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(54) **Title:** HIGH PH PROTEIN REFOLDING METHODS

(57) **Abstract:** Provided herein are methods for refolding denatured protein (e.g., from inclusion bodies) that do not require the use of a denaturing agent. Exemplary methods use a high pH for solubilizing denatured protein, followed by a decrease in pH for refolding the proteins.

HIGH PH PROTEIN REFOLDING METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of provisional patent application
5 61/763,664, filed February 12, 2013, the teachings of which are specifically incorporated
by reference herein.

BACKGROUND

The backbone of an antibody, known as the Fc region, is responsible for
10 pharmacokinetic properties that may be desirable in the case of many therapeutic
biologics (Jeffries, B. *Biotechnol Prog.* 2005; 21: 11-16). The size of the Fc region
makes it resistant to renal filtration and binding to the Fc Neonatal Receptor (FcRn)
allows it to escape endosomal degradation by a recycling mechanism. In addition to the
Fc region that is present in monoclonal antibody therapeutic products, there are Fc fusion
15 products being investigated and developed (Hakim et al. *Mabs.* 2009; 1:281-287). Fc
fusions are the fusion of an Fc region to another protein, peptide, or Active
Pharmaceutical Ingredient (API). The Fc fusion then has both the properties of the Fc
region and the therapeutic properties of the API.

There are many cell lines that are capable of being used to manufacture
20 therapeutic biologics (Jung et al. *Curr Opin Biotechnol.* 2011; 22:1-10). The mammalian
Chinese Hamster Ovary (CHO), insect Sf9, yeast *S. cerevisiae*, and bacterial *E. coli* are
some of the most common cell lines that are discussed for recombinant protein
production. So far, yeast, CHO and *E. coli* have been used for manufacture of Fc
containing therapeutic biologics, including a large number of monoclonal antibodies.
25 Expression in *E. coli* offers three potential and significant advantages over expression in
other cell lines: the cell line development time is much shorter; the bioreactor runs are up
to 7-fold shorter, resulting in a lower capital investment; and there is no need to control
aberrant glycosylation that can occur in yeast and mammalian cell cultures.

Expression of larger proteins, like Fc fusions, in *E. coli* can be a unique challenge.
30 *E. coli* lack the chaperone proteins and other refolding machinery found in a eukaryotic
expression system. The cytoplasm of *E. coli* is also a reducing environment, which is not
favorable for the formation of disulfide bonds. The Fc region of human IgG1 antibodies

contains six disulfide bonds. Two disulfide bonds the hinge region join two peptide chains to form the homodimeric molecule and there are two more disulfide bonds within each of the peptide chains. *E. coli* also have a mechanism to prevent unfolded proteins from interfering with normal cell processes. Unfolded protein is shunted and isolated in insoluble aggregates, called Inclusion Bodies (IB), which can then be isolated in the insoluble fraction following cell lysis. Alternatively, when the rate of recombinant protein production is slowed to allow the protein to fold, a leader sequence may be added to direct soluble protein that is expressed to the periplasmic space. The periplasm is an oxidative environment favorable for the formation of disulfide bonds. However, the reported expression levels of recombinant protein in the periplasm remain low (Liu et al. Protein Expression Purif. 2008; 62:15-20).

In contrast, *E. coli* expression levels in IBs have been reported to be high. Expressing protein in IBs also has the advantages of resistance to protein degradation, and ease of isolation from the cells (Grune et al. Int J Biochem Cell Biol. 2004; 36:2519-2530). Since an IB is an insoluble aggregate, there may be a challenge in restoring the protein of interest to its biologically active conformation (Jungbauer et al. J Biotechnol. 2006; 587-596). Typically, a process is required to break apart and solubilize the IB. Then the protein must be renatured, or refolded, into the biologically active conformation while minimizing losses due to aggregation and precipitation. Current refolding processes may be specific to a given protein, requiring thorough optimization for each case. Many refolding processes require very low protein concentrations and consequently large volumes for the operation. This is difficult because it requires a larger amount of potentially expensive reagents. There is also a challenge in a manufacturing setting, where there is a physical limit to the container size that may be used to refold proteins. Finally, in the case of Fc fusions, the refolding process must correctly form the six disulfide bonds that exist in the native form of the protein.

SUMMARY

Provided herein are methods for refolding a denatured protein, comprising, e.g., (i) suspending a denatured protein in a suspension solution to obtain a composition comprising suspended denatured proteins; (ii) combining the composition comprising suspended denatured proteins with a solubilization buffer having a pH in the range of

10.5 to 13 to thereby obtain a composition comprising solubilized denatured proteins; and
(iii) combining the composition comprising solubilized denatured proteins with a refold
buffer having a pH in the range of 9 to 11 to thereby obtain a composition comprising
refolded proteins. In certain methods, the method does not include the use of a significant
5 amount of denaturing agent and/or reducing agent. The pH of the solubilization buffer
may be in the range of pH 11.5 to 12.8, such as in the range of pH 12.0 to 12.6. The pH
of the refold buffer may be in the range of pH 10 to 10.6, such as in the range of pH 10.3
to 10.5. The suspension solution may consist of water. The composition comprising
solubilized denatured protein may have a pH in the range of 11 to 13, such as a pH in the
10 range of pH 11.5 to 12.8, e.g., pH 12.0 to 12.6. The composition comprising refolded
protein may have a pH in the range of 10 to 11, such as in the range of 10 to 10.6, e.g., pH
10.3 to 10.5.

The denatured proteins may be suspended in suspension solution at a ratio of
weight (g) of denatured proteins:volume (ml) of suspension solution of 1:1-3, e.g., about
15 1:2. The suspension solution may be water. The composition comprising suspended
denatured proteins may be combined with solubilization buffer at a ratio of weight (g;
e.g., weight prior to adding suspension solution) of denatured proteins or volume of
suspension solution (ml): volume (ml) of solubilization buffer of 1:10-30, such as about
1:20. The composition comprising solubilized denatured proteins may be combined with
20 refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer of 1:1-5,
such as about 1:3-4.

In the methods described herein, the denatured proteins may be suspended in
suspension solution at a ratio of weight (g) of denatured proteins:volume (ml) of
suspension solution of 1:1-3; the composition comprising suspended denatured proteins
25 may be combined with solubilization buffer at a ratio of weight (g) of denatured
proteins:volume (ml) of solubilization buffer of 1:10-30; and the composition comprising
solubilized denatured proteins may be combined with refold buffer at a ratio of volume of
solubilization buffer:volume of refold buffer of 1:1-5. The solubilization buffer may have
a pH in the range of 11.5 to 12.8 and the refold buffer may have a pH in the range of 10 to
30 10.9. The denatured proteins may be suspended in suspension solution at a ratio of
weight (g) of denatured proteins:volume (ml) of suspension solution of about 1:2; the
composition comprising suspended denatured proteins may be combined with

solubilization buffer at a ratio of weight (g) of denatured proteins:volume (ml) of solubilization buffer of about 1:20; and the composition comprising solubilized denatured proteins may be combined with refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer of about 1:3-4. The denatured proteins may be suspended
5 in water at a ratio of weight (g) of denatured proteins:volume (ml) of suspension solution of about 1:2; the composition comprising suspended denatured proteins may be combined with solubilization buffer having a pH of about 12.2 at a ratio of weight (g) of denatured proteins:volume (ml) of solubilization buffer of about 1:20; and the composition comprising solubilized denatured proteins may be combined with refold buffer having a
10 pH in the range of 10.2 to 10.6 at a ratio of volume of solubilization buffer:volume of refold buffer of about 1:3-4.

In the methods described herein, the suspended denatured proteins and the solubilization buffer may be combined for 1-10, e.g., 2-5, minutes prior to being combined with the refold buffer. The composition comprising the solubilized denatured
15 proteins may be combined with the refold buffer for 5-60, e.g., 15-25, minutes.

In the methods described herein, the pH of the solution comprising the refolded proteins may be reduced following refolding.

In the methods described herein, the solubilization buffer and/or the refold buffer may comprise Arginine. The refold buffer may comprise an oxidizing agent, e.g.,
20 glutathione, wherein, e.g., glutathione is at an about 5:1 oxidized: reduced ratio.

In certain embodiments, the method does not comprise first suspending the denatured protein in a suspension solution. In certain embodiments, the method does not include the use of a denaturing agent. The denatured proteins may be in the form of inclusion bodies (IBs). The protein that is renatured according to the methods described
25 herein may comprise at least one cysteine. The protein may comprise at least two cysteines that form a disulfide bond in the native protein. The protein may comprise an Fc region, which may comprise a hinge. The protein may comprise a binding domain that specifically binds to a target protein. The binding domain may be an alternative scaffold binding domain, such as a fibronectin based scaffold domain, e.g., a ¹⁰FN3 domain. In
30 certain embodiments, the protein comprises a ¹⁰FN3 protein and an Fc region comprising a hinge, a CH2 and a CH3 domain.

Also provided herein are compositions, e.g., compositions comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and water, wherein the composition does not comprise a buffer or a denaturing agent. Also provided are compositions comprising a suspension of denatured proteins, wherein at least some proteins comprise at least two cysteines that form a disulfide bond under appropriate conditions, and wherein the composition does not comprise a buffer or a denaturing agent. Also provided are compositions comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a solubilization buffer having a pH in the range of pH 10 to 13, wherein the composition does not comprise a denaturing agent. Further provided are compositions comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a refold buffer having a pH in the range of pH 9 to 11 and an oxidizing agent, wherein the composition does not comprise a reducing agent other than a reducing agent that part of an oxidizing agent that is present in the composition. The protein may comprise an Fc region or a portion thereof. The protein may comprise a binding domain, e.g., an FBS domain, e.g., a ¹⁰Fn3 domain. The ¹⁰Fn3 domain may bind specifically to a target, and the ¹⁰Fn3 domain may comprise an amino acid sequence that is at least 50% identical to any of SEQ ID NOs: 1-29. The protein may be present in the composition at a concentration of at least 5 mg/ml or 10 mg/ml.

20

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a diagram showing exemplary steps for refolding a denatured protein.

Figures 2A-C show the results of the use of a G25 buffer exchange method to estimate refold efficiency. A) G25 refold performed with 50 mM Tris pH 8.5 and 0.4 M Arginine. B) G25 refold performed with 50 mM Tris pH 9.0 and 0.4 M Arginine. C) G25 refold performed with 50 mM Tris pH 10.4 and 0.4 M Arginine.

Figures 3A and B show soluble aggregate concentration visualized by SDS-PAGE. SDS PAGE analysis of G25 refolds performed at pH 9.0 (A) and pH 10.4 (B).

Figure 4 shows SDS-PAGE gels of G25 refold reactions incubated for 30 minutes (A) or 4 hours (B) in the absence of TCEP prior to loading on SDS-PAGE.

30

Figure 5 shows dimer formation efficiency after 0, 1 and 2 hour refold incubation periods prior to redox addition. “Dil” refers to the lower concentration of ¹⁰F_{n3}/Fc relative to the concentration of the samples referred to as “neat.”

5 Figures 6A and B show Near and Far UV Circular Dichroism (CD) of ¹⁰F_{n3}/Fc in different conditions. Figure 6A shows Near UV CD representing tertiary structure of the protein at varying pHs or in the presence of guanidine. Figure 6B shows Far UV CD representing secondary structure of the protein at varying pHs and in the presence of guanidine.

10 Figure 7 shows high pH solubilization for refolding ¹⁰F_{n3}/Fc at 1.75, 3.5 and 7 mg/ml with either a 10 µg load (lanes 1-4) or 20 µg load (lanes 5-7).

Figures 8A and B show SPR binding data of (A) mammalian expressed and (B) *E. coli* expressed and refolded ¹⁰F_{n3}/Fc protein. The Tables below the diagrams provide the *k_a*, *k_d* and *K_D* values for each protein.

15 Figure 9 shows the percent inhibition in mice of target induced cytokine secretion by various amounts of *E. coli* expressed and refolded, or mammalian expressed, ¹⁰F_{n3}/Fc protein, indicating that similar levels of inhibition are obtained with the *E. coli* expressed and refolded protein relative to the mammalian expressed protein. “mpk” refers to milligrams of protein per weight of the animal in kg.

20 Figure 10 shows the percent inhibition in mice of target induced cytokine (different from that in Figure 9) secretion by various amounts of *E. coli* expressed and refolded ¹⁰F_{n3}/Fc protein (“*E. coli* Refolded”), or ¹⁰F_{n3}/Fc protein expressed in mammalian cells in a shake flask culture (“Mammalian Shake Flask), or bioreactor (“Mammalian Bioreactor”). Also shown is the percent inhibition obtained with an antibody (“BMS Antibody”) binding to the same target as one of the targets of the
25 ¹⁰F_{n3}/Fc molecule, as well as inhibition by one of the two ¹⁰F_{n3} entities on its own (“mono-adnectin”).

30 Figure 11 shows the percent inhibition in mice of signal transduction by various amounts of *E. coli* expressed and refolded ¹⁰F_{n3}/Fc protein (“*E. coli* Refolded”), or ¹⁰F_{n3}/Fc protein expressed in mammalian cells in a shake flask culture (“Mammalian Shake Flask), or bioreactor (“Mammalian Bioreactor”). Also shown is the percent inhibition obtained with an antibody (“BMS Antibody”) binding to the same target as one of the targets of the ¹⁰F_{n3}/Fc molecule.

DETAILED DESCRIPTION

Provided herein are methods for refolding denatured proteins, such as proteins present in the form of inclusion bodies (IBs). The methods are applicable to, e.g., proteins comprising at least one disulfide bond, such as proteins comprising an Fc region or domain (or portions thereof) of antibodies. A method may comprise combining a composition comprising denatured or unfolded proteins with a composition having a strongly alkaline pH, followed by incubation at reduced pH. Unlike commonly used methods for refolding proteins, e.g., from IBs, the methods described herein do not require the use of a denaturing or chaotropic agent. In addition, the methods described herein allow refolding of denatured proteins, e.g., from IBs, without the use of large volumes of buffer and in generally shorter time frames than those of current commonly used methods.

Definitions

By “polypeptide” is meant any sequence of two or more amino acids, regardless of length, post-translation modification, or function. Polypeptides can include natural amino acids and non-natural amino acids such as those described in U.S. Patent No. 6,559,126, incorporated herein by reference. Polypeptides can also be modified in any of a variety of standard chemical ways (e.g., an amino acid can be modified with a protecting group; the carboxy-terminal amino acid can be made into a terminal amide group; the amino-terminal residue can be modified with groups to, e.g., enhance lipophilicity; or the polypeptide can be chemically glycosylated or otherwise modified to increase stability or *in vivo* half-life). Polypeptide modifications can include the attachment of another structure such as a cyclic compound or other molecule to the polypeptide and can also include polypeptides that contain one or more amino acids in an altered configuration (i.e., R or S; or, L or D).

A “region” of a ¹⁰F_n3 domain (or moiety) as used herein refers to either a loop (AB, BC, CD, DE, EF and FG), a β-strand (A, B, C, D, E, F and G), the N-terminus (corresponding to amino acid residues 1-7 of SEQ ID NO: 1), or the C-terminus (corresponding to amino acid residues 93-101 of SEQ ID NO: 1) of the human ¹⁰F_n3 domain having SEQ ID NO: 1.

A “north pole loop” refers to any one of the BC, DE and FG loops of a human fibronectin type 3 tenth (¹⁰F_n3) domain.

A “south pole loop” refers to any one of the AB, CD and EF loops of a human fibronectin type 3 tenth (¹⁰F_n3) domain.

5 A “scaffold region” refers to any non-loop region of a human ¹⁰F_n3 domain. The scaffold region includes the A, B, C, D, E, F and G β-strands as well as the N-terminal region (amino acids corresponding to residues 1-7 of SEQ ID NO: 1) and the C-terminal region (amino acids corresponding to residues 93-101 of SEQ ID NO: 1).

“Percent (%) amino acid sequence identity” herein is defined as the percentage of
10 amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that
15 are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For purposes herein, however, % amino acid sequence
20 identity values are obtained as described below by using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087, and is publicly available through Genentech, Inc., South
25 San Francisco, Calif. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively
30 be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y where X is the number of amino acid residues scored

as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not
5 equal the % amino acid sequence identity of B to A.

As used herein, an amino acid residue in a polypeptide is considered to “contribute to binding” a target if (1) any of the non-hydrogen atoms of the residue’s side chain or main chain is found to be within five angstroms of any atom of the binding target based on an experimentally determined three-dimensional structure of the complex,
10 and/or (2) mutation of the residue to its equivalent in wild-type ¹⁰F_n3 (e.g., SEQ ID NO: 1), to alanine, or to a residue having a similarly sized or smaller side chain than the residue in question, leads to a measured increase of the equilibrium dissociation constant to the target (e.g., an increase in the k_{on}).

“Moiety” refers to a portion of a protein. For example, a fusion protein may
15 comprise several moieties. In one embodiment, a fusion protein comprises a fibronectin based scaffold moiety and an Fc moiety. An Fc moiety may comprise a CH2 and a CH3 domain, but does not necessarily comprise a hinge.

A “denatured protein” refers to a protein that is not properly folded (i.e., does not have the proper spatial conformation or three dimensional structure). “Denaturation”
20 refers to a process in which the native conformation of the protein is changed but the primary structure (amino acid chain, peptide links) of the protein remains unchanged. To be able to perform its biological function, a protein folds into a specific spatial conformation, by the action of non-covalent interactions such as ionic interactions, Van Der Waals forces, hydrogen bonding, and hydrophobic packing. A protein that is
25 denatured (i.e., not properly folded) may be a protein that does not have a proper secondary, tertiary or quaternary structure. The secondary structure of a protein or polypeptide refers to highly regular local sub-structures, such as the alpha helix and the beta strand or beta sheets, of a protein. The tertiary structure of a protein or a polypeptide refers to the three-dimensional structure of a single protein molecule, in which the folding
30 of the alpha-helices and beta-sheets into a compact globule is driven by the non-specific hydrophobic interactions (the burial of hydrophobic residues from water), salt bridges, hydrogen bonds, and the tight packing of side chains and disulfide bonds. The quaternary

structure of a protein is the three-dimensional structure of subunits of a multi-subunit protein. The subunits of a protein are held together by the same bonds as those that maintain a tertiary structure of a protein. Disulfide bonds contribute to the tertiary and quaternary structure of a protein, polypeptide or polypeptide complex. A denatured
5 protein may be a protein that contains cysteines, but in which the disulfide bonds are not present or are improperly formed. Denatured proteins are generally insoluble and precipitate out of a solution. The presence of one or more disulfide bonds in a protein generally makes its renaturation from a denatured state more challenging. Protein structure can be visualized or determined with various tools, e.g., X-ray crystallography,
10 Nuclear Magnetic Resonance (NMR), circular dichroism and cryo-electron microscopy.

Methods for refolding denatured proteins

When proteins are expressed in certain expression systems, they are produced in a denatured form and must be renatured, i.e., their secondary, tertiary and/or quaternary
15 structure must be reformed. For example, proteins expressed at high levels in *E. coli* are shunted into inclusion bodies (IBs). IBs are essentially made of denatured proteins. The methods described herein may be used to renature unfolded or improperly folded proteins, e.g., present in IBs.

A method for refolding a denatured protein, may comprise: (i) combining
20 denatured protein with a solubilization buffer having a pH in the range of 10 to 13 to thereby obtain a composition comprising solubilized denatured protein; and (ii) combining the composition comprising solubilized denatured protein, with a refold buffer having a pH in the range of 9 to 11 to thereby obtain a composition comprising refolded protein. A method for refolding a denatured protein, may comprise: (i) suspending the
25 denatured protein in a suspension solution to obtain a composition comprising suspended denatured protein; (ii) combining the composition comprising suspended denatured protein with a solubilization buffer having a pH in the range of 10 to 13 to thereby obtain a composition comprising solubilized denatured protein; and (iii) combining the composition comprising solubilized denatured protein, with a refold buffer having a pH in
30 the range of 9 to 11 to thereby obtain a composition comprising refolded protein. When a denatured protein is part of an IB, a method for refolding the protein may comprise: (i) combining the IBs with a solubilization buffer having a pH in the range of 10 to 13 to

thereby obtain a composition comprising solubilized IBs; and (ii) combining the composition comprising solubilized IBs with a refold buffer having a pH in the range of 9 to 11 to thereby obtain a composition comprising refolded protein. A method for refolding a protein may comprise (i) suspending IBs comprising a protein in an IB suspension solution to obtain a composition comprising suspended IBs; (ii) combining the composition comprising suspended IBs with a solubilization buffer having a pH in the range of 10 to 13 to thereby obtain a composition comprising solubilized IBs; and (iii) combining the composition comprising solubilized IBs with a refold buffer having a pH in the range of 9 to 11 to thereby obtain a composition comprising refolded protein. A diagram showing exemplary refolding steps is provided in Figure 1. The first step of the method may be removed.

Certain commonly used methods for refolding denatured proteins known in the art use denaturing agents for solubilizing the denatured proteins. In certain embodiments, the methods described herein or one or more of the compositions, buffers or solutions used in the methods described herein, do not include a significant amount of a denaturing agent. Exemplary denaturing (or chaotropic) agents include: guanidine (or guanidium), guanidium hydrochloride, guanidium chloride, guanidium thiocyanate, urea, thiourea, lithium perchlorate, magnesium chloride, phenol, betain, sarcosine, carbamoyl sarcosine, taurine, dimethylsulfoxide (DMSO); alcohols such as propanol, butanol and ethanol; detergents, such as sodium dodecyl sulfate (SDS), N-lauroyl sarcosine, Zwittergents, non-detergent sulfobetains (NDSB), TRITONTM X-100, NONIDETTM P-40, the TWEENTM series and BRIJTM series; hydroxides such as sodium potassium hydroxide, and combinations thereof. A “significant” amount of a denaturant refers to an amount of denaturant that is sufficient to contribute to the solubilization of denatured protein. Certain concentrations of denaturants denature proteins, and these concentrations are referred to as “denaturing concentrations.” Certain concentrations of denaturants do not denature proteins, but may contribute to the solubilization of proteins, and these concentrations are referred to as “non-denaturing concentrations.” For example, 6 M guanidium is a denaturing concentration, whereas 1M guanidium is not a denaturing concentration. Similarly, urea concentrations of 1-2 M are not considered to be denaturing concentrations. A solution comprising less than a significant amount of a denaturant includes solutions comprising less than a denaturing or non-denaturing

concentration of a denaturant. For example, the methods described herein preferably do not include urea or guanidium at a concentration of 1M or more. In certain embodiments, minor amounts of any denaturing agent that is low enough that it does not contribute to denaturation and/or solubilization of a denatured protein may be present at any time in the methods described herein, e.g., in one or more of the compositions, buffers and solutions used in the methods described herein. As defined herein, when referring to methods which do not include the use of a denaturing agent or a significant amount of a denaturing agent, the agent that produces the alkaline conditions in the solubilization buffer is not considered to be a denaturing agent, or in the alternative, if it is considered to be a denaturing agent, then the statement is intended to mean that no other denaturing agent is added or used.

In certain embodiments, a minor amount of a denaturant is an amount of denaturant that is sufficiently low that its inclusion in a refolding method does not require an additional step to later reduce its concentration or remove it from a solution. For example, in certain embodiments, the methods described herein do not include a dialysis step, e.g., they do not include a dialysis step before or after any of the steps of the methods described herein. For example, no dialysis prior to, or after, adding the suspension solution, solubilization buffer or refold buffer is performed.

In certain embodiments, the concentration of a denaturing agent in the methods described herein or in any step of the methods described herein is less than 1M, 100 mM, 10 mM, 1 mM, 0.1 mM, 10^{-2} mM, 10^{-3} mM, 10^{-4} mM, 10^{-5} mM, or 10^{-6} mM. In certain embodiments, no denaturing agent is added to, or present in, one or more of the following solutions used in the methods described herein: the suspension solution, such as an IB suspension solution; the solubilization buffer; and the refold buffer. In certain embodiments, no denaturing agent is used or present in any step in the methods described herein.

