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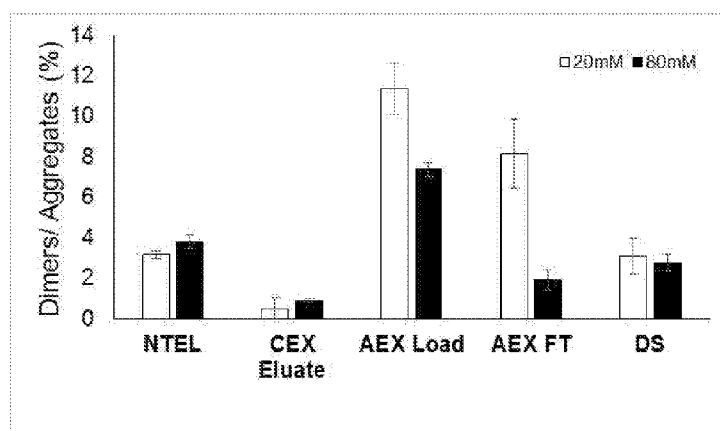


FIG.3

(57) Abstract: The invention provides a method for aggregate or dimer reduction/removal from antibody composition. More particularly, the invention relates to the importance of buffer concentration for reduction/ removal of aggregates in chromatographic purification of antibodies.

METHOD FOR REDUCTION OF AGGREGATES

FIELD OF INVENTION

The present invention relates to the field of preparation and purification of a
5 therapeutic monoclonal antibody mixture. More specifically, the invention relates to the importance of buffer and its concentration for the reduction/removal of antibody dimers or higher molecular weight aggregates during purification of a recombinant antibody.

BACKGROUND OF THE INVENTION

Therapeutic proteins, including monoclonal antibodies are produced by
10 recombinant DNA technology. Proteins/antibodies expressed by recombinant DNA methods are typically associated with impurities such as host cell proteins (HCP), host cell DNA (HCD), endotoxins, variants, viruses etc., including aggregates.

Aggregate formation in antibodies is a common phenomenon which can be
encountered during various stages of a commercial therapeutic antibody
15 manufacturing processes. Aggregates may form during fermentation, purification, final formulation operations, or while storage of the drug substance / drug product.

Aggregates in a therapeutic protein drug can significantly impair its potency. Soluble aggregates can trigger patient immune response to the therapeutic protein product greatly reducing the pharmacodynamics of the drug (Franco R, Daniela G,
20 Fabrizio M, Ilaria G, Detlev H. 1999; Cytotechnology; 29(1):11–25). In addition, aggregates are also known to affect the biological activity of antibody drugs, stability and shelf life. (Rosenberg AS. 2006; AAPS J 8(3): Article 59). Further, the presence of aggregates in drug substances that are administered intravenously has been shown to decrease microcirculation as a consequence of the mechanical blockage of
25 capillaries (Lehr HA, Brunner J, Rangoonwala R, Kirkpatrick CJ.2002; Am J Respir Crit Care Med 165(4):514–520). Specifically, immunoglobulin aggregates have long been known to cause anaphylactic reactions (Rosenberg AS. 2006; AAPS J 8(3): Article 59). Minimizing such risks associated with aggregates is thus an essential and

mandatory requirement from the regulatory agencies approving any therapeutic protein/antibody.

Removal of aggregates from therapeutic protein is often a difficult task as aggregates exhibit similar physical and chemical properties as that of its monomeric form. More challenging are specific immunoglobulin (antibody) types that tend to self-associate e.g., cryoglobulins. Recent studies conclude anti-VEGF antibody to be one such type of antibody wherein reversible self-association, resulting in aggregates, are common. The study disclosed formation of significant amounts of reversible dimers in recombinant humanized anti-VEGF antibodies around neutral or alkaline pH conditions (Moore J. M. R., Patapoff T. W., Cromwell M. E. M.; 1999, *Biochemistry* 38, 13960-13967). Such tendency to form increased aggregates may cause operational difficulties in certain processes, particularly in processes such as fermentation or ion-exchange chromatography that necessitate neutral or alkaline pH conditions for a specific outcome.

