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(54) **Stable formulations of immunoglobulin single variable domains and uses thereof.**

(57) The present invention relates to stable formulations of polypeptides, e.g. immunoglobulin single variable domains, in particular immunoglobulin single variable domains directed against von Willebrand Factor (vWF). The invention provides formulations which are stable upon storage for prolonged periods of time and over a broad range of temperatures. The formulations of the invention ensure a high stability of the polypeptide, allowing multiple freeze-thaw cycles without chemical or physical deterioration, and provide stability in relation to mechanical stress, such as shake, shear or stir stress. They are suitable for pharmaceutical and diagnostic preparations and compatible with pharmaceutically acceptable diluents.

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Title: Stable formulations of immunoglobulin single variable domains and uses thereof

1. Field of the invention

The present invention relates to stable formulations of polypeptides, e.g. immunoglobulin single variable domains, in particular immunoglobulin single variable domains directed against von Willebrand Factor (vWF), such as immunoglobulin single variable domains according to SEQ ID NO:s 1-19, specifically SEQ ID NO: 1, i.e. the Nanobody ALX-0081.

The invention provides formulations which are stable upon storage for prolonged periods of time and over a broad range of temperatures. The formulations of the invention ensure a high stability of the polypeptide, allowing multiple freeze-thaw cycles without chemical or physical deterioration, and provide stability in relation to mechanical stress, such as shake, shear or stir stress. They are suitable for pharmaceutical and diagnostic preparations and compatible with pharmaceutically acceptable diluents, such as saline, Ringer's solution or glucose/dextrose solution.

The present invention also relates to methods of preparation, methods for storage and uses of the formulations. The invention further relates to dosage unit forms, kits and medical uses of the formulations.

2. Background of the invention

Immunoglobulin single variable domains, such as camelid VHH domains, camelized VH domains or humanized VHH domains, represent a rapidly growing class of antibody therapeutics. For example, immunoglobulin single variable domains against vWF have been described in WO2004/015425, WO2004/062551, WO2006/074947, WO2006/122825, WO2009/115614, and WO2011/067160.

Proteins such as immunoglobulin single variable domains (ISVDs) typically must be stored and transported between initial manufacture and use, e.g. administration to a patient. Transport, manufacture, storage and delivery processes can exert manifold stresses on the immunoglobulin single

variable domain, such as chemical and physical stresses. During storage chemical modifications can occur such as, for instance, deamidation, racemization, hydrolysis, oxidation, isomerization, beta-elimination or disulfide exchange. Physical stresses can cause denaturation and unfolding, aggregation, particulate formation, precipitation, opalescence or adsorption.

It is known that these stresses can affect the physicochemical integrity of protein therapeutics, e.g. antibody therapeutics. For example, aggregation, deamidation and oxidation have been described as most common causes of antibody degradation (Cleland et al., 1993, Crit. Rev. Ther. Drug Carrier Systems 10, 307-377). At the same time it is instrumental that formulations are provided which preserve chemical and physical integrity of the immunoglobulin single variable domains. Chemical and physical integrity are required for use as e.g. a therapeutic agent, and typically are also associated with biological activity. Although our knowledge of protein stability is increasing, optimizing formulation conditions to completely suppress or minimize these manifold stresses and ensure a prolonged shelf-life remains a major challenge.

Little is known about suitable formulations of immunoglobulin single variable domains. WO2010/077422 describes a formulation of a TNF binding Nanobody comprising a lyoprotectant, surfactant and a buffer chosen from histidine buffer and Tris-HCl buffer at a pH between 5.0 to 7.5.

Specific vWF binders, and in particular immunoglobulin single variable domains with high affinity to vWF such as ALX-0081 [INN: caplacizumab], have been tested as adjunctive therapy for patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) and are developed as treatment of thrombotic thrombocytopenic purpura (TTP). Phase I clinical trials have been completed successfully and testing in Phase II trials is currently ongoing. Thus far, ALX-0081 has been presented as a phosphate based liquid

formulation containing 5 mg/mL of the active pharmaceutical ingredient (API) in D-PBS, 200 mM glycine and 0.02% Tween-80 (v/v).

Although this formulation proved to be effective, it may be improved in several manners. First, the current concentration would probably necessitate multiple subcutaneous injections (assuming the volume per subcutaneous injection is restricted to about 1 mL) thus reducing patient friendliness and convenience. Secondly, the storage stability and shelf-life of the current formulation of ALX-0081 (hereinafter also referred to as contemporaneous ALX-0081) can be improved at elevated temperatures. The stability in the present formulation at high temperatures is mainly determined by chemical modifications on the polypeptide. Chemical modifications may be linked with potency loss. Although a practicable shelf-life can be achieved by storing the product at -20°C, this is, however, not considered to be a favourable option for most practical purposes.

Freeze-drying is a commonly employed technique for preserving proteins which serves to remove water from the protein preparation of interest. Freeze-drying, or lyophilization, is a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment. An excipient may be included in pre-lyophilized formulations to enhance stability during the freeze-drying process and/or to improve stability of the lyophilized product upon storage (Arakawa et al. Pharm. Res. 8(3):285-291 (1991)).

There remains a need for providing formulations for immunoglobulin single variable domains, e.g. as defined herein, which enhance stability, preserve the active agent against chemical and/or mechanical stress, and hence allow storage and temperature changes without significant physical or chemical deterioration, remain stable for prolonged periods of time, and/or are patient friendly, e.g. in which the active agent is soluble at high concentration.

3. Summary of the invention

The present invention relates to a formulation comprising a von Willebrand Factor (vWF) binder and a citrate or phosphate buffer with a pH in the range of 5.0 to 7.5. In particular, the present invention relates to a formulation as described herein, wherein said vWF binder comprises at least one immunoglobulin single variable domain binding to SEQ ID NO: 20.

Said immunoglobulin single variable domain comprises or essentially consists of, but is not limited to, an immunoglobulin single variable domain that is a heavy chain variable domain sequence, more specifically an immunoglobulin single variable domain which is a heavy chain variable domain sequence which is derived from a conventional four-chain antibody or a heavy chain variable domain sequence which is derived from a heavy chain antibody, or a Nanobody (including but not limited to a VHH sequence), preferably a Nanobody.

In addition, the present invention relates to a formulation as described herein, wherein said vWF binder comprises at least one of SEQ ID NO:s 1-19. Moreover, the present invention relates to a formulation as described herein, wherein said vWF binder is a single chain polypeptide comprising one or more immunoglobulin single variable domains, preferably wherein said vWF-binder is monovalent or multivalent, wherein said vWF-binder is monospecific or multispecific and/or wherein one or more immunoglobulin single variable domains are CDR-grafted, humanized, camelized, de-immunized, and/or in vitro generated (e.g. selected by phage display). The present invention also relates to a formulation as described herein, wherein said vWF binder comprises an amino acid sequence which is at least 90% identical to SEQ ID NO: 1. The present invention relates also to a formulation as described herein, wherein said vWF binder has a concentration in the range of 0.1 to 80 mg/mL, and/or wherein said buffer has a concentration in the range of 5-200 mM.

Additionally, the present invention relates to a formulation as described herein, further comprising an excipient, preferably said excipient has a concentration in the range of 10-500 mM, more preferably, wherein said excipient is selected from the list consisting of sucrose, glycine, mannitol, trehalose and NaCl, even more preferably, wherein said sucrose has a concentration in the range of 1-15%, preferably 2-12%, preferably 4-10%, e.g. 4, 5, 6, 7, 8 or 9% (w/v), most preferably 7%.

The present invention also relates to a formulation as described herein, wherein the buffer is selected from a citrate buffer, preferably said citrate buffer has a pH between 6.0 and 7.0, more preferably 6.5; and a phosphate buffer, preferably said phosphate buffer has a pH in the range of 6.5 to 7.5, preferably 7.1.

In addition, the present invention relates to a formulation as described herein, further comprising a non-ionic detergent, such as Tween-80, preferably in a concentration between 0.001 and 0.5% (v/v), more preferably 0.01-0.02% (v/v).

Furthermore, the present invention relates to a formulation as described herein, wherein said buffer is a citrate buffer at pH 6.5 ±0.5, e.g. 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 or 6.8, more specifically 6.5, and wherein said formulation further comprises sucrose having a concentration in the range of 1-15%, preferably 2-12%, preferably 4-10%, e.g. 4, 5, 6, 7, 8 or 9% (w/v), most preferably 7%, and preferably further comprises a non-ionic detergent such as Tween-80, preferably at a concentration of 0.01% (v/v).

Also, the present invention relates to a formulation as described herein, wherein said formulation has an osmolality in the range of 290 ± 60 mOsm/kg, more preferably in the range of 290 ± 20 mOsm/kg.

The present invention further relates to a formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) an excipient chosen from sucrose, glycine, mannitol, trehalose or NaCl at a concentration of about 1% to about 15% (w/v);

(c) Tween-80 at a concentration of about 0.001% to 0.5% (v/v); and

(d) a buffer chosen from citrate buffer at a concentration of about 5 mM to about 200 mM such that the pH of the formulation is about 6.0 to 7.0 and a phosphate buffer at a concentration of about 10 mM to about 50 mM such that the pH of the formulation is about 6.5 to 7.5, wherein the vWF binder in the formulation retains at least about 80% of its stability after storage for at least 12 months at 5°C.

The invention also relates to a formulation which has less than 5% of high molecular weight (HMW) species after storage for at least 12 months at 5°C; and/or less than 5% of low molecular weight (LMW) species after storage for at least 12 months at 5°C.

The invention further relates to a formulation wherein at least 80%, preferably at least 90%, more preferably at least 95% or even at least 99% of the vWF binder retains it binding activity after storage compared to the binding activity prior to storage, said binding activity as measured by ELISA and/or Biacore.

In addition, the invention relates to a formulation as described herein, wherein said formulation is in a liquid, lyophilized, spray-dried, reconstituted lyophilized or frozen form, more specifically the invention pertains to a liquid or reconstituted lyophilized formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) sucrose at a concentration of about 1% to about 15% (w/v);

(c) Tween-80 at a concentration of about 0.001%-0.5% (v/v); and

(d) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0.

The lyophilized formulation can then be reconstituted as needed by mixing the lyophilized form with a suitable diluent (e.g. water) to resolubilize the original formulation components to a desired concentration.

The present invention also relates to a formulation as described herein, wherein said formulation is a bulk storage formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) sucrose at a concentration of about 1% to about 15%;

(c) Tween-80 at a concentration of about 0.001%-0.5% (w/v); and

(d) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0, wherein at least 100 liters of the formulation are stored at below freezing conditions.

In addition, the present invention relates to a formulation, wherein said formulation is suitable for parenteral administration to a subject, e.g., a human subject (e.g. a patient having a vWF-related disorder). The formulation can be administered to the subject by injection (e.g., intravenous, subcutaneous, intramuscular or intraperitoneal).

Also, the present invention provides a formulation as described herein, for use in a method of treating a human or animal subject, preferably for use in treating vWF- related disorders, such as e.g. acute coronary syndrome (ACS), transient cerebral ischemic attack, unstable or stable angina pectoris, stroke, myocardial infarction or thrombotic thrombocytopenic purpura (TTP), most preferably for use in treating TTP or ACS. Moreover, the present invention pertains to a method or process of preparing the formulation as described herein. The method or process includes expressing the vWF binder in a cell culture; purifying the vWF binder, e.g., by passing the vWF binder through at least one of a chromatography purification step, an ultrafiltration/diafiltration steps; adjusting the concentration of the vWF binder, e.g., to about 0.1 to 80 mg/mL in a formulation containing a lyoprotectant, a surfactant and a

buffer as described herein, e.g., sucrose at a concentration of about 1% to about 15%; Tween-80 at a concentration of about 0.001% to about 0.5% (w/v); and a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0; and optionally comprising a step of confectioning the formulation in a dosage unit form.

The invention also features a method or process for preparing a reconstituted formulation containing a vWF binder, e.g., ALX-0081 as described herein. The method includes: lyophilizing a mixture of a vWF binder, a lyoprotectant, a surfactant and a buffer, thereby forming a lyophilized mixture; and reconstituting the lyophilized mixture in a diluent, thereby preparing a formulation as described herein. In particular, the formulation includes (a) a vWF binder, e.g., ALX-0081 at a concentration of about 0.1 to about 80 mg/mL; (b) sucrose at a concentration of about 1% to about 15% (w/v); (c) Tween-80 at a concentration of about 0.001% to about 0.5% (v/v); and (d) a citrate buffer at a concentration of about 5 to about 200 mM, such that the pH of the formulation is about 6 to 7.0; and optionally comprising a step of confectioning the formulation in a dosage unit form.

The present invention further relates to a method for stabilizing a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19 for storage, comprising preparing a formulation as defined herein.

In addition, the invention relates to a method for storing a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19, comprising preparing a formulation as defined herein.

Also provided are pharmaceutical or diagnostic compositions comprising any of the formulations described herein or obtainable by the methods described herein.

Further, the invention features a method of analyzing a product or a process, e.g., a manufacturing process. The method includes providing a formulation of a vWF binder, e.g., ALX-0081 as described herein, and assessing a parameter of the formulation, such as color, clarity, viscosity or

an amount of one or more HMW, LMW species, as described herein. The evaluation can include an assessment of one or more parameters, such as determining whether the parameter meets a preselected criterion, e.g., determining whether the preselected criterion is present, or is present in a preselected range, thereby analyzing the process. For example, evaluation of the process includes a measure of the stability of the vWF binder formulation. Stability of the ALX-0081 formulation can be measured, for example, by aggregate formation, which is assayed, e.g., by size exclusion high pressure liquid chromatography (SE-HPLC), by color, clarity, or viscosity as described herein.

In addition, the method may further comprise comparing two or more sample formulations in a method of monitoring or controlling batch-to-batch variation, comparing a preparation to a reference standard, classifying, selecting, accepting or discarding, releasing or withholding, processing into a drug product, shipping, moving to a different location, formulating, labelling, or packaging the formulation, based upon the comparison. Also, the method may further comprise providing a record which includes data relating to the assessed parameter of the formulation and optionally includes an identifier for a batch of the formulation; submitting said record to a decision-maker; optionally, receiving a communication from said decision maker; optionally, deciding whether to release or market the batch of formulation based on the communication from the decision maker.

Also provided are kits or articles of manufacture comprising the formulation of the invention and instructions for use by, e.g., a healthcare professional. The kits or articles of manufacture may include a vial or a syringe containing the formulation of the invention as described herein. Preferably, the vial or syringe is composed of glass, plastic, or a polymeric material chosen from a cyclic olefin polymer or copolymer. Furthermore, the

formulation can also be present in an injectable device (e.g., an injectable syringe, e.g. a prefilled injectable syringe).

The invention further provides pharmaceutical unit dosage forms comprising the stable formulations of the invention which are suited for parenteral administration (e.g., intradermally, intramuscularly, intraperitoneally, intravenously and subcutaneously) of the formulation of the invention to a human patient.

Moreover, the formulations of the invention can be used for storage of a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19, such as ALX-0081 as described herein, wherein said storage is 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months, preferably at least 12 months, e.g. at a temperature between -70°C and +40°C, such as -70°C, -20°C, +5°C, +25°C or +40°C, preferably a temperature between -70°C and +25°C.

The present invention also relates to a method of treating or preventing a vWF- related disorder, such as e.g. acute coronary syndrome (ACS), transient cerebral ischemic attack, unstable or stable angina pectoris, stroke, myocardial infarction or thrombotic thrombocytopenic purpura (TTP); said method comprising administering to a subject a pharmaceutical composition comprising the formulation of the invention, thereby reducing one or more symptoms associated with said vWF-related disorder. In particular, said vWF-related disorder is TTP.

4. Brief Description of the Figures

Figure 1 Flow chart representing the different steps of the standard 65h-lyophilization program performed for ALX 0081.

Figure 2A Relevant part of the RP-HPLC chromatograms of ALX-0081 after 1 and 2 months storage at -70°C, +5°C and +25°C; mAU: milli absorbance unit.

Figure 2B Zoom of relevant part of the RP-HPLC chromatograms of ALX-0081 after 0, 4 and 8 weeks incubation at 37°C. Splitting of the RP-HPLC main peak is observed as a result of prolonged incubation at 37°C (0, 4, 8w); mAU: milli absorbance unit.

Figure 3A Overlay of SE-HPLC profiles of blank citrate buffer (bcb) and ALX-0081 in 20 mM citrate pH 7.0 at 55.9 mg/mL before (a) and after 10 freeze-thaw (FT) cycles at -20°C (c) and -70°C (b) (λ = 280 nm). A minor citrate peak was observed for samples pre-diluted in running buffer; mAU: milli absorbance unit.

Figure 3B Overlay of SE-HPLC profiles of blank citrate buffer (bcb) and ALX-0081 in 20 mM citrate pH 7.0 at 55.9 mg/mL after ±1 week storage at +4°C (λ = 280 nm). ALX-0081 was resolved into one main peak (97%) corresponding with intact, unmodified ALX-0081 and small pre peaks representing only 3% of the total surface area. A minor citrate peak was observed for ALX-0081 pre-diluted in running buffer; mAU: milli absorbance unit.

Figure 4A Scatter intensity of stirred ALX-0081 samples in 50 mM citrate pH 6.0, 50 mM citrate pH 6.0 + 0.01% Tween-80 (v/v) and 50 mM citrate pH 6.0 + 0.02% Tween-80 (v/v) at +25°C. '+' represent samples in 50 mM citrate pH 6.0 ($y=0.0044x + 3.5962$, $R^2=0.9549$); 'o' represent samples in 50 mM citrate pH 6.0 + 0.02% Tween-80 (v/v) ($y=0.0002x + 1.0447$, $R^2=0.4673$); 'x' represent samples in 50 mM citrate pH 6.0 + 0.01% Tween-80 (v/v) ($y=0.0004x + 0.5125$, $R^2=0.6804$);(x-axis = time in seconds; y-axis = scatter intensity).

Figure 4B Scatter intensity of stirred ALX-0081 samples in 50 mM citrate pH 6.5, 50 mM citrate pH 6.5 + 0.01% Tween-80 (v/v) and 50 mM citrate pH 6.5 + 0.02% Tween-80 (v/v) at +25°C. '+' represent samples in 50 mM citrate pH 6.5 ($y=0.0041x + 4.7667$, $R^2=0.9431$); 'o' represent samples in 50 mM citrate pH 6.5 + 0.02% Tween-80 (v/v) ($y=0.0004x - 0.0208$, $R^2=0.9391$); 'x' represent samples in 50 mM citrate pH 6.5 + 0.01% Tween-80

(v/v) (y=0.0001x – 1.8853, R^2=0.0376); (x-axis = time in seconds; y-axis = scatter intensity).

Figure 5 Overlay of the cIEF profiles of ALX-0081 at 5 mg/mL in D-PBS + 200 mM glycine + 0.01% Tween-80 after 1 month storage at +40°C (a) and -70°C (b); (λ= 280 nm). AU: absorbance unit; pxlpos: pixelposition.

Figure 6 Picture of lyophilized ALX-0081 formulations (form 3 = citrate/sucrose pH 6.0; form 7 = citrate/sucrose pH 6.5; form 17 = D-PBS/glycine) before (panel A) and after reconstitution with Milli-Q water (panel B).

Figure 7 Picture of lyophilized ALX-0081 citrate/sucrose based formulations.

Figure 8 Pictures of liquid ALX-0081 formulations at 28 mg/mL containing 15, 20, 25, 30, 40, and 50 mM citrate pH 6.5 after 4 days storage at +25°C (panel A) or +5°C (panel B). Blank citrate buffer (50 mM) is included as reference.

Figure 9 Pictures of liquid ALX-0081 formulations at 20 mg/mL containing 15 mM citrate pH 6.5 and different amounts of sucrose and Tween-80 after 4 days storage at +25°C (panel A) or +5°C (panel B). Blank citrate buffer (50 mM) is included as reference.

5. Detailed description of the invention

Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known per se, as will be clear to the skilled person. Reference is for example again made to the standard handbooks and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews: Presta, Adv. Drug Deliv. Rev. 2006, 58 (5-6): 640-56; Levin and Weiss, Mol. Biosyst. 2006, 2(1): 49-57; Irving et al., J. Immunol. Methods,

2001, 248(1-2), 31-45; Schmitz et al., Placenta, 2000, 21 Suppl. A, S106-12, Gonzales et al., Tumour Biol., 2005, 26(1), 31-43, which describe techniques for protein engineering, such as affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

It has now surprisingly been found that vWF binders, and in particular ALX-0081 (SEQ ID NO: 1), can be administered in particular dosing regimens in humans. The vWF binders, and in particular ALX-0081, have been found to produce a pharmacodynamic effect, with a fast onset of action immediately at the end of dosing and maintains its efficacy for up to about 12-24 h. Additionally, vWF binders, and in particular ALX-0081 (SEQ ID NO: 1), have been found to be well tolerated and safe in healthy male volunteers. These results indicate that vWF binders and in particular ALX-0081 (SEQ ID NO: 1) are suitable for acute treatment in patients with stable angina undergoing elective percutaneous coronary intervention (hereinafter also "PCI") and treatment in patients with thrombotic thrombocytopenic purpura (hereinafter also "TTP").

Nevertheless, the current formulations of the vWF binders and in particular ALX-0081 (SEQ ID NO: 1) administered to human recipients were amenable to improvement.

The reformulation invention for the vWF binders, and in particular ALX-0081, described herein rendered a new citrate/sucrose based formulation with increased solubility (up to 80 mg/mL) and significantly improved liquid storage stability (e.g. less oxidation occurs when storing the new formulation to the original formulation in its liquid state). Also, in the lyophilized form, essentially no oxidation or asp-isomerisation could be detected after 12 months storage at +40°C. A residual formation of small amounts of pyroglutamate was still observed. Further optimization of the citrate and sucrose concentration resulted in a reduction of the moisture

content of the lyophilized product, thereby minimizing the rate of residual pyroglutamate formation.

Accordingly the present invention provides stable liquid and lyophilized formulations of anti-vWF binders (e.g. ALX-0081) and uses thereof for treating or preventing vWF-related disorders.

5.1 Polypeptide(s) of the invention

The vWF binders used in the present invention are typically proteins or polypeptides that bind to human von Willebrand Factor (vWF, SEQ ID NO: 20). Preferably, the vWF binders are proteins or polypeptides comprising or consisting of at least one immunoglobulin sequences, such as an immunoglobulin single variable domain (ISVD). Even more preferably, the vWF binders of the present invention are proteins or polypeptides comprising or consisting of SEQ ID NO:s 1-19, and most preferably SEQ ID NO: 1. The vWF binders may be used as adjunctive therapy for patients with ACS undergoing PCI or as treatment of thrombotic thrombocytopenic purpura (TTP). The terms "protein", "polypeptide" and "amino acid sequence" are used interchangeably herein. Thus, an amino acid sequence of the invention is a vWF binder.

Thus, for example, suitable vWF binders for use in the invention may include the compounds in Table A-1, e.g. SEQ ID NO: 1-19, or a compound having 80% or more, more preferably 85% or more, most preferred 90%, 95%, 96%, 97%, 98%, 99% or more, amino acid sequence identity to a compound in Table A-1 (see Definition section for "sequence identity").

Preferably the vWF binders for use in the invention are 12A02H1-like compounds. For the purposes of the present description a 12A02H1-like compound is a compound which comprises 12A02H1 (i.e. SEQ ID NO: 19) or a compound having 80% or more, more preferably 85% or more, most preferably 90%, 95%, 96%, 97%, 98%, 99% or more, amino acid sequence

identity (as further defined herein) to 12A02H1 (SEQ ID NO: 19). A particularly preferred vWF binder is ALX-0081 (SEQ ID NO: 1).

All the vWF binders mentioned above are well known from the literature. This includes their manufacture (see in particular e.g. WO2006/122825 but also WO2004/062551). For example, ALX-0081 is prepared as described e.g. in WO2006/122825 or WO2009/115614.

Unless indicated otherwise, the term "immunoglobulin sequence" - whether used herein to refer to a heavy chain antibody or to a conventional 4-chain antibody - is used as a general term to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as V_{HH} domains or V_H/V_L domains, respectively). The terms antigen-binding molecule or antigen-binding protein are used interchangeably with immunoglobulin sequence, and include immunoglobulin single variable domains, such as Nanobodies®.

Embodiments of the invention relate to immunoglobulin sequences that are immunoglobulin single variable domains, such as light chain variable domain sequences (e.g. a V_L-sequence), or heavy chain variable domain sequences (e.g. a V_H-sequence); more specifically, heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody (e.g. a V_{HH}-sequence).

The term "immunoglobulin single variable domain", defines molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain or suitable fragments thereof. This sets immunoglobulin single variable domains apart from "conventional" immunoglobulins or their fragments, wherein two immunoglobulin domains, in particular two variable domains interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (V_H) and a light chain variable domain (V_L) interact to form an antigen

binding site. In this case, the complementarity determining regions (CDRs) of both V_H and V_L will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation.

In contrast, the antigen binding site of an immunoglobulin single variable domain is formed by a single V_H or V_L domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs, e.g. one, two or three CDRs.

The term "immunoglobulin single variable domain" hence does not comprise conventional immunoglobulins or their fragments which require interaction of at least two variable domains for the formation of an antigen binding site. This is also the case for embodiments of the invention which "comprise" or "contain" an immunoglobulin single variable domain. In the context of the present invention, such embodiments exclude conventional immunoglobulins or their fragments. Thus, a composition that "comprises" or "contains" an immunoglobulin single variable domain may relate to e.g. constructs comprising more than one immunoglobulin single variable domain. Alternatively, there may be further constituents other than the immunoglobulin single variable domains, e.g. auxiliary agents of different kinds, protein tags, colorants, dyes, etc. However, these terms do comprise fragments of conventional immunoglobulins wherein the antigen binding site is formed by a single variable domain.

According to the invention, the polypeptide of the invention, more specifically the immunoglobulin sequences, can consist of, or comprise one or more of the following: domain antibodies, or amino acid sequences that are suitable for use as domain antibodies, single domain antibodies, or amino acid sequences that are suitable for use as single domain antibodies, "dAbs", or amino acid sequences that are suitable for use as dAbs, or Nanobodies®, including but not limited to V_{HH} sequences, and preferably are Nanobodies®.

The present invention encompasses suitable fragments of immunoglobulin single variable domains. "Suitable fragments" of immunoglobulin single variable domains relate to polypeptides which contain fewer amino acids than a native immunoglobulin single variable domain, but still show antigen binding activity (which will then usually contain at least some of the amino acid residues that form at least one of the CDR's, as further described herein). Such immunoglobulin single variable domains and fragments most preferably comprise an immunoglobulin fold or are capable for forming, under suitable conditions, an immunoglobulin fold. More specifically, immunoglobulin single variable domains and their fragments are such that they are capable of binding to the target antigen. As such, the immunoglobulin single variable domain may for example comprise a light chain variable domain sequence (e.g. a V_L-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g. a V_H-sequence or V_{HH}-sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e. a functional antigen binding unit that essentially consists of the immunoglobulin single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit, as is for example the case for the variable domains that are present in for example conventional antibodies and scFv fragments that need to interact with another variable domain – e.g. through a V_H/V_L interaction – to form a functional antigen binding domain).

The immunoglobulin sequences of the invention are preferably in essentially isolated form. The immunoglobulin sequences of the invention may also form part of a protein or polypeptide of the invention (as defined herein), which may comprise or essentially consist of one or more amino acid sequences of the invention and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more

amino acid sequences of the invention may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further amino acid sequences that can serve as a binding unit, so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. Such a protein or polypeptide may also be in essentially isolated form.

The invention relates to immunoglobulin sequences of different origin, comprising mouse, rat, rabbit, donkey, human and camelid immunoglobulin sequences. The invention also includes fully human, humanized or chimeric immunoglobulin sequences. For example, the invention comprises camelid immunoglobulin sequences and humanized camelid immunoglobulin sequences, or camelized domain antibodies, e.g. camelized dAb as described by Ward et al (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996)). Moreover, the invention comprises fused immunoglobulin sequences, e.g. forming a multivalent and/or multispecific construct (for multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to Conrath et al., J. Biol. Chem., Vol. 276, 7346-7350, 2001, as well as to for example WO96/34103 and WO99/23221), and immunoglobulin sequences comprising tags or other functional moieties, e.g. toxins, labels, radiochemicals, etc., which are derivable from the immunoglobulin sequences of the present invention. Immunoglobulin single variable domains have also been described in sharks (also referred to as "IgNARs", as described e.g. in WO03/014161 or Streltsov, 2005).

In a particular embodiment, the immunoglobulin single variable domains of the invention are Nanobodies®, in particular camelid V_{HH} domains, humanized V_{HH} domains or camelized V_H domains. The skilled person is well acquainted with humanization of V_{HH} and/or camelizing V_H domains.

The amino acid sequence and structure of an immunoglobulin sequence, in particular a Nanobody® can be considered - without however being limited thereto - to be comprised of four framework regions or "FR's", which are referred to in the art and herein as "Framework region 1" or "FR1"; as "Framework region 2" or "FR2"; as "Framework region 3" or "FR3"; and as "Framework region 4" or "FR4", respectively; which framework regions are interrupted by three complementary determining regions or "CDR's", which are referred to in the art as "Complementarity Determining Region 1" or "CDR1"; as "Complementarity Determining Region 2" or "CDR2"; and as "Complementarity Determining Region 3" or "CDR3", respectively.

The total number of amino acid residues in a Nanobody® can be in the region of 110-120, is preferably 112-115, and is most preferably 113. It should however be noted that parts, fragments, analogs or derivatives (as further described herein) of a Nanobody® are not particularly limited as to their length and/or size, as long as such parts, fragments, analogs or derivatives meet the further requirements outlined herein and are also preferably suitable for the purposes described herein.

Thus, generally, immunoglobulin single variable domains will be amino acid sequences that consist of, or essentially consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively). "Essentially consist" in this context means that additional elements such as e.g. tags used for purification or labelling may be present, but such additional elements are small as compared to the immunoglobulin single variable domain per se, and do not interfere with the antigen binding activity of the immunoglobulin single variable domain.

As used herein, the term "immunoglobulin sequences" or "immunoglobulin single variable domains" refers to both the nucleic acid

sequences coding for the polypeptide, and the polypeptide per se. Any more limiting meaning will be apparent from the specific context.