In certain embodiments, the concentration of guanidium or salt or analog thereof (e.g., guanidium chloride, guanidium hydrochloride and guanidium thiocyanate) in any step of the methods described herein is less than 1M, 100 mM, 10 mM, 1 mM, 0.1 mM, 10^{-2} mM, 10^{-3} mM, 10^{-4} mM, 10^{-5} mM, or 10^{-6} mM. In certain embodiments, no guanidium, salt or analog thereof (e.g., guanidium chloride, guanidium hydrochloride and guanidium thiocyanate) is added to, or present in, one or more of the following solutions

used in the methods described herein: the suspension solution, such as an IB suspension solution; the solubilization buffer; and the refold buffer. In certain embodiments, no guanidium, salt or analog thereof (e.g., guanidium chloride, guanidium hydrochloride and guanidium thiocyanate) is used or present in any step in the methods described herein. In certain embodiments, the concentration of urea or analog thereof (e.g., dimethylhydroxy urea, dimethylsulphone) in any step of the methods described herein is less than 1M, 100 mM, 10 mM, 1 mM, 0.1 mM, 10^{-2} mM, 10^{-3} mM, 10^{-4} mM, 10^{-5} mM, or 10^{-6} mM. In certain embodiments, no urea or analogs thereof (e.g., dimethylhydroxy urea and dimethylsulphone) is added to, or present in, one or more of the following solutions used in the methods described herein: the suspension solution, such as an IB suspension solution; the solubilization buffer; and the refold buffer. In certain embodiments, no urea or analogs thereof (e.g., dimethylhydroxy urea and dimethylsulphone) is used or present in any step in the methods described herein. In certain embodiments, the concentration of a detergent, e.g., ionic or non-ionic, in any step of the methods described herein is less than 1M, 100 mM, 10 mM, 1 mM, 0.1 mM, 10^{-2} mM, 10^{-3} mM, 10^{-4} mM, 10^{-5} mM, or 10^{-6} mM or less than 10%, 1%, 0.1%, 0.01% or 0.001% final concentration. In certain embodiments, no detergent, e.g., ionic or non-ionic, is added to, or present in, one or more of the following solutions used in the methods described herein: the suspension solution, such as an IB suspension solution; the solubilization buffer; and the refold buffer. In certain embodiments, no detergent, e.g., ionic or non-ionic, is used or present in any step in the methods described herein. Non-ionic detergents include TRITONTM X-100, NONIDETTM P-40, the TWEENTM series and BRIJTM series. Ionic detergents include deoxycholate, SDS, and CTAB.

In certain embodiments, the methods described herein do not use a significant amount of a reducing agent. A reducing agent is an agent that breaks disulfide bonds by reducing one or the two cysteines of the disulfide bond or maintains cysteines in a reduced state (i.e., maintains free sulfhydryl groups so that the intra- or intermolecular disulfide bonds are chemically disrupted). A “significant amount” of a reducing agent is an amount that is sufficient for reducing at least some disulfide bonds in a protein solution or for maintaining at least some cysteines in a protein solution in a reduced state. Exemplary reducing agents include the following: beta-mercaptoethanol (BME), dithiothreitol (DTT), dithioerythritol (DTE), tris(2-carboxyethyl)phosphine (TCEP),

cysteine, cysteamine, thioglycolate, glutathione and sodium borohydride. In certain embodiments, no reducing agent is added to, or present in, one or more of the following solutions used in the methods described herein: the suspension solution, such as an IB suspension solution; the solubilization buffer; and the refold buffer. In certain

5 embodiments, no reducing agent is used or present in any step in the methods described herein. In certain embodiments, the concentration of a reducing agent in any step of the methods described herein is less than 10mM, 1mM, 0.1mM, 10^{-2} mM, 10^{-3} mM, 10^{-4} mM, 10^{-5} mM, or 10^{-6} mM.

10 A method for refolding a denatured protein, e.g., a protein that is present in IBs, may include washing the denatured protein, e.g., IBs, prior to suspending them in a suspension solution. Washing denatured protein, e.g., IBs, may be performed with, e.g., Tris/HCL buffer, phosphate buffer, acetate buffer, citrate buffer or water, or a combination of two or more of these.

15 **Suspension of denatured protein**

Denatured protein, e.g., protein in IBs, which denatured protein may be, e.g., in the form of a pellet (such as a frozen pellet) may be suspended in a suspension solution (or buffer). The denatured protein may be incubated with suspension solution under conditions sufficient to substantially suspend the denatured protein. Incubation may take

20 place under conditions of concentration, incubation time, and incubation temperature to allow suspension of the desired amount or most or substantially all the denatured protein (e.g., at least 70%, 80%, 90%, 95%, 97%, 98% or 99%).

In certain embodiments, the suspension solution is water. Water may be, e.g., tap water, distilled, double distilled, deionized water, reverse osmosed water, or reversed

25 osmosed/deionized (RODI) water. In certain embodiments, a suspension solution comprises low concentrations of a buffer, e.g., TRIS/HCL, e.g., less than about 10 mM, 1 mM, 0.1 mM or less TRIS. A suspension solution may have a pH of 6-10, 6-9, 6-8, 6.5 to 7.5.

In certain embodiments, a pellet of denatured protein is contacted with a

30 suspension solution at a ratio of weight of denatured protein pellet (e.g., IB) (in grams): volume (in ml) of suspension solution (e.g., IB suspension solution) of 1:1-10; 1:1-9; 1:1-8; 1:1-7; 1:1-6; 1:1-5; 1:1-4; 1:1-3; 1:1-2; 1:1; 1:2; 1:3; 1:4; 1:5; 1:6; 1:7; 1:8 1:9 or 1:10.

In certain embodiments, a pellet of denatured protein is contacted with a suspension solution at a ratio of weight of denatured protein pellet (e.g., IB) (in grams): volume (in ml) of suspension solution (e.g., IB suspension solution) of 1:1-3. A weight to volume ratio of "1:3" in this context refers to a ratio of 1 gram of denatured protein to 3 ml of suspension solution. A weight to volume ratio of "1:1-3" in this context refers to a ratio of 1 gram of denatured protein to 1-3 ml (e.g., 1 ml, 2 ml or 3 ml and any values in between) of suspension solution. A weight to volume ratio may also be defined in kgs:liters. The combination of the denatured proteins and the suspension solution is referred to as the "suspension reaction."

10 The suspension reaction may be conducted at a temperature, e.g., ranging from 2 °C to 40 °C; 4 °C to 37 °C; 25 °C to 37 °C; room temperature; or 4 °C to 25 °C. In an exemplary embodiment, a pellet of denatured protein, e.g., an IB pellet, is suspended in water at room temperature (e.g., 25 °C) at a weight (grams) to volume (ml) ratio of denatured protein pellet: suspension solution of 1:1-3, such as 1:1, 1:2 or 1:3.

15 A suspension reaction may be incubated, and optionally stirred, with a suspension solution until most or essentially all denatured protein has been resuspended, and optionally a fine suspension is obtained. Any portion of a pellet of denatured protein that has not fully been suspended will probably not be renatured efficiently. The proportion of denatured protein that is suspended in the suspension solution may be determined optically. In certain embodiments, the denatured protein is incubated and optionally stirred, e.g., for less than 1 minute, in the suspension buffer. In certain embodiments, the denatured protein is incubated and optionally stirred, e.g., for 1-10 minutes; 1-5 minutes or 1-3 minutes in the suspension solution. Longer incubation times, especially at lower temperatures may also be used.

25 In certain embodiments, a pellet of denatured protein is suspended in water at a weight (grams) to volume (ml) ratio of denatured protein pellet: volume of suspension solution of 1:1-3, e.g., 1:2, at room temperature and incubated at room temperature for 1-3 minutes, to thereby obtain a composition comprising a suspension of denatured proteins. In an exemplary embodiment, a pellet of IBs is suspended in water at a weight (grams) to volume (ml) ratio of IB: suspension solution of 1:1-3, e.g., 1:2, at room temperature and incubated at room temperature for 1-3 minutes, to thereby obtain a composition comprising an IB suspension.

30

In certain embodiments, the suspension solution or suspension reaction does not comprise a significant amount of denaturing agent, as further described herein. In certain embodiments, the suspension solution or suspension reaction does not comprise a significant amount of reducing agent, as further described herein. In certain
5 embodiments, the suspension solution or suspension reaction comprises neither a significant amount of denaturing agent nor a significant amount of reducing agent.

Solubilization of denatured protein

A suspension of denatured proteins, e.g., an IB suspension, (obtained, e.g., as
10 described above) may be combined with a solubilization buffer to thereby obtain a composition comprising solubilized denatured protein, e.g., solubilized IBs. In certain embodiments, a the denatured protein, e.g., in the form of a pellet, is directly combined with solubilization buffer, without prior suspension. The denatured protein, e.g., in the form of a suspension of denatured proteins, may be incubated with solubilization buffer
15 under conditions sufficient to substantially solubilize the protein. Incubation may take place under conditions of concentration, incubation time, and incubation temperature to allow solubilization of the desired amount or most or substantially all the protein (e.g., at least 70%, 80%, 90%, 95%, 97%, 98% or 99%).

In certain embodiments, a solubilization buffer comprises a buffering agent
20 suitable for maintaining the pH of the solubilization buffer and/or that of the composition comprising the solubilization buffer and denatured protein (“solubilization reaction”) in a range of pH 10 to 13. The pH of the solubilization buffer and/or the solubilization reaction may also be within the following ranges of pH: pH 10.5 to 13; pH 11 to 13; pH 11 to 12.8; pH 11.5 to 12.8; pH 11.8 to 12.6; pH 12.0 to 12.6; pH 12.0 to 12.4 and pH
25 12.2 to 12.5. Exemplary pHs of solubilization buffers and/or solubilization reactions include pH 12.0; pH 12.1; pH 12.2; pH 12.3; pH 12.4 and pH 12.5.

A solubilization buffer may comprise Arginine (or another positively charged amino acid), e.g., L-arginine/HCl (which is encompassed by the term “Arginine”). A solubilization buffer may comprise Arginine at a concentration that is sufficient for
30 buffering the solubilization buffer at the desired pH, e.g., a pH in the range of pH 10.5 to 13; such as pH 12.0 to pH 12.5. Arginine may be present at concentrations in the range of 50 mM to 500 mM; 100 mM to 500 mM; 200 mM to 500 mM; 300 mM to 500 mM;

350 mM to 450 mM. In certain embodiments, the solubilization buffer includes Arginine at a concentration of 300 mM to 400 mM. In certain embodiments, a solubilization buffer comprises Arginine at 50 mM to 500 mM and has a pH in the range of 10.5 to 13. In certain embodiments, a solubilization buffer comprises Arginine at 200 mM to 500 mM and has a pH in the range of 12 to 12.4.

As Arginine buffers the pH of a solution to an alkaline value, it is not necessary to include another buffer in the solubilization buffer. However, in certain embodiments, one may include one or more of the following buffers: TRIS (Tris[hydroxymethyl]aminomethane), HEPES (N-[2-Hydroxyethyl]piperazine-N'-[3-propane-sulfonic acid]), CAPSO (3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid), AMP (2-Amino-2-methyl-1-propanol), CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), CHES (2-[N-Cyclohexylamino]ethanesulfonic acid), arginine, lysine, and sodium borate. In certain embodiments, the solubilization buffer comprises a buffer, e.g., TRIS, at a concentration of 1 mM to 1 M; 1 mM to 100 mM; 10 mM to 100 nM; 10 mM to 50 mM; 50 mM to 100 mM; 30 mM to 70 mM; or 40 mM to 60 mM. In certain embodiments, the solubilization buffer comprises a buffer, e.g., TRIS, at a concentration of about 50 mM. In certain embodiments, the solubilization buffer comprises TRIS, e.g., at 40-60 mM, and has a pH in the range of pH 12.0 to 12.4.

The solubilization buffer may comprise TRIS and Arginine and have a pH in the range of 12.0 to 12.4. In certain embodiments, the solubilization buffer comprises TRIS at a concentration in the range of 10 mM to 100 mM; Arginine; and have a pH in the range of pH 12.0 to 12.4. The solubilization buffer may comprise TRIS and Arginine, wherein Arginine is at a concentration in the range of 300 mM to 500 mM; and has a pH in the range of 12.0 to 12.4. The solubilization buffer may comprise TRIS at a concentration in the range of 10 mM to 100 mM; Arginine at a concentration in the range of 300 mM to 500 mM; and have a pH in the range of pH 12.0 to 12.4. In certain embodiments, the solubilization buffer comprises TRIS at a concentration in the range of 30 mM to 70 mM; Arginine at a concentration in the range of 300 mM to 500 mM; and has a pH in the range of pH 12.0 to 12.4. In certain embodiments, the solubilization buffer comprises TRIS at a concentration of about 50 mM, Arginine at a concentration of about 400 mM, and has a pH of about 12.2.

The composition comprising the suspended denatured protein, e.g., suspended IBs, may be combined with solubilization buffer at a weight (in grams) to volume (in ml) ratio of weight of denatured protein (e.g., IBs): volume of solubilization buffer of 1:5-50; 1:10-50; 1:10-30; 1:15-25. Exemplary ratios include 1:10, 1:20 and 1:30. For example, for 1 gram of denatured protein (that was suspended in suspension solution), 5-50 ml; 10-50 ml; 10 to 30 ml or 15 to 25 ml of solubilization buffer may be added. In other embodiments, for 1 kg of denatured protein (that was suspended in suspension solution), 5-50 liters; 10-50 liters; 10 to 30 liters or 15 to 25 liters of solubilization buffer may be added.

The solubilization reaction may be conducted at a temperature, e.g., ranging from 2 °C to 40 °C; 4 °C to 37 °C; 25 °C to 37 °C; room temperature; or 4 °C to 25 °C. In certain embodiments, the solubilization reaction is conducted at room temperature (e.g., 25 °C) at a weight (in grams) to volume (in ml) ratio of weight of denatured protein (e.g., IBs): volume of solubilization buffer of 1:10-30, e.g., 1:10, 1:20 or 1:30.

The composition comprising the suspended denatured proteins and the solubilization buffer (the “solubilization reaction”) is incubated, and optionally stirred, for a time sufficient to solubilize essentially all (e.g., at least 70%, 80%, 90%, 95%, 97%, 98% or 99%) of the protein, e.g., prior to adding the refold buffer. The solubilization reaction may be incubated for less than 1 minute; 1 minute to 6 hours; 1 minute to 5 hours; 1 minute to 3 hours; 1 minute to 2 hours; 1 to 60 minutes; 1 to 30 minutes; 1 to 20 minutes; 1 to 10 minutes; 1 to 5 minutes; 1 to 3 minutes; 1 to 2 minutes; 2 to 5 minutes or 2 to 3 minutes, e.g., prior to adding the refold buffer. The solubilization reaction is preferably performed for a time frame sufficient for most proteins to be solubilized. That most or essentially all of the proteins have been solubilized in the solubilization buffer can be determined optically, as the solution becomes clear (transparent) once all or most of the proteins have been solubilized. At the same time, it is preferable to keep the incubation time of the solubilization reaction as short as possible as deamidation occurs at high pH values. For example, incubation of the solubilization reaction may be conducted for a time that results in less than 15%; 12%; 10%; 7%; 5%; 3%; 2% or 1% deamidation (corresponding, e.g., to total additive percentage of deamidation and isoaspartate formation at multiple sites). Deamidation can be measured, e.g., by Liquid Chromatography Mass Spectrometry (LCMS)/peptide map analysis.

In certain embodiments, a suspension of denatured proteins, e.g., IBs, is combined, and optionally mixed, with a solubilization buffer comprising Arginine at a concentration in the range of 300 mM to 500 mM and optionally TRIS at a concentration in the range of 30 mM to 70 mM; and having a pH in the range of 12.0 to 12.4 at a ratio of weight (grams) of denatured protein:volume (ml) of solubilization buffer of 1:10-30, at room temperature; and wherein incubation is conducted for 2 to 5 minutes prior to, e.g., the addition of refold buffer, to thereby obtain a composition comprising solubilized denatured proteins, e.g., solubilized IBs.

In certain embodiments, the solubilization buffer or solubilization reaction does not comprise a significant amount of denaturing agent, as further described herein. In certain embodiments, the solubilization buffer or solubilization reaction does not comprise a significant amount of reducing agent, as further described herein. In certain embodiments, the solubilization buffer or solubilization reaction comprises neither a significant amount of denaturing agent nor a significant amount of reducing agent.

Protein concentration during the solubilization step may be about 1mg/ml to about 60 mg/ml, e.g., about 10-50 mg/ml.

Refolding of denatured protein

A composition comprising solubilized denatured protein, e.g., a composition comprising solubilized IBs, (obtained, e.g., as described above) may be combined with a refold buffer to thereby obtain a composition comprising refolded protein. The composition comprising solubilized denatured proteins may be incubated with refold buffer under conditions sufficient to substantially refold the protein. Incubation may take place under conditions of concentration, incubation time, and incubation temperature to allow refolding of the desired amount or most or substantially all the protein (e.g., at least 70%, 80%, 90%, 95%, 97%, 98% or 99%).

In certain embodiments, a refold buffer comprises a buffering agent suitable for maintaining the pH of the refold buffer and/or that of the composition comprising the solubilized refold buffer and solubilized protein (the "refold reaction") in a range of pH of 9 to 11. The pH of the refold buffer and/or the refold reaction may also be within the following ranges of pH: pH 9 to 11; pH 9.5 to 11; pH 10 to 11; pH 10 to 10.5; pH 10 to

10.8; and pH 10.2 to 10.6. Exemplary pHs of refold buffers and/or refold reactions include pH 10.0; pH 10.1; pH 10.2; pH 10.3; pH 10.4; pH 10.5; pH 10.6 and pH 10.7.

5 Refold buffer may comprise Arginine (or another positively charged amino acid), e.g., L-arginine/HCl (which is encompassed by the term "Arginine"). A refold buffer may comprise Arginine at a concentration that is sufficient for buffering the solubilization buffer at the desired pH, e.g., a pH in the range of pH 9 to 11; such as pH 10 to 10.8. Arginine may be present at concentrations in the range of 50 mM to 500 mM; 100 mM to 500 mM; 200 mM to 500 mM; 300 mM to 500 mM; 350 mM to 450 mM. In certain
10 embodiments, the refold buffer includes Arginine at a concentration of 300 mM to 400 mM. In certain embodiments, a refold buffer comprises Arginine at 50 mM to 500 mM and has a pH in the range of 9 to 11. In certain embodiments, a refold buffer comprises Arginine at 200 mM to 500 mM and has a pH in the range of pH 10 to 10.8.

As Arginine is buffering the refold solution at pH 10.4, it is not necessary to include another buffer. However, if desired, any of the following buffers may be added:
15 TRIS (Tris[hydroxymethyl]aminomethane), HEPPS (N-[2-Hydroxyethyl]piperazine-N'-[3-propane-sulfonic acid]), CAPSO (3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid), AMP (2-Amino-2-methyl-1-propanol), CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), CHES (2-[N-Cyclohexylamino]ethanesulfonic acid), arginine, lysine, and sodium borate. In certain embodiments, the refold buffer includes a buffer,
20 e.g., TRIS, at a concentration of 1 mM to 1 M; 1 mM to 100 mM; 10 mM to 100 nM; 10 mM to 50 mM; 50 mM to 100 mM; 30 mM to 70 mM; or 40 mM to 60 mM. In certain embodiments, the refold buffer comprises a buffer, e.g., TRIS, at a concentration of about 50 mM. In certain embodiments, the refold buffer comprises TRIS, e.g., at 40-60 mM, and has a pH in the range of pH 10.2 to 10.6.

25 The refold buffer may comprise TRIS and Arginine and have a pH in the range of pH 10.2 to 10.6. In certain embodiments, the refold buffer comprises TRIS at a concentration in the range of 10 mM to 100 mM; Arginine; and has a pH in the range of pH 10.2 to 10.6. The refold buffer may comprise TRIS and Arginine, wherein Arginine is at a concentration in the range of 300 mM to 500 mM; and have a pH in the range of
30 pH 10.2 to 10.6. The refold buffer may comprise TRIS at a concentration in the range of 10 mM to 100 mM; Arginine at a concentration in the range of 300 mM to 500 mM; and have a pH in the range of pH 10.2 to 10.6. In certain embodiments, the refold buffer

comprises TRIS at a concentration in the range of 30 mM to 70 mM; Arginine at a concentration in the range of 300 mM to 500 mM; and have a pH in the range of pH 10.2 to 10.6. In certain embodiments, the solubilization buffer comprises TRIS at a concentration of about 50 mM, Arginine at a concentration of about 400 mM, and has a pH of about 10.4.

In embodiments, in which the protein to be refolded comprise one or more disulfide bonds when properly folded, the refold buffer may also comprise an oxidizing agent to facilitate the formation of disulfide bonds. For refolding proteins that do not comprise a disulfide bond, it is not necessary to include an oxidizing agent. In certain embodiments, the oxidizing agent comprises glutathione, e.g., in a ratio of oxidized glutathione: reduced glutathione of about 5:1 or a similar ratio sufficient to facilitate the formation of disulfide bonds. In certain embodiments, a refold buffer comprises 0.1 mM to 10 mM of oxidized glutathione and 0.02 mM to 2 mM of reduced glutathione. In certain embodiments, a refold buffer comprises 0.5 mM to 2 mM of oxidized glutathione and 0.1 to 0.4 mM of reduced glutathione. In certain embodiments, a refold buffer comprises about 1 mM of oxidized glutathione and about 0.2 mM of reduced glutathione. Other oxidizing agents known in the art may also be used.

A composition comprising solubilized protein may be combined with refold buffer at a ratio of volume (ml) of solubilization buffer used to solubilize the denatured protein: volume (ml) of refold buffer of 1:1-50; 1:1-20; 1:1-10; 1:1-5; 1:2-10; 1:2-8; or 1:2-5. Exemplary ratios include about 1:1, 1:2, 1:3, 1:4; 1:5 or 1:6.

The refold reaction may be conducted at a temperature, e.g., ranging from 2 °C to 40 °C; 4 °C to 37 °C; 25 °C to 37 °C; room temperature; or 4 °C to 25 °C. In certain embodiments, the refold reaction is conducted at room temperature (e.g., 25 °C) at a ratio of volume (ml) of solubilization buffer used to solubilize the denatured protein: volume (ml) of refold buffer of 1:1-5, e.g., 1:1, 1:3 or 1:5.

Refold occurs essentially instantaneously, and is generally performed for a time frame sufficient for most proteins to be refolded. In certain embodiments, the refold reaction may be incubated (e.g., with or without stirring), e.g., overnight; for 1 minute to 12 hours; 1 minute to 6 hours; 1 minute to 3 hours; 1 to 120 minutes; 1 to 30 minutes; 1 to 20 minutes; 1 to 10 minutes; 1 to 100 minutes; 10 to 100 minutes; 10 to 80 minutes; 20 to 60 minutes prior to, e.g., adjusting the pH down. Incubation may be performed with or

without stirring. In certain embodiments, the refold reaction is stirred and then incubated without stirring.

In certain embodiments, a composition comprising solubilized proteins is combined with a refold buffer comprising Arginine at a concentration in the range of 300 mM to 500 mM; oxidized glutathione at a concentration in the range of 0.5 mM to 2 mM; (optionally TRIS at a concentration in the range of 30 mM to 70 mM) reduced glutathione at a concentration in the range of 0.1 to 0.4 mM, and having a pH in the range of 10.2 to 10.6, at a volume ratio of solubilization buffer: refold buffer of 1:1-5, and incubated at room temperature for 1 minute to overnight, prior to, e.g., adjusting the pH to a lower value.

In certain embodiments, the refold buffer or refold reaction does not comprise a significant amount of denaturing agent, as further described herein. In certain embodiments, the refold buffer or refold reaction does not comprise a significant amount of reducing agent (except when co-administered together with an oxidant, e.g., when oxidized and reduced glutathione are added together), as further described herein. In certain embodiments, the refold buffer or refold reaction comprises neither a significant amount of denaturing agent nor a significant amount of reducing agent.

Following the refolding reaction, the pH may be adjusted to a lower value, e.g., pH 6 to 8 or pH 7 to 8. In certain embodiments, the pH is adjusted to about pH 8. In certain embodiments, adjusting the pH down to about pH 8 comprises adding 0.3 fold volume of 1M HCl. The addition of the HCl may be conducted slowly, e.g., over 0.5 to 2 minutes.

Following the adjustment to a lower pH, the reaction mixture may be incubated for 30 minutes to 3 hours; for 30 minutes to 2 hours, or overnight, prior to a next step, e.g., a purification step. A refolded protein may be further processed, e.g., purified, according to methods known in the art, e.g., using protein A chromatography and other types of chromatography or purification methods.

Protein concentration during the refold step may be from 1 mg/ml or less to about 10 mg/ml. For example, the protein concentration may be about 5 mg/ml, 6 mg/ml or 7 mg/ml.

