1 (IMGT)	GFTFSGYG (SEQ ID NO: 28)
hvH1 HCDR2 (IMGT)	ITSGGTYT (SEQ ID NO: 29)
hvH1 HCDR3 (IMGT)	ARSLAGNAMDY (SEQ ID NO: 30)

Table 7. Light chain CDR sequences of hB6H12.3 and hB6H12.3 (deamidation mutant) (Kabat).

CDR	Sequence
hvK3 LCDR1 (Kabat)	RASQTISDYLH (SEQ ID NO: 31)
hvK3 LCDR2 (Kabat)	FASQSIS (SEQ ID NO: 32)

hvK3 LCDR3 (Kabat)	QNGHGFPRT (SEQ ID NO: 33)
hvK3 (G91A) LCDR3 (Kabat)	QNAHGFPRT (SEQ ID NO: 34)

Table 8. Light chain CDR sequences of hB6H12.3 and hB6H12.3 (deamidation mutant) (IMGT).

CDR	Sequence
hvK3 LCDR1 (IMGT)	QTISDY (SEQ ID NO: 35)
hvK3 LCDR2 (IMGT)	FAS (SEQ ID NO: 36)
hvK3 LCDR3 (IMGT)	QNGHGFPRT (SEQ ID NO: 37)
hvK3 (G91A) LCDR3 (IMGT)	QNAHGFPRT (SEQ ID NO: 38)

Table 9. Complete heavy and light chain sequences of a masked anti-CD47 antibody according to a preferred embodiment of the invention. Heavy chain and light chain sequences are in plain text, masking sequences are in bold text, and protease cleavage sequences are underlined.

Antibody Chain	Sequence
Heavy Chain version 1	**QGASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG**E VQLLESGGGLVQPGGSLRLSCAASGFTFSGYGMSWVRQAPGKRLEWVATIT SGGTYTYYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYFCARSLAGN AMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQK **(SEQ ID NO: 39)**
Heavy Chain version 2	**QGASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG**E VQLLESGGGLVQPGGSLRLSCAASGFTFSGYGMSWVRQAPGKRLEWVATIT SGGTYTYYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAIYFCARSLAGN AMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQ **(SEQ ID NO: 40)**
Heavy Chain masking sequence	QGASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGS **(SEQ ID NO: 41)**
Light Chain	**QGASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG**E IVMTQSPDFQSVTPKEKVTLTCRASQTISDYLHWYQQKPDQSPKLLIKFASQ SISGVPSRFSGSGSGSDFTLTINSLEAEDAATYYCQNGHGFPRTFGQGTKLEI KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG

	NSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF NRGEC **(SEQ ID NO: 42)**
Light Chain masking sequence	QGASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGS **(SEQ ID NO: 43)**

hB6H12.3

[0169] In certain exemplary embodiments, an anti-CD47 antibody comprises CDRs from a HCVR set forth as SEQ ID NO: 22 and/or CDRs from a LCVR set forth as SEQ ID NO: 23. In other embodiments, an anti-CD47 antibody comprises heavy chain CDRs of SEQ ID NOs: 25, 26 and 27 and/or light chain CDRs of SEQ ID NOs: 31, 32 and 33. In some embodiments, an anti-CD47 antibody comprises heavy chain CDRs of SEQ ID NOs: 28, 29 and 30 and/or light chain CDRs of SEQ ID NOs: 35, 36 and 37. In other embodiments, an anti-CD47 antibody comprises the HCVR / LCVR pair SEQ ID NO: 22 / SEQ ID NO: 23. In other embodiments, an anti-CD47 antibody comprises a HCVR that has at least about 80% homology or identity (e.g., 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) to SEQ ID NO: 22 and/or comprises a LCVR that has at least about 80% homology or identity (e.g., 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) to SEQ ID NO: 23.

hB6H12.3 G91A

[0170] In certain exemplary embodiments, an anti-CD47 antibody comprises CDRs from a HCVR set forth as SEQ ID NO: 22 and/or CDRs from a LCVR set forth as SEQ ID NO: 24. In other embodiments, an anti-CD47 antibody comprises heavy chain CDRs of SEQ ID NOs: 25, 26 and 27 and/or light chain CDRs of SEQ ID NOs: 31, 32 and 34;. In some embodiments, an anti-CD47 antibody comprises heavy chain CDRs of SEQ ID NOs: 28, 29 and 30 and/or light chain CDRs of SEQ ID NOs: 35, 36 and 38. In other embodiments, an anti-CD47 antibody comprises the HCVR / LCVR pair SEQ ID NO: 22 / SEQ ID NO: 24. In other embodiments, an anti-CD47 antibody comprises a HCVR that has at least about 80% homology or identity (e.g., 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) to SEQ ID NO: 22 and/or comprises a LCVR that has at least about 80% homology or identity (e.g., 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) to SEQ ID NO: 24.

[0171] The anti-CD47 antibodies described herein typically bind CD47 with an equilibrium binding constant of ≦1 μM, e.g., ≦100 nM, preferably ≦10 nM, and more preferably ≦1 nM, as measured using standard binding assays, for example, the Biacore®-based binding assay.

[0172] Antibody molecules used in the present formulations may be characterized relative to a reference anti-CD47 antibody, for example, B6H12, 2D3, MABL, CC2C6, or BRIC126.

Antibody B6H12 is described, for example, in U.S. Pat. Nos. 5,057,604 and 9,017,675, is commercially available from Abcam, PLC, Santa Cruz Biotechnology, Inc., and eBioscience, Inc.

Glycosylation Variants

[0173] Antibodies may be glycosylated at conserved positions in their constant regions (Jefferis and Lund, (1997) Chem. Immunol. 65:111-128; Wright and Morrison, (1997) TibTECH 15:26-32). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd et al., (1996) Mol. Immunol. 32:1311-1318; Wittwe and Howard, (1990) Biochem. 29:4175-4180), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (Jefferis and Lund, supra; Wyss and Wagner, (1996) Current Op. Biotech. 7:409-416). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in agalactosylated IgG, the oligosaccharide moiety 'flips' out of the inter-CH2 space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (Malhotra et al., (1995) Nature Med. 1:237-243). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (Boyd et al., (1996) Mol. Immunol. 32:1311-1318), while selective removal of sialic acid residues using neuraminidase resulted in no loss of DMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of α(1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al. (1999) Mature Biotech. 17:176-180).

[0174] Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0175] Glycosylation variants of antibodies are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting one or more carbohydrate moieties found in the antibody, adding one or more carbohydrate moieties to the antibody, changing the composition of glycosylation (glycosylation pattern), the extent of glycosylation, etc.

[0176] Addition of glycosylation sites to an antibody can be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites). Similarly, removal of glycosylation sites can be accomplished by amino acid alteration within the native glycosylation sites of the antibody.

[0177] The amino acid sequence is usually altered by altering the underlying nucleic acid sequence. These methods include isolation from a natural source (in the case of naturally-occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

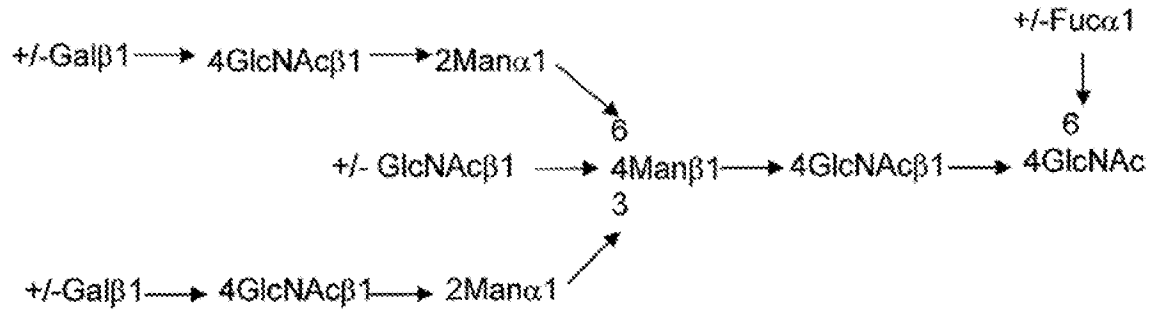
[0178] The glycosylation (including glycosylation pattern) of antibodies may also be altered without altering the amino acid sequence or the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, e.g., antibodies, as potential therapeutics is rarely the native cell, significant variations in the glycosylation pattern of the antibodies can be expected. See, e.g., Hse et al., (1997) J. Biol. Chem. 272:9062-9070. In addition to the choice of host cells, factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide production (U.S. Patent Nos. 5047335; 5510261; 5278299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, e.g., make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

[0179] The glycosylation structure of antibodies can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, Mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical

purposes are also known, and include, without limitation, enzymatic treatment (commonly performed using peptide-N-glycosidase F/endo-β-galactosidase), elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked oligosaccharides.

[0180] A preferred form of modification of glycosylation of antibodies is reduced core fucosylation. "Core fucosylation" refers to addition of fucose ("fucosylation") to N-acetylglucosamine ("GlcNAc") at the reducing terminal of an N-linked glycan.

[0181] A "complex N-glycoside-linked sugar chain" is typically bound to asparagine 297 (according to the number of Kabat). As used herein, the complex N-glycoside-linked sugar chain has a biantennary composite sugar chain, mainly having the following structure:



where +/- indicates the sugar molecule can be present or absent, and the numbers indicate the position of linkages between the sugar molecules. In the above structure, the sugar chain terminal which binds to asparagine is called a reducing terminal (at right), and the opposite side is called a non-reducing terminal. Fucose is usually bound to N-acetylglucosamine ("GlcNAc") of the reducing terminal, typically by an α1,6 bond (the 6-position of GlcNAc is linked to the 1-position of fucose). "Gal" refers to galactose, and "Man" refers to mannose.

[0182] A "complex N-glycoside-linked sugar chain" includes 1) a complex type, in which the non-reducing terminal side of the core structure has one or more branches of galactose-N-acetylglucosamine (also referred to as "gal-GlcNAc") and the non-reducing terminal side of Gal-GlcNAc optionally has a sialic acid, bisecting N-acetylglucosamine or the like; or 2) a hybrid type, in which the non-reducing terminal side of the core structure has both branches of a high mannose N-glycoside-linked sugar chain and complex N-glycoside-linked sugar chain.

[0183] In some embodiments, the "complex N-glycoside-linked sugar chain" includes a complex type in which the non-reducing terminal side of the core structure has zero, one or more branches of galactose-N-acetylglucosamine (also referred to as "gal-GlcNAc") and the non-reducing terminal side of Gal-GlcNAc optionally further has a structure such as a sialic acid, bisecting N-acetylglucosamine or the like.

[0184] According to certain methods, only a minor amount of fucose is incorporated into the complex N-glycoside-linked sugar chain(s) of an antibody. For example, in various embodiments, less than about 60%, less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, or less than about 3% of the molecules of an antibody have core fucosylation by fucose. In some embodiments, about 2% of the molecules of the antibody has core fucosylation by fucose.

[0185] In certain embodiments, only a minor amount of a fucose analog (or a metabolite or product of the fucose analog) is incorporated into the complex N-glycoside-linked sugar chain(s). For example, in various embodiments, less than about 60%, less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, or less than about 3% of the antibodies have core fucosylation by a fucose analog or a metabolite or product of the fucose analog. In some embodiments, about 2% of the antibodies have core fucosylation by a fucose analog or a metabolite or product of the fucose analog.

[0186] Methods of making non-fucosylated antibodies (which may be used to make non-fucosylated masked antibodies) by incubating antibody-producing cells with a fucose analogue are described, e.g., in WO2009/135181. Briefly, cells that have been engineered to express the antibody are incubated in the presence of a fucose analogue or an intracellular metabolite or product of the fucose analog. An intracellular metabolite can be, for example, a GDP-modified analog or a fully or partially de-esterified analog. A product can be, for example, a fully or partially de-esterified analog. In some embodiments, a fucose analogue can inhibit an enzyme(s) in the fucose salvage pathway. For example, a fucose analog (or an intracellular metabolite or product of the fucose analog) can inhibit the activity of fucokinase, or GDP-fucose-pyrophosphorylase. In some embodiments, a fucose analog (or an intracellular metabolite or product of the fucose analog) inhibits fucosyltransferase (preferably a 1,6-fucosyltransferase, e.g., the FUT8 protein). In some embodiments, a fucose analog (or an intracellular metabolite or product of the fucose analog) can inhibit the activity of an enzyme in the de novo synthetic pathway for fucose. For example, a fucose analog (or an intracellular metabolite or product of the fucose analog) can inhibit the activity of GDP-mannose 4,6-dehydratase or/or GDP-fucose synthetase. In some embodiments, the fucose analog (or an intracellular metabolite or product of the fucose analog) can inhibit a fucose transporter (e.g., GDP-fucose transporter).

