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DESCRIPTION

Background of the Invention

Field of the Invention

[0001] This invention pertains to concentrated protein formulations with reduced viscosity, which are particularly suitable for subcutaneous administration. The invention further concerns a method of reducing viscosity of concentrated protein formulations.

Description of the Related Art

[0002] In the past ten years, advances in biotechnology have made it possible to produce a variety of proteins for pharmaceutical applications using recombinant DNA techniques. Because proteins are larger and more complex than traditional organic and inorganic drugs (*i.e.* possessing multiple functional groups in addition to complex three-dimensional structures), the formulation of such proteins poses special problems. One of the problems is the elevated viscosity values of protein formulations, especially at high concentration. The delivery of high protein concentration is often required for subcutaneous administration due to the volume limitations (≤ 1.5 ml) and dose requirements (usually ≥ 50 mg, preferable ≥ 100 mg). For example, if a protein is to be administered to patients at 2 mg/kg on a weekly basis, the average weekly dose will be 130 mg considering 65 kg as the average weight of patients. Since injection volumes of more than 1.5 ml are not well tolerated for subcutaneous administration, the protein concentration for a weekly subcutaneous administration would have to be approximately 100 mg/ml (130 mg protein in less than 1.5 ml volume). However, highly concentrated protein formulations pose several problems. One problem is the tendency of proteins to form particulates during processing and/or storage, which makes manipulation during further processing difficult. In the case of reconstituted liquid formulations, this is usually circumvented by adding a suitable surfactant (*e.g.* a polysorbate) during lyophilization or after lyophilization while reconstituting the formulation. Although surfactants have been shown to significantly reduce the degree of particulate formation of proteins, they do not address another problem associated with manipulating and administering concentrated protein formulations. Proteins tend to form viscous solutions at high concentration because of their macromolecular nature and potential for intermolecular interactions. Moreover, many proteins are often lyophilized in the presence of large amounts of lyoprotectants, such as sugar to maintain their stability. The sugar can enhance the intermolecular interactions and increase the viscosity. Highly viscous formulations are difficult to manufacture, draw into a syringe and inject subcutaneously. The use of force in manipulating the viscous formulations leads to excessive frothing, and the resultant detergent-like action of froth has the potential to denature and inactivate the therapeutically active protein. Moreover, viscous solution increases the back-

pressure during UF/DF process and makes recovery of protein difficult. This can result in considerable loss of protein product. Satisfactory solution of this problem is lacking in the prior art. Therefore, there is a need to develop a method of reducing the viscosity of a formulation containing high concentration of protein.

[0003] Stable isotonic lyophilized protein formulations are disclosed in PCT publication WO 97/04801, published on February 13, 1997. The disclosed lyophilized formulations can be reconstituted to generate high protein-concentration liquid formulations without apparent loss of stability. However, the potential issues associated with the high viscosity of the reconstituted formulations are not addressed.

[0004] Applicants have discovered that the preparation of proteinaceous, lyophilized formulation with 100 mM NaCl diluent can result in a slightly hypertonic solution. It had been previously believed that pharmaceutical formulations must be maintained at physiological pH and be isotonic. This belief was based at least in part on the perception that the administration of hypertonic formulation could lead to dehydration and therefore could damage the tissue at the site of injection. However, the belief of the requirement for absolute isotonicity of a pharmaceutical formulation may not be well-founded. For example, Zietkiewicz et al., Grzyby Drozdopodobne 23: 869-870 (1971) have shown that absolute isotonicity of the drugs is not necessary. It was found to be sufficient to avoid the drug solutions that exceed the critical limits of hypertonicity. For example, tissue damage was observed only when hypertonic solution of 1300 mOsmol/Kg (~650 mM NaCl) or higher was administered subcutaneously or intramuscularly to experimental animals. As a result, formulations which are slightly hypertonic, or outside of the physiological pH range do not appear to present a risk of tissue damage at the site of administration.

[0005] EP 0 661060 describes concentrated immunoglobulin preparations having low osmolarity and low viscosity.

[0006] WO 97/04-801 describes stable isotonic lyophilized antibody formulations.

[0007] Applicants have further found that proteinaceous solutions having a lowered (4.0-5.3) or elevated (6.5-12.0) pH was also effective at reducing the viscosity of high concentration protein formulations.

[0008] The present invention is directed to providing a high concentration immunoglobulin formulation with reduced viscosity, which is easy to handle and is suitable for subcutaneous administration. The present invention is further directed to providing a method of reducing viscosity of concentrated immunoglobulin formulations.

Summary of the Invention

[0009] The present invention concerns a method of lowering the viscosity of concentrated

immunoglobulin composition by: increasing the total ionic strength of the formulation through the addition of salts or buffer components as defined in the claims without significantly compromising stability or biological activity. Accordingly, the invention concerns methods and means for reducing the viscosity of concentrated immunoglobulin formulations, primarily to ensure easy manipulation before and during administration to a patient.

[0010] The present invention is defined in the claims. In one aspect, the present invention provides a stable liquid formulation comprising an immunoglobulin in an amount of at least about 80 mg/ml and a salt and/or a buffer in an amount of at least 100 mM, and having a kinematic viscosity of about 50 mm²/s or less at 25°C, wherein the immunoglobulin is the antibody rhuMAb-E25, rhuMAb-E26, or rhuMAb-E27, and wherein said salt is not sodium chloride, as set out in the claims. The salts and/or buffers are pharmaceutically acceptable and are derived from various known acids (inorganic and organic) or base forming metals and amines. Alternatively, the salts and/or buffers may be derived from amino acids. In a specific aspect, the salts are chosen from the group consisting of arginine hydrochloride, sodium thiocyanate, ammonium thiocyanate, ammonium sulfate, ammonium chloride, calcium chloride, zinc chloride and sodium acetate. In another aspect, the salts or buffers are monovalent. In yet another aspect, the formulation contains the above salt or buffer components in an amount of about 100-200 mM, and has a viscosity of about 2 to 30 mm²/s. In another particular embodiment, the formulation is hypertonic. In yet another particular aspect, the formulation may further comprise a surfactant such as polysorbate. The invention also contemplates a reconstituted formulation that further comprises a lyoprotectant such as sugars. In yet another particular aspect, the lyoprotectant sugar can be, for example, sucrose or trehalose, and may be present in an amount of about 60-300 mM. In another specific aspect, the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

[0011] The invention provides a formulation containing high concentrations of rhuMAbE-25, rhuMAbE-26 or rhuMAbE-27 as set out in the claims. These antibodies are described in WO 99/1556.

[0012] The formulations of the present invention may be pharmaceutical formulations, in particular, formulations for subcutaneous administration.

[0013] In another aspect, the present invention provides a method of reducing the kinematic viscosity of a formulation containing an immunoglobulin in an amount of at least 80 mg/ml, comprising the addition of a salt and/or buffer in an amount of at least 100 mM, wherein the immunoglobulin is the antibody rhuMAb-E25, rhuMAb-E26, or rhuMAb-E27, and wherein said salt is not sodium chloride, as set out in the claims. In a specific aspect the viscosity is reduced to 50 mm²/s or less at 25°C. In a specific aspect, the viscosity is reduced to about 2 to 30 mm²/s. In another specific aspect, the salts or buffer components may be added in an amount of at least about 100 mM, preferably about 100-200 mM, most preferably about 150 mM. The salts and/or buffers are pharmaceutically acceptable and are derived from various known acids

(inorganic and organic) with "base forming" metals or amines. Alternatively, the salts and/or buffers may be derived from amino acids. In yet another specific aspect, the salts and/or buffers are monovalent. In yet another specific aspect, the salts are selected from the group consisting of arginine hydrochloride, sodium thiocyanate, ammonium thiocyanate, ammonium sulfate, ammonium chloride, calcium chloride, zinc chloride and sodium acetate. In yet another aspect, the formulation contains the above salt or buffer components in an amount of about 100-200 mM, and has a viscosity of about 2 to 30 mm²/s. In yet another aspect, the immunoglobulin in the formulation has a molecular weight of at least about 15-20 kD. In another particular embodiment, the formulation may further comprise a surfactant such as polysorbate. The invention also contemplates a reconstituted formulation that further comprises a lyoprotectant such as a sugar. In a particular aspect, the lyoprotectant sugar can be, for example, sucrose or trehalose, and may be present in an amount of about 60-300 mM. In a specific aspect, the formulation can be reconstituted with a diluent comprising the buffer or salt. In a preferred embodiment, the protein concentration in the reconstituted formulation is about 2-40 times greater than the protein concentration in the mixture before lyophilization.

