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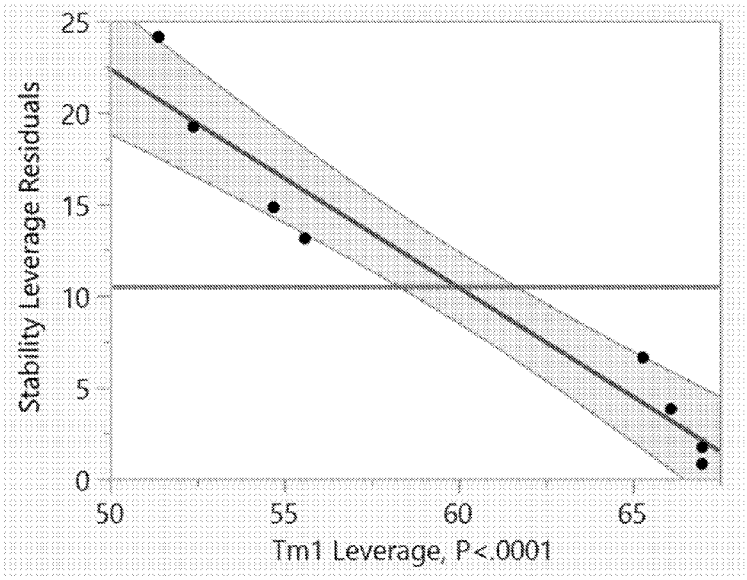
(54) Title: METHODS OF DETERMINING PROTEIN STABILITY

Figure 4



(57) Abstract: The disclosure provides for a method of assessing stability of a therapeutic protein in a formulation comprising the steps of measuring thermal stability of the therapeutic protein wherein the value for thermal stability correlates with the main peak percent change determined by size exclusion chromatography in a statistically significant manner.

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METHODS OF DETERMING PROTEIN STABILITY

[0001] This application claims priority to U.S. Provisional Application No. 62/808,166, filed on February 20, 2019, which is incorporated herein in its entirety.

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

[0002] This application contains, as a separate part of disclosure, a Sequence Listing in computer-readable form (filename: 53583A_SeqListing.txt; 270,082 bytes – ASCII text file created February 19, 2020) which is incorporated by reference herein in its entirety.

FIELD OF INVENTION

[0003] The disclosure provides for methods of assessing stability of a therapeutic protein, optionally in a formulation, comprising the steps of measuring thermal stability of the therapeutic protein, wherein the value for thermal stability correlates with the main peak percent change for the therapeutic protein determined by size exclusion chromatography in a statistically significant manner.

BACKGROUND

[0004] Protein stability has a critical impact on the manufacturability and shelf life of large molecule drug products and is one of the critical attributes for selecting a candidate during early molecule assessments. Selection of candidate molecules with the optimal stability in the formulation buffer reduces the required analysis later in development, which saves time and resources. Identifying stability issues early in development allows for variations in formulation aspects, such as buffering agents, buffer, pH, excipients, *etc.*, in order to improve the protein stability and increase shelf life.

[0005] The current gold standard for measuring protein stability is the Size Exclusion Chromatography (SEC) method. This method is both time consuming and costly. There is a need for more cost-efficient methods of determining protein stability. The present disclosure demonstrates that thermal stability analysis correlates with protein stability determined by SEC methods in a statistically significant manner.

SUMMARY

[0006] The disclosure provides for methods of assessing stability of a therapeutic protein comprising the steps of measuring thermal stability of the therapeutic protein and comparing the thermal unfolding (melting) temperature (T_m) of the therapeutic protein with the T_m of a control protein, wherein an increase in T_m of the therapeutic protein compared to the T_m control protein indicates an increase in stability of the therapeutic protein, and wherein the T_m of the therapeutic protein correlates with

statistical significance with the main peak percent change in protein size determined by size exclusion chromatography (SEC).

[0007] The disclosure also provides for methods of predicting stability of a therapeutic protein as measured by SEC comprising the steps of measuring thermal stability of the therapeutic protein and comparing the unfolding (melting) temperature (T_m) of the therapeutic protein with the T_m of a control protein, wherein the T_m of the therapeutic protein correlates with statistical significance with the main peak percent change in protein size determined by size exclusion chromatography (SEC).

[0008] Any of the disclosed methods may further comprise the step of determining the statistical significance of the correlation between the T_m of the therapeutic protein and the main peak change of the therapeutic protein determined by SEC.

[0009] In any of the disclosed methods, the T_m of the therapeutic protein correlates with the main peak percent change in protein size determined by SEC with a p value of equal to or less than 0.05, or a p value of equal to or less than 0.01, or a p value of equal to or less than 0.005, or a p value of equal to or less than 0.001. In addition, the statistical significance is determined by linear regression analysis or by other methods of fitting data, such as polynomial regression.

[0010] For example, the measurement of T_m value of the therapeutic protein predicts the stability of the therapeutic protein in a formulation for a period of at least one week, for at least two weeks, for at least one month, for at least two months, for at least three months, for at least four months, for at least five months, for at least a six months, for at least nine months, for at least 12 months, for at least 18 months, for at least 2 years, or for at least 3 years.

[0011] In any of the disclosed methods, the thermal stability is measured by differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF). The DSF used in the methods may be nanoDSF.

[0012] In addition, in any of the methods, the T_m of the therapeutic protein predicts the main peak percent change of therapeutic protein as measured on size exclusion chromatography.

[0013] The disclosed methods may be carried out on any therapeutic protein. Therapeutic proteins include monoclonal antibodies, multi-specific antibodies, bi-specific T cell engager (BiTE) molecules, Fc fusion molecules and muteins.

[0014] Exemplary monoclonal antibodies include those presented in Table A.

[0015] Exemplary BiTE molecules include BiTE molecule that specifically bind to one or more of CD33, BCMA, FLT3, CD-19, EGFRvIII, DLL3, and CD-3.

[0016] Exemplary Fc fusion protein include a FC fusion protein or the mutein that specifically binds to IL-2, IL-7, IL-10, IL-12, IL-15, IL-21, CTLA-4, PD-1, PD-L1, PD-L2, B7-H3, B7-H4, CEACAM-1, TIGIT, LAG3, CD112, CD112R, CD96, TIM3, BTLA, ICOS, OX40, 41BB, CD27, GITR.

[0017] In any of the disclosed methods, the therapeutic protein is formulated. For example, the therapeutic protein is formulated at a concentration greater than 50mg/ ml. 60 mg/ml, 70 mg/mL, 80 mg/ml, 90 mg/ml, 100 mg/mL, 110 mg/mL, 120 mg/mL, 130 mg/mL, 140 mg/mL, 150 mg/mL, 160 mg/mL, 170 mg/mL, 180 mg/mL, 190 mg/mL, 200 mg/mL, 210 mg/mL, and 220 mg/mL, and every increment therein.

[0018] In order that the present disclosure can be more readily understood, certain terms are defined herein. Definitions of certain terms are provided at the end of the detailed description. Additional definitions are set forth throughout the detailed description.

BRIEF DESCRIPTION OF DRAWINGS

[0019] Figurer 1 provides a representative leverage plot of different statistical results.

[0020] Figure 2 provides the leverage plot of the SEC and DSC data of a mAb formulation stability study.

[0021] Figure 3 provides the leverage plot of the SEC and DSC data of a BiTE formulation stability study.

[0022] Figure 4 provides the leverage plot of the SEC and DSC data of a Fc mutein stability study.

DETAILED DESCRIPTION

[0023] The disclosure provides for methods of determining protein stability using thermal stability analysis such as differential scanning calorimetry (DSC) and nano differential scanning fluorimetry (DSF or nanoDSF). The experiments described herein demonstrate that analyzing the thermal stability attributes of a protein sample correlates in a statistically significant manner with analyzing the stability of a protein sample using the current standard method in the art: size exclusion chromatography (SEC). Methods of determining thermal stability of a protein sample are less labor intensive; and require less time and less resources (product) to carry out the analysis when compared to SEC methods. Therefore,

the methods of using thermal stability to determine or predict protein stability will cost significantly less than carrying out SEC. For example, SEC requires at least two time points, while the thermal stability methods only require one time point with no waiting period to obtain the results. The methods of the disclosure can be applied to various stages of biopharmaceutical drug development, including molecule assessment, formulation development, protein characterization, and other required experiments for regulatory filings.

[0024] Therapeutic proteins undergo structural changes when stored in a non-physiological environment and/or are exposed to physiochemical stresses. The structural changes introduce variations in the physical properties of the protein, variations in solubility, variations in the tendency to aggregate, and variations in surface activity, which ultimately result in undesirable degradations such as soluble aggregates, insoluble precipitates, unfolded or misfolded folded forms of the protein, denatured forms of the protein, and *etc.* Measuring the stability of the therapeutic protein measures these structural changes within the protein. As demonstrated herein, measuring thermal stability measures these structural changes in response to changes in temperature.

[0025] The current state of the art is to analyze a protein sample using SEC at time zero and at least at a second time point, *e.g.* 6 months at 37°C. A typical SEC analysis requires about 1 mg/ml of therapeutic protein in a sample and a sample size of about 2 ml or more. However, DSC only requires one time point for analysis and requires a sample size of about 0.4 ml. NanoDSF requires an even smaller sample size (~0.01 mL). More importantly, thermal stability assays do not require the long storage times as required by the standard SEC methods.

[0026] Methods of analyzing thermal stability are not currently used in the art to assess the stability of therapeutic proteins within in a formulation. The methods of the disclosure are unique because these methods are based on the data provided herein that demonstrated thermal stability analysis correlated with SEC analysis in a statistically significant manner.

[0027] The methods of the disclosure are useful for analyzing the stability of a therapeutic protein in a formulation at all phases of drug development. For example, at the early stages of drug development, these methods provide a quick analysis to determine if particular formulation components should be further analyzed or whether to omit certain components early on in development. These methods also allow for analysis of many different formulations in a short time, which allows the investigator to focus investigation on particular formulation components that are more suitable for long term use. The

method of the disclosure also allows for quick testing of small adjustments to the formulation without long storage periods.

[0028] The experimental data provided herein demonstrate that the thermal stability values and the protein stability values as measured by SEC are correlated in a statistically significant manner, meaning statistical analysis indicates that these values have a strong linear relationship. The significance level is generally indicated as the probability for the given statistical model (p-value) and statistical significance is indicated by a p-value that is less than 0.05 ($p < 0.05$).

