than without the wash (Gagnon *et al. supra*). Finally, hydroxyapatite with pre-elution washes of urea and/or salt have also reduced hetero-aggregates more effectively than without such washes (Gagnon *supra*). Despite these observations, in general, the use of dissociating agents in pre-elution washes of antibodies bound to chromatography columns has been only moderately successful.

**[0006]** Organic multivalent cations have been indicated for the precipitation of acidic proteins (Farhner et al., U.S. Patent Application No. 20080193981; Ma *et al.*, *J. Chromatogr. B* (2010) 878:798-806; Peram *et al.*, *Biotechnol. Progr.*, (2010) 26:1322-1326; Glynn, in U. Gottschalk (ed.), *Process Scale Purification of Antibodies*, J. T. Wiley and Sons, (2009) Hoboken, 309-324), as well as for precipitation of DNA and endotoxins (Glynn *supra*; Cordes *et al.*, Biotechnol. Progr., (1990) 6:283-285; Dissing *et al.*, *Bioseparation*, (1999) 7 221:9-11) and inactivation of virus (Bernhardt, U.S. Patent. 5,559,250). Multivalent metal cations have also been indicated to remove DNA and endotoxin from some protein preparations (Akcasu *et al.*, *Nature*, (1960) 187:323-324; Matsuzawa *et al.*, *Nucl. Acids Res.*, (2003) 3(3):163-164; Christensen *et al.*, *Prot. Expr. Purif.*, (2004) 37:468-471; Kejnovsky *et al.*, *Nucl. Acids Res.*, (1997) 25:1870-1871; Ongkudon *et al.*, *Anal. Chem.*, (2011) 83 391:13-17).

## SUMMARY OF THE INVENTION

**[0007]** In one aspect, the invention provides a method of reducing the aggregate content of a sample containing an antibody, or antibody fragment; wherein the method comprises the steps of: (i) providing a first component which is a first solid substrate having an electronegative surface: (ii) providing a second component which is a second solid substrate having an electropositive surface; (iii) contacting the sample with ethacridine, or a salt thereof, (iv) contacting the sample with the first and second components, where the first and second components are configured such that the sample may contact both components simultaneously, where the operating conditions substantially prevent binding of the antibody, or antibody fragment, to the first or second components; and (v) separating a resulting sample containing the antibody, or antibody fragment with a reduced aggregate content from the first and second components; wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid and the electropositivity of the surface of the second component is conferred in part by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group.

In still another aspect, the present invention refers to a method of reducing the aggregate content of a sample containing an antibody, or antibody fragment, wherein the method comprises the steps of: (i) providing a first component which is a first solid substrate having an electronegative surface: (ii) providing a second component which is a second solid substrate having an electropositive surface; (iii) contacting the sample with the first and second components, wherein the first and second components are configured such that the sample may contact both components simultaneously, the operating conditions substantially prevent the binding of the antibody, or antibody fragment, to the first or second components, and the conductivity of the sample is greater than 20 mS/cm; and (iv) separating a resulting sample containing the antibody, or antibody fragment, with a reduced aggregate content from the first and second components; wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group. In another aspect, the present invention refers to a method of reducing the aggregate content of a sample containing a desired protein, wherein the method comprises the steps of: (a) providing a composition comprising (i) a first component which is a first solid substrate comprising an electronegative surface and a surface-bound chemical moiety comprising metal affinity functionality and (ii) a second component which is a second solid substrate comprising an electropositive surface and a surface-bound chemical moiety comprising metal affinity functionality; (b) contacting the sample with the composition, where the sample may contact both components (i) and (ii) simultaneously and where operating conditions substantially prevent the binding of the desired protein to the composition; and (c) separating a resulting sample from the composition where the resulting sample comprises the desired protein with a reduced aggregate content; wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid, and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group.

## BRIEF DESCRIPTION OF THE FIGURES

**[0008]** Figure 1 shows a time course plot of the size exchange chromatography (SEC) profiles sampled at indicated intervals in the purification of IgM-529 which was subjected to allantoin-ethacridine batch particle treatment. Aggr: aggregate. HCP: host cell protein. Solid line: 280 nm. Broken line: 254 nm. All time scales same as center panel.

## CLAIMS

1. A method of reducing the aggregate content of a sample containing an antibody, or antibody fragment, wherein the method comprises the steps of: (i) providing a first component which is a first solid substrate having an electronegative surface; (ii) providing a second component which is a second solid substrate having an electropositive surface; (iii) contacting the sample with ethacridine, or a salt thereof; (iv) contacting the sample with the first and second components, where the first and second components are configured such that the sample may contact both components simultaneously, where the operating conditions substantially prevent the binding of the antibody, or antibody fragment, to the first or second components; and (v) separating a resulting sample containing the antibody, or antibody fragment, with a reduced aggregate content from the first and second components; wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid, and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group.