[0014] The invention provides a method of reducing the viscosity of an immunoglobulin formulation as set out in the claims.

In a specific aspect, the method is used to prepare a reconstitutable formulation, specially those that are concentrated to a much greater concentration of immunoglobulin (e.g., 2-40 times) after the concentration step (e.g., lyophilization) compared to before.

[0015] Such formulations are particularly useful for subcutaneous administration.

[0016] Also provided is an article of manufacture comprising a container enclosing a formulation as defined in the claims.

[0017] Also described herein is a method of preventing self-association of proteins in concentrated liquid formulations by (1) adding a salt or a buffer component in an amount of at least about 50 mM; or (2) altering the pH by lowering to (≈ 4.0 to ≈ 5.3) or elevating to (≈ 6.5 to ≈ 12.0). In a specific aspect, the self-association to be prevented is that induced or exacerbated by the presence of sugars (e.g., sucrose or trehalose) that are commonly used as lyoprotectants. Accordingly, this method is particularly useful for preventing self-association of reconstituted lyophilized formulations.

Brief Description of the Drawings

[0018]

Fig. 1 shows the effects of protein concentration on viscosity of reconstituted formulation containing the anti-IgE antibody rhuMAb E25, 16 mM histidine, 266 mM sucrose and 0.03% Polysorbate 20 at 25°C.

Fig. 2 depicts the effects of NaCl concentration on viscosity of reconstituted formulation containing 125 mg/ml of the antibody IgE antibody rhuMAb E25, 16 mM histidine, 266 mM sucrose, 0.03% Polysorbate 20 and various amounts of NaCl at 25 °C.

Fig. 3 shows the effects of various salts on viscosity of reconstituted formulation containing 40 mg/ml of the anti-IgE antibody rhuMAb E25, 10 mM histidine, 250 mM sucrose, 0.01 % Polysorbate 20 and various amounts of salts at 25 °C.

Fig. 4 shows the effects of buffer concentration on viscosity of a liquid formulation containing 80 mg/ml of the anti-IgE antibody rhuMAb E25, 50 mM histidine, 150 mM trehalose, 0.05 % Polysorbate 20 and various amounts of histidine, acetate, or succinate components at 25 °C.

Fig. 5 shows the effects of NaCl concentration on viscosity of a reconstituted formulation containing 21 mg/ml rhuMAb E26, 5 mM histidine, 275 mM sucrose at 6°C.

Fig. 6 shows the effects of pH on viscosity of liquid formulations containing 130 mg/ml rhuMAb E25, 2-17.5 mM of acetate or arginine with and without 150 mM NaCl at 25°C.

Fig. 7 shows the effects of pH on viscosity of reconstituted lyophilized formulations containing 94 mg/ml rhuMAb E25, 250 mM trehalose, 20 mM histidine, at 25°C.

Detailed Description of the Preferred Embodiment

I. Definitions

[0019] By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. Thus, proteins are distinguished from "peptides" which are also amino acid - based molecules that do not have such structure. Typically, an immunoglobulin for use herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD.

[0020] Examples of proteins include mammalian proteins, such as, *e.g.*, growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α -1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIc, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA, *e.g.*, Activase®, TNKase®, Retevase®); bombazine; thrombin; tumor necrosis factor- α and - β ; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1- α); serum albumin such as human serum

albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , any - γ , colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides.

[0021] The immunoglobulin which is formulated is preferably essentially pure and desirably essentially homogeneous (*i.e.* free from contaminating proteins). "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

[0022] The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv).

[0023] The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains. An IgM antibody consists of 5 of the basic heterotetramer unit along with an additional polypeptide called a J chain, and contains 10 antigen binding sites, while IgA antibodies comprise from 2-5 of the basic 4-chain units which can polymerize to form polyvalent assemblages in combination with the J chain. In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an 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. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (V_H) followed by three constant domains (C_H) for each of the α and γ chains and four C_H domains for μ and ϵ isotypes. Each L chain has at the N-terminus, a variable domain (V_L) followed by a constant domain at its other end. The V_L is aligned with the V_H and the C_L is aligned with the first constant domain of the heavy chain (C_H1). Particular amino acid residues

are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a V_H and V_L together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see e.g., Basic and Clinical Immunology, 8th Edition, Daniel P. Sties, Abba I. Terr and Tristram G. Parslow (eds), Appleton & Lange, Norwalk, CT, 1994, page 71 and Chapter 6.

[0024] The L chain from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains (CH), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, having heavy chains designated α , δ , ϵ , γ and μ , respectively. The γ and μ classes are further divided into subclasses on the basis of relatively minor differences in the CH sequence and function, e.g., humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2.

[0025] The term "variable" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines the specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the entire span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of about 15-30 amino acid residues separated by shorter regions of extreme variability called "hypervariable regions" or sometimes "complementarity determining regions" (CDRs) that are each approximately 9-12 amino acid residues in length. The variable domains of native heavy and light chains each comprise four FRs, 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 FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody dependent cellular cytotoxicity (ADCC).

[0026] The term "hypervariable region" (also known as "complementarity determining regions" or CDRs) when used herein refers to the amino acid residues of an antibody which are (usually three or four short regions of extreme sequence variability) within the V-region domain of an immunoglobulin which form the antigen-binding site and are the main determinants of antigen specificity. There are at least two methods for identifying the CDR residues: (1) An approach based on cross-species sequence variability (i.e., Kabat et al., Sequences of Proteins of Immunological Interest (National Institute of Health, Bethesda, MS 1991); and (2) An approach based on crystallographic studies of antigen-antibody complexes (Chothia, C. et al., J. Mol. Biol. 196: 901-917 (1987)). However, to the extent that two residue identification techniques define regions of overlapping, but not identical regions, they can be combined to define a hybrid CDR.

[0027] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

[0028] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

[0029] An "intact" antibody is one which comprises an antigen-binding site as well as a CL and at least the heavy chain domains, C_H1, C_H2 and C_H3.

[0030] An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂ and Fv fragments; diabodies; linear antibodies (see U.S. Patent 5,641,870, Example 2; Zapata et al., *Protein Eng.* 8(10): 1057-1062 [1995]); single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

[0031] Papain digestion of antibodies produced two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (V_H), and the first constant domain of one heavy chain (C_H1). Each Fab fragment is monovalent with respect to antigen binding, *i.e.*, it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large F(ab')₂ fragment which roughly corresponds to two disulfide linked Fab fragments having different antigen-binding activity and

is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having a few additional residues at the carboxy terminus of the C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0032] The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, the region which is also recognized by Fc receptors (FcR) found on certain types of cells.