Aggregates/dimer removal are often effected by chromatographic steps employed during downstream processing of a therapeutic protein/antibody. Size exclusion chromatography, in particular has been used to remove aggregates, as the separation principle of the chromatography is based on the molecular weight difference between a desired protein and undesired compounds such as aggregates (Wang L, Hale G, Ghosh R. 2006; *Anal Chem* 78(19):6863–6867). Others, purification methods for aggregate removal also include mixed mode or other chromatography conditions involving elaborate salt gradients. (Gagnon P. 2009; *Curr Pharm Biotechnol* 10(4):434–439).

Another commonly used method to aggregate reduction or removal is addition of aggregation inhibitor compounds in a therapeutic protein preparation. In addition to the general inclusion of these inhibitors during production/cell culture (fermentation) stage, they are also 'added' to the harvested protein/antibody mixture during purification or drug substance preparation steps. Despite its likely interference in the function and purity of the protein, use of aggregation inhibitors remain, and continue to be the preferred method for reduction/removal of aggregates in a therapeutic protein preparation including antibodies.

The objective of the present invention is to provide a simple and improved method of reducing the formation of aggregates and/or its removal in a therapeutic protein or antibody preparation. In particular, the objective is to provide a method that does not involve addition of any aggregation inhibitors for reduction/removal of aggregates in a protein or antibody composition. A further objective is to effect reduction/ removal of aggregates in antibody types that are known to self-associate or form dimers/aggregates around neutral/alkaline pH conditions. The method also enables operation of specific chromatographic steps around neutral/alkaline pH conditions, alongside reducing/removing aggregates, for such specific self-associating antibody types that form dimers/aggregates around neutral/alkaline pH settings.

SUMMARY OF THE INVENTION

The present invention provides a method for reducing dimer/aggregates formation in a recombinant protein/antibody composition using a buffer comprising specific concentration of phosphoric acid and salts thereof. More particularly, dimer/aggregates formation is minimized (at least about 50 %) when a buffer comprising a concentration of at least 60 mM of phosphoric acid or its salts thereof is used for preparing the protein/antibody mixture. The instant invention also involves preparing and/or delivering the protein/antibody mixture onto an ion-exchange chromatography in the specified concentrations of phosphoric acid or its salts thereof.

Notably, the method does not involve addition of any aggregation inhibitors to the protein/antibody mixture other than the buffering agent i.e., phosphoric acid and its salts thereof.

In addition, the method enables the operation of ion-exchange chromatography at or around neutral/alkaline pH during recombinant antibody purification alongside minimizing dimerization/aggregation, particularly for antibody types that form significant dimers/aggregates around neutral or alkaline pH conditions. Anti-VEGF antibody is one such antibody type that self-associates and form significant dimers in high pH, temperature and ionic strength concentrations and the present invention was therefore effected with example of recombinant anti-VEGF antibody.

Specifically, the disclosed method results in about 50 -80 % reduction in dimer/aggregate content and/or at least about two fold reduction in dimer/aggregate formation in the antibody load mixture i.e., during load preparation and even before subjecting to chromatography. The amount of dimer/aggregate content in the recombinant antibody mixture/composition after chromatographic purification is thus reduced to less than 3%.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG.1: Effect of phosphate buffer concentration on anti-VEGF antibody dimer/aggregates formation during AEX load preparation

FIG.2: Effect of phosphate buffer concentration on anti-VEGF antibody dimer/aggregates formation during AEX load preparation

FIG.3: Effect of phosphate buffer concentrations on anti-VEGF antibody dimer/aggregates content at different stages of downstream purification process (NTEL- Neutralized protein A eluate, CEX eluate- cation exchange chromatography eluate, AEX Load- anion exchange chromatography load, AEX FT- anion exchange chromatography flow-through, DS- Drug substance)