In certain embodiments, total recovery of protein refolded as described herein may be greater than 70% or 80% as measured by reverse phase chromatography. Total

downstream processing recoveries may be as high as 20%, 30%, 40% or more from solubilization of the denatured protein, e.g., IBs, through final chromatography.

During standard expression and purification of recombinant protein comprising an Fc, the disulfide bond of the CH3 loop breaks in about 0.5-5% of the protein composition.

5 Using certain methods described herein, the CH3 open loop has been shown to be in the range of 0.5-3% in the refolded proteins. Accordingly certain Fc containing protein compositions, wherein the proteins have been refolded as described herein, have less than 5%, 4%, 3%, 2%, 1% CH3 open loops. It has also been shown herein that deamidation can be reduced from 40% to 12% (total additive percentage of deamidation and isoasp
10 formation at multiple sites). Accordingly, in certain embodiments, protein refolded using a method described herein comprise less than 40%, 30%, 20%, 15%, or 13% deamidation.

In certain embodiments, refolding denatured protein, e.g., from an IB, is performed in less than 4 hours, 3 hours, or 2 hours.

15 Exemplary methods

A method for refolding a denatured protein, e.g., from an IB, may comprise: (i) suspending a pellet of denatured protein, e.g., an IB pellet, in suspension solution (e.g., water) at a ratio of weight (grams) of denatured protein pellet: volume (ml) of suspension solution of 1:1-3 at room temperature for a time sufficient for most of the denatured
20 protein to be suspended, e.g., 1-10 minutes, to thereby obtain a composition comprising a suspension of denatured protein; (ii) combining (and optionally mixing) the composition comprising a suspension of denatured protein with a solubilization buffer comprising Arginine at a concentration sufficient to buffer the solubilization buffer to a pH in the range of pH 12.0 to 12.4, e.g., in the range of 100 mM to 500 mM (and optionally TRIS
25 at a concentration in the range of 10 mM to 100 mM); at a ratio of weight (grams) of denatured protein: volume (ml) of solubilization buffer of 1:10-30, wherein the incubation is conducted at room temperature for a time sufficient to solubilize most of the denatured protein, e.g., 2 to 5 minutes, to thereby obtain a composition comprising solubilized denatured protein; and (iii) combining the composition comprising solubilized denatured
30 protein with a refold buffer comprising Arginine at a concentration sufficient to buffer the refold buffer to a pH in the range of pH 10.2 to 10.6, e.g., at a concentration in the range of 100 mM to 500 mM (and optionally TRIS at a concentration in the range of 10 mM to

100 mM); a concentration of oxidizing agent sufficient to promote disulfide bond formation, e.g., oxidized glutathione at a concentration in the range of 0.5 mM to 2 mM and reduced glutathione at a concentration in the range of 0.1 mM to 0.4 mM; at a ratio of volume of solubilization buffer used in step (ii): volume of refold buffer of 1:1-5, wherein
5 the incubation is conducted at room temperature for a time sufficient to refold most of the protein, e.g., for 1 minute to overnight; and wherein the method does not comprise using a significant amount of denaturing agent and optionally does not comprise using a significant amount of a reducing agent (other than the reduced agent that is used together with the oxidizing agent).

10 In certain embodiments, a method for refolding a denatured protein, e.g., from an IB, comprises: (i) suspending a pellet of denatured protein, e.g., an IB pellet, in water at a volume ratio of weight (grams) of denatured protein pellet: volume (ml) of water of 1:1-3 at room temperature for 1-10 minutes to thereby obtain a composition comprising a suspension of denatured protein; (ii) combining and mixing the composition comprising a
15 suspension of denatured protein with a solubilization buffer comprising Arginine at a concentration in the range of 300 mM to 500 mM (and optionally TRIS at a concentration in the range of 30 mM to 70 mM); and having a pH in the range of pH 12.0 to 12.4; at a ratio of weight (grams) of denatured protein: volume (ml) of solubilization buffer of 1:10-30, wherein the incubation is conducted at room temperature for 2 to 5 minutes, to
20 thereby obtain a composition comprising solubilized denatured protein; and (iii) combining the composition comprising solubilized denatured protein with a refold buffer comprising Arginine at a concentration in the range of 300 mM to 500 mM (and optionally TRIS at a concentration in the range of 30 mM to 70 mM); oxidized glutathione at a concentration in the range of 0.5 mM to 2 mM; reduced glutathione at a
25 concentration in the range of 0.1 mM to 0.4 mM; and having a pH in the range of pH 10.2 to 10.6; at a ratio of volume of solubilization buffer used in step (ii): volume of refold buffer of 1:1-5, wherein the incubation is conducted at room temperature for 1 to 120 minutes; and wherein the method does not comprise using a significant amount of denaturing agent and optionally does not comprising using a significant amount of a
30 reducing agent (other than the reduced agent that is used together with the oxidizing agent).

Generally, the margin of error of pH for a prepared buffer solution is +/- 0.1 units.

Exemplary proteins

Proteins that may be refolded from a denatured state, e.g., from IBs, using the methods described herein include any protein that is in a denatured form, e.g., proteins comprising at least one disulfide bond in their native state. Proteins without disulfide bonds may also be refolded as described herein. Proteins may comprise a binding domain that specifically binds to a target protein. A protein may be a naturally occurring protein or a genetically engineered or fusion protein. An exemplary protein that may be refolded as described herein is an Fc containing protein, such as an Fc fused to a heterologous domain (e.g., a non-Fc or non-antibody domain). A heterologous protein may be any protein, including an antigen binding portion of an antibody and derivatives thereof, e.g., Fabs, scFvs, bispecific scFvs, single domain antibodies (“sdAbs”) (e.g., V_HH or camelid antibodies and V_{NARS}), diabodies (dAbs), single chain diabodies (scDb), Darpins, anticalins, and fibronectin based scaffolds, such as ¹⁰F_n3, Fibcons and Tencons. A full length antibody may also be refolded as described herein. Heterologous proteins linked to Fc may also be unrelated to antibodies and may be, e.g., TNFR.

Fibronectin Based Scaffolds

As used herein, a “fibronectin based scaffold” or “FBS” protein or moiety refers to proteins or moieties that are based on a fibronectin type III (“Fn3”) repeat. Fn3 is a small (about 10 kDa) domain that has the structure of an immunoglobulin (Ig) fold (i.e., an Ig-like β-sandwich structure, consisting of seven β-strands and six loops). Fibronectin has 18 Fn3 repeats, and while the sequence homology between the repeats is low, they all share a high similarity in tertiary structure. Fn3 domains are also present in many proteins other than fibronectin, such as adhesion molecules, cell surface molecules, e.g., cytokine receptors, and carbohydrate binding domains. For reviews see Bork & Doolittle, Proc Natl Acad Sci USA 89(19):8990-4 (1992); Bork et al., J Mol Biol. 242(4):309-20 (1994); Campbell & Spitzfaden, Structure 2(5):333-7 (1994); Harpez & Chothia, J Mol Biol. 238(4):528-39 (1994)). The term “fibronectin based scaffold” protein or moiety is intended to include scaffolds based on Fn3 domains from these other proteins (i.e., non fibronectin molecules).

An example of fibronectin-based scaffold proteins are Adnectins (Adnexus, a wholly owned subsidiary of Bristol-Myers Squibb). It has been shown that the CDR-like loop regions of the fibronectin based scaffolds can be modified to evolve a protein capable of binding to any compound of interest. For example, U.S. Patent No. 7,115,396 describes Fn3 domain proteins wherein alterations to the BC, DE, and FG loops result in high affinity TNF α binders. U.S. Patent No. 7,858,739 describes Fn3 domain proteins wherein alterations to the BC, DE, and FG loops result in high affinity VEGFR2 binders.

An Fn3 domain is small, monomeric, soluble, and stable. It lacks disulfide bonds and, therefore, is stable under reducing conditions. Fn3 domains comprise, in order from N-terminus to C-terminus, a beta or beta-like strand, A; a loop, AB; a beta or beta-like strand, B; a loop, BC; a beta or beta-like strand, C; a loop, CD; a beta or beta-like strand, D; a loop, DE; a beta or beta-like strand, E; a loop, EF; a beta or beta-like strand, F; a loop, FG; and a beta or beta-like strand, G. The seven antiparallel β -strands are arranged as two beta sheets that form a stable core, while creating two “faces” composed of the loops that connect the beta or beta-like strands. Loops AB, CD, and EF are located at one face (“the south pole”) and loops BC, DE, and FG are located on the opposing face (“the north pole”). Any or all of loops AB, BC, CD, DE, EF and FG may participate in ligand binding.

In exemplary embodiments, the ligand binding fibronectin based scaffold moieties described herein are based on the tenth fibronectin type III domain, i.e., the tenth module of Fn3 (¹⁰Fn3). The amino acid sequence of wild-type human ¹⁰Fn3 (with N-terminal tail (in italics)) is set forth in SEQ ID NO: 1:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKS
TATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIDKPSQ (SEQ ID NO: 1)

(the AB, CD and EF loops are underlined; the BC, FG, and DE loops are emphasized in bold; the β -strands are located between each of the loop regions; and the N-terminal and C-terminal regions are shown in italics). Wild-type ¹⁰Fn3 without the tail set forth in italics in SEQ ID NO: 1 is provided as SEQ ID NO: 5.

In some embodiments, the AB loop corresponds to residues 14-17, the BC loop corresponds to residues 23-31, the CD loop corresponds to residues 37-47, the DE loop corresponds to residues 51-56, the EF loop corresponds to residues 63-67, and the FG loop corresponds to residues 75-87 of SEQ ID NO: 1. The BC, DE and FG loops align

along one face of the molecule, *i.e.* the “north pole”, and the AB, CD and EF loops align along the opposite face of the molecule, *i.e.* the “south pole”. In SEQ ID NO: 1, β -strand A corresponds to residues 8-13, β -strand B corresponds to residues 18-22, β -strand C corresponds to residues 32-36, beta strand D corresponds to residues 48-50, β -strand E corresponds to residues 57-62, β -strand F corresponds to residues 68-74, and β -strand G corresponds to residues 88-92. The β -strands are connected to each other through the corresponding loop, e.g., strands A and B are connected via loop AB in the formation β -strand A, loop AB, β -strand B, etc. The N-terminal and/or C-terminal regions of SEQ ID NO: 1 (italicized above), may be removed or altered to generate a molecule retaining biological activity and comprising, e.g., an amino acid sequence selected from the group consisting of SEQ ID NOs: 2-16. In certain embodiments, the first 8 amino acid residues of SEQ ID NO: 1 and/or the last 7 amino acid residues of SEQ ID NO: 1 (*i.e.*, amino acid residues 1-8 and 95-101 of SEQ ID NO: 1, respectively) may be removed or altered to generate a polypeptide comprising the amino acid sequence of SEQ ID NO: 4.

As described above, amino acid residues corresponding to residues 14-17, 23-31, 37-47, 51-56, 63-67 and 75-87 of SEQ ID NO: 1 define the AB, BC, CD, DE, EF and FG loops, respectively. However, it should be understood that not every residue within a loop region needs to be modified in order to achieve a $^{10}\text{Fn3}$ binding domain having strong affinity for a desired target. Additionally, insertions and deletions in the loop regions may also be made while still producing high affinity $^{10}\text{Fn3}$ binding domains.

Accordingly, in some embodiments, one or more loops selected from AB, BC, CD, DE, EF and FG may be extended or shortened in length relative to the corresponding loop in wild-type human $^{10}\text{Fn3}$. In any given polypeptide, one or more loops may be extended in length, one or more loops may be reduced in length, or combinations thereof. In some embodiments, the length of a given loop may be extended by 2-25, 2-20, 2-15, 2-10, 2-5, 5-25, 5-20, 5-15, 5-10, 10-25, 10-20, or 10-15 amino acids. In some embodiments, the length of a given loop may be reduced by 1-15, 1-11, 1-10, 1-5, 1-3, 1-2, 2-10, or 2-5 amino acids. In particular, the FG loop of $^{10}\text{Fn3}$ is 13 residues long, whereas the corresponding loop in antibody heavy chains ranges from 4-28 residues. To optimize antigen binding in polypeptides relying on the FG for target binding, therefore, the length of the FG loop of $^{10}\text{Fn3}$ may be altered in length as well as in sequence to obtain the greatest possible flexibility and affinity in target binding.

In some embodiments, one or more residues of the integrin-binding motif “arginine-glycine-aspartic acid” (RGD) (amino acids 78-80 of SEQ ID NO: 1) may be substituted so as to disrupt integrin binding. In some embodiments, the FG loop of the polypeptides provided herein does not contain an RGD integrin binding site. In one
5 embodiment, the RGD sequence is replaced by a polar amino acid-neutral amino acid-acidic amino acid sequence (in the N-terminal to C-terminal direction). In another embodiment, the RGD sequence is replaced with SGE. In yet another embodiment, the RGD sequence is replaced with RGE (see, e.g., SEQ ID NO: 16).

As used herein, a “¹⁰F_n3 domain” or “¹⁰F_n3 moiety” refers to wild-type ¹⁰F_n3
10 (e.g., comprising one of SEQ ID NOs: 1-8, 10, 12, 14 or 16) and biologically active variants thereof, e.g., biologically active variants that specifically bind to a target, such as a target protein and biologically active variants having SEQ ID NO: 9, 11, 13 or 15. A wild type ¹⁰F_n3 domain may comprise one of the amino acid sequenced set forth in SEQ ID NO: 1-8, 10, 12, 14 and 16. Biologically active variants of a ¹⁰F_n3 domain include
15 ¹⁰F_n3 domains that comprise at least, at most or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40 or 45 amino acid changes, i.e., substitutions, additions or deletions, relative to a ¹⁰F_n3 domain comprising any one of SEQ ID NOs: 1-16. A biologically active variant of a ¹⁰F_n3 domain may also comprise, or comprise at most, 1-3, 1-5, 1-10, 1-15, 1-10, or 1-25 amino acid changes relative to a
20 ¹⁰F_n3 domain comprising any one of SEQ ID NOs: 1-16. In certain embodiments, a biologically active variant of a ¹⁰F_n3 domain does not comprise more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40 or 45 amino acid changes, i.e., substitutions, additions or deletions, relative to an ¹⁰F_n3 domain comprising any one of SEQ ID NOs: 1-16. Amino acid changes may be in a loop region
25 and/or in a strand. Exemplary degenerate ¹⁰F_n3 amino acid sequences are provided herein as SEQ ID NOs: 17-29.

In some embodiments, a fibronectin based scaffold moiety comprises a ¹⁰F_n3 domain having at least 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% identity to a human ¹⁰F_n3 domain having an amino acid sequence selected from the group
30 of sequence comprising SEQ ID NOs: 1-16. In certain embodiments, the fibronectin based scaffold moiety provided herein have at least 50% identity to an amino acid sequence selected from the group of amino acid sequences comprising SEQ ID NO: 1-16.

In other embodiments, the fibronectin based scaffold moiety has at least 65% identity to an amino acid sequence selected from the group of amino acid sequences comprising SEQ ID NO: 1-16. In certain embodiments, one or more of the loops will not be modified relative to the sequence of the corresponding loop of the wild-type sequence and/or one or more of the β -strands will not be modified relative to the sequence of the corresponding β -strand of the wild-type sequence. In certain embodiments, each of the beta or beta-like strands of a 10 F_n3 domain in a fibronectin based scaffold moiety may comprise, consist essentially of, or consist of an amino acid sequence that is at least 80%, 85%, 90%, 95% or 100% identical to the sequence of a corresponding beta or beta-like strand of SEQ ID NO: 1. Preferably, variations in the β -strand regions will not disrupt the stability of the polypeptide in physiological conditions. In exemplary embodiments, the 10 F_n3 domain binds to a desired target with a K_d of less than 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM or less. In some embodiments, the 10 F_n3 domain of a fibronectin based protein scaffold binds to a desired target with a K_d between 1 pM and 1 μ M, between 100 pM and 500 nM, between 1 nM and 500 nM, or between 1 nM and 100 nM. In exemplary embodiments, the fibronectin based scaffold moiety binds specifically to a target that is not bound by a wild-type 10 F_n3 domain, particularly the wild-type human 10 F_n3 domain having, e.g., SEQ ID NO: 1-8, 10, 12, 14 or 16.

In some embodiments, fusion proteins comprise a fibronectin based scaffold moiety comprising a 10 F_n3 domain, wherein the 10 F_n3 domain comprises a loop, AB; a loop, BC; a loop, CD; a loop, DE; a loop, EF; and a loop, FG; and has at least one loop selected from loop AB, BC, CD, DE, EF and FG with an altered amino acid sequence relative to the sequence of the corresponding loop of the human 10 F_n3 domain of SEQ ID NO: 1-16. In some embodiments, the BC, DE and FG loops are altered. In certain embodiments, the AB, CD and EF loops are altered. In certain embodiments, the FG loop is the only loop that is altered. In other embodiments, the CD and FG loops are both altered, and optionally, no other loops are altered. In certain embodiments, the CD and EF loops are both altered, and optionally, no other loops are altered. In some embodiments, one or more specific scaffold alterations are combined with one or more loop alterations. By "altered" is meant one or more amino acid sequence alterations relative to a template sequence (i.e., the corresponding wild-type human fibronectin domain) and includes amino acid additions, deletions, and substitutions.

In some embodiments, the fibronectin based scaffold moiety comprises a ¹⁰Fn3 domain wherein the non loop regions comprise an amino acid sequence that is at least 80, 85, 90, 95, 98, or 100% identical to the non-loop regions of SEQ ID NO: 1, wherein at least one loop selected from AB, BC, CD, DE, EF and FG is altered. For example, in certain embodiments, the AB loop may have up to 4 amino acid substitutions, up to 10 amino acid insertions, up to 3 amino acid deletions, or a combination thereof; the BC loop may have up to 10 amino acid substitutions, up to 4 amino acid deletions, up to 10 amino acid insertions, or a combination thereof; the CD loop may have up to 6 amino acid substitutions, up to 10 amino acid insertions, up to 4 amino acid deletions, or a combination thereof; the DE loop may have up to 6 amino acid substitutions, up to 4 amino acid deletions, up to 13 amino acid insertions, or a combination thereof; the EF loop may have up to 5 amino acid substitutions, up to 10 amino acid insertions, up to 3 amino acid deletions, or a combination thereof; and/or the FG loop may have up to 12 amino acid substitutions, up to 11 amino acid deletions, up to 25 amino acid insertions, or a combination thereof.

In certain embodiments, a fibronectin based scaffold moiety comprises an amino acid sequence that is at least 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to an amino acid sequence selected from the group of sequences consisting of SEQ ID NOs: 1-16, and the fusion protein binds specifically to a target, e.g., with a *K_d* of less than 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM or less. The proteins may comprise amino acid changes (or alterations) in one or more loops and one or more strands.

In certain embodiments, the fibronectin based scaffold moiety comprises a ¹⁰Fn3 domain that is defined generally by following the sequence:

VSDVPRDLEVVA(X)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVYA(X)_zISINYRT (SEQ ID NO: 17),

or by the sequence having SEQ ID NO: 18-29. In SEQ ID NOs: 17-29, the AB loop is represented by (X)_u, the BC loop is represented by (X)_v, the CD loop is represented by (X)_w, the DE loop is represented by (X)_x, the EF loop is represented by (X)_y and the FG loop is represented by X_z. X represents any amino acid and the subscript following the X represents an integer of the number of amino acids. In particular, u, v, w, x, y and z may each independently be anywhere from 2-20, 2-15, 2-10, 2-8, 5-20, 5-15, 5-

10, 5-8, 6-20, 6-15, 6-10, 6-8, 2-7, 5-7, or 6-7 amino acids. The sequences of the beta strands (underlined) may have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, deletions or additions across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NOs: 17-29. In some embodiments, the sequences of the beta strands may have anywhere from 0 to 10, from 0 to 8, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2, or from 0 to 1 substitutions, e.g., conservative substitutions, across all 7 scaffold regions relative to the corresponding amino acids shown in SEQ ID NO: 17-29. In certain embodiments, the hydrophobic core amino acid residues (bolded residues in SEQ ID NO: 17 above) are fixed, and any substitutions, conservative substitutions, deletions or additions occur at residues other than the hydrophobic core amino acid residues. In some embodiments, the hydrophobic core residues of the polypeptides provided herein have not been modified relative to the wild-type human ¹⁰F_n3 domain (e.g., SEQ ID NO: 1 or 5).

In some embodiments, the amino acid sequences of the N-terminal and/or C-terminal regions of a fibronectin based scaffold moiety may be modified by deletion, substitution or insertion relative to the amino acid sequences of the corresponding regions of ¹⁰F_n3 domains comprising one of SEQ ID NOs: 1-16). In some embodiments, the first eight (i.e., residues 1-8) and the last seven amino acids (i.e., residues 95-101) of SEQ ID NO: 1 are deleted, generating a ¹⁰F_n3 domain having the amino acid sequence of SEQ ID NO: 4. Additional sequences may also be added to the N- or C-terminus of a ¹⁰F_n3 domain having the amino acid sequence of any one of SEQ ID NOs: 1-16. For example, in some embodiments, the N-terminal extension consists of an amino acid sequence selected from the group consisting of: M, MG, and G.

In certain embodiments, the amino acid sequence of the first 1, 2, 3, 4, 5, 6, 7, 8 or 9 residues of SEQ ID NO: 1 may be modified or deleted in the polypeptides provided herein relative to the sequence of the corresponding amino acids in the wild-type human ¹⁰F_n3 domain having SEQ ID NO: 1. In exemplary embodiments, the amino acids corresponding to amino acids 1-8 of SEQ ID NO: 1 are replaced with an alternative N-terminal region having from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Exemplary alternative N-terminal regions include (represented by the single letter amino acid code) M, MG, G, MGVSDVPRDL (SEQ ID NO: 30) and GVSDVPRDL (SEQ ID NO: 31), or N-terminal truncations of any one of SEQ ID NOs: 30 and 31.

Other suitable alternative N-terminal regions include, for example, X_nSDVPRDL (SEQ ID NO: 32), X_nDVPRDL (SEQ ID NO: 33), X_nVPRDL (SEQ ID NO: 34), X_nPRDL (SEQ ID NO: 35), X_nRDL (SEQ ID NO: 36), X_nDL (SEQ ID NO: 37), or X_nL, wherein n = 0, 1 or 2 amino acids, wherein when n = 1, X is Met or Gly, and when n = 2, X is Met-Gly. When a Met-Gly sequence is added to the N-terminus of a ¹⁰Fn3 domain, the M will usually be cleaved off, leaving a G at the N-terminus. In other embodiments, the alternative N-terminal region comprises the amino acid sequence MASTSG (SEQ ID NO: 38).

In certain embodiments, the amino acid sequence corresponding to amino acids 93-101, 94-101, 95-101, 96-101, 97-101, 98-101, 99-101, 100-101, or 101 of SEQ ID NO: 1 are deleted or modified in the polypeptides provided herein relative to the sequence of the corresponding amino acids in the wild-type human ¹⁰Fn3 domain (SEQ ID NO: 1). In exemplary embodiments, the amino acids corresponding to amino acids 95-101 of SEQ ID NO: 1 are replaced with an alternative C-terminal region having from 1-20, 1-15, 1-10, 1-8, 1-5, 1-4, 1-3, 1-2, or 1 amino acids in length. Specific examples of alternative C-terminal region sequences include, for example, polypeptides comprising, consisting essentially of, or consisting of, EIEK (SEQ ID NO: 39), EGSGC (SEQ ID NO: 40), EIEKPCQ (SEQ ID NO: 41), EIEKPSQ (SEQ ID NO: 42), EIEKP (SEQ ID NO: 43), EIEKPS (SEQ ID NO: 44), EIEKPC (SEQ ID NO: 45), or HHHHHH (SEQ ID NO: 46). In some embodiments, the alternative C-terminal region comprises EIDK (SEQ ID NO: 47), and in particular embodiments, the alternative C-terminal region is either EIDKPCQ (SEQ ID NO: 48) or EIDKPSQ (SEQ ID NO: 49).

In certain embodiments, a fibronectin based scaffold moiety comprises a ¹⁰Fn3 domain having both an alternative N-terminal region sequence and an alternative C-terminal region sequence.