[0187] In one embodiment, the fucose analogue is 2-flurofucose. Methods of using fucose analogues in growth medium and other fucose analogues are disclosed, e.g., in WO/2009/135181, which is herein incorporated by reference.

[0188] Other methods for engineering cell lines to reduce core fucosylation included gene knock-outs, gene knock-ins and RNA interference (RNAi). In gene knock-outs, the gene encoding FUT8 (alpha 1,6- fucosyltransferase enzyme) is inactivated. FUT8 catalyzes the transfer of a fucosyl residue from GDP-fucose to position 6 of Asn-linked (N-linked) GlcNac of an N-glycan. FUT8 is reported to be the only enzyme responsible for adding fucose to the N-linked biantennary carbohydrate at Asn297. Gene knock-ins add genes encoding enzymes such as GNTIII or a Golgi alpha mannosidase II. An increase in the levels of such enzymes in cells diverts monoclonal antibodies from the fucosylation pathway (leading to decreased core fucosylation), and having increased amount of bisecting N-acetylglucosamines. RNAi typically also targets FUT8 gene expression, leading to decreased mRNA transcript levels or knocking out gene expression entirely. Any of these methods can be used to generate a cell line that would be able to produce a non-fucosylated antibody.

[0189] Many methods are available to determine the amount of fucosylation on an antibody. Methods include, e.g., LC-MS via PLRP-S chromatography and electrospray ionization quadrupole TOF MS.

V. Antibody-Drug Conjugates

[0190] In certain embodiments, a masked antibody may comprise an antibody drug conjugates (ADCs, also referred to herein as an "immunoconjugate"). Particular ADCs may comprise cytotoxic agents (e.g., chemotherapeutic agents), prodrug converting enzymes, radioactive isotopes or compounds, or toxins (these moieties being collectively referred to as a therapeutic agent). For example, an ADC can be conjugated to a cytotoxic agent such as a chemotherapeutic agent, or a toxin (e.g., a cytostatic or cytocidal agent such as, for example, abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin). Examples of useful classes of cytotoxic agents include, for example, DNA minor groove binders, DNA replication inhibitors, DNA alkylating agents, NAMPT inhibitors, and tubulin inhibitors (i.e., antitubulins). Exemplary cytotoxic agents include, for example, auristatins, camptothecins, calicheamicins, duocarmycins, etoposides, enediyine antibiotics, maytansinoids (e.g., DM1, DM2, DM3, DM4), taxanes, benzodiazepines (e.g., pyrrolo[l,4]benzodiazepines, indolinobenzodiazepines, and oxazolidinobenzodiazepines including pyrrolo[l,4]benzodiazepine dimers, indolinobenzodiazepine dimers, and oxazolidinobenzodiazepine dimers), lexitropsins, taxanes, combretastatins, cryptophysins, and vinca alkaloids. Nonlimiting exemplary cytotoxig agents include auristatin E, AFP, AEB, AEVB, MMAF, MMAE, paclitaxel, docetaxel, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, melphalan, methotrexate, mitomycin C, a CC-1065 analogue, CBI, calicheamicin, maytansine, an analog of dolastatin 10, rhizoxin, or palytoxin, epothilone A,

epothilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, eleutherobin, a tubulysin, a plocabulin, and maytansine.

[0191] An ADC can be conjugated to a pro-drug converting enzyme. The pro-drug converting enzyme can be recombinantly fused to the antibody or chemically conjugated thereto using known methods. Exemplary pro-drug converting enzymes are carboxypeptidase G2, beta-glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β- lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

[0192] Techniques for conjugating therapeutic agents to proteins, and in particular to antibodies, are well-known. (See, e.g., Alley et al., Current Opinion in Chemical Biology 2010 14: 1-9; Senter, Cancer J., 2008, 14(3): 154-169.) The therapeutic agent can be conjugated in a manner that reduces its activity unless it is cleaved off the antibody (e.g., by hydrolysis, by proteolytic degradation, or by a cleaving agent). In some aspects, the therapeutic agent is attached to the antibody with a cleavable linker that is sensitive to cleavage in the intracellular environment of the antigen-expressing cancer cell but is not substantially sensitive to the extracellular environment, such that the conjugate is cleaved from the antibody when it is internalized by the antigen-expressing cancer cell (e.g., in the endosomal or, for example by virtue of pH sensitivity or protease sensitivity, in the lysosomal environment or in the caveolear environment). In some embodiments, the therapeutic agent can also be attached to the antibody with a non-cleavable linker.

[0193] In certain exemplary embodiments, an ADC can include a linker region between a cytotoxic or cytostatic agent and the antibody. As noted supra, typically, the linker can be cleavable under intracellular conditions, such that cleavage of the linker releases the therapeutic agent from the antibody in the intracellular environment (e.g., within a lysosome or endosome or caveolea). The linker can be, e.g., a peptidyl linker that is cleaved by an intracellular peptidase or protease enzyme, including a lysosomal or endosomal protease. Cleaving agents can include cathepsins B and D and plasmin (see, e.g., Dubowchik and Walker, Pharm. Therapeutics 83:67-123, 1999). Most typical are peptidyl linkers that are cleavable by enzymes that are present in antigen-expressing cells. For example, a peptidyl linker that is cleavable by the thiol-dependent protease cathepsin-B, which is highly expressed in cancerous tissue, can be used (e.g., a linker comprising a Phe-Leu or a Val-Cit peptide).

[0194] A cleavable linker can be pH-sensitive, i.e., sensitive to hydrolysis at certain pH values. Typically, the pH-sensitive linker is hydrolyzable under acidic conditions. For example, an acid-labile linker that is hydrolyzable in the lysosome (*e.g.*, a hydrazone, semicarbazone, thiosemicarbazone, cis-aconitic amide, orthoester, acetal, ketal, or the like) can be used. (See, e.g., U.S. Patent Nos. 5,122,368; 5,824,805; 5,622,929; Dubowchik and Walker, Pharm.

Therapeutics 83:67-123, 1999; Neville et al, Biol. Chem. 264: 14653-14661, 1989.) Such linkers are relatively stable under neutral pH conditions, such as those in the blood, but are unstable at below pH 5.5 or 5.0, the approximate pH of the lysosome.

[0195] Other linkers are cleavable under reducing conditions (*e.g.*, a disulfide linker). Disulfide linkers include those that can be formed using SATA (N-succinimidyl-S- acetylthioacetate), SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate), SPDB (N- succinimidyl-3-(2-pyridyldithio)butyrate) and SMPT (N-succinimidyl-oxycarbonyl-alpha- methyl-alpha-(2-pyridyl-dithio)toluene), SPDB and SMPT. (See, *e.g.* , Thorpe et al. , Cancer Res. 47:5924-5931, 1987; Wawrzynczak et al., In Immunoconjugates: Antibody Conjugates in Radioimagery and Therapy of Cancer (C. W. Vogel ed., Oxford U. Press, 1987. See also U.S. Patent No. 4,880,935.)

[0196] The linker can also be a malonate linker (Johnson et al , Anticancer Res. 15: 1387- 93, 1995), a maleimidobenzoyl linker (Lau et al., Bioorg-Med-Chem. 3: 1299-1304, 1995), or a 3'-N-amide analog (Lau et al., Bioorg-Med-Chem. 3: 1305-12, 1995).

[0197] The linker also can be a non-cleavable linker, such as an maleimido-alkylene or maleimide-aryl linker that is directly attached to the therapeutic agent and released by proteolytic degradation of the antibody.

[0198] Typically, the linker is not substantially sensitive to the extracellular environment, meaning that no more than about 20%, typically no more than about 15%, more typically no more than about 10%, and even more typically no more than about 5%, no more than about 3%, or no more than about 1% of the linkers in a sample of the ADC is cleaved when the ADC is present in an extracellular environment (e.g., in plasma). Whether a linker is not substantially sensitive to the extracellular environment can be determined, for example, by incubating independently with plasma both (a) the ADC (the "ADC sample") and (b) an equal molar amount of unconjugated antibody or therapeutic agent (the "control sample") for a predetermined time period (*e.g.*, 2, 4, 8, 16, or 24 hours) and then comparing the amount of unconjugated antibody or therapeutic agent present in the ADC sample with that present in control sample, as measured, for example, by high performance liquid chromatography.

[0199] The linker can also promote cellular internalization. The linker can promote cellular internalization when conjugated to the therapeutic agent (i.e., in the milieu of the linker-therapeutic agent moiety of the ADC or ADC derivate as described herein). Alternatively, the linker can promote cellular internalization when conjugated to both the therapeutic agent and the antibody (i.e., in the milieu of the ADC as described herein).

[0200] The antibody can be conjugated to the linker via a heteroatom of the antibody. These heteroatoms can be present on the antibody in its natural state or can be introduced into the

antibody. In some aspects, the antibody will be conjugated to the linker via a nitrogen atom of a lysine residue. In other aspects, the antibody will be conjugated to the linker via a sulfur atom of a cysteine residue. Methods of conjugating linker and drug-linkers to antibodies are known in the art.

[0201] Exemplary antibody-drug conjugates include auristatin based antibody-drug conjugates meaning that the drug component is an auristatin drug. Auristatins bind tubulin, have been shown to interfere with microtubule dynamics and nuclear and cellular division, and have anticancer activity. Typically the auristatin based antibody-drug conjugate comprises a linker between the auristatin drug and the antibody. The linker can be, for example, a cleavable linker (e.g., a peptidyl linker) or a non-cleavable linker (e.g., linker released by degradation of the antibody). Auristatins include MMAF, and MMAE. The synthesis and structure of exemplary auristatins are described in U.S. Publication Nos. 7,659,241, 7,498,298, 2009-0111756, 2009-0018086, and 7,968, 687 each of which is incorporated herein by reference in its entirety and for all purposes.

[0202] Other exemplary antibody-drug conjugates include maytansinoid antibody-drug conjugates meaning that the drug component is a maytansinoid drug, and benzodiazepine antibody drug conjugates meaning that the drug component is a benzodiazepine (*e.g.*, pyrrolo[l,4]benzodiazepine dimers, indolinobenzodiazepine dimers, and oxazolidinobenzodiazepine dimers).

[0203] In certain embodiments, an antibody may be combined with an ADC with binding specificity to a different target. Exemplary ADCs that may be combined with a masked antibody include brentuximab vedotin (anti-CD30 ADC), enfortumab vedotin (anti-nectin-4 ADC), ladiratuzumab vedotin (anti-LIV-1 ADC), denintuzumab mafodotin (anti-CD19 ADC), glembatumumab vedotin (anti-GPNMB ADC), anti-TIM-1 ADC, polatuzumab vedotin (anti-CD79b ADC), anti-MUC16 ADC, depatuxizumab mafodotin, telisotuzumab vedotin, anti-PSMA ADC, anti-C4.4a ADC, anti-BCMA ADC, anti-AXL ADC, tisotuumab vedotin (anti-tissue factor ADC).

VI. Masked Antibody Expression

[0204] Nucleic acids encoding masked antibodies can be expressed in a host cell that contains endogenous DNA encoding an antibody or masked antibody provided herein. Such methods are well known in the art, e.g., as described in U.S. Pat. Nos. 5,580,734, 5,641,670, 5,733,746, and 5,733,761. Also see, e.g., Sambrook, et al., supra, and Ausubel, et al., *supra*. Those of ordinary skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. Illustrative of cell cultures useful

for the production of the antibodies, masked antibodies, specified portions or variants thereof, are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions or bioreactors can also be used. A number of suitable host cell lines capable of expressing intact glycosylated proteins have been developed in the art, and include the COS-1 (e.g., ATCC CRL 1650), COS-7 (e.g., ATCC CRL-1651), HEK293, BHK21 (e.g., ATCC CRL-10), CHO (e.g., ATCC CRL 1610) and BSC-1 (e.g., ATCC CRL-26) cell lines, hep G2 cells, P3X63Ag8.653, SP2/0-Ag14, HeLa cells and the like, which are readily available from, for example, American Type Culture Collection, Manassas, VA. Yeast and bacterial host cells may also be used and are well known to those of skill in the art. Other cells useful for production of nucleic acids or proteins of the present invention are known and/or available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and hybridomas or other known or commercial sources.