DETAILED DESCRIPTION OF THE INVENTION

Antibody purification process typically involve protein-A affinity chromatography as capture step and one or more ion-exchange chromatography as polishing steps. AEX is popularly used as a polishing step in antibody manufacture because the high pl of most antibodies prevents them from binding to AEX resins/support under the pH conditions (pH 7.0-8.0) typical for AEX, while impurities such HCP, HCD, endotoxins etc., bind to the resin. However, operation of AEX at its typical pH conditions may promote self-association in some antibody types causing increased dimerization or aggregation.

For example, and as said earlier, studies by Moore *et.al.*, disclose that recombinant humanized monoclonal anti-VEGF antibodies self-associate to form dimers/aggregates at physiological or alkaline pH conditions, particularly at pH 7.5 to 8.0, and exists predominately as monomers only at pH 5.5 and below. Specifically, the

anti-VEGF antibodies formed significant amounts of non-covalent and reversible dimers/aggregates at increased pH (pH 7.5 to 8.0), temperature, and ionic strength (Moore J. M. R., Patapoff T. W., Cromwell M. E. M.; 1999, Biochemistry 38, 13960-13967).

5 Thus in addition to the requirement of a simpler solution for the problem of aggregate reduction or removal, there exists a requirement that such solution enables operation of ion-exchange chromatography around 'neutral pH' conditions specifically for self-associating antibody types that increasingly forms dimers/aggregate at or around these pH conditions.

10 The present invention discloses a method wherein, surprisingly, it has been found that altering or increasing the concentration of the buffering agent, such as phosphoric acid and/or its salts thereof, to at least about 60 mM results in reduction of amount of antibody dimers /aggregate in an antibody preparation. Particularly, the antibody mixture that is to be loaded onto a chromatographic support, when prepared
15 in a buffer comprising a concentration of at least 60mM Phosphoric acid and its salts thereof resulted in a mixture with reduced amount of aggregate/ dimers. Most importantly, the aggregate/ dimer reduction was effected during the antibody load preparation viz., even before subjecting to chromatography. The specific concentration of phosphate buffer, thus resulted in about 50 -80 % (at least about two-
20 fold) reduction in aggregates/dimer formation in the antibody load mixture, when compared with the load mixture that was prepared otherwise viz., in a phosphate concentration less than about 60 mM.

In addition, buffer comprising at least 60 mM phosphate when used for preparing and/or delivering the antibody mixture onto an ion-exchange
25 chromatography, resulted in a similar degree of aggregates/dimer reduction in the antibody composition obtained from the ion-exchange chromatographic step. Specifically, the aggregates/dimer content was measured to be consistently less than 3% in the chromatographic output, when a phosphate buffer concentration of at least about 60 mM was used during equilibration, load, wash and /or recovery steps
30 of the chromatography

The term 'antibody' or 'antibodies' mentioned herein can be isolated from various sources, such as murine, human, recombinant etc. It also includes but not limited to truncated antibodies, chimeric, humanized or fully human or pegylated conjugates and fusion proteins which contain an immunoglobulin moiety.

5 The term 'antibody mixture' as used herein refers to a composition comprising an antibody that is obtained from (or after) a cell culture process and can either be a harvested cell culture extract, crude filtered cell culture extract or a partially purified cell culture extract.

10 The term "antibody load" or "antibody load mixture", as used herein can be used interchangeably, refers to an antibody mixture that is loaded onto a chromatographic support

15 The term 'flow-through mode' denotes an operation mode of a purification method, in which a solution containing a substance of interest, e.g. an immunoglobulin in monomeric form, to be purified is brought in contact with a stationary phase, whereby the substance of interest does not bind to the stationary phase. As a result the substance of interest is obtained either in the flow-through (if chromatographic material is resin) or in the filtrate (if chromatographic material is membrane).