In certain embodiments, a fibronectin based scaffold moiety is based on an Fn3 repeat other than the 10th repeat of the type III domain of fibronectin, e.g., human fibronectin. For example, a fibronectin based scaffold moiety may be similar to any of the other fibronectin type III repeats, e.g., the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 11th, 12th, 13th, 14th, 15th, 16th, 17th, and 18th Fn3 repeats. In yet other embodiments, a fibronectin based scaffold moiety may be from a molecule other than fibronectin. Exemplary fibronectin based scaffold moieties may be derived from tenascin, a protein that is

composed of 15 Fn3 domains with similar sequence similarities to one another as found in fibronectin. These repeats are described, e.g., in Jacobs et al. (2012) Protein Engineering, Design & Selection 25:107. Based on the homology of the repeats in the fibronectin molecule and those in the tenascin molecule, artificial molecules based on these homologies have been created. The consensus amino acid sequences based on the homology of the domains in the fibronectin molecule are referred to as Fibcon and FibconB (WO2010/093627 and Jacobs et al. (2012) *supra*) and those based on the homology of the domains in the tenascin molecule are referred to as Tencon. An exemplary Fibcon amino acid sequence comprises the following amino acid sequence:

10 MPAPDLRFTNETPSSLLISWTPPRVQITGYIIRYGPVGS DGRVKEFTVPPSVSSATI
 TGLKPGTEYTISVIALKDNQESEPLRGRVTTGG (FibconB; SEQ ID NO: 50), wherein loop AB consists of amino acids 13-16 (TPSS; SEQ ID NO: 51), loop BC consists of amino acids 22-28 (TPPRVQI; SEQ ID NO: 52), loop CD consists of amino acids 38-43 (VGSDGR; SEQ ID NO: 53), loop DE consists of amino acids 51-54 (PSVS; SEQ ID
 15 NO: 54), loop EF consists of amino acids 60-64 (GLKPG; SEQ ID NO: 55) and loop FG consist of amino acids 75-81 (KDNQESEP; SEQ ID NO: 56). Another Fibcon amino acid sequence comprises the following amino acid sequence:

LDAPDLQVTNVTDTTSITVSWTPPSATITGYRITYTPSNGPGEPKELTVPPSSTSVTI
 TGITPGVEYVVS VYALKDNQESPLVGTCTT (SEQ ID NO: 57; Jacobs et al., *supra*).

20 Tenascin derived Fn3 proteins include Tencons (WO2010/051274, WO2010/051310 and WO2011/137319, which are specifically incorporated by reference herein). An exemplary Tencon protein has the following amino acid sequence:
 LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVG EAINLTVPGSERSY
 DLTGLKPGTEYTVSIYGVKGGHRSNPLSAEFTT (SEQ ID NO: 58; Jacobs et al.,
 25 *supra*, and WO2011/137319), wherein loop AB consists of amino acids 13-16 (TEDS; SEQ ID NO: 59, loop BC consists of amino acids 22-28 (TAPDAAF; SEQ ID NO: 60), loop CD consists of amino acids 38-43 (SEKVGE; SEQ ID NO: 61), loop DE consists of amino acids 51-54 (GSER; SEQ ID NO: 62), loop EF consists of amino acids 60-64 (GLKPG; SEQ ID NO: 63) and loop FG consists of amino acids 75-81 (KGGHRSN;
 30 SEQ ID NO: 64).

A Fibcon, FibconB or Tencon moiety, or target binding variants thereof, whether by themselves or linked to a heterologous moiety, e.g., an Fc, may be refolded as

described herein. Fn3 domains from other proteins, e.g., cell surface hormone and cytokine receptors, chaperonins, and carbohydrate-binding domains, may also be refolded as described herein, either on their own or as part of a fusion protein to, e.g., Fc.

Fibronectin based scaffold proteins or moieties are described, e.g., in
5 WO2010/093627, WO2011/130324, WO2009/083804, WO2009/133208, WO02/04523,
WO2012/016245, WO2009/023184, WO2010/051310, WO2011/020033,
WO2011/051333, WO2011/051466, WO2011/092233, WO2011/100700,
WO2011/130324, WO2011/130328, WO2011/137319, WO2010/051274,
WO2009/086116, WO09/058379 and WO2013/067029 (all of which are specifically
10 incorporated by reference herein, in particular, the various types of molecules are
specifically incorporated by reference herein): any of the fibronectin based scaffold
proteins or moieties described in these publications may be refolded as described herein.

In certain embodiments, a protein that may be refolded as described herein is a
multivalent protein that comprises two or more fibronectin based scaffold moieties, e.g.,
15 ¹⁰F_n3 domains. For example, a multivalent fusion protein may comprise 2, 3 or more
fibronectin based scaffold moieties, e.g., ¹⁰F_n3 domains, that are covalently associated.
In exemplary embodiments, the fusion protein is a bispecific or dimeric protein
comprising two ¹⁰F_n3 domains.

20 **Fc domains**

Proteins that may be refolded as described herein include fusion proteins that
comprise an Fc portion fused to a heterologous portion. In some aspects, the heterologous
portion is a fibronectin based scaffold, e.g., an ¹⁰F_n3 domain, however, the heterologous
portion may be any other protein.

25 As used herein, “Fc portion” encompasses domains derived from the constant
region of an immunoglobulin, preferably a human immunoglobulin, including a fragment,
analog, variant, mutant or derivative of the constant region. Suitable immunoglobulins
include IgG1, IgG2, IgG3, IgG4, and other classes such as IgA, IgD, IgE and IgM. The
constant region of an immunoglobulin is defined as a naturally-occurring or synthetically-
30 produced polypeptide homologous to the immunoglobulin C-terminal region, and can
include a CH1 domain, a hinge, a CH2 domain, a CH3 domain, or a CH4 domain,
separately or in combination. The term “Fc moiety” or “Fc domain” as used herein refers

to any of the combination of CH1, hinge, CH2, CH3 and CH4 domains. Thus, an “Fc domain” or moiety may or may not comprise a hinge.

Shown below is the sequence of a human IgG1 immunoglobulin constant region, and the relative position of each domain within the constant region are indicated based on the EU numbering format:

*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSQVHTEP
AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP
APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
10 KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*
(SEQ ID NO: 65). The core hinge sequence is underlined, and the CH1 region is

italicized; the CH2 and CH3 regions are in regular text. It should be understood that the C-terminal lysine is optional. In certain embodiments, the C-terminal lysine of an IgG
15 sequence may be removed or replaced with a non-lysine amino acid, such as alanine, to further increase the serum half-life of the Fc fusion protein.

In certain embodiments, an Fc fusion protein comprises a human hinge, CH2 and CH3 domains, and may have the following amino acid sequence:

*DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDP
20 EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPGK* (SEQ ID NO: 66), wherein the core hinge sequence is underlined
and the CH2 and CH3 regions are in regular text.

25 In certain embodiments, an Fc fusion protein comprises a CH2 and a CH3 region of a human IgG1 as shown below:

*VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
30 LDDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ
ID NO: 67). It should be understood that the glycine and lysine at the end of a CH3
domain are optional.

Fc fusion proteins may also comprise an Fc domain that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 65-67. An Fc fusion protein may also comprise an Fc domain having at least 50, 100, or 150 contiguous amino acids of SEQ ID NOs: 65-67. Fc fusion proteins may also comprise an Fc domain having from 50-100, 50-150, or 100-150 contiguous amino acids of any one of SEQ ID NOs: 65-67. Fc fusion proteins may comprise an Fc domain comprising any one of SEQ ID NOs: 65-67 with from 1-5, 1-10, 1-15, 1-20, or 1-25 substitutions, e.g., conservative substitutions.

The Fc domain may be a naturally occurring Fc sequence, including natural allelic or splice variants. Alternatively, an Fc domain may be a non-naturally occurring Fc domain, e.g., a hybrid domain comprising a portion of an Fc domain from two or more different Ig isotypes, for example, an IgG2/IgG4 hybrid Fc domain. In exemplary embodiments, the Fc domain is derived from a human immunoglobulin molecule. Alternatively, the Fc domain may be a humanized or deimmunized version of an Fc domain from a non-human animal, including but not limited to mouse, rat, rabbit, camel, llama, dromedary and monkey.

In certain embodiments, the Fc domain is a variant Fc sequence, e.g., an Fc sequence that has been modified (e.g., by amino acid substitution, deletion and/or insertion) relative to a parent Fc sequence (e.g., an unmodified Fc polypeptide that is subsequently modified to generate a variant), to provide desirable structural features and/or biological activity.

For example, one may make modifications in the Fc region in order to generate an Fc variant that (a) has increased or decreased antibody-dependent cell-mediated cytotoxicity (ADCC), (b) increased or decreased complement mediated cytotoxicity (CDC), (c) has increased or decreased affinity for C1q and/or (d) has increased or decreased affinity for a Fc receptor relative to the parent Fc. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc region may include two, three, four, five, etc substitutions therein, e.g., of the specific Fc region positions identified herein. Proteins comprising Fcs that are mutated to modify the biological activity of the Fc may be refolded as described herein. Exemplary Fc mutants are described, e.g., in WO97/34631; WO96/32478; U.S. Pat. No. 5,624,821; U.S.

Pat. No. 5,648,260; U.S. Pat. No. 6,194,551; WO 94/29351; WO00/42072; U.S. Pat. No. 5,624,821; U.S. Pat. No. 6,277,375; U.S. Pat. No. 6,737,056; U.S. Pat. No. 6,194,551; U.S. Pat. No. 7,317,091; U.S. Pat. No. 8,101,720; PCT Patent Publications WO 00/42072; WO 01/58957; WO 02/06919; WO 04/016750; WO 04/029207; WO 04/035752; WO 5 04/074455; WO 04/099249; WO 04/063351; WO 05/070963; WO 05/040217; WO 05/092925; WO 06/020114; and Strohl, 2009, *Current Opinion in Biotechnology* 20:685-691; U.S. Pat. No. 6,277,375; Hinton et al., 2004, *J. Biol. Chem.* 279(8): 6213-6216; Hinton et al. 2006 *Journal of Immunology* 176:346-356; Dall'Acqua et al. *Journal of Immunology*, 2002, 169:5171 -5180, Dall'Acqua et al., 2006, *Journal of Biological* 10 *Chemistry* 281 :23514-23524; Yeung et al., 2010, *J Immunol*, 182:7663-7671; WO88/07054; WO88/07089; U.S. Pat. No. 6,277,375; WO99/051642; WO01/058957; WO2003/074679; WO2004/029207; U.S. Pat. No. 7,317,091 and WO2004/099249.

Exemplary variant Fcs are set forth as SEQ ID NOs: 68-86. In some aspects, an Fc fusion protein described herein comprises an Fc domain having at least 50, 100, or 150 15 contiguous amino acids of any one of SEQ ID NOs: 68-86. In other embodiments, an Fc fusion protein described herein comprises an Fc domain having from 50-100, 50-150, or 100-150 contiguous amino acids of SEQ ID NOs: 68-86. In yet other embodiments, an Fc fusion protein described herein comprises an Fc domain comprising SEQ ID NOs: 68-86 with from 1-5, 1-10, 1-15, 1-20, or 1-25 substitutions, e.g., conservative substitutions.

20 Fc fusion proteins may contain an immunoglobulin hinge region. The hinge region may be derived from antibodies belonging to any of the immunoglobulin classes, i.e. IgA, IgD, IgE, IgG, or IgM. In certain embodiments, the hinge region is derived from any of the IgG antibody subclasses, i.e. IgG1, IgG2, IgG3, and IgG4. In some 25 embodiments, the hinge region may further include residues derived from the CH1 and CH2 regions that flank the core hinge sequence, as discussed further below.

In certain embodiments, a hinge contains a free cysteine residue that is capable of forming a disulfide bond with another monomer to form a dimer. The hinge sequence may naturally contain a cysteine residue, or may be engineered to contain one or more cysteine residues.

30 In certain embodiments, the Fc fusion proteins comprise a hinge region derived from a human IgG1. In some embodiments, the hinge region comprises the core hinge

residues DKTHTCPPCPAPELLG (SEQ ID NO: 87) of IgG1, which corresponds to positions 221-236 according to EU numbering.

In certain embodiments, the hinge sequence may include substitutions that confer desirable pharmacokinetic, biophysical, and/or biological properties. Some exemplary hinge sequences include EPKSSDKTHTCPPCPAPELLGGPS (SEQ ID NO: 88; core hinge region underlined), EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 89; core hinge region underlined), EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 90; core hinge region underlined), DKTHTCPPCPAPELLGGPS (SEQ ID NO: 91; core hinge region underlined), and DKTHTCPPCPAPELLGGSS (SEQ ID NO: 92, core hinge region underlined). In one embodiment, the hinge sequence is a derivative of an IgG1 hinge comprising a P122S substitution (EU numbering 238) (e.g., the Proline residue at position 122 in SEQ ID NO: 22 is substituted with serine). The P122S substitution ablates Fc effector function and is exemplified by the hinges having any one of SEQ ID NOs: 25, 26, and 28. In another embodiment, the hinge sequence is a derivative of an IgG1 hinge comprising D104G and K105S substitutions (EU numbering 221-222). The D104G and K105S substitutions remove a potential cleavage site and therefore increase the protease resistance of the fusion molecule. A hinge having D104G and K105S substitutions is exemplified in SEQ ID NO: 26. In another embodiment, the hinge sequence is a derivative of an IgG1 hinge comprising a C103S substitution (EU numbering 220). The C103S substitution prevents improper cysteine bond formation in the absence of a light chain. Hinges having a C103S substitution are exemplified by SEQ ID NOs: 24-26.

Fc fusion proteins may comprise a hinge sequence that comprises, consists essentially of, or consists of an amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to that of any hinge described herein, e.g., a hinge having SEQ ID NOs: 88-92, or comprises, consists essentially of, or consists of an amino acid sequence of any hinge described herein, e.g., one of SEQ ID NOs: 88-92. Fc fusion proteins may comprise a hinge portion comprises at least or at most 2, 5, 10, 12, 15, 18 or 20 contiguous amino acid residues from any of SEQ ID NOs: 88-92, or a sequence comprising from 1-5, 1-10, 1-15, 1-20, 2-5, 2-10, 2-15, 2-20, 5-10, 5-15, 5-20, 10-15, 10-20, or 15-20 contiguous amino acid residues from any of SEQ ID NOs: 88-92. In exemplary embodiments, the hinge sequence comprises a cysteine residue.

In certain embodiments, an Fc fusion protein does not comprise a hinge. For example, an Fc fusion protein may comprise an Fc domain linked to a heterologous protein, e.g., in the Fc-X or X-Fc format, without comprising a hinge or a core hinge. In one example, an Fc fusion protein does not comprise the sequence

5 EPKSSDKTHTCPPCP (SEQ ID NO: 93) or a variant thereof.

In certain embodiments, an Fc fusion protein does not comprise a linker. For example, an Fc fusion protein may comprise an Fc domain that is linked directly to a heterologous protein, e.g., a ¹⁰Fn3 protein without an intervening sequence. In certain embodiments, there may be 1, 2, 3, 4 or 5 amino acids (e.g., from 1-5 or 1-10 amino

10 acids) between the Fc domain and the heterologous protein. Such Fc fusion proteins may be X-Fc (the heterologous protein is linked at the N-terminus of the Fc) or Fc-X (the heterologous protein is linked at the C-terminus of the Fc) fusion proteins, wherein X is the heterologous protein, and wherein X and Fc are directly linked to each other.

In certain embodiments, an Fc fusion protein comprises neither a hinge nor a

15 linker.

In certain embodiments, an Fc fusion protein is a dimer, wherein each monomer comprises a fusion protein (a homodimer). In certain embodiments, an Fc fusion protein is a heterodimer comprising, e.g., a monomer that comprises an Fc fusion protein and a monomer that comprises an Fc that is not linked to another moiety. The Fc portion of a

20 monomer may comprise one or more amino acid modifications (or mutations) relative to a wild type Fc that favor dimer, e.g., heterodimer, formation with another Fc. For example, an Fc of a dimer may comprise a “hole” and the other Fc of the dimer may comprise a “bump” or “knob,” as described, e.g., in WO96/027011; US 5,731,168 and US 5,821,333. Other modifications, such as electrostatic modifications may be used to

25 enhance dimer formation. Exemplary modifications are described, e.g., in WO2007/110205; WO2009/089004 and WO2010/129304. Such changes are particularly useful for enhancing the association of two heterologous monomers to form a dimer, such as a dimer that comprises a monomer comprising an Fc fusion protein and a monomer comprising an Fc that is different from the Fc fusion protein, e.g., by the lack of a

30 heterologous protein.

In certain embodiments, an Fc fusion protein comprises a monomer comprising the structure X-Fc and a monomer comprising the structure Fc-X (or Fc-Y). An Fc fusion

protein may also comprise two monomers, each comprising the structure X-Fc-X (a “quad” structure), as used, e.g., in Examples 1-3. An Fc fusion protein may also comprise two monomers, each comprising the structure X-Fc-Y, or one monomer comprising the structure X-Fc-Y and a monomer comprising the structure Y-Fc-X. Each monomer may optionally comprise a linker and optionally comprise a hinge.

An Fc fusion protein may comprise a single chain Fc (scFc), wherein the first and the second Fc domain (or the first and the second hinge-Fc domains) are linked through a linker. In one embodiment, a scFc comprises in N- to C-terminal order a first CH2 domain, which first CH2 domain is linked to a first CH3 domain, which CH3 domain is linked to an Fc linker, which Fc linker is linked to a second CH2 domain, which second CH2 domain is linked to a second CH3 domain, wherein the first and the second CH2 and CH3 domains associate to form a dimeric Fc. An scFc may comprise in N- to C-terminal order a first hinge, which first hinge is linked to a first CH2 domain, which first CH2 domain is linked to a first CH3 domain, which first CH3 domain is linked to an Fc linker, which Fc linker is linked to a second hinge, which second hinge is linked to a second CH2 domain, which second CH2 domain is linked to a second CH3 domain, wherein the first and the second hinges, CH2 domains and CH3 domains associate to form a dimeric Fc. scFcS are described, e.g., in WO2008/131242, WO2008/143954 and WO2008/012543.

20

Exemplary linkers for connecting a heterologous protein to an Fc moiety

Any linker may be used for covalently linking a heterologous protein, e.g., a fibronectin based scaffold moiety, to an Fc moiety, provided that the linker allows the fusion protein comprising the heterologous protein to properly fold and be biologically active. For example, the fusion protein should be able to bind efficiently to its target and may have a long half-life in serum relative to the heterologous protein that is not linked to an Fc. A linker is also preferably essentially not immunogenic and not reactive with other proteins (i.e., chemically inert).

A linker may be from 1-6, 1-10, 1-15, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, or 5-50 amino acids long.

Exemplary linkers may comprise, consist of, or consist essentially of GS linkers, e.g., (GS)₁, (GS)₂, (GS)₃, (GS)₄, (GS)₅, (GS)₆, (GS)₇, (GS)₈, (GS)₉ or (GS)₁₀. Linkers

may also comprise, consist of, or consist essentially of G₄S linkers, e.g., (G₄S)₁, (G₄S)₂, (G₄S)₃, (G₄S)₄ or (G₄S)₅. Additional exemplary linkers are provided in WO2012/142515.

5 Exemplary Fc fusion proteins that may be refolded

Fusion proteins comprising a heterologous moiety X, e.g., ¹⁰F_n3, and an Fc moiety are collectively referred to herein as X/Fc fusions regardless of whether they contain a linker or a hinge and regardless of orientation (the “/” indicates that it covers both orientations, i.e., where the Fc is N-terminal or where the Fc is C-terminal to X).

10 In certain embodiments, an Fc is linked directly to X, i.e., without one or more intervening amino acid (e.g., without a linker). In certain embodiment, an Fc is linked indirectly to X, i.e., with one or more intervening amino acids, e.g., a linker.

Exemplary fusion proteins are as follows, and as shown in the N- to C-terminal order:

15 X-hinge-CH₂-CH₃; X-linker-hinge-CH₂-CH₃; X-CH₂-CH₃; X-linker-CH₂-CH₃; hinge-CH₂-CH₃-X; hinge-CH₂-CH₃-linker-X; CH₂-CH₃-Fc; CH₂-CH₃-linker-X, wherein X is a heterologous protein relative to the Fc portion. In either orientation, the X/Fc fusion proteins described herein may further contain an N-terminal methionine and/or a leader sequence (e.g., for expression in mammalian cells).

20 In certain embodiments, a fusion protein comprises (i) a fibronectin based scaffold moiety comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 1-29; and (ii) an Fc moiety, e.g., comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to SEQ ID NO: 65-86, wherein the fusion
25 protein binds specifically to a target (e.g., with a *K_d* of less than 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM or less) that is not bound by a wild-type fibronectin based scaffold moiety (e.g., SEQ ID NOs: 1-8, 10, 12, 14 or 16). In certain embodiments, a fusion protein comprises (i) a fibronectin based scaffold moiety comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99%
30 identical to any one of SEQ ID NOs: 1-29; (ii) an Fc moiety, e.g., comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to SEQ ID NO: 65-86; and (iii) a linker that covalently links the fibronectin

based scaffold moiety to the Fc moiety, wherein the fusion protein binds specifically to a target (e.g., with a K_d of less than 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM or less) that is not bound by a wild-type fibronectin based scaffold moiety (e.g., SEQ ID NOs: 1-8, 10, 12, 14 or 16). In certain embodiments, a fusion protein comprises

5 (i) a fibronectin based scaffold moiety comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 1-29, (ii) an Fc moiety, e.g., comprising an amino acid sequence that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to SEQ ID NO: 65-86; and (iii) a linker that covalently links the fibronectin based scaffold moiety to the Fc

10 moiety, wherein the linker comprises 1-10 amino acids, such as 6 amino acids, and wherein the fusion protein binds specifically to a target (e.g., with a K_d of less than 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, 500 pM, 100 pM or less) that is not bound by a wild-type fibronectin based scaffold moiety (e.g., SEQ ID NOs: 1-8, 10, 12, 14 or 16). Exemplary fibronectin based scaffold moieties linked to an Fc are disclosed in

15 WO2012/142515.

Also provided herein are protein compositions, e.g., compositions comprising one or more protein in one of the solutions or buffers described herein. For example, provided herein are compositions comprising a protein comprising at least two cysteines (wherein, e.g., the protein is a dimer comprising one cysteine on each dimer) that form a

20 disulfide bond under appropriate conditions, and water, wherein the composition does not comprise a significant amount of buffer or a denaturing agent and optionally does not comprise a reducing agent. Also provided herein are compositions comprising a suspension of denatured proteins, wherein at least some proteins comprise at least two cysteines that form a disulfide bond under appropriate conditions, and wherein the

25 composition does not comprise a buffer or a denaturing agent and optionally does not comprise a reducing agent. Further provided herein are compositions comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a solubilization buffer having a pH in the range of pH 10 to 13, wherein the composition does not comprise a denaturing agent and optionally does not comprise a

30 reducing agent. Also provided are compositions comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a refold buffer having a pH in the range of pH 9 to 11 and an oxidizing agent, wherein the composition

does not comprise a reducing agent other than a reducing agent that part of an oxidizing agent that is present in the composition. The protein concentration in any of these compositions may be at least 20 mg/ml, 15 mg/ml, 10 mg/ml, 5 mg/ml or 1 mg/ml. The compositions may comprise at least 70%, 80%, 90%, 95%, 97%, 98% or 99% of the protein of interest, e.g., a fibronectin based scaffold moiety linked to an Fc, relative to the total amount (e.g., in mg/ml) of protein in the composition. In the composition comprising refold buffer, refolded protein may constitute at least 70%, 80%, 90%, 95%, 97%, 98% or 99% of the protein in the sample.

10 Protein Synthesis

Proteins that can be refolded as described herein may be synthesized according to any method known in the art. Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, *E. coli* or *Bacillus* spp. Yeast, preferably from the *Saccharomyces* species, such as *S. cerevisiae*, may also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, (*Bio/Technology*, 6:47, 1988). Purified proteins may be prepared by culturing suitable host/vector systems to express the recombinant proteins. Expressed proteins, e.g., fibronectin based scaffold proteins, may then be purified from culture media or cell extracts.

Proteins may be synthesized chemically, enzymatically or recombinantly. Proteins may also be produced using cell-free translation systems. For such purposes the nucleic acids encoding the fusion protein must be modified to allow *in vitro* transcription to produce mRNA and to allow cell-free translation of the mRNA in the particular cell-free system being utilized (eukaryotic such as a mammalian or yeast cell-free translation system or prokaryotic such as a bacterial cell-free translation system). For chemical synthesis, see, e.g., the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL).