[0029] When discussing the stability of a therapeutic protein, stability can be referred to as "protein stability" or a sub-aspect of this term. For example, "protein stability" refers to an attribute of a protein in its native conformational form and its ability to resist conformational changes to its native form, *e.g.*, degradation, unfolding, aggregation, or changes to other non-native conformation forms, in response to chemical or environmental factors, such as buffer pH, temperature, salts, mechanical agitation, *etc.* The common method for determining the stability of a therapeutic protein in a formulation is SEC.

[0030] A particular aspect of protein stability is thermal stability. "Thermal stability" refers to an attribute of a protein to resist confirmation changes in its form, *e.g.*, degradation, unfolding, aggregation, or other changes to non-native conformation forms, in response to changes in temperature. In any of the methods of the disclosure, thermal stability is measured by any method of measuring thermal stability, such as differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF or nanoDSF).

[0031] Size Exclusion Chromatography (SEC) is the current standard of practice for determining protein stability. In a typical SEC stability study, protein aliquots are stored at different temperatures for various amounts of time. At the end of each time point, the main peak of each protein sample is analyzed using SEC. The SEC method separates molecules based on size using filtration through a matrix consisting of spherical beads containing pores of a specific size distribution. Separation occurs when molecules of different sizes are included or excluded from the pores within the matrix. Small molecules diffuse into the pores and their flow through the column is retarded according to their size, while large molecules do not enter the pores and are eluted in the column's void volume. Consequently, molecules separate based on their size as they pass through the column and are eluted in order of decreasing molecular weight (MW). The main peak represents the bulk of the protein of interest at its correct MW. Any percent change in the amount of protein in the main peak indicates protein instability, such as degradation, unfolding, aggregation, or other non-native conformational forms.

Methods of Determining Thermal Stability

[0032] The methods of the disclosure use steps to carry out a thermal stability (T_m) analysis on a therapeutic protein, optionally in a formulation. "Thermal stability analysis" refers to a technique in which some physical parameter is determined and/or recorded as a function of temperature. Thermal stability refers to an attribute of a protein to resist conformational changes in its native structure, *e.g.*, degradation, unfolding, aggregation, or other non-native confirmation structures, in response to changes in temperature

Differential Scanning Calorimetry (DSC)

[0033] Differential scanning calorimetry (DSC) is a thermodynamic tool for direct assessment of the heat energy uptake, which occurs in a sample within a regulated increase or decrease in temperature. Calorimetry is applied to monitor phase transitions. DSC measures heat capacity as a function of temperature using a thermal analysis instrument that determines the temperature and heat flow associated with material transitions as a function of time and temperature. The term "heat capacity" refers to the amount of energy a unit of matter can hold. Heat capacity increases with temperature.

[0034] During a change in temperature, DSC measures a heat quantity, which is radiated or absorbed excessively by the protein sample on the basis of a temperature difference between the sample and the reference material. In this analysis, a calorimeter measures the heat in and out of the protein sample. During a DSC analysis, the heat capacity of a sample cell (protein sample) is compared to that of a reference cell (lacking protein) while the temperature of both cells is gradually increased. Both the sample and the reference are maintained at nearly the same temperature throughout the analysis. Differences in the energy flows required to heat the cells are recorded and used to calculate the heat capacity of the sample. Plotting heat capacity as a function of temperature produces a DSC thermogram. Protein unfolding transitions are detected as changes in heat capacity. Post-run data analysis yields the midpoint of the thermal unfolding temperature (T_m). Thermal unfolding temperatures obtained from DSC can be used to assess the thermal stability of a protein. Milligram amounts of protein are required to run DSC analysis. See Kodre *et al.* RRJA 3:11-22, 2014 and Gill *et al.* J. Biomed tech., 21(4): 167-193, 2010.

[0035] DSC analysis can be carried out using a heat flow DSC, heat flux DSC, power compensated DSC, modulated DSC, hyper DSC or pressure DSC. In a heat flux DSC, the sample material, enclosed in a pan, and an empty reference pan are placed on a thermoelectric disk surrounded by a furnace. The furnace is heated at a linear heating rate, and the heat is transferred to the sample and reference pan through the

thermoelectric disk. However, owing to the heat capacity (C_p) of the sample, there would be a temperature difference between the sample and reference pans, which is measured by area thermocouples, and the consequent heat flow is determined by the thermal equivalent of Ohm's law (the current through a conductor between two points is directly proportional to the voltage across the two points). In the heat flux DSC, the difference in heat flow into the sample and reference is measured while the sample temperature is changed at the constant rate.

[0036] In a power-compensated DSC, the sample and reference pans are placed in separate furnaces heated by separate heaters. The sample and reference are maintained at the same temperature and the difference in thermal power required to maintain them at the same temperature is measured and plotted as a function of temperature or time. In power compensation DSC, the temperatures of the sample and reference are kept equal to each other while both temperatures are increased or decreased linearly. The power needed to maintain the sample temperature equal to the reference temperature is measured.

[0037] Modulated DSC uses the same heating and cell arrangement as the heat – flux DSC method. Fast scan DSC has the ability to perform valid heat flow measurements with fast linear controlled rates (up to 500 K/min) especially by cooling, where the rates are higher than with the classical powered compensated DSC. Standard DSC operates under 10 K/ min. In pressure DSC, the sample can be submitted to different pressures, which allows the characterization of substances at the pressures of processes or to distinguish between overlapping peaks.

[0038] Additional DSC-based techniques include conventional/basic DSC, microelectromechanical systems (MEMS)-DSC, infrared (IR)-heated DSC, modulated-temperature DSC (MTDSC), gas flow-modulated DSC (GFMDSC), parallel-nano DSC (PNDSC), pressure perturbation calorimetry (PPC), self-reference DSC (SRDSC), and high-performance (HPer) DSC. DSC applications are known to determine structural-phase transition, melting point, heat of fusion, percent of crystallinity, crystallization kinetics and phase transitions, oxidative stability, thermodynamic analysis of biomolecules, and curing kinetics of nonbiological materials. The advantages of DSC are the ease of carrying out the analyses and the speed with which this analysis can be used to observe transition in product.

Differential Scanning Fluorimetry (DSF)

[0039] Differential scanning fluorimetry (DSF) measures the changes in intrinsic fluorescence intensity ratio (350:330 nm) as a function of temperature. Proteins containing aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine) show intrinsic fluorescence. When a molecule unfolds, the

locations of the aromatic amino acid residues change which results in changes in fluorescence spectra. During a DSF or nanoDSF analysis, the intrinsic fluorescence intensity ratio (350:330 nm) is continuously measured and recorded. Plotting the intrinsic fluorescence intensity ratio (or the first derivative of the ratio) as a function of temperature produces a DSF thermogram. Post-run data analysis yields the midpoint of the thermal unfolding temperature (T_m). Thermal unfolding temperatures obtained from DSF can be used to assess the thermal stability of a protein.

Correlation

[0040] A statistical approach is used to evaluate the potential use of the thermal stability attribute to predict protein stability. In statistical hypothesis testing, the significance level defined for a study, α, is the probability of the study rejecting the null hypothesis, given that it is true. The significance level for a study is typically set to 5% (0.05). The p-value is used in the context of null hypothesis testing to quantify the idea of statistical significance. The p-value is the calculated probability for a given statistical model. The result of a given statistical model, *i.e.*, the prediction is considered statistically significant when $p < \alpha$, *i.e.*, $p < 0.05$.

[0041] The methods described herein use the DSC and nanoDSF techniques to analyze thermal stability. The data provided in the Examples demonstrate that the DSC and nanoDSF analysis on a target protein correlates with protein stability analysis carried out using SEC on the same therapeutic protein. The disclosure contemplates using other methods of determining thermal stability that may correlate with the percent change in protein size as determined by SEC analysis, which is the current standard in determine protein stability. For any of these methods, the correlation of the thermal unfolding temperature (T_m) attribute verses the protein stability value (percent change in protein size) as determined by SEC should be evaluated to determine whether the correlation is statistically significant.

[0042] The correlation may be evaluated using linear regression to analyze the data. Linear regression is a linear approach to modelling the relationship between a dependent variable and one or more independent variables (or explanatory variable). The case of one explanatory variable is called simple linear regression. Simple linear regression has an equation of the form $Y = a + bX$, where X is the explanatory variable and Y is the dependent variable. In the present invention, the dependent variable is stability (MP%) and the independent variable is, *e.g.* T_m. The linear regression generates a leverage plot to demonstrate correction. The leverage plot shows if the values are within the model or in this case whether the thermal stability data correlates with statistical significance with the SEC analysis.

When the confidence curve crosses the x axis of the leverage plot, the correlation is statistically significant. In addition, the correlation will be statistically significant when the p value is less than 0.05.

[0043] Examples of data analysis software that may be used to determine linear regression, polynomial regression or other methods to determine correlation include MicroCal Origin 7 software (OriginLab; Northampton, MA), MP® statistical software (SAS, Cary NC), IBM® SPSS software (Armonk, NY), SAS/STAT® (Cary, NC), STATA® (StataCorp, College Station, TX), Python (Wilmington, DE), RStudio (Boston, MA), and PSPP (Boston, MA).

Therapeutic Proteins

[0044] "Therapeutic protein" refers to any protein molecule which exhibits therapeutic biological activity. In one embodiment of the present disclosure, the therapeutic protein molecule is a full-length protein. In other embodiments, the therapeutic protein is an active fragment of the full-length protein. In various embodiments of the present disclosure, the therapeutic protein may be produced and purified from its natural source. Alternatively, according to the present disclosure, the term "recombinant therapeutic protein" includes any therapeutic protein obtained via recombinant DNA technology.