[0033] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0034] "Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the V_H and V_L antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of the sFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0035] The term "diabodies" refers to small antibody fragments prepared by constructing sFv fragments (see preceding paragraph) with short linkers (about 5-10) residues) between the V_H and V_L domains such that inter-chain but not intra-chain pairing of the V domains is achieved, thereby resulting in a bivalent fragment, *i.e.*, a fragment having two antigen-binding sites. Bispecific diabodies are heterodimers of two "crossover" sFv fragments in which the V_H and V_L domains of the two antibodies are present on different polypeptide chains. Diabodies are described in greater detail in, for example, EP 404,097; WO 93/11161; Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993).

[0036] An antibody that "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide is one that binds to that particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

[0037] "Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric

immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) of mostly human sequences, which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (also CDR) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, "humanized antibodies" as used herein may also comprise residues which are found neither in the recipient antibody nor the donor antibody. These modifications are made to further refine and optimize antibody performance. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

[0038] Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g., B cell receptors); and B cell activation.

[0039] "Antibody-dependent cell-mediated cytotoxicity" or ADCC refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., natural killer (NK) cells, neutrophils and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are required for killing of the target cell by this mechanism. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. Fc expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9: 457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ACDD assay, such as that described in U.S. Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in an animal model such as that disclosed in Clynes et al., *PNAS USA* 95:652-656 (1998).

[0040] "Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors, FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic

domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see M. Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9: 457-92 (1991); Capel et al., *Immunomethods* 4: 25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126: 330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus. Guyer et al., *J. Immunol.* 117: 587 (1976) and Kim et al., *J. Immunol.* 24: 249 (1994).

[0041] "Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils, with PBMCs and MNK cells being preferred. The effector cells may be isolated from a native source, *e.g.*, blood.

[0042] "Complement dependent cytotoxicity" of "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, *e.g.*, as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202: 163 (1996), may be performed.

[0043] A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. *Adv. Drug Delivery Rev.* 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40°C for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8°C, generally the formulation should be stable at 30°C or 40°C for at least 1 month and/or stable at 2-8°C for at least 2 years. Where the formulation is to be stored at 30°C, generally the formulation should be stable for at least 2 years at 30°C and/or stable at 40°C for at least 6 months. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. Thus, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein are present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8°C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

[0044] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulations in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation is suitable for administration (e.g. parenteral administration) to a patient to be treated with the immunoglobulin of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

[0045] An "isotonic" formulation is one which has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. The term "hypotonic" describes a formulation with an osmotic pressure below that of human blood. Correspondingly, the term "hypertonic" is used to describe a formulation with an osmotic pressure above that of human blood. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example. The formulations of the present invention are hypertonic as a result of the addition of salt and/or buffer.

[0046] A "pharmaceutically acceptable acid" includes inorganic and organic acids which are non toxic at the concentration and manner in which they are formulated. For example, suitable inorganic acids include hydrochloric, perchloric, hydrobromic, hydroiodic, nitric, sulfuric, sulfonic, sulfinic, sulfanilic, phosphoric, carbonic, etc. Suitable organic acids include straight and branched-chain alkyl, aromatic, cyclic, cycloaliphatic, arylaliphatic, heterocyclic, saturated, unsaturated, mono, di- and tri-carboxylic, including for example, formic, acetic, 2-hydroxyacetic, trifluoroacetic, phenylacetic, trimethylacetic, t-butyl acetic, anthranilic, propanoic, 2-hydroxypropanoic, 2-oxopropanoic, propandioic, cyclopentane-propionic, cyclopentane propionic, 3-phenylpropionic, butanoic, butandioic, benzoic, 3-(4-hydroxybenzoyl)benzoic, 2-acetoxy-benzoic, ascorbic, cinnamic, lauryl sulfuric, stearic, muconic, mandelic, succinic, embonic, fumaric, malic, maleic, hydroxymaleic, malonic, lactic, citric, tartaric, glycolic, glyconic; gluconic, pyruvic, glyoxalic, oxalic, mesylic, succinic, salicylic, phthalic, palmoic, palmeic, thiocyanic, methanesulphonic, ethanesulphonic, 1,2-ethanedisulfonic, 2-hydroxyethanesulfonic, benzenesulphonic, 4-chlorobenzenesulfonic, naphthalene-2-sulphonic, p-toluenesulphonic, camphorsulphonic, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic, glucoheptonic, 4,4'-methylenebis-3-(hydroxy-2-ene-1-carboxylic acid), hydroxynapthoic.

[0047] "Pharmaceutically-acceptable bases" include inorganic and organic bases were are non-toxic at the concentration and manner in which they are formulated. For example, suitable bases include those formed from inorganic base forming metals such as lithium, sodium, potassium, magnesium, calcium, ammonium, iron, zinc, copper, manganese, aluminum, N-methylglucamine, morpholine, piperidine and organic nontoxic bases including, primary, secondary and tertiary amine, substituted amines, cyclic amines and basic ion exchange resins, [e.g., $N(R')_4^+$ (where R' is independently H or C_{1-4} alkyl, e.g., ammonium, Tris)], for example, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine

resins and the like. Particularly preferred organic non-toxic bases are isopropylamine, diethylamine, ethanolamine, trimethamine, dicyclohexylamine, choline, and caffeine.

[0048] Additional pharmaceutically acceptable acids and bases useable with the present invention include those which are derived from the amino acids, for example, histidine, glycine, phenylalanine, aspartic acid, glutamic acid, lysine and asparagine.

[0049] "Pharmaceutically acceptable" buffers and salts include those derived from both acid and base addition salts of the above indicated acids and bases. Specific buffers and or salts include histidine, succinate and acetate.

[0050] A "lyoprotectant" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars and their corresponding sugar alcohols; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher molecular weight sugar alcohols, e.g. glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Plurionics®; and combinations thereof. Additional exemplary lyoprotectants include glycerin and gelatin, and the sugars mellibiose, melezitose, raffinose, mannotriose and stachyose. Examples of reducing sugars include glucose, maltose, lactose, maltulose, iso-maltulose and lactulose. Examples of non-reducing sugars include non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Preferred sugar alcohols are monoglycosides, especially those compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic side group can be either glucosidic or galactosidic. Additional examples of sugar alcohols are glucitol, maltitol, lactitol and iso-maltulose. The preferred lyoprotectant are the non-reducing sugars trehalose or sucrose.

[0051] In preparing the reduced viscosity formulations of the invention, care should be taken using the above enumerated excipients as well as other additives, especially when added at high concentration, so as to not increase the viscosity of the formulation.

[0052] The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the protein in the presence of the lyoprotecting amount of the lyoprotectant, the protein essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0053] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

[0054] A "preservative" is a compound which can be added to the formulations herein to reduce bacterial action. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and *m*-cresol. The most preferred preservative herein is benzyl alcohol.

[0055] A "bulking agent" is a compound which adds mass to a lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and sorbitol. The liquid formulations of the present invention obtained by reconstitution of a lyophilized formulation may contain such bulking agents.

[0056] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

[0057] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, rabbits, cattle, pigs, hamsters, mice, cats, *etc.* Preferably, the mammal is human.

[0058] A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

[0059] A "therapeutically effective amount" is at least the minimum concentration required to effect a measurable improvement or prevention of a particular disorder. Therapeutically effective amounts of known proteins are well known in the art, while the effective amounts of proteins hereinafter discovered may be determined by standard techniques which are well within the skill of a skilled artisan, such as an ordinary physician.