Codon usage may be selected so as to improve expression in a cell. Such codon usage will depend on the cell type selected. Specialized codon usage patterns have been developed for *E. coli* and other bacteria, as well as mammalian cells, plant cells, yeast

cells and insect cells. See for example: Mayfield et al., Proc Natl Acad Sci U S A. 2003 Jan 21;100(2):438-42; Sinclair et al. Protein Expr Purif. 2002 Oct;26(1):96-105; Connell ND. Curr Opin Biotechnol. 2001 Oct;12(5):446-9; Makrides et al. Microbiol Rev. 1996 Sep;60(3):512-38; and Sharp et al. Yeast. 1991 Oct;7(7):657-78.

5 General techniques for nucleic acid manipulation are described for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Laboratory Press, 2 ed., 1989, or F. Ausubel et al., Current Protocols in Molecular Biology (Green Publishing and Wiley-Interscience: New York, 1987) and periodic updates, herein incorporated by reference. The DNA encoding the polypeptide is operably
10 linked to suitable transcriptional or translational regulatory elements derived from prokaryotic, mammalian, viral, or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. The ability to replicate in a host, usually
15 conferred by an origin of replication, and a selection gene to facilitate recognition of transformants are additionally incorporated.

The proteins may comprise a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed
20 (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native signal sequence, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, a factor
25 leader (including *Saccharomyces* and *Kluyveromyces* alpha-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in PCT Publication No. WO90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor regions may be ligated in reading frame to
30 DNA encoding the protein.

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to

antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the fusion protein. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter, beta-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (*trp*) promoter system, and hybrid promoters such as the *tac* promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the protein.

The recombinant DNA can also include any type of protein tag sequence that may be useful for purifying the fusion proteins. Examples of protein tags include but are not limited to a histidine tag, a FLAG tag, a myc tag, an HA tag, or a GST tag. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts can be found in *Cloning Vectors: A Laboratory Manual*, (Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

The expression construct may be introduced into the host cell using a method appropriate to the host cell, as will be apparent to one of skill in the art. A variety of methods for introducing nucleic acids into host cells are known in the art, including, but not limited to, electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent).

Expression in bacterial cells may be conducted essentially as follows or with certain variations therein. DNA encoding a protein of interest, e.g., a ¹⁰Fn3/Fc protein, is inserted into the pET9d (EMD Bioscience, San Diego, CA) vector and are expressed in *E. coli* HMS174 cells. Twenty ml of an inoculum culture (generated from a single plated colony) is used to inoculate 1 liter of LB medium containing 50µg/ml carbenicillin and 34 µg/ml chloromphenicol. The culture is grown at 37 °C until A₆₀₀ 0.6-1.0. After induction with 1mM isopropyl-β-thiogalactoside (IPTG) the culture is grown for 4 hours at 30 °C and is harvested by centrifugation for 30 minutes at ≥10,000 g at 4 °C. Cell pellets are frozen at -80 °C. The cell pellet is resuspended in 25 ml of lysis buffer (20mM NaH₂PO₄, 0.5 M NaCl, 1x Complete Protease Inhibitor Cocktail-EDTA free (Roche), 1mM PMSF,

pH 7.4) using an Ultra-turrax homogenizer (IKA works) on ice. Cell lysis is achieved by high pressure homogenization ($\geq 18,000$ psi) using a Model M-110S MICROFLUIDIZER® (Microfluidics). The insoluble fraction is separated by centrifugation for 30 minutes at 23,300 g at 4 °C. The insoluble pellet recovered from centrifugation of the lysate is washed with 20mM sodiumphosphate/500mM NaCl, pH7.4. The pellet may optionally be further washed with water, and suspended in a suspension solution as further described herein. Other methods are described in WO2012/142515.

Proteins may be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. Non-limiting examples include extraction, recrystallization, salting out (e.g., with ammonium sulfate or sodium sulfate), centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, gel filtration, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution or any combinations of these. After purification, proteins may be exchanged into different buffers and/or concentrated by any of a variety of methods known to the art, including, but not limited to, filtration and dialysis. Methods for expressing fusion proteins in *E. coli* are also provided in WO2012/142515.

The purified protein may be 85%, 95%, 98% or 99% pure. Regardless of the exact numerical value of the purity, the protein may be sufficiently pure for use as a pharmaceutical product.

Exemplary Uses

In one aspect, the application provides proteins, e.g., fusion proteins, comprising a fibronectin based scaffold moiety, useful in the treatment of disorders. The diseases or disorders that may be treated will be dictated by the binding specificity of the fibronectin based scaffold moiety. As described herein, fibronectin based scaffold moieties may be designed to bind to any target of interest. Exemplary targets include, for example, TNF-alpha, VEGFR2, PCSK9, IL-23, EGFR and IGF1R. Merely as an example, fibronectin based scaffold moieties that bind to TNF-alpha may be used to treat autoimmune

disorders such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, and asthma. Fusion proteins described herein may also be used for treating cancer.

The application also provides methods for administering proteins to a subject. In some embodiments, the subject is a human. In some embodiments, the proteins are
 5 pharmaceutically acceptable to a mammal, in particular a human. A "pharmaceutically acceptable" composition refers to a composition that is administered to an animal without significant adverse medical consequences. Examples of pharmaceutically acceptable compositions include compositions, e.g., comprising fibronectin based scaffold moiety, that are essentially endotoxin or pyrogen free or have very low endotoxin or pyrogen
 10 levels.

SEQUENCES

WT ¹⁰F_n3 Domain:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
 15 GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPI SINYRTEIDKPSQ (SEQ ID NO: 1)

¹⁰F_n3 Domain of SEQ ID NO: 1 (with D97E)

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
 20 GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPI SINYRTEIEKPSQ (SEQ ID NO: 2)

WT ¹⁰F_n3 Domain Core Sequence version 1:

LEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKSTAT
 ISGLKPGVDYTITVYAVTGRGDSPASSKPI SINY (SEQ ID NO: 3)

WT ¹⁰F_n3 Domain Core Sequence version 2 :

25 EVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKSTATI
 SGLKPGVDYTITVYAVTGRGDSPASSKPI SINYRT (SEQ ID NO: 4)

WT ¹⁰F_n3 Domain Core Sequence version 3:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
 GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPI SINYRT (SEQ ID NO: 5)

30 WT ¹⁰F_n3 Domain Core Sequence version 4:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
 GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPI SINYRTE (SEQ ID NO: 6)

WT ¹⁰F_{n3} Domain Core Sequence version 5:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEI (SEQ ID NO: 7)

WT ¹⁰F_{n3} Domain Core Sequence version 6:

5 VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEID (SEQ ID NO: 8)

¹⁰F_{n3} Domain Core Sequence version 7 (version 6 with D97E):

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIE (SEQ ID NO: 9)

10 WT ¹⁰F_{n3} Domain Core Sequence version 8:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIDK (SEQ ID NO:
10)

¹⁰F_{n3} Domain Core Sequence version 9 (version 8 with D97E):

15 VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIEK (SEQ ID NO:
11)

WT ¹⁰F_{n3} Domain Core Sequence version 10:

20 VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIDKP (SEQ ID NO:
12)

¹⁰F_{n3} Domain Core Sequence version 11 (version 10 with D97E):

25 VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIEKP (SEQ ID NO:
13)

WT ¹⁰F_{n3} Domain Core Sequence version 12:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIDKPS (SEQ ID
NO: 14)

30 ¹⁰F_{n3} Domain Core Sequence version 13 (version 12 with D97E):

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEIEKPS (SEQ ID NO:
15)

WT ¹⁰F_n3 Domain with D80E Substitution

5 VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVP
GSKSTATISGLKPGVDYTITVYAVTGRGESPASSKPISINYRTEIDKPSQ (SEQ ID
NO: 16)

Degenerate WT ¹⁰F_n3 Domain Core Sequence:

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
10 A(X)_zISINYRT (SEQ ID NO: 17)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTE (SEQ ID NO: 18)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEI (SEQ ID NO: 19)

15 VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEID (SEQ ID NO: 20)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIE (SEQ ID NO: 21)

20 VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIDK (SEQ ID NO: 22)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIEK (SEQ ID NO: 23)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIDKP (SEQ ID NO: 24)

25 VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIEKP (SEQ ID NO: 25)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIDKPS (SEQ ID NO: 26)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
30 A(X)_zISINYRTEIEKPS (SEQ ID NO: 27)

VSDVPRDLEVVA(A)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIDKPSQ (SEQ ID NO: 28)

- VSDVPRDLEVVA(A)(X)_uLLISW(X)_vYRITY(X)_wFTV(X)_xATISGL(X)_yYTITVY
A(X)_zISINYRTEIEKPSQ (SEQ ID NO: 29)
- MGVSDVPRDL (SEQ ID NO: 30)
- GVSDVPRDL (SEQ ID NO: 31)
- 5 X_nSDVPRDL (SEQ ID NO: 32)
- X_nDVPRDL (SEQ ID NO: 33)
- X_nVPRDL (SEQ ID NO: 34)
- X_nPRDL (SEQ ID NO: 35)
- X_nRDL (SEQ ID NO: 36)
- 10 X_nDL (SEQ ID NO: 37)
- MASTSG (SEQ ID NO: 38)
- EIEK (SEQ ID NO: 39)
- EGSGC (SEQ ID NO: 40)
- EIEKPCQ (SEQ ID NO: 41)
- 15 EIEKPSQ (SEQ ID NO: 42)
- EIEKP (SEQ ID NO: 43)
- EIEKPS (SEQ ID NO: 44)
- EIEKPC (SEQ ID NO: 45)
- HHHHHH (SEQ ID NO: 46)
- 20 EIDK (SEQ ID NO: 47)
- EIDKPCQ (SEQ ID NO: 48)
- EIDKPSQ (SEQ ID NO: 49)
- MPAPDLRFTNETPSSLLISWTPPRVQITGYIIRYGPVGS DGRVKEFTVPPS
VSSATITGLKPGTEYTISVIALKDNQESEPLRGRVTTGG (FibconB; SEQ ID NO: 50)
- 25 TPSS (SEQ ID NO: 51)
- TTPRVQI (SEQ ID NO: 52)
- VGSDGR (SEQ ID NO: 53)
- PSVS (SEQ ID NO: 54)
- GLKPG (SEQ ID NO: 55)
- 30 KDNQES EP (SEQ ID NO: 56)
- LDAPDLQVTNVTDT SITVSWTPPSATITGYRITYTPSNGPGEPKELTVPPS
STSVTITGITPGVEYVVS VYALKDNQES PPLVGTCTT (SEQ ID NO: 57)

LPAPKNLVVSEVTEDSLRLSWTAPDAAFDSFLIQYQESEKVGGEAINLTVPG
SERSYDLTGLKPGTEYTVSIYGVKGGHRSNPLSAEFTT (SEQ ID NO: 58)

TEDS (SEQ ID NO: 59)

TAPDAAF (SEQ ID NO: 60)

5 SEKVGE (SEQ ID NO: 61)

GSER (SEQ ID NO: 62)

GLKPG (SEQ ID NO: 63)

KGGHRSN (SEQ ID NO: 64)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV

10 HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP
IEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSL
15 SLSPGK (SEQ ID NO: 65)

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTTPVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALH

20 NHYTQKSLSLSPGK (SEQ ID NO: 66)

VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV
LDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ

25 ID NO: 67)

EPRSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMH

30 EALHNHYTQKSLSLSPGK (SEQ ID NO: 68)

EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY

KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK (SEQ ID NO: 69)

EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
5 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPG (SEQ ID NO: 70)

EPKSCDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
10 HEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK (SEQ ID NO: 71)

EPRSSDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
15 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK (SEQ ID NO: 72)

DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
20 EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV
EWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPGK (SEQ ID NO: 73)

EPKSSDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
25 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK (SEQ ID NO: 74)

EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
30 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS

DIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 75)

EPKSSDKTHTSPPSPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 5 KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 76)

EPKSSDKTHTSPPSPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 10 KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTPPVLDSDGSFFLGSKLTVDKSRWQQGNVFSCSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 77)

EPKSSDKTHTSPPSPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 15 KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTPPVLDSDGSFFLGSKLTVDKSRWQQGNVFSCSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 78)

ESKYGPPCPPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVSQED
 PEVKFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCK
 20 VSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA
 VEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEAL
 HNHYTQKSLSLSLGK (SEQ ID NO: 79)

EPKSSDKTHTCPPAPEPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 25 KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYP
 SDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVM
 HEALHNHYTQKSLSLSLGK (SEQ ID NO: 80)

EPKSSDKTHTCPPAPEPELLGGPSVFLAPPKPKDTLMISRTPEVTCVVVDVVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 30 KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
 EALHNHYTQKSLSLSPGK (SEQ ID NO: 81)

EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
 5 EALHNAYTQKSLSLSPGK (SEQ ID NO: 82)

EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEY
 KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
 10 EALHNHYTQKSLSLSPGK (SEQ ID NO: 83)

EPKSSDKTHTSPPSPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 KCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFALGSKLTVDKSRWQQGNVFSCSVMH
 15 EALHNHYTQKSLSLSPGK (SEQ ID NO: 84)

EPKSSDKTHTSPPSPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
 KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS
 DIAVEWESNGQPENNYKTTPPVLDSDGSFALGSKLTVDKSRWQQGNVFSCSVMH
 20 EALHNHYTQKSLSLSPGK (SEQ ID NO: 85)

EPKSSDKTHTCPPCPAPEAGGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
 SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
 YKCKVSNKALPASIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP
 SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
 25 HEALHNHYTQKSLSLSPGK (SEQ ID NO: 86)

DKTHTCPPCPAPELLG (SEQ ID NO: 87)

EPKSSDKTHTCPPCPAPELLGGPS (SEQ ID NO: 88)

EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 89)

EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 90)

30 DKTHTCPPCPAPELLGGPS (SEQ ID NO: 91)

DKTHTCPPCPAPELLGGSS (SEQ ID NO: 92)

EPKSSDKTHTCPPCP (SEQ ID NO: 93)

The following representative Examples contain important additional information, exemplification and guidance which can be adapted to the practice of this invention in its various embodiments and the equivalents thereof. These examples are intended to help
5 illustrate the invention, and are not intended to, nor should they be construed to, limit its scope.

EXAMPLES

10 **Example 1: High-throughput screening of buffer conditions for refolding denatured proteins**

In order to identify effective refolding conditions for ¹⁰F_n3/Fc fusion proteins produced in *E. coli*, an automated liquid handling platform was used to execute protein refolding by dilution in triplicate in 96-well plates.

The biomass of a ¹⁰F_n3/Fc protein was produced in a 10 L fermentor. The
15 biomass was harvested and the IBs were recovered and washed by centrifugation before being frozen.

The screening study looked at protein concentration, resolubilization buffer, refold pH, temperature, aggregation suppressing excipients, and redox excipients. JMP Design of Experiments (DoE) software was used to design the screening study and analyze data.
20 Data were gathered using a plate reader and SE-HPLC. The data suggested a resolubilization buffer around pH 8 with Guanidine and a refold buffer with Arginine to suppress aggregation, and a Glutathione redox system, around pH 10, favored formation of soluble protein. In addition, these conditions showed protein around the correct
25 ¹⁰F_n3/Fc homodimer.

Scale up of the dilution refold to 50 mL, 100 mL and 200 mL final refold volumes using the above-identified conditions were performed using a calibrated pump and mixing. A variable and heavy precipitation event was observed in all cases and a low recovery of protein was observed. Only around 10 to 20% of the protein was recovered
30 in solution and found to be at the appropriate molecular weight. For a subset of the bench scale dilution refolds, a majority of the protein recovered in solution was found to be at a smaller molecular weight corresponding to ¹⁰F_n3/Fc monomer and indicating that the

disulfide bonds did not form. With these data, it was determined that alternative methods of refolding ¹⁰F_n3/Fc molecules should be evaluated.

Example 2: Effect of pH on refolding proteins from IBs

5 This Example describes that the efficiency of refolding denatured Fc fusion proteins varies with the pH of the buffer used for refolding the proteins, and that refolding is more efficient at higher pH than at lower pH.

The refold efficiency of an Fc fusion protein under different conditions was analyzed using Sephadex G25 chromatography. Briefly, a column is conditioned with
10 refold buffer, following which solubilized IBs are added to the column and the protein from the IBs is recovered by passing the same refold buffer as that used for conditioning the column over the column.

The Fc fusion protein used was a ¹⁰F_n3/Fc molecule having the following amino acid sequence:

15 *MGVSDVPRDLEVVAATPTSLLISWVPPSDDYGYRITYGETGGNSPVQEFTVPIGKGTA*
TISGLKPGVDYTITVYAVEFPWPHAGYYHRPISINYRTEIEPKSSGSTHTCPPCPAPPELL
GGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL
20 *DSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK* (SEQ
ID NO: 94; the ¹⁰F_n3 sequence is shown in italics).

The ¹⁰F_n3/Fc protein was expressed in *E. coli* and the IBs were collected. The IBs were solubilized at a weight (grams):volume (ml) ratio of 1:20 in 50 mM Tris pH 8.0, 6.0
25 M GuHCl, 0.2 mM TCEP. The solubilization reaction was mixed until complete solubilization of IBs occurred. The solubilization reaction was then mixed with a 2x volume of 50mM Tris, 0.4M arginine pH 10.4, and 10ml thereof was loaded onto 2.5x10cm Sephadex G25 columns at 150cm/hr. The G25 Sephadex columns were conditioned with refold buffer consisting of 0.4 M Arginine and 50 mM Tris pH 8.5, 9.0
30 or 10.4 by loading a 10% column volume equivalent of refold buffer on the column at 150cm/hr until stable baseline pH was achieved. Absorbance of the eluate was monitored at 280nm and the major absorbance peak of each column was collected for further analysis.

The chromatograms of the experiments conducted at pH 8.5, 9.0 and 10.4 are shown in Figures 2A, B and C, respectively. Aggregate and precipitated protein can be visualized in the chromatograms as peak shifts or as NaOH strip peaks. The chromatogram of the refold experiment conducted at pH 8.5 (Figure 2A) shows a significant shift as well as a NaOH strip peak, whereas the chromatograms of the refold experiments conducted at pH 9.0 and 10.4 (Figures 2B and C, respectively) show good peak symmetry and no NaOH strip peak, indicating less aggregation and protein precipitation, and higher solubility of the proteins at pH 9.0 and 10.4 than at pH 8.5. As solubility is used as an indicator of proper protein folding in these experiments, the results indicate that refolding denatured protein at pH 9.0 and 10.4 is more efficient than at pH 8.5.

Efficiency of refolding at the different pH values was also analyzed with SDS PAGE. This method shows soluble aggregated protein, which is also indicative of protein misfolding. 10 μ l of protein from the major absorbance peaks that were eluted from the G25 Sephadex columns described above were diluted with 20 μ l of water and 10 μ l LDL sample buffer, and 30 μ l of diluted sample was loaded on a 10 well 4-12% Bis-Tris Gel (Novex). The gel was run at 200V for 35 minutes. The gel was rinsed with water for 10 minutes, stained overnight with thermo gelcode blue stain and destained for 3 hours prior to scanning.

The stained gels are shown in Figures 3A and B. These indicate that significant soluble aggregate is present in the refold done at pH 9.0, whereas at pH 10.4, less soluble aggregate is present and more dimer is present, suggesting that refolding and dimer formation is more efficient at higher pH.

25 **Example 3: A reducing agent is not necessary during refolding**

Certain methods for refolding proteins comprising disulfide bonds use two refolding steps: a first refolding step during which a reducing agent, e.g., TCEP, is used to maintain a reduced conformation of the cysteines, to first refold the monomers; and a second refolding step during which disulfide bonds are formed, to dimerize the properly formed monomers. In this Example the necessity of including a reducing agent during a first refold step was investigated, and the results show that a reducing agent is not necessary for obtaining efficient refolding of a denatured protein.

The same experiment as that described in Example 2 was performed with the refold buffer at pH 10.4. The protein of the major absorbance peak was then incubated at room temperature for 30 minutes or 4 hours in the same buffer (i.e., in the absence of TCEP), prior to being loaded onto an SDS-PAGE gel. The gel was loaded and run and stained essentially as described in Example 2.

The stained SDS-PAGE gel is shown in Figures 4 A and B. The results show that dimer formation does not occur in the absence of TCEP even after the longer (4 hours) incubation time. This finding suggests that it is not necessary to include a reducing agent, e.g., TCEP, during the first refolding step. In addition, eliminating TCEP from the $^{10}\text{Fn3/Fc}$ refolding procedures resulted in significant reduction of CH3 open loop content of $^{10}\text{Fn3/Fc}$ preparations.

Example 4: The first refolding step occurs rapidly

This Example shows that refolding denatured protein during the first refolding step occurs rapidly, and therefore a long incubation time prior to the second refold (oxidizing) step is not necessary.

If proper refolding of monomer is required prior to correct dimer formation and the rate of refolding of the monomer is slow, it may be necessary to conduct the monomer refolding step (i.e., refold step 1) for a long time. The results of the G25 refold experiments described in Examples 1 and 2, however, appear to show that proper folding of $^{10}\text{Fn3/Fcs}$ can be accomplished using an almost instantaneous removal of denaturant (i.e., a short refold step 1). The instant Example confirms this observation by testing different refold step 1 incubation times.

The same experiment as that described in Example 2 was performed with the refold buffer at pH 10.4. The protein of the major absorbance peak was then incubated at room temperature for 0, 1 hour or 2 hours either as eluted or after diluting it 1:1 with 50mM Tris, 0.4M Arginine pH 10.4 refold buffer. After the incubation, Glutathione at a concentration of 1:0.2mM oxidized:reduced was then added to samples. The reactions were then incubated for another 3 hours at room temperature. SDS-PAGE was then performed essentially as described in Example 2.

The stained gels, which are shown in Figure 5, indicate that similar levels of dimer formation occurred in all samples regardless of the incubation time of the samples

(corresponding to refold step 1), suggesting that refolding occurs rapidly after the denaturing agent is removed. Although refold occurred in both the samples directly eluted from the column and those diluted 1:1 with refold buffer, dimer formation appears to be more efficient in the diluted samples.

5

Example 5: Solubilization of IBs at high pH in the absence of guanidine hydrochloride

This Example shows that IBs can be solubilized in a buffer having high pH in the absence of a denaturing agent, such as guanidine hydrochloride.

10 Solubilization efficiency of ¹⁰F_n3/Fc IBs in solubilization buffer with pH values from 10.4 through 12.5 was explored. IBs of the same protein as that in the previous Examples were added to a 20x volume of 50mM Tris, 0.4M Arginine pH 10.4. Mixing was initiated, and the pH was slowly adjusted with 12M NaOH until all IBs were solubilized. The pH of the solution at solubilization was pH 12.2.

15 Later experiments explored reducing the pH of the solubilization using Proline and Glycine as additives. The only condition at lower pH that was successful in solubilizing IBs in a reasonable time frame was 50mM Tris, 0.5M Arginine, 0.1M Proline pH 10.7. Solubilization took 20 minutes in this solution. Thus, at lower pH values, additives were required to fully solubilize the IBs, and the solubilization took
20 significantly longer than at higher pH. Solubilization at pH 12.2 for 3-5 minutes was found to solubilize essentially all IBs in the reaction.

Example 6: Characterization of the protein folded state at different pHs

This Example shows that at least partial tertiary structure of a ¹⁰F_n3/Fc protein is
25 maintained during solubilization of IBs at high pH, but not in a 6M Guanidine solution.

To determine whether a ¹⁰F_n3/Fc protein retains a secondary and tertiary structure during solubilization at high pH, Far and Near UV Circular Dichroism (CD) was used.

A ¹⁰F_n3/Fc protein comprising a human IgG1 Fc linked to the N-terminus of a
¹⁰F_n3 moiety binding to a different target from that bound by the molecule used in
30 Examples 2-5 was expressed in *E. coli* and IBs were isolated. The Fc has the following amino acid sequence:

MGDKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC
 KVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
 AVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHE
 5 ALHNHYTQKSLSLSP (SEQ ID NO: 95).

As Arginine is not compatible with CD, a solubilization buffer different from that in Example 5 was used. A purified high concentration sample of ¹⁰F_n3/Fc protein was diluted into the following buffers at a final concentration of 1mg/ml: 50mM phosphate pH 7.2; 50mM Tris pH 8.0; 50mM carbonate pH 10.2; 50mM phosphate pH 12.2; 50mM
 10 Tris pH 8.0 + 6M GuHCl. A sample of protein diluted into 50mM phosphate pH 12.2 was adjusted back to pH 7.5 using hydrochloric acid. The samples were subjected to Far and Near UV CD.