[0045] Proteins, including those that bind to one or more of the following, can be useful in the disclosed methods. These include CD proteins, including CD3, CD4, CD8, CD19, CD20, CD22, CD30, and CD34; including those that interfere with receptor binding. HER receptor family proteins, including HER2, HER3, HER4, and the EGF receptor. Cell adhesion molecules, for example, LFA-I, MoI, pl50, 95, VLA-4, ICAM-I, VCAM, and alpha v/beta 3 integrin. Growth factors, such as vascular endothelial growth factor ("VEGF"), growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, Mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-I -alpha), erythropoietin (EPO), nerve growth factor, such as NGF-beta, platelet-derived growth factor (PDGF), fibroblast growth factors, including, for instance, aFGF and bFGF, epidermal growth factor (EGF), transforming growth factors (TGF), including, among others, TGF- α and TGF-β, including TGF-βl, TGF-β2, TGF-β3, TGF- β4, or TGF- β 5, insulin-like growth factors-I and -II (IGF-I and IGF-II), des(I-3)-IGF-I (brain IGF-I), and osteoinductive factors. Insulins and insulin-related proteins, including insulin, insulin A-chain, insulin B-chain, proinsulin, and insulin-like growth factor binding proteins. Coagulation and coagulation-related proteins, such as, among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1-antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin;

(vii) other blood and serum proteins, including but not limited to albumin, IgE, and blood group antigens. Colony stimulating factors and receptors thereof, including the following, among others, M-CSF, GM-CSF, and G-CSF, and receptors thereof, such as CSF-1 receptor (c-fms). Receptors and receptor-associated proteins, including, for example, flk2/flt3 receptor, obesity (OB) receptor, LDL receptor, growth hormone receptors, thrombopoietin receptors ("TPO-R," "c-mpl"), glucagon receptors, interleukin receptors, interferon receptors, T-cell receptors, stem cell factor receptors, such as c-Kit, and other receptors. Receptor ligands, including, for example, OX40L, the ligand for the OX40 receptor. Neurotrophic factors, including bone-derived neurotrophic factor (BDNF) and neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6). Relaxin A-chain, relaxin B-chain, and prorelaxin; interferons and interferon receptors, including for example, interferon-α, -β, and -γ, and their receptors. Interleukins and interleukin receptors, including IL-I to IL-33 and IL-I to IL-33 receptors, such as the IL-8 receptor, among others. Viral antigens, including an AIDS envelope viral antigen. Lipoproteins, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES (regulated on activation normally T-cell expressed and secreted), mouse gonadotropin-associated peptide, DNAse, inhibin, and activin. Integrin, protein A or D, rheumatoid factors, immunotoxins, bone morphogenetic protein (BMP), superoxide dismutase, surface membrane proteins, decay accelerating factor (DAF), AIDS envelope, transport proteins, homing receptors, addressins, regulatory proteins, immunoadhesins, antibodies. Myostatins, TALL proteins, including TALL-I, amyloid proteins, including but not limited to amyloid-beta proteins, thymic stromal lymphopoietins ("TSLP"), RANK ligand ("OPGL"), c-kit, TNF receptors, including TNF Receptor Type 1, TRAIL-R2, angiopoietins, and biologically active fragments or analogs or variants of any of the foregoing.

[0046] Exemplary polypeptides and antibodies include Activase® (Alteplase); alirocumab, Aranesp® (Darbepoetin-alfa), Epogen® (Epoetin alfa, or erythropoietin); Avonex® (Interferon β-Ia); Bexxar® (Tositumomab); Betaseron® (Interferon-β); bococizumab (anti-PCSK9 monoclonal antibody designated as L1L3, see U.S.P.N. 8,080,243); Campath® (Alemtuzumab); Dynepo® (Epoetin delta); Velcade® (bortezomib); MLN0002 (anti-α4β7 Ab); MLN1202 (anti-CCR2 chemokine receptor Ab); Enbrel® (etanercept); Eprex® (Epoetin alfa); Erbitux® (Cetuximab); evolocumab; Genotropin® (Somatropin); Herceptin® (Trastuzumab); Humatrope® (somatropin [rDNA origin] for injection); Humira® (Adalimumab); Infergen® (Interferon Alfacon-1); Natrecor® (nesiritide); Kineret® (Anakinra), Leukine® (Sargamostim); LymphoCide® (Epratuzumab); BenlystaTM (Belimumab); Metalyse® (Tenecteplase); Mircera® (methoxy polyethylene glycol-epoetin beta); Mylotarg® (Gemtuzumab ozogamicin); Raptiva® (efalizumab); Cimzia® (certolizumab pegol); SolirisTM (Eculizumab); Pexelizumab (Anti-C5 Complement);

MEDI-524 (Numax®); Lucentis® (Ranibizumab); Edrecolomab (,Panorex®); Trabio® (lerdelimumab); TheraCim hR3 (Nimotuzumab); Omnitarg (Pertuzumab, 2C4); Osidem® (IDM-I); OvaRex® (B43.13); Nuvion® (visilizumab); Cantuzumab mertansine (huC242-DMl); NeoRecormon® (Epoetin beta); Neumega® (Oprelvekin); Neulasta® (Pegylated filgastrim, pegylated G-CSF, pegylated hu-Met-G-CSF); Neupogen® (Filgrastim); Orthoclone OKT3® (Muromonab-CD3), Procrit® (Epoetin alfa); Remicade® (Infliximab), Reopro® (Abciximab), Actemra® (anti-IL6 Receptor Ab), Avastin® (Bevacizumab), HuMax-CD4 (zanolimumab), Rituxan® (Rituximab); Tarceva® (Erlotinib); Roferon-A®-(Interferon alfa-2a); Simulect® (Basiliximab); StelaraTM (Ustekinumab); Prexige® (lumiracoxib); Synagis® (Palivizumab); 146B7-CHO (anti-IL15 antibody, see U.S.P.N. 7.153,507), Tysabri® (Natalizumab); Valortim® (MDX-1303, anti-B. anthracis Protective Antigen Ab); ABthraxTM; Vectibix® (Panitumumab); Xolair® (Omalizumab), ETI211 (anti-MRSA Ab), IL-I Trap (the Fc portion of human IgGl and the extracellular domains of both IL-I receptor components (the Type I receptor and receptor accessory protein)), VEGF Trap (Ig domains of VEGFRl fused to IgGl Fc), Zenapax® (Daclizumab); Zenapax® (Daclizumab), Zevalin® (Ibritumomab tiuxetan), Zetia (ezetimibe), Atacicept (TACI-Ig), anti-α4β7 Ab (vedolizumab); galiximab (anti-CD80 monoclonal antibody), anti-CD23 Ab (lumiliximab); BR2-Fc (huBR3 / huFc fusion protein, soluble BAFF antagonist); SimponiTM (Golimumab); Mapatumumab (human anti-TRAIL Receptor-1 Ab); Ocrelizumab (anti-CD20 human Ab); HuMax-EGFR (zalutumumab); M200 (Volociximab, anti-α5β1 integrin Ab); MDX-010 (Ipilimumab, anti-CTLA-4 Ab and VEGFR-I (IMC-18F1); anti-BR3 Ab; anti-C. difficile Toxin A and Toxin B C Abs MDX-066 (CDA-I) and MDX-1388); anti-CD22 dsFv-PE38 conjugates (CAT-3888 and CAT-8015); anti-CD25 Ab (HuMax-TAC); anti-TSLP antibodies; anti-TSLP receptor antibody (see U.S.P.N. 8,101,182); anti-TSLP antibody designated as A5 (see U.S.P.N. 7,982,016); (see anti-CD3 Ab (NI-0401); Adecatumumab (MT201, anti-EpCAM-CD326 Ab); MDX-060, SGN-30, SGN-35 (anti-CD30 Abs); MDX-1333 (anti- IFNAR); HuMax CD38 (anti-CD38 Ab); anti-CD40L Ab; anti-Cripto Ab; anti-CTGF Idiopathic Pulmonary Fibrosis Phase I Fibrogen (FG-3019); anti-CTLA4 Ab; anti-eotaxinl Ab (CAT-213); anti-FGF8 Ab; anti-ganglioside GD2 Ab; anti-sclerostin antibodies (see, U.S.P.N. 8,715,663 or U.S.P.N.7,592,429) anti-sclerostin antibody designated as Ab-5 (see U.S.P.N. 8,715,663 or U.S.P.N. 7,592,429); anti-ganglioside GM2 Ab; anti-GDF-8 human Ab (MYO-029); anti-GM-CSF Receptor Ab (CAM-3001); anti-HepC Ab (HuMax HepC); MEDI-545, MDX-1103 (anti-IFNα Ab); anti-IGFIR Ab; anti-IGF-IR Ab (HuMax-Inflam); anti-IL12/IL23p40 Ab (Briakinumab); anti-IL-23p19 Ab (LY2525623); anti-IL13 Ab (CAT-354); anti-IL-17 Ab (AIN457); anti-IL2Ra Ab (HuMax-TAC); anti-IL5 Receptor Ab; anti-integrin receptors Ab (MDX-Ol8, CNTO 95); anti-IPIO Ulcerative Colitis Ab (MDX- 1100); anti-LLY antibody; BMS-66513; anti-Mannose Receptor/hCGβ Ab (MDX-1307); anti-mesothelin dsFv-PE38 conjugate (CAT-5001); anti-PDlAb (MDX-1

106 (ONO- 4538)); anti-PDGFRα antibody (IMC-3G3); anti-TGFβ Ab (GC-1008); anti-TRAIL Receptor-2 human Ab (HGS-ETR2); anti-TWEAK Ab; anti-VEGFR/Flt-1 Ab; anti- ZP3 Ab (HuMax-ZP3); NVS Antibody #1; NVS Antibody #2; and an amyloid-beta monoclonal antibody comprising sequences, SEQ ID NO:8 and SEQ ID NO:6 (see U.S.P.N. 7,906,625).