[0205] Expression vectors can include one or more of the following expression control sequences, such as, but not limited to an origin of replication; a promoter (e.g., late or early SV40 promoters, the CMV promoter (U.S. Pat. Nos. 5,168,062; 5,385,839), an HSV tk promoter, a pgk (phosphoglycerate kinase) promoter, an EF-1 alpha promoter (U.S. Pat. No. 5,266,491), at least one human immunoglobulin promoter; an enhancer, and/or processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences). See, e.g., Ausubel et al., supra; Sambrook, et al., supra.

[0206] Expression vectors optionally include at least one selectable marker. Such markers include, e.g., but are not limited to, methotrexate (MTX), dihydrofolate reductase (DHFR, U.S. Pat. Nos. 4,399,216; 4,634,665; 4,656,134; 4,956,288; 5,149,636; 5,179,017), ampicillin, neomycin (G418), mycophenolic acid, or glutamine synthetase (GS, U.S. Pat. Nos. 5,122,464; 5,770,359; and 5,827,739), resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing in E. coli and other bacteria or prokaryotes. Appropriate culture media and conditions for the above-described host cells are known in the art. Suitable vectors will be readily apparent to the skilled artisan. Introduction of a vector construct into a host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other known methods. Such methods are described in the art, such as Sambrook, supra; Ausubel, supra.

[0207] The nucleic acid insert should be operatively linked to an appropriate promoter. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the

beginning and a termination codon (e.g., UAA, UGA or UAG) appropriately positioned at the end of the mRNA to be translated, with UAA and UAG preferred for mammalian or eukaryotic cell expression.

[0208] The nucleic acid insert is optionally in frame with a coiled coil sequence and/or an MMP cleavage sequence, e.g., at the amino-terminus of one or more heavy chain and/or light chain sequences. Alternatively, a coiled coil sequence and/or an MMP cleavage sequence can be post-translationally added to an antibody, e.g., via a disulfide bond or the like.

[0209] When eukaryotic host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript can also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, et al. (1983) J. Virol. 45:773-781). Additionally, gene sequences to control replication in the host cell can be incorporated into the vector, as known in the art.

VII. Therapeutic Applications

[0210] In some embodiments, masked antibody compositions prepared by methods disclosed herein may be used in methods of therapeutic treatment. Nonlimiting exemplary diseases and disorders that may be treated with the formulations provided herein include cancer, autoimmune disorders, and infections. Where the antibodies are anti-CD47 antibodies, for example, the formulations herein may be used for methods of treating disorders associated with cells that express CD47, e.g., cancers. The cells may or may not express elevated levels of CD47 relative to cells that are not associated with a disorder of interest. As a result, a method of treating a subject, for example, a subject with a cancer, is provided using the masked antibodies described herein. The method comprises administering an effective amount of an anti-CD47 masked antibody or a composition comprising an anti-CD47 masked antibody to a subject in need thereof.

[0211] Positive therapeutic effects in cancer can be measured in a number of ways (See, W. A. Weber, J. Null. Med. 50:1S-10S (2009); Eisenhauer et al., supra). In some preferred embodiments, response to a masked antibody is assessed using RECIST 1.1 criteria. In some embodiments, the treatment achieved by a therapeutically effective amount is any of a partial response (PR), a complete response (CR), progression free survival (PFS), disease free survival (DFS), objective response (OR) or overall survival (OS). The dosage regimen of a therapy described herein that is effective to treat a primary or a secondary hepatic cancer patient may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the therapy to elicit an anti-cancer response in the subject. While an embodiment of the

treatment method, medicaments and uses of the present invention may not be effective in achieving a positive therapeutic effect in every subject, it should do so in a statistically significant number of subjects as determined by any statistical test known in the art such as the Student's t-test, the chi2-test, the U-test according to Mann and Whitney, the Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

[0212] "RECIST 1.1 Response Criteria" as used herein means the definitions set forth in Eisenhauer et al., E. A. et al., Eur. J Cancer 45:228-247 (2009) for target lesions or non-target lesions, as appropriate, based on the context in which response is being measured.

[0213] "Tumor" as it applies to a subject diagnosed with, or suspected of having, a primary or a secondary hepatic cancer, refers to a malignant or potentially malignant neoplasm or tissue mass of any size. A solid tumor is an abnormal growth or mass of tissue that usually does not contain cysts or liquid areas. Different types of solid tumors are named for the type of cells that form them. Examples of solid tumors are sarcomas, carcinomas, and lymphomas. Leukemias (cancers of the blood) generally do not form solid tumors (National Cancer Institute, Dictionary of Cancer Terms). Nonlimiting exemplary sarcomas include soft tissue sarcoma and osteosarcoma.

[0214] "Tumor burden" also referred to as "tumor load," refers to the total amount of tumor material distributed throughout the body. Tumor burden refers to the total number of cancer cells or the total size of tumor(s) throughout the body, including lymph nodes and bone narrow. Tumor burden can be determined by a variety of methods known in the art, such as, e.g., by measuring the dimensions of tumor(s) upon removal from the subject, e.g., using calipers, or while in the body using imaging techniques, e.g., ultrasound, bone scan, computed tomography (CT) or magnetic resonance imaging (MRI) scans.

[0215] The term "tumor size" refers to the total size of the tumor which can be measured as the length and width of a tumor. Tumor size may be determined by a variety of methods known in the art, such as, e.g. by measuring the dimensions of tumor(s) upon removal from the subject, e.g., using calipers, or while in the body using imaging techniques, e.g., bone scan, ultrasound, CT or MRI scans.

[0216] Nonlimiting exemplary autoimmune diseases that may be treated with a masked antibody include Crohn's disease, ulcerative colitis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, uveitis, juvenile idiopathic arthritis, multiple sclerosis, psoriasis (including plaque psoriasis), systemic lupus erythematosus, granulomatosis with polyangiitis, microscopic polyangiitis, systemic sclerosis, idiopathic thrombocytopenic purpura, graft-versus-host disease, and autoimmune cytopenias.

[0217] As used herein, the term "effective amount" refers to the amount of a compound (e.g., a masked antibody) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route. Generally, a therapeutically effective amount of active component is in the range of 0.01 mg/kg to 100 mg/kg, 0.1 mg/kg to 100 mg/kg, 1 mg/kg to 100 mg/kg, 0.01 mg/kg to 10 mg/kg, 0.1 mg/kg to 10 mg/kg, 1 mg/kg to 10 mg/kg. The dosage administered can vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; the age, health, and weight of the recipient; the type and extent of disease or indication to be treated, the nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. The initial dosage can be increased beyond the upper level in order to rapidly achieve the desired blood-level or tissue-level. Alternatively, the initial dosage can be smaller than the optimum, and the daily dosage may be progressively increased during the course of treatment. Human dosage can be optimized, e.g., in a conventional Phase I dose escalation study designed to run from 0.5 mg/kg to 20 mg/kg. Dosing frequency can vary, depending on factors such as route of administration, dosage amount, serum half-life of the antibody, and the disease being treated. Exemplary dosing frequencies are once per day, once per week and once every two weeks. Formulation of monoclonal antibody-based drugs is within ordinary skill in the art. In some embodiments, a monoclonal antibody is lyophilized, and then reconstituted in buffered saline, at the time of administration.

[0218] In certain exemplary embodiments, the present invention provides a method for treating cancer in a cell, tissue, organ, animal or patient. In particular embodiments, the present invention provides a method for treating a solid cancer in a human. Exemplary cancers to be treated with anti-CD47 antibodies, for example, are those that possess CD47 expression in a cell having the cancer (i.e., "CD47-expressing cancers"). Examples of cancers include, but are not limited to, solid tumors, soft tissue tumors, hematopoietic tumors that give rise to solid tumors, and metastatic lesions. Examples of hematopoietic tumors that have the potential to give rise to solid tumors include, but are not limited to, diffuse large B-cell lymphomas (DLBCL), follicular lymphoma, myelodysplastic syndrome (MDS), a lymphoma, Hodgkin's disease, a malignant lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, Richter's Syndrome (Richter's Transformation) and the like. Examples of solid tumors include, but are not limited to, malignancies, e.g., sarcomas (including soft tissue sarcoma and osteosarcoma), adenocarcinomas, and carcinomas, of the various organ systems, such as those affecting head and neck (including pharynx), thyroid, lung (small cell or non-small cell lung carcinoma (NSCLC)), breast, lymphoid, gastrointestinal tract (e.g., oral, esophageal, stomach, liver,

pancreas, small intestine, colon and rectum, anal canal), genitals and genitourinary tract (e.g., renal, urothelial, bladder, ovarian, uterine, cervical, endometrial, prostate, testicular), central nervous system (e.g., neural or glial cells, e.g., neuroblastoma or glioma), skin (e.g., melanoma) and the like. In certain embodiments, the solid tumor is an NMDA receptor positive teratoma. In other embodiments, the cancer is selected from breast cancer, colon cancer, pancreatic cancer (e.g., a pancreatic neuroendocrine tumors (PNET) or a pancreatic ductal adenocarcinoma (PDAC)), stomach cancer, uterine cancer, and ovarian cancer.

[0219] In certain embodiments, the cancer is selected from, but not limited to, leukemia's such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), hairy cell leukemia (HCL), T-cell prolymphocytic leukemia (T-PLL), large granular lymphocytic leukemia, adult T-cell leukemia, and acute monocytic leukemia (AMoL).

[0220] In one embodiment, the cancer is a solid tumor that is associated with ascites. Ascites is a symptom of many types of cancer and can also be caused by a number of conditions, such as advanced liver disease. The types of cancer that are likely to cause ascites include, but are not limited to, cancer of the breast, lung, large bowel (colon), stomach, pancreas, ovary, uterus (endometrium), peritoneum and the like. In some embodiments, the solid tumor associated with ascites is selected from breast cancer, colon cancer, pancreatic cancer, stomach, uterine cancer, and ovarian cancer. In some embodiments, the cancer is associated with pleural effusions, e.g., lung cancer.

[0221] Additional hematological cancers that give rise to solid tumors include, but are not limited to, non-Hodgkin lymphoma (e.g., diffuse large B cell lymphoma, mantle cell lymphoma, B lymphoblastic lymphoma, peripheral T cell lymphoma and Burkitt's lymphoma), B-lymphoblastic lymphoma; B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma; lymphoplasmacytic lymphoma; splenic marginal zone B-cell lymphoma (±villous lymphocytes); plasma cell myeloma/plasmacytoma; extranodal marginal zone B-cell lymphoma of the MALT type; nodal marginal zone B-cell lymphoma (±monocytoid B cells); follicular lymphoma; diffuse large B-cell lymphomas; Burkitt's lymphoma; precursor T-lymphoblastic lymphoma; T adult T-cell lymphoma (HTLV 1-positive); extranodal NK/T-cell lymphoma, nasal type; enteropathy-type T-cell lymphoma; hepatosplenic γ-δ T-cell lymphoma; subcutaneous panniculitis-like T-cell lymphoma; mycosis fungoides/sezary syndrome; anaplastic large cell lymphoma, T/null cell, primary cutaneous type; anaplastic large cell lymphoma, T-/null-cell, primary systemic type; peripheral T-cell lymphoma, not otherwise characterized; angioimmunoblastic T-cell lymphoma, multiple myeloma, polycythemia vera or myelofibrosis, cutaneous T-cell lymphoma, small lymphocytic lymphoma (SLL), marginal zone lymphoma,

CNS lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma and the like.

[0222] In particular embodiments, the cancer is sarcoma, colorectal cancer, head and neck cancer, lung cancer, ovarian cancer, pancreatic cancer, gastric cancer, melanoma, and/or breast cancer.

[0223] Anti-CD47 antibodies and masked antibodies as described herein can also be used to treat disorders associated with cancer, e.g., cancer-induced encephalopathy.

[0224] Compositions of the invention can be used in methods of treatment in combination with other therapeutic agents and/or modalities. The term administered "in combination," as used herein, is understood to mean that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disorder, such that the effects of the treatments on the patient overlap at a point in time. In certain embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery." In other embodiments, the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, e.g., an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In some embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive (i.e., a synergistic response). The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered.

