

METHODS FOR CHARACTERIZING PROTEIN COMPLEXES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of and priority to U.S. Provisional Patent Application No. 62/724,700 filed on August 30, 2018, which is incorporated by reference in its entirety.

TECHNICAL FIELD OF THE INVENTION

This invention is generally related to systems and methods of characterizing protein complexes.

BACKGROUND OF THE INVENTION

Monoclonal antibodies (mAbs) are a growing therapeutic field, with over 50 monoclonal antibodies currently on the market. Combination therapy of more than one monoclonal antibody has the potential to improve the efficacy of existing monotherapies. Certain soluble substances, particularly multimeric substances with several repeated epitopes, may bind with two or more antibodies, leading to the formation of large complexes. The production of large, heterogeneous antibody complexes is referred to as “paper-dolling”. Large complexes of antibodies can be rapidly eliminated by phagocytosis, leading to reduced efficacy of the antibody. Large protein complexes can also increase immunogenicity of the mAb.

Protein complexes can range in size from nanometer to visible particles making their characterization by a single analytical technique difficult. One of the most widespread techniques used to determine the size of particles from ~1 nm to ~1 μ m is dynamic light scattering (DLS). DLS is an analytical technique used to determine protein size distribution profile, and is amenable to high throughput applications (Zhou, M., et al., *ChemMedChem*, 11:738-756 (2016)). The Brownian motion of proteins in solution causes light to be scattered, with the resultant scattered intensity fluctuations dependent on particle size. Thus, average radius and the width of the distribution in terms of polydispersity can be determined. However, DLS results are often biased towards larger particles and the particle populations must differ by a factor of at least three to be resolved. Therefore, DLS alone is not sufficient for analyzing protein complexes.

The high polydispersity of many aggregate samples requires separation-based methods to provide more detailed information due to the wide size range of protein complexes. Size exclusion chromatography (SEC) is currently the most commonly used chromatographic

technique for protein separation (Brusotti, et al., *Chromatographia*, 81:3-23 (2018)). In SEC, separation occurs according to hydrodynamic volume or size of molecules. Smaller molecules are retained longer because they are able to diffuse into the pores of the stationary phase, while larger molecules elute first because they are excluded from the pores. However, SEC is limited by upper molecular weight exclusion limits, sample adsorption to the stationary phase, shear degradation at high pressures and flow rates, and an inability to separate analytes based on composition.

Flow field-flow fractionation (FFF) is a promising alternative to SEC when it comes to separation of large proteins and high molar mass polymers. FFF sample separation uses a flow-assisted separation and fractionation method in which the analytes are separated along a ribbon like channel by differences in their diffusion coefficients (Fraunhofer, W. and Winter, G., *Eur. J. Pharm. Biopharm*, 58:369-383 (2004)). FFF can separate analytes in a wide size range (from nanometers to microns). The open channel of FFF renders reduced sample loss, low pressures, and low shear rates. While FFF has been used in combination with other molecular techniques such as light scattering to detect protein aggregates, there is still a growing need to more fully characterize heterogeneity and conformation of protein complexes, including mAb and soluble ligand complexes.

Therefore, it is an object of the invention to provide methods for identifying protein drug products that have the ability to form large protein complexes.

It is another object of the invention to provide methods of identifying and characterizing protein drug product and soluble ligand complexes.

SUMMARY OF THE INVENTION

Methods for characterizing protein complexes in a sample are provided. One embodiment provides a method for assessing the stoichiometry and size distribution of protein complexes in a sample by fractionating the sample by asymmetrical flow field flow fractionation (A4F), and determining the molar mass, stoichiometry, and size distribution of the protein complexes in the sample using Multi-Angle Laser Light Scattering (MALLS). In some embodiments, the protein complexes contain an antibody or fusion protein bound to its ligand. The ligand is typically a soluble ligand. The ligand can be monomeric or multimeric. In one embodiment the ligand can be a homodimer or heterodimer. In another embodiment the protein complexes comprise or consist of antibody:ligand complexes or fusion protein:ligand complexes.

Another embodiment provides a method for selecting a lead protein drug product by adding a first protein drug product to its target or ligand to produce protein:ligand complexes, and adding a second protein drug product to the first protein drug product:ligand complexes to form multiple protein:ligand complexes. The method includes separating the protein:ligand
5 complexes and determining the molar mass, stoichiometry, and size distribution of protein:ligand complexes using asymmetrical flow field flow fractionation– MALLS. The method also includes selecting the protein drug product that forms fewer large protein:ligand complexes as the lead target protein drug. Typically, the protein drug product is an antibody or antigen binding fragment thereof, a fusion protein, or a recombinant protein. In some embodiments, the ligand is
10 a soluble ligand. The ligand can be monomeric or multimeric. In some embodiments the large protein complexes are heteromeric.

Another embodiment provides a pharmaceutical composition containing the lead protein drug product selected using the method described above.

In some embodiments, the disclosed methods can be used to determine if two individual
15 antibodies targeting the same ligand will form large, heterogeneous complexes.

Still another embodiment provides a method for characterizing protein complexes formed between protein drug products and soluble ligands by preparing a sample containing the protein drug product and its ligand to produce protein drug product:ligand complexes. The method includes fractionating the protein drug:ligand complexes and analyzing the fractionated protein
20 drug:ligand complexes by multi-angle laser light scattering to determine the molar mass and heterogeneity of protein complexes. In one embodiment, fractionating the total protein is performed by asymmetrical flow field flow fractionation. The concentration of the protein is determined with UV/Vis.

The differences in conformation of protein complexes formed by different protein drug
25 products to the same ligand can be determined by comparing the elution profile/time of protein drug product:ligand complexes formed by the different protein drug products. Different elution profiles/times of complexes with the same molar mass indicate that the complexes may have different conformations or shapes. In one embodiment, each protein drug product and the same soluble ligand is analyzed separately in order to calculate a molar mass for each individual
30 component, wherein the molar mass of each component is used to determine the estimated stoichiometry of said components in each protein complex.

Another embodiment provides a method for characterizing protein complexes formed between protein drug products and soluble ligands by fractionating protein drug product:ligand complexes using asymmetrical flow-field flow fractionation, analyzing the fractionated protein drug product:ligand complexes by multi-angle laser light scattering to characterize the molar mass, stoichiometry, or both of protein drug:ligand complexes, and determining the heterogeneity of the protein complexes by comparing the size of each protein complex to the estimated size of each individual component to determine the components that make up each complex.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is an illustration of a complex formed between one antibody and one ligand. Figure 1B is an illustration of a complex formed between one antibody and two ligands. Figure 1C is an illustration of a complex formed between four antibodies and five ligands, representative of “paper dolling”.

Figures 2A-2B are an illustration of a complex formed between antibody 1 (black), two ligands, and antibody 2 (gray) in a non-linear conformation. Figures 2C-2D are an illustration of a complex formed between antibody 1 (black), two ligands, and antibody 2 (gray) in a linear conformation.

Figure 3A is a chromatogram from SEC-MALLS analysis of Ab1, Protein X, and combinations of Ab1 and Protein X at molar ratios of 5:1, 1:1, and 1:5. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 280nm (AU). Figure 3B is a fractogram from asymmetrical flow field flow fractionation -MALLS (A4F-MALLS) analysis of combinations of Ab1 and Ab2 at molar ratios of 5:1, 1:1, and 1:5. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol). The right Y axis represents absorbance at 215 nm (AU).

Figure 4A is a fractogram from A4F-MALLS analysis of lead compound A (Lead A), Lead A + Protein Y (1:3), and Lead compound B (Lead B) + Protein Y (1:3). Figure 4B is a fractogram from A4F-MALLS analysis of Lead A, Lead A + Protein Y (1:1), and Lead B + Protein Y (1:1). Figure 4C is a fractogram from A4F-MALLS analysis of Lead A, Lead A + Protein Y (3:1), and Lead B + Protein Y (3:1). The X axis represents time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 5A and 5B are line graphs of mouse anti-human antibody titer of anti-Protein Y complexes with Lead A (Figure 5A) and Lead B (Figure 5B). The X axis represents time (days) and the Y axis represents concentration ($\mu\text{g/ml}$).

Figure 6A and 6B are line graphs showing percent hemolysis of rabbit red blood cells with increasing concentrations of various anti-Protein Z mAbs (Figure 6A) or combinations of Ab3 and various anti-Protein Z mAbs (Figure 6B). The X axis represents concentration of mAb (Log [M]). The Y axis represents percent hemolysis.