[0047] Examples of antibodies suitable for the disclosed methods include the antibodies shown in Table A. Other examples of suitable antibodies include infliximab, bevacizumab, ranibizumab, cetuximab, ranibizumab, palivizumab, abagovomab, abciximab, actoxumab, adalimumab, afelimomab, afutuzumab, alacizumab, alacizumab pegol, ald518, alemtuzumab, alirocumab, alemtuzumab, altumomab, amatuximab, anatumomab mafenatox, anrukinzumab, apolizumab, arcitumomab, aselizumab, altinumab, atlizumab, atorolimiumab, tocilizumab, bapineuzumab, basiliximab, bavituximab, bectumomab, belimumab, benralizumab, bertilimumab, besilesomab, bevacizumab, bezlotoxumab, biciromab, bivatuzumab, bivatuzumab mertansine, blinatumomab, blosozumab, brentuximab vedotin, briakinumab, brodalumab, canakinumab, cantuzumab mertansine, cantuzumab mertansine, caplacizumab, capromab pendetide, carlumab, catumaxomab, cc49, cedelizumab, certolizumab pegol, cetuximab, citatuzumab bogatox, cixutumumab, clazakizumab, clenoliximab, clivatuzumab tetraxetan, conatumumab, crenezumab, cr6261, dacetuzumab, daclizumab, dalotuzumab, daratumumab, demcizumab, denosumab, detumomab, dorlimomab aritox, drozitumab, duligotumab, dupilumab, ecromeximab, eculizumab, edobacomab, edrecolomab, efalizumab, efungumab, elotuzumab, elsilimomab, enavatuzumab, enlimomab pegol, enokizumab, enokizumab, enoticumab, enoticumab, ensituximab, epitumomab cituxetan, epratuzumab, erlizumab, ertumaxomab, etaracizumab, etrolizumab, exbivirumab, exbivirumab, fanolesomab, faralimomab, farletuzumab, fasinumab, fbta05, felvizumab, fezakinumab, ficlatuzumab, figitumumab, flanvotumab, fontolizumab, foralumab, foravirumab, fresolimumab, fulranumab, futuximab, galiximab, ganitumab, gantenerumab, gavilimomab, gemtuzumab ozogamicin, gevokizumab, girentuximab, glembatumumab vedotin, golimumab, gomiliximab, gs6624, ibalizumab, ibritumomab tiuxetan, icrucumab, igovomab, imciromab, imgatuzumab, inclacumab, indatuximab ravtansine, infliximab, intetumumab, inolimomab, inotuzumab ozogamicin, ipilimumab, iratumumab, itolizumab, ixekizumab, keliximab, labetuzumab, lebrikizumab, lemalesomab, lerdelimumab, lexatumumab, libivirumab, ligelizumab, lintuzumab, lirilumab, lorvotuzumab mertansine, lucatumumab, lumiliximab, mapatumumab, maslimomab, mavrilimumab, matuzumab, mepolizumab, metelimumab, milatuzumab, minretumomab, mitumomab, mogamulizumab, morolimumab, motavizumab, moxetumomab pasudotox, muromonab-cd3, nacolomab tafenatox, namilumab, naptumomab estafenatox, narnatumab, natalizumab, nebacumab,

necitumumab, nerelimomab, nesvacumab, nimotuzumab, nivolumab, nofetumomab merpentan, ocaratuzumab, ocrelizumab, odulimomab, ofatumumab, olaratumab, olokizumab, omalizumab, onartuzumab, oportuzumab monatox, oregovomab, orticumab, otelixizumab, oxelumab, ozanezumab, ozoralizumab, pagibaximab, palivizumab, panitumumab, panobacumab, parsatuzumab, pascolizumab, pateclizumab, patritumab, pemtumomab, perakizumab, pertuzumab, pexelizumab, pidilizumab, pintumomab, placulumab, ponezumab, priliximab, pritumumab, PRO 140, quilizumab, racotumomab, radretumab, rafivirumab, ramucirumab, ranibizumab, raxibacumab, regavirumab, reslizumab, rilotumumab, rituximab, robatumumab, roledumab, romosozumab, rontalizumab, rovelizumab, ruplizumab, samalizumab, sarilumab, satumomab pendetide, secukinumab, sevirumab, sibrotuzumab, sifalimumab, siltuximab, simtuzumab, siplizumab, sirukumab, solanezumab, solitomab, sonepcizumab, sontuzumab, stamulumab, sulesomab, suvizumab, tabalumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tanezumab, taplitumomab paptox, tefibazumab, telimomab aritox, tenatumomab, tefibazumab, telimomab aritox, tenatumomab, teneliximab, teplizumab, teprotumumab, TGN1412, tremelimumab, ticilimumab, tildrakizumab, tigatuzumab, TNX-650, tocilizumab, toralizumab, tositumomab, tralokinumab, trastuzumab, TRBS07, tregalizumab, tremelimumab, tucotuzumab celmoleukin, tuvirumab, ublituximab, urelumab, urtoxazumab, ustekinumab, vapaliximab, vatelizumab, vedolizumab, veltuzumab, vepalimomab, vesencumab, visilizumab, volociximab, vorsetuzumab mafodotin, votumumab, zalutumumab, zanolimumab, zatuximab, ziralimumab and zolimomab aritox.

[0048] Most preferred antibodies for use in the disclosed formulations and methods are adalimumab, bevacizumab, blinatumomab, cetuximab, conatumumab, denosumab, eculizumab, erenumab, evolocumab, infliximab, natalizumab, panitumumab, rilotumumab, rituximab, romosozumab, and trastuzumab, and antibodies selected from Table A.

TABLE A

Examples of therapeutic antibodies

Target (informal name)	Conc. (mg/ml)	Viscosity (cP)	HC Type (including allotypes)	LC Type	pI	LC SEQ ID NO	HC SEQ ID NO
anti-amyloid	142.2	5.0	IgG1 (f) (R;EM)	Kappa	9.0	1	2
GMCSF (247)	139.7	5.6	IgG2	Kappa	8.7	3	4
CGRPR	136.6	6.3	IgG2	Lambda	8.6	5	6
RANKL	152.7	6.6	IgG2	Kappa	8.6	7	8
Sclerostin (27H6)	145.0	6.7	IgG2	Kappa	6.6	9	10
IL-1R1	153.9	6.7	IgG2	Kappa	7.4	11	12
Myostatin	141.0	6.8	IgG1 (z) (K;EM)	Kappa	8.7	13	14
B7RP1	137.5	7.7	IgG2	Kappa	7.7	15	16
Amyloid	140.6	8.2	IgG1 (za) (K;DL)	Kappa	8.7	17	18
GMCSF (3.112)	156.0	8.2	IgG2	Kappa	8.8	19	20
CGRP (32H7)	159.5	8.3	IgG2	Kappa	8.7	21	22
CGRP (3B6.2)	161.1	8.4	IgG2	Lambda	8.6	23	24
PCSK9 (8A3.1)	150.0	9.1	IgG2	Kappa	6.7	25	26
PCSK9 (492)	150.0	9.2	IgG2	Kappa	6.9	27	28
CGRP	155.2	9.6	IgG2	Lambda	8.8	29	30
Hepcidin	147.1	9.9	IgG2	Lambda	7.3	31	32
TNFR p55)	157.0	10.0	IgG2	Kappa	8.2	33	34
OX40L	144.5	10.0	IgG2	Kappa	8.7	35	36
HGF	155.8	10.6	IgG2	Kappa	8.1	37	38
GMCSF	162.5	11.0	IgG2	Kappa	8.1	39	40
Glucagon R	146.0	12.1	IgG2	Kappa	8.4	41	42
GMCSF (4.381)	144.5	12.1	IgG2	Kappa	8.4	43	44
Sclerostin (13F3)	155.0	12.1	IgG2	Kappa	7.8	45	46
CD-22	143.7	12.2	IgG1 (f) (R;EM)	Kappa	8.8	47	48
INFgR	154.2	12.2	IgG1 (za) (K;DL)	Kappa	8.8	49	50
Ang2	151.5	12.4	IgG2	Kappa	7.4	51	52
TRAILR2	158.3	12.5	IgG1 (f) (R;EM)	Kappa	8.7	53	54

Target (informal name)	Conc. (mg/ml)	Viscosity (cP)	HC Type (including allotypes)	LC Type	pI	LC SEQ ID NO	HC SEQ ID NO
EGFR	141.7	14.0	IgG2	Kappa	6.8	55	56
IL-4R	145.8	15.2	IgG2	Kappa	8.6	57	58
IL-15	149.0	16.3	IgG1 (f) (R;EM)	Kappa	8.8	59	60
IGF1R	159.2	17.3	IgG1 (za) (K;DL)	Kappa	8.6	61	62
IL-17R	150.9	19.1	IgG2	Kappa	8.6	63	64
Dkk1 (6.37.5)	159.4	19.6	IgG2	Kappa	8.2	65	66
Sclerostin	134.8	20.9	IgG2	Kappa	7.4	67	68
TSLP	134.2	21.4	IgG2	Lambda	7.2	69	70
Dkk1 (11H10)	145.3	22.5	IgG2	Kappa	8.2	71	72
PCSK9	145.2	22.8	IgG2	Lambda	8.1	73	74
GIPR (2G10.006)	150.0	23.0	IgG1 (z) (K;EM)	Kappa	8.1	75	76
Activin	133.9	29.4	IgG2	Lambda	7.0	77	78
Sclerostin (2B8)	150.0	30.0	IgG2	Lambda	6.7	79	80
Sclerostin	141.4	30.4	IgG2	Kappa	6.8	81	82
c-fms	146.9	32.1	IgG2	Kappa	6.6	83	84
α4β7	154.9	32.7	IgG2	Kappa	6.5	85	86
PD-1	-	-	IgG1	Kappa	-	88	87

* An exemplary concentration suitable for patient administration; ^HC – antibody heavy chain; LC – antibody light chain.

Mutein

[0049] Mutein is a protein having at least amino acid change due to a mutation in the nucleic acid sequence, such as a substitution, deletion or insertion. Exemplary muteins comprise amino acid sequences having at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, or has greater than about 90% (*e.g.*, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%) sequence identity to the wild type amino acid sequence. In addition, the mutein may be a fusion protein as described above.

[0050] In exemplary embodiments, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the amino acid substitution(s) is/are conservative amino acid substitution(s). As used herein, the term "conservative

amino acid substitution" refers to the substitution of one amino acid with another amino acid having similar properties, *e.g.*, size, charge, hydrophobicity, hydrophilicity, and/or aromaticity, and includes exchanges within one of the following five groups:

I. **Small aliphatic, nonpolar or slightly polar residues**: Ala, Ser, Thr, Pro, Gly;

II. **Polar, negatively charged residues and their amides and esters**: Asp, Asn, Glu, Gln, cysteic acid and homocysteic acid;

III. **Polar, positively charged residues**: His, Arg, Lys; Ornithine (Orn)

IV. **Large, aliphatic, nonpolar residues**: Met, Leu, Ile, Val, Cys, Norleucine (Nle), homocysteine

V. **Large, aromatic residues**: Phe, Tyr, Trp, acetyl phenylalanine.

[0051] In exemplary embodiments, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the amino acid substitution(s) is/are non-conservative amino acid substitution(s). As used herein, the term "non-conservative amino acid substitution" is defined herein as the substitution of one amino acid with another amino acid having different properties, *e.g.*, size, charge, hydrophobicity, hydrophilicity, and/or aromaticity, and includes exchanges outside the above five groups.