[0225] In one embodiment, the methods of the invention include administering to the subject a masked antibody as described herein, e.g., a composition or preparation, in combination with one or more additional therapies, e.g., surgery or administration of another therapeutic preparation. In the case of cancer, for example, the additional therapy may include chemotherapy, e.g., a cytotoxic agent. In one embodiment the additional therapy may include a targeted therapy, e.g. a tyrosine kinase inhibitor, a proteasome inhibitor, or a protease inhibitor. In one embodiment, the additional therapy may include an anti-inflammatory, anti-angiogenic, anti-fibrotic, or anti-proliferative compound, e.g., a steroid, a biologic immunomodulatory, such as an inhibitor of an immune checkpoint molecule, a monoclonal antibody, an antibody fragment, an aptamer, an siRNA, an antisense molecule, a fusion protein, a cytokine, a cytokine

receptor, a bronchodilator, a statin, an anti-inflammatory agent (e.g. methotrexate), or an NSAID. In another embodiment, the additional therapy could include combining therapeutics of different classes. The antibody or masked antibody preparation and the additional therapy can be administered simultaneously or sequentially.

[0226] An "immune checkpoint molecule," as used herein, refers to a molecule in the immune system that either turns up a signal (a stimulatory molecule) or turns down a signal (an inhibitory molecule). Many cancers evade the immune system by inhibiting T cell signaling. Hence, these molecules may be used in cancer treatments as additional therapeutics. In other cases, a masked antibody may be an immune checkpoint molecule.

[0227] Exemplary immune checkpoint molecules include, but are not limited to, programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), PD-L2, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin and mucin domain containing 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM-1), CEACAM-5, V-domain Ig suppressor of T cell activation (VISTA), B and T lymphocyte attenuator (BTLA), T cell immunoreceptor with Ig and ITIM domains (TIGIT), leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), CD160, TGFR, adenosine 2A receptor (A2AR), B7-H3 (also known as CD276), B7-H4 (also called VTCN1), indoleamine 2,3-dioxygenase (IDO), 2B4, killer cell immunoglobulin-like receptor (KIR), and the like.

[0228] An "immune checkpoint inhibitor," as used herein, refers to a molecule (e.g., a small molecule, a monoclonal antibody, an antibody fragment, etc.) that inhibit and/or block one or more inhibitory checkpoint molecules.

[0229] Exemplary immune checkpoint inhibitors include, but are not limited to, the following monoclonal antibodies: PD-1 inhibitors such as pembrolizumab (Keytruda, Merck) and nivolumab (Opdivo, Bristol-Myers Squibb); PD-L1 inhibitors such as atezolizumab (Tecentriq, Genentech), avelumab (Bavencio, Pfizer), durvalumab (Imfinzi, AstraZeneca); and CTLA-1 inhibitors such as ipilimumab (Yervoy, Bristol-Myers Squibb).

[0230] Exemplary cytotoxic agents include anti-microtubule agents, topoisomerase inhibitors, antimetabolites, protein synthesis and degradation inhibitors, mitotic inhibitors, alkylating agents, platinating agents, inhibitors of nucleic acid synthesis, histone deacetylase inhibitors (HDAC inhibitors, e.g., vorinostat (SAHA, MK0683), entinostat (MS-275), panobinostat (LBH589), trichostatin A (TSA), mocetinostat (MGCD0103), belinostat (PXD101), romidepsin (FK228, depsipeptide)), DNA methyltransferase inhibitors, nitrogen mustards, nitrosoureas, ethylenimines, alkyl sulfonates, triazenes, folate analogs, nucleoside analogs, ribonucleotide reductase inhibitors, vinca alkaloids, taxanes, epothilones, intercalating agents, agents capable of

interfering with a signal transduction pathway, agents that promote apoptosis and radiation, or antibody molecule conjugates that bind surface proteins to deliver a toxic agent. In one embodiment, the cytotoxic agent that can be administered with a preparation described herein is a platinum-based agent (such as cisplatin), cyclophosphamide, dacarbazine, methotrexate, fluorouracil, gemcitabine, capecitabine, hydroxyurea, topotecan, irinotecan, azacytidine, vorinostat, ixabepilone, bortezomib, taxanes (e.g., paclitaxel or docetaxel), cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, vinorelbine, colchicin, anthracyclines (e.g., doxorubicin or epirubicin) daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, adriamycin, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, ricin, or maytansinoids.

[0231] The methods and compositions can be used in the treatment of subjects with CD47 positive cancer. In one embodiment, the CD47 positive cancer expresses one or more Matrix Metalloproteinases (MMPs). Exemplary MMPs include, but are not limited to, MMP1 through MMP28. Particularly exemplary MMPs include MMP2 and MMP9. In one embodiment, the CD47 positive cancer is a tumor in which infiltrating macrophages are present.

[0232] The methods and compositions of the invention can be used in the treatment of subjects with a CD47 positive cancer that expresses one or more MMPs and contains infiltrating macrophages.

[0233] Methods of determining the presence of CD47 positive cancers, MMP expression, and the presence of tumor infiltrating macrophages are known in the art.

[0234] Assessment of CD47 positive cancers in a subject can be determined by conventional methods that include immunohistochemistry (IHC), Western blot, flow cytometry, or RNA sequencing methods. IHC, Western blot, and flow cytometry may be analyzed with any anti-CD47 antibody know in the art, as well as the anti-CD47 antibodies disclosed herein.

[0235] Assessment of macrophage infiltration in tissues can be conducted by monitoring for surface markers of macrophages, including F4/80 for mouse macrophages or CD163, CD68, or CD11b by conventional methods that include immunohistochemistry (IHC), Western blot, flow cytometry, or RNA sequencing methods.

[0236] Assessment of proteases in tissues can be monitored using a variety of techniques, including both those that monitor protease activity as well as those that can detect proteolytic activity. Conventional methods that can detect the presence of proteases in a tissue, which could include both inactive and active forms of the protease, include IHC, RNA sequencing, Western blot, or ELISA-based methods. Additional techniques can be used to detect protease activity in tissues, which includes zymography, in situ zymography by fluorescence microscopy, or the use

of fluorescent proteolytic substrates. In addition, the use of fluorescent proteolytic substrates can be combined with immuno-capture of specific proteases. Additionally, antibodies directed against the active site of a protease can be used by a variety of techniques including IHC, fluorescence microscopy, Western blotting, ELISA, or flow cytometry (See, Sela-Passwell et al. Nature Medicine. 18:143-147. 2012; LeBeau et al. Cancer Research. 75:1225-1235. 2015; Sun et al. Biochemistry. 42:892-900. 2003; Shiryaev et al. 2:e80. 2013.)

VIII. Pharmaceutical Compositions and Formulations

[0237] For therapeutic use, a masked antibody is preferably combined with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" means buffers, carriers, and excipients suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The carrier(s) should be "acceptable" in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient. Pharmaceutically acceptable carriers include buffers, solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art.

[0238] Accordingly, purified masked antibody formulations made by the processes of the present invention can be formulate to comprise at least one of any suitable excipients, such as, but not limited to, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like. Pharmaceutically acceptable excipients are preferred. Non-limiting examples of, and methods of preparing such sterile solutions are well known in the art, such as, but not limited to, those described in Gennaro, Ed., Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co. (Easton, Pa.) 1990. Pharmaceutically acceptable carriers can be routinely selected that are suitable for the mode of administration, solubility and/or stability of the antibody molecule, fragment or variant composition as well known in the art or as described herein.

[0239] In some embodiments, compositions of masked antibodies are aqueous formulations. In other embodiments, the compositions are lyophilized. In either case, the compositions may comprise a buffer as well as masked antibodies comprising a first and a second masking domain, these domains being linked to the heavy chain variable region and to the light chain variable region of the antibody, respectively. In some embodiments, the masking domains comprise coiled-coil forming polypeptides. Accordingly, in some embodiments, the masked antibodies purified by the processes herein and then formulated comprise a first masking domain comprising a coiled-coil domain, which is linked to a heavy chain variable region of the

antibody and a second masking domain comprising a coiled-coil domain, which is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1). In some embodiments, the first masking domain comprises the sequence GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG (SEQ ID NO: 4) and/or the second masking domain comprises the sequence GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG (SEQ ID NO: 3).

[0240] Pharmaceutical formulations prepared for the purified masked antibodies as disclosed herein can be presented in a dosage unit form, or can be stored in a form suitable for supplying more than one unit dose. A pharmaceutical composition should be formulated to be compatible with its intended route of administration. Lyophilized formulations are typically reconstituted in solution prior to administration or use, whereas aqueous formulations may be "ready to use," meaning that they are administered directly, without being first diluted for example, or can be diluted in saline or another solution prior to use.

[0241] Examples of routes of administration are intravenous (IV), intradermal, intratumoral, inhalation, transdermal, topical, transmucosal, and rectal administration. The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, subcutaneous, intraarterial, intrathecal, intracapsular, intraorbital, intravitreous, intracardiac, intradermal, intraperitoneal, transtracheal, inhaled, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

[0242] Pharmaceutical formulations are preferably sterile. Sterilization can be accomplished by any suitable method, e.g., filtration through sterile filtration membranes. Where the composition is lyophilized, filter sterilization can be conducted prior to or following lyophilization and reconstitution.

[0243] The present invention also provides a kit, comprising packaging material and at least one vial comprising an aqueous formulation of masked antibody as described herein. The kit may further comprise instructions for use and/or a diluent solution if the antibody formulation must be diluted prior to use. The present invention also provides a kit, comprising packaging material and at least one vial comprising a lyophilized formulation of masked antibody as described herein. The kit may further comprise instructions for use, a reconstitution solution for

reconstituting the antibody into solution, and/or a diluent solution if the antibody formulation must be further diluted after reconstitution.

EXAMPLES

Example 1: Stability of an anti-CD47 masked antibody Vel-IPV-hB6H12.3 in formulations with different pH

[0244] The pH dependence of aggregation was evaluated in formulations of a masked antibody against CD47, Vel-IPV-hB6H12.3, also called "CD47M" herein. An increase in the percentage of high molecular weight (HMW) antibody species over time suggests aggregation is occurring.

[0245] Vel-IPV-hB6H12.3 was buffer exchanged via dialysis into the following formulations (each pH condition was studied with and without 150 mM sodium chloride): 20 mM acetate pH 4, 20 mM histidine pH 5, 20 mM histidine pH 6, 20 mM potassium phosphate pH 7, and 20 mM potassium phosphate pH 8. Samples were diluted to approximately 5 mg/mL with the appropriate buffer, filled into glass vials and stored at 25°C until the indicated time points. Analysis was performed by size-exclusion ultra performance liquid chromatography (SE-UPLC), as follows.

[0246] SE-UPLC analysis was used to measure high molecular weight (HMW), main peak (MP), and low molecular weight (LMW) forms of Vel-IPV-hB6H12.3. For SE-UPLC analysis, size distribution of Vel-IPV-hB6H12.3 was achieved using ACQUITY Protein BEH SEC Column (4.6x 300 mm) connected to a U-HPLC (Waters I-Class) via isocratic separation with 86% 25 mM sodium phosphate, 480 mM sodium chloride, pH 6.6 plus 14% isopropyl alcohol. Total run time was 20 minutes at a flow rate of 0.3 mL/minute. Detection was at 220 nm.

[0247] The formulation at pH 4 controlled HMW aggregation and promoted stability of Vel-IPV-HB6H12.3 after incubation for 3 days at 25 °C (Figure 1A), particularly in low salt. In contrast, relatively high HMW levels were observed in formulations at pH 5-8. Addition of salt increased HMW levels for the formulation at pH 4, did not affect HMW levels for the formulation at pH 5, and decreased HMW levels for the formulations at pH 6-8.

[0248] Stability over time was determined for formulations of at pH 4 (20 mM acetate) and at pH 6 (20 mM histidine) with Vel-IPV-hB6H12.3 concentrations of approximately 5 mg/mL (Figure 1B). The formulation at pH 6 is a typical antibody formulation, but increasing HMW Vel-IPV-hB6H12.3 levels were seen over time with incubation at 25 °C. Thus, in standard formulations at pH 6, Vel-IPV-hB6H12.3 had insufficient liquid stability during the processing times typically required for manufacturing.