Figure 7 is a fractogram from A4F-MALLS analysis of Free anti-Protein Z mAb1, Protein Z, and $1\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1 and Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 8 is a fractogram from A4F-MALLS analysis of Free mAb, $1\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1 and Protein Z, $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, anti-Protein Z mAb5, and Protein Z, and $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, anti-Protein Z mAb7, and Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 9 is a fractogram from A4F-MALLS analysis of Free mAb combo, $1\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1 and Protein Z, $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, anti-Protein Z mAb3 combo, and Protein Z, and $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, anti-Protein Z mAb6 combo, and Protein Z, and $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb6, anti-Protein Z mAb7, and Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 10 is a fractogram from A4F-MALLS analysis of free mAb combo, $1\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1 and Protein Z, $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, anti-Protein Z mAb2, and Protein Z, and $0.5\mu\text{M}:0.5\mu\text{M}:1\mu\text{M}$ combination of anti-Protein Z mAb1, COMP1 mAb, and Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 11 is a fractogram from A4F-MALLS analysis of Free mAb combo, 1 μ M:1 μ M combination of anti-Protein Z mAb1 and Protein Z, 0.5 μ M:0.5 μ M:1 μ M combination of anti-Protein Z mAb1, anti-Protein Z mAb4, and Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 12A and 12B are fractograms from A4F-MALLS analysis of simultaneous addition (Figure 12A) or sequential addition (Figure 12B) of 0.3 μ M:1 μ M:1 μ M anti-Protein Z mAb1:COMP1 mAb:Protein Z, 1 μ M:1 μ M:1 μ M anti-Protein Z mAb1:COMP1 mAb:Protein Z, and 3 μ M:1 μ M:1 μ M anti-Protein Z mAb1:COMP1 mAb:Protein Z. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 13 is a fractogram from A4F-MALLS analysis of a representative mAb, Protein W, and combinations of mAb and Protein W at ratios of 1 μ M:1 μ M. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 14 is a fractogram from A4F-MALLS analysis of a representative mAb, Protein W, and combinations of mAb2 and Protein W, mAb3 and Protein W, and COMP1 and Protein W at ratios of 1 μ M:1 μ M. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 15 is a fractogram from A4F-MALLS analysis of COMP2, Protein W, and combinations of COMP2 and Protein W at ratios of 1 μ M:1 μ M. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

Figure 16 is a fractogram from A4F-MALLS analysis of a representative mAb, Protein W, and combinations of mAb4 and Protein W, and COMP3 and Protein W at ratios of 1 μ M:1 μ M. The X axis represents elution time (minutes). The left Y axis represents molar mass (g/mol) and the right Y axis represents absorbance at 215 nm (AU).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

It should be appreciated that this disclosure is not limited to the compositions and methods described herein as well as the experimental conditions described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing certain embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any compositions, methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications mentioned are incorporated herein by reference in their entirety.

The use of the terms "a," "an," "the," and similar referents in the context of describing the presently claimed invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

Use of the term "about" is intended to describe values either above or below the stated value in a range of approx. +/- 10%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 5%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 2%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 1%. The preceding ranges are intended to be made clear by context, and no further limitation is implied. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless

otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

“Protein” refers to a molecule comprising two or more amino acid residues joined to each other by a peptide bond. Protein includes polypeptides and peptides and may also include
5 modifications such as glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, alkylation, hydroxylation and ADP-ribosylation. Proteins can be of scientific or commercial interest, including protein-based drugs, and proteins include, among other things, enzymes, ligands, receptors, antibodies and chimeric or fusion proteins. Proteins are produced by various types of recombinant cells using well-known cell culture methods, and
10 are generally introduced into the cell by genetic engineering techniques (e.g., such as a sequence encoding a chimeric protein, or a codon-optimized sequence, an intronless sequence, etc.) where it may reside as an episome or be intergrated into the genome of the cell.

“Antibody” refers to an immunoglobulin molecule consisting of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy
15 chain has a heavy chain variable region (HCVR or VH) and a heavy chain constant region. The heavy chain constant region contains three domains, CH1, CH2 and CH3. Each light chain has a light chain variable region and a light chain constant region. The light chain constant region consists of one domain (CL). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions
20 that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The term “antibody” includes reference to both glycosylated and non-glycosylated immunoglobulins of any isotype or subclass. The term “antibody” includes antibody molecules prepared, expressed, created or isolated by recombinant
25 means, such as antibodies isolated from a host cell transfected to express the antibody. The term antibody also includes bispecific antibody, which includes a heterotetrameric immunoglobulin that can bind to more than one different epitope. Bispecific antibodies are generally described in US Patent No. 8,586,713, which is incorporated by reference into this application.

“Fc fusion proteins” comprise part or all of two or more proteins, one of which is an Fc
30 portion of an immunoglobulin molecule, which are not otherwise found together in nature. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various

portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88: 10535, 1991; Byrn et al., Nature 344:677, 1990; and Hollenbaugh et al., "Construction of Immunoglobulin Fusion Proteins", in Current Protocols in Immunology, Suppl. 4, pages 10.19.1 - 10.19.11, 1992. "Receptor Fc fusion proteins"

5 comprise one or more extracellular domain(s) of a receptor coupled to an Fc moiety, which in some embodiments comprises a hinge region followed by a CH2 and CH3 domain of an immunoglobulin. In some embodiments, the Fc-fusion protein comprises two or more distinct receptor chains that bind to one or more ligand(s). For example, an Fc-fusion protein is a Trap, such as for example an IL-1 Trap or VEGF Trap.

10 As used herein, "soluble ligand" refers to polar or charged ligands that cannot readily cross the plasma membrane of a cell. Most soluble ligands bind to the extracellular domains of cell-surface receptors.

"A4F" represents asymmetrical flow field-flow fractionation which is a fractionating technique in which separation of analytes is achieved through the interaction of the sample with
15 an external, perpendicular physical field.

Multi angle light scattering (MALS) describes a technique for measuring the light scattered by a sample into a plurality of angles. It is used for determining both the absolute molar mass and the average size of molecules in solution, by detecting how they scatter light. Collimated light from a laser source is most often used, in which case the technique can be
20 referred to as multi angle laser light scattering (MALLS). In practice the terms MALS and MALLS are used interchangeably.

As used herein, "Brownian motion" refers to the continuous motion of particles suspended in liquid.

II. Methods For Characterizing Protein Complexes

25 Monoclonal antibody combination therapy has emerged as a promising therapeutic strategy for diseases such as cancer and inflammatory conditions in which multiple signaling pathways are involved. In addition, administration of more than one monoclonal antibody targeting the same pathway could be beneficial to completely block pathways involved in the pathogenesis of diseases when monotherapy alone does not fully inhibit the pathway. However,
30 binding of therapeutic mAbs to soluble, multimeric targets can lead to the formation of large heterogeneous complexes. The size, shape, and conformation of a protein complex can affect

immunogenicity and antibody clearance time, among other factors. Analysis of protein complexes is important to provide insight into the pharmacokinetics of a mAb during drug development.

Therefore, methods and systems for characterizing protein complexes are provided. One embodiment provides a method for assessing the stoichiometry and size distribution of heterogeneous protein drug product:ligand complexes in a sample by fractionating the sample using asymmetrical flow field flow fractionation and determining the molar mass, stoichiometry, and size distribution of heterogeneous protein complexes in the sample using multi-angle laser light scattering. The protein complexes typically are comprised of a protein that specifically binds to a protein of interest also referred to as a target or ligand. In one embodiment the protein that specifically binds to the target is an antibody or fusion protein.

When an antibody or fusion protein is combined with its target or ligand in vivo or in vitro, a heterogeneous mixture of antibody:ligand or fusion protein:ligand can form. In one embodiment, binding of therapeutic proteins such as monoclonal antibodies (mAbs) or fusion proteins to soluble, multimeric targets lead to large heterogeneous complexes or large heteromeric complexes. For example, the large protein complexes can be characterized as a protein complex with a protein:ligand ratio selected from the group consisting of 3:2, 2:3, 4:4, 6:6, or [2:2]_n. Large heterogeneous complexes refer to complexes formed between multiple multimeric ligand molecules and multiple protein drug product molecules. The term large heteromeric complex refers to ligand bound by two different protein drug products, for example two different antibodies, two different fusion proteins, or an antibody and a fusion protein binding the same ligand at different sites.

Another embodiment provides a method of identifying protein drug products that form large heterogeneous complexes with soluble targets in vivo, in vitro, or both. The method includes preparing a sample containing a protein drug product and its soluble ligand to produce protein drug product:ligand complexes, fractionating the sample to separate the protein drug product:ligand complexes and analyzing the fractionated protein drug product:ligand complexes by multi-angle laser light scattering to determine the size and heterogeneity of protein complexes. In one embodiment, the protein sample is fractionated using asymmetrical flow field-flow fractionation.

Further details of the disclosed methods and systems are provided below.

A. System for Characterizing Protein Complexes

In one embodiment, the system includes an asymmetrical flow field-flow fractionation (A4F) system and a multi-angle laser light scattering (MALLS) system. An example of a commercially available A4F system is an Eclipse™ 3+ A4F Separation System. An example of a commercially available MALLS system is the Wyatt Technology Dawn HELEOS® II laser light scattering instrument. The system typically includes a UV/VIS detector and/or a refractive index detector. An exemplary commercially available UV/VIS detector is Agilent 1260 Infinity UV detector. An exemplary commercially available refractive index detector is Optilab® T-rEX refractive index detector. In one embodiment the A4F system includes an A4F short channel fitted with a 350W spacer and a 4 kDa MWCO NADIR® hydrophilic PES (PESH) membrane. In another embodiment, the A4F short channel is fitted with a 490W spacer and a 10kDa MWCO Nadir® regenerated cellulose membrane. Exemplary mobile phases include 10 mM phosphate and 500 mM NaCl at pH 7.0. However, an advantage of A4F over column chromatography separation is that there are no limitations on the type of mobile phase, or carrier fluid that can be used. In one embodiment, the samples are separated using a linear gradient over 60 minutes. In one embodiment, the channel flow and cross-flow program are specifically optimized to achieve a desired resolution on a case-by-case basis. It is to be understood that a person of skill in the art could modify and optimize the elution profile according to the resolution being required of the specific sample being separated using A4F.