[0052] In exemplary aspects, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the substitute amino acid is a naturally-occurring amino acid. By “naturally-occurring amino acid” or “standard amino acid” or “canonical amino acid” is meant one of the 20 alpha amino acids found in eukaryotes encoded directly by the codons of the universal genetic code (Ala, Val, Ile, Leu, Met, Phe, Tyr, Trp, Ser, Thr, Asn, Gln, Cys, Gly, Pro, Arg, His, Lys, Asp, Glu). In exemplary aspects, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the substitute amino acid is a non-standard amino acid, or an amino acid which is not incorporated into proteins during translation. Non-standard amino acids include, but are not limited to: selenocysteine, pyrrolysine, ornithine, norleucine, β-amino acids (*e.g.*, β-alanine, β-aminoisobutyric acid, β-phenlyalanine, β-homophenylalanine, β-glutamic acid, β-glutamine, β-homotryptophan, β-leucine, β-lysine), homo-amino acids (*e.g.*, homophenylalanine, homoserine, homoarginine, monocysteine, homocystine), *N*-methyl amino acids (*e.g.*, L-abrine, *N*-methyl-alanine, *N*-methyl-isoleucine, *N*-methyl-leucine), 2-aminocaprylic acid, 7-aminocephalosporanic acid, 4-aminocinnamic acid, alpha-aminocyclohexanepropionic acid, amino-(4-hydroxyphenyl)acetic acid, 4-amino-nicotinic acid, 3-aminophenylacetic acid, and the like.

BiTE Molecule

[0053] Bispecific T cell engager (BiTE) molecules are a bispecific antibody construct or bispecific fusion protein comprising two antibody binding domains (or targeting regions) linked together. One arm of the molecule is engineered to bind with a protein found on the surface of cytotoxic T cells, and the other arm is designed to bind to a specific protein found primarily on tumor cell. When both targets are engaged, the BiTE molecule forms a bridge between the cytotoxic T cell and the tumor cell, which enables the T cell to recognize the tumor cell and fight it through an infusion of toxic molecules. For example, the tumor-binding arm of the molecule can be altered to create different BiTE antibody constructs that target different types of cancer

[0054] The term "binding domain" in regards to a BiTE molecule refers to a domain which (specifically) binds to / interacts with / recognizes a given target epitope or a given target site on the target molecules (antigens). The structure and function of the first binding domain (recognizing the tumor cell antigen), and preferably also the structure and/or function of the second binding domain (cytotoxic T cell antigen), is/are based on the structure and/or function of an antibody, *e.g.* of a full-length or whole immunoglobulin molecule.

[0055] The "epitope" refers to a site on an antigen to which a binding domain, such as an antibody or immunoglobulin or derivative or fragment of an antibody or of an immunoglobulin, specifically binds. An “epitope” is antigenic and thus the term epitope is sometimes also referred to herein as “antigenic structure” or “antigenic determinant”. Thus, the binding domain is an “antigen interaction site”. Said binding/interaction is also understood to define a “specific recognition”.

[0056] For example, the BiTE molecule comprises a first binding domain characterized by the presence of three light chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VL region) and three heavy chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VH region). The second binding domain preferably also comprises the minimum structural requirements of an antibody which allow for the target binding. More preferably, the second binding domain comprises at least three light chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VL region) and/or three heavy chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VH region). It is envisaged that the first and/or second binding domain is produced by or obtainable by phage-display or library screening methods rather than by grafting CDR sequences from a pre-existing (monoclonal) antibody into a scaffold.

[0057] A binding domain may typically comprise an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH); however, it does not have to comprise both. Fd fragments,

for example, have two VH regions and often retain some antigen-binding function of the intact antigen-binding domain. Examples of (modified) antigen-binding antibody fragments include (1) a Fab fragment, a monovalent fragment having the VL, VH, CL and CH1 domains; (2) a F(ab')2 fragment, a bivalent fragment having two Fab fragments linked by a disulfide bridge at the hinge region; (3) an Fd fragment having the two VH and CH1 domains; (4) an Fv fragment having the VL and VH domains of a single arm of an antibody, (5) a dAb fragment (Ward *et al.*, (1989) Nature 341 :544-546), which has a VH domain; (6) an isolated complementarity determining region (CDR), and (7) a single chain Fv (scFv) , the latter being preferred (for example, derived from an scFV-library).

[0058] The terms “(specifically) binds to”, (specifically) recognizes", “is (specifically) directed to”, and “(specifically) reacts with” regarding a BiTE molecule refers to a binding domain that interacts or specifically interacts with one or more, preferably at least two, more preferably at least three and most preferably at least four amino acids of an epitope located on the target protein or antigen.

[0059] The term "variable" refers to the portions of the antibody or immunoglobulin domains that exhibit variability in their sequence and that are involved in determining the specificity and binding affinity of a particular antibody (*i.e.*, the "variable domain(s)"). The pairing of a variable heavy chain (VH) and a variable light chain (VL) together forms a single antigen-binding site. The CH domain most proximal to VH is designated as CH1. Each light (L) chain is linked to a heavy (H) chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype.

[0060] Variability is not evenly distributed throughout the variable domains of antibodies; it is concentrated in sub-domains of each of the heavy and light chain variable regions. These sub-domains are called “hypervariable regions” or "complementarity determining regions" (CDRs). The more conserved (*i.e.*, non-hypervariable) portions of the variable domains are called the "framework" regions (FRM) and provide a scaffold for the six CDRs in three dimensional space to form an antigen-binding surface. The variable domains of naturally occurring heavy and light chains each comprise four FRM regions (FR1, FR2, FR3, and FR4), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRM and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site (see Kabat *et al.*, loc. cit.). The constant domains are not directly involved in antigen binding, but

exhibit various effector functions, such as, for example, antibody-dependent, cell-mediated cytotoxicity and complement activation.

[0061] The CDR3 of the light chain and, particularly, the CDR3 of the heavy chain may constitute the most important determinants in antigen binding within the light and heavy chain variable regions. In some antibody constructs, the heavy chain CDR3 appears to constitute the major area of contact between the antigen and the antibody. In vitro selection schemes in which CDR3 alone is varied can be used to vary the binding properties of an antibody or determine which residues contribute to the binding of an antigen. Hence, CDR3 is typically the greatest source of molecular diversity within the antibody-binding site. H3, for example, can be as short as two amino acid residues or greater than 26 amino acids.

[0071] The sequence of antibody genes after assembly and somatic mutation is highly varied, and these varied genes are estimated to encode 1010 different antibody molecules (Immunoglobulin Genes, 2nd ed., eds. Jonio *et al.*, Academic Press, San Diego, CA, 1995). Accordingly, the immune system provides a repertoire of immunoglobulins. The term "repertoire" refers to at least one nucleotide sequence derived wholly or partially from at least one sequence encoding at least one immunoglobulin. The sequence(s) may be generated by rearrangement in vivo of the V, D, and J segments of heavy chains, and the V and J segments of light chains. Alternatively, the sequence(s) can be generated from a cell in response to which rearrangement occurs, *e.g.*, in vitro stimulation. Alternatively, part or all of the sequence(s) may be obtained by DNA splicing, nucleotide synthesis, mutagenesis, and other methods, see, *e.g.*, U.S. Patent 5,565,332. A repertoire may include only one sequence or may include a plurality of sequences, including ones in a genetically diverse collection.

[0062] The term “bispecific” as used herein refers to an antibody construct which is “at least bispecific”, *i.e.*, it comprises at least a first binding domain and a second binding domain, wherein the first binding domain binds to one antigen or target, and the second binding domain binds to another antigen or target. Accordingly, antibody constructs within a BiTE molecule comprise specificities for at least two different antigens or targets. The term “bispecific antibody construct” of the invention also encompasses multispecific antibody constructs such as trispecific antibody constructs, the latter ones including three binding domains, or constructs having more than three (*e.g.* four, five…) specificities.

[0063] The at least two binding domains and the variable domains of the antibody construct within a BiTE molecule may or may not comprise peptide linkers (spacer peptides). The term “peptide linker” defines in accordance with the present invention an amino acid sequence by which the amino acid

sequences of one (variable and/or binding) domain and another (variable and/or binding) domain of the antibody construct of the invention are linked with each other. An essential technical feature of such peptide linker is that said peptide linker does not comprise any polymerization activity. Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233 or WO 88/09344.

[0064] In the event that a linker is used, this linker is preferably of a length and sequence sufficient to ensure that each of the first and second domains can, independently from one another, retain their differential binding specificities. For peptide linkers which connect the at least two binding domains in the antibody construct within a BiTE molecule (or two variable domains), those peptide linkers are preferred which comprise only a few number of amino acid residues, *e.g.* 12 amino acid residues or less. Thus, peptide linker of 12, 11, 10, 9, 8, 7, 6 or 5 amino acid residues are preferred. An envisaged peptide linker with less than 5 amino acids comprises 4, 3, 2 or one amino acid(s) wherein Gly-rich linkers are preferred. A particularly preferred "single" amino acid in context of said "peptide linker" is Gly. Accordingly, said peptide linker may consist of the single amino acid Gly. Another preferred embodiment of a peptide linker is characterized by the amino acid sequence Gly-Gly-Gly-Gly-Ser, *i.e.* Gly4Ser, or polymers thereof, *i.e.* (Gly4Ser)x, where x is an integer of 1 or greater. The characteristics of said peptide linker, which comprise the absence of the promotion of secondary structures are known in the art and are described *e.g.* in Dall'Acqua *et al.* (Biochem. (1998) 37, 9266-9273), Cheadle *et al.* (Mol Immunol (1992) 29, 21-30) and Raag and Whitlow (FASEB (1995) 9(1), 73-80). Peptide linkers which also do not promote any secondary structures are preferred. The linkage of said domains to each other can be provided by, *e.g.* genetic engineering, as described in the examples. Methods for preparing fused and operatively linked bispecific single chain constructs and expressing them in mammalian cells or bacteria are well-known in the art (*e.g.* WO 99/54440 or Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).

[0065] The BiTE molecules of the disclosure may comprise an antibody construct in a format selected from the group consisting of (scFv)2, scFv-single domain mAb, diabodies and oligomers of any of the aforermentioned formats.