[0060] "Viscosity" as used herein may be "kinematic viscosity" or "absolute viscosity." "Kinematic viscosity" is a measure of the resistive flow of a fluid under the influence of gravity. When two fluids of equal volume are placed in identical capillary viscometers and allowed to flow by gravity, a viscous fluid takes longer than a less viscous fluid to flow through the capillary. If one fluid takes 200 seconds to complete its flow and another fluid takes 400 seconds, the second fluid is twice as viscous as the first on a kinematic viscosity scale. "Absolute viscosity", sometimes called dynamic or simple viscosity, is the product of kinematic viscosity and fluid density:

$$\text{Absolute Viscosity} = \text{Kinematic Viscosity} \times \text{Density}$$

Absolute viscosity = Kinematic viscosity x Density

The dimension of kinematic viscosity is L^2/T where L is a length and T is a time. Commonly, kinematic viscosity is expressed in centistokes (cSt). The SI unit of kinematic viscosity is mm^2/s , which is 1 cSt. Absolute viscosity is expressed in units of centipoise (cP). The SI unit of absolute viscosity is the milliPascal-second (mPa-s), where 1 cP = 1 mPa-s.

II. Modes for Carrying out the Invention

A. Protein Preparation

[0061] The immunoglobulin protein to be formulated may be produced by any known technique, such as by culturing cells transformed or transfected with a vector containing nucleic acid encoding the protein, as is well known in art, or through synthetic techniques (such as recombinant techniques and peptide synthesis or a combination of these techniques) or may be isolated from an endogenous source of the protein.

[0062] Preparation of the immunoglobulin protein to be formulated by the method of the invention by recombinant means may be accomplished by transfecting or transforming suitable host cells with expression or cloning vectors and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in *Mammalian Cell Biotechnology: A Practical Approach*, M. Butter, Ed. (IRL Press, 1991) and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Press. Methods of transfection are known to the ordinarily skilled artisan, and include for example, CaPO_4 and CaCl_2 transfection, electroporation, microinjection, etc. Suitable techniques are also described in Sambrook *et al.*, *supra*. Additional transfection techniques are described in Shaw et al., *Gene* 23: 315 (1983); WO 89/05859; Graham et al., *Virology* 52: 456-457 (1978) and U.S.P. 4,399,216.

[0063] The nucleic acid encoding the desired immunoglobulin protein for formulation according to the present method may be inserted into a replicable vector for cloning or expression. Suitable vectors are publicly available and may take the form of a plasmid, cosmid, viral particle or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, and enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

[0064] Forms of immunoglobulin the protein to be formulated may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent or through enzymatic cleavage. Cells employed for expression can also be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption or cell lysing agents.

[0065] Purification of the immunoglobulin protein to be formulated may be effected by any suitable technique known in the art, such as for example, fractionation on an ion-exchange column, ethanol precipitation, reverse phase HPLC, chromatography on silica or cation-exchange resin (e.g., DEAE), chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, gel filtration using protein A Sepharose columns (e.g., Sephadex® G-75) to remove contaminants such as IgG, and metal chelating columns to bind epitope-tagged forms.

B. Antibody preparation

(i) Polyclonal antibodies.

[0066] Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

[0067] One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies.

[0068] Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for

possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

[0069] For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

[0070] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

[0071] The immunizing agent will typically include the protein to be formulated. Generally either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, *Monoclonal antibodies: Principles and Practice*, Academic Press (1986), pp. 59-103. Immortalized cell lines are usually transformed mammalian cell, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0072] Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0073] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or

by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

[0074] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

[0075] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

[0076] The immunizing agent will typically include the epitope protein to which the antibody binds. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press (1986), pp. 59-103.

[0077] Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[0078] Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, Marcel Dekker, Inc., New York, (1987) pp. 51-63.

[0079] The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against the protein to be formulated. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be

determined by the Scatchard analysis of Munson and Pollard, *Anal. Biochem.*, 107:220 (1980).

[0080] After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Goding, *supra*. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

[0081] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0082] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthum, *Immunol. Revs.* 130:151-188 (1992).

[0083] In a further embodiment, antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Biol/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

[0084] The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

[0085] Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having

specificity for a different antigen.

[0086] Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

(iii) Humanized and human antibodies.

[0087] The antibodies subject to the formulation method may further comprise humanized or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domain, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Jones et al., Nature 321: 522-525 (1986); Riechmann et al., Nature 332: 323-329 (1988) and Presta, Curr. Opin. Struct. Biol. 2: 593-596 (1992).

[0088] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers, Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science 239:1534-1536 (1988), or through substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0089] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody. Sims et al., J. Immunol., 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 (1987). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies. Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993).

[0090] It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[0091] Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991)).

[0092] Human antibodies can also be produced using various techniques known in the art, including phage display libraries. Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991); Marks et al., J. Mol. Biol. 222: 581 (1991). The techniques of Cole *et al.*, and Boerner *et al.*, are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol. 147(1): 86-95 (1991). Similarly, human antibodies can be made by introducing human

immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resemble that seen in human in all respects, including gene rearrangement, assembly and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806, 5,569,825, 5,625,126, 5,633,425, 5,661,016 and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-13 (1994), Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996), Neuberger, *Nature Biotechnology* 14: 826 (1996) and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

(iv) Antibody Dependent Enzyme-Mediated Prodrug Therapy (ADEPT)

[0093] The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g. a peptidyl chemotherapeutic agent, see WO 81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U. S. Patent No. 4,975,278.

[0094] The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

[0095] Enzymes that are useful in the method of this invention include, but are not limited to, glycosidase, glucose oxidase, human lysozyme, human glucuronidase, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases (e.g., carboxypeptidase G2 and carboxypeptidase A) and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β -lactamase useful for converting drugs derivatized with β -lactams into free drugs; and penicillin amidases, such as penicillin Vamidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes" can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

[0096] The enzymes of this invention can be covalently bound to the anti-IL-17 or anti-LIF antibodies by techniques well known in the art such as the use of the heterobifunctional cross-linking agents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of the antibody of the invention linked to at least a functionally active portion of

an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g. Neuberger et al., Nature 312: 604-608 (1984)).

(iv) Bispecific and polyspecific antibodies

[0097] Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different epitopes. Such antibodies can be derived from full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies).

[0098] Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities. Millstein et al., Nature, 305:537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

[0099] Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions, and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. For further details of generating bispecific antibodies, see, for example, Suresh et al., Methods in Enzymology 121: 210 (1986).

[0100] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0101] According to another approach described in WO 96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 region of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chains(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0102] In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690, published March 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

[0103] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

[0104] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. For example, Fab' fragments recovered from *E. coli* can be chemically coupled *in vitro* to form bivalent antibodies. See, Shalaby et al., *J. Exp. Med.*, 175:217-225 (1992).

[0105] Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies). Techniques for generating bispecific antibodies from antibody fragments have been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB

derivatives is then reconverted to the Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

[0106] Fab' fragments may be directly recovered from *E. coli* and chemically coupled to form bispecific antibodies. Shalaby et al., J. Exp. Med. 175: 217-225 (1992) describes the production of fully humanized bispecific antibody F(ab')₂ molecules. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

[0107] Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelny et al., J. Immunol., 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993) has provided an alternative mechanism for making bispecific/bivalent antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific/bivalent antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

[0108] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., J. Immunol. 147: 60 (1991).

[0109] Exemplary bispecific antibodies may bind to two different epitopes on a given molecule. Alternatively, an anti-protein arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.*, CD2, CD3, CD28 or B7), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the cell expressing the particular protein. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express a particular protein. Such antibodies possess a protein-binding arm and an arm which binds a cytotoxic agent or a radionuclide chelator, such as EOTUBE, DPTA, DOTA or TETA. Another bispecific antibody of interest binds the protein of interest and further binds tissue factor (TF).

(v) Heteroconjugate Antibodies

[0110] Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example; been proposed to target immune system cells to unwanted cells, U.S.P. 4,676,980, and for treatment of HIV infection. WO 91/00360, WO 92/200373 and EP 03089. It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

B. Preparation of Lyophilized Formulations

[0111] Although the formulations herein are not limited to reconstituted lyophilized formulations, in a particular embodiment, the immunoglobulin proteins are lyophilized and then reconstituted to produce the reduced-viscosity stable liquid formulations of the invention. In this particular embodiment, after preparation of the immunoglobulin protein of interest as described above, a "pre-lyophilized formulation" is produced. The amount of immunoglobulin protein present in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration *etc.* For example, the starting concentration of an intact antibody can be from about 2 mg/ml to about 50 mg/ml, preferably from about 5 mg/ml to about 40 mg/ml and most preferably from about 20-30 mg/ml.