20 In an embodiment, the invention describes an antibody composition with reduced aggregate/dimer content at neutral or alkaline pH conditions, wherein said antibody being pre-disposed to form aggregates/dimers at neutral or alkaline pH conditions, obtained by a process comprising preparing the antibody composition in a buffer comprising a concentration of at least 60 mM of phosphoric acid or its salt thereof, wherein the antibody composition so prepared demonstrates reduction in the aggregate/dimer content when compared with the antibody composition at
25 neutral/alkaline pH that was prepared in a buffer comprising a concentration of less than 60mM of phosphoric acid or its salt thereof.

30 In another embodiment, the invention describes a method for reduction of aggregate /dimer content in an antibody composition at neutral/alkaline pH, wherein the said antibody being predisposed to form aggregates/dimers at neutral or alkaline pH, the said method comprising preparing the antibody composition in a buffer comprising a concentration of at least 60 mM of phosphoric acid or its salt thereof,

wherein the antibody composition so prepared demonstrates reduction in the aggregate/dimer content when compared with the antibody composition at neutral/alkaline pH that was prepared in a buffer comprising a concentration of less than 60mM of phosphoric acid or its salt thereof.

5 In another embodiment the invention describes a method for reducing the aggregate/dimer formation in an antibody load mixture at neutral or alkaline pH conditions, during chromatographic purification, wherein the said antibody being pre-disposed to form aggregates / dimers at neutral or alkaline pH, by preparing the said antibody load mixture in a buffer comprising a concentration of at least 60 mM of
10 phosphoric acid and/or its salts thereof and wherein there is a reduction in aggregate/dimer formation when compared with the antibody load mixture prepared in a neutral or alkaline pH buffer comprising a concentration of less than 60 mM of phosphoric acid or its salt thereof.

15 In another embodiment the above said chromatographic purification include steps of load, wash and/or recovery that are operated at neutral or alkaline pH.

In a further embodiment the invention provides a method for reduction of aggregates/dimer formation in an antibody composition comprising steps of;

- a. preparing the antibody load mixture in a buffer comprising phosphoric acid and/or its salts thereof in a concentration range of at least about 60 mM,
- 20 b. contacting the antibody load mixture in the said phosphate buffer onto an ion-exchange chromatographic support at a pH of about 7 to about 7.5,
- c. allowing the antibody to flow-through the support, and
- d. recovering the antibody.

25 wherein, there is a reduction of aggregate/dimer formation in antibody load mixture at step a, than the antibody load mixture prepared in phosphoric acid and/or its salt thereof at concentration less than 60mM.

In another embodiment the invention describes a method, wherein the said chromatographic procedure may include one or more wash buffer steps.

In another embodiment, the invention describes a method wherein the reduction of aggregate/dimer formation in the said antibody load at least about 50 %.

In another embodiment the invention describes a method wherein the aggregate/dimer content in the recovered antibody composition is less than 3%.

5 In another embodiment the invention describes a method wherein, the reduction in aggregate/dimer content in antibody load mixture is at least about two-fold.

In another embodiment, the said antibody is an anti-VEGF antibody.

10 In another embodiment, the said the chromatography or the chromatographic support is an anion exchange chromatography or anion exchange chromatographic support.

The method described herein result in about 50 – 80 % reduction in aggregates/dimer formation, and/or at least about two-fold reduction of aggregates/dimer formation during antibody load preparation. In fact in the described
15 method herein, the aggregate/dimer level is reduced to less than 3% in antibody composition.

In another embodiment, the pH of the said buffer in any of the above mentioned methods in the range of about 7 to about 7.5.

20 In the method described herein, the antibody mixture does not involve treatment with, or addition of any aggregation inhibitors. Examples of a few commonly known aggregation inhibitors are chaotropes, non-ionic surfactants, ionic surfactants, detergents, amino acids such as arginine, proline, methionine, glutamic acid etc., polyethylene glycols, polyhydric alcohols, glycerol, sorbitol, sugars such as sucrose, glucose.