In particular, the amino acid sequence of the invention may be a Nanobody® or a suitable fragment thereof. For a further description of VHH's and Nanobodies, reference is made to the review article by Muyldermans in Reviews in Molecular Biotechnology 74(2001), 277-302; as well as to the following patent applications, which are mentioned as general background art: WO94/04678, WO95/04079 and WO96/34103 of the Vrije Universiteit Brussel; WO94/25591, WO99/37681, WO00/40968, WO00/43507, WO00/65057, WO01/40310, WO01/44301, EP1134231 and WO02/48193 of Unilever; WO97/49805, WO01/21817, WO03/035694, WO03/054016 and WO03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO03/050531 of Algonomics N.V. and Ablynx N.V.; WO 01/90190 by the National Research Council of Canada; WO03/025020 (= EP1433793) by the Institute of Antibodies; as well as WO04/041867, WO04/041862, WO04/041865, WO04/041863, WO04/062551, WO05/044858, WO06/40153, WO06/079372, WO06/122786, WO06/122787 and WO06/122825, by Ablynx N.V. and the further published patent applications by Ablynx N.V. Reference is also made to the further prior art mentioned in these applications, and in particular to the list of references mentioned on pages 41-43 of the International application WO06/040153, of which the list and references are incorporated herein by reference. As described in these references, Nanobodies (in particular VHH sequences and partially humanized Nanobodies) can in particular be characterized by the presence of one or more "Hallmark residues" in one or more of the framework sequences. A further description of the Nanobodies, including humanization and/or camelization of Nanobodies, as well as other modifications, parts or fragments, derivatives or "Nanobody fusions", multivalent constructs (including some non-limiting examples of linker

sequences) and different modifications to increase the half-life of the Nanobodies and their preparations can be found e.g. in WO07/104529.

The immunoglobulin single variable domains provided by the invention are preferably in isolated form or essentially isolated form. The immunoglobulin sequences of the invention may also form part of a protein or polypeptide of the invention, which may comprise or essentially consist of one or more immunoglobulin single variable domains and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers). For example, and without limitation, the one or more immunoglobulin single variable domains may be used as a binding unit in such a protein or polypeptide, which may optionally contain one or more further amino acid sequences that can serve as a binding unit, so as to provide a monovalent, multivalent or multispecific polypeptide of the invention, respectively, all as described herein. Such a protein or polypeptide may also be in isolated or essentially isolated form. Thus, according to the invention, immunoglobulin single variable domains comprise constructs comprising two or more antigen binding units in the form of single domains, as outlined above. For example, two (or more) immunoglobulin single variable domains with the same or different antigen specificity can be linked to form e.g. a bivalent, trivalent or multivalent construct. By combining immunoglobulin single variable domains of two or more specificities, bispecific, trispecific etc. constructs can be formed. For example, a polypeptide according to the invention may comprise two immunoglobulin single variable domains directed against target A, and one immunoglobulin single variable domain against target B, making it bivalent for A and monovalent for B. Such constructs and modifications thereof, which the skilled person can readily envisage, are all encompassed by the present invention. In particular embodiments, the invention relates to bi-paratopic constructs comprising at least two

immunoglobulin single variable domains directed to different epitopes within the same target antigen.

All these molecules are also referred to as "polypeptide of the invention", which is synonymous with "immunoglobulin sequences" or "immunoglobulin single variable domains" of the invention.

In addition, the term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "variable domain sequence", "VHH-sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

According to one non-limiting embodiment of the invention, the immunoglobulin sequences, Nanobody® or polypeptide of the invention is glycosylated. According to another non-limiting embodiment of the invention, the immunoglobulin sequences, Nanobody® or polypeptide of the invention is non-glycosylated.

5.2 "Binding" to an antigen

The invention relates to immunoglobulin sequences that can bind to and/or have affinity for an antigen as defined herein, e.g. von Willebrand Factor. In the context of the present invention, "binding to and/or having affinity for" a certain antigen has the usual meaning in the art as understood e.g. in the context of antibodies and their respective antigens.

In particular embodiments of the invention, the term "binds to and/or having affinity for" means that the immunoglobulin sequence specifically interacts with an antigen, and is used interchangeably with immunoglobulin sequences "against" the said antigen.

The term "specificity" refers to the number of different types of antigens or antigenic determinants to which a particular immunoglobulin sequence, antigen-binding molecule or antigen-binding protein (such as an

immunoglobulin single variable domain, a Nanobody® or a polypeptide of the invention) can bind. The specificity of an antigen-binding protein can be determined based on affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (KD), is a measure for the binding strength between an antigenic determinant and an antigen-binding site on the antigen-binding protein: the lesser the value of the KD, the stronger the binding strength between an antigenic determinant and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (KA), which is 1/KD). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known per se, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as an immunoglobulin single variable domain, a Nanobody® or polypeptide of the invention) and the pertinent antigen. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

Typically, immunoglobulin sequences of the present invention (such as the amino acid sequences, immunoglobulin single variable domains, Nanobodies® and/or polypeptides of the invention) will bind to their antigen with a dissociation constant (KD) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (KA) of 10^{5} to 10^{12} liter/moles or more, and preferably 10^{7} to 10^{12} liter/moles or more and more preferably 10^{8} to 10^{12} liter/moles), and/or bind to their antigen as defined herein with a kon-rate of between 10^{2} $M^{-1}s^{-1}$ to about 10^{7} $M^{-1}s^{-1}$, preferably between 10^{3} $M^{-1}s^{-1}$ and 10^{7} $M^{-1}s^{-1}$, more preferably between 10^{4} $M^{-1}s^{-1}$ and 10^{7} $M^{-1}s^{-1}$, such as between 10^{5} $M^{-1}s^{-1}$ and 10^{7} $M^{-1}s^{-1}$; and/or bind to their antigen as defined herein with a koff rate between $1s^{-1}$ (t1/2=0.69 s) and 10^{-6}

s^{-1} (providing a near irreversible complex with a t1/2 of multiple days), preferably between 10^{-2} s^{-1} and 10^{-6} s^{-1}, more preferably between 10^{-3} s^{-1} and 10^{-6} s^{-1}, such as between 10^{-4} s^{-1} and 10^{-6} s^{-1}.

Any KD value greater than 10^{-4} M (or any KA value lower than 10^{4} M^{-1}) is generally considered to indicate non-specific binding.

Preferably, a monovalent immunoglobulin sequence of the invention will bind to the desired antigen with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM.

Specific binding of an antigen-binding protein to an antigen or antigenic determinant can be determined in any suitable manner known per se, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein.

The dissociation constant (KD) may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned herein. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/liter or 10^{-3} moles/liter (e.g., of 10^{-2} moles/liter). Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant (KA), by means of the relationship [KD = 1/KA].

The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the KD, or dissociation constant, which has units of mol/liter (or M). The affinity can also be expressed as an association constant, KA, which equals 1/KD and has units of $(mol/liter)^{-1}$ (or M^{-1}). In the present specification, the stability of the

interaction between two molecules (such as an amino acid sequence, immunoglobulin sequence, immunoglobulin single variable domain, Nanobody® or polypeptide of the invention and its intended target) will mainly be expressed in terms of the KD value of their interaction; it being clear to the skilled person that in view of the relation KA =1/KD, specifying the strength of molecular interaction by its KD value can also be used to calculate the corresponding KA value. The KD-value characterizes the strength of a molecular interaction also in a thermodynamic sense as it is related to the free energy (DG) of binding by the well-known relation DG=RT.ln(KD) (equivalently DG= RT.ln(KA)), where R equals the gas constant, T equals the absolute temperature and ln denotes the natural logarithm.

The KD for biological interactions, such as the binding of the immunoglobulin sequences of the invention to vWF as defined herein, which are considered meaningful (e.g. specific) are typically in the range of 10^{-10} M (0.1 nM) to 10^{-5} M (10000 nM). The stronger an interaction is, the lower its KD is.

The KD can also be expressed as the ratio of the dissociation rate constant of a complex, denoted as koff, to the rate of its association, denoted kon (so that KD =koff/kon and KA = kon/koff). The off-rate koff has units s^{-1} (where s is the SI unit notation of second). The on-rate kon has units $M^{-1}s^{-1}$.

As regards immunoglobulin sequences of the invention, the on-rate may vary between 10^2 $M^{-1}s^{-1}$ to about 10^7 $M^{-1}s^{-1}$, approaching the diffusion-limited association rate constant for bimolecular interactions. The off-rate is related to the half-life of a given molecular interaction by the relation t1/2=ln(2)/koff . The off-rate of immunoglobulin sequences of the invention may vary between 10 6 s-1 (near irreversible complex with a t1/2 of multiple days) to 1 s^{-1} (t1/2=0.69 s).

The affinity of a molecular interaction between two molecules can be measured via different techniques known per se, such as the well-known

surface plasmon resonance (SPR) biosensor technique (see for example Ober et al., Intern. Immunology, 13, 1551-1559, 2001) where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding kon, koff measurements and hence KD (or KA) values. This can for example be performed using the well-known Biacore instruments.

It will also be clear to the skilled person that the measured KD may correspond to the apparent KD if the measuring process somehow influences the intrinsic binding affinity of the implied molecules for example by artefacts related to the coating on the biosensor of one molecule. Also, an apparent KD may be measured if one molecule contains more than one recognition sites for the other molecule. In such situation the measured affinity may be affected by the avidity of the interaction by the two molecules.

Another approach that may be used to assess affinity is the 2-step ELISA (Enzyme-Linked Immunosorbent Assay) procedure of Friguet et al. (J. Immunol. Methods, 77, 305-19, 1985). This method establishes a solution phase binding equilibrium measurement and avoids possible artefacts relating to adsorption of one of the molecules on a support such as plastic.

However, the accurate measurement of KD may be quite labour-intensive and as consequence, often apparent KD values are determined to assess the binding strength of two molecules. It should be noted that as long as all measurements are made in a consistent way (e.g. keeping the assay conditions unchanged) apparent KD measurements can be used as an approximation of the true KD and hence in the present document KD and apparent KD should be treated with equal importance or relevance.

Finally, it should be noted that in many situations the experienced scientist may judge it to be convenient to determine the binding affinity relative to some reference molecule. For example, to assess the binding strength between molecules A and B, one may e.g. use a reference molecule

C that is known to bind to B and that is suitably labelled with a fluorophore or chromophore group or other chemical moiety, such as biotin for easy detection in an ELISA or FACS (Fluorescent activated cell sorting) or other format (the fluorophore for fluorescence detection, the chromophore for light absorption detection, the biotin for streptavidin-mediated ELISA detection). Typically, the reference molecule C is kept at a fixed concentration and the concentration of A is varied for a given concentration or amount of B. As a result an IC50 value is obtained corresponding to the concentration of A at which the signal measured for C in absence of A is halved. Provided KD ref, the KD of the reference molecule, is known, as well as the total concentration cref of the reference molecule, the apparent KD for the interaction A-B can be obtained from following formula: KD =IC50/(1+cref/ KD ref). Note that if cref << KD ref, KD ≈ IC50. Provided the measurement of the IC50 is performed in a consistent way (e.g. keeping cref fixed) for the binders that are compared, the strength or stability of a molecular interaction can be assessed by the IC50 and this measurement is judged as equivalent to KD or to apparent KD throughout this text.

5.3 Target antigen

The immunoglobulin single variable domains of the present invention bind to and/or have affinity for vWF. In the context of the present invention, "vWF" includes, but is not limited, to cynomolgus, baboon, pig, guinea pig, mouse, and/or human vWF and most preferred human vWF, i.e. SEQ ID NO: 20 or GenBank entry: NP_000543.

5.4 Specific embodiments of immunoglobulin sequences

The present invention relates to immunoglobulin single variable domains described in, or obtainable by the methods as disclosed in WO2004/015425, WO2004/062551, WO2006/074947, WO2006/122825,

WO2009/115614, or WO2011/067160, all in the name of the present applicant.

The invention also encompasses optimized variants of these amino acid sequences. Generally, an "optimized variant" of an amino acid sequence according to the invention is a variant that comprises one or more beneficial substitutions such as a substitutions increasing i) the degree of "humanization", ii) the chemical stability, and/or iii) the level of expression; while the potency (measured e.g. by the potency assay as described in the experimental part of WO2006/122825 remains comparable (i.e. within a 10% deviation) to the wild type 12A02 (as defined in WO2006/122825) or comparable to the variant 12A02H1 (SEQ ID NO: 19), also as defined in WO2006/122825. Preferably, compared to the wild-type sequence of 12A02, an amino acid sequence of the invention contains at least one such substitution, and preferably at least two such substitutions, and preferably at least three humanizing substitutions and preferably at least 10 such humanizing substitutions.

In a particular aspect, the amino acid sequences of the invention contain a total of between 1 and 15, preferably between 2 and 14, such as between 9 and 13, e.g. 10, 11 or 12 amino acid substitutions compared to the wild-type sequence 12A02. As mentioned, these differences preferably at least comprise one and preferably at least two, such as three, four or five or ten humanizing substitutions, and may optionally comprise one or more further substitutions (such as any one of, or any suitable combination of any two or more of, the further substitutions (a) to (c) as mentioned herein). Again, based on the disclosure herein and optionally after a limited degree of trial and error, the skilled person will be able to select (a suitable combination of) one or more such suitable humanizing and/or further substitutions.

The present invention encompasses polypeptide sequences that are highly similar to any of the specific examples provided herein, or any of the

specific examples defined by reference above. Highly similar means an amino acid identity of at least 90%, e.g. 95, 97, 98 or 99%. The highly similar polypeptide sequences will have the same function as the sequence they are derived from, i.e. they will bind to vWF, more specifically bind to and inhibit interaction between vWF and platelets.

In a particular embodiment, the invention relates to sequences highly similar to any one of SEQ ID NO:s 1-19, in particular SEQ ID NO: 1. However, for each variant sequence stability in the formulation as defined herein has to be evaluated, such that the invention in particular refers to variants or highly similar sequences which are stable in the formulations as defined herein.

Methods to generate polypeptide sequences of the invention are widely known and include e.g. recombinant expression or synthesis. The skilled person is well acquainted with suitable expression technology, e.g. suitable recombinant vectors and host cells, e.g. bacterial or yeast host cells. The skilled person is also well acquainted with suitable purification techniques and protocols.

5.5 Formulations of the invention

The present invention provides formulations of polypeptides directed against vWF, e.g. immunoglobulin single variable domains (ISVDs) or polypeptides comprising at least one immunoglobulin single variable domain, which are stable, and preferably suitable for pharmaceutical uses, comprising the preparation of medicaments.

A formulation of a vWF binder, e.g., an ISVD, includes an ISVD, a compound that can serve as a cryoprotectant and/or lyoprotectant, and a buffer. The pH of the formulation is generally pH 5 - 7.5. In some embodiments, a formulation is stored as a liquid. In other embodiments, a formulation is prepared as a liquid and then is dried, e.g., by lyophilization or spray-drying, prior to storage. A dried formulation (i.e. the lyophilisate)

can be used as a dry compound, e.g., as an aerosol or powder, or reconstituted to its original or another concentration, e.g., using water, a buffer, or other appropriate liquid (diluent).

The vWF binder purification process is designed to permit transfer of the vWF binder into a formulation suitable for long-term storage, e.g. as a frozen liquid and/or subsequently for freeze-drying (e.g., using a citrate/sucrose formulation). The formulation is lyophilized with the protein, e.g. vWF binder at a specific concentration. The lyophilized formulation can then be reconstituted as needed with a suitable diluent (e.g., water) to resolubilize the original formulation components to a desired concentration, generally the same or higher concentration compared to the concentration prior to lyophilization. The lyophilized formulation may be reconstituted to produce a formulation that has a concentration that differs from the original concentration (i.e., before lyophilization), depending upon the amount of diluent added to the lyophilisate relative to the volume of liquid that was originally freeze-dried. Suitable formulations can be identified by assaying one or more parameters of vWF binder integrity. The assayed parameters are generally the percentage of High Molecular Weight (HMW) species or the percentage of Low Molecular Weight (LMW) species by Size Exclusion HPLC (SE-HPLC).

Accordingly, the present invention provides formulations characterized by a suitable degree of purity and at suitable concentrations as required e.g. for pharmaceutical purposes. The formulations provide the polypeptides, e.g. immunoglobulin single variable domains or polypeptides comprising at least one immunoglobulin single variable domain as defined herein in a stable form over a large range of concentrations, and a large range of storage conditions, e.g. temperatures, including stressed conditions such as elevated temperatures (e.g. +25°C or higher), lyophilization, shaking or other forms of physical stress.

The formulation comprises an aqueous carrier. The aqueous carrier is in particular a buffer.

The invention, however, also encompasses products obtainable by further processing of a liquid formulation, such as a frozen, lyophilized or spray-dried product. Upon reconstitution, these solid products can become liquid formulations as described herein (but are not limited thereto). In its broadest sense, therefore, the term "formulation" encompasses both liquid and solid formulations. However, solid formulations are understood as derivable from the liquid formulations (e.g. by freezing, freeze-drying or spray-drying), and hence have various characteristics that are defined by the features specified for liquid formulations herein. The invention does not exclude reconstitution that leads to a composition that deviates from the original composition before e.g. freeze- or spray drying.

The formulations of the invention comprise at least one vWF binder, in particular immunoglobulin single variable domains or a polypeptide comprising at least one immunoglobulin single variable domain as defined herein. In particular embodiments, the formulation comprises one or more polypeptides selected from SEQ ID NO:s 1-19, preferably SEQ ID NO: 1. The polypeptides may in addition be half-life extended e.g. by incorporating a serum-albumin binding peptide or binding domain, which may be any suitable serum-albumin binding peptide or binding domain capable of increasing the half-life of the construct (compared to the same construct without the serum-albumin binding peptide or binding domain), and may in particular be serum albumin binding peptides as described in WO2008/068280 by applicant (and in particular WO2009/127691 and WO2011/095545, both by applicant), or a serum-albumin binding immunoglobulin single variable domain (such as a serum-albumin binding Nanobody; for example Alb-1 or a humanized version of Alb-1 such as Alb-8, for which reference is for example made to WO06/122787). Alternative means for extending half-life which are also encompassed by the present

invention include e.g. pegylation (PEG) as widely known in the art, including site specific or random pegylation, preferably site specific pegylation. PEG can be used with a molecular weight above 5000, e.g. between 10.000 and 200.000, preferably in the range between 20.000 and 100.000. In any aspect of half-life extension, it is envisaged that the activity of the polypeptide as defined herein is not compromised, e.g. retains at least 75%, 80%, 85%, 90% or 95% of the activity of the same polypeptide without half-life extension. Activity can relate to e.g. binding to the target antigen, and/or potency in a bioassay. The skilled person will also ascertain that the chosen half-life extension technology is suitable in that it does not increase, or even decreases immunogenicity.

5.5.1 Buffer

The formulation of the invention comprises a buffer selected from at least one of citrate or phosphate buffer, preferably a citrate buffer. In a particular embodiment, the citrate buffer is prepared using citric acid monohydrate and tri-sodium citrate dehydrate, e.g. 0.2154 g/L citric acid monohydrate and 5.5805 g/L tri-sodium citrate dehydrate. As determined by measuring melting temperatures in a non-limiting example, these buffers enhance the stability of the vWF binders, compared to other tested buffers.

The formulation according to the invention comprises a citrate buffer at a concentration in the range of 5-200 mM, e.g. 5, 7.5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 mM, preferably 5-100 mM, more preferably 7.5-80 mM, even more preferably 10-50, e.g. 10, 15, 20, 25 or 30 mM, and most preferably 20 mM, wherein each value is understood to optionally encompass a range of ±5 mM. The formulation according to the invention may comprise a phosphate buffer at a concentration in the range of 5-200 mM, e.g. 5, 7.5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 mM, preferably 5-80 mM, more preferably 7.5-60 mM, even more

preferably 10-40, e.g. 10, 15, 20, 25 or 30 mM, and most preferably 10 mM, wherein each value is understood to optionally encompass a range of ±5 mM. It will be understood that a lower concentration of the buffer has an effect on the final osmolality, and correspondingly on the additional solutes that may have to be added.

The pH of the formulation of the invention is in the range 5.0 to 7.5, wherein each value is understood to encompass a range of ±0.2. Specific examples of preferred pH values for formulations of the invention can be selected from the non-limiting list comprising pH of 5.0, 5.5, 5.8, 6.0, 6.2, 6.5, 6.7, 7.0, 7.1, 7.2 or 7.5, preferably 6.0 to 7.0, more preferably 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8 or 6.9, e.g. 6.5, wherein each value is understood to optionally encompass a range of ±0.2.

Unexpectedly, the citrate and phosphate buffers have overlapping pH ranges with e.g. histidine and Tris-HCl buffers, yet favour stability.

The most advantageous pH will depend on the buffer comprised in the formulation. Hence, the invention relates particularly to a formulation comprising a phosphate buffer, which preferably has a pH in the range of 6.5 to 7.5, preferably 6.9, 7.0, 7.1, e.g. 7.1.

It was shown that a formulation comprising a citrate buffer was outstandingly suitable for storage and use. However, in contrast to conventional wisdom, liquid formulations comprising a citrate buffer were most stable at a pH of about 6.0, while lyophilized formulations comprising a citrate buffer were most stable at a pH of about 6.5. Hence, the present invention relates to a formulation comprising a citrate buffer, which preferably has a pH between 6.0 and 7.0, more preferably 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8 or 6.9, e.g. 6.5, wherein each value is understood to optionally encompass a range of ±0.2.

5.5.2 Concentration

The formulations of the invention will comprise the vWF binders as defined herein, in particular the immunoglobulin single variable domains or

polypeptides comprising at least one immunoglobulin single variable domain at a concentration that is suitable for clinical purposes, which includes concentrations used in stock solutions for dilution prior to use on the patient. Apart from improved stabilization, the formulations of the invention enable higher concentrations of the vWF binders, e.g. ISVDs or polypeptides. In particular, the formulations of the invention remained physically stable, i.e. the absence of turbidity and/or small particle formation, as confirmed by visual inspection, microscopy, SE-HPLC and DLS. Storage at elevated temperatures for prolonged times and repeated freeze-thaw cycles did apparently not affect the physical stability of the vWF binders in these formulations.

Typical concentrations of the active agent, e.g. vWF binders or the polypeptides of the invention, in formulations of the invention comprise the non-limiting examples of concentrations in the range of 0.1 to 80 mg/mL, preferably 1-70 mg/mL, 5-60 mg/mL, 7.5-50 mg/mL, or 10-40 mg/mL, such as 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 50 or 60 mg/mL, preferably 12.5 mg/mL or 10 mg/mL, wherein each value is understood to optionally encompass a range of ±20% (e.g. a value of 10 optionally encompasses a range of 8 to 12 mg/mL).

5.5.3 Excipients

The formulations according to the invention may also optionally comprise one or more excipients. The term "excipient" as used herein refers to an inert substance which is commonly used as a diluent, vehicle, preservative, lyoprotectant, binder or stabilizing agent for compounds which impart a beneficial physical property to a formulation. The skilled person is familiar with excipients suitable for pharmaceutical purposes, which may have particular functions in the formulation, such as lyoprotection, stabilization, preservation, etc. Commonly used stabilizers and preservatives are well known to the skilled person (see e.g.

WO2010/077422). Pharmaceutically acceptable carriers that may be used in these compositions include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene polyoxypropylene block polymers, polyethylene glycol and wool fat. In advantageous embodiments, the excipient may be one or more selected from the list consisting of NaCl, trehalose, sucrose, mannitol or glycine.

In appreciating the invention, the skilled person can readily determine suitable concentrations of the excipients to be added to the formulations. In exemplary embodiments, NaCl has a concentration in the range of 10-500 mM, such as 25, 30, 40, 50, 60, 70, 100, 150, 250 or 500 mM, preferably 50-150 mM, e.g. 75 or 140 mM, wherein each value is understood to optionally encompass a range of ±5 mM; and/or mannitol has a concentration of 1-10%, preferably 2-4%, e.g. 2, 3 or 4% (w/w), wherein each value is understood to optionally encompass a range of ±0.5%; and/or sucrose has a concentration of 1-15%, preferably 2-12% or 4-10%, e.g. 4, 5, 6, 7, 8 or 9% (w/w), and most preferably 7%, wherein each value is understood to optionally encompass a range of ±0.5%; and/or glycine has a concentration in the range of 10-500 mM, such as 25, 30, 40, 50, 60, 70, 75, 100, 150, 250 or 500 mM, preferably 50-400 mM, 75-300 mM, 100-250 mM, e.g. 140 or 200 mM, wherein each value is understood to optionally encompass a range of ±5 mM; and/or trehalose has a concentration in the range of 10-500 mM, such as 25, 30, 40, 50, 60, 70, 75, 100, 150, 250 or 500 mM, preferably 100-300

mM, 150-280 mM, e.g. 160 mM or 260 mM, wherein each value is understood to optionally encompass a range of ±5 mM.

In a preferred embodiment, the formulations according to any aspect of the invention are isotonic in relation to human blood. Isotonic solutions possess the same osmotic pressure as blood plasma, and so can be intravenously infused into a subject without changing the osmotic pressure of the subject's blood plasma. Tonicity can be expressed in terms of osmolality, which can be a theoretical osmolality, or preferably an experimentally determined osmolality. Typically, osmolality will be in the range of 290 ± 60 mOsm/kg, preferably 290 ± 20 mOsm/kg.

Thus, in the selection of excipients (if any) the skilled person will consider buffer concentration and the concentrations of the one or more excipients and preferably arrive at a formulation with an osmolality in the ranges as specified above. The skilled person is familiar with calculations to estimate osmolality (see e.g. WO2010/077422). If required, the skilled person can also further include a compound to adjust the osmolality of the formulation. Exemplary compounds include, but are not limited to the above mentioned excipients, and/or one or more of sorbitol, methionine, dextrose, inositol, arginine, or arginine hydrochloride.

It has been shown that a formulation comprising sucrose was particularly suited for maintaining the physical stability, during e.g. storage and freeze-thawing, of the polypeptides. Accordingly, the present invention relates to formulations comprising about 5-9% more preferably 6-8% and even more preferably 7% sucrose, wherein each value is understood to optionally encompass a range of ±0.5%.

The formulations of the invention may also comprise compounds that are specifically useful for protecting the polypeptide of the invention during freeze-drying. Such compounds are also known as lyoprotectants, and are well known to the skilled person. Specific examples include, but are not limited to sugars like sucrose, sorbitol or trehalose; amino acids such as

glutamate, in particular monosodium glutamate or histidine; betain, magnesium sulfate, sugar alcohols, propylene glycol, polyethylene glycols and combinations thereof. By appreciating the invention, the required amount of such a compound to be added can readily be determined by the skilled person under consideration of stability of the formulation in liquid form and when undergoing lyophilization. Formulations that are particularly suitable for freeze-drying may furthermore comprise bulking agents. Suitable agents are widely known to the skilled person. It has been shown that a formulation comprising sucrose was not only particularly suited for maintaining the physical stability, during e.g. storage and freeze-thawing, of the vWF binders, but also as lyoprotectant.

5.5.4 Detergent

In a further embodiment of the invention, the formulation according to any aspect of the invention may further comprise a detergent or surfactant. Suitable detergents or surfactants for use with the invention include, but are not limited to, polyoxyethylene sorbitan fatty acid esters e.g. polysorbate-20, -40, -60, -65, -80 or -85. Common brand names for polysorbates include Alkest, Canarcel and Tween. The skilled person knows further non-limiting examples of detergents, such as those listed e.g. in WO2010/077422. In a preferred embodiment, the detergent is a non-ionic detergent. More specifically, the detergent is polysorbate-80, also designated Tween-80 hereafter. The skilled person can readily determine a suitable concentration of detergent for a formulation of the invention. Typically, the concentration will be as low as possible, whilst maintaining the beneficial effects of the detergents, e.g. a stabilizing effect under conditions of shear stress, e.g. stirring, which reduces aggregation of the formulated vWF binders. In exemplary, non-limiting embodiments, the concentration of the detergent may be in the range of 0.001 to 0.5%, e.g. 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%,

0.045%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4% or 0.5%, preferably in a concentration between 0.01 and 0.05%, more preferably between 0.01 and 0.02%, e.g. 0.01% (v/v).

5.5.5 Combinations

The various embodiments as described above in Sections 5.5.1 to 5.5.4 can be combined in formulations of the invention without limitations. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included. However, preferable non-limiting examples of formulations include formulations wherein the buffer is a citrate buffer at about pH 6.5, preferably at a concentration of 20 mM, and the formulation further comprises sucrose, preferably at a concentration of about 7% (w/v), and optionally further comprises a non-ionic detergent such as Tween-80, preferably at a concentration of 0.01% (v/v).

5.6 Further processing

As outlined, any of the above formulations can be further processed e.g. by lyophilization, spray-drying or freezing, e.g. bulk freezing. The resulting processed product has characteristics derived from the liquid starting formulation, as defined above. Where necessary, additional agents may be included for further processing, such as, for instance, lyoprotectants, etc.

5.6.1 Freezing

In some cases, formulations containing vWF binders are frozen for storage. Accordingly, it is desirable that the formulation be relatively stable under such conditions, such as freeze thaw (FT) cycles. One method of determining the suitability of a formulation is to subject a sample formulation to at least two, e.g., three, four, five, eight, ten, or more cycles of

freezing (at, for example -20°C or -70°C) and thawing (for example by fast thaw in a 25°C water bath or slow thaw at +2°C to +8°C), determining the mass recovery of the original product and the presence and/or amount of LMW species and/or HMW species that accumulate after the FT cycles and comparing it to the amount of LMW species or HMW species present in the sample prior to the FT procedure, e.g. by SE-HPLC. An increase in the LMW or HMW species indicates decreased stability.

5.6.2 Lyophilization

Formulations can be stored after lyophilization. Therefore, testing a formulation for the stability of the polypeptide component of the formulation after lyophilization is useful for determining the suitability of a formulation. The method is similar to that described, supra, for freezing, except that the sample formulation is lyophilized instead of frozen, reconstituted to its original volume, and tested for the presence of LMW species and/or HMW species. The lyophilized sample formulation is compared to a corresponding sample formulation that was not lyophilized. An increase in LMW or HMW species in the lyophilized sample compared to the corresponding sample indicates decreased stability in the lyophilized sample. In general, a lyophilization protocol includes loading a sample into a lyophilizer or freeze-dryer, a pre-cooling period, freezing, vacuum initiation, ramping to the primary drying temperature, primary drying, ramping to the secondary drying temperature, secondary drying, and stoppering of the sample. Although the process of freeze-drying is well known in the art, various factors determine the freeze-drying characteristics of a sample, including: the glass transition temperature (Tg′) and collapse temperature (Tc). Additional parameters that can be selected for a lyophilization protocol include vacuum (e.g., in microns) and condenser temperature.

Suitable ramp rates for temperature are between about 0.1°C/min. to 2°C/min., for example 0.1°C/min. to 1.0°C/min., 0.1°C/min. to 0.5°C/min.,

0.2°C/min. to 0.5°C/min., 0.1°C/min., 0.2°C/min., 0.3°C/min., 0.4°C/min., 0.5°C/min., 0.6°C/min., 0.7°C/min., 0.8°C/min., 0.9°C/min., and 1.0°C/min. Suitable shelf temperatures during freezing for a lyophilization cycle are generally from about -55°C to -5°C, -25°C to -5°C, -20°C to -5°C, -15°C to -5°C, -10 C to -5°C, -10°C, -11°C, -12°C, 13°C, -14°C, 15°C, -16°C, -17°C, -18°C, -19°C, -20°C, -21°C, -22°C, -23°C, -24°C, or -25°C. Shelf temperatures can be different for primary drying and secondary drying, for example, primary drying can be performed at a lower temperature than secondary drying. In a non-limiting example, primary drying can be executed at 0°C or alternatively at +5°C and the secondary drying at +25°C. In some cases, an annealing protocol is used during freezing and prior to vacuum initiation. In such cases, the annealing time must be selected and the temperature is generally above the glass transition temperature of the composition. In general, the annealing time is about 2 to 20 hours, about 3 to 19 hours, about 2 to 10 hours, about 3 to 5 hours, about 3 to 4 hours, about 2 hours, about 3 hours, about 5 hours, about 8 hours, about 10 hours, about 12 hours, about 15 hours or about 19 hours. The temperature for annealing is generally from about -35°C to about -5°C, for example from about -25°C to about -8°C, about -20°C to about -10°C, about -25°C, about -20°C, about -15°C, about 0°C, or about -5°C. In some cases, the annealing temperature is generally from 35°C to +5°C, for example from -25°C to -8°C, -20°C to -10°C, -25°C, -20°C, -15°C, 0°C, +5°C.