2. The method of claim 1 wherein the first and second components are combined prior to contacting the sample.

3. The methods of any one of claims 1-2, wherein the ethacridine, or salt thereof, is removed from the sample in the step of separating the sample with a reduced aggregate content from the first and second components to the extent that the ethacridine, or salt thereof, is associated with a substrate of the first or second component.

4. The method of any one of claims 1-5, wherein the first substrate is particulate or wherein the second substrate is particulate.

5. The method of claim 4, wherein the particulate substrate or substrates is each provided as a plurality of particles.

6. The method of claim 5, wherein the particles are porous and the average pore size of the porous particles is between 10nm and 100nm.

7. The methods of any one of claims 4-6, wherein the particles are sandwiched between porous membranes or monoliths, or wherein the particles are sandwiched between woven or amorphous fibrous filters, or wherein the particles are sandwiched between crystalline frits, or wherein the particles are embedded in a reticular polymer network.

8. The method of any one of claims 1-7 wherein the conductivity of the sample is at a level to avoid precipitation of the antibody, or antibody fragment, from the sample while contacting the ethacridine, or salt thereof.

9. The method of claim 8, wherein the conductivity of the sample is greater than 20mS/cm or greater than 30 mS/cm, or greater than about 40 mS/cm, or greater than about 100 mS/cm.

10. The method of any one of claims 1-9, wherein the aggregates comprise homo-aggregates of the antibody, or antibody fragment.

11. The method of claim 10, wherein the presence of homo-aggregates of the antibody, or antibody fragment, is substantially eliminated.

12. The method of any one of claims 1-11, wherein the aggregates comprise hetero-aggregates of the antibody, or antibody fragment, and a contaminant.

13. The method of claim 12, wherein the hetero-aggregates are of substantially the same hydrodynamic size as the antibody, or antibody fragment.

14. The method of claim 12, wherein the contaminant is a nucleic acid, nucleotide, endotoxin, metal ion, protein, lipid, or cell culture media component.

15. The method of any one of claims 12-14, wherein the presence of hetero-aggregates of the antibody, or antibody fragment, and a contaminant is substantially eliminated.

16. The method of any one of claims 1-15, wherein the antibody, or antibody fragment,

is a recombinant protein.

17. The method of any one of claims 1-16, wherein the sample is a cell culture harvest, a cell culture supernatant, an antibody-containing solution derived from a cell culture, or an antibody-containing solution from a previous stage of protein purification.

18. The method of claim 20, wherein the cultured cells are mammalian cells, bacterial cells, or yeast cells.

19. The method of any one of claims 1-18, wherein the sample is contacted with the first and second components by flowing the preparation through the first and second components.

20. The method of any one of claims 1-19, wherein the electronegativity of the surface of the first component is conferred through one or more kinds of complex chemical moieties that embody more than one chemical functionality, or through a combination of simple and complex chemical groups mixed on the surface, or a combination of surfaces of differing chemical composition.

21. The method of any one of claims 1-20, wherein the electropositivity of the surface of the second component is conferred through one or more kinds of complex chemical groups that embody more than one chemical functionality, or through a combination of simple and complex chemical groups mixed on the surface, or a combination of surfaces of differing chemical composition.

22. The method of any one of claims 1-21, wherein the electronegativity of the surface of the first component is conferred, in part, by iminodiacetic acid and the electropositivity of the surface of the second component is conferred, in part, by tris(2-aminoethyl)amine.

23. The method of any one of claims 1-22, wherein the first component has a surface-bound chemical moiety possessing metal affinity functionality.

24. The method of any one of claims 1-23, wherein the electropositive surface of the second component includes a surface-bound chemical moiety possessing metal affinity functionality.

25. The method of any one of claims 23-24, wherein at least one of the substrates has one or more chemical moieties in addition to the surface-bound chemical moiety possessing metal

affinity functionality wherein such additional chemical moieties enhance the capacity of one or more of the components to participate in hydrogen bonding, hydrophobic interactions, or pi-pi binding with a protein of the protein preparation.

26. The method of any one of claims 23-25, wherein the surface-bound chemical moiety possessing metal affinity functionality is a multidentate metal chelating moiety.

27. The method of any one of claims 1-26, wherein the electropositive surface of the first component includes a surface-bound chemical moiety possessing metal affinity functionality, the electronegative surface of the second component includes a surface-bound chemical moiety possessing metal affinity functionality, and another surface includes a surface-bound chemical moiety possessing an elevated hydrophobic functionality.

28. The method of any one of claims 1-27, wherein the ethacridine, or a salt thereof, is present in an amount between 0.01% and 0.05%.