[0112] The immunoglobulin protein to be formulated is generally present in solution. For example, in the elevated ionic strength reduced viscosity formulations of the invention, the protein may be present in a pH-buffered solution at a pH from about 4-8, and preferably from about 5-7. The buffer concentration can be from about 1 mM to about 20 mM, alternatively from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired tonicity of the formulation (e.g. of the reconstituted formulation). Exemplary buffers and/or salts are those which are pharmaceutically acceptable and may be created from suitable acids, bases and salts thereof, such as those which are defined under "pharmaceutically acceptable" acids, bases or buffers.

[0113] In one embodiment, a lyoprotectant is added to the pre-lyophilized formulation. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. However, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, alternatively from about 30 mM to about 300 mM, alternatively from about 50 mM to about 100 mM. Exemplary lyoprotectants include sugars and sugar alcohols such as sucrose, mannose, trehalose, glucose, sorbitol, mannitol. However, under particular circumstances, certain lyoprotectants may also contribute to an

increase in viscosity of the formulation. As such, care should be taken so as to select particular lyoprotectants which minimize or neutralize this effect. Additional lyoprotectants are described above under the definition of "lyoprotectants".

[0114] The ratio of protein to lyoprotectant can vary for each particular immunoglobulin protein or antibody and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (e.g., sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectant to antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about 200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

[0115] In a preferred embodiment, it may be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20 or 80); polyoxamers (e.g. poloxamer 188); Triton; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (e.g. lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUA™ series (Mona Industries, Inc., Paterson, New Jersey), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronic, PF68 *etc.*). The amount of surfactant added is such that it reduces particulate formation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in the pre-lyophilized formulation in an amount from about 0.001-0.5%, alternatively from about 0.005-0.05%.

[0116] A mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (e.g. mannitol or glycine) may be used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein *etc.* Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

[0117] The formulation herein may also contain more than one immunoglobulin protein as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the other protein. For example, it may be desirable to

provide two or more antibodies which bind to the HER2 receptor or IgE in a single formulation. Such proteins are suitably present in combination in amounts that are effective for the purpose intended.

[0118] The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120°C for about 30 minutes, for example.

[0119] After the immunoglobulin protein, optional lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50™ (Hull, USA) or GT20™ (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about -30 to 25°C (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (e.g. 40-60 hrs). Optionally, a secondary drying stage may also be performed depending upon the desired residual moisture level in the product. The temperature at which the secondary drying is carried out ranges from about 0-40°C, depending primarily on the type and size of container and the type of protein employed. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15-30°C (e.g., about 20°C). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, e.g., on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours (e.g. 10-15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

C. Reconstitution of a Lyophilized Formulation

[0120] Prior to administration to the patient, the lyophilized formulation is reconstituted with a pharmaceutically acceptable diluent such that the immunoglobulin protein concentration in the reconstituted formulation is at least about 80 mg/ml, for example from about 80 mg/ml to about 400 mg/ml, alternatively from about 80 mg/ml to about 300 mg/ml, alternatively from about 90 mg/ml to about 150 mg/ml. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the

protein concentration in the reconstituted formulation may be about 2-40 times, alternatively 3-10 times, alternatively 3-6 times (e.g. at least three fold or at least four fold) that of the pre-lyophilized formulation.

[0121] Reconstitution generally takes place at a temperature of about 25°C to ensure complete hydration, although other temperatures may be employed as desired. The time required for reconstitution will depend, e.g., on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFJ), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1-2.0% and preferably from about 0.5-1.5%, but most preferably about 1.0-1.2%.

[0122] Preferably, the reconstituted formulation has less than 6000 particles per vial which are $\geq 10 \mu\text{m}$ in size.

D. Administration of the Formulation

[0123] The formulations of the present invention, including but not limited to reconstituted formulations, may be administered to a mammal in need of treatment with the immunoglobulin protein, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[0124] As described herein the formulations may be administered to the mammal by subcutaneous (*i.e.* beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease™ and Genject™ devices); injector pens (such as the GenPen™), needleless devices (e.g. MediJector™ and BioJector™); and subcutaneous patch delivery systems.

[0125] The appropriate dosage ("therapeutically effective amount") of the immunoglobulin protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. The immunoglobulin protein is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The immunoglobulin protein may be administered as the

sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question.

[0126] Where the immunoglobulin protein of choice is an antibody, from about 0.1-20 mg/kg is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques.

[0127] Uses for an anti-IgE formulation (e.g., rhuMAbE-25, rhMAbE-26) include the treatment or prophylaxis of IgE-mediated allergic diseases, parasitic infections, interstitial cystitis and asthma, for example. Depending on the disease or disorder to be treated, a therapeutically effective amount (e.g. from about 1-15 mg/kg) of the anti-IgE antibody is administered to the patient.

E. Articles of Manufacture

[0128] In another embodiment of the invention, an article of manufacture is provided which contains the formulation as defined in the claims and preferably provides instructions for its use. The article of manufacture comprises a container. Suitable containers include, for example, bottles, vials (e.g. dual chamber vials), syringes (such as dual chamber syringes) and test tubes. The container may be formed from a variety of materials such as glass or plastic. The container holds the formulation as defined in the claims and the label on, or associated with, the container may indicate directions for use. The label may further indicate that the formulation is useful or intended for subcutaneous administration. The container holding the formulation may be a multi-use vial, which allows for repeat administrations (e.g. from 2-6 administrations) of the formulation. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

[0129] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. **Examples outside the scope of the claims are for reference.**

EXAMPLE 1

[0130] The effects of protein concentrations on the viscosity of a recombinant anti-IgE monoclonal antibody formulation (rhuMAb E25) were studied at 25°C. This antibody is a humanized anti-IgE monoclonal antibody that has been developed by Genentech Inc. as a potential therapeutic agent to treat allergic rhinitis and allergic asthma (Presta et al., J. Immunol. 151(5): 2623-2632 (1993)(PCT/US92/06860). The formulated rhuMAb E25 was formulated to a final concentration of 40 mg/ml, 85 mM Sucrose, 5mM Histidine, 0.01%

Polysorbate 20 and filled into 5 cc vials. The samples were then frozen from 5°C to -50°C in 45 minutes and followed by a sequential increase in the lyophilizer shelf temperature 10°C per hour from -50°C to 25°C. A drying step was conducted at a shelf temperature of 25°C and a chamber pressure of 50 mTorr for 39 hours. The lyophilized rhuMAb E25 was reconstituted with SWFI to produce a solution with rhuMAb E25 at 125 mg/ml, 266 mM sucrose, 16 mM histidine, 0.03% polysorbate 20.

[0131] The viscosity of reconstituted samples were measured in Cannon-Fenske Routine capillary viscometer (Industrial Research Glassware LTD). The samples were measured approximately at 8 ml with a glass pipette and loaded into a capillary viscometer of size 50 for liquid samples with kinematic viscosity ranging from 0.8 to 4 cs or size 200 for those ranging from 20 to 100 cs. The sample temperature was maintained at 25°C in a waterbath with a digitized temperature control system. The viscometer was placed into the holder vertically and inserted into the waterbath that was maintained at a fixed temperature. The efflux time was measured by allowing the liquid sample to flow freely down past marks. The kinematic viscosity of liquid sample in centistokes was calculated by multiplying the efflux time in seconds by the viscometer constants (0.004 for size 50 and 0.015 for size 100). The viscosity of E25 solution is highly dependent on the concentration of protein molecules (Figure 1). It increases exponentially with increase of rhuMAb E25 concentration. At 25°C, the reconstituted rhuMAb E25 at 125 mg/ml is about 80 fold more viscous than water.