Typically, the sample is injected into the sample inlet port of the A4F channel. The sample is then focused by allowing the carrier fluid to flow into the channel from both the inlet and outlet ports, meeting at a point in the channel, typically near the sample inlet port, to form a focusing zone. During the focusing period, particles from the injected sample are held in this focusing zone to allow for relaxation prior to fractionation. The final step is fractionation of the particles. As particles flow along the channel, the perpendicularly-opposed cross-flow separation field pushes the molecules towards the bottom of the channel. As they accumulate near the bottom of the channel, they undergo a counter acting diffusion back into the channel against the cross-flow field. The extent to which the molecules can diffuse back into the channel is dictated by their natural Brownian motion, a characteristic defined by the translational diffusion coefficient, which, in turn, is related to the size and shape unique to each individual species. Generally, smaller particles have a faster diffusion coefficient than larger ones and are

able to diffuse higher into the channel against the cross-flow field. The rate of laminar flow within the channel is not uniform. It travels in a parabolic pattern with the speed of the flow increasing towards the center of the channel and decreasing towards the upper and lower walls of the channel. Therefore, the rate at which particles will be carried through will depend on their position within the channel. Those with faster diffusion, located near the center of the channel, will be transported with a greater velocity. The larger particles in the shallow, slower moving stream near the bottom accumulation wall of the channel are transported with lower flow velocity and elute later than smaller particles. This results in a gentle separation of particles based on mass with the elution order of smallest to largest.

As the sample is flowing through the A4F channel, the leading portion of sample exits the channel through an outlet port. The multi-angle laser light scattering (MALLS) detector is in fluid communication with the A4F system and receives sample from the A4F outlet port. In some embodiments, the sample first flows through a UV/VIS detector to measure sample concentration as a function of absorbance. The MALLS system focuses a beam of polarized light (or a laser) onto the sample molecule and the scattered light is detected with a photo detector.

Multi angle light scattering (MALS) measures light being scattered from a sample containing molecules, particles, or protein complexes. This scattering depends on the optical configuration of the setup, and in a typical experimental realization, the light is then detected at one or several different angles. In the one-scattering-angle solution, the three most popular designs are 90 degrees (also right angle light scattering or RALS), 7 degrees (also low angle light scattering or LALS), or 173 degrees (also non-invasive back scattering or NIBS). In the multi-angle setup there are in principle those where the angles are fixed (this is most often called the MALS or MALLS setup) and those where the angles are variable (typically referred to as a light scattering goniometer or spectrometer). MALS usually refers to a system with multiple fixed angles used as part of a particle separation setup, for example A4F. The most widespread application of MALS is as an absolute molar mass detector in conjunction with a concentration detector (like RI or single-wavelength UV).

MALS can be used to measure: M_w – weight-averaged molar mass of a protein complex; R_g – average radius of protein complex; and A_2 – solubility of protein in solution.

B. Methods of Characterizing Protein Complexes

The disclosed systems and methods can be used to characterize protein complexes, for example protein drug product:ligand complexes in a sample. One embodiment provides a method for assessing the stoichiometry and size distribution of heterogeneous protein complexes in a sample by fractionating the sample by asymmetrical flow field flow fractionation (A4F), and determining the molar mass, stoichiometry and size distribution of protein complexes in the sample using Multi-Angle Laser Light Scattering (MALLS), wherein the complexes comprise or consist of antibody:ligand complexes or fusion protein:ligand complexes. In some embodiments, the ligand is a soluble ligand. Typically, the antibody is a monoclonal antibody. In one embodiment, the protein complex is characterized by its antibody or fusion protein to ligand ratio. In a non-limiting example, the antibody or fusion protein to ligand ratio can be selected from the group consisting of 1:0, 0:1, 1:1, 1:2, 2:1, 2:2, 3:2, 2:3, 4:4, 6:6, or $[2:2]_n$. It is to be understood that the antibody or fusion protein to ligand ratio will be dependent on the specific antibody or fusion protein and ligand that are being tested.

1. Mixtures of Protein Complexes

To determine the characteristics of protein complexes, a molar mass can be experimentally determined for each component of the complex to calculate an expected molar mass of complexes with varying stoichiometries. In one embodiment, each protein and ligand in the mixture is analyzed separately to determine a molar mass for each component. In one embodiment, a protein drug product and its ligand are mixed to form protein drug product:ligand complexes and the complexes are then characterized. The fractionated protein drug product:ligands are subjected to A4F-MALLS to determine molar mass of the complexes. The calculated values of the fractionated complexes are then compared to the experimentally determined molar masses of the individual components to determine the likely stoichiometric ratio of individual components present in each complex. In one embodiment, the methods can detect a 1:1 protein drug product:ligand complex. In another embodiment, the methods can detect any ratio of protein drug product:ligand. In a non-limiting example, the methods can detect a protein drug product:ligand complex of 1:0, 0:1, 2:1, 1:2, 2:2, 3:2, 2:3, 4:4, 6:6, or $[2:2]_n$. It is to be understood that the antibody or fusion protein to ligand ratio will be dependent on the specific antibody or fusion protein and ligand that are being tested. In some embodiments, the

complex contains multiple different protein drug products complexed with a common soluble ligand.

a. Ligands

The ligand in the protein drug product:ligand complex can be a monomeric or multimeric
5 ligand. In one embodiment, the ligand is a soluble ligand. In some embodiments the soluble
ligands correspond to the extracellular portions of transmembrane proteins including but not
limited to transmembrane receptor proteins.

Monomeric ligands contain only one protein or one protein unit. Multimeric ligands can
be for example dimeric, trimeric, etc. containing multiple proteins or protein subunits. For
10 example the ligands can be homodimers or heterodimers. In some embodiments, the multimeric
ligands bind to more than one molecule of a protein drug product. Figure 1A shows an
exemplary 1:1 antibody:ligand complex. Figure 1B shows an exemplary 1:2 antibody:ligand
complex, and Figure 1C shows an example of the “paper dolling” effect wherein each arm of an
interior antibody binds to a different ligand creating a large, heterogeneous complex.

15 In one embodiment, the large, heterogeneous protein drug product:ligand complex has a
size of 500 kDa or greater. In another embodiment, the heterogeneous protein drug
product:ligand complex has a size of 500 – 4000 kDa. In another embodiment, the large,
heterogeneous protein drug product:ligand has a ratio of protein drug product:ligand of 3:2, 2:3,
4:4, or 6:6.

20 In one embodiment, the disclosed methods are used to determine if a lead protein drug
product designed to target a multimeric ligand will form large, heterogeneous protein drug
product:ligand complexes.

In one embodiment, the disclosed methods can be used to determine if a multimeric
ligand will form complexes with more than one protein drug product or fusion protein. The
25 complexes that can be formed include but are not limited to protein:ligand ratios of 1:0, 0:1, 2:1,
1:2, 2:2, 3:2, 2:3, 4:4, 6:6, or $[2:2]_n$. Figures 1A-1C illustrate exemplary complexes that could be
formed between a multimeric ligand and a monoclonal antibody.

b. Combination of Multiple mAbs

Combination therapy using multiple protein drug products to target the same pathway or
30 the same ligand is growing in popularity. In some embodiments, the disclosed methods can be
used to distinguish between different combinations of antibodies targeting the same ligand based

on the stoichiometry and size distributions of protein complexes formed by the protein drug products. When two protein drug products are mixed together with a monomeric ligand, the protein drug products have the potential to form a heteromeric complex. A heteromeric complex, as defined herein, refers to two different protein drug products binding the same target molecule. In addition, each of the two arms of the same antibody have the ability to bind two ligands which can also be bound by a second antibody to form a heteromeric complex. Figures 2A-2D show representative heteromeric complexes. Figures 2A and 2C show complexes formed when one protein drug product (black), or antibody, binds two ligands and one of the ligands is also bound by a second, unique protein drug product (gray). If the gray protein is bound by another ligand which is then bound by the black protein, larger, more heterogeneous complexes can form, as represented in Figures 2B and 2D.

2. Selecting Lead Protein Drug Product

Another embodiment provides a method for selecting a lead protein drug product by adding a first protein drug product to a first sample comprising a target of the first protein drug product to produce heterogeneous protein:ligand complexes and adding a second protein drug product to the a second sample containing the target to form protein:ligand complexes. The method includes separating the heterogeneous protein:ligand complexes and determining the size distribution and stoichiometry of heterogeneous protein:ligand complexes using Asymmetrical flow field flow fractionation– Multi-Angle Laser Light Scattering. The methods also include selecting the protein drug product that forms fewer heterogeneous protein:ligand complexes as the lead target protein drug. In some embodiments, the ligand is a soluble ligand. The soluble ligand can be a monomeric ligand or a multimeric ligand. Typically, the protein drug product is an antibody or antigen binding fragment thereof, a fusion protein, or a recombinant protein. The protein complex can be characterized as a protein complex with an antibody or fusion protein to ligand ratio selected from the group consisting of but not limited to 1:0, 0:1, 1:1, 1:2, 2:1, 2:2, 3:2, 2:3, 4:4, 6:6, or $[2:2]_n$. Another embodiment provides a pharmaceutical composition containing the lead protein drug product selected using the method above.