[0249] In contrast, the formulation at pH 4 did not show increases in HMW Vel-IPV-hB6H12.3 levels over time with incubation at 25 °C. These data suggest that Vel-IPV-hB6H12.3 is less susceptible to aggregation at low pH.

Example 2a. Exemplary antibody purification process

[0250] A purification process is developed for the Vel-IPV-hB6H12.3 masked antibody including steps of harvesting, a series of filtration steps, and formulation of the antibody. This purification process for a masked antibody includes chilling of purification steps that are performed at neutral pH. For steps occurring at room-temperature, the product pool is held at low pH. These modifications are found to improve masked antibody stability and decrease aggregation.

Harvesting

[0251] A 2000 L sample of CHO cells is harvested. The harvest process produces a cell lysate containing the masked antibody. The sample is then filtered to remove cell debris, contaminants and impurities. The filtration is performed with Cuno 05SP01A, Cuno 90ZB08A, and Emphaze Hybrid Purifier, and a 0.2 μm filter. Harvested cell culture fluid is held chilled before further processing to mitigate microbial growth. All other solutions are held at room temperature, and processing is at room temperature (18-25°C).

[0252] After the harvest filtration, the sample is subjected to Protein A chromatography.

Protein A chromatography

[0253] Protein A chromatography is performed with GE MabSelect® SuRe packed into a BPG30 column. Protein A chromatography parameters are provided in Table 2.

Table 2. Protein A chromatography parameters

Step	Buffer	Volume (CV)	Flowrate (cm/h)
Equilibration	25 mM Tris, 50 mM NaCl, pH 7.5	5.0	300
Load	HCCF (Harvested cell culture fluid)	(to 25 g/L resin loading)	300
Wash 1	25 mM Tris, 50 mM NaCl, pH 7.5	2.5	300
Wash 2	25 mM Tris, 500 mM NaCl, pH 7.5	3.0	300
Wash 3	25 mM Tris, 50 mM NaCl, pH 7.5	2.5	300
Wash 4	25 mM sodium acetate, pH 5.0	3.0	300
Elution	0.1 M acetic acid	4.0*	300

CV = column volume

*Eluate collected from A280 200 mAU ascending – 200 mAU descending at 2 mm path length

[0254] Resin loading is limited in order to mitigate aggregation, with a maximum loading of 25 g/L.

[0255] A final wash step (wash 4) during the Protein A chromatography at pH 5 improves results. The combination of wash 4 and the elution solution (0.1 M acetic acid) reduces antibody aggregation in comparison to chromatography performed without one or both of these steps.

[0256] Following elution, the eluate is titrated to pH 3.6 with 1 M acetic acid and held for 90 minutes (18-25°C) for low pH viral inactivation. At completion of viral inactivation, the eluate is held for 12-24 hours at room temperature (18-25°C) at pH 3.6. This extended hold at low pH further de-aggregates the antibody in the eluate. After the low pH hold, the pH is adjusted to 4.0 with 1M Tris Base, followed by depth filtration.

Depth filtration

[0257] A depth filter is utilized for additional impurity clearance (for example, for clearance of host cell protein and DNA). The filter loading is limited to ensure sufficient impurity reduction going into the downstream steps. Depth filtration at pH 4 helps maintain antibody stability and reduces aggregation.

[0258] Depth filtration is performed over a Millipore® X0HC POD equilibrated with 20 mM sodium acetate, pH 4. The sample is collected by volume. The depth filtrate pool is held at 2-8°C to prepare for hydrophobic interaction chromatography (HIC).

Hydrophobic interaction chromatography

[0259] Optional in-line filtration may be performed using a 0.22µm polyethersulfone (PES) filter prior to loading onto HIC. The HIC membrane is Sartobind® Phenyl. The HIC process is operated in flow-through mode. The Load, Equilibration and Wash Buffer, and Flow-Through are maintained at 2-8°C throughout processing.

[0260] The chilled (2-8°C) X0HC depth filtrate is titrated to pH 7.5 with 1 M Tris base. The titrated product pool is conditioned by adding 25 mM Tris, 1.05 M sodium citrate, pH 7.5, chilled to 2-8°C, in a 1:2 addition (i.e., the conditioning buffer volume is added at 0.5X neutralized X0HC depth filtrate volume). The conditioning brings the product pool to 0.35 M sodium citrate at a pH between 7.5-8.1. Membrane loading is limited in order to mitigate aggregation, with a maximum loading of 25 g/L.

[0261] Chilling the HIC process improves antibody stability compared to performing the process at room temperature. Maintaining the solution during the processing either at low pH or at neutral pH but at low temperature mitigates antibody aggregation.

[0262] Table 4 provides representative parameters for the HIC process.

Table 4. HIC parameters

Step	Buffer	Volume (MV)	Flowrate (MV/min)
Equilibration	25 mM Tris, 0.35 M sodium citrate, pH 8.0 (Chilled)	10.0	1.5
Load*	Conditioned HIC load (Chilled)	(to 25 g/L loading)	1.5
Wash*	25 mM Tris, 0.35 M sodium citrate, pH 8.0 (Chilled)	10.0	1.5

*Product collected from A280 100 mAU ascending – 200 mAU descending at 2 mm path length

Tangential flow filtration

[0263] After HIC, tangential flow filtration (TFF) is performed to buffer exchange the antibody into 40 mM acetic acid. A Pall Omega Centrasette® T-Series 30 kDa membrane is used, which is equilibrated with 20 mM potassium phosphate, pH 7 buffer. The retentate is chilled to 2-15°C. After titration to pH 4, the process is operated at room temperature. The TFF process includes two diafiltration (DF) steps (DF 1 and DF 2) performed with a titration to acidic pH between the two, followed by ultrafiltration (UF).

[0264] In the DF 1 step, 6 diavolumes are introduced into 20 mM potassium phosphate, pH 7.0. This step reduces salt prior to titration to pH 4, since masked antibodies may aggregate when salt is present at low pH. The DF 1 buffer and retentate are chilled to 2-15°C to maintain product stability. In the titration step, 25% volume/volume (v/v) glacial acetic acid is added to obtain a target pH of 4. In the DF 2 step, 8 diavolumes are introduced into 40 mM acetic acid. The DF 2 buffer and retentate are held at room temperature (18-25°C). UF is then performed to concentrate the product pool to 25-30 mg/mL protein. After UF, the retentate is held at 18-25°C.

[0265] Although typically an antibody is concentrated by UF prior to performing diafiltration, this may lead to aggregation of a masked antibody, because aggregation of the masked antibody is sensitive to antibody concentration, which may be further exacerbated when the masked antibody is not in an acidic, low salt solution. By first reducing the pH and salt using diafiltration, UF may be performed at room temperature.

Viral filtration

[0266] Viral filtration (nanofiltration) is performed after UF using a Sartopore® 2 (0.1 μm nominal) pre-filter and an Asahi® BioEX 4 m^2 viral filter. The viral filtration is performed at 18-25°C with target operating pressure is 45 psig (30 – 49 psig). While typically, nanofiltration is performed earlier in a purification process, nanofiltration is performed at the end of the present process at low pH and room temperature.

Example 2b: Further exemplary antibody purification process

Harvesting

[0267] A 2000 L sample of CHO cells is harvested. The harvest process produces a cell lysate containing the masked antibody. The sample is then filtered to remove cell debris, contaminants and impurities. The filtration is performed with Millipore D0SP, Millipore X0SP, and Emphaze Hybrid Purifier, and a 0.2 µm filter. Harvested cell culture fluid is held chilled before further processing to mitigate microbial growth. All other solutions are held at room temperature, and processing is at room temperature (18-25°C). After the harvest filtration, the sample is subjected to Protein A chromatography.

Protein A

[0268] Protein A chromatography is performed with GE MabSelect® SuRe packed to 20 cm bed height. Protein A chromatography parameters are provided in Table 5.

Table 5. Protein A chromatography parameters

Step	Buffer	Volume (CV)	Flowrate (cm/h)
Equilibration	25 mM Tris, 50 mM NaCl, pH 7.5	5.0	300
Load	HCCF (harvested cell culture fluid)	(to 37 g/L resin loading)	300
Wash 1	25 mM Tris, 50 mM NaCl, pH 7.5	2.5	300
Wash 2	25 mM Tris, 0.5 M Arginine, pH 9.0	3.0	300
Wash 3	25 mM Tris, 50 mM NaCl, pH 7.5	2.5	300
Wash 4	20 mM glutamate, pH 5.0	3.0	300
Elution	40 mM glutamic acid	4.0*	300

CV = column volume

*Eluate collected from A280 200 mAU ascending – 200 mAU descending at 2 mm path length

[0269] Intermediate wash steps may reduce impurities such as host cell proteins and DNA. A final wash step (wash 4) at pH 5, for example in glutamate or acetate buffer, may further improve results. The combination of wash 4 and the elution solution (40 mM glutamic acid)

reduces antibody aggregation in comparison to chromatography performed without one or both of these steps.

[0270] Following elution, the eluate is titrated to pH 3.6 with 0.5 M phosphoric acid and held for 90 minutes (18-25°C) for low pH viral inactivation. At completion of viral inactivation, the eluate is held for 12-24 hours at room temperature (18-25°C) at pH 3.6. This extended hold at low pH further de-aggregates the antibody in the eluate. After the low pH hold, the pH is adjusted to 4.0 with 1M Tris base, followed by depth filtration.

Depth Filtration

[0271] A depth filter is utilized for additional impurity clearance (for example, for clearance of host cell protein and DNA). The filter loading is limited to ensure sufficient impurity reduction going into the downstream steps. Depth filtration at ≤ pH 4, such as pH 3.6 to pH 4, helps maintain antibody stability and reduces aggregation.

[0272] Depth filtration is performed over a Millipore® X0SP POD equilibrated with 40 mM glutamate, pH 4. The sample is collected by volume. The depth filtrate pool is held at 2-8°C or room temperature to prepare for hydrophobic interaction chromatography (HIC).

Hydrophobic Interaction Chromatography (HIC)

[0273] Optional in-line filtration may be performed using a 0.22µm polyethersulfone (PES) filter prior to loading onto HIC. The HIC resin used is Butyl Sepharose HP. The HIC process is operated in flow-through mode. The Load, Equilibration and Wash Buffer, and Flow-Through are maintained at room temperature throughout processing. The process utilizes 40 mM glutamate, pH 4.0 as the equilibration and wash buffer. The X0SP depth filtrate at pH 4.0 can be loaded directly onto the HIC column without manipulation. Resin loading is limited in order to mitigate aggregation, with a maximum loading of 25 g/L. Table 6 provides representative parameters for the HIC process.

Table 6: representative HIC parameters

Step	Buffer	Volume (CV)	Flowrate (cm/h)
Equilibration	40 mM Glutamate, pH 4.0	5.0	150
Load	Depth Filtrate	(to 25 g/L resin)	150
Wash	40 mM Glutamate, pH 4.0	5.0	150

CV = column volume

*Eluate collected from A280 100 mAU ascending – 100 mAU descending at 2 mm path length

Viral Filtration

[0274] In this workflow, viral filtration occurs prior to tangential flow filtration rather than after it. Viral filtration (nanofiltration) is performed after HIC using a Sartopore® 2 (0.1 μm nominal) pre-filter and an Asahi® BioEX 4 m2 viral filter. The viral filtration is performed at 18-25°C with target operating pressure is 45 psig (30 – 49 psig).

Tangential Flow Filtration

[0275] After viral filtration, tangential flow filtration (TFF) is performed to buffer exchange the antibody into 40 mM glutamate, pH 3.6 to target a formulated mAb in 40 mM glutamate at pH 3.8. A Pall Omega Centrasette® T-Series 30 kDa membrane is used, which is equilibrated with 40 mM glutamate, pH 3.6 buffer. The process is operated at room temperature. If the nanofiltrate pH is > 3.6, it is titrated to pH 3.6 prior to starting the ultrafiltration (concentration) step to mitigate aggregation as the antibody concentration increases. The nanofiltrate at pH 3.6 is concentrated to 30 mg/mL, then diafiltered into 40 mM glutamate, pH 3.6 for 8 diavolumes.

Example 3: Evaluation of aggregation after demasking of Vel-IPV-hB6H12.3

[0276] The impact of mask removal was evaluated for Vel-IPV-hB6H12.3. Vel-IPV-hB6H12.3 was enzymatically demasked using matrix metalloproteinase 2 (MMP2, EMD Millipore) in a digestion buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.5). Demasking was performed at 37°C for up to 2 hours followed by quenching of MMP2 activity with tissue inhibitor of metalloproteinases 2 (TIMP2, EMD Millipore). Demasked samples were analyzed by SE-UPLC.