The results, which are shown in Figures 6A and B, indicate that at high pH some tertiary structure is maintained while with 6M Guanidine all the secondary and tertiary
 15 structure is lost (see Figures 6A and B). At pH 10.4 most of the secondary and tertiary structure is maintained. Bringing the protein from pH 12.2 to pH 7.5 showed partial refolding of the molecule. Thus, the results indicate that a denatured protein that is solubilized in a solubilizing buffer at high pH but in the absence of a denaturing agent maintains some tertiary structure, and that this may facilitate refolding of the protein
 20 relative to a solubilization/refolding method using a denaturing agent, such as guanidine hydrochloride.

Example 7: High pH IB solubilization and refold of ¹⁰F_n3/Fc proteins

This Example describes a method for solubilizing and refolding denatured
 25 proteins in the absence of a denaturing agent. The method was used to solubilize and refold several different ¹⁰F_n3/Fc proteins, some of which were in the ¹⁰F_n3-Fc format and others in the Fc-¹⁰F_n3 format.

All steps were performed at room temperature.

15 grams of IBs were suspended in 30 mls of Milli Q water. The suspension was
 30 mixed until a uniform appearance was observed and then placed aside.

Solubilization buffer was prepared by adding 1.398ml of 12.5M NaOH to 300 mls of 50 mM Tris, 0.4M Arginine pH 10.4. Final pH after addition was 12.2.

Refold buffer was prepared as follows. A total of 0.915g of oxidized glutathione and 0.0915g of reduced glutathione was added to 900ml of 50 mM Tris pH, 0.4 M Arginine pH 10.4. A total of 1.701ml of 12.0M HCl was spiked into the refold buffer + glutathione.

- 5 Table 1 provides the amount of HCl to be added to various volumes of Refold buffer to achieve a pH around 10.4.

Table 1: volumes of 12 M HCl to add to refold buffer to obtain a pH of about 10.4

Volume of solubilization buffer	Volume of refold buffer at 1:3 dilution	Volume of 12.0 M HCl to add to refold buffer prior to dilution
1 ml	3 ml	5.67 μ l
250 ml	750 ml	1.42 ml
750 ml	2.25 L	4.25 ml
1 L	3 L	5.67 ml

- Solubilization was conducted as follows. Mixing of inclusion body suspension was initiated on a stir plate. The 300 ml of solubilization buffer were added to the mixing
 10 IB suspension and a timer was started.

Mixing of the 900ml of refold buffer was initiated at 2 minutes.

After 2.5 minutes of mixing the IB suspension with the solubilization buffer, the mixing solubilization solution (or solubilization reaction) appeared transparent and there were no intact IBs present in the solution.

- 15 The solubilization reaction was then poured into the mixing refold buffer as quickly as possible (<5 seconds). Mixing was continued for 30 seconds after the solubilization reaction was added so that complete mixing of the two solutions was accomplished.

- 20 The refold reaction was then removed from the stir plate and allowed to sit static at room temperature for 1 hour. After 1 hour the refold reaction was placed back on the stir plate and mixing was again initiated. Once mixing occurred, 380 ml of a 0.1M HCl solution was added to the refold reaction as quickly as possible (<5 seconds). Refolding was accomplished at this point, and the refolded protein can be loaded onto protein A for purification.

25

Example 8: Refolding of a ¹⁰F_{n3}/Fc protein

This Example describes a method for solubilizing and refolding denatured proteins in the absence of a denaturing agent.

Cell paste from the induction phase of fermentation of an *E. coli* culture
5 expressing the ¹⁰F_{n3}/Fc protein of Example 6 was removed from -80°C storage and
suspended in 20 mM Sodium Phosphate pH 6.2, 250 mM Sodium Chloride, 5 mM EDTA
at a ratio of 1:10 (W/W solids/buffer) using an UltraTurrax. The suspended material was
passed through a microfluidizer twice at a psi of 18,000. The disrupted suspension was
centrifuged at 10,000 x g for 30 minutes to isolate the insoluble fraction. This fraction
10 was resuspended, washed, and isolated twice. Wash Buffer was 20 mM Sodium
Phosphate pH 6.2, 250 mM Sodium Chloride, 5 mM EDTA, 1% Triton X-100. The
isolation was performed via centrifugation 10,000 x g. The remaining insoluble fraction
was then washed twice more in DI water. Isolation was performed via centrifugation at
10,000 x g for 30 minutes. The isolated insoluble fraction (IBs) was stored at -20°C.

15 The ¹⁰F_{n3}/Fc protein fusion in the IBs was refolded as follows. Frozen IBs were
thawed in RODI water. Once the IB solution was fully homogenous, the IBs were
resolubilized by dilution with 50 mM Tris 0.4M Arginine pH 12.2 with stirring. This
solution was left stirring for 2-5 minutes, until fully dissolved and no large particles were
visible. After solubilization, the sample was diluted further with the addition of 50 mM
20 Tris 0.4M Arginine pH 10.4, with the extra addition of 12M HCl to bring the pH of the
solution to 10.4. To refold the protein, a redox reaction was performed using a 1mM:
0.2mM oxidized to reduced ratio of Glutathione. Redox reaction was left for 1 hour at
room temperature. After 1 hour, the pH of the reaction was dropped to about pH 8.0 with
the addition of 0.3x volume of 1M HCl.

25

**Example 9: Effect of protein concentration on efficiency of high pH solubilization
and refolding**

This Example shows that the high pH method for solubilizing and refolding
denatured protein can be used with concentrations of proteins of up to at least 7 mg/ml.

30 The high pH solubilization method described Examples 7 and 8 was used for
refolding ¹⁰F_{n3}/Fc protein (used in Examples 5 and 7) at 1.75, 3.5 and 7 mg/ml. Protein
concentration was determined at the solubilization stage and adjusted for the later

dilution. The concentration was measured by absorbance at 280nm. 10 or 20 µg of refolded protein were subjected to SDS-PAGE analysis. The gel, which is shown in Figure 7, indicates that dimer formation occurred at each of these concentrations.

Therefore, concentrations of protein up to at least 7 mg/ml at the solubilization stage can be refolded using this method. Dimer formation was, however, more efficient at lower refold concentrations.

Example 10: Control of deamidation that occurs at high pH

A concern with using high pH to solubilize and refold ¹⁰Fn3/Fcs is deamidation of the protein. This Example shows that limiting the time of incubation at high pH for solubilization reduces deamidation.

In early experiments, solubilization at pH 12.2 as described in Examples 7 and 8 was conducted for one hour at room temperature and the refold (at pH 10.4) was incubated overnight at room temperature. The refold showed Iso-asp formation at a level of 28.1% and 17.8% deamidation by lys-C digestion followed by peptide map analysis.

To try to reduce deamidation levels, a solubilization and refold performed as in Examples 7 and 8 with the ¹⁰Fn3/Fc protein of Example 5 was conducted with a solubilization time of 5 minutes and a refold of 18-20 hours at pH of 10.4. The refolded protein showed only 5.6% iso-asp, 3.7% deamidation and 2.7% imide by lys-C peptide map.

Thus, by minimizing exposure to both pH 12.2 during the solubilization and pH 10.4 in the refold buffer, deamidation was minimized. The high pH solubilization and refolding method may provide more control over refolding time and therefore deamidation, considering that chemical denaturant removal is not required and simply reducing the pH will reduce deamidation.

Example 11: Determination of pH of solubilization and refold reactions

This Example describes the pH of the solubilization and refold reactions when performing the solubilization and refold method described in Examples 7 and 8.

The ¹⁰Fn3/Fc protein used in Examples 6 and 7 was expressed in *E. coli* and IBs were obtained. 0.5 grams of IBs were suspended in 1 ml of Milli Q water. The suspension was mixed until a uniform appearance was observed and then placed aside.

Solubilization buffer was prepared by adding 46.6 μ l of 12.5M NaOH to 10 mls of 50 mM Tris, 0.4 M Arginine pH 10.4. The final pH of this solubilization buffer was 12.3.

A total of 0.0305 g of oxidized glutathione and 0.003 g of reduced glutathione was added to 30 ml of 50 mM Tris, 0.4 M Arginine pH 10.4. The final pH of this refold
5 buffer was 10.4.

A total of 56.7 μ l of 12.0 M HCl was spiked into the refold buffer containing glutathione.

The entire 10 ml of solubilization buffer was added to the IB suspension. After 3 minutes, the mixing solubilization solution appeared transparent and there were no intact
10 IBs present in the solution. The pH of this solubilization reaction was 12.5.

The solubilization reaction was then poured into the mixing refold buffer as quickly as possible (less than 5 seconds). Mixing was continued for 30 seconds after the solubilization reaction was added so that complete mixing of the two solutions was accomplished. The pH of this refold reaction was 10.3.

15 All steps of the method described in this Example were performed at room temperature.

Thus, the pH of the solubilization and refold reactions vary slightly from the pH of the solubilization and refold buffers, respectively. The pH of the solubilization reaction was pH 12.05, compared to the buffer, which was 12.3.

20 The pH of the refold reaction is also affected by the protein in solution. The pH of the refold reaction was measured at 10.3. Fc dimerization should be effective for any pH above 10.0, although a pH of as low as 9.0 can be used, although at such a pH protein aggregation may occur.

25 **Example 12: Comparison of target binding of refolded and mammalian expressed ¹⁰F_n3/Fc protein**

This experiment shows that a ¹⁰F_n3/Fc protein that was expressed in *E. coli* and refolded as described herein binds similarly to its target protein relative to the same protein that was expressed in mammalian cell culture.

30 A ¹⁰F_n3-¹⁰F_n3-Fc (i.e., a bispecific molecule having two ¹⁰F_n3 entities binding to two different targets) protein was expressed both in *E. coli* and refolded essentially as described in Example 8 and 9. The same protein was also expressed in mammalian cells

HEK293-6E. Binding of both $^{10}\text{Fn3}$ entities to their target was determined by SPR. The following format was used. Protein A was covalently linked to a chip. $1.5\text{nM }^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ was captured on the protein A. Binding to one of the two target proteins was measured by SPR. $0.15\text{-}5\text{ nM}$ of the target was used.

5 The results, which are shown in Figures 8A and B, indicate that the $^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ protein expressed in *E. coli* and refolded has similar target binding kinetics to those of the $^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ protein that was expressed in mammalian cells. Thus, $^{10}\text{Fn3/Fc}$ proteins expressed in *E. coli* and refolded as described herein is at least sufficiently refolded to allow binding to its target.

10

Example 13: Comparison of inhibition of biological activity of refolded and mammalian expressed $^{10}\text{Fn3/Fc}$ protein

 This Example shows that a $^{10}\text{Fn3/Fc}$ protein that was expressed in *E. coli* and refolded as described herein has similar biological activity relative to the same protein
15 that was expressed in mammalian cell culture.

 In this experiment mice are injected with the bispecific $^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ of Example 12 made either in *E. coli* or in mammalian cells, and the level of activity of the $^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ molecule was determined by determining the level of inhibition of the biological activity of the target. The mammalian protein was produced in a mammalian
20 shake flask culture or in a mammalian bioreactor. For comparison purposes, the experiment also included an antibody to one of the two targets and a the adnectin binding to one of the targets alone (i.e. a mono-adnectin). Different concentrations of each were tested. Three different biological activities of the target protein were tested: two of the
25 biological activities consisted of the induced secretion of two different cytokines and the third biological activity of the target protein was stimulation of signal transduction.

 Inhibition of the secretion of one cytokine is shown in Figure 9. The results indicate that the $^{10}\text{Fn3-}^{10}\text{Fn3-Fc}$ protein expressed in *E. coli* and refolded as described herein inhibit cytokine secretion induced by the target to a similar level. A similar result was seen when measuring the level of inhibition of secretion of the second cytokine
30 (Figure 10).

Inhibition of signal transduction is shown in Figure 11. The results indicate that the ¹⁰F_{n3}-¹⁰F_{n3}-Fc protein expressed in *E. coli* and refolded as described herein inhibit signal transduction induced by the target to a similar level.

Thus, these results taken together with those of Example 12 indicate that the
5 ¹⁰F_{n3}-¹⁰F_{n3}-Fc protein expressed in *E. coli* and refolded as described herein is at least sufficiently refolded to have biological activity that is similar to that of the same protein expressed in a mammalian cell culture system.

The entire disclosures of each document cited (including patents, patent
10 applications, journal articles, abstracts, laboratory manuals, books, GENBANK® Accession numbers, SWISS-PROT® Accession numbers, or other disclosures) in the instant patent application, including the Background, Detailed Description, Brief Description of the Drawings, and Examples, are hereby incorporated herein by reference
15 in their entirety.

The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any that are functionally equivalent are within the scope of the invention. Various
20 modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

25

CLAIMS

1. A method for refolding a denatured protein, comprising
suspending a denatured protein in a suspension solution to obtain a
composition comprising suspended denatured proteins;
5 combining the composition comprising suspended denatured proteins with a
solubilization buffer having a pH in the range of 10.5 to 13 to thereby obtain a
composition comprising solubilized denatured proteins; and
combining the composition comprising solubilized denatured proteins with a
refold buffer having a pH in the range of 9 to 11 to thereby obtain a
10 composition comprising refolded proteins;
wherein the method does not include the use of a significant amount of
denaturing agent.
2. The method of claim 1, wherein the pH of the solubilization buffer is a pH in the
range of 11.5 to 12.8.
- 15 3. The method of claim 2, wherein the pH of the solubilization buffer is a pH in the
range of 12.0 to 12.6.
4. The method of any one of claims 1-3, wherein the pH of the refold buffer is a pH
in the range of 10 to 10.6.
5. The method of claim 4, wherein the pH of the refold buffer is a pH in the range of
20 10.3 to 10.5.
6. The method of any one of claims 1-5, wherein the suspension solution consists of
water.
7. The method of any one of claims 1-6, wherein the composition comprising
solubilized denatured protein has a pH in the range of 11 to 13.
- 25 8. The method of claim 7, wherein the composition comprising solubilized denatured
protein has a pH in the range of 11.5 to 12.8.
9. The method of claim 8, wherein the composition comprising solubilized denatured
protein has a pH in the range of 12.0 to 12.6.
10. The method of any one of claims 1-9, wherein the composition comprising
30 refolded protein has a pH in the range of 10 to 11.
11. The method of claim 10, wherein the composition comprising refolded protein has
a pH in the range of 10 to 10.6.

12. The method of claim 11, wherein the composition comprising refolded protein has a pH in the range of 10.3 to 10.5.
13. The method of any one of claims 1-12, wherein the denatured proteins are suspended in suspension solution at a ratio of weight (g) of denatured proteins:volume (ml) of suspension solution of 1:1-3.
14. The method of claim 13, wherein the denatured proteins are suspended in suspension solution at a ratio of weight (g) of denatured proteins:volume (ml) of suspension solution of about 1:2.
15. The method of claim 14, wherein the suspension solution is water.
16. The method of any one of claims 1-15, wherein the composition comprising suspended denatured proteins is combined with solubilization buffer at a ratio of weight (g) of denatured proteins: volume (ml) of solubilization buffer of 1:10-30.
17. The method of claim 16, wherein the composition comprising suspended denatured proteins is combined with solubilization buffer at a ratio of weight (g) of denatured proteins: volume (ml) of solubilization buffer of about 1:20.
18. The method of any one of claims 1-17, wherein the composition comprising solubilized denatured proteins is combined with refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer of 1:1-5.
19. The method of claim 18, wherein the composition comprising solubilized denatured proteins is combined with refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer of 1:3-4.
20. The method of any one of claims 1-19, wherein the denatured proteins are suspended in suspension solution at a ratio of weight (g) of denatured proteins:volume (ml) of suspension solution of 1:1-3; the composition comprising suspended denatured proteins is combined with solubilization buffer at a ratio of weight (g) of denatured proteins:volume (ml) of solubilization buffer of 1:10-30; and the composition comprising solubilized denatured proteins is combined with refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer of 1:1-5.
21. The method of claim 20, wherein the solubilization buffer has a pH in the range of 11.5 to 12.8 and the refold buffer has a pH in the range of 10 to 10.9.

22. The method of claim 21, wherein
the denatured proteins are suspended in suspension solution at a ratio of weight
(g) of denatured proteins:volume (ml) of suspension solution of about 1:2;
the composition comprising suspended denatured proteins is combined with
5 solubilization buffer at a ratio of weight (g) of denatured proteins:volume (ml) of
solubilization buffer of about 1:20; and
the composition comprising solubilized denatured proteins is combined with
refold buffer at a ratio of volume of solubilization buffer:volume of refold buffer
of about 1:3-4.
- 10 23. The method of claim 22, wherein
the denatured proteins are suspended in water at a ratio of weight (g) of denatured
proteins:volume (ml) of suspension solution of about 1:2;
the composition comprising suspended denatured proteins is combined with
solubilization buffer having a pH of about 12.2 at a ratio of weight (g) of
15 denatured proteins:volume (ml) of solubilization buffer of about 1:20; and
the composition comprising solubilized denatured proteins is combined with
refold buffer having a pH in the range of 10.2 to 10.6 at a ratio of volume of
solubilization buffer:volume of refold buffer of about 1:3-4.
- 20 24. The method of any one of claims 1-23, wherein the suspended denatured proteins
and the solubilization buffer are combined for 1-10 minutes prior to being
combined with the refold buffer.
- 25 25. The method of claim 24, wherein the suspended denatured proteins and the
solubilization buffer are combined for 2-5 minutes prior to being combined with
the refold buffer.
- 26 26. The method of any one of claims 1-25, wherein the composition comprising the
solubilized denatured proteins is combined with the refold buffer for 5-60
minutes.
- 27 27. The method of claim 26, wherein the composition comprising the solubilized
denatured proteins is combined with the refold buffer for 15-25 minutes.
- 30 28. The method of any one of claims 1-27, comprising reducing the pH of
composition comprising refolded proteins.

29. The method of any one of claims 1-28, wherein the solubilization buffer comprises Arginine.
30. The method of any one of claims 1-29, wherein the refold buffer comprises Arginine.
- 5 31. The method of any one of claims 1-30, wherein the refold buffer comprises an oxidizing agent.
32. The method of claim 31, wherein the oxidizing agent is glutathione.
33. The method of any one of claims 1-32, wherein glutathione is at an about 5:1 oxidized: reduced ratio.
- 10 34. The method of any one of claims 1-33, wherein the method does not comprise first suspending the denatured protein in a suspension solution.
35. The method of any one of claims 1-34, wherein the method does not include the use of a denaturing agent.
36. The method of any one of claims 1-35, wherein the denatured proteins are in the form of inclusion bodies (IBs).
- 15 37. The method of any one of claims 1-36, wherein the protein comprises at least one cysteine.
38. The method of claim 37, wherein the protein comprises at least two cysteines that form a disulfide bond in the native protein.
- 20 39. The method of any one of claims 1-38, wherein the protein comprises an Fc region.
40. The method of claim 39, wherein the Fc region comprises a hinge.
41. The method of any one of claims 1-40, wherein the protein comprises a binding domain that specifically binds to a target protein.
- 25 42. The method of claim 41, wherein the binding domain is an alternative scaffold binding domain.
43. The method of claim 42, wherein the alternative scaffold binding domain is a fibronectin based scaffold domain.
44. The method of claim 43, wherein the fibronectin based scaffold domain is a ¹⁰FN3 domain.
- 30 45. The method of any one of claim 1-44, wherein the protein comprises a ¹⁰FN3 protein and an Fc region comprising a hinge, a CH2 and a CH3 domain.

46. A composition comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and water, wherein the composition does not comprise a buffer or a denaturing agent.
- 5 47. A composition comprising a suspension of denatured proteins, wherein at least some proteins comprise at least two cysteines that form a disulfide bond under appropriate conditions, and wherein the composition does not comprise a buffer or a denaturing agent.
- 10 48. A composition comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a solubilization buffer having a pH in the range of pH 10 to 13, wherein the composition does not comprise a denaturing agent.
- 15 49. A composition comprising a protein comprising at least two cysteines that form a disulfide bond under appropriate conditions, and a refold buffer having a pH in the range of pH 9 to 11 and an oxidizing agent, wherein the composition does not comprise a reducing agent other than a reducing agent that part of an oxidizing agent that is present in the composition.
50. The composition of any one of claims 46-49, wherein the protein comprises an Fc region or a portion thereof.
- 20 51. The composition of any one of claims 46-49, wherein the protein comprises a binding domain.
52. The composition of claim 51, wherein the binding domain is an FBS domain.
53. The composition of claim 52, wherein the FBS is a ¹⁰Fn3 domain.
- 25 54. The composition of claim 53, wherein the ¹⁰Fn3 domain binds specifically to a target, and wherein the ¹⁰Fn3 domain comprises an amino acid sequence that is at least 50% identical to any of SEQ ID NOs: 1-29.
55. The composition of any one of claims 46-55, wherein the protein is present in the composition at a concentration of at least 5 mg/ml.
- 30 56. The composition of claim 55, wherein the protein is present in the composition at a concentration of at least 10 mg/ml.