The stability of the formulations described herein can be tested using a variety of lyophilization parameters including: the primary drying shelf temperatures from -25°C to +30°C, and secondary drying durations of 2 hours to 33 hours at 0° to +30°C. The temperature for secondary drying should be as high as possible, without causing degradation of the active pharmaceutical ingredient.

An excipient to be used in a formulation of this invention should preferably satisfy one or more of the following parameters: be

pharmacologically inert; be compatible with processing requirements; be well tolerated by the patient; be non-damaging to the active material; provide a soluble, absorbable product; provide a shelf-stable product; and provide a commercially acceptable product.

In an embodiment, the formulation of the present invention is prepared by freeze-drying, for instance as outlined in Figure 1 or Table 14.

It was demonstrated that lyophilization of citrate/sucrose based formulations dramatically improves stability of the vWF-binders. In particular, the citrate/sucrose based formulations essentially prevent chemical modifications that occur in the liquid form, but with the exception of small quantities of pyroglutamate formation. Unexpectedly, lowering the citrate concentration and at the same time increasing the sucrose concentration improved chemical stability, e.g. decreased pyroglutamate formation. The vWF binder was demonstrated to be robust after lyophilization to extremes in product temperature. Indeed, the stability profile was identical for material that had been prepared using a variety of freeze-drying cycles.

In general, a lyophilization cycle can run from 10 hours to 100 hours, e.g., 20 hours to 80 hours, 30 hours to 70 hours, 40 hours to 60 hours, 45 hours to 50 hours, 50 hours to 66 hours.

In a non-limiting example, a formulation of 20 mM citrate, 7% sucrose, 0.01% Tween-80, pH 6.5, at a protein concentration of 12.5 mg/mL vWF-binder was formulated in bulk and lyophilized.

Non-limiting examples of the temperature range for storage of a formulation of the invention are about -20°C to about +50°C, e.g., about -15°C to about +40°C, about -15°C to about +30°C, about -15°C to about +20°C, about +5°C to about +25°C, about +5°C to about +20°C, about +5°C to about +15°C, about +2°C to about +12°C, about +2°C to about +10°C, about +2°C to about +8°C, about +2°C to about +6°C, or about +2°C, +3°C, +4°C, +5°C, +6°C, +7°C, +8°C, +10°C, +15°C, +25°C, +30°C or +40°C.

Notwithstanding the storage temperatures, in certain cases, samples are stable under temperature changes that may transiently occur during storage and transportation conditions that can be anticipated for such compositions.

It has been established that by working with the formulations of the invention as defined herein, it is possible to obtain a resultant dried powder which exhibits a particle size suitable for comfortable retention and a fast dissolution of the active material. The dried formulation according to the invention comprises particles which remain stable and uniform throughout processing, final finishing, storage and distribution. The formulation is shelf-stable and free-flowing, presents no problems when dispensed into its final container and is simple to administer by the patient.

5.6.3 Spray-drying

In some cases, a formulation is spray-dried and then stored. Spray-drying is conducted using methods known in the art, and can be modified to use liquid or frozen spray-drying (e.g., using methods such as those from Niro Inc. (Madison, WI), Upperton Particle Technologies (Nottingham, England), or U.S. Patent Publ. Nos. 2003/0072718 and 2003/0082276), or Buchi (Brinkman Instruments Inc., Westbury, NY).

5.6.4 Diluent

The lyophilized formulations as described herein may be reconstituted as needed by mixing the lyophilized form with a suitable diluent to resolubilize the original formulation components to a desired concentration. The term "diluent" as used herein refers to a pharmaceutically acceptable (safe and non-toxic for administration to a human) solvent for altering or achieving an appropriate concentration as described herein. Exemplary diluents include, but are not limited to, sterile

water (e.g. WFI, Milli-Q water), saline, glucose, dextrose, Ringer and aqueous buffer solutions.

5.7 Pharmaceutical compositions

The formulations of the present invention are preferably suitable for use in methods of therapy of the animal or human body. Hence, the invention pertains to pharmaceutical or diagnostic compositions comprising a formulation of the polypeptide according to any aspect of the invention or obtainable by any method or process of the invention.

The formulations of the invention are preferably pharmaceutical formulations. In particular, the formulations are suitable for parenteral administration to a human, e.g. subcutaneous, intravenous, intramuscular, intradermal or intraperitoneal administration, preferably intravenous or subcutaneous administration. Administration encompasses any way of administering a liquid formulation, in particular injection. Other forms of systemic administration, e.g. via implantable devices, micro-infusion pumps (optionally implantable), and/or (implantable) sustained release formulations, e.g. deposits, gels, biodegradable polymer formulations are also within the scope of the present invention. Pharmaceutical compositions are sterile and stable during manufacture and storage, as derivatives/degradation products of the vWF binders are undesired in a clinical setting. The composition will also be of high purity, e.g. exclude the presence of bacterial products such as LPS. The formulations can be sterilized by any suitable means, e.g. sterile filtration, irradiation and combinations thereof, etc. Preferably, the pharmaceutical compositions are adapted to parenteral (especially intravenous, intra-arterial or transdermal) administration. Intravenous administration is considered to be of particular importance. Preferably the vWF binder is in the form of a parenteral form, most preferably intravenous and subcutaneous forms.

To be suitable as a pharmaceutical formulation, the formulation of the invention will typically comprise the polypeptide of the invention (i.e. the active agent) in a suitable ratio to the volume. For example, for subcutaneous injection the concentration of active agent may be higher, in order to allow the necessary pharmaceutical dose to be administered in a smaller volume, as compared to a formulation for intravenous injection. However, in some embodiments the concentration of active agent will be identical for subcutaneous or intravenous injection, and can be in the exemplary ranges as defined herein.

In some embodiments, the formulations of the invention may comprise additional agents, e.g. additional active agents, excipients, stabilizers, preservatives such as antimicrobial agents, etc.

The formulations of the invention are preferably in a dose applied to a patient in need thereof. Nevertheless, the particular mode of administration and the dosage may be selected by the attending physician taking into account the particulars of the patient, especially age, weight, life style, activity level, and general medical condition as appropriate. More specifically, ALX-0081 is administered intravenously or subcutaneously in a 24 h dose interval. Even more preferably, ALX-0081, is administered intravenously or subcutaneously in a 24 h dose interval upon consideration of the aggregation activity, e.g. measured by RIPA, ristocetin induced platelet aggregation (Favaloro EJ. Clin Haematol 2001; 14: 299-319) and/or Ristocetin Cofactor Platelet Agglutination Assay – (Howard MA, Firkin BG. Ristocetin – a new tool in the investigation of platelet aggregation. Thrombosis et Diathesis Haemorrhagica 1971; 26: 362-9). For example, a further dose is not administered if the aggregation activity is estimated to stay below 10% measured by RIPA or stay below 20% measured by RICO for the next 6 hours (Clinically relevant inhibition).

However, in general the dosage of the vWF binders may depend on various factors, such as effectiveness and duration of action of the active

ingredient, warm-blooded species, and/or sex, age, weight and individual condition of the warm-blooded animal.

Normally the dosage is such that a single dose of a vWF binder is for instance estimated based on in vitro results, or for instance based on results from a dose escalating study to test subchronic toxicity in cynomolgus monkeys. Based on such a preclinical data set, a starting and subsequent escalating dose for a vWF binder can be determined. For instance a dose may be from 0.5 – 50 mg, especially 1 – 30 mg, and is administered to a warm-blooded animal weighing approximately 75 (+/-30) kg (but can be different as well to this norm). If desired, this dose may also be taken in several, optionally equal, partial doses ("mg" means mg drug per mammal - including human - to be treated).

The dose mentioned above - either administered as a single dose (which is one embodiment) or in several partial doses - may be repeated, as mentioned above for example once every six hours, once every 12 hours, or once daily. In other words, the pharmaceutical compositions may be administered in regimens ranging from continuous 6 hourly therapy to longer interval dosing therapy.

Preferably, the vWF binders are administered in doses which are in the same order of magnitude as those used in the adjunct treatment in patients in need for PCI as herein suggested for ALX-0081. For example, for the preferred 12A02H1-containing vWF binders, e.g. ALX-0081 and functional variants thereof, doses of vWF binders in the range from about 0.5 to about 40 mg, preferably from about 1 to about 35 mg, or from about 2 to about 30 mg, even more preferably from about 3 to about 25 mg or from about 4 to about 20 mg, or from about 5 to about 17.5 mg, or even from about 6 to about 16 mg, or from about 7.5 to about 15 mg, or even from about 10 to about 14 mg, more preferably about 10 or about 12.5 mg, may be used for acute treatment in human patients.

Formulations in single dose unit form contain preferably from about 0.5 to about 40 mg, preferably from about 1 to about 35 mg, or from about 2 to about 30 mg, even more preferably from about 3 to about 25 mg or from about 4 to about 20 mg, or from about 5 to about 17.5 mg, or even from about 6 to about 16 mg, or from about 7.5 to about 15 mg, or even from about 10 to about 14 mg, more preferably about 10 or about 12.5 mg and formulations not in single dose unit form contain preferably from about 0.5 to about 40 mg, preferably from about 1 to about 35 mg, or from about 2 to about 30 mg, even more preferably from about 3 to about 25 mg or from about 4 to about 20 mg, or from about 5 to about 17.5 mg, or even from about 6 to about 16 mg, or from about 7.5 to about 15 mg, or even from about 10 to about 14 mg, more preferably about 10 or about 12.5 mg of the active ingredient.

Pharmaceutical preparations for parenteral administration are, for example, those in dosage unit forms, such as ampoules. They are prepared in a manner known per se, for example by means of conventional mixing, dissolving or lyophilizing processes.

Parenteral formulations are especially injectable fluids that are effective in various manners, such as at site of PCI, intra-arterially, intramuscularly, intraperitoneally, intranasally, intradermally, subcutaneously or preferably intravenously. Such fluids are preferably isotonic aqueous solutions or suspensions which can be prepared before use, for example from lyophilized preparations or concentrate which contain the active ingredient alone or together with a pharmaceutically acceptable carrier. The pharmaceutical preparations may be sterilised and/or contain adjuncts, for example preservatives, stabilisers, wetting agents and/or emulsifiers, solubilisers, salts for regulating the osmotic pressure and/or buffers.

Suitable formulations for transdermal application include an effective amount of the active ingredient with carrier. Advantageous

carriers include absorbable pharmacologically acceptable solvents to assist passage through the skin of the host. Characteristically, transdermal devices are in the form of a bandage comprising a backing member, a reservoir containing the compound optionally with carriers, optionally a rate controlling barrier to deliver the active ingredient of the skin of the host at a controlled and predetermined rate over a prolonged period of time, and means to secure the device to the skin.

The following table provides some non-limiting examples of citrate and phosphate buffer based formulations of the present invention. All formulations can be adjusted to an osmolality of 290 ± 60 mOsm/kg by adding a suitable excipient, if desired. The formulations can comprise any one or more of the polypeptides of the present invention, e.g. SEQ ID NO:s 1-19, particularly SEQ ID NO: 1.

Buffer	Buffer conc (mM)	pH		Buffer	Buffer conc (mM)	pH
Citrate	10	6.0		phosphate	10	6.5
Citrate	10	6.5		phosphate	10	7.0
Citrate	10	7.0		phosphate	10	7.5
Citrate	20	6.0		phosphate	20	6.5
Citrate	20	6.5		phosphate	20	7.0
Citrate	20	7.0		phosphate	20	7.5
Citrate	30	6.0		phosphate	30	6.5
Citrate	30	6.5		phosphate	30	7.0
Citrate	30	7.0		phosphate	30	7.5
Citrate	40	6.0		phosphate	40	6.5
Citrate	40	6.5		phosphate	40	7.0
Citrate	40	7.0		phosphate	40	7.5
Citrate	50	6.0		phosphate	50	6.5
Citrate	50	6.5		phosphate	50	7.0
Citrate	50	7.0		phosphate	50	7.5

The buffer concentrations in this table are understood to optionally encompass ± 5 mM. The pH values are understood to optionally encompass ± 0.2. Each of the above buffers can be combined with one or more excipients selected from e.g. NaCl at a concentration of e.g. 25, 30, 40, 50, 60, 70, 100, 150, 250 or 500 mM; mannitol at a concentration of e.g. 2, 3 or 4 % (w/v); glycine at a concentration of e.g. 25, 30, 40, 50, 60, 70, 100, 150, 250 or 500 mM; trehalose at a concentration of e.g. 25, 30, 40, 50, 60, 70, 100, 150, 250 or 500 mM and sucrose at a concentration of e.g. 4, 5, 6, 7, 8 or 9% (w/v), and/or a surfactant, e.g. Tween-80 at a concentration of 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4% or 0.5% (v/v).

5.8 Effects of the invention

The invention provides stable formulations of the vWF binders, e.g. the immunoglobulin single variable domains as defined herein, e.g. SEQ ID NO:s 1-19, in particular SEQ ID NO: 1. "Stable" generally means that the immunoglobulin single variable domains do not suffer from significant physical or chemical changes upon storage for prolonged periods of time, e.g. 1 month to 36 months, even if exposed to one or more chemical or physical stresses such as elevated temperatures (equal to or higher than +25°C), or physical stress such as shaking or stirring. More in particular, "stable" means that upon storage for prolonged periods (as defined) under conditions (as defined) there is only a limited formation (as defined) of one or more of degradation products, e.g. low molecular weight (LMW) derivatives (fragments) of the polypeptides of the invention; and/or chemical derivatives or modifications such as e.g. pyroglutamate variants; and/or high molecular weight (HMW) derivatives (oligomers or polymers) formed e.g. by aggregation.

The skilled person is well acquainted with techniques to assess protein size, e.g. size exclusion chromatography-HPLC or to assess the

formation of chemical derivatives, e.g. reversed phase HPLC. The skilled person is also familiar with commonly used apparatuses and software tools for performing such analyses. For example, the skilled person knows commonly used software to analyse chromatographic runs e.g. in terms of relative peak area. Examples include (but are not limited to) Agilent 1200 HPLC system equipped with ChemStation software (Agilent Technologies, Palo Alto, USA, Rev B) or Dionex Ultimate 3000 HPLC system equipped with Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA, V6.8).

General techniques that can be used to assess stability of a protein, e.g. an immunoglobulin single variable domain include static light scattering, tangential flow filtration, Fourier transform infrared spectroscopy, circular dichroism, urea induced protein unfolding, intrinsic tryptophan fluorescence and/or 1-anilin- 8-naphtalenesulfonic acid protein binding. In addition, the formulation of the invention shows little or no loss of potency/biological activity in the course of storage and/or under influence of one or more stresses as defined herein. Biological activity and/or potency can be determined e.g. as described in WO2006/122825.

5.8.1 Thermal stability

The formulations of the present invention are characterized by providing a high thermal stability of the vWF binders, e.g. the immunoglobulin single variable domains as defined herein. Thermal stability can be evaluated e.g. by determining the melt temperature (Tm). Suitable techniques for determining the melt temperature are known and include e.g. a thermal shift assay (TSA) e.g. as described herein. More specifically, the formulations of the present invention lead to an increase of Tm for the immunoglobulin single variable domains as determined by TSA in comparison to other formulations. This effect is exemplified in Table 1 of the experimental section.

As can be ascertained from the experimental section, high thermal stability, i.e. high Tm can be taken as an indication for storage stability.

According to the present invention, the formulations of the invention have a positive influence on Tm over a broad range of pH values, e.g. between 6.0 and 7.0 for citrate buffer, and 6.5 to 7.5 for phosphate buffer. The most advantageous effect on Tm can be observed for citrate buffer at pH 6-7, and in particular for pH 6.5 ± 0.2 and phosphate buffer at pH 6.5 to 7.5, in particular pH 7.1 ± 0.2.

The addition of excipients can have a further positive or negative effect on Tm (Table 1). For example, trehalose can increase Tm (in the context of a particular buffer) e.g. between 150 mM and 300 mM. Also mannitol or sucrose had a clear positive effect on Tm. These excipients can find use in particular embodiments of the invention, e.g. formulations where a bulking agent or lyoprotectants are advantageous. These exemplary embodiments do not preclude the use of further known lyoprotectants or bulking agents, either alone or in combination with mannitol or sucrose.

As evidenced by the experimental section of this description, Tm as determined by TSA serves as a valuable indicator for stability of the vWF binders, e.g. the immunoglobulin single variable domains of the invention.

5.8.2 Stability as concerns mechanical stress

The formulations of the invention are characterized by a high stability as concerns mechanical stress, such as stirring, shaking or shear stress. A possible assay to evaluate stability under mechanical stress is monitoring 500 nm scatter signal in a spectrofluorometer or via UV spectrophotometry e.g. at 340 nm. An increase in scatter or UV absorption reflects the formation of aggregates. When aggregates are formed (HMW), the increase over time follows a linear curve for which a slope (scatter intensity/time or absorbance units/s) can be determined. Preferably, the formulations of the present invention are characterized by a slope of less

than 0.0006, e.g. less than 0.0005, e.g. between 0 and 0.0004 (cf. Figures 4 A and B).

The formulations comprising citrate buffers are particularly preferred and have a positive effect on protein recovery after e.g. stirring as defined above. For example, mass recovery is at least 90%, 95%, 98% or 100%. Protein recovery is determined in comparison to the total protein content before stressing the sample e.g. by stirring. The formulations comprising phosphate buffers result in a recovery of at least 75%, 80%, 85% or even more after stirring as defined above.

At an exemplary, non-limiting concentration of 5 mg/mL, the formulations of the invention only form reversible aggregates in response to stirring in the absence of Tween. Thus, the formulations of the invention prevent the formation of irreversible aggregates under mechanical stress. Accordingly, in a further embodiment of the invention, the formulations of the invention may comprise a non-ionic detergent as defined above, e.g. Tween-80, e.g. at a concentration as defined above, e.g. between 0.01% and 0.02% (v/v). The addition of the detergent can further improve physical stability of the formulation. For instance, at a non-limiting exemplary concentration of 5 mg/mL, the addition of the detergent can prevent the formation of aggregates (reversible and irreversible) as determined e.g. by monitoring 500 nm scatter signal in a spectrofluorometer or by UV spectrophotometry (340nm) (Figures 4A and B).

The physical stability of the formulations of the present invention can also be demonstrated by SE-HPLC. Different non-limiting formulations of immunoglobulin single variable domains of the present invention can withstand mechanical stress, e.g. stirring stress, without forming oligomers (HMW) or degradation products (LMW). The formulations of the invention remain stable without degradation or oligomerization, as determined e.g. after 1.5 hours of stirring by SE-HPLC analysis.

No oligomerization or degradation (e.g. as determined by RP-HPLC (only degradation) or SE-HPLC profile) is detected in any of the formulations. Thus, according to a preferred embodiment of the invention, the formulations comprise a citrate buffer and show a recovery of at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, or even about 100%, e.g. under conditions as described above, wherein recovery is determined e.g. by RP-HPLC or SE-HPLC in comparison with a non-stressed sample. Advantageously, the excipient in the context of a citrate buffer can be sucrose, and recovery as defined above is at least 80%, 85%, 90%, 95%, 98%, or even about 100%.

5.8.3 Stability testing of liquid formulations

5.8.3.1 Storage stability

The liquid formulations of the invention provide for good stability when stored, e.g. at a temperature of -70°C, -20°C, +5°C, +25°C or +40°C, e.g. for 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months. The most advantageous results can be obtained with citrate buffer based formulations as exemplified in Table 5.

The skilled person will further recognize that storage at +25°C, and more in particular +40°C represent stressed storage conditions. Such conditions are expected to increase and accelerate any signs of instability, e.g. chemical or physical instability. Hence, relatively short storage at e.g. +25 or +40°C provides a good indication for long term storage stability under milder conditions (e.g. +5°C or frozen).

5.8.3.2 Storage stability in terms of protein recovery

For example, the formulations of the present invention provide for a protein recovery of at least 95%, e.g. at least 96, 97, 98, 99 or even about 100% after storage at a temperature between -70°C and +40°C. Protein recovery can be determined by any known means to quantify proteins, e.g.

by RP-HPLC or SE-HPLC, as exemplified in Table 5 as compared to a reference sample kept at -70°C. These results can be observed e.g. after storage at the indicated temperature of 1 month, 1.5 months, 3 months, 6 months, 9 months, 12 months, 18 months, 24 months, 30 months or even at 36 months.

5.8.3.3 Storage stability in terms of chemical derivatives / degradation products

Moreover, the formulations of the present invention minimize production of chemical derivatives, e.g. pyroglutamate variants, of less than 5.0% in peak size as determined e.g. by RP-HPLC (cf. Table 5). In this type of analysis, the area of a given peak is compared to the total area of the chromatogram, and a relative area is allocated to each peak. The skilled person knows suitable analyzing means, e.g. suitable software, to analyze the chromatograms (specific, non-limiting examples include Agilent 1200 HPLC system equipped with ChemStation software (Agilent Technologies, Palo Alto, USA, Rev B) or Dionex Ultimate 3000 HPLC system equipped with Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA, V6.8). Thus, preferably, the pyroglutamate variant contributes to a peak area of less than 5%, preferably less than 4.6%, e.g. 4.5, 4.3, 4.2, 4.0 or even less than 3.8% as determined by RP-HPLC upon storage at temperatures between -70°C and +40°C, e.g. +40°C, e.g. after storage for a duration as defined above, e.g. 1 month.

The formulations of the present invention also minimize oxidation, such as the formation of oxidized products (as determined e.g. by RP-HPLC) over a storage period as defined above, e.g. 1 month at a temperature between -70°C and +40°C (cf. Table 5). Thus, the formulations of the present invention result in oxidation variants with a peak area of less than 3%, preferably less than 2.7%, preferably less than 2.5%, e.g. less than 2.3%, 2.2%, e.g. 2.0, or even less such as 1.7% or 1.5% upon storage at

temperatures between -70°C and +40°C, e.g. +40°C, e.g. after storage for a duration as defined above, e.g. 1 month (as determined e.g. by RP-HPLC) .

5.8.3.4 Storage stability in terms of oligomerization

The formulations of the invention also provide for storage stability, such that no apparent soluble oligomeric material is formed (as defined e.g. by SE-HPLC) at storage temperatures between -70°C and +40°C, after storage durations as defined above, e.g. 1 month; or less than 1%, preferably less than 0.5%, e.g. 0.3% soluble oligomeric material is formed (as defined e.g. by SE-HPLC) at storage temperatures between -70°C and +40°C, e.g. +40°C, after storage durations as defined above, e.g. 1 month.

The present invention also has the effect of providing an aggregation index as determined by absorbance values [(100xA340)/(A280-A340)] which remains below 0.15, preferably below 0.1 after storage at -70°C or +40°C for storage of a duration as defined above, e.g. 1 month.

5.8.3.5 Storage stability as reflected in recovery of main product

The formulations of the invention have the effect that the main product peak area, as determined e.g. by RP-HPLC (cf. Table 5) is about 90% after storage between -70°C and +40°C after a storage duration as indicated above, e.g. 1 month; or the main product peak, as determined e.g. by RP-HPLC (cf. Table 5) is at least 85%, or more, such as 86%, 87% or 88%. More preferably, the main peak is 90%, 92% or 95%, e.g. at least 97%, more preferably 100% after storage between -70°C and +40°C, e.g. +40°C after a storage duration as indicated above, e.g. 1 month; or the main product peak, as determined e.g. by SE-HPLC is at least 85%, at least 90%, preferably at least 95%, e.g. at least 98% or even about 100% after storage between -70°C and +40°C, e.g. +40°C, after a storage duration as indicated above, e.g. 1 month.

The formulations according to the present invention also have the effect that the main peak area as determined by RP-HPLC after storage e.g. at a concentration up to 20 mg/mL at between -70°C and +25°C for between 1 and 3 months remains unchanged as compared to the formulation prior to storage, and represents at least 90%, more preferably at least 95% of the total peaks, wherein the reference sample has a main peak of e.g. 95%. Upon storage at +40°C for 1 month the formulation of the present invention retains the main peak as determined by RP-HPLC of at least 80%, 85% or 90%; after storage for 2 months of at least 80%, or 85 %, and after storage for 3 months of at least 75% or 80%.

Moreover, as determined by cIEF, the formulation of the present invention has the effect of providing recovery of the main product after storage at a concentration of e.g. up to 20 mg/mL for between 1-3 months at a temperature between -70°C and +40°C that is comparable to the reference sample (formulation without storage, main peak is at least 98%), e.g. the main peak is at least 85%, or more, such as 86%, 87% or 88%. More preferably, the main peak is 90%, 92% or 95%, e.g. at least 97%, more preferably 100% after storage between -70°C and +40°C.

5.8.3.6 Stability under freeze-thaw conditions

Apart from providing stability of the formulations under conditions of storage that remain constant over time (e.g. storage at +5°C), or include a single FT cycle (e.g. storage at -20°C or -70°C), a further effect of the invention is stability under conditions of repeated FT cycles. Every transition between frozen and liquid state and vice versa imposes particularly stressful conditions upon the immunoglobulin single variable domains.

The formulations of the invention also have the effect of providing good stability under FT conditions. For example the formulations of the invention can be subjected to e.g. 10 FT cycles between -70°C and room

temperature (e.g. +25°C), or -20°C and room temperature. The immunoglobulin single variable domains comprised in the formulations will withstand these conditions without significant deterioration, as ascertained e.g. by RP-HPLC or SE-HPLC. The effect of repetitive FT cycles on different non-limiting embodiments of formulations of the invention were evaluated, and reveal that in all cases chemical and physical integrity of the vWF binders, e.g. immunoglobulin single variable domains has been preserved. Overall recovery was in the range between 95 and 100%, preferably at least 95, 98 or 99%. The relative proportion of the different peaks remained unchanged in comparison to a control subjected to only one FT cycle.

More specifically, at a concentration of between 5 mg/mL and 20 mg/mL, 10 FT cycles resulted in a recovery (as determined on the basis of e.g. total peak area, i.e. AU of polypeptide, as determined either by RP-HPLC or SE-HPLC that is at least 90%, 95%, 98% or 100%; wherein in a particular embodiment the RP-HPLC or SE-HPLC profile was unchanged as compared to a reference sample (1 FT cycle).

5.8.3.7 Stability in terms of potency

The skilled person knows various ways to determine potency of vWF binders, in particular immunoglobulin single variable domains, more specifically polypeptides according to any one of SEQ ID NO:s 1-19, e.g. SEQ ID NO: 1 (see, for example, experimental section of WO2006/122825, e.g. Examples 3-6, 18 and 19, or the experimental section of WO2009/115614).

In one embodiment, potency of the polypeptide of the present invention can be determined by binding to its antigen by a conventional assay, e.g. ELISA, Biacore, RIA, FACS, etc.

The potency of vWF binders remained acceptable in the formulations of the invention as tested under stressed conditions, i.e. 4 weeks storage at +40°C.

5.8.3.8 Stability in terms of compatibility

The formulations of the present invention also are compatible with a range of different diluents. For instance, the formulations can be mixed/diluted with such diluents, without affecting chemical and physical stability of the immunoglobulin single variable domains.

Thus, the formulations of the present invention also provide stability over a broad range of concentrations, as defined herein.

5.8.3.9 Summary of stabilizing effects

The formulations of the present invention have the effect of maintaining the chemical and physical integrity of the polypeptides of the present invention even after prolonged storage, e.g. for durations as defined above, at temperatures between -70°C and +25°C.

Storage of immunoglobulin single variable domains as defined herein, in particular ALX-0081 at -70°C for 1 month did not affect their physicochemical characteristics for any of the formulations of the invention, in particular the non-limiting examples of buffers tested in the experimental section. Storage did not have a significant effect on RP-HPLC, SE-HPLC or cIEF profiles.

5.8.4 Stability testing of lyophilized formulations

In addition, the invention provides stable formulations of the vWF binders, e.g. the immunoglobulin single variable domains as defined herein, e.g. SEQ ID NO:s 1-19, preferably SEQ ID NO: 1, which are particularly useful for lyophilization. The formulations of the invention resulted in improved solubility and improved storage stability after lyophilization.

5.8.4.1 Storage stability

The formulations of the invention may provide for good stability after lyophilization when stored, e.g. at a temperature of -70°C, -20°C, +5°C, +25°C or +40°C, e.g. for 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or

36 months. The most advantageous results can be obtained with citrate buffer based formulations, e.g. formulations 3 and 7 as exemplified in the experimental section (e.g. good cake formation and no visuals signs of decay, Figure 6). The skilled person can recognize that in the below discussion the preferred values reflect citrate buffer compositions, e.g. as exemplified in Table 8.

The skilled person will also recognize that storage at +25°C, and more in particular +40°C represent stressed storage conditions. Such conditions are expected to increase and accelerate any signs of instability, e.g. chemical or physical instability. Hence, relatively short storage at e.g. +25°C or +40°C provides a good indication for extended storage stability under milder conditions (e.g. +5°C or frozen).

5.8.4.2 Storage stability in terms of protein recovery

For example, the formulations of the present invention provide for a protein recovery after lyophilization of at least 95%, e.g. at least 96, 97, 98, 99 or even about 100% after storage at a temperature between -70°C and +40°C. Protein recovery can be determined by any known means to quantify proteins, e.g. by content, RP-HPLC or SE-HPLC. These results can be observed e.g. after storage at the indicated temperature of 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months.

5.8.4.3 Storage stability in terms of chemical derivatives / degradation products

Moreover, the formulations of the present invention may prevent and minimize production of chemical derivatives after lyophilization, as confirmed by e.g. by SE-HPLC.

5.8.4.4 Storage stability in terms of oligomerisation

The formulations of the invention may also provide for storage stability after lyophilization, such that no apparent soluble oligomeric material is formed (as defined e.g. by SE-HPLC) at storage temperatures between -70°C and +40°C, after storage durations as defined above, e.g. 1 month; or less than 1%, preferably less than 0.5%, e.g. 0.3% soluble oligomeric material is formed (as defined e.g. by SE-HPLC) at storage temperatures between -70°C and +40°C, e.g. +40°C, after storage durations as defined above, e.g. 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months.