3. Determining Size and Shape of Protein Complexes

In one embodiment, the disclosed methods can be used to determine the size of protein complexes. A mixture of protein drug products, and optionally soluble ligands, are separated using an A4F fractionation. The size and stoichiometry of the protein complexes can then be

determined using MALLS analysis. In MALLS analysis, a beam of polarized light (or a laser) is focused onto the sample molecule and the scattered light is detected with a photo detector. The scattered light is detected at various different angles simultaneously. The intensity of the scattered light at each angle is proportional to the molar mass of the complex. In one
5 embodiment, UV/Vis spectrometry is used to determine the concentration of each protein complex.

In another embodiment, the shape/conformation of a protein complex formed between different protein drug products to a common ligand can be distinguished using the disclosed methods. Differences in elution time or elution profile between complexes with the same molar
10 mass suggest differences in shape or conformation of the protein complexes. Complexes with the same or similar molar mass but with different elution times indicate that the complex with the slower elution time has an increased hydrodynamic volume due to a difference in shape or conformation of the complex.

The molar mass and size heterogeneity of the protein complexes can be used to predict
15 the clearance of the protein drug product. In one embodiment, the larger the protein complex, the faster the protein drug product is cleared from the body.

C. Proteins in the Protein Complexes

In one embodiment one of the proteins in the protein complex is a protein drug product or is a protein of interest suitable for expression in prokaryotic or eukaryotic cells. For example,
20 the protein in the protein complexes can be an antibody or antigen-binding fragment thereof, a chimeric antibody or antigen-binding fragment thereof, an ScFv or fragment thereof, an Fc-fusion protein or fragment thereof, a growth factor or a fragment thereof, a cytokine or a fragment thereof, or an extracellular domain of a cell surface receptor or a fragment thereof. Proteins in the complexes may be simple polypeptides consisting of a single subunit, or complex
25 multisubunit proteins comprising two or more subunits. The protein of interest may be a biopharmaceutical product, food additive or preservative, or any protein product subject to purification and quality standards.

In some embodiments, the protein in the protein complexes is an antibody, a human antibody, a humanized antibody, a chimeric antibody, a monoclonal antibody, a multispecific
30 antibody, a bispecific antibody, an antigen binding antibody fragment, a single chain antibody, a diabody, triabody or tetrabody, a dual-specific, tetravalent immunoglobulin G-like molecule,

termed dual variable domain immunoglobulin (DVD-IG), an IgD antibody, an IgE antibody, an IgM antibody, an IgG antibody, an IgG1 antibody, an IgG2 antibody, an IgG3 antibody, or an IgG4 antibody. In one embodiment, the antibody is an IgG1 antibody. In one embodiment, the antibody is an IgG2 antibody. In one embodiment, the antibody is an IgG4 antibody. In another embodiment, the antibody comprises a chimeric hinge. In still other embodiments, the antibody comprises a chimeric Fc. In one embodiment, the antibody is a chimeric IgG2/IgG4 antibody. In one embodiment, the antibody is a chimeric IgG2/IgG1 antibody. In one embodiment, the antibody is a chimeric IgG2/IgG1/IgG4 antibody.

In some embodiments, the antibody is selected from the group consisting of an anti-
 10 Programmed Cell Death 1 antibody (e.g. an anti-PD1 antibody as described in U.S. Pat. Appln. Pub. No. US2015/0203579A1), an anti-Programmed Cell Death Ligand-1 (e.g., an anti-PD-L1 antibody as described in in U.S. Pat. Appln. Pub. No. US2015/0203580A1), an anti-Dll4 antibody, an anti-Angiopoetin-2 antibody (e.g., an anti-ANG2 antibody as described in U.S. Pat. No. 9,402,898), an anti- Angiopoetin-Like 3 antibody (e.g., an anti-AngPtI3 antibody as
 15 described in U.S. Pat. No. 9,018,356), an anti-platelet derived growth factor receptor antibody (e.g., an anti-PDGFR antibody as described in U.S. Pat. No. 9,265,827), an anti-Erb3 antibody, an anti- Prolactin Receptor antibody (e.g., anti-PRLR antibody as described in U.S. Pat. No. 9,302,015), an anti-Complement 5 antibody (e.g., an anti-C5 antibody as described in U.S. Pat. Appln. Pub. No US2015/0313194A1), an anti-TNF antibody, an anti-epidermal growth factor
 20 receptor antibody (e.g., an anti-EGFR antibody as described in U.S. Pat. No. 9,132,192 or an anti-EGFRvIII antibody as described in U.S. Pat. Appln. Pub. No. US2015/0259423A1), an anti-Proprotein Convertase Subtilisin Kexin-9 antibody (e.g., an anti-PCSK9 antibody as described in U.S. Pat. No. 8,062,640 or U.S. Pat. No. 9,540,449), an Anti-Growth and Differentiation Factor-8 antibody (e.g. an anti-GDF8 antibody, also known as anti-myostatin antibody, as described in
 25 U.S. Pat Nos. 8,871,209 or 9,260,515), an anti-Glucagon Receptor (e.g. anti-GCGR antibody as described in U.S. Pat. Appln. Pub. Nos. US2015/0337045A1 or US2016/0075778A1), an anti-VEGF antibody, an anti-IL1R antibody, an interleukin 4 receptor antibody (e.g., an anti-IL4R antibody as described in U.S. Pat. Appln. Pub. No. US2014/0271681A1 or U.S. Pat Nos. 8,735,095 or 8,945,559), an anti-interleukin 6 receptor antibody (e.g., an anti-IL6R antibody as
 30 described in U.S. Pat. Nos. 7,582,298, 8,043,617 or 9,173,880), an anti-IL1 antibody, an anti-IL2 antibody, an anti-IL3 antibody, an anti-IL4 antibody, an anti-IL5 antibody, an anti-IL6 antibody,

an anti-IL7 antibody, an anti-interleukin 33 (e.g., anti-IL33 antibody as described in U.S. Pat. Nos. 9,453,072 or 9,637,535), an anti-Respiratory syncytial virus antibody (e.g., anti-RSV antibody as described in U.S. Pat. Appln. Pub. No. 9,447,173), an anti-Cluster of differentiation 3 (e.g., an anti-CD3 antibody, as described in U.S. Pat. Nos. 9,447,173 and 9,447,173, and in U.S. Application No. 62/222,605), an anti-Cluster of differentiation 20 (e.g., an anti-CD20 antibody as described in U.S. Pat. Nos. 9,657,102 and US20150266966A1, and in U.S. Pat. No. 7,879,984), an anti-CD19 antibody, an anti-CD28 antibody, an anti-Cluster of Differentiation-48 (e.g. anti-CD48 antibody as described in U.S. Pat. No. 9,228,014), an anti-Fcγd1 antibody (e.g. as described in U.S. Pat. No. 9,079,948), an anti-Middle East Respiratory Syndrome virus (e.g. an anti-MERS antibody as described in U.S. Pat. Appln. Pub. No. US2015/0337029A1), an anti-Ebola virus antibody (e.g. as described in U.S. Pat. Appln. Pub. No. US2016/0215040), an anti-Zika virus antibody, an anti-Lymphocyte Activation Gene 3 antibody (e.g. an anti-LAG3 antibody, or an anti-CD223 antibody), an anti-Nerve Growth Factor antibody (e.g. an anti-NGF antibody as described in U.S. Pat. Appln. Pub. No. US2016/0017029 and U.S. Pat. Nos. 8,309,088 and 9,353,176) and an anti-Protein Y antibody. In some embodiments, the bispecific antibody is selected from the group consisting of an anti-CD3 x anti-CD20 bispecific antibody (as described in U.S. Pat. Appln. Pub. Nos. US2014/0088295A1 and US20150266966A1), an anti-CD3 x anti-Mucin 16 bispecific antibody (e.g., an anti-CD3 x anti-Muc16 bispecific antibody), and an anti-CD3 x anti-Prostate-specific membrane antigen bispecific antibody (e.g., an anti-CD3 x anti-PSMA bispecific antibody). In some embodiments, the protein of interest is selected from the group consisting of abciximab, , adalimumab, adalimumab-atto, ado-trastuzumab, alemtuzumab, alirocumab, atezolizumab, avelumab, basiliximab, belimumab, benralizumab, bevacizumab, bezlotoxumab, blinatumomab, brentuximab vedotin, brodalumab, canakinumab, capromab pendetide, certolizumab pegol, cemiplimab, cetuximab, denosumab, dinutuximab, dupilumab, durvalumab, eculizumab, elotuzumab, emicizumab-kxwh, emtansinealirocumab, evinacumab, evolocumab, fasinumab, golimumab, guselkumab, ibritumomab tiuxetan, idarucizumab, infliximab, infliximab-abda, infliximab-dyyb, ipilimumab, ixekizumab, mepolizumab, necitumumab, nesvacumab, nivolumab, obiltoxaximab, obinutuzumab, ocrelizumab, ofatumumab, olaratumab, omalizumab, panitumumab, pembrolizumab, pertuzumab, ramucirumab, ranibizumab, raxibacumab, reslizumab, rinucumab,

rituximab, sarilumab, secukinumab, siltuximab, tocilizumab, tocilizumab, trastuzumab, trevogrumab, ustekinumab, and vedolizumab.