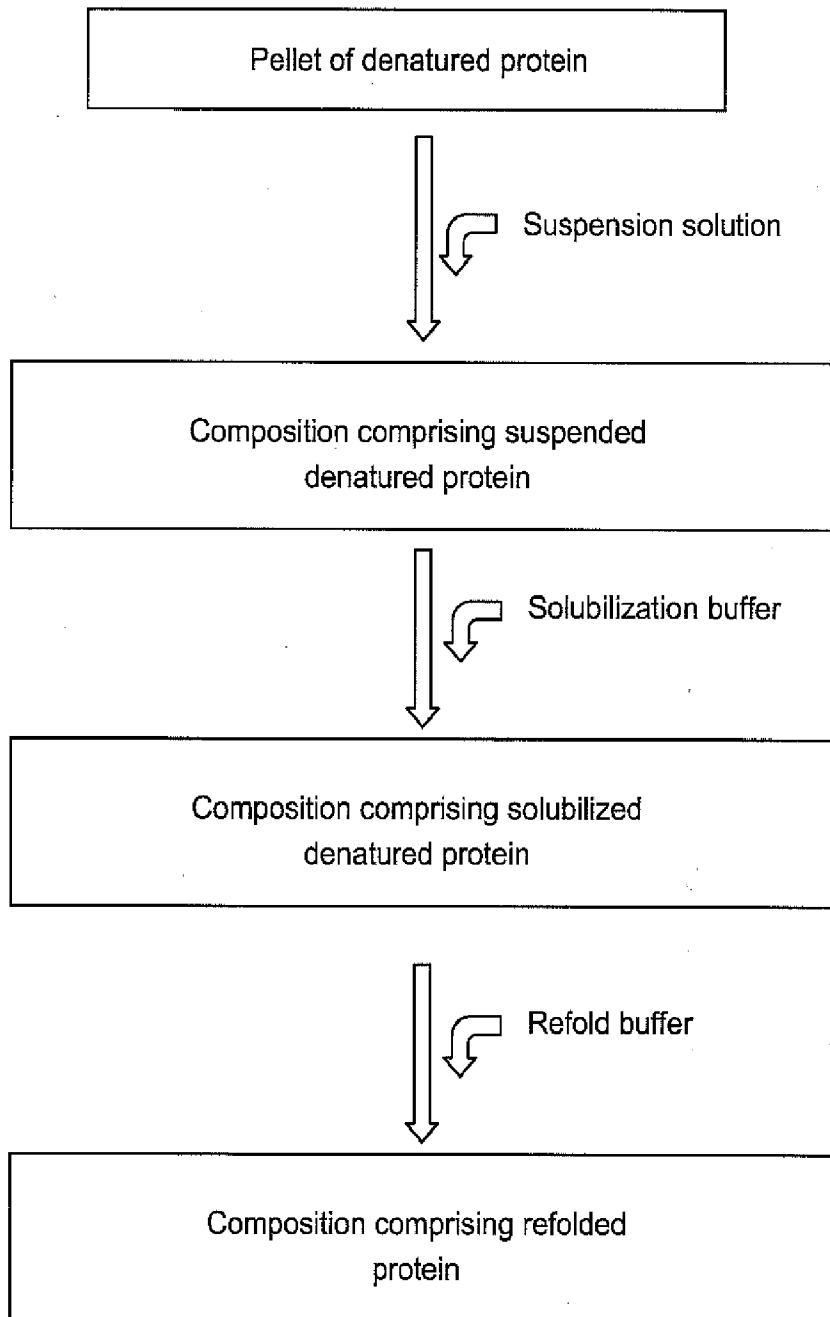


FIG. 1

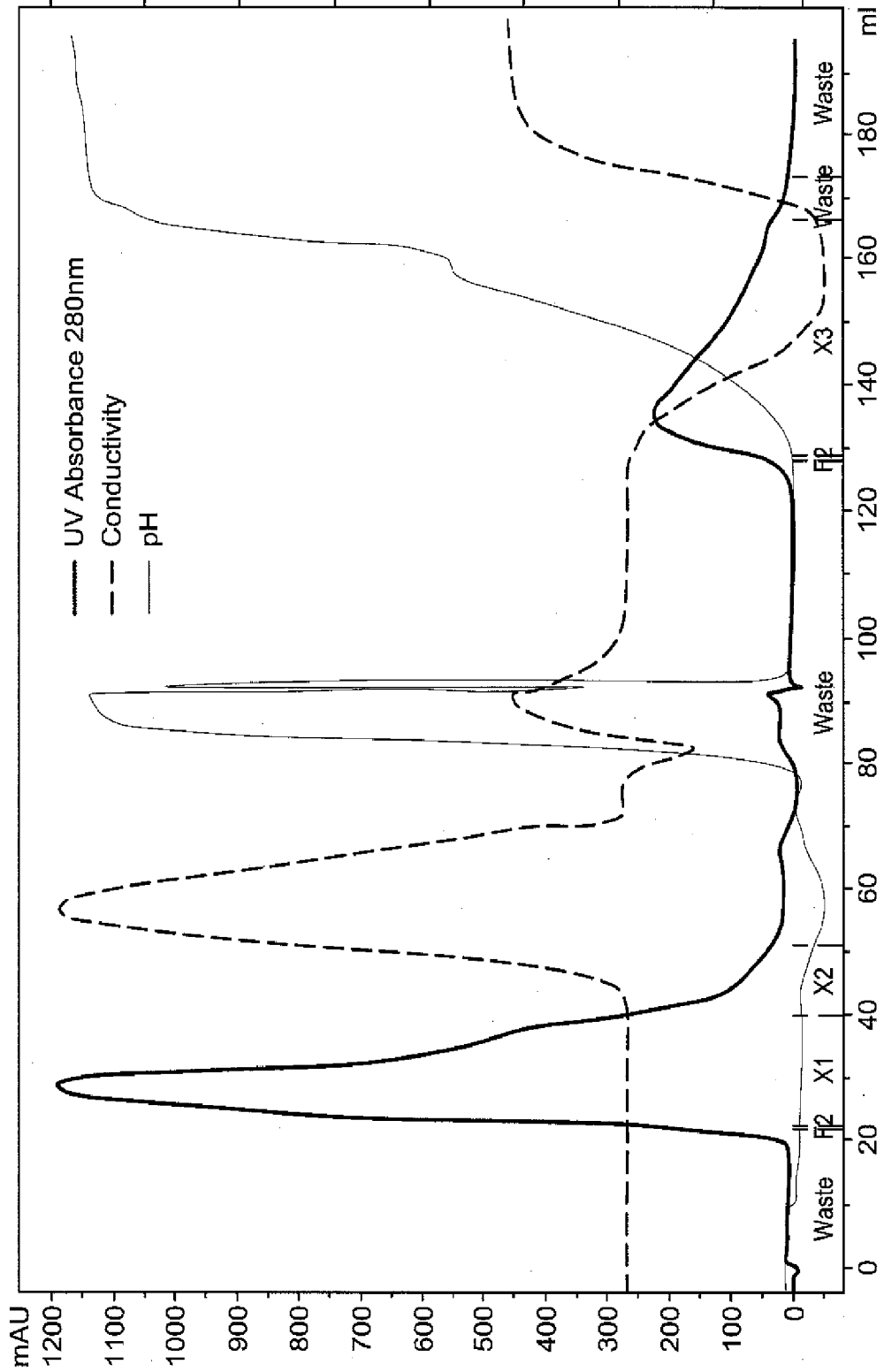


FIG. 2A

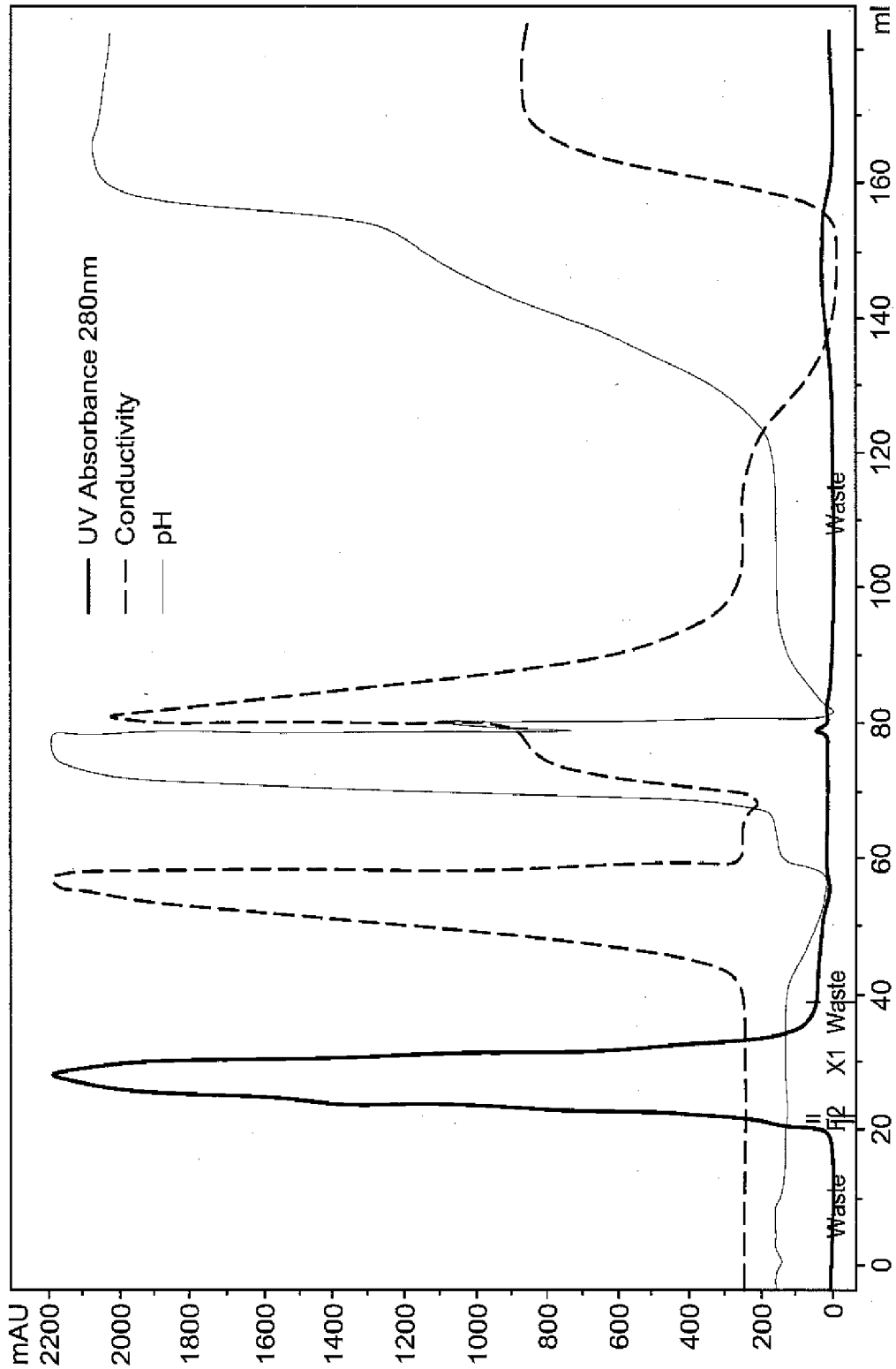


FIG. 2B

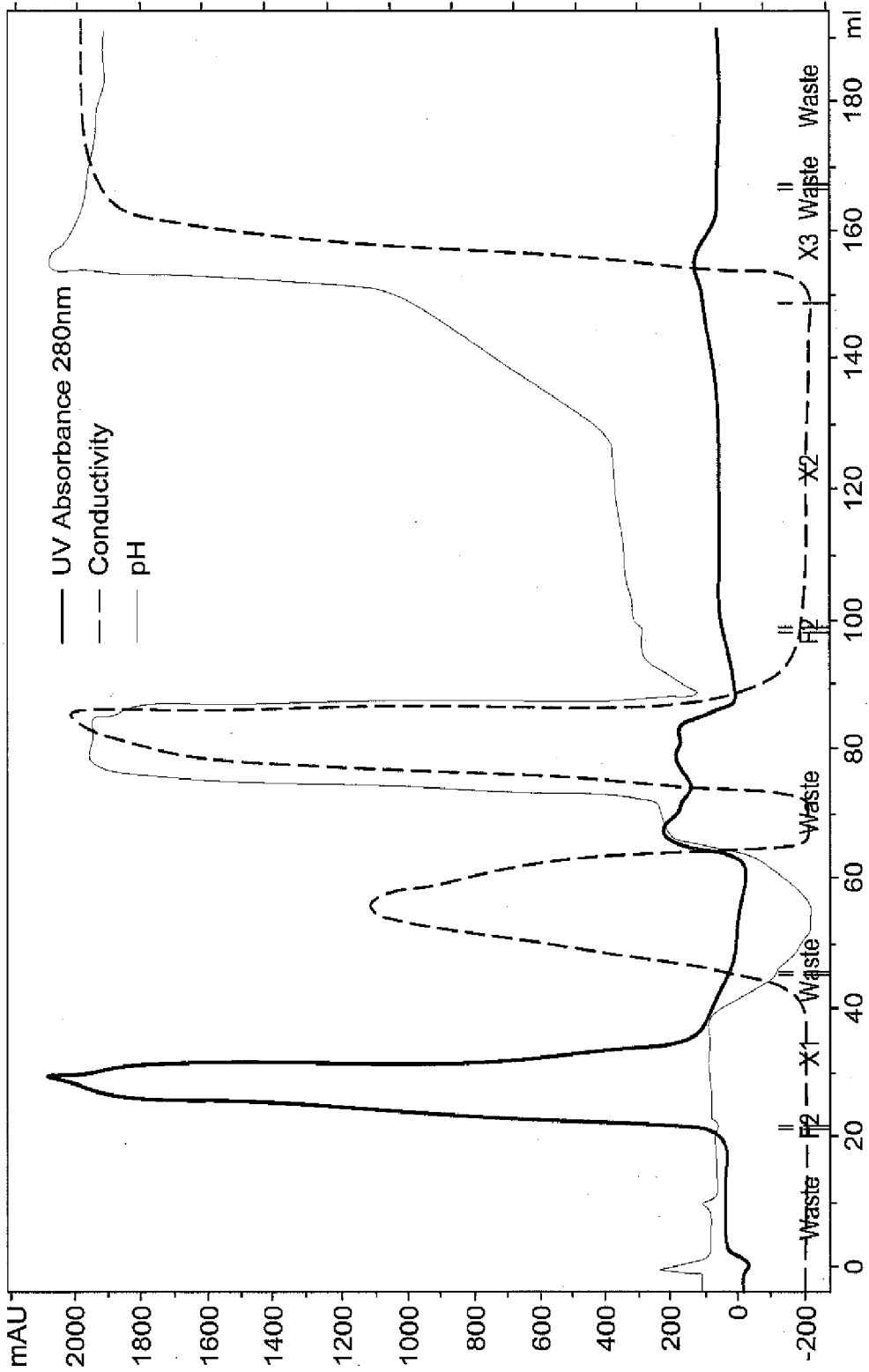


FIG. 2C

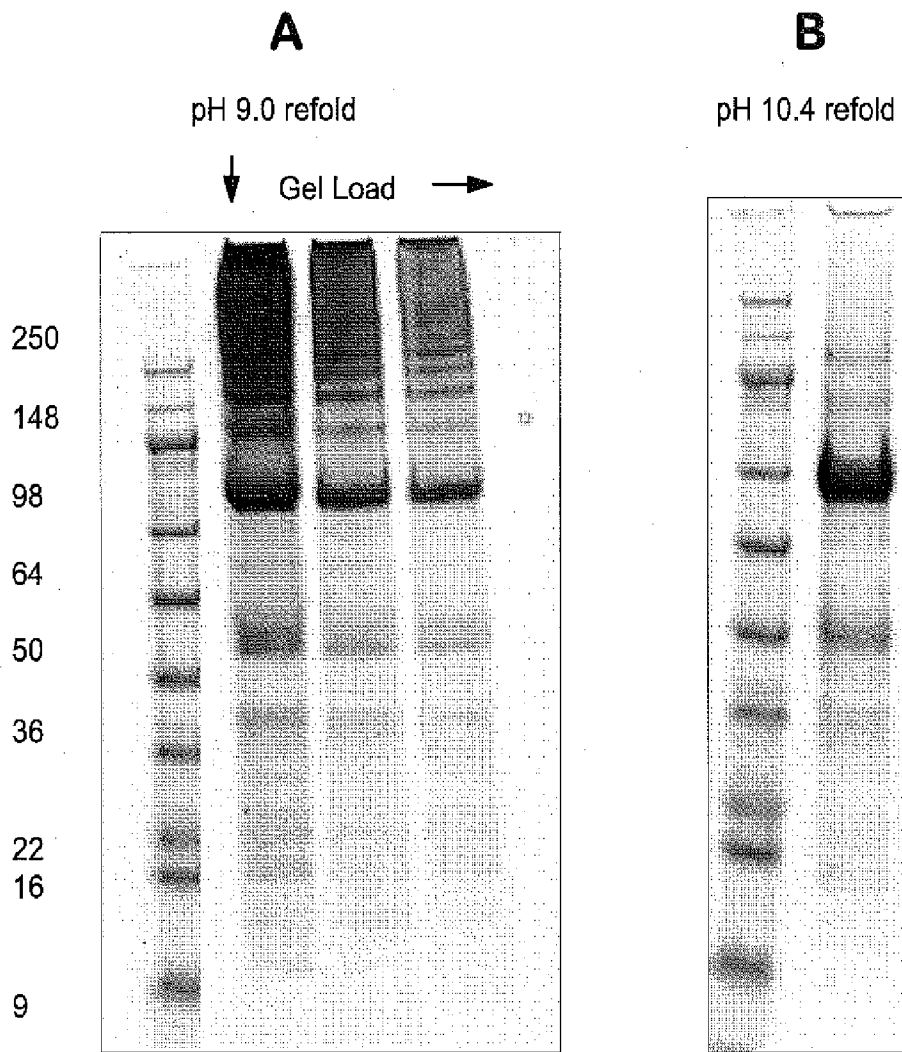


FIG. 3

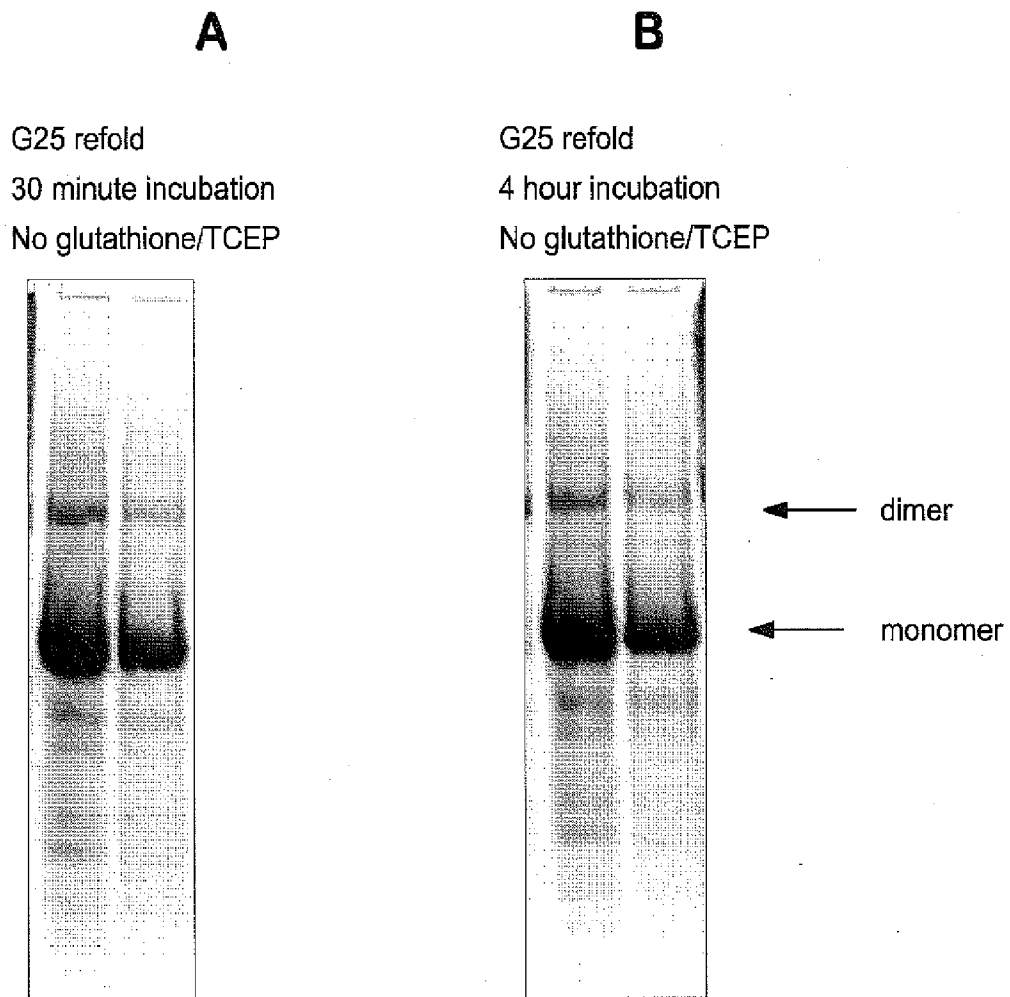


FIG. 4

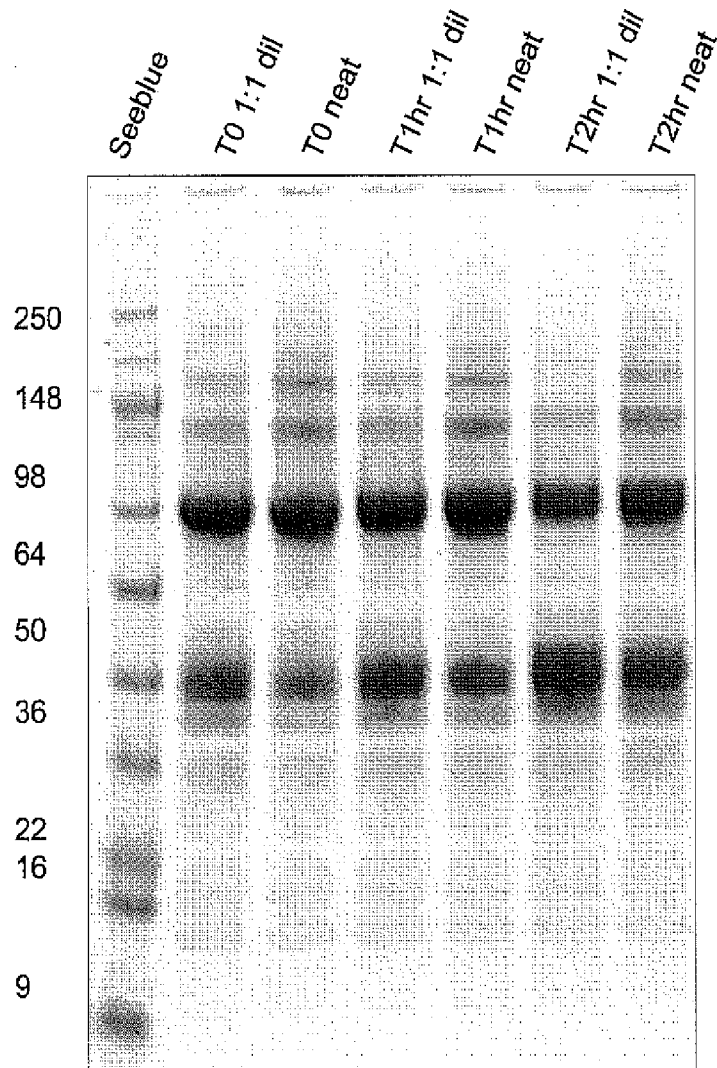


FIG. 5

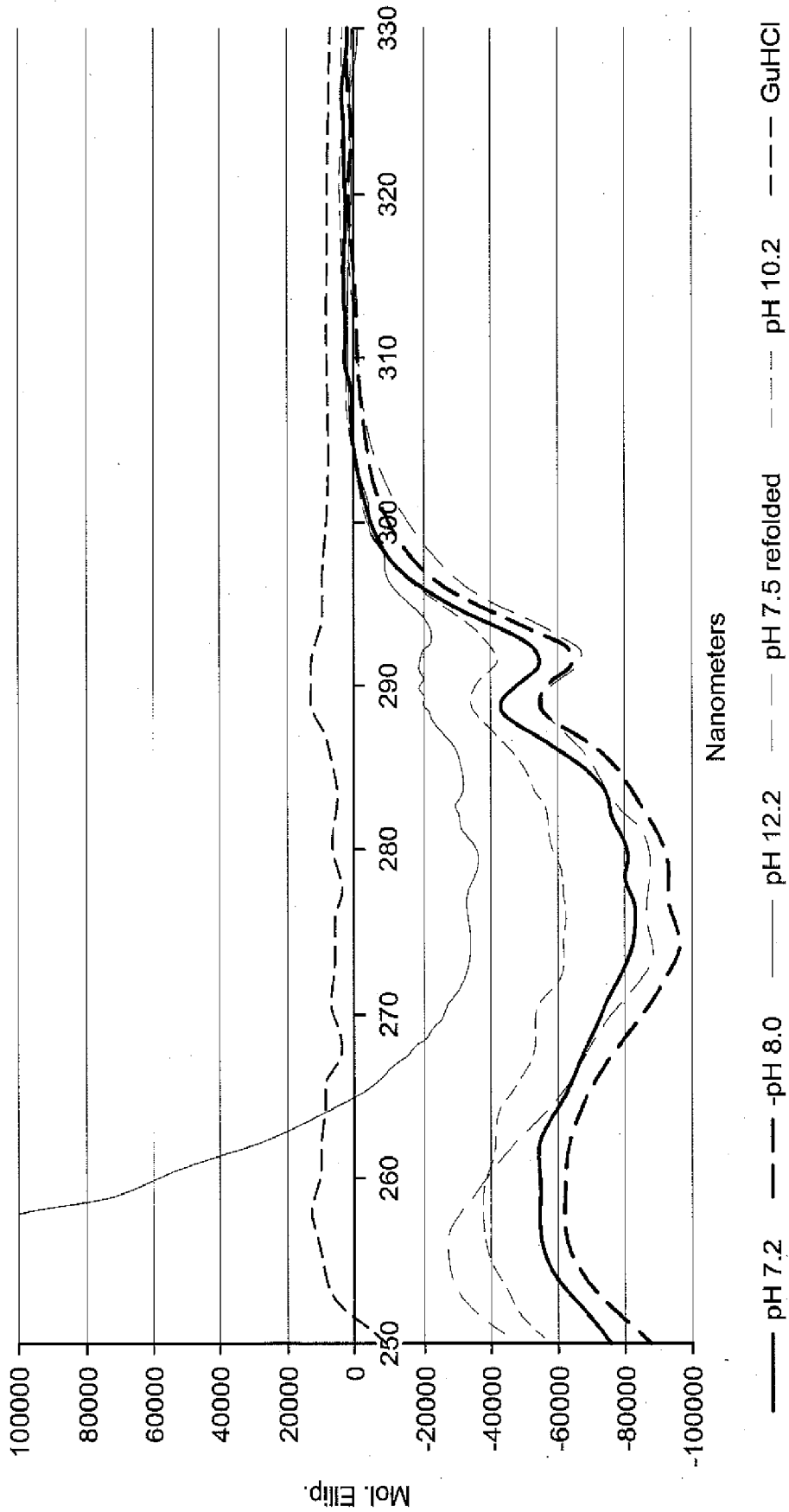


FIG. 6A

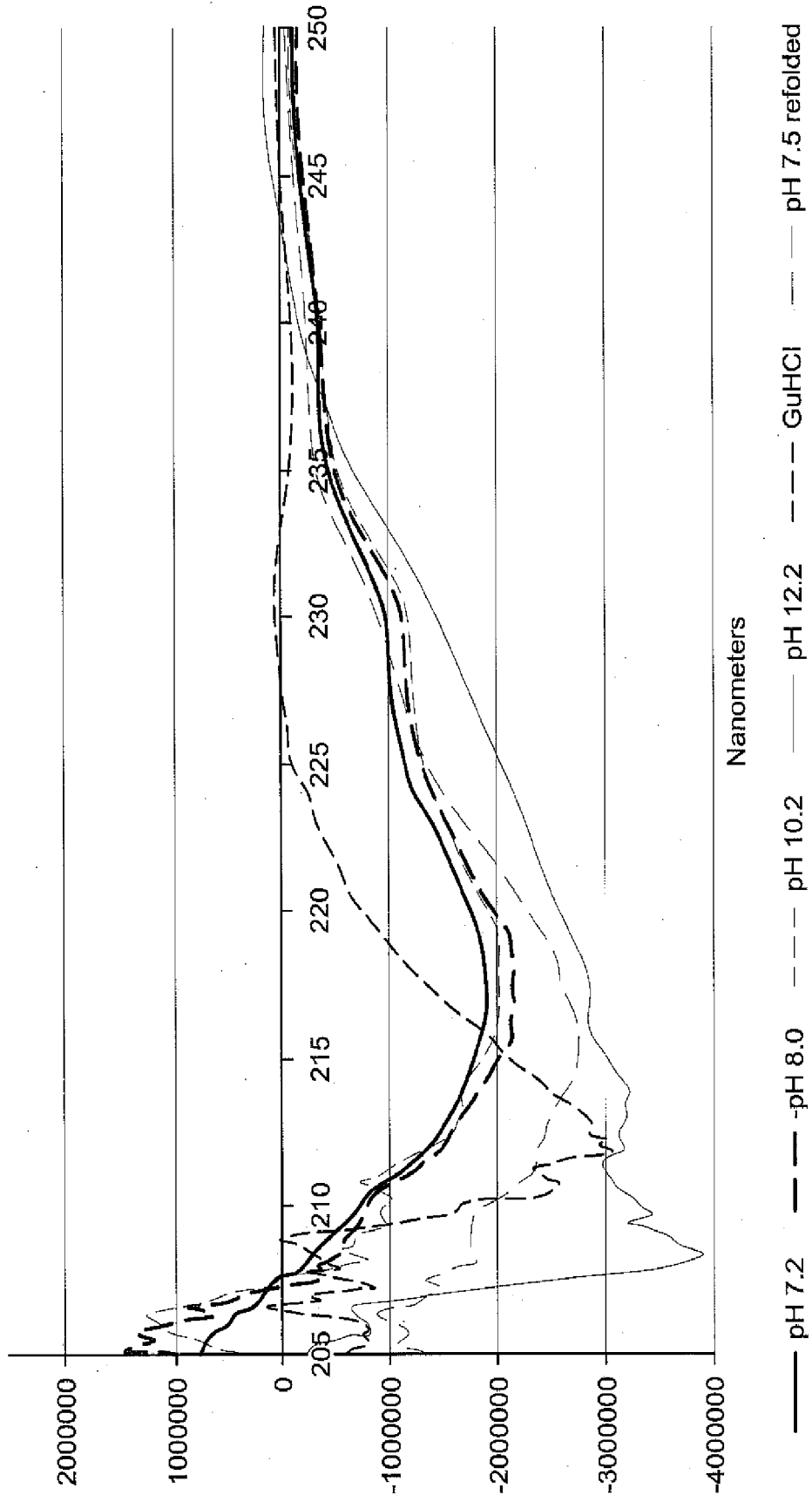


FIG. 6B

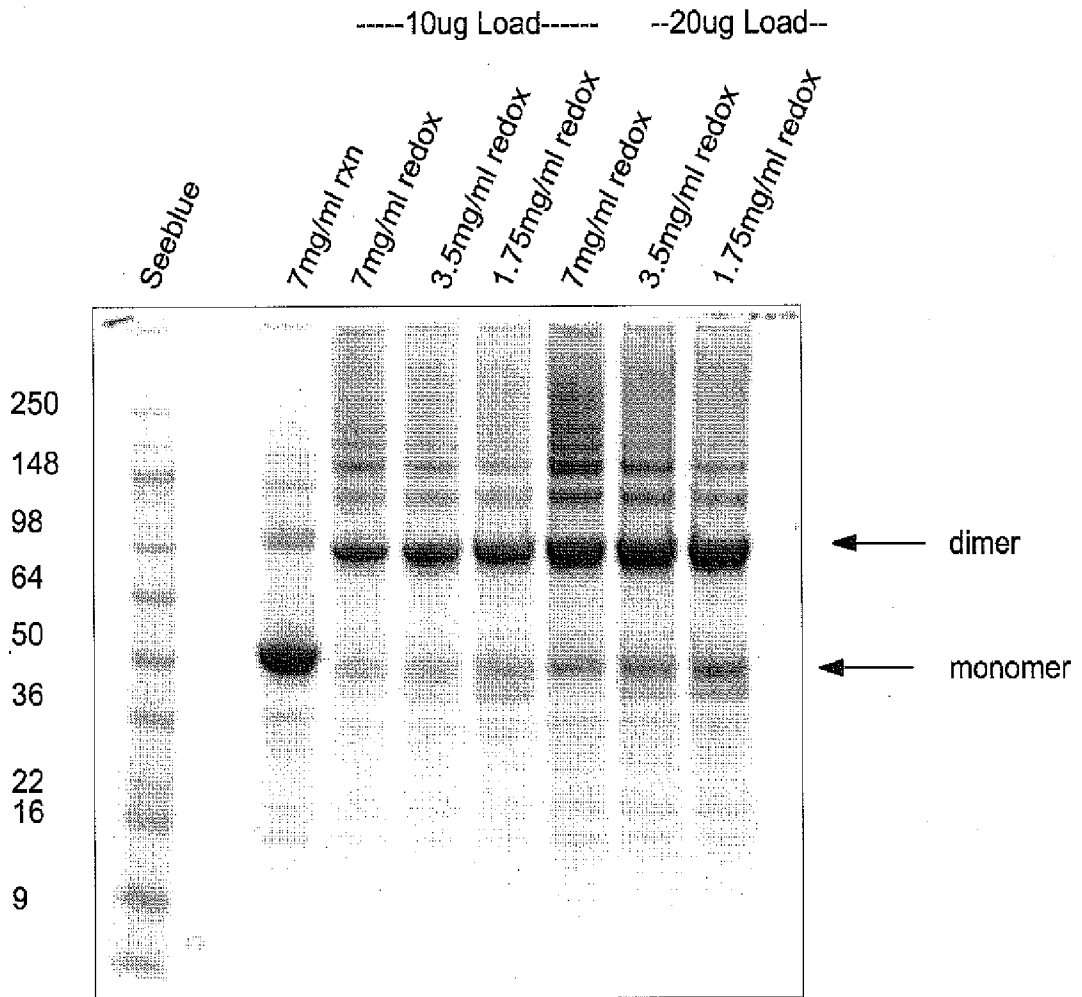


FIG. 7

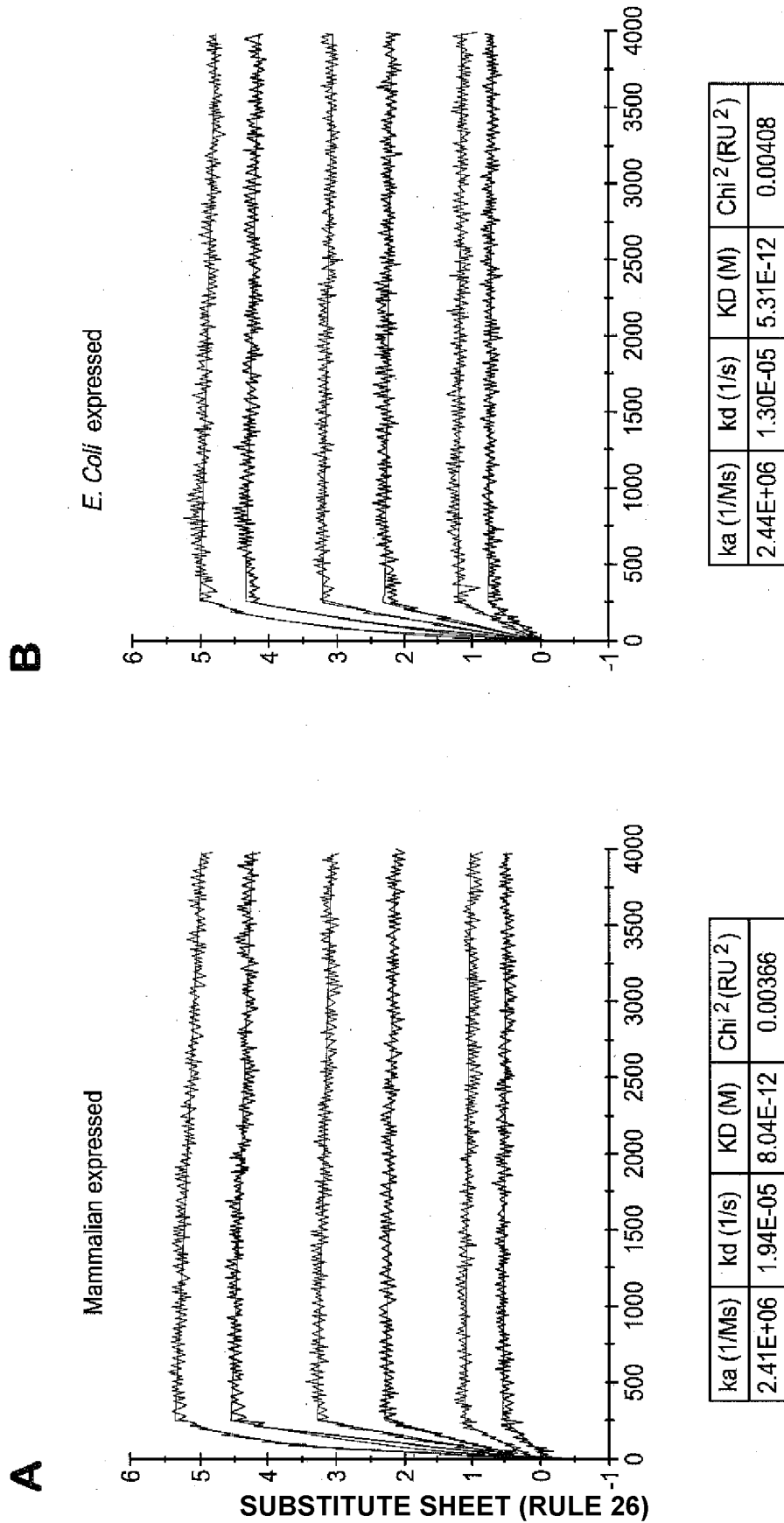


FIG. 8

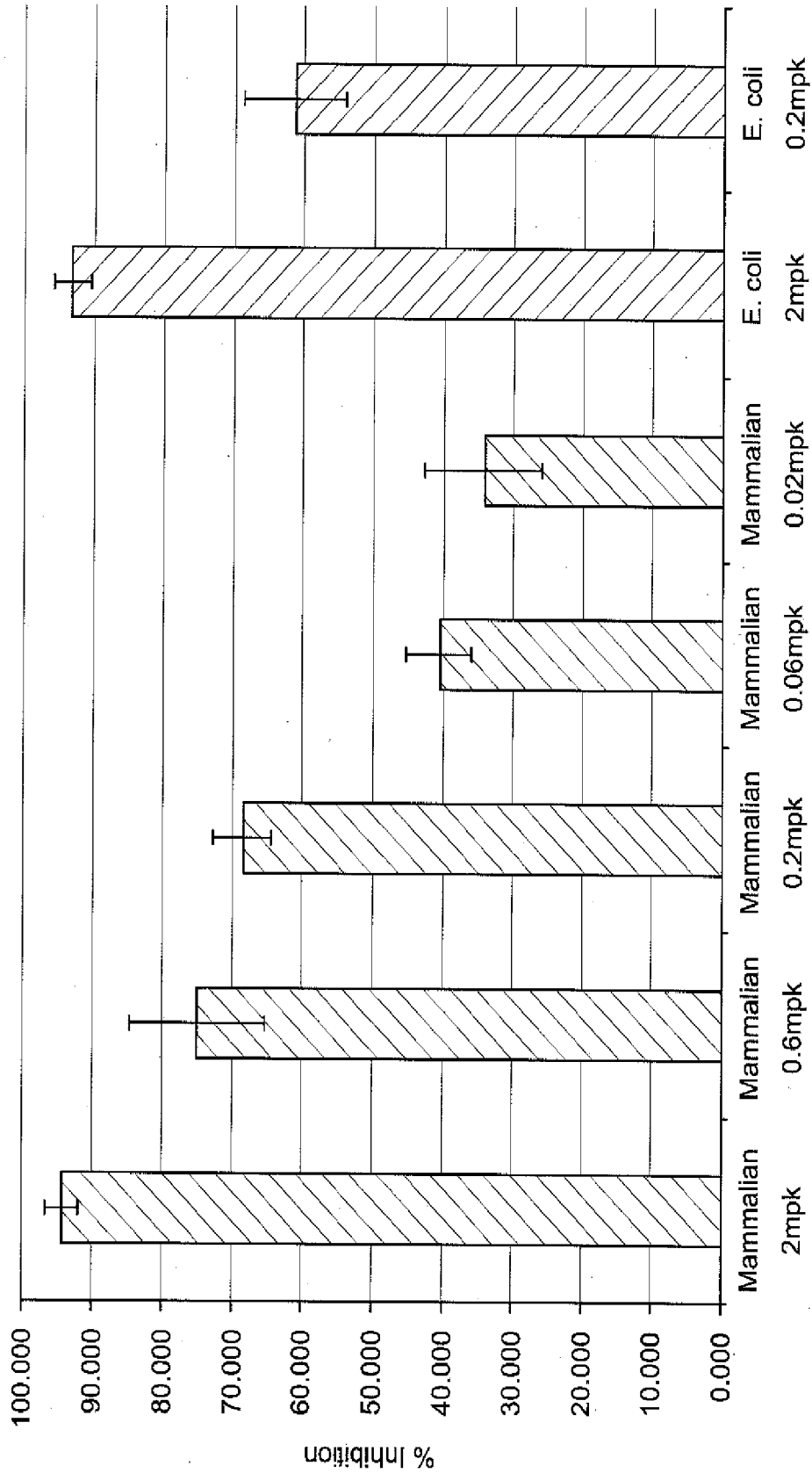
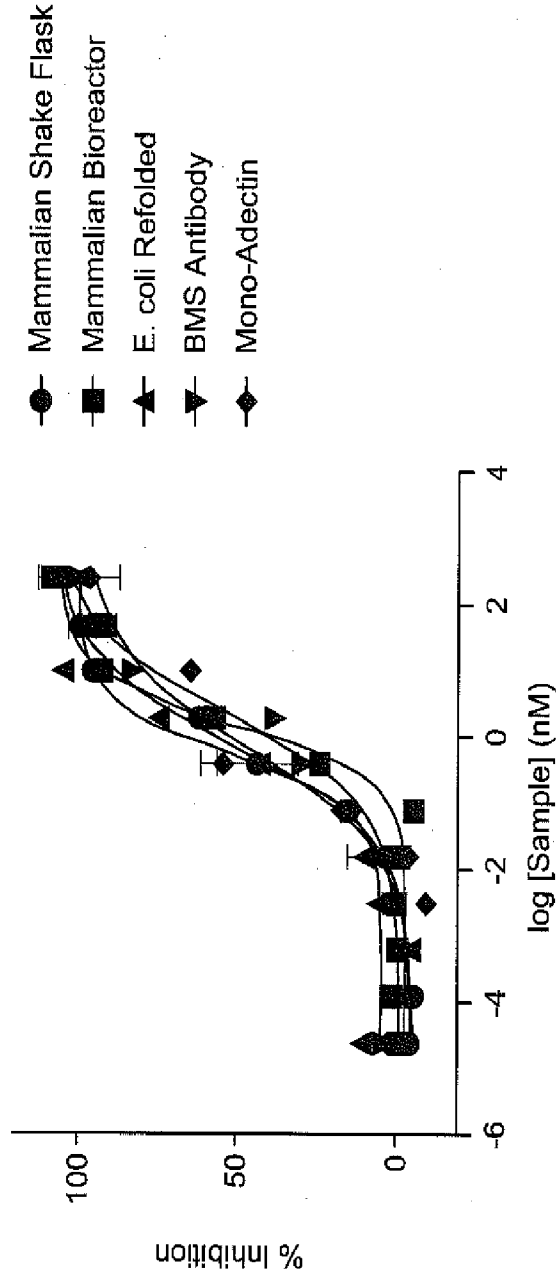


FIG. 9



	IC50
BMS Antibody	2.154
Mammalian Shake Flask	0.7492
Mammalian Bioreactor	1.45
E. coli Refolded	0.515
Mono-Adectin	0.6122

FIG. 10

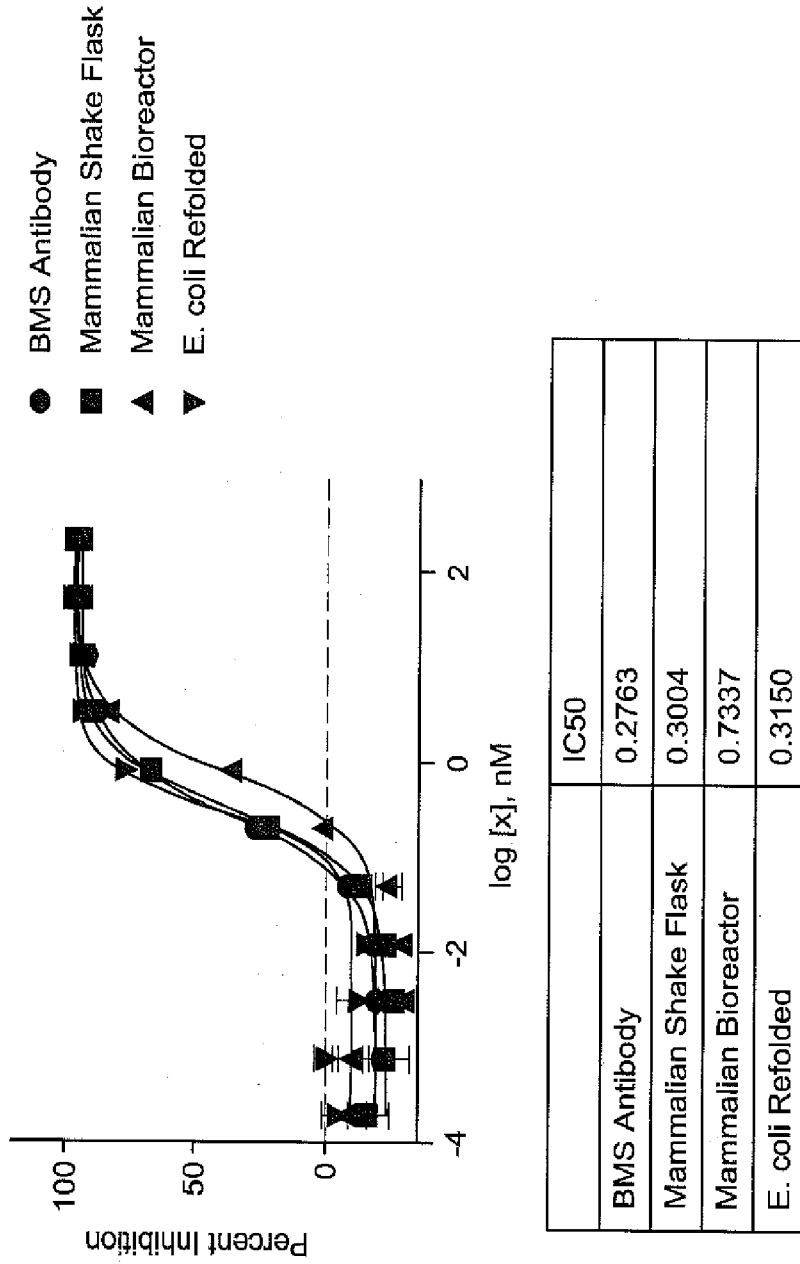


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No PCT/US2014/015702
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A. CLASSIFICATION OF SUBJECT MATTER INV. C07K1/113 C07K14/00 ADD.				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED				
Minimum documentation searched (classification system followed by classification symbols) C07K				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y	VALLEJO L F ET AL: "STRATEGIES FOR THE RECOVERY OF ACTIVE PROTEINS THROUGH REFOLDING OF BACTERIAL INCLUSION BODY PROTEINS", MICROBIAL CELL FACTORIES, BIOMED CENTRAL, LONDON, NL, vol. 3, 2 September 2004 (2004-09-02), pages 1-12, XP001205592, ISSN: 1475-2859, DOI: 10.1186/1475-2859-3-11 abstract ----- -/--	1-45		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.				
* Special categories of cited documents : <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; border: none; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search	Date of mailing of the international search report			
15 May 2014	16/07/2014			
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Rosin, Oliver			

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/015702

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RUDOLPH R ET AL: "IN VITRO FOLDING OF INCLUSION BODY PROTEINS", FASEB JOURNAL, FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, US, vol. 10, no. 1, 1 January 1996 (1996-01-01), pages 49-56, XP000605154, ISSN: 0892-6638 abstract	1-45
X	US 6 001 604 A (HARTMAN JACOB R [IL] ET AL) 14 December 1999 (1999-12-14)	1-3,7-9, 26,28, 35-38,41
Y	examples; claims 1-5,13	1-45
X	ZHIZHOU ZHANG ET AL: "Mechanism of enhancement of prochymosin renaturation by solubilization of inclusion bodies at alkaline pH", SCIENCE IN CHINA SERIES C LIFE SCIENCES, vol. 40, no. 2, 1 April 1997 (1997-04-01), pages 169-175, XP055117189, chapter 2; figures 1-3	1-3, 7-10,13, 26,28, 36-38,41