5.8.4.5 Storage stability as reflected in recovery of main product

The formulations of the invention may also have the effect that after lyophilization the main product peak, as determined e.g. by SE-HPLC (cf. Table 18) is about 100% after storage between -70°C and +40°C after a storage duration as indicated above, e.g. 1, 3, 6, 9 or 12 months; or the main product peak, as determined e.g. by SE-HPLC (cf. Table 18) is at least 85%, or more, such as 86%, 87% or 88%. More preferably, the main peak is 90%, 92% or 95%, e.g. at least 97%, more preferably 100% after storage between -70°C and +40°C, e.g. +25°C after a storage duration as indicated above, e.g. 1, 3, 6, 9 or 12 months; or the main product peak, as determined e.g. by SE-HPLC is at least 85%, at least 90%, preferably at least 95%, e.g. at least 98% or even about 100% after storage between -70°C and +40°C, e.g. +40°C, after a storage duration as indicated above, e.g. 1, 3, 6, 9 or 12 months.

The formulations according to the present invention also have the effect that after lyophilization the main peak as determined by RP-HPLC after storage e.g. at a concentration of 12.5 mg/mL at between -70°C and +40°C for between 1 and 12 months remains unchanged as compared to the formulation prior to storage, and represents at least 90%, more preferably at least 93% of the total peaks, wherein the reference sample has a main peak

of e.g. 93% (cf. Table 15). Upon storage after lyophilization at +40°C for up to 12 months the formulation of the present invention retains the main peak as determined by RP-HPLC of at least 91%, 92% or 93%.

Moreover, as determined by cIEF (cf. Table 8), the formulations of the present invention have the effect of providing after lyophilization recovery of the main product after storage at a concentration of e.g. 20 mg/mL for between 1-3 months at a temperature between -70°C and +40°C that is comparable to the reference sample (formulation without storage, main peak is at least 98%), e.g. the main peak is at least 85%, or more, such as 86%, 87% or 88%. More preferably, the main peak is 90%, 92% or 95%, e.g. at least 97%, more preferably 100% after storage between -70°C and +40°C.

5.8.4.6 Stability under freeze-thaw conditions

The formulations of the invention also have the effect of providing good stability after lyophilization under FT conditions. For example the formulations of the invention can be subjected to e.g. 5 FT cycles between -20°C and room temperature (e.g. +25°C). The immunoglobulin single variable domains comprised in the formulations will withstand these conditions without significant deterioration, as ascertained e.g. by RP-HPLC or SE-HPLC. In all cases chemical and physical integrity of the vWF binders, e.g. immunoglobulin single variable domains has been preserved. Overall recovery was in the range between 95 and 100%, preferably at least 95, 98 or 99% compared to a liquid control sample stored at -70°C.

More specifically, at a concentration of 16 mg/mL, 5 FT cycles resulted in a recovery (as determined on the basis of e.g. total area, i.e. AU) of polypeptide, as determined either by RP-HPLC or SE-HPLC that is at least 90%, 95%, 98%, 99% or 100%; wherein in a particular embodiment the RP-HPLC or SE-HPLC profile was unchanged as compared to a reference sample (liquid control sample stored at -70°C)(cf. Table 12).

5.8.4.7 Stability in terms of potency

The skilled person knows various ways to determine potency of vWF binders, in particular immunoglobulin single variable domains, more specifically polypeptides according to any one of SEQ ID NO:s 1-19, e.g. SEQ ID NO: 1 (see, for example, experimental section of WO2006/122825, e.g. Examples 3-6, 18 and 19, or the experimental section of WO2009/115614). The potency of the vWF binders after lyophilization was not affected after repeated FT cycles in the formulations. In particular, the potency of vWF binders remained stable in the formulations of the invention as tested under stressed conditions, i.e. up to 12 months storage at +40°C (Table 23). In one embodiment, potency of the polypeptide of the present invention after lyophilization can be determined by binding to its antigen by a conventional assay, e.g. ELISA, Biacore, RIA, FACS, etc. More specifically, in the formulations of the present invention at least 80%, preferably at least 90%, more preferably at least 95% or even at least 99% of the vWF binder retains it binding activity after storage under the above stress conditions compared to the binding activity prior to storage.

In a further aspect, the formulations of the present invention exhibit almost no loss in biological activity when comparing the liquid formulation of ALX-0081 to the lyophilized formulation, as assessed by various immunological assays including, but not limited to Biacore assay, enzyme-linked immunosorbent assay (ELISA), ristocetin induced cofactor activity assay (RICO) and/or Gyrolab-based assay (see Section 7.13 and Table 24).

5.8.4.8 Summary of stabilizing effects

The formulations of the present invention have the effect after lyophilization of maintaining the chemical and physical integrity of the polypeptides of the present invention, in particular ALX-0081, i.e. even after prolonged storage, e.g. for durations as defined above, at temperatures

between -70°C and +40°C, the purity/impurity profile of the product is essentially not changing. For example, prolonged storage after lyophilization did not have a significant effect on RP-HPLC, SE-HPLC or cIEF profiles as supported by the experimental section.

5.9 Methods of the invention

The vWF binders of the invention can be produced by any commonly used method. Typical examples include the recombinant expression in suitable host systems, e.g. bacteria or yeast. The vWF binders will undergo a suitable purification regimen prior to being formulated in accordance to the present invention.

The present invention encompasses methods of producing the formulations as defined herein.

The purification and formulation steps may coincide, e.g. when the vWF binders of the invention are eluted from a column using a buffer according to the present invention. Alternatively, the formulations of the invention can be prepared by exchanging a buffer by any suitable means, e.g. means widely used in the art such as dialyzing, ultrafiltration, etc.

In some embodiments the method of producing a formulation of the invention may also relate to the reconstitution of a lyophilized or spray-dried formulation, e.g. by addition of water or a suitable buffer (which may optionally comprise further excipients).

The methods for preparing a formulation according to the present invention may encompass further steps, such as filling it into vials suitable for clinical use, such as sealed containers and/or confectioning it in a dosage unit form. The methods may also comprise further steps such as spray-drying, lyophilization, or freezing, e.g. bulk freezing. The invention also encompasses the containers, dosage unit forms, or other products obtainable by any of the methods recited herein.

The formulations of the present invention can be used to store the vWF binders, e.g. ISVDs as defined herein. Thus, the invention encompasses a method of storage of a vWF binder as used herein, characterized by the use of a formulation as defined herein. More specifically, the invention encompasses methods for stabilizing a vWF binder as defined herein for storage, comprising e.g. the preparation of a formulation as described herein. Storage can be 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months, e.g. at least 12 months, optionally at a temperature between -70°C and +40°C, such as -70°C, -20°C, +5°C, +25°C or +40°C, preferably a temperature between -70°C and +25°C, more preferably at a temperature between -20°C and +5°C. Thus, storage may encompass freezing, freeze-drying (lyophilization) and/or spray-drying. The storage methods may furthermore comprise the assessment of physical and chemical integrity of the vWF binders as defined herein.

The present invention also relates to methods for analyzing formulations comprising at least one of the vWF binders as defined herein. The formulations can be analyzed for any signs of chemical or physical instability of the vWF binders as defined herein. For example, the formulations can be assessed for the presence of degradation products, e.g. low molecular weight derivatives such as proteolytic fragments; and/or for chemical derivatives, e.g. pyroglutamate variants; and/or for high molecular weight derivatives such as aggregates, agglomerates, etc. The formulation can also be assessed for total protein content and/or potency. Each of the various assay methods as referred to herein can be used in the analysis method of the present invention.

Thus, the present invention also relates to a method for monitoring and/or assessing the quality and/or stability of a formulation, e.g. during one or more of manufacture, storage and use. The invention also relates to a method of quality control of a formulation, e.g. to assess that the formulation meets product specifications as further described herein. The

invention in any of these aspects comprises one or more selected from the comparison with one or more reference samples, the analysis of batch to batch variation, and the ongoing monitoring of a production process.

The present invention relates to any product that is associated with the formulations of the present invention, e.g. by comprising them, or by being necessary for their production or confectioning, without any limitations.

For example, the present invention relates to an article of manufacture, e.g. a sealed container comprising one or more of the formulations according to the present invention. The invention also relates to a pharmaceutical unit dosage form, e.g. a dosage form suitable for parenteral administration to a patient, preferably a human patient, comprising one or more of the formulation according to any embodiment described herein. The dosage unit form can be e.g. in the format of a prefilled syringe, an ampoule, cartridge or a vial. The syringe, ampoule, cartridge or vial can be manufactured of any suitable material, such as glass or plastic and may include rubber materials, such as rubber stoppers for vials and rubber plungers and rubber seals for syringes and cartridges. The invention also relates to a kit comprising one or more of the formulations according to the present invention. The kit may further comprise instructions for use and/or a clinical package leaflet. In any embodiment of the products as defined herein, the invention also encompasses the presence of packaging material, instructions for use, and/or clinical package leaflets, e.g. as required by regulatory aspects.

5.10 Definitions

5.10.1 Identity

For the purposes of comparing two or more amino acid sequences, the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid

identity") may be calculated by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), i.e. as an "amino acid difference" as defined herein.

Alternatively, the degree of sequence identity between two or more amino acid sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0. using standard settings.

Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO04/037999, EP0967284, EP1085089, WO00/55318, WO00/78972, WO98/49185 and GB2357768-A.

Usually, for the purpose of determining the percentage of "sequence identity" between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the "first" amino acid sequence, and the other amino acid sequence will be taken as the "second" amino acid sequence.

Also, in determining the degree of sequence identity between two amino acid sequences, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO04/037999, GB2357768-A,

WO98/49185, WO00/46383 and WO01/09300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO04/037999 as well as WO98/49185 and from the further references cited therein. Such conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) – (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu. Any amino acid substitutions applied to the polypeptides described herein may also be based on the analysis of the frequencies of amino acid variations between homologous proteins of different species developed by Schulz et al., Principles of Protein Structure, Springer-Verlag, 1978, on the analyses of structure forming potentials developed by Chou and Fasman, Biochemistry 13: 211, 1974 and Adv. Enzymol., 47: 45-149, 1978, and on the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al., Proc. Natl. Acad. Sci. USA 81: 140-144, 1984; Kyte & Doolittle; J Molec. Biol. 157: 105-132, 1981, and Goldman et al., Ann. Rev. Biophys. Chem. 15: 321-353, 1986, all incorporated herein in their entirety by reference. Information on the primary, secondary and tertiary structure of Nanobodies® is given in the description herein and in the general background art cited above. Also, for this purpose, the crystal structure of a

V_{HH} domain from a llama is for example given by Desmyter et al., Nature Structural Biology, Vol. 3, 9, 803 (1996); Spinelli et al., Nature Structural Biology (1996); 3, 752-757; and Decanniere et al., Structure, Vol. 7, 4, 361 (1999). Further information about some of the amino acid residues that in conventional V_H domains form the V_H/V_L interface and potential camelizing substitutions on these positions can be found in the prior art cited above.

6. Abbreviations

API	Active Pharmaceutical Ingredient
cIEF	Capillary IsoElectric Focusing
DLS	Dynamic Light Scattering
DOE	Design of Experiments
DP	Drug Product
DS	Drug Substance
FT	Freeze-Thaw
HMW	High Molecular Weight
LMW	Low Molecular Weight
MALS	Multi-Angle Light Scattering
RH	Relative Humidity
RPC	Reverse Phase Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SE-HPLC	Size Exclusion High Performance Liquid Chromatography
SOP	Standard Operating Procedure
Tm	Melting Temperature (°C)
TSA	Thermal Shift Assay
vWF	von Willebrand Factor
WFI	Water For Injection

7. EXAMPLES

A set of experiments was designed in order to obtain an improved formulation buffer, intended to satisfy a broad range of different and seemingly incongruous objectives. In particular, exemplary formulations are provided herein which are capable of maintaining the stability, biological activity, purity and quality of ALX-0081 and this over an extended period of time, stable to various stresses such as freezing, lyophilization, heat and/or reconstitution.

Contemporaneous ALX-0081 DS has been presented as a liquid formulation containing 5 mg/mL of the active pharmaceutical ingredient (API) in a phosphate-based (D-PBS) buffer containing 200 mM glycine and 0.02% Tween-80 (v/v), pH 7.1 (DS). Although this formulation has been applied during initial clinical trials, it may be improved in several manners. First, the relatively low concentration would probably necessitate multiple subcutaneous injections (assuming the volume per subcutaneous injection is restricted to about 1 mL) thus reducing patient usage friendliness. Secondly, the storage stability at 3-8°C or room temperature of the current formulation of ALX-0081 is limited. The limited shelf-life in the present formulation is mainly determined by chemical modification (cf. Section 7.2). Chemical modifications may be linked with potency loss. Although a practicable shelf-life can be achieved by storing the product at -20°C, this is, however, not considered to be a favourable option for most practical purposes.

7.1 Methods

Samples were analyzed essentially according to standard operating procedures for assessing content, potency and purity, precipitation, concentration, degradation, aggregation and potency. In addition, all samples were visually inspected for turbidity or the presence of protein

aggregates or precipitation. The residual moisture content of specific lyophilized samples was determined by means of Karl-Fischer titration.

Three different lyophilization programs were used in the present study to freeze-dry ALX-0081 – a standard 65h run (Figure 1), a shortened 37h run and a longer lyophilization cycle of 66h optimized to reduce residual moisture content as described in Table 14.

Briefly, at the start of the shortened 37h lyophilization process the shelf temperature was +20°C and was brought to -50°C in 2 hours. Next, a vacuum of 0.04 mbar was created in 1 hour. After reaching the vacuum of 0.04 mbar the shelf temperature was kept at -50°C for 4 hours. After these 4 hours the temperature was gradually increased to 0°C in 15 hours (i.e. primary drying step, removing frozen water). The shelf temperature of 0°C was kept for 7 hours while keeping a vacuum of 0.04 mbar. After 7 hours the temperature was raised to +25°C in 3 hours and subsequently kept at 25°C for 5 hours (i.e. secondary drying step, removing unfrozen water). The vials were closed under a vacuum of ±0.400 mbar after which normal pressure was restored.

The same method was applied for the standard 65h run and only differs in the second drying step which was prolonged with 28h at +25°C under vacuum resulting in a total cycle time of about 65h. A schematic overview of the different steps in the standard 65h lyophilization run is shown in Figure 1. During the lyophilisation process, product temperature of three vials in strategic positions were monitored. Finally, the standard 65h run was modified during the run according to the reading of the temperature probes resulting in a prolonged lyophilization cycles as described in Table 14.

7.2 Chemical stability of contemporaneous ALX-0081 formulation

RP-HPLC is one of the most informative methods to assess the chemical stability of a drug substance (DS).

RP-HPLC resolved the ALX-0081 DS into a number of different species. In addition to the main peak, pre-peaks (substance eluting before the intact unmodified material) and a number of post-peaks could be discerned. In the batches produced so far, the pre-peaks and post-peak 1 consistently represented about 2% and 3.6% of the DS respectively, whereas the other post-peaks accounted for less than 1% of the DS.

During storage under accelerated (+5°C) or stressed (+25°C and +37°C/+40°C) conditions however, the relative abundance of certain product related variants increased with time and temperature, as depicted in Figure 2A. Additionally, the RP-HPLC main peak appears to divide into several different species upon prolonged incubation, especially at elevated temperatures (≥+25°C) (Figure 2B). The data indicate that some earlier eluting new molecular species are generated during storage.

The most important modifications which were present in ALX-0081 DS at the time of manufacturing or which arose during storage are the following: (i) pre-peak 1 (oxidation); (ii) post-peak 1 (nor-leu variant); (iii) post-peak 2 (formation of pyroglutamate), and (iv) splitting of the main peak (isomerization). The modifications (i), (ii), and (iii) did not significantly affect the potency (data not shown). In contrast, the isomerization of the aspartic acid residues at position 105 and 236 of SEQ ID NO: 1, which are located in the CDR3 region, were shown to be the predominant molecular mechanism underlying a potential loss of potency of ALX-0081 (cf. (iv) above).

Some of the ALX-0081 product related variants, present at the time of manufacturing or arising during storage, could also be detected by cIEF. This was the case for the pyroglutamate modification which appeared as a post-peak (cf. (iii) above). Also, similar to what was observed in the RP-

HPLC analysis, the isomerization events at position 105 in both 12A02H1 domains resulted in main peak broadening and eventually splitting of the main cIEF peak (cf. (iv) above).

7.3 Buffer and excipient screening

In order to further develop the formulation of vWF binders, a complex set of experiments was designed elaborating various parameters which all influence each other, including (i) different buffers, (ii) at different concentrations, (iii) each buffer at various pH; and (iv) each combined with different excipients.

Buffer systems should have an as low buffering capacity as feasible, so as not to significantly disturb the body's buffering system when injected. In addition, the buffer type and concentration on the activity of the active pharmaceutical ingredient (API) must be evaluated very carefully.

Generally, increased levels of protein stability have been attributed to high melting temperatures. Accordingly, thermal properties of ALX-0081 were monitored in the presence of various compositions. In particular, a TSA experiment was performed in 192 different isotonic formulations, for which the results were fed into a design of experiments (DOE) to evaluate the effect of buffer, concentration, ionic strength, pH and excipients on the thermal stability of ALX-0081. The read-out was the melting temperature (Tm) of ALX 0081 which is indicative for the thermal stability of the protein in the various tested compositions.

Briefly, the employed thermal shift assay (TSA) follows the signal changes of a fluorescence dye, such as Sypro Orange, while the protein undergoes thermal unfolding. When Sypro Orange is added to a properly folded protein solution, it cannot bind to any surface on the protein and its fluorescence signal is quenched. When the temperature rises, the protein undergoes thermal unfolding and exposes its hydrophobic core region. Sypro Orange then binds to the hydrophobic regions and unquenches, which

results in the increase of the fluorescence signal. The assay was performed on solutions containing different formulations to be tested, ALX-0081 at 0.2 mg/mL and 10x Sypro Orange. The program consisted of the following steps: heat to 37°C at a ramp rate of 4.4°C/s and hold for 10s ; heat to 90°C at a continuous ramp rate of 0.02°C/s (20 acquisitions per °C); and cool to 37°C at a ramp rate of 2.2°C/s and hold for 10s.

The following set of buffers with varying concentrations (10-200 mM), pH values and excipients were herein explored:

- citrate pH 6.0-6.5-7.0
- histidine pH 5.5-6.0-6.5
- phosphate pH 6.5-7.0-7.5
- Tris-HCl pH 7.4-7.7-8.0
- NaCl 0-140 mM concentration range
- glycine 0-270 mM concentration range
- mannitol 0-270 mM concentration range
- sucrose 0-270 mM concentration range
- trehalose 0-270 mM concentration range

The obtained melting temperatures (Tm) were imported in the Design Expert program for analysis of the factorial screening experiment to predict 50 formulations resulting in the highest thermal stability (see Table 1).

The highest Tm values were predicted for phosphate (pH 7.0-7.5) and citrate (pH 6.2-7.0) containing trehalose, sucrose, mannitol or glycine. Wholly unexpectedly, the results of the study suggest that Tris-HCl (pH 7.8-8.0) and histidine-HCl (pH 6.5) based buffers return significantly lower melting temperatures, although they were previously elected as the buffer system of choice for controlling solution pH of immunoglobulin single variable domains as decribed in WO2010/077422.

Accordingly, it was concluded that phosphate and citrate formulations containing trehalose, sucrose, glycine or mannitol, performed especially well in stabilizing vWF binders, e.g. ALX-0081.

7.4 Solubility testing

In order to evaluate whether the solubility of ALX-0081 could be further enhanced, an initial screening was performed in several formulations. ALX-0081 was buffer exchanged to the formulation of interest (excluding Tween-80) and further concentrated in a stirring cell (e.g. type Amicon) equipped with 5 kDa cut off filter. As soon as visible precipitation or turbidity occurred, the sample was filtered and the protein concentration was measured. Table 2 shows a summary of the obtained results.

Concentration in phosphate and histidine based buffers resulted in sample turbidity and formation of precipitation at relatively low protein concentrations (<10 mg/mL). In contrast, ALX 0081 remained physically stable in citrate buffer, even after reaching a concentration of ~56 mg/mL. In addition to the visual inspection, the absence of particulate matter or HMW species was confirmed by fluorescence microscopy (staining with Nile Red, by SE-HPLC and DLS). Furthermore, subjecting the ~56 mg/mL solution to either 10 FT cycles at -20°C or -70°C, or storage at +4°C for about 1 week did not appear to affect the physical stability of the molecule as evidenced by SE-HPLC analysis (see Figures 3A and 3B respectively).

The comparatively high solubility of ALX-0081 in the citrate buffer was corroborated by a PEG precipitation assay (data not shown).

7.5 Tween-80

In order to determine whether the non-ionic surfactant polysorbate, also designated Tween, (Polyoxyethylene (N) sorbitan monolaurate; wherein N=20, 40, 60, 65, 80 or 85) is required in the formulation of ALX-0081, several stir stress experiments were performed in 50 mM citrate buffer at

pH 6.0 and 6.5. The effect of different concentrations of Tween-80 (no Tween-80 vs. 0.01% vs. 0.02% (v/v)) on the physical stability of ALX 0081 was evaluated at 5 mg/mL by monitoring 500 nm scatter signal in a spectrofluorometer.

Tween-80 prevented an increase in the scatter signal in both buffers demonstrating its protective effect (Figures 4A and 4B). No significant differences were observed between samples containing 0.01% or 0.02% Tween-80 (v/v). Furthermore, the SE-HPLC profiles of samples before and after stirring did not show any differences: 95-100% recovery was achieved and no oligomerization or degradation could be detected.

Based on these results, it was decided to include 0.01% Tween-80 (v/v) in the formulation of vWF binders, e.g. ALX-0081.

7.6 Tween

In order to determine whether the other members in the polysorbate range, which differ by the length of the polyoxyethylene chain and the fatty acid ester moiety, e.g. Tween-20, Tween-40, Tween-60, Tween-65 and Tween-85 are required in the formulation of anti-vWF binders, several stir stress experiments are performed in 50 mM citrate buffer at pH 6.0 and 6.5, essentially as described in Section 7.5 above. The effect of different concentrations of the various Tween-members (no Tween vs. 0.01% vs. 0.02% (v/v)) on the physical stability of the vWF binder is evaluated at 5 mg/mL by monitoring 500 nm scatter signal in a spectrofluorometer.

Tween-20, Tween-40, Tween-60, Tween-65 and Tween-85 give substantially the same beneficial result as Tween-80.

7.7 Stability testing of liquid formulations

A more comprehensive study was performed to assess the stability of ALX-0081 in different citrate-based isotonic formulations at a

concentration of 20 mg/mL. Table 3 gives an overview of the different formulations which were tested.

The main goal was to evaluate the effect of pH (6.0-6.5-7.0) and excipient type (NaCl, mannitol, sucrose or glycine) on the stability of the liquid product. For control and direct comparison purposes the study also included contemporaneous ALX-0081 formulated at 5 mg/mL in D-PBS and glycine (identical to current formulation, except for the lower Tween-80 concentration) as well as the previously mentioned different citrate-based isotonic ALX-0081 solutions but formulated at a concentration of 5 mg/mL instead of 20 mg/mL. Altogether, this resulted in 17 different liquid formulations (formulation no. 1-17) which were subjected to profound stability studies. In order to exclude the effect of differences in Tween concentration, all formulations contained 0.01% Tween-80 (v/v).

7.7.1 Freeze-Thaw stability

The effect of repetitive FT cycles on the stability of ALX-0081 as a liquid formulation was evaluated. Aliquots of the different formulations (0.5 mL/ tube) were subjected to up to 10 FT cycles at -70°C or -20°C. One cycle included freezing for ±20 min followed by thawing during 5 min in a water bath at +25°C. After this treatment, all formulations remained visibly clear. RP-HPLC analyses showed good recovery (95-100%) and no significant difference in profile could be detected suggesting that the quality of vWF binders, e.g. ALX-0081 is not affected by repeated freeze-thawing in the 17 different liquid formulations tested.

7.7.2 Storage stability

The stability of the 17 different formulations was also assessed by storing aliquots (0.5 mL/ tube) under stressed conditions, i.e. +40°C; the -70°C long term storage condition was included as reference. The analyses focused on RP-HPLC because this method is generally known as a particularly informative method for revealing chemical modifications that occur during storage (see Table 4). This section gives an overview of the data obtained after 1 month storage; the results confirm the findings at the earlier time points, i.e. after 1 week and 2 weeks.

(a) *RP-HPLC*

As previously indicated in Section 7.2 above, RP-HPLC analysis resolved contemporaneous ALX-0081 DS (D PBS/glycine formulation) into some product related variants and impurities. Briefly, under stressed conditions (e.g. +40°C), the purity (% main peak) decreased concomitantly with an increase in some of the existing pre/post-peaks as well as the formation of additional ones.

The RP-HPLC data obtained in the present study are summarized in Table 5.

Overall, the obtained results indicated that essentially the same modifications took place in the different citrate buffers as observed in the present formulation buffer (i.e. D-PBS/glycine), although some differences in relative peak area could be observed. In particular, the increase in pre-peak area (oxidation) was slower in the citrate formulations (especially at pH 6.0) compared to the D PBS/glycine formulation. With respect to this pre-peak build up, glycine appeared to be the least favorable amongst the different excipients. The profile of the different post-peaks after storage for 1 month at +40°C was comparable for all formulations, although the second post peak (i.e. the pyroglutamate variant) appeared to be more pronounced at pH 7.0 than at pH 6.0 - 6.5. The extent of broadening/splitting of the main peak

– the result of asp isomerization – is difficult to quantify due to poor resolution; the area percentage of the shoulder peak could not be accurately estimated and was therefore included in the relative surface area reported in Table 5 for the main peak. Nonetheless, the corresponding RP-HPLC chromatograms (data not shown) allowed making a qualitative assessment; these data suggest that the extent of isomerization is quite similar in the various formulations.

(b) *cIEF*

Similar to RP-HPLC, the cIEF method permits the detection of certain product variants that occur during storage under stressed conditions (see Section 7.2 for details). This is exemplified in Figure 5, comparing the electropherograms of contemporaneous ALX-0081 after storage during one month at -70°C and +40°C.

The cIEF data obtained in the present study (data not shown) basically confirm the conclusions reached by the RP-HPLC analysis, i.e. the same type of modifications take place to roughly the same extent in the different citrate buffers as observed in the present formulation buffer, represented herein by formulation 17 as depicted in Figure 5 (i.e. D-PBS/glycine). However, some differences in relative peak area could be observed. In particular, the post peak (i.e. the pyroglutamate variant) appeared to be more pronounced at pH 7.0 than at pH 6.0 - 6.5, which is in agreement with the findings by RP-HPLC as previously summarized in Table 5.

(c) *SE-HPLC*

SE-HPLC analysis was performed to examine the physical stability of ALX-0081, i.e. to detect HMW species and/or degradation products that could have formed during storage under stressed conditions. For all of the

formulations which were tested here, the stress test did not appear to have a significant effect on the SE-HPLC chromatograms.

(d) *Conclusion*

A summary of the most important findings regarding the storage stability of the different liquid ALX-0081 formulations is shown in Table 5. Only the most informative data based on RP-HPLC analysis were listed. These data suggest a higher chemical stability in 50 mM citrate at pH 6.0-6.5. With the exception of glycine, the type of excipient did not have a significant effect on stability. With respect to the physical stability, no differences could be observed between the different formulations. The latter was evidenced by the ± 100% recovery observed for all samples in the various HPLC analyses as well as by the SE-HPLC chromatograms demonstrating the absence of aggregation/degradation.

Based on the above results, it was decided to further explore the potential of the citrate/sucrose formulations at pH 6.0-6.5.

7.8 Stability testing of lyophilized formulations

The effect of lyophilization was assessed by comparing the storage stability of ALX-0081 in liquid and lyophilized citrate/sucrose formulations (20 mg/mL API at pH 6.0 6.5). An overview of the formulations tested is given in Table 6. The prior art D PBS/glycine based formulation (5 mg/mL API) was included for comparison. Liquid (i.e. prior to lyophilization) and lyophilized ALX-0081 were kept frozen (-70°C for the liquid samples and -20°C for the lyophilized formulations) as well as at +5°C, +25°C and +40°C and samples were analyzed after 2 weeks and 1.5 months storage.

Panel A of Figure 6 shows a picture of the vials after the lyophilization process using the standard 65h run as depicted in Figure 1. Lyophilization of the formulations containing citrate/sucrose resulted in good cake formation, whereas samples formulated in D-PBS/glycine did not

produce a decent cake. All samples could readily be resolubilized with Milli-Q water and solutions were clear and colorless (Figure 6, panel B).

7.8.1 Evaluation of product before and after lyophilization

RP-HPLC and SE-HPLC analysis revealed no significant differences in terms of physicochemical characteristics between the liquid starting product (kept at ≤ -70°C) and the product after lyophilization and reconstitution for any of the tested formulations. Furthermore, full sample recovery was demonstrated for all formulations (Table 7).

7.8.2 Evaluation of lyophilized product after 1.5 month storage

(a) ***Visual inspection and content***

The cake of the lyophilized samples showed no visual signs of decay after 1.5 month storage at -20°C, +5°C, +25°C or +40°C.

Samples were clear and colorless after reconstitution with Milli-Q water. Also, storage did not have a significant effect on content, measured after reconstitution (Table 8).

(b) ***RP-HPLC***

The profiles of the 3 different lyophilized formulations (no 3, 7 and 17) were compared after 1.5 months storage at -20°C, +5°C, +25°C and +40°C respectively. A comparison at the most strenuous conditions (+40°C) best reveals the impact of the lyophilization on the chemical stability.

Corresponding results are summarized in Table 8. As can be seen, storage in the frozen form does not appear to affect ALX-0081 in any of the formulations tested in the present study.

Overall, the prevailing conclusion from the obtained data is that lyophilization of a citrate/sucrose based formulation essentially prevents the chemical modifications that occur in the liquid form, with the exception of some minor amounts of pyroglutamate modification. In these lyophilized

formulations, there was neither an increase in the area percentage of the pre-peaks nor a sign of main peak broadening/splitting. In contrast, lyophilization of the D-PBS/glycine based formulation did not result in a significant improvement of the chemical stability. The pyroglutamate formation in the citrate/sucrose lyophilized formulation appeared to be slightly more pronounced at pH 6.0 than at pH 6.5. This is substantiated by the data at +25°C, at which temperature the rate of pyroglutamate formation is lower but shows the same pH dependence. As expected, storage for up to 1.5 months at -20°C or +5°C did not cause any detectable deterioration of lyophilized ALX-0081 (data not shown).

Surprisingly, at +40°C improved stability was obtained in the citrate/sucrose based formulations compared to the formulation based on D-PBS/glycine, the latter showing significantly higher susceptibility to chemical modifications.

For the liquid formulations, storage of up to 1.5 months at -70°C, +5°C and +25°C did not have a significant effect on ALX-0081 (data not shown). The deterioration observed after 1.5 months of storage at +40°C, is roughly in agreement with earlier observations (see Section 7.7.2).

(c) *cIEF*

The results obtained by cIEF analysis are in agreement with those of RP-HPLC. Most notably, lyophilization of a citrate/sucrose based formulation is not able to prevent the pyroglutamate modification completely. Indeed, storing the lyophilized product at +40°C for 1.5 months resulted in an increase in the post peak. Again, faster pyroglutamate formation was observed in citrate/sucrose at pH 6.0 than at pH 6.5.