In some embodiments, the protein in the complexes is a recombinant protein that contains an Fc moiety and another domain, (e.g., an Fc-fusion protein). In some embodiments, an Fc-fusion protein is a receptor Fc-fusion protein, which contains one or more extracellular domain(s) of a receptor coupled to an Fc moiety. In some embodiments, the Fc moiety comprises a hinge region followed by a CH2 and CH3 domain of an IgG. In some embodiments, the receptor Fc-fusion protein contains two or more distinct receptor chains that bind to either a single ligand or multiple ligands. For example, an Fc-fusion protein is a TRAP protein, such as for example an IL-1 Trap (e.g., rilonacept, which contains the IL-1RAcP ligand binding region fused to the IL-1RI extracellular region fused to Fc of hIgG1; see U.S. Pat. No. 6,927,004, which is herein incorporated by reference in its entirety), or a VEGF Trap (e.g., aflibercept or ziv-aflibercept, which comprises the Ig domain 2 of the VEGF receptor Flt1 fused to the Ig domain 3 of the VEGF receptor Flk1 fused to Fc of hIgG1; see U.S. Pat. Nos. 7,087,411 and 7,279,159). In other embodiments, an Fc-fusion protein is a ScFv-Fc-fusion protein, which contains one or more of one or more antigen-binding domain(s), such as a variable heavy chain fragment and a variable light chain fragment, of an antibody coupled to an Fc moiety.

EXAMPLES

Example 1: A4F analysis offers superior resolution for samples containing large, heterogeneous complexes compared to SEC fractionation.

Methods

SEC-MALLS Mobile Phase Buffer

For SEC-MALLS analysis, the composition of SEC mobile phase buffer was 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0, and filtered (0.2 µm) before use.

SEC-MALLS Analysis

The SEC-MALLS system is composed of a Superose 6 GL column (10 mm x 300 mm; GE Healthcare, cat# 17-5172-01), coupled to an Agilent 1200 Series HPLC system equipped with a ultraviolet (UV) diode array detector, Wyatt Technology miniDawn TREOS® laser light scattering instrument (LS), and an Optilab® T-rEX differential refractometer (RI) detector. The

detectors were connected in series in the following order: UV-LS-RI. LS and RI detectors were calibrated according to instructions provided by Wyatt Technology.

Defined amounts of anti-ProteinX mAb were each combined with recombinant ProteinX and diluted in 1X DPBS, pH 7.4 to yield the following molar ratios: 5 μ M anti-ProteinX mAb: 1 μ M ProteinX, 1 μ M anti-ProteinX mAb: 1 μ M ProteinX, and 1 μ M anti-ProteinX mAb: 5 μ M ProteinX. All samples were incubated at ambient temperature for 2 hours and maintained unfiltered at 4°C prior to injection into the column. The column was pre-equilibrated with the mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 $\pm$ 0.1) at a flow rate of 0.3 mL/min, prior to the injection of each sample. Bovine serum albumin (BSA; 2 mg/mL; 150 μ g sample load) was injected separately and included as a system suitability control.

The SEC-MALLS mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 $\pm$ 0.1) was used throughout the fractionation. Each sample (100–200 μ g) was injected, and was eluted with a flow rate of 0.3 mL/min. BSA was fractionated using the same parameter settings.

15 *A4F-MALLS Mobile Phase Buffer*

For SEC-MALLS analysis, the composition of SEC mobile phase buffer was 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0, and filtered (0.2 μ m) before use.

A4F-MALLS

The A4F-MALLS system was composed of an Eclipse™ 3+ A4F Separation System coupled to an Agilent 1200 Series HPLC system equipped with a ultraviolet (UV) diode array detector, Wyatt Technology Dawn HELEOS® II laser light scattering instrument (LS), and an Optilab® T-rEX differential refractometer (RI) detector. The detectors were connected in series in the following order: UV-LS-RI. LS and RI detectors were calibrated according to instructions provided by Wyatt Technology.

25 Defined amounts of anti-ProteinX mAb were each combined with recombinant ProteinX and diluted in 1X DPBS, pH 7.4 to yield the following molar ratios: 5 μ M anti-ProteinX mAb: 1 μ M ProteinX, 1 μ M anti-ProteinX mAb: 1 μ M ProteinX, and 1 μ M anti-ProteinX mAb: 5 μ M ProteinX. All samples were incubated at ambient temperature for 2 hours and maintained unfiltered at 4°C prior to injection into an Eclipse™ short channel fitted with a W350 spacer foil (350 μ m spacer thickness, 2.2 cm spacer width) and using a 10 kDa MWCO Nadir regenerated cellulose membrane. The channel was pre-equilibrated with the mobile phase buffer (10 mM

sodium phosphate, 500 mM sodium chloride, pH 7.0 ± 0.1), prior to the injection of each sample. Bovine serum albumin (BSA; 2 mg/mL; 10 µg sample load) was injected separately and included as a system suitability control.

The fractionation method consisted of four steps: injection, focusing, elution, and a channel “wash-out” step. The A4F-MALLS mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 ± 0.1) was used throughout the fractionation method. Each sample (7 µg) was injected at a flow rate of 0.2 mL/min for 1 min and subsequently focused for 2 min with a focus flow rate of 1.5 mL/min. The sample was eluted with a channel flow rate of 1.0 mL/min with the linear gradient cross flow from 3.0 mL/min to 0 mL/min over 45 min. Finally, the cross flow was held at 0 mL/min for an additional 5 min to wash out the channel. BSA was fractionated using the same parameter settings.

MALLS Data Analysis

Data were analyzed using ASTRA V software (version 5.3.4.14, Wyatt Technology). The data were fit to the equation that relates the excess scattered light to the solute concentration and weight-average molar mass, M_w , (Wyatt, 1993; Kendrick, 2001)

$$\text{Equation 1: } \frac{K * c}{R(\theta, c)} = \frac{1}{M_w P(\theta)} + 2A_2 c$$

where c is the solute concentration, $R(\theta, c)$ is the excess Raleigh ratio from the solute as a function of scattering angle and concentration, M_w is the molar mass, $P(\theta)$ describes the angular dependence of scattered light (~1 for particles with radius of gyration < 50 nm), A_2 is the second virial coefficient in the expansion of osmotic pressure (which can be neglected since measurements are performed on dilute solutions) and

$$\text{Equation 2: } K^* = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \left(\frac{dn}{dc} \right)^2$$

where n_0 represents the solvent refractive index, N_A is Avogadro's number, λ_0 is the wavelength of the incident light in a vacuum, and dn/dc represents the specific refractive index increment for the solute.

The molar mass of BSA monomer served to evaluate the calibration constants of the light scattering and differential refractive index detectors during data collection (system suitability check). The relative standard deviation (%RSD) of the average molar mass of BSA determined from the UV and RI detectors was ≤ 5.0%.

The normalization coefficients for the light scattering detectors, inter-detector delay volume and band broadening terms were calculated from the BSA chromatograms collected for the A4F-MALLS condition employed. These values were applied to the data files collected for all the other samples to correct for these terms.

The dn/dc value and the extinction coefficient at 215 nm or 280 nm (corrected for glycosylation) were experimentally determined using the protein conjugate analysis provided in the Astra software. The corrected extinction coefficient and dn/dc value was used to analyze all protein-protein complex samples.

Results

SEC-MALLS analysis of the samples showed poor resolution of higher order complexes (elution volume = 8 – 14 mL) and no distinction of intermediate complexes (Figure 3A, Table 1). In contrast, A4F-MALLS analysis of the samples showed superior resolution of higher order complexes (elution time = ~11 – 30 min) and clear distinction of intermediate complexes (Figure 3B, Table 2).

Table 1. Approximate molar mass and retention volume for mAb:Protein X complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		Higher Order Complexes		[Intact Antibody] ₂ : [ProteinX] ₁ Complex		[Intact Antibody] ₁ : [ProteinX] ₁ Complex		[Intact Antibody] ₁ : [ProteinX] ₂ Complex	
		EV, min	M _w , kDa	EV, min	M _w , kDa	EV, min	M _w , kDa	EV, min	M _w , kDa
mAb1:ProteinX	5:1	9.0-12.6	~390-1000	13.1	331	NA	NA	NA	NA
mAb1:ProteinX	1:1	8.0-13.4	~400-3000	NA	NA	15.4	167	NA	NA
mAb1:ProteinX	1:5	8.5-13.8	~430-2000	NA	NA	NA	NA	16.0	216

EV: Elution Volume; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons;

Table 2. Approximate molar mass and retention times for mAb:Protein X complexes

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
		[Intact Antibody] ₁ : [ProteinX] ₁₋₂ Complex		[Intact Antibody] ₂ : [ProteinX] ₁ Complex		[Intact Antibody] ₃ : [ProteinX] ₂ Complex		[Intact Antibody] ₄ : [ProteinX] ₃ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1:ProteinX	5:1	NA	NA	10.8	321	12.4	498	13.7	674	14.6	830-1190
mAb1:ProteinX	1:1	NA	NA	10.7	333	12.5	515	13.7	671	14.6	780-1620
mAb1:ProteinX	1:5	9.4	191	NA	NA	11.8	461	13.3	600	14.4	720-1180

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min: minutes; kDa: kiloDaltons;

Example 2: Anti-Protein Y Complexes.