Y	CLARK E D B: "Protein refolding for industrial processes", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 12, no. 2, 1 April 2001 (2001-04-01), pages 202-207, XP002485975, ISSN: 0958-1669, DOI: 10.1016/S0958-1669(00)00200-7 the whole document	1-45
Y	WO 03/102013 A2 (GONZALEZ-VILLASENOR LUCIA IREN [US]) 11 December 2003 (2003-12-11) the whole document	1-45
Y	PATRA A K ET AL: "Optimization of Inclusion Body Solubilization and Renaturation of Recombinant Human Growth Hormone from Escherichia coli", PROTEIN EXPRESSION AND PURIFICATION, ACADEMIC PRESS, SAN DIEGO, CA, vol. 18, no. 2, 1 March 2000 (2000-03-01), pages 182-192, XP004435566, ISSN: 1046-5928, DOI: 10.1006/PREP.1999.1179 the whole document	1-45
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/015702

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WALSH DANIEL J ET AL: "Non-reducing alkaline solubilization and rapid on-column refolding of recombinant prion protein", PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY : AN INTERNATIONAL JOURNAL FOR RAPID COMMUNICATION, TAYLOR & FRANCIS, US, vol. 42, no. 1, 1 January 2012 (2012-01-01), pages 77-86, XP008169250, ISSN: 1532-2297, DOI: 10.1080/10826068.2011.564256 [retrieved on 2012-01-12] the whole document</p>	1-45
Y	<p>----- US 5 340 926 A (LOWE PETER A [GB] ET AL) 23 August 1994 (1994-08-23) the whole document</p>	1-45
Y	<p>----- WO 2008/153745 A2 (AMGEN INC [US]; WALKER KENNETH W [US]; GEGG COLIN V JR [US]) 18 December 2008 (2008-12-18) paragraph [0308]; sequence 2 -----</p>	1-45

INTERNATIONAL SEARCH REPORT

International application No.
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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-45

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-45

Methods for refolding denatured proteins.

2. claims: 46-56

Compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/US2014/015702

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6001604	A	14-12-1999	NONE
WO 03102013	A2	11-12-2003	CA 2444480 A1 23-08-2002
			US 2003166062 A1 04-09-2003
			WO 03102013 A2 11-12-2003
US 5340926	A	23-08-1994	NONE
WO 2008153745	A2	18-12-2008	AU 2008262490 A1 18-12-2008
			CA 2687141 A1 18-12-2008
			CA 2840407 A1 18-12-2008
			EP 2162540 A2 17-03-2010
			EP 2738257 A1 04-06-2014
			JP 2010527607 A 19-08-2010
			JP 2014064575 A 17-04-2014
			US 2009118181 A1 07-05-2009
			US 2013209466 A1 15-08-2013
			US 2013217625 A1 22-08-2013
			WO 2008153745 A2 18-12-2008