(d) *SE-HPLC/MALS/DLS*

Storage for up to 1.5 months at -70°C/-20°C, +5°C and +25°C did not have an effect on the SE-HPLC profiles of lyophilized or liquid

formulations of ALX 0081 (data not shown). However, at +40°C peak broadening and formation of shoulder peaks could be observed in all liquid formulations. MALS analysis showed that these shoulder peaks correspond to monomeric ALX-0081 (data not shown). The data hint at a conformational change in a subpopulation of ALX-0081 as a result of the stressed storage. Surprisingly, the SE-HPLC profile of lyophilized citrate/sucrose formulations was not affected by the +40°C stress test indicating that these lyophilized formulations also improve the physical stability of ALX-0081. This however was not the case for the lyophilized D-PBS/glycine formulation; stressing this formulation at +40°C not only resulted in a shoulder peak but apparently also in some higher molecular weight species, visible as a broad pre-peak (Table 8). DLS analysis did not detect any large oligomeric species in any of the formulations (data not shown).

(e) ***Conclusion***

A summary of the most important findings regarding the storage stability of the tested lyophilized ALX-0081 formulations is shown in Table 8. Overall, only limited differences in stability were observed between the citrate/sucrose formulations, although unexpectedly ALX-0081 appeared to be less prone to pyroglutamate formation at pH 6.5 than at pH 6.0. Therefore, further reformulation work for ALX-0081 was focused on citrate/sucrose based formulations at pH 6.5.

7.9 Further optimization of the citrate/sucrose based formulation

The data collected so far show that a citrate/sucrose based formulation improves the solubility and that lyophilization of this formulation dramatically improves the storage stability of ALX-0081. However, storage of lyophilized ALX 0081 at higher temperatures, albeit limited, still results in pyroglutamate formation. It is reasonable to assume

that this modification may limit the shelf-life of the lyophilized product (even when stored at +5°C). It remains to be elucidated why lyophilization was not able to prevent this modification.

It was hypothesized that water remaining in the freeze-dried product plays a key role.

If this hypothesis would be true, then the remaining water may be minimized by optimizing the physical lyophilization parameters, such as drying time, temperatures, vacuum etc, as listed above, but at the same time the other parameters of the vWF binder should remain constant. Another approach is modifying the formulation, but again at the same time the other parameters of the vWF binder should remain constant. In addition, adjusting the physical lyophilization parameters in combination with modifying the formulation may be used.

7.9.1 Optimizing lyophilization parameters

Optimizing the physical lyophilization parameters, including (i) drying time times, (ii) temperatures of the different steps, (iii), vacuum, and a combination of (i) - (iii) was not satisfactory, i.e. no or inadequate effect on residual moisture content or affecting the parameters of the vWF binders.

7.9.2 Optimizing formulation for lyophilization

The effect of moisture content on the chemical stability of the lyophilized product was investigated by adjusting the concentrations of the citrate buffer and the sucrose excipient. In addition, a secondary drying time during the lyophilization program was investigated.

7.10 Effect of moisture content on stability of lyophilized product

Three different isotonic formulations of ALX-0081 with varying citrate and sucrose concentrations (all three at pH 6.5) were subjected to

two different lyophilization programs: the standard 65h run on the one hand and a shortened 37h run on the other hand. An overview of the formulations tested is given in Table 9. Figure 7 shows the vials obtained after lyophilization. Lyophilization resulted in good cake formation for all formulations.

Lyophilized samples of ALX-0081 were analyzed after 2 and 4 weeks of storage at both -20°C and +40°C. In the present experiment, it was decided to perform an exhaustive testing so as to further substantiate the usefulness of the formulations.

First, during storage at +40°C for up to 4 weeks the cake of the lyophilized samples remained intact and reconstitution yielded clear solutions. The lyophilization cycle appeared to have no significant effect on content (measured spectrophometrically at 277 nm) or osmolality. In agreement with earlier experiments, 4 weeks storage at +40°C did not have an effect on the physical stability of ALX-0081, based on SE-HPLC, MALS, and DLS analyses (data not shown). Additionally, it was found that the potency of ALX-0081 as determined by the Biacore-based assay was unaffected by the lyophilization process and the subsequent storage (data not shown). However, the RP-HPLC analyses demonstrated that storage did again result in the formation of -albeit minor- quantities of the pyroglutamate variant. This was slightly more pronounced for the formulation containing the highest concentration of citrate and lowest concentration of sucrose (Table 10). In addition, for each lyophilized formulation, the total moisture content was determined by means of Karl Fisher titration. A summary of these data together with the amount of pyroglutamate detected in the corresponding stressed samples is shown in Table 10. It appears from the data obtained for each lyophilization program separately that a higher moisture content results in a higher susceptibility to pyroglutamate formation. This suggests that residual water present in the lyophilized product promotes chemical modifications.

In conclusion, the results indicate that reducing the moisture content of lyophilized vWF binders, e.g. ALX-0081, is beneficial for its chemical stability.

7.11 Effect of reducing buffer strength and increasing sucrose content

The data obtained in the previous section show that reducing the citrate concentration while increasing the sucrose concentration (thereby maintaining an isotonic solution) is beneficial for the stability of the lyophilized product. At the same time, evidence was obtained that ALX-0081 required a sufficiently high concentration of citrate to obtain improved solubility. It was therefore decided to assess the effect of the citrate and sucrose concentrations on the appearance of the solution during storage at +5°C and +25°C, and to re-evaluate the freeze-thaw stability in the presence of lower concentrations of citrate.

7.11.1 Assessing impact of citrate/sucrose concentration

In a first experiment, 12 different formulations of ALX-0081 were stored at +5°C and +25°C for up to 4 days. Samples were inspected on a regular basis for turbidity or presence of precipitate. Pictures taken of the samples after 4 days of storage are shown in Figures 8 and 9. An overview of the different formulations and corresponding results is presented in Table 11. After 4 days of storage at +25°C all samples remained clear and colorless (Figure 8, panel A). In contrast, at +5°C most citrate formulations without excipients became hazy (Figure 8, panel B). Clearly, the degree of haziness is inversely proportional to the citrate concentration, with the formulation of 50 mM citrate remaining clear. Also, sample recovery for the sample containing 15 mM citrate was 68% (based on A277 after 20h storage), while other recoveries varied from 90 to 100% (data not shown). Adding sucrose to the 15 mM citrate formulation prevented sample haziness, although at the

lowest concentration of sucrose (i.e. 5%) some minor turbidity was detected at +5°C (Figure 9, panel B).

The observations confirm the importance of a sufficiently high concentration of citrate in maintaining ALX-0081 soluble, particularly at low temperature. Nevertheless, increasing the citrate concentration resulted in increased moisture content. Unexpectedly, reducing the citrate concentration can be compensated for by the addition of sucrose. No effect of Tween-80 on solubility was observed.

7.11.2 Assessing FT stability

A follow-up experiment focused on the FT stability of several citrate/sucrose based formulations. Nine different formulations of ALX-0081 were subjected to 5 consecutive FT cycles at -20°C. An overview of the tested formulations and corresponding results is shown in Table 12. All samples remained clear and FT cycles did not affect the physical stability of vWF binders, e.g. ALX 0081, based on content analysis and the SE-HPLC data.

7.11.3 Optimizing sucrose and citrate concentration in view of isotonicity

Based on the above mentioned storage and FT results the optimal concentration of citrate buffer was selected as 20 mM. A final experiment was performed on three formulations differing in sucrose concentration. The aim of this experiment was to establish the optimal sucrose concentration for achieving an isotonic formula and to confirm FT stability of ALX-0081 at 20 mg/mL. In addition to 5 consecutive FT cycles, each formulation was also subjected to 1 FT cycle followed by 24h storage at +25°C and an additional FT cycle in order to mimic the handling steps during manufacturing.

A summary of the results is given in Table 13. All tested formulations were clear and the various handlings did not have an effect on

content/recovery or osmolality. Based on the osmolality values it seems that a concentration of 7% sucrose is optimal to obtain an isotonic solution.

7.12 Stability study of lyophilized ALX-0081 formulations stored at various temperatures for up to 12 months

ALX-0081 formulated at 12.5 mg/mL in 20 mM citrate buffer pH 6.5, 7% sucrose (w/v) and 0.01% Tween-80 (v/v) was lyophilized according to the conditions set out in Table 14. Samples were subsequently stored at -20°C (±5°C), +5°C (±3°C), +25°C (±2°C/60±5% RH) and +40°C (±2°C/75±5% RH).

The stability of the lyophilized formulations was assessed at different timepoints, i.e. initial, 1 month, 3 months, 6 months, 9 months and 12 months, and was evaluated for purity, appearance, physicochemical properties and potency.

Detailed sample characterization data are provided in Tables 15 to 23.

Purity of the samples was assessed by RP-HPLC in which the percentage of mean peak area was determined as well as the percentage of pre-and post-peak areas. The protein concentration was determined by UV absorbance.

Further, the lyophilized samples were visually inspected, reconstituted, and the reconstituted formulation was visually inspected. The pH of the samples after reconstitution was measured and the moisture content of the lyophilized powder was determined by coulometric titration (Karl Fischer). Particulate matter count measurements were performed to count particles ≥10 µm and ≥25 µm. The samples were further characterized for biological function using a biacore-based assay. Potency was expressed as percent relative potency of reference material.

The obtained stability data show that the characteristics of the lyophilized ALX-0081 product are not significantly affected by 12 months

storage at either -20°C or +5°C. The data collected throughout the stability study at those temperatures were found to be comparable to those generated at time zero.

Several minor changes were observed for samples stored at +25°C or +40°C which can be attributed to the accelerated or stressed storage conditions. The main observations were:

- At +25°C and at +40°C an increase in post peak 2 was observed on RP-HPLC during the 12 months storage, corresponding with the formation of the pyroglutamate variant from 0.7% to 1.1% or to 2.4% respectively.
- At +40°C an increase in moisture content from 0.7% to 2.1% (w/w) after 12 months storage was noted. This could potentially be attributed to intake of moisture from the storage environment (i.e. 75% RH) by the stopper, with the subsequent gradual diffusion to the product.

The results obtained under stressed conditions suggest a correlation between the moisture content and the chemical stability of the product; this corresponds with the data previously reported under Section 7.10.

Hence, these data indicate the importance of controlling the moisture content of the DP product during storage.

Considering that +40°C storage can be regarded predictive for the long term stability at +25°C, the 12-month stability data included herein provide a good indication for long term storage stability at room temperature (such as 18, 24, 30 or 36 months) and even prolonged stabilities when stored under milder conditions (e.g. +5°C or frozen).

7.13 *In vitro* comparability study on the biological activity of the liquid and lyophilized drug product formulation of the anti-vWF Nanobody caplacizumab (ALX-0081)

7.13.1 Objective

A number of assays were used to evaluate the *in vitro* comparability of contemporaneous ALX-0081 DP [liquid formulation containing 5 mg/mL of the active pharmaceutical ingredient (API) in a phosphate-based (D-PBS) buffer containing 200 mM glycine and 0.02% Tween-80 (v/v), pH 7.1] and the lyophilized ALX-0081 DP formulation as presented above [formulated at 12.5 mg/mL in 20 mM citrate buffer pH 6.5, 7% sucrose (w/v) and 0.01% Tween-80 (v/v)] with regard to biological activity and target binding:

a) Biacore-based potency assay

b) ELISA-based potency assay

c) Ristocetin Induced Cofactor Activity (RICO) pharmacodynamic biomarker assay

d) Gyrolab-based affinity determination

These assays allowed a side-by-side comparison of the liquid and lyophilized drug product of ALX-0081 (caplacizumab). Predefined comparability criteria were used to evaluate comparability for each assay, and are listed in Table 24.

7.13.2 Methods

a) The Biacore assay is based on the surface plasmon resonance (SPR) technology, and measures avid binding of ALX-0081 to human vWF A1-domain immobilized on a sensor chip. The assay has been selected for potency testing at release and on stability.

b) The ELISA-based potency assay is an orthogonal method for potency testing of ALX-0081 that has been developed for further

characterisation of the target neutralisation capacity of caplacizumab. This assay measures the inhibition of ristocetin-induced binding of von Willebrand Factor (vWF) to bound platelet by caplacizumab.

c) The RICO assay is used as pharmacodynamics marker for the pharmacological activity of caplacizumab. The assay measures the rate and degree to which human lyophilized platelets form aggregates after the addition of the antibiotic ristocetin, which mimics shear-induced activation of vWF.

d) Gyrolab-based assay analyses the kinetic interactions of caplacizumab with its multimeric target vWF and determines the affinity constant of caplacizumab to human multimeric vWF.

Briefly, affinity determination on the Gyroloab platform was established as follows: Gyrolab Bioaffy 1000 CDs were used. As capture tool, 3000 nM in-house biotinylated purified vWF (purified HaemateP using size-exclusion chromatography) was applied on the columns which were prepacked with streptavidin-coated beads. Filter-sterilized D-PBS containing 0.01% Tween-20 was used for dilution of the capture tool. A 1/3 dilution series of purified vWF HaemateP was pre-incubated for 24 hours at RT (+20°C) in a 96-well plate on a rotor at 600 rpm with a fixed concentration of caplacizumab (5 pM) in AD1 buffer (Assay Diluent buffer for dose response curve). After 24 hours, the plate was centrifuged for 1 min at 200g. 70 µL of the pre-incubation mixture, containing the free caplacizumab molecules, was brought into a deep well PCR plate. Then, this mixture was flowed over the column so that free caplacizumab could bind to biotinylated vWF immobilized on the column. The Gyrolab system automatically transferred the mixture in triplicate to the CDs. Free caplacizumab was detected with 50 nM AlexaFluor647-labeled anti-caplacizumab monoclonal antibody diluted in Rexxip F buffer (commercially available detection buffer). Three independent experiments were performed to determine final KD. The fluorochromes were excited by the red laser so

fluorescent signals were obtained and amplified by a photo multiplier tube (PMT). The amplification level of this assay was 1% PMT. An unknown ligand analysis model was used for the KD-determination of caplacizumab. Analysis was performed with the XL fit software of the Gyrolab workstation.

7.13.3 Results

a) The relative potency of the liquid and lyophilized ALX-0081 test samples was measured in a Biacore potency assay, relative to the ALX-0081 reference material used in the potency assay, also designated master reference standard 2 (MRS-2). The relative potency was 102.8% and 102.9%, respectively, indicating full comparability with regard to biological potency determined via Biacore (see Table 24).

b) The relative potency of the liquid and lyophilized ALX-0081 test samples was determined in the ELISA-based potency assay, relative to MRS-2. The relative potency values were 99.4% and 109.5%, respectively, and thus well within the comparability criteria (see Table 24). Therefore, these results indicate that both formulations are comparable with respect to potency determined via ELISA.

c) RICO-activity of the liquid and lyophilized ALX-0081 test samples was measured in a side-by-side comparison, and the concentration to completely block RICO activity (<20%) was determined. The concentration to completely block RICO activity (<20%) was ≤ 0.4 µg/mL for both formulations. These results are well within comparability criteria (see Table 24) and indicate full comparability with respect to pharmacodynamic activity of both formulations.

d) The affinity constant (K_D-value) of the liquid and lyophilized ALX-0081 test samples was also determined in a side-by-side comparison in the Gyrolab-based assay. K_D-values were 6.84 pM and 4.46 pM, respectively, with overlapping confidence intervals. Therefore, these

results indicate full comparability of both formulations with respect to affinity for the multimeric target vWF (see Table 24).

7.13.4 Conclusion

The objective of this study was to evaluate the in vitro comparability of the liquid and lyophilized drug product of ALX-0081 (caplacizumab) by means of four assays, capable of assessing in vitro biological activity and target binding:

a) Biacore-based potency assay

b) ELISA-based potency assay

c) Ristocetin Induced Cofactor Activity (RICO) pharmacodynamic biomarker assay

d) Gyrolab-based affinity determination

All in vitro assays met predefined acceptance criteria and showed that both formulations of ALX-0081 are comparable in terms of biological activity and target binding (see Table 24). The tested liquid and lyophilized ALX-0081 DP formulations showed:

- a similar relative potency determined via Biacore and ELISA assay
- a comparable pharmacodynamic activity in vitro (target neutralization) via RICO assay
- a comparable target affinity via Gyrolab assay.

7.14 General conclusion

The reformulation invention for vWF binders, and especially ALX-0081 described herein rendered a new citrate/sucrose based formulation with improved solubility (up to 80 mg/mL) and significantly improved liquid storage stability (e.g. less oxidation compared to its original formulation). Also, in the lyophilized form, essentially no oxidation or asp-isomerisation could be detected after 12 months storage at +40°C. Further optimization of

the citrate and sucrose concentration resulted in a reduction of the moisture content of the lyophilized product, thereby minimizing the rate of pyroglutamate formation. It was shown that each physicochemical characteristic of the vWF binder was differently influenced by the different constituents, physical as well as chemical, of the formulation, such as buffer choice, pH, concentration, excipient, etc. Various formulations are provided herein optimized for remedying or preventing different chemical and/or physical stresses.

One formulation buffer was designed that met most critical criteria: 20 mM citrate pH 6.5 + 7.0% sucrose (w/v) + 0.01% Tween-80 (v/v). Using this formulation, ALX-0081 was shown to be stable for at least 12 months at -20°C, +5°C, +25°C and +40°C. These data clearly point to a considerably longer shelf-life at +5°C than the current liquid formulation.

In addition the inventors have extensively shown that the contemporaneous formulation of ALX-0081 which has been used in clinical studies to date is comparable to the newly optimized lyophilized ALX-0081 formulation presented herein in terms of *in vitro* biological activity and target binding.

Table A-1: Examples of vWF binders

Name	SEQ ID NO	Sequence
12A02H1-3a-12A02H1 (ALX-0081)	1	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGRELVAAISRTGGSTYYP DSVEGRFTISRDNAKRMVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYTFWGQG TQVTVSSAAAEVQLVESGGGLVQPGGSLRLS CAASGRTFSYNPMGWFRQAPGKGRELVAAI SRTGGSTYYPDSVEGRFTISRDNAKRMVYLQ MNSLRAEDTAVYYCAAAGVRAEDGRVRTLP SEYTFWGQGTQVTVSS
12A02-3a-12A02	2	QVKLEESGGGLVQAGGALRLSCAASGRTFS YNPMGWFRQAPGKERDLVAAISRTGGSTYY PDSVEGRFTISRDNAKRMVYLQMNNLKPED TAVYYCAAAGVRAEDGRVRTLPSEYTFWGQ GTQVTVSSAAAEVQLVESGGGLVQAGGALR LSCAASGRTFSYNPMGWFRQAPGKERDLVA AISRTGGSTYYPDSVEGRFTISRDNAKRMVY LQMNNLKPEDTAVYYCAAAGVRAEDGRVRT LPSEYTFWGQGTQVTVSS

12A02-GS9-12A02	3	QVKLEESGGGLVQAGGALRLSCAASGRTFS YNPMGWFRQAPGKERDLVAAISRTGGSTYY PDSVEGRFTISRDNAKRMVYLQMNNLKPED TAVYYCAAAGVRAEDGRVRTLPSEYTFWGQ GTQVTVSSGGGGSGGGSEVQLVESGGGLVQ AGGALRLSCAASGRTFSYNPMGWFRQAPGK ERDLVAAISRTGGSTYYPDSVEGRFTISRDN AKRMVYLQMNNLKPEDTAVYYCAAAGVRA EDGRVRTLPSEYTFWGQGTQVTVSS
12A02-GS30-12A02	4	QVKLEESGGGLVQAGGALRLSCAASGRTFS YNPMGWFRQAPGKERDLVAAISRTGGSTYY PDSVEGRFTISRDNAKRMVYLQMNNLKPED TAVYYCAAAGVRAEDGRVRTLPSEYTFWGQ GTQVTVSSGGGGSGGGGSGGGGSGGGGSGG GGSGGGGSEVQLVESGGGLVQAGGALRLSC AASGRTFSYNPMGWFRQAPGKERDLVAAIS RTGGSTYYPDSVEGRFTISRDNAKRMVYLQ MNNLKPEGTAVYYCAAAGVRAEDGRVRTLP SEYTFWGQGTQVTVSS
12A05-3a-12A05	5	AVQLVESGGGLVQPGGSLRLSCLASGRIFSIG AMGMYRQAPGKQRELVATITSGGSTNYADP VKGRFTISRDGPKNTVYLQMNSLKPEDTAV YYCYANLKQGSYGYRFNDYWGQGTQVTVSS AAAEVQLVESGGGLVQPGGSLRLSCLASGRI FSIGAMGMYRQAPGKQRELVATITSGGSTNY ADPVKGRFTISRDGPKNTVYLQMNSLKPED TAVYYCYANLKQGSYGYRFNDYWGQGTQV TVSS

12A05-GS9-12A05	6	AVQLVESGGGLVQPGGSLRLSCLASGRIFSIGAMGMYRQAPGKQRELVATITSGGSTNYADPVKGRFTISRDGPKNTVYLQMNSLKPEDTAVYYCYANLKQGSYGYRFNDYWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCLASGRIFSIGAMGMYRQAPGKQRELVATITSGGSTNYADPVKGRFTISRDGPKNTVYLQMNSLKPEDTAVYYCYANLKQGSYGYRFNDYWGQGTQVTVSS
12A05-GS30-12A05	7	AVQLVESGGGLVQPGGSLRLSCLASGRIFSIGAMGMYRQAPGKQRELVATITSGGSTNYADPVKGRFTISRDGPKNTVYLQMNSLKPEDTAVYYCYANLKQGSYGYRFNDYWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCLASGRIFSIGAMGMYRQAPGKQRELVATITSGGSTNYADPVKGRFTISRDGPKNTVYLQMNSLKPEDTAVYYCYANLKQGSYGYRFNDYWGQGTQVTVSSS
12B06-3a-12B06	8	QVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSSAAAEVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSS

12B06-GS9-12B06	9	QVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSSGGGGSGGGSEVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSS
12B06-GS30-12B06	10	QVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQAGGALRLSCAASGRTFSYNPMGWFRQAPGKERDVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNALKPEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSS
12A02H4-3a-12A02H4	11	EVQLVESGGGLVQPGGSLRLSCAASGRTFSYNPMGWFRQAPGKGRELVAAISRTGGSTYYPDSVEGRFTISRDNAKRSVYLQMNSLRAEDTAVYYCAAAGVRAEDGRVRTLPSEYTFWGQGTQVTVSSAAAEVQLVESGGGLVQPGGSLRLSCAASGRTFSYNPMGWFRQAPGKGRELVAAISRTGGSTYYPDSVEGRFTISRDNAKRSVYLQMNSLRAEDTAVYYCAAAGVRAEDGRVRTLPSEYTFWGQGTQVTVSS

12B06H2-3a-12B06H2	12	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGREVVAAISRTGGSTYYA RSVEGRFTISRDNAKRMVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYNFWGQG TQVTVSSAAAEVQLVESGGGLVQPGGSLRLS CAASGRTFSYNPMGWFRQAPGKGREVVAAI SRTGGSTYYARSVEGRFTISRDNAKRMVYLQ MNSLRAEDTAVYYCAAAGVRAEDGRVRTLP SEYNFWGQGTQVTVSS
12A02H1-GS9-12A02H1	13	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGRELVAAISRTGGSTYYP DSVEGRFTISRDNAKRMVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYTFWGQG TQVTVSSGGGGSGGGSEVQLVESGGGLVQP GGSLRLSCAASGRTFSYNPMGWFRQAPGKG RELVAAISRTGGSTYYPDSVEGRFTISRDNA KRMVYLQMNSLRAEDTAVYYCAAAGVRAE DGRVRTLPSEYTFWGQGTQVTVSS
12A02H4-GS9-12A02H4	14	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGRELVAAISRTGGSTYYP DSVEGRFTISRDNAKRSVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYTFWGQG TQVTVSSGGGGSGGGSEVQLVESGGGLVQP GGSLRLSCAASGRTFSYNPMGWFRQAPGKG RELVAAISRTGGSTYYPDSVEGRFTISRDNA KRSVYLQMNSLRAEDTAVYYCAAAGVRAED GRVRTLPSEYTFWGQGTQVTVSS

12B06H2-GS9-12B06H2	15	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGREVVAAISRTGGSTYYA RSVEGRFTISRDNAKRMVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYNFWGQG TQVTVSSGGGGSGGGSEVQLVESGGGLVQP GGSLRLSCAASGRTFSYNPMGWFRQAPGKG REVVAAISRTGGSTYYARSVEGRFTISRDNA KRMVYLQMNSLRAEDTAVYYCAAAGVRAE DGRVRTLPSEYNFWGQGTQVTVSS
12A02H1-GS30-12A02H1	16	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGRELVAAISRTGGSTYYP DSVEGRFTISRDNAKRMVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYTFWGQG TQVTVSSGGGGSGGGGSGGGGSGGGGSGGG GSGGGGSEVQLVESGGGLVQPGGSLRLSCA ASGRTFSYNPMGWFRQAPGKGRELVAAISR TGGSTYYPDSVEGRFTISRDNAKRMVYLQM NSLRAEDTAVYYCAAAGVRAEDGRVRTLPS EYTFWGQGTQVTVSS
12A02H4-GS30-12A02H4	17	EVQLVESGGGLVQPGGSLRLSCAASGRTFSY NPMGWFRQAPGKGRELVAAISRTGGSTYYP DSVEGRFTISRDNAKRSVYLQMNSLRAEDT AVYYCAAAGVRAEDGRVRTLPSEYTFWGQG TQVTVSSGGGGSGGGGSGGGGSGGGGSGGG GSGGGGSEVQLVESGGGLVQPGGSLRLSCA ASGRTFSYNPMGWFRQAPGKGRELVAAISR TGGSTYYPDSVEGRFTISRDNAKRSVYLQMN SLRAEDTAVYYCAAAGVRAEDGRVRTLPSEY TFWGQGTQVTVSS

12B06H2-GS30-12B06H2	18	EVQLVESGGGLVQPGGSLRLSCAASGRTFSYNPMGWFRQAPGKGREVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNSLRAEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGRTFSYNPMGWFRQAPGKGREVVAAISRTGGSTYYARSVEGRFTISRDNAKRMVYLQMNSLRAEDTAVYYCAAAGVRAEDGRVRTLPSEYNFWGQGTQVTVSS
12A02H1	19	EVQLVESGGGLVQPGGSLRLSCAASGRTFSYNPMGWFRQAPGKGRELVAAISRTGGSTYYPDSVEGRFTISRDNAKRMVYLQMNSLRAEDTAVYYCAAAGVRAEDGRVRTLPSEYTFWGQGTQVTVSS

Table A-2

Name	SEQ ID NO	Sequence
Human vWF	20	MIPARFAGVLLALALILPGTLCAEGTRGRSSTARCS LFGSDFVNTFDGSMYSFAGYCSYLLAGGCQKRSF SIIGDFQNGKRVSLSVYLGEFFDIHLFVNGTVTQG DQRVSMPYASKGLYLETEAGYYKLSGEAYGFVARI DGSGNFQVLLSDRYFNKTCGLCGNFNIFAEDDFM TQEGTLTSDPYDFANSWALSSGEQWCERASPPSS SCNISSGEMQKGLWEQCQLLKSTSVFARCHPLVD PEPFVALCEKTLCECAGGLECACPALLEYARTCAQ EGMVLYGWTDHSACSPVCPAGMEYRQCVSPCAR TCQSLHINEMCQERCVDGCSCPEGQLLDEGLCVE STECPCVHSGKRYPPGTSLSRDCNTCICRNSQWIC SNEECPGECLVTGQSHFKSFDNRYFTFSGICQYLL ARDCQDHSFSIVIETVQCADDRDAVCTRSVTVRLP GLHNSLVKLKHGAGVAMDGQDIQLPLLKGDLRIQ HTVTASVRLSYGEDLQMDWDGRGRLLVKLSPVYA GKTCGLCGNYNGNQGDDFLTPSGLAEPRVEDFG NAWKLHGDCQDLQKQHSDPCALNPRMTRFSEEA CAVLTSPTFEACHRAVSPLPYLRNCRYDVCSCSDG RECLCGALASYAAACAGRGVRVAWREPGRCELNC PKGQVYLQCGTPCNLTCRSLSYPDEECNEACLEG CFCPPGLYMDERGDCVPKAQCPCYYDGEIFQPEDI FSDHHTMCYCEDGFMHCTMSGVPGSLLPDAVLSS PLSHRSKRSLSCRPPMVKLVCPADNLRAEGLECT KTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV ALERCPCFHQGKEYAPGETVKIGCNTCVCRDRKW NCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECQ

		YVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKR VTILVEGGEIELFDGEVNVKRPMKDETHFEVVES GRYIILLLGKALSVVWDRHLSISVVLKQTYQEKVC GLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKV SSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRI LTSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDC ACFCDTIAAYAHVCAQHGKVVTWRTATLCPQSCE ERNLRENGYECEWRYNSCAPACQVTCQHPEPLAC PVQCVEGCHAHCPPGKILDELLQTCVDPEDCPVC EVAGRRFASGKKVTLNPSDPEHCQICHCDVVNLT CEACQEPGGLVVPPTDAPVSPTTLYVEDISEPPLH DFYCSRLLDLVFLLDGSSRLSEAEFEVLKAFVVDM MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRP SELRRIASQVKYAGSQVASTSEVLKYTLFQIFSKID RPEASRIALLLMASQEPQRMSRNFVRYVQGLKKK KVIVIPVGIGPHANLKQIRLIEKQAPENKAFVLSSV DELEQQRDEIVSYLCDLAPEAPPPTLPPHMAQVTV GPGLRNSMVLDVAFVLEGSDKIGEADFNRSKEFM EEVIQRMDVGQDSIHVTVLQYSYMVTVEYPFSEA QSKGDILQRVREIRYQGGNRTNTGLALRYLSDHSF LVSQGDREQAPNLVYMVTGNPASDEIKRLPGDIQ VVPIGVGPNANVQELERIGWPNAPILIQDFETLPR EAPDLVLQRCCSGEGLQIPTLSPAPDCSQPLDVILL LDGSSSFPASYFDEMKSFAKAFISKANIGPRLTQV SVLQYGSITTIDVPWNVVPEKAHLLSLVDVMQRE GGPSQIGDALGFAVRYLTSEMHGARPGASKAVVIL VTDVSVDSVDAAADAARSNRVTVFPIGIGDRYDAA QLRILAGPAGDSNVVKLQRIEDLPTMVTLGNSFLH KLCSGFVRICMDEDGNEKRPGDVWTLPDQCHTV TCQPDGQTLLKSHRVNCDRGLRPSCPNSQSPVKV

		EETCGCRWTCPCVCTGSSTRHIVTFDGQNFKLTG SCSYVLFQNKEQDLEVILHNGACSPGARQGCMKS IEVKHSALSVELHSDMEVTVNGRLVSVPYVGGNM EVNVYGAIMHEVRFNHLGHIFTFTPQNNEFQLQL SPKTFASKTYGLCGICDENGANDFMLRDGTVTTD WKTLVQEWTVQRPGQTCQPILEEQCLVPDSSHCQ VLLLPLFAECHKVLAPATFYAICQQDSCHQEQVCE VIASYAHLCRTNGVCVDWRTPDFCAMSCPPSLVY NHCEHGCPRHCDGNVSSCGDHPSEGCFCPPDKV MLEGSCVPEEACTQCIGEDGVQHQFLEAWVPDH QPCQICTCLSGRKVNCTTQPCPTAKAPTCGLCEVA RLRQNADQCCPEYECVCDPVSCDLPPVPHCERGL QPTLTNPGECRPNFTCACRKEECKRVSPPSCPPH RLPTLRKTQCCDEYECACNCVNSTVSCPLGYLAST ATNDCGCTTTTCLPDKVCVHRSTIYPVGQFWEEG CDVCTCTDMEDAVMGLRVAQCSQKPCEDSCRSG FTYVLHEGECCGRCLPSACEVVTGSPRGDSQSSW KSVGSQWASPENPCLINECVRVKEEVFIQQRNVSC PQLEVPVCPSGFQLSCKTSACCPSCRCERMEACM LNGTVIGPGKTVMIDVCTTCRCMVQVGVISGFKLE CRKTTCNPCPLGYKEENNTGECCGRCLPTACTIQ LRGGQIMTLKRDETLQDGCDTHFCKVNERGEYF WEKRVTGCPPFDEHKCLAEGGKIMKIPGTCCDTC EEPECNDITARLQYVKVGSCKSEVEVDIHYCQGK CASKAMYSIDINDVQDQCSCCSPTRTEPMQVALH CTNGSVVYHEVLNAMECKCSPRKCSK

Table 1. Overview of 50 different buffer/excipient combinations predicted by the Design Expert program to yield the highest melting temperatures for ALX-0081. The buffer/excipient combinations are ranked according to the Tm value. Different buffer types are shown in different shadings of grey.