[0277] Demasked Vel-IPV-hB6H12.3 increased over the reaction time with MMP2, with a corresponding decrease in masked Vel-IPV-hB6H12.3 (Figure 2A). Vel-IPV-hB6H12.3 aggregate levels initially increased due to dilution in the pH 7.5 digestion buffer. By the end of the 2-hour MMP2 treatment, the demasked sample showed very low levels of aggregation as measured by percentage HMW (Figure 2B). Thus, aggregation levels decrease after removal of mask from Vel-IPV-hB6H12.3. These data support the hypothesis that the mask of Vel-IPV-hB6H12.3 plays a role in inducing aggregation in certain formulations.

Example 4: Cytokine production in response to hB6H12.3

[0278] Samples of the fresh whole blood from cancer patients (10 sarcoma, 3 NSCLC, 3 colon cancer, and 1 melanoma) were incubated with increasing concentrations (maximum concentration, 20 μg/ml) of FITC labeled hB6H12.3 or FITC labeled Vel-IPV-hB6H12.3, or with 0.1 μg/mL LPS for 20 hours at 37°C. Cytokine levels were assessed using a 38-plex cytokine and chemokine magnetic bead panel.

[0279] In a majority of patient samples tested, modest cytokine production was induced by hB6H12.3, but minimal cytokine production was induced by Vel-IPV-hB6H12.3. Cytokines IP-10, IL1-Ra, MIP-1α, and MIP-1α were most commonly induced by hB6H12.3. The levels of IL1-Ra (Figure 3B), MIP-1α, and MIP-1β were below 200 pg/mL at the maximum concentration of hB6H12.3 tested, whereas IP-10 levels reached 4000-5000 ng/mL (Figure 3A). Cytokine levels produced by Vel-IPV-hB6H12.3 were lower than those produced by hB6H12.3 in all cases, and were typically 100-1000 fold lower.

Example 5: hB6H12.3 induces apoptosis *in vivo*

[0280] Nude mice bearing human HT1080 fibrosarcoma xenografts were administered a 5 mg/kg IP dose of hB6H12.3, Vel-IPV-hB6H12.3, or a hIgG1 isotype control when tumors reached 200 mm^3. At given time points (24 and 96 hrs), mice were sacrificed and tumors collected. Tumors were homogenized and human HT1080 xenograft fibrosarcoma tumor cells were re-suspended at 1 million cells/ml in 1X Annexin V staining buffer (10x staining buffer containing 50mM HEPES, 700mM NaCl, 12.5mM CaCl2 pH7.4 diluted 1:10 in water). Cells were transferred to a round bottom 96 well plate (100µl/well) and 5µl of FITC Annexin V staining reagent and 1µl of 100µg/ml ultraviolet Live/Dead staining buffer were added to each well. Cells were stained for 30 minutes at room temperature. Samples were spun at 1550g for 5 minutes, supernatant were removed, and cells were washed 3X with 1X ice cold Annexin V staining buffer. Cells were re-suspended in 100µl of 1X Annexin V staining buffer. Apoptosis was assessed by flow cytometry on an LSRII cytometer as percent of cells positive for Annexin V binding to surface phosphatidyl serine. Cells that stained positive with the Live/Dead stain were excluded from the analysis.

[0281] As shown in Figure 4, tumors treated with both hB6H12.3 and Vel-IPV-hB6H12.3 exhibited increased Annexin V+ apoptotic cells 96 hours post treatment when compared to untreated and isotype control-treated tumor samples.

WHAT IS CLAIMED IS:

1. A process for purifying a masked antibody, comprising:
   a) loading a starting composition comprising the masked antibody onto a protein A chromatography column under conditions suitable for binding the masked antibody to the protein A chromatography column;
   b) washing the protein A chromatography column comprising the bound masked antibody at least once with an acidic wash buffer at pH 4.5-5.5; and
   c) eluting the masked antibody from the protein A column in an acidic elution buffer at pH 2.5-4 to form a protein A eluate comprising the masked antibody;
   d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

2. The process of claim 1, wherein the starting composition is a cell lysate.

3. The process of claim 1 or 2, wherein the protein A chromatography is performed at room temperature.

4. The process of any one of claims 1-3, wherein the acidic wash buffer is an acetate or glutamate buffer.

5. The process of claim 4, wherein the acidic wash buffer comprises 10-100 mM, 10-90 mM, 10-80 mM, 10-70 mM, 10-60 mM, 10-50 mM, 10-40 mM, 15-30 mM, 20-30 mM, or 25 mM acetate, or wherein the acidic wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate.

6. The process of any one of claims 1-4, wherein the at least one acidic wash buffer of step (b) is at pH 4.7-5.4, pH 4.8, pH 4.9, pH 5, pH 5.1, or pH 5.2.

7. The process of any one of claims 1-5, wherein the acidic elution buffer comprises 0.05-0.2M, 0.07-0.15M, 0.07-0.13M, 0.08-0.12M, 0.09M, 0.1M, or 0.11M acetic acid, or wherein the acidic elution buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamic acid.

8. The process of any one of claims 1-7, wherein the acidic elution buffer of step (c) is at pH 2.5-5, pH, 3-5, pH 3-4.5, pH 3.5-4, pH 2.5-3.8, pH 2.7-3.8, or pH 2.5-3.5, pH 2.6, pH 2.7, pH 2.8, pH 2.9, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5.

9. The process of any one of claims 1-8, wherein the process comprises washing the column at least once between (a) and (b) with a neutral wash buffer at pH 6-8, optionally wherein the neutral wash buffer is a Tris buffer, which is optionally at pH 7.5, and/or wherein the process comprises washing the column at least once between (a) and (b) with a basic wash buffer at pH 8.5-9.5, optionally wherein the basic wash buffer is an arginine buffer, which is optionally at pH 9.

10. The process of any one of claims 1-9, further comprising adjusting the pH of the protein A eluate to pH 3-4.2, pH 3-4, pH 3.5-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4, to form an acidified eluate.

11. The process of claim 10, wherein the pH is adjusted using acetic acid, optionally 1M acetic acid, or using phosphoric acid, optionally 0.5 M phosphoric acid.

12. The process of any one of claim 10 or claim 11, comprising incubating the acidified eluate for 4-30 hours, 6-30 hours, 10-30 hours, 4-20 hours, 6-20 hours, 8-20 hours, 10-20 hours, 4-18 hours, 6-18 hours, 8-18 hours, 10-18 hours, 8-16 hours, 10-16 hours, 8-14 hours, 10-14 hours, 11-13 hours, 10 hours, 11 hours, 12 hours, 13 hours, or 14 hours after adjusting the pH..

13. A process for purifying a masked antibody, comprising:

a) subjecting a starting composition comprising the masked antibody to one or more chromatography purification steps, to form a chromatography eluate;

b) adjusting the pH of the chromatography eluate to pH 3-4.2, pH 3-4, pH 3.5-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4, to form an acidified eluate; and

c) incubating the acidified eluate for 4-30 hours, 6-30 hours, 10-30 hours, 4-20 hours, 6-20 hours, 8-20 hours, 10-20 hours, 4-18 hours, 6-18 hours, 8-18 hours, 10-18 hours, 8-16 hours, 10-16 hours, 8-14 hours, 10-14 hours, 11-13 hours, 10 hours, 11 hours, 12 hours, 13 hours, or 14 hours;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain

comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

14. The process of claim 13, wherein the starting composition is a cell lysate.

15. The process of any one of claims 12-14, wherein the acidified eluate is incubated at room temperature.

16. The process of any one of claims 12-15, comprising further adjusting the pH of the acidified eluate to pH 3.5-4.5, pH 3.5-4.3, pH 3.7-4.2, pH 3.6-4, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, or pH 4.2 after incubation.

17. The process of claim 16, wherein the pH is adjusted using tris base, optionally 1M tris base.

18. The process of any one of claims 12-17, comprising filtering the acidified eluate on a depth filter.

19. The process of any one of claims 12-18, comprising chilling the acidified eluate to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C after incubation or depth filtration.

20. The process of claim 19, comprising adjusting the pH of the chilled acidified eluate to pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9, to form a chilled neutral eluate.

21. The process of claim 20, wherein the pH is adjusted using tris base, optionally 1M tris base.

22. The process of claim 20 or claim 21, further comprising loading the chilled neutral eluate on a hydrophobic interaction chromatography (HIC) column or membrane.

23. The process of claim 22, comprising washing the HIC column or membrane with a HIC wash buffer that has been chilled to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C.

24. A process for purifying a masked antibody, comprising:

a) chilling a starting composition comprising the masked antibody, wherein the pH of the starting composition is adjusted to pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9 before or after chilling, to form a chilled starting composition;

b) loading the chilled starting composition on a hydrophobic interaction chromatography (HIC) column or membrane; and

c) washing the HIC column or membrane with a HIC wash buffer that has been chilled to a temperature of 1-15°C, 1-10°C, or 1-9°C, or 2-8°C;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain

variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

25. The process of claim 24, wherein the starting composition is a cell lysate.

26. The process of any one of claims 23-25, wherein the HIC wash buffer is a tris/sodium citrate buffer.

27. The process of any one of claims 23-26, wherein the HIC wash buffer is at pH 7-9, pH 7-8.5, pH 7-8, pH 7.1, pH 7.2, pH 7.3, pH 7.4, pH 7.5, pH 7.6, pH 7.7, pH 7.8, or pH 7.9.

28. The process of any one of claims 23-27, wherein the HIC wash buffer comprises sodium citrate.

29. The process of claim 28, wherein the concentration of sodium citrate in the HIC wash buffer is 200-700 mM, or 200-600 mM, or 200-500 mM, or 250-500 mM, or 300-500 mM, or 300-400 mM.

30. The process of any one of claims 23-29, comprising collecting a HIC effluent comprising the masked antibody.

31. The process of claim 30, further comprising a first diafiltration following HIC to reduce the concentration of sodium citrate to below 40 mM, or below 35 mM, or below 30 mM, or below 25 mM, or below 20 mM, or below 15 mM, or below 10 mM, or below 5 mM, to form a diafiltered HIC effluent.

32. The process of claim 31, wherein the first diafiltration is performed at 1-15°C, 1-10°C, or 1-9°C, or 2-8°C.

33. The process of claim 31 or claim 32, further comprising a second diafiltration in an acetate buffer, wherein the second diafiltration is performed at room temperature, optionally 15-28°C, or 18-25°C.

34. The process of claim 33, wherein the acetate buffer comprises 20-100 mM, 20-90 mM, 20-80 mM, 20-70 mM, 30-50 mM, 35 mM, 40 mM, or 45 mM acetate.

35. The process of claim 33 or claim 34, wherein prior to the second diafiltration, the pH of the diafiltered HIC effluent is adjusted to pH 3.5-4.5, pH 3.7-4.5, pH 3.7-4.3, pH 3.8, pH 3.9, pH 4, pH 4.1, or pH 4.2.

36. The process of claim 35, wherein the pH is adjusted using 25% v/v glacial acetic acid, to form an acidified diafiltered HIC effluent.

37. The process of any one of claims 33-36, wherein the acidified diafiltered HIC effluent is subjected to ultrafiltration to form a concentrated masked antibody composition.

38. The process of claim 37, wherein the concentration of the masked antibody in the concentrated masked antibody composition is 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL.

39. The process of any one of claims 30-38, further comprising performing virus removal.

40. The process of claim 39, wherein virus removal is performed by nanofiltration.

41. The process of claim 40, wherein the nanofiltration is performed at acidic pH.

42. The process of claim 41, wherein the acidic pH is pH 3-4.4, pH 3.5-4.4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4.

43. The process of any one of claims 39-42, wherein the virus removal is performed at room temperature.

44. The process of any one of claims 39-43, wherein virus removal follows the ultrafiltration.

45. The process of any one of claims 10-18, optionally comprising adjusting the pH of the acidified eluate to pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5.

46. The process of claim 45, wherein the pH is adjusted using tris base, optionally 1M tris base.

47. The process of claim 45 or claim 46, further comprising loading the optionally pH adjusted acidified eluate on a hydrophobic interaction chromatography (HIC) column or membrane.