29. The method of any one of claims 1-27, wherein the ethacridine, or a salt thereof, is present in an amount between 0.020 and 0.025%.

30. The method of any one of claims 1-29, further comprising treating the sample with an organic multivalent cation selected from the group consisting of polyethyleneimine, polyallyamine, and chlorhexidine, and salts thereof, prior to the step of contacting the sample with the first and second components.

31. The method of claim 30, wherein the organic multivalent cation is provided in a concentration between 0.01% and 0.05%.

32. The method of claim 30, wherein the organic multivalent cation is provided in a concentration between 0.020 and 0.025%.

33. The method of any one of claims 1-32, wherein the sample is additionally contacted with a soluble organic modulator selected from the group consisting of nonionic organic polymers, organic solvents, surfactants, and allantoin, prior to the step of contacting the sample with the first and second components.

34. The method of any one of claims 1-33, wherein the sample is additionally contacted with an antiviral agent, prior to the step of contacting the sample with the first and second

components.

35. The method of claim 34, wherein the antiviral agent is a non-multivalent organic cation, or wherein the antiviral agent is selected from the group consisting of benzalkonium chloride, methylene blue and tri (n-butyl) phosphate.

36. The method of any one of claims 1-35, comprising the additional steps of, prior to the step of contacting the sample with the first and second components, contacting the sample with allantoin in an amount sufficient for the allantoin to be supersaturated in the protein preparation, and separating the supernatant containing the antibody, or antibody fragment, from the solid or undissolved portions of the sample.

37. The method of claim 36, wherein the allantoin is present in an amount greater than 0.5% (w/v), or wherein the allantoin is present in an amount greater than approximately 1% (w/v), or wherein the allantoin is present in an amount greater than approximately 2% (w/v), or wherein the allantoin is present in an amount greater than approximately 5% (w/v), or wherein the allantoin is present in an amount greater than approximately 10% (w/v).

38. The method of claim 33, wherein the organic modulator is a nonionic organic polymer selected from the group consisting of glycerol, polyethylene glycol, polypropylene glycol and polybutylene glycol, or wherein the organic modulator is an organic solvent selected from the group consisting of ethylene glycol, propylene glycol, butylene glycol, dimethylsulfoxide, ethanol, isopropanol, and phenoxyethanol, or wherein the organic modulator is a surfactant selected from the group consisting of Tween, triton, CHAPS, CHAPSO and octyl glucoside, or wherein the organic modulator is allantoin provided in a subsaturating amount.

39. The method of claim 38, wherein, in case the organic modulator is a nonionic organic polymer, the nonionic organic polymer has an average molecular weight of approximately 500 D or less.

40. A method of reducing the aggregate content of a sample containing an antibody, or antibody fragment, wherein the method comprises the steps of: (i) providing a first component which is a first solid substrate having an electronegative surface: (ii) providing a second component which is a second solid substrate having an electropositive surface; (iii) contacting the sample with the first and second components, wherein the first and second components are configured such that the sample may contact both components

simultaneously, the operating conditions substantially prevent the binding of the antibody, or antibody fragment, to the first or second components, and the conductivity of the sample is greater than 20 mS/cm; and (iv) separating a resulting sample containing the antibody, or antibody fragment, with a reduced aggregate content from the first and second components; wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group.

41. A method of reducing the aggregate content of a sample containing a desired protein, wherein the method comprises the steps of:

(a) providing a composition comprising (i) a first component which is a first solid substrate comprising an electronegative surface and a surface-bound chemical moiety comprising metal affinity functionality and (ii) a second component which is a second solid substrate comprising an electropositive surface and a surface-bound chemical moiety comprising metal affinity functionality;

(b) contacting the sample with the composition, where the sample may contact both components (i) and (ii) simultaneously and where operating conditions substantially prevent the binding of the desired protein to the composition; and

(c) separating a resulting sample from the composition where the resulting sample comprises the desired protein with a reduced aggregate content;

wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, sulfurous acid, sulfonate, and phosphoric acid, and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group

consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, a secondary amino group, a tertiary amino group, and a quaternary amino group.

42. The method of claim 41, wherein the electronegativity of the surface of the first component is conferred, in part, by a moiety from the group consisting of iminodiacetic acid, ethylene glycol(aminoethylether)diacetic acid, nitriloacetic acid, aspartic acid, glutamic acid, a carboxylic acid, and phosphoric acid, and the electropositivity of the surface of the second component is conferred, in part, by a moiety from the group consisting of tris(2-aminoethyl)amine, diethylenetriamine, triethylenetetramine, tetraethylenepentamine, polypropylenimine tetramine, PAMAM dendrimer (ethylenediamine core), deferoxamine, a primary amino group, and a secondary amino group.