Run	Buffer		Excipient 1		Glycine	NaCl	Tm
	Name	Conc (mM)	Name	Conc (mM)	Conc (mM)	Conc (mM)	°C
1	Phosphate pH 6.92	17.24	Trehalose	239.25	0.00	0.00	77.2772
2	Phosphate pH 6.98	16.29	Trehalose	242.04	0.00	0.00	77.2742
3	Phosphate pH 6.95	9.47	Trehalose	262.12	0.00	0.00	77.2641
4	Phosphate pH 7.50	9.47	Mannitol	0.00	273.04	0.00	77.2096
5	Phosphate pH 7.50	25.19	Mannitol	0.00	224.82	0.00	77.1483
6	Phosphate pH 7.50	9.47	Sucrose	0.00	273.04	0.00	77.1399
7	Phosphate pH 7.50	28.79	Mannitol	0.00	213.78	0.00	77.1262
8	Phosphate pH 7.50	30.88	Mannitol	0.00	207.39	0.00	77.1117
9	Phosphate pH 7.50	32.96	Mannitol	0.00	201.00	0.00	77.0953
10	Citrate pH 6.23	48.16	Trehalose	162.85	0.00	0.00	77.0307
11	Citrate pH 6.22	48.38	Trehalose	162.29	0.00	0.00	77.0307
12	Phosphate pH 6.87	19.89	Mannitol	231.44	0.00	0.00	76.9832

Run	Buffer		Excipient 1		Glycine	NaCl	Tm
	Name	Conc (mM)	Name	Conc (mM)	Conc (mM)	Conc (mM)	°C
13	Phosphate pH 7.50	9.47	Trehalose	0.00	273.04	0.00	76.9483
14	Citrate pH 7.00	60.84	Sucrose	129.38	0.00	0.00	76.9338
15	Citrate pH 7.00	57.67	Sucrose	137.75	0.00	0.00	76.9312
16	Phosphate pH 7.50	50.01	Mannitol	0.00	148.72	0.00	76.9295
17	Citrate pH 7.00	84.92	Sucrose	65.25	0.00	0.00	76.7979
18	Phosphate pH 7.06	36.18	Sucrose	183.48	0.00	0.00	76.7972
19	Citrate pH 6.44	10.56	Trehalose	262.12	0.00	0.00	76.7449
20	Citrate pH 7.00	77.11	Mannitol	0.00	90.04	0.00	76.7297
21	Citrate pH 7.00	75.42	Mannitol	0.00	94.69	0.00	76.7291
22	Citrate pH 7.00	79.01	Mannitol	81.42	0.00	0.00	76.6192
23	Citrate pH 6.17	53.45	Mannitol	146.67	2.90	0.00	76.5956
24	Citrate pH 6.18	53.23	Mannitol	149.46	0.00	0.00	76.5955
25	Citrate pH 6.18	53.23	Mannitol	149.46	0.00	0.00	76.5955
26	Citrate pH 6.16	53.66	Mannitol	148.35	0.00	0.00	76.5955
27	Tris pH 7.77	17.13	Trehalose	134.96	0.00	69.29	76.1017
28	Tris pH 8.00	89.75	Mannitol	0.00	15.10	70.51	76.0374

Run	Buffer		Excipient 1		Glycine	NaCl	Tm
	Name	Conc (mM)	Name	Conc (mM)	Conc (mM)	Conc (mM)	°C
29	Tris pH 8.00	92.83	Mannitol	0.00	16.27	67.17	76.0343
30	Tris pH 8.00	93.86	Mannitol	0.00	0.00	74.76	76.0322
31	Tris pH 7.83	17.13	Sucrose	142.77	0.00	65.04	76.0321
32	Tris pH 7.82	17.13	Sucrose	142.21	0.00	65.34	76.0321
33	Tris pH 8.00	82.56	Mannitol	0.00	31.37	68.38	76.0298
34	Tris pH 8.00	97.63	Mannitol	0.00	0.00	71.42	76.0285
35	Tris pH 8.00	95.23	Sucrose	0.00	0.00	73.55	75.9714
36	Tris pH 8.00	97.63	Sucrose	0.00	0.00	71.42	75.97
37	Tris pH 7.77	17.13	Mannitol	121.58	0.00	76.59	75.5801
38	Tris pH 8.00	61.32	Trehalose	0.00	53.45	75.37	75.3047
39	Tris pH 8.00	67.14	Trehalose	0.00	47.06	73.85	75.3027
40	Tris pH 7.81	17.13	Trehalose	0.00	140.00	69.60	75.2196
41	Histidine pH 6.50	20.03	Sucrose	0.00	142.33	68.38	74.9111
42	Histidine pH 6.50	20.03	Sucrose	0.00	136.52	71.42	74.91
43	Histidine pH 6.50	20.03	Sucrose	0.00	124.90	77.50	74.9012
44	Histidine pH 6.50	20.03	Mannitol	0.00	127.22	76.28	74.8582

Run	Buffer		Excipient 1		Glycine	NaCl	Tm
	Name	Conc (mM)	Name	Conc (mM)	Conc (mM)	Conc (mM)	°C
45	Histidine pH 6.50	20.03	Mannitol	0.00	131.29	74.16	74.8576
46	Histidine pH 6.50	20.03	Mannitol	0.00	118.51	80.84	74.8558
47	Histidine pH 6.50	20.03	Mannitol	0.00	136.52	71.42	74.8553
48	Histidine pH 6.50	20.83	Mannitol	0.00	109.21	85.10	74.8397
49	Histidine pH 6.49	20.03	Mannitol	0.00	146.98	65.95	74.8281
50	Histidine pH 6.50	20.03	Trehalose	0.00	144.07	67.47	74.4053

Table 2. Solubility testing of ALX-0081 in different formulation buffers.

formulation	pH	visual	measured conc. (mg/mL)	recovery (%)
D-PBS + 200 mM glycine	7.4	turbid + small particles	8.1	88.6
10 mM phosphate + 200 mM glycine	7.4	turbid + small particles	8.7	88.3
20 mM phosphate	7.4	turbid + particles	4.9	96.4
20 mM histidine	6.5	turbid + particles	<3.4	N.D.
20 mM citrate	7.0	clear	55.9	97.6

N.D. = not determined

Table 3. Overview of liquid ALX-0081 formulations evaluated in a storage and FT stability trial, together with measured pH and osmolality values.

formulation no.	conc. (mg/mL)	buffer		excipient		Tween-80 (v/v)	pH measured	osmolality mOsm/kg
		type	pH	strength	type			
1	20	50 mM citrate	6.0	75 mM	NaCl	0.01%	5.9	281
2				2.0%	mannitol		6.0	253
3				4.0%	sucrose		6.0	272
4				140 mM	glycine		6.0	273
5			6.5	75 mM	NaCl		6.5	288
6				2.0%	mannitol		6.5	266
7				4.0%	sucrose		6.6	280
8				140 mM	glycine		6.6	280
9			7.0	75 mM	NaCl		6.9	279
10				2.0%	mannitol		7.0	259
11				4.0%	sucrose		7.1	271
12				140 mM	glycine		7.0	278
13	5			75 mM	NaCl		6.9	274
14				2.0%	mannitol		7.0	254
15				4.0%	sucrose		7.0	267
16				140 mM	glycine		7.0	274
17		D-PBS	7.1	137/200 mM	NaCl/glycine		7.2	470

Table 4. Overview of the storage stability study of different ALX-0081 formulations. Time points, storage temperatures and methods are indicated.

time point	temperature		formulation no. as indicated in Table 3	methods		
	-70°C	+40°C		RP-HPLC	cIEF	SE-HPLC
1 week	X	X	1-17	X		
2 weeks	X	X	1-17	X		
1 month	X	X	1-17	X	X	X

Table 5. Storage stability data for the different liquid ALX-0081 formulations. The relative surface areas of the most relevant RP-HPLC peaks after 1 month storage at +40°C are shown. Pyro = pyroglutamate, main = main peak (including the shoulder peak, when present), oxidation = pre-peaks collectively. Color coding indicates the relative sample purity: highest purity in white, intermediate purity in grey, and lowest purity in black. Recovery was ±100% for all samples.

formulation	conc.	buffer		excipient		Tween-	% peak area RP-HPLC		
no.	(mg/mL)	type	pH	strength	type	80 (v/v)	oxidation	main	pyro
1	20	50 mM citrate	6.0	75 mM	NaCl	0.01%	1.5	88.6	3.7
2				2.0%	mannitol		1.7	88.1	4.3
3				4.0%	sucrose		1.7	88.1	4.6
4				140 mM	glycine		2.2	88.3	3.9
5			6.5	75 mM	NaCl		2.5	88.5	3.6
6				2.0%	mannitol		2.7	87.5	4.5
7				4.0%	sucrose		2.3	88.0	4.2
8				140 mM	glycine			85.2	4.3
9			7.0	75 mM	NaCl		2.4	86.8	
10				2.0%	mannitol		2.6	86.0	
11				4.0%	sucrose		2.8	85.9	6.1
12				140 mM	glycine				6.2
13	5			75 mM	NaCl		2.3	86.8	
14				2.0%	mannitol		2.4	86.5	

formulation no.	conc. (mg/mL)	buffer		excipient		Tween-80 (v/v)	% peak area RP-HPLC		
		type	pH	strength	type		oxidation	main	pyro
15				4.0%	sucrose		2.1	87.7	
16				140 mM	glycine				6.4
17		D-PBS	7.1	137/200 mM	NaCl/ glycine		9.0	75.2	6.4

Table 6. Overview of lyophilized/liquid ALX-0081 formulations evaluated in a storage stability trial.

formulation no.	conc. (mg/mL)	buffer		excipient		Tween-80 (v/v)
		type	pH	strength	type	
3	20	50 mM citrate	6.0	4.0% (w/v)	sucrose	0.01%
7			6.5	4.0% (w/v)	sucrose	
17	5	D-PBS	7.1	137/200 mM	NaCl/glycine	

Table 7. Recovery of ALX-0081 in different formulations after lyophilization and reconstitution based on total areas reported by RP-HPLC and SE-HPLC.

recovery after lyophilization/ reconstitution (%)	**citrate pH 6.0 + sucrose (formulation 3)**	**citrate pH 6.5 + sucrose (formulation 7)**	**D-PBS + glycine (formulation 17)**
RP-HPLC	104.3	105.4	103.2
SE-HPLC	101.3	99.6	102.8

Table 8. Overview of storage stability data of the different lyophilized ALX-0081 formulations (1.5 months storage at -20°C, +5°C, +25C and +40°C). The color code represents a qualitative assessment of the sample stability, ranging from white (highest stability) over shades of grey to black (lowest stability).

N.T. = not tested. (* compared to liquid control sample kept at -70°C)

1.5 months storage		**citrate pH 6.0 + sucrose (formulation 3)**				**citrate pH 6.5 + sucrose (formulation 7)**				**D-PBS + glycine (formulation 17)**			
		-20°C	**+5°C**	**+25°C**	**+40°C**	**-20°C**	**+5°C**	**+25°C**	**+40°C**	**-20°C**	**+5°C**	**+25°C**	**+40°C**
visual		cake not affected by storage + reconstitution with Milli-Q water renders clear solution in all samples											
content	**conc. (mg/mL)**	N.T.	N.T.	N.T.	21.1	N.T.	N.T.	N.T.	21.2	N.T.	N.T.	N.T.	4.89
	recovery* (%)	N.T.	N.T.	N.T.	106	N.T.	N.T.	N.T.	105	N.T.	N.T.	N.T.	101
pH		N.T.	N.T.	N.T.	6.1	N.T.	N.T.	N.T.	6.6	N.T.	N.T.	N.T.	7.0
osmolality		N.T.	N.T.	N.T.	289	N.T.	N.T.	N.T.	295	N.T.	N.T.	N.T.	487

(mOsm/kg)													
RP-HPLC	area % main peak	92.9	92.9	92.3	89.7	93.0	92.9	92.8	91.4	92.3	92.1		65.8
	area % pre peaks	2.4	2.2	2.2	2.5	2.3	2.3	2.2	2.4	2.8	3.0		16.1
	area % pyro	0.9	1.0	1.6		0.8	0.9	1.2	2.2	1.0	1.1		12.6
	recovery* (%)	104	102	113	100	105	102	103	102	103	105	112	96.5
cIEF	area % pyro	N.T.	N.T.	N.T.		N.T.	N.T.	N.T.	1.1	N.T.	N.T.	N.T.	12.7
SE-HPLC	area % main peak	99.9	99.9	99.9	99.8	99.9	99.9	99.9	99.9	99.9	100	99.9	95.8
	area %	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	4.2

	pre peaks (HMWs)												
	recovery* (%)	101	98.4	108	100	99.7	95.0	96.2	99.0	103	98.1	101	89.6

Table 9. Overview of ALX-0081 formulations evaluated in a storage stability trial.

conc. (mg/mL)	lyo cycle time (hours)	citrate buffer		sucrose	Tween-80 (v/v)
		strength (mM)	pH		
20	±65	50	6.5	4.0%	5
		32		5.5%	
		15		7.0%	
	±37	50		4.0%	
		32		5.5%	0.01%
		15		7.0%	

Table 10. Moisture content of lyophilized ALX-0081 samples and relative amounts of pyroglutamate detected on RP-HPLC after 4 weeks storage at +40°C.

conc. (mg/mL)	lyo cycle time (hours)	citrate buffer		sucrose	Tween-80 (v/v)	moisture content	RP-HPLC
		Strength (mM)	pH				pyro
20	±65	50	6.5	4.0%	0.01%	4.87%	1.7%
		32		5.5%		2.32%	1.4%
		15		7.0%		1.27%	1.2%
	±37	50		4.0%		4.40%	1.6%
		32		5.5%		NA	1.3%
		15		7.0%		2.43%	1.1%

NA= not available

Table 11. Results of visual inspection of ALX-0081 formulation during storage at +5°C and +25°C. " +" = clear, "+/-" = slightly turbid, "-" = hazy "h" = hour, "d" = days.

Formulation				storage at +5°C					storage at +25°C				
conc. (mg/mL)	citrate pH 6.5 (mM)	sucrose w/v (%)	Tween-80 v/v (%)	1h	2h	19h	24h	4d	1h	2h	19h	24h	4d
28	15	----	----	-	-	-	-	-	+	+	+	+	+
	20	----	----	+	+	+	-	-	+	+	+	+	+
	25	----	----	+	+	+	-	-	+	+	+	+	+
	30	----	----	+	+	+	-	-	+	+	+	+	+
	40	----	----	+	+	+	-	-	+	+	+	+	+
	50	----	----	+	+	+	+	+	+	+	+	+	+
20	15	5.0	----	+	+	+	+	+/-	+	+	+	+	+
	15	6.0	----	+	+	+	+	+	+	+	+	+	+
	15	7.0	----	+	+	+	+	+	+	+	+	+	+
	15	5.0	0.01	+	+	+	+	+/-	+	+	+	+	+
	15	6.0	0.01	+	+	+	+	+	+	+	+	+	+

Formulation				storage at +5°C					storage at +25°C				
conc. (mg/mL)	citrate pH 6.5 (mM)	sucrose w/v (%)	Tween-80 v/v (%)	1h	2h	19h	24h	4d	1h	2h	19h	24h	4d
	15	7.0	0.01	+	+	+	+	+	+	+	+	+	+

Table 12. Results of visual inspection, content and SE-HPLC analysis of ALX-0081 formulations after 5 consecutive FT cycles at -20°C (*compared to liquid control sample kept at ≤-70°C).

Formulation				5 FT cycles at -20°C			
conc. (mg/mL)	citrate pH 6.5 (mM)	sucrose w/v (%)	Tween-80 (v/v) (%)	visual	content	SE-HPLC	
					recovery (%)*	profile	recovery (%)*
16	20	5.0	0.01	clear	100	no effect	105.3
		6.0			102		99.4
		7.0			99.0		103.4
	25	5.0			101		103.9
		6.0			102		98.5
		7.0			100		98.4
	30	5.0			97.2		98.8
		6.0			103		99.3
		7.0			97.5		102.8

Table 13. Results of visual inspection, recovery and osmolality measurements of ALX-0081 formulations after 5 consecutive FT cycles at -20°C or after 1 FT cycle + 24h storage + 1 FT cycle (*compared to liquid control sample kept at ≤-70°C).

Formulation				5 FT cycles at -20°C			1 FT cycle + 24h at 25°C + 1 FT cycle		
conc. (mg/mL)	citrate pH 6.5 (mM)	sucrose w/v (%)	Tween-80 (v/v) (%)	visual	recovery (%)*	osmolality (mOsm/kg)	visual	recovery (%)	osmolality (mOsm/kg)
20	20	5.0	0.01	clear	103	236	clear	101	236
		6.0			104	273		101	271
		7.0			103	304		99.4	306

Table 14. Lyophilization parameters.

Step No	Description	Temperature (°C)	Pressure	Time (hh:mm)
1	Load	20	Atmospheric	N.A
2	Freezing	20 → -50	Atmospheric	02:00
3	Freezing	-50	Atmospheric	02:00
4	Evacuation	-50	0.130 mbar	00:10
5	Primary drying	-50 → -20	0.130 mbar	1:00
6	Primary drying	-20	0.130 mbar	19:00
7	Primary drying	-20 → 5	0.130 mbar	00:50
8	Primary drying	5	0.130 mbar	05:00
7	Primary drying	5 → 25	0.130 mbar	03:00
7	Secondary drying	25	0.130 mbar	33:00
9	Pre-Aeration with nitrogen	15	0.8 bar	N.A.
10	Stoppering	15	0.8 bar	N.A.
11	Aeration with nitrogen	15	Atmospheric	N.A.
	Total length (without stoppering)			66:00

Table 15. RP-HPLC analysis main peak (purity) ALX-0081 formulation [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Time point (months)	Storage condition	Avg. purity (% area main peak)
Initial	-	93.3
1	-20°C	93.0
	+5°C	93.0
	+25°C/60%RH	93.0
	+40°C/75%RH	92.7
3	-20°C	93.3
	+5°C	93.3
	+25°C/60%RH	93.1
	+40°C/75%RH	92.6
6	-20°C	93.2
	+5°C	93.3
	+25°C/60%RH	93.0
	+40°C/75%RH	92.4
9	-20°C	93.4
	+5°C	93.3
	+25°C/60%RH	93.1
	+40°C/75%RH	91.8
12	-20°C	93.2

Time point (months)	Storage condition	Avg. purity (% area main peak)
	+5°C	93.1
	+25°C/60%RH	92.8
	+40°C/75%RH	91.3

Table 16. RPC pre en post peak analysis of ALX-0081 formulation [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Time point (month)	Storage Condition	Replicate	Pre-peaks (% area)				Post-peaks (% area)				
			3	2	1	Avg. 1+2+3	Avg .1	Avg. 2	Avg. 3	Avg. 4	Avg. 3+4
Initial	-	01	0.07	0.47	1.86	**2.34**	**3.54**	**0.70**	0.09	0.19	**0.19**
		02	0.08	0.47	1.88						
1	**-20°C**	01	0.09	0.58	1.78	**2.37**	**3.67**	**0.85**	0.11	0.21	**0.11**
		02	0.09	0.60	1.77						
	+5°C	01	0.09	0.60	1.79	**2.39**	**3.64**	**0.84**	0.11	0.22	**0.11**
		02	0.09	0.59	1.80						
	+25°C/60%RH	01	0.09	0.60	1.77	**2.40**	**3.66**	**0.91**	0.11	0.22	**0.11**
		02	0.08	0.60	1.83						
	+40°C/75%RH	01	0.09	0.56	1.88	**2.45**	**3.68**	**1.11**	0.11	0.23	**0.11**
		02	0.09	0.63	1.83						
3	**-20°C**	01	0.09	0.51	1.91	**2.37**	**3.45**	**0.72**	0.09	0.26	**0.26**
		02	0.09	0.49	1.82						

Time point (month)	Storage Condition	Replicate	Pre-peaks (% area)				Post-peaks (% area)				
			3	2	1	Avg. 1+2+3	Avg .1	Avg. 2	Avg. 3	Avg. 4	Avg. 3+4
	+5°C	01	0.09	0.51	1.83	**2.35**	**3.41**	**0.73**	0.09	0.28	**0.28**
		02	0.09	0.50	1.85						
	+25°C/60%RH	01	0.09	0.49	1.86	**2.38**	**3.47**	**0.84**	0.09	0.27	**0.27**
		02	0.09	0.53	1.87						
	+40°C/75%RH	01	0.09	0.53	1.89	**2.41**	**3.44**	**1.32**	0.09	0.31	**0.31**
		02	0.09	0.51	1.88						
6	-20°C	01	0.07	0.52	1.85	**2.40**	**3.49**	**0.71**	0.08	0.21	**0.21**
		02	0.07	0.54	1.89						
	+5°C	01	0.06	0.53	1.79	**2.33**	**3.47**	**0.72**	0.09	0.22	**0.22**
		02	0.07	0.54	1.80						
	+25°C/60%RH	01	0.07	0.54	1.82	**2.40**	**3.50**	**0.93**	0.09	0.22	**0.22**
		02	0.06	0.53	1.91						
	+40°C/75%RH	01	0.07	0.54	1.84	**2.43**	**3.47**	**1.62**	0.11	0.21	**0.32**
		02	0.07	0.55	1.92						

Time point (month)	Storage Condition	Replicate	Pre-peaks (% area)				Post-peaks (% area)				
			3	2	1	Avg. 1+2+3	Avg .1	Avg. 2	Avg. 3	Avg. 4	Avg. 3+4
9	-20°C	01	0.07	0.47	1.83	**2.30**	**3.43**	**0.70**	0.09	0.22	**0.22**
		02	0.07	0.47	1.83						
	+5°C	01	0.07	0.45	1.87	**2.31**	**3.45**	**0.69**	0.09	0.22	**0.22**
		02	0.07	0.47	1.83						
	+25°C/60%RH	01	0.08	0.50	1.89	**2.44**	**3.42**	**0.73**	0.11	0.21	**0.32**
		02	0.07	0.51	1.97						
	+40°C/75%RH	01	0.07	0.49	1.84	**2.32**	**3.46**	**2.07**	0.13	0.24	**0.37**
		02	0.08	0.47	1.84						
12	-20°C	01	0.07	0.50	1.66	**216**	**3.64**	**0.70**	0.10	0.25	**0.35**
		02	0.07	0.49	1.66						
	+5°C	01	0.08	0.47	1.69	**2.19**	**3.64**	**0.74**	0.11	0.25	**0.36**
		02	0.06	0.49	1.72						
	+25°C/60%RH	01	0.08	0.48	1.74	**2.21**	**3.55**	**1.07**	0.12	0.26	**0.38**
		02	0.09	0.47	1.73						
	+40°C/75%RH	01	0.09	0.46	1.78	**2.26**	**3.63**	**2.37**	0.16	0.29	**0.44**

Time point (month)	Storage Condition	Replicate	Pre-peaks (% area)				Post-peaks (% area)				
			3	2	1	Avg. 1+2+3	Avg .1	Avg. 2	Avg. 3	Avg. 4	Avg. 3+4
		02	0.09	0.48	1.79						

Table 17. Protein concentration results by UV for ALX-0081 formulation [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Time point (months)	Storage Condition	Avg Conc of diluted sample (mg/mL)	Avg Conc Corrected by dilution factor (mg/vial)
Initial	-	0.534	13.4
1	-20°C	0.532	13.3
	+5°C	0.530	13.3
	+25°C/60%RH	0.525	13.1
	+40°C/75%RH	0.516	12.9
3	-20°C	0.501	12.5
	+5°C	0.524	13.1
	+25°C/60%RH	0.530	13.3
	+40°C/75%RH	0.534	13.4
6	-20°C	0.530	13.3
	+5°C	0.528	13.2
	+25°C/60%RH	0.531	13.3
	+40°C/75%RH	0.523	13.1
9	-20°C	0.505	12.6
	+5°C	0.504	12.6
	+25°C/60%RH	0.511	12.8
	+40°C/75%RH	0.519	13.0
12	-20°C	0.504	12.6
	+5°C	0.505	12.6
	+25°C/60%RH	0.497	12.4
	+40°C/75%RH	0.510	12.7

Table 18. SE-HPLC analysis of ALX-0081 formulation [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Time point (months)	**Storage condition**	**Avg. Pre Peak** (% Area)	**Avg. Main Peak (% Area)**
Initial	-	0.55	99.5
1	**-20°C**	0.51	99.5
	+5°C	0.53	99.5
	+25°C/60%RH	0.55	99.4
	+40°C/75%RH	0.56	99.5
3	**-20°C**	0.47	99.6
	+5°C	0.47	99.5
	+25°C/60%RH	0.47	99.5
	+40°C/75%RH	0.48	99.5
6	**-20°C**	0.60	99.4
	+5°C	0.63	99.4
	+25°C/60%RH	0.65	99.4
	+40°C/75%RH	0.68	99.3
12	**-20°C**	0.66	99.4
	+5°C	0.68	99.3
	+25°C/60%RH	0.67	99.3
	+40°C/75%RH	0.71	99.3

Table 19. Results physical tests on lyophilized ALX-0081 stored at -20°C [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Test	Unit	initial	1M	3M	6M	9M	12M
Appearance of the lyophilisate	-	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles
Appearance of the reconstituted solution	-	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles
Reconstitution time*	seconds	50	60	50	48	41	41
Osmolality	mOsm/kg	298	298	297	280	-	296
pH of the reconstituted solution	-	6.8	6.6	6.8	6.7	-	6.6
Moisture	% w/w	0.65	0.72	0.83	0.74	0.62	0.63

Test	Unit	initial	1M	3M	6M	9M	12M
content							
Subvisible particles by HIAC	particles/mL	105 ø ≥ 10µm;	73 ø ≥ 10µm;	79 ø ≥ 10µm;	50 ø ≥ 10µm;	-	-
	particles/mL	3 ø ≥ 25µm	3 ø ≥ 25µm	7 ø ≥ 25µm	4 ø ≥ 25µm	-	-

* (reconstitute with 1 mL of WFI)

Table 20. Results physical tests on lyophilized ALX-0081 stored at +5°C [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Test	Unit	initial	1M	3M	6M	9M	12M
Appearance of the lyophilisate	-	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles
Appearance of the reconstituted solution	-	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles
Reconstitution time*	seconds	50	60	55	50	43	43
Osmolality	mOsm/kg	298	298	294	279	-	293
pH of the reconstituted solution	-	6.8	6.6	6.8	6.7	-	6.6

Test	Unit	initial	1M	3M	6M	9M	12M
Moisture content	% w/w	0.65	0.76	0.72	0.72	0.80	0.68
Subvisible particles by HIAC	particles/ mL	105 ø ≥ 10µm;	88 ø ≥ 10µm;	49 ø ≥ 10µm;	109 ø ≥ 10µm;	-	-
	particles/ mL	3 ø ≥ 25µm	5 ø ≥ 25µm	4 ø ≥ 25µm	7 ø ≥ 25µm	-	-

* (reconstitute with 1 mL of WFI)

Table 21. Results physical tests on lyophilized ALX-0081 stored at +25°C/60%RH [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Test	Unit	initial	1M	3M	6M	9M	12M
Appearance of the lyophilisate	-	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles
Appearance of the reconstituted solution	-	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles
Reconstitution time *(reconstitute with 1 mL of WFI)*	seconds	50	70	52	50	40	40
Osmolality	mOsm/kg	298	300	299	280	-	302
pH of the reconstituted solution	-	6.8	6.6	6.8	6.7	-	6.6

Test	Unit	initial	1M	3M	6M	9M	12M
Moisture content	% w/w	0.65	0.83	0.68	0.93	0.99	0.89*
Subvisible particles by HIAC	particles/mL	105 ø ≥ 10µm;	58 ø ≥ 10µm;	34 ø ≥ 10µm;	45 ø ≥ 10µm;	-	-
	particles/mL	3 ø ≥ 25µm	3 ø ≥ 25µm	4 ø ≥ 25µm	0 ø ≥ 25µm	-	-

Note: *Average value of 2 instead of 3 independent measurements

Table 22. Results physical tests on lyophilized ALX-0081 stored at +40°C/75%RH [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Test	Unit	initial	1M	3M	6M	9M	12M
Appearance of the lyophilisate	-	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles	White cake with no dark particles
Appearance of the reconstituted solution	-	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles	Clear colorless solution free of visible particles
Reconstitution time *(reconstitute with 1 mL of WFI)*	seconds	50	70	50	52	44	43
Osmolality	mOsm/kg	298	300	299	279	-	292
pH of the reconstituted solution	-	6.8	6.7	6.8	6.7	-	6.6

Test	Unit	initial	1M	3M	6M	9M	12M
Moisture content	% w/w	0.65	0.83	1.13	1.48	1.83	2.09*
Subvisible particles by HIAC	particles/mL	105 ø ≥ 10µm;	35 ø ≥ 10µm;	94 ø ≥ 10µm;	52 ø ≥ 10µm;	-	-
	particles/mL	3 ø ≥ 25µm	3 ø ≥ 25µm	20 ø ≥ 25µm	2 ø ≥ 25µm	-	-

Note: * Average value of 2 instead of 3 independent measurements

Table 23. Potency results ALX-0081 formulation [12.5 mg/mL API, 0.01% Tween-80 (v/v) and 7% sucrose (w/v) in 20 mM citrate buffer at pH 6.5].