25 The buffer used in the method described herein, consists either sodium or potassium salt of phosphoric Acid.

The method according to the present invention can be combined with one or more chromatography steps such as protein A affinity chromatography or a protein G

affinity chromatography or cation exchange chromatography or a hydrophobic charge induction chromatography (HCIC), or a hydrophobic interaction chromatography (HIC) or a mixed mode. The chromatography material or support used can be resin or a membrane or a monolith. The method as described in the instant invention may be extended to other related antibody types, particularly in downstream ion-chromatography steps where operation around specific, i.e., neutral/alkaline pH conditions, becomes inevitable.

The method according to the present invention may include one or more tangential flow filtration, concentration, diafiltration or ultrafiltration steps.

The method according to the present invention may include one or more viral inactivation steps or sterile filtration or nano filtration steps.

The invention is more fully understood by reference to the following examples. These examples should not, however, be construed as limiting the scope of the invention.

EXAMPLE 1

Protein A chromatography

Anti-VEGF antibody was cloned and expressed in a CHO cell line as described in U.S. Patent No. 7,060,269, which is incorporated herein by reference. The cell culture broth containing the expressed antibody was harvested, clarified and subjected to protein A affinity chromatography as described below.

The clarified cell culture broth was loaded onto a protein A chromatography column (Mabselect, VL44x250, 205 mL) that was pre-equilibrated with Tris buffer solution (pH 7.0). The column was then washed with equilibration buffer, followed by a wash with Tris buffer (pH 7.0) of high conductivity. The bound antibody was eluted using Acetic acid, pH 2.5 – 3.5. Protein A eluate pH was adjusted to pH 6.2 using 1M Tris chloride solution of pH 9.

EXAMPLE 2

Cation exchange chromatography

The neutralized protein A eluate (NTEL) obtained from the procedure described in Example 1 was loaded onto a cation exchange resin (POROS HS 50, VL32X250, 160 ml) pre-equilibrated with phosphate buffer, pH 6.2, followed by washing the resin with a wash buffer of 35 mM phosphate buffer, pH 6.2. The bound antibody was then
5 eluted using phosphate, pH 6.2, at a conductivity between 7 to 8 mS/cm.

EXAMPLE 3

Anion exchange chromatography

The eluate containing the antibody mixture, obtained from the cation exchange chromatography procedure described in Example 2 was buffer exchanged with a 20
10 mM phosphate buffer of pH about 7.0 to 7.5 for loading onto an anion-exchange chromatography. The antibody mixture prepared in buffer of 20 mM phosphate was then loaded onto an anion exchange resin (Q-Sepharose FF, VL32x250, 80 mL) pre-equilibrated with 5 CV of equilibration buffer consisting of 20 mM phosphate buffer, about pH 7.0 to 7.5. This was followed by a post load wash with equilibration buffer
15 and the flow-through from the column obtained during load and wash step was collected.

Alternatively, the above procedure was repeated with varying concentrations of buffer containing about 60 mM to about 200 mM of phosphate concentration, in AEX load, pH about 7.2, to determine reduction/removal in the aggregate/dimer content.
20 During these procedures, the equilibration and post load wash buffers concentrations were maintained similar to AEX load buffer concentration. Impact of phosphate concentration in aggregate/dimer reduction, during AEX load preparation, is depicted in Fig.1 and Fig 2. Comparison of relative outcome of 20 mM and 80 mM phosphate concentration in aggregate/dimer reduction, during load preparation and in the flow-
25 through of the anion-exchange chromatography step, is depicted in Fig.3.