5 Methods

A4F MALLS Mobile Phase Buffer

1X DPBS, pH 7.4, was prepared by diluting 500 mL of 10X DPBS with HPLC grade water to a total volume of 4.9 L. A solution of 0.0025% (w/v) sodium azide was added as an antimicrobial agent. Hydrochloric acid (12 M) was slowly added in small volume increments to adjust the pH to 7.4 before bringing the final volume to 5.0 L. The final, measured pH of the buffer was 7.4. The buffer solution was prepared fresh and filtered (0.2 μm) prior to use.

A4F MALLS Analysis

Defined amounts of anti-Protein Y mAbs (Lead A and Lead B) were each combined with recombinant human Protein Y and diluted in 1X DPBS, pH 7.4 to yield the following molar ratios: 1 μM anti-Protein Y mAb: 3 μM hActA, 1 μM anti-Protein Y mAb: 1 μM Protein Y, and 3 μM anti-Protein Y mAb: 1 μM Protein Y. All samples were incubated at ambient temperature for 2 hours and maintained unfiltered at 4°C prior to injection into an Eclipse™ short channel fitted with a W490 spacer foil (490 μm spacer thickness, 2.2 cm spacer width) and using a 10 kDa MWCO Nadir regenerated cellulose membrane. The channel was pre-equilibrated with 1X

DPBS buffer, pH 7.4, prior to the injection of each sample. Bovine serum albumin (BSA; 2 mg/mL; 10 µg sample load) was injected separately and included as a system suitability control.

The fractionation method consisted of four steps: injection, focusing, elution, and a channel “wash-out” step. The A4F-MALLS mobile phase buffer (1X DPBS, pH 7.4) was used throughout the fractionation method. Each sample (10 µg) was injected at a flow rate of 0.2 mL/min for 1 min and subsequently focused for 2 min with a focus flow rate of 1.5 mL/min. The sample was eluted with a channel flow rate of 1.0 mL/min with the linear gradient cross flow from 1.2 mL/min to 0 mL/min over 20 min. Finally, the cross flow was held at 0 mL/min for an additional 5 min to wash out the channel. BSA was fractionated using the same parameter settings.

Mouse Anti-Human Antibody Titer

Mouse anti-human antibody (MAHA) titers were determined using a sandwich ELISA specific for the detection of mAb A or mAb B mouse IgG. Briefly, mAb A or mAb B at 1 µg/mL in phosphate-buffered saline (PBS) were passively adsorbed to a microtiter plate overnight at 4°C followed by a nonspecific binding block with 5% bovine serum albumin (BSA) in PBS. Serial dilutions of serum samples were prepared in dilution buffer (0.5% BSA in PBS) starting from 1:100. Therefore, the corresponding dilution factor (100) was defined as the assay’s lower limit of detection (LOD). Samples were then added to the mAb A or mAb B coated plate (100 µL/well) and incubated 16-18 hours at 4°C. Wells with addition of dilution buffer only were included to determine background signal. Subsequently, plate-captured mAb A or mAb B-specific MAHA was detected using horseradish peroxidase (HRP)-conjugated anti-mouse Fcγ at 40 ng/mL. The chromogenic HRP-substrate, 3,3',5,5'-tetramethylbenzidine (TMB) was used to detect HRP activity; and the resultant optical density at 450nm (OD₄₅₀) was read on a Perkin Elmer Victor X4 Multimode Plate Reader. Data of binding signal versus dilution factor were analyzed by non-linear regression using GraphPad Prism software and titers were calculated. The MAHA titer was defined as the calculated dilution factor of the serum sample corresponding to a binding signal equivalent to twice the background signal of the assay.

Results

The two lead mAbs formed distinctly different complexes with Protein Y. Under all conditions tested, mAb Lead-A formed smaller, less heterogeneous complexes with Protein Y than mAb Lead-B (Figures 4A-4C, Tables 3-5).

Table 3. Approximate molar mass and retention times for mAb:Protein Y complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3	
		[Intact Antibody] ₁ : [ProteinY] ₂ Complex		[Intact Antibody] ₂ : [ProteinY] ₃ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
Lead-A: ProteinY	1:3	10.1	216	NA	NA	13.1	~500-1000
Lead-B: ProteinY	1:3	10.1	219	12.5	390	14.3	~550-2000

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons;

Table 4. Approximate molar mass and retention times for mAb:Protein Y complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		[Intact Antibody] ₂ : [ProteinY] ₁₋₂ Complex		[Intact Antibody] ₂₋₃ : [ProteinY] ₃₋₄ Complex		[Intact Antibody] ₄ : [ProteinY] ₃ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
Lead-A: ProteinY	1:1	11.9	348	13.5	589	NA	NA	16.5	~850-1400
Lead-B: ProteinY	1:1	NA	NA	14.0	449	15.3	636	16.4	~815-2300

5

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons;

Table 5. Approximate molar mass and retention time for mAb:Protein Y complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2	
		[Intact Antibody] ₂ : [ProteinY] ₁ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa
Lead-A: ProteinY	3:1	11.3	265	13.0	~400-900
Lead-B: ProteinY	3:1	11.6	307	12.9	~480-1600

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons;

5 The size and heterogeneity of anti-Protein Y complexes correlated well with mouse PK observations (Figures 5A-5B). Larger complexes observed for Lead-B with Protein Y correlated with faster clearance (Figure 5B).

Example 3: Anti-Human Protein Z Complexes.

Methods

10 Sample Preparation

Samples were prepared in 1X DPBS, pH 7.4 and allowed to incubate at room temperature for 2 hrs. prior to fractionation of total protein by A4F-MALLS. The samples were as follows: 1 mM anti-Protein Z mAb1 + Secondary mAb (0.5 μM + 0.5 μM) + 1 μM complement Protein Z (7 combinations) or 1 mM anti-Protein Z mAb6 + anti-Protein Z mAb7 (0.5 μM + 0.5 μM) + 15 1 μM complement Protein Z. The list of secondary antibodies can be found in Table 6.

Table 6. Sample Nomenclature.

Secondary mAbs Tested	Nomenclature of Combo with anti-Protein Z mAb1
anti-Protein Z mAb2	mAb2 Combo
anti-Protein Z mAb3	mAb3 Combo
anti-Protein Z mAb4	mAb4 Combo
anti-Protein Z mAb5	mAb5 Combo
anti-Protein Z mAb6	mAb6 Combo
anti-Protein Z mAb7	mAb7 Combo
COMP1 mAb	COMP1 Combo

A4F MALLS Analysis

All samples were incubated at ambient temperature for total of 2 hours and maintained unfiltered at 4°C prior to injection into an Eclipse™ short channel fitted with a W350 spacer foil (350 µm spacer thickness, 2.2 cm spacer width) and using a 4 kDa MWCO hydrophilic PES (PESH) membrane. The channel was pre-equilibrated with the mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 ± 0.1), prior to the injection of each sample. BSA (2 mg/mL; 10 µg sample load) was injected separately and included as a system suitability control.

The fractionation method consisted of four steps: injection, focusing, elution, and a channel “wash-out” step. The A4F-MALLS mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 ± 0.1) was used throughout the fractionation method. Each sample (7 µg complex or 4 µg individual component) was injected at a flow rate of 0.2 mL/min and focused for 5 min with a focus flow rate of 1 mL/min. The sample was eluted with a channel flow rate of 1 mL/min with the linear gradient cross flow from 2 mL/min to 0 mL/min over 45 min. Finally, the cross flow was held at 0 mL/min for an additional 5 min to wash out the channel. BSA was fractionated using the same parameter settings.

RBC Hemolysis Assay

Alternative pathway (AP) hemolysis assay was used as the measure of complement activation to evaluate the ability of anti-Protein Z mAbs to block the lysis of rabbit red blood cells (RbRBCs). Lysis of rabbit red blood cells by membrane attack complex is the basis of the assay by which complement activation is experimentally measured.

A desired number of RbRBCs are washed in GVB-Mg²⁺/EGTA buffer and re suspended at 2x10⁸ cells/ml. To test the efficacy of either single anti-C5 mAb or combination of anti-C5 mAbs, normal human serum was diluted to 50-96% in GVB-Mg²⁺/EGTA buffer to achieve a final concentration of 25-48% when added to RBC. Round bottom 96 well plates were used to measure hemolysis activity. A total of 100 µL RbRBCs (2x10⁸ cells/ml) were plated into 96-well plate at 37°C followed by addition of 100 µL of diluted serum. Cells were gently mixed and incubated at 37°C for 30-120 minutes. After incubation time, the cells were spun down by centrifugation at 1250xg at 4°C. A total of 100 µL of the supernatant was transferred to a fresh 96 flat bottom plate and read at 412nm on a Spectramax microplate reader. The calculation of percent of hemolysis was done as described below.