Time point (months)	**Storage condition**	**Potency result** (%)*	**Lower limit** (%)**	**Upper limit** (%)***	**Pass/Fail**	**Acceptance criteria**
Initial	-	91.4	88.1	94.8	Pass	80% -120% (compared to reference)
1	**-20°C**	94.8	91.5	98.2	Pass	
	+5°±3°C	94.5	90.3	98.8		
	+25°C/60%RH	97.4	94.2	100.7		
	+40°C/75%RH	97.7	94.0	101.6		
3	**-20°C**	105.5	101.7	109.5	Pass	
	+5°±3°C	99.3	95.3	103.4		
	+25°C/60%RH	97.0	93.1	101.1		
	+40°C/75%RH	97.8	94.4	101.3		
6	**-20°C**	93.2	90.2	96.2	Pass	
	+5°±3°C	93.1	90.0	96.2		
	+25°C/60%RH	97.5	94.1	101.0		
	+40°C/75%RH	100.2	96.9	103.6		

Time point (months)	Storage condition	Potency result (%)*	Lower limit (%)**	Upper limit (%)***	Pass/Fail	Acceptance criteria
9	-20°C	101.0	95.2	107.2	Pass	
	+5°±3°C	101.1	94.8	107.7		
	+25°C/60%RH	101.6	96.2	107.3		
	+40°C/75%RH	98.7	93.8	103.9		
12	-20°C	101.3	98.7	103.9	Pass	
	+5°±3°C	101.2	98.3	104.2		
	+25°C/60%RH	105.7	103.0	108.5		
	+40°C/75%RH	100.4	95.5	105.5		

Table 24. In vitro comparability results of caplacizumab.

Study type	**Method**	**Criterion for comparability**	**Contemporaneous ALX-0081**	**Lyophilized ALX-0081**
Biological activity (potency)	Surface plasmon resonance (Biacore)	Relative potency of 80 – 120% (compared to Master Reference Standard)	102.8%	102.9%
Biological activity (potency)	vWF neutralising ELISA	Relative potency of 80 – 120% (compared to Master Reference Standard)	99.4%	109.5%
Biological activity (biomarker)	RICO	Concentrations of both formulations needed to completely block RICO (< 20%) do not differ by a factor > 5	0.4 µg/mL	0.4 µg/mL
Affinity	Gyrolab	K_D values of both formulations do not statistically differ (by means of 95% CI around K_D estimation)	6.84 pM (2.74 – 10.95)	4.46 pM (-0.18 – 9.10)

8. Aspects

1. A formulation comprising a von Willebrand Factor (vWF) binder and a citrate or phosphate buffer with a pH in the range of 5.0 to 7.5.

2. The formulation according to aspect 1, wherein said vWF binder comprises at least one immunoglobulin single variable domain binding to SEQ ID NO: 20.

3. The formulation according to aspect 2, wherein said immunoglobulin single variable domain comprises a heavy chain variable domain sequence which is derived from a conventional four-chain antibody or a heavy chain variable domain sequence which is derived from a heavy chain antibody or a Nanobody (including but not limited to a VHH sequence).

4. The formulation according to any one of aspects 1-3, wherein said vWF binder comprises at least one of SEQ ID NO:s 1-19.

5. The formulation according to any one of aspects 1-4, wherein said vWF binder is a single chain polypeptide comprising one or more immunoglobulin single variable domains.

6. The formulation according to aspect 5, wherein said vWF-binder is monovalent or multivalent.

7. The formulation according to aspect 5, wherein said vWF-binder is monospecific or multispecific.

8. The formulation according to aspect 5, wherein one or more immunoglobulin single variable domains are CDR-grafted, humanized, camelized, de-immunized, or selected by phage display.

9. The formulation according to any one of aspects 1-8, wherein said vWF binder comprises an amino acid sequence which is at least 90% identical to SEQ ID NO: 1.

10. The formulation according to any one of aspects 1 to 9, wherein said vWF binder has a concentration in the range of 0.1 to 80 mg/mL.

11. The formulation according to any one of aspects 1 to 10, wherein said buffer has a concentration in the range of 5-200 mM.

12. The formulation according to any one of aspects 1 to 11, further comprising an excipient.

13. The formulation according to aspect 12, wherein said excipient has a concentration in the range of 10-500 mM.

14. The formulation according to aspect 12 or 13, wherein said excipient is selected from the list consisting of sucrose, glycine, mannitol, trehalose and NaCl.

15. The formulation according to aspect 14, wherein said sucrose has a concentration in the range of 1-15%, preferably 2-12%, preferably 4-10%, e.g. 4, 5, 6, 7, 8 or 9% (w/v).

16. The formulation according to any one of aspects 1 to 15, wherein the buffer is selected from a citrate buffer, preferably having a pH between 6.0 and 7.0, more preferably 6.5; and a phosphate buffer, preferably having a pH in the range of 6.5 to 7.5, preferably 7.1.

17. The formulation according to any one of aspects 1 to 16, further comprising a non-ionic detergent, such as Tween-80, preferably in a concentration between 0.001 and 0.5% (v/v), more preferably 0.01-0.02% (v/v).

18. The formulation according to any one of aspects 1 to 17, wherein said buffer is a citrate buffer at pH 6.5 ± 0.5, e.g. 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 or 6.8, more specifically 6.5, said formulation further comprising sucrose having a concentration in the range of 1-15%, preferably 2-12%, preferably 4-10%, e.g. 4, 5, 6, 7, 8 or 9% (w/v), and preferably further comprising a non-ionic detergent such as Tween-80, preferably at a concentration of 0.01% (v/v).

19. The formulation according to any one of aspects 1 to 18, which has an osmolality in the range of 290 ± 60 mOsm/kg, more preferably in the range of 290 ± 20 mOsm/kg.

20. A formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) an excipient chosen from sucrose, glycine, mannitol, trehalose or NaCl at a concentration of about 1% to about 15% (w/v);

(c) Tween-80 at a concentration of about 0.001% to 0.5% (v/v); and

(d) a buffer chosen from citrate buffer at a concentration of about 5 mM to about 200 mM such that the pH of the formulation is about 6.0 to 7.0 and a phosphate buffer at a concentration of about 10 mM to about 50 mM such that the pH of the formulation is about 6.5 to 7.5,

wherein the vWF binder in the formulation retains at least about 80% of its stability after storage for at least 12 months at 5°C.

21. The formulation according to any one of aspects 1-20, which has:

(i) less than 5% of high molecular weight (HMW) species after storage for at least 12 months at 5°C; and/or

(ii) less than 5% of low molecular weight (LMW) species after storage for at least 12 months at 5°C.

22. The formulation according to any of aspects 1-20, wherein at least 80%, preferably at least 90%, more preferably at least 95% or even at least 99% of the vWF binder retains its binding activity after storage compared to the binding activity prior to storage, said binding activity as measured by ELISA and/or Biacore.

23. The formulation according to any of aspects 1-22, which is a liquid or reconstituted lyophilized formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) sucrose at a concentration of about 1% to about 15% (w/v);

(c) Tween-80 at a concentration of about 0.001%-0.5% (v/v); and

(d) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0.

24. The formulation according to any of aspects 1-22, which is a bulk storage formulation comprising:

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) sucrose at a concentration of about 1% to about 15%;

(c) Tween-80 at a concentration of about 0.001%-0.5% (w/v); and

(d) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0,

wherein at least 100 liters of the formulation are stored at below freezing conditions.

25. The formulation according to any one of aspects 1 to 23, suitable for parenteral administration, such as one or more selected from intravenous injection, subcutaneous injection, intramuscular injection or intraperitoneal injection.

26. The formulation according to any one of aspects 1 to 23, which is in liquid, lyophilized, spray-dried, reconstituted lyophilized or frozen form.

27. The formulation according to any one of aspects 1 to 26 for use in a method of treating a human or animal subject.

28. The formulation according to any one of aspects 1 to 27, for use in treating TTP or ACS.

29. A method of preparing a formulation according to any one of aspects 1 to 28.

30. A method or process of preparing a formulation comprising a vWF binder, said method or process comprising the steps of:

- expressing the vWF binder in a cell culture;
- purifying the vWF binder by passing the vWF binder through at least one of a chromatography purification step and an ultrafiltration/diafiltration step;
- adjusting the concentration of the vWF binder to about 0.1 to 80 mg/mL in a formulation containing:

(i) sucrose at a concentration of about 1% to about 15%;

(ii) Tween-80 at a concentration of about 0.001%-0.5% (w/v); and

(iii) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0.

31. A method of preparing a reconstituted formulation containing a vWF binder molecule, said method comprising the steps of: (i) lyophilizing a mixture of a vWF binder, a lyoprotectant, a surfactant and a buffer, thereby forming a lyophilized mixture; and (ii) reconstituting the lyophilized mixture in a diluent, thereby preparing the formulation, wherein the reconstituted formulation comprises

(a) a vWF binder at a concentration from about 0.1 mg/mL to about 80 mg/mL;

(b) sucrose at a concentration of about 1% to about 15% (w/v);

(c) Tween-80 at a concentration of about 0.001%-0.5% (v/v); and

(d) a citrate buffer at a concentration of about 5 mM to about 200 mM, such that the pH of the formulation is about 6.0 to 7.0.

32. The method according to any of aspects 29-31, further comprising a step of confectioning the formulation in a dosage unit form.

33. Method for stabilizing a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19 for storage, comprising preparing a formulation according to any one of aspects 1 to 24.

34. A method for storing a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19, comprising preparing a formulation according to any one of aspects 1 to 24.

35. A method of analyzing a manufacturing process, comprising:

- providing a sample of the formulation of any of aspects 1-24;
- assessing a parameter of the formulation chosen from color, clarity, viscosity, or an amount of one or more HMW or LMW species;
- determining whether the parameter meets a preselected criterion,

thereby analyzing the process.

36. The method of aspect 35, further comprising comparing two or more sample formulations in a method of monitoring or controlling batch-to-batch variation or to compare the sample to a reference standard.

37. The method of aspect 35, further comprising classifying, selecting, accepting or discarding, releasing or withholding, processing into a drug product, shipping, moving to a different location, formulating, labelling, packaging the formulation, based upon the comparison.

38. The method of aspect 37, further comprising providing a record which includes data relating to the assessed parameter of the formulation and optionally includes an identifier for a batch of the formulation; submitting said record to a decision-maker; optionally, receiving a communication from said decision maker; optionally, deciding whether to release or market the batch of formulation based on the communication from the decision maker.

39. A kit or an article of manufacture, comprising a container containing the formulation of any of aspects 1-24, and instructions for use.

40. The kit or article of manufacture of aspect 39, wherein the formulation is present in a vial or an injectable syringe.

41. The kit or article of manufacture of aspect 39, wherein the formulation is present in a prefilled injectable syringe.

42. The kit or article of manufacture of aspect 40 , wherein the syringe or a vial is composed of glass, plastic, or a polymeric material chosen from a cyclic olefin polymer or copolymer.

43. Pharmaceutical unit dosage form suitable for parenteral administration to a patient, preferably a human patient, comprising a formulation according to any one of aspects 1 to 23.

44. Use of a formulation according to any one of aspects 1 to 24 for storage of a vWF binder, preferably a polypeptide comprising at least one of SEQ ID NO:s 1-19.

45. Use according to aspect 44, wherein storage is 1-36 months, such as 1, 1.5, 3, 6, 9, 12, 18, 24, 30 or 36 months, preferably at least 12 months, e.g. at a temperature between -70°C and +40°C, such as -70°C, -20°C, +5°C, +25°C or +40°C, preferably a temperature between 70°C and +25°C.

46. Pharmaceutical or diagnostic composition comprising a formulation comprising a vWF binder according to any one of aspects 1 to 24, or obtainable by the method according to aspects 28 to 33.

47. A method of treating or preventing a vWF-related disorder, comprising administering to a subject, a pharmaceutical composition that comprises the formulation of any of aspects 1-24, thereby reducing one or more symptoms associated with the vWF-related disorder.

48. The method of aspect 47, wherein the vWF-related disorder is TTP.

CONCLUSIES

1. Een stabiele formulering die een pH heeft van 6.0 – 7.0 en omvat:

(a) 0.1 – 80 mg/mL van een von Willebrand Factor VHH dat de aminozuur-sequentie volgens SEQ ID NO: 1 omvat;

(b) sucrose in een concentratie van 1 – 15% (w/v);

(c) polysorbaat-80 in een concentratie van 0.001 – 0.5% (v/v); en

(d) citraat buffer in een concentratie van 5 – 200 mM.

2. De formulering volgens conclusie 1, waarin het genoemde VHH een concentratie heeft van 12.5 mg/mL.

3. De formulering volgens elk van de conclusies 1-2, waarin sucrose een concentratie heeft van 7% (w/v).

4. De formulering volgens elk van de conclusies 1-3, waarin polysorbaat-80 een concentratie heeft van 0.01% (v/v).

5. De formulering volgens elk van de conclusies 1-4, waarin citraat buffer een concentratie heeft van 20 mM.

6. De formulering volgens elk van de conclusies 1-5, waarin de formulering een pH heeft van 6.5.

7. De formulering volgens elk van de conclusies 1-6, waarin

a) de formulering een pH heeft van 6.5;

b) het genoemde VHH een concentratie heeft van 12.5 mg/mL;

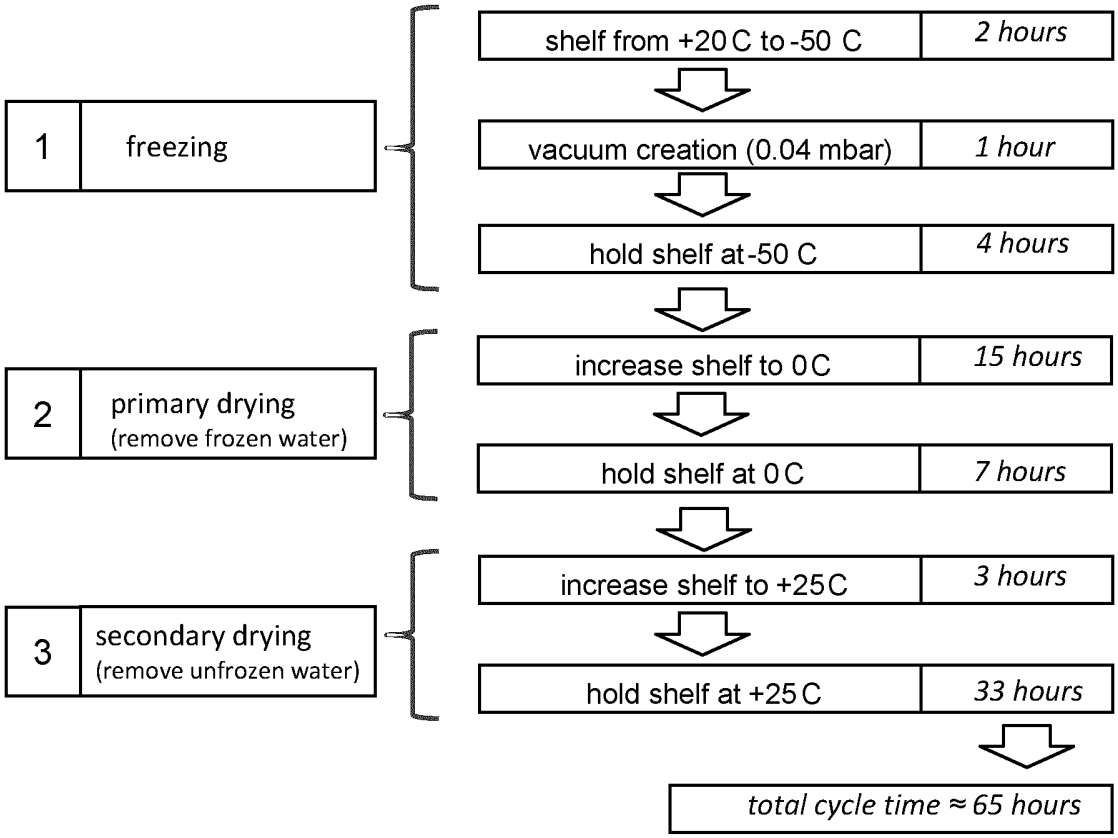
c) sucrose een concentratie heeft van 7% (w/v);

d) polysorbaat-80 een concentratie heeft van 0.01% (v/v); en

e) citraat buffer een concentratie heeft van 20 mM.

8. De formulering volgens elk van de conclusies 1-7, die bij 5°C gedurende ten minste 12 maanden stabiel is.

9. De formulering volgens elk van de conclusies 1-8, waarin de formulering vloeibaar is, gelyofiliseerd is, of opnieuw vloeibaar werd gemaakt na lyofilisatie.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freezing
shelf from +20 C to -50 C
2 hours
vacuum creation (0.04 mbar)
1 hour
hold shelf at -50 C
4 hours
2
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(remove frozen water)
increase shelf to 0 C
15 hours
hold shelf at 0 C
7 hours
3
secondary drying
(remove unfrozen water)
increase shelf to +25 C
3 hours
hold shelf at +25 C
33 hours
total cycle time ≈ 65 hours

FIG. 1

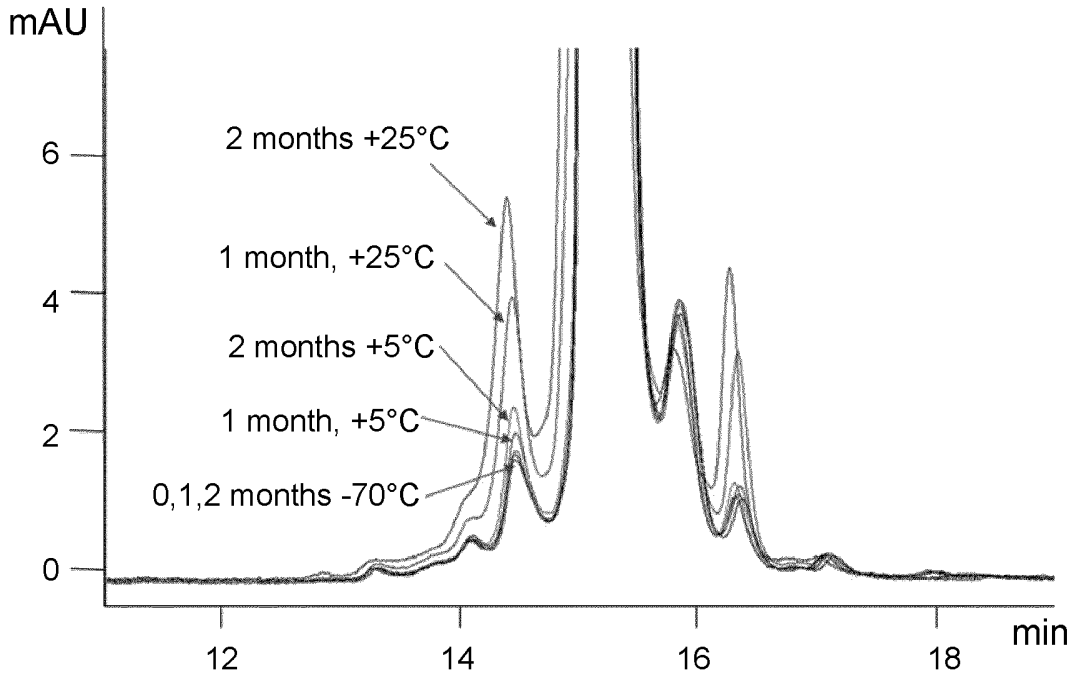

mAU
6
4
2
0
2 months +25°C
1 month, +25°C
2 months +5°C
1 month, +5°C
0,1,2 months -70°C
12
14
16
18
min

FIG. 2A

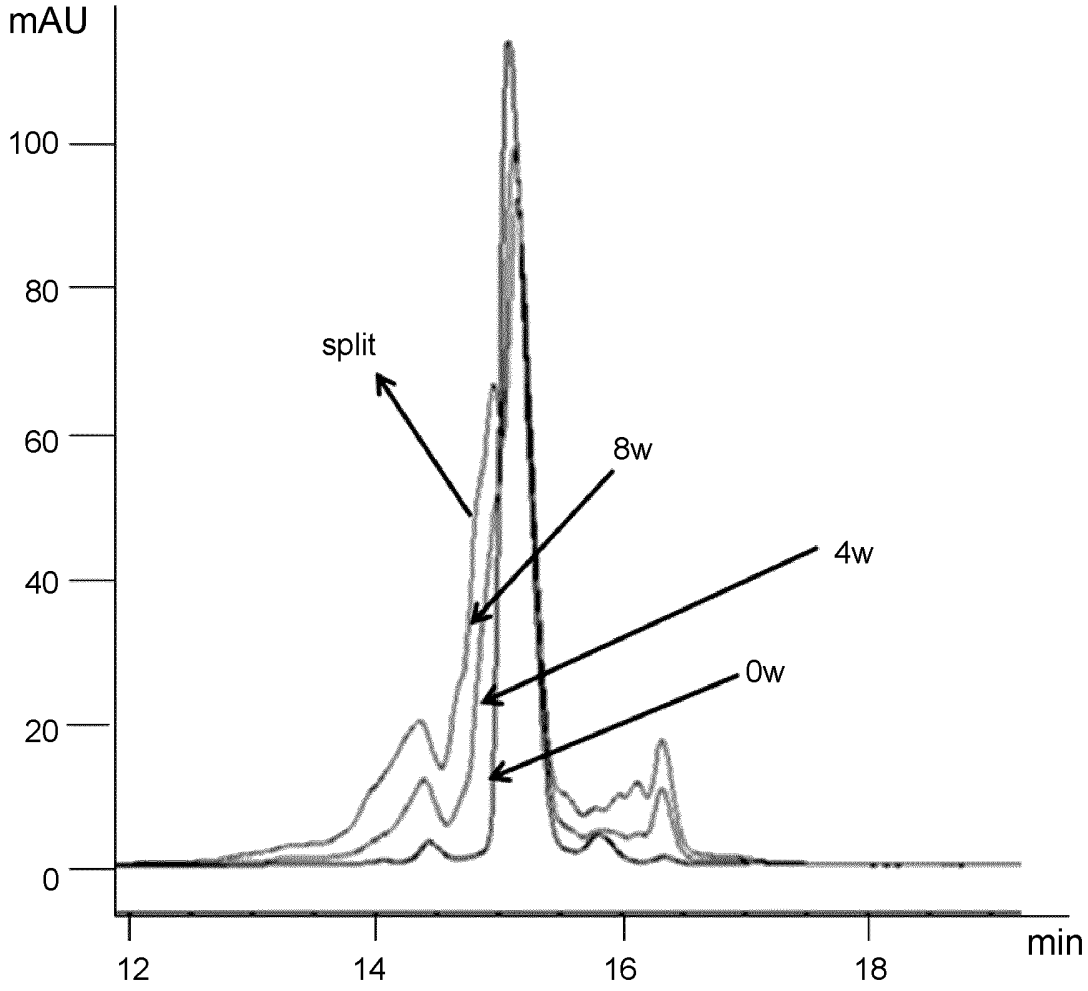
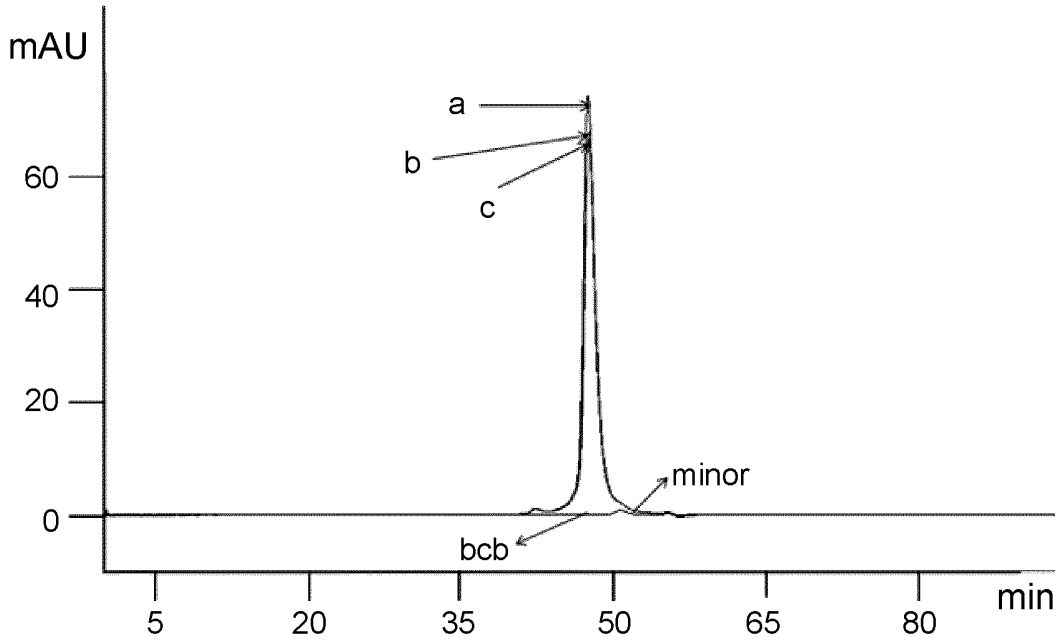


FIG. 2B


mAU
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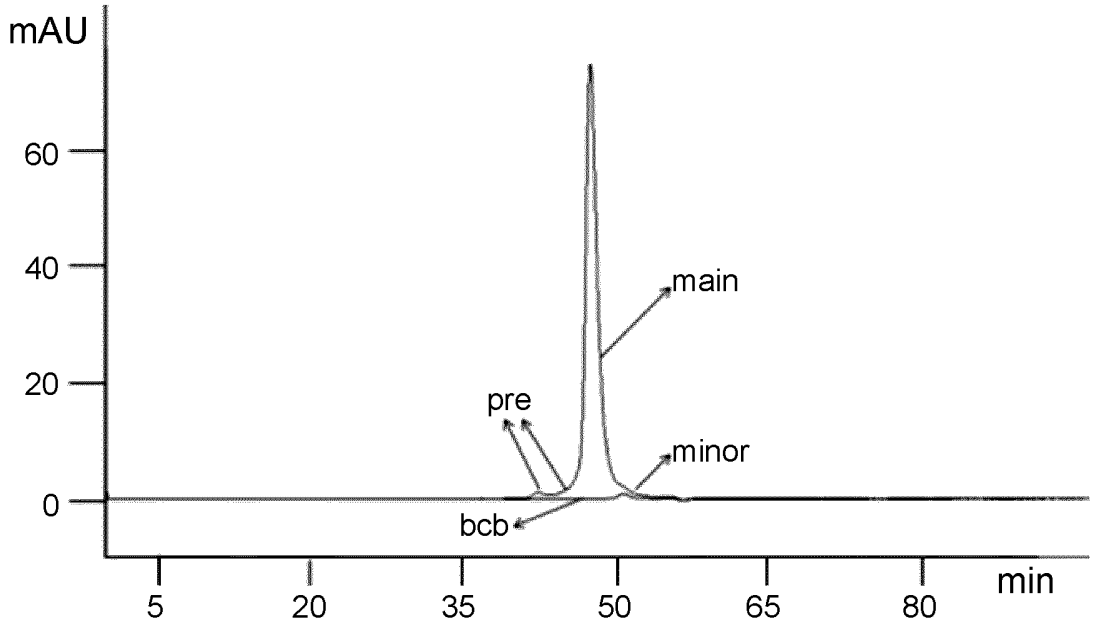
40
20
0
a
b
c
minor
bcb
5
20
35
50
65
80
min

FIG. 3A


mAU
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40
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main
pre
minor
bcb
5
20
35
50
65
80
min

FIG. 3B

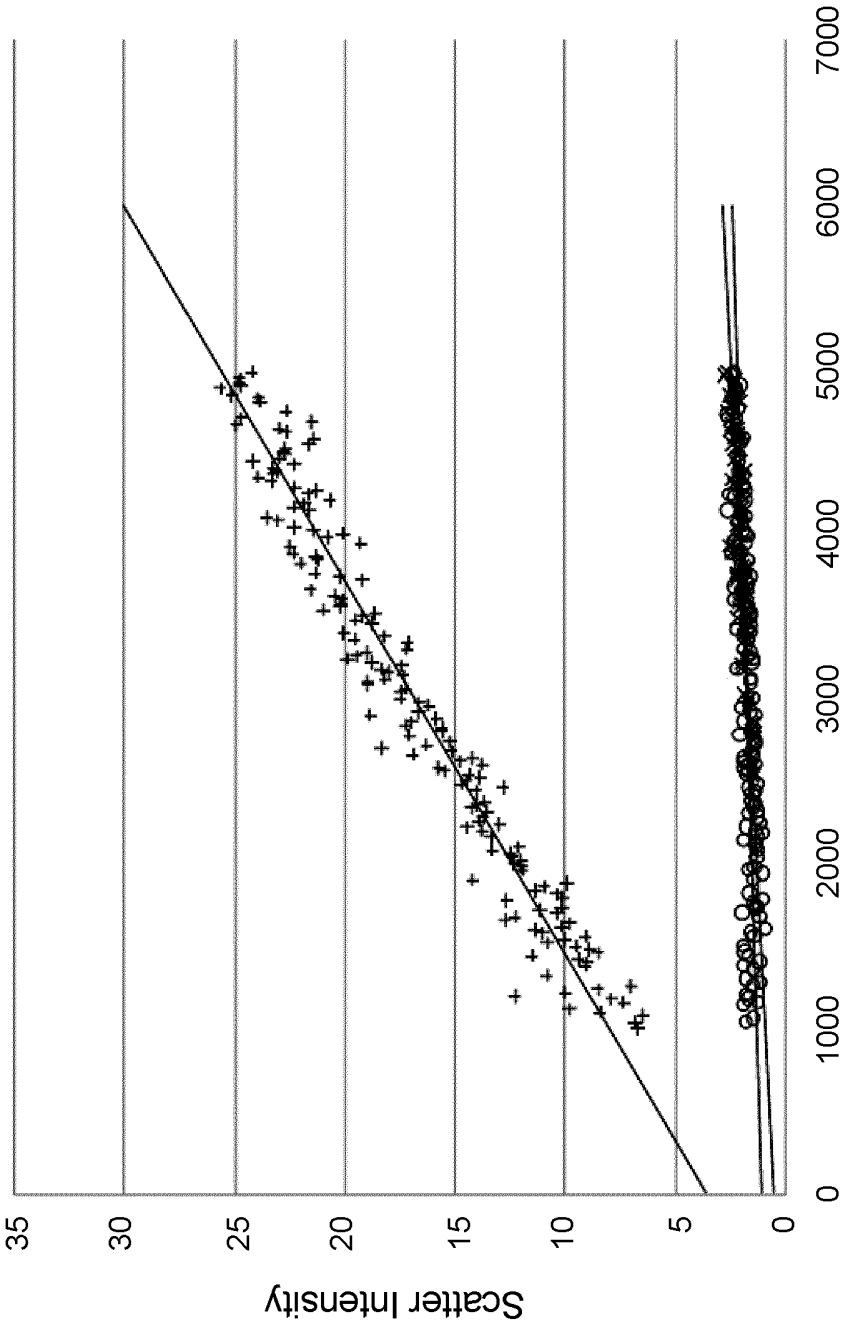

Scatter Intensity
35
30
25
20
15
10
5
0
0
1000
2000
3000
4000
5000
6000
7000
time (s)