EXAMPLE 4

Analysis of antibody dimers/aggregate by size exclusion chromatography

NTEL, cation exchange chromatography eluate, anion exchange chromatography load, anion exchange chromatography flow through and drug

substance were analysed using size exclusion chromatography high performance liquid chromatography (SEC-HPLC). Details of the SEC-HPLC conditions are as follow,

Instrument: WATERS HPLC system

5 Resin: TSK-GEL G3000SW_{XL}

Column: 7.8mm x 30cm

Flow rate: 1 mL/min

Buffer: 200mM Potassium phosphate buffer + 250mM KCl (pH-6.2±1)

Wavelength: 280nm

Claims:

1. An antibody composition with reduced aggregate/dimer content at neutral or alkaline pH conditions, wherein said antibody being pre-disposed to form aggregates/dimers at neutral or alkaline pH conditions, obtained by a process comprising preparing the antibody composition in a buffer comprising a concentration of at least 60 mM of phosphoric acid or its salt thereof, wherein the antibody composition so prepared demonstrates reduction in the aggregate/dimer content when compared with the antibody composition at neutral/alkaline pH that was prepared in a buffer comprising a concentration of less than 60mM of phosphoric acid or its salt thereof.
2. An antibody composition according to claim 1 wherein, the said antibody is anti-VEGF antibody.
3. An antibody composition according to claim 1, wherein the reduction of aggregate/dimer formation in the said antibody composition is at least about 50 %.
4. An antibody composition according to claim 1 wherein, the reduction in aggregate/dimer content in the said antibody composition is at least about two-fold.
5. A method for reduction of aggregate /dimer content in an antibody composition at neutral/alkaline pH, wherein the said antibody being predisposed to form aggregates/dimers at neutral or alkaline pH, the said method comprising preparing the antibody composition in a buffer comprising a concentration of at least 60 mM of phosphoric acid and/or its salt thereof, wherein the antibody composition so prepared demonstrates reduction in the aggregate/dimer content when compared with the antibody composition at neutral/alkaline pH

that was prepared in a buffer comprising a concentration of less than 60mM of phosphoric acid and/or its salt thereof.

6. A method according to claim 5 wherein, the said antibody is anti-VEGF antibody.

5 7. A method according to claim 5 wherein, the reduction of aggregate/dimer formation in the said antibody composition is at least about 50 %.

8. A method according to claim 5 wherein, the reduction in aggregate/dimer content in the said antibody composition is at least about two-fold.

9. A method for reducing the aggregates/dimer formation in an antibody load
10 mixture at neutral or alkaline pH conditions, during chromatographic purification, wherein the said antibody being pre-disposed to form aggregates / dimers at neutral or alkaline pH, the said method comprising, preparing the said antibody load mixture in a buffer comprising a concentration of at least 60 mM of phosphoric acid and/or its salts thereof and wherein there is a reduction
15 in aggregates/dimer formation in the said antibody load mixture when compared with the antibody load mixture prepared in a neutral or alkaline pH buffer comprising a concentration of less than 60 mM of phosphoric acid and/or its salts thereof.

10. A method according to claim 9, wherein the said chromatographic purification
20 with its steps of load, wash and/or recovery are operated at neutral or alkaline pH.

11. A method according to claim 9 wherein, the said chromatography purification involves an anion exchange chromatography or support.

12. A method according to claim 10, wherein the said chromatographic procedure
25 may include one or more wash buffer steps.

13. A method according to claim 9 wherein, the said antibody is anti-VEGF antibody.

14. A method according to claim 9 wherein, the reduction of aggregate/dimer formation in the said antibody load mixture is at least about 50 %.

5 15. A method according to claim 9 wherein, the reduction in aggregate/dimer content in the said antibody load mixture is at least about two-fold.

16. A method for reduction of aggregates/dimer formation in an antibody composition comprising steps of;

- 10 a) preparing the antibody load mixture in a buffer comprising phosphoric acid and/or its salts thereof in a concentration range of at least about 60 mM,
- b) contacting the said antibody load mixture onto an ion-exchange chromatographic support at a pH of about 7 to about 7.5,
- c) allowing the antibody to flow-through the support, and
- d) recovering the antibody, and

15 wherein, there is a reduction of aggregate/dimer formation in antibody load mixture at step a, than the antibody load mixture prepared in phosphoric acid and/or its salt thereof at concentration less than 60mM.