[0066] According to a particularly preferred embodiment, and as documented in the appended examples, the antibody construct within a BiTE molecule is a "bispecific single chain antibody construct", more prefereably a bispecific "single chain Fv" (scFv). Although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to

form a monovalent molecule; see *e.g.*, Huston *et al.* (1988) Proc. Natl. Acad. Sci USA 85:5879-5883). These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are evaluated for function in the same manner as are whole or full-length antibodies. A single-chain variable fragment (scFv) is hence a fusion protein of the variable region of the heavy chain (VH) and of the light chain (VL) of immunoglobulins, usually connected with a short linker peptide of about ten to about 25 amino acids, preferably about 15 to 20 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and introduction of the linker.

[0067] Bispecific single chain molecules are known in the art and are described in WO 99/54440, Mack, J. Immunol. (1997), 158, 3965-3970, Mack, PNAS, (1995), 92, 7021-7025, Kufer, Cancer Immunol. Immunother., (1997), 45, 193-197, Löffler, Blood, (2000), 95, 6, 2098-2103, Brühl, Immunol., (2001), 166, 2420-2426, Kipriyanov, J. Mol. Biol., (1999), 293, 41-56. Techniques described for the production of single chain antibodies (see, inter alia, US Patent 4,946,778, Kontermann and Dübel (2010), loc. cit. and Little (2009), loc. cit.) can be adapted to produce single chain antibody constructs specifically recognizing (an) elected target(s).

[0068] Bivalent (also called divalent) or bispecific single-chain variable fragments (bi-scFvs or di-scFvs having the format (scFv)2) can be engineered by linking two scFv molecules. If these two scFv molecules have the same binding specificity, the resulting (scFv)2 molecule will preferably be called bivalent (*i.e.* it has two valences for the same target epitope). If the two scFv molecules have different binding specificities, the resulting (scFv)2 molecule will preferably be called bispecific. The linking can be done by producing a single peptide chain with two VH regions and two VL regions, yielding tandem scFvs (see *e.g.* Kufer P. *et al.*, (2004) Trends in Biotechnology 22(5):238-244). Another possibility is the creation of scFv molecules with linker peptides that are too short for the two variable regions to fold together (*e.g.* about five amino acids), forcing the scFvs to dimerize. This type is known as diabodies (see *e.g.* Hollinger, Philipp *et al.*, (July 1993) Proceedings of the National Academy of Sciences of the United States of America 90 (14): 6444-8.).

[0069] Single domain antibodies comprise merely one (monomeric) antibody variable domain which is able to bind selectively to a specific antigen, independently of other V regions or domains. The first single domain antibodies were engineered from havy chain antibodies found in camelids, and these are called VHH fragments. Cartilaginous fishes also have heavy chain antibodies (IgNAR) from which single

domain antibodies called VNAR fragments can be obtained. An alternative approach is to split the dimeric variable domains from common immunoglobulins *e.g.* from humans or rodents into monomers, hence obtaining VH or VL as a single domain Ab. Although most research into single domain antibodies is currently based on heavy chain variable domains, nanobodies derived from light chains have also been shown to bind specifically to target epitopes. Examples of single domain antibodies are called sdAb, nanobodies or single variable domain antibodies.

[0070] A (single domain mAb)2 is hence a monoclonal antibody construct composed of (at least) two single domain monoclonal antibodies, which are individually selected from the group comprising VH, VL, VHH and VNAR. The linker is preferably in the form of a peptide linker. Similarly, an "scFv-single domain mAb" is a monoclonal antibody construct composed of at least one single domain antibody as described above and one scFv molecule as described above. Again, the linker is preferably in the form of a peptide linker.

[0071] Exemplary BiTE molecules include anti-CD33 and anti-CD3 BiTE molecule, anti-BCMA and anti-CD3 BiTE molecule, anti-FLT3 and anti-CD3 BiTE, anti-CD19 and anti-CD3 BiTE, anti-EGFRvIII and anti-CD3 BiTE molecule, anti-DLL3 and anti-CD3 BiTE, BLINCYTO (blinatumomab) and Solitomab.

EXAMPLES

Example 1

Methods

Size Exclusion Chromatography

[0072] Size exclusion chromatography (SEC) experiments were performed using an Agilent 1100 HPLC system with a TSK-GEL G3000SWxl, 5 µm particle size, 7.8 mm ID x 300 mm length column (Tosoh Biosep) with an Agilent UV detector with wavelength set at 280 nm (Agilent; Santa Clara, CA). The SEC-LS runs were performed at room temperature, with 100 mM potassium phosphate, 250 mM potassium chloride, pH 6.8 ± 0.1 buffer used as the mobile phase and the flow rate was 0.5 mL/min.

Differential Scanning Calorimetry

[0073] Differential Scanning Calorimetry (DSC) experiments were performed on a MicroCal VP-Capillary DSC system (Malvern Panalytical Inc.; Westborough, MA). The sample concentrations used in the DSC experiments were approximately 1 mg/mL by diluting the original samples using the corresponding formulation buffer. The samples were heated from 10°C to 100°C at a rate of 60°C/hour. The pre-scan was 15 minutes, the filtering period 10 seconds, and the feedback mode none. An

endothermic thermal transition in the sample cell causes positive deflection of the signal. The data analysis was performed using MicroCal Origin 7 software (OriginLab; Northampton, MA).

Nano Differential Scanning Fluorimetry

[0074] The Nano Differential Scanning Fluorimetry (nanoDSF) experiments were performed on a nanoTemper nanoDSF system (Nanotemper; South San Francisco, CA). The sample concentrations used in the DSC experiments were approximately 1 mg/mL by diluting the original samples using the corresponding formulation buffer. The samples were heated from 20°C to 95°C at a rate of 60°C/hour. The data analysis was performed using PR.ThermControl software (Nanotemper).

Statistical analysis

[0075] The statistical analysis was done using the statistical software, JMP® (Cary, North Carolina). The p-values and leverage plots were obtained from the JMP data analysis. The typical results of leverage plots of different statistical results are shown in Figure 1.

Example 2

Thermal Stability Data Predicts Stability of Monoclonal Antibodies

[0076] Formulation comprising a monoclonal antibody (mAb-1) were analyzed using the SEC and the DSC method as described in Example 1. The sample information as well as the SEC and DSC data are shown in Table 1. The main peak consisted of mAb-1 in its native form.

[0077] The data were analyzed by the JMP statistical software. The P-value obtained from the JMP statistical analysis was P = 0.0159, which was less than 0.05. This suggests that there was a statistically significant correlation between the 6-month SEC stability data and the T_m data (T=0). The leverage plot obtained from JMP statistical analysis is shown in Figure 2. The confidence curve in Figure 2 crosses the solid gray horizontal line. This also confirmed that there was a statistically significant correlation between the 6-month SEC stability data and the T_m data (T=0). The statistical analysis results of the mAb-1 data indicates that the thermal stability attribute value can be used to predict the mAb-1 SEC stability data.

Table1. The SEC and DSC data of the mAb-1 formulation stability study

ID	Buffer*	MP% (T=0)	MP% (6m37C)**	MP Change% (6m37C)	T_{m1} (T=0)
1	A52SuT	99.6	96.4	3.2	70.0
2	A52SoT	99.6	97.1	2.6	68.6
3	A52So	99.6	97.5	2.0	69.9
4	A45SoT	99.7	94.6	5.1	58.6
5	A52SoT	99.6	97.5	2.1	69.7
6	G45SoT	99.7	97.7	2.0	63.0
7	G48SoT	99.7	97.9	1.8	65.6
8	G50SoT	99.6	97.8	1.9	67.6
9	G52SoT	99.6	97.8	1.8	68.9
10	G55SoT	99.5	97.4	2.1	72.6
11	G58SoT	99.4	97.1	2.4	72.9
12	S45SoT	99.7	95.0	4.8	61.9
13	S52SoT	99.6	96.7	2.9	67.9

* A = Acetate buffer, G = Glutamate buffer, S (the 1st letter) = Succinate buffer, So = Sorbitol, and Su = Sucrose; ** MP = main peak, 6m = 6 months, and 37C = 37°C

Example 3

Thermal Stability Data Predicts Stability of Bispecific T Cell Engager Molecule

[0078] Formulations comprising different constructs of a bispecific T Cell engager (BiTE-1) molecule were analyzed using the SEC and the DSC method as described in Example 1. The sample information is provided in Table 2 and the SEC and the DSC data are shown in Table 3.

[0079] The data were analyzed by the JMP statistical software. The P-value obtained from the JMP statistical analysis was P = 0.0014, which was less than 0.05. This suggests that there was a statistically significant correlation between the 2-week SEC stability data and the T_m data. The leverage plot obtained from JMP is shown in Figure 2. The confidence curve in Figure 3 crosses the solid gray horizontal line. This also confirmed that there was a statistically significant correlation between the 6-week SEC stability data and the T_m data. The statistical analysis results of the BiTE-1 data indicates that the thermal stability attribute value can be used to predict the BiTE-1 SEC stability data.

Table 2. Sample and Buffer Info of the BiTE-1 Samples

ID	Sample and Buffer Info
1	1 = PL-31767 (Maxibody BiTE-1)
2	2 = PL-31776 (Fc-Crossbody BiTE-1)
3	3 = P-144537.6 (Maxibody BiTE-1)
4	4 = P-144539.6 (Fc-Crossbody BiTE-1)
A	A = 10mM NaCitrate, 50mM Arginine, 50mM Glutamic Acid, 4% Sucrose 0.01% PS80 pH 6.0
B	B = 10mM NaCitrate, 75mM Lysine, 4% Trehalose, 0.01% PS80 pH 7.0
C	C = 10mM KPO4, 161mM Arginine, 151mM NaCl, pH 7.0

Table 3. The SEC and DSC data of the BiTE-1 stability study

Sample and Buffer ID	MP Change% (2w40C, SEC)*	T_{m1} (T=0)
1A	2.8	55.7
2A	8.9	48.2
3A	0.3	63.5
4A	4.7	57.4
1B	3.8	55.2
2B	13.9	49.8
3B	1.3	63.1
4B	4.7	57.3
1C	3.7	56.4
2C	20.7	49.9
3C	0.9	62.3
4C	4.0	57.2

** MP = main peak, 2w = 2 weeks, and 40C = 40°C

Example 4

Thermal Stability Data Predicts Stability of Fc Fusion Protein

[0080] Formulations comprising a muteins Fc fusion protein (mutein-1) were analyzed using the SEC and the DSC method as described in Example 1. The sample information is provided in Table 4 and the SEC and the DSC data are shown in Table 5.