EXAMPLE 2

[0132] The lyophilized rhuMAb E25 of Example 1 was reconstituted with different concentrations of NaCl solution. The viscosity of reconstituted solution was measured at 25°C in a Cannon-Fenske Routine capillary viscometer using the same method as described in Example 1.

[0133] The results as shown in Figure 2 demonstrate that the addition of NaCl can significantly reduce the viscosity of the protein formulation. The reconstituted rhuMAb E25 with 100 mM NaCl will give the solution that is about 4 fold less viscous than that reconstituted with SWFI.

[0134] The preparation of a rhuMAb E25 lyophilized formulation with 100 mM NaCl resulted in a slightly hypertonic solution. However, as reported previously, strict isotonicity is not absolutely necessary in that tissue damage was detected only when at extremely high tonicity levels (1300 mOsmol/Kg).

[0135] Thus, the administration of a formulation containing higher concentration of salt (100 mM to 200 mM NaCl resulting in osmolarity of ~600 to ~700 mOsmol/Kg for current rhuMAb E25 lyophilized materials) as contemplated herein, for reducing the viscosity of the formulation, does not appear to present a risk of tissue damage at the site of administration.

EXAMPLE 3

[0136] The effects of different salts on the viscosity of rhuMAb E25 solution were studied at 25°C. The lyophilized rhuMAb E25 was first reconstituted with SWFI to produce a solution with rhuMAb E25 at 125 mg/ml, 266 mM sucrose, 16 mM histidine, 0.03% Polysorbate 20. The samples were then diluted with 10 mM histidine, 250 mM sucrose, pH 6.0 to a final concentration of 40 mg/ml. Various concentrated salts were then added into solution to bring the final salt concentration ranging from 0 - 200 mM. The viscosity of solution was determined in a Cannon-Fenske Routine capillary viscometer using the same method as described in Example 1.

[0137] The results were demonstrated in Figure 3. Although each salt shows slightly different impact on change of viscosity, they appear to follow a similar trend whereby the viscosity of the solution decreases with increase in buffer concentration and ionic strength.

EXAMPLE 4

[0138] Also studied were the effects of different buffers on viscosity of rhuMAbE25 solution at 25°C. A liquid formulation containing 80 mg/ml of rhuMAb E25, 50 mM histidine, 150 mM trehalose and 0.05 % of Polysorbate 20 was added with different amounts of histidine, acetate, or succinate buffer components. The pH of sample was maintained at ~6.0 for all the preparation. The viscosity of solution was determined in a Cannon-Fenske Routine capillary viscometer using the same method as described in Example 1.

[0139] As shown in Figure 4, the viscosity of solution containing either histidine or acetate buffers decreases with increasing buffer concentration up to 200 mM. However, for succinate buffer, the viscosity of solution decreases only at low buffer concentration (<100 mM), but not at high buffer concentration (>200 mM). Similar results have also been observed in other buffers that contain negatively charged multivalent buffer components, such as phosphate, citrate and carbonate.

EXAMPLE 5

[0140] This example used a second generation of anti-IgE monoclonal antibody, rhuMAb E26. This monoclonal antibody is a homologous to rhuMAb E25 with five amino acid residue differences in CDR I region in the light chain and is described in WO 99/01556. The recombinant rhuMAb E26 was also expressed in CHO cell line and purified with similar chromatography methods as described above for rhuMAb E25. The samples were formulated into 5 mM histidine and 275 mM sucrose with concentration of rhuMAb E25 at 21 mg/ml. The viscosity of samples was measured at 6°C in a Cannon-Fenske Routine capillary viscometer

using the same method as described in Example 1.

[0141] The effects of NaCl on viscosity of rhuMAb E26 were shown in Figure 5. The result demonstrates that the increase of NaCl concentration can effectively reduce the viscosity of rhuMAb E26 solution.

EXAMPLE 6

[0142] The effects of pH on viscosity of a highly concentrated anti-IgE monoclonal antibody, rhuMAb E25 in liquid formulations have been examined in both hypotonic and isotonic conditions. The hypotonic solutions were prepared by adding small amounts of 10% acetic acid or 0.5 M arginine into an unbuffered rhuMAb E25 solution that has been concentrated to ~130 mg/ml in Milli-Q water. The final concentrations of total buffer and salt were maintained at 17.5 mM. The hypotonic solutions were then mixed with a small volume of 5 M NaCl to produce the isotonic solutions with final NaCl concentration around 150 mM NaCl. The viscosity of solution was determined at 25°C in a Cannon-Fenske Routine capillary viscometer using the same method as described in Example 1. As shown in figure 6, the viscosity of rhuMAb E25 solution is highly dependent on the pH of buffer, especially in very hypotonic solutions. The addition of ionic species, such as NaCl, can significantly reduce such pH effects.

EXAMPLE 7

[0143] The effects of pH on viscosity of a reconstituted anti-IgE monoclonal antibody, rhuMAb E25 have also been examined in the presence of other excipients, such as trehalose. The lyophilized rhuMAb E25 was reconstituted with SWFI and then dialyzed against 20 mM Histidine, 250 mM Trehalose, at pH 5. The protein concentration is about 94 mg/ml. The pH of solution was adjusted with 1 M NaOH. The viscosity of solution was determined at 25°C in a Cannon-Fenske Routine capillary viscometer using the same method as described in Example 1. The results, as shown in figure 7, demonstrated that the viscosity of antibody can be significantly altered by pH of solution.

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P A T E N T K R A V

1. Stabil flydende formulering omfattende et immunoglobulin i en mængde på mindst 80 mg/ml og et salt og/eller en buffer i en mængde på mindst 100 mM og har en kinematisk viskositet på 50 mm²/s eller mindre ved 25°C, hvor immunoglobulinet er antistoffet rhuMAb-E25, rhuMAb-E26 eller rhuMAb-E27, og hvor saltet ikke er natriumchlorid.
2. Formulering ifølge krav 1 omfattende:
 - (a) saltet og/eller bufferen i en mængde på 100-200 mM; eller
 - (b) saltet og/eller bufferen i en mængde på 200 mM.
3. Formulering ifølge krav 1, hvor saltet og/eller bufferen er afledt af:
 - (a) en organisk eller uorganisk syre og et basedannende metal eller amin; eller
 - (b) en aminosyre.
4. Formulering ifølge krav 3, hvor:
 - (a) det basedannende metal er valgt fra gruppen bestående af alkalimetaller, jordalkalimetaller, Al, Zn og Fe; eller
 - (b) den basedannende amin er NR₄⁺, hvor R uafhængigt er H eller C₁₋₄-alkyl.
5. Formulering ifølge krav 1, hvor saltet og/eller pufferen er afledt fra en aminosyre.
6. Formulering ifølge krav 1, hvor saltet er valgt fra gruppen bestående af natriumthiocyanat, ammoniumthiocyanat, ammoniumsulfat, ammoniumchlorid, calciumchlorid, argininhydrochlorid, zinkchlorid og natriumacetat.
7. Formulering ifølge krav 1, hvilken
 - (a) har en kinematisk viskositet på 40 mm²/s eller mindre ved 25°C; eller
 - (b) har en kinematisk viskositet på 30 mm²/s eller derunder ved 25°C; eller
 - (c) har en kinematisk viskositet på 20 mm²/s eller mindre ved 25°C; eller
 - (d) har en kinematisk viskositet på 10 til 30 mm²/s ved 25°C.
8. Formulering ifølge krav 1, der yderligere omfatter en lyoprotektant.
9. Formulering ifølge krav 8, hvor lyoprotektanten er et sukker.
10. Formulering ifølge krav 9, hvor sukkeret er saccharose eller trehalose.
11. Formulering ifølge krav 9, der omfatter sukkeret i en mængde på 60-300 mM.
12. Formulering ifølge krav 1, der yderligere omfatter et overfladeaktivt stof.
13. Formulering ifølge krav 1, som er hypertonisk.
14. Formulering ifølge krav 1, som er en rekonstitueret formulering.
15. Formulering ifølge krav 14, hvor immunoglobulinproteinkoncentrationen i den rekonstituerede formulering er 2-40 gange større end immunoglobulinproteinkoncentrationen i blandingen inden lyofilisering.
16. Formulering ifølge krav 1, som er en flydende farmaceutisk formulering.
17. Formulering ifølge krav 16, som er til subkutan indgivelse.
18. Fremgangsmåde til reduktion af den kinematiske viskositet af en formulering indeholdende et immunoglobulin i en mængde på mindst 80 mg/ml, omfattende tilsætning af et salt og/eller en buffer i en mængde på mindst 100 mM, hvor immunoglobulinet er anti-