FIG. 4A

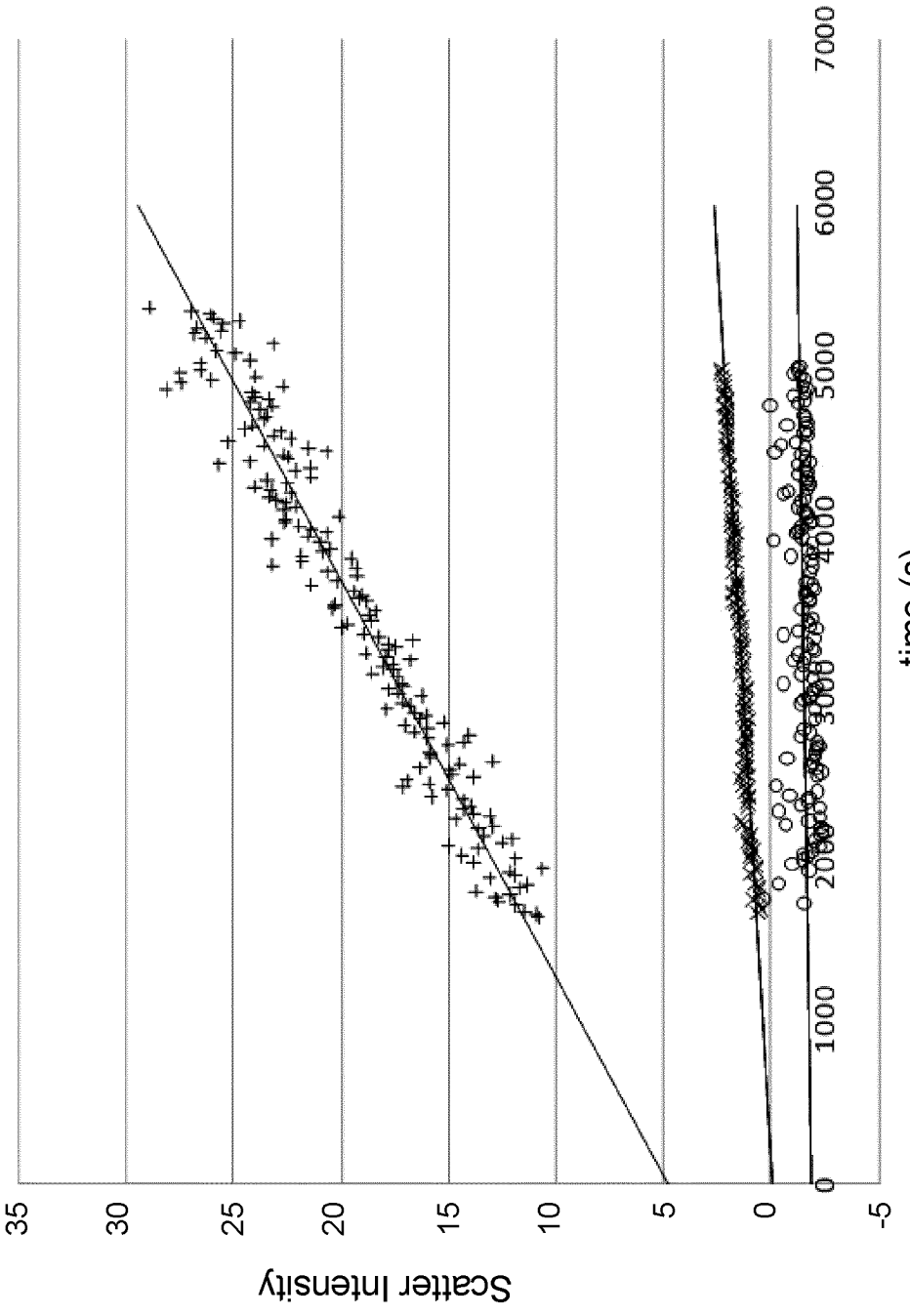

Scatter Intensity
35
30
25
20
15
10
5
0
-5
0
1000
2000
3000
4000
5000
6000
7000
time (s)

FIG. 4B

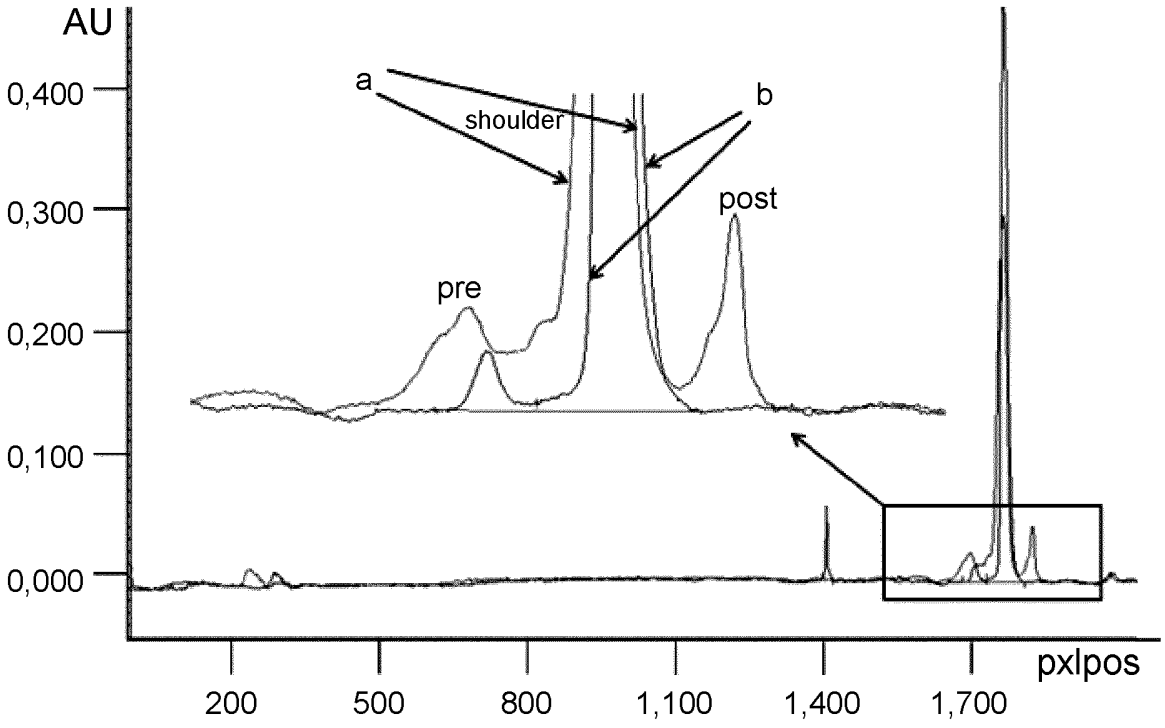
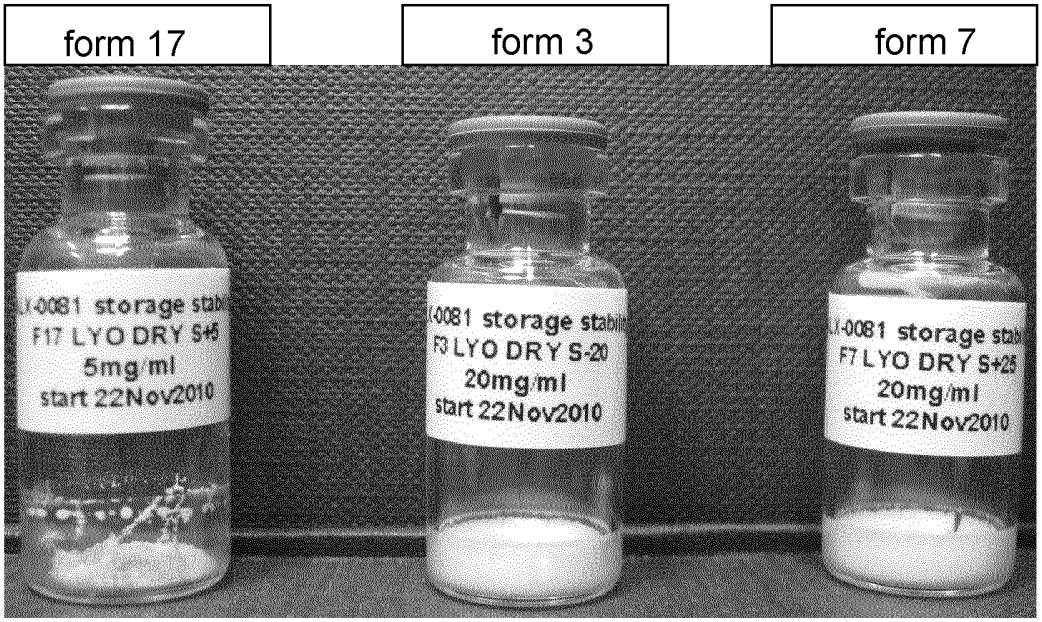

AU
0,400
0,300
0,200
0,100
0,000
a
shoulder
b
post
pre
pxlpos
200
500
800
1,100
1,400
1,700

FIG. 5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form 3
form 7
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5mg/ml
start 22Nov2010
F3 LYO DRY S-20
20mg/ml
start 22Nov2010
F7 LYO DRY S+25
20mg/ml
start 22Nov2010

FIG. 6A

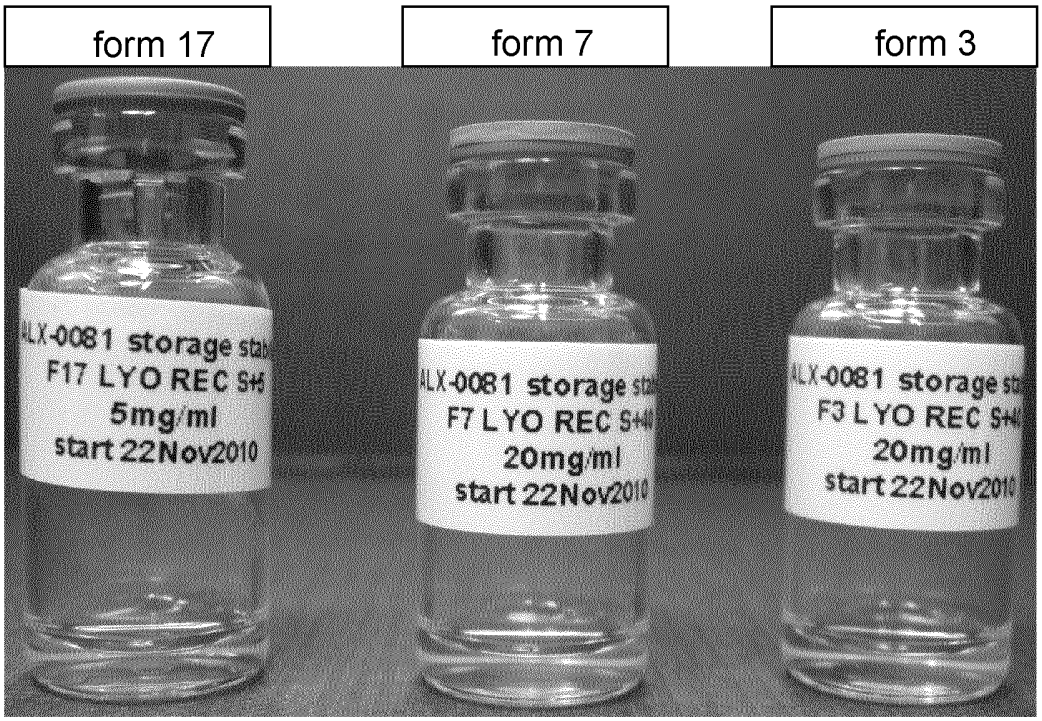
form 17
form 7
form 3
F17 LYO REC S+5
5mg/ml
start 22Nov2010
F7 LYO REC S+40
20mg/ml
start 22Nov2010
F3 LYO REC S+40
20mg/ml
start 22Nov2010

FIG. 6B

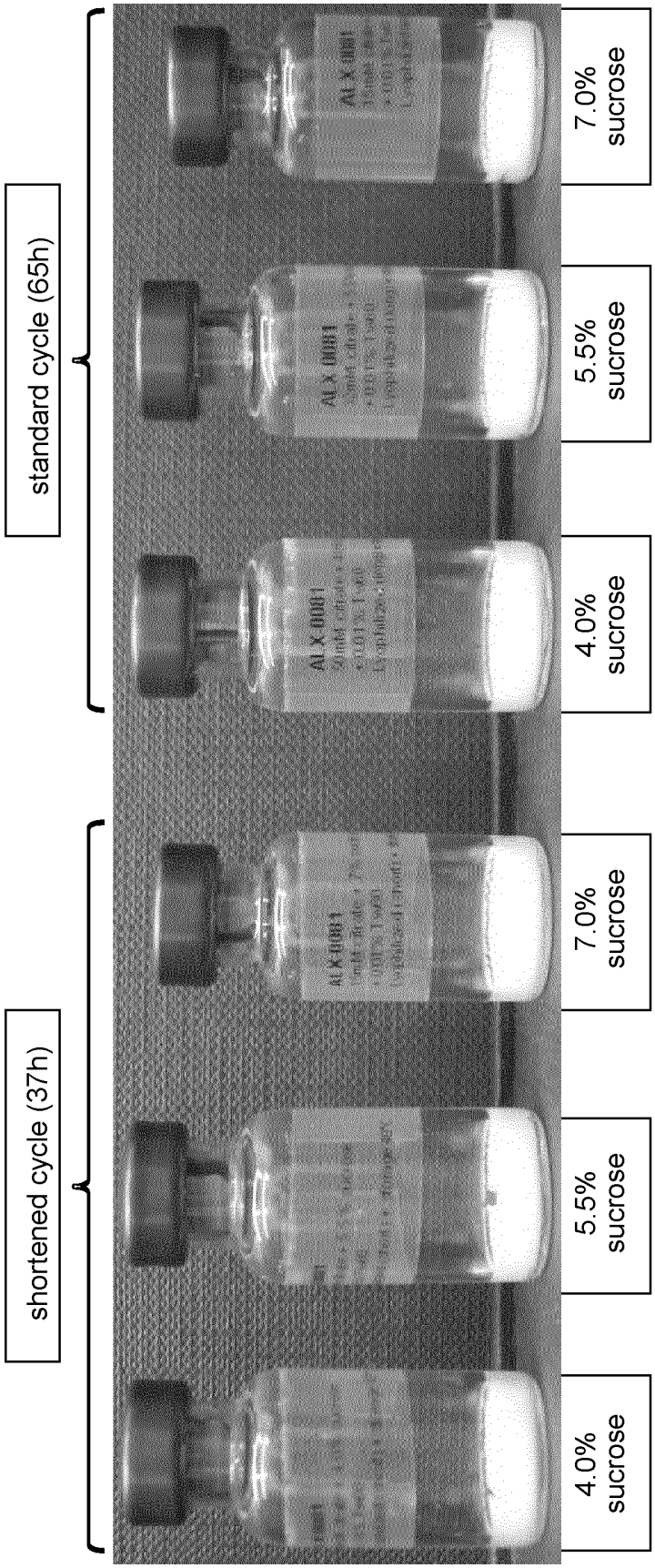
shortened cycle (37h)
standard cycle (65h)
4.0% sucrose
5.5% sucrose
7.0% sucrose
4.0% sucrose
5.5% sucrose
7.0% sucrose

FIG. 7

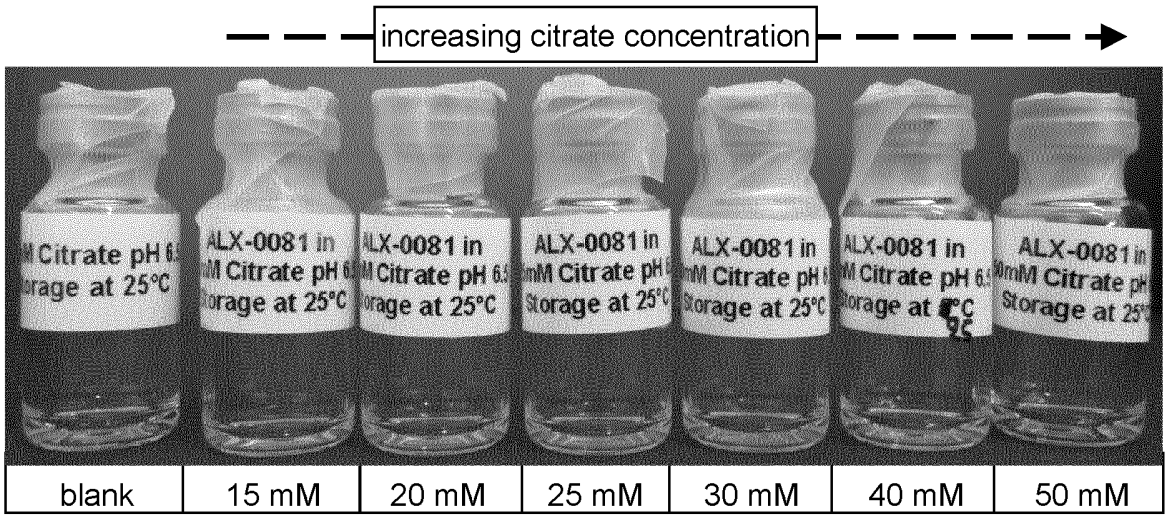
increasing citrate concentration
blank
15 mM
20 mM
25 mM
30 mM
40 mM
50 mM

FIG. 8A

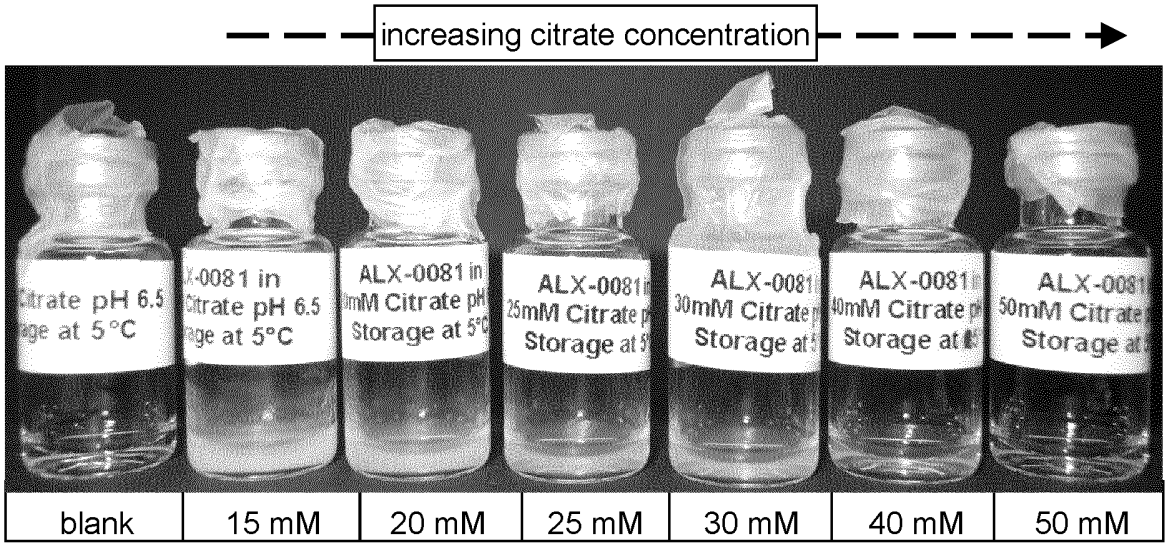
increasing citrate concentration
blank
15 mM
20 mM
25 mM
30 mM
40 mM
50 mM

FIG. 8B

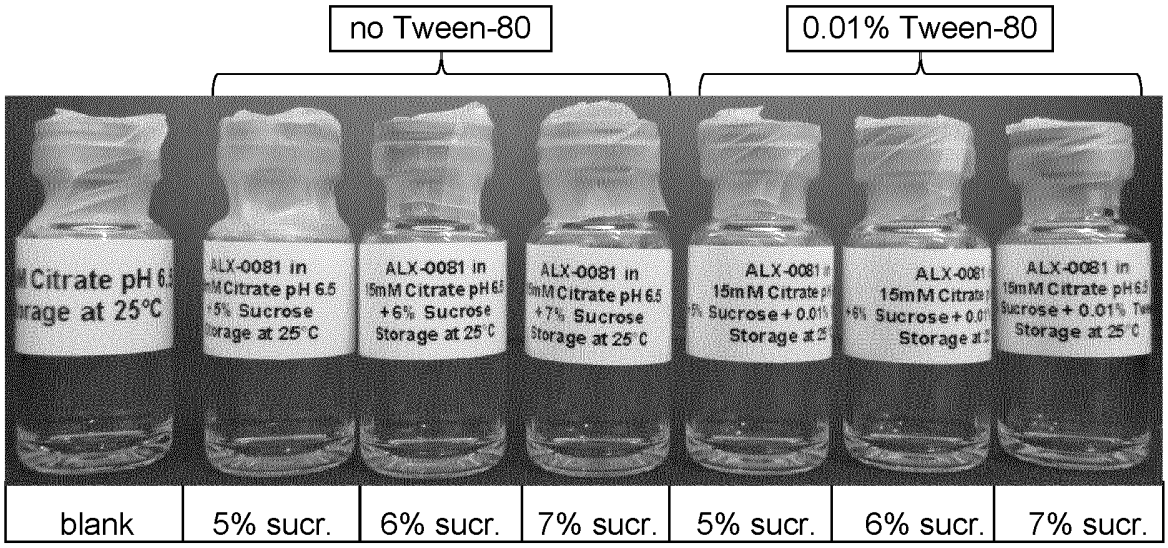
no Tween-80
0.01% Tween-80
blank
5% sucr.
6% sucr.
7% sucr.
5% sucr.
6% sucr.
7% sucr.

FIG. 9A

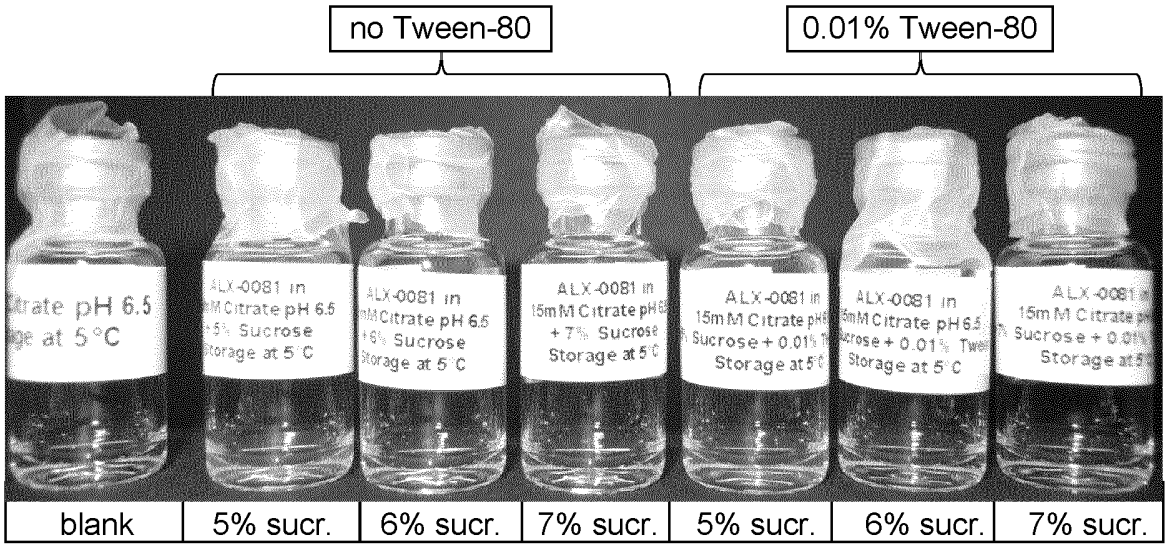
no Tween-80
0.01% Tween-80
blank
5% sucr.
6% sucr.
7% sucr.
5% sucr.
6% sucr.
7% sucr.

FIG. 9B

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
Nederlands aanvraag nr. **1040254**	Indieningsdatum **14-06-2013**
	Ingeroepen voorrangsdatum **17-05-2013**
Aanvrager (Naam) **Ablynx N.V.**	
Datum van het verzoek voor een onderzoek van internationaal type **12-10-2013**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN 60822**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

A61K39/335 **A61K9/08**

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**A61K**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	☐ **GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES**	(opmerkingen op aanvullingsblad)
IV.	☐ **GEBREK AAN EENHEID VAN UITVINDING**	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 1040254

A. CLASSIFICATIE VAN HET ONDERWERP
INV. A61K39/395 A61K9/08
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
A61K

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
Y	F. CALLEWAERT ET AL: "Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura", BLOOD, deel 120, nr. 17, 4 september 2012 (2012-09-04), bladzijden 3603-3610, XP055109473, ISSN: 0006-4971, DOI: 10.1182/blood-2012-04-420943 * samenvatting * * bladzijde 3604 * ----- -/--	1-9

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
26 maart 2014	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vandenbogaerde, Ann

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 1040254

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
Y	H. ULRICHTS ET AL: "Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs", BLOOD, deel 118, nr. 3, 16 mei 2011 (2011-05-16), bladzijden 757-765, XP055109919, ISSN: 0006-4971, DOI: 10.1182/blood-2010-11-317859 * samenvatting * * bladzijde 758 *	1-9
Y	US 2012/225072 A1 (MEYVIS YVES [BE] ET AL) 6 september 2012 (2012-09-06) * conclusies *	1-9
Y	EP 2 196 476 A1 (NOVARTIS AG [CH]) 16 juni 2010 (2010-06-16) * alinea [0002] - alinea [0010] *	1-9

1

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 1040254

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
US 2012225072 A1	06-09-2012	GEEN	
EP 2196476 A1	16-06-2010	AU 2009324371 A1	23-06-2011
		CA 2745938 A1	17-06-2010
		CN 102245639 A	16-11-2011
		CO 6361952 A2	20-01-2012
		EC SP11011192 A	31-08-2011
		EP 2196476 A1	16-06-2010
		EP 2376533 A1	19-10-2011
		JP 2012511540 A	24-05-2012
		KR 20120009421 A	31-01-2012
		MA 33023 B1	01-02-2012
		NZ 592918 A	21-12-2012
		PE 03422012 A1	24-04-2012
		RU 2011127913 A	20-01-2013
		US 2011236398 A1	29-09-2011
		US 2012315285 A1	13-12-2012
		WO 2010066762 A1	17-06-2010

OCTROOICENTRUM NEDERLAND

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN60822	14.06.2013	17.05.2013	NL1040254

International Patent Classification (IPC)
INV. A61K39/395 A61K9/08

Applicant
Ablynx N.V.

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☐ Box No. VIII Certain observations on the application

	Examiner
	Vandenbogaerde, Ann

Application number

WRITTEN OPINION

NL1040254

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☒ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-9
	No:	Claims	
Inventive step	Yes:	Claims	
	No:	Claims	1-9
Industrial applicability	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 F. CALLEWAERT ET AL: "Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura",
BLOOD,
deel 120, nr. 17, 4 september 2012 (2012-09-04), bladzijden 3603-3610, XP055109473,
ISSN: 0006-4971, DOI: 10.1182/blood-2012-04-420943

D2 H. ULRICHTS ET AL: "Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs",
BLOOD,
deel 118, nr. 3, 16 mei 2011 (2011-05-16), bladzijden 757-765, XP055109919,
ISSN: 0006-4971, DOI: 10.1182/blood-2010-11-317859

D3 US 2012/225072 A1 (MEYVIS YVES [BE] ET AL) 6 september 2012 (2012-09-06)

D4 EP 2 196 476 A1 (NOVARTIS AG [CH]) 16 juni 2010 (2010-06-16)

- D1 discloses (cf. abstract, Methods p.3604 Materials) the efficacy of subcutaneous administration of ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura; ALX-0681 is formulated in D-PBS, 0.2 M glycine, 0.02% Tween, pH 7.1.
- D2 describes (cf. abstract, Methods p.758 Materials) the efficacy of intravenous administration of ALX-0081 as anti-thrombotic drug in a preclinical baboon model; ALX-0081 is formulated in 0.9% saline.
- D3 discloses (cf. claims) the preparation of a stable formulation comprising the 4CXCR104 Nanobody® (0.1-150 mg/ml), wherein the formulation comprises a phosphate or citrate buffer (5-100 mM) having a pH between 5.0 and 7.5, an excipient selected from NaCl (10-500 mM), mannitol (1-10% w/w) or sucrose (1-12% w/w), and Tween-80 (0.005-0.1% w/w) as non-ionic detergent.

- D4 discloses (cf. [0002]-[0003], [0006]-[0010]) the preparation of stable formulation comprising an antibody, in particular antibodies to human IL-1beta, which comprises a citrate, histidine, sodium succinate or phosphate buffer having pH 5.5-7.5, as stabilizer sucrose, trehalose or mannitol, and polysorbate 20 or polysorbate 80 as surfactant.

1 **Novelty**

1.1 The subject-matter of claims relates to a formulation of pH 6.0-7.0 comprising 0.1-80 mg/ml Caplacizumab (= Nanobody® ALX-0081 = Nanobody® ALX-0681) of SEQ. 1, 1-15% w/w sucrose, 0.001-0.5% v/v polysorbate 80 (= Tween-80) and 5-200 mM citrate buffer.

1.2 The subject-matter of claims 1-9 novel over the teaching of the cited prior art documents.

2 **Inventive step**

However, the subject-matter of claims 1-9 cannot be considered as involving an inventive step for the following reasons. Document D3, which is considered to represent the most relevant state of the art, discloses (cf. claims) the preparation of a stable formulation comprising the 4CXCR104 Nanobody® (0.1-150 mg/ml), wherein the formulation comprises a phosphate or citrate buffer (5-100 mM) having a pH between 5.0 and 7.5, an excipient selected from NaCl (10-500 mM), mannitol (1-10% w/w) or sucrose (1-12% w/w), and Tween-80 (0.005-0.1% w/w) as non-ionic detergent.

The subject-matter of claims 1-9 differs from the closest prior art document D3 in that a stable formulation for a different Nanobody® is prepared. The problem to be solved by the present invention may therefore be regarded as the provision of a stable formulation for an alternative Nanobody®.

As it is already known from closest prior art document D3 which are the ingredients used for the stabilisation of a Nanobody®, it would be a matter of routine for the skilled person to optimise the formulation by simple experiments using the standard ingredients in order to arrive at the most preferred formulation for the Nanobody® ALX-0061 of SEQ No. 1, which is known from D1 and D2.

Furthermore, it seems that citrate buffer, sucrose and polysorbate-80 are generally used in stability studies for optimising the formulation of antibodies, as can be seen from for instance document D4.

Therefore, the subject-matter of claims 1-9 cannot be considered as involving an inventive step.