[0081] The data were analyzed by the JMP statistical software. The P-value obtained from the JMP statistical analysis was $P < 0.0001$, which was less than 0.05. This suggests that there was a statistically significant correlation between the 2-week SEC stability data and the T_m data. The leverage plot obtained from JMP is shown in Figure 4. The confidence curve in Figure 4 crosses the solid gray horizontal line. This confirms that there was a statistically significant correlation between the 2-week SEC stability data and the T_m data. The statistical analysis results of the mutein data indicate that the

thermal stability attribute value can be used to predict the Fc fusion protein stability data as well as the stability data of the muteins of other structure families, such as mAbs.

Table 4. Sample and Buffer Info of the Mutein Samples

BioReg ID	Mutation#1	Mutation#2	Buffer
2066-1	H16K	C125A	A52SuT
2066-2	H16K	C125A	KP6RT
2067-1	H16R	C125A	A52SuT
2067-2	H16R	C125A	KP6RT
2068-1	V91D	C125A	A52SuT
2068-2	V91D	C125A	KP6RT
2069-1	V91R	C125A	A52Su
2069-2	V91R	C125A	KP6RT

Table5. The SEC and DSC data of the IL-2 Mutein stability study

BioReg ID	MP Change% (2w25C, SEC)*	T_{m1}
2066-1	24.1	51.4
2066-2	14.8	54.7
2067-1	19.2	52.4
2067-2	13.1	55.6
2068-1	6.6	65.3
2068-2	3.8	66.1
2069-1	0.8	67.0
2069-2	1.7	67.0

[0082] ** MP = main peak, 2w = 2q11 weeks, and 25C = 25°C

Conclusions

[0083] Using the statistical approach demonstrated that protein stability measured by the SEC method can be predicted using thermal stability attribute (T_m) with statistical significance. The method of measuring the thermal stability attribute (T_m) to predict protein stability, as measured by SEC, saves significant time and resources during drug development analysis.

[0084] As used herein, the terms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. The terms “a” (or “an”), as well as the terms “one or more,” and “at least one” can be used interchangeably herein. Furthermore, “and/or” where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term “and/or” as used in a phrase such as “A and/or B” herein is intended to include “A and B,” “A

or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0085] The term "comprise" or "comprising" is generally used in the inclusive sense, that is, to permit the presence of one or more features or components.

[0086] The term "about" as used in connection with a numerical value throughout the specification and the claims denotes an interval of accuracy, familiar and acceptable to a person skilled in the art. In general, such interval of accuracy is ±10%.

[0087] 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 is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure. The following procedures and techniques may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the specification.

[0088] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or aspects of the disclosure, which can be had by reference to the specification as a whole.

What is Claimed:

1. A method of assessing stability of a therapeutic protein comprising the steps of measuring thermal stability of the therapeutic protein and comparing the thermal unfolding temperature T_m of the therapeutic protein with the T_m of a control protein, wherein an increase in T_m of the therapeutic protein compared to the T_m of the control protein indicates an increase in stability of the therapeutic protein, and wherein the T_m of the therapeutic protein correlates with statistical significance with the main peak percent change determined by size exclusion chromatography (SEC).

2. A method of predicting stability of a therapeutic protein as measured by size exclusion chromatography (SEC) comprising the steps of measuring thermal stability of the therapeutic protein and comparing the thermal unfolding temperature T_m of the therapeutic protein with the T_m of a control protein, wherein the T_m of the therapeutic protein correlates with statistical significance with the main peak percent change determined by size exclusion chromatography (SEC).

3. The method of claim 1 or 2, wherein the T_m of the therapeutic proteins correlates with the main peak percent change determined by SEC with a p value of less than 0.05.

4. The method of claim 1 or 2, wherein the T_m of the therapeutic proteins correlates with the main peak percent change determined by SEC with a p value of less than 0.01.

5. The method of claim 1 or 2, wherein the T_m of the therapeutic proteins correlates with the main peak percent change determined by SEC with a p value of less than 0.005.

6. The method of any one of claims 1-5, wherein thermal stability is measured by differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF).

7. The method of any one of claims 1-6, further comprising the step of determining the statistical significance of the correlation between the T_m value of the therapeutic protein and the main peak percent change determined by SEC.

8. The method of claim 7, wherein statistical significance is determined by linear regression analysis or polynomial regression.

9. The method of any one of claims 1-8, wherein the T_m value of the therapeutic protein predicts the stability of the therapeutic protein for at least a six-month period.

10. The method of any one of claims 1-9, wherein the T_m value of the therapeutic protein predicts the main peak percent change - of therapeutic protein as measured on size exclusion chromatography.

11. The method of any one of claims 1-10, wherein the therapeutic protein is a monoclonal antibody or a fragment or derivative thereof, a multi-specific antibody, a bi-specific T cell engager (BiTE®) molecule, an Fc fusion molecule, or a mutein.

12. The method of claim 11, wherein the monoclonal antibody is selected from the group consisting of those presented in Table A.

13. The method of claim 11 wherein the BiTE molecule specifically binds to one or more of CD33, BCMA, FLT3, CD-19, EGFRvIII, DLL3, and CD-3.

14. The method of claim 11 wherein the Fc fusion molecule or the mutein specifically binds to IL-2, IL-7, IL-10, IL-12, IL-15, IL-21, CTLA-4, PD-1, PD-L1, PD-L2, B7-H3, B7-H4, CEACAM-1, TIGIT, LAG3, CD112, CD112R, CD96, TIM3, BTLA, ICOS, OX40, 41BB, CD27, GITR.

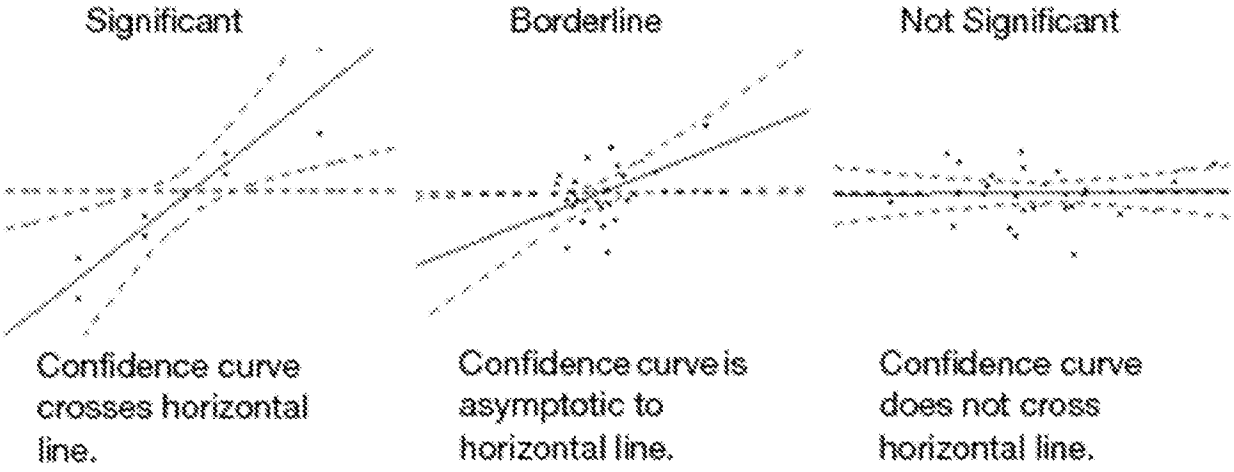
15. The method of any previous claim, wherein the therapeutic protein is formulated.

16. The method of claim 15, wherein the therapeutic protein is formulated at a concentration greater than 70 mg/mL .

17. The method of claim 15, wherein the therapeutic protein is formulated a concentration of less than 70 mg/ml.

18. The method of claim 15, wherein the therapeutic protein is at a concentration in mg/mL selected from the group consisting of 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, and 220, and every increment therein.

Figure 1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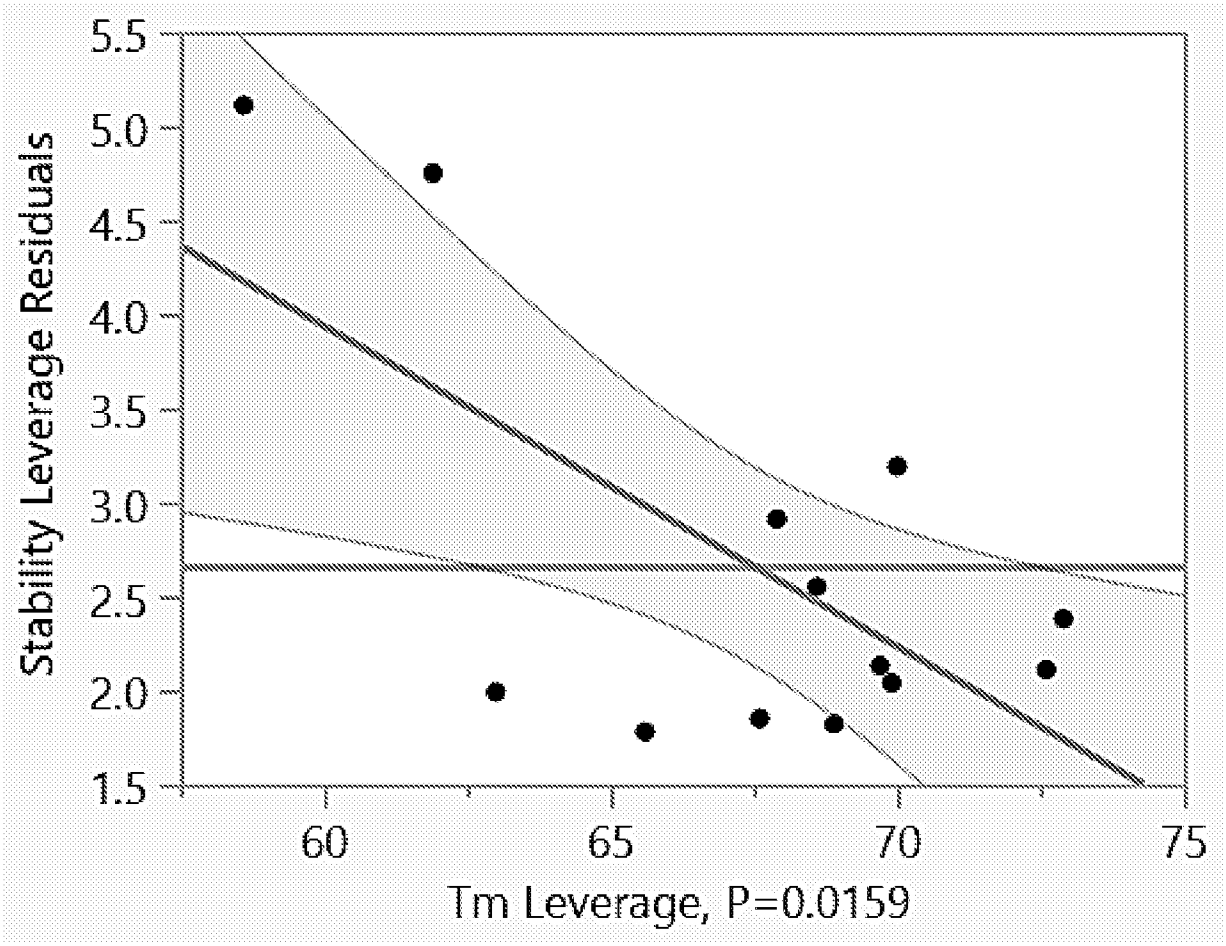
Not Significant
Confidence curve crosses horizontal line.
Confidence curve is asymptotic to horizontal line.
Confidence curve does not cross horizontal line.