17. A method according to claim 16, wherein the aggregate/dimer content in the recovered antibody composition is less than 3%.

20 18. A method according to claim 16 wherein, the said chromatographic support involves an anion exchange chromatography or support.

19. A method according to claim 16 wherein, the said chromatographic procedure may include one or more wash buffer steps.

25 20. A method according to claim 16 wherein, the said antibody is anti-VEGF antibody.

21. A method according to claim 16, wherein the reduction of aggregate/dimer formation in the said antibody load mixture is at least about 50 %.
22. A method according to claim 16 wherein, the reduction in aggregate/dimer content in the said antibody load mixture is at least about two-fold.

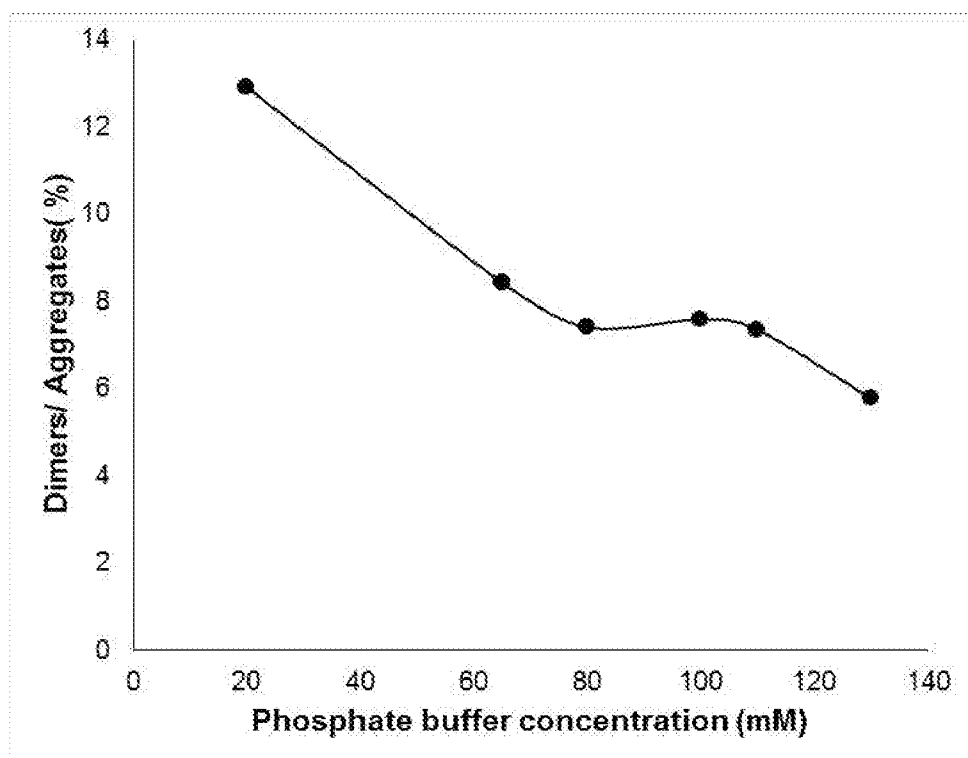


FIG.1

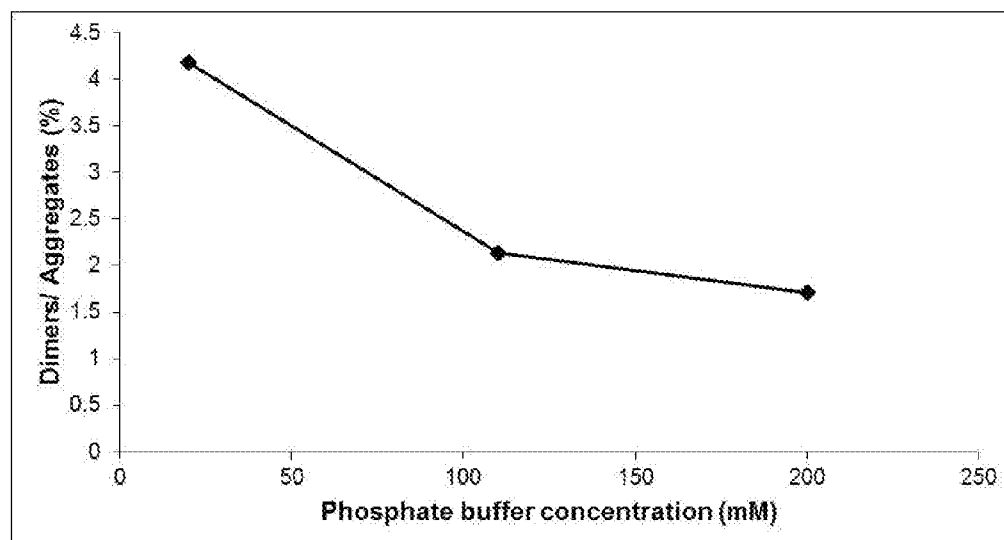


FIG.2

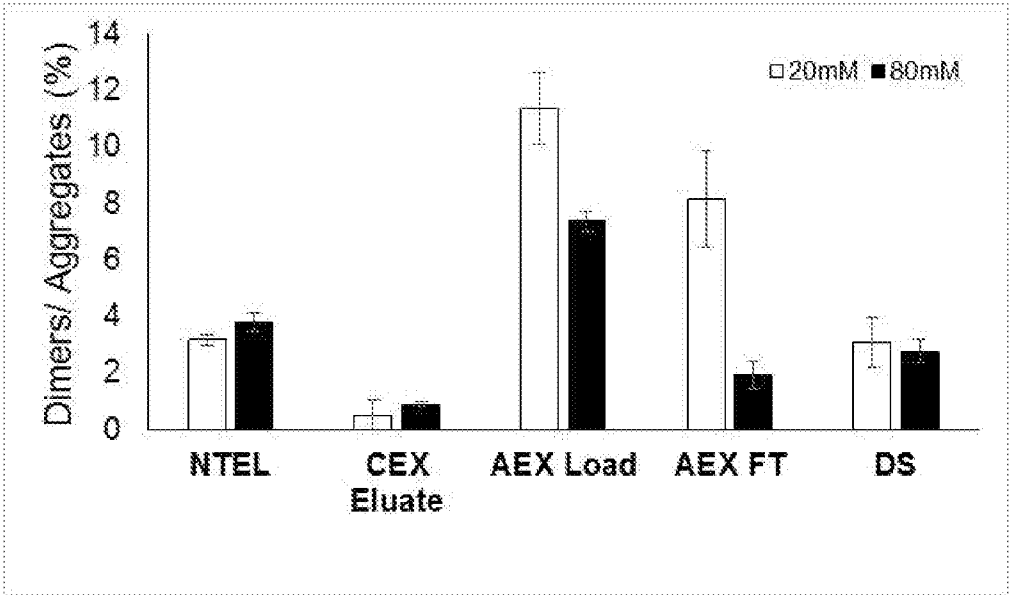


FIG.3

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2013102822 A1, (DR. REDDY'S LABORATORIES LIMITED), 11-07-2013 (11 July 2013). Page 2, para 6 & 7; examples.	1-4
Y	claims ; example 2; example 3. -----	5-22
Y	WO2011090719 A2, (DR. REDDY'S LABORATORIES LIMITED), 28-07-2011 (28 July 2011). Claims; page 1, para 2; examples. -----	5-22
Y	WO2004076485 A1, (LONZA BIOLOGICS PLC.), 10-09-2004 (10 september 2004). Claims; page 13, lines 1-3; page 24, lines 14-15.	5-22

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT
Information on patent family members

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