The percentage of hemolysis was calculated with the absorbance values by using the following equation:

$$\text{Equation 3: } \% \text{ Hemolysis} = 100 \times \frac{(\text{Experimental Cell Lysis} - \text{Background Cell Lysis})}{(\text{Maximum Cell Lysis} - \text{Background Cell Lysis})}$$

In this equation “background cell lysis” is the OD at A412nm from the cells incubated in GVB-Mg²⁺/EGTA buffer only containing no serum. The “maximum cell lysis” is the OD at A412nm from the cells treated with water. Maximum inhibition of lysis was calculated as a difference between bottom and top values in the curve expressed as a percentage of top value. Data represented as mean $\pm$ standard error of mean.

Results

Anti-Protein Z mAb1 (lead anti-Protein Z mAb) in combination with COMP1 mAb or other Protein Z mAbs completely blocks hemolysis of rabbit RBCs via alternative pathway activation (Figure 6B, Table 8), compared to monotherapies which do not completely block hemolysis (Figure 6A, Table 7). Because all of the anti-Protein Z mAb1:anti-Protein Z mAb combinations completely blocked hemolysis of rabbit RBC, it was of importance to determine if there are differences in complex formation, such as size, shape and orientation which can provide insight into the pharmacokinetics (PK) of a mAb during drug development, such as immunogenicity and/or target-mediated clearance.

Table 7. Effect of anti-Protein Z antibodies on rabbit RBC hemolysis.

Protein ID	AP, IC50 [M]	AP, IC80 [M]	% Max Inhibition of Lysis
Anti-Protein Z mAb1	8.896e-008	1.970e-007	81.25
Anti-Protein Z mAb2	8.366e-008	1.813e-007	88.66
Anti-Protein Z mAb3	5.252e-008	9.390e-008	59.24
Anti-Protein Z mAb4	4.942e-008	5.963e-008	36.63
Anti-Protein Z mAb5	7.419e-008	1.467e-007	77.83
Anti-Protein Z mAb6	9.346e-008	2.260e-007	62.80
Anti-Protein Z mAb7	7.424e-008	1.414e-007	76.21
COMP1 mAb	6.291e-008	1.185e-007	61.14
COMP2 mAb	4.568e-008	5.600e-008	88.83

Table 8. Effect of anti-Protein Z antibody combinations on rabbit RBC hemolysis.

Protein ID	AP, IC50 [M]	AP, IC80 [M]	% Max Inhibition of Lysis
Anti-Protein Z mAb1 + Anti-Protein Z mAb2	7.182e-008	7.528e-008	97.59
Anti-Protein Z mAb1 + Anti-Protein Z mAb3	7.709e-008	8.749e-008	98.11
Anti-Protein Z mAb1 + Anti-Protein Z mAb4	9.751e-008	1.064e-007	98.16
Anti-Protein Z mAb1 + Anti-Protein Z mAb5	9.571e-008	1.051e-007	98.10
Anti-Protein Z mAb1 + Anti-Protein Z mAb6	7.737e-008	8.461e-008	97.29
Anti-Protein Z mAb1 + Anti-Protein Z mAb7	8.353e-008	9.432e-008	98.14
Anti-Protein Z mAb1 + COMP1 mAb	7.372e-008	7.776e-008	98.26
Anti-Protein Z mAb1 + COMP2 mAb	7.306e-008	8.017e-008	98.07

In the absence of secondary mAbs, anti-Protein Z mAb1 formed canonical 1:1 and 1:2 complexes with Protein Z when mixed in equimolar amounts (Figure 7, Table 9).

5 **Table 9.** Approximate molar mass and retention times for mAb:Protein Z complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2	
		[Intact Antibody] ₁ : [ProteinZ] ₁		[Intact Antibody] ₁ : [ProteinZ] ₂	
		Complex		Complex	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1:ProteinZ	1:1	13.7	341	15.3	499

R_t: Retention Time; M_w: weight average molar mass; min:minutes; kDa: kiloDaltons;

Most secondary mAb combinations with anti-Protein Z mAb1 favored smaller, well defined complexes consistent with a heteromeric 2:2 mAb:Protein Z complex (Figures 8 and 9 and Tables 10-12).

Table 10. Molar mass of mAb:Protein Z complex.

mAb:Protein Z Complex	Theoretical Molar Mass (kDa)
1:0	150
0:1	195
1:1	345
2:1	495
1:2	540
2:2	690
3:2	840
2:3	885
4:4	1380
6:6	2070

Table 11. Approximate molar mass and retention time for mAb:Protein Z complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		[Intact Antibody] ₂ : [ProteinZ] ₂ Complex		[Intact Antibody] ₄ : [ProteinZ] ₄ Complex		[Intact Antibody] ₆ : [ProteinZ] ₆ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1:mAb5: ProteinZ	0.5:0.5:1	16.0	684	18.5	1342	20.1	1876	21.5	~2250-3560
mAb1:mAb7: ProteinZ	0.5:0.5:1	16.1	688	18.5	1327	20.1	1866	21.5	~2380-4250

5 R_t: Retention Time; M_w: weight average molar mass; min:minutes; kDa: kiloDaltons.

Table 12. Approximate molar mass and retention times for mAb:Protein Z complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		[Intact Antibody] ₂ : [ProteinZ] ₂ Complex		[Intact Antibody] ₄ : [ProteinZ] ₄ Complex		[Intact Antibody] ₆ : [ProteinZ] ₆ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1:mAb3:ProteinZ	0.5:0.5:1	16.4	685	18.4	1262	NA	NA	20.2	~1700-2700
mAb1:mAb6:ProteinZ	0.5:0.5:1	15.7	686	17.7	1320	19.4	1850	20.6	~2300-3800
mAb6:mAb7:ProteinZ	0.5:0.5:1	15.8	688	17.8	1334	19.3	1872	20.6	~2300-3600

Although anti-Protein Z mAb1/ anti-Protein Z mAb3 combination formed similar sized complexes with Protein Z, differences in elution time/profile suggested that complexes formed had differences in shape/orientation compared to other combinations (Figure 9). Combinations of anti-Protein Z mAb1 with anti-Protein Z mAb2 and COMP1 mAb favored larger, more heterogeneous complexes with Protein Z indicative of “paper-dolling” (Figure 10, Table 13).

Table 13. Approximate molar mass and retention times for mAb:Protein Z complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		[Intact Antibody] ₂ : [ProteinZ] ₂ Complex		[Intact Antibody] ₄ : [ProteinZ] ₄ Complex		[Intact Antibody] ₆ : [ProteinZ] ₆ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1: mAb2: ProteinZ	0.5:0.5:1	15.5	665	19.2	1304	21.9	1901	23.6	~2300-4100
mAb1: COMP1: ProteinZ	0.5:0.5:1	15.4	714	18.8	1346	21.5	2002	24.2	~2500-5000

R_t: Retention Time; M_w: weight average molar mass; min: minutes; kDa: kiloDaltons.

Anti-Protein Z mAb4 combination with anti-Protein Z mAb1 displayed a reduced tendency to form heteromeric complexes with Protein Z (Figure 11, Table 14). Presence of free mAb and 1:1 mAb:Protein Z species indicated incomplete formation of heteromeric complexes with Protein Z. Mixtures of homomeric and heteromeric complexes with Protein Z were also evident.

Table 14. Approximate molar mass and retention time for mAb:Protein Z complexes.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3	
		[Intact Antibody] ₂ : [ProteinZ] ₂ Complex		[Intact Antibody] ₄ : [ProteinZ] ₄ Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
mAb1:mAb4: ProteinZ	0.5:0.5:1	15.9	650	19.1	1288	20.6	~1700-2300

R_t: Retention Time; M_w: weight average molar mass; min:minutes; kDa: kiloDaltons

Example 4: Order of addition does not significantly impact the molar mass and distribution of complexes formed between anti-Protein Z mAb1, COMP1 mAb, and Protein Z.

Methods

To determine whether order of addition impacts complex formation, equimolar combinations of COMP1 mAb and Protein Z were prepared in 1X DPBS, pH 7.4 to yield a molar ratio of 1 μ M COMP1 mAb: 1 μ M Protein Z and allowed to incubate at ambient temperature for 1 hr. Following incubation, varying amounts of Anti-Protein Z mAb1 was added to the pre-formed COMP1 mAb:Protein Z complexes and diluted in 1X DPBS, pH 7.4 to yield the following molar ratios: 0.3 μ M Anti-Protein Z mAb1: 1 μ M COMP1 mAb: 1 μ M Protein Z, 1 μ M Anti-Protein Z mAb1: 1 μ M COMP1 mAb: 1 μ M Protein Z, and 3 μ M Anti-Protein Z mAb1: 1 μ M COMP1 mAb: 1 μ M Protein Z. and incubated for an additional hour prior to injection onto the instrument. The A4F MALLS analysis methods of Example 3 were followed.

Results

Similar complexes, with respect to molar mass and distribution, were formed between anti-Protein Z mAb1, COMP1 mAb, and Protein Z regardless of whether anti-Protein Z mAb1 was added simultaneously (Figure 12A, Table 15) or sequentially (Figure 12B, Table 15) to the

other components. The slight shift in elution time observed between the two datasets is indicative of the variability inherent to the method and is not considered unusual.

Table 15. Approximate molar mass and retention times for mAb:Protein Z complexes.