Figure 2


Stability Leverage Residuals
5.5
5.0
4.5
4.0
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2.0
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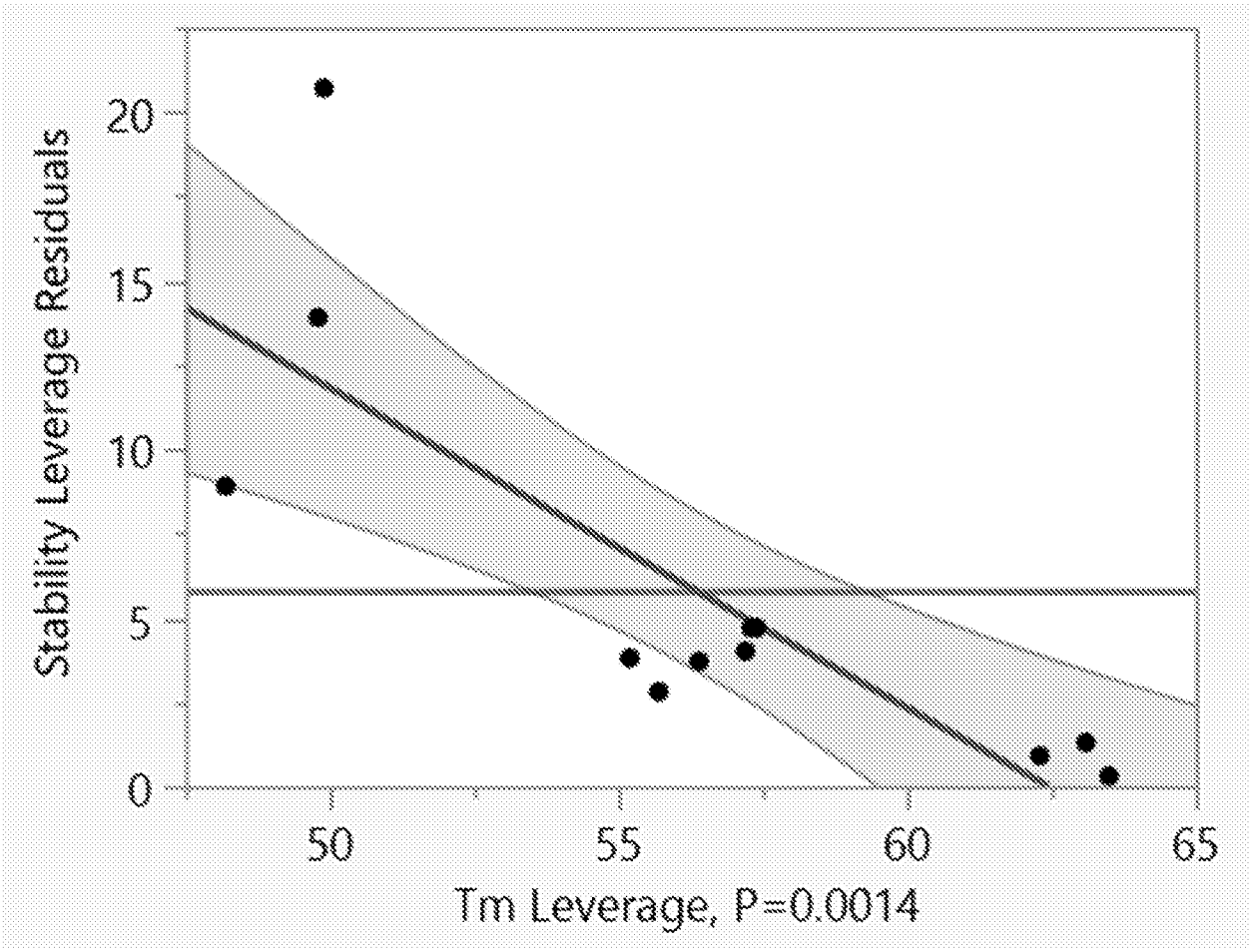
Tm Leverage, P=0.0159

Figure 3


Stability Leverage Residuals
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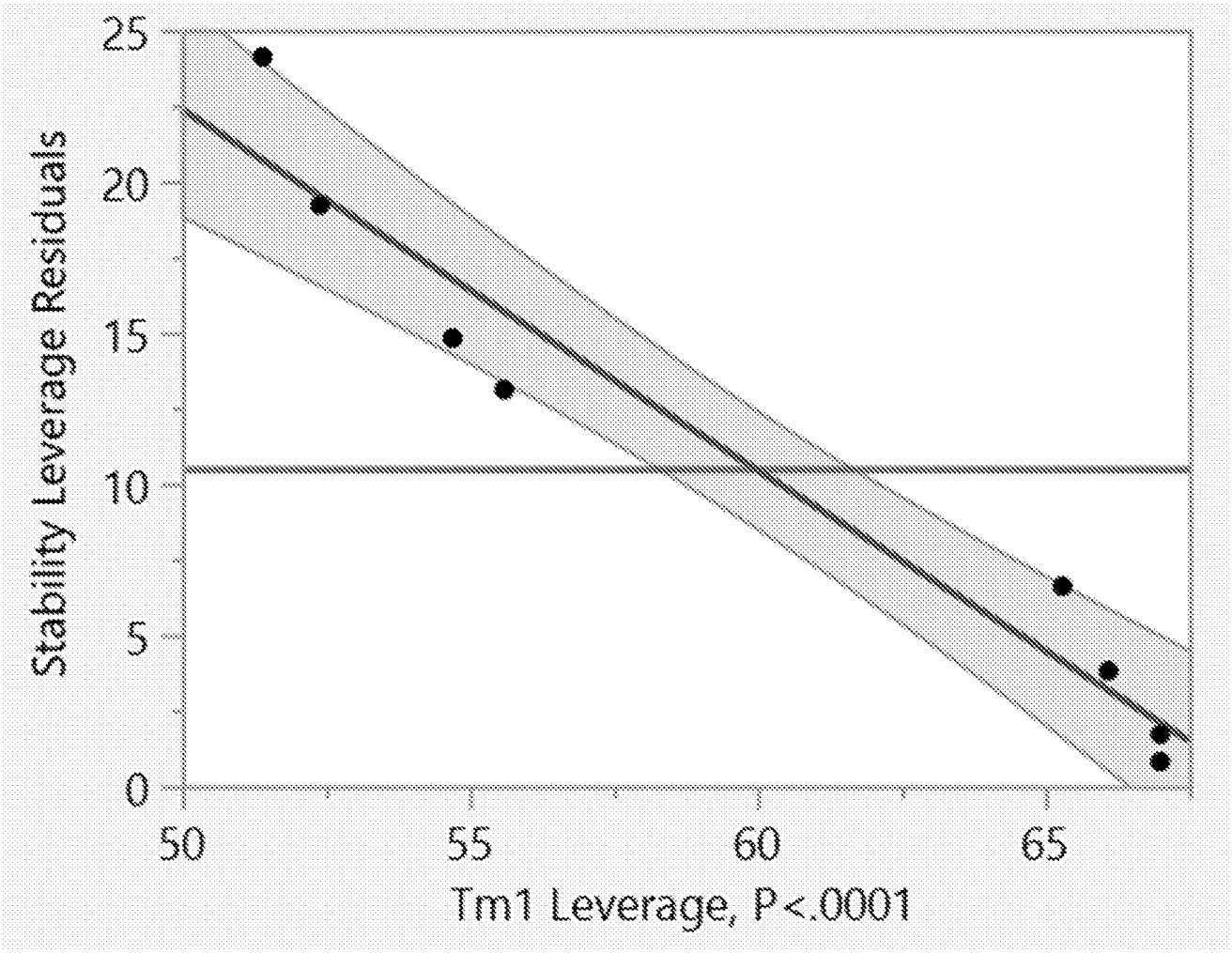
15
10
5
0
50
55
60
65
Tm Leverage, P=0.0014

Figure 4


Stability Leverage Residuals
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20
15
10
5
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50
55
60
65
Tm1 Leverage, P<.0001

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/018847

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 G01N21/64
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)
G01N

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/135427 A1 (HAYS ANNA-MARIA [US] ET AL) 22 June 2006 (2006-06-22) paragraphs [0400], [0406]; figure 5; example 2; tables 13,33-74	1-17

-/--

[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

"E" earlier application or patent but published on or after the international filing date

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

"P" document published prior to the international filing date but later than the priority date claimed

"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2020	04/06/2020

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	Gundlach, Björn

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/018847

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BANKS DOUGLAS D ET AL: "Removal of cysteinylation from an unpaired sulfhydryl in the variable region of a recombinant monoclonal IgG1 antibody improves homogeneity, stability, and biological activity", JOURNAL OF PHARMACEUTICAL SCIENCES, AMERICAN CHEMICAL SOCIETY AND AMERICAN PHARMACEUTICAL ASSOCIATION, US, vol. 97, no. 2, 1 February 2008 (2008-02-01), pages 775-790, XP002539396, ISSN: 0022-3549, DOI: 10.1002/JPS.21014 abstract; figure 4 page 767, column 1, paragraph 2 - column 2, paragraph 1 page 768, column 2	1-17
Y	US 2013/011378 A1 (YANG TZUNG-HORNG [US] ET AL) 10 January 2013 (2013-01-10) abstract; table 22 paragraph [0732] - paragraph [0735]	1-17
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