48. The process of claim 47, comprising conducting HIC at room temperature.

49. A process for purifying a masked antibody, comprising:

a) obtaining a starting composition comprising the masked antibody, wherein the pH of the starting composition is adjusted to pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5;

b) loading the starting composition on a hydrophobic interaction chromatography (HIC) column or membrane; and

c) washing the HIC column or membrane with a HIC wash buffer at pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5;

d) wherein the masked antibody comprises a first masking domain comprising a first coiled-coil domain, wherein the first masking domain is linked to a heavy chain variable region of an antibody and a second masking domain comprising a second coiled-coil domain, wherein the second masking domain is linked to a light chain variable region of the antibody, wherein the first coiled-coil domain comprises the sequence VDELQAEVDQLEDENYALKTKVAQLRKKVEKL (SEQ ID NO: 2), and the second coiled-coil domain comprises the sequence VAQLEEKVKTLRAENYELKSEVQRLEEQVAQL (SEQ ID NO: 1).

50. The process of any one of claims 47-49, wherein the starting composition is a cell lysate.

51. The process of any one of claims 47-50, wherein the HIC wash buffer is a glutamate buffer.

52. The process of claim 51, wherein the HIC wash buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate.

53. The process of any one of claims 47-52, wherein the HIC wash buffer is at pH 3.6 to 4, pH 3.6, pH 3.7, pH 3.8, pH 3.9, or pH 4.

54. The process of any one of claims 47-53, wherein the HIC step is conducted at room temperature.

55. The process of any one of claims 47-54, comprising collecting a HIC effluent comprising the masked antibody.

56. The process of claim 55, further comprising exchanging the buffer of the HIC effluent to a glutamate buffer at pH pH 3-5, pH 3-4.5, pH 3.5-4.5, 3.6-4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4, or pH 4.5, wherein exchanging the buffer is through diafiltration or tangential flow filtration.

57. The process of claim 56, wherein the glutamate buffer comprises 10-60 mM, 10-50 mM, 20-60 mM, 20-50 mM, 10-40 mM, 20-40 mM, 30-50 mM, 20-40 mM, 30-40 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 60 mM glutamate.

58. The process of claim 56 or 57, further comprising ultrafiltration prior to diafiltration or tangential flow filtration to concentrate the masked antibody to 10-40 mg/mL, 15-35 mg/mL, 20-35 mg/mL, or 25-35 mg/mL.

59. The process of any one of claims 49-58, further comprising performing virus removal.

60. The process of claim 59, wherein virus removal is performed by nanofiltration.

61. The process of claim 60, wherein the nanofiltration is performed at acidic pH.

62. The process of claim 61, wherein the acidic pH is pH 3-4.4, pH 3.5-4.4, pH 3.6-4, pH 3, pH 3.1, pH 3.2, pH 3.3, pH 3.4, pH 3.5, pH 3.6, pH 3.7, pH 3.8, pH 3.9, pH 4, pH 4.1, pH 4.2, pH 4.3, pH 4.4.

63. The process of any one of claims 59-62, wherein the virus removal is performed at room temperature.

64. The process of any one of claims 59-63, wherein virus removal precedes the diafiltration and ultrafiltration.

65. The process of any one of claims 1-64, wherein each pH >5 step of the process is performed at a temperature of 1°C to 15°C, and/or wherein each room temperature step of the process is performed at pH <4.5.

66. The process of any one of claims 1-65, wherein each masking domain comprises a protease-cleavable linker and is linked to the heavy chain or light chain via the protease-cleavable linker.

67. The process of claim 66, wherein the protease-cleavable linker comprises a matrix metalloprotease (MMP) cleavage site, a urokinase plasminogen activator cleavage site, a matriptase cleavage site, a legumain cleavage site, a Disintegrin and Metalloprotease (ADAM) cleavage site, or a caspase cleavage site.

68. The process of claim 67, wherein the protease-cleavable linker comprises a matrix metalloprotease (MMP) cleavage site.

69. The process of claim 68, wherein the MMP cleavage site is selected from an MMP2 cleavage site, an MMP7 cleavage site, an MMP9 cleavage site and an MMP13 cleavage site.

70. The process of claim 67 or claim 68, wherein the MMP cleavage site comprises the sequence IPVSLRSG (SEQ ID NO: 19) or GPLGVR (SEQ ID NO: 21).

71. The process of any one of claims 1-70, wherein the first masking domain comprises the sequence GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG (SEQ ID NO: 4).

72. The process of any one of claims 1-71, wherein the second masking domain comprises the sequence GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG (SEQ ID NO: 3).

73. The process of any one of claims 1-72, wherein the first masking domain comprises the sequence GASTSVDELQAEVDQLEDENYALKTKVAQLRKKVEKLGSIPVSLRSG (SEQ ID NO: 4), and the second masking domain comprises the sequence GASTTVAQLEEKVKTLRAENYELKSEVQRLEEQVAQLGSIPVSLRSG (SEQ ID NO: 3).

74. The process of any one of claims 1-73, wherein the first masking domain is linked to the amino-terminus of the heavy chain and the second masking domain is linked to the amino-terminus of the light chain.

75. The process of any one of claim 1-74, wherein the antibody binds an antigen selected from CD47, CD3, CD19, CD20, CD22, CD30, CD33, CD34, CD40, CD44, CD52, CD70, CD79a, CD123, Her-2, EphA2, lymphocyte associated antigen 1, VEGF or VEGFR, CTLA-4, LIV-1, nectin-4, CD74, SLTRK-6, EGFR, CD73, PD-L1, CD163, CCR4, CD147, EpCam, Trop-2, CD25, C5aR, Ly6D, alpha v integrin, B7H3, B7H4, Her-3, folate receptor alpha, GD-2, CEACAM5, CEACAM6, c-MET, CD266, MUC1, CD10, MSLN, sialyl Tn, Lewis Y, CD63, CD81, CD98, CD166, tissue factor (CD142), CD55, CD59, CD46, CD164, TGF beta receptor 1 (TGFβR1), TGFβR2, TGFβR3, FasL, MerTk, Axl, Clec12A, CD352, FAP, CXCR3, and CD5.

76. The process of claim 75, wherein the antibody binds CD47.

77. The process of claim 76, wherein the antibody comprises a light chain variable region and a heavy chain variable region, wherein the heavy chain variable region comprises HCDR1 comprising SEQ ID NO: 25; HCDR2 comprising SEQ ID NO: 26; and HCDR3 comprising SEQ ID NO: 27; wherein the light chain variable region comprises LCDR1 comprising SEQ ID NO: 31; LCDR2 comprising SEQ ID NO: 32; and LCDR3 comprising SEQ ID NO: 33 or 34.

78. The process of claim 77, wherein the heavy chain variable region comprises an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence selected from SEQ ID NO: 22.

79. The process of claim 77 or claim 78, wherein the light chain variable region comprises an amino acid sequence with at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 23 or 24.

80. The process of any one of claims 76-79, wherein the antibody comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising SEQ ID NOs: 25, 26, 27, 31, 32, and 33.

81. The process of claim 76, wherein the antibody comprises a light chain variable region and a heavy chain variable region, wherein the heavy chain variable region comprises HCDR1 comprising SEQ ID NO: 28; HCDR2 comprising SEQ ID NO: 29; and HCDR3 comprising SEQ ID NO: 30; and wherein the light chain variable region comprises LCDR1 comprising SEQ ID NO: 35; LCDR2 comprising SEQ ID NO: 36; and LCDR3 comprising SEQ ID NO: 37 or 38.

82. The process of claim 81, wherein the heavy chain variable region comprises an amino acid sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 22.

83. The process of claim 81 or claim 82, wherein the light chain variable region comprises an amino acid sequence with at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence of SEQ ID NO: 23 or 24.

84. The process of any one of claims 81-83, wherein the antibody comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising SEQ ID NOs: 28, 29, 30, 35, 36, and 37.

85. The process of any one of claims 76-84, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 22.

86. The process of any one of claims 76-85, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 23 or 24.

87. The process of any one of claims 76-86, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 22 and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 23.

88. The process of claim 76, wherein the masked antibody comprises a first masking domain linked to a heavy chain and a second masking domain linked to a light chain, wherein the first masking domain and the heavy chain comprises or consists of the sequence of SEQ ID NO: 39 or SEQ ID NO: 40, and the second masking domain and the light chain comprises or consists of the sequence of SEQ ID NO: 42.

89. The process of any one of claims 76-88, wherein the antibody blocks an interaction between CD47 and SIRPα.

90. The process of any one of claims 1-89, wherein the antibody has reduced core fucosylation.

91. The process of any one of claims 1-89, wherein the antibody is afucosylated.

92. The process of any one of claims 1-91, wherein the masked antibody is conjugated to a cytotoxic agent.

93. The process of claim 92, wherein the cytotoxic agent is an antitubulin agent, a DNA minor groove binding agent, a DNA replication inhibitor, a DNA alkylator, a topoisomerase inhibitor, a NAMPT inhibitor, or a chemotherapy sensitizer.

94. The process of claim 92 or claim 93, wherein the cytotoxic agent is an anthracycline, an auristatin, a camptothecin, a duocarmycin, an etoposide, an enediyine antibiotic, a lexitropsin, a taxane, a maytansinoid, a pyrrolobenzodiazepine, a combretastatin, a cryptophysin, or a vinca alkaloid.

95. The process of any one of claims 92-94, wherein the cytotoxic agent is auristatin E, AFP, AEB, AEVB, MMAF, MMAE, paclitaxel, docetaxel, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, melphalan, methotrexate, mitomycin C, a CC-1065 analogue, CBI, calicheamicin, maytansine, an analog of dolastatin 10, rhizoxin, or palytoxin, epothilone A, epothilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, eleutherobin, a tubulysin, a plocabulin, or maytansine.

96. The process of claim 95, wherein the cytotoxic agent is an auristatin.

97. The process of claim 96, wherein the cytotoxic agent is MMAE or MMAF.

98. The process of any one of claims 1-91, wherein following purification of the masked antibody, the masked antibody is conjugated to a cytotoxic agent.

99. The process of claim 98, wherein the cytotoxic agent is an antitubulin agent, a DNA minor groove binding agent, a DNA replication inhibitor, a DNA alkylator, a topoisomerase inhibitor, a NAMPT inhibitor, or a chemotherapy sensitizer.

100. The process of claim 98 or claim 99, wherein the cytotoxic agent is an anthracycline, an auristatin, a camptothecin, a duocarmycin, an etoposide, an enediyine antibiotic, a lexitropsin, a taxane, a maytansinoid, a pyrrolobenzodiazepine, a combretastatin, a cryptophysin, or a vinca alkaloid.

101. The process of any one of claims 98-100, wherein the cytotoxic agent is auristatin E, AFP, AEB, AEVB, MMAF, MMAE, paclitaxel, docetaxel, doxorubicin, morpholino-doxorubicin, cyanomorpholino-doxorubicin, melphalan, methotrexate, mitomycin C, a CC-1065 analogue, CBI, calicheamicin, maytansine, an analog of dolastatin 10, rhizoxin, or palytoxin, epothilone A, epothilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, eleutherobin, a tubulysin, a plocabulin, or maytansine.