stof rhuMAb-E25, rhuMAb-E26 eller rhuMAb-E27, og hvor saltet ikke er natriumchlorid.

19. Fremgangsmåde ifølge krav 18, hvor saltet er valgt fra gruppen bestående af natriumthiocyanat, ammoniumthiocyanat, ammoniumsulfat, ammoniumchlorid, calciumchlorid og argininhydrochlorid.

5 20. Fremgangsmåde ifølge krav 18, hvor formuleringen er en rekonstitueret formulering.

21. Fremgangsmåde ifølge krav 18, hvor formuleringen er hypertonisk.

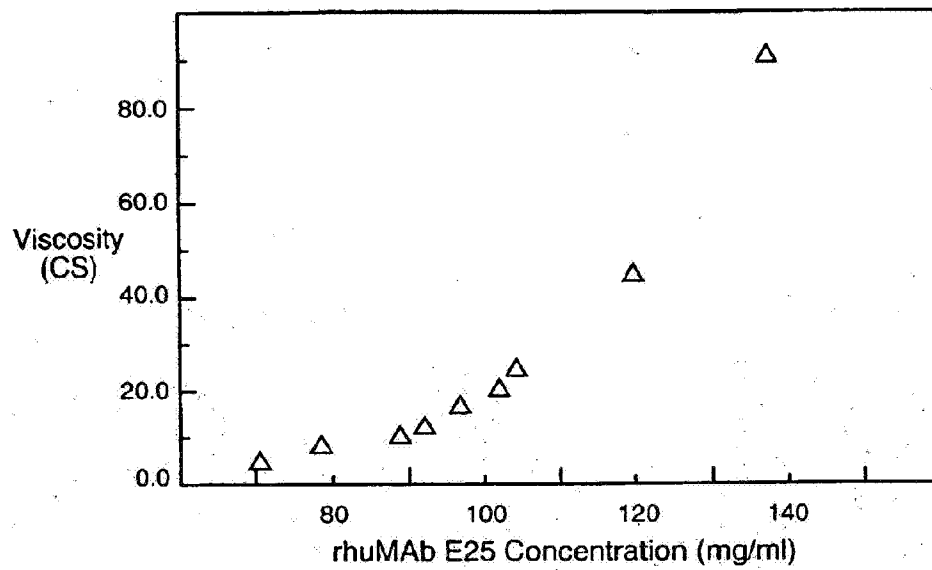
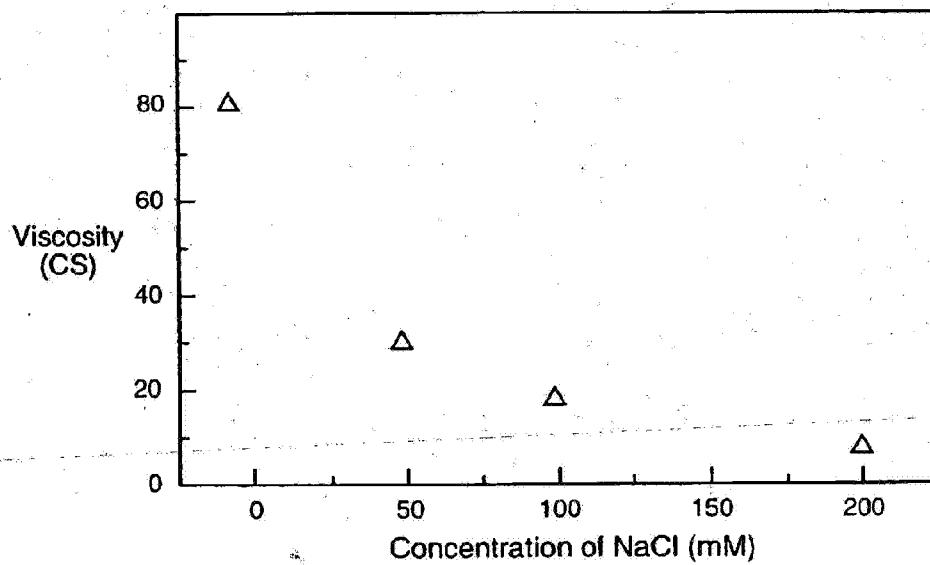
22. Fremgangsmåde ifølge krav 20, hvor proteinkoncentrationen i den rekonstituerede formulering er 2-40 gange større end proteinkoncentrationen i blandingen inden lyofilisering.
10

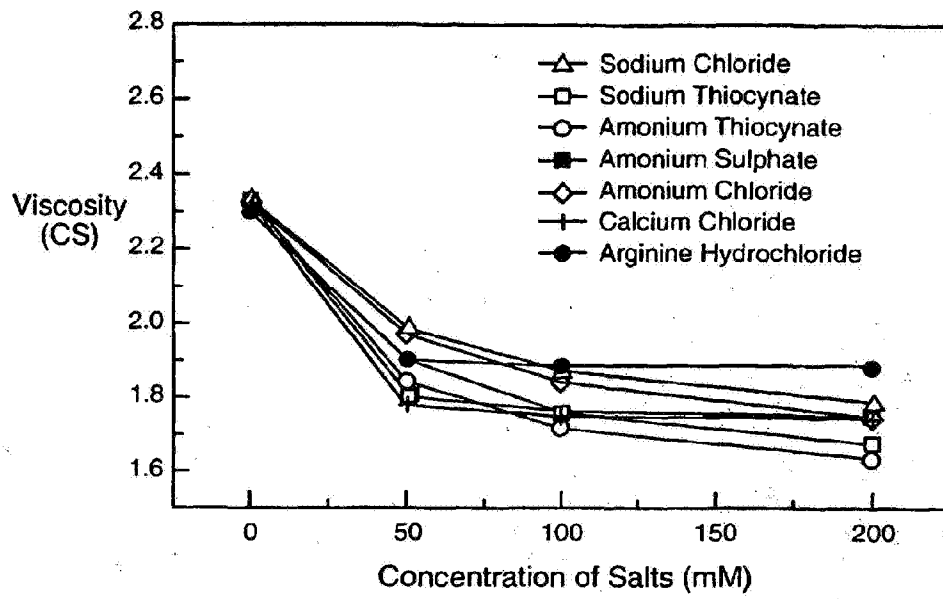
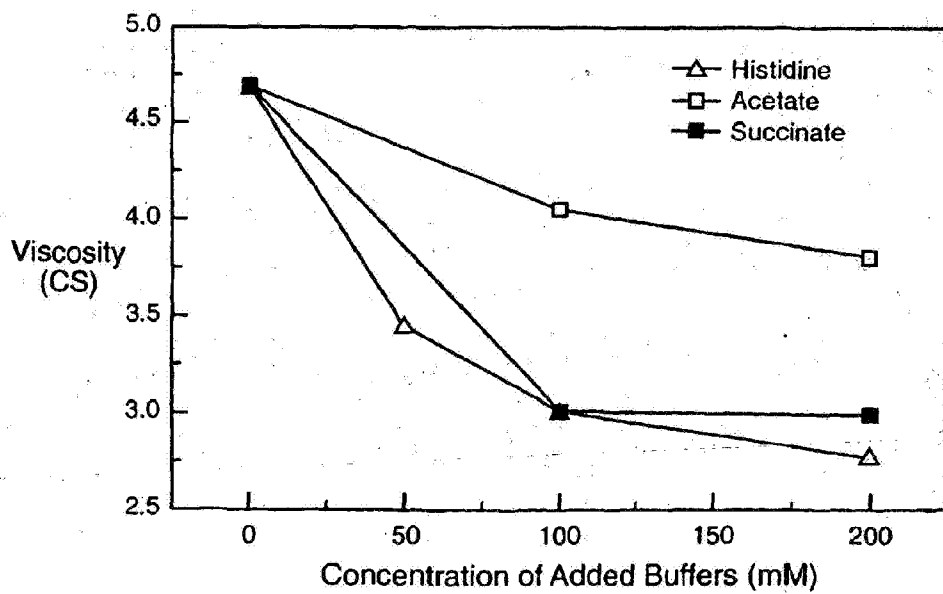
23. Fremgangsmåde ifølge krav 18, hvor den kinematiske viskositet af formuleringen reduceres til 50 mm²/s eller mindre som bestemt ved 25°C.