Sample	mAb1: COMP1: ProteinZ Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4	
		[Intact Antibody] ₁ : [ProteinZ] ₁ Complex		[Intact Antibody] ₁ : [ProteinZ] ₂ Complex		[Intact Antibody] _{2:3} : [ProteinZ] _{2:3} Complex		Higher Order Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
Simultaneous Addition	3:1:1	NA	NA	14.5	537	16.7	89	18.0	~1200-1950
	1:1:1	NA	NA	14.6	523	16.7	793	18.6	~1400-2300
	0.3:1:1	13.3	344	15.0	541	16.6	750	18.4	~1000-1800
Sequential Addition	3:1:1	NA	NA	15.6	500	18.0	817	20.0	~1200-1800
	1:1:1	NA	NA	15.7	496	18.0	806	20.0	~1500-2400
	0.3:1:1	14.2	380	16.4	663	17.9	818	19.9	~1500-2600

5 Example 5: Anti-Protein W Complexes.

Methods

A4F-MALLS Mobile Phase Buffer

The mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 ± 0.1) was prepared by combining 1.4 g sodium phosphate monobasic monohydrate, 10.7 g sodium phosphate dibasic heptahydrate, and 500 mL 5 M sodium chloride; the solution was then brought to a volume to 5.0 L with HPLC grade water. The final measured pH of the buffer was 7.0. The mobile phase buffer was filtered (0.2 µm) before use.

A4F MALLS Analysis

The A4F-MALLS system was composed of an Eclipse™ 3+ A4F Separation System coupled to an Agilent 1200 Series HPLC system equipped with a ultraviolet (UV) diode array detector, Wyatt Technology Dawn HELEOS® II laser light scattering instrument (LS), and an Optilab® T-REX differential refractometer (RI) detector. The detectors were connected in series

in the following order: UV-LS-RI. LS and RI detectors were calibrated according to instructions provided by Wyatt Technology.

Defined amounts of anti-Protein W mAb were each combined with Protein W and diluted in 1X DPBS, pH 7.4 to yield the equimolar ratio: 1 μ M anti-Protein W mAb: 1 μ M Protein W.

5 All samples were incubated at ambient temperature for 2 hours and maintained unfiltered at 4°C prior to injection into an Eclipse™ short channel fitted with a W350 spacer foil (350 μ m spacer thickness, 2.2 cm spacer width) and using a 10 kDa MWCO regenerated cellulose membrane. The channel was pre-equilibrated with the mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 $\pm$ 0.1), prior to the injection of each sample. Bovine serum
10 albumin (BSA; 2 mg/mL; 10 μ g sample load) was injected separately and included as a system suitability control.

The fractionation method consisted of four steps: injection, focusing, elution, and a channel “wash-out” step. The A4F-MALLS mobile phase buffer (10 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 $\pm$ 0.1) was used throughout the fractionation method. Each
15 sample (7 μ g) was injected at a flow rate of 0.2 mL/min for 1 min and subsequently focused for 3 min with a focus flow rate of 1.0 mL/min. The sample was eluted with a channel flow rate of 1.0 mL/min with the linear gradient cross flow from 3.0 mL/min to 0 mL/min over 25 min. Finally, the cross flow was held at 0 mL/min for an additional 5 min to wash out the channel. BSA was fractionated using the same parameter settings.

20 Results:

A4F-MALLS was used to assess the relative size distribution of complexes formed between Protein W, a dimeric, multi-domain ligand, and several anti-Protein W antibodies that specifically bind to different domains within the ligand. The theoretical molar mass and predicted stoichiometry of potential antibody complexes with Protein W are provided in Table

25 16.

Table 16. Theoretical Molar Mass of mAb:Protein W Complexes.

mAb:ProteinW Complex	Molar Mass (kDa)
1:0	146
0:1	146
1:1	292
1:2 [†]	438
2:2	584
2:3 [†]	730
3:3	876
3:4 [†]	1022
4:4	1168

[†] Unequal ratios (such as 1:2 and 2:1) cannot be differentiated, because they will have same MW.

Overall, out of the panel of mAbs, mAb1 targeting Domain A formed the highest proportion of lower-order complexes with the predominant species representing a discrete 1:1 and 2:2 complex with Protein W when combined at equimolar ratios (Peak 2, ~289 kDa; and peak 3, ~562 kDa, Figure 13, Table 17).

Table 17. Molar Masses and Retention Times of Human Protein W Complexes with mAb1 Targeting Domain A.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
		Free mAb/ProteinW		[mAb] ₁ : [ProteinW] ₁ Complex		[mAb] ₂ : [ProteinW] ₂ Complex		[mAb] ₃ : [ProteinW] ₃ Complex		Higher Order Heteromeric Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
ProteinW	-	9.6	1476	ND	ND	ND	ND	ND	ND	ND	ND
mAb1	-	9.5	145	ND	ND	ND	ND	ND	ND	ND	ND
mAb1:ProteinW	1:1	9.3	146	10.7	289	12.6	562	13.7	824	14.5	~1000-2000

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons.

Each of the mAbs targeting DomainB (mAb2, mAb3 and COMP1) predominantly formed a discrete 2:2 complex with ProteinW (Peak 3, ~563-580 kDa, Figure 14, Table 18) with mAb2 and COMP1 forming the most homogeneous distribution of complexes relative to other mAbs tested. While COMP2 targeting DomainA primarily favored a mixture of 1:2 and 2:2

complexes with ProteinW (Peak 3, ~550 kDa, Figure 15, Table 19), a moderate degree of large, heterogeneous complexes was also observed. This suggests that unlike mAb1, which also targeted DomainA, COMP2 binds to a unique epitope on ProteinW that allows for the formation of extended antibody-antigen lattices in a process termed “paper-dolling”. In this sample, a distinct peak (Peak 4) having a molar mass of approximately 835 kDa was observed, followed by a series of broad, poorly-resolved species (peak 5) with a wide molar mass distribution ranging from ~1000-1900 kDa (Figure 15, Table 19).

Table 18. Molar Masses and Retention Times of Human Protein W Complexes with mAbs targeting the Domain B.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
		Free mAb/ ProteinW		[mAb] ₁ : [ProteinW] ₁ Complex		[mAb] ₂ : [ProteinW] ₂ Complex		[mAb] ₃ : [ProteinW] ₃ Complex		Higher Order Heteromeric Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
ProteinW	~	9.6	147	ND	ND	ND	ND	ND	ND	ND	ND
mAb2	~	9.6	152	ND	ND	ND	ND	ND	ND	ND	ND
mAb2:ProteinW	1:1	9.4	144	10.9	283	13.2	574	13.9	731	15.4	~1000-1900
mAb3:ProteinW	1:1	9.3	144	10.7	26.8	12.9	563	14.0	831	14.6	~1000-1900
COMP1:ProteinW	1:1	9.4	143	10.8	298	13.0	580	13.9	765	15.0	~1000-1900

10 R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min:minutes; kDa: kiloDaltons.

Table 19. Molar Masses and Retention Times of Human Protein W Complexes with COMP2 targeting Domain A.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
		Free ProteinW		Free mAb		[mAb] ₁₋₂ : [ProteinW] ₂ Complex		[mAb] ₂₋₃ : [ProteinW] ₃ Complex		Higher Order Heteromeric Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
ProteinW	-	9.6	147	ND	ND	ND	ND	ND	ND	ND	ND
COMP2	-	10.5	122	ND	ND	ND	ND	ND	ND	ND	ND
COMP2:ProteinW	1:1	ND	ND	9.4	147	13.1	550	15.2	835	16.9	~1000-1900

R_t: Retention Time; M_w: weight average molar mass; NA: Not Applicable; min: minutes; kDa: kiloDaltons.

Based on the calculated molar masses of the individual components, peak 4 likely represents complexes containing at least 3 molecules of mAb coordinating 2-3 molecules of Protein W, whereas peak 5 corresponds to a heterogeneous distribution of higher order heteromeric complexes composed of ≥ 3 molecules of mAb coordinating ≥ 4 molecules of Protein W (Table 18). In contrast, mAbs targeting Domain C (mAb4 and COMP3) formed a broad distribution of large, heterogeneous complexes (molar mass ranging from ~700-8000 kDa) with mAb4 displaying the most extensive “paper-dolling” amongst the panel of mAbs tested (Figure 16, Table 20).

Table 20. Molar Masses and Retention Times of Human ProteinW Complexes with mAbs targeting Domain C.

Sample	Molar Ratio (mol:mol)	Peak 1		Peak 2		Peak 3	
		Free mAb/ ProteinW		[mAb] ₁₋₂ : [ProteinW] ₂ Complex		Higher Order Heteromeric Complexes	
		R _t , min	M _w , kDa	R _t , min	M _w , kDa	R _t , min	M _w , kDa
ProteinW	-	9.6	147	ND	ND	ND	ND
mAb4	-	9.5	148	ND	ND	ND	ND
mAb4:ProteinW	1:1	9.5	158	ND	ND	28.5	1400-4000
COMP3:ProteinW	1:1	9.7	161	13.9	528	14.6	700-8000

While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been put forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein can be varied considerably without
5 departing from the basic principles of the invention.

All references cited herein are incorporated by reference in their entirety. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than
10 to the foregoing specification, as indicating the scope of the invention.