102. The process of claim 101, wherein the cytotoxic agent is an auristatin.

103. The process of claim 102, wherein the cytotoxic agent is MMAE or MMAF.

104. A masked antibody purified by the process of any one of claims 1-103.

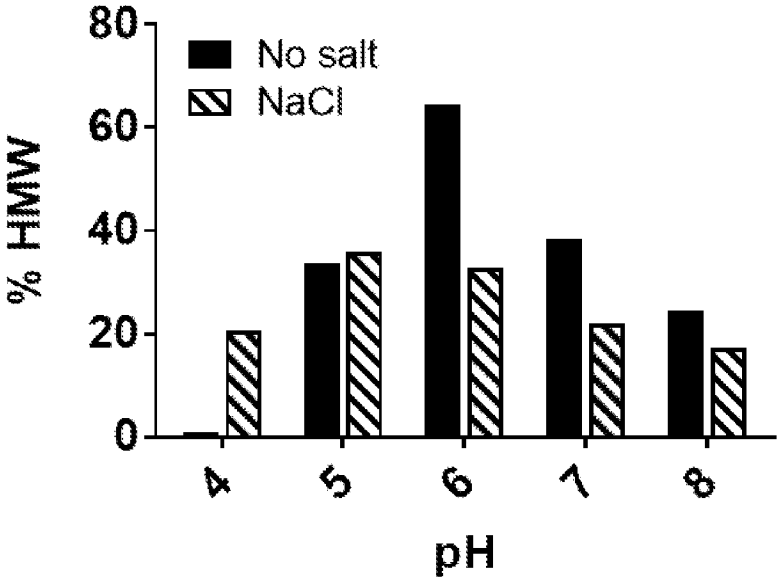


Figure 1A

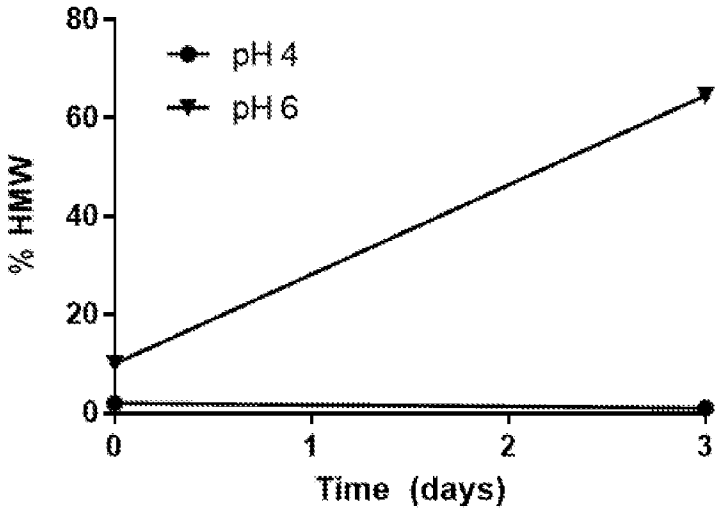


Figure 1B

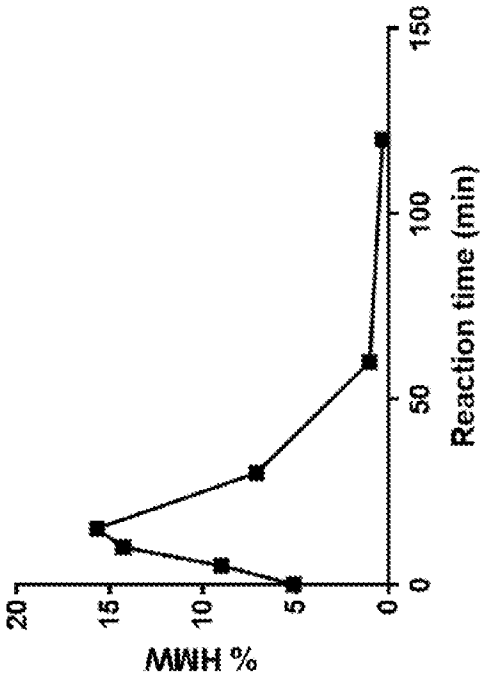


Figure 2B

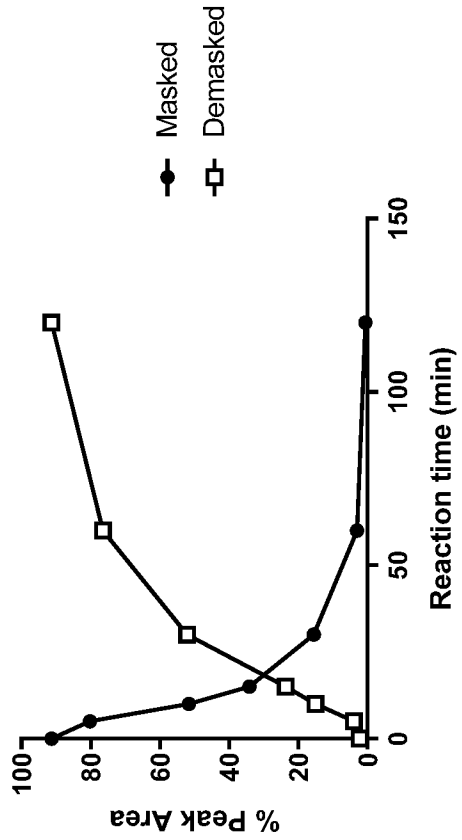


Figure 2A

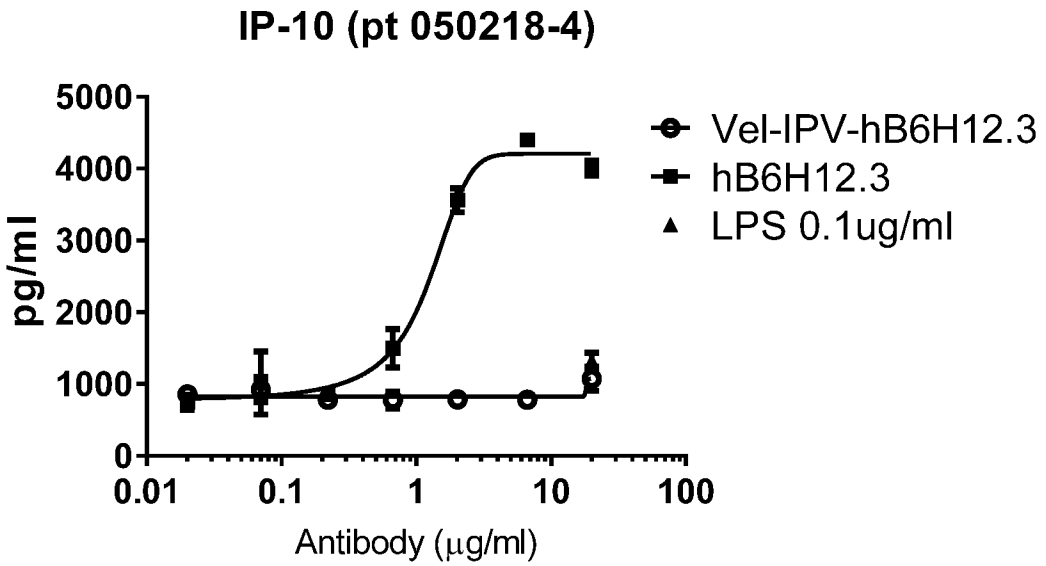


Figure 3A

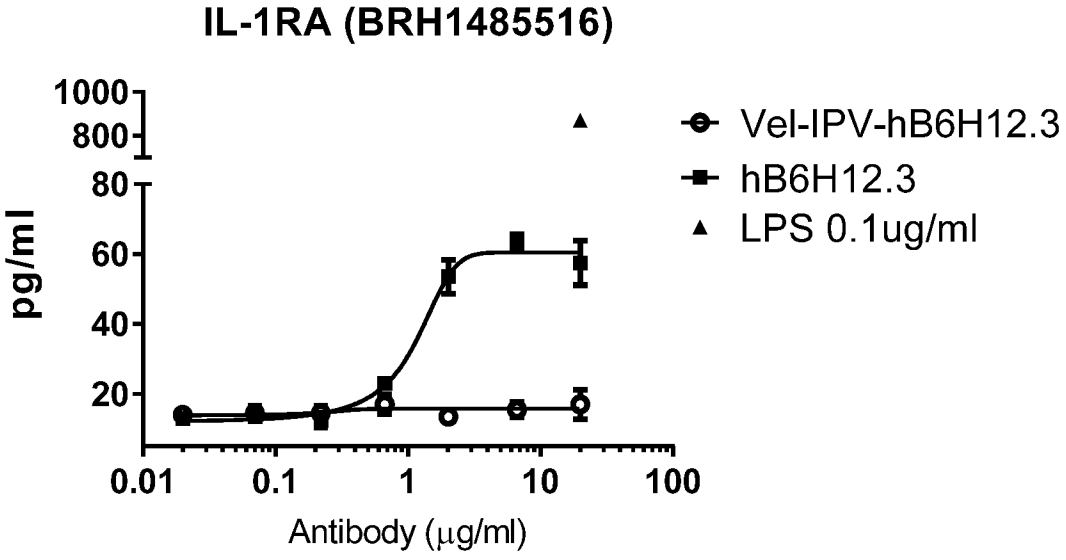


Figure 3B

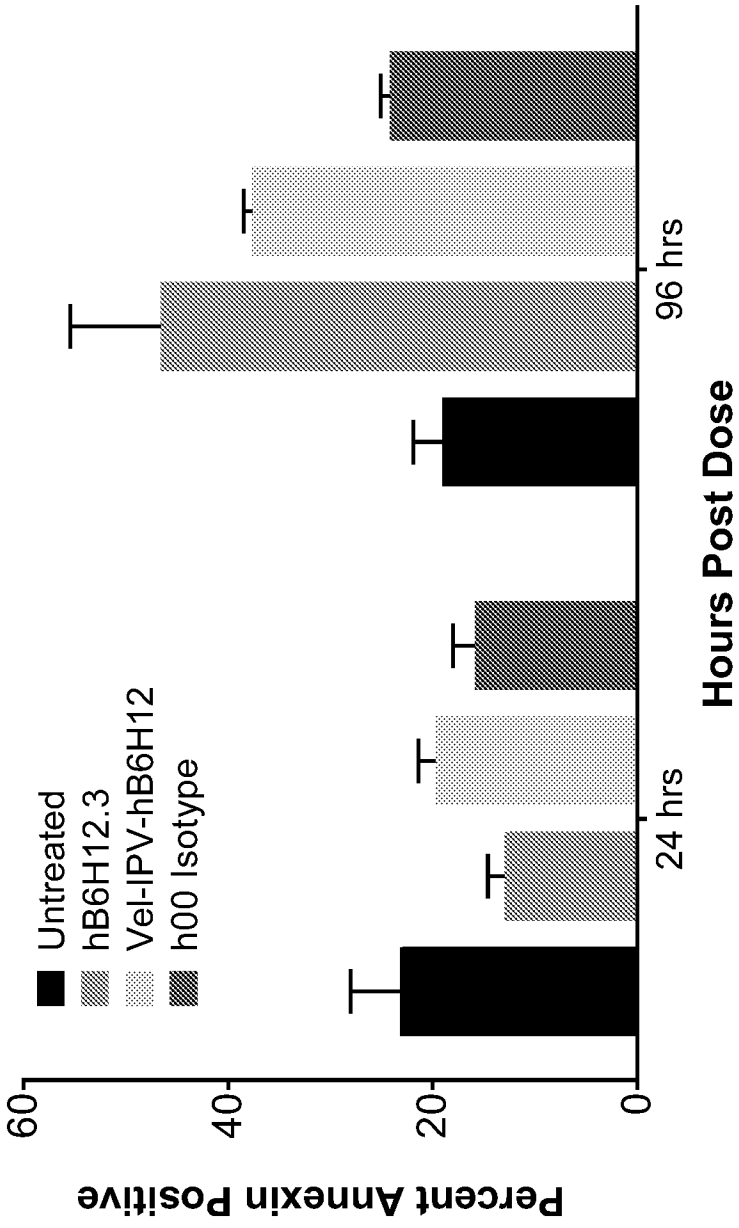

Annexin V Staining on Ht1080 Tumor Cells
Untreated
hB6H12.3
Vel-IPV-hB6H12
h00 Isotype
Percent Annexin Positive
0
20
40
60
24 hrs
96 hrs
Hours Post Dose

Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/036037

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 C07K16/06 C07K16/28 C07K1/20 C07K1/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/107125 A1 (SEATTLE GENETICS INC [US]) 14 June 2018 (2018-06-14) page 32, paragraph [0110]; claims; examples; sequences 31,32,34,35,44,45,46,47,64,66 -----	1-104
A	WO 2011/050071 A2 (ABBOTT LAB [US]; HICKMAN ROBERT K [US]) 28 April 2011 (2011-04-28) page 63, line 12 - line 15; examples ----- -/--	1-104

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 2020	22/10/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Loubradou, Gabriel

1

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A	ISHIKAWA TOMOYOSHI ET AL: "Influence of pH on Heat-Induced Aggregation and Degradation of Therapeutic Monoclonal Antibodies", BIOLOGICAL & PHARMACEUTICAL BULLETIN, PHARMACEUTICAL SOCIETY OF JAPAN, TOKYO, JP, vol. 33, no. 8, 1 August 2010 (2010-08-01), pages 1413-1417, XP009157095, ISSN: 0918-6158, DOI: 10.1248/BPB.33.1413 abstract	1-104
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International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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