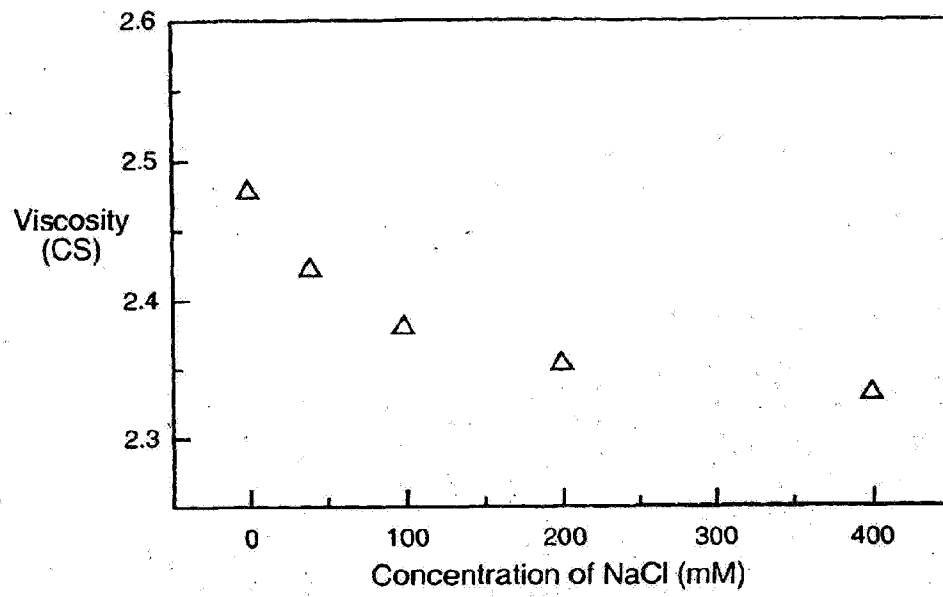
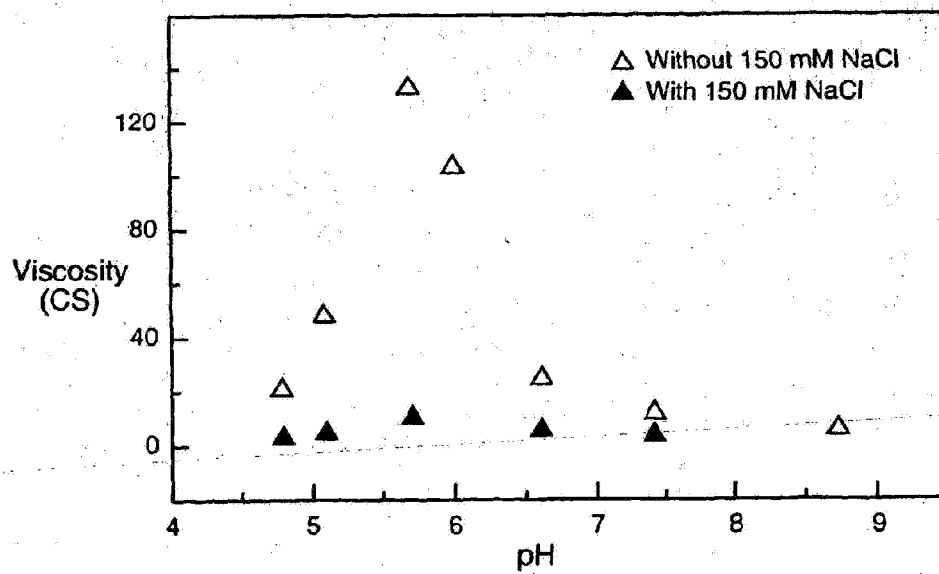
24. Produkt omfattende en beholder indeholdende formuleringen ifølge et hvilket som helst af kravene 1 til 17.

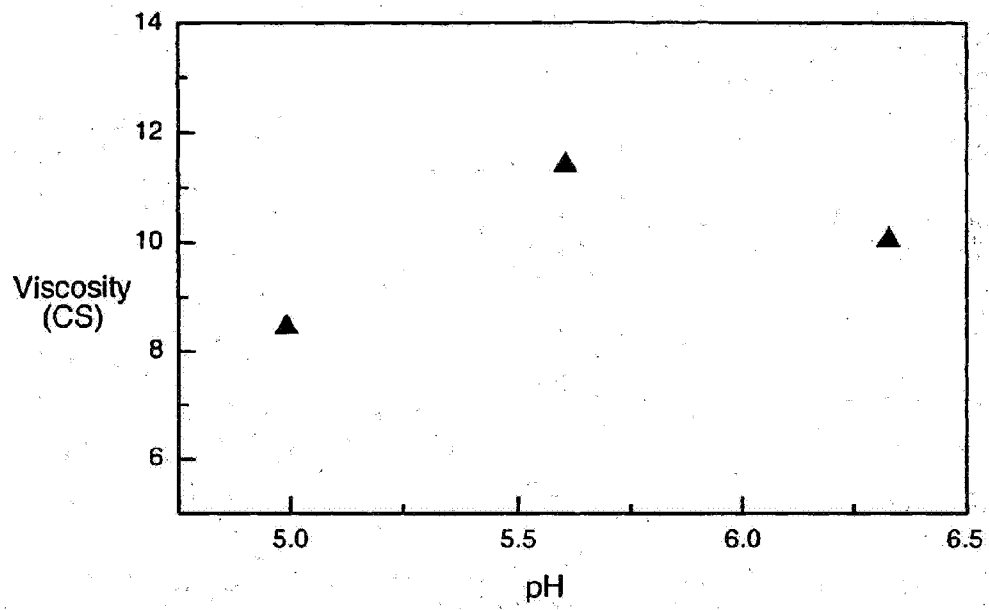
15 25. Produkt ifølge krav 24, der yderligere omfatter retningslinier for indgivelse af formuleringen.

DRAWINGS

**FIG._1****FIG._2**

**FIG._3****FIG._4**

**FIG._5****FIG._6**

**FIG. 7**